Amino Acids (2014) 46:1805–1818
DOI 10.1007/s00726-014-1759-2

INVITED REVIEW

Current knowledge of D-aspartate in glandular tissues

Maria Maddalena Di Fiore · Alessandra Santillo · Gabriella Chieffi Baccari

Received: 24 April 2014 / Accepted: 28 April 2014 / Published online: 17 May 2014

Abstract Free D-aspartate (D-Asp) occurs in substantial amounts in glandular tissues. This paper reviews the existing work on D-Asp in vertebrate exocrine and endocrine glands, with emphasis on functional roles. Endogenous D-Asp was detected in salivary glands. High D-Asp levels in the parotid gland during development suggest an involvement of the amino acid in the regulation of early developmental phases and/or differentiation processes. D-Asp has a prominent role in the Harderian gland, where it elicits exocrine secretion through activation of the ERK1/2 pathway. Interestingly, the increase in NOS activity associated with D-Asp administration in the Harderian gland suggests a potential capability of D-Asp to induce vasodilatation. In mammals, an increase in local concentrations of D-Asp facilitates the secretion of anterior pituitary hormones, i.e., PRL, LH and GH, whereas it inhibits the secretion of POMC/α-MSH from the intermediate pituitary and of oxytocin from the posterior pituitary. D-Asp also acts as a negative regulator for melatonin synthesis in the pineal gland. Further, D-Asp can stereo-specifically modulate the production of sex steroids, thus taking part in the endocrine control of reproductive activity. Although D-Asp receptors remain to be characterized, gene expression of NR1 and NR2 subunits of NMDAr responds to D-Asp in the testis.

M. M. Di Fiore (✉) · A. Santillo · G. Chieffi Baccari
Dipartimento di Scienze e Tecnologie Ambientali, Biologiche e Farmaceutiche, Seconda Università di Napoli, via Vivaldi 43, 80100 Caserta, Italy
e-mail: mariam.difiore@unina2.it

Keywords D-Aspartate · Salivary glands · Harderian gland · Endocrine glands · D-Aspartate oxidase · Aspartate racemase

Introduction

D-amino acids, either in free form or peptide-bound, are present in several tissues of vertebrates and invertebrates (Furuchi and Homma 2005; Homma 2007; D'Aniello 2007; Ota et al. 2012). Among these, D-aspartate (D-Asp), D-serine, D-alanine and D-glutamate are the free D-enantiomers occurring in substantial levels in mammalian tissues. D-Asp has received attention because of its presence in animal nervous and reproductive systems. D'Aniello and Giuditta (1977) first demonstrated the presence of high free D-Asp concentrations in the brain of cephalopods. Then, D-Asp was also detected in the nervous system of frog (Burrone et al. 2012a; Santillo et al. 2013), chicken, rat and man (D'Aniello 2007; Errico et al. 2012; Ota et al. 2012). D-Asp occurs at high levels in the rat embryo nervous system, whereas in adult animals it occurs at relatively low concentrations (Wolosker et al. 2000), but increases in endocrine glands, particularly the pituitary, adrenal and pineal glands, and in the gonads, where it has been suggested to play an important role as a messenger molecule (D'Aniello 2007). The literature on D-Asp counts numerous reports and reviews but attention has so far been focused on the role of this molecule in nervous and neuroendocrine systems (D'Aniello 2007; Errico et al. 2012; Ota et al. 2012). Here we review the existing work on D-Asp in vertebrate exocrine and endocrine tissues, with emphasis on functional roles and molecular mechanisms.

Occurrence and functions of D-aspartate in glandular tissues

Exocrine glands

Salivary glands

Imai et al. (1997) first demonstrated that a small level of D-Asp naturally occurs in rat salivary glands and that intravenously administered D-Asp is accumulated in this gland. In male adult rat, relatively high levels of D-Asp were found in the parotid gland and submandibular gland whereas lower level was present in the sublingual gland (Masuda et al. 2003) (Table 1). The ratio of D^- to total Asp ($D^- + L^-$) was also high in both parotid (37.9 %) and submandibular glands (30.0 %). In female rat, D-Asp levels were not more than half of those in male rats in both parotid and submandibular glands, and no D-Asp was detected in the sublingual gland (Table 1). Immunohistochemical studies showed that free D-Asp is predominantly localized in the acinous region of the parotid gland and the striated duct cells in the submandibular gland. In line with biochemical data, D-Asp immunoreactivity was found to be almost absent in the sublingual gland.

A distinct emergence pattern of D-Asp during development was observed in rat salivary glands. D-Asp concentration in the parotid gland increased transiently at 3 weeks of age, then it decreased and its level at 14 weeks was lower than in 1-week-old rat (Masuda et al. 2003). In contrast, D-Asp level in the submandibular gland gradually increased from 1 to 7 weeks of age, when the levels of the amino acid were comparable to those of the adult (Masuda et al. 2003). These results suggest that D-Asp performs different physiological functions in the parotid and submandibular glands. In the former, D-Asp could participate in the regulation of early developmental phases and/or differentiation processes. High D-Asp levels in the submandibular gland of the adult rat suggest a role of the amino acid in exocrine (saliva) and endocrine secretion (kallikreins and epidermal growth factor). In contrast to parotid and submandibular glands, D-Asp level in sublingual gland kept constantly low through the age, with only a negligible increase at 3 weeks.

D-Asp i.p. administration to 3-week-old rats induced a dramatic increase of the amino acid in all salivary glands. In contrast, D-Asp administration to 7-week-old rats elicited no change in the amino acid levels in any glands. This strongly suggests that D-Asp transfer from blood to glandular cells is present in young rats but disappears during aging (Masuda et al. 2003).

Table 1 Endogenous D-Asp concentrations in exocrine glands

	D-Asp (nmol/g tissue)
Salivary glands	
Rat male	
PG	212
SMG	233
SLG	38
Rat female	
PG	89
SMG	103
SLG	n.d.
Harderian glands	
Frog	130
Lizard	19
Rat	190

PG parotid gland, *SMG* submandibular gland, *SLG* sublingual gland

Harderian gland

High level of D-Asp has been demonstrated by HPLC in the Harderian gland (HG) of the frog *Rana esculenta* (Raucci et al. 2005a), the lizard *Podarcis s. sicula* (Santillo et al. 2006) and the rat (Monteforte et al. 2009) (Table 1). The HG is an orbital gland found in many tetrapod species that possess the nictating membrane (Di Matteo et al. 1989; Chieffi Baccari et al. 1990, 1992; Chieffi et al. 1996). Although the main role of HG is to lubricate the eye through seromucous and/or lipoprotein secretions, in rodents the gland also plays an endocrine role through melatonin production. Immunohistochemical staining with a polyclonal anti-D-Asp antibody in frog (Raucci et al. 2003) and lizard HG (Santillo et al. 2006) demonstrated D-Asp localization in the perinuclear cytoplasm of glandular cells. Numerous in vivo experiments employing i.p. D-Asp (2 μmol/g bw) administration demonstrated that the HG has the capacity to take up and accumulate the amino acid (Raucci et al. 2005a; Santillo et al. 2006; Monteforte et al. 2009; Di Giovanni et al. 2010a). Notably, in frog HG the administered amino acid accumulated at the apex of the cells beneath the plasma membrane (Raucci et al. 2003). Exogenous D-Asp appeared to modulate the secretory activity of *Rana esculenta* HG by exerting a stimulatory or inhibitory effect, respectively, when the gland had low or high secretory activity (Raucci et al. 2005a). Particularly, when secretory activity was low, i.p. administration of D-Asp induced a rapid increase in the number of cells active in RNA synthesis (Chieffi Baccari et al. 1992). The increase in transcriptional activity was followed by a significant increase in mucous secretion (Fig. 1). Consistent with activation of HG secretion was a powerful hyperaemia associated with D-Asp administration. By contrast, under condition of high secretory activity, D-Asp induced a

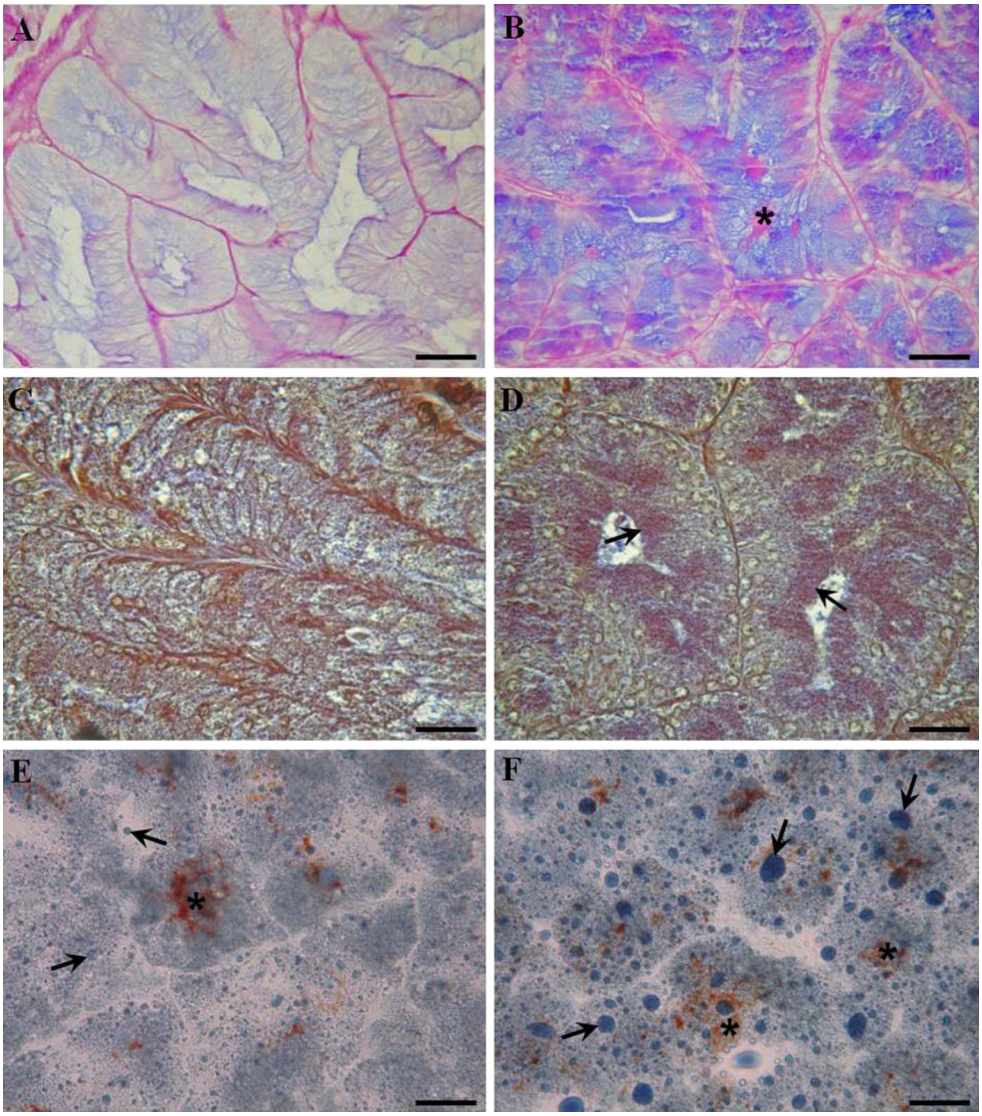

Fig. 1 **a**, **b** Paraffin sections of *R. esculenta* HG. **a** Glandular cells of a control frog are weakly Alcian blue/PAS (AB/PAS)-positive; **b** After D-Asp treatment, an increase in AB/PAS positivity in the glandular epithelium and in the lumina (*asterisk*) is observed. AB/PAS reaction. **c**, **d** Paraffin sections of the lateral part of *P. s. sicula* HG. **c**, The glandular cells of control are weakly positive to mercury bromophenol blue reaction for protein; **d** After D-Asp injection, a strong positivity for mercury bromophenol blue is observed at the apex of glandular cells (*arrows*). Mercury bromophenol blue reaction. **e**, **f** Cryostat sections from rat HG. **e** Small Sudan black-positive vacuoles (*arrows*) and porphyrin accretions (*asterisk*) are present in glandular cells of control. **f** Large vacuoles stained with Sudan black (*arrows*) are observed within and outside the acini in the D-Asp-treated HG. Note the numerous porphyrin accretions (*asterisks*). Sudan black stain. *Scale bars* 30 μm

decrease in HG transcriptional activity and a rapid reduction of mucous secretion.

The most notable effect of D-Asp administration in the *Podarcis s. sicula* HG was the increased serous secretion in the lateral part of the gland (Fig. 1), where immunohistochemical staining revealed the highest amino acid accumulation (Santillo et al. 2006). Two hours after D-Asp treatment, an increase in protein fractions between 10 and 20 kDa was observed, resulting in a total protein increase of about 60 %. D-Asp treatment induced an increase in mucous secretion also in the HG medial part, with the accumulation of highly sulfated mucosubstances (Santillo et al. 2006).

D-Asp administration in rat HG induced the rapid activation of massive lipid and porphyrin secretion (Fig. 1) with consequent increased gland levels of fatty acid peroxides and reactive oxygen species (Monteforte et al. 2009; Santillo et al. 2011). Uncoupling protein 3 (UCP3) expression strongly increased in D-Asp-treated rat HG (Santillo et al. 2011). UCP3 is a member of the subfamily

of mitochondrial anion carriers involved in the export of fatty acid peroxides and reactive oxygen species out of the mitochondria to counteract damaging effects (Chieffi Baccari et al. 2004; Santillo et al. 2008). Enhanced oxidative stress in D-Asp-treated rat HG could explain the activation of the SAPK/JNK pro-apoptotic pathway, an event most likely directed at attenuating cellular damage and preserving gland integrity (Monteforte et al. 2009).

The observed decrease in α-tubulin expression, most likely reflecting microtubule depolymerization and α-tubulin degradation induced by hydroperoxide accumulation, correlates well with an increased oxidative status (Santillo et al. 2011).

Interestingly, histochemical and electron microscopy analyses revealed a more abundant connective tissue among the glandular acini in D-Asp-treated rat HG. Such increase could be associated with the potential capability of D-Asp to stimulate the secretory activity of fibroblasts, thereby enhancing the production of extracellular matrix. D-Asp-treated rat HG also showed morphological evidence of a powerful hyperaemia (Monteforte et al. 2009).

Endocrine glands

Pituitary gland

The highest concentration of D-Asp in the pituitary gland has been found in pig (Yamamoto et al. 2010), and the lowest concentrations in the adult mouse (Topo et al. 2010) and rat (Dunlop et al. 1986; Hashimoto et al. 1993; D'Aniello et al. 1996, 2000a; Topo et al. 2009; Han et al. 2011a) (Table 2). Pituitary D-Asp levels in the rat were reported to increase gradually from 2 days to 8 weeks of age (Dunlop et al. 1986; Hashimoto et al. 1995). Mutant mice (Ddo −/−) with targeted deletion of Ddo showed pronounced increases of immunoreactive D-Asp in the pituitary intermediate lobe, normally devoid of D-Asp from endogenous Ddo expression (Huang et al. 2006). Immunohistochemical analysis demonstrated that in rats D-Asp was concentrated in the posterior lobe, with few stained cells widely scattered in the intermediate and anterior lobes (Schell et al. 1997; Lee et al. 1999). In the posterior lobe, naturally occurring free D-Asp was detected in the pericytes and in the heterochromatin beneath the nuclear envelope of microglia cells; moreover, extremely intense staining was observed in nerve processes and terminals, which derive primarily from the supraoptic and paraventricular nuclei of the hypothalamus (Wang et al. 2002). These observations are in line with the report of intense and highly localized staining for D-Asp in magnocellular neurons of both nuclei (Schell et al. 1997). Injection of D-Asp into the hypothalamus for 7 days induced significantly higher levels of oxytocin mRNA, thus indicating that D-Asp participates in the control of oxytocin synthesis and secretion in vivo (Wang et al. 2000) (Table 3). D-Asp also increases levels of vasopressin mRNA and could have a general role in the modulation of gene expression or hormone production (Wang et al. 2000). On the other hand, it is well known that D-Asp is involved in the regulation of neurosecretion from hypothalamic nerve terminals in the posterior pituitary to the systemic circulation in mammals. Further details and references on this topic can be found in recent papers significantly dedicated to the role of D-Asp in the neurosecretion (D'Aniello 2007; Ota et al. 2012).

In the anterior lobe, intense immunoreactivity was found throughout the lobe, being specifically localized in prolactin-containing cells or cell types closely related to these (Lee et al. 1997, 1999, 2001). Both D-Asp levels and prolactin-producing cells were more abundant in females than

Table 2 Endogenous D-Asp concentrations in vertebrate endocrine glands

	D-Asp (nmol/g tissue)
Pituitary gland	
Mouse	100
Rat	100–132
Pig	270–350/protein
Pineal gland	
Rat	130–3524
Pig	270–350/protein
Thyroid	
Mouse	100
Rat	90–105
Adrenal glands	
Chicken	23–30
Mouse	Very low
Rat	200–574
Pancreas	
Pigeon	15–18
Chicken	6–10
Rat	20
Ovary	
Frog	3–58
Lizard	2–8
Mouse	Very low
Rat	30
Human	11–19 (*f.f.*)
Testis	
Frog	140–236
Lizard	3–30
Duck	11–23
Mouse	140
Rat	129–220
Pig	22
Boar	40

f.f. follicular fluid

Table 3 Serum hormone concentrations before and after D-Asp treatment

	Hormones	Concentration (ng/ml serum)	
		Endogenous	After D-Asp treatment[a]
Rat	Oxytocin	520	158
	PRL	11	40
	LH	4	9
	GH	31	82
	TSH	6	8
	T_4	50	62
	T_3	1	1
	Progesterone	10	30
	Testosterone	5	18
	17β-estradiol	2	3
Sheep	LH	0.5	4
Human	LH	4	6
	Testosterone	4	6

[a] In the table are reported the maximum values of serum hormone levels after D-Asp treatment. The reader can find all details in relative papers

males (Lee et al. 1999). D-Asp levels in the pituitary gland were enhanced by estrogen implantation, which in turn increased the number of prolactin-producing cells (Lee et al. 1999).

The presence and synthesis of D-Asp have also been demonstrated in the cytoplasm of a prolactin (PRL) secreting clonal strain of rat pituitary tumor cells (GH3) (Long et al. 2000). Further, TRH-stimulated PRL secretion by these cells was increased in a dose-dependent fashion by D-Asp. When anterior pituitary cells were cultured in the presence of posterior pituitary cells, D-Asp increased PRL secretion and decreased GABA release in these co-cultures (Pampillo et al. 2002). The data suggest that D-Asp could stimulate PRL release directly, through NMDA receptors (NMDAr), or indirectly, by decreasing GABA release from the posterior pituitary. However, contrasting results by Lee et al. (2001) demonstrated that exogenous D-Asp accumulated in endothelial cells but not in PRL-containing cells of the pituitary gland. Northern and Western blot analyses and immunohistochemistry showed that developmental changes in Glu transporter expression did not correlate with tissue levels of D-Asp and that the Glu transporter was not expressed in PRL-containing cells. Therefore, in contrast to other endocrine tissues, most of the D-Asp in the pituitary gland of adult rats should originate within the gland itself.

D-Asp administration to rat and sheep resulted in a significant uptake of the amino acid by the pituitary gland, followed by an increase in luteinizing hormone levels (D'Aniello et al. 1996, 2000a; Boni et al. 2006) (Table 3). The release of luteinizing hormone following D-Asp treatment has also been reported in humans (Topo et al. 2009). In addition, in rats exogenous D-Asp induced a significant, dose- and time-dependent serum prolactin release (D'Aniello et al. 2000b) and a rise in growth hormone level whereas it did not significantly affect thyroid stimulating hormone (D'Aniello et al. 2000a) (Table 3). However, in vitro experiments showed that D-Asp was able to induce luteinizing hormone release from adenohypophysis only when this gland was co-incubated with the hypothalamus. This is because D-Asp also induces the release of gonadotropin releasing hormone (GnRH) from the hypothalamus, which in turn is directly responsible for the D-Asp-induced luteinizing hormone secretion from the pituitary gland (D'Aniello et al. 2000a).

Negligible levels of D-Asp have been reported for the intermediate pituitary lobe (IL) (Schell et al. 1997; Lee et al. 1999). IL contains almost exclusively melanotropes, which generate proopiomelanocortin (POMC) as the sole source of pituitary α-MSH, a member of the melanocortin peptide family (Hadley and Haskell-Luevano 1999). Elevated D-Asp levels in IL of Ddo −/− (mice with targeted deletion of D-aspartate oxidase) led to diminished POMC/α-MSH and melanocortin-dependent behaviors, thereby firmly pointing to D-Asp as a mediator of physiologic functions (Huang et al. 2006). D-Asp presumably decreases pituitary α-MSH levels by regulating POMC biosynthesis, which then leads to alterations in behaviors known to be mediated by α-MSH.

Pineal gland

High D-Asp levels have been detected in the pineal gland of the rat (Hamase et al. 1997; Schell et al. 1997; Topo et al. 2010; Han et al. 2011a, b) and pig (Yamamoto et al. 2010). Low D-Asp levels have been demonstrated in the pineal gland of different strains of mice (Han et al. 2011b). D-Asp levels in rat were relatively low at 2 weeks of age, increased significantly from 4 to 10 weeks, and then gradually decreased up to 36 weeks (Imai et al. 1995). Imai et al. (1995) reported that in 6-week-old rats the concentration of pineal D-Asp did not differ between the sexes and was higher at night (at 2.00 a.m. 2,830 ± 485 pmol per pineal gland) than during the day (at 10.00 a.m. 1,030 ± 200 and at 3:00 p.m. 682 ± 194 pmol per pineal gland), suggesting that the biosynthesis of D-Asp in the pineal gland occurs at night. Variations in pineal D-Asp levels with no daily rhythm were observed in male littermates trained on a 12-h light/dark cycle for 3 weeks, possibly reflecting a shorter ultradian cycle or simply random fluctuations (Schell et al. 1997). By contrast, L-Asp levels displayed a daily rhythmicity, with levels doubling during the dark phase.

Immunohistochemical staining of the rat pineal gland evidenced D-Asp in the cytoplasm of pinealocytes, the parenchymal cells of the pineal gland, sometimes with

higher levels in islands of cells near blood vessels (Schell et al. 1997). D-Asp level was higher in the distal (caudal) than proximal (rostral) area of the gland (Lee et al. 1997). Since pinealocytes in the distal area are closely involved in the synthesis and secretion of melatonin, D-Asp distribution seems to be consistent with involvement of the amino acid in the regulation of melatonin secretion.

D-Asp administered intra-peritoneally or intravenously is incorporated into the pineal gland of rat (Imai et al. 1997) and sheep (Boni et al. 2006). Intense staining with anti-D-Asp antibody was evident in 15 min throughout the cytoplasm of rat pinealocytes (Schell et al. 1997). The L-Glu transporter, known to occur in the rat pineal gland, may be responsible for D-Asp uptake (Yamada et al. 1997).

Cultured rat pinealocytes contained D-Asp in their cytoplasm, for an amount corresponding to $\sim$30 % of the total free aspartate (Yatsushiro et al. 1997). D-Asp was efficiently taken up into cells, in a time- and dose-dependent manner (Takigawa et al. 1998). Interestingly, L-Asp levels in the cells and culture medium decreased in parallel with the uptake of D-Asp (Takigawa et al. 1998). Rat pinealocytes pre-treated with D-Asp released D-Asp as well as L-Asp in response to norepinephrine stimulation, whereas norepinephrine-induced secretion of melatonin was suppressed (Takigawa et al. 1998). In agreement with these observations, Ishio et al. (1998) reported that exogenous D-Asp strongly inhibited norepinephrine-dependent melatonin synthesis in the rat pineal gland, through the inhibitory cAMP cascade, possibly reflecting a reduction of norepinephrine-dependent serotonin *N*-acetyltransferase activity. The body of information now available suggests that D-Asp acts as a negative regulator for melatonin synthesis in the pineal gland.

Thyroid

D-Asp concentration has been determined in mammalian thyroid gland (Boni et al. 2006; Topo et al. 2009, 2010) (Table 2). In rat and sheep the amino acid levels underwent a threefold increase after D-Asp administration (Boni et al. 2006; Topo et al. 2009). In rat, serum levels of T_4 and T_3 were not significantly affected by D-Asp administration (D'Aniello et al. 2000a) (Table 3).

Adrenal gland

The highest concentration of D-Asp in the adrenal gland has been found in rat (Hashimoto et al. 1993, 1995; Han et al. 2011a), the lowest concentrations in mouse (Huang et al. 2006) and chicken (Kera et al. 1996) (Table 2). In both 4-week-old and 4-month-old Ddo −/− mice, D-Asp levels increased 25- to 300-fold in adrenals (Huang et al. 2006).

D-Asp levels in rat adrenal gland have been shown to undergo a transient increase at 3 weeks of age, followed by a decrease and stabilization at an adult level after 8 weeks (Hashimoto et al. 1995). An immunocytochemical study by Sakai et al. (1997) described developmental changes in cellular localization of D-Asp in rat adrenal. At 1–3 weeks of age, immunoreactivity was intense in the cytoplasm of cells in the zona fasciculata and zona reticularis of the adrenal cortex and relatively weak in the zona glomerulosa. Conversely, at 8 weeks of age, immunoreactivity showed an opposite pattern, with a more intense staining in the zona glomerulosa than in the zona fasciculata and zona reticularis. In the adrenal medulla, a positive reaction to the antibody was observed in large clusters of cells identified as adrenaline-storing cells. The emergence of D-Asp in specific types of cells at distinct periods of development of rat adrenal gland suggests that this amino acid has a physiological role in the maturation of the organ (Sakai et al. 1997). Schell et al. (1997) found that in the adrenal gland D-Asp was selectively concentrated in chromaffin epinephrine-producing cells, whereas D-Asp oxidase activity was detected in norepinephrine cells. These findings were in partial agreement with results by Sakai et al. (1997).

Male mice lacking the gene for D-AspO have D-Asp immunoreactive cells in the adrenal gland that are not observed in wild-type mice (Weil et al. 2006).

Lee et al. (2001) found that exogenous D-Asp administered i.p. was incorporated into the same region of the adrenal cortex in which endogenous D-Asp was present. The glutamate transporter transiently increased at 3 weeks of age and its localization pattern in the gland tissue closely mirrored that of endogenous D-Asp, thereby suggesting that D-Asp in 3-week-old rats is primarily acquired by uptake from the vascular system (Imai et al. 1997; Lee et al. 2001).

In the rat adrenal medulla, D-Asp is depleted by intraperitoneal nicotine injections (Wolosker et al. 2000). In adrenal slices, D-Asp is released by depolarization with KCl or acetylcholine, implying physiological release by activation of the cholinergic innervation of the adrenal.

Interestingly, rat pheochromocytoma PC12 cells contained detectable amounts of D-Asp (257 ± 31 pmol/10^7 cells) (Nakatsuka et al. 2001). Since PC12 cells are deficient in the Glu transporter necessary for D-Asp uptake, this amino acid could be constitutively secreted from PC12 cells by a distinct mechanism that does not involve reversed uptake through the transporter (Long et al. 1998, 2002; Moriyama et al. 1998). PC12 cells store D-Asp in dopamine-containing secretory granules and secrete it through a Ca^{2+}-dependent exocytotic mechanism (Nakatsuka et al. 2001). Exocytosis of D-Asp further supports the functioning of this molecule as a chemical transmitter in neuroendocrine cells. D-Asp is also synthesized in MPT1 cells (a subclone of PC12 cells) and D-Asp levels respond to the cell density of the culture (Long et al. 2002).

Pancreas

D-Asp was detected in the pancreas of pigeon, chicken (Kera et al. 1996) and rat (Hashimoto et al. 1993; Han et al. 2011a) (Table 2). The content of free D-Asp was significantly different between genders in the chicken (Kera et al. 1996).

Immunohistochemical analysis showed that D-Asp was present in all cells of rat Langerhans islets, with higher levels in α cells and a subpopulation of F cells (Hiasa and Moriyama 2006). Consistently, anti-glutamate/aspartate transporter immunoreactivity showed that the rat islets glutamate/aspartate transport activity was localized to the non-β cell islet mantle (Weaver et al. 1998). Quantitatively, the content of D-Asp was 0.13 pmol/islet which is about 8 % of total aspartate in the islet (Iharada et al. 2007).

Interestingly, INS-1 E clonal β cells also contain D-Asp (1.50 ± 0.48 nmol/10^7 cells), which accounts for about 2 % of total cellular free aspartate. Immunohistochemistry indicated that the amino acid was localized in insulin-containing secretory granules and co-secreted with insulin by exocytosis (Iharada et al. 2007). Cell swelling, induced by hyposmotic shock, increased the fractional release of D-Asp from INS-1 cells (Grant et al. 2000).

Ovary

Free D-Asp has been detected in the ovary of the frog, *R. esculenta* (Di Fiore et al. 1998), lizard, *P. s. sicula* (Assisi et al. 2001) and rat (Hashimoto et al. 1993); very low levels of D-Asp have been found in the ovary of mouse (Huang et al. 2006) (Table 2). In Ddo −/− mice ovary D-Asp level was about 1,000 nmol/g (Huang et al. 2006). In the sheep D-Asp administration induced an increase of serum luteinizing hormone levels, suggesting a role of D-Asp in the ovarian cycle of the sheep (Boni et al. 2006) (Table 3).

In seasonally breeding vertebrates, i.e., the green frog *R. esculenta* and the lizard *P. s. sicula*, an inverse correlation has been demonstrated between D-Asp ovary concentration and serum/ovary testosterone levels (Di Fiore et al. 1998; Assisi et al. 2001). It is well known that, in *R. esculenta*, serum testosterone levels are higher in the female than the male. Testosterone levels are higher during the recovery period and the early phases of the reproductive period, whereas in concomitance D-Asp levels are very low. In vivo and in vitro experiments demonstrated that exogenous D-Asp accumulated in the ovary and induced a decrease of testosterone levels (Di Fiore et al. 1998). D-Asp, chronically administered to female frogs, enhanced the maturation of ovaries and the accumulation of carbohydrate yolk plates in the ooplasm (Raucci and Di Fiore 2011).

In the lizard *P. s. sicula*, D-Asp ovarian concentrations were found to vary significantly during the reproductive cycle, being inversely correlated with testosterone levels and directly with oestradiol levels in the ovary and plasma (Assisi et al. 2001). In vivo and in vitro experiments demonstrated that exogenous D-Asp induced an increase of aromatase activity (Assisi et al. 2001). Aromatase is the key enzyme which converts testosterone into 17β-estradiol (Simpson et al. 1994).

An immunohistochemical study demonstrated that D-Asp is localized in pre-vitellogenetic pyriform cells, intermediate cells, some cells of the granulose and some thecal elements of lizard *Podarcis sicula* (Raucci and Di Fiore 2010). During vitellogenesis, D-Asp is localized in the proximity of the zona pellucida, in the theca, and in the ooplasm. Therefore, D-Asp may be related to the synchrony of reproduction, either enhancing the growth and maturation of follicular epithelium or influencing its endocrine functions (Raucci and Di Fiore 2010).

D'Aniello et al. (2007) reported the presence of endogenous D-Asp in pre-ovulatory human ovarian follicular fluid (Table 2). D-Asp concentration seems to be related to the patient's age and the quality of oocytes since it occurs at higher concentrations in younger women (19.11 ± 1.91 nmol/ml) than in older women (10.86 ± 1.22 nmol/ml). Further, a relationship has been suggested between D-Asp concentration and fertility outcome parameters (D'Aniello et al. 2007).

Testis

Free D-Asp has been detected in the testes of the frog, *R. esculenta* (Raucci et al. 2004), lizard, *P. s. sicula* (Raucci et al. 2005b), duck, *Anas platyrhynchos* (Di Fiore et al. 2008), mouse (Topo et al. 2010; Han et al. 2011b), rat (Hashimoto et al. 1993; Han et al. 2011a, b), pig (Kato et al. 2012) and boar (Lamanna et al. 2006, 2007a, b) (Table 2). In Ddo −/− mice, D-Asp concentration was twofold higher than that in wild type (Huang et al. 2006).

With the use of an antibody against D-Asp, the amino acid was prevalently localized in Leydig cells, sertoli cells and germ cells, particularly in spermatogonia, elongate spermatids and spermatozoa in several vertebrate species (D'Aniello et al. 1996, 2005; Sakai et al. 1998; Raucci and Di Fiore 2009; Lamanna et al. 2006, 2007a, b; Di Fiore et al. 2008; Santillo et al. 2014).

A detailed biochemical analysis of the different testis compartments carried out by D'Aniello et al. (1998) demonstrated that the highest concentrations of D-Asp (about 120 nmol/ml) were found in testicular venous blood plasma, with slightly lower concentrations in the rete testis

fluid (95 nmol/ml) and epididymal spermatozoa (80 nmol/g wet weight). Lower levels were found in testicular parenchymal cells, luminal fluid from the seminiferous tubules, and interstitial extracellular fluid (26, 23 and 11 nmol/ml, respectively). However, these values were all higher than in peripheral blood plasma (6 nmol/ml). Therefore, the authors hypothesized that, since D-Asp is poured by testis mostly into the venous blood of the interstitial compartment, it is possible that through the rete testis fluid it would be incorporated into the spermatozoa.

D'Aniello et al. (1996) observed a strong correlation between D-Asp concentration and testosterone levels in rat testes throughout life. The testes of embryonic rats start synthesizing D-Asp (55 ± 8 nmol/g) and testosterone (200 ± 30 ng/g) in the later part of the fetal life. At birth, both D-Asp and testosterone levels were very low and increased progressively, reaching maximum levels at sexual maturity (D'Aniello et al. 1996; Sakai et al. 1998). At 80 days after birth, the concentration of D-Asp and testosterone in testes was about 150–200 nmol/g and 380 ± 40 ng/g, respectively (D'Aniello et al. 2000a, b).

When D-Asp (2.0 μmol/g body weight) was i.p. administered to adult rats, it accumulated in the testis at high concentration (D'Aniello et al. 2000a, b). At 2 and 5 h, D-Asp concentration was about fourfold higher than in controls (D'Aniello et al. 2000a, b). When the animals were allowed to drink 20 mM sodium D-Asp for 12 days, D-Asp concentration in rat testes were about seven times higher (Topo et al. 2009; Santillo et al. 2014). Both intra-peritoneal and oral D-Asp administration to adult rats induced an increase of serum progesterone and luteinizing hormone as well as testis/serum testosterone levels, whereas 17β-estradiol levels did no change (D'Aniello et al. 1996, 2000a, b; Topo et al. 2009; Santillo et al. 2014) (Table 3). Furthermore, exogenous D-Asp induced up-regulation of androgen receptor and down-regulation of estrogen receptor expression (Santillo et al. 2014).

However, Chandrashekar and Muralidhara (2010) demonstrated that the administration of massive amounts of D-Asp (100 and 500 mg/kg bw/d, i.p. 7 days) to prepuberty rats may induce oxidative imbalance in testis.

Topo et al. (2009) reported that the D-Asp administration in human enhanced serum luteinizing hormone and testosterone levels (Table 3). Furthermore, treatment of subfertile humans with sodium D-Asp improved the number and motility of spermatozoa (D'Aniello et al. 2012). Macchia et al. (2010) reported that DL-Asp administration improved sperm quality in bucks, with high levels of D-Asp in seminal plasma suggesting a primary role for this D-amino acid in reproductive activity.

In vitro experiments performed on testicular homogenates of boar incubated with D-Asp demonstrated that D-Asp induced testosterone release as well as 17β-estradiol synthesis through aromatase activation (Lamanna et al. 2006, 2007a). Further, it has been found that the addition of D-Asp (0.2 mM) to rat-isolated testes induced testosterone but not progesterone synthesis (D'Aniello et al. 1996).

Specific stimulation of testosterone synthesis by D-Asp in purified rat Leydig cells was demonstrated by Nagata and co-workers (1999a). Leydig cells were cultured for different time intervals with D-Asp (200 μM) in the presence or absence of human chorionic gonadotropin (hCG) (5 mIU/ml). D-Asp and hCG acted synergically to increase testosterone production, and D-Asp stimulated testosterone synthesis even in the absence of hCG stimulation (Nagata et al. 1999a). Notably, D-Asp increased testosterone production by stimulating gene and protein expression of steroidogenic acute regulatory (StAR), which is a key regulatory factor of cholesterol translocation to the inner mitochondrial membrane (Nagata et al. 1999b).

Experiments carried out on isolated rat hypothalamus showed that D-Asp was able to induce the release of GnRH, which in turn elicited luteinizing hormone secretion from the pituitary gland (D'Aniello et al. 2000a). The bulk of evidence from in vivo and in vitro studies suggests that the D-Asp might induce testosterone production by acting either directly on Leydig cells or indirectly on the hypothalamus–pituitary–testis axis.

Studies on seasonal-breeding vertebrates have confirmed the involvement of D-Asp in the endocrine control of reproductive activity. Because of their cyclic pattern of reproductive activity, seasonal breeders are good models for studying the effect of D-Asp on the testis. In both frog, *R. esculenta*, and lizard, *P. s. sicula*, D-Asp concentration in the testis showed significant variations during the reproductive cycle with the highest levels in sexually active animals (Raucci et al. 2004, 2005b; Raucci and Di Fiore 2009, 2011). D-Asp content in the testis is directly linked with gonadal and plasmatic levels of testosterone. Intra-peritoneal injection of D-Asp (2.0 μmol/g body weight) induced a rise of testosterone and a fall in 17β-estradiol in frog and lizard, both in pre-reproductive and post-reproductive phases (Raucci et al. 2004, 2005b; Raucci and Di Fiore 2009, 2011). Exogenous D-Asp in reproductive frogs increased 17β-estradiol levels (Raucci et al. 2004; Burrone et al. 2012b).

Increased spermatogonial mitotic activity has been reported in the testis of D-Asp-treated frog (Raucci et al. 2004) and lizard prevalently in post-reproductive phase (Raucci et al. 2005b).

In the testis of the duck, *A. platyrhynchos,* D-Asp concentration was higher in the reproductive than non-reproductive phase, paralleling similar fluctuations in testicular

testosterone levels (Di Fiore et al. 2008). When slices of *A. platyrhynchos* testes were incubated in a medium containing D-Asp, a significant increase of testosterone was observed in the culture medium.

Interestingly, high D-Asp levels have been found in the gonads of *Ciona intestinalis*, a marine protochordate which shares common ancestry with vertebrates (D'Aniello et al. 2003).

Aspartate racemase and D-aspartate oxidase in glandular tissues

The occurrence in glandular tissues of an aspartate racemase, a pyridoxal 5′-phosphatase-dependent enzyme that converts L-Asp to D-Asp, strongly suggests that D-Asp is synthesized endogenously where it is needed. Aspartate racemase activity has been reported for rat pituitary (Wolosker et al. 2000; Topo et al. 2009) and thyroid (Topo et al. 2010) as well as in the testis of frog *R. esculenta* (Raucci et al. 2004), lizard *P. s. sicula* (Raucci et al. 2005b) and rat (Topo et al. 2009). An aspartate racemase that specifically generates D-Asp using only L-Asp as a substrate is present in the Harderian gland of both frog (Raucci et al. 2005a) and rat (Monteforte et al. 2009).

D-Asp oxidase (D-AspO, Ddo; EC 1.4.3.1) is a flavin-dependent enzyme that catalyzes the oxidative deamination of D-Asp to produce oxaloacetate, ammonia and hydrogen peroxide. Localizations of D-AspO are reciprocal to D-Asp, suggesting that the enzyme depletes endogenous stores of the amino acid. Therefore, D-AspO-enriched glandular tissues, low in D-Asp, may represent areas of high turnover where D-Asp may be physiologically important. Mutant mice (Ddo −/−) with targeted deletion of Ddo showed selective increase of D-Asp levels in numerous glandular tissues, i.e., adrenal gland, testis and ovary (Huang et al. 2006). The pituitary intermediate lobe, normally devoid of D-Asp from endogenous Ddo expression, manifests pronounced increases of immunoreactive D-Asp in Ddo −/− mice (Huang et al. 2006). D-AspO is present in the frog Harderian gland (Di Giovanni et al. 2010a) as well as in rat salivary glands (Osamura 2000). Specifically, D-AspO was located in the peroxisomal membrane or core and the enzyme activities were stronger in submandibular and sublingual glands than in the parotid gland. Among endocrine glands, D-AspO activity is expressed at highest levels in the pituitary gland, exclusively in the intermediate lobe, with staining concentrated in the outermost cells adjacent to the anterior lobe in Ddo −/− mice (Huang et al. 2006). In porcine pituitary gland, almost all ACTH and POMC-positive cells (corticotrophs and melanotrophs) express D-AspO whereas the other hormone-secreting cells, except a subgroup of thyrotrophs, had no detectable levels (Yamamoto et al. 2010). Using immunohistochemistry, Schell et al. (1997) described an inverse localization of D-AspO to endogenous D-Asp in the rat pineal gland. The adrenal gland apparently contains very low levels of D-AspO activity (Schell et al. 1997). Frog testis contains D-AspO, and specifically expresses D-AspO activity in response to D-Asp (Burrone et al. 2010; Di Giovanni et al. 2010a).

Finally, endogenous D-Asp, D-AspO, and D-aspartate racemase have been described by Topo et al. (2010) in rat thyroid gland. Since production of H_2O_2 in the thyroid gland is by oxidation of endogenous D-Asp by D-AspO, and D-Asp racemase catalyzes the in vivo production of D-Asp from L-Asp, interaction of endogenous D-Asp, D-AspO and D-aspartate racemase in thyroid gland provides an additional biochemical pathway for the production of H_2O_2 and consequently for the synthesis of thyroid hormones (Topo et al. 2010).

Molecular pathways elicited by D-aspartate

Harderian gland

Numerous studies reported that D-Asp is recognized by receptors for NMDA, as are L-Asp and L-Glu (D'Aniello 2007). Di Giovanni et al. (2010b) reported the presence of putative glutamate-binding sites of NMDA type in the rat HG; particularly, NR1-NR2A-NR2B-NR2D subunits were expressed (Monteforte et al. 2009). When activated, the NMDA receptor opens a channel that allows Ca^{2+} to move into the cell. It has been proved that D-Asp treatment markedly increases ERK activity in rat (Monteforte et al. 2009) and frog HG (Raucci et al. 2005a) (Fig. 2). In frog, at a time when the gland shows relatively low secretory activity, i.p. administration of D-Asp rapidly induces the activation of ERK1 and an increase in the number of cells active in RNA synthesis (Chieffi Baccari et al. 1992). The rise in transcriptional activity was followed by a significant augmentation of mucous secretion. By contrast, administration of exogenous D-Asp when HG was showing high activity rapidly inhibited both ERK1 and transcriptional activity. In addition, D-Asp administration elicited an increase in NOS activity as well as of cGMP levels in the rat (Fig. 2). NOS activity reflects the NO levels. NO is an endothelial-derived relaxing factor which diffuses in smooth muscle cells by activating a guanylyl cyclase that, in turn, produces cGMP from GTP with consequent muscle relaxation and vasodilatation. Consistently, D-Asp-treated HG showed morphological evidence of a powerful hyperaemia (Raucci et al. 2005a; Monteforte et al. 2009). Furthermore, Monteforte et al. (2009) demonstrated that D-Asp administration activated cAMP pathways in the rat HG (Fig. 2).

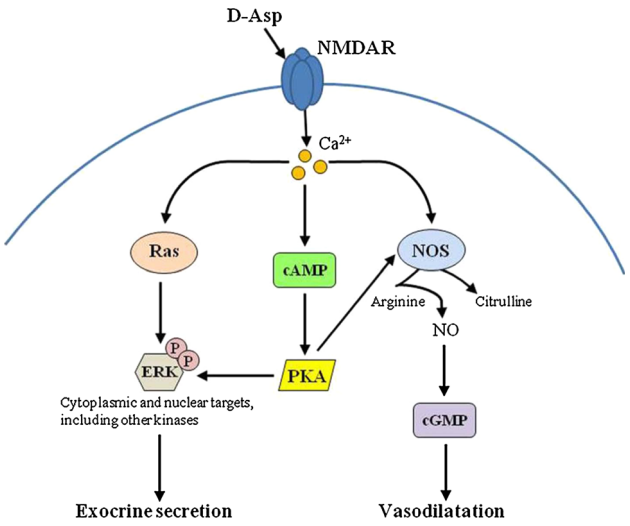

Fig. 2 Schematic representation of molecular pathways activated by D-Asp in the HG. D-Asp is recognized by NMDAR whose activation allows the entry of Ca^{2+}. The resulting increase of intracellular Ca^{2+} concentration induces the phosphorylation of ERK protein; ERK is also phosphorylated by cAMP-activated protein kinase A (PKA). Exocrine secretion could be activated by both pathways. Furthermore, D-Asp administration elicits an increase in NOS activity, reflecting the levels of NO, which activates a guanylyl cyclase to produce cGMP from GTP, with consequent vasodilatation

Testis

Although D-Asp receptors remain to be characterized, gene expression of NR1 and NR2 subunits of NMDAr responds to D-Asp in the rat testis. NR1 immunoreactivity has been detected in both interstitium and spermatogonia (Santillo et al. 2014) (Fig. 3). In vivo and in vitro studies have demonstrated that D-Asp up-regulates testosterone production in rat Leydig cells by increasing cAMP levels and activating StAR protein and gene expressions (Nagata et al. 1999a, b; Topo et al. 2009; Burrone et al. 2012a) (Fig. 3). Furthermore, D-Asp affected the gene expression of other key enzymes involved in the steroidogenic pathway, i.e., 5α-reductase, which converts testosterone into 5α-dihydrotestosterone (DHT), a more potent androgen, as well as cytochrome P450 aromatase, which converts testosterone into 17β-estradiol (Fig. 3). On the other hand, D-Asp affects 17β-estradiol levels, by influencing P450 aromatase activity in boar and frog testis (Lamanna et al. 2006, 2007a; Burrone et al. 2012b). In rat testis, D-Asp treatment enhances the androgen receptor expression (Santillo et al. 2014) (Fig. 3).

Santillo et al. (2014) demonstrated that D-Asp treatment increased the expression of both NR1 and NR2A subunits of NMDAr in rat testis, resulting in an increase of ERK1/2

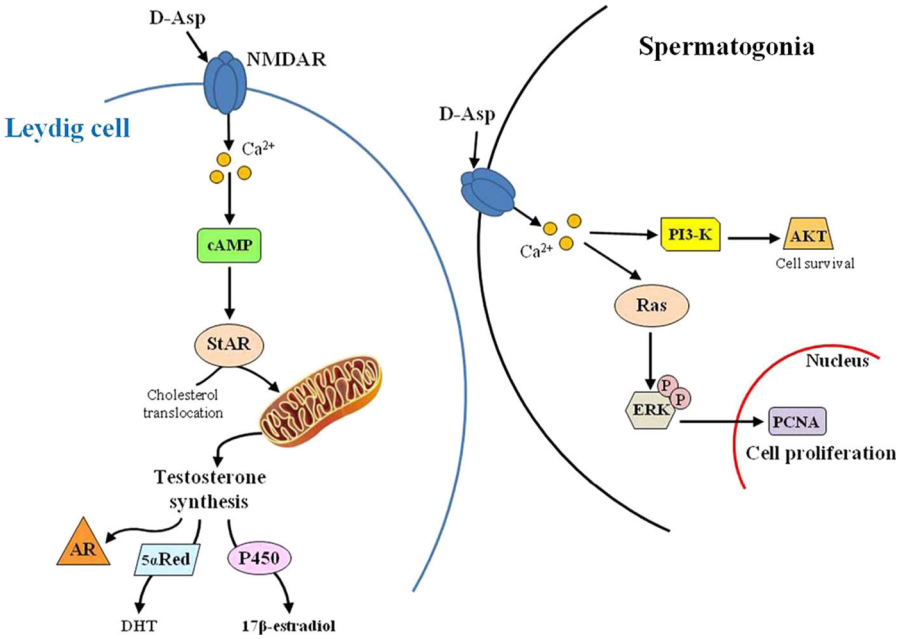

Fig. 3 Schematic representation of molecular pathways activated by D-Asp in the Leydig cells and spermatogonia. Through the activation of NMDAR, D-Asp up-regulates testosterone production in Leydig cells by increasing StAR expression through cAMP. StAR is needed for the translocation of the cholesterol to the inner mitochondrial membrane and to synthesize testosterone (T). The increased T levels determine up-regulation of androgen receptor (AR) gene expressions. D-Asp up-regulates also (1) 5α-reductase (5αRed), which converts T into 5α-dihydrotestosterone (DHT), and (2) cytochrome P450 aromatase (P450), which converts testosterone into 17β-estradiol. In parallel, D-Asp treatment induces spermatogonia proliferation by activation of ERK–proliferation cell nuclear antigen (PCNA) pathway. Further, D-Asp activates PI3-K/AKT, a cell survival pathway

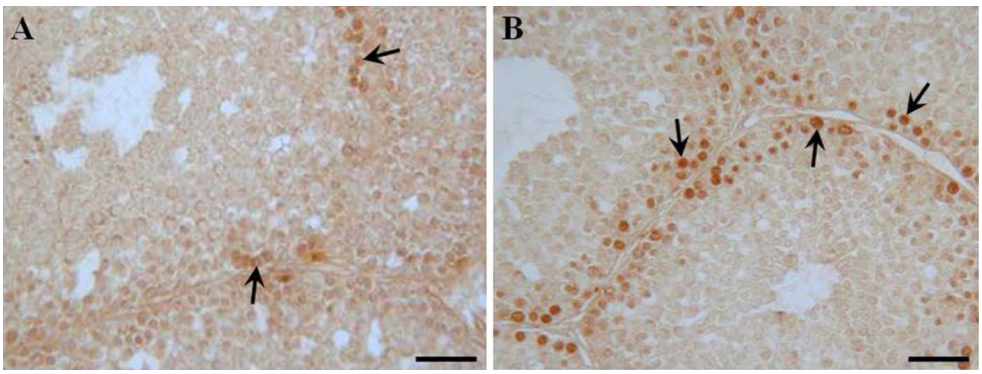

Fig. 4 **a** Immunohistochemistry for PCNA in the testis of *P. s. sicula*, during pre-reproductive period. **a** A weak immunopositivity is observed in the nuclei of the spermatogonia in control testis (*arrows*). **b** After D-Asp treatment, numerous spermatogonia show a strong immunopositivity for PCNA. *Scale bars* 30 μm

phosphorylation. Immunohistochemical studies revealed that both NR1 subunit and P-ERK1/2 protein are prevalently localized in the spermatogonia. These findings suggest that D-Asp could be involved in both steroidogenesis and spermatogenesis through NMDAR activation (Fig. 3). There is strong evidence of D-Asp direct participation in spermatogenesis. In vivo D-Asp administration induced an increase of c-kit receptor expression and of tyrosine kinase activity in the spermatogonia of *P. s. sicula* (Raucci and Di Fiore 2009). It is well known that stem cell factor/c-kit signal stimulates spermatogonial proliferation (Rossi et al. 1991). An intense immunopositivity for the proliferation cell nuclear antigen (PCNA), a mitotic activity marker, was observed in *R. esculenta* (Raucci et al. 2004) and *P. s. sicula* spermatogonia following D-Asp treatment (Raucci et al. 2005b; Raucci and Di Fiore 2009) (Figs. 3, 4).

The direct involvement of D-Asp in spermatogenesis is strongly supported by in vitro studies on a mouse spermatogonia cell line (GC-1spg), showing that the amino acid activated ERK and PCNA as well as AKT, a survival cellular pathway (unpublished data) (Fig. 3).

Conclusions and future perspectives

Glands are likely candidates for modulation by D-Asp, since they contain the highest tissue levels of D-Asp and possess systems of biosynthesis and degradation of this amino acid. In contrast to substantial evidence for a major role of D-Asp in the activity of endocrine glands, the role of this amino acid in exocrine glands remains little documented. High D-Asp levels in the parotid gland during development suggest involvement in the regulation of early developmental phases and/or differentiation processes. Studies of the Harderian gland have unveiled a prominent role in modulating exocrine secretion through activation/inhibition of the ERK1/2 pathway. Further, the increase in NOS activity elicited by D-Asp administration in the Harderian gland suggests a potential capability to induce vasodilatation. If demonstrated in other tissue, this property might be of paramount interest for potential pharmacological application.

The bulk of information available strongly points to D-Asp as a physiological modulator of the neuroendocrine system and reproductive activity. It acts as an excitatory molecule inducing the release of hormones by the anterior pituitary gland (LH, PRL, GH) but, at the same time, it exhibits a inhibitory effect on hormone release by the intermediate (MSH) and posterior pituitary (oxytocin) and the pineal gland (melatonin).

As for any new field of investigation, open questions are by far more numerous than answers. Which are the molecular mechanisms evoked by D-Asp for modulating endocrine secretion? Which is the role of D-Asp in the thyroid, adrenal gland and pancreas?

The greatest efforts have so far been focused on the function of D-Asp in the gonads. Studies carried out in mammals and in seasonal-breeding vertebrates demonstrated that D-Asp can stereo-specifically regulate steroidogenesis. D-Asp increases T production directly by stimulating protein expression of StAR in Leydig cells and/or indirectly through the hypothalamus–pituitary–testis axis by inducing GnRH release. Further, D-Asp is involved in dihydrotestosterone (DHT) and 17β-estradiol synthesis through 5αRed and P-450 aromatase activation, respectively. In line with these data, it has been suggested that neuro-steroid biosynthesis is a possible target for neuronal D-Asp. Preliminary approaches to the topic appear to be promising, notably the recent demonstration by Burrone et al. (2012a), which D-Asp enhances brain aromatase expression through the CREB pathway, with consequent production of 17β-estradiol from testosterone. Taken together, these data strongly support a prominent role of D-Asp in the endocrine control of reproductive activity.

Noteworthy, preliminary in vitro studies demonstrated that D-Asp directly affects spermatogenesis by activation of spermatogonial proliferation through the ERK-PCNA pathway. Recently, Talevi et al. (2013) demonstrated that D-Asp exerts a direct protective effect on human spermatozoa by preventing the reduction of motility, DNA fragmentation and lipid peroxidation. However, a great effort is still needed to understand the in vivo role of D-Asp in spermatogenesis and the potential value of this molecule in the male fertility.

Conflict of interest The authors declare no conflict of interest.

References

Assisi L, Botte V, D'Aniello A, Di Fiore MM (2001) Enhancement of aromatase activity by D-aspartic acid in the ovary of the lizard *Podarcis s. sicula*. Reproduction 121(5):803–808

Boni R, Santillo R, Macchia G, Spinelli P, Ferrandino G, D'Aniello A (2006) D-Aspartate and reproductive activity in sheep. Theriogenology 65(7):1265–1278

Burrone L, Di Giovanni M, Di Fiore MM, Baccari GC, Santillo A (2010) Effects of D-aspartate treatment on D-aspartate oxidase, superoxide dismutase, and caspase 3 activities in frog (*Rana esculenta*) tissues. Chem Biodivers 7(6):1459–1466

Burrone L, Santillo A, Pinelli C, Baccari GC, Di Fiore MM (2012a) Induced synthesis of P450 aromatase and 17β-estradiol by D-aspartate in frog brain. J Exp Biol 215(Pt 20):3559–3565

Burrone L, Raucci F, Di Fiore MM (2012b) Steroidogenic gene expression following D-aspartate treatment in frog testis. Gen Comp Endocrinol 175(1):109–117

Chandrashekar KN, Muralidhara (2010) D-Aspartic acid induced oxidative stress and mitochondrial dysfunctions in testis of prepubertal rats. Amino Acids 38:817–827

Chieffi Baccari G, Minucci S, Di Matteo L, Chieffi G (1990) Harderian gland and the lacrimal gland of the lizard *Podarcis s. sicula*: histology, histochemistry, and ultrastructure. Anat Rec 226(3):269–278

Chieffi Baccari G, Marmorino C, Minucci S, Di Matteo L, Varriale B, d'Istria M, Chieffi G (1992) Mallory stain may indicate differential rates of RNA synthesis: I. A seasonal cycle in the harderian gland of the green frog (*Rana esculenta*). Eur J Histochem 36(1):81–90

Chieffi Baccari G, Monteforte R, de Lange P, Raucci F, Farina P, Lanni A (2004) Thyroid hormone affects secretory activity and uncoupling protein-3 expression in rat harderian gland. Endocrinology 145:3338–3345

Chieffi G, Chieffi Baccari G, Di Matteo L, d'Istria M, Minucci S, Varriale B (1996) Cell biology of harderian gland. Int Rev Cytol 168:1–80

D'Aniello A, Giuditta A (1977) Identification of D-aspartic acid in the brain of *Octopus vulgaris* Lam. J Neurochem 29(6):1053–1057

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

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

D'Aniello G, Ronsini S, Notari T, Grieco N, Infante V, D'Angelo N, Mascia F, Di Fiore MM, Fisher G, D'Aniello A (2012) D-Aspartate, a key element for the improvement of sperm quality. Adv Sex Med 2:47–53

D'Aniello A (2007) D-Aspartic acid: an endogenous amino acid with an important neuroendocrine role. Brain Res Rev 53(2):215–234

D'Aniello A, Di Cosmo A, Di Cristo C, Annunziato L, Petrucelli L, Fisher G (1996) Involvement of D-aspartic acid in the synthesis of testosterone in rat testes. Life Sci 59:97–104

D'Aniello A, Di Fiore MM, D'Aniello G, Colin FE, Lewis G, Setchell BP (1998) Secretion of D-aspartic acid by the rat testis and its role in endocrinology of the testis and spermatogenesis. FEBS Lett 436(1):23–27

D'Aniello A, Spinelli P, De Simone A, D'Aniello S, Branno M, Aniello F, Fisher GH, Di Fiore MM, Rastogi RK (2003) Occurrence and neuroendocrine role of D-aspartic acid and *N*-methyl-D-aspartic acid in *Ciona intestinalis*. FEBS Lett 552:193–198

D'Aniello G, Ronsini S, Guida F, Spinelli P, D'Aniello A (2005) Occurrence of D-aspartic acid in human seminal plasma and spermatozoa: possible role in reproduction. Fertil Steril 84(5):1444–1449

D'Aniello G, Grieco N, Di Filippo MA, Cappiello F, Topo E, D'Aniello E, Ronsini S (2007) Reproductive implication of D-aspartic acid in human pre-ovulatory follicular fluid. Hum Reprod 22(12):3178–3183

Di Fiore MM, Assisi L, Botte V, D'Aniello A (1998) D-Aspartic acid is implicated in the control of testosterone production by the vertebrate gonad. Studies on the female green frog, *Rana esculenta*. J Endocrinol 157(2):199–207

Di Fiore MM, Lamanna C, Assisi L, Botte V (2008) Opposing effects of D-aspartic acid and nitric oxide on tuning of testosterone production in mallard testis during the reproductive cycle. Reprod Biol Endocrinol 6:28

Di Giovanni M, Burrone L, Chieffi Baccari G, Topo E, Santillo A (2010a) Distribution of free D-aspartic acid and D-aspartate oxidase in frog *Rana esculenta* tissues. J Exp Zool A Ecol Genet Physiol 313(3):137–143

Di Giovanni M, Topo E, Santillo A, D'Aniello A, Chieffi Baccari G (2010b) D-Aspartate binding sites in rat Harderian gland. Amino Acids 38(1):229–235

Di Matteo L, Minucci S, Chieffi Baccari G, Pellicciari C, d'Istria M, Chieffi G (1989) The harderian gland of the frog, *Rana esculenta*, during the annual cycle: histology, histochemistry and ultrastructure. Basic Appl Histochem 33(2):93–112

Dunlop DS, Neidle A, McHale D, Dunlop DM, Lajtha A (1986) The presence of free D-aspartic acid in rodents and man. Biochem Biophys Res Commun 141(1):27–32

Errico F, Napolitano F, Nisticò R, Usiello A (2012) New insights on the role of free D-aspartate in the mammalian brain. Amino Acids 43(5):1861–1871

Furuchi T, Homma H (2005) Free D-aspartate in mammals. Biol Pharm Bull 28(9):1566–1570

Grant AC, Thomson J, Zammit VA, Shennan DB (2000) Volume-sensitive amino acid efflux from a pancreatic beta-cell line. Mol Cell Endocrinol 162(1–2):203–210

Hadley ME, Haskell-Luevano C (1999) The proopiomelanocortin system. Ann NY Acad Sci 885:1–21

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

Han H, Miyoshi Y, Ueno K, Okamura C, Tojo Y, Mita M, Lindner W, Zaitsu K, Hamase K (2011a) Simultaneous determination of D-aspartic acid and D-glutamic acid in rat tissues and physiological fluids using a multi-loop two-dimensional HPLC procedure. J Chromatogr B 879:3196–3202

Han H, Miyoshi Y, Oyama T, Konishi R, Mita M, Hamase K (2011b) Enantioselective micro-2D-HPLC determination of aspartic acid in the pineal glands of rodents with various melatonin contents. J Sep Sci 34:2847–2853

Hashimoto A, Nishikawa T, Oka T, Hayashi T, Takahashi K (1993) Widespread distribution of free D-aspartate in rat periphery. FEBS Lett 331(1–2):4–8

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

Hiasa M, Moriyama Y (2006) Immunohistochemical localization of D-aspartate in islets of Langerhans. Biol Pharm Bull 29(6):1251–1253

Homma H (2007) Biochemistry of D-aspartate in mammalian cells. Amino Acids 32(1):3–11

Huang AS, Beigneux A, Weil ZM, Kim PM, Molliver ME, Blackshaw S, Nelson RJ, Young SG, Snyder SH (2006) D-aspartate regulates melanocortin formation and function: behavioral alterations in D-aspartate oxidase-deficient mice. J Neurosci 26(10):2814–2819

Iharada M, Hiasa M, Kobara A, Moriyama Y (2007) Exocytosis of D-aspartate from INS-1E clonal beta cells. Biol Pharm Bull 30(7):1329–1331

Imai K, Fukushima T, Hagiwara K, Santa T (1995) Occurrence of D-aspartic acid in rat brain pineal gland. Biomed Chromatogr 9(2):106–109

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

Ishio S, Yamada H, Hayashi M, Yatsushiro S, Noumi T, Yamaguchi A, Moriyama Y (1998) D-Aspartate modulates melatonin synthesis in rat pinealocytes. Neurosci Lett 249(2–3):143–146

Kato S, Ikuta T, Hemmi H, Takahashi S, Kera Y, Yoshimura T (2012) Enzymatic assay for D-aspartic acid using D-aspartate oxidase and oxaloacetate decarboxylase. Biosci Biotechnol Biochem 76(11):2150–2152

Kera Y, Aoyama H, Watanabe N, Yamada RH (1996) Distribution of D-aspartate oxidase and free D-glutamate and D-aspartate in chicken and pigeon tissues. Comp Biochem Physiol B Biochem Mol Biol 115(1):121–126

Lamanna C, Assisi L, Botte V, Di Fiore MM (2006) Endogenous testicular D-aspartic acid regulates gonadal aromatase activity in boar. J Endocrinol Invest 29(2):141–146

Lamanna C, Assisi L, Botte V, Di Fiore MM (2007a) Involvement of D-Asp in P450 aromatase activity and estrogen receptors in boar testis. Amino Acids 32(1):45–51

Lamanna C, Assisi L, Vittoria A, Botte V, Di Fiore MM (2007b) D-Aspartic acid and nitric oxide as regulators of androgen production in boar testis. Theriogenology 67(2):249–254

Lee JA, Homma H, Sakai K, Fukushima T, Santa T, Tashiro K, Iwatsubo T, Yoshikawa M, Imai K (1997) Immunohistochemical localization of D-aspartate in the rat pineal gland. Biochem Biophys Res Commun 231(2):505–508

Lee JA, Homma H, Tashiro K, Iwatsubo T, Imai K (1999) D-Aspartate localization in the rat pituitary gland and retina. Brain Res 838(1–2):193–199

Lee JA, Long Z, Nimura N, Iwatsubo T, Imai K, Homma H (2001) Localization, transport, and uptake of D-aspartate in the rat adrenal and pituitary glands. Arch Biochem Biophys 385(2):242–249

Long Z, Homma H, Lee JA, Fukushima T, Santa T, Iwatsubo T, Yamada R, Imai K (1998) Biosynthesis of D-aspartate in mammalian cells. FEBS Lett 434(3):231–235

Long Z, Lee JA, Okamoto T, Nimura N, Imai K, Homma H (2000) D-Aspartate in a prolactin-secreting clonal strain of rat pituitary tumor cells (GH(3)). Biochem Biophys Res Commun 276(3):1143–1147

Long Z, Sekine M, Adachi M, Furuchi T, Imai K, Nimura N, Homma H (2002) Cell density inversely regulates D- and L-aspartate levels in rat pheochromocytoma MPT1 cells. Arch Biochem Biophys 404(1):92–97

Macchia G, Topo E, Mangano N, D'Aniello E, Boni R (2010) DL-Aspartic acid administration improves semen quality in rabbit bucks. Anim Reprod Sci 118(2–4):337–343

Masuda W, Nouso C, Kitamura C, Terashita M, Noguchi T (2003) Free D-aspartic acid in rat salivary glands. Arch Biochem Biophys 420(1):46–54

Monteforte R, Santillo A, Di Giovanni M, D'Aniello A, Di Maro A, Chieffi Baccari G (2009) D-Aspartate affects secretory activity in rat Harderian gland: molecular mechanism and functional significance. Amino Acids 37:653–664

Moriyama Y, Yamada H, Hayashi M, Oda T, Yamaguchi A (1998) Identification of D-aspartate in rat pheochromocytoma PC12 cells. Neurosci Lett 248(1):57–60

Nagata Y, Homma H, Lee J-A, Imai K (1999a) D-Aspartate stimulation of testosterone synthesis in rat Leydig cells. FEBS Lett 444:160–164

Nagata Y, Homma H, Matsumoto M, Imai K (1999b) Stimulation of steroidogenic acute regulatory (StAR) gene expression by D-aspartate in rat Leydig cells. FEBS Lett 454:317–320

Nakatsuka S, Hayashi M, Muroyama A, Otsuka M, Kozaki S, Yamada H, Moriyama Y (2001) D-Aspartate is stored in secretory granules and released through a Ca(2+)-dependent pathway in a subset of rat pheochromocytoma PC12 cells. J Biol Chem 276(28):26589–26596

Osamura S (2000) Studies on peroxisomal D-asparate oxidase in rat salivary glands. J Kyushu Dent Soc 54:86–94

Ota N, Shi T, Sweedler JV (2012) D-Aspartate acts as a signaling molecule in nervous and neuroendocrine systems. Amino Acids 43(5):1873–1886

Pampillo M, Theas S, Duvilanski B, Seilicovich A, Lasaga M (2002) Effect of ionotropic and metabotropic glutamate agonists and D-aspartate on prolactin release from anterior pituitary cells. Exp Clin Endocrinol Diabetes 110(3):138–144

Raucci F, Di Fiore MM (2009) The reproductive activity in the testis of *Podarcis s. sicula* involves D-aspartic acid: a study on c-kit receptor protein, tyrosine kinase activity and PCNA protein during annual sexual cycle. Gen Comp Endocrinol 161(3):373–383

Raucci F, Di Fiore MM (2010) The maturation of oocyte follicular epithelium of *Podarcis s. sicula* is promoted by D-aspartic acid. J Histochem Cytochem 58(2):157–171

Raucci F, Di Fiore MM (2011) D-Asp: a new player in reproductive endocrinology of the amphibian *Rana esculenta*. J Chromatogr B Anal Technol Biomed Life Sci 879(29):3268–3276

Raucci F, Monteforte R, Di Fiore MM, Chieffi Baccari G (2003) D-Aspartic acid induces merocrine secretion in the frog harderian gland. Rend Fis Acc Lincei 14:205–215

Raucci F, Assisi L, D'Aniello S, Spinelli P, Botte V, Di Fiore MM (2004) Testicular endocrine activity is upregulated by D-aspartic acid in the green frog, *Rana esculenta*. J Endocrinol 182:365–376

Raucci F, Santillo A, D'Aniello A, Chieffi P, Chieffi Baccari G (2005a) D-Aspartate modulates transcriptional activity in Harderian gland of frog, *Rana esculenta*: morphological and molecular evidence. J Cell Physiol 204(2):445–454

Raucci F, D'Aniello S, Di Fiore MM (2005b) Endocrine roles of D-aspartic acid in the testis of lizard *Podarcis s. sicula*. J Endocrinol 187(3):347–359

Rossi P, Albanesi C, Grimaldi P, Geremia R (1991) Expression of the mRNA for the ligand of c-kit in mouse Sertoli cells. Biochem Biophys Res Com 176:910–914

Sakai K, Homma H, Lee JA, Fukushima T, Santa T, Tashiro K, Iwatsubo T, Imai K (1997) D-Aspartic acid localization during postnatal development of rat adrenal gland. Biochem Biophys Res Commun 235(2):433–436

Sakai K, Homma H, Lee J-A, Fukushima T, Santa T, Tashiro K, Iwatsubo T, Imai K (1998) Localization of D-aspartic acid in elongate spermatids in rat testis. Arch Biochem Biophys 351:96–105

Santillo A, Monteforte R, Raucci F, D'Aniello A, Chieffi Baccari G (2006) Occurrence of D-aspartate in the harderian gland of *Podarcis s. sicula* and its effect on gland secretion. J Exp Zool A Comp Exp Biol 305(8):610–619

Santillo A, Monteforte R, De Lange P, Lanni A, Farina P, Chieffi Baccari G (2008) Dimorphic expression of uncoupling protein-3 in golden hamster harderian gland: effects of castration and testosterone administration. J Cell Physiol 215:481–487

Santillo A, Burrone L, Senese R, Cioffi F, Lanni A, Chieffi Baccari G (2011) Effect of D-aspartate uptake on uncoupling protein-3 and α-tubulin expressions in rat Harderian gland. J Chromatogr B Analyt Technol Biomed Life Sci 879:3344–3348

Santillo A, Pinelli C, Burrone L, Chieffi Baccari G, Di Fiore MM (2013) D-Aspartic acid implication in the modulation of frog brain sex steroid levels. Gen Comp Endocrinol 181:72–76

Santillo A, Falvo S, Chieffi P, Burrone L, Chieffi Baccari G, Longobardi S, Di Fiore MM (2014) D-Aspartate affects NMDA receptor-extracellular signal-regulated kinase pathway and upregulates androgen receptor expression in the rat testis. Theriogenology 81(5):744–751

Schell MJ, Cooper OB, Snyder SH (1997) D-Aspartate localizations imply neuronal and neuroendocrine roles. Proc Natl Acad Sci USA 94(5):2013–2018

Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD et al (1994) Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev 15:342–355

Takigawa Y, Homma H, Lee J-A, Fukushima T, Santa T, Iwatsubo T, Imai K (1998) D-Aspartate uptake into cultured rat pinealocytes and the concomitant effect on L-aspartate levels and melatonin secretion. Biochem Biophys Res Commun 248:641–647

Talevi R, Barbato V, Fiorentino I, Braun S, Longobardi S, Gualtieri R (2013) Protective effects of in vitro treatment with zinc, D-aspartate and coenzyme q10 on human sperm motility, lipid peroxidation and DNA fragmentation. Reprod Biol Endocrinol 11:81–88

Topo E, Soricelli A, D'Aniello A, Ronsini S, D'Aniello G (2009) The role and molecular mechanism of D-aspartic acid in the release and synthesis of LH and testosterone in humans and rats. Reprod Biol Endocrinol 7:120

Topo E, Fisher G, Sorricelli A, Errico F, Usiello A, D'Aniello A (2010) Thyroid hormones and D-aspartic acid, D-aspartate oxidase, D-aspartate racemase, H2O2, and ROS in rats and mice. Chem Biodivers 7(6):1467–1478

Wang H, Wolosker H, Pevsner J, Snyder SH, Selkoe DJ (2000) Regulation of rat magnocellular neurosecretory system by D-aspartate: evidence for biological role(s) of a naturally occurring free D-amino acid in mammals. J Endocrinol 167(2):247–252

Wang H, Wolosker H, Morris JF, Pevsner J, Snyder SH, Selkoe DJ (2002) Naturally occurring free D-aspartate is a nuclear component of cells in the mammalian hypothalamo-neurohypophyseal system. Neuroscience 109(1):1–4

Weaver CD, Gundersen V, Verdoorn TA (1998) A high affinity glutamate/aspartate transport system in pancreatic islets of Langerhans modulates glucose-stimulated insulin secretion. J Biol Chem 273(3):1647–1653

Weil ZM, Huang AS, Beigneux A, Kim PM, Molliver ME, Blackshaw S, Young SG, Nelson RJ, Snyder SH (2006) Behavioural alterations in male mice lacking the gene for D-aspartate oxidase. Behav Brain Res 171(2):295–302

Wolosker H, D'Aniello A, Snyder SH (2000) D-Aspartate disposition in neuronal and endocrine tissues: ontogeny, biosynthesis and release. Neuroscience 100(1):183–189

Yamada H, Yatsushiro S, Yamamoto A, Hayashi M, Nishi T, Futai M, Yamaguchi A, Moriyama Y (1997) Functional expression of a GLT-1 type Na^+-dependent glutamate transporter in rat pinealocytes. J Neurochem 69(4):1491–1498

Yamamoto A, Tanaka H, Ishida T, Horiike K (2010) D-Aspartate oxidase localisation in pituitary and pineal glands of the female pig. J Neuroendocrinol 22(11):1165–1172

Yatsushiro S, Yamada H, Kozaki S, Kumon H, Michibata H, Yamamoto A, Moriyama Y (1997) L-Aspartate but not the D form is secreted through microvesicle-mediated exocytosis and is sequestered through Na^+-dependent transporter in rat pinealocytes. J Neurochem 69(1):340–347