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Altered expression of neurotrophic factors in patients with major depression

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Abstract

There is an abundance of evidence suggesting the involvement of altered levels of expression of neurotrophic factors in the pathophysiology of neuropsychiatric disorders. Although postmortem brain studies have indicated the alterations in the expression levels of neurotrophic factors in mood disorder patients, it is unclear whether these changes are state- or trait-dependent. In this study, we examined the expression levels of the members of the glial cell line-derived neurotrophic factor (GDNF) family (GDNF, artemin (ARTN), neurturin, and persephin), brain-derived neurotrophic factor, nerve growth factor, neurotrophin-3 (NT-3), and neurotrophin-4 mRNAs by using quantitative real-time PCR method in peripheral blood cells of patients with major depressive and bipolar disorders in both a current depressive and a remissive states. Reduced expression levels of GDNF, ARTN, and NT-3 mRNAs were found in patients with major depressive disorder in a current depressive state, but not in a remissive state. Altered expressions of these mRNAs were not found in patients with bipolar disorder. Our results suggest that the changes in the expression levels of GDNF, ARTN, and NT-3 mRNAs might be state-dependent and associated with the pathophysiology of major depression.

Keywords: Major depression; Bipolar disorder; Neurotrophic factor; GDNF; State marker

1. Introduction

Brain imaging studies have indicated the altered volumes of the hippocampus, prefrontal cortex, cingulate cortex, or amygdala in patients with major depression or bipolar disorder (Drevets et al., 1997; Sheline et al., 1999; Bremner et al., 2000; Caetano et al., 2006). Postmortem brain studies have also reported anatomical alterations such as neuronal/glial cell atrophy or reduction in cortical brain regions in depressed patients (Ongur et al., 1998; Rajkowska et al., 1999; Cotter et al., 2001). These studies suggest the alteration of structural and neuronal plasticity in mood disorders.

Neurotrophic factors have many effects on the nervous system such as neurogenesis, neuronal growth, differentiation, and plasticity. There is a growing body of evidence demonstrating that neurotrophic factors have crucial roles in stress response, the action of antidepressants, and pathophysiology of mood disorders (Smith et al., 1995; Nibuya et al., 1999; Karege et al., 2002; Nestler et al., 2002; Duman and Monteggia, 2006; Govindarajan et al., 2006). For example, the expression level of brain-derived neurotrophic factor (BDNF) was significantly decreased in animals subjected to social defeat, immobilization, and maternal deprivation stresses (Smith et al., 1995; Nibuya et al., 1999; Roceri et al., 2002; Tsankova et al., 2006). In addition, chronic antidepressant treatment increases the expression levels of BDNF and neurogenesis in rodents (Malberg et al., 2000; Duman and Monteggia, 2006; Tsankova et al., 2006). Furthermore, low levels of serum BDNF is

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suggested to be associated with major depression (Duman et al., 1997; Karege et al., 2002; Nestler et al., 2002). In addition to BDNF, the expression levels of other neurotrophic factors, including: nerve growth factor (NGF); glial cell line-derived neurotrophic factor (GDNF); neurotrophin-3 (NT-3); and neurotrophin-4 (NT-4) were also reported to be associated with stress response and neurogenesis in rodents and depression in humans (Smith et al., 1995; Ueyama et al., 1997; Aloe et al., 2002; Dwivedi et al., 2005; Shimazu et al., 2006). However, little is known about whether the changes in the expression levels of these molecules are state- or trait-dependent in mood disorders.

Another well known characteristic of the pathophysiology of mood disorders is the dysregulation of hypothalamic-pituitary–adrenal (HPA) axis (Arborelius et al., 1999; Holsboer, 2001; Nestler et al., 2002; de Kloet, 2003). Although the molecular mechanisms of the aberrant regulation of the HPA axis in mood disorders remains unclear, one candidate is the dysfunction of the glucocorticoid receptor (GR), which plays an important role in the negative feedback of the HPA axis and the adaptation to stress (Holsboer, 2000; Pariante and Miller, 2001). In response to stress, glucocorticoid hormone and GR are associated with stress-induced effects on the brain, including shrinkage of neural dendrites, suppressed neurogenesis, and reduced serotonin metabolism (Lopez et al., 1998; McEwen, 2000; Sapolsky et al., 2000; de Kloet, 2003). In addition, previous studies have indicated the reduced expression of GRα mRNA in the brain and peripheral white blood cells of patients with mood disorders (Webster et al., 2002; Knable et al., 2004; Perlman et al., 2004; Matsubara et al., 2006). Several lines of evidence have suggested that glucocorticoid hormone or GR affect the expression levels of neurotrophic factors (Barbany and Persson, 1992; Chao and McEwen, 1994; Smith et al., 1995; Mocchetti et al., 1996; Ridder et al., 2005; Schulte-Herbruggen et al., 2006). For example, adrenalectomy significantly decreased the mRNA levels of BDNF, NT-3, and NGF in the rat cerebral cortex and hippocampus (Barbany and Persson, 1992). In addition, GR knock-out heterozygous mice exhibited decreased levels of BDNF protein in the hippocampus, whereas GR overexpressing mice exhibited increased levels of BDNF protein in the amygdala and hippocampus (Ridder et al., 2005; Schulte-Herbruggen et al., 2006). These observations suggest that the reduced levels of GR may affect the expression levels of neurotrophic factors in patients with mood disorders.

In this study, we aimed to determine whether there are alterations in the expression levels of multiple neurotrophic factors, including GDNF family members (GDNF, artemin (ARTN), neurturin (NRTN), and persephin (PSPN)), BDNF, NGF, NT-3, and NT-4 mRNAs in peripheral white blood cells of mood disorder patients. Furthermore, to examine whether the altered expression of neurotrophic factor mRNAs are state- or trait-dependent, the mRNA levels of these factors were examined in both current depressive and remissive states.

2. Materials and methods

2.1. Subjects

Major depressive and bipolar disorder patients were diagnosed according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV; American Psychiatric Association, 1994). These included both outpatients and inpatients of the Division of Neuropsychiatry of the Yamaguchi University Hospital. The extent of the depressive state was assessed by a 21-item "Hamilton Depression Rating Scale" (HDRS). Subjects were classified as under a current depressive state when they showed a score of more than 18 on HDRS and met the DSM-IV criteria for major depressive episode. Subjects were classified as being in remission when they showed a score of less than 6 on HDRS and did not show any symptoms of the major depressive episode in the DSM-IV criteria for more than 2 months. Individuals were excluded from the present study if they had abnormal physical examinations or abnormal results for routine medical laboratory tests such as a complete blood count, renal, liver or thyroid function. Female subjects who were pregnant or took oral contraceptives were also excluded. All healthy control subjects were screened to exclude significant current or past medical or neurological illnesses, significant alcohol or drug abuse and past or current Axis I psychiatric illnesses. This protocol was approved by the Institutional Review Board of Yamaguchi University Hospital. Informed written consent was obtained for all subjects.

2.2. Blood sample preparation, RNA isolation, and cDNA synthesis

Blood sample preparation, total RNA isolation and cDNA synthesis were performed as previously described (Matsubara et al., 2006). In brief, blood was obtained by venipuncture between 10:00 a.m. and 11:00 a.m. and processed for total RNA purification from peripheral blood cells by using QIAamp RNA Blood Mini Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's manual. The quality of RNA was determined based on A_{260}/A_{280} ratio, which was found as 1.7–2.0 for all RNA preparations. One microgram of total RNA was used for cDNA synthesis using random hexamer primers and Omniscript Reverse Transcriptase (Qiagen). The cDNA was stored at −80 °C until further use.

2.3. Serum cortisol determination

Serum cortisol concentration was measured via radioimmunoassay by the SRL Corporation (Tokyo, Japan).

2.4. Quantitative real- time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in an Applied Biosystems 7300 Fast

Real-Time PCR System with SYBR green PCR master mix (Applied Biosystems, CA, USA) according to the manufacturer's manual. PCR conditions were 15 min at 95 °C, 45 cycles of 15 s at 95 °C and 30 s at 60 °C. The primer sequences are described in Table 1. The amplification of a single PCR product was confirmed by monitoring the dissociation curve and electrophoresis on 1.5% agarose gels stained with ethidium bromide. Amplification curves were visually inspected to set a suitable baseline range and threshold level. To generate standard curves, various concentrations of cDNA, made from total RNA isolated from human lymphoma-derived Jurkat cells, were used in each PCR reaction. The number of cycles required to reach the threshold fluorescence level was scored and used for generating standard curves and interpolating mRNA concentration levels. The relative quantification method, in which the ratio between the amount of target molecule and a reference molecule within the same sample was calculated, was employed for quantifying target molecules according to the manufacturer's protocol. All measurements were performed at least in duplicate. Levels of GAPDH mRNA was used to normalize the relative expression levels of target mRNA. Expression values for mood disorder subjects were normalized by the mean of the value for control subjects.

2.5. Dex/CRH test

A few days after blood sampling for the RNA isolation and basal cortisol determination, Dexamethasone (Dex)/corticotropin-releasing hormone (CRH) test was performed as previously reported (Matsubara et al., 2006). Mood disorder patients were pretreated with an oral dose of 1 mg of Dex (Asahikasei Pharmaceutical Corporation, Tokyo, Japan) at 11:00 p.m. The next day intravenous cannulation was carried out at 12:30 p.m. and 100 μg of human CRH (hCRH, Mitsubishi Pharma Corporation, Tokyo, Japan) was administered intravenously at 1:00 p.m., immediately after the first blood collection. Blood specimens were drawn through the intravenous catheter 15 min, 30 min, 60 min and 120 min later. Blood samples were immediately centrifuged and stored at −20 °C. Serum level of cortisol and plasma level of ACTH were measured with radioimmunoassay (SRL). We defined non-suppressors as those individuals whose post-CRH plasma cortisol levels were more than 5 μg/dl.

2.6. Statistical analysis

Commercial software (SPSS version 11.5; Chicago, Illinois) was used to perform data analysis. All data are expressed as the mean ± standard error of the mean (SEM). Two group comparisons were performed using the Student's *t*-test. The other data were subjected to one-way analysis of variance (ANOVA) followed by post hoc analysis (Tukey test). In all cases, comparisons were considered statistically significant for $p < 0.05$.

3. Results

Table 2 shows the demographic and clinical characteristics of the subjects. The majority of the patients were on medications. The mean ages were not significantly different among major depressive disorder patients, bipolar depressive patients or healthy control subjects ($F = 1.228$, $df = 4.125$, $p = 0.303$).

We first examined the expression levels of the GDNF family members (GDNF, ARTN, NRTN, and PSPN) in patients with mood disorder in a current depressive state (Fig. 1). The qRT-PCR revealed that the expression of GDNF mRNA was significantly decreased in major depressive disorder patients compared with healthy control subjects ($F = 5.472$, $df = 2.58$, $p = 0.005$) (Fig. 1A). In addition, ARTN mRNA was significantly decreased in major depressive disorder patients compared with healthy control subjects and bipolar disorder patients ($F = 4.147$, $df = 2.58$, $p = 0.037$; $p = 0.049$) (Fig. 1B). There was no significant difference in the expression levels of GDNF and ARTN mRNA between patients with bipolar disorder in a current depressive state and healthy control subjects (GDNF: $F = 5.472$, $df = 2.58$, $p = 0.214$, ARTN: $F = 4.147$, $df = 2.58$, $p = 0.932$) (Fig. 1A and B). There was no significant difference in the expression levels of NRTN and PSPN mRNAs among major depressive, bipolar disorder patients and healthy control subjects (NRTN: $p = 0.67$; PSPN: $p = 0.282$) (Fig. 1C and D).

Next, we examined the expression levels of BDNF, NGF, NT-3 and NT-4 mRNAs in patients with mood

Table 1
Primer sequences used for quantitative real-time PCR

	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size (bp)
BDNF	TGGCTGACACTTTCGAACAC	AGAAGAGGAGGCTCCAAAGG	145
NGF	CAACAGGACTCACAGGAGCA	GTCTGTGGCGGTGGTCTTAT	115
NT-3	GAAACGCGATGTAAGGAAGC	CCAGCCCACGAGTTTATTGT	138
NT-4	CCTCCGCCAGTACTTCTTTG	TCAGATACCCAGTGCCTCCT	114
GDNF	CCAACCCAGAGAATTCCAGA	AGCCGCTGCAGTACCTAAAA	150
ARTN	CTCTCCACACGACCTCAGC	ATGAAGGAGACCGCTTGGTA	123
NRTN	ACGAGACGGTGCTGTTCC	GCAGCCCGAGGTCGTAGA	72
PSPN	CCGTAGGGAAGTTCCTGCT	CCTTTGCCACCTGTTCAGAC	126
GAPDH	CAGCCTCAAGATCATCAGCA	TGTGGTCATGAGTCCTTCCA	106

Table 2
Demographic and clinical characteristics of subjects

	Control	Patients			
		MDD		BPD	
	$n = 28$	Depressive $n = 20$	Remissive $n = 40$	Depressive $n = 13$	Remissive $n = 29$
Age (years)	50.0 ± 1.8	52.3 ± 3.5	57.1 ± 2.2	55.5 ± 3.5	53.6 ± 2.6
Gender (Male/Female)	15/13	10/10	15/25	2/11	5/24
HDRS		25.9 ± 1.9	3.3 ± 0.2	24.6 ± 1.1	2.9 ± 0.2
Serum cortisol (μg/dl)	8.6 ± 0.8	10.3 ± 1.3	11.6 ± 1.1	10.9 ± 4.5	10.5 ± 1.0
Dex-CRH test					
Suppressor		8	7	3	2
Non-suppressor		8	3	7	5
Medication					
No medication	28	3	4	1	0
Antidepressants	0	17	36	9	14
Mood stabilizers	0	0	0	10	27

MDD, major depressive disorder; BPD, bipolar disorder; HDRS, Hamilton Depression Rating Scale.

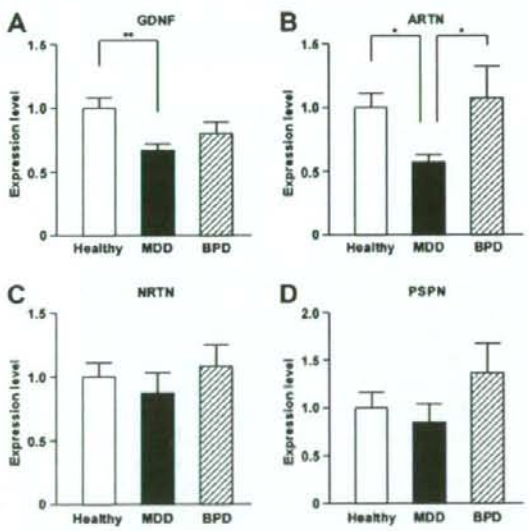

Fig. 1. The mRNA expression of GDNF family members in mood disorder patients in a depressive state. Quantitative real-time PCR revealed decreased GDNF and ARTN mRNA expression only in MDD patients ($n = 20$) compared with healthy control subjects ($n = 28$). MDD, major depressive disorder; BPD, bipolar disorder. Values are mean ± standard error. *: $p < 0.05$; **: $p < 0.01$.

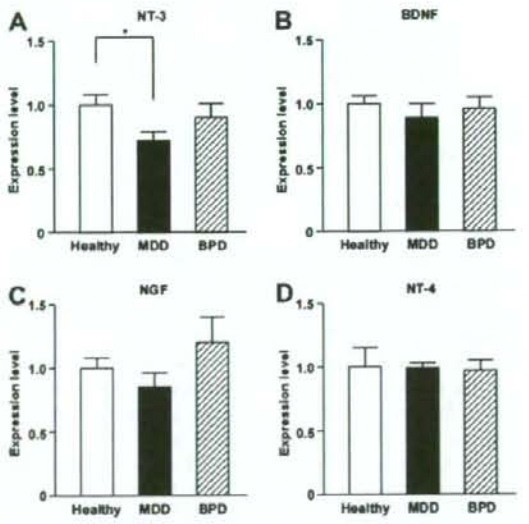

Fig. 2. The mRNA expression of NT-3, BDNF, NGF, and NT-4 in mood disorder patients in a depressive state. Quantitative real-time PCR revealed decreased NT-3 mRNA expression only in MDD patients ($n = 20$) compared with healthy control subjects ($n = 28$). There was no significant difference in the levels of BDNF, NGF, and NT-4 mRNAs among the three groups studied. MDD, major depressive disorder; BPD, bipolar disorder. Values are mean ± standard error. *: $p < 0.05$.

disorder in a current depressive state (Fig. 2). The expression levels of NT-3 mRNA was significantly decreased in major depressive patients compared with healthy control subjects ($F = 3.310$, df = 2.58, $p = 0.034$) (Fig. 2A). There was no significant difference in the expression level of NT-3 mRNA between patients with bipolar disorder in a current depressive state and healthy control subjects ($F = 3.31$, df = 2.58, $p = 0.675$) (Fig. 2A). There was no significant difference in the expression levels of BDNF, NGF and NT-4 mRNAs among the three groups (BDNF: $p = 0.615$; NGF: $p = 0.162$, NT-4: $p = 0.245$) (Fig. 2B–D).

We analyzed the effect of gender on the mRNA levels of GDNF, ARTN, and NT-3 in patients with major depressive disorder in a current depressive state and healthy controls. Gender difference did not produce a significant effect on GDNF, ARTN, and NT-3 mRNA expression of healthy control subjects (GDNF: $t = -0.970$, df = 26, $p = 0.341$, ARTN: $t = -1.187$, df = 26, $p = 0.246$, NT-3: $t = -0.386$, df = 26, $p = 0.703$) and those of major depressive disorder patients in a current depressive state (GDNF: $t = -0.919$, df = 18, $p = 0.370$, ARTN: $t = -0.984$, df = 18, $p = 0.338$, NT-3: $t = -0.659$, df = 18, $p = 0.519$).

To examine whether the altered expression levels of GDNF, ARTN, and NT-3 mRNAs observed in a current depressive state are state- or trait-dependent, the mRNA levels of these factors were also examined in a remissive state. The expression levels of GDNF, ARTN, and NT-3 mRNAs in major depressive disorder patients in a remissive state showed no significant differences compared with healthy control subjects or bipolar disorder patients (GDNF: $F = 2.296$, df = 2.94, $p = 0.106$; ARTN: $F = 2.619$, df = 2.94, $p = 0.078$; NT-3:$F = 0.980$, df = 2.94, $p = 0.379$) (Fig. 3A–C). In addition, the expression levels of GDNF, ARTN, and NT-3 mRNAs in bipolar disorder patients in a remissive state showed no significant differences compared with healthy control subjects or major depressive disorder patients (GDNF: $F = 2.296$, df = 2.94, $p = 0.997$; ARTN: $F = 2.619$, df = 2.94, $p = 0.063$; NT-3:$F = 0.980$, df = 2.94, $p = 0.522$) (Fig. 3A–C). We also examined the expression levels of GDNF, ARTN, and NT-3 mRNA in same patients with major depressive ($n = 15$) or bipolar disorder ($n = 9$) before and after remission. qRT-PCR revealed that the expressions of these three mRNA were significantly decreased in patients with major depressive disorder in a current depressive state compared with those in a current remissive state (GDNF: $t = -2.704$, df = 28, $p = 0.012$, ARTN: $t = -3.241$, df = 28, $p = 0.030$, NT-3: $t = -2.151$, df = 28, $p = 0.040$). There was no significant difference in the expression levels GDNF, ARTN, and NT-3 mRNA between patients with bipolar disorder in a current depressive and depressive state (GDNF: $t = -0.095$, df = 16, $p = 0.925$, ARTN: $t = -0.655$, df = 16, $p = 0.522$, NT-3: $t = 0.778$, df = 16, $p = 0.448$). Importantly, there was no significant difference in the treatment periods before remission between major depressive disorder (2.93 ± 0.36 months) and bipolar disorder patients (2.78 ± 0.41 months) ($t = 0.274$, df = 22, $p = 0.787$). In addition, there was no difference in the medication (imipramine equivalent) between patients with major depressive disorder in a current depressive and remissive state ($t = 0.120$, df = 28, $p = 0.906$). Furthermore, there was no correlation between expression levels of GDNF, ARTN, or NT-3 mRNA and medication (imipramine equivalent) in same patients with major depressive disorder (GDNF: $r = 0.186$, $p = 0.326$, ARTN: $r = -0.130$, $p = 0.493$, NT-3: $r = 0.048$, $p = 0.801$). These data suggest that the observed effects are likely to be due to the disease states.

There was no significant correlation between GDNF, ARTN, or NT-3 mRNA levels and the serum cortisol concentration for healthy control subjects, major depressive disorder patients or bipolar disorder patients (data not shown).

To examine the association between GDNF, ARTN or NT-3 mRNA levels and HPA axis activity; the mRNAs levels for these three genes of mood disorder patients in a current depressive state were compared between suppressors and non-suppressors of the Dex/CRH test. There was no significant difference in the expression level of the mRNAs for these three genes between suppressors and non-suppressors (data not shown).

4. Discussion

In the present study, we found the reduced expression of GDNF, ARTN, and NT-3 mRNAs in peripheral white blood cells of major depressive disorder patients in a current depressive state. In contrast, these reductions of GDNF, ARTN, and NT-3 mRNA expression were not observed in major depressive disorder patients in a remissive state. These results suggest that reduced expression of GDNF, ARTN, and NT-3 mRNAs are state-dependent.

Importantly, this represents the first study showing a reduced expression of ARTN mRNA in a depressive state, but not in a remissive state of major depressive disorder. ARTN is expressed in structures of the basal ganglia (subthalamic nucleus, putamen and the substantia nigra), thalamus and many peripheral adult human tissues (Baloh et al., 1998), and is a potent neurotrophic factor that may play an important role in the structural development and plasticity of ventral mesencephalic dopaminergic neurons (Zihlmann et al., 2005). It is known that the GDNF family ligands exert their effects through GDNF family receptor α (GFRα) and Ret (Airaksinen et al., 1999). Four distinct GFRαs (GFRα1-4) have been reported (Airaksinen et al., 1999). These molecules form a complex, containing the GDNF family ligand and GFRα homodimers, that

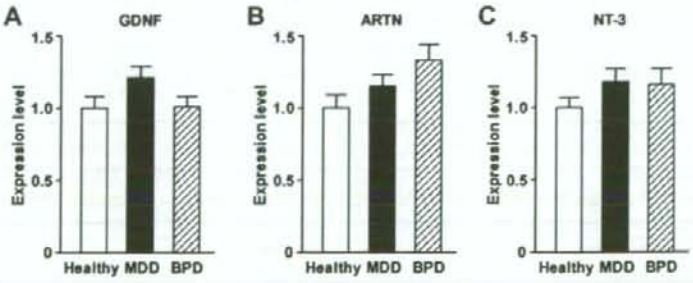

Fig. 3. The mRNA expression of GDNF, ARTN, and NT-3 in mood disorder patients in a remissive state. There was no significant difference in the levels of GDNF, ARTN, and NT-3 mRNAs among the three groups studied. MDD, major depressive disorder; BPD, bipolar disorder. Values are mean ± standard error.

brings two molecules of Ret together (Coulpier and Ibanez, 2004). Ret can then activate a variety of intracellular signaling pathways, including Ras/mitogen-activated protein kinase (MAPK); PI3K/AKT; RAC1/Jun NH2-terminal kinase (JNK); p38MAPK and phospholipase Cγ pathways (Ichihara et al., 2004; Kodama et al., 2005), resulting in cell survival, inflammation, differentiation and apoptosis. Concerning the relationship between ARTN and disease, the upregulation of ARTN mRNA expression is observed in smooth muscle cells of arteries, Schwann cells, and neural ganglia in chronic pancreatitis (Ceyhan et al., 2007), and in the auditory nerve of deafened rats (Wissel et al., 2006). To our knowledge, however, there is no report indicating the involvement of ARTN in the pathophysiology of neuropsychiatric disorders. It is important to note that both GDNF and ARTN bind to GFRα1 (Airaksinen et al., 1999). Our present data showed both GDNF and ARTN expression levels were reduced in patients with major depressive disorder, suggesting that dysfunctions of GFRα1-mediated pathways may be involved in the pathophysiology of major depression.

Our major finding is the reduced expression of two GDNF family gene (GDNF and ARTN) mRNAs in major depressive disorder. Recent postmortem brain studies have shown a reduction of glia in different brain structures of patients with mood disorders (Ongur et al., 1998; Rajkowska et al., 1999; Hamidi et al., 2004). Although there are no reports showing an altered expression of GDNF in the brains of patients with mood disorders, a report showed the decreased levels of whole blood GDNF in a remissive state of major depressive and bipolar disorder patients (Takebayashi et al., 2006). In contrast, increased serum GDNF immunocontent in bipolar disorders was observed (Rosa et al., 2006). The reason for this discrepancy is unclear, but one possible speculation is racial differences between the Japanese and Brazilian populations. Differences in the methodology used for sample preparation and determination of GDNF levels, such as ELISA (Takebayashi et al., 2006) and Western blotting (Rosa et al., 2006) may also account for this discrepancy.

A growing body of evidence has implicated a role for chronic or moderate oxidative stress in the pathogenesis of mood disorders. Previous studies showed that increased levels of malondialdehyde (a marker of lipid peroxidation) (Kuloglu et al., 2002; Khanzode et al., 2003; Ozcan et al., 2004), and decreased catalase levels (Ranjekar et al., 2003; Ozcan et al., 2004) were observed in the peripheral blood of mood disorder patients. Moreover, increased levels of oxidative DNA damage was reported in the peripheral blood of major depressive disorder patients (Forlenza and Miller, 2006). These results suggest an increased oxidative stress status in mood disorders. Importantly, GDNF blocked the DNA cleavage induced by bleomycin sulphate and L-buthionine-[*S*,*R*]-sulfoximine exposure in cultured mesencephalic neurons (Sawada et al., 2000), and reduced oxidative stress-induced cell death (Gong et al., 1999; Toth et al., 2002; Ugarte et al., 2003). Interestingly, increased levels of apoptosis have been observed in peripheral blood lymphocytes of depressive patients (Eilat et al., 1999; Ivanova et al., 2007). In addition, in a postnatal primary culture of mesencephalic dopaminergic neurons, GDNF supports their viability by suppressing apoptosis (Burke et al., 1998). Taken together, these data indicate that the reduced expression of GDNF mRNA may contribute to oxidative stress-induced cell death and apoptosis in peripheral blood cells of mood disorders, and this might be one of the mechanisms of immune inadequacy in mood disorders (Riccardi et al., 2002).

NT-3 mRNA and protein levels have been observed to be decreased in the hippocampus in a postmortem study (Dwivedi et al., 2005). In addition, increased levels of NT-3 in the cerebrospinal fluid of major depressive patients were reported (Hock et al., 2000). In peripheral blood, serum NT-3 levels were increased in bipolar disorder patients during a depressive state (Walz et al., 2007), and this data could not be replicated by our present study. Walz et al. (2007) speculated that the increased levels of NT-3 was due to a compensation to the decreased BDNF level. Our data showed decreased levels of NT-3 in a depressive state in major depressive disorder, but not for BDNF. The reason for this discrepancy is unclear.

A limitation of our study is that the majority of the patients were on medication, thus we can not exclude the influence of medication on the expression levels of the neurotrophic factors studied. A previous report indicated no alteration of the level of GDNF in the hippocampus of mice upon chronic treatment with antidepressants (Chen et al., 2001), whereas the increased expression of GDNF has been reported in cultured cells upon treatment with both antidepressants and mood stabilizers (Hisaoka et al., 2001; Castro et al., 2005). In addition, NT-3 levels have been reported to be decreased by chronic treatment with antidepressants which blocked norepinephrine uptake in the locus coeruleus of rats (Smith et al., 1995). In our study, however, expression levels of GDNF, ARTN, and NT-3 mRNA are only affected in the depressive state, but not in the remissive state. In addition, we found that there was significant difference in the expression levels of the GDNF, ARTN, and NT-3 mRNA in same patients with major depressive disorder before and after remission, although these two patient groups received similar medications. It is important to note that there was no correlation between expression levels of GDNF, ARTN, or NT-3 mRNA and medications in same patients with major depressive disorder before and after remission. These results suggest that there is no effect of medications on the expression of these three mRNAs, and the observed effects are likely to be due to the disease states.

Several lines of evidence have suggested that glucocorticoid hormone or GR affect the expression levels of GDNF and NT-3 mRNA (Barbany and Persson, 1992; Verity et al., 1998; Baecker et al., 1999; Hansson et al., 2000). It

is important to note that reduced expression of GRα has also been observed in the cerebral cortex, hippocampus, and amygdala in mood disorder patients (Webster et al., 2002; Knable et al., 2004; Perlman et al., 2004). Furthermore, we previously reported that the expression of GRα mRNA is also reduced in peripheral white blood cells of mood disorder patients (Matsubara et al., 2006). These observations raise the possibility that dysfunction of GR in peripheral blood plays a causal role in the aberrant GDNF and NT-3 expression in mood disorder patients. However, present our study indicated that there was no difference in the expression levels of GDNF and NT-3 mRNA between suppressors and non-suppressors of the Dex/CRH test or even in cortisol levels between mood disorders and healthy controls. Furthermore, it is important to mention that some antidepressants alter the expression of GR (Pariante and Miller, 2001). Thus, the involvement of GR in the aberrant transcriptional regulation of certain neurotrophic factors in patients with major depressive disorder remains unclear. Further studies are needed to clarify the aberrant transcriptional mechanisms of both GDNF and NT-3 expression in patients with major depressive disorder.

In conclusion, our results suggest that the changes in the expression levels of GDNF, ARTN, and NT-3 mRNAs in peripheral white blood cells might be state-dependent and associated with the pathophysiology of major depression.

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6. Conflict of interest

There are no conflicts of interest including any financial, personal, or other relationships with people for any of the coauthors related to the work described in the article.

7. Contributors

K. Otsuki, S. Uchida, and Y. Watanabe designated the research. K. Otsuki, T. Watanuki, Y. Wakabayashi, M. Fujimoto, H. Funato, and T. Matsubara performed the experiments. The manuscript was written by K. Otsuki, S. Uchida and Y. Watanabe. All authors discussed results and commented on the manuscript.

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Reduced expression of glyoxalase-1 mRNA in mood disorder patients

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ABSTRACT

Glyoxalase-1 (Glo1) is an antioxidant enzyme which detoxifies α-ketoaldehydes to prevent the accumulation of pro-oxidant compounds, such as methylglyoxal, in all cell types. Glo1 has been suggested to be involved in anxiety disorders, autism, and Alzheimer's disease. Mood disorders have a high rate of comorbidity with anxiety disorders although, to date, little is known of the involvement of Glo1 in the pathophysiology of these conditions. In the present study, we examined the expression levels of Glo1 mRNA in peripheral white blood cells of mood disorder patients to understand the role of Glo1 in mood disorders. Quantitative real-time polymerase chain reaction experiments revealed that reduced expression of Glo1 mRNA was observed in major depressive and bipolar disorder patients in a current depressive state, as compared with healthy control subjects. In contrast, the expression of Glo1 mRNA in major depressive and bipolar patients, in a remissive state, showed no significant alteration when compared with healthy control subjects. These results suggest that the aberrant expression of Glo1 might be involved in the pathophysiology of mood disorders.

A growing body of evidence has implicated a role of chronic or moderate oxidative stress in the pathogenesis of anxiety in humans [2]. Previous clinical investigations have reported an imbalance of antioxidant enzyme activities in patients with social phobia and obsessive-compulsive disorder [17]. Glyoxalase-1 (Glo1) is an antioxidant enzyme that, together with the cofactor glutathione, is involved in the detoxification of α-ketoaldehydes, thereby preventing the accumulation of pro-oxidant compounds such as methylglyoxal [27,28]. The association between altered Glo1 expression levels and anxiety disorders in mice supports the hypothesis that Glo1 is involved in the pathogenesis of these conditions [11,15].

The manifestation of anxiety in a number of psychiatric disorders such as generalized anxiety disorder, depressive disorder, panic disorder, phobia, obsessive-compulsive disorder and post-traumatic stress disorder [6] highlights the importance of gaining a better understanding of common biomarkers for these disorders. The significant association between anxiety and depression in behavioral studies [15] resembles the clinical situation of a high comorbidity between anxiety disorders and major depressive disorder [18]. Although Glo1 has been reported to be associated with anxiety [22], little is known about the involvement of Glo1 in the pathophysiology of mood disorders. To investigate the role of Glo1 in the pathophysiology of mood disorders, we examined the expression levels of Glo1 mRNA in the peripheral white blood cells of major depressive and bipolar disorder patients in a depressive, as well as a remissive, state.

Major depressive and bipolar disorder patients were diagnosed according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) [1]. These included both outpatients and inpatients of the Division of Neuropsychiatry of the Yamaguchi University Hospital. The extent of the depressive state was assessed by a 21-item "Hamilton depression rating scale" (HDRS). Subjects were classified as being under a current depressive state when they showed a score of more than 18 on HDRS and met the DSM-IV criteria for a major depressive episode. Subjects were classified as being in remission when they showed a score of less than six on HDRS and did not show any symptoms of a major depressive episode in the DSM-IV criteria for more than 2 months. Individuals were excluded from the present study if they had abnormal physical examinations or abnormal results for routine medical laboratory tests such as a complete blood count and renal, liver or thyroid function. Female subjects who were pregnant or took oral contraceptives were also excluded. All healthy control subjects were screened to exclude significant current or past medical or neurological illnesses, significant alcohol or drug abuse and past or current axis I psychiatric illnesses. This protocol was approved by the Institutional Review Board of Yamaguchi University Hospital. Informed written consent was obtained for all subjects.

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Blood sample preparation, total RNA isolation and cDNA synthesis were performed as previously described [19]. In brief, blood was obtained by vein puncture between 10:00 a.m. and 11:00 a.m. and total RNA was isolated using the QIAamp RNA blood mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's manual. One microgram of total RNA was used for cDNA synthesis using random hexamer primers and omniscript reverse transcriptase (Qiagen). The cDNA was stored at −80 °C until use. Quantitative real-time polymerase chain reaction (PCR) was performed in an Applied Biosystems 7300 fast real-time PCR system with SYBR green PCR master mix (Applied Biosystems, Foster City, CA), as previously reported [19]. PCR conditions were 15 min at 95 °C, 45 cycles of 15 s at 95 °C and 30 s at 60 °C. Amplification of the single PCR product was confirmed by monitoring the dissociation curve and electrophoresis on 1% agarose gels stained with ethidium bromide. The expression level of GAPDH mRNA was used for normalization and the expression value was normalized by dividing the mean of the value for control subjects. All measurements were performed in duplicate and two-independent experiments were conducted. The following PCR primers were used: Glo1 forward, 5′-CGAGGATTCGGTCATATTGG-3′; Glo1 reverse, 5′-CCAGGCCTTTC-ATTTTACCA-3′; GAPDH forward, 5′-CAGCCTCAAGATCATCAGCA-3′; GAPDH reverse, 5′-TGTGGTCATGAGTCCTTCCA-3′. A subgroup of subjects in a current depressive state underwent the dexamethasone (Dex)/corticotropin-releasing hormone (CRH) test as previously reported [19]. All data are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed with commercial software (SPSS version 16.0; Chicago, IL). Multivariable analysis was conducted using Glo1 mRNA level as a dependent variable and with age, gender, state (depressive and remissive states) and type of drugs used (antidepressants and mood stabilizers) as independent variables. Gender distribution was analyzed by the χ^2-test. The data of Glo1 mRNA levels were subjected to a factorial analysis of variance (ANOVA) followed by *post hoc* comparison (Dunnett test). The Spearman rank correlation was calculated to assess the correlation between data. Two group comparisons, such as suppressors and non-suppressors of the Dex/CRH test on Glo1 mRNA expression, were performed using the Student's *t*-test. In all cases, *p*-values were two-tailed, and comparisons were considered to be statistically significant for $p < 0.05$.

Table 1 shows the demographic and clinical characteristics of the subjects used in this study. The mean ages were not significantly different among major depressive disorder patients, bipolar disorder patients and healthy control subjects ($F_{(2, 104)} = 1.84$, $p = 0.16$). Regarding the gender distribution, bipolar disorder patients showed a significantly larger ratio of females to males

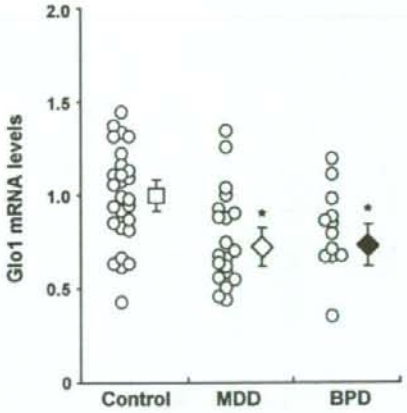

Fig. 1. Expression levels of Glo1 mRNA for mood disorder patients in a current depressive state. Quantitative real-time PCR experiments revealed reduced expression levels of Glo1 mRNA (open circles) for major depressive disorder patients in a current depressive state (MDD, $n = 20$) and bipolar disorder patients (BPD, $n = 13$), as compared to normal control subjects ($n = 28$). Data is represented as means ± S.E.M. (control, open square; MDD, open diamond; BPD, closed diamond). Asterisk represents statistically significant difference at $p < 0.05$.

($\chi^2 = 11.77$, $p = 0.001$). Multivariable analyses demonstrated that the variable "state (depressive and remissive states)" was solely and significantly associated with the expression level of Glo1 mRNA ($p = 0.004$), when analyzed together with the control variables: age, gender, and type of drugs used (antidepressants and mood stabilizers). Quantitative real-time PCR experiments revealed that reduced expression of Glo1 mRNA was observed in major depressive disorder patients ($F_{(2, 58)} = 5.70$, $p < 0.01$) and bipolar disorder patients in a current depressive state ($F_{(2, 58)} = 5.70$, $p < 0.05$), compared with healthy control subjects (Fig. 1). In a remissive state, by contrast, there was no significant difference in the expression levels of Glo1 mRNA in major depressive disorder patients ($F_{(2, 98)} = 0.19$, $p = 0.82$) or bipolar disorder patients ($F_{(2, 98)} = 0.19$, $p = 1.00$), compared with healthy control subjects (Fig. 2). There was a significant correlation between Glo1 mRNA levels and HDRS scores in major depressive disorder patients ($r = -0.358$, $p = 0.005$) (Fig. 3), but not in bipolar disorder patients ($r = -0.198$, $p = 0.187$).

Dysfunction of the hypothalamic–pituitary–adrenal (HPA) system is the most characteristic biological alteration found in the majority of depressed patients. Accumulating evidence suggests

Table 1
Demographic and clinical characteristics of subjects

	Controls	Patients			
		MDD		BPD	
		Depressive	Remissive	Depressive	Remissive
Number of subjects	28	20	40	13	33
Mean age (years)	50.0 ± 1.8	52.3 ± 3.5	57.2 ± 2.2	55.5 ± 3.5	52.7 ± 2.6
Gender (male/female)	15/13	10/10	15/25	2/11	7/26
HDRS		25.9 ± 1.9	3.3 ± 0.2	24.6 ± 1.0	2.8 ± 0.2
Medication					
No medication	28	3	4	1	0
SSRI/SNRI	0	10	38	9	9
TCA/other antidepressants	0	23	28	6	14
Li	0	0	2	4	17
VPA	0	0	0	7	15
CBZ	0	0	0	2	8

MDD, major depressive disorder; BPD, bipolar disorder; HDRS, Hamilton depression rating scale; SSRI, selective serotonin reuptake inhibitor; SNRI, serotonin–noradrenaline reuptake inhibitor; TCA, tricyclic antidepressant; Li, lithium; VPA, valproic acid; CBZ, carbamazepine.

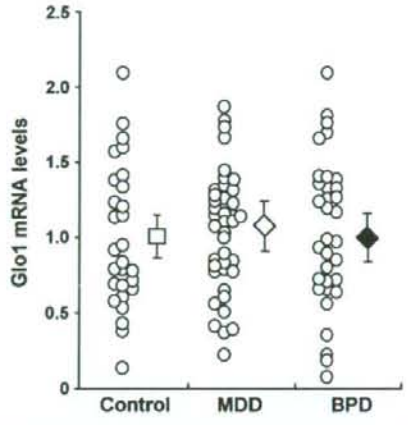

Fig. 2. Expression levels of Glo1 mRNA for mood disorder patients in a remissive state. Quantitative real-time PCR experiments revealed that expression of Glo1 mRNA (open circles) for major depressive disorder patients in a remissive state (MDD, $n = 40$) and bipolar disorder patients (BPD, $n = 33$) were not significantly different to that of normal control subjects ($n = 28$). Data is represented as means ± S.E.M. (control, open square; MDD, open diamond; BPD, closed diamond).

that the combined Dex/CRH test is highly sensitive and is able to detect HPA system abnormalities [10]. ACTH and cortisol responses to this test are exaggerated in depressed patients [7,8]. To examine the association between Glo1 mRNA levels and HPA axis activity, the mRNA levels for Glo1 of mood disorder patients in a current depressive state were compared between suppressors ($n = 11$; 8 major depressive disorder patients and three bipolar disorder patients) and non-suppressors ($n = 15$; 8 major depressive disorder patients and seven bipolar disorder patients) of the Dex/CRH test. There was no significant difference in the expression levels of Glo1 mRNA between suppressors and non-suppressors ($F_{(1,24)} = 3.68$, $p = 0.67$). In addition, there was no significant correlation between Glo1 mRNA levels and the plasma cortisol concentration in healthy control subjects ($r = -0.09$, $p = 0.72$), major depressive disorder patients ($r = 0.42$, $p = 0.27$) or bipolar disorder patients ($r = -0.50$, $p = 0.39$).

Previous reports have suggested the involvement of Glo1 in neuropsychiatric disorders, including anxiety disorders and autism. A significant association of the Glo1 Ala111Glu polymorphism has been observed in a subgroup of patients with panic disorder without agoraphobia [22] and patients with autism [12]. Reduced Glo1 enzyme activity has also been observed in the brains of patients

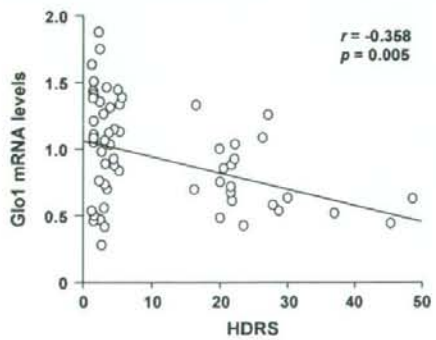

Fig. 3. Significant inverse correlation between HDRS scores and Glo1 mRNA levels was found in the major depressive disorder patients ($n = 60$). HDRS, Hamilton depression rating scale.

with autism [12]. Moreover, a possible association between Glo1 and mood disorders has been found in a linkage study of families with mood disorders [26]. There is a wealth of data demonstrating the comorbidity of mood disorders with anxiety disorders [3,31,24], including panic disorder [5,13,25]. Genetic data with regard to panic disorder and major depressive disorder have been inconsistent, although there is some evidence for a shared diathesis for anxiety and depression [29]. These data suggest an important role for Glo1 in the pathophysiology of many neuropsychiatric disorders, especially with regard to the anxiety symptoms of these conditions.

Krömer et al. [15] have reported an association between reduced Glo1 expression and high anxiety-like behaviors in mice. Importantly, the reduced expression of Glo1 was observed not only in the amygdala, but also in peripheral red blood cells [15], suggesting that the expression levels of Glo1 in the brain is well correlated with that in peripheral blood cells. These data and our present study raise the possibility that the expression levels of Glo1 in mood disorder patients may be reduced in multiple systems. However, a recent study has shown that local overexpression of Glo1 in various brain regions, e.g. cingulate cortex, resulted in increased anxiety-like behavior [11]. This finding is discordant with that of Krömer et al. [15] and thus, it is still unclear how Glo1 is involved in the pathophysiology of anxiety and depression.

A previous report has shown that the number of Glo1 immunopositive neurons and astroglia increase up to, approximately, 55 years of age and decrease progressively thereafter in humans [16]. Glo1 mRNA levels also showed a biphasic course similar to those observed with protein determination [16], suggesting that the expression of Glo1 is primarily regulated at the transcriptional level. The promoter region of the human Glo1 gene contains several consensus sequences for known transcriptional regulatory elements, including: insulin responsive element, metal responsive element and glucocorticoid responsive element [23]. The existence of the glucocorticoid responsive element in the human Glo1 promoter is particularly interesting, because the glucocorticoid receptor (GR) has been shown to be associated with mood disorders and in the adaptation to stress [4,9,20]. Reduced expression of GRα has been observed in the cerebral cortex, hippocampus and amygdala in mood disorder patients [30,14,21]. In addition, we have previously reported that the expression of GRα mRNA is also reduced in the peripheral white blood cells of mood disorder patients [19]. This raises the possibility that dysfunction of GR plays a causal role in the aberrant Glo1 expression observed in mood disorder patients.

Considering our results from multivariable analysis and the significant correlation between Glo1 mRNA levels and HDRS scores in major depressive disorders, it could be interpreted that the reduced expression of Glo1 mRNA is "state-dependent" at least in major depressive disorders. However, our study has the limitation that all the patients were on medication; therefore, we cannot exclude completely the influence of medication on the expression levels of Glo1 mRNA. To our knowledge, however, there is no evidence showing altered levels of Glo1 expression by treatment with antidepressants or mood stabilizers *in vitro* or *in vivo*. Further study conducted in medication-free subjects is needed to elucidate this issue.

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State-dependent changes in the expression levels of NCAM-140 and L1 in the peripheral blood cells of bipolar disorders, but not in the major depressive disorders

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ABSTRACT

Recent postmortem brain and imaging studies provide evidence for disturbances of structural and synaptic plasticity in patients with mood disorders. Several lines of evidence suggest that the cell adhesion molecules (CAMs), neural cell adhesion molecules (NCAM) and L1, play important roles in both structural and synaptic plasticity. Although postmortem brain studies have indicated altered expression levels of NCAM and L1, it is still unclear whether these changes are state- or trait-dependent. In this study, the mRNA levels for various CAMs, including NCAM and L1, were measured using quantitative real-time PCR in peripheral blood cells of major depressive disorder patients, bipolar disorder patients and normal healthy subjects. Reduced expression levels of NCAM-140 mRNA were observed in bipolar disorder patients in a current depressive state. In contrast, L1 mRNA levels were increased in bipolar disorder patients in a current depressive state. NCAM-140 and L1 mRNA levels were not changed in bipolar disorder patients in a remissive state, or in major depressive disorder patients. In addition, there were no significant changes in the expression levels of intercellular adhesion molecule -1, vascular cell adhesion molecule -1, E-cadherin, or integrin αD among healthy controls, major depressive or bipolar disorder patients. Our results suggest that the reciprocal alteration in the expression of NCAM-140 and L1 mRNAs could be state-dependent and associated with the pathophysiology of bipolar disorder.

1. Introduction

Brain imaging studies have indicated altered volumes of the hippocampus, prefrontal cortex or amygdala in patients with major depression, bipolar disorder or schizophrenia (Drevets et al., 1997; Nelson et al., 1998; Sheline et al., 1999; Wright et al., 2000). Furthermore, postmortem brain studies have reported anatomical alterations such as neuronal/glial atrophy and a reduction in the central nervous system of patients with mood disorders (Ongur et al., 1998; Cotter et al., 2001; Drevets, 2003). These data suggest the aberrant alteration of structural and synaptic plasticity in mood disorders. However, the exact pathways and mechanisms for the pathophysiology of mood disorders have not been fully characterized.

Cell adhesion molecules (CAMs) play crucial roles in cell–cell interactions, which form the basis for the organization of cells and tissues. Neural cell adhesion molecule (NCAM) and L1 are members of the immunoglobulin superfamily and play important roles in structural and synaptic plasticity in the brain (Martin and Kandel, 1996; Schachner, 1997; Benson et al., 2000; Sandi, 2004).

NCAM is composed of three major isoforms: NCAM-180, -140 and -120 (Hemperly et al., 1990; Ricard et al., 1999). These molecules are involved in multiple neurobiological functions (Kiss and Muller, 2001; Sandi, 2004). The NCAM-180 and -140 isoforms are transmembrane proteins, whereas the NCAM-120 isoform is an extracellular polypeptide attached to the cell surface via a glycosyl phosphatidyleinositol lipid anchor. The NCAM-180 and -120 isoforms are mainly expressed in the brain, whereas the NCAM-140 isoform is widely expressed in a number of organs (Gennarini et al., 1986; He et al., 1986; Hemperly et al., 1986; Murray et al., 1986; Sadoul et al., 1986). NCAM and L1 have important roles in cell–cell adhesion, intracellular signaling and interactions with growth factor receptors through homophilic (NCAM–NCAM or L1–L1) and heterophilic (NCAM–L1) binding (Sandi, 2004).

Several lines of evidence have indicated that altered expression of NCAM and L1 was observed in patients with mood disorders. Variable alternative spliced exon (VASE)–NCAM, which leads to a down-regulation of synaptic plasticity, was increased in the prefrontal cortex and hippocampus of patients with bipolar disorder (Vawter et al.,

Abbreviations: BPD, bipolar disorder; CAM, cell adhesion molecule; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HDRS, Hamilton depression rating scale; HPA, hypothalamic-pituitary-adrenal; MDD, major depressive disorder; NCAM, neural cell adhesion molecule; PBMCs, peripheral blood monocyte cells.

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1998). In addition, levels of secreted exon (SEC)–NCAM were increased in the hippocampus of patients with bipolar disorder (Vawter et al., 1999). Furthermore, NCAM protein levels were increased in the cerebrospinal fluid (CSF) of patients with major depressive disorder and bipolar disorder (Poltorak et al., 1996). Alterations in the protein levels of L1 in the brains of patients with mood disorders are controversial. The expression of L1 mRNA and protein was reported to be increased in the prefrontal cortex, but decreased in the ventral parieto-occipital cortex, in patients with major depression (Laifenfeld et al., 2005b). However, another study reported a lack of any significant change of L1 protein in the prefrontal cortex of patients with major depressive disorder and bipolar disorder (Webster et al., 1999). Also, it is still unclear whether alterations in the levels of NCAM and L1 in mood disorders are state- or trait-dependent.

Previously, we and others have reported that the reduced expression of glucocorticoid receptor (GR), which plays important roles in the negative feedback of the HPA axis and adaptation to stress (Holsboer, 2000; Pariante and Miller, 2001), was observed in peripheral blood cells and postmortem brains of patients with mood disorders (Webster et al., 2002; Knable et al., 2004; Perlman et al., 2004; Torrey et al., 2005; Matsubara et al., 2006). Aberrant regulation and function of GR are thought to be involved in the pathophysiology of stress-related disorders including mood disorders, anxiety disorder and post-traumatic stress disorder in human, and are also associated with stress-induced alterations of neural plasticity such as shrinkage of neural dendrites and suppressed neurogenesis in rodents (de Kloet et al., 1998; McEwen, 1999; Sapolsky, 2000; Gass et al., 2001). Importantly, the expression levels of NCAM and L1 are considered to be regulated through glucocorticoid-mediated pathways, although the detailed molecular mechanisms of this regulation are still unclear (Grant et al., 1996; Simpson and Morris, 2000; Sandi, 2004). These observations prompt us to speculate the possibility that the mRNA levels of NCAM and L1 would be altered by the reduced expression of GR observed in patients with mood disorder.

In the present study, we assessed the mRNA levels of NCAM, L1 and other CAMs in peripheral white blood cells of depressive patients with mood disorders. Furthermore, to examine whether the altered mRNA levels of CAMs are state- or trait-dependent, we also assessed them in patients in a remissive state.

2. Materials and methods

2.1. Subjects

Major depressive and bipolar disorder patients were diagnosed according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV; American Psychiatric Association, 1994). These included both outpatients and inpatients of the Division of Neuropsychiatry of the Yamaguchi University Hospital. The extent of the depressive state was assessed by a 21-item "Hamilton Depression Rating Scale" (HDRS). Subjects were classified as under a current depressive state when they showed a score of more than 18 on HDRS and met the DSM-IV criteria for a major depressive episode. Subjects were classified as being in remission when they showed a score of less than 6 on HDRS and did not show any symptoms of a major depressive episode in the DSM-IV criteria for more than 2 months. A group of individuals with mood disorder in a current depressive state was assessed every two months for 6 months to investigate alterations in gene expression during recovery from depressive states. Individuals were excluded from the present study if they had abnormal physical examinations or abnormal results for routine medical laboratory tests such as a complete blood count, renal, liver and thyroid function. Female subjects who were pregnant or took oral contraceptives were also excluded. All healthy control subjects were screened to exclude significant current or past medical or neurological illness, significant alcohol or drug abuse and past or current Axis I psychiatric illness. This protocol was approved by the Institutional Review Board of Yamaguchi University Hospital. Informed written consent was obtained for all subjects.

2.2. Blood sample preparation, RNA isolation and cDNA synthesis

Blood sample preparation, total RNA isolation and cDNA synthesis were performed as previously described (Matsubara et al., 2006). In brief, blood was obtained by venipuncture between 10:00 a.m. and 11:00 a.m. and processed for total RNA purification from peripheral blood cells using the QIAamp RNA Blood Mini Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's manual. One microgram of total RNA was used for cDNA synthesis by random hexamer primers and Ominiscript Reverse Transcriptase (Qiagen). Human brain cDNA was commercially obtained

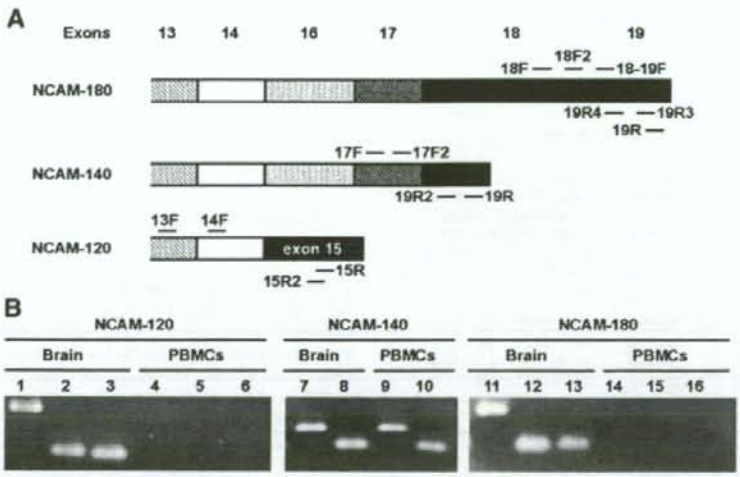

Fig. 1. Schematic diagram of the 3' region of the three NCAM isoforms mRNA (A). The commonly used exons for each isoform are filled with the same patterns. The positions of PCR primers used in RT-PCR are indicated with a black line with the primer name. RT-PCR revealed that the expression of mRNA for the three NCAM isoforms was detected in human brain. In contrast, the expression of NCAM-120 and -180 mRNA was not observed in human PBMCs, although NCAM-140 mRNA expression was detected (B). PBMCs, peripheral blood monocyte cells.

(FirstChoice™ PCR-Ready Human Brain cDNA; Ambion, Foster City, CA, USA). The cDNA was stored at −80°C until further use.

2.3. Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in an Applied Biosystems 7300 Fast Real-Time PCR System with SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's manual. PCR conditions were 15 min at 95°C, 40 to 50 cycles of 15 s at 95°C and 30 s at 60°C. The position and sequences of the PCR primers on the NCAM gene to detect each specific-NCAM isoform are described in Fig. 1A and Table 1, respectively. Primer sequences for other CAMs were: L1 forward, GCCAAAGGAGACAGTGAAGC; L1 reverse, GTCCTGCTTGATGTGCAAGA; ICAM-1 forward, CAAGGCCTCAGTCAGTGTGA; ICAM-1 reverse, CCTCTGGCTTCGTCAGAATC; VCAM-1 forward, AAGATGGTCGTGATCCTTGG; VCAM-1 reverse, GGTGCTGCAAGTCAATGAGA; E-cadherin forward, GAACGCATTGCCACATACAC; E-cadherin reverse, ATTCGGGCTTGTTGTCATTC; ITGAD forward, ATTTGGGGGATACAGGAAGG; ITGAD reverse, AGAAGCAGCTGCAGGAGAAG. The amplification of the single PCR product was confirmed by monitoring the dissociation curve and electrophoresis on 1.5% agarose gels stained with ethidium bromide. Amplification curves were visually inspected to set a suitable baseline range and threshold level. To generate standard curves, various concentrations of cDNA, made from total RNA isolated from human lymphoma-derived Jurkat cells, were used in each PCR reaction. The number of cycles required to reach the threshold fluorescence level was scored and used for generating standard curves and interpolating mRNA concentration levels. The relative quantification method, in which the ratio between the amount of target molecule and a reference molecule within the same sample was calculated, was employed for quantifying target molecules according to the manufacturer's protocol. All measurements were performed, at least, in duplicate. GAPDH mRNA levels were used for normalization. The PCR primers of GAPDH were: forward, CAGCCTCAAGATCATCAGCA; reverse, TGTGGTCATGAGTCCTTCCA. Expression values for mood disorder subjects were normalized by the mean of the value for control subjects.

2.4. Serum cortisol determination

Serum cortisol concentrations were measured via radioimmunoassay by the SRL Corporation (Tokyo, Japan).

Table 1
NCAM PCR primers

Lane	Primer name	Exon	Direction	Sequence (5'–3')
NCAM-120				
1, 4	13F	13	Forward	GGGAACCCAGTGCACCTAAG
	15R	15	Reverse	GAGCAAAAGAAGAGTCAC
2, 5	14F	14	Forward	GAACCAGCAAGGAAAATCCA
	15R	15	Reverse	GAGCAAAAGAAGAGTCAC
3, 6	14F	14	Forward	GAACCAGCAAGGAAAATCCA
	15R2	15	Reverse	GAAGAGTCACTGCAGAGAAAAGC
NCAM-140				
7, 9	17F	17	Forward	GAGTCCAAGGAGCCCATC
	19R	19	Reverse	TCTGTGTGGCGTCATTG
8, 10	17F2	17	Forward	AACCATGATGGAGGGAAACA
	19R2	19	Reverse	GGCTTCGTTTCTGTCTCCTG
NCAM-180				
11, 14	18F	18	Forward	AGATATTGACCTTGCAAAGGATG
	19R	19	Reverse	TCTGTGTGGCGTCATTG
12, 15	18–19F	18–19	Forward	CCAGCAAAGACCGAGAAG
	19R3	19	Reverse	ATTGGGGACCGTCTTGACTT
13, 16	18F2	18	Forward	CTGAGCTTGCTCCTTCCACT
	19R4	19	Reverse	TTCTGTCTCCTGGCACTCTG

The positions of the primers are shown in Fig. 1.

Table 2
Demographic and clinical characteristics of subjects

Control		Patients			
		MDD		BPD	
		Depressed	Remissive	Depressed	Remissive
	$n=28$	$n=20$	$n=40$	$n=13$	$n=29$
Age (years)	50.0±1.8	52.3±3.6	57.1±2.2	55.5±3.7	53.8±2.7
Gender (male/female)	15/13	10/10	15/25	2/11	6/23
HDRS		25.9±1.9	3.3±0.2	24.6±1.1	2.8±0.2
Serum cortisol (μg/dl)	8.5±0.9	10.3±1.4	11.1±1.0	10.9±5.5	10.1±1.1
Dex-CRH test					
Suppressor		8	7	3	2
Nonsuppressor		8	3	7	5
Medication					
No medication	28	3	4	1	0
Antidepressants	0	17	36	9	14
Mood stabilizers	0	0	0	10	27

MDD, major depressive disorder; BPD, bipolar disorder; HDRS, Hamilton Depression Rating Scale.

2.5. Dex/CRH test

A subgroup of subjects in a current depressive state underwent the Dex/CRH test as previously reported, with minor modifications (Matsubara et al., 2006; Heuser et al., 1996). Mood disorder patients were pretreated with an oral dose of 1 mg of Dex (Dexamethasone, Asahikasei Pharmaceutical Corporation, Tokyo, Japan) at 11:00 p.m. The next day intravenous cannulation was carried out at 12:30 p.m. and 100 μg of human CRH (hCRH, Mitsubishi Pharma Corporation, Tokyo, Japan) was administered intravenously at 1:00 P.M., immediately after the first blood collection. Blood specimens were drawn through the intravenous catheter 15 min, 30 min, 60 min and 120 min later. Blood samples were immediately centrifuged and stored at −20°C. Serum levels of cortisol and ACTH were measured with radioimmunoassay (SRL). We defined non-suppressors as those individuals whose post-CRH serum cortisol levels were more than 5 μg/dl.

2.6. Statistical analysis

Commercial software (SPSS version 11.5; Chicago, Illinois) was used to perform data analysis. All data are expressed as the mean± standard error of mean (SEM). For analysis of two groups, data were subjected to the Student's *t* test. For analysis of three or more groups, data were subjected to one-way analysis of variance (ANOVA) followed by *post hoc* analysis (Dunnett test). The Spearman correlation was calculated to assess the correlation between data. In all cases, comparisons were considered statistically significant for $p<0.05$.

3. Results

3.1. The mRNA expression pattern of three NCAM isoforms in peripheral blood cells

The three major isoforms of NCAM, generated by alternative splicing from a single NCAM pre-mRNA, are expressed in the brain (Hemperly et al., 1990; Ricard et al., 1999). However, the mRNA expression pattern of these NCAM isoforms in peripheral white blood cells is not known. Therefore, we examined the expression of the mRNA for these NCAM isoforms in peripheral white blood cells using reverse transcription-PCR (RT-PCR). The locations and sequences of the PCR primer sets are described in Fig. 1A and Table 1, respectively. To detect the expression of specific-NCAM isoform mRNA, multiple PCR primers were constructed in the positions of isoform-specific exons or splice junctions (Fig. 1A). In addition, human brain cDNA was used as a positive control. RT-PCR showed that the expression of NCAM-180, -140 and -120 isoform mRNAs were detected in human

brain, while only NCAM-140 isoform mRNA was detected in human peripheral white blood cells (Fig. 1B).

3.2. Demographic and clinical characteristics of subjects

Table 2 shows the demographic and clinical characteristics of the subjects used in this study. The mean ages were not significantly different among major depressive disorder patients, bipolar depressive patients and healthy control subjects ($F=1.228$, $df=4,125$, $p=0.303$). Levels of serum cortisol were not significantly different among the three groups ($F=0.833$, $df=4,55$, $p=0.510$). Table 3 shows the summarized profile of medication for longitudinally followed subjects with bipolar disorder.

3.3. The expression levels of NCAM-140 and L1 mRNA in mood disorder patients

The expression levels of NCAM-140 mRNA were examined in patients with mood disorder in a current depressive state. qRT-PCR revealed that the expression levels of NCAM-140 mRNA were significantly decreased in bipolar disorder patients compared with healthy control subjects and major depressive disorder patients ($F=5.22$, $df=2,58$, $p=0.008$, post hoc $p=0.008$) (Fig. 2A). There were no significant differences in the expression levels of NCAM-140 mRNA between major depressive disorder patients and healthy control subjects ($p=1.00$) (Fig. 2A). In contrast, in a remissive state, bipolar disorder patients did not show any differences in the expression levels of NCAM-140 mRNA compared with healthy control subjects and major depressive disorder patients. ($F=0.724$, $df=2,94$, $p=0.590$) (Fig. 2A). There was no significant correlation between NCAM-140 mRNA levels and serum cortisol concentrations in healthy control subjects, major depressive disorder patients and bipolar disorder patients in a current depressive state (data not shown).

In contrast to NCAM-140, the expression levels of L1 mRNA were significantly increased in bipolar disorder patients in a current depressive state compared with healthy control subjects and major depressive disorder patients in a current depressive state ($F=8.307$, $df=2,58$, $p=0.001$, post hoc $p=0.001$) (Fig. 2B). There were no significant differences in the expression levels of L1 mRNA between major depressive disorder patients in a current depressive state and healthy control subjects ($p=0.923$) (Fig. 2B). The expression levels of L1 mRNA in bipolar disorder patients in a remissive state showed no significant difference compared with healthy control subjects and major depressive disorder patients in a remissive state ($F=0.783$, $df=2,94$, $p=0.460$) (Fig. 2B). There was no significant correlation between L1 mRNA levels and serum cortisol concentrations of healthy control subjects, major depressive disorder patients and bipolar disorder patients in a current depressive state (data not shown).

Table 3
Summarized profile of medication for longitudinally followed subjects with bipolar disorder

Mood stabilizers		Treatment period (Months)			
		0	2	4	6
Antidepressants alone	–	2	4	3	2
Antidepressants+	Li	2	2	2	2
mood stabilizers	VPA	3		1	
	Li+VPA	2	1		
	VPA+CBZ		1		
Mood stabilizers alone	VPA	1	2	1	1
	CBZ	1			
	Li+VPA				1
	Li+CBZ			1	1
	VPA+CBZ	1		1	1
No medication	–	1			
Total number		13	10	9	8

Li, lithium carbonate; VPA, sodium valproate; CBZ, carbamazepine.

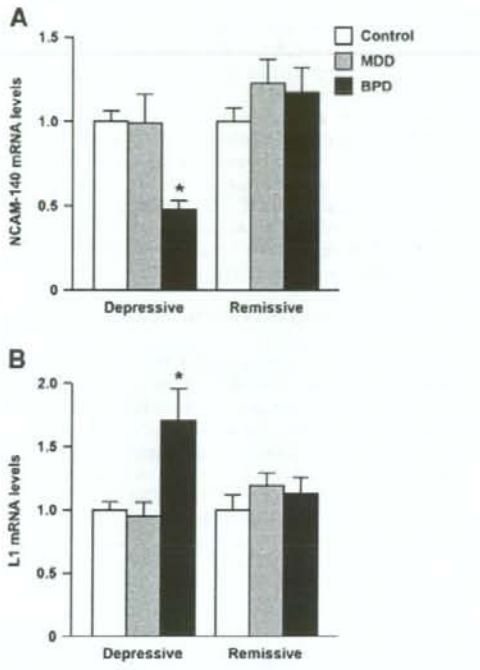

Fig. 2. The expression levels of NCAM-140 and L1 mRNA in mood disorder patients. Reduced expression levels of NCAM-140 mRNA were shown in bipolar disorder patients in a current depressive state, but not in bipolar disorder patients in a remissive state, major depressive patients in current depressive state and major depressive patients in a remissive state compared with healthy control subjects (A). Increased expression levels of L1 mRNA were shown in bipolar disorder patients in a current depressive state, but not in bipolar disorder patients in a remissive state, major depressive patients in a current depressive state and major depressive patients in a remissive state compared with healthy control subjects (B). MDD, major depressive disorder; BPD, bipolar disorder. Values are mean ± standard error. *$p<0.01$.

To further investigate whether NCAM-140 and L1 mRNA levels vary during recovery from a depressive state, bipolar disorder patients in a current depressive state were assessed for NCAM-140 and L1 mRNA expression every 2 months for 6 months (0 M, 2 M, 4 M and 6 M indicate 0, 2, 4 and 6 months recovery period, respectively). The decreased expression levels of NCAM-140 mRNA were observed in 0 M compared with healthy controls ($F=4.518$, $df=4,63$, $p=0.003$, post hoc $p=0.001$), but not in 2 M, 4 M and 6 M (2 M, $p=0.385$; 4 M, $p=0.070$; 6 M, $p=0.553$) (Fig. 3A). Similar to NCAM-140, the increased expression levels of L1 mRNA compared with healthy controls were observed in only 0 M ($F=3.387$, $df=4,63$, $p=0.014$, post hoc $p=0.008$), but not in 2 M, 4 M and 6 M (2 M, $p=1.00$; 4 M, $p=0.944$; 6 M, $p=0.146$) (Fig. 3B). The Hamilton Depression Rating Scale (HDRS) score was significantly reduced at 2 months in bipolar disorder patients ($F=19.6$, $df=3,35$, $p<0.01$) (Fig. 3C). Thus, the reduced expression of NCAM-140 and the increased expression of L1 mRNAs would be state-dependent in bipolar disorder patients.

3.4. Relationship between CAMs (NCAM-140, L1) and the HPA axis

Next, we examined whether the altered expression levels of NCAM-140 and L1 mRNAs are correlated with GRα mRNA levels or the HDRS score. There was no significant correlation between GRα mRNA levels and NCAM-140 or L1 mRNA levels in bipolar disorder patients in a current depressive state ($r=-0.198$, $p=0.517$, $r=-0.052$, $p=0.865$, respectively) (data not shown). There was a significant correlation

between HDRS scores and NCAM-140 mRNA levels in patients with bipolar disorders ($r=-0.411$, $p=0.008$), whereas no significant correlation was observed between HDRS scores and L1 mRNA levels ($r=-0.042$, $p=0.797$) (data not shown).

To examine the association between NCAM-140 or L1 mRNA levels and HPA axis regulation, NCAM-140 and L1 mRNA levels of patients with major depressive disorder and bipolar disorder in a current depressive state were compared between suppressors and non-suppressors of the Dex/CRH test. Non-suppressors showed a significant decrease of NCAM-140 mRNA expression, but not L1 mRNA, compared with suppressors ($t=2.346$, $df=24$, $p=0.028$, $t=-0.531$, $df=24$, $p=0.600$, respectively) (Fig. 4).

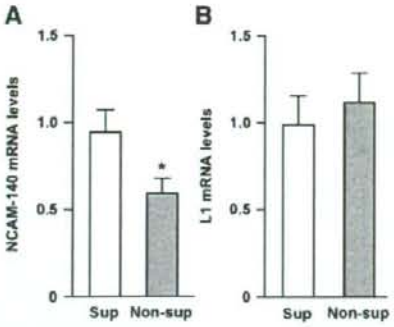

Fig. 4. Comparison of the expression levels of NCAM-140 and L1 mRNA between suppressors and non-suppressors of Dex/CRH test. There was a significant difference in NCAM-140 mRNA expression of mood disorder patients, but not for L1. Sup, suppressors; Non-Sup, non-suppressors. Values are mean ± standard error. *$p<0.05$.

3.5. The expression levels of other CAMs mRNA in mood disorder patients

Previous reports have indicated the increased expression of intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) in post mortem brains of bipolar disorder patients or major depression in the elderly patients (Thomas et al., 2002, 2004). In addition, cadherin and integrin are also well known to be associated with structural plasticity in the brain. Therefore, we also examined the expression levels of the mRNA for these CAMs in mood disorder patients. There were no significant differences in the expression levels of ICAM-1 ($F=0.338$, $df=2,58$, $p=0.715$), VCAM-1 ($F=0.760$, $df=2,58$, $p=0.472$), E-cadherin ($F=1.005$, $df=2,58$, $p=0.372$) or integrin α D ($F=0.751$, $df=2,58$, $p=0.477$) between the mood disorder patients in a current depressive state and healthy controls (data not shown).

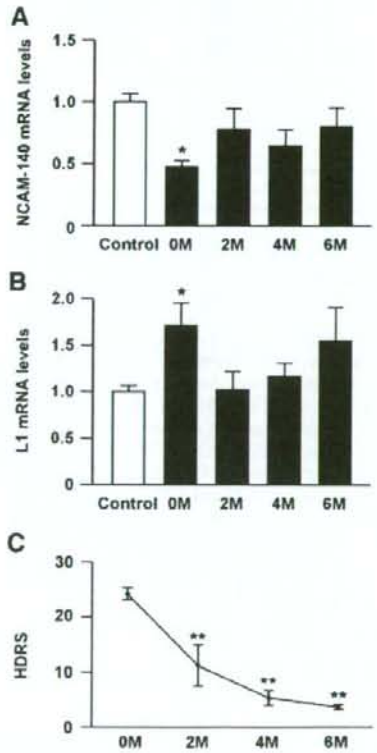

Fig. 3. The expression levels of NCAM-140 and L1 mRNA in bipolar disorder patients during recovery from a depressive state. Reduced expression levels of NCAM-140 mRNA were not observed at 2, 4 and 6 months in bipolar disorder patients (A). Similarly, increased expression levels of L1 mRNA were not observed at 2, 4 and 6 months in bipolar disorder patients (B). HDRS value of bipolar disorder patients was significantly reduced at 2, 4 and 6 months compared with 0 month (C). HDRS, Hamilton Depressive Rating Scale; 0 M, 2 M, 4 M and 6 M, 0, 2, 4 and 6 months recovery period, respectively. Values mean ± standard error. *$p<0.05$; **$p<0.01$.

4. Discussion

In the present study, we examined the mRNA expression levels of structural and synaptic plasticity-associated CAMs in mood disorder patients. Our main finding is the significant alterations in the expression levels of NCAM-140 and L1 mRNAs in bipolar disorder patients. Even in this group of subjects, the decreased expression levels of NCAM-140 mRNA and increased expression levels of L1 mRNA were specifically observed in a depressive state. These results suggest that the reciprocal alteration of NCAM-140 and L1 mRNA expression in peripheral white blood cells is state-dependent in bipolar disorders. Moreover, a significant correlation between HDRS scores and NCAM-140 mRNA levels in bipolar disorders supports this idea.

In contrast, any significant difference was not observed in the expression levels of NCAM-140 or L1 mRNA of major depressive disorder patients compared with healthy control subjects. Recent studies have demonstrated the pathophysiologic distinctions between bipolar and major depression (Soares and Mann, 1997; Vawter et al., 2000; Beyer and Krishnan, 2002). For instance, increased expression of VASE–NCAM and SEC–NCAM isoforms were shown in postmortem brains of bipolar disorder patients (Vawter et al., 1998, 1999; Vawter, 2000). Furthermore, a genetic study has indicated that a single nucleotide polymorphism of the NCAM gene displays a significant association with bipolar disorder but not with major depressive disorder in Japanese individuals (Arai et al., 2004). Thus, these results support the idea that NCAM is more relevant to the pathophysiology of bipolar disorder than that of major depressive disorder.

We have previously shown a reduced expression of GRα mRNA in mood disorder patients in both a depressive and a remissive state (Matsubara et al., 2006). In this study, the expression levels of NCAM and L1 mRNA was not changed in a remissive state. In addition, we did not find any significant correlation in the expression levels between GRα mRNA and NCAM or L1 mRNA in mood disorder patients in a current depressive state. These data suggest that the alteration of NCAM-140 and L1 mRNA expression in bipolar disorder patients is not likely to be due to aberrant regulation of GR, although previous reports have suggested that NCAM expression is regulated by a GR-mediated pathway (Sandi, 2004). It has been reported that transforming growth factor-β (TGF-β) (Roubin et al., 1990) and neural restrictive silencer factor (NRSF; also known as REST) (Schoenherr and Anderson,

1995; Schoenherr et al., 1996; Kallunki et al., 1997) modulate expression of NCAM and L1 mRNAs, respectively. Thus, the observed alteration of NCAM and L1 mRNA expression in our bipolar disorder patients may due to the aberrant regulation of other transcriptional mechanisms such as TGF-β and NRSF. Further studies are needed to elucidate the molecular mechanisms responsible for the altered expression of these genes in bipolar disorder patients.

Another important finding of our study are the significant differences in the expression levels of NCAM-140 mRNA between suppressors and non-suppressors of Dex/CRH test in mood disorder patients. Non-suppression of the Dex/CRH test is thought to be indicative of a blunted response to increased glucocorticoid via GR (Holsboer, 2000; Pariante and Miller, 2001). Our previous report indicated the lack of a significant difference in the expression levels of GRα or GRβ mRNAs between suppressors and non-suppressors of Dex/CRH test in mood disorder patients (Matsubara et al., 2006). Accordingly, these findings also suggest that the decreased expression of NCAM-140 mRNA in non-suppressors of mood disorder patients might be induced by a GR-independent mechanism. It is known that many neurotransmitters and hormones such as arginine vasopressin, gamma-aminobutyric acid and glutamate are also involved in the regulation of the HPA axis. Further studies should examine whether NCAM-140 mRNA reduction is associated with an aberrant regulation of the HPA axis in mood disorder patients.

Recent report has been suggested that the problems in information processing within neural networks might underlie depression (Castren, 2005). It should be noted that NCAM and L1 are thought to be important players on neural connectivity and structural remodeling related to stress and mood disorders (Sandi, 2004; Sandi and Bisaz, 2007). Actually, animal studies have suggested roles for NCAM and L1 in the pathophysiology of mood disorders through a dysregulation of neuronal structural plasticity (Tomasiewicz et al., 1993; Cremer et al., 1994; Dahme et al., 1997; Fransen et al., 1998). NCAM knock-out (KO) mice showed deficits in learning and memory, impaired hippocampal long-term potentiation (Cremer et al., 1994; Bukalo et al., 2004), reduced exploratory activity (Stork et al., 1997) and increased anxiety behavior (Stork et al., 1999). Conversely, conditional L1 KO mice showed increased basal synaptic activity in CA1 of the hippocampus and decreased anxiety behavior (Law et al., 2003). Thus, NCAM and L1 may have opposite roles in the regulation of synaptic plasticity and emotional behavior. In addition, we previously reported that chronically stressed rats showed a significant shortening and debranching of apical dendrites in CA3 pyramidal neurons of the hippocampus (Watanabe et al., 1992). Interestingly, chronically stressed rats showed a specific reduction of NCAM-140 mRNA and protein levels without any change in NCAM-180 mRNA levels and increased expression of L1 mRNA and protein in the hippocampus (Sandi et al., 2001; Touyarot and Sandi, 2002; Venero et al., 2002). Although these results were observed in the rodent brain, the altered expression of NCAM-140 and L1 mRNA were similar to our data in peripheral white blood cells of bipolar disorder patients in a depressive state.

It is not known what effects the altered expression of NCAM and L1 has in the peripheral tissues of bipolar disorder patients. NCAM-140 is also known as CD56 in immune system, and natural killer (NK) cells are characterized by their expression of CD56 and/or CD16 antigen. Several reports have indicated decreased numbers of $CD56^+$ NK cells or lower NK activity in peripheral blood of major depressive disorder patients (Seidel et al., 1996; Pike and Irwin, 2006), suggesting the altered function of the immune system in a depressed state of bipolar disorders. At the molecular level, it has been shown that glial cell line-derived neurotrophic factor (GDNF) binds directly to NCAM-140 with high affinity (Paratcha et al., 2003). Furthermore, decreased levels of whole blood GDNF in bipolar disorder patients was reported (Takebayashi et al., 2006), suggesting a possible involvement of a dysregulation of GDNF–NCAM pathway in bipolar disorders. In addition, increased levels of oxidative DNA damage and apoptosis have been observed in blood lymphocytes of depressive patients (Forlenza and Miller, 2006; Ivanova et al., 2007). It has been reported that GDNF may contribute to reduce oxidative stress-induced cell death (Burke et al., 1998). Moreover, a protective role against oxidative stress-related cell damage has been proposed for NCAM-140 (Feng et al., 2002).

A limitation of our study is that most patients were on medication, thus we could not exclude the influence of medication on the expression levels of CAMs. A previous report indicated that treatment with valproic acid increased the expression levels of NCAM mRNA and protein in vitro (Lampen et al., 2005), whereas the treatment of mood disorder patients with medication did not change the concentrations of NCAM in CSF (Poltorak et al., 1996) and the expression levels of NCAM were not affected in lithium-treated rats (Plenge et al., 1992). On the other hand, the expression of L1 mRNA in the hippocampus of rats was increased by chronic administration of antidepressants (Laifenfeld et al., 2005a). In addition, postmortem study of major depression indicated that the protein levels of L1 in unmedicated subjects were lower than in medicated subjects (Laifenfeld et al., 2005b). These findings were not consistent with our study and further study conducted with medication-free subjects is needed.

5. Conclusion

Our results suggest that the reciprocal alteration in the expressions of NCAM-140 and L1 mRNA could be state-dependent and more relevant to the pathophysiology of bipolar disorder rather than that of major depressive disorder.

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