

## A CSF and postmortem brain study of D-serine metabolic parameters in schizophrenia

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### Abstract

Clinical trials demonstrated that D-serine administration improves schizophrenia symptoms, raising the possibility that altered levels of endogenous D-serine may contribute to the N-methyl D-aspartate receptor hypofunction thought to play a role in the disease. We hypothesized that cerebro-spinal fluid (CSF) D-serine levels are decreased in the patients due to reduced synthesis and/or increased degradation in brain. We now monitored amino acid levels in CSF from 12 schizophrenia patients vs. 12 controls and in postmortem parietal-cortex from 15 control subjects and 15 each of schizophrenia, major-depression and bipolar patients. In addition, we monitored postmortem brain serine racemase and D-amino acid oxidase protein levels by Western-blot analysis. We found a 25% decrease in D-serine levels and D/L-serine ratio in CSF of schizophrenia patients, while parietal-cortex D-serine was unaltered. Levels of L-serine, L-glutamine and L-glutamate were unaffected. Frontal-cortex (39%) and hippocampal (21%) serine racemase protein levels and hippocampal serine racemase/D-amino acid oxidase ratio (34%) were reduced. Hippocampal D-amino acid-oxidase protein levels significantly correlated with duration of illness ( $r=0.6$ ,  $p=0.019$ ) but not age. D-amino acid oxidase levels in patients with DOI>20 years were 77% significantly higher than in the other patients and controls. Our results suggest that reduced brain serine racemase and elevated D-amino acid oxidase protein levels may contribute to the lower CSF D-serine levels in schizophrenia.

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**Keywords:** Serine racemase; D-Amino acid oxidase; Schizophrenia; Postmortem brain; CSF; D-Serine

### 1. Introduction

Accumulating evidence suggests that N-methyl D-aspartate (NMDA) receptor hypofunction contributes to the symptomatic features of schizophrenia, particularly regarding the negative and cognitive symptoms of the disease (Coyle et al., 2003). A unique feature of the NMDA receptor is that the channel only operates when the sites of both glutamate and a coagonist are occupied

*Abbreviations:* CSF, cerebro-spinal fluid; DAAO, D-amino acid oxidase; NMDA, N-methyl D-aspartate; SR, serine racemase.

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(Danysz and Parsons, 1998). D-Serine has been shown to be a major endogenous coagonist of the NMDA receptors (Mothet et al., 2000; Shleper et al., 2005; Stevens et al., 2003). Based on the NMDA receptor hypofunction hypothesis, several clinical trials investigated whether administration of NMDA receptor coagonists is beneficial for the patients. When combined with conventional neuroleptics or newer atypical antipsychotics D-serine improved positive, negative and cognitive symptoms of schizophrenia patients (Heresco-Levy et al., 2005; Javitt, 2004; Tsai et al., 1998).

Endogenous D-serine is synthesized from L-serine by serine racemase (SR) (De Miranda et al., 2002; Foltyn et al., 2005; Wolosker et al., 1999). Degradation of D-serine in the cerebellum and brainstem is mediated by D-amino acid oxidase (DAAO) that specifically oxidizes D-amino acids (Schell et al., 1995). The homeostasis of extracellular brain D-serine levels is also affected by glial (Hayashi et al., 1997; Ribeiro et al., 2002) and neuronal (Helboe et al., 2003; Javitt et al., 2002; Yamamoto et al., 2001) transport systems. Despite the pharmacological use of D-serine in clinical trials in schizophrenia little is known about brain D-serine metabolism in this disease.

The possible involvement of D-serine in the etiology of schizophrenia is further suggested by the association of the disease with single nucleotide polymorphisms (SNPs) in the SR, the DAAO and its regulator (G72) (Chumakov et al., 2002; Detera-Wadleigh and McMahon, 2006; Goltsov et al., 2006; Schumacher et al., 2004). Yet, Yamada et al. (2005) did not replicate association between SR or DAAO and schizophrenia. Decreased serum D-serine levels were previously found in schizophrenic subjects (Hashimoto et al., 2003) and recently replicated (Yamada et al., 2005). However, it is not known whether serum D-serine levels reflect the levels in the brain. D-serine content and D/L-serine ratio were reported to be unchanged in postmortem (PM) schizophrenia patients' prefrontal or parietal cortex (Kumashiro et al., 1995). Very recently, Hashimoto et al. (2005b) found reduced D/total (D+L) serine ratio, but not D-serine levels *per-se*, in CSF samples from schizophrenia patients compared with control subjects.

The present study was carried out to further elucidate whether the metabolism of D-serine is involved in the pathophysiology and treatment of schizophrenia. The study is comprised of two parts: 1) D-serine and other amino acid level measurements in CSF samples from schizophrenia patients and control subjects. 2) Evaluation of SR and DAAO protein levels in postmortem frontal cortex and hippocampus of schizophrenia patients and control subjects. CSF as well as postmortem brain samples from psychiatric patients are hard to obtain

and usually contain small number of specimens in each cohort. Nevertheless, it provides a first-hand evidence for the pathology. We therefore combined two cohorts of CSF samples and a well-controlled brain collection.

## 2. Materials and methods

### 2.1. Cerebro-spinal fluid samples

Lumbar cerebro-spinal fluid (CSF) aliquots were obtained from 12 psychiatrically healthy subjects and 12 schizophrenia patients originated from two collections. Four age and sex-matched pairs were collected by Dr. Regenold's clinics, University of Maryland School of Medicine, Baltimore, MD., and eight pairs — from Dr. Torrey's collection (Kozlovsky et al., 2004; Levine et al., 2005). Patients of both cohorts were diagnosed by a well trained psychiatrist as meeting DSM-IV criteria for chronic schizophrenia. The study was approved by the IRB committees of each of the sites.

Table 1 summarizes the demographic and clinical data of the subjects.

### 2.2. Brain samples for serine levels

Postmortem (PM) parietal cortex blocks (0.5 g each) were obtained from the well-matched Stanley Medical Research Institute's Brain Collection. Detailed information about the group demographics were described elsewhere (Torrey et al., 2000). Briefly, 60 samples consisted of 15 schizophrenia patients, 15 bipolar patients, 15 unipolar patients and 14 normal controls. None of the control subjects had a history of psychiatric disorder or received anti-psychotic medication nor did any die of suicide or neurological disorders. The four groups were matched by age, sex, race, postmortem interval (PMI), mRNA quality, pH, and side of the brain. The study was approved by our hospital Helsinki committee (IRB).

### 2.3. Brain samples for serine racemase and D-amino acid oxidase protein levels

Postmortem brain samples from 15 schizophrenia patients and 15 matched normal controls were obtained from the Rebecca L. Cooper Research Laboratories at the Mental Health Research Institute of Victoria, Australia. Samples studied were of frontal cortex (FC) [Brodmann's Area (BA) 9] and hippocampus. Prior to commencement, permission to carry out this study was obtained from the Ethics Committee of the Victorian Institute of Forensic Medicine and the North Western

Table 1  
Demographic and clinical data of the schizophrenic patients and the matched healthy controls

	Sex	Age (years)	Race	CSF total protein	DOI (years)	Acuteness of symptoms	Locus	Medication
Schizophrenia patients	Male	39	A	28	25	2	1	1
	Female	53	C	53	32	1	4	1
	Female	61	C	27	40	2	3	2
	Male	45	A	52	20	2	1	1
	Male	52	C	27	25	3	4	3
	Male	45	C	27	23	2	4	1
	Male	16	C	26	2	2	2	1
	Female	33	C	33	12	3	4	1
	Male	23	C	34	0	3	1	0
	Female	31	C	23	9	2	3	1
	Male	42	C	34	5	1	4	1
	Female	27	C	27	0.1	1	2	1
Healthy controls	Male	42	A	31				
	Male	53	A	35				
	Male	46	A	44				
	Female	70	C	35				
	Male	25	C	39				
	Male	40	C	29				
	Male	25	C	19				
	Female	26	C	14				
	Female	45	C	33				
	Female	37	C	15				
	Female	28	C	27				
	Male	49	C	37				

C = Caucasian, A = Afro-American, DOI = duration of illness (years).

Acuteness of symptoms: 1. Minimal; 2. Moderate; 3. Marked.

Locus:

1. At beginning of psychotic episode
2. Peak of psychotic episode
3. Just recovered from psychotic episode
4. Chronic symptoms, no recent relapse.

Medication: 0. None; 1. Typical neuroleptics; 2. Atypical neuroleptics; 3. Washout for 2 months.

Mental Health Program Behavioral and Psychiatric Research and Ethics Committee. The final diagnosis according to DSM-IV criteria for chronic schizophrenia was established by consensus by two senior psychiatrists and a psychologist following an extensive case history review using the Diagnostic Instrument for Brain Studies (Hill et al., 1996; Roberts et al., 1998). Control subjects had no contact with any psychiatric service prior to death, had not received anti-psychotic medication, had not died by suicide or had any neurological disorders. The groups were matched by age, sex, postmortem interval (PMI) and pH of brain tissue.

#### 2.4. Antibodies

Serine racemase (SR) antibody was generated and characterized as previously described (Panizzutti et al., 2001). D-amino acid oxidase (DAAO) antibody was

generated by immunizing rabbits with purified hog DAAO Lot 85236822 (Roche, Indianapolis).

#### 2.5. D-serine measurement

To extract the free amino acids, protein was precipitated by addition of trichloroacetic acid (TCA) to 5% final concentration. After removing TCA by extraction with diethyl ether, D-serine was monitored by HPLC analysis, as described (Hashimoto et al., 1992). Since CSF D-serine levels are in the low micromolar range, the identity of the D-serine peak was confirmed by treating the samples with 10 µg/ml purified recombinant D-serine deaminase for one hour in 20 mM KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.5). The latter enzyme specifically oxidizes D-serine into pyruvate and ammonia and was purified as described (Shleper et al., 2005). This treatment disclosed a background peak eluting at D-serine's position

that accounted for about 10% of the signal, which was subtracted from each sample (Fig. 2A).

### 2.6. Crude homogenate preparation and Western-blot analysis of serine racemase and DAAO protein levels

Crude homogenates used in the present study have been prepared for a previously described study (Nadri et al., 2004). Sodium-dodecyl sulfate/polyacrylamide gel electrophoretic separation (SDS-PAGE) and immunoblotting of SR and DAAO were performed using previously described procedure (Nadri et al., 2004) with modifications. Aliquots (35–50 µg total protein for hippocampus or 50–100 µg total protein for frontal cortex, within the linear range of quantitative detection) were separated (12% gel, 150 V), blotted, and probed over-night at 4 °C with diluted polyclonal anti SR antiserum (1:1000) and anti DAAO antiserum (1:100). Secondary Horse Reddish Peroxidase-anti-rabbit antibody (1:10,000) was then added for one hour for detection. Bands were detected with Chemiluminescence Western-blot detection Kit (Amersham, Oakville, Ont). Fig. 1 shows representative blots of FC and hippocampus serine racemase and DAAO. Densities of the immunoreactive bands were quantified using AIDA-2D image analysis system (Dinco, Israel). Results are given as percent of mean control value in each gel.

Since DAAO activity measured histochemically in the forebrain has been reported to be very low or absent in the forebrain (Schell et al., 1995), to assure the specificity of the DAAO antibody we ran a comparative Western blot of human postmortem FC, hippocampal

and cerebellar crude homogenate. The 37 kDa band density of DAAO of the cerebellum [29 Arbitrary Units (AU)+9 (SD)/50 µg total protein was about 3 fold higher than that of the FC (7 AU+3) and the hippocampus (10+3)]. This is qualitatively, but not quantitatively in accordance with the enrichment of mRNA (Yoshikawa et al., 2005) and activity of D-amino acid oxidase in the cerebellum (Schell et al., 1995). Our data is in agreement with previous immunocytochemical study indicating the presence of significant levels of DAAO in prefrontal areas of rat brain (Moreno et al., 1999). Moreover, Verrall et al. (2006) have also recently reported DAAO protein abundance in postmortem human dorsolateral prefrontal cortex (DLPFC). To further substantiate the specificity of the antibodies, 0.1 ml of the antiserum were preabsorbed with 50 µg purified DAAO preparation (Roche, Indianapolis), diluted to the same degree as routinely used in our assays and then used for Western blot analysis. No 37 kDa band was detected with the preabsorbed antibody (data not shown) confirming that the bands monitored with the antibodies are those of DAAO.

The investigators who carried out HPLC analysis (I. B., R.P., J.D.M) and Western-blotting (C.N. and S.A.) were blind to the group's identity of the samples. Analyses were carried out in a matched manner so that an equal number of samples of all diagnoses were assayed concomitantly.

### 2.7. Statistical analysis

ANOVA with posthoc Fisher's LSD test and Student's *t*-test were carried out to compare among

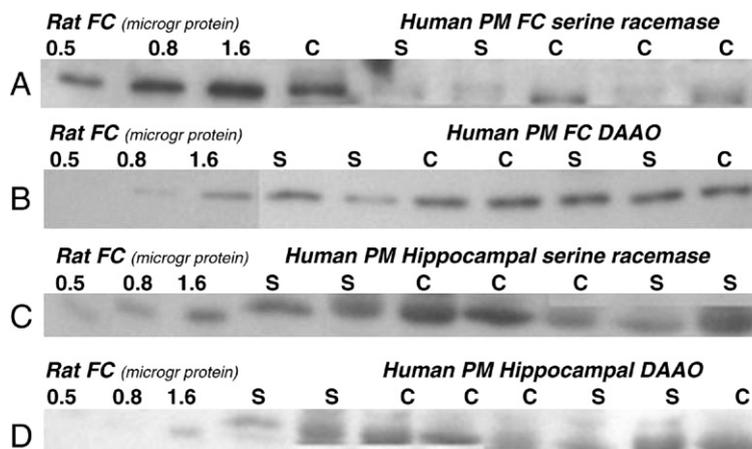


Fig. 1. Representative blots of FC and hippocampal serine racemase and DAAO. Frontal cortex (A and B) serine racemase (A) and DAAO (B). Hippocampal (C and D) serine racemase (C) and DAAO (D). A rat FC sample served as a calibration curve for all human PM brain samples. S = Schizophrenia patients, C = Control subjects.

Table 2  
CSF amino acid levels

	D-Serine ( $\mu\text{M}$ )	L-Serine ( $\mu\text{M}$ )	D-Serine/L-Serine	L-Glutamate ( $\mu\text{M}$ )	L-Glutamine ( $\mu\text{M}$ )	L-Glutamine/L- Glutamate
Control	1.81 $\pm$ 0.37	37.6 $\pm$ 5.8	0.049 $\pm$ 0.009	8.9 $\pm$ 2.4	509 $\pm$ 90	59.2 $\pm$ 10.5
Schizophrenia	1.35 $\pm$ 0.48	37.3 $\pm$ 8.4	0.037 $\pm$ 0.013	8.2 $\pm$ 2.6	453 $\pm$ 91	54.2 $\pm$ 19.6
<i>p</i>	0.015	0.93	0.022	0.48	0.14	0.45

mean values and correlations were analyzed by Spearman's correlation test using the software STATISTICA.

### 3. Results

#### 3.1. D-serine levels

##### 3.1.1. Human CSF

A significant 25% decrease in CSF D-serine levels was observed in schizophrenia patients vs. healthy control subjects (ANOVA,  $F=6.98$ ,  $p=0.015$ ; Table 2

and Fig. 2B). D-serine levels did not differ between males and females in the whole cohort [females ( $n=10$ ) 1.43  $\mu\text{M}\pm$ 0.56 (S.D); males ( $n=14$ ) 1.69 $\pm$ 0.41;  $F=1.73$ ,  $p=0.201$ ]; D-serine levels in female schizophrenia patients differed significantly from healthy female controls (schizophrenia patients 1.06 $\pm$ 0.33; controls 1.8 $\pm$ 0.49,  $F=7.88$ ,  $p=0.023$ ) whereas in males the difference was not statistically significant (schizophrenia patients 1.56 $\pm$ 0.48; controls 1.82 $\pm$ 0.31,  $F=1.52$ ,  $p=0.242$ ). Introducing sex as a covariate did not affect the significance of the difference in D-serine

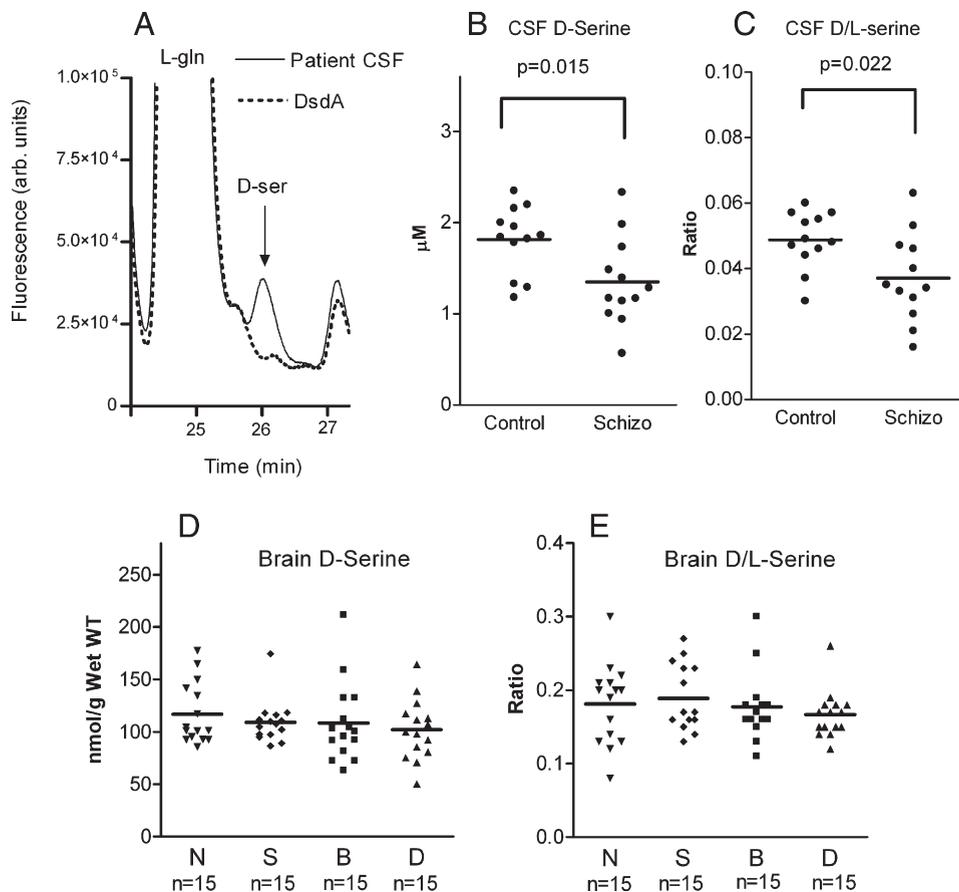


Fig. 2. CSF and postmortem parietal cortex D-serine levels. A. HPLC analysis reveals a discrete D-serine peak in CSF, which disappears following treatment with D-serine deaminase (DsdA). B. CSF D-serine levels. C. CSF D/L-serine ratio. D. D-serine levels in postmortem parietal cortex of normal controls (N), schizophrenia (S), bipolar (B) and major depression without psychosis (D) patients. E. D/L-Serine ratio in postmortem parietal cortex in the same groups as in D.

levels between the two diagnostic groups ( $F=7.37$ ,  $p=0.013$ ) and two-way ANOVA with diagnosis and sex as independent factors showed only a significant effect of diagnosis, no effect of sex and no interaction (diagnosis:  $F_{(1,20)}=8.84$ ,  $p=0.008$ ; sex:  $F_{(1,20)}=2.34$ ,  $p=0.142$ ; interaction:  $F_{(1,20)}=1.98$ ,  $p=0.175$ ). There was no correlation between D-serine levels and age ( $r=0.027$ ,  $p=0.902$ ), CSF total protein ( $r=-0.178$ ,  $p=0.405$ ) and duration of illness ( $r=0.601$ ,  $p=0.853$ ). There was no effect of race in the whole cohort [Caucasians ( $n=19$ )  $1.54 \mu\text{M} \pm 0.53$  (SD); Afro-Americans ( $n=5$ )  $1.72 \pm 0.26$ ;  $F=-0.726$ ,  $p=0.476$ . Since there were only five samples derived from Afro-Americans (2 patients, 3 controls), excluding them from the analysis did not affect the significance of the difference in D-serine levels between the two diagnostic groups ( $F=4.74$ ,  $p=0.044$ ). Two-way ANOVA with diagnosis and race as independent factors showed a marginally significant effect of diagnosis, no effect of race and no interaction (diagnosis:  $F_{(1,20)}=3.35$ ,  $p=0.08$ ; race:  $F_{(1,20)}=0.32$ ,  $p=0.577$ ; interaction:  $F_{(1,20)}=0.07$ ,  $p=0.796$ ). There was no effect of acuteness of symptoms (defined under Table 1) [ANOVA,  $F_{(2,9)}=0.68$ ,  $p=0.530$ ], locus of CSF withdrawal (defined under Table 1) [ANOVA,  $F_{(3,8)}=1.72$ ,  $p=0.24$ ] and medication (defined under Table 1) [ANOVA,  $F_{(3,8)}=0.601$ ,  $p=0.632$ ]. Two-way ANOVA with diagnosis and cohort (Dr. Regenold's vs. Dr. Torrey's collections) as independent factors showed a significant effect of diagnosis, no effect of cohort and no interaction (diagnosis:  $F_{(1,20)}=5.32$ ,  $p=0.032$ ; cohort:  $F_{(1,20)}=0.152$ ,  $p=0.7$ ; interaction:  $F_{(1,20)}=0.06$ ,  $p=0.81$ ). Three-way ANOVA with diagnosis, sex and cohort as independent factors showed a significant effect of diagnosis, no effect of cohort or sex and no interaction (diagnosis:  $F_{(1,16)}=4.38$ ,  $p=0.05$ ; sex:  $F_{(1,16)}=2.83$ ,  $p=0.11$ ; cohort:  $F_{(1,16)}=0.29$ ,  $p=0.6$ ; interaction:  $F_{(1,16)}=1.26$ ,  $p=0.28$ ).

The D/L-serine ratio was also 23% significantly lower in the patients (ANOVA,  $F_{(1,22)}=6.12$ ,  $p=0.022$ , Table 2 and Fig. 2C). D/L-serine ratio did not differ between males and females in the whole cohort [females ( $n=10$ )  $0.039 \pm 0.01$  (SD); males ( $n=14$ )  $0.046 \pm 0.01$ ;  $F_{(1,22)}=1.26$ ,  $p=0.221$ ]; D/L-serine ratio in female schizophrenia patients differed significantly from healthy females (schizophrenia patients  $0.03 \pm 0.01$ ; controls  $0.048 \pm 0.01$ ,  $F_{(1,8)}=-2.39$ ,  $p=0.044$ ) whereas in males the difference was not statistically significant (schizophrenia patients  $0.042 \pm 0.01$ ; controls  $0.049 \pm 0.01$ ,  $F_{(1,12)}=-1.24$ ,  $p=0.239$ ). However, two-way ANOVA with diagnosis and sex as independent factors showed a significant effect only of diagnosis, no effect of sex and no interaction (diagnosis:  $F_{(1,20)}=7.42$ ,  $p=0.013$ ; sex:  $F_{(1,20)}=2.02$ ,  $p=0.171$ ; interaction:  $F_{(1,20)}=1.47$ ,  $p=0.24$ ). There was no effect of race on D/L-serine ratio in the whole cohort [Caucasians ( $n=19$ )  $0.42 \pm 0.01$  (SD); Afro-Americans ( $n=5$ )  $0.46 \pm 0.01$ ;  $F_{(1,22)}=-0.633$ ,  $p=0.533$ ; the difference between Caucasian patients and controls was statistically non-significant [schizophrenia patients ( $n=10$ ),  $0.37 \pm 0.01$ ; controls ( $n=9$ )  $0.47 \pm 0.01$ ;  $F_{(1,17)}=-1.69$ ,  $p=0.108$ ] while between Afro-American patients and controls the difference was statistically different [schizophrenia patients ( $n=2$ ),  $0.36 \pm 0.01$ ; controls ( $n=3$ )  $0.53 \pm 0.01$ ;  $F_{(1,3)}=-3.43$ ,  $p=0.042$ ]. Two-way ANOVA with diagnosis and race as independent factors showed a significant effect of diagnosis, no effect of race and no interaction (diagnosis:  $F_{(1,20)}=5.04$ ,  $p=0.036$ ; race:  $F_{(1,20)}=0.12$ ,  $p=0.733$ ; interaction:  $F_{(1,20)}=0.38$ ,  $p=0.543$ ). Introducing both sex and race as covariates did not affect the significance of the difference in D/L-serine ratio between the two diagnostic groups ( $F_{1,20}=6.107$ ,  $p=0.023$ ). There was no correlation between D/L-serine ratio and age ( $r=0.176$ ,  $p=0.411$ ), CSF total protein ( $r=0.08$ ,  $p=0.970$ ) and duration of illness ( $r=0.172$ ,  $p=0.592$ ). There was no effect of acuteness of

Table 3  
Parietal cortex amino acid levels

	D-Serine (nmol/g Wet WT)	L-Serine (nmol/g Wet WT)	D-Serine/L-Serine	L-Glutamate (nmol/g Wet WT)	L-Glutamine (nmol/g Wet WT)	L-Glutamine/L-Glutamate
Control	117±30	734±428	0.181±0.055	5099±880	2084±642	0.409±0.070
Schizophrenia	109±21	626±250	0.189±0.045	4792±715	1732±321	0.477±0.250
Bipolar	109±36	636±243	0.177±0.046	4737±1293	2705±2194	0.526±0.368
Depression	102±29	638±222	0.167±0.032	4497±984	2049±965	0.408±0.137
<i>p</i> (ANOVA)	0.56	0.85	0.62	0.40	0.18	0.46

symptoms [ANOVA,  $F_{(2,9)}=1.76$ ,  $p=0.226$ ], locus of CSF withdrawal [ANOVA,  $F_{(3,8)}=1.68$ ,  $p=0.247$ ] and medication [ANOVA,  $F_{(3,8)}=2.02$ ,  $p=0.190$ ]. Two-way ANOVA with diagnosis and cohort (Dr. Regenold's vs. Dr. Torrey's collections) as independent factors showed a significant effect of diagnosis, no effect of cohort and no interaction (diagnosis:  $F_{(1,20)}=5.48$ ,  $p=0.03$ ; cohort:  $F_{(1,20)}=0.24$ ,  $p=0.63$ ; interaction:  $F_{(1,20)}=0.09$ ,  $p=0.77$ ). Three-way ANOVA with diagnosis, sex and

cohort as independent factors showed a significant effect of diagnosis, no effect of cohort or sex and no interaction (diagnosis:  $F_{(1,16)}=6.2$ ,  $p=0.024$ ; sex:  $F_{(1,16)}=1.12$ ,  $p=0.307$ ; cohort:  $F_{(1,16)}=0.36$ ,  $p=0.557$ ; interaction:  $F_{(1,16)}=2.93$ ,  $p=0.106$ ).

The differences in D-serine levels and D/L-serine ratio are specific, since levels of L-serine [ $F_{(1,22)}=0.009$ ,  $p=0.926$ ] as well as L-glutamate [ $F_{(1,22)}=0.033$ ,  $p=0.857$ ], L-glutamine [ $F_{(1,22)}=2.29$ ,  $p=0.144$ ] and L-

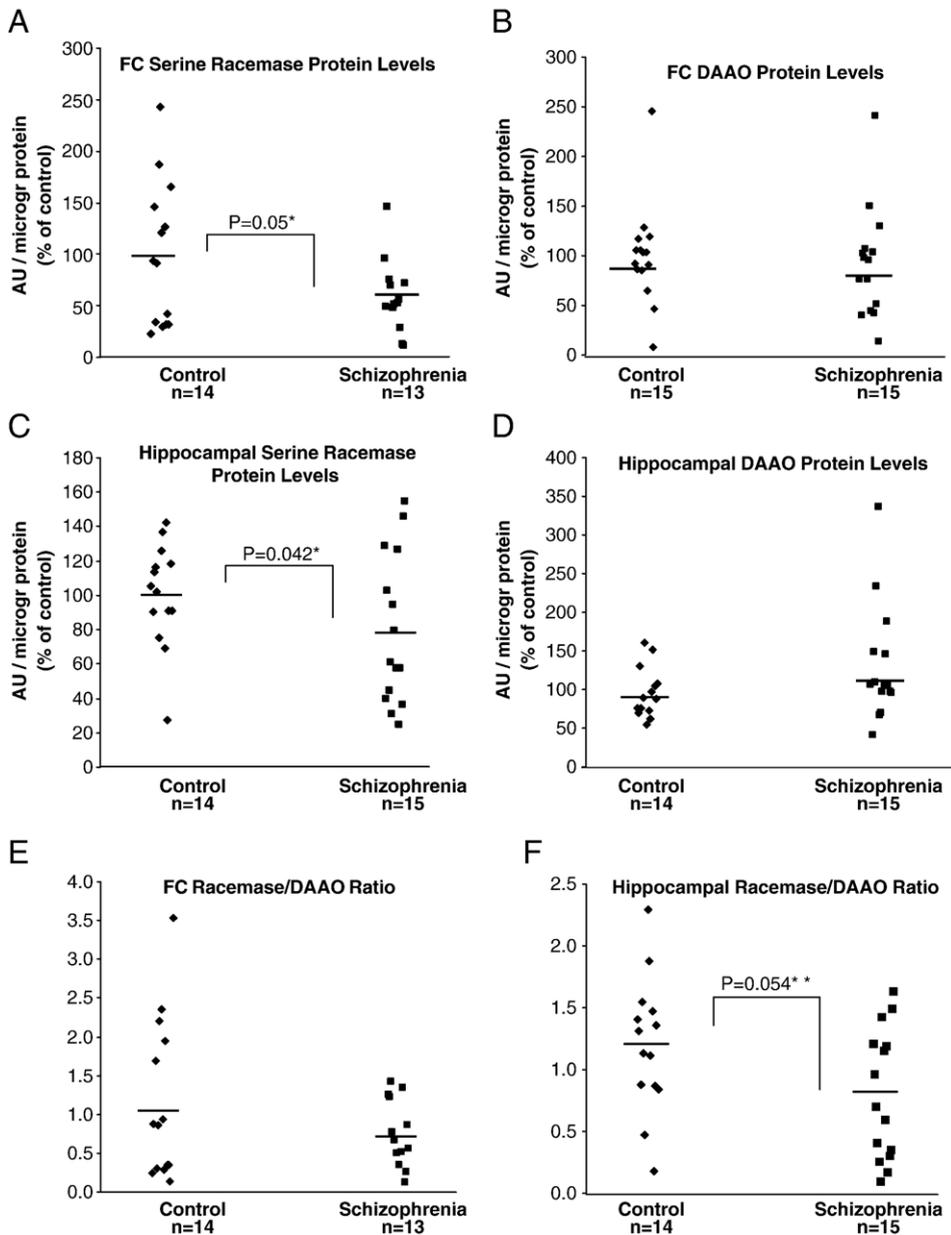


Fig. 3. Postmortem brain serine racemase and DAAO protein levels. Frontal cortex (A and B) serine racemase (A) and DAAO (B) protein levels. Hippocampal (C and D) serine racemase (C) and DAAO (D) protein levels. Serine racemase/DAAO ratio in the FC (E) and in the hippocampus (F).

\* Marginally significant *t*-test. \*\* Statistically significant *t*-test.

glutamine/L-glutamate ratio [ $F_{(1,22)}=0.87, p=0.361$ ] in the patients did not differ from controls (Table 2).

### 3.1.2. Human postmortem parietal cortex

There were no statistically significant differences in PM parietal cortex D-serine, L-serine, D/L-serine ratio, L-glutamate and L-glutamine levels in 15 patients each of schizophrenia, bipolar disorder and major depressive disorder without psychotic features, and 15 normal controls (Fig. 2D and 2E and Table 3). There was no correlation between D-serine levels and age ( $r=-0.041, p=0.755$ ), age of onset ( $r=-0.166, p=0.276$ ), duration of disease ( $r=0.044, p=0.776$ ), pH of the tissue ( $r=-0.002, p=0.9897$ ), post-mortem interval (PMI) ( $r=0.072, p=0.584$ ) and tissue storage time ( $r=0.067, p=0.609$ ) and no effect of gender ( $r=-0.08, p=0.546$ ), cause of death ( $r=0.134, p=0.308$ ), neuroleptic use ( $r=0.084, p=0.658$ ) and history of alcohol and substance abuse ( $r=0.199, p=0.127$ ) on D-serine levels. Mean PMI values of the four diagnostic groups did not statistically differ from each other [ANOVA,  $F_{(3,56)}=1.86; p=0.147$ ] but schizophrenia patients' mean PMI was 42% significantly higher than that of the control group ( $t=-2.18, p=0.038$ ). We therefore introduced PMI values as a covariate but it did not change the lack of difference in D-serine levels among the diagnostic groups (ANOVA,  $F_{(3,55)}=0.74; p=0.741$ ).

L-glutamate, L-glutamine and L-glutamine/L-glutamate ratio did not differ among the four diagnostic groups [ANOVA, L-glutamate:  $F_{(3,56)}=0.507; p=0.679$ ; L-glutamine:  $F_{(3,56)}=1.2; p=0.319$ ; L-glutamine/L-glutamate ratio:  $F_{(3,56)}=0.885; p=0.454$ ] (Table 3).

## 3.2. Serine racemase and DAAO protein levels

### 3.2.1. Frontal cortex

Frontal cortex (BA9) serine racemase protein levels were 39% marginally significantly lower in the schizophrenia patients' group compared with the control group [59 arbitrary units (AU)/ $\mu\text{g}$  protein  $\pm 36$  (SD),  $n=13$  vs.  $98 \pm 70, n=14$ , respectively,  $t$ -test,  $t=2.9, p=0.1$  covariating for age (serine racemase protein levels correlated with age ( $n=27, r=0.45, p=0.02$ )] (Fig. 3A). DAAO protein levels in the schizophrenia patients did not differ from control values ( $91 \pm 56, n=15$  vs.  $100 \pm 51, n=15$ , respectively,  $t=0.46, p=0.648$ ) (Fig. 3B). Serine racemase/DAAO protein levels ratio in the schizophrenia patients also did not differ significantly from control values [ $0.8 \pm 0.4$  (SD),  $n=13$  vs.  $1.1 \pm 1.0, n=15$ , respectively,  $t=1.02, p=0.296$ ] (Fig. 3E).

Serine racemase protein levels did not correlate with PMI ( $r=0.14, p=0.494$ ), pH ( $r=-0.09, p=0.966$ ) or DOI ( $r=0.04, p=0.904$ ) and did not differ between males and females ( $77 \pm 48, n=20$  vs.  $86 \pm 86, n=7$ , respectively,  $t=-0.34, p=0.733$ ). DAAO protein levels did not correlate with age ( $r=-0.06, p=0.771$ ), PMI ( $r=-0.22, p=0.237$ ), pH ( $r=0.29, p=0.117$ ) or DOI ( $r=0.16, p=0.564$ ) and did not differ between males and females ( $97 \pm 44, n=22$  vs.  $91 \pm 74, n=8$ , respectively,  $t=0.30, p=0.767$ ). Serine racemase/DAAO ratio did not correlate with age ( $r=0.22, p=0.259$ ), PMI ( $r=0.29, p=0.135$ ), pH ( $r=-0.38, p=0.845$ ) or DOI ( $r=-0.19, p=0.538$ ) and did not differ between males and females ( $0.9 \pm 0.6, n=20$  vs.  $1.0 \pm 1.2, n=8$ , respectively,  $t=-0.44, p=0.662$ ).

### 3.2.2. Hippocampus

Hippocampal serine racemase protein levels were 21% marginally significantly lower in the schizophrenia patients' group [ $79 \pm 44, n=15$  vs.  $100 \pm 30, n=14$ , respectively,  $t=3.21, p=0.084$ , covariating for sex (male values significantly differed from those of females:  $81 \pm 37, n=22$  vs.  $115 \pm 36, n=7$ , respectively,  $t=4.524, p=0.043$ )] (Fig. 3C). Schizophrenia patients' hippocampal DAAO levels (Fig. 3D) significantly correlated with DOI ( $r=0.6, p=0.019$ ) but not age ( $r=0.27, p=0.332$ ). DAAO levels in patients with DOI > 20 years were 77% significantly higher than in the other patients and controls (Table 4). Serine racemase/DAAO protein levels ratio in the schizophrenia patients was 34% significantly lower from control values ( $0.8 \pm 0.5, n=15$  vs.  $1.2 \pm 0.5, n=14$ , respectively,  $t=2.01, p=0.05$ ) (Fig. 3F).

Hippocampal serine racemase protein levels did not correlate with age ( $r=-0.28, p=0.142$ ), PMI ( $r=0.07, p=0.709$ ), pH ( $r=-0.15, p=0.440$ ) or DOI ( $r=-0.15, p=0.587$ ). Hippocampal DAAO protein levels did not correlate with age ( $r=0.17, p=0.368$ ), PMI ( $r=-0.04,$

Table 4  
Increased hippocampal DAAO protein levels in schizophrenia patients with DOI  $\geq 20$  years

	Control	Schizophrenia	
		DOI < 20 years	DOI $\geq 20$ years
N	14	10	5
Age (years, mean $\pm$ SD)	41.8 $\pm$ 15.7	46.6 $\pm$ 15.4	56.2 $\pm$ 9.4 <sup>a</sup>
DAAO (AU)	106 $\pm$ 40	96 $\pm$ 33	177 $\pm$ 109 <sup>b</sup>

<sup>a</sup> ANOVA:  $F=1.585, df=2,27, p=0.223$ .

<sup>b</sup> ANOVA:  $F=4.35, df=2,26, p=0.0235$ ; post-hoc LSD:  $p=0.0075$  from controls and  $p=0.023$  from schizophrenia patients with DOI < 20 years.

$p=0.944$ ) or pH ( $r=0.27$ ,  $p=0.152$ ) and did not differ between males and females ( $116\pm 66$ ,  $n=22$  vs.  $106\pm 39$ ,  $n=7$ , respectively,  $t=0.38$ ,  $p=0.707$ ). Serine racemase/DAAO ratio did not correlate with age ( $r=-0.2$ ,  $p=0.302$ ), PMI ( $r=-0.07$ ,  $p=0.732$ ), pH ( $r=-0.24$ ,  $p=0.223$ ) or DOI ( $r=-0.23$ ,  $p=0.407$ ) and did not differ between males and females ( $1.0\pm 0.6$ ,  $n=21$  vs.  $1.2\pm 0.5$ ,  $n=7$ , respectively,  $t=-1.11$ ,  $p=0.277$ ).

#### 4. Discussion

The present study found a specific and statistically significant 25% decrease in CSF D-serine levels in schizophrenia patients vs. control subjects. This finding provides an indication that D-serine disposition in the nervous system may be altered in this disease. Since the coagonist site of NMDA receptors of several brain regions may be unsaturated *in vivo* (Danysz and Parsons, 1998), decreased extracellular D-serine may contribute to NMDA hypofunction thought to occur in schizophrenia.

Hashimoto and colleagues reported a decrease in serum D-serine associated with increased serum L-serine in schizophrenia subjects (Hashimoto et al., 2003). This was confirmed in a subsequent study with a larger sample population (Yamada et al., 2005). We did not detect significant changes in L-serine levels in our CSF samples. This difference may be related to the limitations of using serum to ascertain brain levels of amino acids. L-serine values in the serum are 10 fold higher than those we found in the CSF (Table 2) and than those reported in brain extracellular fluid (Hashimoto et al., 1995). Serum L-serine concentration is intimately related to the amino acid metabolism of peripheral tissues, such as the catabolic L-serine dehydratase enzyme in the liver (Xue et al., 1999). In addition, several factors (e.g., renal clearance and hepatic metabolism) may also affect serum levels of D-serine regardless of its brain values. On the other hand, CSF levels of amino acids are more indicative of their extracellular brain concentration. Hashimoto and colleagues have recently reported reduced D/total (D+L) serine, but not D-serine levels *per-se*, in CSF samples from schizophrenia patients compared with control subjects (Hashimoto et al., 2005b). Although the changes observed by Hashimoto et al. (2005b) in CSF were less striking than in our present study, their report further highlights a possible change in D-serine disposition in schizophrenia.

Hashimoto et al. (2005a) have recently reported increased CSF L-glutamine/L-glutamate ratio in first episode and drug naïve schizophrenia patients but no difference in the levels of each of these amino acids compared with healthy controls. The patients from

whom CSF was obtained for the present study were neither first episode nor drug naïve and no difference in L-glutamine/L-glutamate ratio was obtained. In parallel, PM parietal cortex L-glutamate, L-glutamine and L-glutamine/L-glutamate ratio did not differ between the four diagnostic groups in this study. The schizophrenia patients from whom postmortem brain samples were obtained were also neither first episode nor drug naïve.

In agreement with a previous study that used a different PM brain bank (prefrontal and parietal cortex) (Kumashiro et al., 1995), no difference was now found between normal controls and schizophrenia patients in parietal cortex D-serine levels. Kumashiro et al. (1995) reported that elongated PMI results in decreased D-serine levels. Mean PMI values of the four diagnostic groups studied presently were not statistically different from each other (Torrey et al., 2000). If any, the mean PMI in the schizophrenia patients group was the highest of all groups and nevertheless their D-serine levels were not reduced. Since we also did not find an effect of chronic antipsychotic treatment of rats on frontal cortex D-serine levels, the decrease in CSF D-serine observed in the present study is conceivably not due to global changes in brain D-serine synthesis or degradation and not due to neuroleptic treatment of the patients. Yet, the results do not rule out the possibility that localized changes in D-serine levels occur in discrete areas of the brain of schizophrenia patients.

Reduction in CSF D-serine levels may result from reduced serine racemase levels and/or elevated DAAO levels so that serine racemase/DAAO ratio would be reduced. Our results partially support these possibilities since frontal cortex and hippocampal serine racemase protein levels in schizophrenia patients were found 39% and 21%, respectively, significantly reduced, and hippocampal serine racemase/DAAO ratio 34% marginally significantly reduced. Verrall et al. (2006) have recently reported increased SR protein levels in PM DLPFC of schizophrenia patients from two UK brain collections and Steffek et al. (2006) report increased SR protein levels in PM hippocampus in schizophrenia patients. These results support the notion of dysregulation of D-serine metabolism in schizophrenia patients. The difference between the results of the present study and the two other groups may not be attributed to antipsychotic medication since, as found in the present study for D-serine levels (data not shown), Verrall et al. (2006) did not find an effect of haloperidol and clozapine treatment on rat FC SR protein levels.

The role of DAAO in metabolizing D-serine in forebrain areas has been debatable. In rats, DAAO activity monitored in brain sections is restricted to the

cerebellum and brainstem (Schell et al., 1995). Moreover, mice expressing catalytically inactive DAAO do not display increased levels of D-serine in the forebrain, suggesting that DAAO would not play a role in D-serine metabolism in these areas (Hashimoto et al., 1993; Nagata, 1992). However, using immunohistochemical methods Ceru and co-workers detected DAAO immunoreactivity in rat forebrain areas (Moreno et al., 1999), and Verrall et al. (2006) have recently been able to detect mRNA as well as protein (using Western blotting) of DAAO in postmortem human DLPFC. Almond et al. (2006) characterized a naturally occurring mutant mouse strain which lacks DAAO activity accompanied by increased occupancy of the NMDA receptor glycine site due to elevated extracellular D-serine levels. These mice also exhibit enhanced NMDA receptor function *in vivo*. Our detection of DAAO expression in human forebrain will allow the investigation of its role in schizophrenia. Interestingly, we found that hippocampal DAAO protein levels in the patients significantly correlated with DOI but not with age, suggesting the possibility that increased breakdown of hippocampal D-serine is associated with the progressive nature of the severity of schizophrenia.

Another possible factor contributing to the altered CSF D-serine levels is dysregulation of D-serine transport. Neutral amino acid transporters were previously shown to mediate D-serine uptake and release from neural cells, but the molecular identity of the D-serine transporter in the brain is still not clear (Javitt, 2002; Ribeiro et al., 2002). It is conceivable that the transport of D-serine is functionally altered in schizophrenia and this might contribute to decreased extracellular D-serine levels. Further studies of the identity of D-serine transport systems and their expression in tissue from schizophrenia patients will shed light on the mechanisms regulating brain and CSF D-serine levels.

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