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Brain D-serine and tyrosine levels in ataxic mutant mice

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Summary. Since D-serine occurs at high concentrations in mammalian forebrains, the brain D-serine content was analyzed in hyperkinetic and ataxic mutant mice as well as normal control mice in a search for a physiological role. The concentrations of free D-serine (nmol/g wet weight) were 392 ± 114 (mean ± S.D.), 43 ± 17 and 18 ± 8.4 in the cerebrum, brain stem and cerebellum of the BUS mouse, respectively; and 336 ± 93, 58 ± 11 and 18 ± 8.5 in the cerebrum, brain stem and cerebellum of the Rolling mouse, respectively. These values were not significantly different from those for each control animal. The present results suggest that brain D-serine may not be a cause of the abnormal movements of the mutant mice. On the contrary, among many amino acids examined, tyrosine level was found to be lower in the brain stem of BUS mouse compared to the normal control animal by amino acid analysis.

Keywords: Amino acids – D-Serine – Tyrosine – Brain – Ataxia – Mutant mouse

Introduction

Endogenous free D-serine has been recently found in mammalian forebrains (Hashimoto et al., 1992; Nagata, 1992; Nagata et al., 1994). Up to 30% of free serine was found to be of D-configuration in the mouse and rat cerebra. The physiological function of the endogenous D-serine is unknown while [^{3}H]D-serine has been shown to bind to the strychnine-insensitive glycine receptor by in vitro experiments (Danysz et al., 1990; Snell et al., 1988). Currently the endogenous D-serine is hypothesized to be a neurotransmitter/neuromodulator based on a co-localization of D-serine and the N-methyl-D-aspartate (NMDA) receptors (Chouinard et al., 1993; Hashimoto et al., 1993; Schell et al., 1995). D-Serine has been shown to affect rat behavior after i.c.v. administration (Contreras, 1990). In order to investigate the above hypoth-

esis, we carried out experiments using two ataxic mouse strains which exhibit movement disorders.

An inbred strain of hyperkinetic mutant mice bearing a single autosomal recessive gene (*bus*) has been established by Shoji et al. (1988), and named BUS after the bustling behavior. Anatomical, biochemical and pharmacological studies on this mouse brain are currently under way. Rolling mouse Nagoya is an ataxic mutant found and developed by Oda (1973) as an animal model of human cerebellar degeneration diseases. The mouse has a gait disturbance characterized by creeping and rolling, and frequently falls over. The mutation is transmitted by a single autosomal recessive gene (*rol*) on chromosome number 8 (Oda, 1978). No gross histopathological abnormalities were observed except for smaller cerebellar size comparing to that of the littermate control animal. The mechanism of the abnormal movements has not been elucidated completetly, whereas some biochemical findings have been accumulated.

In the present investigation, we have measured free D-serine in the cerebra, brain stems and cerebella of these mice, in order to know whether D-serine is a cause of the neurological phenotype. We also assayed other free amino acids in the brain of BUS mouse under the same purpose.

Materials and methods

Preparation of free amino acids

The BUS mouse, *bus/bus* and the normal control, *bus/+* mice from inbred JSR/Idr strain have been established and maintained by Shoji at Institute for Developmental Research (Idr), Aichi Human Service Center. Ten-week old male *bus/bus* and *bus/+* (normal heterozygote, as control) mice were used. The Rolling mouse Nagoya (*rol/rol*) with heterogenous genetic background has been maintained at Laboratory of Animal Management, School of Agricultural Sciences, Nagoya University. Four-month old female rolling mice (*rol/rol*), and as the control, the sex- and age-matched normal littermates (*rol/+*) were used in the present experiment. The animals were given food (MF; Oriental Yeast, Tokyo, Japan) and water ad libitum and were housed under a standardized light: dark cycle (lights: 8 a.m. to 8 p.m.) at 23°C. The mice were starved but allowed water, for 16 h before being killed by collecting blood from the axillary vessels under anesthesia with diethyl ether. The brains were quickly removed from the mice. Each brain was separated on an ice-cooled glass plate into the cerebrum, cerebellum and the remaining region (designated as brain stem). The spinal cord was not examined. The brain sections were frozen at −80°C until use.

Analysis of D- and L-serine

The analysis was performed as described previously (Nagata et al., 1992; Nagata et al., 1994), with minor modifications. Briefly, each brain section was homogenized with 4 vol of phosphate-buffered saline (150 mM NaCl in 10 mM sodium phosphate, pH 7.4) in a glass homogenizer in an ice bucket at 1,100 rpm for 1 min. Cold trichloroacetic acid solution was added to a final concentration of 5% of the supernatant extract obtained by a centrifugation at 14,500 g for 30 min at 4°C. The resultant supernatant was passed through a Dowex 50W-x8 column (200–400 mesh, ammonium form) to remove trichloroacetic acid. The column was washed with 5 vol of water, and eluted with 3 vol of 2 M NH_4OH. The eluate was evaporated to dryness in vacuo in a centrifugal evaporator. The

dried eluate was derivatized with 1-fluoro-2, 4-dinitropheny1-5-L-alanine amide (FDAA, Marfey's reagent, Pierce, Rockford, IL, USA; Marfey, 1984) to determine D- and L-serine. In order to avoid any minimal contamination with other amino acids, FDAA-serine was separated from other FDAA-amino acids by thin-layer chromatography on a silica gel-plate, prior to high-performance liquid-chromatography (HPLC).

FDAA-serine recovered from the plate was analyzed for the D- and L-enantiomers by HPLC with a reversed-phase column, Nova-Pak C18 (150 × 3.9 mm ID, Waters, Milford, MA, USA). The sample was eluted with a linear gradient of acetonitrile in 50 mM triethylamine-phosphate buffer (pH 3.5) from 10 to 25% over 20 min at a flow rate of 1.0 ml/min at 23°C. The eluate was monitored at 340 nm with a D-2500 Chromato-Integrator (Hitachi, Tokyo, Japan), and peak areas of FDAA-D- and FDAA-L-serine were obtained automatically. Amounts of D- and L-serine in the brain sample were calculated on the basis of the peak areas and internal controls. The internal controls were known amounts of D- and L-serine added to the brain extracts before the trichloroacetic acid treatment, and were treated similarly as other brain samples. No racemization was caused during the course of extraction, derivatization with FDAA, thin-layer chromatography and HPLC of free amino acids (Nagata et al., 1992).

Amino acid analysis

The brain extract was treated with 5% trichloroacetic acid. The resultant supernatant was passed through the Dowex 50W-x8-column. The eluate was dried in vacuo in the centrifugal evaporator, and dissolved in double distilled water. Aliquots of 100 μl of the solution containing 5 to 10 nmols of amino acids were used for the analysis, using Hitachi high-performance amino-acid analyzer L-8500 equipped with an ion-exchange column Hitachi 2622SC. The sample was eluted with Na-citrate buffers of 0.1 M (pH 3.3), 0.135 M (pH 4.3) and 1.26 M (pH 5.5) in that order over 80 min at 65°C. The eluate was monitored at 570 nm and 440 nm with the D-2500 Chromato-Integrator.

Chemicals

D- and L-serine were purchased from Sigma (St Louis, MO, USA), and all other reagents used were of HPLC- or analytical grade obtained from Wako (Osaka, Japan) or Nacalai (Kyoto, Japan).

Statistics

The significance of differences between any two groups was assessed by Student's *t*-test. Differences were considered significant when $P < 0.05$, for the two-tailed test.

Results

The brain levels of free D- and L-serine in the BUS and Rolling mice, and the ratio of D-serine to L-serine (D/L ratio) are shown in Table 1. No difference was indicated in the free D-serine levels of cerebrum, brain stem and cerebellum between the mutant and the normal control groups. Although heterozygotes were used as the control animals in this experiment, the values for each control are similar to those for other normal mice such as BALB/c and IQI/J (Nagata et al., 1994). We have used the present HPLC-method for the analysis of D-serine for years, and obtained values comparable to those by other investigators (Hashimoto et al., 1992) with other methods such as gas-chromatography. We also confirmed that the "D-serine" measured in this

Table 1. Free D- and L-serine levels (nmol/g wet wt), and the D/L ratios in BUS and Rolling mouse brains

		D	L	D/L
BUS mouse				
Cerebrum	*bus/bus*	392 ± 114	833 ± 214	0.463 ± 0.062
	bus/+	332 ± 44	761 ± 140	0.452 ± 0.057
Brain Stem	*bus/bus*	43 ± 17	337 ± 40	0.128 ± 0.048
	bus/+	34 ± 23	313 ± 65	0.104 ± 0.050
Cerebellum	*bus/bus*	18 ± 8.4	522 ± 126	0.035 ± 0.013
	bus/+	20 ± 15	536 ± 137	0.038 ± 0.032
Rolling mouse				
Cerebrum	*rol/rol*	336 ± 93	882 ± 67	0.381 ± 0.103
	rol/+	270 ± 79	815 ± 121	0.331 ± 0.123
Brain Stem	*rol/rol*	58 ± 11	308 ± 89	0.187 ± 0.029
	rol/+	62 ± 32	385 ± 118	0.162 ± 0.037
Cerebellum	*rol/rol*	18 ± 8.5	500 ± 167	0.036 ± 0.020
	rol/+	18 ± 9.2	522 ± 212	0.034 ± 0.006

Values for the BUS mouse are the means ± S.D. for eight animals. Values for the Rolling mouse are the means ± S.D. for five mutant mice and four control mice.

experiment was a D-amino acid, based on reduction of the HPLC-peak area by addition of D-amino acid oxidase (EC 1.4.3.3) to the Dowex-column eluates (data not shown).

We investigated amino acid levels in the cerebrum, brain stem and cerebellum of the BUS mouse. As a result, no difference was found in aspartate, threonine, serine, glutamate, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, phenylalanine, γ-aminobutyric acid, lysine, histidine and proline levels between the mutant and the control groups (data not shown). Only tyrosine level of the mutant mouse was significantly lower ($p < 0.01$, $p < 0.001$) than that of the control animal in the brain stem (Table 2), but not in the cerebrum and cerebellum.

Discussion

A malfunction of the cerebellum generally causes a movement disorder. The low level of D-serine in the cerebellum may suggest that the endogenous free D-serine is rapidly catabolized in the cerebellum where much more D-amino acid oxidase is localized than in the cerebrum. In the mutant mouse (Konno and Yasumura, 1983) lacking D-amino acid oxidase, the D-serine level in the cerebellum was comparable to that in the cerebrum (Nagata, 1992). Nothing is known about the source of endogenous D-serine.

In the present study, it has been found that there is no difference in the brain D-serine level between the ataxic mutant and normal mice. In addition, no abnormal movement is observed with the mutant mouse lacking D-amino acid oxidase. It therefore appears that the endogenous D-serine may not be involved in the abnormal movements. Although D-serine administered i.c.v.

Table 2. Free tyrosine levels in the brain stem of BUS mouse

	nmol/g wet wt		
Experiment	BUS	Control	
1	41.5 ± 10.1	72.6 ± 9.3	$p < 0.001$
2	53.7 ± 8.7	78.9 ± 12.6	$p < 0.01$

Values are the means ± S.D. for five animals.

antagonized the phencyclidine-induced stereotyped behavior and ataxia by possibly binding to the NMDA receptor-channel complex in rats (Contreras, 1990), the present results may not support the possibility that the endogenous brain D-serine is a neurotransmitter or a modulator.

Several neurotransmitters or candidates have been of concern for the movement disorder of Rolling mouse: significant decrease of binding sites for $GABA_A$ (Onodera et al., 1988; Yamaguchi et al., 1984a) and kainic acid (Yamaguchi et al., 1984b); decrease of taurine and glycine levels and increase of glutamate (Muramoto et al., 1981) in the cerebellum; increase of tyrosine hydroxylase and dopamine-β-hydroxylase activities (Konagaya et al., 1980; Nagatsu et al., 1980) and noradrenaline level (Konagaya et al., 1980) in the brain stem and cerebellum comparing to the control animal. As for the BUS mouse, We have shown that levels of glutamate, glycine and γ-aminobutyric acid were comparable to the normal control mouse in the three brain regions. Among other amino acids, only free tyrosine was found to be reduced in the brain stem comparing to the control animal (Table 2). The result might indicate a possible involvement of catecholamine systems in the phenotype of the BUS mouse.

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