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Review

D-Aspartate—An important bioactive substance in mammals: A review from an analytical and biological point of view☆

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ABSTRACT

It was long believed that D-amino acids were either unnatural isomers or laboratorial artifacts and that the important functions of amino acids were exerted only by L-amino acids. However, recent investigations have shown that a variety of D-amino acids are present in mammals and that they play important roles in physiological functions in the body. Among the free D-amino acids that have been identified in mammals, D-aspartate (D-Asp) has been shown to play a crucial role in the neuroendocrine and endocrine systems as well as in the central nervous system. Here, we present an overview of recent studies of free D-Asp, focusing on the analytical methods in real biological matrices, expression and localization in tissues and cells, biological and physiological activities, biosynthesis, degradation, cellular transport, and possible relevance to disease. In addition to frequently used techniques for the enantiomeric determination of amino acids, including high-performance liquid chromatography and enzymatic methods, the recent development of analytical methods is also described.

Contents

1. Introduction

All proteinogenic amino acids, with the exception of glycine, contain asymmetric α-carbon atoms and can exist as one of two stereoisomers: L-forms and D-forms. In the past, D-amino acids were regarded as either unnatural isomers or laboratory artifacts, despite their discovery in lower animals, such as bacteria and insects. Consequently, it was believed that D-amino acids did not play a significant role in the physiology of most organisms. However, recent advances in analytical methods for separating chiral amino acids have revealed that living organisms contain several D-amino acids, either free or as components of proteins. In particular, free D-serine (D-Ser) and D-aspartate (D-Asp) have been identified in a wide variety of mammalian tissues and cells at relatively high

Abbreviations: Boc-L-Cys, *N*-*tert*-butyloxycarbonyl-L-cysteine; BTCC, *N*-(*tert*-butylthiocarbamoyl)-L-cysteine ethyl ester; *D*%, proportion of D-amino acid in total amino acid [D-form/(D-form + L-form) × 100%]; DAO, D-amino acid oxidase; DDO, D-aspartate oxidase; FAD, flavin adenine dinucleotide; fEPSP, field excitatory post-synaptic potential; hCG, human chorionic gonadotropin; HPLC, high-performance liquid chromatography; LTD, long-term depression; LTP, long-term potentiation; α-MSH, α-melanocyte-stimulating hormone; NAC, *N*-acetyl-L-cysteine; NBD-F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; NMDA, *N*-methyl-D-aspartate; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; ODS, octadecylsilyl silica gel; OPA, *o*-phthalaldehyde; PLP, pyridoxal phosphate; POMC, proopiomelanocortin; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; StAR, steroidogenic acute regulatory protein; VSOC, volume-sensitive organic anion channel.

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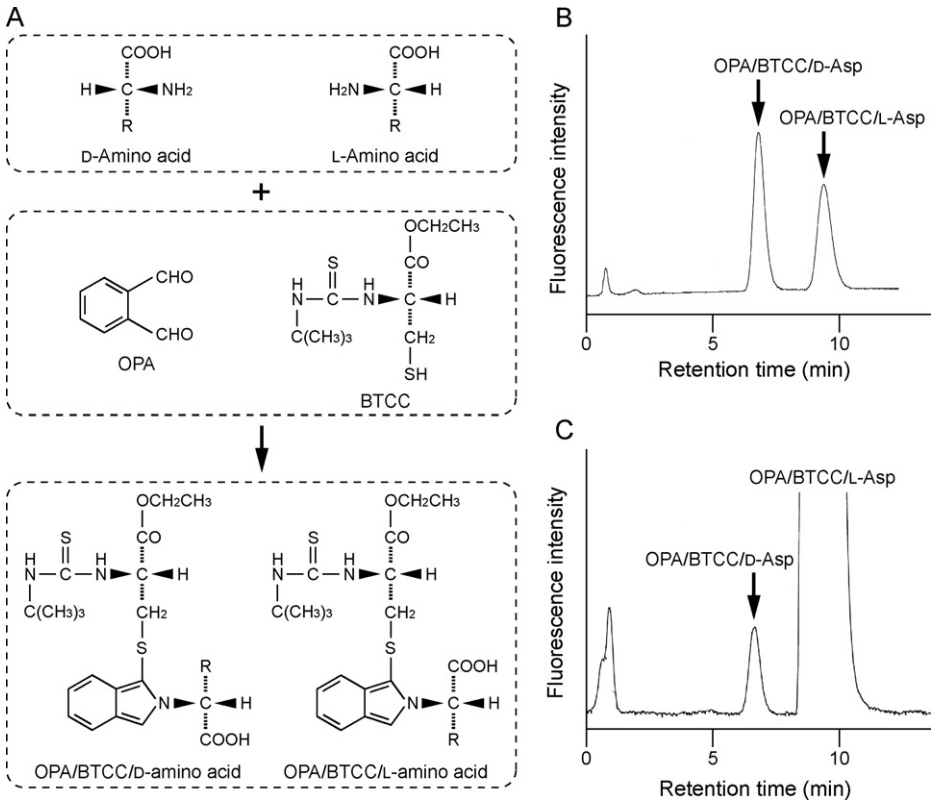

Fig. 1. Enantiomeric separation and determination of amino acids by conventional reversed-phase HPLC following fluorogenic derivatization with OPA and BTCC. (A) Fluorogenic derivatization of amino acids with OPA and BTCC is shown schematically. (B) Chromatogram of the derivatives formed from standard D- and L-Asp and OPA/BTCC. OPA/BTCC-labeled D- and L-Asp were separated at a flow rate of 0.5 mL/min on an ODS column of Mightysil RP-18 GP [75 mm × 3.0 mm I.D. (5 μm), Kanto Kagaku, Tokyo, Japan] maintained at 20 °C with a mobile phase of 50 mM sodium phosphate buffer (pH 6.8):methanol:acetonitrile (70:10:20). The fluorescence was detected at an excitation wavelength of 335 nm and an emission wavelength of 420 nm. The amount of 125 pmol each of the derivatives was injected. (C) Chromatogram of the reaction mixture of Asp racemase from archaebacteria *Thermoplasma acidophilum*. The analytical conditions were the same as in (B). A small amount of D-Asp enzymatically formed from L-Asp was sensitively detected, even in the presence of a large amount of L-Asp, the substrate. The sample included 18 pmol and approx. 5.9 nmol of OPA/BTCC-labeled D- and L-Asp, respectively. For details, see text and [24].
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concentrations [1–11]. D-Ser has been proposed to be a neuromodulator that binds to the glycine-binding site of the *N*-methyl-D-Asp (NMDA) receptor, a subtype of the L-glutamate (L-Glu) receptor, and potentiates glutamatergic neurotransmission in the central nervous system. Details of the findings on D-Ser have been reviewed extensively in a number of excellent articles [12–14]. In the present article, we review recent advances in studies on free D-Asp as well as analytical methods for the enantiomeric determination of amino acids.

2. Analytical methods for the determination of D-amino acids

Chromatographic and electrophoretic separations of amino acids are effective methods for determining D-amino acids in biological samples [15], and enzymes that are stereospecific for D-amino acids are also frequently used. In this section, we briefly describe the techniques routinely used for the determination of D-Asp in biological matrices, including high-performance liquid chromatography (HPLC) and enzymatic methods. Other methodologies for the enantiomeric determination of amino acids, such as those that utilize gas chromatography, have been reviewed elsewhere [16–18].

HPLC is the most widely used technique for the determination of D-amino acids in biological samples. Two methods are primarily used for the enantiomeric separation of amino acids by HPLC: (1) diastereomeric derivatization of amino acids followed by separation on non-chiral stationary phases, and (2) direct enantiomeric separation on chiral stationary phases. We have used both of these methods to analyze D-amino acids in mammalian tissues and cells. A method for the enantiomeric separation of amino acids on non-chiral stationary phases with chiral mobile phases has also been developed; however, there are a few reports on the application of this method for the determination of D-amino acids in biological samples [19,20].

For diastereomeric derivatization of amino acids followed by the separation on non-chiral stationary phases, amino acids are first derivatized to pairs of diastereomers with *o*-phthalaldehyde (OPA) and an optically active thiol reagent, most often *N*-acetyl-L-cysteine (NAC) or *N*-*tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys). Subsequently, the diastereomers are separated on non-chiral stationary phases, such as an octadecylsilyl silica gel (ODS) column. Since the diastereomers produced by OPA/NAC or OPA/Boc-L-Cys derivatization are fluorescent, they can be detected by conventional reversed-phase HPLC and a fluorometric detector [21–23]. Our laboratory synthesized a novel chiral thiol compound, *N*-(*tert*-butylthiocarbamoyl)-L-cysteine ethyl ester (BTCC), which improved the enantiomeric separation of amino acids by HPLC, particularly acidic amino acids [24]. Similar to NAC and Boc-L-Cys reagents, BTCC reacts with amino acids in combination with OPA to produce fluorescent diastereomers that can be detected with high sensitivity (Fig. 1A and B). Because the OPA/BTCC derivatization reaction proceeds rapidly at room temperature, a fully-automated system has been established for derivatization and sample injec-

tion. This automated method is practical and has been successfully applied to assays of archaebacterial Asp-specific amino acid racemase activity (Fig. 1C) [24].

For direct enantiomeric separation on chiral stationary phases, Pirkle-type enantioselective column are used. These columns retain enantiomeric amino acid derivatives as the chiral center of the ligands, which are immobilized on silica-based stationary phases [25]. First, the amino acids are fluorescently derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) (Fig. 2A) and then subjected to HPLC. Due to their differing affinity for the chiral stationary phases, NBD-labeled D- and L-amino acids can be effectively separated. However, it is difficult to accurately determine small amounts of D-amino acids in mammalian tissues and cells, since large amounts of matrix substances, including L-amino acids in biological samples, obscure the peak of the target D-amino acids. To overcome this problem, two-dimensional HPLC is employed [26]. In this method, NBD-labeled amino acids are first separated by reversed-phase HPLC with an octylsilyl silica gel column, and the fractions that contain both D- and L-forms of each amino acid are isolated (Fig. 2B). Subsequently, the enantiomers in the isolated fractions are separated and determined by a second round of HPLC with the Pirkle-type enantioselective column. Our laboratory has developed a reliable and efficient HPLC system in which the two chromatographic steps are linked by automated column-switching. This method enabled us to accurately determine small amounts of D-Asp in several biological samples (Fig. 2C) [26].

Recently, a capillary liquid chromatography system coupled with tandem mass spectrometry has been reported for the determination of D-amino acids [27]. In this method, amino acids are first derivatized with NBD-F, after which the NBD-labeled amino acids are trapped on an ODS extraction microcolumn. Next, the amino acids are enantiomerically separated on a chiral stationary phase that is comprised of teicoplanin aglycon-bonded silica gel particles, followed by detection with tandem mass spectrometry. With the use of a stable isotope-labeled amino acid as an internal standard, sensitive and reliable measurements of D-amino acids in biological samples have been achieved [27,28]. In addition, a capillary electrophoresis system with laser-induced fluorescence detection for the determination of D-amino acids has also been described [29]. In this method, amino acids are fluorescently derivatized with naphthalene-2,3-dicarboxaldehyde in the presence of KCN, after which the derivatized amino acids are enantiomerically separated by capillary electrophoresis with a separation buffer containing β-cyclodextrin and sodium dodecyl sulfate. Using an argon ion laser as the light source, the fluorescently derivatized amino acids can be detected with high sensitivity. In fact, D-Asp in the process and soma of a single sea slug neuron (*Aplysia californica*) was detected [29].

For the enzymatic determination of D-amino acids, D-amino acid oxidase (DAO, also abbreviated DAAO; EC 1.4.3.3) or D-Asp oxidase (DDO, also abbreviated DASPO; EC 1.4.3.1) are the most frequently used enzymes [15]. DAO and DDO are flavin adenine dinucleotide (FAD)-containing flavoproteins that catalyze the oxidative deamination of D-amino acids to generate the corresponding 2-oxo acids, as well as hydrogen peroxide and ammonia (Fig. 3A) [30,31]. These two enzymes are stereospecific, and neither DAO nor DDO acts on L-amino acids. DAO displays broad substrate specificity and recognizes several neutral and basic D-amino acids [32]. In contrast, DDO is highly specific for acidic D-amino acids, such as D-Asp, NMDA and D-Glu, none of which are substrates for DAO. Enzymatic methods using DAO or DDO are simple techniques by which to determine D-amino acid content, since the enzymatic reaction with substrate D-amino acid(s) can easily be followed using several assay methods. For example, 2-oxo acids produced in the reaction are conjugated to 2,4-dinitrophenylhydrazine, and the resultant hydrazone derivatives can be measured spectrophotometrically (Fig. 3B). Alternatively, hydrogen peroxide, another reaction product, can be measured spectrophotometrically through the chromogenic reaction of certain reductants with peroxidase and hydrogen peroxide. Furthermore, an oxygen consumption assay and an amperometric biosensor assay have also been developed [15]. Notably, these methods can determine several D-amino

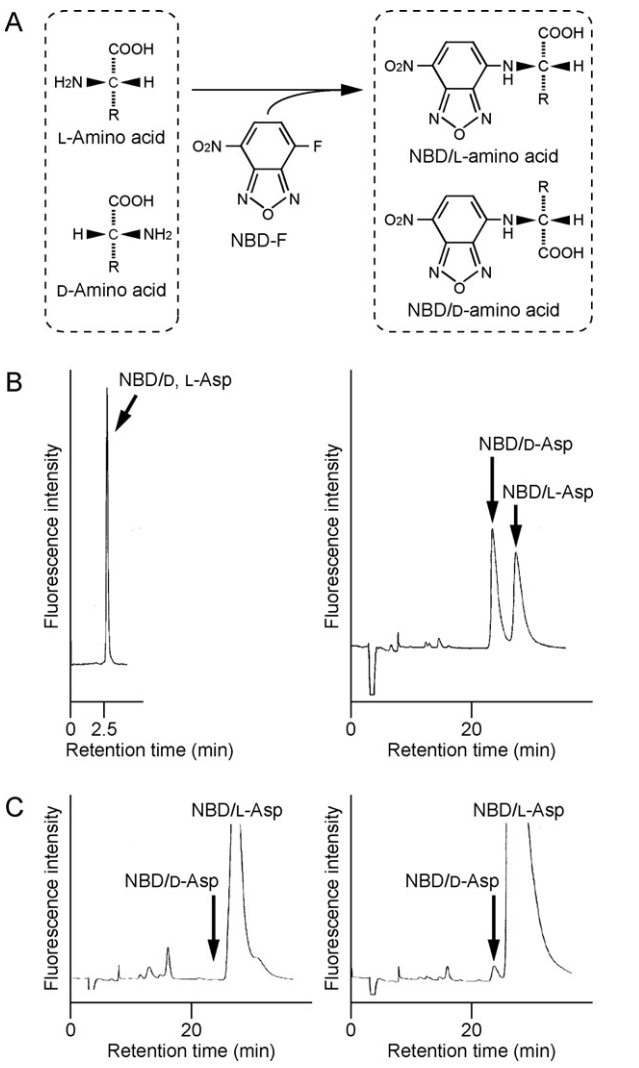

Fig. 2. Enantiomeric separation and determination of amino acids by two-dimensional HPLC following fluorogenic derivatization with NBD-F. (A) Fluorogenic derivatization of amino acids with NBD-F is shown schematically. (B) Chromatograms of the derivatives formed from standard D- and L-Asp and NBD-F. The fraction containing the NBD-labeled D- and L-Asp was first isolated by reversed-phase HPLC with an octylsilyl silica gel column of LiChroCART Superspher RP-8 [125 mm × 4.0 mm I.D. (5 μm), Merck, Darmstadt, Germany] (left). The column was maintained at 40 °C and eluted at 1 mL/min, with a mobile phase of 50 mM sodium acetate buffer (pH 5.1). Subsequently, the derivatives in the isolated fraction were enantiomerically separated by a second round of HPLC with a Pirkle-type enantioselective column of Sumichiral OA-3100 [250 mm × 4.6 mm I.D. (5 μm), Sumika Chemical Analysis Service, Osaka, Japan] (right). The column was maintained at 40 °C and eluted at 1 mL/min, with a mobile phase of 7 mM citric acid in methanol. The fluorescence was detected at an excitation wavelength of 470 nm and an emission wavelength of 530 nm. The amount of 500 fmol each of the derivatives was injected. (C) Chromatograms of cell culturing media. The analytical conditions were the same as in (B). The D-Asp level in the media in which rat pheochromocytoma MPT1 cells were cultured for 1 day was below the detection limit, but approx. 4.7 μM of L-Asp could be detected (left). In contrast, when the MPT1 cells were treated with L-cysteine sulfinic acid, an inhibitor of the L-Glu transporters that have an affinity for D-Asp as well as for L-Glu and L-Asp, the levels of D- and L-Asp in the culture media were observed to be increased to approx. 220 nM and 26.3 μM, respectively (right). For details, see text and [26].
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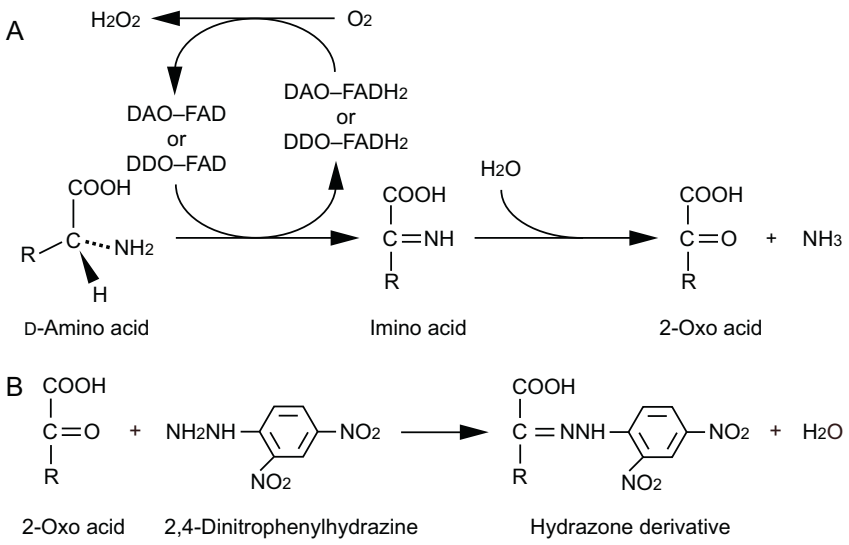

Fig. 3. Determination of D-amino acids by stereospecific enzymes. (A) Reactions catalyzed by DAO or DDO are shown schematically. DAO and DDO catalyze the dehydrogenation of a D-amino acid to generate the corresponding imino acid, coupled with the reduction of FAD. FAD reoxidizes spontaneously in the presence of oxygen, producing hydrogen peroxide, while the imino acid is nonenzymatically hydrolyzed to the 2-oxo acid and ammonia. (B) Derivatization of the 2-oxo acid with 2,4-dinitrophenylhydrazine is shown schematically. The resultant hydrazone derivatives can be measured spectrophotometrically.

acids in many samples at once, although it is difficult to measure the level of each individual D-amino acid in complex samples without prior separation.

3. Expression and localization of D-Asp in mammalian tissues and cells

Through the use of several different methods, including HPLC analysis, immunohistochemical staining with anti-D-Asp antibody, and reactivity of DDO (D-Asp-specific oxidase), expression and localization of D-Asp in mammalian tissues and cells have been studied. In 1986, Dunlop et al. first reported the existence of free D-Asp in mammals, including humans [2]. Since then, substantial amounts of D-Asp have been found in a wide variety of mammalian tissues, particularly the central nervous, neuroendocrine, and endocrine systems. Alterations in D-Asp levels during development and localization of D-Asp in these tissues have been investigated in detail. In several regions of the brain, concentrations of D-Asp are elevated during early development. For instance, D-Asp levels in the rat cerebrum are relatively high just after birth (approx. 140 nmol/g wet weight), and the proportion of D-Asp [*D*%: $D/(D+L) \times 100\%$] is approx. 11% (Fig. 4A) [7]. The D-Asp content then rapidly decreases to trace levels by 3 weeks of age. Similarly, high concentrations of D-Asp (approx. 70 nmol/g wet weight) are present in the rat cerebellum just after birth, and gradually decrease thereafter (Fig. 4B) [7]. This pattern has also been observed in the human prefrontal cortex, where remarkably high concentrations of D-Asp (approx. 0.36 μmol/g wet weight) are present at 14 weeks gestation (Fig. 4C) [4]. This content exceeds that of L-Asp (approx. 0.21 μmol/g wet weight), with *D*% reaching more than 60%, and then rapidly decreases to trace levels at birth and remains low thereafter.

Immunohistochemical analysis of the rat embryonic brain with a specific anti-D-Asp antibody revealed that D-Asp is initially expressed in the hindbrain, after which it spreads into the forebrain and then throughout the entire brain [33]. Within the nerve cells, D-Asp first occurs in the cell bodies of neuroblasts in the outer layer of the neuronal epithelium and then appears in the processes of the cells. Once a distinct axonal layer has been established, D-Asp is localized exclusively to the axons of the cells. Thus, the intracellular localization of D-Asp in nerve cells changes during cell

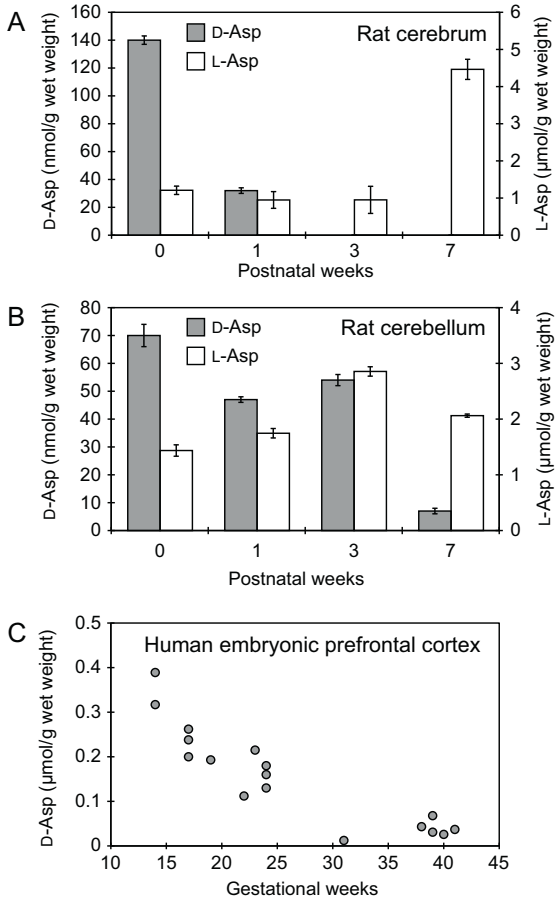

Fig. 4. Alterations in D-Asp levels in the mammalian brain during early development. (A) D-Asp (grey bars) and L-Asp (open bars) levels in rat cerebrum were determined at the indicated ages. The figure was drawn based on data from [7]. For details, see text and [7]. (B) D-Asp (grey bars) and L-Asp (open bars) levels in rat cerebellum were determined at the indicated ages. The figure was drawn based on data from [7]. For details, see text and [7]. (C) D-Asp levels in human prefrontal cortex were determined at the indicated gestational ages. For details, see text and [4].
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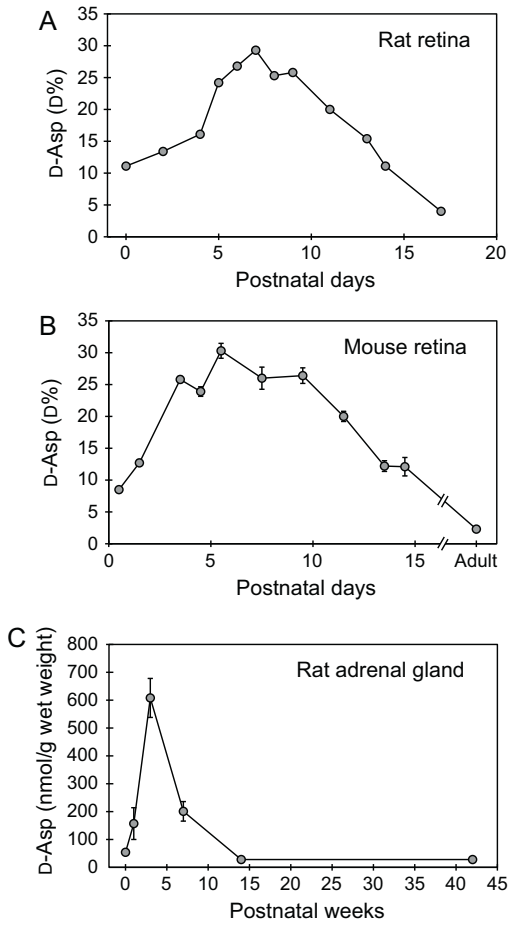

Fig. 5. Alterations in D-Asp levels in the mammalian retina and adrenal gland during development. (A) D-Asp levels in the rat retina were determined at the indicated ages. The vertical axis represents the *D*%. The figure was modified from [11]. For details, see text and [11]. (B) D-Asp levels in the mouse retina were determined at the indicated ages. The vertical axis represents the *D*%. The figure was modified from [35]. For details, see text and [35]. (C) D-Asp levels in the rat adrenal gland were determined at the indicated ages. The figure was drawn based on data from [7]. For details, see text and [7].
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differentiation. Furthermore, in the rat cerebral cortex just after birth, intense D-Asp immunoreactivity is observed in the cortical plate and subventricular zone, which suggests that it plays a major role in regulating neural development [34]. Taken together, these lines of evidence indicate that D-Asp is important in the development and neurogenesis of the brain.

In the rat retina, transient emergence of D-Asp has been observed during the early stages of development. D-Asp levels rapidly increase after birth, and the highest concentrations of D-Asp (approx. 0.35 μmol/g wet weight) are present at seven days, with *D*% reaching approx. 29% (Fig. 5A) [11]. The D-Asp content then rapidly decreases thereafter. Similar ontogenic changes in D-Asp content have also been observed in the mouse retina (Fig. 5B) [35]. Immunohistochemical localization of D-Asp in the rat retina revealed that it is expressed in the innermost ganglion cell and nerve fiber region at three days of age [36]. In contrast, no D-Asp immunoreactivity is evident in the inner plexiform layer or neuroblastic cell layer. At seven days of age, however, intense D-Asp immunoreactivity is observed in the differentiating bipolar cell layer, especially in the innermost zone of this layer, and in the region between the differentiating bipolar cell layer and the photoreceptor cell layer, in addition to the innermost ganglion cell and nerve fiber region. In these regions, nerve cells presumably begin to make contact with each other. These observations suggest that D-Asp is involved in synaptic formation and neuronal survival in the retina.

As in the retina, transient emergence of D-Asp has been observed in the rat adrenal gland. Specifically, D-Asp levels at one week of age are relatively low but increase markedly at three weeks of age, followed by a rapid decline (Fig. 5C) [7]. The D-Asp content at three weeks of age is approx. 600 nmol/g wet weight, with *D*% reaching approx. 46%. In the rat adrenal cortex, D-Asp occurs in different cell types at distinct periods during development. At three weeks of age, D-Asp immunoreactivity is prominent in the cytoplasm of cells in the two inner zones, the zona fasciculate and zona reticularis, but is negligible in the outermost zone, the zona glomerulosa [37]. In contrast, at eight weeks of age, intense D-Asp immunoreactivity is observed in the zona glomerulosa, and less is observed in the zona fasciculate and zona reticularis. Thus, the patterns of D-Asp distribution appear to reverse as development proceeds. The two inner zones (zona fasciculate and zona reticularis) secrete glucocorticoids, while the outermost zone (zona glomerulosa) secretes mineralocorticoids. Moreover, the maturation periods of these regions are different. Therefore, the above-mentioned temporal and site-specific patterns of D-Asp distribution suggest that it is involved in the development and maturation of steroidogenesis in the adrenal cortex.

In the rat adrenal medulla, D-Asp immunoreactivity is observed in the cytoplasm of epinephrine cells, but not in norepinephrine cells [37,38]. Epinephrine cells and norepinephrine cells make and store epinephrine and norepinephrine, respectively, and constitute approx. 80% and 20% of the total number of chromaffin cells in the adrenal medulla. Notably, enzyme histochemical analysis of the rat adrenal medulla revealed that DDO, an enzyme that degrades D-Asp, is present in norepinephrine cells but not in epinephrine cells [38]. Thus, this suggests that the decrease in D-Asp levels in norepinephrine cells is due to the DDO-mediated metabolism of D-Asp. Notably, unlike rat, D-Asp immunoreactivity is undetectable in the mouse adrenal medulla. However, immunohistochemical analysis confirmed that D-Asp is present in the epinephrine cells of DDO-deficient mice, as described below (see Section 6) [39].

In contrast to the developmental changes described above, D-Asp levels in other neuroendocrine and endocrine tissues, such as the pineal gland, pituitary gland and testis, increase as development proceeds (Fig. 6) [3,7,40]. The D-Asp content in the adult rat pineal gland is very high (approx. 3.5 μmol/g wet tissue) with a *D*% of approx. 33% [3], but there are regional differences in D-Asp immunoreactivity. Specifically, intense immunoreactivity is observed in the distal (caudal) region of the gland, while little staining is visible in the proximal (rostral) region [41]. In addition, D-Asp is localized to the cytoplasm of pinealocytes, which constitute approx. 80% of the cells in this gland.

The pituitary gland consists of three different lobes: an anterior lobe, an intermediate lobe, and a posterior lobe. In the pituitary gland of adult rats, D-Asp immunoreactivity is evident in the anterior and posterior lobes, but not in the intermediate lobe [36,38]. Quantitative analysis revealed that the D-Asp content in the anterior lobe (approx. 140 nmol/g wet weight) is approx. seven-fold higher than in the posterior lobe (approx. 20 nmol/g wet weight) [36], and the *D*% in the anterior and posterior lobes is approx. 5% and 1%, respectively. In the anterior lobe of the rat pituitary gland, five different hormones are produced and secreted by distinct cell types: growth hormone, prolactin, adrenocorticotropic hormone, thyroid-stimulating hormone, and gonadotropic hormone are secreted by somatotrophs, mammotrophs, corticotrophs, thyrotrophs, and gonadotrophs, respectively. Of these cell types, D-Asp immunoreactivity is observed predominantly in prolactin-producing mammotrophs or in a closely related cell type [36]. In the posterior lobe of the rat pituitary gland, intense D-Asp

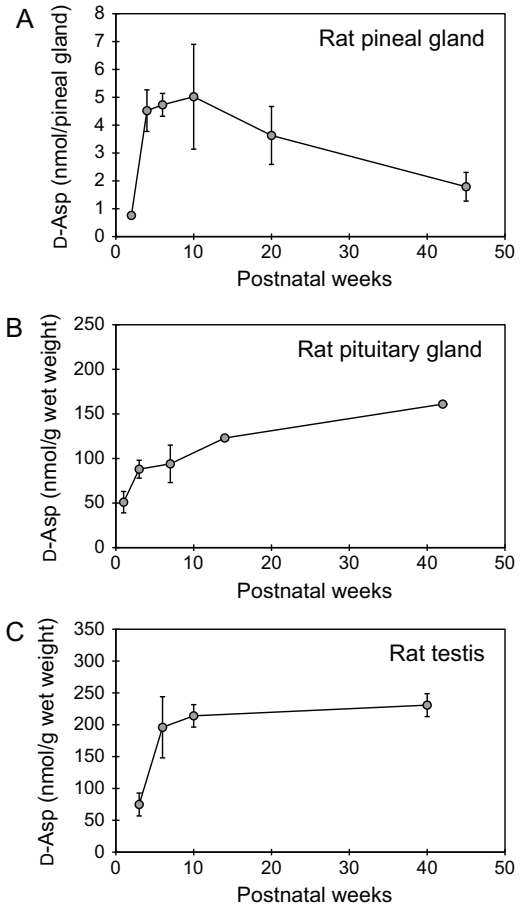

Fig. 6. Alterations in D-Asp levels in the mammalian pineal gland, pituitary gland and testis during development. (A) D-Asp levels in the rat pineal gland were determined at the indicated ages. For details, see text and [3]. (B) D-Asp levels in the rat pituitary gland were determined at the indicated ages. The figure was drawn based on data from [7]. For details, see text and [7]. (C) D-Asp levels in the rat testis were determined at the indicated ages. For details, see text and [40].
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immunoreactivity is observed in the nerve processes and terminals of magnocellular neurons [38,42]. The cell bodies of magnocellular neurons are present in the supraoptic and paraventricular nuclei of the hypothalamus, where D-Asp immunoreactivity is also observed.

Substantial amounts of D-Asp (approx. 220 nmol/g wet weight) are present in the adult rat testis, and the *D*% is approx. 35% (Fig. 6C) [40]. In this tissue, intense D-Asp immunoreactivity is observed in the cytoplasm of germ cells within the seminiferous tubules, particularly in the elongate spermatids, the most mature germ cells. By contrast, almost no staining is observed in cells other than germ cells, such as Sertoli cells and interstitial cells. It is worth noting that each seminiferous tubule in a cross section of the testis contains germ cells at distinct stages of development, and therefore the intensity of the D-Asp immunoreactivity varies between the seminiferous tubules. This stage-specific pattern of D-Asp expression has been confirmed by an assay that uses toxic chemicals to destroy specific testicular cell populations. In this assay, a significant decrease in D-Asp levels is observed when the elongate spermatids are selectively depleted [40].

Recently, the content and localization of D-Asp in exocrine tissues has been investigated. The pancreas is an exocrine tissue that produces digestive zymogens and enzymes, but also retains endocrine function. A number of specific hormones are secreted from the Langerhans islet, which constitutes approx. 1% of the cells in this tissue. In the adult rat pancreas, D-Asp immunoreactivity is predominantly observed in the Langerhans islet [43], with a *D*% of approx. 8% [44]. The Langerhans islet comprises at least four major types of endocrine cells: glucagon-secreting α cells, insulin-secreting β cells, somatostatin-secreting δ cells, and pancreatic polypeptide-secreting PP cells. Of these cell types, D-Asp has been shown to occur in α cells and a subpopulation of PP cells [43]. On the other hand, D-Asp also occurs in the Harderian gland, an intraorbital exocrine gland found in various vertebrates. High concentrations of D-Asp (approx. 0.19 μmol/g wet weight) are present in the adult rat Harderian gland, with a *D*% of approx. 36% [45]. In addition, D-Asp has also been found in human gastric juice [46], human saliva [47], and human and rat salivary glands [47–49]. The salivary gland is an exocrine tissue comprised of three kinds of major salivary glands (parotid gland, submandibular gland and sublingual gland) and several minor salivary glands. Immunohistochemical analysis of rat major salivary glands revealed selective localization of D-Asp to acinar cells in the parotid gland and striated duct cells in submandibular gland [49]. Moreover, quantitative analysis revealed that D-Asp expression changed dramatically during development, suggesting tissue- and/or cell type-specific roles of D-Asp in the development and maturation of these glands.

4. Biological and physiological activities of D-Asp

In the rat pineal gland, D-Asp is concentrated in the cytoplasm of pinealocytes in the distal region, as described above (Section 3) [41]. Since pinealocytes in the distal region of the pineal gland are involved in the synthesis and secretion of melatonin, the expression of D-Asp in pinealocytes suggests that D-Asp regulates this process (the proposed functions of D-Asp in selected neuroendocrine and endocrine tissues are summarized in Table 1). Indeed, the addition of D-Asp to primary cultured rat pinealocytes suppresses the norepinephrine-induced secretion of melatonin in a dose-dependent manner [50,51]. Moreover, cultured pinealocytes are able to efficiently take up D-Asp from the culture medium, and D-Asp is released from the cells by stimulation with norepinephrine. It thus appears that both melatonin secretion and D-Asp release from pinealocytes are enhanced by stimulation with norepinephrine, after which melatonin secretion is suppressed by the action of the released D-Asp on the cells. This negative feedback mechanism may regulate norepinephrine-induced melatonin secretion in the pineal gland.

In the anterior lobe of the rat pituitary gland, D-Asp is localized to prolactin-producing mammotrophs or a closely related cell type, as described above (Section 3) [36]. Expression of D-Asp in this cell type suggests that D-Asp plays a regulatory role in the synthesis and secretion of prolactin in the anterior lobe of the gland. This is further supported by the observation that the addition of D-Asp to GH_3 cells, which are a prolactin-producing clonal strain of a rat pituitary tumor cell line, enhances thyrotropin–releasing hormone-induced secretion of prolactin in a dose-dependent manner [52]. The secretion of prolactin from isolated pituitary glands or dispersed anterior pituitary cells in the adult rat is also enhanced by incubation with D-Asp [53,54]. Interestingly, GH_3 cells contain D-Asp, and levels of D-Asp increase with culture duration, even if D-Asp is not added to the culture medium [52]. This observation clearly indicates that D-Asp is indeed synthesized in mammalian cells. Biosynthesis of D-Asp also occurs in rat pheochromocytoma PC12 cells, MPT1 cells (a PC12 subclone), and primary cultured rat embryonic neurons, as described in the next section [34,55,56]. In addition, it is likely that prolactin-producing cells in the anterior lobe of the rat pituitary gland synthesize their own D-Asp rather than take it up after it is synthesized elsewhere. Prolactin-producing cells do not express any of the L-Glu transporters known to date, which have an affinity for D-Asp as well as for L-Glu and L-Asp. The affinity of the L-Glu

Table 1
Biological and physiological activities of D-Asp in select mammalian neuroendocrine and endocrine tissues.

Tissue	Target cells	Proposed functions	Ref.
Pineal gland	Pinealocytes	Suppression of the synthesis and secretion of melatonin	[41,50,51]
Anterior pituitary gland	Mammotrophs	Enhancement of the synthesis and secretion of prolactin	[36,52–54]
Posterior pituitary gland and hypothalamus	Magnocellular neurons	Modulation of the production of oxytocin and/or vasopressin	[42,62,63]
Intermediate pituitary gland	Melanotrophs	Suppression of α-MSH levels by regulating the biosynthesis of POMC	[39]
Testis	Leydig cells	Enhancement of the synthesis of testosterone	[65–69]

transporters for D-Asp is almost similar to that of L-Glu or L-Asp, and the transporters are involved in the uptake of these amino acids, whereas the affinity for D-Glu is so low that it is essentially not transported [57–60]. In fact, intraperitoneally administered D-Asp is not incorporated into prolactin-producing cells in the pituitary gland, while it is taken up by endothelial cells [61]. Furthermore, alterations in the expression levels of L-Glu transporters in the pituitary gland do not correlate with the development-related changes in D-Asp levels. Collectively, it appears that D-Asp is synthesized in the anterior lobe of the pituitary gland where it acts on prolactin-producing cells as a regulator in an autocrine or paracrine fashion to enhance prolactin secretion in the cells (Table 1).

In the rat posterior pituitary gland and hypothalamus, intense D-Asp immunoreactivity is observed in the magnocellular neurons, as described above (Section 3) [38,42], and D-Asp appears to modulate the production of oxytocin and/or vasopressin in these neurons (Table 1). Indeed, D-Asp stimulates the release of oxytocin from rat hypothalamic explants [42]. Moreover, intraperitoneal injection of D-Asp increases the expression of oxytocin and vasopressin genes in the supraoptic and paraventricular nuclei of the rat hypothalamus [62]. Within the magnocellular neurons of the rat supraoptic nucleus, D-Asp immunoreactivity is observed in the nucleoli [63]. This immunoreactivity is predominantly associated with heterochromatin but not with other subcellular structures of the nucleus and soma, including the nucleoplasm and cytoplasm. These observations have led to the proposal that D-Asp directly interacts with DNA and/or acts on nuclear protein(s) involved in the regulation of gene transcription, and thereby contributes to the regulation of gene expression in the hypothalamo-neurohypophyseal system [63].

In contrast to the anterior and posterior lobes, D-Asp immunoreactivity is not observed in the intermediate lobe of the rat pituitary gland [36,38], while DDO is prominently expressed in the intermediate lobe, but not in the anterior or posterior lobes [38]. This reciprocal localization of D-Asp and DDO is also evident in the mouse pituitary gland [39]. Interestingly, in DDO-deficient mice, intense D-Asp immunoreactivity is observed in the intermediate lobe, as well as in the anterior and posterior lobes [39]. The intermediate lobe of the pituitary gland contains melanotrophs almost exclusively, which produce proopiomelanocortin (POMC) as the sole source of pituitary α-melanocyte-stimulating hormone (α-MSH), a member of the melanocortin peptide family. In DDO-deficient mice, expression levels of POMC, as well as of α-MSH, are substantially decreased in the intermediate lobe of the gland [39]. Furthermore, alterations associated with α-MSH, such as elevated body mass, sexual deficits, and decreased autogrooming, are observed in DDO-deficient mice. It thus appears that D-Asp suppresses α-MSH expression by regulating the biosynthesis of POMC in the intermediate lobe of the pituitary gland (Table 1), and thereby participates in the regulation of several phenotypes mediated by α-MSH.

In the rat testis, D-Asp is localized in germ cells within the seminiferous tubules, particularly in the elongate spermatids [40]. However, intraperitoneally administered D-Asp is not incorporated into the seminiferous tubules but instead accumulates in the interstitial spaces of the testis (unpublished observations). It thus appears that germ cells in the testicular seminiferous tubules, similar to prolactin-producing cells in the anterior lobe of the pituitary gland described above, synthesize their own D-Asp rather than take it up after its synthesis elsewhere. The gene that encodes Asp racemase (which converts L-Asp into D-Asp) was identified very recently in mouse and has been shown to be highly expressed in elongate spermatids of the mouse testis [64]. Furthermore, it has been reported that D-Asp levels in rat testicular venous blood plasma are higher than those in peripheral blood plasma, suggesting that D-Asp synthesized in the testicular seminiferous tubules is then secreted from the tubules [65]. In addition, secreted D-Asp appears to act on interstitial Leydig cells to modulate testosterone synthesis. Indeed, the addition of D-Asp to primary cultured rat testicular Leydig cells enhances human chorionic gonadotropin (hCG)-induced synthesis of testosterone in a dose-dependent manner [66]. This effect is specific for D-Asp, as no increase in hCG-induced testosterone synthesis is observed following treatment with L-Asp, L-Glu or D-Glu. Moreover, cultured Leydig cells are able to efficiently take up D-Asp from the culture medium through the L-Glu transporter, and the amount of D-Asp in the cells correlates with the increase in the testosterone production (Fig. 7A). When the cultured Leydig cells are treated with L-cysteine sulfinic acid, an inhibitor that prevents the uptake of amino acids by the L-Glu transporter, both the uptake of D-Asp and the D-Asp-mediated enhancement of testosterone production are concomitantly suppressed in a dose-dependent manner (Fig. 7B) [66]. Therefore, it appears that D-Asp is incorporated into Leydig cells through the L-Glu transporter, after which D-Asp enhances the testosterone synthesis in the cells (Table 1). Recently, the enhancement of testicular testosterone synthesis by D-Asp has also been demonstrated in pigs and humans [67,68].

In Leydig cells, testosterone synthesis is initiated by the transport of intracellular cholesterol to the inner mitochondrial membrane, where it is converted to pregnenolone by intramitochondrial cytochrome P450 side-chain cleavage. Subsequently, pregnenolone is converted to testosterone via the steroidogenic pathway in the endoplasmic reticulum. Cholesterol delivery to the inner mitochondrial membrane is the rate-limiting step in testosterone synthesis, and the key regulatory protein that facilitates this process is steroidogenic acute regulatory protein (StAR). Interestingly, the addition of D-Asp to cultured Leydig cells enhances the steady state mRNA and protein levels of StAR, which increases testosterone synthesis in the cells [69]. Thus, it appears that D-Asp enhances testosterone synthesis in Leydig cells by stimulating the StAR gene expression, after which testosterone is released from the cells and acts on germ cells within the seminiferous tubules. Through this feedback mechanism, D-Asp in the germ cells may regulate the production and secretion of testosterone, which subsequently promotes the differentiation and maturation of the germ cells in the testis.

5. Biosynthesis of D-Asp

Since the discovery of free D-Asp in mammals, much attention has been paid to the origins and synthetic pathways of this D-amino acid. As recently reviewed extensively by Friedman [70],

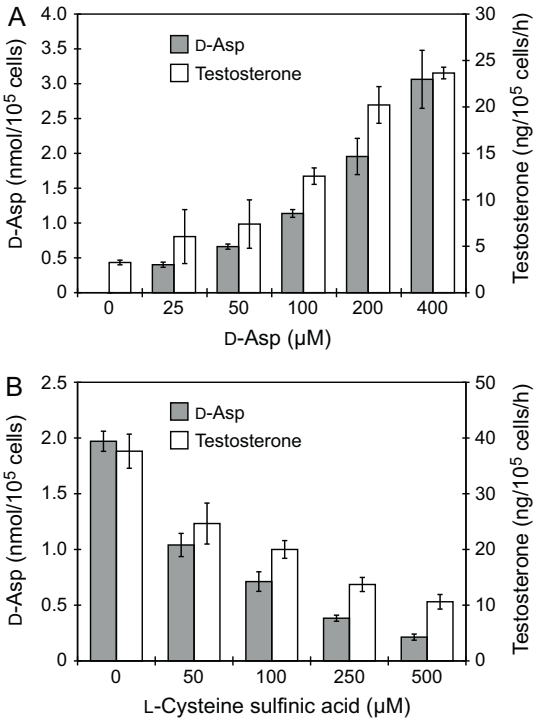

Fig. 7. Enhanced testosterone synthesis in primary cultured rat testicular Leydig cells by treatment with D-Asp. (A) Purified Leydig cells were cultured with hCG and the indicated doses of D-Asp. The left and right vertical axes represent the D-Asp content in the cells (grey bars) and the testosterone content in the media (open bars), respectively. For details, see text and [66]. (B) Purified Leydig cells were incubated with D-Asp and hCG and the indicated doses of L-cysteine sulfinic acid. The left and right vertical axes represent the D-Asp content in the cells (grey bars) and the testosterone content in the media (open bars), respectively. For details, see text and [66].
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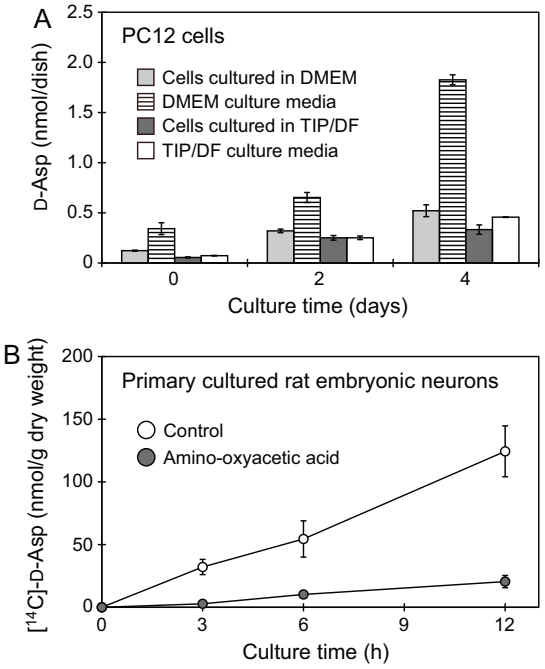

Fig. 8. Biosynthesis of D-Asp in cultured mammalian cells. (A) PC12 cells were cultured in DMEM or TIP/DF medium. The level of D-Asp in cells cultured in DMEM (light grey bars), DMEM culture media (striped bars), cells cultured in TIP/DF (dark grey bars), and TIP/DF culture media (open bars) were determined at the indicated culture times. For details, see text and [55]. (B) Purified rat embryonic neurons were cultured with [^{14}C]-L-Asp in the absence (open circles) or presence (grey circles) of amino-oxyacetic acid. The [^{14}C]-D-Asp contents in the cells were determined at the indicated culture times. For details, see text and [34].
Reproduced with permission from Elsevier [34,55].

substantial amounts of D-Asp have been detected in various lower organisms. In addition, ingested D-Asp can be absorbed by the intestine and transported to several tissues in mammals, as described in the next section [71]. Therefore, mammalian D-Asp can be partially derived from enterobacterium and/or diet. On the other hand, Neidle and Dunlop observed that the D-Asp content is remarkably increased in fertilized chicken eggs during incubation [11]. Since the egg is a closed system without dietary supplementation, this observation strongly suggested that the biosynthesis of D-Asp occurs in chicken embryos and therefore perhaps also in the mammalian body.

In cultured mammalian cells, biosynthesis of D-Asp was first demonstrated in PC12 cells [55]. PC12 cells contain D-Asp, and D-Asp levels in both the cells and the culture media increase with the duration of culture (Fig. 8A). D-Asp was not added to the culture medium, nor can exogenous D-Asp be taken up into the cells, since PC12 cells do not express the L-Glu transporter. In fact, radiolabeled D-Asp was not incorporated into the cells [55]. Thus, these observations clearly indicate that D-Asp is indeed synthesized in mammalian cells. In PC12 cells, the highest *D*% is approx. 14% and no D-amino acids other than D-Asp are detected [55]. In addition to PC12 cells, GH_3 cells and MPT1 cells have also been found to synthesize D-Asp [52,56], whereas the biosynthesis of D-Asp is not observed in mouse fibroblast Swiss 3T3 cells or human neuroblastoma NB-1 cells [55].

Primary cultured rat embryonic neurons express high levels of endogenous D-Asp, and biosynthesis of [^{14}C]-D-Asp occurs when the cultured neurons are treated with [^{14}C]-L-Asp as the precursor molecule (Fig. 8B) [34]. Moreover, the production of [^{14}C]-D-Asp is markedly inhibited by treatment with amino-oxyacetic acid, which is a potent inhibitor of pyridoxal phosphate (PLP)-dependent enzymes. Thus, it appears that the biosynthesis of D-Asp in mammalian cells is mainly mediated by Asp-specific amino acid racemase, which converts L-Asp to D-Asp in a PLP-dependent manner. Indeed, the gene that encodes Asp racemase was recently identified in mouse [64]. The recombinant form of this enzyme has been shown to exhibit PLP-dependent Asp racemase activity. In mammals, Ser racemase, the synthetic enzyme that creates D-Ser from L-Ser in a PLP-dependent manner, has also been detected [72,73]. Besides mammals, a PLP-dependent Asp racemase of animal origin has been identified in bivalves (*Scapharca broughtonii*) [74]. Notably, the deduced amino acid sequence of mouse Asp racemase is more homologous to that of mammalian Asp aminotransferase than to the mammalian Ser racemases and *S. broughtonii* Asp racemase, whereas the *S. broughtonii* Asp racemase shows relatively high amino acid sequence identity with the mammalian Ser racemases. Therefore, it appears that the mouse and *S. broughtonii* Asp racemases have evolved convergently from different ancestral proteins to acquire a similar catalysis.

In mice, Asp racemase is abundant in the brain, heart and testis, followed by the adrenal gland, and is expressed at negligible levels in the liver, lung, kidney and spleen [64]. Immunohistochemical analysis revealed coincident localization of Asp racemase and D-Asp in the mouse brain, pituitary gland, hippocampus, pineal gland, adrenal medulla and testis. Interestingly, the depletion of Asp racemase in the adult mouse hippocampus by retrovirus-mediated expression of Asp racemase-targeting short-hairpin RNA has been shown to elicit profound defects in dendritic development and neuron survival [64]. Thus, the current data suggest that Asp racemase plays an essential role in neuronal development, consistent with the previous proposal that D-Asp is important for the development and neurogenesis of the brain [33,34].

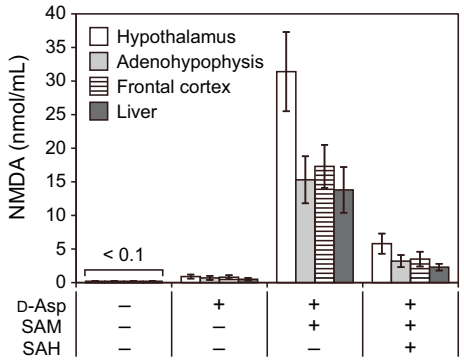

Fig. 9. Synthesis of NMDA in rat tissue homogenates. The vertical axis represents the concentration of NMDA produced by the incubation with tissue homogenates of the rat hypothalamus (open bars), adenohypophysis (light grey bars), frontal cortex (striped bars), and liver (dark grey bars) in the absence or presence of D-Asp, SAM, and/or SAH.
The figure was drawn based on data from [90]. For details, see text and [90].

Considering an alternative pathway of D-Asp synthesis in mammals, the involvement of D-amino acid aminotransferase may be possible. This enzyme transfers an amino group from another D-amino acid to oxaloacetic acid, resulting in the production of D-Asp. In fact, cDNA of D-amino acid aminotransferase was recently cloned from *Arabidopsis thaliana*, and the recombinant form of this enzyme has been shown to catalyze transamination from D-Glu to oxaloacetic acid to produce D-Asp [75]; however, D-amino acid aminotransferase has not been detected in mammals. On the other hand, free D-Asp can be derived from D-aspartyl residue-containing proteins. It is known that L-asparaginyl and L-aspartyl residues in proteins undergo spontaneous isomerization under physiological conditions to generate D-aspartyl and/or isoaspartyl residues [76–86]. In addition, D-aspartyl endopeptidase, a protease that can generate free D-Asp through the degradation of D-aspartyl residue-containing proteins, has been identified in rabbit and mouse [87].

In 1987, Sato et al. first reported the existence of NMDA in muscle extracts of *S. broughtonii* [88]. Since then, NMDA has been detected in various organisms, such as mollusks, cephalopods, crustaceans, cephalochordates, tunicates, fishes, amphibians, birds and mammals [89–94]. In mammalian tissues, the synthesis of NMDA from D-Asp has been reported [90]. Specifically, in tissue homogenates of rat hypothalamus, adenohypophysis, frontal cortex and liver, low concentrations of NMDA are produced by the addition of D-Asp (Fig. 9). The amount of NMDA synthesized increases remarkably when *S*-adenosyl-L-methionine (SAM), the universal methyl donor in transmethylation reactions, is concomitantly added to the homogenates. Moreover, this increase in the NMDA production is significantly suppressed by the addition of *S*-adenosyl-L-homocysteine (SAH), a potent transmethylation inhibitor. Collectively, these observations suggest the existence of unidentified methyltransferase that synthesizes NMDA from D-Asp in mammals, although further study is certainly necessary.

6. Degradation of D-Asp

As noted above (Section 5), the enzymes postulated to be involved in the synthesis of D-Asp in mammals have been very recently identified. In contrast, DDO has long been known as a degradative enzyme that stereospecifically acts on D-Asp. In 1949, Still et al. first reported the activity of DDO in rabbit kidney extracts [31]. This enzyme is stereospecific for acidic D-amino acids and degrades not only D-Asp, but also D-Glu and NMDA. DDO is a FAD-containing flavoprotein in which one molecule of FAD noncovalently binds to one molecule of DDO. In the first step of the reaction, which is the only enzymatic step of the entire reaction scheme, DDO catalyzes the dehydrogenation of a D-amino acid to generate the corresponding imino acid, coupled with the reduction of FAD (Fig. 3A). Subsequently, FAD reoxidizes spontaneously in the presence of molecular oxygen, producing hydrogen peroxide, while the imino acid nonenzymatically hydrolyzes to 2-oxo acid and ammonia. When NMDA is the substrate, the product is methylamine instead of ammonia. Thus, DDO catalyzes the oxidative deamination of D-amino acids to generate the corresponding 2-oxo acids, along with hydrogen peroxide and ammonia (or methylamine).

DDO is present in a wide variety of eukaryotes, including humans, and its activity has been detected in tissue homogenates from organisms such as cephalopods, gastropods, fishes, amphibians, birds, and mammals, as well as yeast [31,95–110]. In contrast, DDO is not found in bacteria or plants. In mammals, DDO activity is highest in the kidney, followed by the liver and brain, and is low in other peripheral tissues [106,107]. Mammalian DDOs have been shown to localize to the subcellular peroxisome, where the toxic hydrogen peroxide produced in the DDO reaction can be detoxified by peroxisomal catalase, a hydrogen peroxide-degrading enzyme [106,111–114]. DDO cDNAs have been cloned from bovine, human, mouse and pig, as well as yeast (*Cryptococcus humicola*) and nematode (*Caenorhabditis elegans*), and the recombinant forms of these enzymes have been functionally characterized [109,115–125].

DDO expression in the kidney and liver of rat are relatively low at birth and rapidly increase thereafter [71,100,106]. In contrast, D-Asp levels in these tissues decrease as development proceeds [2,71]. Similar ontogenic changes in DDO and D-Asp have also been observed in the kidney and liver of mouse [71]. In addition, although the D-Asp content in the rat cerebrum rapidly decreases as development proceeds (Fig. 4A) [7], the DDO content rapidly increases after birth [106]. Histochemical analysis of rat brain tissues revealed that DDO is abundant in regions where D-Asp immunoreactivity is not observed, whereas DDO activity is not detected in regions where the D-Asp content is very high [38]. The reciprocal localization of DDO and D-Asp has also been observed in several brain tissues in mouse and pig [39,126]. Taken together, these observations suggest that DDO in mammals degrades endogenous D-Asp as a physiological substrate and thereby regulates the cellular levels of this D-amino acid.

Ingestion of D-Asp by rats increases the D-Asp levels in blood; levels peak within 1–2 h, after which they decrease progressively to baseline levels within 24–48 h [71]. Approx. 10–20% of the ingested D-Asp is excreted in the urine and feces within a 48 h period, and the rest is absorbed by the intestine. Because the levels of oxaloacetic acid and ammonia, which are the products of the enzymatic reaction catalyzed by DDO, increase substantially in the blood, liver, and kidney after ingestion of D-Asp, it appears that D-Asp absorbed by the intestine is transferred through the blood to the liver and kidneys, where it is metabolized by DDO. Interestingly, it has been reported that DDO activity in the livers of adult mice is increased by oral or intraperitoneal administration of D-Asp, but not L-Asp [127,128]. In addition, oral administration of D-Asp during pregnancy and suckling has been shown to increase DDO activity in the liver and kidneys of newborn rats [71]. Thus, these results suggest that DDO activity is induced by D-Asp. In fact, DDO activity in *C. humicola* has been shown to increase strikingly when the yeast cells are grown on medium containing D-Asp as the sole nitrogen source, the sole carbon source, or the sole nitrogen and carbon source [129]. Moreover, an increase in DDO mRNA is also observed, suggesting that expression is regulated at the transcriptional level. This induction of DDO expression is specific to D-Asp, since L-Asp, D-Glu or D-alanine (D-Ala) do not increase DDO mRNA levels. It is likely that DDO gene expression in mammals is regulated in a similar way; however, the detailed mechanisms are still unclear.

Table 2
D-Asp contents in seminal plasma and spermatozoa from normospermic, oligoasthenoteratospermic, and/or azoospermic donors.

Donor type	D-Asp content [a]	
	Seminal plasma (nmol/mL seminal plasma)	Spermatozoa (fmol/spermatozoa)
Normospermic donors	80 ± 10 (15.5)	130 ± 15 (28.5)
Oligoasthenoteratospermic donors	26 ± 6 (8.5)	61 ± 5 (10.4)
Azoospermic donors	12 ± 1.5 (3.4)	–[b]

Source: Data are from [150]. For details, see text and [150]. Reproduced with permission from Elsevier [150].
[a] Data are shown as the mean ± standard deviation ($n = 10$). Values in parentheses indicate *D%*.
[b] Not determined.

Recently, two independent groups established DDO-deficient mice and showed that these mice have significantly elevated levels of D-Asp in several tissues [39,130]. Although the DDO-deficient mice do not show any change in life span, several phenotypes have been observed in these mice. Behavioral phenotypes in DDO-deficient mice have been summarized previously (see Table 2 in [131]). Immunohistochemical analysis of DDO-deficient mice revealed that D-Asp is present in the intermediate pituitary gland, renal Bowman's capsule, renal thin limbs of Henle's loop, testicular Leydig cells, the vasculature traversing the adrenal cortex, adrenal medulla epinephrine cells, and cerebellar cortex Bergmann glia, while D-Asp immunoreactivity is undetectable in these tissues in wild-type mice [39,132]. As noted above (Section 4), POMC and α-MSH levels are substantially decreased in the intermediate lobe of the pituitary gland in DDO-deficient mice [39]. This decrease is location-specific, and no change is observed in either POMC expression, or the number of POMC-positive neurons in the anterior lobe of the gland or in the arcuate nucleus of the hypothalamus in DDO-deficient mice. These observations further support the idea that D-Asp is closely involved in the neuroendocrine and endocrine regulation of hormone production in mammals.

The findings obtained from studies using DDO-deficient mice collectively suggest an important role(s) for D-Asp as a novel neuromodulator. For instance, in tests that evaluate learning and memory, such as the Morris water maze and contextual fear conditioning, altered behavior is observed in DDO-deficient mice [133]. The hippocampus is a region closely involved in learning and memory, and in DDO-deficient mice, the D-Asp content in this region (approx. 400 nmol/g tissue) is approx. 13-fold higher than that in wild-type mice [134]. In addition, electrophysiological analysis of the induction of long-term potentiation (LTP) in hippocampal slices revealed that synaptic depotentiation following LTP induction is significantly suppressed in DDO-deficient mice compared to wild-type mice (Fig. 10A) [134]. Long-term synaptic plasticity, such as LTP and long-term depression (LTD), is a basic process of learning and memory, and the NMDA receptor is believed to play a pivotal role in this process. D-Asp is known to stimulate the NMDA receptor by binding to the L-Glu-binding site [135,136]; therefore, it is likely that NMDA receptor-mediated neurotransmission is enhanced by the elevated concentrations of D-Asp in the hippocampus of DDO-deficient mice. Similar findings have also been reported in studies using corticostriatal slices. Specifically, the D-Asp content in the striatum is remarkably increased in DDO-deficient mice, similar to the hippocampus, and high-frequency stimulation of corticostriatal fibers induces LTD in corticostriatal slices of wild-type mice but not DDO-deficient mice (Fig. 10B) [133]. In the corticostriatal system, it is believed that enhanced NMDA receptor signaling blocks the induction of LTD. It thus appears that NMDA receptor-mediated neurotransmission is enhanced by high concentrations of D-Asp in DDO-deficient mice, resulting in the disappearance of LTD. Collectively, these lines of evidence suggest that D-Asp functions as a regulator of NMDA receptor-mediated neurotransmission; however, the mechanisms and molecular nature of this regulation remain to be clarified.

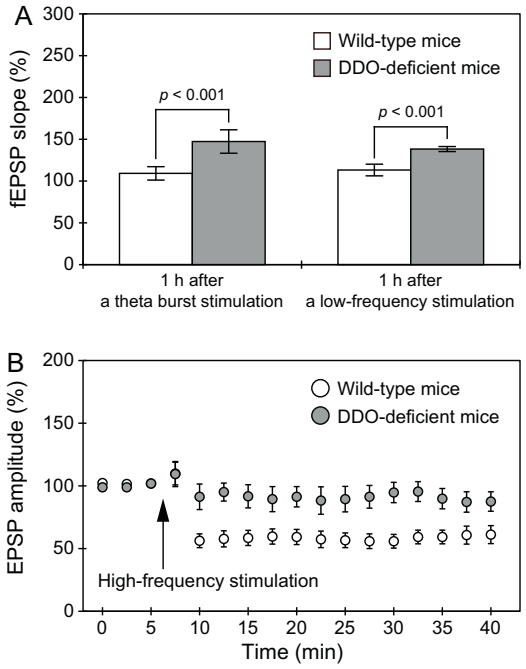

Fig. 10. Alterations in long-term synaptic plasticity in DDO-deficient mice. (A) The vertical axis represents a percentage of the field excitatory postsynaptic potential (fEPSP) slopes relative to the baselines. fEPSP slopes were measured 1 h after a theta burst stimulation (left). Potentiation recovers almost to baseline in hippocampal slices from wild-type mice (open bar), whereas the induced LTP is maintained in slices from DDO-deficient mice (grey bar). fEPSP slopes were measured 1 h after low-frequency stimulation following the induction of LTP (right). Synaptic depotentiation is observed in slices from wild-type mice (open bar) but not in slices from DDO-deficient mice (grey bar). The figure was drawn based on data from [134]. For details, see text and [134]. (B) The vertical axis represents a percentage of the EPSP amplitudes relative to that recorded before high-frequency stimulation. High-frequency stimulation of corticostriatal fibers induces LTD in corticostriatal slices from wild-type mice (open circles), but not in slices from DDO-deficient mice (grey circles). For details, see text and [133].
Reproduced with permission from the Society of Neuroscience [133].

7. Cellular transport of D-Asp

It has been reported that endogenous D-Asp is released from rat adrenal tissue slices by depolarization with a high concentration of KCl [34]. This depolarization also evokes the release of L-Asp from the slices, but the magnitude of release is much lower. The release of D-Asp is Ca^{2+}-dependent and completely suppressed by the addition of EGTA, a Ca^{2+} chelating agent. Interestingly, treatment with EGTA also decreases the basal release of D-Asp in the absence of stimulation by KCl. Treatment with acetylcholine also evokes the release of D-Asp from the slices, suggesting that D-Asp secretion is regulated by cholinergic neurotransmission in the adrenal gland. On the other hand, primary cultured rat pinealocytes are able to efficiently take up D-Asp from the culture medium, and D-Asp is released from the cells by stimulation with norepinephrine [51]. In

addition, and as noted in the section above (Section 4), it is believed that D-Asp is synthesized in and secreted from rat testicular seminiferous tubules [65]. Taken together, these observations strongly imply that mammalian cells are equipped with molecular mechanism(s) for the efflux of intracellular D-Asp into the extracellular space. Detailed characterization of the specific pathway(s) of D-Asp release could provide an important clue to understanding the physiological significance of D-Asp in mammals.

In our studies, we have used three cell lines to study the efflux of D-Asp: PC12, MPT1 and 2068 cells. MPT1 and 2068 cells are spontaneously-occurring flat cell variants of PC12 cells [137,138]. In contrast to PC12 cells, MPT1 and 2068 cells express the L-Glu transporter and are able to efficiently take up D-Asp from the culture medium [137–139]. In the culture of PC12 cells that are able to synthesize their own D-Asp, the D-Asp contents in the cells and culture media increase with the duration of culture (Fig. 8A) [55]. These observations suggest that D-Asp synthesized in PC12 cells is spontaneously and continuously released into the culture medium without specific stimulation. Immunocytochemical analysis of PC12 cells with an anti-D-Asp antibody revealed that D-Asp is distributed homogeneously throughout the cytoplasm [140]. In contrast, it is well known that dopamine is concentrated in intracellular vesicles in mammalian cells and that it is released into the extracellular space via an exocytotic pathway. There is an obvious contrast between the release of D-Asp and the release of dopamine in PC12 cells. Specifically, while dopamine is rapidly released from the cells following stimulation with a high concentration of KCl, the release of D-Asp is slow under the same conditions [140]. In addition, the D-Asp release is insensitive to nifedipine, a voltage-gated Ca^{2+} channel blocker, whereas the dopamine release is inhibited by treatment with nifedipine in a dose-dependent manner. Furthermore, knockdown of SNAP-25, a SNARE protein involved in the exocytotic machinery, results in a significant decrease in dopamine release, whereas the D-Asp release is increased, rather than decreased, by SNAP-25 silencing. Therefore, it appears that the spontaneous and continuous release of endogenous D-Asp occurs through the efflux of cytoplasmic D-Asp in PC12 cells, and that the D-Asp release is mediated by an unidentified pathway(s) that is distinct from vesicular exocytosis.

Similar to PC12 cells, MPT1 cells have also been shown to synthesize D-Asp, which is spontaneously and continuously released into the culture medium [56]. However, in contrast to PC12 cells, MPT1 cells express the L-Glu transporter, and the released D-Asp is actively taken up into the cells [139,141]. Thus, there is a dynamic exchange of D-Asp between the intra- and extracellular spaces during the culture of MPT1 cells. In fact, the addition of substrates other than D-Asp or inhibitors of the L-Glu transporter competitively inhibits the uptake of D-Asp by MPT1 cells, resulting in the accumulation of D-Asp in the culture medium (Fig. 2C) [26,139,141]. Therefore, it appears that in cells that exhibit dynamic D-Asp homeostasis, as observed in MPT1 cells, L-Glu transporter substrates or inhibitors regulate the concentration of D-Asp in the extracellular space.

Interestingly, immunocytochemical analysis of 2068 cells demonstrated that D-Asp immunoreactivity is predominantly associated with granule-like structures around the nuclei in the cells [142]. Because 2068 cells express the L-Glu transporter, extracellular D-Asp can be taken up into the cells through this transporter. When 2068 cells are preloaded with [^{14}C]-D-Asp, the release of [^{14}C]-D-Asp from the cells is significantly enhanced by stimulation with a high concentration of KCl or treatment with a Ca^{2+} ionophore [142]. These observations strongly suggest that D-Asp is released from 2068 cells via an exocytotic pathway, which is markedly different from the pathway of D-Asp release in PC12 cells. Further studies to clarify the details of the release mechanism(s) in 2068 cells are needed, including the identification of the vesicular transporter that accumulates D-Asp in intracellular vesicles. In contrast to these findings, Nakatsuka et al. also reported the release of D-Asp from mammalian cells via an exocytotic pathway [143]. However, these studies were performed in PC12 cells and the results conflict with the results obtained in our studies using PC12 cells. This contradiction has been discussed in our previous review article [144].

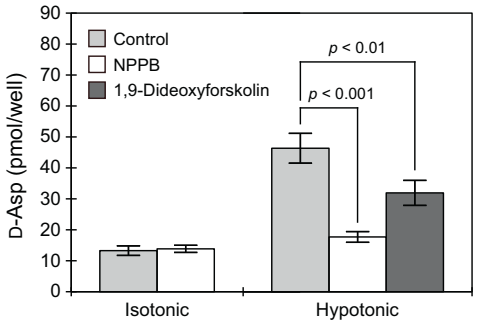

Fig. 11. Hypotonic stimulus-induced efflux of endogenous D-Asp in PC12 cells. PC12 cells were incubated in isotonic (316 mOsM) or hypotonic (216 mOsM) solutions in the absence (light grey bars) or presence of NPPB (open bars) or 1,9-dideoxyforskolin (dark grey bars), and the D-Asp efflux was determined after 20 min. For details, see text and [140].
Reproduced with permission from Elsevier [140].

Detailed investigation of D-Asp efflux in PC12 cells revealed a third pathway: the release of D-Asp from the cells through the volume-sensitive organic anion channel (VSOC), which exists on the cell membrane, connects the cytoplasmic and extracellular spaces, and opens in response to changes in osmotic pressure. When PC12 cells are exposed to hypotonic medium, substantial amounts of endogenous D-Asp are released from the cells through VSOC (Fig. 11) [140]. Moreover, the amount of released D-Asp significantly increases as the osmolarity is further reduced. In contrast, dopamine is not osmo-dependently released from the cells. Furthermore, the observed efflux of D-Asp is substantially suppressed by treatment with putative VSOC blockers, such as 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and 1,9-dideoxyforskolin (Fig. 11). It is worth noting that these VSOC blockers do not inhibit the spontaneous release of D-Asp from the cells, which suggests that this pathway is distinct from that of D-Asp efflux through VSOC. Consequently, at least three different pathways have been proposed to contribute to the release of D-Asp in PC12 cells and its subclones: (1) a spontaneous and continuous release of cytoplasmic D-Asp, (2) an exocytotic discharge of vesicular D-Asp, and (3) a release of cytoplasmic D-Asp through VSOC. These pathways may be selectively employed in distinct cell types *in vivo*, depending on their physiological and pathological states.

As noted in Section 4, it appears that D-Asp synthesized in germ cells of the testicular seminiferous tubules is secreted from the tubules, after which it acts on the interstitial Leydig cells to enhance testosterone synthesis (Table 1). It is well known that, in the mammalian testis, spontaneous germ cell apoptosis occurs during spermatogenesis. Interestingly, the treatment of PC12 cells with apoptotic inducers, including staurosporin, tumor necrosis factor-α, hydrogen peroxide, and C2-ceramide, evokes the release of D-Asp (Fig. 12) [145]. Since the increase in the D-Asp release following the treatment with apoptotic inducers is almost completely inhibited by putative VSOC blockers, such as NPPB and 4,4′-diisothiocyanostilbene-2,2′-sulfonic acid, D-Asp efflux in this case is conceivably mediated through the VSOC pathway. It is noteworthy that this apoptosis-stimulated D-Asp release is observed under hypotonic conditions but not under isotonic conditions (Fig. 12) [145]. Although the molecular nature and heterogeneity of VSOC has not yet been clarified, several lines of evidence show that

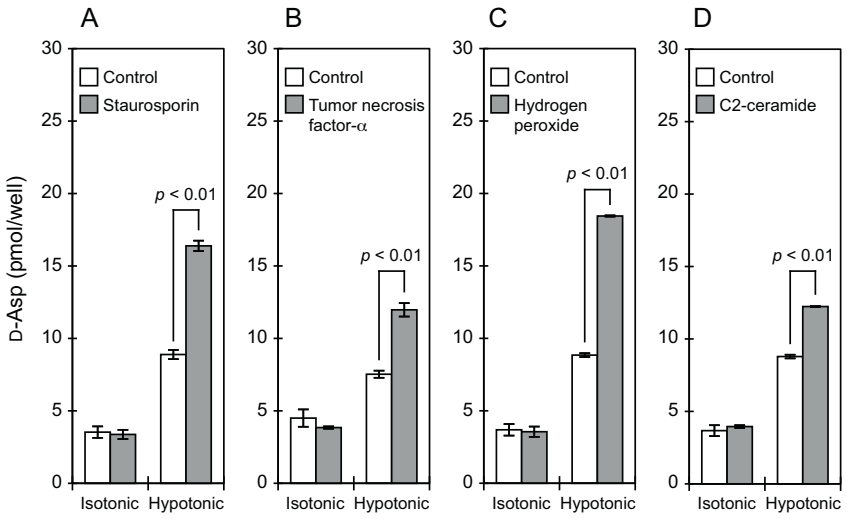

Fig. 12. Effect of apoptotic inducers on the efflux of endogenous D-Asp in PC12 cells. PC12 cells were incubated in isotonic (316 mOsM) or hypotonic (216 mOsM) solutions in the absence (open bars) or presence of apoptotic inducers (grey bars: staurosporin, tumor necrosis factor-α, hydrogen peroxide, and C2-ceramide for A, B, C, and D, respectively), and the D-Asp efflux was determined after 20 min. For details, see text and [145].
Reproduced with permission from Elsevier [145].

Table 3
Comparison of D-Asp content in pre-ovulatory follicular fluid, oocyte quality, and fertilization competence between age groups.

Age group	D-Asp in follicular fluid (nmol/mL)[a]	Number of oocytes at metaphase II[a]		Fertilization rate (%)[b]
		Good quality	Bad quality	
Ages 22–34	19.1 ± 1.9	11.5 ± 1.8	0.4 ± 0.1	96.7
Ages 35–40	10.8 ± 1.2	2.4 ± 0.4	4.4 ± 1.2	87.0

Source: Data are from [151]. For details, see text and [151]. Reproduced with permission from Oxford Journals [151].
[a] Data are shown as the mean ± standard deviation ($n = 10$).
[b] The fertilization rate was calculated for only two oocytes at metaphase II for each patient.

different types of VSOC may exist in mammals and that the activation of VSOC occurs in certain types of cells under physiologically normal conditions [146–148]. Therefore, it is likely that apoptotic stimulation enhances D-Asp efflux in spermatogenic germ cells under physiologically normal conditions. It is well known that an insufficient supply of testosterone from the Leydig cells results in the acceleration of germ cell apoptosis in the seminiferous tubules. Therefore a feedback mechanism may operate in the testis as follows: decreased testosterone supply stimulates increased apoptosis of germ cells in the seminiferous tubules, which in turn increases D-Asp efflux, thereby enhancing testosterone synthesis in the interstitial Leydig cells [145].

8. Role of D-Asp in disease

Dysfunctional NMDA receptor-mediated neurotransmission has been implicated in the onset of various mental disorders, including depression and schizophrenia. Therefore, a substance that can activate NMDA receptor function may be a novel antipsychotic drug. As noted in the section above (Section 6), D-Asp stimulates the NMDA receptor by binding to the L-Glu-binding site of the receptor [135,136]. Thus, D-Asp could be considered a candidate drug for the treatment of NMDA receptor-related diseases. Indeed, DDO-deficient mice, which have elevated concentrations of D-Asp in several brain regions [133,134], exhibit a reduced immobility time in the Porsolt forced-swim test, a model of depression, suggesting that the genetic ablation of DDO has a specific antidepressant effect [132]. In addition, deficits in prepulse inhibition induced by amphetamine, a potent dopamine releaser, or by MK-801, a noncompetitive antagonist of the NMDA receptor, are attenuated in DDO-deficient mice [133]. This suggests that D-Asp protects against sensorimotor gating deficits, which are observed in schizophrenic patients [149]. It should be noted, however, that another research group has reported that increased levels of D-Asp in the brain have the opposite effect on sensorimotor gating modulation; namely, DDO-deficient mice display significant deficits in prepulse inhibition [132]. In this regard, further studies are necessary to elucidate the true effect of D-Asp and/or DDO on sensorimotor gating.

Recently, D-Asp has been reported in human seminal plasma, spermatozoa, and pre-ovulatory follicular fluid [150,151]. As noted in the previous sections (Sections 4 and 7), D-Asp in germ cells appears to regulate the endocrine system, and thereby participates in the maturation of the reproductive organs. Interestingly, the amount of D-Asp in human seminal plasma and spermatozoa is significantly reduced in oligoasthenoteratospermic and/or azoospermic donors compared with normospermic donors (Table 2) [150]. On the other hand, it has been reported that the ingestion of D,L-Asp by rabbit bucks significantly increases the D-Asp content in seminal plasma, whereas the L-Asp content in seminal plasma remains almost unchanged [152]. Administration of D,L-Asp also results in a significant increase in several kinetic parameters of motile spermatozoa that are indicative of sperm quality. Furthermore, it has also been found that in human female patients undergoing *in vitro* fertilization, the D-Asp content of the pre-ovulatory follicular fluid is lower in older patients than in younger ones (Table 3) [151]. This decrease in D-Asp content appears to reflect a decrease in oocyte quality and fertilization competence. Taken together, these lines of evidence suggest that D-Asp is closely involved in the quality control of germ cells in mammals.

9. Concluding remarks

In summary, the progress in analytical methods for the enantiomeric determination of amino acids has enabled us to effectively and sensitively analyze D-amino acids in biological samples. Consequently, it has been shown that mammalian cells possess the molecular mechanisms necessary to synthesize, release, take up, and degrade D-Asp. In mammalian tissues, D-Asp is present in specific cells, and the above-mentioned mechanisms may be selectively employed in distinct cell types. Although the target molecule(s) of D-Asp action have not been clearly identified, this D-amino acid appears to play a crucial role in the regulation of neurotransmission and the endocrine system in mammals. Moreover, the relevance of D-Asp in several human diseases has also been studied; however, the details of these relationships warrant further investigation. As noted in Section 1, D-Ser has been proposed to be a neuromodulator that binds to the glycine-binding site of the NMDA receptor and potentiates glutamatergic neurotransmission in the central nervous system [12–14]. In addition, D-Ala, another D-amino acid found in mammals at relatively low levels, has been postulated to be involved in endocrine activity in the anterior pituitary gland and pancreas [153–155]. Furthermore, it has recently been shown that aromatic D-amino acids, such as D-phenylalanine and D-tryptophan, elicit chemotactic responses in human neutrophils via the activation of GPR109B, an orphan G protein-coupled receptor [156]. Collectively, these findings demonstrate that D-amino acids function as important bioactive substances in the mammalian body.

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