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Simultaneous two-dimensional HPLC determination of free D-serine and D-alanine in the brain and periphery of mutant rats lacking D-amino-acid oxidase☆

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ABSTRACT

A fully automated two-dimensional HPLC system combining a microbore-ODS column and a narrowbore-enantioselective column was designed and validated, and the amounts of D-serine (D-Ser) and D-alanine (D-Ala) in various tissues and physiological fluids of Long–Evans agouti/SENDAI (LEA/Sen) rats lacking D-amino-acid oxidase (DAO) were determined. Intra- and inter-day precision was less than 4.3% and accuracy ranged between 99.9 and 104%. LEA/Sen rats were reported to lack DAO in their kidneys and expected to be a novel mutant animal lacking DAO, however, the amounts of D-amino acids in the LEA/Sen rats have not been investigated. In the present study, the intrinsic amounts of D-Ser and D-Ala, which are neuromodulators of the *N*-methyl-D-aspartate (NMDA) receptors, were determined in seven brain tissues, four peripheral tissues, plasma and urine of the LEA/Sen rats and compared to those of the control (Wistar and SD) rats having normal DAO activity. The levels of D-Ser in the tissues and physiological fluids of the LEA/Sen rats were significantly higher than those of the Wistar and SD rats except for the frontal brain regions. Concerning D-Ala, the amounts in the tissues and physiological fluids of the LEA/Sen rats were drastically increased compared to those of the Wistar and SD rats. These results indicate that the intrinsic amounts of D-Ser and D-Ala in the tissues of rats are regulated by DAO, and that LEA/Sen rats would be useful for the study of NMDA receptor-related diseases in which DAO is implicated.

1. Introduction

It has been believed for a long time that only L-amino acids are present in higher animals. However, with the recent advances and improvement in analytical methods, some free D-amino acids, such as D-serine (D-Ser) [1,2], D-aspartic acid (D-Asp) [3–5], D-alanine (D-Ala) [6–8], D-proline (D-Pro) [9,10] and D-leucine (D-Leu) [7,11,12], have been found in mammals including humans [13–15]. Their origins, distributions, metabolisms and functions have also been gradually clarified, and D-amino acids are now recognized as novel candidates of physiologically active substances and biomarkers [16,17].

A large amount of D-Ser has been reported to be localized in the central nervous system (CNS), such as the cerebral cortex and hippocampus in mammals [1,2], and D-Ala has also been found in the brain and widely in peripheral tissues [8,18,19]. Both D-Ser and D-Ala were noted to bind to the glycine site of the *N*-methyl-D-aspartate (NMDA) receptor and to modulate neurotransmission [20,21]. These D-amino acids have been shown to be implicated in diseases associated with NMDA receptor dysfunction [22], such as schizophrenia [23,24], depression [25], Alzheimer's disease [26] and amyotrophic lateral sclerosis [27]. Therefore, modulation of the intrinsic amounts of D-Ser and D-Ala is expected as one of the key targets for developing novel drugs for these neuronal diseases.

D-Ser and D-Ala are metabolized by D-amino-acid oxidase (DAO, EC 1.4.3.3) [28]. DAO is distributed in kidney, liver, brain (cerebellum, medulla oblongata) and spinal cord in mammals [29,30], and catalyzes the oxidative deamination of neutral D-amino acids to the corresponding 2-oxo acids [31]. DAO is a major enzyme which regulates the level of D-amino acids, and the relationships between DAO

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and several diseases related to NMDA receptor dysfunction have been reported [32,33]. DAO is considered to control the neurotransmission via NMDA receptors by regulating the intrinsic amounts of D-Ser and D-Ala. Therefore, the change in the intrinsic levels of D-Ser and D-Ala by changing the DAO activity is of major interest for the treatment of neuronal diseases.

The aim of the present study is to design a 2D-HPLC system for the simultaneous determination of D-Ser and D-Ala, and to investigate the intrinsic amounts of these D-amino acids in the brain and peripheral tissues of LEA/Sen rats lacking DAO. Until now, only one mouse strain (ddY/DAO$^{-/-}$) is known to have a deficiency of DAO activity [34]. The amounts of D-Ser and D-Ala in the tissues and physiological fluids have been investigated in these mice [35–39]. However, mouse tissues are not sufficiently large enough for the particular regions to be precisely discriminated, and the experiments involving the detailed tissue distribution of intrinsic D-Ser and D-Ala, as well as the region-focused studies, such as immunostaining, in situ hybridization, microdialysis, electrophysiological investigation or cell purification, have been difficult. In contrast, rat tissues are larger than those of mice, and are practically useful for detailed investigations. Therefore, the LEA/Sen rats are interesting as novel model animals lacking DAO. In the present study, we have determined for the first time the intrinsic amounts of D-Ser and D-Ala in the brain and peripheral tissues of LEA/Sen rats. Concerning the methodology, this is also the first report to show the 2D-HPLC procedure for the simultaneous determination of D-Ser and D-Ala.

2. Experimental

2.1. Materials

The enantiomers of Ser, Ala and HPLC grade acetonitrile (MeCN) were obtained from Nacalai Tesque (Kyoto, Japan). Methanol (MeOH) of HPLC grade, trifluoroacetic acid (TFA), citric acid monohydrate and boric acid were purchased from Wako (Osaka, Japan). The derivatizing reagent, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), was from Tokyo Kasei (Tokyo, Japan). Water was purified using a Milli-Q gradient A 10 system (Millipore, Bedford, MA, USA). All other reagents were of the highest reagent grade and were used without further purification.

2.2. Animals

The LEA/Sen rat was established at Tohoku University, Graduate School of Medicine, and has been maintained at National Center for Global Health and Medicine (NCGM). Male LEA/Sen rats (7 weeks of age) from NCGM were used for this study. Male Wistar and SD rats (7 weeks of age) were purchased from Kyudo (Kumamoto, Japan). These rats were reared at the animal center of Kyushu University for at least 1 week before use under a 12-h light/12-h dark cycle (light on at 07:00 a.m.) with free access to food (NMF Diet, Oriental Yeast, Tokyo, Japan) and water. All experiments were performed with permission (A21-005-0) from the Animal Care and Use Committee of Kyushu University.

2.3. Sample preparation

The samples of rat tissues and physiological fluids were prepared according to the previous report [17] with minor modifications. Briefly, the rats were anesthetized with diethyl ether and euthanized by exsanguination from the abdominal aorta. The blood was collected in a heparinized tube (type 365985, Nippon Becton Dickinson, Tokyo, Japan), centrifuged at 8000 × *g* for 15 min at 4 °C and the plasma was obtained. The urine was collected from the urinary bladder. To the plasma and urine, 20-fold volumes of MeOH were added and mixed for 2 min. These mixtures were centrifuged at 12,100 × *g* for 10 min, and then the supernatants were collected. The tissues (olfactory bulb, cerebral cortex, hippocampus, hypothalamus, cerebellum, medulla oblongata, spinal cord, anterior pituitary, pancreas, liver and kidney) were quickly excised and stored at −80 °C after being weighed. The tissues were homogenized in 20-fold volumes of MeOH (anterior pituitary was homogenized in 100-fold volumes of MeOH) using a micro-homogenizing system (Micro Smash™ MS-100R, Tomy, Tokyo, Japan) at 4 °C and centrifuged at 12,100 × *g* for 10 min to obtain the supernatants. An aliquot of the supernatant (50 μL was used for the anterior pituitary, and 10 μL for the other tissues, plasma and urine) was evaporated to dryness under reduced pressure at 40 °C. To the residue, 20 μL of 200 mM sodium borate buffer (pH 8.0) and 5 μL of 40 mM NBD-F in anhydrous MeCN were added, then heated at 60 °C for 2 min. An aqueous 2% (v/v) TFA solution (75 μL) was then added and 2 μL of the reaction mixture was subjected to HPLC.

2.4. HPLC system for the determination of Ser and Ala enantiomers

The Ser and Ala enantiomers were quantified using a two-dimensional HPLC system combining a microbore-monolithic ODS column and a narrowbore-enantioselective column. Ser and Ala derivatized with NBD-F were separated using a microbore-reversed-phase column maintained at 40 °C (monolithic ODS column, 750 mm × 0.53 mm i.d., prepared in a fused silica capillary, provided from Shiseido, Tokyo, Japan) with the gradient elution using mobile phase A (an aqueous solution containing 5% (v/v) MeCN and 0.05% (v/v) TFA) and mobile phase B (an aqueous solution containing 20% (v/v) MeCN and 0.05% (v/v) TFA). The gradient program was as follows: 0–40 min, 100% A; 40–45 min, linear gradient from 100% A to 100% B; 45–70 min, 100% B. The flow rate was 30 μL/min. The target amino acid fractions were automatically transferred to the enantioselective column via a column-switching valve with a loop of 150 μL, and the enantiomers were separated by a Sumichiral OA-2500S column (250 mm × 1.5 mm i.d., self-packed, material was from Sumika Chemical Analysis Service, Osaka, Japan) maintained at 25 °C. The mobile phase for NBD-Ser was 3 mM citric acid in the mixed solution of MeOH–MeCN (25:75, v/v), and that for NBD-Ala was 4 mM citric acid in the mixed solution of MeOH–MeCN (50:50, v/v). The flow rate was 150 μL/min. The fluorescence detection of NBD-Ser and NBD-Ala was carried out at 530 nm with excitation at 470 nm.

3. Results

3.1. Simultaneous 2D-HPLC determination of D-Ser and D-Ala

For the sensitive determination of D-Ser and D-Ala in various tissues and physiological fluids of the rats, a two-dimensional HPLC system combining a reversed-phase column and an enantioselective column was established. For the measurement, the amino acids were pre-column derivatized with NBD-F and separated using a microbore-monolithic ODS column (750 mm × 0.53 mm i.d.) as the mixtures of D and L enantiomers. The fractions of NBD-Ser and NBD-Ala were separately transferred to a loop, and introduced to a narrowbore-enantioselective column (Sumichiral OA-2500S, 250 mm × 1.5 mm i.d.) in which the enantiomers were separated. Because the enantiomer separations were performed after the reversed-phase isolation of the NBD-Ser and NBD-Ala fractions, the selective determination of the small amounts of D-Ser and D-Ala could be carried out even in the complex biological samples.

Fig. 1 shows the chromatograms obtained from analysis of plasma samples of the Wistar, SD and LEA/Sen rats. As shown

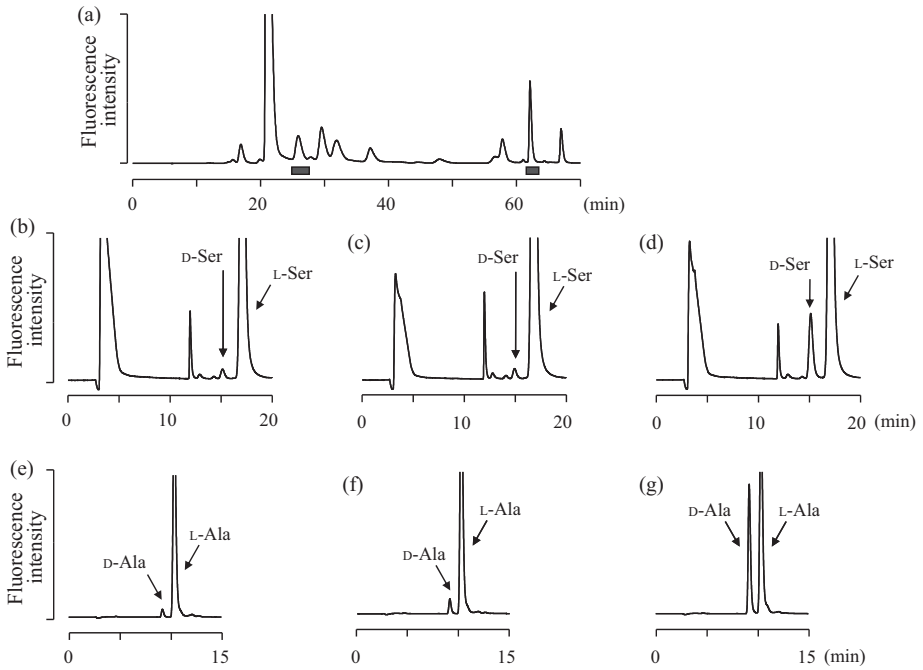

Fig. 1. Reversed-phase separation of NBD-Ser and NBD-Ala in the plasma of Wistar rat (a), and enantiomer separations of NBD-Ser in the plasma of Wistar (b), SD (c) and LEA/Sen rat (d) obtained by the two-dimensional HPLC system. Enantiomer separations of NBD-Ala in the plasma of Wistar (e), SD (f) and LEA/Sen rat (g). The fractions of NBD-Ser and NBD-Ala were separated from the other substances using a microbore-ODS column in the first dimension, and the enantiomers were separated using a Sumichiral OA-2500S enantioselective column in the second dimension.

in Fig. 1(b)–(g), small amounts of D-Ser and D-Ala were clearly detected in the biological matrices using the 2D-HPLC procedure, and the amounts of D-Ser and D-Ala in the LEA/Sen rats were significantly greater than those in the Wistar and SD rats. The amounts of D-Ser and D-Ala in the plasma of LEA/Sen rats (D-Ser; 14 μM, D-Ala; 161 μM) were confirmed by the reversal of retention orders using Sumichiral OA-2500(R) column (D-Ser; 15 μM, D-Ala; 174 μM). Validation results are summarized in Tables 1 and 2. These results indicate that the present 2D-HPLC procedure shows good linearity and precision for the determination of D-Ser and D-Ala in biological samples.

3.2. Intrinsic amount of free D-Ser in the tissues and physiological fluids of rats lacking DAO

The amounts of D-Ser in seven brain tissues (olfactory bulb, cerebral cortex, hippocampus, hypothalamus, cerebellum, medulla oblongata and anterior pituitary), four peripheral tissues (spinal cord, pancreas, liver and kidney), plasma and urine of the LEA/Sen rats were determined. As the control rats having normal DAO activities, Wistar and SD rats were used. The amounts of D-Ser in various tissues, plasma and urine of the Wistar, SD and LEA/Sen rats are summarized in Fig. 2(a). Relatively large amounts of D-Ser were present in the olfactory bulb, cerebral cortex, hippocampus and hypothalamus of all strains (250–350 nmol/g in the cerebral cortex and hippocampus, 80–200 nmol/g in the olfactory bulb and hypothalamus). In the cerebellum, medulla oblongata and spinal cord, where high DAO activities are observed in the normal rats, the amounts of D-Ser in the LEA/Sen rats were 20-25 times greater than those of the Wistar and SD rats (4-8 nmol/g for Wistar and SD rars, 90-200 nmol/g for LEA/Sen rats). In the peripheral tissues, plasma and urine, the amounts of D-Ser in the LEA/Sen rats were also greater than those in the Wistar and SD rats. Especially in the kidney, the content in the LEA/Sen rat was 439 nmol/g, being 70 times higher than those in the Wistar and SD rats. The amounts of L-Ser in the LEA/Sen rats were almost the same as those in the Wistar and SD rats in every tissue and body fluid (Fig. 2b).

3.3. Intrinsic amount of free D-Ala in the tissues and physiological fluids of rats lacking DAO

The amounts of D-Ala in various tissues and physiological fluids of the Wistar, SD and LEA/Sen rats are shown in Fig. 3(a). Although both D-Ala and D-Ser are major substrates of DAO, the distribution of D-Ala was quite different from that of D-Ser. In the brain and peripheral tissues of the Wistar and SD rats, large

Table 1
Calibration lines of standard amino acids and precision of the method.

Amino acids	Calibration line[a]			Precision (RSD, %)[b]					
	Calibration range (pmol)	Equation	Correlation coefficient	Intra-day			Inter-day		
				Low	Middle	High	Low	Middle	High
D-Ser	0.0005–5	$y = 61.6x - 0.3$	0.99999	1.57	0.89	2.81	2.79	3.19	2.95
L-Ser	0.01–10	$y = 49.4x + 0.3$	0.99998	1.54	0.94	2.72	2.00	3.04	2.70
D-Ala	0.0005–5	$y = 76.3x + 0.1$	1.0000	1.59	2.39	2.64	2.81	2.17	2.76
L-Ala	0.01–10	$y = 64.4x - 0.2$	0.99999	1.43	1.73	2.53	1.82	1.70	2.37

[a] Regression equations were obtained by plotting (y) the fluorescence intensity (peak height, mV) versus (x) the amount of amino acids (injection amount, pmol). Injection amounts of D-amino acids: 0.0005, 0.001, 0.01, 0.05, 0.1, 1 and 5 pmol. Those for L-amino acids: 0.01, 0.1, 0.5, 1 and 10 pmol. The LLOD values of D-Ser and D-Ala are 500 amol.
[b] Intra- and inter-day precision ($n = 4$; RSD, %) were determined using 0.01, 0.1 and 1 pmol (injection amount) of D-amino acids and 0.1, 1 and 10 pmol of L-amino acids.

Table 2
Validation of the method for Ser and Ala enantiomers in mouse serum.

Amino acids	Standard line[a]			Precision (RSD, %)[b]		Mean accuracy (%)[c]
	Spiked amount (pmol)	Equation	Correlation coefficient	Intra-day	Inter-day	
D-Ser	0.01–5	$y = 64.1x + 0.9$	0.99994	4.29	3.74	104.0
L-Ser	0.1–10	$y = 50.0x + 69.4$	0.99999	3.28	2.35	101.2
D-Ala	0.01–5	$y = 76.2x + 9.5$	0.99999	3.69	1.64	99.9
L-Ala	0.1–10	$y = 64.7x + 333.7$	0.99935	1.93	2.00	100.5

[a] Regression equations were obtained by plotting (y) the fluorescence intensity (peak height, mV) versus (x) the spiked amounts of D-amino acids (0.01, 0.05, 0.1, 1 and 5 pmol) or L-amino acids (0.1, 0.5, 1 and 10 pmol). Amino acids were spiked to a mouse serum representing typical biological matrices.
[b] Values represent RSD (%) of 4 analyses. Spiked amount; 0.1 pmol for D-amino acids and 1 pmol for L-amino acids.
[c] Mean accuracy was calculated by the comparison of the slopes of the regression equations reported in this table with those of Table 1.

amounts of D-Ala were found in the anterior pituitary and pancreas (55–65 nmol/g in the anterior pituitary, 55–75 nmol/g in the pancreas), while the amounts were low in the other tissues. On the other hand, the amounts of D-Ala in the LEA/Sen rats were much higher than those in the Wistar and SD rats. Especially, large amounts of D-Ala were observed in the kidney, pancreas, anterior pituitary and urine of the LEA/Sen rats, and the amounts were 3432, 752, 437 and 2530 nmol/g or mL, respectively. Concerning L-Ala, the amounts were almost the same between the control and LEA/Sen rats (Fig. 3b).

4. Discussion

The microarray analysis between the Long–Evans cinnamon (LEC) rat (an animal model for Wilson's disease characterized by a copper metabolism disorder) and the LEA rat was performed to examine the expression profile during the development of hepatitis [40]. Compared to the LEA rat liver, a marked increase (158-fold) in the expression of DAO gene was observed in the LEC rat liver. Since it was postulated that the exceptionally high expression of DAO in the LEC rats relative to the LEA rats was considered to be strain-dependent rather than copper-related, Konno et al. [41,42] revealed that the DAO activity in the LEA/Sen rat kidney was much lower (below the detection limit of the assay) than that of the normal rats. However, the amounts of D-amino acids in the tissues and physiological fluids of the LEA/Sen rats have not been determined. Therefore, in the present paper, a 2D-HPLC method for the simultaneous determination of D-Ser and D-Ala has been designed and the amounts of these D-amino acids in the LEA/Sen rats were clarified. As control rats having normal DAO activity, the Wistar and SD rats were used and the amounts of the D-amino acids were compared to those in the LEA/Sen rats.

Concerning the methodology for the simultaneous determination of D-Ser and D-Ala in mammals, reversed-phase HPLC procedures using chiral derivatization reagents [16] such as *o*-phthaldialdehyde plus *N*-acetyl-L-cysteine, *N*-isobutyryl-L-cysteine, *N*-isobutyryl-D-cysteine, *N*-*tert*-butyloxycarbonyl-L-cysteine, (1)-1-(9-fluorenyl)ethyl chloroformate or 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide have been reported. A GC method using chiral stationary phase (Chirasil-L-Val) is also widely used [14]. However, a more selective method is required for the determination of small amounts of D-forms in complex biological samples, and in the present report, a 2D-HPLC approach for the simultaneous determination of D-Ser and D-Ala is reported for the first time. In addition to the selectivity, the sensitivity of the present method is high. The values of lower limit of quantitation of D-Ser and D-Ala using the present 2D-HPLC system were 500 amol/injection (0.05 nmol/g wet tissue or mL physiological fluid), which is sensitive enough for the determination of trace amounts of the intrinsic D-Ser and D-Ala. Concerning the precision and accuracy, the intra- and inter-day precision of the present method is less than 4.3% and the accuracy is around 100%. These results indicate that present method is useful for the precise, accurate and sensitive determination of small amounts of D-Ser and D-Ala in biological matrices.

Using the 2D-HPLC system, the amounts of D-Ser and D-Ala in the brain and peripheral tissues, plasma and urine of the LEA/Sen, Wistar and SD rats were determined. In the cerebral cortex and hippocampus of all strains, large amounts of D-Ser (250–350 nmol/g)

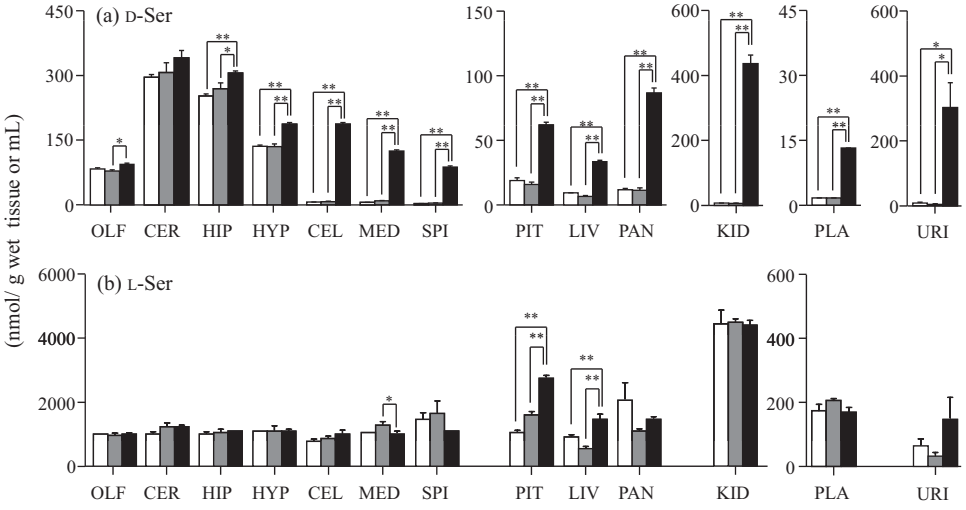

Fig. 2. Content of D-Ser (a) and L-Ser (b) in the tissues and physiological fluids of Wistar (open bars), SD (gray bars) and LEA/Sen (closed bars) rats. Values represent mean ± SE (nmol/g wet tissue or mL) of 3–6 rats. OLF, olfactory bulb; CER, cerebral cortex; HIP, hippocampus; HYP, hypothalamus; CEL, cerebellum; MED, medulla oblongata; SPI, spinal cord; PIT, anterior pituitary; LIV, liver; PAN, pancreas; KID, kidney; PLA, plasma; URI, urine. $^{**}P < 0.01$, $^{*}P < 0.05$, significant difference determined by Dunnett test between Wistar or SD rats and LEA/Sen rats.

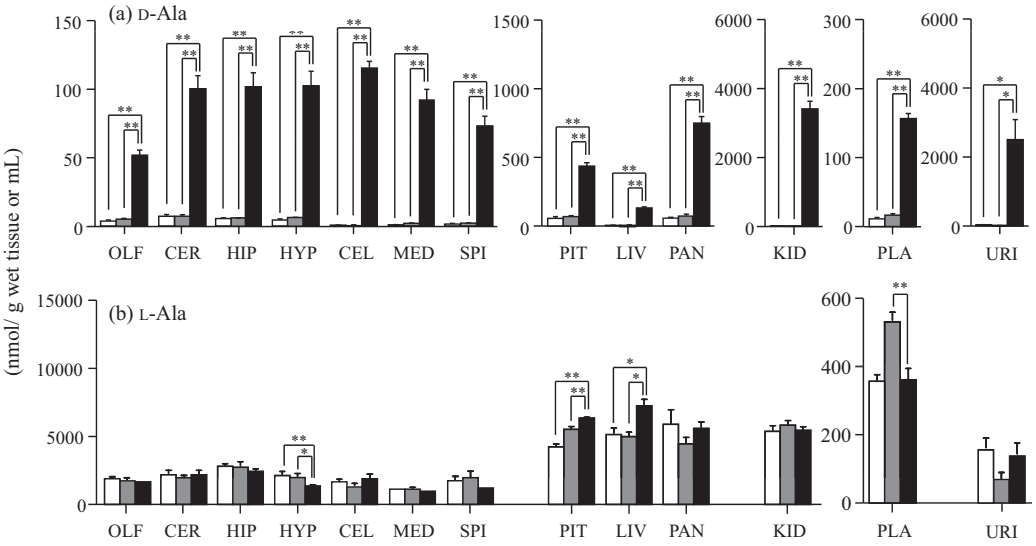

Fig. 3. Content of D-Ala (a) and L-Ala (b) in the tissues and physiological fluids of Wistar (open bars), SD (gray bars) and LEA/Sen (closed bars) rats. Values represent mean ± SE (nmol/g wet tissue or mL) of 3–6 rats. OLF, olfactory bulb; CER, cerebral cortex; HIP, hippocampus; HYP, hypothalamus; CEL, cerebellum; MED, medulla oblongata; SPI, spinal cord; PIT, anterior pituitary; LIV, liver; PAN, pancreas; KID, kidney; PLA, plasma; URI, urine. $^{**}P < 0.01$, $^{*}P < 0.05$, significant difference determined by Dunnett test between Wistar or SD rats and LEA/Sen rats.

were observed; D-Ser is considered to be produced by serine racemase in these tissues [43,44]. In the cerebellum, medulla oblongata and spinal cord, low amounts of D-Ser (4–8 nmol/g) were present in the Wistar and SD rats. On the other hand, the amounts of D-Ser in the LEA/Sen rats were about 100–200 nmol/g, which were about 25 times higher than those in the Wistar and SD rats. The reported values of D-Ser in the cerebral cortex and cerebellum of the Wistar and SD rats were 200–400 (cerebral cortex) and ranged between not detectable to 20 (cerebellum) nmol/g, respectively [2,7,45,46], and the present values are consistent with these reports. In the LEA/Sen rats, the amounts of D-Ser in the cerebral cortex and hippocampus were almost equal to those in the Wistar and SD rats. The DAO activity in the frontal brain regions, such as the cerebral cortex and hippocampus, is reported to be low, therefore, the amounts of intrinsic D-Ser in these regions appear to be not affected by the DAO. The significant difference in the amount of D-Ser in the hippocampus and hypothalamus may reflect the strain-dependent genetic factors except for DAO. In the other tissues of the LEA/Sen rats, especially in the cerebellum, medulla oblongata and spinal cord where DAO is abundantly present, a significant increase in the amounts of D-Ser was observed when compared to the Wistar and SD rats. These results strongly indicate that the intrinsic D-Ser amounts in these tissues are regulated by DAO.

Concerning D-Ala, relatively high amounts of D-Ala were observed in the pancreas and anterior pituitary of the Wistar and SD rats (55–80 nmol/g). D-Ala was reported to be localized in the anterior pituitary and pancreas of rats (26–86 nmol/g in the anterior pituitary and 29 nmol/g in the pancreas [7,8]), and the results obtained in the present study are in good agreement with previous reports. On the other hand, the amounts of D-Ala in all the tested tissues and physiological fluids were significantly higher in the LEA/Sen rats compared to the Wistar and SD rats. The amounts of D-Ala in the tissues were reported to be affected by the amounts of D-Ala in the serum [38], and the D-Ala amounts in the serum were considered to be affected by the DAO activity in the kidney [39]. Because the amounts of D-Ala in the plasma were high in the LEA/Sen rats due to the lack of DAO activity, the present results showing that the amounts of D-Ala in all the tissues of the LEA/Sen rats are higher than those of the Wistar and SD rats are conceivable.

In the present study, the amounts of D-Ser and D-Ala in the LEA/Sen rats lacking DAO were determined for the first time. The amounts of D-Ser and D-Ala were examined in ddY/DAO$^{-/-}$ mice lacking DAO [35–39]. The significant difference in the D-Ser amounts in the cerebral cortex and hippocampus were not observed between the ddY/DAO$^{-/-}$ mice and the control ddY/DAO$^{+/+}$ mice. On the other hand, the amounts of D-Ser in the other tissues and physiological fluids of ddY/DAO$^{-/-}$ mice were significantly increased compared to those of the ddY/DAO$^{+/+}$ mice. The amounts of D-Ala in the ddY/DAO$^{-/-}$ mice were significantly higher than those in the ddY/DAO$^{+/+}$ mice in all the tested tissues and physiological fluids (six brain tissues, five peripheral tissues, serum and urine). The changes in the amounts of D-Ser and D-Ala due to the lack of DAO were almost the same in the ddY/DAO$^{-/-}$ mice and LEA/Sen rats. The results obtained in the present study strongly indicate that DAO is one of the major modulators of the amounts of D-Ser and D-Ala in mammals. Since rats are larger than mice, LEA/Sen rats should be useful for the studies of the neuronal diseases related to NMDA receptors, especially for the analysis in the small area of the brain.

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