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Cerebrospinal fluid BDNF pro-peptide levels in major depressive disorder and schizophrenia

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ABSTRACT

The role of brain-derived neurotrophic factor (BDNF) and its related molecules has been extensively studied in the context of psychiatric disorders. In the present study, we focused on the newly identified BDNF pro-peptide, which is generated together with mature BDNF by proteolytic processing of their precursor, proBDNF. Here, we report, for the first time, that BDNF pro-peptide is present in human cerebrospinal fluid (CSF) and quantifiable by western blotting. We measured CSF BDNF pro-peptide levels in 27 patients with schizophrenia, 18 patients with major depressive disorder (MDD), and 27 healthy controls matched for age, sex, and ethnicity (Japanese). The ratio of the BDNF pro-peptide level to the total protein level in MDD patients was significantly lower than that in controls (Kruskal-Wallis with Dunn's multiple comparisons test; $p = 0.046$). When men and women were examined separately, males with MDD had a significantly lower BDNF pro-peptide/protein ratio than male controls ($p = 0.047$); this difference was not found in female subjects. The ratio tended to be lower in male schizophrenia patients ($p = 0.10$). Although we tried to measure the levels of mature BDNF in CSF, they were below the limit of detection of the ELISA and multiple analyte profiling technology. Taken together, the results suggest that reduced CSF BDNF pro-peptide levels are associated with MDD, particularly in males. Further studies involving a larger sample size are warranted.

1. Introduction

Brain-derived neurotrophic factor (BDNF) is a widely distributed neurotrophic factor in the brain. It has multiple biological functions, including the promotion of neuronal survival and differentiation, modulation of synaptic transmission, and plasticity (Bibel and Barde, 2000; Huang and Reichardt, 2003; Park and Poo, 2013). Since proteolytic cleavage of precursor BDNF (proBDNF) leads to the production of mature BDNF (mBDNF) and BDNF pro-peptide, BDNF pro-peptide is a by-product that is generated by the proteolytic processing of proBDNF. Altered BDNF functions caused by reduced mBDNF expression as well as biological actions of proBDNF and its pro-peptide may be implicated in the pathophysiology of major depressive disorder (MDD) and schizophrenia (Castren and Kojima, 2017).

MDD is a widespread psychiatric illness whose core symptoms include depressed mood, anhedonia, difficulties in concentrating, and abnormalities in appetite and sleep. MDD is a chronic, recurring illness that affects up to 20% of the world's population (Ferrari et al., 2013). Although the pathophysiology of depressive disorder remained elusive, Nibuya et al. (1996) demonstrated that chronic (but not acute) antidepressant treatments increase mBDNF levels in the rat hippocampus (Nibuya et al., 1996).

Since this report, how mBDNF levels change in patients with psychiatric disorders in particular MDD has been explored extensively. Studies on post-mortem brains and blood levels of mBDNF in depressed patients have suggested the important role of mBDNF in depression (reviewed in Duman and Monteggia (2006)). In post-mortem studies of suicide victims with depression, mBDNF expression has consistently been reduced in the hippocampus (Duman and Monteggia, 2006; Dwivedi et al., 2003). Similar reduction was shown in prefrontal cortex

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as well (Karege et al., 2005). Furthermore, in the hippocampus of suicide victims with antidepressants, mBDNF expression was unchanged or even increased (Chen et al., 2001; Karege et al., 2005), which suggests that antidepressants increase the level of mBDNF. Furthermore, since chronic stress plays a causal role in the onset of MDD, the interplay between chronic stress and reduced mBDNF could be important (reviewed by Kunugi et al. (2010)).

As regards blood mBDNF levels, drug naive patients with depression often showed decreased mBDNF, while they were increased in patients treated with antidepressants (Shimizu et al., 2003a). Meta-analytic studies confirmed such findings (Sen et al., 2008; Brunoni et al., 2008). Furthermore, human and rodent studies show a significant correlation between changes in mBDNF levels after antidepressant medication and changes in depression scores (Brunoni et al., 2008; Autry and Monteggia, 2012).

Although the blood mBDNF level may be useful as a biomarker for depression, mBDNF is abundant in platelets and other peripheral tissues. It is still unclear how the blood mBDNF level reflects that of the brain. Accumulating evidence suggests that CSF is mainly produced by the brain parenchyma, and it directly reflects brain activity (Slavik and Dolezal, 2012). In Alzheimer's disease, for example, the level of phosphorylated tau in the CSF is a diagnostic marker that reflects the intensity of neurodegeneration and the severity of acute neuronal damage in the brain (Blennow and Zetterberg, 2018). Likewise, CSF BDNF pro-peptide levels may reflect impaired BDNF function in the brains of patients with MDD.

Schizophrenia is a serious mental illness that affects approximately 1% of the population worldwide, and is characterized by distortion of thinking and perception, cognitive impairments, difficulty in communication, and restricted affective expression. Post-mortem studies have revealed that mRNA levels of mBDNF and its receptor TrkB are decreased in the prefrontal cortex of patients with schizophrenia (Weickert et al., 2003, 2005). Serum, plasma, and CSF mBDNF levels are also decreased in patients with schizophrenia (Grillo et al., 2007; Pirildar et al., 2004; Tan et al., 2005; Toyooka et al., 2002). One report demonstrated a positive correlation between plasma and CSF mBDNF levels in drug-naive first-episode psychotic subjects (Pillai et al., 2010), which indicates that plasma mBDNF levels may reflect the changes in brain mBDNF levels in schizophrenia. However, several other reports found no significant association between serum mBDNF and schizophrenia (Huang and Lee, 2006; Jockers-Scherubl et al., 2004; Shimizu et al., 2003b). One possible reason for this conflicting evidence is that mBDNF levels in CSF are very low; thus, CSF mBDNF levels are unlikely to be a biomarker for psychiatric disorders (Carlino et al., 2011).

In the present study, we hypothesized that BDNF pro-peptide, a by-product of proBDNF proteolytic processing (Fig. 1a) might be an alternative biomarker to the mature form. In a recent study, we demonstrated that BDNF pro-peptide is a facilitator of long-term depression (LTD) in hippocampal slices (Mizui et al., 2015). Prior to this, studies revealed that BDNF pro-peptide is endogenously detectable (Dieni et al., 2012) and released in a neuronal activity-dependent manner (Anastasia et al., 2013; Mizui et al., 2015). Recently, using western blotting, it was shown that protein levels of BDNF pro-peptide are significantly lower in the post-mortem cerebellum of patients with MDD and schizophrenia than in healthy controls, while BDNF pro-peptide levels are increased in the parietal cortex (Yang et al., 2016). Given these reports together, BDNF pro-peptide may play a pathophysiological role in MDD and schizophrenia and could be a biomarker of these psychiatric disorders.

In the present study, we investigated whether 1) BDNF pro-peptide is present in CSF, and 2) CSF BDNF pro-peptide levels are different in patients with MDD and those with schizophrenia compared with healthy controls. We demonstrate that BDNF pro-peptide was present in human CSF. The ratio of the BDNF pro-peptide level to the total protein level was significantly lower in males, but not in females, and significantly lower in patients with MDD than in same-sex controls. These results suggest that decreased CSF BDNF pro-peptide is associated with MDD in male patients.

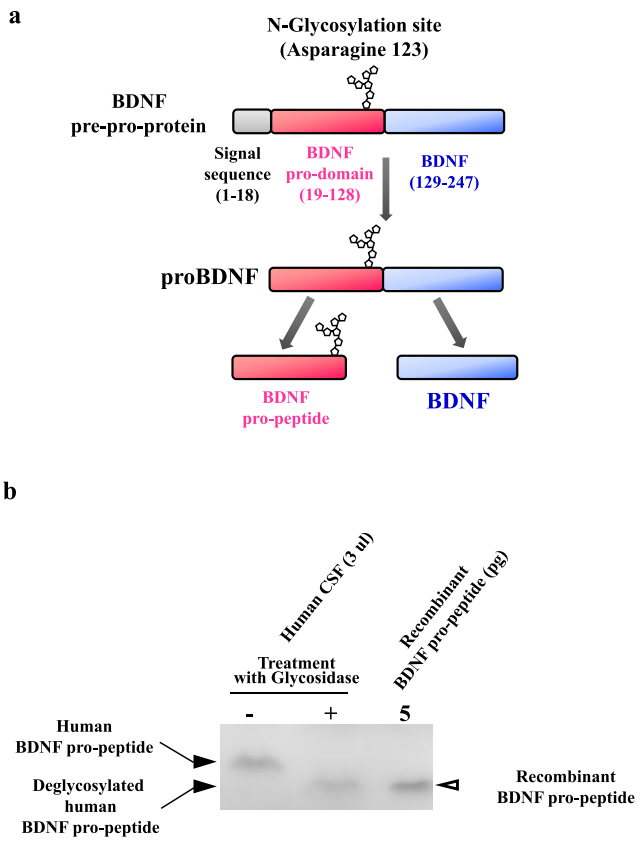

Fig. 1. BDNF pro-peptide is present in CSF. (a) Processing of pre-proBDNF leads to the generation of BDNF pro-peptide and BDNF. The different domains of pre-pro BDNF are shown (pre-pro-protein: 249 aa; signal sequence: 18 aa; pro-domain: 112 aa; BDNF: 119 aa). The intracellular protein convertases (PCs) furin and plasmin, as well as extracellular tPA, cleave proBDNF at position 130, and a putative n-glycosylation site located at position 123 (Lessmann and Brigadski, 2009). Recently, it was demonstrated that, similar to BDNF, BDNF pro-peptide is endogenously present and secreted by neurons (Anastasia et al., 2013; Dieni et al., 2012; Mizui et al., 2015). **(b) Detection of BDNF pro-peptide in human CSF.** The immunoreactive band corresponding to the BDNF pro-peptide is shown by the arrow. Treatment of human CSF samples with *N*-glycosidase F reduced the molecular weight to that of recombinant BDNF pro-peptide (middle and right lanes).

2. Materials and methods

2.1. Participants

Subjects were 27 patients with schizophrenia, 18 patients with MDD, and 27 healthy controls. The mean age and sex ratio were matched across the three diagnostic groups. Majority of the patients were on antipsychotic or antidepressant treatment. Antipsychotic and antidepressant doses were converted to chlorpromazine and imipramine (IMI) equivalent doses, respectively, according to the published guidelines (Inagaki et al., 2013). All subjects were Japanese and biologically unrelated. Patients were recruited from the National Center Hospital, National Center of Neurology and Psychiatry (NCNP), Kodaira, Tokyo, Japan, and control subjects were recruited through advertisements in free local information magazines and by our website announcement. The study protocol was approved by the ethics committee of the NCNP and AIST, and the study was conducted according to the Declaration of Helsinki (World Medical Association, 2000). After

an explanation of the study and its aims, all participants provided signed informed consent.

2.2. Clinical assessments

All participants underwent a structured interview using the Japanese version of the Mini-International Neuropsychiatric Interview (M.I.N.I.) (Amorim et al., 1998; Otsubo et al., 2005), which was administered by a trained psychologist or a research psychiatrist. Consensus diagnosis by at least two psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition criteria (American Psychiatric Association, 1994), on the basis of the M.I.N.I. results, additional unstructured interviews, and medical records if available. The control subjects were healthy volunteers with no current or past history of psychiatric treatment, who were screened using the M.I.N.I. to rule out any axis I psychiatric disorders.

Participants were excluded if they had prior medical histories of central nervous system disease or severe head injury, if they met the criteria for substance abuse or dependence, or mental retardation, if they were currently taking anti-inflammatory medication, or if they suffered from any inflammatory, infectious, or systemic immune diseases, based on self-reports, at the time of assessment.

Depression severity was assessed using the Japanese version of the 17-item Hamilton Depression Rating Scale (HAMD-17) (Hamilton, 1960), and the cut-off score for remission was set at ≤7 (Zimmerman et al., 2013). Remitted patients with MDD were not enrolled in the study. Schizophrenia symptoms were assessed using the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987).

2.3. CSF sample collection

Sample collection, storage, and distribution were supported by the NCNP biobank (a member of the National Center Biobank Network: NCBN Project). CSF samples were obtained by lumbar puncture, as described previously (Hattori et al., 2015). The procedures were performed between 10:00 and 16:00. After neurologic examinations, each participant received local anesthesia followed by a lumbar puncture at L3-4 or L4-5 using an atraumatic pencil point needle (Uniever 22G, 75 mm, Unisis Corp, Tokyo, Japan). The initial 2 mL of CSF was used for laboratory tests, including those to determine the number of cells, total protein levels, and glucose levels. Then, 8–10 mL of CSF was collected in a low protein adsorption tube (PROTEOSAVE SS 15 mL Conicaltube, Sumitomo Bakelite Co., Japan) and immediately chilled on ice. The CSF was centrifuged (4000 g × 10 min, 4 °C), and the supernatant was dispensed into 0.5 mL aliquots in low protein adsorption tubes (PROTEOSAVE SS 1.5 mL Slimtube, Sumitomo Bakelite Co.) and stored in a deep freezer (−80 °C). Selected samples were thawed and dispensed in low protein adsorption tubes (PROTEOSAVE SS 0.5 mL Slimtube, Sumitomo Bakelite Co.).

2.4. Deglycosylation of BDNF pro-peptide

The deglycosylation study of BDNF pro-peptide was performed according to the manufacturer's protocols (New England Biolabs, Ipswich, MA, USA). Briefly, human CSF samples (10 μL) were solubilized with denaturing buffer and heat-denatured at 100 °C for 10 min. The samples were then incubated with 1 μL of *N*-glycosidase F (NEB) at 37 °C for 1 h, and analyzed by immunoblotting as described below.

2.5. Western blotting

Western blot analysis of BDNF pro-peptide was performed according to the report of Mizui et al. (2015). Briefly, the protein concentration in human CSF was determined using a BCA assay kit (Thermo Scientific, Waltham, MA, USA). CSF samples were mixed with an equal volume of SDS sample buffer (0.125 M Tris–HCl, pH 6.8, 20% (wt/vol) glycerol, 4% (wt/vol) SDS, and 10% vol/vol) 2-mercaptoethanol), and then heat-denatured at 100 °C for 3 min. In this study, the polyacrylamide gels (stacking gel, 5% acrylamide; running gel, 20%) were prepared using the Mini-PROTEAN Tetra hand cast system (BioRad, Hercules, CA, USA). CSF samples (3 μL) were loaded onto the stacking gel and electrophoresed at 20 mA/gel. The electrophoresis was stopped when the lower-most protein marker almost reached the foot line of the glass plate. Separated proteins were transferred to 0.45 μm Immobilon-P membranes (Millipore, Billerica, MA, USA) and immunoblotted with a mouse monoclonal antibody specific for the pro-domain of human BDNF (mAb287, GeneCopoeia, Rockville, MD, USA).

To detect the endogenous BDNF pro-peptide, membranes were fixed with 2.5% (vol/vol) glutaraldehyde (electron microscopy grade; Kishida Chemical Co., Osaka, Japan) for 30 min as described previously (Karey and Sirbasku, 1989; Dieni et al., 2012; Mizui et al., 2015). Karey and Sirbasku reported that pre-treatment with glutaraldehyde prior to blocking increased the western blotting signal of low molecular weight proteins with both acidic and basic isoelectric points (p*I*) (Karey and Sirbasku, 1989).

After blocking with 5% (wt/vol) skimmed milk-containing buffer (0.1% [vol/vol] Tween-20 prepared in TBS; TBST), the membranes were incubated with TBST solution containing the mAb287 antibody (1:2000) and 3% (wt/vol) BSA overnight at 4 °C (Anastasia et al., 2013). After washing, membranes were incubated with TBST solution containing an anti-mouse HRP-conjugated secondary antibody (1:1000, GE Healthcare, Amersham, UK) and 3% BSA for 1 h at room temperature.

To determine the amounts of BDNF pro-peptide in CSF, various concentrations of recombinant BDNF pro-peptide (Alomone Labs, Jerusalem, Israel) were loaded along with CSF samples onto the same gel (Loaded recombinant BDNF pro-peptide in Supplementary Fig. 3 (a)). Recombinant BDNF pro-peptide (1.25–50 pg) was loaded side-by-side with the CSF samples (Supplementary Fig. 3 (a)). After Western blotting described above, immunoreactive protein bands were visualized and quantitative analysis of band intensity was performed as described previously (Dieni et al., 2012; Mizui et al., 2015).

To test the reliability of the mAb287 antibody for quantitative analysis of the BDNF pro-peptide, cerebral cortex tissue lysates prepared from *bdnf*$^{+/+}$ mice and *bdnf*$^{-/-}$ littermates were obtained from Jackson Laboratories, and western blot analysis was performed as described (Yang et al., 2009).

To confirm that the amount of BDNF pro-protein correlated with the total protein level, various volumes of CSF samples containing BDNF pro-peptide and recombinant BDNF pro-peptide at the indicated concentrations were loaded on the same gel and western blot analysis was performed as described above (Supplementary Fig. 3). Quantitative analysis of the band intensity was performed using ImageQuant software.

2.6. CSF mBDNF measurement

The CSF mBDNF level was measured using an ELISA kit for human mBDNF (Duoset, DY248, R&D Systems, Minneapolis, MN, USA) and the chemiluminescent reagent ELISA Femto Maximum Sensitivity Substrate (37075, Thermo Fisher Scientific, Rockford, IL, USA). CSF mBDNF levels were also measured using the MAGPIX system and a magnetic on-bead antibody for mBDNF (HNDG3MAG-36K, Merck Millipore, Darmstadt, Germany).

2.7. Statistical analysis

All data are presented as the median ± interquartile ranges. Normality was examined using the Kolmogorov-Smirnov test. Since distribution of CSF pro-peptide levels was deviated from the normal distribution, between-group comparisons of these levels were performed using the Kruskal-Wallis test with Dunn's multiple comparisons

Table 1
Demographic and Clinical data. The Table is partially modified from Ishiwata et al. (2017).

	HC (n = 27)	SZ (n = 27)	MDD (n = 18)	Statistic
N				
CSF	27	27	18	
Age (years)	42.0 (34.0–50.0)	41.0 (37.0–46.0)	42.0 (37.0–47.3)	N.S.
Gender				
Female	13 (48.1%)	13 (48.1%)	8 (44%)	
Male	14 (51.9%)	14 (51.9%)	10 (56%)	
Drug free		3	4	
CP equivalent value		926.5 (437.5–1438) (n = 16)		
IMI equivalent value			37.5 (0–187.5) (n = 12)	
PANSS positive score		11.1 (8.8–17.0) (n = 26)		
PANSS negative score		18.0 (14.5–23.0) (n = 26)		
PANSS general psychopathology score		30.0 (23.8–36.5) (n = 26)		
PANSS total score		62.5 (55.0–70.0) (n = 26)		
HAMD17 score (only 7 <)			12.5 (9.0–16.3) (n = 18)	
BDNF-pp (pg/mL)	420.0 (297.9–735.3) (n = 27)	309.6 (107.5–575.0) (n = 27)	354.6 (136.9–444.7) (n = 18)	N.S. (SZ), N.S. (MDD)
BDNF-pp/Protein	464.6 (340.7–767.7) (n = 27)	344.0 (83.4–678.4) (n = 27)	335.4 (163.2–438.7) (n = 18)	N.S. (SZ), * (MDD)
Protein (mg/mL)	0.86 (0.76–0.97) (n = 27)	0.88 (0.80–1.09) (n = 27)	1.09 (0.78–1.16) (n = 18)	N.S. (SZ), * (MDD)

*p < 0.05.

test or Mann-Whitney *U* test. The relationships between CSF pro-peptide levels and clinical variables (age, PANSS, HAMD-17, and drug equivalent doses) were analyzed using Spearman's rank order correlation test. A p-value < 0.05 was considered significant. All analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA).

3. Results

Table 1 shows the demographic and clinical characteristics of participants. There were no significant differences in terms of age or sex distribution between the patient groups and the healthy control group.

3.1. BDNF pro-peptide is present in human CSF

Initially, we examined whether BDNF pro-peptide is present in human CSF. We detected an immunoreactive band corresponding to the BDNF pro-peptide with a predicted molecular mass higher than 15 kDa (Fig. 1b, human BDNF pro-peptide). Since the BDNF pro-domain corresponding to the BDNF pro-peptide (Fig. 1a) is glycosylated (Mowla et al., 2001), we tested if the molecular weight of BDNF pro-peptide was reduced on SDS-PAGE gels when the CSF samples were pre-treated with *N*-glycosidase F. To this end, we loaded three samples on the same gel: (1) the human CSF sample un-treated, (2) the human CSF sample pre-treated with *N*-glycosidase F, and (3) *Escherichia coli*-derived recombinant BDNF pro-peptide.

Immunoblotting with a mouse monoclonal antibody against the pro-domain of human BDNF (mAb287, GeneCopoeia) showed that the molecular weight of BDNF pro-peptide in human CSF was shifted to the same molecular weight as that of *E. coli*-derived recombinant non-glycosylated BDNF pro-peptide (Fig. 1b, Deglycosylated N-glycosylated human BDNF pro-peptide). Examination of the entire immunoblot (Supplementary Fig. 1) revealed two additional bands with molecular masses > 15 kDa (arrows). These bands are likely to be non-specific because their molecular weight was not decreased by glycosidase treatment.

To further confirm that the mAb287 antibody reliably recognized the endogenous BDNF pro-peptide, we performed western blot analysis with hippocampal lysates derived from *bdnf*$^{+/+}$ mice and *bdnf* $^{-/-}$ littermates (Supplementary Fig. 2). Immunoblotting with the mAb287 antibody detected the BDNF pro-peptide band in samples from *bdnf*$^{+/+}$ but not *bdnf* $^{-/-}$mice, although non-specific bands were observed in lysates from both genotypes; these results confirm that the mAb287 antibody can be used to detect BDNF pro-peptide in CSF.

In addition, given that the total protein content of CSF samples varies, we tested the relationship between total protein and BDNF pro-protein within individual CSF samples prior to the quantitative analysis. Notably, the amount of total protein correlated with the amount of BDNF pro-protein in individual CSF samples (Supplementary Figs. 3a and b).

These results provide convincing evidence that BDNF pro-peptide is present in human CSF and is detectable by western blotting.

3.2. The ratios of CSF BDNF pro-peptide level to total protein level are different by diagnostic group and sex

Since blood-brain barrier hyperpermeability has been reported in psychiatric patients, including those with schizophrenia and MDD (Chen et al., 2017; Greene et al., 2017; Najjar et al., 2017), we first tested whether CSF total protein concentrations were higher in psychiatric patients than in control subjects. Protein levels varied significantly between the three groups (Kruskal-Wallis test; H = 6.09, p = 0.048). Total protein concentrations in the CSF of patients with MDD were significantly higher than those in healthy controls (Dunn's multiple comparison test; p = 0.043). We found no significant difference between patients with schizophrenia and controls (p = 0.51), or between MDD and schizophrenic groups (p = 0.67) (Table 1 and Supplementary Fig. 4). The total protein concentrations in CSF were not significantly different between males and females in any diagnostic group (Control, p = 0.92; Schizophrenia, p = 0.11; MDD, p = 0.17).

Because CSF total protein levels were different between diagnosis groups, we adjusted the levels of BDNF pro-peptide by the protein concentrations of the CSF. The BDNF pro-peptide/protein ratios multiplied by 10^6 are presented (BDNFPPR). CSF BDNFPPR levels varied significantly between the three diagnostic groups (Kruskal-Wallis test; H = 6.42, p = 0.04). Compared with the control group, CSF BDNFPPR was significantly lower in the MDD group (post hoc Dunn's test; p = 0.046), but not in the schizophrenia group (p = 0.25) (Fig. 2). There was no significant difference in CSF BDNFPPR between MDD and schizophrenic groups (p = 0.99).

We further evaluated the CSF BDNFPPR in male and female patients. A significant variation of CSF BDNFPPR was found in the male patients with MDD, schizophrenia and controls, but not in the females (Male: H = 7.16, p = 0.028, Female: H = 0.83, p = 0.66, by Kruskal-

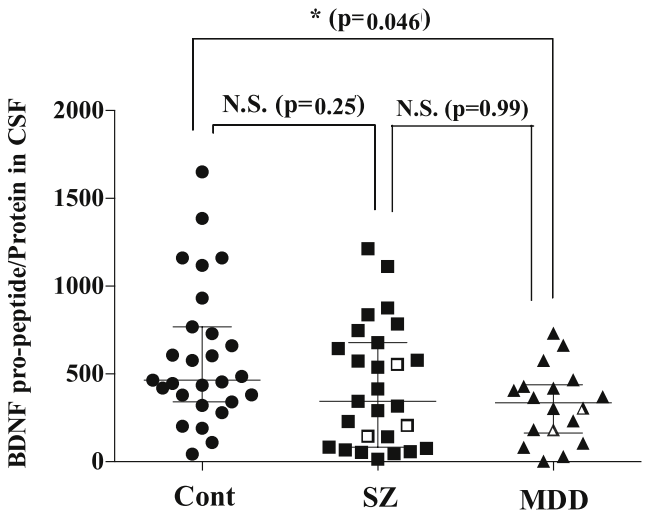

Fig. 2. BDNF pro-peptide/protein ratio in cerebrospinal fluid (CSF) are significantly lower in patients with MDD than in healthy controls. BDNF pro-peptide/protein ratios in healthy controls (Cont), patients with schizophrenia (SZ) (n = 27), and patients with MDD (n = 18).

Wallis test). BDNFPPR in the CSF was significantly lower in male patients with MDD than in male healthy controls (post hoc Dunn's test; p = 0.047). We did not find any difference between male patients with schizophrenia and controls (p = 0.10), and male patients with schizophrenia and MDD (p = 0.99) (Fig. 3a). There was no significant difference in CSF BDNFPPR in female patients with schizophrenia (p = 0.99) or female patients with MDD (p = 0.99) compared with female controls. No significant difference between female patients with schizophrenia and MDD (p = 0.99) was shown (Fig. 3b). We also examined differences in CSF BDNF pro-peptide levels between male and female healthy controls. There was no significant difference in BDNF pro-peptide levels between the genders (Mann-Whitney *U* test: p = 0.88). Furthermore, there was no significant correlation between BDNF pro-peptide levels and age (data not shown).

3.3. CSF mBDNF level is below the detection limit

Despite the highly sensitive methods used (see Materials and methods), CSF mBDNF levels were below the detection limit, which indicates that the human CSF mBDNF concentration is very low compared with that of BDNF pro-peptide (data not shown).

3.4. The relationship between BDNFPPR and clinical severity

In patients with MDD and schizophrenia, there was no significant correlation between BDNFPPR and the HAMD-17 score (ρ = 0.16, p = 0.54) or the PANSS total score (ρ = −0.05, p = 0.81) (Fig. 4a and b).

IMI equivalent values significantly correlated with BDNFPPR in the MDD patients (ρ = 0.61, p = 0.038; Fig. 4c). However, the CP equivalent values did not correlate with BDNFPPR in the schizophrenia patients (ρ = −0.08, p = 0.73; Fig. 4d). The mean IMI or CP equivalent values did not differ between males and females (Mann-Whitney *U* test: IMI equivalent value, p = 0.88; CP equivalent value, p = 0.67). Thus, it is unlikely that the observed gender differences in BDNF pro-peptide levels (Fig. 3) are attributable to medication.

We next attempted to examine the difference in BDNF pro-peptide concentration between treated and drug-free patients, but the numbers of drug-free patients were too small to compare the two groups (n = 2 for MDD and n = 3 for schizophrenia). In order to clarify the distribution of drug-free patients, these patients are indicated by open squares or triangles in Figs. 2 and 3 and Supplementary Fig. 4.

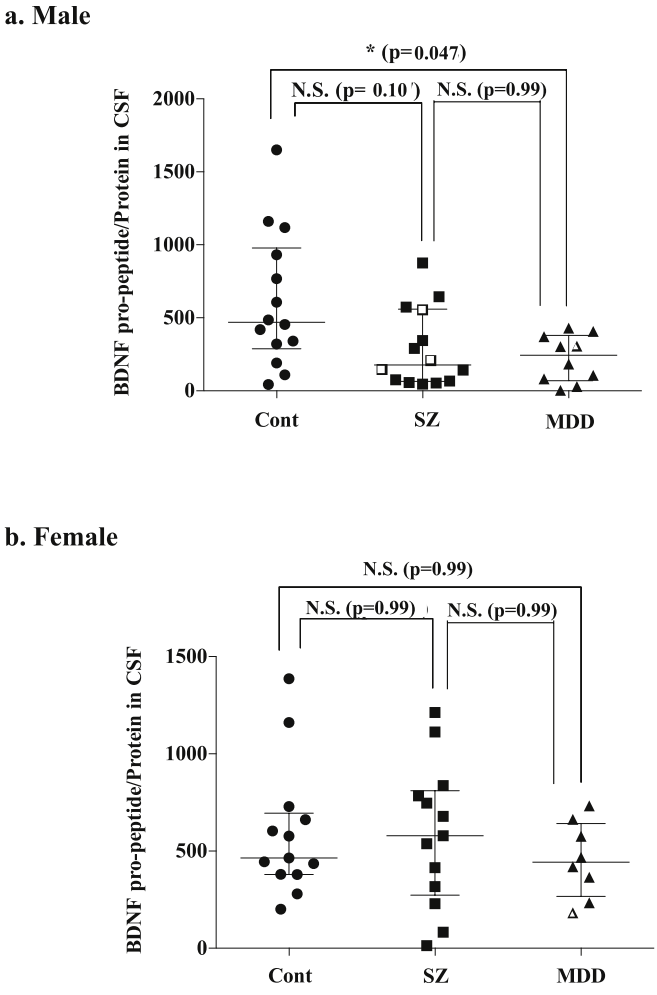

Fig. 3. The BDNF pro-peptide/protein ratio in cerebrospinal fluid is lower in male patients with major depressive disorder (MDD) and schizophrenia (SZ) than in male healthy controls (Cont), but does not differ between female patients and controls. The BDNF pro-peptide/protein ratio (BDNFPPR) in male patients with MDD was significantly lower than that in male controls, and tended to decrease in male patients with schizophrenia (a). However, the BDNFPPR did not differ between female patients and female controls (b).

4. Discussion

This is the first report on CSF BDNF pro-peptide levels in patients with MDD, those with schizophrenia, and healthy controls. We demonstrated that BDNF pro-peptide was present and measurable in human CSF. We also found that CSF BDNFPPR was significantly lower in patients with MDD than in controls. Furthermore, BDNFPPR was significantly lower in males, but not in females, patients with MDD than in same-sex controls. These results suggest an association of reduced levels of the BDNF pro-peptide with MDD in males.

First, using immunoblotting with an anti-BDNF pro-peptide antibody, we demonstrated that BDNF pro-peptide was present in human CSF. A recent immunoblotting study examined the presence of the BDNF pro-peptide was contained in human brain tissues (Yang et al., 2016); however, it was unclear whether the immunodetectable band was derived specifically from the endogenous BDNF pro-peptide (Yang et al., 2016). In a previous mouse study, knock-out mice lacking the *bdnf* gene were used to confirm that the immunoreactive band was endogenously derived (Dieni et al., 2012).

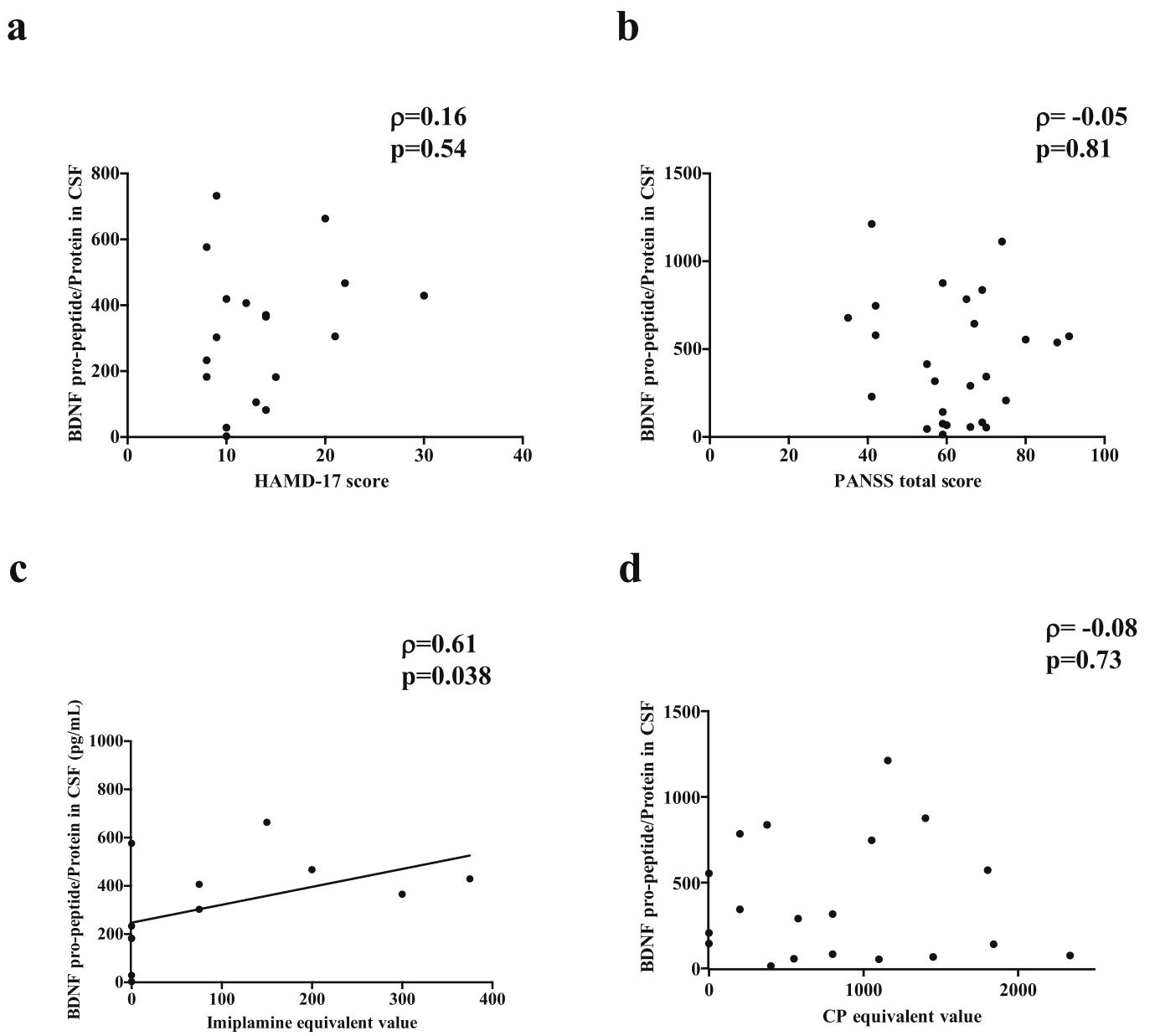

Fig. 4. Correlations between the cerebrospinal fluid (CSF) BDNF pro-peptide/protein ratio (BDNFPPR) and clinical data. Correlations of the CSF BDNFPPR with 17-item Hamilton Depression Rating Scale (HAMD-17) scores **(a)** and imipramine (IMI) equivalent values **(c)** in patients with major depressive disorder. Correlations of the CSF BDNFPPR with Positive and Negative Syndrome Scale (PANSS) scores **(b)** and chlorpromazine (CP) equivalent values **(d)** in patients with schizophrenia.

We designed the present study to overcome this issue and sought to ensure that the immunoreactive band with a molecular weight higher than 15 kDa was derived from endogenous BDNF pro-peptide. Since the BDNF pro-domain corresponding to the BDNF pro-peptide (Fig. 1a) is glycosylated (Mowla et al., 2001), it may migrate as a broad band on the gel. Thus, to avoid any confusion caused by such experimental concerns, the CSF samples were pre-treated with N-glycosidase F and subjected to immunoblot analysis. As expected, the band corresponding to the BDNF pro-peptide shifted to the same molecular weight position as that of the *E. coli*-derived recombinant non-glycosylated BDNF pro-peptide, indicating that the immunoreactive band corresponding to the BDNF pro-peptide was endogenously derived, and that the BDNF pro-peptide is present in human CSF.

CSF is a crucial body fluid for understanding molecular alterations in the brain (Schwarz and Bahn, 2008). In accordance with previous studies (Chen et al., 2017; Greene et al., 2017; Najjar et al., 2017), total protein levels were higher in psychiatric patients than in controls, which is indicative of the increased permeability of the blood-brain barrier in psychiatric patients. Thus, we consider the possibility that CSF BDNF pro-peptide levels may be affected by the protein concentration of CSF, and, to this end, adjusted the BDNF pro-peptide concentrations to by the protein levels of the CSF. The CSF BDNFPPR was significantly lower in patients with MDD than in the controls.

A previous study indicates that altered blood BDNF levels reflect the pathology of psychiatric disorders, including depression (Autry and Monteggia, 2012). One study reported that mBDNF levels measured by an ELISA in both the plasma and CSF are significantly lower in patients with first-episode psychosis than in controls (Pillai et al., 2010). However, in the present study, despite being measured by two sensitive methods, an ELISA kit for human mBDNF using a chemiluminescent reagent and a MAGPIX system using a magnetic on-bead antibody for mBDNF, CSF mBDNF levels were below the detection limit (data not

shown). This indicates that the CSF mBDNF concentration is very low compared with that of BDNF pro-peptide concentration.

Interestingly, we found that patients with MDD had lower CSF BDNF pro-peptide levels than healthy controls. While the mechanism underlying this difference is unclear, a hypothetical explanation is possible. Considering that mBDNF expression is decreased in MDD patients and animal models of depression (Castren and Kojima, 2017), and that BDNF expression is controlled by neuronal activity (Park and Poo, 2013), low concentrations of BDNF pro-peptide in CSF peptide may be a result of lower neuronal activity in the depressive brain (Castren and Kojima, 2017). A recent post-mortem study reported that BDNF pro-peptide protein levels are significantly decreased in the cerebellum of patients with MDD and schizophrenia, but increased in the parietal cortex of these patients (Yang et al., 2016). Although the reasons for these regional differences are not yet clear, future work (using techniques such as immunoblotting and immunohistochemistry, for example) could elucidate whether BDNF pro-peptide protein levels are altered in other brain regions. Furthermore, the distinct role of BDNF pro-peptide in psychiatric disorders should be investigated in future studies.

The CSF BDNFPPR was significantly lower in male, but not female, patients with MDD than in controls. Total CSF protein concentration was not different between males and females and therefore did not affect the observed sex differences in BDNFPPR. These results suggest that sex is related to the reduction of the BDNF pro-peptide level. Sex differences in the severity of psychiatric disorders, such as schizophrenia and MDD, have been reported previously (Cyranowski et al., 2000). For example, symptoms of schizophrenia are likely to be more severe in males than females. Actually, the PANSS negative scores were significantly higher in male patients with schizophrenia than female patients with those in our study (male: 20.0 (18.5–24.0), female: 15.0 (10.5–18.0); $p = 0.001$, Mann-Whitney U test). Indeed, alterations of hormonal status have been associated with sex differences in depression pathology and the induction of mBDNF. For example, compared with control mice, mBDNF levels have been reported to be significantly reduced in the prefrontal cortex of intact female mice with chronic variable mild stress, but not of ovariectomized mice with chronic variable mild stress (Karisetty et al., 2017a, 2017b). Another study found that cerebellar mBDNF levels are elevated by injections of estradiol benzoate and that estradiol-mediated induction of mBDNF is prevented by co-treatment with the estrogen receptor antagonist (Sasahara et al., 2007). On the other hand, in fertile women, plasma BDNF levels in luteal phase were significantly higher than those in follicular phase. Amenorrhoeic women showed a significant lower mBDNF levels compared with fertile females (Begliuomini et al., 2007). Furthermore, individuals with premenstrual dysphoric disorder were significantly higher luteal serum BDNF levels than the control subjects (Oral et al., 2015). These results have shown that plasma mBDNF levels in women were influenced by hormonal status. Therefore, hormonal status in women might be associated with the BDNF pro-peptide concentrations in our sample. Further investigations are required to elucidate the mechanisms underlying the sex differences in the CSF BDNF pro-peptide level.

BDNFPPR significantly correlated with the IMI equivalent values, but not with the CP equivalent values. BDNF mRNA expression in the rat hippocampus was significantly increased by chronic (21 days), but not acute (1 day), administration of various antidepressant drugs (Nibuya et al., 1995). Furthermore, antidepressants increased BDNF levels in the hippocampus of post-mortem brains (Chen et al., 2001). It is possible that antidepressant drugs may have minimized the difference in BDNF pro-peptide levels between patients with MDD and controls. Further studies in drug-free patients are warranted.

This study has some limitations. Because the sample size was small, false negative results may have occurred. Moreover, most patients were on medication; therefore, an effect of medication on BDNF pro-peptide concentrations cannot be excluded. To clarify the relationship between CSF levels of BDNF pro-peptide and psychiatric disorders, further investigations in a large sample of drug-free patients with schizophrenia and MDD will be needed.

The positive correlation between the imipramine equivalent value and BDNFPPR suggests that antidepressant medications would have minimized rather than exaggerated the difference in BDNFPPR between patients with MDD and controls. Therefore, the observed decrease in BDNF pro-peptide levels in MDD patients is unlikely to be ascribed to antidepressant medications. Further studies are required to elucidate the effects of antidepressant drugs on BDNF pro-peptide levels.

In conclusion, the CSF BDNF pro-peptide/total protein ratio was significantly lower in patients with MDD than in healthy controls. Additionally, the ratio was significantly lower in male, but not female, patients with MDD than in controls. Given that mBDNF concentrations seem to be very low, CSF BDNF pro-peptide may be implicated in the pathophysiology of MDD. Further investigations are needed to clarify the association between BDNF pro-peptide and psychiatric disorders.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpsychires.2019.03.024.

Author disclosure

Conflict of interest

The authors declare no conflict of interest.

Contributors

Three authors (TM, HK and MK) designed research. Two authors (TM and MK) performed the biochemical assays and analyzed the data. Four authors (HK, SH, SY and HK) recruited and diagnosed the patients; Four authors (HK, SH, SY and HK) collected CSF samples. Four authors (HK, SH, SY and HK) evaluated the clinical assessments. Five authors (TM, KH, SI, HK and MK) analyzed the data. Five authors (TM, KH, SI, HK and MK) wrote the paper.

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