a combination of methylphenidate and atomoxetine (18 and 60 mg/day, respectively, n=1), a combination of atomoxetine and aripiprazole (35 and 9 mg/day, respectively, n=1), and a combination of methylphenidate and risperidone (18 and 6 mg/day, respectively, n=1), while three subjects were drug-naïve. Treatment with these drugs was stable for the 4 weeks prior to enrollment, and was stable during the trial. The Mini-International Neuropsychiatric Interview for Children and Adolescents (MINI-KID)[20] was conducted to exclude any current, past, personal, or familial history of mental illness. Two subjects were also diagnosed as having a learning disorder: one subject was diagnosed with tic disorders, and one with learning and tic disorders, according to the DSM-IV criteria.[19]

Measurement of clinical symptoms

All patients were assessed using the ADHD Rating Scale IV (ADHD-RS), Japanese version.[21] The ADHD-RS is a reliable and easy-to-administer instrument both for diagnosing ADHD in children and adolescents and for assessing treatment response. It consists of 18 items, with the scale being linked directly to DSM-IV diagnostic criteria for ADHD.[22] The Das–Naglieri Cognitive Assessment System (DN-CAS), Japanese version,[23] was used to assess cognitive function. The DN-CAS is an assessment battery designed to evaluate cognitive processing. It was developed to integrate theoretical and applied psychological knowledge, using cognitive processing theory and tests, designed to measure planning, attention, and simultaneous and successive processing (PASS) in individuals aged 5–17 years. The Wechsler Intelligence Scale for Children third/fourth editions (WISC-III/IV),[24,25] Japanese versions, were used to assess the full intelligent quotient of all patients.

Statistical analysis

Statistical analyses were performed using the software package SPSS version 21.0 for Macintosh (IBM Armonk, NY, USA). The data show means ± standard deviation. Student's paired *t*-test was used to compare changes from baseline to 4 weeks. The Wilcoxon signed-rank test was used as a post hoc test to compare changes from baseline to 4 weeks. Values of $P<0.05$ were considered statistically significant.

Results

The baseline scores and mean changes of primary and secondary outcomes from the 4-week trial of tipepidine in ADHD subjects are shown in Table 2. A comparison of baseline scores and 4-week end-point scores showed that all the ADHD-RS scores (total scores, hyperimpulsive subscores, and inattentive subscores) improved significantly ($P<0.001$). The Wilcoxon signed-rank test also detected statistical significance in all ADHD-RS scores ($P<0.005$). However, a comparison of baseline scores and 4-week end-point scores found that none of the DN-CAS scores (total scores and planning, attention, simultaneous, and successive subscores) showed significant change. The Wilcoxon signed-rank test also failed to detect statistical significance in any DN-CAS score changes.

Table 2 Baseline scores and mean changes of primary and secondary outcomes after a 4-week trial of tipepidine in ADHD subjects

	Baseline mean ± SD	Week 4 mean ± SD	*P*-value (*df*, *t*-score)
ADHD-RS total score	30.2±9.9	16.4±8.4	<0.001* (9, 11.8)
ADHD-RS hyperimpulsive subscore	11.2±7.1	5.0±4.1	<0.001* (9, 5.7)
ADHD-RS inattentive subscore	19.0±3.6	10.6±3.8	<0.001* (9, 10.6)
DN-CAS total score	81.1±20.0	87.6±21.6	0.093 (9, −1.88)
DN-CAS planning subscore	89.2±16.6	94.0±16.4	0.164 (9, −1.52)
DN-CAS attention subscore	81.3±22.4	89.5±23.1	0.262 (9, −1.20)
DN-CAS simultaneous subscore	83.8±18.3	91.8±25.0	0.137 (9, −1.63)
DN-CAS successive subscore	89.8±20.0	89.9±14.4	0.981 (9, −0.03)

Notes: *$P<0.05$. Student's paired *t*-test was used to compare changes from baseline to 4 weeks. Wilcoxon signed-rank test was used as a post hoc test to compare changes from baseline to 4 weeks.

Abbreviations: ADHD, attention deficit/hyperactivity disorder; SD, standard deviation; *df*, degrees of freedom; ADHD-RS, Attention Deficit/Hyperactivity Disorder-Rating Scale IV (Japanese version); DN-CAS, Das–Naglieri Cognitive Assessment System (Japanese version).

Tipepidine was well tolerated, with no patients discontinuing medication because of side effects. No significant effects were revealed in blood parameters, urine analysis, weight, height, blood pressure, or cardiac frequency during the 4-week follow-up period.

Discussion

Tipepidine improved the ADHD symptoms of inattention and hyperimpulsivity, as shown by ADHD-RS scores. To our knowledge, this is the first report demonstrating the beneficial effect of tipepidine in treating pediatric ADHD subjects.

Comparisons of all baseline DN-CAS scores (total scores and planning, attention, simultaneous, and successive subscores) and 4-week end-point scores detected no

significant differences. However, a comparison of baseline DN-CAS total scores and 4-week end-point scores did show a mild trend of improvement (P=0.093).

The lower improvement in DN-CAS symptomatology may have been partly due to the relatively low dosage of tipepidine used in this study (1.288±0.349 mg/kg/day, Table 1), compared with that of the Fujieda et al trial in mice (20 mg/kg).[17] Higher dosages may be more beneficial for ADHD symptoms, as they are associated with higher monoaminergic neurotransmission through GIRK channels. At present, the optimal dosage of tipepidine for ADHD is unknown, and defining this dosage should be the primary focus in the treatment of these patients.

The inhibition of GIRK channels by tipepidine is predicted to modulate brain monoamine levels in a similar manner to psychostimulants and selective norepinephrine-reuptake inhibitors. However, this trial found none of the side effects typically associated with psychostimulants and selective norepinephrine-reuptake inhibitors. Tipepidine has been used safely as an over-the-counter antitussive for children and adults in Japan since 1959. Therefore, safety issues will be no of no concern if this is used as a new treatment for ADHD.

Very recently, Hamasaki et al showed that tipepidine activated dopamine neurons in the ventral tegmental area through the inhibition of GIRK channel-activated currents, and their preliminary microdialysis study showed that tipepidine dramatically increased dopamine levels in the shell of the nucleus accumbens (NAc).[26] In addition, Costa Dias et al identified the possible involvement of NAc connections in the pathophysiology of impulsive decision making in ADHD, using functional connectivity magnetic resonance imaging.[27] Therefore, further detailed studies of tipepidine use in ADHD are needed to investigate dopamine activation in the NAc and its neural pathways.

The main limitation of this study was the small sample size (n=10). The second limitation was the low proportion of drug-naïve subjects. Further studies with greater analytical power, larger sample sizes, and more drug-naïve subjects will be necessary.

Conclusion

In conclusion, our pilot study suggests that tipepidine therapy may prove to be an effective alternative treatment for pediatric patients with ADHD, and since this drug is already in wide clinical use for other conditions, there should be no ensuing safety issues. However, the safety of long-term tipepidine use needs to be evaluated carefully, since many antitussive medications are completed within 1 week. Nonetheless, more detailed randomized, double-blind studies are needed to confirm tipepidine's efficacy and safety.

Author contributions

T Sasaki and K Hashimoto drafted the manuscript. K Hashimoto is the principal investigator of this study. All authors recruited the patients, revised the article, conducted the statistical analysis, approved the final manuscript, and agreed to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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A positive correlation between serum levels of mature brain-derived neurotrophic factor and negative symptoms in schizophrenia

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ABSTRACT

A meta-analysis study reported serum brain-derived neurotrophic factor (BDNF) levels as a potential biomarker for schizophrenia. However, at the time, commercially available human ELISA kits were unable to distinguish between pro-BDNF (precursor BDNF) and mature BDNF, because of limited antibody specificity. Here, we used new ELISA kits, to examine serum levels of mature BDNF and matrix metalloproteinase-9 (MMP-9), which converts pro-BDNF to mature BDNF in schizophrenia. Sixty-three patients with chronic schizophrenia and 52 age- and sex-matched healthy controls were enrolled. Patients were evaluated using the Brief Psychiatry Rating Scale, the Scale for the Assessment of Negative Symptoms (SANS) and neuropsychological tests. Neither serum mature BDNF nor MMP-9 levels differed between patients and controls. In male subgroups, serum MMP-9 levels of smoking patients were higher than those of non-smoking patients, but this was not observed in male controls or the female subgroup. In patients, serum mature BDNF levels were associated with SANS total scores and the Information subtest scores of the Wechsler Adult Intelligence Scale Revised (WAIS-R), while serum MMP-9 levels were associated with smoking and category fluency scores. These findings suggest that neither mature BDNF nor MMP-9 is a suitable biomarker for schizophrenia, although further studies using large samples are needed.

1. Introduction

Accumulating evidence implicates brain-derived neurotrophic factor (BDNF) in the pathophysiology of schizophrenia (Autry and Monteggia, 2012; Favalli et al., 2012; Martinotti et al., 2012; Nurjono et al., 2012). A meta-analysis suggested reduced blood BDNF levels in patients with schizophrenia, regardless of medication exposure and gender, and an association between reduced BDNF levels in schizophrenia and increasing age (Green et al., 2011). A number of studies have also reported reduced blood BDNF levels in patients with schizophrenia (Chen da et al., 2009; Fernandes et al., 2010; Pillai et al., 2010; Rizos et al., 2010a; Rizos et al., 2010b; Lee et al., 2011; Rizos et al., 2011; Yang et al., 2011; Zhang et al., 2012a; Zhang et al., 2012b; Zhang et al., 2012c). In contrast, several case-control studies have found increased peripheral blood BDNF levels in patients with schizophrenia (Reis et al., 2008; Domenici et al., 2010). We previously reported that serum levels of BDNF in schizophrenics were indistinguishable from those of healthy controls (Shimizu et al., 2003; Niitsu et al., 2011), a finding replicated by other studies (Huang and Lee, 2006; Mackin et al., 2007; Goto et al., 2011). As yet, there is no plausible explanation for this heterogeneity of findings and thus the role of BDNF in schizophrenia pathophysiology remains unclear.

Mature BDNF is synthesized as a precursor protein, pre-pro-BDNF, in the endoplasmic reticulum. Following cleavage of the signal peptide, pro-BDNF is converted to mature BDNF, by extracellular proteases, such as matrix metalloproteinase-9 (MMP-9) and plasmin (Lu, 2003; Hwang et al., 2005; Lu et al., 2005; Ethell and Ethell, 2007; Hashimoto, 2007, 2010, 2013). It was initially thought that only secreted mature BDNF was biologically active, and that pro-BDNF, which localizes intracellularly, served as an inactive precursor. However, new evidence shows that pro-BDNF and mature BDNF elicit opposing effects via the p75NTR and TrkB

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receptors, respectively, and that both pro- and mature BDNF play important roles in several physiological functions (Lu, 2003; Lu et al., 2005; Hashimoto, 2007, 2010, 2013). Considering the physiological importance of both proteins, it would be informative to measure individual levels of pro-BDNF and mature BDNF in human body fluids (Hashimoto, 2010, 2012, 2013). A previous study reported increased serum levels of mature- and pro-BDNF, and decreased serum levels of truncated BDNF in patients with schizophrenia, as measured by western-blotting (Carlino et al., 2011). Although BDNF levels in human blood can be measured using newer commercially available human BDNF, enzyme-linked immunosorbent assay (ELISA) kits, earlier versions of these kits were unable to distinguish between pro-BDNF and mature BDNF due to the limited specificity of the BDNF antibody (Yoshida et al., 2012a; Yoshida et al., 2012b). It is highly possible that the limited specificity of these ELISA kits has contributed to the heterogeneity of results in previous studies examining blood BDNF levels in schizophrenics.

MMP-9 plays a key role in synaptic plasticity of the brain, and acts by converting pro-BDNF to mature BDNF (Hwang et al., 2005; Ethell and Ethell, 2007). A study using MMP-9 knock-out mice demonstrated that MMP-9 plays a role in the development of pentylenetetrazole-induced kindling, by converting pro-BDNF to mature BDNF in the hippocampus (Mizoguchi et al., 2009). Another study suggested that serum levels of MMP-9 increased in patients with major depressive disorder and schizophrenia (Domenici et al., 2010). Therefore, it is plausible that serum levels of both mature BDNF and MMP-9 could play roles in the pathophysiology of schizophrenia.

Considering the evidence presented above, we hypothesized that in patients with schizophrenia, serum levels of mature BDNF and MMP-9 would be higher than those of the healthy controls. In this study, we examined serum levels of mature BDNF and MMP-9 in patients with chronic schizophrenia, and their association with demographic and clinical variables, including cognition.

2. Methods

2.1. Study design

The ethics committee of Chiba University Graduate School of Medicine approved the present study. All subjects provided written informed consent for participation in the study, after the procedure had been fully explained. This study is an exploratory, cross-sectional, and case-control design.

2.2. Participants

Sixty-three Japanese patients with schizophrenia (DSM-IV) were recruited from the outpatient departments of Chiba University Hospital and its affiliated hospitals, in Chiba, Japan. Fifty-two age- and sex-matched healthy Japanese subjects were recruited as healthy controls. Entry criteria of participants are described in detail elsewhere, and this study used the same sample as our previous study (Niitsu et al., 2011).

2.3. Clinical assessments

Clinical symptoms were assessed using the Brief Psychiatry Rating Scale (BPRS) and the Scale for the Assessment of Negative Symptoms (SANS). Drug-induced extrapyramidal symptoms were evaluated using the Drug Induced Extrapyramidal Symptoms Scale (DIEPSS). Intelligence quotient (IQ) scores were estimated using the short version of the Japanese Wechsler Adult Intelligence Scale Revised (WAIS-R), which consisted of the Information, Digit Span, and Picture Completion subtests. Age at onset, duration of illness, duration of untreated psychosis and smoking status were evaluated.

2.4. Cognitive assessments

Cognitive assessments of participants were performed by neuropsychological tests. Details of cognitive assessments and results are available elsewhere (Niitsu et al., 2011). Briefly, participants were assessed using the Verbal Fluency Test (letter, category) (Sumiyoshi et al., 2005), the Wisconsin Card Sorting Test (WCST, Keio version) (the number of achieved categories and perseverative errors) (Igarashi et al., 2002; Hori et al., 2006), the Trail Making Test (Part A and Part B), and the Stroop Test (Part D, a list of 24 colored dots; Part C, 24 words naming a color, written in an incongruent color) (Carter et al., 1995; Chan et al., 2004).

2.5. Measurement of mature BDNF and MMP-9 levels from serum

Serum samples of participants were collected between 10:00 and 13:00 h and stored at −80 °C until assayed. Levels of mature BDNF and MMP-9 were measured using a human BDNF ELISA Kit (Adipo Bioscience, Santa Clara, CA, USA) and a human MMP-9 ELISA Kit (R&D Systems, Minneapolis, MN, USA), respectively. To minimize assay variance, serum levels of mature BDNF and MMP-9 from each subject were measured on the same day. All experiments were performed in duplicate. Protocols were performed according to the manufacturer's instructions. The optical density of each well was measured using an automated microplate reader (Emax; Molecular Devices, Sunnyvale, CA, USA).

2.6. Statistical analysis

For the comparisons between groups, the Chi-squared test was employed for categorical variables, and Student's *t*-test for continuous variables. Two-way analysis of variance (ANOVA) was employed to examine the effects of diagnosis and gender on serum levels of mature BDNF and MMP-9. Effects of smoking status stratified by gender were also examined. Bonferroni correction was used for post hoc tests. Associations between serum levels of mature BDNF and MMP-9, and clinical and cognitive variables were tested for, using Pearson's correlation coefficients and stepwise multiple regression analysis. Since serum levels of MMP-9 did not show normal distribution, the logarithm transformation was used for this variable. Statistical analyses were performed in two-sided tests using SPSS, version 18.0 J software (IBM, Tokyo, Japan). The statistical significance was set at $P<0.05$ with power $(1-\beta)=0.80$. ANOVAs with a total of 115 samples and 52 male samples would have enabled us to detect the following effect sizes: $f=0.31$ (medium-to-large) and 0.48 (large).

3. Results

3.1. Demographic data and clinical variables

Characteristics of the participants are shown in Table 1. Gender, age, education and smoking status did not differ between patients and healthy controls. The proportions of smokers between patients and controls differed in the male (Fisher's exact test, $P=0.04$) but not female subgroup ($P>0.05$) (Tables 2 and 3). The correlations of cognitive data with serum levels of mature BDNF

Table 1
Sample characteristics.

	Controls ($n=52$)	Patients ($n=63$)	*P*
Gender (Male/female)	25/27	26/37	NS[a]
Age (years)	34.9 (7.3)	35.9 (8.2)	NS
Education duration (years)	14.7 (2.7)	13.8 (2.3)	NS
Smoking status (No/yes)	43/9	45/18	NS[a]
Estimated IQ	110.2 (12.0)	102.4 (13.9)	<0.01
Age at onset of illness (years)	–	26.8 (7.0)	–
Duration of illness (years)	–	9.1 (7.3)	–
DUP (months)	–	8.1 (13.4)	–
BPRS	–	25.5 (7.5)	–
SANS	–	70.4 (11.8)	–
DIEPSS	–	2.7 (2.7)	–
Antipsychotic dose (mg/day) [#]	–	323.9 (184.2)	–
Mature BDNF (ng/ml)	28. 10 (7.18)	29.79 (6.09)	NS
MMP-9 (ng/ml)	672.49 (378.36)	700.92 (330.81)	NS

Values represent mean (S.D.). NS, not significant.
Abbreviations: DUP, Duration of Untreated Psychosis; BPRS, Brief Psychiatric Rating Scale; SANS, Scale for the Assessment of Negative Symptoms; and DIEPSS, Drug Induced Extra-Pyramidal Symptoms Scale.

[a] χ^2 test. Other *p*-values are calculated by Student's *t*-test.
[#] Chlorpromazine equivalent dose ($n=60$).

Table 2
Serum levels of mature BDNF (ng/ml).

	Controls ($n=52$)		Patients ($n=63$)		Diagnosis		Gender		Diagnosis × gender	
		n		*n*	*F*	*P*	*F*	*P*	*F*	*P*
Gender					2.09	NS	1.17	NS	0.03	NS
Male	28.70 (7.97)	25	30.70 (6.61)	26						
Female	27.54 (6.46)	27	29.16 (5.71)	37						
							Smoking		**Diagnosis × smoking**	
							F	*P*	*F*	*P*
Total					3.48	NS	0.25	NS	1.66	NS
Nonsmokers	28.57 (7.58)	43	29.45 (4.89)	45						
Smokers	25.85 (4.48)	9	30.66 (8.51)	18						
Male ($n=51$)					1.80	NS	0.16	NS	1.45	NS
Nonsmokers	29.44 (8.67)	20	29.77 (4.62)	13						
Smokers	25.70 (3.11)	5	31.64 (8.23)	13						
Female ($n=64$)					0.65	NS	0.46	NS	0.02	NS
Nonsmokers	27.80 (6.58)	23	29.32 (5.06)	32						
Smokers	26.04 (6.37)	4	28.10 (9.67)	5						

Values represent mean (S.D.). NS, not significant. Statistical values are calculated by two-way ANOVA.
Abbreviation: BDNF, Brain-derived neurotrophic factor.

Table 3
Serum levels of MMP-9 (ng/ml).

	Controls ($n=52$)		Patients ($n=63$)		Diagnosis		Gender		Diagnosis × gender	
		n		*n*	*F*	*P*	*F*	*P*	*F*	*P*
Gender					0.37	NS	0.16	NS	0.01	NS
Male	676.5 (451.1)	25	706.3 (369.8)	26						
Female	668.8 (304.9)	27	697.2 (305.7)	37						
							Smoking		**Diagnosis × smoking**	
					F	*P*	*F*	*P*	*F*	*P*
Total ($n=115$)					1.33	NS	2.54	NS	2.14	NS
Nonsmokers	675.5 (400.1)	43	637.2 (305.5)	45						
Smokers	658.2 (267.9)	9	860.3 (346.3)	18						
Male ($n=51$)					0.23	NS	2.39	NS	4.60	0.037 [a]
Nonsmokers	688.8 (479.3)	20	527.9 (290.3)	13						
Smokers	627.5 (354.7)	5	884.6 (362.9)	13						
Female ($n=64$)					0.18	NS	0.92	NS	0.001	NS
Nonsmokers	664.0 (327.0)	23	681.5 (304.6)	32						
Smokers	696.4 (142.2)	4	797.3 (328.5)	5						

Values represent mean (S.D.). NS, not significant. Statistical values are calculated by two-way ANOVA with log-transformed serum levels of MMP-9.
Abbreviation: MMP-9, Matrix Metalloproteinase-9.
[a] Post hoc test revealed a significant difference between smokers and non-smokers in male patients with schizophrenia ($P=0.004$).

and MMP-9 are shown in Table 4. Mean estimated IQ of patients with schizophrenia was significantly lower than that of the healthy controls ($P<0.01$). Furthermore, patients with schizophrenia showed significantly lower scores in all neuropsychological tests ($P<0.05$) (Niitsu et al., 2011).

3.2. Serum mature BDNF levels and MMP-9

Serum levels of mature BDNF did not differ between the diagnosis groups (Table 1). Two-way ANOVA on serum levels of mature BDNF showed no significant effects for diagnosis and gender, or for diagnosis and smoking status (Table 2). After stratification by gender, two-way ANOVA on serum levels of mature BDNF showed no significant effects for diagnosis and smoking status, and their interaction (Table 2). In patients with schizophrenia, serum levels of mature BDNF showed no significant differences among the four types of antipsychotic medications: risperidone (31.20 ± 6.81 [mean $\pm$ S.D., ng/mL], $n=25$); olanzapine (28.34 ± 5.76, $n=18$); aripiprazole (30.48 ± 5.46, $n=9$); and quetiapine (27.25 ± 5.43, $n=8$); ($P=0.296$).

Serum levels of MMP-9 did not differ between the diagnosis groups (Table 1). Two-way ANOVA on serum levels of MMP-9 showed no significant effects for diagnosis and gender, or diagnosis and smoking status (Table 3). However, in the male subgroup, after stratification by gender, a significant interaction effect for diagnosis and smoking status was observed in MMP-9 serum levels ($P=0.037$). This effect was not observed in the female subgroup (Table 3). Simple effects analysis revealed that serum levels of MMP-9 in male smoking patients were significantly higher than those in male non-smoking patients ($P=0.004$). In patients with schizophrenia, serum levels of MMP-9 showed no significant differences among the four types of antipsychotic medication:

Table 4
Cognitive data and their correlation coefficients with serum levels of mature BDNF and MMP-9.

	Cognitive data		Correlation coefficients			
	Controls (n=52)	Patients (n=63)	Mature BDNF		MMP-9 [a]	
			Controls	Patients	Controls	Patients
Estimated IQ	110.2 (12.0)	102.4 (13.9)	−0.167	0.245	−0.108	-0.131
Information	11.1 (2.6)	10.1 (2.7)	−0.245	0.271*	−0.081	−0.123
Digit span	11.7 (2.9)	10.6 (2.9)	−0.029	0.199	−0.087	−0.138
Picture completion	11.0 (1.9)	10.5 (2.2)	0.033	−0.034	0.054	0.066
Letter fluency test (words)	35.2 (9.0)	28.0 (8.9)	−0.102	−0.021	−0.096	−0.170
Category fluency test (words)	49.1 (6.8)	39.9 (6.9)	−0.163	−0.017	−0.017	−0.323**
WCST, accomplished categories (n) [b]	4.9 (1.5)	3.3 (2.2)	−0.154	0.157	0.071	0.080
WCST, perseverative errors (n) [b]	0.9 (1.8)	4.5 (6.7)	0.149	−0.064	−0.120	−0.079
Trail making test A (s)	27.2 (7.7)	33.8 (10.1)	0.081	0.254*	0.141	−0.090
Trail making test B (s)	52.9 (16.0)	80.5 (27.1)	0.001	−0.034	0.083	0.071
Stroop Test part D (s)	12.7 (2.5)	14.2 (2.6)	0.259	0.146	0.200	0.110
Stroop test part C (s)	18.6 (5.3)	22.7 (5.9)	0.078	0.157	0.157	0.182

Cognitive data represent mean (S.D.).
Abbreviations: BDNF, Brain-derived neurotrophic factor; MMP-9, Matrix Metalloproteinase-9; WCST, Wisconsin Card Sorting Test.
[a] Log-transformed serum levels of MMP-9 are used.
[b] Spearman's correlation coefficients are calculated for WCST. The other statistical values represent Pearson's correlation coefficients.
* $P<0.05$.
** $P<0.01$.

risperidone (747.1 ± 318.8 [mean ± S.D., ng/mL], n=25); olanzapine (585.3 ± 284.7, n=18); aripiprazole (843.3 ± 454.8, n=9); and quetiapine (662.6 ± 332.4, n=8); (P=0.172).

3.3. Association between mature BDNF levels and clinical variables

Regression analysis revealed no association between serum levels of mature BDNF and MMP-9 in the combined samples, healthy controls, or patients with schizophrenia ($P>0.05$). In schizophrenics, serum levels of mature BDNF were positively associated with SANS total scores (β=0.40, P=0.001) and the Information subtest of WAIS-R (β=0.29, P=0.014), suggesting that higher serum levels of mature BDNF were independently associated with more severe negative symptoms and better performance in the Information subtest. The Trail Making Test Part A was excluded from the stepwise regression model. These associations continued to be significant after adjustment for age, gender and education (SANS total score, β=0.41, P=0.001; Information subtest, β=0.31, P=0.014). In healthy controls, there was no significant association between serum levels of mature BDNF and any other variables. In combined samples, serum levels of mature BDNF were positively associated with Stroop D test scores (β=0.23, P=0.015), implying that higher serum levels of mature BDNF were associated with lower performances in the Stroop D test (naming colors of dots). However, this association was not significant after adjustment for diagnosis, age, and gender.

3.4. Association between MMP-9 levels and clinical variables

In combined samples, serum levels of MMP-9 were positively associated with age (β=0.20, P=0.029) and smoking status (β=0.18, P=0.049), indicating that higher serum levels of MMP-9 were independently associated with older age and smoking. These associations continued to be significant after adjustment for diagnosis and gender (age, β=0.20, P=0.031; smoking status, β=0.20, P=0.035). In healthy controls, serum levels of MMP-9 were positively associated with age (β=0.49, $P<0.001$). In schizophrenics, serum levels of MMP-9 showed positive association with smoking status (β=0.28, P=0.031) and negative association with the Category fluency test (β=−0.25, P=0.047), suggesting that higher serum levels of MMP-9 were independently associated with smoking and lower performance in the Category fluency test. These associations continued to be significant after adjustment for age, gender and education (smoking status, β=0.35, P=0.011; the Category fluency test, β=−0.29, P=0.025).

4. Discussion

We found no differences in serum levels of mature BDNF or MMP-9 between chronically medicated patients with schizophrenia and healthy controls. In the male but not female subgroup, we detected an interaction effect between diagnosis and smoking status for serum levels of MMP-9. Post hoc analysis revealed that serum levels of MMP-9 in male smoking schizophrenics were significantly higher than those in male non-smoking patients.

The mature BDNF ELISA kits used in this study are able to distinguish between the two forms of BDNF and identify mature BDNF from human serum (Yoshida et al., 2012a; Yoshida et al., 2012b). Although a previous study suggested increased mature BDNF levels in patients with schizophrenia, as measured by western-blotting (Carlino et al., 2011), we found no difference between serum mature BDNF levels in patients and healthy controls. This finding is consistent with several previous studies (Shimizu et al., 2003; Huang and Lee, 2006; Mackin et al., 2007; Goto et al., 2011; Niitsu et al., 2011), although in these studies there was no distinction between BDNF isoforms. In addition, serum levels of mature BDNF and MMP-9 remained the same, independent of the atypical antipsychotic treatment. To determine the effects of antipsychotics on serum levels of mature BDNF in schizophrenic patients, further studies using drug-naïve samples are needed.

The influence of smoking on serum BDNF levels remains controversial. A previous study which did not distinguish between BDNF isoforms, showed that serum BDNF levels were significantly higher in male smoking schizophrenic patients compared with male non-smoking patients (Zhang et al., 2010). However, we found no such effects of smoking on mature BDNF serum levels, in total samples or gender specific subsamples. Therefore, additional studies that distinguish between BDNF isoforms will be needed to determine the effects of smoking on serum BDNF levels.

No association between levels of mature BDNF and MMP-9 was found, although MMP-9 plays an important role in the conversion of pro-BDNF to mature BDNF (Hwang et al., 2005; Ethell and

Ethell, 2007). We found that higher serum levels of mature BDNF in schizophrenics were associated with more severe negative symptomology. This finding is consistent with previous studies examining the same sample set (Niitsu et al., 2011) and a completely different sample set (Reis et al., 2008), although these studies did not distinguish between BDNF isoforms. Additionally, we found that higher levels of mature BDNF in patients were associated with a better performance on the Information subtest of WAIS-R, but not on other cognitive tests. This finding may support a recent study which suggested that serum BDNF levels were positively associated with immediate memory performance in chronic patients with schizophrenia (Zhang et al., 2012b), although again, this study did not distinguish between BDNF isoforms. With regards to other proteolytic BDNF isoforms, a previous study suggested that reduced serum levels of truncated-BDNF (a proteolytic BDNF isoform of 28 kDa), but not mature BDNF, correlated with higher positive and lower negative PANSS scores and a poorer performance in all cognitive assays (Carlino et al., 2011). Recently, Carlino et al. proposed a hypothesis stating that deficits in pro and mature BDNF expression may lead to different types of cognitive deficits (Carlino et al., 2013), and our findings go some way to support this hypothesis. Thus, further studies examining the association between the BDNF isoforms and clinical symptoms and cognitive impairment are still needed.

We found no difference in serum MMP-9 levels between patients with schizophrenia and healthy controls. This finding is inconsistent with the results of a proteomic study, using plasma samples from a large case-control collection, which found increased levels of MMP-9 in both male and female patients with schizophrenia (Domenici et al., 2010). It is worth noting however, that this study included a significantly higher percentage of active smokers in the schizophrenia group, compared with controls. In this study, the proportion of male smoking schizophrenics was higher than that of male smoking controls, although this difference was not present in the female subgroup. Interestingly, we found that, in male patients, serum MMP-9 levels in smokers were higher than those of non-smokers, while this was not the case in male controls. It is likely therefore that MMP-9 serum levels in male patients with schizophrenia are affected by smoking. In females, the discrepancy in MMP-9 levels between our results and a previous study (Domenici et al., 2010) may be attributed to the difference in the proportion of smokers between the two studies. In summary, we found that serum MMP-9 levels were associated with age in healthy controls, and smoking status and performance of the Category fluency test in patients with schizophrenia. While the role of serum MMP-9 in the pathophysiology of schizophrenia is currently unclear, further studies are needed to confirm its association with these factors.

As with similar studies, this study has a number of limitations, the most prominent being the small sample size. It is clear that future studies will need larger cohort numbers. Next, the proportion of smokers in the male subgroup differed between patients and controls. This limitation may produce biases in our observations. Finally, we did not examine the levels of pro-BDNF and truncated-BDNF. It is possible that serum levels of BDNF isoforms play a role in the pathophysiology of schizophrenia (Carlino et al., 2011), and therefore it would be of great interest to study the relationship between serum levels of BDNF isoforms, as well as levels of extracellular peptidases (e.g., MMP-9, plasmin) that convert pro-BDNF to other BDNF isoforms.

In conclusion, we found that serum levels of mature BDNF and MMP-9 showed no difference between patients with schizophrenia and controls. However, in male patients only, serum MMP-9 levels of smokers were higher than those of non-smokers. We also found that in patients with schizophrenia, serum mature BDNF levels were associated with negative symptoms and Information subscale scores of WAIS-R, while serum MMP-9 levels were associated with smoking status and Category fluency performance. Further studies measuring the serum levels of mature BDNF and MMP-9 using larger cohorts are needed to examine the results of this study.

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Competing interests

The authors declare that they have no competing interests.

Author contributions

Conception and design: TN, DM, YS, KH, MI. Serum sampling and assay: TI, TY, TH. Analysis and interpretation of data: TN, KH. Drafting the article: TN. Critical review: TY, DM, YS, MN, ES, KH, MI. Final approval of the article: TN, TI, TY, TH, DM, YS, MN, ES, KH, MI.

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Changes in Self-Regulation-Related Prefrontal Activities in Eating Disorders: A Near Infrared Spectroscopy Study

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Abstract

Objective: The aim of this study is to clarify the symptomatology of the eating disorders examining the prefrontal function and activity associated with self-regulation among participants with or without eating disorders.

Methods: Ten patients with anorexia nervosa, fourteen with bulimia nervosa, and fourteen healthy control participants performed two cognitive tasks assessing self-regulatory functions, an auditorily distracted word fluency task and a rock-paper-scissors task under the measurements on prefrontal oxyhemoglobin concentration with near infrared spectroscopy. The psychiatric symptoms of patient groups were assessed with several questionnaires.

Results: Patients with bulimia nervosa showed decreased performances and prefrontal hyper activation patterns. Prefrontal activities showed a moderate negative correlation with task performances not in the patient groups but only in the healthy participants. The prefrontal activities of the patient groups showed positive correlations with some symptom scale aspects.

Conclusions: The decreased cognitive abilities and characteristic prefrontal activation patterns associated with self-regulatory functions were shown in patients with bulimia nervosa, which correlated with their symptoms. These findings suggest inefficient prefrontal self-regulatory function of bulimia nervosa that associate with its symptoms.

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Introduction

Eating disorders (EDs) are life-threatening mental diseases that include anorexia nervosa (AN), bulimia nervosa (BN), and related disorders. ED patients take extreme preoccupation with foods and desire for thinness. They make an effort to achieve significant weight loss through restricting fattening food, exercising, and/or purging behaviors (self-induced vomiting, laxatives/diuretics abuse), but they sometimes overeat impulsively – bingeing.

Personality traits are important predisposition for ED. Both AN and BN patients are characterized by their personality traits, such as perfectionism, obsessive-compulsiveness, neuroticism, negative emotionality, harm avoidance, low self-directedness, and low cooperativeness [1,2]. Patients with these features causing an impairment of daily life are diagnosed as having comorbid personality disorders, which can affect symptoms [3], dysregulation of dietary behavior [4], and the onset and course [5–7] of ED. One highlighted aspect is self-regulation with its potential contribution to the symptoms of ED. Changes in self-regulation have been discussed in the context of characteristic personality and pathologic behavior of ED, especially related with impulsivity. Binge eating/purging type of AN and BN show loss of self-control [1,3,8,9] while restrictive type of AN accompanies overly controlled behavioral style [10,11]. Binge eating/purging type of AN and BN, which have in common bulimic behaviors and sensitivity to substance use problems, are thus considered to be related to difficulties in self-regulation seen as an impulsivity [1,12]. According to a review paper, changes in self-regulation seem relevant to deficits in the executive functioning such as set-shifting that is also observed in ED [13].

Abnormal prefrontal function of ED had been shown in previous investigations with functional neuroimaging methods. Typically, studies have used tasks/stimuli provoking core symptoms and shown abnormal activations of brain areas including the prefrontal cortex (PFC) [14–16]. These authors explained the observed involvement of prefrontal areas as representations of compulsive feature of behavior [14] and emotional processing and aversive response to the provocative stimuli [14,17,18]. Provocation studies with distorted body images induced incongruent results in the PFC activations of patient groups [19–21]. These authors explained such inconsistent decreased activities of patient groups associating with a realistic perception of own body size and shape [20], however possibly including influence of difference in stimuli [19,21].

Table 1. Demographics of all participants, and scores of symptom scales relevant to the ED group.

		HC (N = 14)		ED (total N = 24)									η^2
				Total			AN (N = 10)			BN (N = 14)			
		mean	SD	mean	SD	N	mean	SD	N	mean	SD	N	
Age		24.1	3.0	26.1	7.1	24	27.7	8.4	10	25.0	6.1	14	0.06
Height		159.7	4.9	157.4	4.6	23	157.1	4.7	10	157.7	4.7	13	0.06
Weight		53.7	7.5	45.8	8.2	23	42.2	8.7	10**	48.6	6.9	13	0.28
BMI		21.0	2.2	18.5	3.2	23	17.0	3.1	10**	19.6	3.0	13	0.27
Period of illness (months)				67.6	53.5	21	42.7	34.6	7	80.1	57.9	14	
Treatment period (months)				36.6	42.9	20	19.4	16.8	7	45.9	50.1	13	
Antidepressants treatment						4			0			4	
Antipsychotics treatment						3			2			1	
Anxiolytics treatment						8			2			6	
History of hospitalization				(11 of 20 patients had)			(5 of 7 patients had)			(6 of 13 patients had)			
HADS	Anxiety	3.3	1.9	11.1	3.0	24	10.8	3.0	10***	11.4	3.1	14***	0.69
	Depression	1.8	1.6	9.3	3.7	24	6.8	3.4	10***	11.1	2.8	14***	0.72
EDI				127.9	41.4	15	107.3	42.4	6	141.6	36.8	9	
EDE-Q	Global			3.9	1.3	16	3.3	1.5	6	4.2	1.1	10	
	Restraint			3.3	1.7	16	3.4	1.8	6	3.2	1.6	10	
	Eating			3.9	1.5	17	3.4	1.8	7	4.2	1.4	10	
	Weight			4.2	1.6	17	3.4	1.7	7	4.7	1.3	10	
	Shape			4.4	1.3	16	3.8	1.6	6	4.7	1.1	10	
BITE	Severity			13.4	5.8	13	12.0	7.1	4	14.0	5.5	9	
	Symptom			22.9	3.5	13	22.0	5.3	4	23.3	2.6	9	
TAS-20				65.4	5.2	15	63.4	3.5	5	66.4	5.7	10	

Notes: One-way analysis of variances was performed to compare age, height, weight, BMI and HADS subscales between the HC, AN and BN groups. HC, healthy control; ED, eating disorder; AN, anorexia nervosa; BN bulimia nervosa; SD, standard deviation; BMI, body mass index; HADS, Hospital Anxiety and Depression Scale; EDI, the Eating Disorder Inventory; EDE-Q, the Eating Disorder Examination Questionnaire, BITE, the Bulimic Investigatory Test; TAS-20, the 20-item Toronto Alexithymia Scale. Significant differences between each patient group with the HC group were shown with ***$p<0.001$, **$p<0.01$ or *$p<0.05$.

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PFC is known to be involved in self-regulation of irrelevant behavior [22–26] and emotion [27]. The cognitive down-regulation of craving was associated with activity in prefrontal regions associated with regulating emotion and cognitive control in general, including dorsomedial, dorsolateral, and ventrolateral prefrontal cortices [28]. To examine associations between self-regulation, symptoms, and brain function of ED patients, researchers have used neuroimaging studies in combination with unique tasks that relate seemingly "indirectly" with symptoms. For instance, a monetary reward gambling task was used to reflect an improper value judgement of ED patients [29]. With the Simon spatial incompatibility task associated with self-regulatory control, BN patients showed an impaired activation pattern in their prefrontal neuronal network [30]. Despite the proposed importance to understand pathophysiology of characteristic self-regulation of ED, little has been shown addressing their relationships with prefrontal function.

The aim of the present study was to investigate prefrontal functions associated with self-regulation and symptoms of ED. We utilized two cognitive tasks related with self-regulation, an auditorily-distracted word fluency task (WFT) to assess inhibitory processing for emotional distraction, and a rock-paper-scissors task with intentional loss ($RPST_{loss}$) to assess self-regulatory control on habitual behavior [23,25,31] and emotion. To assess the prefrontal activity evoked with the cognitive tasks, we measured prefrontal oxyhemoglobin concentrations ([oxyHb]) with near infrared spectroscopy (NIRS).

Our hypotheses were that (i) ED patients had changed cognitive abilities seen in the task performances associated with self-regulation; (ii) the expected abnormal cognitive functions were accompanied by changes in prefrontal blood perfusion; and (iii) the cognitive abnormalities and prefrontal activity were associated with the severity of ED symptoms measured with symptom scales.

Methods

Participants

Twenty-four female patients fulfilling the DSM-IV-TR criteria of EDs were recruited from the inpatient (N = 5) and outpatient (N = 19) units of Chiba University Hospital (Table 1) and divided into the AN group (10 patients; four restrictive type and six binge eating/purging type) and the BN group (14 patients; 13 purging type and one non-purging type). Among the patients with AN, two patients were medicated with antipsychotics (olanzapine and sulpiride). Among the patients with BN, three were medicated with antidepressants only (sertraline, setiptiline and lofepramine), one with both antidepressant (trazodone) and antipsychotics (olanzapine and risperidone), and ten without either antidepressants or antipsychotics. Anxiolytics, hypnotics, and tranquilizing drugs were prescribed for two patients with AN and six patients with BN.

One patient with AN had comorbid major depression and another with AN had dissociative disorder, and no other participant was diagnosed as having comorbid psychiatric disorder. Three patients classified into the BN group had past history of AN. Fourteen age-matched healthy control (HC) females who had no history of psychiatric disorder were also recruited. Exclusion criteria for the participants were left-handed, comorbid progressive physical disorder, and a history of personality disorder, head injury, drug dependency, or alcoholic dependency. All participants were native Japanese and very familiar with the ordinary rock-paper-scissors game for a long time since a young age. All subjects participated after written informed consent for study participation was obtained. The study was approved by the Institutional Review Board of the Graduate School of Medicine, Chiba University and conformed to the provisions of the Declaration of Helsinki in 1995.

Cognitive Tasks

The cognitive tasks to evoke prefrontal cortical activity, the WFT and the $RPST_{loss}$, were performed by all participants. The subjects sat on a comfortable chair in a room lit by daylight. The tasks were computed with RSVP Experiment ver. 1.1 [32]. Subjects were visually instructed regarding the performance of all task procedures and were cued on a PC display placed in front of them.

1) WFT. In the previous researches, WFT has been used as "a general frontal activating task" that focus on evidence of neural functions (e.g., activating areas) and experimental usability than on relations with traits or symptoms [33,34]. We modified it aiming at exposing emotion inhibition adding auditory distracters during performing ordinary WFT. The subjects performed two WFT trials, each consisting of a 30-sec pretask baseline, a 60-sec task, and a 60-sec posttask (Figure 1A). During task periods of the WFT, the subjects were instructed to generate as many words as they could that had a particular initial syllable [34]. There were two syllable-sets prepared to be presented: (A)/a/,/ka/, and/sa/, and (B)/i/,/ki/, and/shi/. In each 60-sec task period, the three syllables within one of the two syllable-sets were presented, changing in turn every 20 sec (Figure 1B). The number of words generated during the task was determined as a task performance. In pretask and posttask periods, the subjects were instructed to repeat the syllables,/a/,/i/,/u/,/e/, and/o/at approximately 1 Hz.

During each WFT trial, one of two auditory distracters, baby crying or white noise; was presented with a headphone at approximately 70 dB(A) at peaks. Baby crying was expected to be a stronger distracter than white noise, to arouse not only auditory neuronal processing but also emotion-inhibitory processing. Baby crying was also expected as a non-verbal noise that reduces the risk of giving participants any verbal "cues" to answer WFT [35–37]. The two trials were separated by an interval of at least 2 min. The presenting order of stimuli consisting of two syllable-sets and two distracters was determined in a pseudorandom order among the participants.

2) $RPST_{loss}$. Use of the ordinary rock-paper-scissors game is a simple and easy method to divide two or more players into winner and loser, and it is more familiar and played more frequently than a coin toss for most Japanese. Players inwardly choose one of three hand shapes, a fist for a rock, a flat palm for a piece of paper, or splayed second and third fingers for scissors, and then simultaneously present their hands while forming the chosen shapes. When two players participate, the relationship between the hands of the two players decides their outcomes of winning, losing, or having a draw. Rock beats scissors because a rock break metal, paper beats rock because paper wraps around a rock, and scissors beats paper because scissors cut a paper. If the same hand shapes are presented, the result is a draw and the game is replayed until a player wins.

In order to assess self-regulatory control, we developed the $RPST_{loss}$ (Figure 1C) based on an ordinary rock-paper-scissors game. In this task, the three pictures of hand shapes were shown one by one in a random order (Figure 1D). The subjects were instructed to respond by hitting any one of three buttons corresponding to each of three hand shapes, at which point the picture disappeared and the next picture was shown after 300 ms of a blank display. The subjects were told to respond so that they would intentionally lose the games during a task period and so that they would reach a draw during pretask and posttask periods as fast and correctly as possible [31]. Each of such three periods lasted until it met predefined requirements for both time and the number of games played, >30 sec and >30 games for the pretask, >60 sec and >48 games for the task, and >60 sec and >60 games for the posttask. For these numbers of games (30, 48, and 60 games), the ratio of correct responses ("accuracy") and the mean reaction time were determined as the subjects' performances. To analyze the [oxyHb] data of $RPST_{loss}$, the time lengths of which varied with the individual participants, 30 sec of initial pretask period, 60 sec of initial task period, and 60 sec of initial posttask period were extracted to consecutively combined. Hence, the time periods used for the analyses of [oxyHb] overlapped but were strictly incongruent with those for the performances.

NIRS Measurements

During the tasks, prefrontal [oxyHb] changes were measured with two-channel NIRS (NIRO-200, Hamamatsu Photonics K.K., Hamamatsu, Japan) at three wavelengths of near infrared light (775, 810 and 850 nm) with a sampling rate of 2 Hz. The detection probes were placed on the Fp1 and Fp2, and the emission probes were 3 cm lateral from the detection probes along the T3-Fpz-T4 line, according to the international 10/20 system used in electroencephalography. The points measured with this setup were projected to the lower dorsolateral PFC to the orbitofrontal areas (BA 9–10, Figure 2) [38,39].

The acquired [oxyHb] data were preprocessed as described below. Five-sec moving average methods were done to attenuate high-frequency components such as motion artifacts. Data were transformed into the z score against the mean [oxyHb] of the pretask period in each channel. Linear data-trends between the mean of a pretask period and the last twenty sec of a posttask period were removed from the z scores of the WFT to remove a low-frequency band as noise. Removal of linear data-trend was not performed on data of the $RPST_{loss}$ to avoid removal of different frequency band by the individual participants, because of varying time length spent for this task. The z scores of task and posttask periods were each divided into first and last halves (task1, task2, posttask1, and posttask2), averaged respectively, and subjected to statistic analyses.

Assessments of Clinical Symptoms

All participants completed the Hospital Anxiety and Depression Scale (HADS) [40], and ED group were assessed using the Eating Disorder Inventory-3 (EDI) [41], the Eating Disorder Examination Questionnaire (EDE-Q) [42], the Bulimic Investigatory Test, Edinburgh (BITE) [43], and the 20-item Toronto Alexithymia Scale (TAS-20) [44,45].

Statistical Analyses

The experimental values were statistically analyzed with SPSS 12.0 (SPSS, Inc., Chicago, IL). One-way analysis of variances and

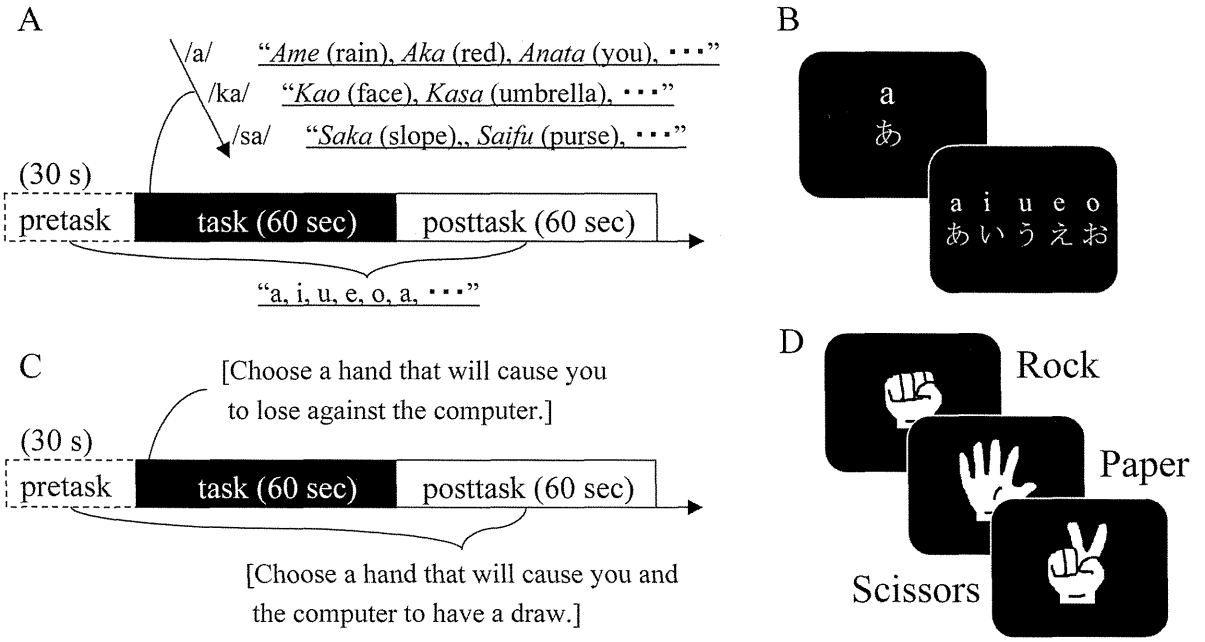

Figure 1. Task procedures of the word fluency task (WFT) and the rock-paper-scissors intentional loss task ($RPST_{loss}$). (A) Time schedule of one trial of the WFT. (B) Visual instructions presented in the WFT. A set of three different syllables was presented for 20 sec each during a 60-sec task period, to serve as the initial syllables for word generation. During the pretask and posttask periods, participants were instructed to repeat the presented five syllables in turn. (C) Time schedule and instructions of one trial of the $RPST_{loss}$. (D) Three hand shape pictures presented in the $RPST_{loss}$. The same pictures were used during the pretask, task, and posttask periods.
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Bonferroni's comparisons were performed for the variables measured in all participant groups, that is, demographics (age, height, weight and BMI) and scores of symptom scales (two subscales of HADS). To extenuate possible effects of malnutrition for cognitive functioning (e.g., effects of cortisol [46] and Triiodothyronine [47]), the analyses for task performances were done controlling for BMI as a covariate of no interest only if these parameters met prerequisites to be controlled; 1) showing no significant interaction with BMI and 2) showing significant linear correlation with BMI. Consequently, no task performance parameter was controlled for BMI. Repeated measures of analysis of variances and Bonferroni's comparisons were performed for task performances and [oxyHb] data. Exploratory correlation analyses were performed between [oxyHb] data, task performances, and symptom scales. As correlation coefficients we used Spearman's rho for data pair including one non-normal variable (or both) and Pearson's r for data pairs with only normal variables. Bonferroni's correction was not performed for the probabilities of correlation coefficients in order to avoid attenuating power of

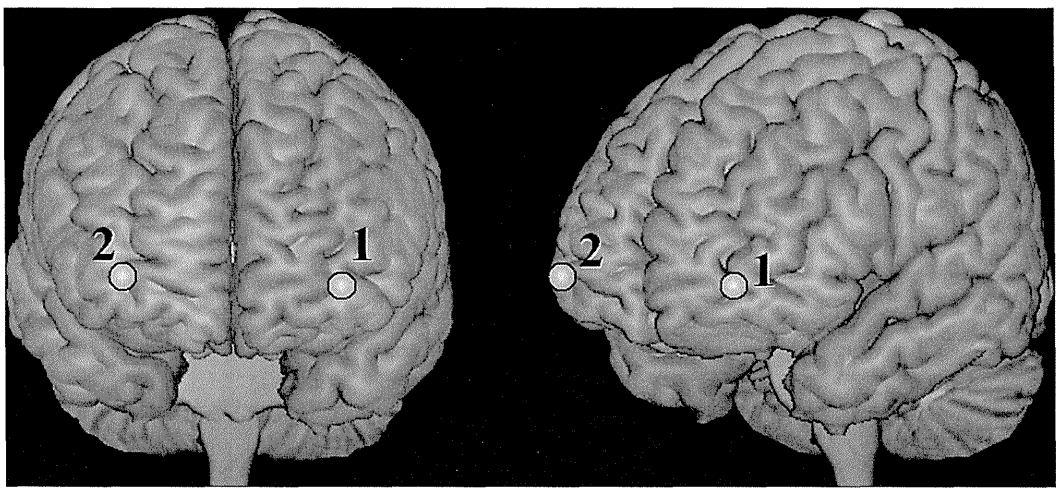

Figure 2. Brain sites observed with near infrared spectroscopy. The oxyhemoglobin concentrations in the left and right regions (channels 1 and 2, respectively) over the lower dorsolateral prefrontal to orbitofrontal areas were measured. These regions are labelled in the standard brain space [38,39].
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detection in this study. Values with $p<0.05$ were considered statistically significant.

Results

Demographics and Clinical Characteristics

The demographics and scores of symptom scales of participants are listed in Table 1. The AN, BN, and also the group with combined ED showed elevated scores in both HADS subscales compared to the HC.

WFT (Figures 3, 4)

Regarding the number of words produced during the WFT, a significant interaction was observed between the group factor and the distracter factor ($F(2,34)=3.389$, $p=0.045$, partial $\eta^2=0.166$). Simple main effect of the group factor during hearing baby crying ($F(2,34)=3.750$, $p=0.034$, partial $\eta^2=0.181$) suggested that the BN group generated significantly fewer words than did the HC ($p=0.033$) and no difference was seen between the AN group and the HC during the task ($p=1.000$). A significant main effect of the auditory distracter factor was seen ($F(1,34)=15.900$, $p<0.001$, partial $\eta^2=0.319$), as expected. Comparing the two auditory distracters, baby crying was found to affect word generation significantly than white noise in the patients with HC ($F(1,34)=7.277$, $p=0.011$, partial $\eta^2=0.176$) and BN ($F(1,34)=18.771$, $p<0.001$, partial $\eta^2=0.356$), whereas the AN group showed no significant difference ($F(1,34)=0.066$, $p=0.799$, partial $\eta^2=0.002$). This analysis was performed not controlled for BMI because there was no significant linear correlation (with white noise, $t=0.817$, $p=0.420$; with baby crying, $t=-0.438$, $p=0.664$) between BMI and the distracter factor, while there was a significant interaction ($F(1,33)=4.564$, $p=0.040$).

The [oxyHb] changes in bilateral prefrontal regions during the WFT depicted a regular arching pattern within all groups and conditions commonly, which increased during the task and decreased during the posttask period. Among the auditory distracter factor, the task phase factor, and the group factor, there was a significant secondary interactions in the left ($F(6,102)=2.331$, $p=0.038$, partial $\eta^2=0.118$) but right ($F(6,102)=1.468$, $p=0.197$, partial $\eta^2=0.079$) channels. However, no significant intergroup difference was found in *post hoc* multiple comparisons bilaterally. There was no significant primary interaction [left, (group*distracter, $F(2,34)=3.129$, $p=0.057$, partial $\eta^2=0.155$; group*task phase, $F(6,102)=0.417$, $p=0.866$, partial $\eta^2=0.024$; distracter*task phase, $F(3,102)=0.702$,

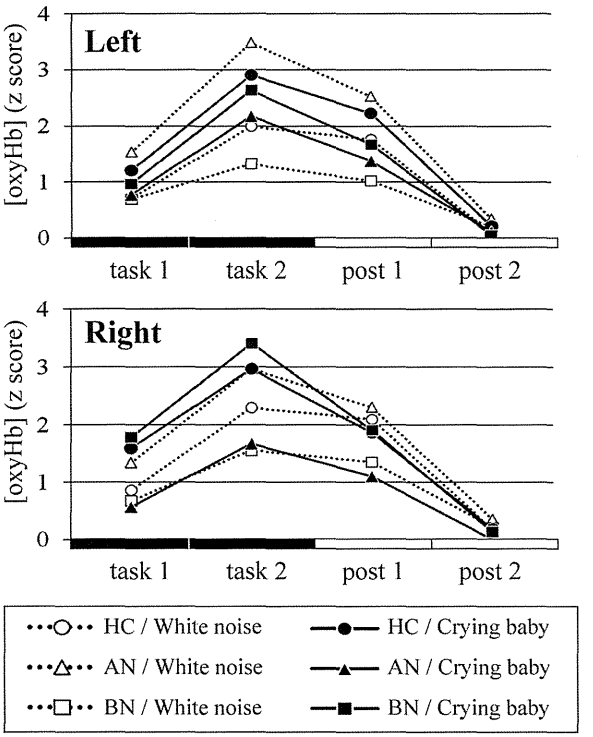

Figure 4. Oxyhemoglobin concentration changes during the word fluency task in the bilateral prefrontal cortices. Mean z scores of [oxyHb] during the task and posttask periods each divided into first and last halves are shown. Circular, triangular, and square plots represent the healthy control, anorexia nervosa, and bulimia nervosa groups, respectively. Outlined plots with dashed lines and filled plots with solid lines represent [oxyHb] with white noise and baby crying as an auditory distracter, respectively. There was no significant difference between groups or between auditory distracters. HC, healthy control; AN, anorexia nervosa; BN, bulimia nervosa.
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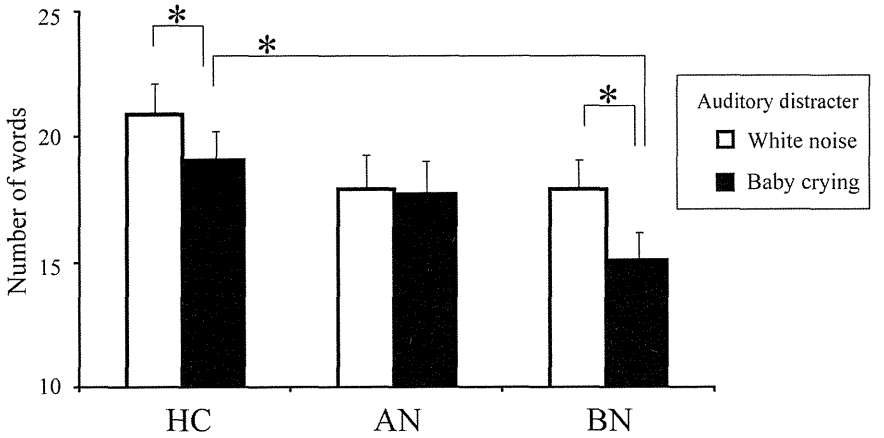

Figure 3. Mean numbers of generated words during task periods of the word fluency task (WFT). Two WFT trials were performed with two types of auditory distracters, respectively: white noise (outlined bars) and baby crying (filled bars). Baby crying significantly affected word generation by the HC and BN groups. The BN group generated a significantly lower number of words than the HC when during hearing the crying baby distracter. Error bars represent the standard error of the mean. HC, healthy control; AN, anorexia nervosa; BN, bulimia nervosa; *$p<0.05$.
doi:10.1371/journal.pone.0059324.g003

$p = 0.553$, partial $\eta^2 = 0.020$); right, (group*distracter, $F(2,34) = 2.314$, $p = 0.114$, partial $\eta^2 = 0.120$; group*task phase, $F(6,102) = 0.090$, $p = 0.997$, partial $\eta^2 = 0.005$; distracter*task phase, $F(3,102) = 1.564$, $p = 0.203$, partial $\eta^2 = 0.044$)]. A significant main effect of the task phase factor was detected (left, $F(3,102) = 30.560$, $p<0.001$, partial $\eta^2 = 0.473$; right, $F(3,102) = 20.918$, $p<0.001$, partial $\eta^2 = 0.381$) and the [oxyHb] differed between all four time points. No significant main effect of the auditory distracter factor (left, $F(1,34) = 0.046$, $p = 0.831$, partial $\eta^2 = 0.001$; right, $F(1,34) = 0.062$, $p = 0.805$, partial $\eta^2 = 0.002$) or the group factor (left, $F(2,34) = 0.365$, $p = 0.697$, partial $\eta^2 = 0.021$; right, $F(2,34) = 0.051$, $p = 0.950$, partial $\eta^2 = 0.003$) was seen in the bilateral channels. In each patient group, classifying and comparing the [oxyHb] data of participants with or without each type of drug medication suggested that medication did not contribute our findings.

$RPST_{loss}$ (Figures 5, 6)

During interviews after the examinations, almost all of the participants reported that intentionally losing the games was more difficult than playing to a draw and that they felt chagrined at erroneously winning. Regarding the accuracy of responses, there were no significant interaction ($F(4,64) = 0.981$, $p = 0.424$, partial $\eta^2 = 0.056$) or main effect found among the task phase factor ($F(2,66) = 0.830$, $p = 0.441$, partial $\eta^2 = 0.025$) and the group factor ($F(2,33) = 0.698$, $p = 0.505$, partial $\eta^2 = 041$). No difference was observed between groups in *post hoc* comparisons. Analyses on the reaction time in $RPST_{loss}$ suggested that the BN group had a longer reaction time than that of the HC during the task period ($F(2,33) = 4.165$, $p = 0.024$, partial $\eta^2 = 0.202$), even though no significant interaction was detected between the group factor and the task phase factor ($F(4,64) = 2.186$, $p = 0.090$, partial $\eta^2 = 0.117$). The main effects of the group factor ($F(2,33) = 4.167$, $p = 0.024$, partial $\eta^2 = 202$) and the task phase factor ($F(2,66) = 106.079$, $p<0.001$, partial $\eta^2 = 0.763$) were found. The accuracy variable was not controlled for BMI because BMI showed no significant interaction ($F(2,64) = 0.008$, $p = 0.992$) or a significant linear correlation (the pretask, $t = 1.237$, $p = 0.225$; the task, $t = 0.687$, $p = 0.497$; the posttask, $t = 0.351$, $p = 0.728$) with the task phase factor. The reaction time variable was also not controlled for BMI because of no significant interaction ($F(2,64) = 0.001$, $p = 0.999$) or a significant linear correlation (the pretask, $t = -1.774$, $p = 0.086$; the task, $t = -0.590$, $p = 0.559$; the posttask, $t = -0.908$, $p = 0.371$) between BMI and the task phase factor.

The [oxyHb] changes during the $RPST_{loss}$ were analyzed using the task phase factor and the group factor. In the [oxyHb] data, no interaction between the two factors (left, $F(6,102) = 1.557$, $p = 0.167$, partial $\eta^2 = 0.084$; right, $F(6,102) = 1.185$, $p = 0.320$, partial $\eta^2 = 0.065$) or a main effect of group factor (left, $F(2,34) = 1.007$, $p = 0.376$, partial $\eta^2 = 0.056$; right, $F(2,34) = 0.376$, $p = 0.690$, partial $\eta^2 = 0.022$) was observed. Multiple comparisons using both the task phase and group factors revealed a characteristic [oxyHb] change pattern in the BN group which is an increasing and decreasing activation pattern linked to the task course (left, $F(3,32) = 7.053$, $p<0.001$, partial $\eta^2 = 0.398$; right, $F(3,32) = 8.240$, $p<0.001$, partial $\eta^2 = 0.436$). The [oxyHb] of the BN group showed a significant elevation (left, $p = 0.004$; right, $p<0.001$) toward a peak at the latter half of the task period, and then a decrease (left, $p = 0.008$; right, $p = 0.062$; between the peak and the latter half of the posttask period). At this peak period, but not at other three periods, weak group difference of distribution was suggested in the left channel (left, $F(2,34) = 2.547$, $p = 0.093$, partial $\eta^2 = 0.130$; right, $F(2,34) = 1.592$, $p = 0.218$, partial $\eta^2 = 0.086$). Such activation pattern was not detected in the AN group (left, $F(3,32) = 0.531$, $p = 0.664$, partial $\eta^2 = 0.047$; right, $F(3,32) = 0.940$, $p = 0.433$, partial $\eta^2 = 0.081$) and the HC group (left, $F(3,32) = 0.237$, $p = 0.870$, partial $\eta^2 = 0.022$; right, $F(3,32) = 0.817$, $p = 0.494$, partial $\eta^2 = 0.071$). The patterns of the [oxyHb] changes associated with the $RPST_{loss}$ were relatively flat, unlike those associated with the WFT, because of different preprocessing [oxyHb] data for each task (see 2.3 NIRS measurements). In each patient group, classifying and comparing the [oxyHb] data of participants with or without each type of drug medication suggested that medication did not contribute to our findings.

Correlation Coefficients of Prefrontal Activity with Task Performances and Symptom Scales (Table 2)

To investigate the relationships of prefrontal activity with cognitive functions and symptoms in ED patients, we assessed the correlation coefficients between [oxyHb] data, task performance, and symptom ratings. Although no significant main group effect was pointed in any [oxyHb] data, we took notice of data at the latter half of the task period of $RPST_{loss}$ for correlation analyses according to a result of a weak group difference of distribution suggested, because it showed characteristic values that could have different features between groups and be a biomarker of ED. At this phase the [oxyHb] data of only the HC showed a moderate negative correlation with the accuracy (left, Spearman's rho = -0.548, $p = 0.065$; right, rho = -0.354, $p = 0.259$), while did not those of the AN group (left, rho = -0.031, $p = 0.933$; right, rho = 0.117, $p = 0.747$) and the BN group (left, rho = 0.297, $p = 0.324$; right, rho = -0.014, $p = 0.963$). No significant correlation was found between task performance variables and the [oxyHb] data at other task phases.

In the patient groups, the [oxyHb] data positively correlated with some ED symptom scales (Table 2). The left channel [oxyHb] data of the AN group positively correlated with the severity subscale of BITE (Pearson's $r = 0.977$, $p = 0.023$). The [oxyHb] of the BN group correlated significantly positively with the global score (right; $r = 0.690$, $p = 0.027$) and the restraint subscale (left, $r = 0.675$, $p = 0.032$; right, $r = 0.896$, $p<0.001$) of EDE-Q, and the severity subscale (right, $r = 0.811$, $p = 0.008$) of BITE.

Discussion

Different profiles in the tasks associated with self-regulation were found between the BN group and the HC group: the BN group showed decreased cognitive functions in the WFT through the emotion inhibition task and the $RPST_{loss}$, and the latter was accompanied by characteristic prefrontal activity. Moreover, prefrontal activity seen in the $RPST_{loss}$ correlated with symptom scales in both the AN and BN groups. The data supports our first hypothesis that ED patients had changed cognitive abilities seen in the task performances associated with self-regulation.

WFT

In the WFT, the BN patients hearing a baby crying showed lower verbal fluency than that of the HC, suggesting disordered emotion inhibition in the BN group. Previous research suggested that verbal fluency of ED patients did not differ from that of the HC but reflected frontal lobe dysfunction and ED symptoms [33]. We were able to measure the difference in verbal fluency between the groups that was not observed previously, probably because of using an additional factor, an auditory distracter. Neuropsychological studies have revealed various aspects of cognitive deficits in BN patients, such as executive functioning, visual-spatial ability,

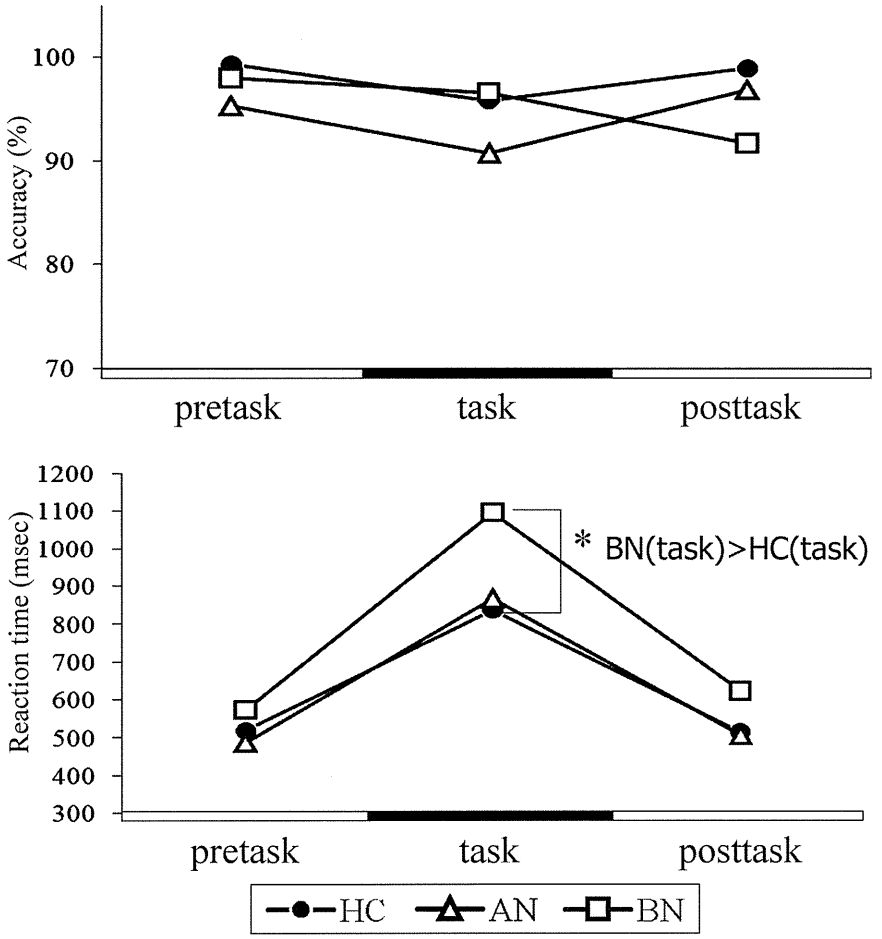

Figure 5. Mean values of accuracy and reaction time of the rock-paper-scissors intentional loss task. Mean values of accuracy and reaction time of pretask and posttask (instructed to be draw games) and task (instructed to lose intentionally) periods are shown. Circles, triangles, and squares represent the healthy control, anorexia nervosa, and bulimia nervosa groups, respectively. There was no group difference in accuracy. The bulimia group showed a significantly longer reaction time than that of the healthy controls during the task period. BN, bulimia nervosa; HC, healthy control; *$p<0.05$.
doi:10.1371/journal.pone.0059324.g005

divided and sustained attention, verbal functioning, learning, and memory [13,48,49]. The decreased verbal fluency under existence of emotional auditory distracter in the BN group observed in this study should be a newly revealed aspect of this multidimensional cognitive impairment. This vulnerability of BN might reflect strong perceived expressed emotion [50] or distress tolerance which was associated with eating attitudes [51]. Meanwhile, the AN group showed no evidence of cognitive impairment, but another feature of the AN was a lack of auditory distracting effect expected, i.e., both sounds had an equal distractive effect. Since there is no difference in TAS-20 between the AN and BN groups, the effect of alexithymia on processing of both distracters was inapparent.

To our knowledge, this was the first study to measure prefrontal function measured with baby crying stimuli in the patients with ED. Prefrontal blood flow showed strong peaks reflecting the time course of the WFT. Unlike the result of task performance, no group difference was clarified in prefrontal activation. Word generation performed in the WFT is known to involve widespread brain areas including inferior frontal gyrus, medial frontal, supplementary motor area, premotor area, cingulate cortex, putamen, etc. [52–56]. Because of innate limitation of NIRS, [oxyHb] observation is limited to narrow and superficial regions. Taken together, brain areas other than those measured with NIRS but relating to word generation and/or emotional self-regulation might be involved with the decreased verbal fluency in the BN group only under an emotional auditory distracter. These results could propose a prudent view to usage of WFT just as "a common prefrontal activation task", not as a task of verbal fluency. Further studies using techniques such as functional magnetic resonance imaging and positron emission tomography are required to investigate the involvement of other cortical areas than PFC as well as deep brain structures.

$RPST_{loss}$

The longer reaction time during the task period than during the pretask and posttask periods and impressions by participants suggest that it was more difficult to lose than to be draw for subjects who were familiar with an ordinary rock-paper-scissors game. In previous studies, the authors believed that the difficulty of intentionally losing reflected a malfunction of stereotypical response inhibition, since wanting to win the game is the stereotypical response [23,25,31]. The authors of these studies using the game have interpreted intentional loss trials as triggers of cognitive conflict and self-regulatory control associated with

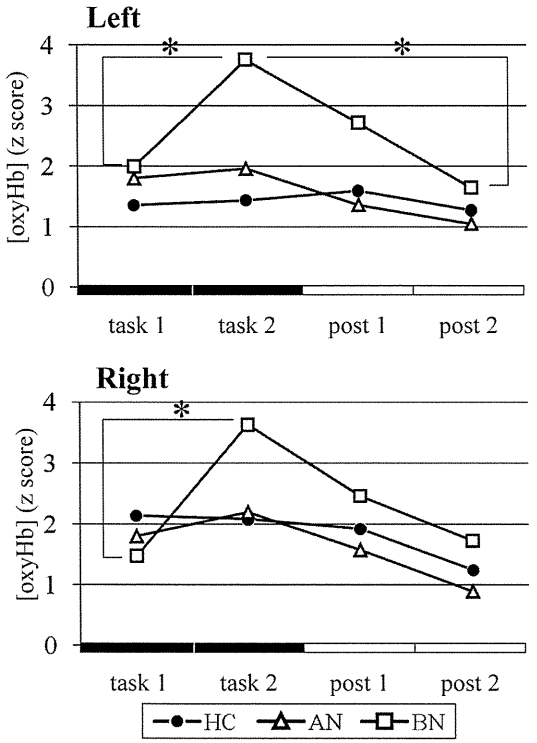

Figure 6. Oxyhemoglobin concentration changes during the rock-paper-scissors intentional loss task in the bilateral prefrontal cortices. Mean z scores of [oxyHb] during the task and posttask periods each divided into first and last halves are shown. Circles, triangles, and squares represent the healthy control, anorexia nervosa, and bulimia nervosa groups, respectively. Only the bulimia group showed increasing and decreasing activation patterns of [oxyHb]. *$p<0.05$.
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habitual behavior. Moreover, most participants reported self-accusation and feelings of inefficiency as part of their introspections associated with erroneous winning. In addition to the act of trying to win being habitual behavior, we propose that the self-regulation was also directed to emotions such as self-accusation, unpleasantness, or anger; i.e., reactions to perfectionistic ideas. Hence, the most likely reason for the prolonged reaction time during the task period compared to the pretask/posttask periods was the requirement for additional information processing within the brain.

The BN patients showed two specific features revealed with the $RPST_{loss}$. First, they required a prolonged reaction time compared to that of the HC only during the task period. We propose that, applying the abovementioned concepts, BN patients had more difficulty self-regulating and required more effort to achieve self-regulation to achieve an equal level of accuracy to that of HC during task periods. Adversely, since no group difference was seen during the pretask and posttask periods, no difference was demonstrated in brain functions when playing to a draw, such as cognition of presented hand shapes, decision of the same hand shape, and choice and hitting the appropriate buttons.

Secondly, only the BN group had the increasing and decreasing [oxyHb] change pattern in PFC linked to the task course. Why were the prefrontal areas activated, and why in the BN group? The cognitive concept tested in the $RPST_{loss}$ was self-regulation on habitual stereotypical behavior [23,25]. Greater impulsivity and disinhibition (lack of control) are often seen in BN patients compared with AN patients as episodes of eating behaviors [3,8,9], and the DSM-IV diagnostic description of BN requires disinhibition over binge eating episodes. Moreover, PFC carries self-regulation of irrelevant behavioral responses [22,24,26], habitual stereotypical behavior [23,25], and also emotion [27] by exerting an inhibitory physiological influence over other brain areas. A neuroimaging study showed that adolescent ED patients with binge eating/purging had greater activation during a response inhibition task in hypothalamus and right dorsolateral PFC compared to both HC and patients with restrictive type of AN

Table 2. Correlation coefficients of prefrontal activity with task performances and symptom scales.

		AN				BN			
		left		right		Left		Right	
		r	*p*	*R*	*p*	*R*	*p*	*r*	*p*
HADS	Anxiety	0.400	0.253	0.177	0.625	−0.267	0.357	−0.187	0.522
	Depression	0.465	0.176	0.461	0.180	−0.049	0.868	0.080	0.786
EDI		−0.040	0.940	−0.188	0.721	0.054	0.890	0.619	0.075
EDE-Q	Global	−0.377	0.462	−0.415	0.413	0.210	0.561	0.690	0.027*
	Restraint	−0.190	0.718	−0.442	0.380	0.675	0.032*	0.896	0.000*
	Eating concern	−0.337	0.460	−0.403	0.371	−0.109	0.764	0.385	0.271
	Weight concern	−0.249	0.590	−0.213	0.647	0.019	0.959	0.490	0.150
	Shape concern	−0.371	0.468	−0.389	0.446	−0.062	0.865	0.310	0.383
BITE	severity	0.977	0.023*	0.810	0.190	0.632	0.068	0.811	0.008*
	symptom	−0.494	0.506	−0.695	0.305	0.342	0.368	0.662	0.052
TAS-20		−0.541	0.347	0.101	0.872	−0.263	0.463	−0.005	0.990
BMI		−0.432	0.213	−0.209	0.563	−0.156	0.610	−0.329	0.273

Notes: Correlation coefficients were calculated between the symptom scales and mean z scores of [oxyHb] data during the latter half of the task period of the $RPST_{loss}$. AN, anorexia nervosa; BN, bulimia nervosa; HADS, Hospital Anxiety and Depression Scale; EDI, the Eating Disorder Inventory; EDE-Q, the Eating Disorder Examination Questionnaire; BITE, the Bulimic Investigatory Test; TAS-20, the 20-item Toronto Alexithymia Scale; BMI, body mass index. *r*: Pearson's correlation coefficient, *$p<0.05$.
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[57]. In line with this context, we propose that the BN patients have lower and inefficient self-regulatory function of PFC that consequently appears as impulsivity. The term "inefficient" was derived from that, despite blunted functioning of PFC, the BN patients might require strong prefrontal activation to perform a difficult "to lose" task with the high accuracy equal to that of the HC. The prefrontal hyperactivity pattern of these patients might represent compensatory activation, i.e., the recruitment of additional brain regions, and/or discrepant brain activation patterns with impaired cognitive ability [58].

Bingeing has been considered to be related to impulsivity [1]. Patients with restrictive type of AN, i.e., without bingeing, are less impulsive than are HC [10,11]. Indeed, in the present study, if the six binge eating/purging type of AN patients and all BN patients were combined into a "bingeing group", their combined [oxyHb] data had a similar activation pattern to that of the original BN group, and the task performance variables (accuracy and reaction time) had no group difference (data not shown) supposedly because of limited participation of patients with restrictive type of AN (N = 4), following the findings in the previous study [57]. These results suggest that BN and binge eating/purging type of AN have a common (or similar) mechanism of prefrontal self-regulatory function representing patients' impulsivity and eating behavior.

Meanwhile, the AN group showed no specific feature of task performance variables or prefrontal activation in the $RPST_{loss}$ compared to the HC. This might be because restricting type of AN patients were included, who often show overly controlled behavioral style and less impulsive than binge eating/purging type of AN patients [9].

Correlation Coefficients

Correlation analyses also revealed specific features of ED. First, both the AN and BN groups showed no significant correlation between $RPST_{loss}$ task accuracy and the prefrontal [oxyHb], while the HC showed a moderate negative correlation like those of a previous study [25]. According to the aforementioned interpretations of the $RPST_{loss}$, the correlation of the HC could be reworded to state that whoever could get a higher score needed less self-inhibition, while those with lower accuracy and felt difficulty required a higher prefrontal activity. Additionally, the lack of significance in these correlations in the AN and BN groups can be explained by inefficient self-regulatory function of their PFC. That is, their PFC might have reached upper limit of self-regulatory function and then run over a function-dependent activity control range seen in the HC, so even who could get a high score showed high activity.

Secondly, prefrontal activity during the $RPST_{loss}$ task period correlated with some symptom scales. In the BN group, the correlation between the severity subscale of BITE and prefrontal activity suggested that the inefficient prefrontal self-regulatory function could associate with their symptoms. Interestingly, the restraint subscale of EDE-Q in the BN group showed positive correlation with the prefrontal activity. And at the same time, the severity subscale of BITE in the AN group positively correlated with the prefrontal activity, suggesting a correlation between a bulimic tendency and the functional inefficacy in prefrontal self-regulation, which may signal latent binge eating and purging behavior in the AN group. Thus, these findings suggest a common etiology of both binge eating/purging type of AN and BN with regard to patients' prefrontal self-regulatory function.

There have been fewer functional neuroimaging studies in BN patients as compared with AN patients [18,59,60]. Our findings suggest a new potential investigational approach to understanding the pathophysiology of abnormal self-regulation of BN.

Limitations

There are several methodological limitations in this study. The use of the two-channel NIRS, i.e. inexhaustive imaging method, could incur unavoidable misalignment of probes than areas of "ideal" activation peak pointed in the previous fMRI/PET/multi-channel NIRS researches thus might limit the symptom-related sensitivity. However, this choice with predetermined setup was not entirely a shortcoming but made this study stricter with respect to channel setting and analyzing process. Interchannel (not interprobe) distances are fixed with most of the available multi-channel probe sets, which can induce inappropriate measurements being not adjusted for individuals with different head sizes. There is another limitation that raw NIRS data should basically not be used for comparisons because of its fundamental that two measurements have different photo-pathlengths. Even if careful preprocessings and analyses are performed, NIRS data requires careful interpretation for comparisons between different channels, between different experimental days, or between individuals.

The number of subjects examined was relatively small for discussing group differences of brain functions. The limited number of participation might diminish the value of the controlling task performance parameters for BMI by not meeting prerequisites for analysis for covariance. Thus these analyses might not strictly rule out possible effects of malnutrition. The patient groups should consist of only drug-free patients to eliminate the possible effects of psychotropic medications known to modulate cerebral hemodynamic functions. We found no significant differences between patients with and without psychotropic medications, but the possibility of medication effects cannot be ruled out with such small sample. Some limitations were given by the use of NIRS, one of which is that brain regions observed with it are limited to narrow and superficial regions, but not localized. Furthermore, [oxyHb] data obtained from different pathlengths should not be compared between individuals or channels, and there is currently no standard method to standardize data. We attempted to avoid such limitations by using the z score.

Conclusions

In conclusion of this preliminary study, our first hypothesis that the ED patients would have changes in cognitive abilities associated with self-regulation was partly confirmed in the BN group. The results of the BN group could support our second hypothesis, namely that the abnormal cognitive functions were accompanied by changes in prefrontal blood perfusion as a characteristic activation pattern, though the NIRS data showed no significant group difference directly. Third hypothesis was partly confirmed as that prefrontal activities associated with self-regulation in the $RPST_{loss}$ of the AN and BN groups showed no significant correlation with the task accuracy, which the HC group showed a moderate negative correlation, and showed positive correlations with some symptom scales.

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Author Contributions

Conceived and designed the experiments: CS MN DM ES. Performed the experiments: CS MN KT TN MI. Analyzed the data: CS MN DM KT. Contributed reagents/materials/analysis tools: CS. Wrote the paper: CS MN DM.

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