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Review

# D-Aspartic acid: An endogenous amino acid with an important neuroendocrine role

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ABSTRACT

D-Aspartic acid (D-Asp), an endogenous amino acid present in vertebrates and invertebrates, plays an important role in the neuroendocrine system, as well as in the development of the nervous system. During the embryonic stage of birds and the early postnatal life of mammals, a transient high concentration of D-Asp takes place in the brain and in the retina. D-Asp also acts as a neurotransmitter/neuromodulator. Indeed, this amino acid has been detected in synaptosomes and in synaptic vesicles, where it is released after chemical ($K^+$ ion, ionomycin) or electric stimuli. Furthermore, D-Asp increases cAMP in neuronal cells and is transported from the synaptic clefts to presynaptic nerve cells through a specific transporter. In the endocrine system, instead, D-Asp is involved in the regulation of hormone synthesis and release. For example, in the rat hypothalamus, it enhances gonadotropin-releasing hormone (GnRH) release and induces oxytocin and vasopressin mRNA synthesis. In the pituitary gland, it stimulates the secretion of the following hormones: prolactin (PRL), luteinizing hormone (LH), and growth hormone (GH) In the testes, it is present in Leydig cells and is involved in testosterone and progesterone release. Thus, a hypothalamus–pituitary–gonads pathway, in which D-Asp is involved, has been formulated. In conclusion, the present work is a summary of previous and current research done on the role of D-Asp in the nervous and endocrine systems of invertebrates and vertebrates, including mammals.



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## 1. Introduction: stereochemical consideration on D-amino acids

Stereochemical configuration of the α-carbon atom of amino acids is fundamental to all living systems. All the amino acids found in proteins (except for glycine) are optically active and have the same stereochemical configuration as the α-carbon atom. The prefix L attached to these amino acids goes back to half a century ago when Louis Pasteur in 1851 (Pasteur, 1851) observed that asparagine, the first natural amino acid discovered by Vauquelin and Robiquet in 1806 (Vauquelin and Robiquet, 1806), was "levorotatory", i.e., able to disperse the polarized light to the left. On the other hand, the prefix D indicated that the amino acids were able to rotate the polarized light to the right. Although Pasteur forcefully pointed out that the natural asparagine was different from the synthetic one, he failed to recognize that the synthetic molecule was a racemic mixture, which recalled the racemic form of tartaric acid that he himself had previously studied. However, Greenstein (1954) and Greenstein et al. (1953) later observed that not all natural amino acids had the same effect on the dispersion of light. Therefore, in an attempt to clarify the nomenclature of the amino acids, the capitals L- and D- were used to refer to the configurations of the α-carbon atom: the L-amino acid (L-form) designation was given to the natural amino acids, whereas the D-amino acid (D-form) designation was given to those amino acids having opposite spatial configurations of the α-carbon atom (obtained by X-ray diffraction) (Greenstein et al., 1953). Furthermore, since L-amino acids have the same spatial form as L-glyceraldehyde, all compounds with an asymmetric carbon atom similar to L-glyceraldehyde were designed as L-forms, whereas those opposite in configuration were designed as D-forms (Fig. 1). In addition to the chemical considerations, it has also been observed that only the L-amino acids are biologically oxidized by L-amino acid oxidase (L-AAO; EC 1.4.3.2) (Bender et al., 1949; Blaschko and Newkim, 1952). By contrast, the D-amino acids are oxidized by other oxidases. Specifically, D-aspartic acid (D-Asp), D-glutamic acid (D-Glu), and *N*-methyl-D-aspartic acid (NMDA) are oxidized by a D-aspartate oxidase (D-AspO; EC 1.4.3.1) (D'Aniello et al., 1993c; Dixon et al., 1967), whereas all the other D-amino acids are oxidized by a D-amino acid oxidase (D-AAO; EC 1.4.3.3) (D'Aniello et al., 1993c; Dixon and Kleppe, 1965). Then, since no free D-amino acids were found in plants or animals, it was believed that only the L-amino acids were natural compounds. However, during the last half of the 20th century, by using specific enzymatic methods based on the use of D-AspO, D-AAO and HPLC techniques, many D-amino acids, in particular D-Asp, D-Ser and D-Ala, were discovered in free compounds or bound to peptides and proteins in bacteria, moulds, vertebrates, and invertebrates (Corrigan, 1969; Meister et al., 1965). The present study has gleaned some of the most recent breakthroughs in the study of D-Asp in an attempt to highlight its prominent role in the development of the nervous and endocrine systems during the embryonic stages or the early postnatal stages of animals.

## 2. D-Amino acids in peptides and proteins

The first D-amino acids detected in living organisms were discovered in some plants and bacteria about 50 years ago (Corrigan, 1969; Meister et al., 1965). These compounds were either found in a free state or were incorporated in peptides and protein linkages. Indeed, a number of antibiotics (e.g., polymixin, bacitracin, gramicidin, actinomycins, etc.) and bacterial cell walls contain D-amino acid residues in peptides bound to L-amino acids. In the last case, D-amino acids seem to constitute a measure of protection against peptidase and protease attacks, since, so far, no known protease has been shown to cleave peptide bonds, characterized by a sequence of amino acids in D-D or D-L conformation (Corrigan, 1969; Meister et al., 1965). Only one peptidase, capable of hydrolyzing bonds involving D-amino acids, has been purified from the intestinal sac of some marine mollusks (D'Aniello and Strazzullo, 1984). D-Phe and D-Asn occur in the two peptides: Gly-D-Phe-L-Ala-L-Asp and L-Phe-D-Asn-L-Glu-L-Phe-L-Val. Because they are both found in the cerebral ganglia of the mollusk *Achatina fulica* (Corrigan, 1969; Meister et al., 1965), they contribute to the

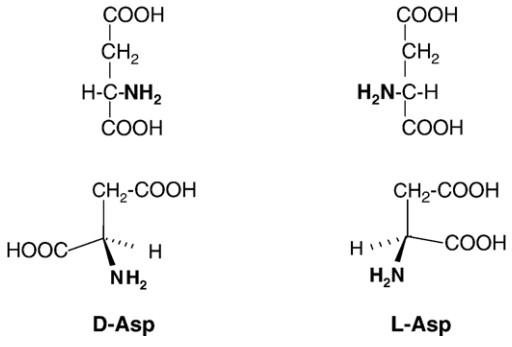



**Fig. 1 – Stechiometric representation of D-Asp and L-Asp using a linear formulae (upper panel) and carbonium presentation based on the tetrahedral configuration (lower panel).**

neurobiological and neuroendocrine properties of the peptides. D-Ala, which occurs in the peptide L-Tyr-D-Ala-L-Phe-Gly-L-Tyr-L-Pro-L-Ser is found in the skin of the frog *Phyllomedusa sawvagei* (Montecucchi et al., 1981), where it has potent opiate-like activity. D-Asp has also been demonstrated in proteins of human dentine (Helfman and Bada, 1975), in eye lenses, in cataracts (D'Aniello et al., 1987; Master et al., 1977), in brain (Fisher et al., 1992; Man et al., 1983), and in cartilages (Maroudas et al., 1992). Recently, using a novel method to hydrolyze the proteins without racemizing the amino acids (D'Aniello et al., 1993b), we have found D-Asp in horse bone proteins (Di Bernardo et al., 2004) and in membrane proteins of human erythrocytes (Perna et al., 1997). Although the function of D-amino acids in proteins is not yet known, we could advance the hypothesis that such presence is probably correlated with the degeneration of proteins due to aging or other causes.

## 3. D-Amino acids in animal tissues

The principal free D-amino acids found in significant quantities in animal tissues are D-Ala, D-Ser and D-Asp. D-Ala was first discovered in the blood of the milkweed bug *Oncopeltus fasciata* and in the larva of the monarch butterfly *Danaus plexippus* (Meister et al., 1965). Later, it was also found in marine crustaceans (Abe et al., 1999; D'Aniello and Giuditta, 1980; Okuma and Abe, 1994), in eggs and embryos of echinoderms (D'Aniello et al., 1990), and in bivalve mollusks (Okuma et al., 1998). In mammals, D-Ala has been found in human plasma and in the of mice (Nagata et al., 1992a,b). In mutant mice, lacking D-amino acid oxidase, D-Ala concentrations are significantly higher than those in normal mice, thus implying a possible role of D-amino acid oxidase in metabolizing D-amino acids (Morikawa et al., 2001). D-Ala also occurs in the rat brain (D'Aniello et al., 1993a; Fisher et al., 1991) and in the pituitary gland (Hashimoto et al., 1995). The biological role of D-Ala in mammals is not yet known. In marine animals, it has been hypothesized that D-Ala could have a role in the regulation of animal osmolarity with the external environment (Okuma et al., 1998).

D-Ser occurs in mice, rats (Hashimoto et al., 1995; Mothet et al., 2000; Schell et al., 1995, 1997a), and humans (Fisher et al., 1998; Hashimoto et al., 1993a; Nagata et al., 1995). In mice, D-Ser has been found in the brain together with D-Ala and D-Asp (Morikawa et al., 2001), whereas in rats, it has been detected in various regions of the brain at concentrations of about 200–300 nmol/g of tissue (Hashimoto et al., 1995). This amino acid is also localized in pineal and pituitary glands, but to a much lesser degree than that found in the nervous system (Hashimoto et al., 1995), thus implying that it has a greater role in the nervous system than in the endocrine system. Using an antibody against D-Ser, immunohistochemical studies have demonstrated that this amino acid is concentrated in the gray-matter, in the hippocampus, in the anterior olfactory nucleus, and in the amygdala (Schell et al., 1995, 1997a). It has been seen that D-Ser increases the affinity of NMDA sensitive glutamate binding sites in rat brain synaptic membrane (Fadda et al., 1988; Mothet et al., 2000). Its presence has also been reported in the prefrontal cortex of human embryos and adults at concentrations ranging from 50–80 nmol/g of tissue (Hashimoto et al., 1993a; Nagata et al., 1995). No significant differences in concentration levels have been found between the brains of normal subjects and those of Alzheimer's patients (Nagata et al., 1995). Interestingly, although D-Ser has been found in the CSF of both normal and Alzheimer's patients, it was significantly higher in the latter (Nagata et al., 1995).

## 4. D-Aspartic acid in animal tissues

D-Asp is the mostly widespread amino acid in animal tissues. Back in 1977, we discovered its presence in the brain and optic lobes of the cephalopod mollusk *Octopus vulgaris* (common octopus) (D'Aniello and Giuditta, 1977). Later, it was found not only in the peripheral nervous system (stellate ganglia and in the axoplasm fluid of the giant axon) of the cephalopods *Sepia officinalis* (common cuttlefish) and *Loligo vulgaris* (common squid) (D'Aniello and Giuditta, 1978; D'Aniello et al., 1995b), but also in their reproductive system (D'Aniello et al., 1995a). Subsequently, D-Asp was detected in the nervous and endocrine tissues of many other animal phyla. For example, it has been detected in the circumsoesophageal ganglia of the opistobranchia sea hare *Aplysia fasciata* (D'Aniello et al., 1992a) and *Aplysia limacina* (Spinelli et al., 2006), in the gametes of the embryo and adult *Ciona intestinalis* (sea squirt) (D'Aniello et al., 1992b, 2003), in the nervous ganglia of the crustacean *Jasus lalandii* (Rock lobster) (Okuma and Abe, 1994), in the gonads and in the Harderian glands of the amphibian *Rana esculenta* (green frog) (Di Fiore et al., 1998; Raucci et al., 2005), in the ovary of the reptile *Podarcis sicula* (Italian wall lizard) (Assisi et al., 2001), in the nervous system of the fish *Merlucius merlucius* (European hake) and *Solea solea* (D'Aniello et al., 1995b), and, finally, in the brains of chickens (*Gallus gallus*) (Neidle and Dunlop, 1990). Plus, it has also been described in the neuroendocrine system of mammals, specifically in mice (*Mus musculus*) (Morikawa et al., 2001), rats (*Rattus norvegicus*) (D'Aniello et al., 1993a, 1996; Dunlop et al., 1986; Hamase et al., 1996; Hashimoto et al., 1993b; Imai et al., 1995; Lee et al., 1999; Masuda et al., 2003) and humans (*Homo sapiens*) (D'Aniello et al., 1998b; Fisher et al., 1991), as well as in cultured rat pinealocytes (Takigawa et al., 1998) and pituitary tumor GH3 cells (Long et al., 2000) (Table 1).

In parallel with the biochemical studies, the immunohistochemical approach, using an anti-D-Asp antibody, has revealed its presence in the rat frontal cortex and hippocampus (Schell et al., 1997b; Wang et al., 2000). Instead, in the endocrine system, this amino acid has been located in pituitary and adrenal glands (Lee et al., 1997b; Sakai et al., 1997), in the pineal gland (Lee et al., 1997a), in testes (D'Aniello et al., 1996), in the elongated spermatids (Sakai et al., 1998b), as well as in various differentiating neurons of the rat CNS (Sakai et al., 1998a) and retina (Lee et al., 1999). In addition, D-Asp has also been detected in a nuclear component of cells in the mammalian hypothalamus–neurohypophyseal system (Wang et al., 2002) and in the retina of the cephalopod *S. officinalis*, where it is implicated in vision (D'Aniello et al., 2005). All the above results thus

**Table 1 – D-Aspartate content in nervous and endocrine tissues in various animal phyla**

| Species | Animals | Tissues | nmol/g tissues | References |
|---|---|---|---|---|
| Mollusks | *Octopus vulgaris* | Brain | 8000–15,000 | (D'Aniello and Giuditta, 1977; D'Aniello and Giuditta, 1978) |
| | *Octopus vulgaris* | Retina | 3000–4000 | (D'Aniello et al., 2005) |
| | *Octopus vulgaris* | Reproductive organs | 2000–3000 | (D'Aniello et al., 1995a) |
| Opistobranks | *Aplysia fasciata* | Esophageal ganglia | 600–800 | (D'Aniello et al., 1992a) |
| | *Aplysia limacina* | Cerebral ganglia | 3100–4600 | (Spinelli et al., 2006) |
| Tunicates | *Ciona intestinalis* | Cerebral ganglia | 300–500 | (D'Aniello et al., 1992b; D'Aniello et al., 2003) |
| Crustaceans | *Rock lobster* | Brain | 4000–5000 | (Okuma and Abe, 1994) |
| | *Rock lobster* | Retina | 800–900 | (Okuma and Abe, 1994) |
| Amphibians | *Rana esculenta* | Testes | 200–300 | (Di Fiore et al., 1998) |
| | | Harderian gland | 100–150 | (Raucci et al., 2005) |
| Reptiles | *Podarcis s. sicula* | Reproductive organs | 20–30 | (Assisi et al., 2001) |
| Fishes | *Merlucius merlucius* | Brain | 30–40 | (D'Aniello et al., 1995b) |
| Birds | *Gallus gallus* | Brain, retina (embryo) | 300–600 | (Neidle and Dunlop, 1990) |
| | | Brain, retina (adult) | 20–60 | (Neidle and Dunlop, 1990) |
| Mammals | *Rattus* | Brain (embryo) | 250–350 | (Dunlop et al., 1986) |
| | | Brain (adult) | 15–30 | (D'Aniello et al., 1993b; Dunlop et al., 1986; Hamase et al., 1996; Hashimoto et al., 1993b; Imai et al., 1995) |
| | | Retina (6 days) | 300–400 | (Neidle and Dunlop, 1990) |
| | | Retina (adult) | 30–60 | (Lee et al., 1999; Neidle and Dunlop, 1990) |
| | | Pituitary (2 days old) | 10–20 | (D'Aniello et al., 1996; Lee et al., 1999) |
| | | Pituitary (adult) | 120–140 | (D'Aniello et al., 2000a; Hashimoto et al., 1993a,b; Lee et al., 1999; Lee et al., 1997b) |
| | | Pineal gland | 650–3000 | (Imai et al., 1995; Lee et al., 1997a) |
| | | Testes (embryo) | 50–70 | (D'Aniello et al., 1996; Hashimoto et al., 1993a) |
| | | Testes (adult) | 220–250 | (D'Aniello et al., 1996; Hashimoto et al., 1995; Hashimoto et al., 1993a) |
| | | Liver | 5–15 | (D'Aniello et al., 1993a; D'Aniello et al., 2000a; Hashimoto et al., 1995) |
| | *Human* | Brain (embryo) | 340–380 | (Hashimoto et al., 1995) |
| | | Brain (adult) | 20–40 | (D'Aniello et al., 1998a; Fisher et al., 1991; Hashimoto et al., 1993a) |

Values represent the mean concentration in nmol of D-Asp/g of fresh tissue taken from the respective references.

indicate that D-Asp plays a role in both the nervous and endocrine systems.

## 5. D-Aspartic acid in the nervous system: general consideration

Many data suggest that D-Asp promotes the synthesis of proteins involved in the development of the nervous system and acts as a neurotransmitter or neuromodulator at synapses. For instance, it has been observed that in the brain and retina of 13- to 14-day-old chicken embryos, transient high concentrations of D-Asp occur (about 230–260 nmol/g tissue and 550–650 nmol/g tissue, respectively). After this stage, the D-Asp concentrations rapidly decrease to very low levels (about 20–40 nmol/g tissue) and remain the same for the rest of the chicken's life (Dunlop et al., 1986; Hashimoto et al., 1993b; Neidle and Dunlop, 1990). Such phenomena also occur in the mammalian brain and retina. Similarly, a high transient accumulation of D-Asp takes place in the brain (approximately 300 nmol/g tissue) of 17- to 19-day-old rat embryos. However, after this phase, it progressively decreases, reaching levels as low as 15–20 nmol/g of tissue. By contrast, in the mammalian retina, D-Asp does not emerge during the embryonic life but during the early postnatal stage, specifically 5–7 days after birth, reaching values of about 350 nmol/g tissue. Then, similarly to the other species, it rapidly declines to marked low levels (about 30–50 nmol/g tissue) and remains the same thereafter (Dunlop et al., 1986; Neidle and Dunlop, 1990). Thus, it is clear that both in chickens and in rats, D-Asp is among one of the many underlying factors involved in the development of the central nervous system, particularly during fetal life or soon after birth. Interestingly, in the retina of both chickens and rats, the highest concentrations of D-Asp occur 4–7 days before the animals develop their sight. Indeed, the chicken, already possesses a complete vision system from birth, i.e., about 5–6 days after D-Asp peaks in the retina (Okuma and Abe, 1994). On the other hand, the rat begins to open its eyes on the 12–13th day of life, i.e., 4–5 days after D-Asp reaches peak concentrations in the retina (Dunlop et al., 1986) (Fig. 2).

### 5.1. D-Aspartic acid in the nervous system: neurotransmitter/neuromodulator role

Many experimental results have supported the hypothesis that D-Asp has a role in neurotransmission or neuromodulator. For example, very high quantities of D-Asp are present in the nervous tissues of many marine animals. Particularly, in the brains of *O. vulgaris*, *S. officinalis* and *L. vulgaris*, D-Asp occurs at the concentration of 8–15 μmol/g tissue, that is, a concentration as high as that of the two neuroexcitatory amino acids L-Asp and L-Glu (D'Aniello and Giuditta, 1977, 1978). In the cerebral ganglia of *A. fasciata*, D-Asp is found at a concentration of 0.6–0.8 μmol/g tissue (D'Aniello et al., 1992a), whereas, in the cerebral ganglia of the *Rock lobster*, it is found at a concentration of 2.0–4.0 μmol/g tissue (Okuma and Abe, 1994). The same pattern also occurs in the retina. Indeed, in the retina of *S. officinalis*, D-Asp occurs at relative high concentrations (2.0–3.0 μmol/g tissue) (D'Aniello et al., 2005). Accordingly, it has been suggested that D-Asp plays a role in vision. Briefly, when the animal is left in the dark, the concentrations of D-Asp significantly decrease in the retina; intriguingly, however, when the animals are exposed to the light again, D-Asp returns to the previous levels (D'Aniello et al., 2005). The aspartate racemase, the enzyme that converts L-Asp into D-Asp, also decreases its activity in the dark, thus indicating that a biochemical pathway does exist for D-Asp synthesis and that it may have a function in vision (Fig. 3). Concerning the aspartate racemase, this enzyme has also been found in the rat brain (Wolosker et al., 2000), where it transforms L-Asp into D-Asp.

Altogether, these data indicate that D-aspartate is implicated in neurological activities. This possibility has been confirmed by other studies as well and has led to the following important findings:

(1) In the goldfish retina, D-Asp is capable of potentiating the effects of L-glutamate in the light response by about 15-fold (Ishida and Gordon, 1981).

(2) In cultured cells from goldfish retina, D-Asp acts as a selective NMDA receptor agonist, thus eliciting GABA release (Cha et al., 1986).

(3) D-Asp is recognized by the $Na^+$-dependent L-glutamate transporters (Bouvier et al., 1992; Kanai and Hediger, 1992). This carrier protein, which transports L-Glu from the glial cells to the terminal axons, is also efficiently capable of transporting D-Asp from the synaptic clefts to the neurons (Bouvier et al., 1992).

(4) Endogenous D-Asp or accumulated [$^3$H]D-Asp is released from the rat cerebellum slices or from cultured astrocytes by depolarizing stimuli induced by potassium ions via a calcium channel-dependent mechanism, just as it occurs for other neurotransmitters (Davies and Johnton, 1976; Hopainen et al., 1990; Levi et al., 1982; Malthe-Sorenssen et al., 1979). The same events occur in the guinea pig spinal cord (Potashner et al., 1988).

(5) When *Xenopus* oocytes, expressing a complementary DNA that encodes an electrogenic $Na^+$-dependent affinity glutamate transporter (EAAC1), are exposed to a bath containing D-Asp, a large inward current occurs. This specific protein transporter, which removes glutamate from the synaptic cleft, can also efficiently recognize D-Asp and, thus, transport it to the nerve ending (Kanai and Hediger, 1992). This finding thus demonstrates that when D-Asp is released into the synaptic cleft following depolarization of the presynaptic nerve ending (as it occurs for neurotransmitters), EAAC1 rapidly eliminates D-Asp from the synaptic cleft and carries it to the presynaptic neuron (Fig. 4).

(6) When cultured L-7 motor neurons of *Aplysia californica* are used, D-Asp, at a concentration of 0.1 mM, shows a moderate agonist action under the current clamp (Dale and Kandel, 1993). However, since D-Asp has been tested only at 0.1 mM, but not at 10 mM (as done for L-Glu), it is very likely that D-Asp could have the same agonist response as L-Glu. In addition, it has been found that D-

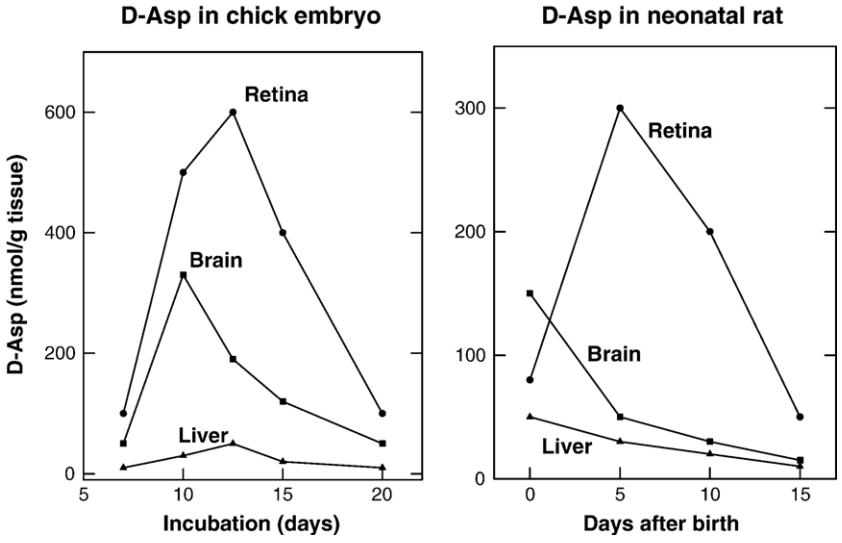



**Fig. 2 – D-Asp concentration in the chick embryo and neonatal rat. Left panel: Transient high concentration of D-Asp in the retina and in the brain of the chick embryo during development. Right panel: Transient high concentration of D-Asp in the retina and brain of the rat during development. Values are recalculated from Ref. Dunlop et al. (1986).**

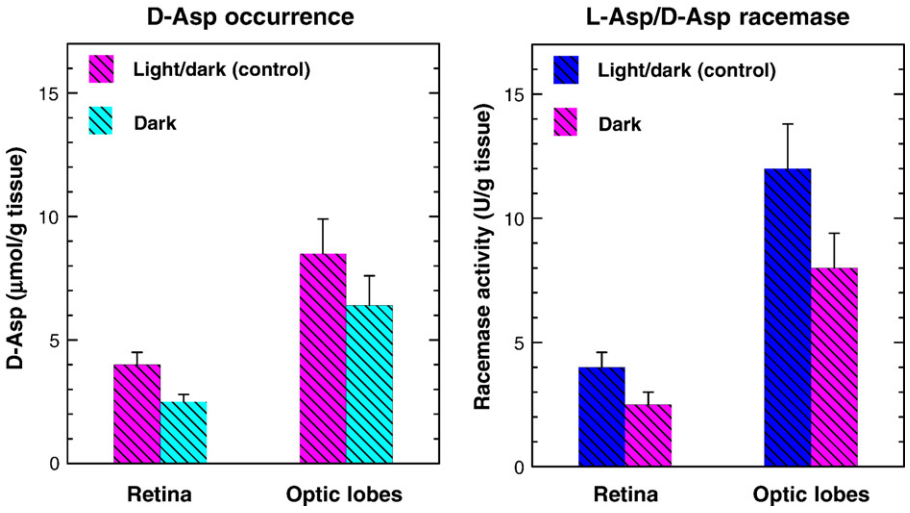



**Fig. 3 – D-Aspartic acid and aspartate racemase in the retina and optic lobes of *Sepia officinalis* during the light-dark cycles. Left panel: Concentration of D-Asp in the retina and optic lobes of *Sepia officinalis* under light/dark conditions of control animals and animals left under dark conditions for 5 days. Right panel: racemase activity (L-Asp → D-Asp) in the retina and optic lobes of *Sepia officinalis* under light/dark conditions of control animals and of animals left under dark conditions for 5 days. The values are from Ref. D'Aniello et al. (2005).**

Asp blocks not only the synaptic current response of motor neurons at L-7 but also the response of the inward current to the natural L-glutamate transmitter in a dose-dependent manner (Dale and Kandel, 1993).

(7) D-Asp has been found in the olfactory bulb, hypothalamic, supraoptic and paraventricular nuclei of rats (Wolosker et al., 2000). Plus, because D-aspartate oxidase (D-AspO), the enzyme which metabolizes D-Asp, has been localized in the neurons of the hippocampus, cerebral cortex, and in other parts of the brain, the enzyme might inactivate synaptically released D-Asp (Wolosker et al., 2000). Depolarization experiments conducted on rat adrenal slices have demonstrated that D-Asp is released by depolarization with KCl or acetylcholine (Table 2). Furthermore, nicotine treatments in rats (10 mg/kg, i.p.) have resulted in a pronounced depletion of endogenous D-Asp, thereby implying the role of D-Asp in the activation of cholinergic innervation (Table 3).

(8) Lastly, we have recently found that D-Asp acts as a neurotransmitter/neuromodulator in *A. limacina* (Spinelli et al., 2006). In this animal, in fact, high concentrations of D-Asp in the cerebral ganglia (5.10±0.65 µmol/g tissue), buccal ganglia (4.70±0.68 µmol/g tissue), pleural ganglia (4.60±0.62 µmol/g tissue), pedal ganglia (4.55±0.55 µmol/g tissue), and abdominal ganglia (4.90±0.70 µmol/g tissue) have been detected. In non-nervous tissues, like the hepatopancreas and the muscle, D-Asp

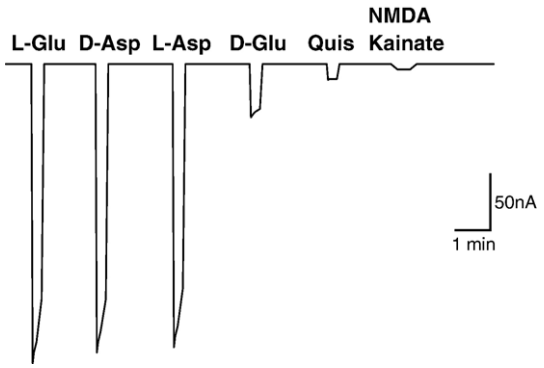



**Fig. 4 – Membrane current response evoked by D-aspartate. Measurement of membrane current by 100 µM L-Glu, D-Asp and other compounds on *Xenopus oocytes* encoding of the glutamate transporter. Membrane current was measured in standard bath solution at a holding potential of –60 mV. L-Glutamate, D-aspartate and L-aspartate evoked almost the same amplitude of inward current. The values are from Ref. Dale and Kandel (1993).**

**Table 2 – Release of D- and L-aspartate from adrenal slices**

| Treatment | D- and L-aspartate levels in the medium | |
|---|---|---|
| | D-Aspartate | L-Aspartate |
| None (control) | 158±37 | 406±60 |
| KCl (55 mM) | 469±43* | 727±65* |
| KCl (55 mM)+EGTA (0.2 mM) | 55±15* | 165±70* |
| Acetylcholine (100 mM) | 279±28* | 584±45* |

Results indicating pmol/mg of protein (Wolosker et al., 2000) and refers to the adrenal glands from Sprague-Dawley rats (21 days old) cut in 400-µm slices. The slices were washed three times and equilibrated with oxygenated Krebs–Heinseleit buffer for 30 min at 37 °C. The release of L- or D-aspartate was elicited by adding the reagent in the medium (see table). After 10 min, the medium was analyzed for amino acid content.

* Different from the control ($P<0.01$).

**Table 3 – Nicotine releases D-aspartate from the adrenal gland**

| Treatment | D-Aspartate | L-Aspartate | L-Glutamate |
|---|---|---|---|
| None (control) | 409±98 | 883±108 | 2460±256 |
| Nicotine | 135±38* | 918±146 | 3007±336* |

Results indicate nmol/g weight tissue (Wolosker et al., 2000). Groups of four Sprague-Dawley rats (21 days old) were treated with nicotine (10 mg/kg, i.p.) for 3 days, and the adrenal gland was analyzed for amino acid content.
* Different from saline ($P<0.01$).

occurs at a very low concentration, as opposed to the nervous system (0.25±0.1 μmol/g tissue). The other D-amino acids are found at very low values compared to D-Asp (Table 4).

Light immunohistochemical studies, conducted on cultured neurons using an anti-D-aspartate antibody, have demonstrated that D-Asp occurs in the soma, in dendrites, and in synaptic varicosities (Fig. 5). The electron microscopy, performed on isolate synaptosomes and on purified synaptic vesicles – previously prepared from the cerebral ganglia of this mollusk – has revealed an intense quantity of D-Asp in these nerve ending structures (Fig. 6), therefore implying the role of D-Asp in neurotransmission. HPLC analyses have shown that, in these cellular organelles, high concentrations of D-Asp, together with L-aspartate and L-glutamate (Fig. 7), are present. Interestingly, in synaptic vesicles, D-Asp is the most highly concentrated amino acid, representing about 25% of the total amino acids present in these cellular organelles. In these vesicles, $K^+$ depolarization or ionomycin promotes its release from synaptosomes. Lastly, it has been shown that if D-Asp is injected into live animals or added to incubation media of cultured neurons, it will trigger an increase in cAMP (Fig. 8). On the whole, these findings suggest that because D-Asp acts as a neurotransmitter/neuromodulator, it may have an important role in neurotransmission not only in *Aplysia limacine* but also in other animals. Some of the possible interactions of D-Asp with glutamatergic neurotransmission and the processing of amino acids are diagrammatically shown in Fig. 9. In this scheme, D-Asp is released from presynaptic terminals and acts on postsynaptic receptors that are able to increase cAMP. As a result, D-Asp, oxidized by a D-aspartate oxidase present on the postsynaptic membrane, releases $H_2O_2$ and $NH_3$. Moreover, it is known that free D-Asp is also transported by the L-glutamate transporters from the synaptic cleft to the presynaptic axon (Fig. 9). This last event could constitute an alternative way to eliminate D-Asp from synaptic cleft.

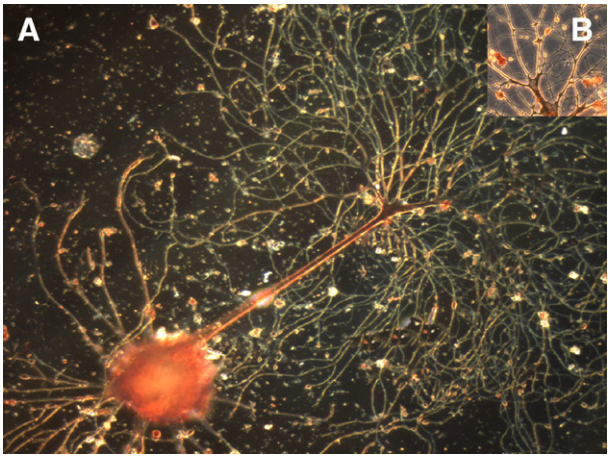



**Fig. 5 – D-Asp immunostaining in cultured neurons of *Aplysia limacina*. (A) Cultured neurons from a juvenile *Aplysia limacina* treated with an antibody–anti-D-aspartate raised in rabbit (primary antibody) and with a biotinylated anti-rabbit IgG raised in goat (secondary antibody). Subsequently, cultured neurons were immunoassayed with a complex avidin–biotin-peroxidase system and stained with DAB. The immunopositivity of D-Asp is evident in the neuron cell body, as well as along the nerve fibbers and at nerve endings (40×). (B) Insert of a particular area of nerve ending (100×). The values are from Ref. Spinelli et al. (2006).**

**Table 4 – D-Aspartic acid and other D-amino acids in the nervous system and non-nervous tissues of *Aplysia limacina***

| | D-Asp+NMDA+D-Glu [a] | D-Asp [b] | D-Glu+NMDA [c] | Total other D-amino acids [d] |
|---|---|---|---|---|
| *Nervous tissues* | | | | |
| Cerebral ganglia | 5.10±0.65 | 4.60±0.45 | 0.50 | 0.48±0.06 |
| Buccal ganglia | 4.70±0.68 | 4.15±0.35 | 0.55 | 0.55±0.07 |
| Pleural ganglia | 4.60±0.62 | 3.95±0.40 | 0.45 | 0.45±0.06 |
| Pedal ganglia | 4.55±0.55 | 4.05±0.35 | 0.50 | 0.44±0.08 |
| Abdominal ganglia | 4.90±0.70 | 4.45±0.45 | 0.45 | 0.42±0.05 |
| *Non nervous tissues* | | | | |
| Hepathopancreas | 0.25±0.10 | 0.15±0.05 | 0.10 | 0.18±0.08 |
| Muscle | 0.20±0.05 | 0.12±0.03 | 0.10 | 0.15±0.06 |

Results represent μmol/g tissue (mean±SD) obtained from 10 *Aplysia limacina* (Spinelli et al., 2006),
[a] D-Glu was determined by enzymatic colorimetric method based on the determination of ketoacids developed after treatment with beef D-AspO.
[b] D-Asp was determined by the specific HPLC method.
[c] NMDA was obtained by subtracting the mean values of the first column from those of the second column.
[d] D-Amino acids values indicate the total amount of D-amino acids (D-Pro, D-Met, D-Leu, D-Ile, D-Phe, D-Tyr, D-Ala, D-Trp, D-Ser, D-Thr, D-His, D-Arg, D-Val, and D-Lys). They were determined by the enzymatic colorimetric method based on the determination of ketoacids after the use of D-AAO.

## 6. D-Aspartic acid in the endocrine system: general considerations

It has been demonstrated that in the nervous system of chicken, rat and human embryos, D-Asp occurs at high concentrations. By contrast, it practically disappears from the nervous tissues of adult animals but increases in endocrine glands (Table 1). The latter phenomenon, clearly highlighting D-Asp involvement in the endocrine activity of

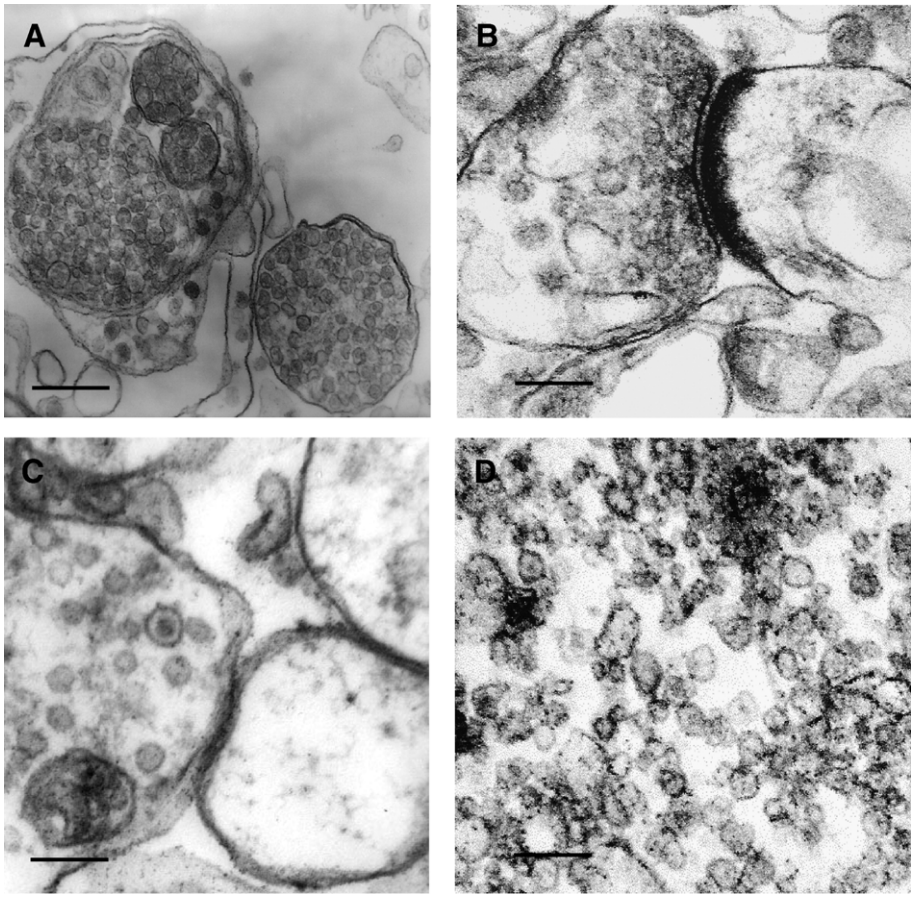



**Fig. 6 – Electron microscopy of synaptosomes and synaptic vesicles from *Aplysia limacina* cerebral ganglia. (A) Morphology of two synaptosomes filled with synaptic vesicles obtained from cerebral ganglia (80,000×). (B) Synapse between a presynaptic nerve ending filled with synaptic vesicles and a postsynaptic ending without vesicles. The postsynaptic density is very evident (80,000×). (C) A couple of pre- and post-synaptosomes after lyses with distilled water (80,000×). The synaptic vesicles have disappeared owing to osmotic lyses. (D) Isolated synaptic vesicles obtained by lyses of synaptosomes (120,000×). Panels A–C scale bars: 0.2 μm; D: 0.130 μm. The values are from Ref. Spinelli et al. (2006).**

the adult animal, and, more generally, in endocrinology, has been substantially supported by the rich array of studies burgeoning in this field in the past few decades. In 1996, we first demonstrated that whenever an adult male rat received an i.p. injection of D-Asp, this amino acid accumulated in the adenohypophysis. Such accumulation led to a significant increase in LH (luteinizing hormone), testosterone, and progesterone levels in the blood (D'Aniello et al., 1996). Consistently, we later demonstrated that D-Asp was also capable of inducing the release of various hormones such as prolactin (PRL), growth hormone (GH), and LH (D'Aniello et al., 2000a,b) (Table 5). Thus, these data indicate that D-Asp is capable of stimulating the synthesis and release of LH, which, in turn, induces the release of testosterone and progesterone in the testes. Immunohistochemical studies, which have used an antibody against D-aspartic acid, have evidenced that D-Asp in testes is localized in Leydig cells – the cells that synthesize testosterone (D'Aniello et al., 1996) – whereas biochemical analyses conducted with an enzymatic HPLC method have demonstrated that D-Asp is highly concentrated in the testicular venous blood plasma, in the rete testis fluid, in epididymal spermatozoa, in testicular parenchymal cells, and in seminiferous tubules (D'Aniello et al., 1998a). Actually, because D-Asp is essentially secreted into the venous blood by the testes, it probably passes into the rete testis fluid, where it is finally incorporated into the spermatozoa (D'Aniello et al., 1998a). Other researchers have demonstrated that D-Asp is also localized in the elongated spermatids, the most mature germ cells of the adult rat (Sakai et al., 1998b). However, traces of D-Asp have also been found in young rats, who, in fact, have not yet developed elongated spermatids in the seminiferous tubules. Indeed, D-Asp has been detected in the spermatocytes, the most mature population of germ cells occurring in young rats (Sakai et al., 1998b). Evidence that D-Asp is synthesized into the Leydig cells is that when a toxicant agent, such as EDS (ethane dimethane sulfonate), is used to destroy these cells, D-Asp levels markedly decrease in the rats' testes 4 days after the beginning of the treatment, thus indicating that D-Asp may be synthesized in Leydig cells (Sakai et al., 1998b).

Besides the fact that an injection of D-Asp into rats induces a rise in the serum levels of LH, testosterone, progesterone, GH, and PRL, experiments *in vitro* – involving the incubation of isolated hypothalamus with D-Asp – have indicated that it can

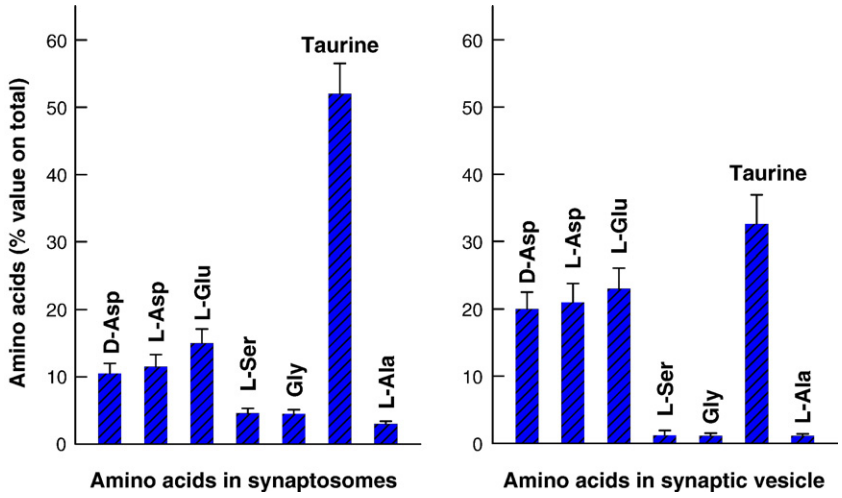



**Fig. 7 – Amino acids content in the synaptosomes and synaptic vesicles from the cerebral ganglia of *Aplysia limacina*. Synaptosomes were obtained from a pool of *Aplysia limacina* cerebral ganglia using a sucrose gradient. Synaptic vesicles were prepared by homogenizing synaptosomes in distilled water and then collecting the vesicles at 100,000 × *g* for 2 h. Synaptosomes and synaptic vesicles were both homogenized in 0.1 M HCl and then centrifuged. A suitable aliquot of the supernatant was analyzed by HPLC. Left panel: Amino acid composition found in synaptosomes. Right panel: Amino acid composition found in the synaptic vesicles. In both cases, values are expressed as percentage concentration of each amino acids compared to the concentration of total amino acids. The values are from Ref. Spinelli et al. (2006).**

elicit the release of mGnRH (mammalian gonadotropin-releasing hormone), the peptide hormone responsible for LH and FSH release from the pituitary cells (D'Aniello et al., 2000a). Interestingly, in the rat brain, we have also detected the presence of endogenous NMDA (N-methyl-D-aspartic acid), which enhances the release of the GnRH from the hypothalamus (D'Aniello et al., 2000a,b). In fact, NMDA ability to enhance the release of the hypothalamic factors had already been discovered by Downing et al. (1996), Gay and Plant (1987), Ondo et al. (1988), Pohl et al. (1989) and Price et al. (1978). They observed that NMDA, which posses the capability

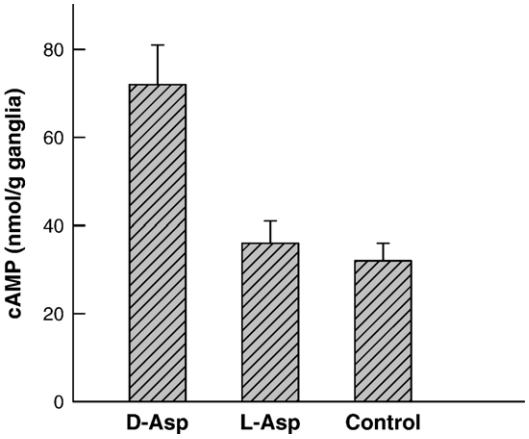



**Fig. 8 – Effects of D-Asp on cAMP synthesis in *Aplysia limacina* cerebral ganglia. Concentrations of cAMP in cerebral ganglia after intracoelomic injection of either D-Asp, L-Asp, or sea water. Values represent mean ± SD for each group (10 animals). ($P<0.01$). CAMP levels significantly increased ($P<0.01$) in the animal's ganglia injected with D-Asp. The values are from Ref. Spinelli et al. (2006).**

of increasing the release of the GnRH in *Macaca mulatta* (Gay and Plant, 1987), led to an increase in the two hypothalamus hormones – prolactin (PRL) (Downing et al., 1996; Pohl et al., 1989) and LH (Ondo et al., 1988; Pohl et al., 1989; Price et al., 1978) – in rats and ewes. Later we found that this methylated amino acid was synthesized by an enzyme by us called D-aspartic acid methyltransferase (or NMDA synthetase). It is present in the rat and in other animals (D'Aniello et al., 2002) and catalyzes the conversion of D-Asp into NMDA (D'Aniello et al., 2000a,b). Experiments *in vitro* have indeed revealed that when adenohypophysis is incubated with D-Asp 1 mM, LH and GH release is significantly increased (Table 6). However, when D-Asp is incubated with both adenohypophysis and hypothalamus, the rise in LH and GH release increases to a much greater degree (Table 6). What lends support to this observation is the fact that D-Asp in adenohypophysis cells is transformed into NMDA [28;29] which, in turn, induces a much higher release of these hormones. Further, when adenohypophysis is incubated with both D-Asp and NMDA, the release of hormones is significantly higher. This action is probably due to the stimulation of the NMDA-type L-glutamate receptors, since in the presence of D-AP5, a known molecule that inhibits glutamate receptors, the hormonal release is abolished (Table 6).

### 6.1. *Involvement of D-aspartic acid in the hormone regulation of the hypothalamus-pituitary-gonads axis in rat*

Further studies have indicated that, in the testes, D-Asp is capable of boosting the action of hCG (human chorionic gonadotropin), thereby inducing testosterone synthesis in purified rat Leydig cells (Nagata et al., 1999a). It has been proposed that D-Asp is taken up into the cells to increase the steroidogenesis activity. In fact, L-cysteine sulfinilic acid, an

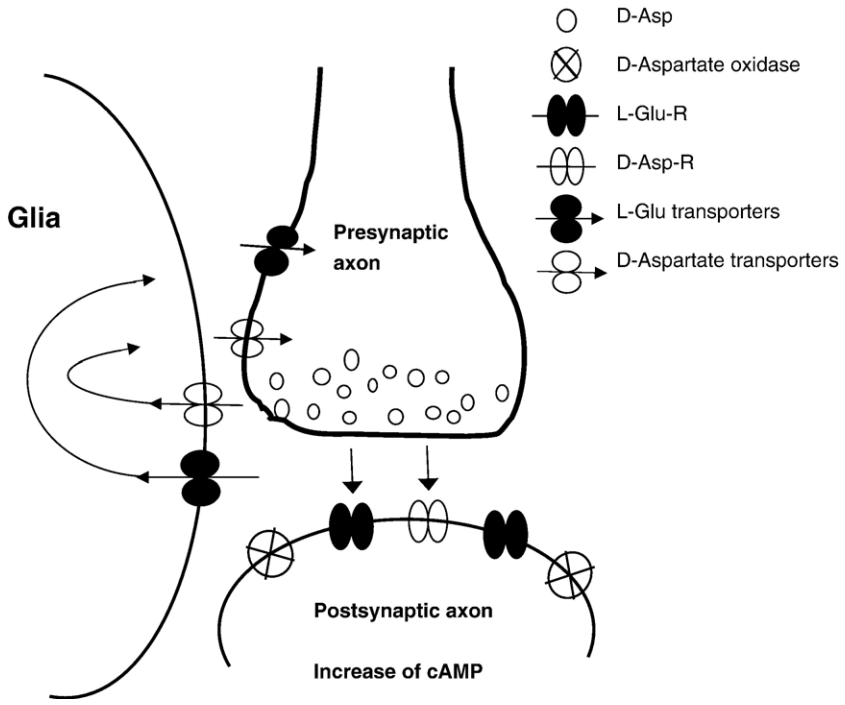



**Fig. 9 – Diagram outlining some of the possible roles of D-Asp in synaptic signaling. Release of D-Asp could act on postsynaptic targets such as L-Glu receptors and putative D-Asp receptors. The action of D-Asp is terminated by a D-aspartate oxidase located at the postsynaptic densities. In addition to this action, D-Asp enters neurons and glia through L-Glu transporters and, by doing so, interferes with the synaptic dynamics of L-Glu neurotransmission (e.g., increased half life of L-Glu in the synaptic cleft). In addition to these roles, there may be an unidentified transporters for D-Asp. The values are from Ref. Spinelli et al. (2006).**

inhibitor of D-aspartate uptake, suppresses both testosterone production and intracellular D-Asp levels (Nagata et al., 1999a). The action of D-Asp in hormone release is highly specific, since none of the other amino acids tested, including L-Asp, D-Glu and L-Glu, etc., can induce testosterone synthesis in Leydig cells (Nagata et al., 1999a). The molecular mechanism by which D-Asp stimulates testosterone synthesis is regulated by an mRNA of StAR gene expression (stimulating steroidogenic acute regulatory protein) (Nagata et al., 1999b). The involvement of D-Asp in endocrine activity has also been clearly observed in non-mammalian animals. For example, in the gonads of the green frog *R. esculenta*, where D-Asp controls testosterone production (Di Fiore et al., 1998), changes in D-Asp concentrations occur in the different phases of the sexual cycle. However, contrary to what happens in mammals, in this animal's gonads, D-Asp shows an inverse correlation between D-Asp concentration and testosterone production. Specifically, during the early spring period (March), a time when D-Asp concentrations in the ovary are low (2–4 nmol/g ovary), testosterone levels are high (36.9±4.8 ng/g ovary). In contrast, in autumn (October), when D-Asp concentrations in the ovary are relatively high (50–60 nmol/g ovary), testosterone levels are rather low (1–2 ng/g ovary). Thus, by inhibiting testosterone production in October, D-Asp blocks the fertilization process of amphibians during the cold season (Di Fiore et al., 1998). Laboratory experiments, involving the injection of D-Asp into the dorsal lymphatic sac of adult females, have confirmed that D-Asp increases in the ovary and decreases in the testes, lowering therefore testosterone levels (Di Fiore et al., 1998). Another biological function of D-Asp has been observed in the ovary of the lizard *Podarcis s. sicula*. In this

**Table 5 – Effects *in vivo* of D-Asp on different hormone release in the blood of adult male rat**

| Hormones | ng/ml Serum | |
|---|---|---|
| | Control | Between 1 and 5 h after i.p. injection of 2 mM D-Asp |
| Prolactin (PRL) | 12.0±3.5 | 30.2±4.5* |
| Growth hormone (GH) | 31.6±6.5 | 82.5±17.8* |
| Luteinizing hormone (LH) | 3.7±0.6 | 9.1±1.3* |
| Testosterone | 5.5±1.3 | 18.5±5.4* |
| Progesterone | 10.8±3.2 | 29.4±6.7* |
| 17β-Estradiol | 2.5±0.5 | 2.8±0.7 |
| 17α-Hydroxyprogesterone | 20.1±5.2 | 22.3±4.8 |
| Androstenedione | 1.0±0.2 | 1.4±0.3 |
| Cortisol | 7.0±2.3 | 7.8±1.2 |

Results indicate ng/ml serum of adult rat (D'Aniello et al., 2000a,b). Each result represents the mean and standard deviation of hormones released after 1 h (prolactin) and after 5 h (the other hormones) following i.p. injection of D-Asp (2 mM).
* Different from control ($P<0.01$).

**Table 6 – Effects of D-Asp, NMDA and D-AP5 on GH and LH release from isolated adenohypophysis and adenohypophysis plus hypothalamus**

| Concentration in the medium | Adenohypophysis | | Adenohypophysis + Hypothalamus | |
|---|---|---|---|---|
| | LH | GH | LH | GH |
| Control (1 mM NaCl) | 3.7±0.6 | 31±6 | 8.4±2.1 | 80±11 |
| D-Asp 1 mM | 4.3±1.0* | 82±10** | 15±4 | 120±20* |
| D-Asp 1 mM+NMDA 0.1 mM | 8.8±1.9** | 110±15** | 25.3±4.7** | 190±23** |
| D-Asp 1 mM+NMDA 0.1 mM+D-AP5 0.1 mM | 4.1±0.6 | 50±8 | 12.3±2.4 | 90±11 |

Results indicate ng/ml medium (D'Aniello et al., 2000a,b). Adenohypophysis was incubated alone or with hypothalamus in 2 ml of medium at 37 °C for 60 min under moderate agitation. Values represent the mean±SD of 5 experiments. D-AP5 is a specific NMDA receptors antagonist.
* Different from control ($P<0.05$).
** Different from control ($P<0.01$).

animal, in fact, D-Asp is capable of inducing an increase in aromatase activity, the enzyme responsible for the conversion of testosterone into 17β-estradiol (Assisi et al., 2001).

All the above results clearly indicate that D-Asp, either alone or together with NMDA, is involved in the mechanisms underlying hormone release and synthesis in rats (Fig. 10). The endocrine pathway observed in rats has also been recently observed in *C. intestinalis*, a marine animal belonging to the class of the tunicates (animals which are considered vertebrates as larvae and invertebrates as adults). Indeed, we found that D-Asp and NMDA are both present in the nervous ganglia, in the neural gland, and in the gonads of *C. intestinalis*, and that NMDA is biosynthesized by D-Asp. Thus, in this species, the neural gland NMDA elicits a release of GnRH which, in turn, stimulates the release of testosterone and progesterone from the gonads (D'Aniello et al., 2003).

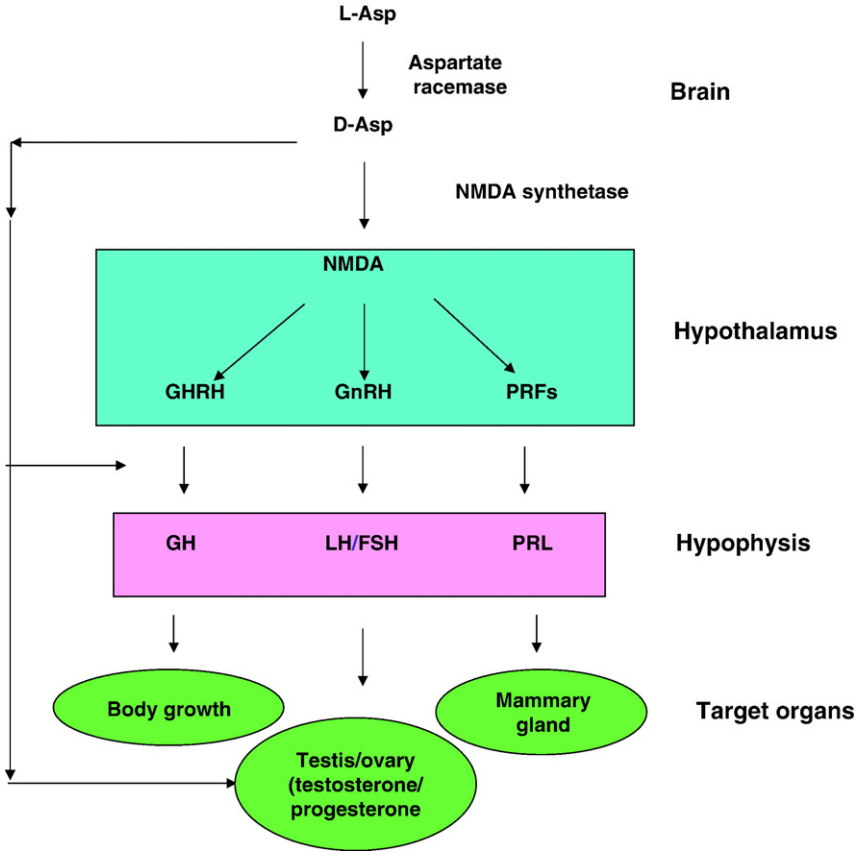



**Fig. 10 – Pathway of the involvement of D-Asp and NMDA in the hormone release of the hypothalamic–hypophysis–gonad axis. In the brain, L-Asp is converted into D-Asp by an aspartate racemase and, in turn, D-Asp is converted into NMDA by a D-aspartate methyltransferase (NMDA synthetase). In the hypothalamus NMDA induces the release of GHRH, GnRH and PRFs factors, which at the pituitary gland, stimulate the release of GH, LH, FSH, and PRL. LH and FSH, in turn, stimulate testosterone and progesterone in the testis or ovary. However, D-Asp has also a minor direct action on the pituitary gland in the release of LH and FSH, as well as on testes or ovary in the release of testosterone and progesterone, respectively.**

### 6.2. *Involvement of D-aspartic acid in the activity of other endocrine glands*

Recently Pampillo et al. (2002) have found that 1 mM D-Asp is capable of enhancing the release of GnRH, and α-MSH (α-melanocytes-stimulating hormone) from rat adenohypophysis. Specifically, GnRH increases by about 1.5 times (about 3.8 pg/mg protein in stimulated sample versus about 2.8 pg/mg protein in control) and α-MSH release increases by about 1.31 times (about 50 pg/mg protein in stimulated sample versus about 38 pg/mg protein in control). Evidence that the stimulatory effect of D-Asp is exerted through NMDA receptors is that in the presence of D-AP5 (D-(–)2-amino-5-phosphonopentanoic acid) – a competitive antagonist of NMDA receptor – the release of both factors is blocked. Furthermore, when the hypothalamus is pre-incubated with 1.0 mM of D-Asp and then stimulated with 40 mM $K^+$ ion, GABA is readily released, whereas dopamine is inhibited (Pampillo et al., 2002). Notably, other researchers obtained evidence that D-Asp could have a role in the release of other hormones. For instance, Imai et al. (1995) found that the concentration of secretion from the pineal gland in 6-week-old Sprague-Dawley rats reached very high values at night (mean value 2830 pmol/pineal gland at 2:00 a. p.), whereas very significant low values were found during the day (682 pmol/pineal gland at 3:00 p.m.) (Table 7). This concentration reflects that of melatonin during the nocturnal–diurnal cycle. In fact, the same authors found that at 2:00 a.m. the secretion of this hormone rose at 5.7 pmol/pineal gland; whereas at 3:00 p.m. it dropped at 1.6 pmol/pineal gland. Similar results were found by Schell et al. (Schell et al., 1997b). Indeed, they reported that D-Asp cyclically increases and decreases in the pineal gland, depending on the light or dark cycle. They found, in fact, that the very high peaks of D-Asp (2.0–2.2 μmol/g tissue) occurring during the night are then followed by low ones (0.2–0.3 μmol/g tissue) in the day (Table 7).

### 6.3. *Involvement of D-aspartic acid in the activity of exocrine glands*

In salivary glands of rats, D-Asp has been reported to be present in relative high levels (Masuda et al., 2003). High concentrations have been found in the parotid glands (PG) and in submadibular glands (SMG) of 4- to 7-week-old rats (about 400 and 250 nmol/g tissue in PG and SMG respectively) (Masuda et al., 2003). Recently, Raucci et al. (2005) have found, in the frog *R. esculenta*, high levels of D-Asp in the Harderian glands (HG), which are orbital seromucoid glands displaying seasonal changes in secretory activity. The maximum concentration of D-Asp was found during the spring. Like the rat pituitary gland, after an injection of D-Asp in *R. esculenta*, D-Asp rises in the HG and is capable of stimulating the secretion of mucous compounds (Fig. 11). This activity – mediated by the activation of ERK1, a phosphoprotein kinase involved in the transcriptional process – is accompanied by the increase in RNA synthesis (Raucci et al., 2005). In conclusion, the effects of D-Asp on exocrine gland activity appear to be specific for this amino acids, for no other D- or L-amino acid shows the same secretory effects (Table 8).

**Table 7 – D-Asp and melatonin concentration in rat pineal gland during day/night cycle**

| Dissection time | D-Asp (pmol/pineal gland) | Melatonin (pmol/pineal gland) |
|---|---|---|
| 2:00 a.m. | 2830 ± 485 | 5.7 ± 1.1 |
| 3:00 p.m. | 682 ± 194 | 1.6 ± 0.3 |
| 10:00 a.m. | 1030 ± 200 | 1.6 ± 0.1 |

Results are taken from Reference Imai et al. (1995). Groups of 6-week-old male Sprague-Dawley rats were sacrificed at different times. Pineal glands were used for the D-Asp and melatonin determination.

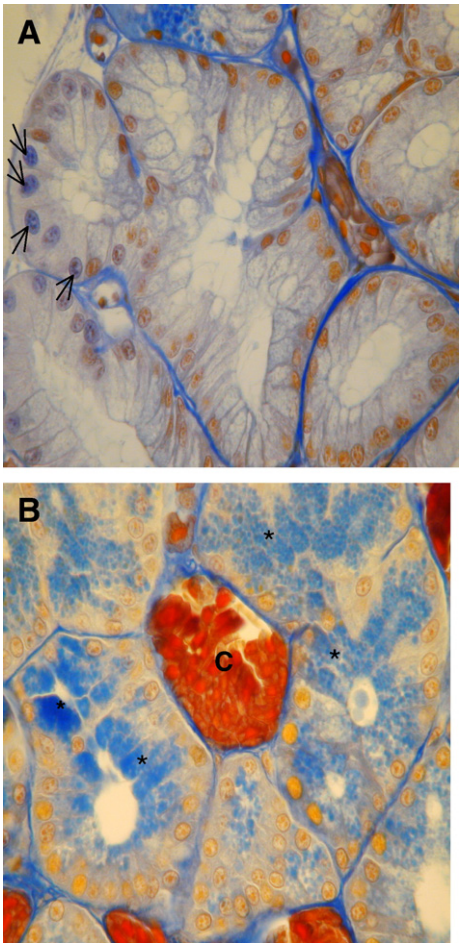



**Fig. 11 – Paraffin sections of HG of *Rana esculenta*. (A) The glandular cells of a March frog are columnar, with weakly basophilic cytoplasm. Note "blue nuclei" among orange-stained nuclei of glandular cells (arrows). (B) Two hours after D-Asp injection, numerous blue nuclei (asterisks) are present in the HG acini of a March frog. (C) Fifteen hours after D-Asp treatment, glandular cells of March frogs are filled with basophilic secretory granules. Mallory stain; ×500. The values are from Ref. Raucci et al. (2005).**

**Table 8 – Effects of D-aspartate on hormone release from isolated endocrine and exocrine glands**

| Animal | Tissues | Effects | Reference |
|---|---|---|---|
| *Endocrine glands* | | | |
| Rat | Hypothalamus | Increase GnRH release | (Pampillo et al., 2002) |
| | Hypothalamus | Increase α-MSH and GABA release | (Pampillo et al., 2002) |
| | Hypothalamus | Inhibit dopamine release | (Pampillo et al., 2002) |
| | Pineal Inhibit | melatonin release | (Ishio et al., 1998; Takigawa et al., 1998) |
| | Pituitary | Increase LH and GH release | (D'Aniello et al., 1996; D'Aniello et al., 2000a) |
| | Pituitary | Increase PRL release | (D'Aniello et al., 2000b) |
| | Testes | Increase testosterone and Progesterone release | (D'Aniello et al., 1996; D'Aniello et al., 2000a) |
| | Leydig cells | Stimulate testosterone synthesis | (Nagata et al., 1999a) |
| *Rana esculenta* | Ovary | Inhibit testosterone release | (Di Fiore et al., 1998) |
| *Podarcis s. sicula* | Ovary | Increase 17β-estradiol release | (Assisi et al., 2001) |
| *Ciona intestinalis* | Ovary | Increase testosterone and progesterone release | (D'Aniello et al., 2003) |
| *Exocrine glands* | | | |
| *Rana esculenta* | Harderian gland | Increase secretion of seromucoid | (Raucci et al., 2005) |

GnRH (gonadotropin releasing hormone); LH (luteinizing hormone); GH (growth hormone); PRL (prolactin); α-MSH (melanocytes-stimulating hormone).

### 6.4. *Hormonal effects of D-aspartic acid on isolated cells*

Other studies, conducted on rat pituitary tumor cells (GH3), have reported that D-Asp promotes the activity of thyrotropin-releasing hormone (TRH) in stimulating PRL release from cells (Long et al., 2000). When GH3 cells are incubated with 1.0 μM TRH and with 50 μM D-Asp, PRL secretion is 2 times higher than that in the control (Long et al., 2000). Furthermore, D-Asp has been found in the magnocellular neurons of rat hypothalamus, the neurons that produce oxytocin. Actually, oxytocin (the hormone responsible for milk production during lactation) is elevated in magnocellular neurons during lactation but returns to normal levels thereafter (Wang et al., 2000). Indeed injections of D-Asp into the hypothalamus for 7 days in a row induce significantly higher levels of oxytocin mRNA, thus indicating that D-Asp regulates the synthesis and secretion of oxytocin *in vivo*. Furthermore, D-Asp increases levels of vasopressin mRNA and could have a general role in the modulation of gene expression or hormone production (Wang et al., 2000).

## 7. D-Aspartic acid as a precursor for the synthesis of NMDA

Recently, we have demonstrated that *N*-methyl-D-aspartic acid (NMDA) is a molecule present in the rat neuroendocrine system (D'Aniello et al., 2000a,b), in *C. intestinalis* (D'Aniello et al., 2003), and in other animal phyla (D'Aniello et al., 2002). Synthetic NMDA is well known for its stimulatory action on the NMDA-type L-glutamate receptor. Then, in addition to having this stimulatory effect, NMDA is also known to induce hormone release in the hypothalamus and hypophysis. In fact, as far back as 1978, Price et al. had already observed that a very low amount of NMDA injected into the rat increased LH levels (Price et al., 1978). After that, Gay et al., and Downic et al., also reported that a minute quantity of NMDLA (*N*-methyl-DL-aspartic acid) injected into Rhesus monkey (*M. mulatta*) induced the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Gay and Plant, 1987). Other researchers have demonstrated that synthetic NMDA is also able to elicit the release of LH in rats (Pampillo et al., 2002), PRL in ewes (Downing et al., 1996) and rats (Pohl et al., 1989), and GH in ewes (Downing et al., 1996). Consistently, our studies have indicate that the endogenous NMDA is biosynthesized *in vivo* and *in vitro* and that D-Asp is its biological precursor (D'Aniello et al., 1998a,b). Specifically, we have detected an NMDA methyltransferase which possesses the ability to transfer a methyl group from SAM (S-adenosyl-methyonine) to D-Asp so as to form NMDA. Furthermore, our rat studies have elucidated the fact that endogenous D-Asp and NMDA are both involved in neuroendocrine activity, particularly in the control of the hormone release axis: hypothalamus–pituitary–gonad (Fig. 10). In an attempt to clarify the biological mechanism taking place in this pathway, we are going to trace the cascade of interconnected biological events in the brain, in the hypothalamus, in the pituitary glands, and, finally, in the gonads: (1) in the brain (above the whole cortex and hypothalamus) L-Asp is racemized in D-Asp; (2) in the hypothalamus, NMDA is synthesized from D-Asp, and, in turn, NMDA induces the release of GnRH; (3) in the pituitary gland, GnRH induces LH release; and (4) in the gonads, LH induces testosterone and progesterone release (D'Aniello et al., 2000a,b) (Fig. 10). The same biological pathway has been found in the invertebrate *C. intestinalis* (D'Aniello et al., 2003). Indeed, in the animal's cerebral ganglia, D-Asp is synthesized and transferred to the neuronal ganglia where it is then transformed into NMDA. Next, NMDA induces GnRH release, which, in turn, is transported to the gonads, thereby inducing the increase in testosterone production (Fig. 10).

## 8. Conclusion on the role of D-aspartic acid and NMDA

In conclusion, the evidence so far accumulated suggests that D-Asp plays a dual role: a role in the nervous system and a role in the endocrine system.

In the nervous system, some studies have pinpointed that in the chicken brain and retina a transient high concentration of D-Asp occurs in the last stage of the animal's embryonic life. The same event occurs in the rat brain and retina during the early postnatal life (Dunlop et al., 1986; Hashimoto et al., 1993b; Neidle and Dunlop, 1990). Moreover, evidence that D-Asp acts as a neuromodulator or neurotransmitter is that it is present in nerve endings (synaptosomes and synaptic vesicles), it is released by potassium and ionomycin stimuli, and it is capable of increasing intracellular cAMP levels in cultured neurons (Spinelli et al., 2006). In addition, a number of studies have detected specific transporters for D-Asp, which specifically transfer it from the synaptic cleft to glia and to presynaptic neurons (Bouvier et al., 1992; Kanai and Hediger, 1992). Lastly, it has been reported that an enzymatic system present in nerve endings transforms L-Asp into D-Asp (Spinelli et al., 2006).

In the endocrine system, D-Asp has a role in the regulation of hormone release from various endocrine glands (D'Aniello et al., 1996; D'Aniello et al., 2000a,b). In fact, it is able to induce the release of: (1) GnRH from the hypothalamus, (2) LH, GH and PRL from the pituitary gland, and (3) testosterone and progesterone from the gonads (D'Aniello et al., 1996; D'Aniello et al., 2000a,b). Notably, D-Asp also constitutes the precursor for the synthesis of the endogenous NMDA (D'Aniello et al., 2000a,b), which at nM concentration levels is able to induce the release of hypothalamic factors and pituitary hormones (Downing et al., 1996; Gay and Plant, 1987; Pohl et al., 1989; Price et al., 1978).

## 9. D-Aspartate oxidase: the key enzyme for measuring the concentration of D-Asp and NMDA

The last part of the present review contains a brief summary of the methods we developed to detect low quantities of D-Asp and NMDA. Since D-aspartate oxidase constituted the essential tool for the determination of D-Asp and NMDA, we will first describe some of its main characteristics and then each specific method.

D-Aspartate oxidase (D-AspO; EC 1.4.3.1), a peroxysomal enzyme with a molecular weight of about 40 kDa, is a flavoprotein that containing two FAD (flavin–adenin-dinucleotide) residues as prosthetic group of protein molecules (D'Aniello and Rocca, 1972; De Marco et al., 1969; Dixon et al., 1967; Still and Sperling, 1950). It was first purified from the hepatopancreas of *O. vulgaris* with an isoelectric point of pH 5.8, Km versus D-Asp of 5.2 mM, and an optimum pH of 8.2 (D'Aniello and Rocca, 1972). Later, it was also purified from beef kidney (Negri et al., 1987). Because of its presence in various mammals (Davies and Johnston, 1975; De Marco et al., 1969; Yusko et al., 1973), as well as in *Criptococcus hymicolus* (Takahashi et al., 2004; Yamada et al., 1996), this enzyme has been studied for its peculiar characteristics. In effects, D-AspO is similar in structure but not in specificity to the another cognate oxidative enzyme, *viz*, D-amino acid oxidase (D-AAO; EC 1.4.3.3.). This latter enzyme, in fact, is capable of oxidizing all the other D-amino acids except for D-Asp, D-Glu and NMDA (Dixon and Kleppe, 1965; Konno et al., 1982; Simonetta Pollegioni et al., 1989). The D-AspO is ubiquitously distributed in bacteria, mould, and in high animals. Notably, it is present in various tissues of mammals, particularly in the kidneys, liver and brain (Davies and Johnston, 1975; Zaar et al., 2002). The mechanism of reaction by which D-AspO oxidizes D-Asp is the following (D'Aniello et al., 1993a; D'Aniello et al., 2005; D'Aniello et al., 2000a; De Marco et al., 1969; Dixon and Kleppe, 1965).

$$\text{D-Asp} + O_2 + H_2O \xrightarrow{\text{D-AspO}} \alpha\text{-ketoacids} + H_2O_2 + NH_3$$

Notably, D-AspO has an intriguing enzymatic specificity that varies depending on the animal species. The enzyme from *O. vulgaris* is able to oxidize D-Asp and D-Glu at very high rates, but NMDA at low ones (D'Aniello et al., 1993c; Tedeschi et al., 1994). The one from beef kidney, instead, is able to oxidize D-Asp and NMDA at very high rates, but D-Glu at very low ones. However, regardless of its source, it reacts only to D-Asp, D-Glu and NMDA (D'Aniello et al., 1993c; Dixon and Kleppe, 1965; Tedeschi et al., 1994) and uses either oxygen or potassium ferricyanide ($K_3Fe(CN)_6$) as the electron acceptor (De Marco et al., 1969; Dixon et al., 1967). Recently, the D-AspO from beef kidney, obtained by overexpression and purified from *Escherichia coli* (D'Aniello et al., 2005; Negri et al., 1999), has revealed the same chemical and enzymatic characteristic of the enzyme purified from beef kidney.

Two principal methods are frequently used for the determination of the enzymatic activity: (1) an indirect method, based on the oxidization of $K_3Fe(CN)_6$, and (2) a direct method, based on the determination of the α-oxaloacetate derived from D-Asp oxidation.

(1) The indirect method is based on the fact that when D-AspO reacts with its substrate, the FAD (flavin adenine dinucleotide, oxidized form), which constitutes the prosthetic group of the enzyme, is reduced to FADH (flavin adenine dinucleotide, reduced form) as follows:
   (a) D-amino acid + D-AspO-FAD + $2H^+$ = oxidized D-amino acid + D-AspO-$FADH^+$
   (b) D-AspO-$FADH^+$ $O_2$ = D-AspO-FAD + $2H^+$
   If in the assay mixture another redox molecule, like $K_3Fe(CN)_6$, is present, D-AspO-FADH reacts with $K_3Fe(CN)_6$ and not with oxygen and returns to D-AspO-FAD (oxidized). Instead, $K_3Fe(CN)_6$ is transformed into $K_4Fe(CN)_6$. The $K_3Fe(CN)_6$ is yellow and has maximum absorbance at 420 nm, whereas the $K_4Fe(CN)_6$ is colorless. Such reduction is taken for the measurement of the enzymatic activity (D'Aniello and Giuditta, 1977; Dixon et al., 1967; Dixon and Kleppe, 1965).
(2) The direct method is based on the measurement of the α-oxaloacetate produced by the oxidation of D-Asp as follow:
   (1) α-oxaloacetate + 2,4-dinitrophenylhydrazine = α-oxaloacetate-dinitrophenylhydrazine
   (2) α-oxaloacetate-dinitrophenylhydrazine complex + NaOH = dinitrophenylhydrazone (purple colour).

The enzymatic assay is carried out as previously described (D'Aniello et al., 1993a, 2005). One enzymatic unity (E.U.) is defined as the amount of the enzyme which catalyzes the production of 1.0 nmol of oxaloacetate in a 1-min incubation at 37 °C.

## 10. Determination of total D-Asp, D-Glu and NMDA and specific analysis for D-Asp or NMDA

The total amount of D-Asp, D-Glu and NMDA is determined by various colorimetric or fluorimetric methods based on the determination of the α-ketoacid or $H_2O_2$ (Gross and Sirer, 1959) developed by the oxidation of one of the above amino acids by D-AspO according to the following reaction:

$$\text{D-Asp or D-Glu or NMDA} + O_2 + H_2O_2 \xrightarrow{\text{D-AspO}} \alpha\text{-ketoacids} + H_2O_2 + NH_3\ (\text{or } CH_3\text{-}NH_2)$$

$NH_3$ is developed if the substrate is D-Asp or D-Glu, whereas $CH_3$-$NH_2$ (methylamine) is developed if the substrate is NMDA (D'Aniello and Giuditta, 1977; D'Aniello et al., 1993a,b, 2000a,b, 2003, 2005; Spinelli et al., 2006).

Other studies, instead, have used an HPLC method combined with D-AspO to determine specifically either D-Asp than NMDA. The determination of specific D-Asp is obtained by using a chromatographic enzymatic HPLC method based on the separation of D-Asp from other amino acids at HPLC (Aswad, 1984) and its oxidation with D-AspO (D'Aniello et al., 2000a,b, 2002, 2005; Spinelli et al., 2006). The determination of specific NMDA is based on the determination of $CH_3$-$NH_2$

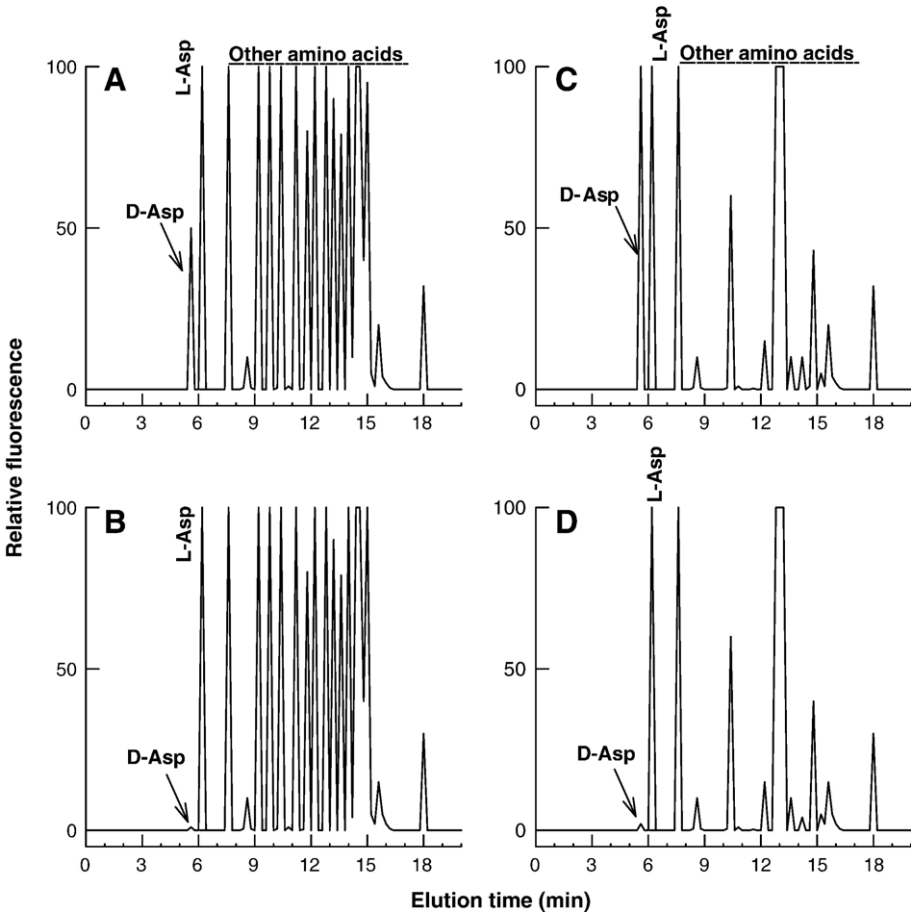
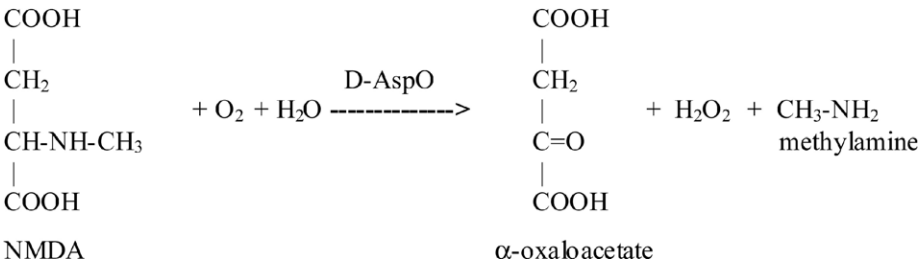



**Fig. 12 – Typical example of determination of D-Asp by HPLC. (A) HPLC profile of a mixture of free amino acids contained 25 nmol of D-Asp and 50 nmol of 19 different L-amino acids. The HPLC column used was an ODS-$C_{18}$ (0.45×25 cm) and the OPA-NAC (O-phthaldialdehyde-*N*-acetylcysteine) was used as the derivative agent. (B) Same sample but after its incubation with D-AspO (the enzyme which specifically destroy D-Asp). After D-Asp treatment, the peak corresponding to D-Asp is completely abolished or strongly reduced. (C) HPLC profile of an TCA homogenate of cerebral ganglia of *Aplysia limacina*. The peaks indicate amino acid compositions from 0.05 mg tissue. (D) Profile indicates the same sample used after incubation with D-AspO. The peak corresponding to D-Asp is abolished or strongly reduced, demonstrating that this peak actually corresponded to D-Asp (Spinelli et al., 2006).**

(methylamine) at HPLC, which is generated by the oxidation of NMDA with D-AspO according to the following reaction (D'Aniello et al., 1998a, 2000a,b). The Figs. 12 and 13 show two typical examples used for the determination of D-Asp and NMDA from a standard mixture of amino acids and from a biological sample, respectively.




## 11. Future prospects

On the basis of what has been discovered so far on D-Asp and NMDA, it would be of great scientific interest to delve deeper into the molecular mechanisms and signal transduction mechanisms that are elicited by these two amino acids in the nervous and endocrine systems. The theory that D-Asp is involved in the nervous system has been based on the fact that in chickens, during the last embryonic stage (Dunlop et al., 1986), and in rats, during the early neonatal life (Neidle and Dunlop, 1990), a transient increase in D-Asp occurs. Actually, during the embryonic or the early postnatal life, two important biological events take place. The first entails the activation of a specific gene that promotes the synthesis of D-aspartate racemase—the enzyme responsible for D-Asp synthesis (D'Aniello et al., 2005; Spinelli et al., 2006) and for converting L-Asp into D-Asp. The second regards the activation of a second specific gene that promotes the synthesis of a protein receptor for D-Asp. Such novel receptor, implicated in the capture of the input signal on the cell membrane, stimulates membrane signal transduction to trigger gene expression and protein synthesis, both involved in the development of the nervous system. By depleting the endogenous free D-Asp during the animal's embryonic stage, future studies may be able to examine whether phenotypes are altered in the animal's nervous system. One possible approach will entail the elimination of free endogenous D-Asp by overexpressing D-AspO, the natural enzyme which metabolizes D-Asp (Davies and Johnston, 1975; D'Aniello and Giuditta, 1977; D'Aniello et al., 1993a; Dixon et al., 1967; Negri et al., 1987).

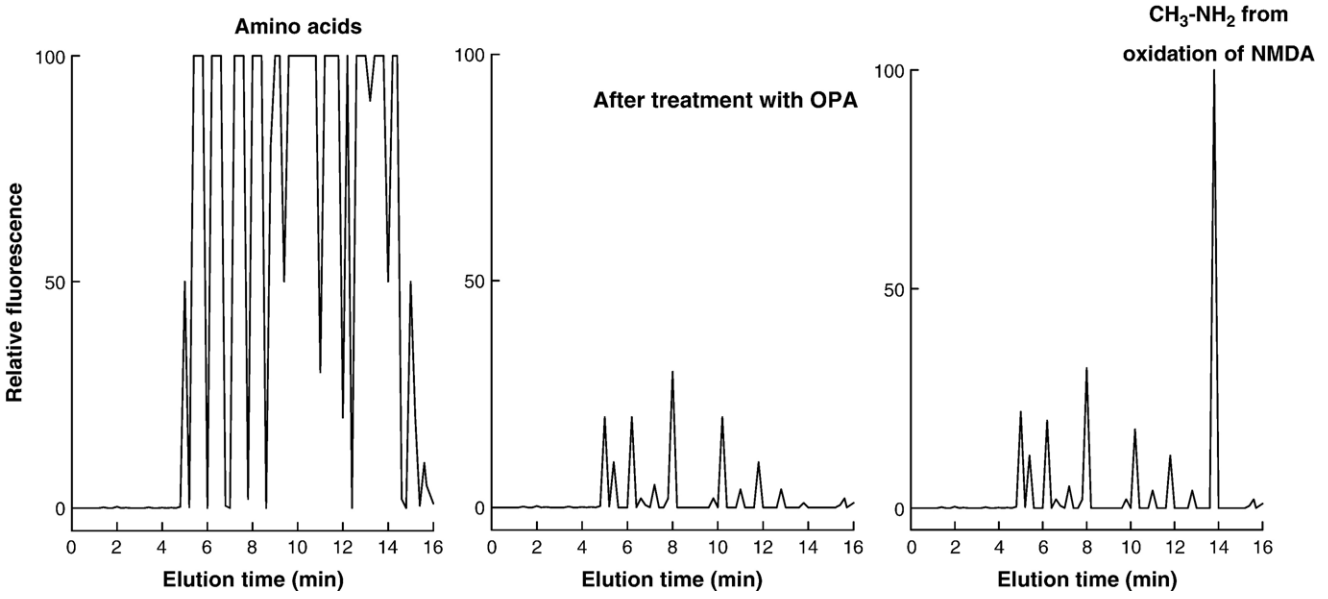



**Fig. 13 – Typical example of NMDA determination at HPLC through the quantification of methylamine ($CH_3$-$NH_2$). Left panel: A typical example of a free amino acid profile from a TCA supernatant of the cerebral ganglia of the tunicate *Ciona intestinalis*, treated with OPA-mercaptoethanol. Peaks correspond to the amino acids from 0.2 mg of tissue. Middle panel: HPLC analysis of the same sample, but after purification with OPA reagent to eliminate almost all free amino acids (but not NMDA). The HPLC profile of the amino acids corresponds to that contained in an amount of tissues from 2 mg of original tissue. Almost 95–98% of free amino acids have been eliminated by OPA treatment. Right panel: NMDA, which remained in the purified sample, reacts with D-AspO to produce methylamine ($CH_3$-$NH_2$), which in turn reacts with OPA-mercaptoethanol giving a sharp peak at 13.6–13.7 min.**

Among the various animal models, the zebra fish, as the vertebrate model, and *C. intestinalis*, as the invertebrate model, should be used owing to their well-documented genome and embryonic development.

A second important goal will be to determine whether D-Asp and NMDA can be regarded as two novel neurotransmitter/neuromodulator. Concerning D-Asp, we have recently found that it is present, at substantially high concentrations, in the terminal axons and in the synaptic vesicles of *A. limacina*. In addition, D-Asp is released from vesicles following $K^+$ ions or ionomycin stimuli (Spinelli et al., 2006). Thus, because these results clearly indicate that D-Asp possesses the role of a neurotransmitter/neuromodulator, it will be interesting to verify, via electrophysiological experiments, whether it also possesses similar, or better yet, the same properties in neurons isolated from *A. limacine* or other animals. To test such hypothesis, it will be necessary to prepare cultured motoneurons (L-7) and sensory neurons from juvenile animals. Then, following motoneuron stimulation, the current in the sensory neurons will be measured. Finally, the current response will indicate whether or not D-Asp inhibits the transmission and, consequently, whether or not D-Asp is a neurotransmitter.

Concerning NMDA, we have found that NMDA is present as an endogenous compound in the nervous and the endocrine tissues (D'Aniello et al., 2000a,b, 2002). Moreover, previous authors have demonstrated that very low levels of synthetic NMDA are able to induce the release of some hypothalamic and pituitary hormones (Downing et al., 1996; Gay and Plant, 1987; Ondo et al., 1988; Pampillo et al., 2002; Pohl et al., 1989; Price et al., 1978). Accordingly, it has been hypothesized that the endogenous NMDA could play a role in neuroendocrine transmission by specifically stimulating either nervous or hormonal cells for hormonal release. Therefore, by means of electrophysiological experiments, it might be intriguing to test whether NMDA possesses such ability in cultured hypothalamic and hypophysial cells from vertebrate animals. In addition to the electrophysiological experiments, equally intriguing will be to verify whether specific receptors for NMDA, other than the well-known NMDA-type L-Glu receptors, are present on the neuroendocrine cell membrane. To this aim, it will be necessary to do binding experiments on hypothalamic and pituitary cellular membranes using radiolabeled NMDA.

A third study may be to create an antibody anti-NMDA in the rabbit so as to localize the endogenous presence of NMDA in the various compartmental cells and in synaptic vesicles by means of optic and electron microscopy. However, because NMDA alone does not provoke an immunological response, in order to obtain an antibody anti-NMDA, the preparation of a complex conjugating NMDA with carbodiimide and a protein carrier (i.e.,. BSA) will be necessary. Eventually, the injection of such complex into the rabbit should be able to produce an antibody anti-NMDA.

A fourth and most important study may be to verify whether D-Asp can have a role in learning and memory. Indeed, we have previously found that in Alzheimer's brains, D-Asp is reduced compared to normal brains (D'Aniello et al., 1998b). This result, thus, could indicate that D-Asp (and/or NMDA, which is synthesized in vivo by D-Asp) is implicated in learning and memory. To verify this hypothesis, experiments on Aplysia species (i.e., *A. limacina*) and on rats will have to be conducted. The advantage of using Aplysia is that it possesses large neurons and can be biochemically analyzed. A possible approach could be to subject it to learning experiments. By doing so, researchers will be able to assess whether D-Asp (and NMDA) increases in the cerebral neurons and in nervous ganglia. The same experiment should be conducted on the rat. Briefly, after treating the rats with a D-Asp solution for several days, we will be able to assess memory improvement by using the Holton system as an indicator for learning and memory. Moreover, it would be equally insightful to conduct a biochemical study so as to detect the areas of the brain in which D-Asp is accumulated and to examine whether NMDA increases in these same areas as well.

In conclusion, the present review was written on the grounds that the physiological and biochemical mechanisms underlying D-Asp involvement in the development of embryonic and early postnatal life of animal, though largely documented, are far from being clear, not least its role in learning and memory processes. We hope that the host of evidence here reported, and, more important, our speculations on prospective investigations will further today's highly insightful findings by inspiring more promising and revealing research on the role of D-Asp and its mechanisms of action.

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