episode of stress acutely reduces the sensitivity of human appetitive instrumental performance to outcome devaluation [38–40].

In the present study, despite the fact that stressed animals have demonstrated augmented response to psychostimulants [41], acute cocaine administration attenuated the responses in Group CHX. Although cocaine binds to the DA transporter (DAT) and serotonin and norepinephrine transporters with almost equal affinity [42], its major motor effects are provoked by the blockade of DAT at low or moderate concentrations and a consequent elevation of extracellular DA levels (DA tone) in all terminal fields expressing DAT [43–44]. The extent of DA efflux following psychostimulant administration in the NAc, the terminus of the mesoaccumbal DA pathway, is implicated in hyperlocomotion [44–47]. Meanwhile, increased DA transmission in the dSt, the terminus of the nigrostriatal DA pathway, is associated with augmented species-specific stereotyped behaviors [46, 48–49]. Rearing of rats is often described as a landmark of cocaine-induced stereotyped behaviors [50–51]. Thus, our results suggest that repeated CHX administration interfered with the acute cocaine-induced DAergic signaling pathway in the NAc and dSt.

A plausible neural mechanism underlying the attenuated response to cocaine is that repeated CHX administration decreased the number of functional DATs in the mesoaccumbal/nigrostriatal DAergic pathways. Indeed, DAT knockout (KO) mice do not exhibit an increase in locomotor activity in response to cocaine [43, 52]. In addition, compared with wild-type mice, DAT-KO mice exhibit attenuated habituation [43, 53–54], which is consistent with our findings (Fig. 2A). However, similar phenotypes may be generated when tyrosine hydroxylase mRNA is upregulated because both events may result in hyperactivation of D2 receptor-mediated signaling pathways followed by counterbalancing desensitization of functional D2 receptors [55].

Another finding opposite to the typical stressed model was that CHX-treated rats displayed antidepressant-like behavior in the forced swimming test. Reduced immobility of rodents is a validated experimental index of a potential antidepressant-like effect [56], and it is directly controlled, at least in part, by mesoaccumbal DAergic projections [57], although some research suggests that immobility in the forced swimming test is a readily learned adaptation to stress that conserves energy opposed to an impairment of coping with the stress [58]. The DAT hypothesis described previously may also be applicable because DAT-KO animals exhibit increased mobility than wild-type animals in the forced swimming test [43, 59].

Because we sought to evaluate the interactions between numerous behavioral variables and the effects of CHX on these variables, we employed multivariate analyses using PCA. These analyses revealed functional relationships among behavioral variables consolidated in each PC as well as the specific effects of repeated CHX administration on PC II, which included instrumental performance under the devalued condition, open-field activities before and after acute cocaine administration, and the immobility ratio of the forced swimming test (Table 1, Fig. 4, and S6 Figure). As described previously, in the outcome devaluation test

following the instrumental learning, a reduction in lever pressing during the devalued condition reflects the action controlled predominantly by goal-directed process that is sensitive to the current value of the outcome, whereas the outcome insensitivity manifests the predominance of habitual process to goal-directed process. Thus, the lower PC II score of CHX-treated animals versus controls indicates that the former are prone to habitual performance. Then, a raised question is whether outcome devaluation is related to striatal DA tone, as proposed for other variables in this component. A study using DAT KO mice illustrated that the outcome devaluation effect is unaltered [60]. However, because the DAT expression in this transgenic line was inhibited at all times, other neural mechanisms may compensate for the lack of DAT function, at least in part [61]. In addition, late-developing long-term neuroadaptation induced by repeated exposure to CHX, as noted for repeated psychostimulant administration [62], could be responsible for unknown mechanism.

Although the detailed mechanism by which each PC is functionally segregated or how CHX specifically influences PC II remains unknown, striatal DA-mediated regulation may play a pivotal role in integrating most of the behavioral variables of PC II and making them sensitive to repeated CHX administration. Supporting this idea, repeated administration of methamphetamine, which induces much stronger oxidative stress than cocaine, reduces the number of DAT molecules in the rat striatum [63]. In addition, methamphetamine promotes habit formation in rats [64]. It is possible that repeated CHX administration works on DAT in a similar manner as methamphetamine. Nevertheless, the effect of CHX on neural substrates other than the DA system is also possible, and the possibility that CHX affects molecules other than glutathione must be fairly considered.

At this point, it is not conclusive that how much the alterations we observed in the present work were due to the direct effect of oxidative stress. However, because the effects of repeated CHX administration were observed when there was no change in the levels of glutathione (S1 Figure) or apoptosis (S2 Figure), it is possible that these effects were developed at a later stage as long-lasting neuroadaptations of the redox system and not by oxidative stress itself. In accordance with this hypothesis, at present, the concept that oxidative stress always acts invasively whereas the redox system always works protectively in mitigating disease is under revision [65]. Therefore, investigating the effect of repeated CHX administration on the striatal DA system and decision-making will be more important in the future. In addition, studies are required to answer the question of whether CHX-induced alterations are restored by manipulating the redox system.

In this study, we were unable to detect an effect of CHX on the performance of response discrimination, its reversal learning, or any significant group difference in PCs that were significantly loaded by these variables. In contrast, it is proposed that oxidative stress may affect reversal learning, as reported in a recent study of transgenic mice expressing a putative dominant-negative disrupted in schizophrenia 1 (DISC1). This report implicates that oxidative stress may be involved in the loss of motivation in instrumental performance with a PR schedule,

accelerated habit formation of instrumental learning, and degradation of a reversal learning performance [66]. Another model exhibiting some discrepancy with our findings is glutathione-cysteine-ligase modifier (GCLM) KO mice in which glutathione synthesis is reduced. Whereas the mice were less immobile in the forced swimming tests similar to our findings, psychostimulants induced an excessive hyperlocomotion in the KO mice [13]. However, it is noteworthy that these studies employed transgenic mice with compromised DICS1 or GCLM function from the early neonatal stage but not from adulthood, and the level of glutathione reduction was far greater than that observed in our model, particularly in GCLM-KO mice. Those factors could explain the differences between these reports and our findings.

Another point to be mentioned is the difference of our findings from those in animal models of depression. Although there are some studies reporting the induction of oxidative stress in rodents exposed to chronic mild stress [67–68], Group CHX displayed a mixture of pro-depression- (attenuated habituation in novel environment and habit-prone in decision making) and antidepression-like phenotypes (blunted immobility in forced swimming and attenuated response to acute cocaine). This could be attributed to the systemic administration of CHX, which may affect the entire brain, whereas in major depression, oxidative stress may be induced in more specific brain regions or circuits.

In conclusion, repeated CHX administration produced a habit-prone phenotype by prolonging the effect of CHX challenge on outcome devaluation, although it did not affect the acquisition or motivation of an instrumental learning trained under a PR schedule. Response discrimination and reversal learning were not affected by CHX. Repeated CHX administration reduced immobility in the forced swimming tests and blunted cocaine-induced behaviors. Combined with the results of multivariate analyses, these findings suggest that repeated CHX administration to adult rats did not generate an animal model of a certain specific mental disorder, but it may have persistently altered a group of functionally related behavioral and cognitive variables.

Supporting Information

S1 Figure. Dose-, timing-, and brain region-dependent effect of CHX on total glutathione levels in the nucleus accumbens (NAc) and dorsal striatum (dSt). (A) Effects of various doses of CHX on total glutathione levels in the NAc at 1 h after acute CHX administration. Data are represented as relative ratios of the concentration of total glutathione after an acute administration of varying doses of CHX (12.5, 25, and 50 mg/kg, i.p.) to that of saline (N=4–5 each: $^{*}p<0.05$ vs. saline). (B) Effects of an excess amount of the glutathione precursor *N*-acetylcysteine on total glutathione levels in the NAc when co-administrated with CHX. *N*-acetylcysteine (1 g/kg) was i.p. administrated 1.5 h before acute injection of 25 mg/kg CHX (N=4–5: $^{*}p<0.05$, vs. saline). (C-D) Temporal effects of an acute CHX administration (25 mg/kg) on the total glutathione levels in the NAc

(C; N=4–5) and dSt (D; N=5). Data are represented as the relative ratios of the concentrations of total glutathione at 1 and 3 h after the acute administration of 25 mg/kg of CHX to that at 1 h after acute administration of saline ($^{*}p<0.05$, vs. saline). (E–F) Effects of repeated CHX administration for 7 days on total glutathione levels in the NAc and dSt at 1 day (E; N=5 each; dSt, $^{*}p<0.05$, vs. saline) or 3 weeks (F; N=8 each) after the final CHX administration. Data are represented as relative ratios compared with the concentration of total glutathione after repeated saline administration for 7 days in the same region of the brain. Error bars represent SEM.
doi:10.1371/journal.pone.0114024.s001 (PDF)

S2 Figure. TUNEL assay of the NAc and dSt of the animals which received 25 mg/kg of CHX for 7 consecutive days. Animals were perfused on the day following the last Vehicle/CHX administration. The brain sections from the core and shell subregions of the NAc and the dSt were compared with sections of the dSt where kainic acid was microinjected. KA: kainic acid, Veh: vehicle. Green: TUNEL, Blue: DAPI.
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S3 Figure. Repeated CHX administration did not affect performance in response discrimination learning and its reversal. (A) Mean number of trials to reach the criterion of the response discrimination training. A one-way ANOVA (Group: vehicle vs. CHX) did not reveal any significant group difference ($F[1, 16]=1.67$). (B) Mean number of errors committed during the response discrimination training without a significant group difference ($F[1, 16]=1.58$). (C) Mean number of trials to reach the criterion of the response reversal learning without a significant group difference ($F[1, 16]<1$). (D) Mean numbers of the two error subtypes, perseverative and regressive, recorded during the response reversal learning. A two-way ANOVA, 2 (Group: vehicle vs. CHX) × 2 (Error type: perseverative vs. regressive), did not demonstrate significant main effects of Group and Error type ($Fs[1, 16]<1$) or interaction between factors ($F[1, 16]=2.10$). Data represent the mean + SEM.
doi:10.1371/journal.pone.0114024.s003 (TIF)

S4 Figure. Repeated CHX administration did not affect spontaneous activity in a novel environment. The spontaneous activities in actimeter, locomotor activity (A), rearing counts (B), and stereotypic counts (C) were monitored on the preceding day of the acute cocaine administration (apparatus habituation training, 25 days after repeated vehicle/CHX administration). Separate two-way ANOVAs, 2 (Group) × 12 (Time bin), revealed only significant main effects of Time bin ($F[11, 176]=79.77$, 121.16, and 43.87; $ps<0.001$) for locomotor activity, rearing, and stereotypy, respectively, without the Group effects (main effect or interactions: $Fs<1.72$). Data represent the mean ± SEM.
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S5 Figure. Repeated CHX administration did not affect body weights. Robust group differences were not detected during repeated CHX administration (7

days), food deprivation, or subsequent *ad-libitum* feeding. Analysis using two-way ANOVA of 2 (Group) $\times$7 (Time point) revealed only a significant main effect of Time point, $F(6, 96)=1560.48$, $p<0.001$. Data represent the mean $\pm$ SEM.
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S6 Figure. Structure of principal component (PC) II. PC II accounted for 20.5% of the variance. This component was negatively loaded by the response rate in the devalued condition of devaluation test 1 as well as open field activity before acute cocaine injection, and positively loaded by open field activity after acute cocaine injection as well as the immobility ratio of the forced swimming test. Analysis of the PC scores calculated for individual animals in Groups Veh and CHX revealed that the scores of animals in Group CHX were significantly lower on PC II than those of animals in Group Veh, $t(16)=2.96$, $p<0.05$.
doi:10.1371/journal.pone.0114024.s006 (TIF)

S1 Table. Intercorrelations (Pearson correlation coefficient) between behavioral phenotypes.
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S2 Table. Principal component scores calculated for each animal in Groups Veh and CHX.
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S1 Text. Supporting materials and methods.
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Author Contributions

Conceived and designed the experiments: YI YM ST. Performed the experiments: YI ZL SK HN ST. Analyzed the data: YI ST. Contributed reagents/materials/analysis tools: YI ZL SK HN ST. Wrote the paper: YI YM ST.

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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.

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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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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

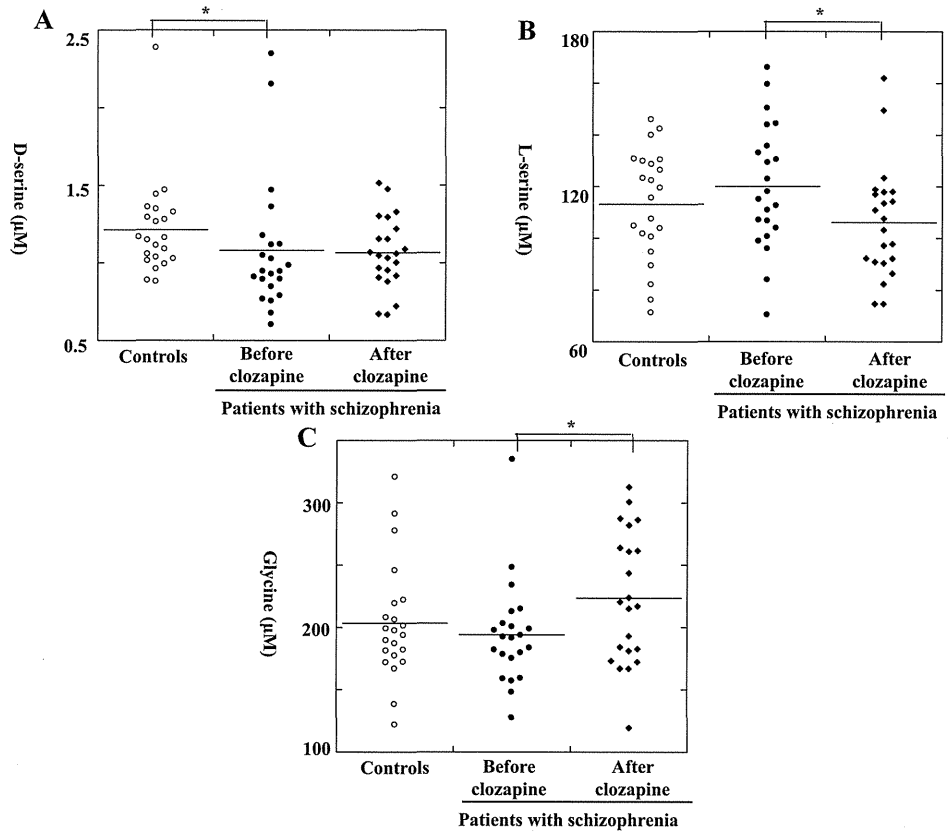

Fig. 1. Plasma levels of D-serine, L-serine, and glycine in treatment-resistant schizophrenia before and after clozapine treatment. The plasma levels of D-serine, L-serine, and glycine in the controls and patients with treatment-resistant schizophrenia before and after clozapine treatment (controls, $n = 22$; 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.

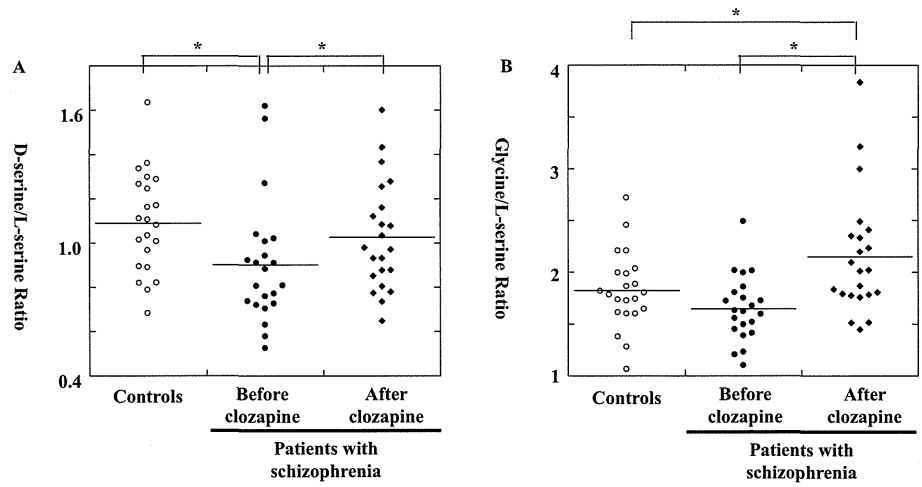

Fig. 2. Plasma D-/L-serine and glycine/L-serine ratio in treatment-resistant schizophrenia before and after clozapine treatment. The plasma D-/L-serine and glycine/L-serine ratios in the controls and patients with treatment-resistant schizophrenia before and after clozapine treatment (controls, $n = 22$; 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.

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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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