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Localization, Transport, and Uptake of D-Aspartate in the Rat Adrenal and Pituitary Glands

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Large amounts of D-aspartate (D-Asp) are present in the rat adrenal and pituitary glands. D-Asp is thought to be synthesized in the mammalian body and also accumulates in various tissues following intraperitoneal or intravenous administration. This report examines the origins of D-Asp in the adrenal and pituitary glands. We administered D-Asp to male rats intraperitoneally and immunolocalized this exogenous D-Asp in adrenal and pituitary tissue, using an anti-D-Asp antiserum which was previously developed in our laboratory. D-Asp levels in the rat adrenal gland have been shown to undergo a transient increase at 3 weeks of age and to decrease rapidly thereafter. We found that in the adrenal gland, exogenous D-Asp administered intraperitoneally was incorporated into the same region of the adrenal cortex in which endogenous D-Asp was present. By Northern and Western blot analysis and immunohistochemistry of glutamate (Glu) transporter, we also found that expression of the Glu transporter (GLAST), which has an affinity for D-Asp, transiently increased at 3 weeks of age and that localization patterns of the Glu transporter within the tissue were almost coincident with those of endogenous D-Asp. These observations suggest that D-Asp in the adrenal cortex of 3-week-old male rats is primarily acquired by uptake from the vascular system. We have previously shown that D-Asp is specifically localized in prolactin (PRL)-containing cells in the anterior lobe of the adult rat pituitary gland. Here we report that in the pituitary gland, exogenous D-Asp accumulated in endothelial cells, but not in PRL-containing cells. Northern and Western blot analysis and immunohistochemistry of Glu transporter revealed that developmental changes in the Glu transporter (GLAST) expression did not correlate with tissue levels of D-Asp and that the Glu transporter was not expressed in PRL-containing cells. These observations suggest that, in contrast to the adrenal gland, most of the D-Asp in the pituitary gland of adult male rats originates inside the gland itself.

Key Words: **D-aspartate; D-amino acids; glutamate transporter; adrenal gland; pituitary gland; immunohistochemistry.**

Recent investigations have demonstrated the involvement of D-aspartate (D-Asp)[2] in a variety of biological activities in the mammalian body. D-Asp suppresses melatonin secretion in cultured rat pinealocytes (1) and isolated rat pineal gland (2), presumably via activation of the glutamate (Glu) receptor (mGlu R3) (3–5), and increases testosterone production in isolated rat Leydig cells by stimulating the expression of steroidogenic acute regulatory protein (StAR) (6, 7). This stimulation of StAR expression is apparently Glu receptor-independent and requires D-Asp to be taken up by the cells (6, 7). Moreover, it has been demonstrated that D-Asp is actually synthesized in mammalian cells (8). These lines of evidence suggest that D-Asp

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[2] Abbreviations used: D-Asp, D-aspartate; Glu, glutamate; PRL, prolactin; IR, immunoreactivity; ZF, zona fasciculata; ZR, zona reticularis; ZG, zona glomerulosa; Star, steroidogenic acute regulatory protein; Ser, serine; SD, Sprague–Dawley; PBS, phosphate-buffered saline; NA, nonadrenaline; A, adrenaline; Mops, 3-[N-morpholino]propanesulfonic acid; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ECL, enhanced chemiluminescence.

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plays an important role as a messenger in mammalian endocrine and neuroendocrine tissues (9).

D-Asp is synthesized in rat pheochromocytoma (PC12) cells (8), and a serine (Ser)-specific racemase which was recently cloned from rat brain is assumed to be involved in the synthesis of D-Ser (10, 11), another D-amino acid which is found in abundance in mammals. It therefore appears likely that D-Asp is also synthesized in the mammalian body, although the precise synthetic route has yet to be elucidated. On the other hand, when D-Asp is administered to rats either intraperitoneally (12) or intravenously (13), it accumulates via the vascular system in various tissues, including testis and the pineal, pituitary, and adrenal glands. It therefore remains to be determined whether D-Asp is synthesized in almost every organ, or is produced by a restricted range of tissues and subsequently accumulated in other tissues via the vascular system.

The D-Asp content of a number of tissues has been shown to change markedly during development. D-Asp levels are transiently increased during the development of human (14) and rat brain (15), rat retina (16) and adrenal gland (17), and chicken embryonic brain (16). In previous reports (18–22), we used an anti-D-Asp antibody to study developmental changes in D-Asp localization within various rat tissues. As development proceeds, D-Asp appears and becomes localized in specific types of cells within these tissues, and both the tissue localization and the intracellular distribution of D-Asp change during development. However, the origin of the D-Asp observed in these tissues remains unknown. In addition, it is unclear how the levels and localization of D-Asp within the tissues are regulated. It has been reported that the localization pattern of D-Asp is the inverse of that of D-Asp oxidase in several rat tissues: D-Asp content is low in cells in which D-Asp oxidase activity is high and vice versa (9, 23–25). However, D-Asp is not detectable in some tissues which lack D-Asp oxidase activity, so tissue levels of D-Asp are apparently determined not only by degradative D-Asp oxidase activity but also by other factors, possibly including regulation of endogenous synthesis and/or uptake from the vascular system.

Rat adrenal and pituitary glands apparently contain very low levels of D-Asp oxidase activity (9). D-Asp levels in the adrenal gland transiently increase at 3 weeks of age, decrease thereafter, and remain at an adult level after 8 weeks of age (17). In contrast, the D-Asp level in the pituitary gland continues to increase gradually from 1 to 8 weeks of age (15, 17). In 3-week-old rats, D-Asp is predominantly localized in most regions of the adrenal cortex, whereas in 8-week-old rats it is localized primarily in the adrenal medulla (19). In the pituitary gland, D-Asp is localized in prolactin (PRL)-containing cells or some other very closely related type of cells in the anterior lobe of the gland (22). In this report, D-Asp which was administered intraperitoneally to 3-week-old and 8-week-old rats was incorporated into various tissues via the vascular system. We subsequently localized this exogenous D-Asp within the adrenal and pituitary glands by immunohistochemistry and compared the localization patterns of exogenous and endogenous D-Asp.

It seems likely that D-Asp is incorporated into cells via the L-Glu transporter, which has an affinity for D-Asp in addition to L-Glu and L-Asp (26–28). We therefore also analyzed the localization of the Glu transporter and developmental changes in its expression in the adrenal and pituitary glands. We then compared these results with the localization of endogenous D-Asp and developmental changes in the D-Asp content of these tissues in order to examine the origins of D-Asp in the rat adrenal and pituitary glands.

MATERIALS AND METHODS

Chemicals. D,L-Asp was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium pentobarbital was from Abbott Laboratories (IL). Glutaraldehyde and paraformaldehyde used in immunohistochemical studies were obtained from EM Science (PA) and sodium cacodylate was from TAAB Laboratories (Reading, UK). FITC-conjugated goat anti-rabbit IgG antibody was obtained from Organon Teknika (Durham, NC, UK), and mouse monoclonal anti-rat PRL antibody (IgG1 fraction) were obtained from QED Bioscienc Inc. (USA). Texas red-conjugated goat anti-mouse IgG (H + L) antibody, were obtained from Jackson ImmunoResearch (West Grove, PA). The Dako PAP Kit was purchased from DAKO (Denmark). [α-^{32}P]dCTP (110 TBq/mmol) was a product of Amersham Pharmacia Biotech Inc. (Piscataway, NJ). Oligonucleotide primers were prepared by Sawady Technology Inc. (Tokyo, Japan). Restriction enzymes and *Taq* DNA polymerase were purchased from TAKARA (Kyoto, Japan). Other chemicals were of the highest grade available.

Animals. Male Sprague–Dawley (SD) rats (specific pathogen free) purchased from Charles River Japan Inc. (Kanagawa, Japan) were kept in a constant 12-h light/12-h dark cycle (lights on at 7:00 AM) with free access to food and water.

Determination of the D-Asp content of rat adrenal and pituitary gland by HPLC. D-Asp in saline (1.0 μmol/g body weight, approx. 0.5 ml, neutralized) or saline as control was administered to 8-week-old male SD rats by intraperitoneal (ip) injection (12). Fifteen min or 5 h after ip injection, the rats were anesthetized with diethyl ether and sacrificed by exsanguination from the abdominal aorta. Asp levels in the adrenal and pituitary glands were determined by HPLC with a Pirkle-type chiral stationary phase and fluorometric detection as described previously (29). D-Asp was administered to two rats and Asp levels in the glands were represented as average ± half range.

Immunohistochemistry. Rats were anesthetized by ip injection of sodium pentobarbital solution (50 mg/kg body weight). After 1–2 min of transcardial perfusion with Ringer's solution, animals were fixed by perfusion with fixative solution (2.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M cacodylate, pH 7.4) at a rate of 10 ml/min for 20 min. The tissues were removed and postfixed in the fixative solution for 2 h at 4°C and cryoprotected in 10, 15, and 20% sucrose in PBS before being frozen in embedding medium (OCT Compound, Miles Laboratories, Naperville, IL). Cryostat tissue sections (10 μm thickness) were mounted on poly(L)-lysine-treated slides (Matsunami Glass Ind., Japan) and air-dried. Sections were pretreated for 20 min with 0.5% $NaBH_4$ in PBS to inactivate residual glutaraldehyde and then blocked with 10% calf serum containing 0.1% sodium azide for

30 min and incubated overnight at 25°C with anti-D-Asp antibody prepared in this laboratory (at a dilution of between 1:30 and 1:1000 in PBS containing 10% calf serum and 0.1% sodium azide) (18, 20). Immunoreactivity was visualized by the peroxidase–antiperoxidase method using the PAP complex (DAKO). The sections were counterstained with hematoxylin. Preabsorption of the antibody with a liquid phase conjugate of glutaraldehyde and D-Asp (1 mM) abolished D-Asp immunoreactivity in all the cases described in this study.

For staining with anti-Glu transporter antisera, animals were fixed by perfusion with 4% paraformaldehyde solution in PBS, pH 7.4. Then the immunostaining of GLAST was carried out as that of D-Asp described above. The sections were probed with 1:50 anti-GLAST antiserum (CovalAb, France), and visualized by the peroxidase–antiperoxidase method or fluorescent secondary antibodies (FITC-conjugated anti-rabbit IgG antibodies). We also determined the localization of GLAST with the anti-GLAST antiserum which was kindly donated by Prof. M. Watanabe (Univ. Hokkaido, School of Medicine). There are no differences between the localization determined by these two anti-GLAST antisera.

Localization of exogenous D-Asp, which was administered ip and accumulated abundantly in the tissues, was determined using a much higher dilution of the anti-D-Asp antiserum than was used to detect endogenous D-Asp. The antiserum dilution used to detect exogenous D-Asp did not detect any endogenous D-Asp in control animals injected with saline.

The noradrenaline (NA)- and adrenaline (A)-storing cells of the adrenal medulla were distinguished as described in a previous report (19). After identification of NA cells by fixation of tissue sections in 50% Karnovisky solution, the sections were further probed with anti-D-Asp antiserum.

Reverse transcription–polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from isolated rat glands with Isogen reagent (Wako Chemical Ind., Osaka, Japan), a monophasic solution of phenol and guanidine isothiocyanate. One microgram of total RNA was transcribed into cDNA followed by PCR amplification using a TaKaRa RNA PCR kit (AMV) Ver. 2.1 (TaKaRa, Kyoto, Japan). The primers used were based on published sequences: GLT-1: sense: GGGAAGAAGAACGACGAGGTG (bases 466–486), antisense, ACCTCCATCCAGGATGACCCCATTC (bases 1242–1266) (30); GLAST: sense: TCGTGCAGGTGACTGCCGCAG (bases 482–502), antisense: CTGTCCAAAATTCAGGTCAAAG (bases 1254–1275) (31); EAAC1: sense: GACGCCATGTTGGATCTGATCAGGAA (bases 418–443), antisense: GCTTCATAGAGCGCAGTGCCGTCCAT (bases 1096–1121) (32); EAAT4: sense: CGAGTGGTAACAAGGACGAT (bases 757–776), antisense: GTGTGTTACCCCTCATCTAC (bases 1211–1230) (33). The PCR products amplified by these primer pairs were 801, 794, 704, and 474 base pairs long, respectively.

Northern blot analysis. The rat Glu transporter cDNA fragments described above were cloned into the pT7Blue-2 T-vector (Novagen, Madison, WI). The resulting inserts were extracted and purified using a QIAEX II kit (QIAGEN Inc., Valencia, CA), and labeled with 1.85 MBq of [α-^{32}P]dCTP (10^9 dpm/mg) using a DNA labeling kit (Ready To Go, Amersham Pharmacia Biotech Inc., NJ), according to the respective manufacturers' instructions. Labeled probes were separated from free nucleotides with G50 spin columns (ProbeQuant, Amersham Pharmacia Biotech). mRNA was extracted from adrenal or pituitary glands with the QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech). Approximately 5 μg of mRNA was separated by electrophoresis on 1.0% agarose/18% formaldehyde/Mops gels and transferred onto Hybond N^+ nylon membranes (Amersham Pharmacia Biotech). After prehybridization for 1 h at 65°C, filters were hybridized with the labeled probes (Glu transporter cDNA or rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA) at 1–2 $\times$ 10^6 dpm/cm^2 for 2 h at 65°C in hybridization buffer (Rapid hyb buffer, Amersham Pharmacia Biotech). Following hybridization, membranes were rinsed with 2 $\times$ SSPE, 0.1% SDS, washed in 0.1 $\times$ SSPE, 0.1% SDS, and then exposed to Kodak X-OMAT AR films at −80°C for 1–2 days. Intensities of autoradiographic bands were estimated by densitometric scanning.

Western blot analysis. A total membrane fraction of rat pituitary or adrenal gland was prepared as follows with a slight modification of the method of Yamada *et al.* (34). Male SD rats (1, 3, 8, and 13 weeks of age) were anesthetized with diethyl ether and sacrificed by exsanguination from the abdominal aorta. The pituitary and adrenal glands were dissected and homogenized separately in 2 ml SME buffer (20 mM Mops–Tris (pH 7.0) containing 0.3 M sucrose, 5 mM EDTA, 5 μg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 4 μg/ml aprotinin). The homogenate was centrifuged at 900*g* for 10 min, and the supernatant was centrifuged at 100,000*g* for 30 min. The resulting pellet was suspended in the same buffer and the protein content was determined using a BioRad protein assay with bovine serum albumin as standard. Proteins (50 μg per lane) were separated on 12% SDS/polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked in 10 mM phosphate-buffered saline (PBS) containing 5% skim milk and 0.2% Tween 20 at 4°C overnight and then probed with antibodies (GLT-1 antibody 1 μg/ml, GLAST antibody 0.5 μg/ml) for 1 h at room temperature. These anti-rat Glu transporter antisera were kindly donated by Prof. M. Watanabe (Univ. Hokkaido, School of Medicine). The membranes were then washed, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (at a dilution of 1:5000) for 1 h, and immunoreactive bands detected using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech Inc.).

RESULTS

In Vivo Uptake of D-Asp into Rat Adrenal and Pituitary Glands

When D-Asp is intraperitoneally administered to rats, it accumulates via the vascular system in various tissues, including the testis and pituitary gland (12). However, the precise localization of the exogenous D-Asp within these tissues has yet to be determined. Intravenous administration of radiolabeled D-Asp also resulted in the accumulation of radioactivity in these tissues as well as the pineal and adrenal glands (13). However, the radioactivity detected in tissues does not necessarily represent intact D-Asp alone, but may also include metabolites, and the results of this study are therefore difficult to interpret. Thus the localization of the exogenous D-Asp within the tissues cannot be determined precisely even by autoradiography of the tissue sections. In the present study, we administered D-Asp to rats by ip injection and localized this exogenous intact D-Asp in the tissues using anti-D-Asp antiserum at a dilution which did not detect endogenous D-Asp in control animals. The localization patterns of exogenous and endogenous D-Asp were then compared, in order to determine whether the exogenous D-Asp was incorporated into the same tissue regions in which endogenous D-Asp was found.

The D-Asp levels in the adrenal and pituitary glands were, respectively, 21.9 $\pm$ 18.7 and 13.8 $\pm$ 11.8 nmol/gland 15 min after D-Asp injection and 36.4 $\pm$ 1.7 and 48.3 $\pm$ 0.68 nmol/gland 5 h after injection. In contrast,

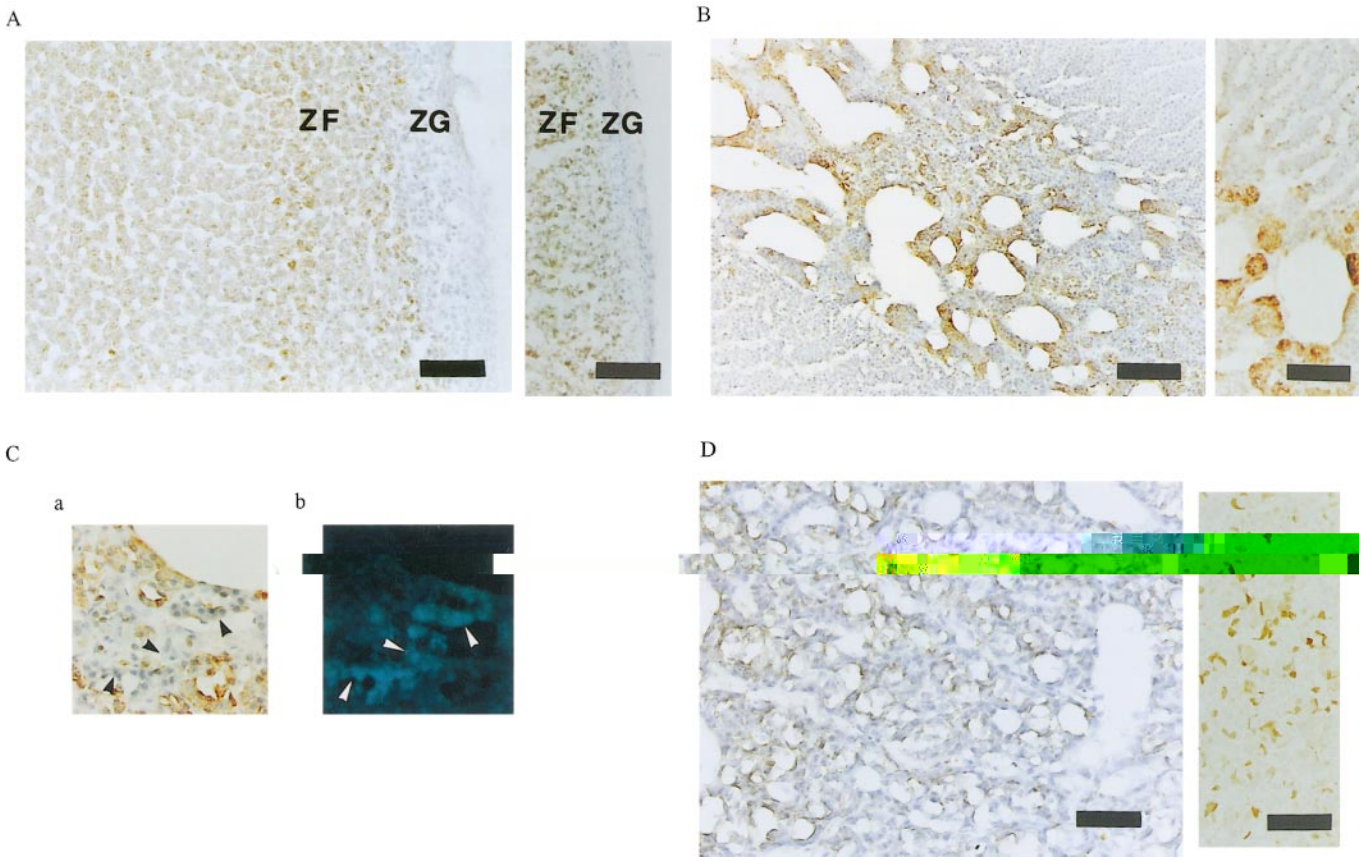

FIG. 1. *In vivo* uptake of D-Asp into rat adrenal and pituitary glands. D-Asp was administered intraperitoneally to male rats (3 or 8 weeks of age) and this exogenous D-Asp was localized in the adrenal and pituitary glands 5 h after injection using anti-D-Asp antiserum. (A) Adrenal cortex of 3-week-old rat. IR to exogenous D-Asp is prominent in the ZF and ZR (ZR is not shown in this figure) of the cortex, but almost completely absent from the ZG. Anti-D-Asp antiserum was diluted 1:1500, at which concentration endogenous D-Asp was not stained. Bar, 60 μm. (Inset) Endogenous D-Asp in the adrenal cortex of 3-week-old rat. Localization pattern of exogenous D-Asp is almost identical to that of endogenous D-Asp. Anti-D-Asp antiserum was diluted 1:100. Bar, 120 μm. (B) Adrenal medulla of 8-week-old rat. Exogenous D-Asp is evident in the adrenal medulla, not in the ZF and ZR of the cortex. Anti-D-Asp antiserum was diluted 1:300. Bar, 120 μm. (Inset) Endogenous D-Asp in the adrenal medulla of 8-week-old rat. Localization of exogenous D-Asp is similar to that of endogenous D-Asp. Anti-D-Asp antiserum was diluted 1:50. Bar, 60 μm. (C) (a) Immunolocalization of exogenous D-Asp in the adrenal medulla of 8-week-old rat. (b) Fluorescent photomicrograph of the section shown in (a) Noradrenaline (NA)-storing cells of the medulla are stained and adrenaline (A)-storing cells are not. The cells that are stained in b (NA cells, white arrows) are negative for exogenous D-Asp (arrows in a). (D) Anterior lobe of the pituitary gland of 8-week-old rat. Exogenous D-Asp is primarily evident in the endothelial cells of the blood vessels. Anti-D-Asp antiserum was diluted 1:1000. Bar, 60 μm. (Inset) Endogenous D-Asp in the anterior lobe of the pituitary gland of 8-week-old rat. Anti-D-Asp antiserum was diluted 1:300. Bar, 60 μm. Endogenous D-Asp is present in PRL-containing cells or some other very closely related type of cells (22). The cells positive for exogenous D-Asp differ in both morphology and distribution from those positive for endogenous D-Asp.

endogenous D-Asp levels in the adrenal and pituitary glands were 0.56 $\pm$ 0.076 and 0.49 $\pm$ 0.092 nmol/gland, respectively. The localization of exogenous D-Asp was examined in the adrenal gland of 3- and 8-week-old rats, and in the pituitary gland of 8-week-old rats. The endogenous D-Asp concentration in rat adrenal glands shows a transient increase at 3 weeks of age, and markedly decreases thereafter, remaining at an adult level after 8 weeks of age (17). In contrast, the level in the pituitary gland continues to increase gradually from 1 to 8 weeks of age (17).

When D-Asp was administered intraperitoneally to 3-week-old rats, immunoreactivity (IR) to exogenous D-Asp in the adrenal gland appeared primarily in the cytoplasm of cells in the zona fasciculata (ZF) and zona reticularis (ZR) of the cortex, but was almost undetectable in the zona glomerulosa (ZG) (Fig. 1A). In the adrenal medulla, exogenous D-Asp IR was detected in scattered, irregularly shaped groups of cells (data not shown). This localization of exogenous D-Asp is almost identical to the localization of endogenous D-Asp in the adrenal cortex (Fig. 1A, inset) and in the adrenal medulla (19).

At 8 weeks of age, IR to exogenous D-Asp was intense in large clusters of cells in the adrenal medulla, but was less intense in the ZF and ZR of the cortex (Fig.

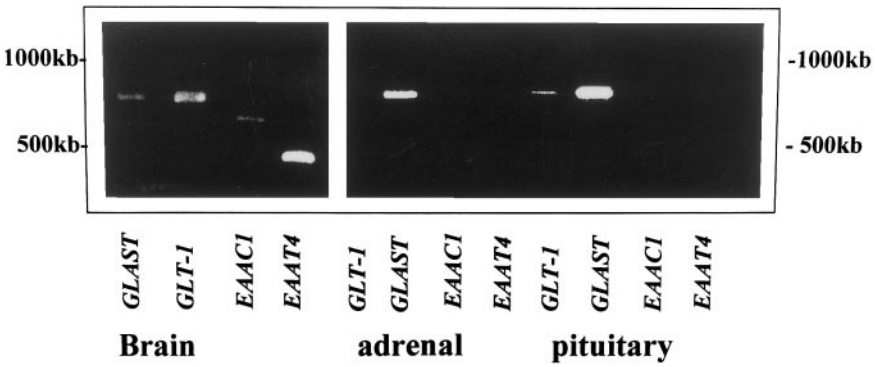

FIG. 2. RT-PCR of Glu transporter subtypes in the rat adrenal and pituitary glands. Total RNA (1 μg) extracted from the brain and the adrenal and pituitary glands of 8-week-old rats was transcribed into cDNA, followed by PCR amplification with primer pairs specific for GLT-1, GLAST, EAAC1, and EAAT4. The amplified products were resolved on 1.2% agarose gels and stained with ethidium bromide.

1B). This staining pattern is similar to that of endogenous D-Asp (Fig. 1B, inset) (19). The adrenal medulla comprises NA-storing and A-storing cells, and IR to exogenous D-Asp was evident in A cells (Fig. 1C), while in some sections the IR was also associated with NA cells (data not shown). Endogenous D-Asp is specifically localized to the cytoplasm of A cells in the adrenal medulla (9, 19). In the rat adrenal medulla, D-Asp is presumably acquired by local synthesis in addition to uptake, since it is produced in a clonal strain of rat pheochromocytoma (PC12) cells, which is derived from the rat adrenal medulla (8). The selective localization of D-Asp to A cells is assumed to be due to D-Asp oxidase activity, since D-Asp oxidase activity is localized in the medulla and selectively associated with NA cells (9).

In the anterior lobe of the pituitary gland of 8-week-old rats, IR to exogenous D-Asp was primarily evident in the endothelial cells of the blood vessels (Fig. 1D), whereas endogenous D-Asp was detected in PRL-containing cells or some other very closely related cell type (Fig. 1D, inset) (22). In contrast to the adrenal gland, exogenous D-Asp in the pituitary gland was mostly incorporated into different cells from those which contained endogenous D-Asp. Moreover, D-Asp oxidase is exclusively localized in the intermediate lobe and not detected in the anterior lobe (9).

Developmental Changes in Glu Transporter Expression in Rat Adrenal and Pituitary Glands

D-Asp is likely to be taken up into cells by the L-Glu transporter, which has an affinity for D-Asp in addition to L-Glu and L-Asp (26–28). We therefore examined developmental changes in the expression of the Glu transporter in the adrenal and pituitary glands and compared the results with developmental changes in D-Asp concentrations in the same tissues. RT-PCR demonstrated that GLAST is the predominant isoform in the rat adrenal and pituitary glands, while GLT-1 is also detected in the pituitary at a very low level (Fig. 2). In the adrenal gland, steady-state levels of GLAST mRNA were transiently increased at 3 weeks of age (Fig. 3A), consistent with the transient increase in adrenal D-Asp content at the same age. In contrast, in the pituitary, GLAST mRNA levels remained almost constant from 1 to 13 weeks of age (Fig. 3B). This result is in marked contrast to developmental changes in pituitary D-Asp concentration, which continues to increase gradually from 1 to 8 weeks of age (17). GLT-1 mRNA was not detected in the pituitary by Northern blot, presumably due to its low level of expression (data not shown), although it was detected at a very low level by RT-PCR.

Western blotting was also used to detect GLAST in the rat adrenal and pituitary glands (Figs. 3C and 3D). The level of GLAST protein in the adrenal gland increased significantly at 3 weeks of age, and several forms of different molecular mass (glycosylated monomer, dimer, and glycosylated dimer) (35) were readily discernible at that age (Fig. 3C). This result is comparable with the transient increase of adrenal D-Asp content at 3 weeks of age. In the pituitary, GLAST protein levels were almost constant during development (Fig. 3D); this is consistent with the mRNA levels described above and contrasts with the gradual increase in D-Asp concentration in the pituitary.

Localization of Glu Transporter in the Rat Adrenal and Pituitary Glands

Figure 4A shows the spatial distribution of GLAST protein in the rat adrenal gland. In 3-week-old rats, GLAST was localized in the ZF and ZR of the adrenal cortex, but was almost undetectable in the ZG (Fig. 4A). This result is consistent with the observation described above, that exogenous and endogenous D-Asp are both localized primarily in the ZF and ZR at 3 weeks of age. In the adrenal medulla of 8-week-old

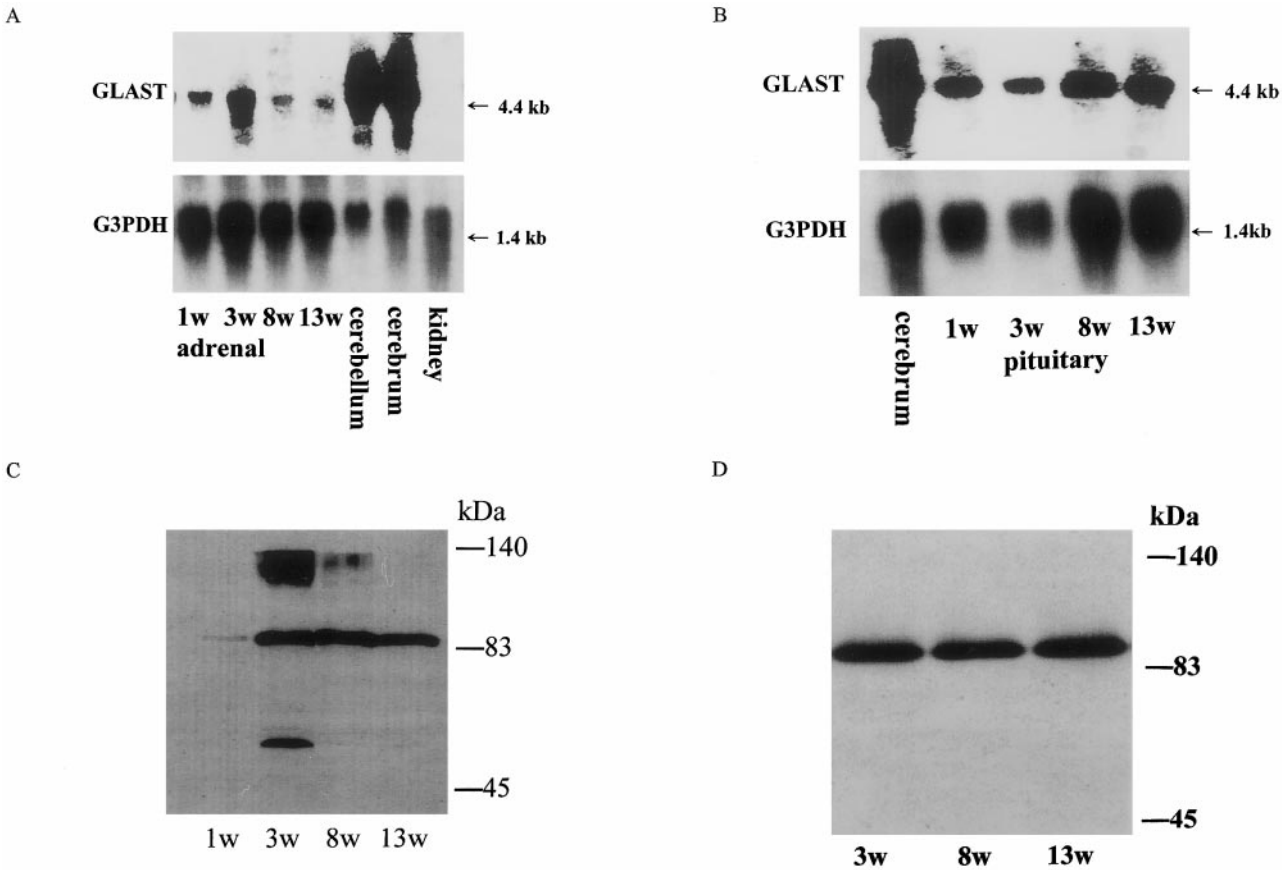

FIG. 3. Developmental changes of Glu transporter expression in the rat adrenal and pituitary glands. (A, B) Northern blot analysis of GLAST mRNA in the rat adrenal (A) and pituitary (B) glands at various ages (1, 3, 8, and 13 weeks). Analysis of mRNA from the cerebellum, cerebrum, and kidney was also carried out to provide positive and negative controls, and the blot was reprobed with G3PDH cDNA as a loading control. (C, D) Western blot analysis of GLAST in the rat adrenal (C) and pituitary (D) glands at various ages (1, 3, 8, and 13 weeks).

rats, IR to GLAST was localized not only in A cells but also in NA cells (data not shown). Endogenous D-Asp is specific to A cells in the adrenal medulla, and this selective localization is presumably due to D-Asp oxidase activity, since the oxidase activity is selectively associated with NA cells (9).

Double staining with anti-GLAST and anti-PRL antibodies was used to examine whether GLAST is associated with PRL-containing cells, which also contain D-Asp, in the pituitary gland of 8-week-old rats (Fig. 4B). PRL-positive cells (red) were only rarely superimposed (yellow) on GLAST-positive cells (green), suggesting that PRL-containing cells do not contain the Glu transporter.

DISCUSSION

In a previous report we demonstrated that, in the course of primary culture of parenchymal cells from the rat pineal gland, D-Asp was not synthesized but was efficiently taken up by the cells (1). Given that the pineal gland contains a large amount of D-Asp *in vivo*, this result suggests that D-Asp in the pineal gland is derived from other tissue(s) and acquired by cells of the pineal gland from the vascular system, although it does not rule out loss of D-Asp synthesis activity during the primary culture of the cells. A recent report indicated that a high concentration of D-Asp is found in rat testicular venous blood plasma, suggesting that D-Asp is produced in the testis and secreted into the venous blood (36). These lines of evidence indicate that D-Asp may be produced in certain specific mammalian tissue(s), and subsequently accumulated in other tissues by uptake from the vascular system. In the present study, we examined the origins of D-Asp in the rat adrenal and pituitary glands.

D-Asp concentration in the rat adrenal gland increases transiently at 3 weeks of age (17). It had been presumed that this result was brought about by transient increase of D-Asp synthesis at 3 weeks of age in the gland. Here we present evidence that suggests that this endogenous D-Asp in the adrenal cortex of 3-week-old rats is primarily acquired by uptake from the vas-

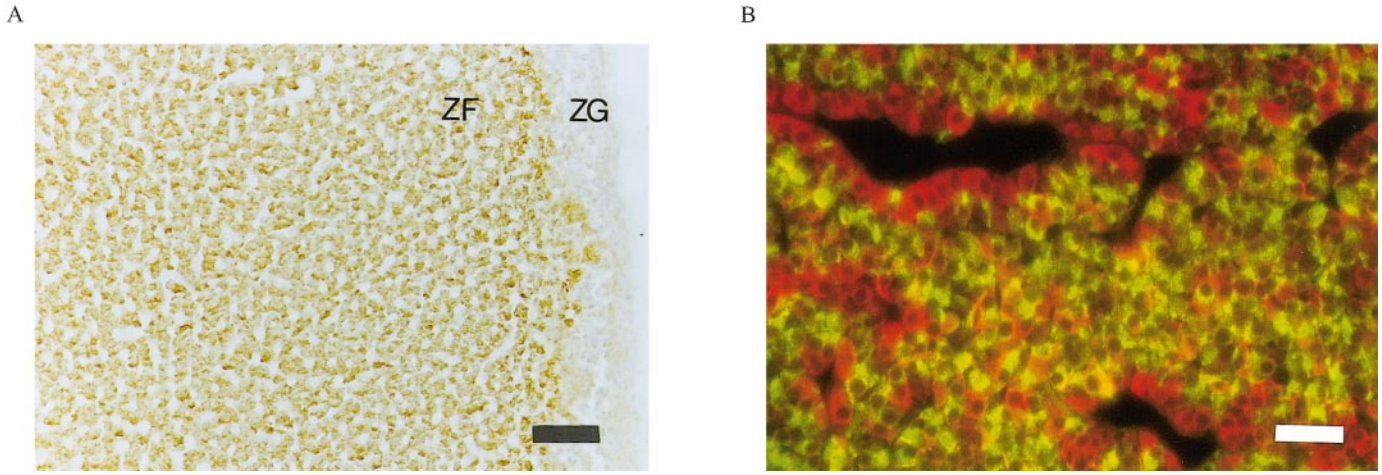

FIG. 4. Localization of Glu transporter in the rat adrenal and pituitary glands. (A) Immunolocalization of GLAST in the adrenal cortex of a 3-week-old rat. GLAST reactivity is predominantly present in the ZF and ZR (ZR is not shown in this figure), and weak in the ZG. Bar, 120 μm. This GLAST localization pattern is almost identical to those of both endogenous and exogenous D-Asp. (B) Double staining of GLAST and PRL in the anterior lobe of the pituitary gland of an 8-week-old rat. The lobe was probed with anti-PRL antiserum (red) and anti-GLAST antiserum (green). PRL-positive cells are only rarely superimposed (yellow) on GLAST-positive cells, suggesting that PRL-containing cells do not contain the Glu transporter. Bar, 60 μm.

cular system. However, the primary tissue(s) in which D-Asp synthesis takes place is not clear at present. D-Asp in the adrenal medulla of 8-week-old rats is probably metabolized by D-Asp oxidase following uptake from the vascular system or local synthesis, as described under Results.

In contrast, our observations suggest that most of the D-Asp in the pituitary gland originates inside the tissue, although they do not rule out the possibility that other type(s) of Glu transporter are present in the pituitary gland, which has an affinity for D-Asp but has not been cloned yet. At present, four different types of Glu transporter (EAAT1(GLAST), EAAT2(GLT-1), EAAT3(EAAC1), and EAAT4) have been characterized, while another retina-specific isoform with a low affinity for D-Asp (EAAT5) is also recognized (37).

Recently we found that D-Asp is actually synthesized in a PRL-secreting clonal strain of rat pituitary tumor cells (GH3) and that it promotes thyrotropin-releasing hormone (TRH)-induced PRL secretion from the cells (38). Thus, D-Asp in the rat anterior pituitary gland appears to act as an autacoid in an autocrine or paracrine fashion, whereas D-Asp in the rat adrenal gland might be a messenger that acts in an endocrine fashion, thereby stimulating steroid production (6, 7).

D-Asp is also found in other rat tissues, including the cerebrum, cerebellum, and retina of newborn rats and the spleen and thymus of adult rats, and is present in a variety of mouse and human tissues (39, 40). The localization of D-Asp within these tissues has not been examined. Furthermore, the origin of the endogenous D-Asp in these tissues is totally unknown. Identification and cloning of the enzyme(s) responsible for the synthesis of D-Asp would greatly advance the current understanding of the role and regulation of D-Asp in the mammalian body.

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REFERENCES

1. Takigawa, Y., Homma, H., Lee, J.-A., Fukushima, T., Santa, T., Iwatsubo, T., and Imai, K. (1998) *Biochem. Biophys. Res. Commun.* **248,** 641–647.
2. Ishio, S., Yamada, H., Hayashi, M., Yatsushiro, S., Noumi, T., Yamaguchi, A., and Moriyama, Y. (1998) *Neurosci. Lett.* **249,** 143–146.
3. Govitrapong, P., and Ebadi, M. (1988) *Neurochem. Int.* **13,** 223–230.
4. Sato, K., Kiyama, H., Shimada, S., and Tohyama, M. (1993) *Neuroendocrinology* **58,** 77–79.
5. Yamada, H., Yatsushiro, S., Ishio, S., Hayashi, M., Nishi, T., Yamamoto, A., Futai, M., Yamaguchi, A., and Moriyama, Y. (1998) *J. Neurosci.* **18,** 2056–2062.
6. Nagata, Y., Homma, H., Lee, J.-A., and Imai, K. (1999) *FEBS Lett.* **444,** 160–164.
7. Nagata, Y., Homma, H., Matsumoto, M., and Imai, K. (1999) *FEBS Lett.* **454,** 317–320.
8. Long, Z., Homma, H., Lee, J.-A., Fukushima, T., Santa, T., Iwatsubo, T., Yamada, R., and Imai, K. (1998) *FEBS Lett.* **434,** 231–235.
9. Schell, M. J., Cooper, O. B., and Snyder, S. H. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 2013–2018.

10. Wolosker, H., Sheth, K. N., Takahashi, M., Mothet, J.-P., Brady J., R. O., Ferris, C. D., and Snyder, S. H. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 721–725.
11. Wolosker, H., Blackshaw, S., and Snyder, S. H. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 13409–13414.
12. D'Aniello, A., Di Cosmo, A., Di Cristo, C., Annunziato, L., Petrucelli, L., and Fisher, G. (1996) *Life Sci.* **59,** 97–104.
13. Imai, K., Fukushima, T., Santa, T., Homma, H., Suguhara, J., Kodama, H., and Yoshikawa, M. (1997) *Proc. Jn Acad.* **73**B, 48–52.
14. Hashimoto, A., Kumashiro, S., Nishikawa, T., Oka, T., Takahashi, K., Mito, T., Takashima, S., Doi, N., Mizutani, Y., Yamazaki, T., Kaneko, T., and Ootomo, E. (1993) *J. Neurochem.* **61,** 348–351.
15. Dunlop, D., S., Neidle, A., McHale, D., Dunlop D., M., and Lajtha, A. (1986) *Biochem. Biophys. Res. Commun.* **141,** 27–32.
16. Neidle, A., and Dunlop, D. S. (1990) *Life Sci.* **46,** 1517–1522.
17. Hashimoto, A., Oka, T., and Nishikawa, T. (1995) *Eur. J. Neurosci.* **7,** 1657–1663.
18. Lee, J., Homma, H., Sakai, K., Fukushima, T., Santa, T., Tashiro, K., Iwatsubo, T., Yoshikawa, M., and Imai, K. (1997) *Biochem. Biophys. Res. Commun.* **231,** 505–508.
19. Sakai, K., Homma, H., Lee, J.-A., Fukushima, T., Santa, T., Tashiro, K., Iwatsubo, T., and Imai, K. (1997) *Biochem. Biophys. Res. Commun.* **235,** 433–436.
20. Sakai, K., Homma, H., Lee, J.-A., Fukushima, T., Santa, T., Tashiro, K., Iwatsubo, T., and Imai, K. (1998) *Arch. Biochem. Biophys.* **351,** 96–105.
21. Sakai, K., Homma, H., Lee, J.-A., Fukushima, T., Santa, T., Tashiro, K., Iwatsubo, T., and Imai, K. (1998) *Brain Res.* **808,** 65–71.
22. Lee, J., Homma, H., Tashiro, K., Iwatsubo, T., and Imai, K. (1999) *Brain Res.* **838,** 193–199.
23. D'Aniello, A., D'Onofrio, G., Pischetola, M., D'Aniello, G., Vetere, A., Petrucelli, L., and Fisher, G. H. (1993) *J. Biol. Chem.* **268,** 26941–26949.
24. Nagasaki, H. (1994) *Int. J. Biochem.* **26,** 415–423.
25. Kera, Y., Aoyama, H., Matsumura, H., Hasegawa, A., Nagasaki, H., and Yamada, R.-H. (1995) *Biochim. Biophys. Acta* **1243,** 282–286.
26. Balcar, V. J., and Johnston, G. A. R. (1972) *J. Neurochem.* **19,** 2657–2666.
27. Christensen, H., Greene, A. A., Kakuda, D. K., and MacLeod, C. L. (1994) *J. Exp. Biol.* **196,** 297–305.
28. Cooper, B., Chebib, M., Shen, J., King, N. J. C., Darvey, I. G., Kuchel, P. W., Rothstein, J. D., and Balcar, V. J. (1998) *Arch. Biochem. Biophys.* **353,** 356–364.
29. Hamase, K., Homma, H., Takigawa, Y., Fukushima, T., Santa, T., and Imai, K. (1997) *Biochim. Biophys. Acta* **1334,** 214–222.
30. Pines, G., Danbolt, N. C., Bjørås, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathisen, J., Seeberg, E., and Kanner, B. I. (1992) *Nature* **360,** 464–467.
31. Storck, T., Schulte, S., Hofmann, K., and Stoffel, W. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10955–10959.
32. Kanai, Y., and Hediger, M. (1992) *Nature* **360,** 467–471.
33. Fairman, W. A., Vandenberg, R. J., Arriza, J. L., Kavanaugh, M. P., and Amara, S. G. (1995) *Nature* **375,** 599–603.
34. Yamada, H., Yatsushiro, S., Yamamoto, A., Hayashi, M., Nishi, T., Futai, M., Yamaguchi, A., and Moriyama, Y. (1997) *J. Neurochem.* **69,** 1491–1498.
35. Furuta, A., Rothstein, J. D., and Martin, L. J. (1997) *J. Neurosci.* **17,** 8363–8375.
36. D'Aniello, A., Di Fiore, M. M., D'Aniello, G., Colin, F. E., Lewis, G., and Setchell, B. P. (1998) *FEBS Lett.* **436,** 23–27.
37. Arriza, J. L., Eliasof, S., Kavanaugh, M. P., and Amara, S. G. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4155–4160.
38. Long, Z., Lee, J.-A., Okamoto, T., Nimura, N., Imai, K., and Homma, H. (2000) *Biochem. Biophys. Res. Commun.*, **276,** 1143–1147, doi:10.1006/bbrc.2000.3573.
39. Imai, K., Fukushima, T., Santa, T., Homma, H., Hamase, K., Sakai, K., and Kato, M. (1996) *Biomed. Chromatogr.* **10,** 303–312.
40. Hashimoto, A., and Oka, T. (1997) *Prog. Neurobiol.* **52,** 325–353.