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The presence of free D-serine in rat brain

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Free amino acid enantiomers in adult rat brain extracts were analyzed as their *N,O*-pentafluoropropionyl isopropyl derivatives by gas chromatography on a capillary column of Chirasil-L-Val. A peak X, which exhibited the same retention time as the *N,O*-pentafluoropropionyl isopropyl derivative of authentic D-serine, was detected in the brain extracts. Electron impact and positive chemical ionization mass spectra of the peak X of the brain extracts were identical to those of authentic D-serine. The concentration of free D-serine and the ratio of D-serine/total serine in the brain were estimated to be 0.27 and 0.23 μmol/g of wet weight, respectively. These data provide the first evidence that substantial quantities of free D-serine are present in mammalian brain tissues.

D-Serine; Amino acid enantiomer; Chirasil-L-Val; Gas chromatography; Mass spectrometry; Rat brain

1. INTRODUCTION

Several lines of evidence have demonstrated that D-amino acids such as D-serine and D-alanine potentiate *N*-methyl-D-aspartate (NMDA)-type excitatory amino acid receptor-mediated response through interaction with the strychnine-insensitive glycine binding site in mammalian brains in vitro [1–3]. Furthermore, an intracerebroventricular administration of these D-amino acids enhances the NMDA-induced seizure susceptibility [4] and inhibits phencyclidine- and methamphetamine-induced locomotor stimulation and stereotyped behaviors in rats [5–7]. Despite these significant functional roles in the central nervous system, the presence of free D-amino acids in mammalian brain has not yet been demonstrated, with the exception of the small amounts of free neutral amino acids in mice and human brains [8,9] and of free D-aspartate in adult rat and human brains (less than 3% of total aspartate) [10–12]. In the present study, we report the identification and the concentration of the free D-enantiomer of serine in adult rat brain using gas chromatographic (GC) analysis and GC-electron-impact (EI)- and positive chemical ionization (PCI)-mass spectroscopic (MS) analyses.

Abbreviations: GC, gas chromatography; MS, mass spectrometry; PFPA, pentafluoropropionic anhydride; TCA, trichloroacetic acid; EI, electron-impact ionization; PCI, positive chemical ionization; NMDA, *N*-methyl-D-aspartate; L-NL, L-norleucine.

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2. MATERIALS AND METHODS

2.1. *Reagents*

All amino acids and trichloroacetic acid (TCA) were obtained from Nakarai Tesque (Japan). All solvents were HPLC grade and purchased from Kanto Chemicals (Japan). A pentafluoropropionic anhydride (PFPA) was purchased from G.L.C. Science (Japan).

Amino acid stock solutions were prepared by dissolving amino acids in 0.01 M HCl to a concentration of 10 μmol/ml each. The standard amino acid stock solutions after appropriate dilution with 0.01 M HCl were used for calibration. L-Norleucine (L-NL) was used as an internal standard.

2.2. *GC analysis*

The gas chromatographic analysis was carried out using a Hewlett Packard 5890 (Hewlett Packard, Japan) equipped with nitrogen-phosphorus detector. Samples were analyzed on a Chirasil-L-Val capillary column (25 m × 0.25 mm, film thickness 0.12 μm, Gasukuro Kogyo, Japan).

The chromatographic conditions were: injection temperature, 250°C; detector temperature, 300°C; temperature program, 80°C, isothermal for 5 min, then 80–140°C at 2°C/min and split ratio, 1:10. The column head pressure was 110 kPa, and the helium flow-rate on the column was 0.4 ml/min; the flow-rate (plus auxiliary gas) was 30 ml/min. The hydrogen flow-rate and the air flow-rate at the detector were 3.5 and 110 ml/min, respectively. Peak area was determined using a SIC chromatocorder 11 integrator (System Instruments Co.).

2.3. *MS analysis*

The GC-MS analysis was performed on a V.G. Masslab Trio-1 mass spectrometer (Jasco, International Co., Japan), equipped with a Hewlett Packard 5890A gas chromatograph. The GC conditions were the same as for the GC analysis with the exception of a splitless injection. The mass spectrometer conditions were: electron accelerating voltage, 70 eV; interface temperature, 170°C; photomultiplier

voltage, 400 V; ion source temperature, 200°C (EI) and 190°C (PCI); trap current, 150 μA (EI); emission current, 150 μA (PCI). PCI-MS was performed using isobutane as a reactant gas.

2.4. *Animals and tissue extraction*

The subjects were 50-day-old male Wistar rats (ST strain, Shizuoka Laboratory Animals, Japan) weighing 215–235 g at the time of experiments. The animals were housed at 22.0 ± 0.5°C in a humidity controlled room under a 12-h light-dark cycle (lights on at 08:00 h) and were allowed food and water ad libitum.

The perfusion of rat brain by 0.9% NaCl solution was carried out under pentobarbital anesthesia (40 mg/kg, i.p.) to eliminate contamination by amino acids in the blood. The perfused brain tissue, which contains telencephalon, diencephalon and midbrain was dissected out on ice and stored at −80°C until use.

After the addition of L-NL as an internal standard, the perfused tissue was homogenized at 4°C in 5 vols of 5% TCA and the homogenate was centrifuged at 18 000 × *g* for 30 min at 4°C. To remove TCA, the supernatant was washed three-times with water-saturated diethyl ether. The aqueous layer was then passed through a Millipore filter, HV (0.45 mm) and stored at −80°C until derivatization.

2.5. *Preparation of amino acid derivatives*

The 5% TCA extract was evaporated under a gentle stream of nitrogen gas at room temperature. The last trace of water was removed azeotropically using methylene chloride. The residue was dissolved in 2 ml of isopropanol–2N HCl. After the mixture was heated at 100°C for 1 h, the excess reagent and solvent were removed under a gentle stream of nitrogen gas at room temperature. 400 μl of methylene chloride and 100 μl of PFPA were added to the residue, which was heated at 100°C for 20 min. The excess reagent and solvent were then evaporated under a gentle stream of nitrogen gas at room temperature. After addition of 2 ml distilled water, the derivatized sample was extracted three-times with ethyl acetate. To remove any trace amount of water in the combined ethyl acetate extract, an appropriate amount of dry sodium sulfate was added to the combined extract and allowed to stand for 20 min. After filtrating, the extract was carefully evaporated in the same way. Finally, the derivatized sample was dissolved in an appropriate volume of ethyl acetate and stored at 4°C until analysis. 1–2 μl of the solution was injected into the gas chromatograph.

2.6. *Calculations*

The concentrations of amino acids (AA) in samples were determined as follows:

AA (μmol/g wet wt.) = (AA area/L-NL area in sample) × (L-NL area/AA area in standard) × (μmol L-NL added/g wet wt. of sample).

3. RESULTS

Fig. 1a and Table I show a representative chromatogram and retention times of *N,O*-PFP-isopropyl standard amino acid derivatives. *N,O*-PFP-isopropyl amino acid derivatives gave good resolution for most of the amino acid enantiomers and non-chiral amino acids such as glycine, β-alanine and γ-aminobutyric acid. Fig. 1b shows a typical chromatogram of *N,O*-PFP-isopropyl free amino acid derivatives in the rat brain extracts. As can be seen, a peak X at 11.5 min, which co-eluted with the D-serine derivative, was detected in the brain extracts. In addition, only trace amounts of D-alanine and D-aspartic acid were detected in the brain extracts (data not shown).

In principle, the determination of D-amino acids by GC alone may be falsified by a compound with the same retention time as one of the unknown compounds in the rat brain. To confirm the presence of free D-serine in

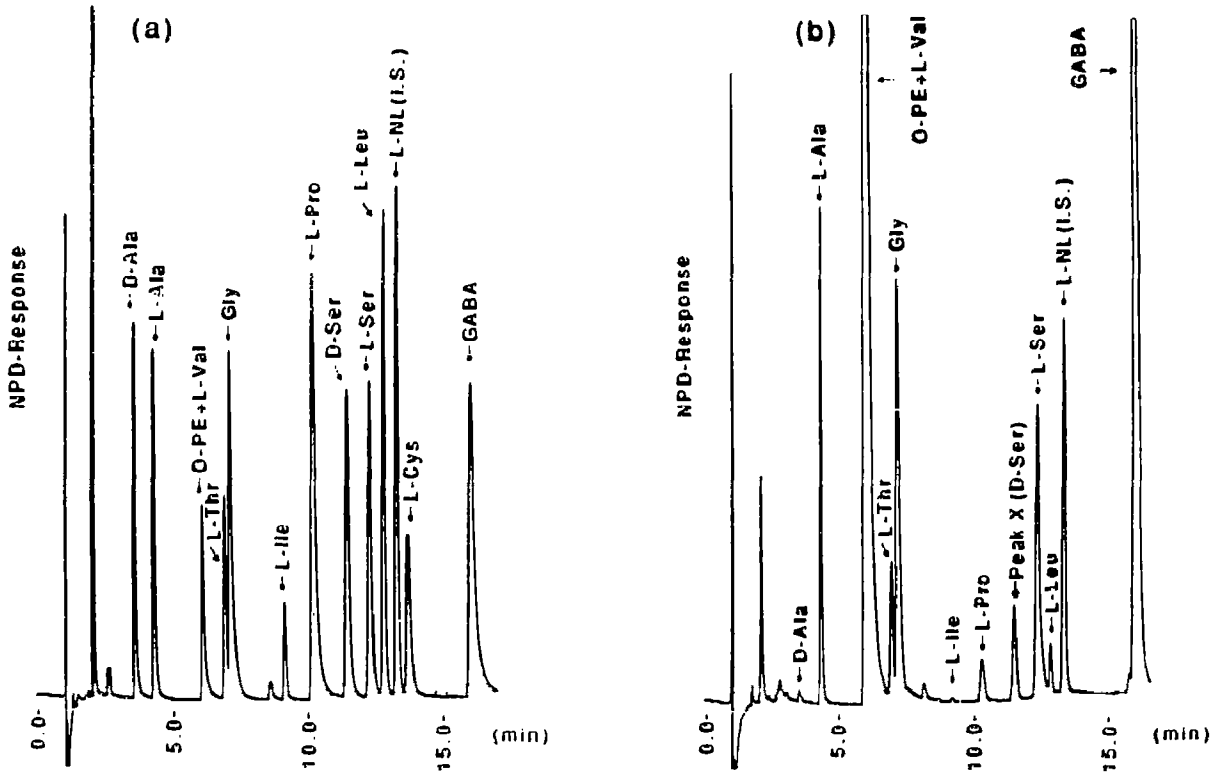

Fig. 1. Gas chromatograms of *N,O*-PFP-isopropyl derivatives of standard amino acids (a) and free amino acids in the brain extracts (b).

Table I

Retention times of *N,O*-PFP-isopropyl derivatives of standard amino acids injected on a Chirasil-L-Val capillary column

Amino acids	Retention time (min)	
	D	L
Alanine	3.63	4.32
Valine	5.41	6.15
Threonine	6.26	7.00
Isoleucine	7.37	9.20
Proline	9.97	10.22
Serine	11.50	12.34
Cysteic acid	12.63	13.80
Leucine	10.41	12.88
Norleucine (I.S.)		13.37
O-Phosphoethanolamine	6.04	
Glycine	7.15	
β-Alanine	8.02	
GABA	16.00	

Table II

The concentration of free D-serine and the ratio of free D-serine/total free serine in the rat brain extracts

Concentration (μmol/g wet weight)			Ratio
D-serine	L-serine	Total serine	
0.27 ± 0.01	0.89 ± 0.02	1.16 ± 0.03	0.23 ± 0.01

Values are expressed as means ± SEM of 5 samples.

the rat brain, we performed GC-MS analysis of the peak X on the chromatogram obtained from the brain samples. Fig. 2a and b show the EI mass spectra of the *N,O*-PFP-isopropyl derivative of authentic D-serine (a) and the peak X in the brain extracts (b). The EI mass spectrum of the peak X, which showed a base peak ion at m/z 189, $[CH_2CHNHCOCF_2CF_3]^+$ and 2 weak fragment ions at m/z 352, $[M\text{-}COOCH(CH_3)_2]^+$ and m/z 380 $[M\text{-}OCH(CH_3)_2]^+$, was almost identical to that of the authentic D-serine. Furthermore, the PCI mass spectrum of the peak X showed a quasimolecular ion at m/z 440 $[M+1]^+$, which was identical to that of the authentic D-serine (data not shown). Consequently, the peak X was identified as D-serine.

The concentration of free D-serine in the brain extract was estimated to be 0.27 ± 0.01 μmol/g of wet wt. and the ratio of free D-serine/total serine was 0.23 ± 0.01 (Table II). The concentrations of amino acids in brain extracts such as total serine, L-alanine and glycine were 1.16 ± 0.03, 0.57 ± 0.03 and 0.79 ± 0.02 (μmol/g wet wt.), respectively, which were in accordance with those of previous results [13].

4. DISCUSSION

The present results demonstrate that peak X, which exhibited the identical retention time as the *N,O*-PFP-isopropyl authentic D-serine derivative, is detected in the rat brain samples and that the EI and PCI mass spectra of the peak X of the brain extracts were identical to those of the authentic D-serine derivative. These findings indicated that a considerable amount of the free D-enantiomer of serine naturally occurs in the brain of a mature rat.

The possibility cannot be excluded that a part of the free D-serine detected in the present study would be racemized from the L-form during the assay process. This is, however, unlikely because a previous report indicated that there is no detectable amount of racemization of amino acids under similar assay conditions [10]. Indeed, no racemization of L-serine was observed during the present derivatization process (data not shown). The fact that a similar content of free D-serine in rat brain was confirmed using HPLC analysis following derivatization with *O*-phthalaldehyde and Boc-L-cysteine (unpublished data) also argues against the possibility that the presence of D-serine in the brain extracts is due to an artifact.

The concentration of free D-serine appears to be higher than those of the other free D-amino acids ever determined in mammalian brain tissues. Free neutral amino acids have been shown to be present in mice and human brains at the amounts of 0.063 μmol/g of wet wt. and 0.012 μmol/g of wet wt., respectively [8,9]. Although the brain of a newborn rat contains a much higher level of free D-aspartate (0.164 μmol/g of wet wt.) than that of the adult rat (0.013 μmol/g of wet wt.) [11,12], the free aspartate content of the neonatal rat brain is still lower than the free D-serine content in the present study.

The origin and metabolizing systems of free D-serine in the brain are not yet known. The transport of neutral

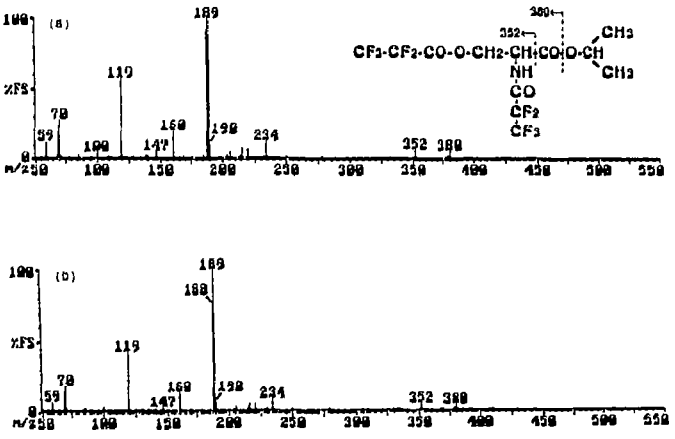

Fig. 2. Electron impact mass spectra of *N,O*-PFP-isopropyl derivative of authentic D-serine (a) and the peak X in the brain extracts (b) injected on a Chirasil-L-Val capillary column.

amino acids such as glycine, serine and alanine through the blood–brain barrier is slow [14,15] and the D-forms were less permeable than the L-forms [16]. In addition, the D-enantiomers of free neutral amino acids are considered to be catalyzed in the liver and kidneys and are excreted into the urine [17]. These observations suggested that free D-serine in rat brain may not be derived from the diet.

It has been well established that D-serine is a potent and selective allosteric agonist for the NMDA–receptor complex [1–3]. Thus, like glycine, the D-amino acid is 100-times more potent than the L-isomer in displacing [^{3}H]glycine binding to the allosteric regulation site. In contrast, D-serine has a lower affinity for the inhibitory glycine receptor than L-serine [18]. It has been generally accepted that the D-enantiomers of amino acids are assumed to be selectively excluded from a source of proteins [19]. Taken together, it is proposed that free D-serine in brain tissues might be a novel candidate of an endogenous neurotransmitter for the strychnine-insensitive glycine binding site associated with the NMDA–receptor complex.

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