Neuroscience Letters 556 (2013) 37–41

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Plasma levels of mature brain-derived neurotrophic factor (BDNF) and matrix metalloproteinase-9 (MMP-9) in treatment-resistant schizophrenia treated with clozapine☆

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HIGHLIGHTS

- Plasma levels of mature BDNF in schizophrenia were measured for the first time.
- No significant difference was observed in mature BDNF levels in schizophrenia.
- MMP-9 plasma levels were significantly increased in patients with schizophrenia.
- Plasma mature BDNF levels were significantly correlated with plasma MMP-9 levels.

ARTICLE INFO

Article history:
Received 12 August 2013
Received in revised form 9 September 2013
Accepted 25 September 2013

Keywords:
Treatment-resistant schizophrenia
Brain-derived neurotrophic factor (BDNF)
Matrix metalloproteinase-9 (MMP-9)
Clozapine

ABSTRACT

Brain-derived neurotrophic factor (BDNF) regulates the survival and growth of neurons, and influences synaptic efficiency and plasticity. Peripheral BDNF levels in patients with schizophrenia have been widely reported in the literature. However, it is still controversial whether peripheral levels of BDNF are altered in patients with schizophrenia. The peripheral BDNF levels previously reported in patients with schizophrenia were total BDNF (proBDNF and mature BDNF) as it was unable to specifically measure mature BDNF due to limited BDNF antibody specificity. In this study, we examined whether peripheral levels of mature BDNF were altered in patients with treatment-resistant schizophrenia. Matrix metalloproteinase-9 (MMP-9) levels were also measured, as MMP-9 plays a role in the conversion of proBDNF to mature BDNF. Twenty-two patients with treatment-resistant schizophrenia treated with clozapine and 22 age- and sex-matched healthy controls were enrolled. The plasma levels of mature BDNF and MMP-9 were measured using ELISA kits. No significant difference was observed for mature BDNF however, MMP-9 was significantly increased in patients with schizophrenia. The significant correlation was observed between mature BDNF and MMP-9 plasma levels. Neither mature BDNF nor MMP-9 plasma levels were associated clinical variables. Our results do not support the view that peripheral BDNF levels are associated with schizophrenia. MMP-9 may play a role in the pathophysiology of schizophrenia and serve as a biomarker for schizophrenia.

Abbreviations: BDNF, brain-derived neurotrophic factor; MMP-9, matrix metalloproteinase-9; MDD, major depressive disorder; DSM-IV, diagnostic and statistical manual of mental disorders, fourth edition; PANSS, positive and negative syndrome scale.

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http://dx.doi.org/10.1016/j.neulet.2013.09.059

1. Introduction

Schizophrenia is a severe psychiatric disease characterized by delusions, hallucinations, impairment of cognitive function and incoherent behavior. It affects approximately 1% of the general population worldwide. Mounting evidence suggests that a deficit in neurotrophin supply to cortical neurons may be an underlying factor in the pathophysiology of schizophrenia as adequate neurotrophic support is required for normal brain development, maturation and function [3,4].

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that regulates neuronal survival, differentiation and growth during brain development, with important effects on neurogenesis and neuroplasticity. It is also important for hippocampal-related learning and memory [17]. A common single nucleotide polymorphism (SNP) of the BDNF gene has impact on episodic memory, hippocampal morphology and memory-related hippocampal activity in human [9,16]. Mature BDNF is initially synthesized as a precursor protein, preproBDNF. Following cleavage of the signal peptide, proBDNF is converted to mature BDNF by extracellular proteases, such as matrix metalloproteinase-9 (MMP-9). Mature BDNF and poBDNF each plays important roles in several physiological functions. Recent studies show that mature BDNF and pro BDNF elicit opposing effects via the TrkB and $p75^{NTR}$ receptors respectively. Mature BDNF preferentially binds to the TrkB receptor and plays an important role through BDNF-TrkB signaling which fulfills wide variety of functions such as cell survival, migration, outgrowth of neuritis and synaptic plasticity. In contrast, pro BDNF preferentially binds to the $p75^{NTR}$ receptors and elicit apoptosis rather than cell survival [8,11]. Considering the important roles of mature BDNF, it would be informative to specifically measure mature BDNF. Although BDNF levels in human blood can be measured using commercially available human BDNF ELISA kits, due to the limited specificity of the BDNF antibody, it has not been possible to distinguish between proBDNF and mature BDNF. Recently, peripheral levels of mature BDNF have been reported to be measurable using newly available human BDNF ELISA kits [23].

It is of great interest to assess the potential contribution of BDNF to the pathophysiology of schizophrenia. Several studies report altered BDNF mRNA and protein in prefrontal cortical regions and hippocampus of post-mortem brain tissues [13,21,22]. Peripheral BDNF levels in patients with schizophrenia have also been widely reported in the literature. However, there is no widespread agreement on the degree of peripheral BDNF levels in patients with schizophrenia, as measured in blood serum or plasma. A recent meta-analysis reported that peripheral BDNF levels were reduced in schizophrenia. However, there was considerable heterogeneity in the results [5]. Considering the important roles of mature BDNF such as cell survival, migration, outgrowth of neuritis and synaptic plasticity, it would be informative to specifically measure mature BDNF in patients with schizophrenia because dysfunction of these mature BDNF roles might be an underlying factor in the pathophysiology of schizophrenia. The peripheral BDNF levels previously reported in patients with schizophrenia were total BDNF (proBDNF and mature BDNF); peripheral levels of mature BDNF specifically have not been investigated in patients with schizophrenia. This study aimed to determine whether peripheral levels of mature BDNF were altered in patients with treatment-resistant schizophrenia. We also investigated Matrix metalloproteinase-9 (MMP-9) levels, as MMP-9 plays a role in the conversion of proBDNF to mature BDNF [8].

Table 1
Demographic variables for subjects.

Variables	Control $n=22$	Patients with schizophrenia $n=22$
Age (years)	38.1 ± 12.9	38.1 ± 13.2
Gender (male/female)	(12/10)	(12/10)
Age at onset	–	21.9 ± 8.4
Duration of illness	–	17.2 ± 11.1
PANSS positive	–	23.0 ± 4.6
PANSS negative	–	25.5 ± 5.5
PANSS general	–	52.9 ± 9.6
Clozapine dose (mg)	–	448.6 ± 130.0

Means ± SD are shown.

2. Materials and methods

2.1. Subjects

Twenty-two patients with treatment-resistant schizophrenia who were treated with clozapine were included in this study. Twenty-two age- and sex-matched healthy controls also participated in this study (Table 1). Cases were recruited at Osaka University hospitals. Each subject had been diagnosed and assessed by at least two trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria based on structured clinical interview. Treatment-resistant schizophrenia was defined according to the following criteria mentioned in clozapine drug information in Japan: (1) Non- or little response to treatment from at least two adequately dosed antipsychotic trials for at least 4 weeks (including at least one second-generation antipsychotic, >600 mg/day of chlorpromazine equivalent) and patients never had the Global Assessment of Functioning (GAF) scores that were higher than 40. (2) Intolerance to at least two second-generation antipsychotics because of uncontrolled extrapyramidal symptoms. All subjects included in this study met the criteria of non- or little response. Symptoms of schizophrenia were assessed using the Positive and Negative Syndrome Scale (PANSS). Cases of schizophrenia with the comorbidities of substance-related disorders or mental retardation were excluded. Controls were recruited through local advertisements. Psychiatrically, medically and neurologically healthy controls were evaluated using the DSM-IV structured clinical interview, non-patient version. Subjects were excluded if they had neurological or medical conditions that could potentially affect the central nervous system, such as atypical headache, head trauma with loss of consciousness, chronic lung disease, kidney disease, chronic hepatic disease, thyroid disease, active stage cancer, cerebrovascular disease, epilepsy or seizures. Written informed consent was obtained for all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University and Chiba University.

2.2. Measurement of mature BDNF and MMP-9

Plasma levels of mature BDNF and MMP-9 were measured using the human BDNF ELISA Kit (Adipo Bioscience, Santa Clara, CA, USA), and the human MMP-9 ELISA Kit (R&D Systems, Minneapolis, MN, USA), respectively. To minimize assay variance, plasma levels of mature BDNF and MMP-9 were measured in each subject 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). As plasma levels of proBDNF are not measurable by the newly available proBDNF ELISA kit due to low sensitivity, we measured only mature BDNF.

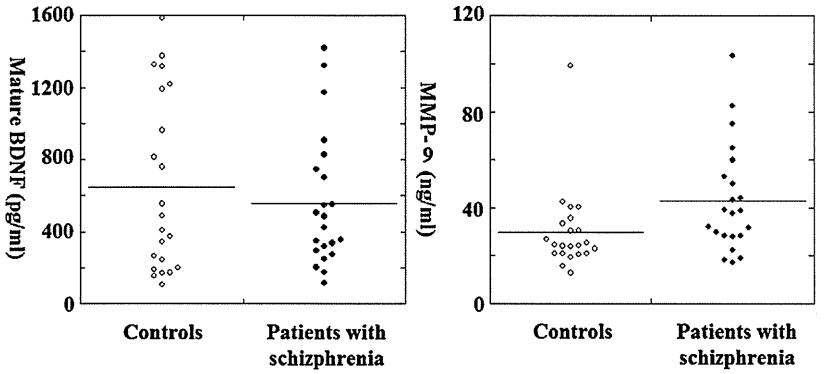

Fig. 1. Plasma levels of mature BDNF and MMP-9 in treatment-resistant schizophrenia treated with clozapine. The plasma levels of mature BDNF and MMP-9 in the controls and treatment-resistant patients with schizophrenia who were treated with clozapine (control, $n=22$, schizophrenia, $n=22$).

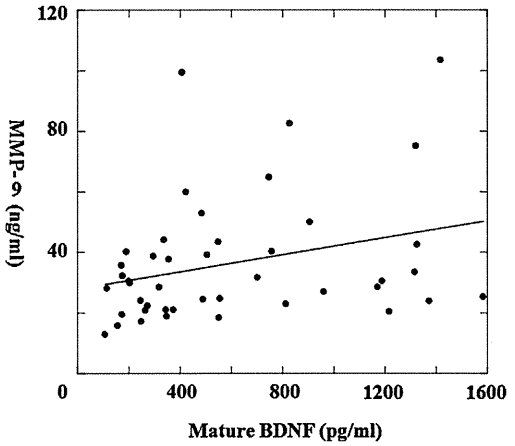

Fig. 2. Correlation between plasma levels of mature BDNF and MMP-9. Positive correlation was observed between plasma levels of mature BDNF and MMP-9 (patients with schizophrenia and controls, $n=44$, $r=0.333$, $p=0.027$).

2.3. Statistical analysis

Statistical analyses were performed using SPSS 20.0J software (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls were analyzed using χ^2 tests for categorical variables. The groups did not differ with respect to age or gender (Table 1). Kolmogrov–Smirnov test was used to test the normality of data distribution. Mature BDNF did not normally distribute in both patients with schizophrenia and controls (patients with schizophrenia; $p=0.041$, controls; $p=0.042$). MMP-9 distributed normally in patients with schizophrenia, however did not distribute normally in controls (patients with schizophrenia; $p=0.130$, controls; $p=0.012$). And differences between patients and controls were analyzed using the Mann–Whitney U-test for continuous variables. Homogeneity of variance was assessed by Levene's test. The assumption of homogeneity of group variance was not violated in both mature BDNF and MMP-9 levels (mature BDNF; $p=0.052$, MMP-9; $p=0.112$). Test of rejection of Smirnoff–Grubbs was performed. Spearman rank order correlation test was performed to assess the possible correlation between plasma levels of mature BDNF and MMP-9 and clinical characteristics. The significant level for statistical tests was set at $p<0.05$.

3. Results

The plasma levels of mature BDNF and MMP-9 were compared between patients with treatment-resistant schizophrenia who were treated with clozapine and controls, and no significant difference was observed for mature BDNF (Fig. 1, Mann–Whitney test; $U=238$, $p=0.925$). However, MMP-9 was significantly increased in patients with schizophrenia (Fig. 1, Mann–Whitney test; $U=139$, $p=0.016$). When we exclude each one sample in both groups by test of rejection of Smirnoff–Grubbs, MMP-9 was still significantly increased in patients with schizophrenia (Mann–Whitney test; $U=118$, $p=0.010$). As MMP-9 plays a role in the conversion of proBDNF to mature BDNF, the correlation between the levels of mature BDNF and MMP-9 was examined. There were significant correlation between the levels of mature BDNF and MMP-9 in (Fig. 2, patients with schizophrenia and controls, $n=44$, $r=0.333$, $p=0.027$). When we investigate this correlation in patients and controls groups separately, significant correlation was observed in patients with schizophrenia ($n=22$, $r=0.585$, $p=0.004$) but not in controls ($n=22$, $r=0.322$, $p=0.143$). To determine the effect of clozapine on mature BDNF and MMP-9 levels, we also examined the correlation between the plasma levels of mature BDNF or MMP-9 and clozapine dosage. No significant correlation was observed between the plasma levels of mature BDNF or MMP-9 and clozapine dosage (Table 2, BDNF and clozapine dosage; $n=22$, $r=0.028$, $p=0.901$, MMP-9 and clozapine dosage; $n=22$, $r=0.131$, $p=0.562$). The correlations between the plasma levels of mature BDNF or MMP-9 and positive and negative symptom scores on the PANSS were also investigated; no significant correlations were observed (Table 2, BDNF and PANSS positive; $n=22$, $r=-0.014$, $p=0.952$, BDNF and PANSS negative; $n=22$, $r=-0.079$, $p=0.726$, MMP-9 and PANSS positive; $n=22$, $r=0.306$, $p=0.167$, BDNF and PANSS negative; $n=22$, $r=0.127$, $p=0.574$). The correlations between the plasma levels of mature BDNF or MMP-9 and duration of illness were also investigated; no significant correlations were observed (Table 2, BDNF and duration of illness; $n=22$, $r=0.121$, $p=0.592$, MMP-9 and duration of illness; $n=22$, $r=0.087$, $p=0.699$).

Table 2
Correlation analysis.

	Clozapine dosage	PANSS positive	PANSS negative	PANSS general	Age at onset	Duration of illness
Mature BDNF	0.901	0.952	0.726	0.865	0.332	0.592
MMP-9	0.562	0.167	0.574	0.454	0.685	0.699

p values are shown.

4. Discussion

In this study, for the first time, we measured the plasma levels of mature BDNF in patients with schizophrenia. The plasma levels of mature BDNF were decreased in treatment-resistant schizophrenia, however the difference did not reach statistical significance. Our result was consistent with some previous studies that investigated the serum levels of total BDNF in patients with schizophrenia [10,20]. Treatment-resistant schizophrenia patients treated with clozapine were enrolled because some studies suggest that peripheral BDNF levels increase in association with antipsychotics treatment including clozapine which is used for the treatment of poorly responsive patients with schizophrenia [6,10] and serum BDNF levels were reported to be significantly correlated with clozapine daily dose but not with typical antipsychotics [15]. However, we found no effect of clozapine treatment on the plasma levels of mature BDNF. A possible explanation would be the difference in race. This is the first study investigating the effect of clozapine treatment on the plasma levels of mature BDNF in Japanese population. Accumulating evidence suggests that BDNF plays a key role in the pathophysiology of major depressive disorder (MDD). It was reported that BDNF serum levels in patients with MDD were significantly lower than those of healthy controls, and that there was a negative correlation between BDNF serum levels and the severity of depression in patients [19]. Furthermore, decreased serum levels of BDNF in antidepressant naive patients with MDD, recovered to levels associated with amelioration of depressive symptoms, after antidepressant treatment. Three meta-analyses and a study using a large sample size confirmed these findings [7]. Recently, peripheral levels of mature BDNF have been reported to be decreased in MDD [23]. Further study using larger samples is needed to see whether peripheral levels of mature BNDF are not altered in schizophrenia and mature BDNF levels are not associated with clozapine.

We also investigated MMP-9 plasma levels, as MMP-9 plays a role in the conversion of proBDNF to mature BDNF [8]. The significant correlation was observed between mature BDNF and MMP-9 plasma levels, suggesting that MMP-9 plays a role in the conversion of proBDNF to mature BDNF in the samples of this study. The serum levels of MMP-9 have been reported to be increased in patients with schizophrenia [2]. A higher frequency of positive MMP-9 activity in serum from patients with schizophrenia has also been reported [1]. We confirmed the presence of elevated plasma MMP-9 levels in patients with treatment-resistant schizophrenia. In patients with schizophrenia, MMP9 might be induced to recover the decreased mature BDNF. The finding that significant correlation between mature BDNF and MMP-9 was observed only in patients with schizophrenia but not in controls supports this idea. Plasma levels of MMP-9 have been proposed to be a useful biomarker for assessing pathological event in brain. It was reported that levels of MMP-9 in plasma and brain were significantly correlated after cerebral ischemia in rats [14]. MMP-9 is an enzyme implicated in a number of pathological conditions including neuropsychiatric disorders [18]. A role of MMP-9 in the plasticity of the central nervous system has been investigated in experimental studies and MMP-9 is reported to be required for hippocampal long-term potentiation and memory [12]. MMP-9 may have some roles in pathophysiology of schizophrenia.

Our study must be interpreted in lights of its limitations. Firstly, the sample size of this study is small. Secondly, only treatment-resistant schizophrenia patients treated with clozapine were included and patients treated with other antipsychotics or patients without antipsychotics treatment were not included in this study. Further studies are needed to evaluate the relationship between plasma levels of mature BDNF and schizophrenia and clozapine treatment.

Acknowledgements

We thank all individuals who participated in this study.

This work was supported in part by research grants from the Japanese Ministry of Health, Labor and Welfare (H22-seishin-ippan-010); the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) KAKENHI [25461730-Grant-in-Aid for Scientific Research (C), 22390225-Grant-in-Aid for Scientific Research (B), 23659565-Grant-in-Aid for Challenging Exploratory Research and 221S0003-Grant-in-Aid for Scientific Research on Innovative Areas (Comprehensive Brain Science Network)]; and the Japan Foundation for Neuroscience and Mental Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Neuroscience Letters 582 (2014) 93–98

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Changes in plasma D-serine, L-serine, and glycine levels in treatment-resistant schizophrenia before and after clozapine treatment

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HIGHLIGHTS

- The plasma D-/L-serine ratio was lower in schizophrenia before clozapine treatment.
- The plasma D-/L-serine ratio increased in response to clozapine treatment.
- The plasma glycine/L-serine ratio increased in response to clozapine treatment.
- The glycine/L-serine ratio was higher in schizophrenia after clozapine treatment.

ARTICLE INFO

Article history:
Received 10 April 2014
Received in revised form 31 July 2014
Accepted 30 August 2014
Available online 10 September 2014

Keywords:
Clozapine
Glycine
Plasma
Serine
Treatment-resistant schizophrenia

ABSTRACT

Hypofunction of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors may be involved in the pathophysiology of schizophrenia. Many studies have investigated peripheral NMDA receptor-related glutamatergic amino acid levels because of their potential as biological markers. Peripheral D-serine levels and the ratio of D-serine to total serine have been reported to be significantly lower in patients with schizophrenia than in controls. Peripheral D-serine levels and the D-/L-serine ratio have also been reported to significantly increase in patients with schizophrenia as their clinical symptoms improve from the time of admission to the time of discharge. In this study, we examined whether peripheral NMDA receptor-related glutamatergic amino acids levels were altered in patients with treatment-resistant schizophrenia compared to controls and whether these peripheral amino acids levels were altered by clozapine treatment. Twenty-two patients with treatment-resistant schizophrenia and 22 age- and gender-matched healthy controls were enrolled. The plasma levels of D-serine, L-serine, glycine, glutamate, and glutamine were measured before and after clozapine treatment. We found that the plasma levels of D-serine and the D-/L-serine ratio were significantly lower in the patients before clozapine treatment than in the controls. The D-/L-serine ratio was significantly increased by clozapine treatment in patients, and no significant difference was observed in the plasma levels of D-serine and the D-/L-serine ratio between the patients after clozapine treatment and the controls. We also found that plasma glycine levels and the glycine/L-serine ratio were significantly increased following clozapine treatment in the patients, and the glycine/L-serine ratio was significantly higher in the patients after clozapine treatment than in the controls. There was no significant difference in the plasma levels of glutamate and glutamine both between the controls and

Abbreviations: CSF, cerebrospinal fluid; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, fourth edition; GAF, Global Assessment of Functioning; HPLC, high-performance liquid chromatography; NMDA, N-methyl-D-aspartate; PANSS, Positive and Negative Syndrome Scale.

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http://dx.doi.org/10.1016/j.neulet.2014.08.052

patients and between before and after clozapine treatment. This study firstly demonstrated changes of D-/L-serine and glycine/L-serine ratio between before and after clozapine treatment, suggesting that the plasma D-/L-serine ratio and glycine/L-serine ratio could be markers of therapeutic efficacy or clinical state in treatment-resistant schizophrenia.

1. Introduction

Recent investigations of schizophrenia have focused on hypofunction of N-methyl-D-aspartate (NMDA)-type glutamate receptors, in part, because of clinical evidence that phencyclidine, a non-competitive antagonist of the NMDA receptor, produces schizophrenia-like symptoms in normal controls [16].

A few studies investigated amino acids levels that are related to neurotransmission via the NMDA receptors; D-serine, L-serine, glycine, glutamate, and glutamine in postmortem brains of patients with schizophrenia [13,17,23]. No changes of these amino acids levels have been reported in postmortem brains of schizophrenia patients. Among these amino acids, glycine and glycine precursor, serine, co-agonists at NMDA receptors, and thus, increases glutamatergic neurotransmission, have drawn particular attention in schizophrenia research [7]. Because substantial quantities of D-serine have been found to be present in the mammalian brain [10] and because D-serine has a stronger affinity for the glycine site of NMDA receptors than does glycine [19], the importance of D-serine in the pathophysiology of schizophrenia has become the focus of the research field. Several studies have investigated CSF levels of these amino acids in patients with schizophrenia, and reduced D-serine levels and D-serine to total serine ratio in patients with schizophrenia has been reported [2,3,11,21].

There is evidence that the venous plasma and CSF levels of amino acids, including serine and glycine, are significantly correlated in human subjects [5], indicating that the plasma levels of these amino acids reflect, to some extent, those in the central nervous system. Serum/plasma glycine and serine levels have been investigated as biological markers for schizophrenia. First, total plasma serine and glycine levels have been found to be significantly higher in patients with schizophrenia than in controls [1]. An association between plasma glycine levels and negative symptoms in patients with schizophrenia has also been reported [24]. It has been reported that serum/plasma D-serine levels and the ratio of D-serine to total serine were significantly lower in patients with schizophrenia than in controls [4,12,25]. Many other studies have also investigated the serum/plasma glycine and serine levels in patients with schizophrenia, but these studies produced inconsistent results [3,21]. Moreover, only a few studies have investigated the plasma levels of these amino acids during the clinical course [22]. Ohnuma et al. reported that the D-serine level and the D-/L-serine ratio were significantly increased in patients with schizophrenia as their clinical symptoms improved from the time of admission to the time of discharge [22]. In addition, the increase in the plasma D-serine levels of drug-naïve patients was reported to be correlated with improvements in positive symptoms. In another study, it was reported that patients with schizophrenia taking clozapine had different serine and glycine metabolisms from the patients taking other antipsychotics [14]. The plasma levels of amino acids have not been investigated in treatment-resistant schizophrenia and the plasma levels of these amino acids have not been compared before and after clozapine treatment.

The aims of this study were to determine whether (1) plasma D-serine, L-serine, glycine, glutamate, and glutamine levels were altered in patients with treatment-resistant schizophrenia compared to controls and (2) these amino acids levels were altered by clozapine treatment.

2. Materials and methods

2.1. Subjects

Twenty-two patients with treatment-resistant schizophrenia who were treated with clozapine were included in this study. Twenty-two age- and gender-matched healthy controls also participated in this study. Detailed information is shown in Table 1. Blood samples were collected before and after clozapine treatment of the patients. Cases were recruited at the Osaka University hospitals. Each subject had been diagnosed and assessed by at least two trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria based on a structured clinical interview. Treatment-resistant schizophrenia was defined according to the following criteria mentioned in the clozapine drug information in Japan: (1) no or little response to treatment from at least two adequately dosed antipsychotic trials for at least 4 weeks (including at least one second-generation antipsychotic, >600 mg/day of chlorpromazine equivalent) and Global Assessment of Functioning (GAF) scores that were never higher than 41, or (2) Intolerance to at least two second-generation antipsychotics because of extrapyramidal symptoms [26]. All subjects included in this study met the criterion of no or little response. All patients were inpatients when they start to take clozapine and were taking other antipsychotic drugs. Each patient was taking different drugs including typical and atypical antipsychotic drugs and average dosage and duration of treatment are shown in Table 1. The start dosage was 12.5 mg of once daily. The dosage was increased to 200 mg in 3 weeks or more. The dosage more than 50 mg was taken twice daily. Maintenance dosage was from 200 mg to 400 mg. The interval of dosage increase was 4 days or more and maximum dosage increase/day was 100 mg. Maximum dosage was 600 mg. Other antipsychotic withdrawal was performed within 4 weeks from the start of clozapine. Symptoms of schizophrenia were assessed using the Positive and Negative Syndrome Scale (PANSS). Patients with schizophrenia with comorbidities of substance-related disorders or mental retardation were excluded. Controls were recruited through local advertisements. Psychiatrically, medically and neurologically healthy controls were evaluated using the DSM-IV structured clinical interview, non-patient version. Subjects were excluded if they had neurological or medical conditions that could potentially affect the central nervous system, such as atypical headache, head trauma with loss of consciousness, chronic lung disease, kidney disease, chronic hepatic disease, thyroid disease, active stage cancer, cerebrovascular disease, epilepsy or seizures. Written informed consent was obtained from all subjects after the procedures had been fully explained. This study was conducted in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University, Tokushima University and Chiba University.

2.2. Determination of plasma levels of amino acids

Measurement of total, D- and L-serine levels in the plasma was carried out using a column-switching high performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) as previously reported [9,25]. Measurement of glycine,

Table 1
Demographic variables for subjects.

Variables	Control ($n=22$)	Patients with schizophrenia ($n=22$)
Age (years)	38.1 ± 12.9	38.1 ± 13.2
Gender (male/female)	(12/10)	(12/10)
Schizophrenia type (paranoid/disorganized/catatonic/undifferentiated)	–	(15/7/0/0)
Outpatients/inpatients	–	(0/22)
Duration of illness (years)	–	17.2 ± 11.1
Duration of medication (years)	–	12.6 ± 7.8
Clozapine dose (mg)	–	448.6 ± 130.0
Antipsychotic dose before clozapine (CPZ equivalent doses) (mg)	–	1229 ± 642.9
Antipsychotic before clozapine (atypical only/atypical + typical)	–	(18/4)
PANSS positive (before/after clozapine treatment)	–	(29.8 ± 5.2/23.0 ± 4.6)
PANSS negative (before/after clozapine treatment)	–	(32.4 ± 7.7/25.5 ± 5.5)
PANSS general (before/after clozapine treatment)	–	(63.6 ± 13.0/52.9 ± 9.6)

Means ± SD are shown. CPZ, chlorpromazine.

glutamine, and glutamate was carried out using a HPLC system with fluorescence detection, as previously reported [11]. The researchers responsible for the measurements were blinded to the respective groups (controls and patients).

2.3. Statistical analysis

The statistical analyses were performed using SPSS 20.0J software (SPSS Japan Inc., Tokyo, Japan). Differences in the clinical characteristics between the patients and controls were analyzed using χ^2 tests for categorical variables. The groups did not differ with respect to age or gender (Table 1). Test of normality was performed by Shapiro–Wilk test and D-serine levels, Glycine levels in patients, D-/L-serine ratios in patients, and glycine/L-serine ratios in patients were not distributed normally and differences in the plasma amino acids levels between the patients and controls were analyzed using Mann–Whitney *U*-test. The differences in plasma amino acids levels and PANSS scores of the patients before and after treatment were analyzed by the Wilcoxon rank sum test. The positive, negative, and general symptom scores on the PANSS were significantly improved in the patients by clozapine treatment (Table 1). The Spearman rank order correlation test was performed to assess the possible correlation between the plasma levels of amino acids and clinical characteristics. The significance level for the statistical tests was set at $p<0.05$.

3. Results

Plasma levels of D-serine, L-serine, glycine, glutamate, glutamine, and the D-/L-serine and glycine/L-serine ratios were compared between patients with treatment-resistant schizophrenia and controls (i.e., between controls and patients before clozapine treatment, and between controls and patients after clozapine treatment). The differences in the plasma levels of D-serine, L-serine, glycine, glutamate, glutamine, and the D-/L-serine and glycine/L-serine ratios before and after clozapine treatment were also compared.

The plasma levels of D-serine were significantly lower in the patients before clozapine treatment than in the controls (Fig. 1A and Table 2, Mann–Whitney *U*-test; $U=141, Z=-2.4, p=0.016$). No significant difference was observed in the plasma D-serine levels in the patients before and after clozapine treatment. The difference in the plasma D-serine levels between the controls and patients after clozapine treatment was not significant (Fig. 1A and Table 2). No significant difference was observed in the plasma levels of L-serine and glycine between the controls and patients before or after clozapine treatment (Fig. 1B and C, Table 2). The plasma levels of L-serine were significantly decreased in the patients after clozapine treatment (Fig. 1B and Table 2, Wilcoxon rank sum test; $Z=-2.8, p=0.006$). The plasma levels of glycine were significantly increased in the patients after clozapine treatment (Fig. 1C and Table 2, Wilcoxon rank sum test; $Z=-2.3, p=0.022$). There was no significant difference in the plasma levels of glutamate and glutamine between the controls and patients before or after clozapine treatment (Table 2). The plasma levels of glutamate and glutamine did not differ in the patients before and after clozapine treatment (Table 2).

The D-/L-serine ratio was significantly lower in the patients before clozapine treatment than in the controls (Fig. 2A and Table 2, Mann–Whitney *U*-test; $U=123, Z=-2.8, p=0.005$). The D-/L-serine ratio was significantly increased in the patients after clozapine treatment (Fig. 2A and Table 2, Wilcoxon rank sum test; $Z=-2.3$, $p=0.02$), and the difference in the D-/L-serine ratio between the controls and patients after clozapine treatment was not significant (Fig. 2A and Table 2). The glycine/L-serine ratio did not differ between the controls and the patients before clozapine treatment (Fig. 2B and Table 2). The glycine/L-serine ratio was significantly increased in the patients after clozapine treatment (Fig. 2B and Table 2, Wilcoxon rank sum test; $Z=-3.8, p=0.0002$) and was significantly higher in the patients after clozapine treatment than in the controls (Fig. 2B and Table 2, Mann–Whitney *U*-test; $U=157$, $Z=-2.0, p=0.046$).

The correlations between the plasma levels of these amino acids and clinical variables including duration of illness, clozapine dosage and positive, negative, and general symptom scores on the PANSS were also investigated; no significant correlation was observed (Supplementary Table 1).

4. Discussion

In this study, we measured the plasma amino acids levels before and after clozapine treatment in treatment-resistant schizophrenia; this is the first study, which investigated changes before and after clozapine treatment. We made the following findings: (1) The plasma levels of D-serine and the D-/L-serine ratio were lower in patients before clozapine treatment than in the controls, the D-/L-serine ratio increased in the patients in response to clozapine treatment and the plasma levels of D-serine and the D-/L-serine ratio in the patients after clozapine treatment were similar to those in the controls. (2) The plasma L-serine levels were decreased by clozapine treatment in the patients. (3) The plasma glycine levels and glycine/L-serine ratio were increased by clozapine treatment in the patients, and the glycine/L-serine ratio was higher in the patients after clozapine treatment than in the controls.

It has been reported that D-serine levels and the ratio of D-serine to total serine in CSF are lower in patients with schizophrenia than in controls [2,11]. Decreased serum D-serine levels and the ratio of D-serine to total serine in patients with schizophrenia were also reported [12,25]. We confirmed the lower plasma D-serine levels and D-/L-serine ratio in treatment-resistant schizophrenia

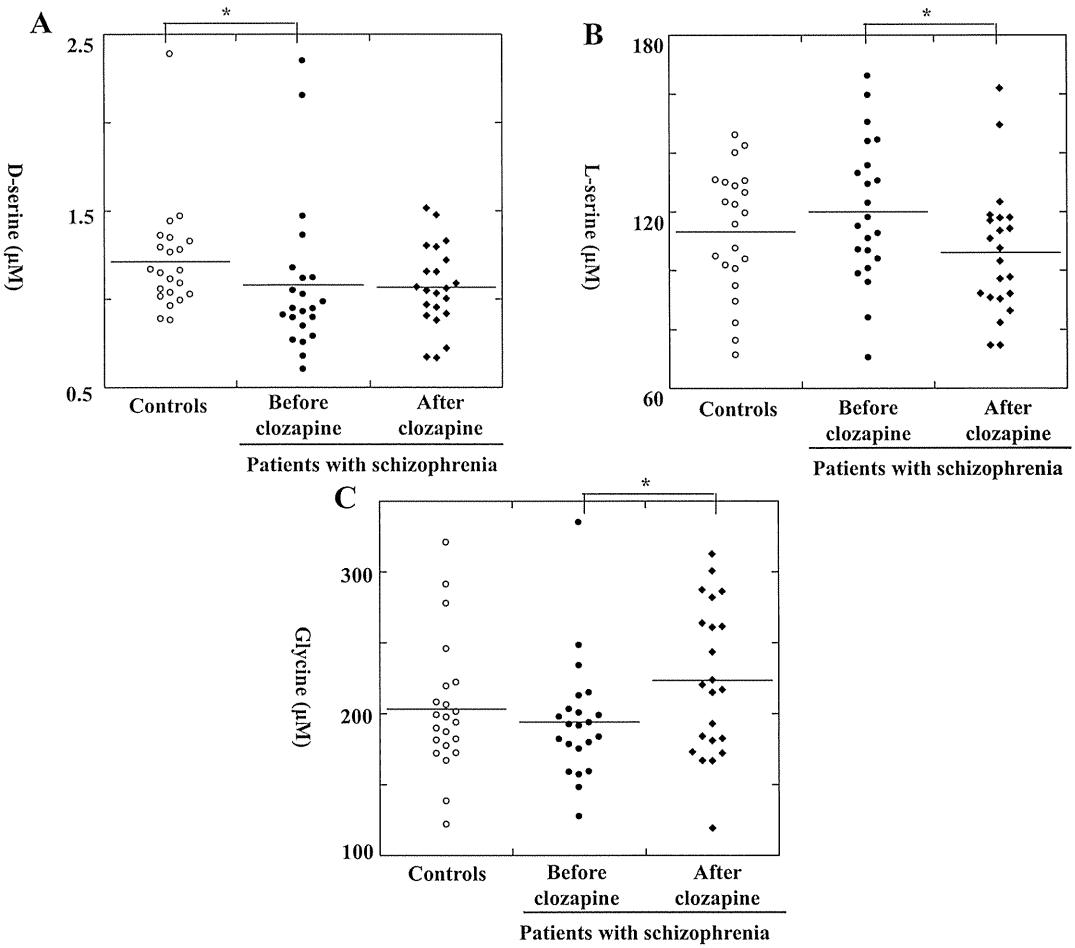

Fig. 1. Plasma levels of D-serine, L-serine, and glycine in treatment-resistant schizophrenia before and after clozapine treatment. The plasma levels of D-serine, L-serine, and glycine in the controls and patients with treatment-resistant schizophrenia before and after clozapine treatment (controls, $n = 22$; patients with schizophrenia, $n = 22$). The bars represent mean values. $^{*}p < 0.05$.

compared to controls. It has been reported that plasma D-serine levels and the D-/L-serine ratio increase during progression from the acute stage of schizophrenia to the remission stage and that L-serine levels decrease during this period [22]. Consistent with previous findings, we found that the D-/L-serine ratio increased and the L-serine levels decreased in response to clozapine treatment. Ohnuma et al. reported no significant difference in the plasma glycine levels between patients with different stages of schizophrenia, from the acute stage to the remission stage [22]. However, we found that the plasma glycine levels and the glycine/L-serine ratio increased in response to clozapine treatment and that the glycine/L-serine ratio was higher in patients after clozapine treatment. The increase in plasma glycine levels and the glycine/L-serine ratio may be specifically related to clozapine because clozapine was not used in the previous report by Ohnuma et al.

Many studies have investigated serum/plasma L-serine levels [21] and these studies have produced conflicting results. Several studies reported elevated L-serine levels in patients with schizophrenia [1,21], but other studies did not [8,21]. Serum/plasma glycine levels and glycine/serine ratio were also investigated by many studies because glycine acts as an

Table 2
Amino acids levels in patients with schizophrenia before and after clozapine treatment and in controls.

	Control ($n = 22$)	Patients with schizophrenia ($n = 22$)		*P* value		
		Before clozapine treatment	After clozapine treatment			
D-Serine (μM)	1.21 ± 0.31	1.08 ± 0.43	1.07 ± 0.23	0.018[a]	0.133[b]	0.485[c]
L-Serine (μM)	113.1 ± 21.5	120.0 ± 24.2	106.1 ± 21.8	0.385[a]	0.166[b]	0.006[c]
Glycine (μM)	203.3 ± 46.8	194.2 ± 41.8	223.4 ± 52.9	0.526[a]	0.260[b]	0.022[c]
Glutamate (μM)	35.8 ± 16.2	39.3 ± 13.5	33.8 ± 15.4	0.197[a]	0.907[b]	0.140[c]
Glutamine (μM)	510.9 ± 69.0	507.0 ± 75.3	475.0 ± 111.1	0.734[a]	0.348[b]	0.082[c]
D-/L-Serine ratio × 100	1.09 ± 0.23	0.90 ± 0.28	1.03 ± 0.24	0.005[a]	0.280[b]	0.020[c]
Glycine/L-serine ratio	1.82 ± 0.37	1.65 ± 0.32	2.15 ± 0.58	0.067[a]	0.046[b]	≤0.001[c]

Means ± SD are shown. Significant *p* values are underlined.

[a] The comparison between controls and patients before treatment with clozapine was performed by Mann–Whitney *U* test.

[b] The comparison between controls and patients after treatment with clozapine was performed by Mann–Whitney *U* test.

[c] The comparison between before and after treatment with clozapine was performed by Wilcoxon rank sum test.

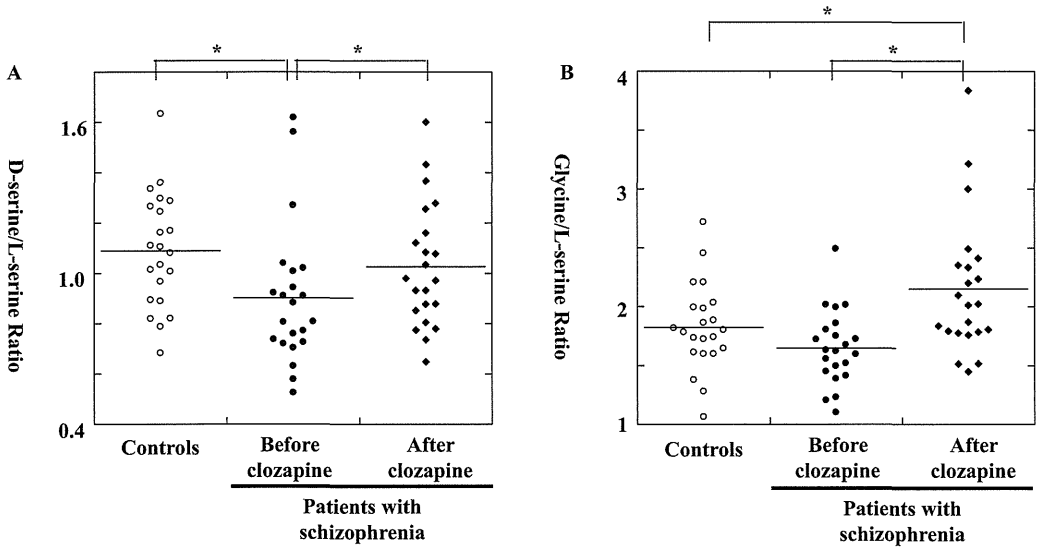

Fig. 2. Plasma D-/L-serine and glycine/L-serine ratio in treatment-resistant schizophrenia before and after clozapine treatment. The plasma D-/L-serine and glycine/L-serine ratios in the controls and patients with treatment-resistant schizophrenia before and after clozapine treatment (controls, $n=22$; patients with schizophrenia, $n=22$). The bars represent mean values. $^{*}p<0.05$.

endogenous, selective, full co-agonist at the glycine site of the NMDA receptor and modulates glutamatergic neurotransmission, and some studies found normal glycine levels [18,21], other studies reported increased concentrations in patients with schizophrenia [1,21] and other studies reported decreased levels in patients with schizophrenia [15,20,24]. In most of the previous studies, amino acids levels were measured at various times throughout clinical course, and the patients investigated were medicated with various antipsychotics or were medication-free. In this study, the only antipsychotic used in the treatment of patients was clozapine, and amino acids levels were measured before and after clozapine treatment, as the patients' clinical symptoms improved. We found no significant difference in the plasma L-serine and glycine levels in patients with schizophrenia, but we found significant change in the plasma L-serine and glycine levels in response to clozapine treatment. This change in the amino acids levels in response to treatment or clinical course may explain the inconsistencies between previous studies.

It has been reported that peripheral glutamate and glutamine levels were not changed in schizophrenia patients in comparison to controls [6,21]. Our result was consistent with previous studies.

Our study must be interpreted in light of its limitations. First, the sample size of the study is small. Second, only treatment-resistant patients with schizophrenia treated with clozapine were included, and patients treated with other antipsychotics or patients who were not treated with antipsychotics were not included in this study. Third, the antipsychotics used before clozapine treatment differ among the patients. Further studies are needed to evaluate the relationship between plasma amino acids levels, schizophrenia, and clozapine treatment.

Acknowledgments

We thank all of the individuals who participated in this study. This work was supported in part by research grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) KAKENHI [25461730-Grant-in-Aid for Scientific Research (C), 22390225-Grant-in-Aid for Scientific Research (B), 23659565-Grant-in-Aid for Challenging Exploratory Research and 221S0003-Grant-in-Aid for Scientific Research on Innovative Areas (Comprehensive Brain Science Network)]; and the Japan Foundation for Neuroscience and Mental Health. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2014.08.052.

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