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Amino Acids

Alterations in D-amino acid levels in the brains of mice and rats after the administration of D-amino acids

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Summary. To mutant ddY/DAO$^-$ mice lacking D-amino-acid oxidase activity and normal ddY/DAO$^+$ mice, five D-amino acids (D-Asp, D-Ser, D-Ala, D-Leu and D-Pro) were orally administered for two weeks, and the D-amino acid levels were examined in seven brain regions. The levels of D-Asp markedly increased in the pituitary and pineal glands in both strains. In the ddY/DAO$^+$ mice, the levels of the other D-amino acids did not significantly change in most of the brain regions. While in the ddY/DAO$^-$ mice the levels of D-Ser significantly increased in most of the brain regions except for the cerebrum and hippocampus. The levels of D-Ala and D-Leu increased in all regions but the levels of D-Pro did not significantly change. The same five D-amino acids were intravenously injected into Wistar rats and the D-amino acid levels in their brains were examined for 60 min after the administration. The levels of D-Asp markedly increased in the pineal gland 3 min after the administration, while the levels of D-Ser, D-Ala, and D-Pro increased both in the pineal and pituitary glands, the levels of D-Leu increased in all brain regions. These results are useful for the elucidation of the origins and regulation of D-amino acids in the mammalian body.

Keywords: D-Amino acid – Brain distribution – D-Amino-acid oxidase – HPLC

Introduction

D-Amino acids (D-AAs) are the stereoisomers of widely observed L-amino acids, and recently, some D-AAs are reported to be naturally occurring in the mammalian brain (Hashimoto and Oka, 1997; Hamase et al., 2002). The intrinsic D-AAs are localized to particular brain regions in specific manners; D-serine (D-Ser) is highly localized to the frontal brain, while D-aspartic acid (D-Asp) is concentrated in the pineal gland (Hashimoto et al., 1995; Hamase et al., 1997; Morikawa et al., 2001). In addition, the specific localization of D-alanine (D-Ala) in the pituitary gland has been reported (Hamase et al., 1997; Morikawa et al., 2001, 2003), and relatively high levels of D-leucine (D-Leu) (Inoue et al., 2000) and D-proline (D-Pro) (Hamase et al., 2001) have been found in the pituitary gland and the pineal gland. Concerning the physiological functions of the D-AAs, D-Ser has a high affinity to the *N*-methyl-D-aspartic acid subtype of the glutamate receptor (NMDA receptor) and stimulates the neurotransmission as an endogenous modulator (Hashimoto and Oka, 1997; Snyder and Kim, 2000). The usefulness of D-Ser for the treatment of schizophrenia has been reported (Tsai et al., 1998). D-Asp regulates the release of several hormones, such as the growth hormone (D'Aniello et al., 2000a), prolactin (D'Aniello et al., 2000b; Long et al., 2000), and melatonin (Ishio et al., 1998). The alterations of the serum and brain levels of various D-AAs have been reported to correlate with some diseases (Hamase et al., 2002), such as schizophrenia (Hashimoto et al., 2003), Alzheimer's disease (Fishier et al., 1991), and renal disease (Brückner and Haush, 1993).

The D-AAs are thus becoming noticed as the candidates of novel physiologically active substances, and the origins of these D-AAs are the matters of interest. The biosynthesis of D-Ser and D-Asp has been suggested (Hashimoto and Oka, 1997; Snyder and Kim, 2000), and Wolosker et al. (1999) have purified serine racemase from rat brains. The exogenous D-AAs are also proposed to be one of the major origins of D-AAs in the mammalian body. Konno et al. (1990, 1993) reported that D-Ala and D-Met in the mouse urine are derived from intestinal bacteria and their diet, respectively. Because some kinds of foods, beverages and intestinal bacteria contain D-AAs in relatively large amounts (Friedman, 1999), it is probable that mammals

including humans are ingesting these D-AAs from exogenous origins. The incorporated D-AAs would affect our physiological conditions, however, the effect of exogenous D-AAs on the D-AA levels in the mammalian tissues, especially in the brain, has not been well investigated. Although some researchers have reported that administered D-AAs are absorbed into the tissues (Nagata et al., 1994; Imai et al., 1997, 1998; Hasegawa et al., 2004; Hashimoto and Chiba, 2004; Lee et al., 2001; Takahashi et al., 1997; Schiber et al., 1997), most results have been obtained regarding a single D-AA in the limited brain regions.

The present research was designed to obtain information about the effects of the exogenous D-AAs on the D-AA levels in wide brain regions. In the natural world, the animals ingest several D-amino acids at a time, therefore multiple D-AAs were simultaneously administered to mice and rats, and the D-amino acids were determined in seven brain regions. Another purpose of the present investigation was to clarify the contribution of the D-amino-acid oxidase (DAO, EC 1.4.3.3) to the elimination of the exogenous D-AAs. In mammals, DAO is usually highly expressed in the kidney, liver and particular brain regions, catalyzing the oxidative deamination of neutral D-AAs to the corresponding α-keto acids (Pilone, 2000). Several studies using mutant ddY/DAO$^-$ mice established by Konno et al. (1983) have demonstrated that the lack of the DAO activity causes augmentation of several D-AAs in particular tissues and physiological fluids (Morikawa et al., 2001; Hamase et al., 2001; Konno et al., 1990, 1993; Nagata et al., 1994). Therefore, DAO is considered to be a major enzyme to regulate the amounts of naturally occurring D-AAs in the mammalian body. In addition, Chumakov et al. (2002) have reported that the protein coded by a schizophrenia-related gene (G72) activates the DAO, suggesting that the decrease in the D-Ser level caused by activation of DAO is a trigger of schizophrenia. The effects of the DAO activity on the D-AA levels in mammalian body are thus very important to clarify. However, the contribution of DAO to the regulation of the brain D-AAs derived from exogenous origins has been scarcely investigated.

Therefore, in the present investigation, we selected 5 D-amino acids (widely observed D-Asp and D-Ser, plus 3 D-amino acids of small amounts we reported previously, D-Ala, D-Leu, and D-Pro, Hamase et al., 1997, 2001; Morikawa et al., 2001, 2003; Inoue et al., 2000), and orally administered them to the mutant ddY/DAO$^-$ mice and normal ddY/DAO$^+$ mice for 2 weeks. Five D-AAs in the seven brain regions (cerebrum, cerebellum, hippocampus, hypothalamus, medulla oblongata, pituitary gland and pineal gland) and serum were then determined by HPLC. In addition, the five D-AAs were intravenously administered to rats, and the changes in the brain D-AA levels were investigated.

Materials and methods

Materials

D- and L-Amino acids of guaranteed grade, acetonitrile (MeCN), methanol (MeOH) and tetrahydrofuran (THF) of HPLC grade were purchased from Nacalai Tesque (Kyoto, Japan). *o*-Phthalaldehyde (OPA), citric acid monohydrate and trifluoroacetic acid (TFA) were obtained from Wako (Osaka, Japan). *N-tert*-Butyloxycarbonyl-L-cysteine (Boc-L-Cys) and 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) were the products of Novabiochem (Läufelfingen, Switzerland) and Tokyo Kasei (Tokyo, Japan), respectively. Water was purified by a Milli-Q system (Millipore, Billerica, MA, USA). All other reagents and solvents were of reagent grade.

Animals

Male mice (9–13 weeks old, specific pathogen-free) of the mutant strain lacking DAO activity (ddY/DAO$^-$) and the control strain (ddY/DAO$^+$) established by Konno et al. (1983) were used. Male Wistar rats (8 weeks old, specific pathogen-free) were purchased from Seac Yoshitomi (Fukuoka, Japan). These animals were reared under a 12-h light/12-h dark cycle (lights on at 07:00 a.m.) at the animal center of Kyushu University, Graduate School of Pharmaceutical Sciences. For the oral administration of D-AAs to the ddY/DAO$^-$ and ddY/DAO$^+$ mice, the water containing 10 mM each of D-Asp, D-Ala, D-Leu, D-Ser, and D-Pro was given instead of the tap water for two weeks. For the intravenous administration of D-AAs to the rats, a saline solution containing 50 mM each of D-Asp, D-Ala, D-Leu, D-Ser, and D-Pro was administered from the tail vein (50 μmol/kg body weight).

Sample preparation

The animal was anesthetized with diethyl ether and killed by exsanguination from the abdominal aorta, and the brain was quickly excised and separated into seven regions (cerebrum, cerebellum, hippocampus, hypothalamus, medulla oblongata, pituitary gland and pineal gland). The brain tissues and serum were deproteinized in 20 × volumes (or 500 μl in case of small tissue) of MeOH on ice and centrifuged at 4500 *g* for 5 min. A portion of the supernatant was evaporated to dryness under reduced pressure and was used for the HPLC determination.

HPLC determination of amino acids

D-Asp, D-Ser, and D-Ala were determined by a reversed-phase HPLC system according to our previous report (Morikawa et al., 2001). Briefly, the amino acids were derivatized with OPA and Boc-L-Cys under basic conditions (pH 9.0) and were applied to a reversed-phase column (ODS-80TsQA, 250 × 4.6 mm i.d., Tosoh, Tokyo, Japan). The amino acid derivatives were eluted using 0.1 M Na-acetate buffer (pH 6.0) containing MeCN (9–16% linear gradient from 0 to 35 min, 16% from 35 to 80 min) and were determined by their fluorescence (Ex. 344 nm, Em. 443 nm).

D-Leu and D-Pro were determined using a column-switching HPLC system (Inoue et al., 2000; Hamase et al., 2001). Briefly, amino acids were derivatized with NBD-F under basic conditions (pH 8.0) and the NBD-amino acids were purified as DL mixtures from other materials in the

tissue using a micro-reversed phase column (Mightysil RP-18 GP, 100 × 1.0 mm i.d., Kanto Chemical, Tokyo, Japan). The mobile phase was the mixed solution of MeCN, THF, TFA and water. The purified fraction was automatically introduced into a chiral column (Sumichiral OA-2500S or Sumichiral OA-2500R, 250 × 4.6 mm i.d., Sumika Analytical Center, Osaka, Japan) for the separation of the enantiomers. The mobile phase for enantioseparation was citric acid in MeOH. The fluorescence (Ex. 470 nm, Em. 530 nm) was monitored to determine the NBD-amino acids.

Results

The D-AA levels in the brains of ddY/DAO$^+$ and ddY/DAO$^-$ mice after the oral administration of the D-AAs

Figures 1–5 summarize the amounts of the five D-AAs in the seven brain regions (cerebrum, hippocampus, hypothalamus, pituitary gland, cerebellum, medulla oblongata, and pineal gland) of normal ddY/DAO$^+$ and mutant ddY/DAO$^-$ mice lacking DAO activity. After the oral administration of D-AAs, the levels of five D-AAs changed in the different brain regions. The levels of D-Asp markedly increased in the pituitary gland in both strains, being 300–400 nmol/g wet tissue higher after administration (Fig. 1A and B). The increases in D-Asp in the pineal gland were 635 pmol/gland in the ddY/DAO$^+$ mice and 943 pmol/gland in the ddY/DAO$^-$ mice (because the tissue wet weight of mouse pineal gland is too small to measure accurately, the amount of D-amino acid is expressed as pmol/whole gland). These values were remarkable because the weight of the mouse pineal gland is less than 1 mg. The augmentations of D-Asp in this organ are calculated to correspond to more than 600 nmol/g wet tissue.

The levels of D-Ser did not significantly change in any of the brain regions in the ddY/DAO$^+$ mice (Fig. 2A). In the ddY/DAO$^-$ mice, the D-Ser levels in the cerebrum, hippocampus, and pineal gland did not significantly change, whereas the D-Ser levels in the other brain regions significantly increased after the administration of the D-AAs (Fig. 2B). Concerning D-Ala, the D-Ala levels did not significantly increase in any brain regions except for the cerebrum in the ddY/DAO$^+$ mice (Fig. 3A), whereas the D-Ala levels were 100–200 nmol/g wet tissue higher after the administration in the ddY/DAO$^-$ mice (Fig. 3B).

The D-Leu levels of the ddY/DAO$^+$ mice increased in all brain regions. However, the increases were not significant because of the large SE values (Fig. 4A). In the ddY/DAO$^-$ mice, the D-Leu levels markedly increased in all of the brain regions, and the values were more than 40-fold greater after the administration (Fig. 4B). On the other hand, the amounts of D-Pro did not significantly increase in any brain tissues in either strain, although relatively high amounts have been observed in the pituitary gland and pineal gland (31 nmol/g wet tissue and 40 pmol/pineal gland, respectively) in the ddY/DAO$^-$ mice (Fig. 5A and B).

The concentrations of D-Asp, D-Ser, D-Ala, D-Leu, and D-Pro in the serum after the oral administration of the five D-AAs are shown in Table 1. In the ddY/DAO$^+$ mice, the serum levels of all D-AAs increased although a significant increase was observed only in the D-Leu level. On the contrary, in the ddY/DAO$^-$ mice, remarkable increases were observed in all the neutral D-AAs ($p < 0.01$ for D-Ser, D-Leu and D-Pro; $p = 0.04$ for D-Ala). The increases of neutral D-AAs were much greater in the ddY/DAO$^-$ mice

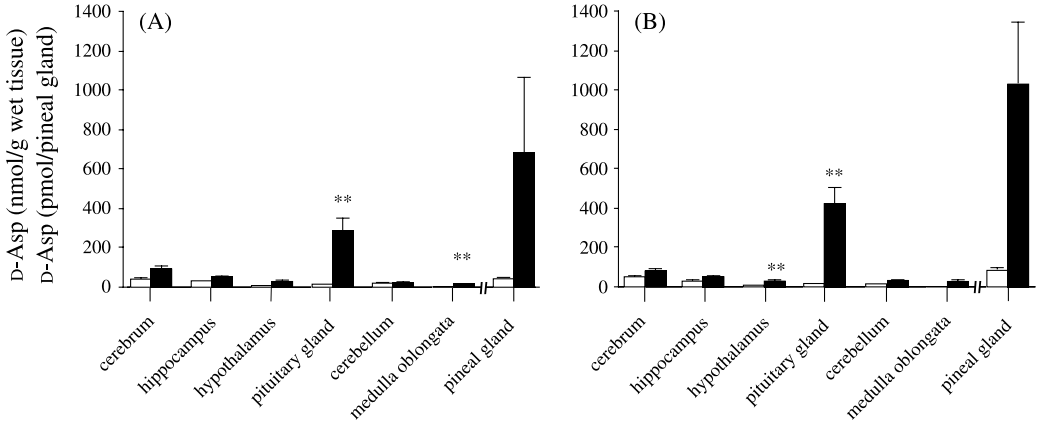

Fig. 1. The levels of D-Asp in the brain of **A** ddY/DAO$^+$, **B** ddY/DAO$^-$ mice after the oral administration of the D-AAs. Values represent means ± SE (nmol/g wet tissue or pmol/pineal gland). Open bars indicate the D-Asp amounts in the control mice ($n = 4$ or 5), and closed bars indicate the D-Asp amounts in the D-AA-administered mice ($n = 3$). $^{**}P < 0.01$, significant difference from the values of control mice. Data for control mice are taken from our previous report (Morikawa et al., 2001)

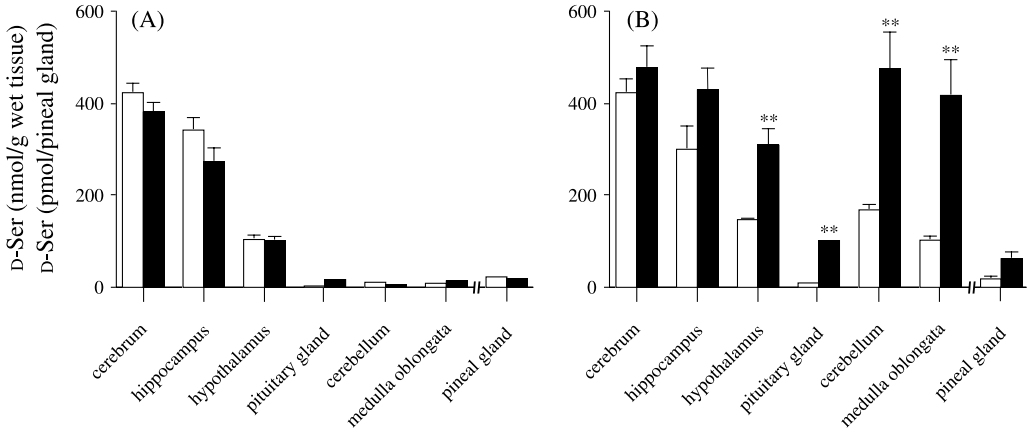

Fig. 2. The levels of D-Ser in the brain of **A** ddY/DAO$^+$, **B** ddY/DAO$^-$ mice after the oral administration of the D-AAs. Values represent means ± SE (nmol/g wet tissue or pmol/pineal gland). Open bars indicate the D-Ser amounts in the control mice ($n = 4$ or 5), and closed bars indicate the D-Ser amounts in the D-AA-administered mice ($n = 3$). $^{**}P < 0.01$, significant difference from the values of control mice. Data for control mice are taken from our previous report (Morikawa et al., 2001)

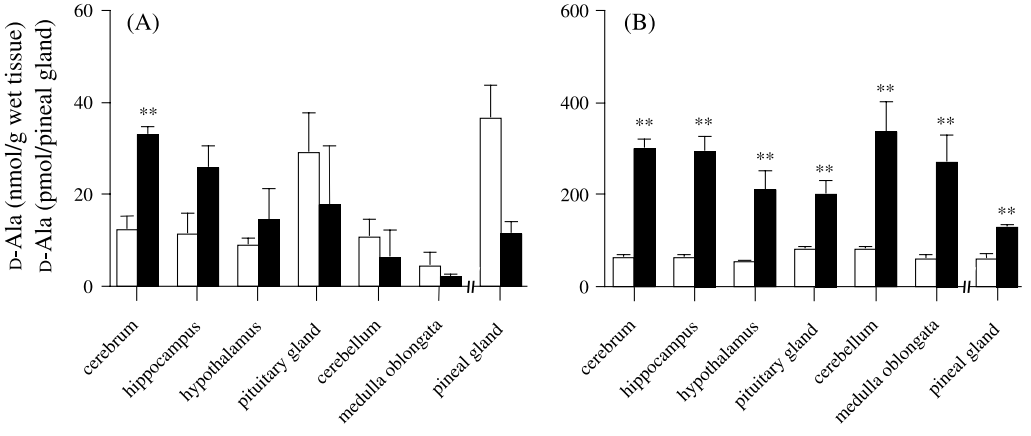

Fig. 3. The levels of D-Ala in the brain of **A** ddY/DAO$^+$, **B** ddY/DAO$^-$ mice after the oral administration of the D-AAs. Values represent means ± SE (nmol/g wet tissue or pmol/pineal gland). Open bars indicate the D-Ala amounts in the control mice ($n = 4$ or 5), and closed bars indicate the D-Ala amounts in the D-AA-administered mice ($n = 3$). $^{**}P < 0.01$, significant difference from the values of control mice. Data for control mice are taken from our previous report (Morikawa et al., 2001). Note the difference in the scale of ordinate between **A** and **B**

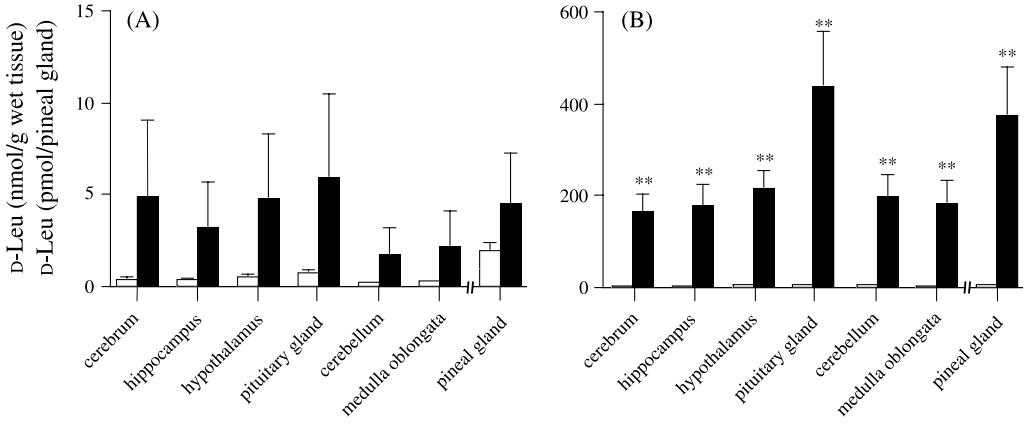

Fig. 4. The levels of D-Leu in the brain of **A** ddY/DAO$^+$, **B** ddY/DAO$^-$ mice after the oral administration of the D-AAs. Values represent means ± SE (nmol/g wet tissue or pmol/pineal gland). Open bars indicate the D-Leu amounts in the control mice ($n = 4$), and closed bars indicate the D-Leu amounts in the D-AA-administered mice ($n = 3$). $^{**}P < 0.01$, significant difference from the values of control mice. Data for control mice are taken from our previous report (Hamase et al., 2001)

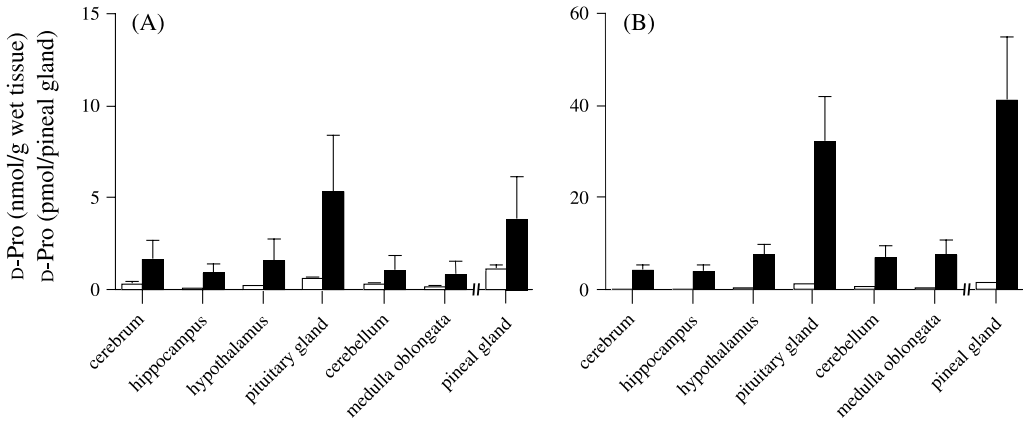

Fig. 5. The levels of D-Pro in the brain of **A** ddY/DAO$^+$, **B** ddY/DAO$^-$ mice after the oral administration of the D-AAs. Values represent means ± SE (nmol/g wet tissue or pmol/pineal gland). Open bars indicate the D-Pro amounts in the control mice ($n = 4$), and closed bars indicate the D-Pro amounts in the D-AA-administered mice ($n = 3$). $^{**}P < 0.01$, significant difference from the values of control mice. Data for control mice are taken from our previous report (Hamase et al., 2001)

Table 1. Serum D-AA levels of D-AAs-administered ddY/DAO$^+$ and ddY/DAO$^-$ mice

Strain	D-AA	D-AA concentration (nmol/ml)		D-AA increase over control (nmol/ml)
		Control mice	D-AAs administered mice	
ddY/DAO$^+$	D-Asp	1.0 ± 0.1 (7.1)	8.7 ± 5.6 (39.8)	7.7
	D-Ser	2.1 ± 0.1 (1.1)	21.7 ± 14.0 (8.4)	19.6
	D-Ala	8.8 ± 1.4 (1.2)	37.5 ± 15.5 (5.0)	28.7
	D-Leu	0.39 ± 0.11 (0.3)	14.1 ± 11.8** (7.4)	13.7
	D-Pro	0.45 ± 0.05 (0.5)	6.6 ± 6.0 (4.7)	6.1
ddY/DAO$^-$	D-Asp	1.7 ± 0.3 (11.9)	8.6 ± 2.5 (41.9)	6.9
	D-Ser	11.6 ± 1.2 (6.1)	98.5 ± 16.0** (37.6)	86.9
	D-Ala	134.6 ± 16.5 (15.0)	255.0 ± 35.0 (32.1)	120.4
	D-Leu	10.4 ± 0.4 (7.3)	571.4 ± 64.9** (76.5)	561.0
	D-Pro	2.2 ± 0.5 (2.0)	39.7 ± 7.3** (24.2)	37.5

Data for control mice are taken from our previous reports (Morikawa et al., 2001; Hamase et al., 2001). Values for D-AAs administered mice represent means ± SE (nmol/ml serum) of 3 mice. Values in parentheses are the proportions of D-AAs (D/(L + D) × 100). $^{**}P < 0.01$, significant difference from the values of control mice

than those in the ddY/DAO$^+$ mice. However, the increases in the levels of D-Asp were similar in the ddY/DAO$^+$ and ddY/DAO$^-$ mice.

The time courses of the alteration of D-AA levels in the brains after the intravenous administration of the D-AAs to rats

The concentrations of D-Asp, D-Ser, D-Ala, D-Leu, and D-Pro in the rat brain were determined at 0, 3, 5, 15, and 60 min after the intravenous administration of the five D-AAs (Fig. 6). Because commercially available ddY mouse has the variation in DAO gene, and it is difficult to obtain sufficient numbers of ddY/DAO$^+$ and ddY/DAO$^-$ mice. Also, the DAO gene is well preserved in Wistar rats, and strong DAO activity is observed in these rats. Therefore, Wistar rats were used in this experiment. The D-Asp amount markedly increased in the pineal gland; the level reached approximately 5000 nmol/g wet tissue 3 min after the administration, and decreased to almost half of the maximum levels within 60 min (Fig. 6A). The D-Ser levels readily increased in the pituitary and pineal glands by 4–8-fold of the control levels at 3 min and the levels did not significantly change until 60 min after the administration (Fig. 6B). Remarkable increases in the D-Ala levels were observed only in the pituitary and pineal glands; the amounts after the administration were 2–3-fold higher than the control levels at 3–15 min and decreased to almost the control levels at 60 min (Fig. 6C). The D-Leu amounts increased in all the brain regions; the high levels of D-Leu in the frontal brain regions were observed at 3–60 min, while the D-Leu

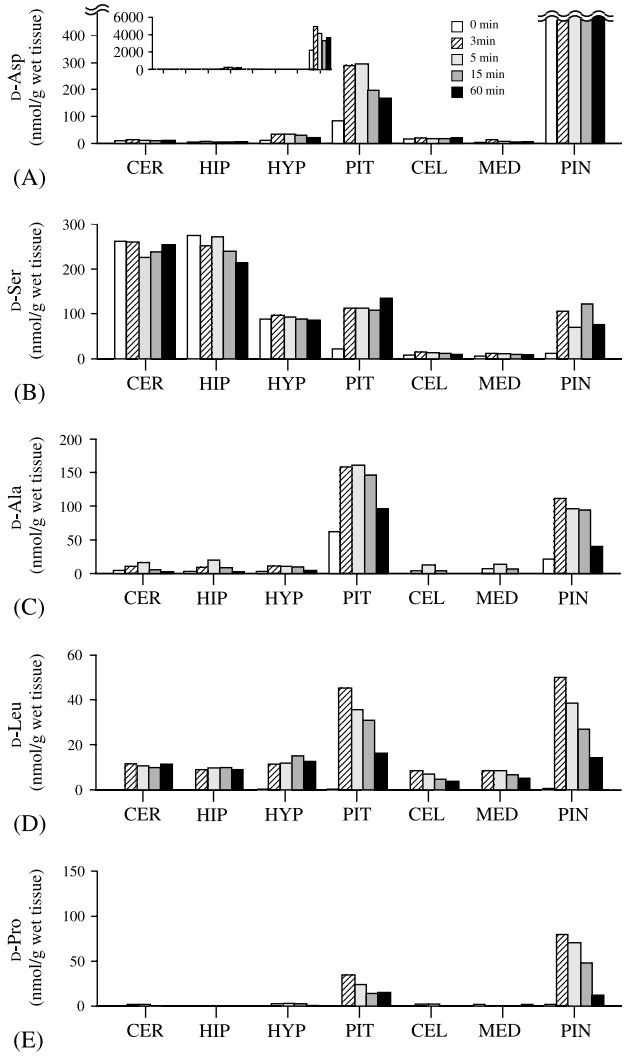

Fig. 6. The time courses of the **A** D-Asp, **B** D-Ser, **C** D-Ala, **D** D-Leu, **E** D-Pro levels in various rat brain regions after the intravenous administration of the five D-AAs to the rats. Values represent means (nmol/g wet tissue) of 3 (D-Asp, D-Ser, D-Ala) or 2 (D-Leu, D-Pro) rats. The reduced graph of **A** is also shown. *CER* Cerebrum, *HIP* hippocampus, *HYP* hypothalamus, *PIT* pituitary gland, *CEL* cerebellum, *MED* medulla oblongata, *PIN* pineal gland

levels in the pituitary and pineal glands were the highest at 3 min and gradually decreased to less than half of the maximum levels within 60 min (Fig. 6D). As for D-Pro, notable increases were observed only in the pituitary and pineal glands; the levels were the highest 3 min after the administration and decreased to less than half of the maximum values within 60 min (Fig. 6E).

The plasma levels of the five D-AAs were less than 10 nmol/ml before the administration; the values increased to 200–1000 nmol/ml 3 min after the administration and decreased to less than half of the maximum values within 15 min.

Discussion

In the present investigation, the levels of D-Asp, D-Ser, D-Ala, D-Leu, and D-Pro in the seven brain regions were examined after their oral and intravenous administrations of the five D-AAs. Different increasing profiles were observed for each D-AA. As for D-Asp, remarkable augmentations occurred in the pineal and pituitary glands of both the ddY/DAO$^+$ and ddY/DAO$^-$ mice after the oral administration; the augmentations of the D-Asp levels in both strains occurred to similar extents, suggesting that the DAO activity did not affect the levels of D-Asp in vivo. These results are in good agreement with the reports that the DAO has low affinity to acidic D-AAs in vitro (D'Aniello et al., 1993), and that the activity of the D-asparatic acid oxidase, the metabolizing enzyme of D-Asp, is not different between the ddY/DAO$^+$ and ddY/DAO$^-$ mice (Nagasaki et al., 1990). The immediate and considerable increases in D-Asp were also observed in the rat pineal and pituitary glands after the intravenous administration. These results are in agreement with the report by Imai et al. (1997) showing that high radioactivities are observed in the pineal and pituitary glands 30 min after the intravenous administration of ^{14}C-D-Asp. It has been reported that intrinsic D-Asp is highly localized to the pineal and pituitary glands, and regulates the hormonal secretion from these endocrine glands (Hashimoto and Oka, 1997; Hamase et al., 2002). The present results suggest that the D-Asp levels in the pineal and pituitary glands are easily affected by the intake of exogenous D-Asp, indicating the possibility that the exogenous D-Asp derived from intestinal bacteria and food affects the hormonal secretion from these glands.

The amounts of D-Ser in the serum and all brain tissues did not significantly increase in the ddY/DAO$^+$ mice. While in the ddY/DAO$^-$ mice, the levels of D-Ser markedly increased in the serum and brain except for the frontal brain regions. These results suggest that the DAO have crucial roles to eliminate the exogenous D-Ser in the serum and particular brain regions, such as the cerebellum and medulla oblongata in the normal mammals. In addition, it is also suggested that the exogenous D-Ser is readily incorporated into some brain regions. To date, a large number of studies have indicated that the intrinsic D-Ser is localized to the frontal brain regions, such as cerebrum and hippocampus. The origins and regulatory mechanisms of highly concentrated D-Ser are important subjects of interest. In the present study, we have found that the D-Ser levels in the frontal brain regions are not significantly affected by the oral administration even in the

ddY/DAO$^-$ mice as well as in the ddY/DAO$^+$ mice. Combined with the result that the D-Ser did not increase in the frontal brain regions after the intravenous administration to rats, the findings obtained in the present investigation suggest that the exogenous D-Ser does not significantly affect the levels of D-Ser in the frontal brain regions, which gives support to the hypothesis that D-Ser is synthesized by the serine racemase in the frontal brain regions (Wolosker et al., 1999). On the other hand, Hashimoto and Chiba (2004) and Takahashi et al. (1997) reported that the D-Ser levels in the frontal brain regions increase by the administration of D-Ser. This would be caused by the differences in 1) dose; they administered 180–200-fold higher D-Ser than we administered in the present research, and 2) the method of administration; they intraperitoneally administered only D-Ser, while we orally and intravenously administered the mixture of the five D-AAs.

The levels of D-Ala in the cerebrum and cerebellum of the ddY/DAO$^-$ mice have been already reported to increase after the oral administration of D-Ala (Nagata et al., 1994). In the present research, we found that D-Ala was incorporated into the wide brain areas of the ddY/DAO$^-$ mice, although, in the normal mice, the chronic (for two weeks) oral administration of D-Ala did not significantly affect the brain levels of D-Ala. Besides, by the acute intravenous administration of D-AAs to rats, we demonstrated that a large amount of D-Ala was rapidly and transiently incorporated into the pituitary and pineal glands.

Concerning D-Leu, Hasegawa et al. (2004) has reported that the serum levels of D-[$^2H_7$]Leu are significantly different between the ddY/DAO$^+$ and ddY/DAO$^-$ mice 60 min after the intravenous administration of D-[$^2H_7$]Leu. In our present study, it was found that the increase in the serum D-Leu level in the ddY/DAO$^-$ mice was significantly greater than that in the ddY/DAO$^+$ mice after the chronic, oral administration of the D-AAs, suggesting that DAO plays a crucial role in eliminating the exogenous D-Leu. In addition, rapid and notable increases in the levels of D-Leu were first found in wide brain areas of the rat after the intravenous administration. Different from other neutral D-AAs, the levels of D-Pro in all the brain regions did not significantly increase either in the ddY/DAO$^+$ mice or in the ddY/DAO$^-$ mice by the oral administration of the D-AAs. Moreover, the intravenous administration of the D-AAs hardly affected the D-Pro levels in almost all the brain regions of the rats. These results are in accordance with the report by Schiber et al. (1997) that the brain level of D-Pro does not increase by the oral administration of D-Pro.

In addition to the DAO, other regulation mechanisms, such as urinary secretion and amino acid transporters, should also be considered for the control of the D-AA levels in the mammalian body. Schiber et al. (1997) have reported that the level of D-Pro in the rat urine remarkably increases after the oral D-Pro administration, while the urinary D-Asp level does not increase after the oral D-Asp administration. These results suggest that renal excretion is important for controlling the D-AA levels. On the other hand, amino acid transporters that have a high affinity to D-Asp (Kanai and Hediger, 2003), D-Ser, D-Ala (Fukasawa et al., 2000), and D-Leu (Kanai et al., 1998) have been reported, although their contributions to the regulation of the brain D-AA levels in vivo are not clear. The data obtained in the present research would be useful for investigating the physiological functions of these amino acid transporters.

In conclusion, the present investigation has clarified the effects of the D-AAs administration on the brain D-AA levels and the contribution of DAO to the metabolism of the orally and intravenously administered D-AAs. These findings would be useful for the investigations related to the origins and regulation mechanism of D-AAs in mammals, also indicate the importance to use genetically pure animals in terms of DAO on the research of various neutral D-amino acids.

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References

Brückner H, Haush M (1993) Gas chromatographic characterization of free D-amino acids in the blood serum of patients with renal disorders and of healthy volunteers. J Chromatogr 614: 7–17

Chumakov I, Blumenfeld M, Guerassimenko O, Cavarec L, Palicio M, Abderrahim H, Bougueleret L, Barry C, Tanaka H, Rosa PL, Puech A, Tahri N, Akenine AC, Delabrosse S, Lissarrague S, Picard FP, Maurice K, Essioux L, Millasseau P, Grel P, Debailleul V, Simon AM, Caterina D, Dufaure I, Malekzadeh K, Belova M, Luan JJ, Bouillot M, Sambucy JL, Primas G, Saumier M, Boubkiri N, Saumier SM, Nasroune M, Peixoto H, Delaye A, Pinchot V, Bastucci M, Guillou S, Chevillon M, Fuertes RS, Meguenni S, Aurich-Costa J, Cherif D, Gimalac A, Duijn CV, Gauvreau D, Ouellette G, Fortier I, Raelson J, Sherbatich T, Riazanskaia N, Rogaev E, Raeymaekers P, Aerssens F, Konings W, Luyten F, Macciardi PC, Sham RE, Straub DR, Weinberger J, Cohen N, Cohen D, Ouelette G, Realson J (2002) Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. Proc Natl Acad Sci USA 99: 13675–13680

D'Aniello A, Vetere A, Petrucelli L (1993) Further study on the specificity of D-amino acid oxidase and D-aspartate oxidase and time course for

complete oxidation of D-amino acids. Comp Biochem Physiol B 105: 731–734

D'Aniello A, Fiore MMD, Fisher GH, Milone A, Seleni A, D'Aniello S, Perna AF, Ingrosso D (2000a) Occurrence of D-aspartic acid and *N*-methyl-D-aspartic acid in rat neuroendocrine tissues and their role in the modulation of luteinizing hormone and growth hormone release. FASEB J 14: 699–714

D'Aniello G, Tolino A, D'Aniello A, Errico F, Fisher GH, Fiore MMD (2000b) The role of D-aspartic acid and *N*-methyl-D-aspartic acid in the regulation of prolactin release. Endocrinology 141: 3862–3870

Fisher GH, D'Aniello A, Vetere A, Padula L, Cusano GP, Man EH (1991) Free D-aspartate and D-alanine in normal and Alzheimer brain. Brain Res Bull 26: 983–985

Friedman M (1999) Chemistry, nutrition, and microbiology of D-amino acids. J Agr Food Chem 47: 3457–3479

Fukasawa Y, Segawa H, Kim JY, Chairoungdua A, Kim DK, Matsuo H, Cha SH, Endou H, Kanai Y (2000) Identification and characterization of a Na^+-independent neutral amino acid transporter that associates with the 4F2 heavy chain and exhibits substrate selectivity for small neutral D- and L-amino acids. J Biol Chem 275: 9690–9698

Hamase K, Homma H, Takigawa Y, Fukushima T, Santa T, Imai K (1997) Regional distribution and postnatal changes of D-amino acids in rat brain. Biochim Biophy Acta 1334: 214–222

Hamase K, Inoue T, Morikawa A, Konno R, Zaitsu K (2001) Determination of free D-proline and D-leucine in the brains of mutant mice lacking D-amino acid oxidase activity. Anal Biochem 298: 253–258

Hamase K, Morikawa A, Zaitsu K (2002) D-Amino acids in mammals and their diagnostic value. J Chromatogr B 781: 73–91

Hasegawa H, Matsukawa T, Shinohara Y, Konno R, Hashimoto T (2004) Role of renal D-amino-acid oxidase in pharmacokinetics of D-leucine. Am J Physiol 287: E160–E165

Hashimoto A, Oka T, Nishikawa T (1995) Anatomical distribution and postnatal changes in endogenous free D-aspartate and D-serine in rat brain and periphery. Eur J Neurosci 7: 1657–1663

Hashimoto A, Oka T (1997) Free D-aspartate and D-serine in the mammalian brain and periphery. Prog Neurobiol 52: 325–353

Hashimoto A, Chiba Y (2004) Effect of systemic administration of D-serine on the levels of D- and L-serine in several brain areas and periphery of rat. Eur J Pharmacol 495: 153–158

Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N, Nakazato M, Kumakiri C, Okada S, Hasegawa H, Imai K, Iyo M (2003) Decreased serum levels of D-serine in patients with schizophrenia. Arch Gen Psychiatry 60: 572–576

Imai K, Fukushima T, Santa T, Homma H, Sugihara J, Kodama H, Yoshikawa M (1997) Accumulation of radioactivity in rat brain and peripheral tissues including salivary gland after intravenous administration of ^{14}C-D-aspartic acid. Proc Jpn Acad 73: 48–52

Imai K, Fukushima T, Santa T, Homma H, Huang Y, Shirao M, Miura K (1998) Whole body autoradiographic study on the distribution of ^{14}C-D-serine administered intravenously to rats. Amino Acids 15: 351–361

Inoue T, Hamase K, Morikawa A, Zaitsu K (2000) Determination of minute amounts of D-leucine in various brain regions of rat and mouse using column-switching high-performance liquid chromatography. J Chromatogr B 744: 213–219

Ishio S, Yamada H, Hayashi M, Yatsushiro S, Noumi T, Yamaguchi A, Moriyama Y (1998) D-Aspartate modulates melatonin synthesis in rat pinealocytes. Neurosci Lett 249: 143–146

Kanai Y, Segawa H, Miyamoto K, Uchino H, Takeda E, Endou H (1998) Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98). J Biol Chem 273: 23629–23632

Kanai Y, Hediger MA (2003) The glutamate and neutral amino acid transporter family: physiological and pharmacological implications. Eur J Pharmacol 479: 237–247

Konno R, Yasumura Y (1983) Mouse mutant deficient in D-amino acid oxidase activity. Genetics 103: 277–285

Konno R, Niwa A, Yasumura Y (1990) Intestinal bacterial origin of D-alanine in urine of mutant mice lacking D-amino-acid oxidase. Biochem J 268: 263–265

Konno R, Oowada T, Ozaki A, Iida T, Niwa A, Yasumura Y, Mizutani T (1993) Origin of D-alanine present in urine of mutant mice lacking D-amino-acid oxidase activity. Am J Physiol 265: G699–G703

Lee J-A, Long Z, Nimura N, Iwatsubo T, Imai K, Homma H (2001) Localization, transport, and uptake of D-aspartate in the rat adrenal and pituitary glands. Arch Biochem Biophys 385: 242–249

Long Z, Lee J-A, Okamoto T, Nimura N, Imai K, Homma H (2000) D-Aspartate in a prolactin-secreting clonal strain of rat pituitary tumor cells (GH_3). Biochem Biophys Res Commun 276: 1143–1147

Morikawa A, Hamase K, Inoue T, Konno R, Niwa A, Zaitsu K (2001) Determination of free D-aspartic acid, D-serine and D-alanine in the brain of mutant mice lacking D-amino-acid oxidase activity. J Chromatogr B 757: 119–125

Morikawa A, Hamase K, Zaitsu K (2003) Determination of D-alanine in the rat central nervous system and periphery using column-switching high-performance liquid chromatography. Anal Biochem 312: 66–72

Nagasaki H, Yamada R, Konno R, Yasumura Y, Iwashima A (1990) D-Aspartate oxidase activity and D-aspartate content in a mutant mouse strain lacking D-amino acid oxidase. Experientia 46: 468–470

Nagata Y, Konno R, Niwa A (1994) Amino acid levels in D-alanine-administered mutant mice lacking D-amino acid oxidase. Metabolism 43: 1153–1157

Pilone MS (2000) D-Amino acid oxidase: new findings. Cell Mol Life Sci 57: 1732–1747

Schieber A, Brückner H, Rupp-Classen M, Specht W, Nowitzki-Grimm S, Classen HG (1997) Evaluation of D-amino acid levels in rat by gas chromatography-selected ion monitoring mass spectrometry: no evidence for subacute toxicity of orally fed D-proline and D-aspartic acid. J Chromatogr B 691: 1–12

Snyder SH, Kim PM (2000) D-Amino acids as putative neurotransmitters: focus on D-serine. Neurochem Res 25: 553–560

Takahashi K, Hayashi F, Nishikawa T (1997) In vivo evidence for the link between L- and D-serine metabolism in rat cerebral cortex. J Neurochem 69: 1286–1290

Tsai G, Yang P, Chung LC, Lange N, Coyle JT (1998) D-Serine added to antipsychotics for the treatment of schizophrenia. Biol Psychiatry 44: 1081–1089

Wolosker H, Sheth KN, Takahashi M, Mothet JP, Brady Jr. RO, Ferris CD, Snyder SH (1999) Purification of serine racemase: biosynthesis of the neuromodulator D-serine. Proc Natl Acad Sci USA 96: 721–725

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