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Amino Acids

D-Aspartic acid and L-amino acids in the neural system of the amphioxus *Branchiostoma lanceolatum*

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Summary. The lancelet (amphioxus), a cephalochordate, is the closest invertebrate relative to vertebrates, with a simple vertebrate-like body plan and a prototypical genome. We have determined D-aspartic acid (D-Asp) and major free L-amino acids (L-AAs) content in the nervous system (neural tube) of the European amphioxus *Branchiostoma lanceolatum*, and have compared these values with those of molluscs and human brain. The *B. lanceolatum* neural tube contains relatively high amounts of L-Glu, L-Asp, L-Ala and L-Gly. Thus, the amphioxus neural tube has in common with the molluscan and human nervous systems the presence of appreciable amounts of L-Glu and L-Asp, which suggests that they are the most common neurotransmitters among these phylogenetically distant animal groups. The relatively high concentration of L-Ala in amphioxus is consistent with that found in molluscs and the low concentration of taurine is consistent with that described in the human brain.

The D-Asp concentration, very high in the molluscan nervous system, was rather low in amphioxus, although a little higher than the extremely low amounts observed in the human brain. Our data on free amino acids composition is in agreement with the intermediate phylogenetic position of cephalochordates, in terms of the evolutionary transition from simple to complex neural systems.

Keywords: Amphioxus – D-aspartic acid – L-amino acids – Nervous system – GABA – Chordates – Evolution

Introduction

The cephalochordate amphioxus represents a key animal model, with a privileged position at the invertebrate/vertebrate metazoan transition (Fig. 1). It is widely considered to be the closest living invertebrate relative of the vertebrates. Like vertebrates, the amphioxus body plan is characterised by a dorsal, hollow nerve cord, a notochord, segmental muscles, pharyngeal gill slits and a post-anal tail. Amphioxus embryonic development is also very close to that of the vertebrate, and increasing evidence indicates that embryonic patterning in amphioxus and vertebrates makes use of similar molecular pathways. However, its simpler genome generally contains single-copy genes for most gene families found in vertebrates genomes (Holland et al., 2004). This feature greatly advances an understanding of the gene evolution leading to vertebrates, since similar gene pathways in cephalochordates are supported by less complex information than in vertebrates, which is thus easier to analyse since it by-passes vertebrate genome redundancy.

Although many studies from different biological fields have been conducted on amphioxus, a biochemical study on its metabolism and role of free amino acids has not been carried out to our knowledge. Concentrations of free amino acids in animal tissues, particularly in the nervous system, are extremely important from a biochemical and physiological point of view. Indeed, one of the main roles of free amino acids is to act as substrates for important enzymatic reactions, as well as to regulate the balance of nitrogen compounds. Moreover, alterations in the concentration of some amino acids or their derivatives lead to important metabolic disorders. In humans, for example, high concentrations of cystine in the urine are a sign of cystinuria, a congenital defect in the amino acid transport system, which controls the passage of small molecular mass substances in and out of cells, ultimately resulting in cystine precipitation in the renal pelvis, forming cystine stones (calculi). An excess of phenylalanine in the blood can cause a disease called "phenylketonuria", a mental retardation characterised by the absence of L-phenylalanine hydroxylase, which transforms L-phenylalanine into tyrosine. High levels of tyrosine in the blood are a symptom of tyrosinosis, a disease characterized by hepatic cirrhosis and renal damage. Nevertheless, mental retardation can

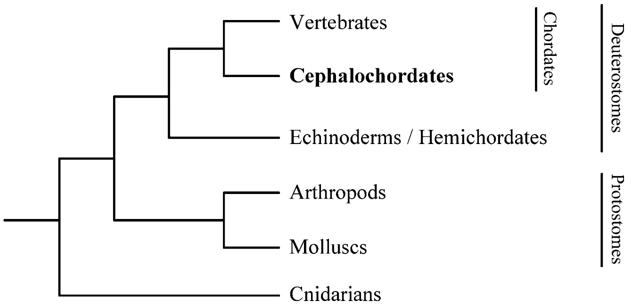

Fig. 1. Major phylogenetic relationships among some metazoan clades. Amphioxus (Cephalochordates) occupies a key position, predating the origin of vertebrates. Mammalian and molluscan data are discussed in the text

result from high concentrations of any of the following amino acids: glycine, valine, lysine, homocystine and hydroxyproline (Kachmar et al., 1976).

On the other hand, some amino acids play an important role in neurotransmission; for example, L-Glu and L-Asp are known to be important neurotransmitters, whereas L-Glycine and GABA are neuroinhibitors (Danbolt, 2001). In addition, recent evidence demonstrates that D-Asp plays an important role in hormone release from the neuroendocrine system (D'Aniello et al., 2000) and acts as a "novel" neurotransmitter (Danbolt, 2001). This amino acid is present in high concentrations in the nervous system tissues of opistobranchia (Spinelli et al., 2006), molluscs, tunicates, crustaceans, amphibians, reptiles, fishes, birds and mammals (D'Aniello et al., 2005; Spinelli et al., 2006). In addition, D-Asp has also been detected as a nuclear compound of cells in the mammalian hypothalamus-neurohypophyseal system (Wang et al., 2002) and in the retina of *Sepia officinalis*, where it is implicated in vision (D'Aniello et al., 2005). Therefore, in the present study, we determined not only the concentration of free L-amino acids, but also the concentration of D-Asp in the amphioxus nervous system.

Materials and methods

Animal collection and tissue homogenisation

Adult amphioxus (*Branchiostoma lanceolatum*) were collected in the bay of Argelès-sur-Mer (southern coast of France) at a depth of approximately 10 meters, between October and November 2005. Adult animals were kept for about a week in tanks in the laboratory before dissection, at a water temperature of 15 °C and a 10/14 day-night light cycle (corresponding to natural conditions). Filtered seawater was changed daily and antibiotics (Streptomycin 100 mg/l; Penicillin 10 mg/l) were provided to prevent bacterial contamination. Specimens were dissected under an optic microscope using a micro-fine knife with a 4 mm cutting edge (F.S.T.) on ice. No anaesthetic was used to immobilize the animals, in order to avoid drug-induced alterations in HPLC measurements. Neural tubes (Fig. 2C) from

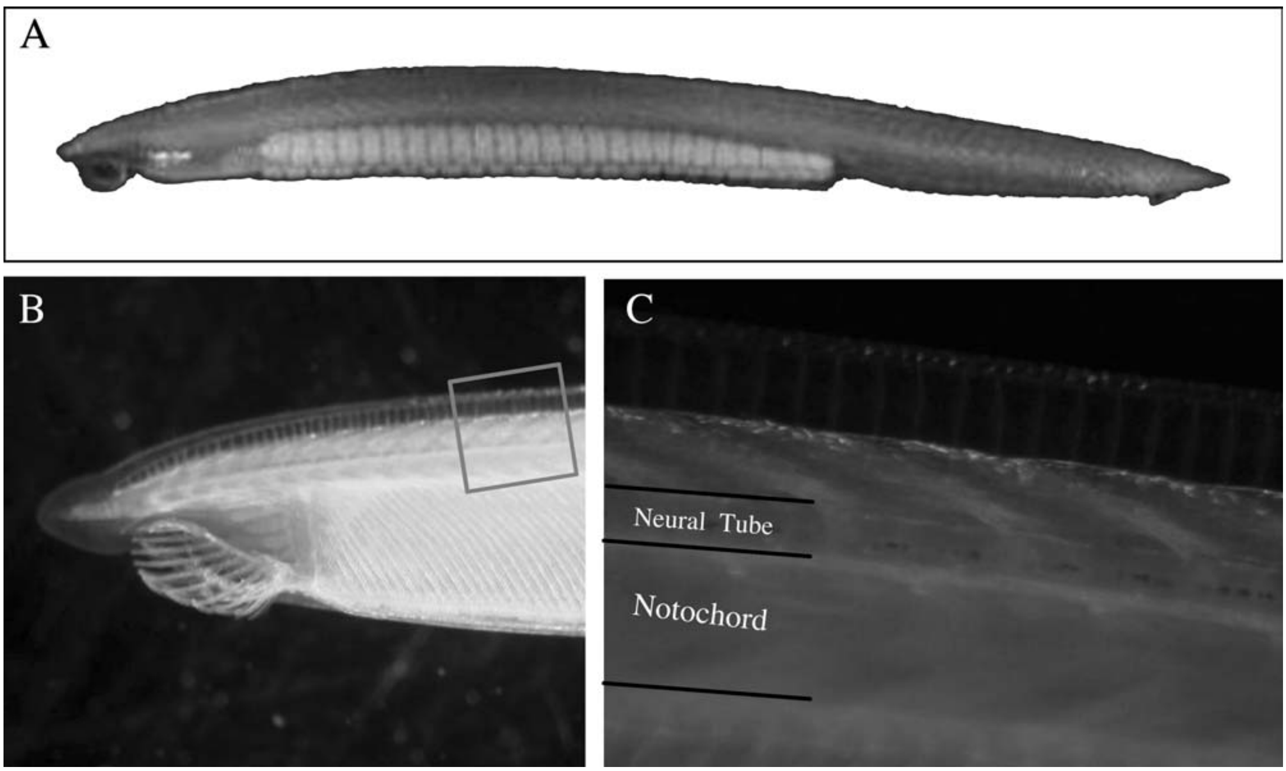

Fig. 2. **A** Lateral view of an adult amphioxus. Anterior is to the left and dorsal is at top. **B** Detail of the anterior part. **C** Enlargement of the frame highlighting the neural tube, dorsal to the notochord. Neural tube and notochord extend for nearly the entire length of the animal

60 adult animals were collected and divided into 6 groups. Each group, containing a pool of 10 neural tubes weighing a total of about 3 mg (approximately 0.3 mg each), was homogenised in 0.1 M TCA (trichloroacetic acid) at a ratio of 1:20 and centrifuged at 15,000 *g* for 15 minutes.

HPLC analysis of D-Asp and L-amino acids

20 µl of the TCA-homogenised pool (1 mg of tissues) of neural tubes was neutralised with 20 µl of 0.1 M NaOH and mixed with 100 µl of 0.5 M pyrophosphate buffer (pH 9.5) and 20 µl of OPA-NAC reagent, which was obtained by mixing 20 mg OPA (*o*-phthaldialdehyde) with 10 mg NAC (*N*-acetyl-L-cysteine) in 2.0 ml of methanol. After 2 min, distilled water was added to a final volume of 1.0 ml, and 100 µl of this mixture was injected into a Supelcosil C-18 HPLC column (0.45 × 25 cm in length and 5 µm beads; Supelco, Bellefonte, PA, USA). Amino acids were eluted using two buffers, A (10% acetonitrile in 30 mM citrate phosphate buffer, pH 5.6) and B (90% acetonitrile in double-distilled water), using the following gradient: 0 to 40% B over 40 min, 40–100% B over 10 min, maintaining at 100% B for 5 min, and then returning to 100% A for 1 min. The flow rate was 1.2 ml/min. Amino acids were detected fluorometrically at an excitation wavelength of 325 nm and an emission wavelength of 415 nm. D-Asp eluates with a peak at 4.6 min were followed by L-Asp (0.9–1 min later) and remained well separated from other amino acids (Fig. 3). In order to confirm that the peak eluting at 4.6 min was D-Asp, 20 µl of the TCA supernatant was neutralised with 20 µl of 0.1 M NaOH and mixed with 100 µl of 0.5 M pyrophosphate buffer (pH 8.2) and 2.0 µl of bovine D-aspartate oxidase (D-AspO, 1–2 mg/ml purified by overexpression in *E. coli*) (D'Aniello et al., 2000) and incubated at 37 °C for 20–30 min. Subsequently, 200 µl of 0.1 M sodium pyrophosphate (pH 10.0) was added and the HPLC analysis was repeated as above. The near complete disappearance of the peak at 4.6 min confirmed the presence of D-Asp (data not shown). To determine the concentration of D-Asp and L-amino acids, a standard curve was made with known amounts of amino acids purchased from Sigma Chemical Co (St. Louis, MO) as follows: 10 µl of a mixture of 20 different L-amino acids, each at a concentration of 0.1µmol/ml, and D-Asp at a concentration of 0.05mol/ml was mixed with 100 µl of 0.5 M pyrophosphate buffer (pH 9.5) and 20 µl of OPA-NAC. After 2 min, distilled water was added to obtain a final volume of 1.0 ml, and 100 µl of the sample was injected into the HPLC column. 100 µl of injected mixture contained 0.05 nmoles of D-Asp and 0.1 nmoles of each of the other amino acids.

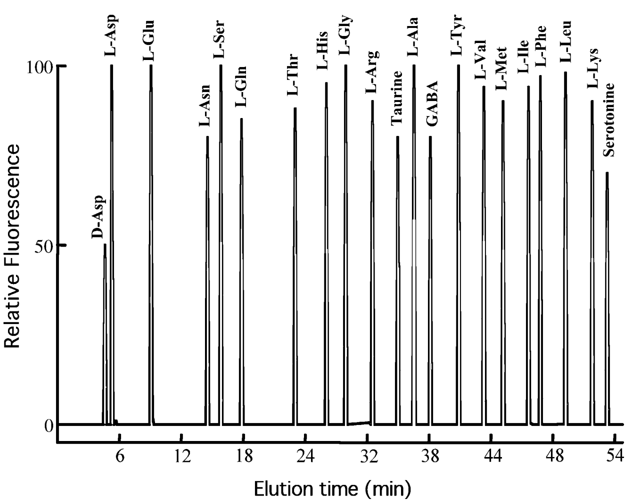

Fig. 3. HPLC chromatogram of a mixture of standard D-Asp and L-amino acids (Sigma Co.). The area of the peaks corresponds to the amount of amino acids: 10 pmoles of D-Asp and 20 pmoles of each L-amino acid

Statistical analyses

Statistical analyses were performed using Statistical Package (StatSoft, 98th Edition, 1997).

Results

The nervous system of *Branchiostoma lanceolatum* mainly consists of a small anterior cerebral vesicle and a neural tube (or spinal cord) that extends for nearly the animal's entire length (Fig. 2). The amino acids were derivatised with a mixture consisting of *o*-phthaldialdehyde and *N*-acetylcysteine and then injected into the HPLC column. Using the derivatising mixture, we obtained a clean separation of D-Asp from L-Asp, as well as from other amino acids. Figure 3 shows a typical chromatographic example of a separation of standard D-Asp (10 pmoles) and other L-amino acids (20 pmoles each).

Table 1 shows the concentration of free amino acid concentrations found in the neural tube of the amphioxus *B. lanceolatum* detected in the present study, compared with the concentration values both in the brain of the *Octopus vulgaris*, which was taken as representative of molluscs (D'Aniello et al., 1995), and in the human brain, which was taken as representative of mammals (Tarbit

Table 1. Concentrations of D-aspartate (D-Asp) and major free L-amino acids in the nervous system of amphioxus compared to that of molluscs and humans

Amino acids	Molluscs (*O. vulgaris*) Brain[a]	Amphioxus (*B. lanceolatum*) Neural tube[b]	Mammalians (*H. sapiens*) Frontal cortex[c]
D-aspartate	9.10 ± 2.20	0.28 ± 0.04	0.02[d]
Glutamate	16.2 ± 2.10	4.50 ± 0.60	9.15
Glutamine	2.80 ± 0.40	0.42 ± 0.05	5.50
Aspartate	9.00 ± 1.10	3.90 ± 0.30	1.09
Asparagine	0.92 ± 0.20	0.20 ± 0.03	0.09
Taurine	80.20 ± 7.10	0.35 ± 0.05	1.85
Glycine	2.40 ± 0.30	2.35 ± 0.35	0.67
GABA	6.10 ± 1.10	0.27 ± 0.04	0.79
Alanine	15.30 ± 2.50	2.50 ± 0.40	0.39
Serine	1.50 ± 0.30	1.10 ± 0.15	0.62
Threonine	1.30 ± 0.20	0.25 ± 0.05	0.14
Histidine	2.60 ± 0.70	0.30 ± 0.02	0.09
Valine	1.00 ± 0.30	0.11 ± 0.03	0.15
Arginine	2.80 ± 0.70	0.38 ± 0.05	0.06
Tyrosine	0.50 ± 0.20	0.15 ± 0.02	0.03
Methionine	0.20 ± 0.10	0.22 ± 0.03	0.01
Isoleucine	0.60 ± 0.20	0.23 ± 0.02	0.05
Leucine	0.70 ± 0.20	0.30 ± 0.15	0.01
Phenylalanine	0.04 ± 0.01	0.23 ± 0.03	0.03
Lysine	0.40 ± 0.10	0.35 ± 0.04	0.10

Results are expressed in µmol/g tissue (mean ± SD)
[a] From D'Aniello (1995); [b] present work; [c] from Tarbit (1980); [d] from Fisher (1991)

et al., 1980). All the results are expressed as μmol/g of wet tissues. As is shown in Table 1, in the amphioxus neural tube L-glutamic acid (4.50 ± 0.6 μmol/g) and L-aspartic acid (3.90 ± 0.3 μmol/g) were found in the highest concentrations, followed by L-alanine, L-glycine and L-serine at concentrations of 2.50 ± 0.4, 2.35 ± 0.35 and 1.10 ± 0.15 μmol/g, respectively. In contrast, the amino acid at the higher concentration in the mollusc *O. vulgaris* was essentially the taurine (80.2 ± 7.1 μmol/g). Relatively high amounts of L-glutamic acid, L-alanine, D-aspartic acid, L-aspartic acid and GABA were observed (16.2 ± 2.1, 15.3 ± 2.5, 9.1 ± 2.2, 9.0 ± 1.1 and 6.1 ± 1.1 μmol/g tissue, respectively). In the frontal region of the human brain, L-glutamic acid and L-glutamine were the predominant free amino acids (9.15 and 5.50 μmol/g tissue, respectively) followed by taurine, L-aspartate, GABA, glycine and L-serine (1.85, 1.09, 0.79, 0.67 and 0.62 μmol/g tissue, respectively). Thus, amphioxus has, in common with mollusc and mammal brains, high amounts of L-Glu and L-Asp, which act as excitatory neurotransmitters and which are present in all of the animal nervous systems examined thus far. Therefore, they may be presumed to be the most common neurotransmitters despite the long phylogenetical distances involved. The relatively high concentration of L-Ala in amphioxus is consistent with that found in molluscs, while the low concentration of taurine is consistent with that described in the human brain.

Amphioxus was also found to contain D-aspartic acid. This amino acid has been found in high concentrations in the nervous system of the lowest animal phyla, such as in the mollusc *O. vulgaris*, where its concentration ranges between 9.0 and 10.0 μmol/g (D'Aniello et al., 1995). In contrast, its concentration in mammals is very low, as shown by data from human samples with concentrations ranging between 0.020 and 0.030 μmol/g (Fisher et al., 1991). We detected D-aspartic acid in the amphioxus nervous system at a concentration of 0.30–0.40 μmol/g tissue. This data allow us to speculate on the fact that even if amphioxus is a primitive chordate, such values represent a transitory state from invertebrates to vertebrates: amphioxus contains D-aspartate at a concentration that

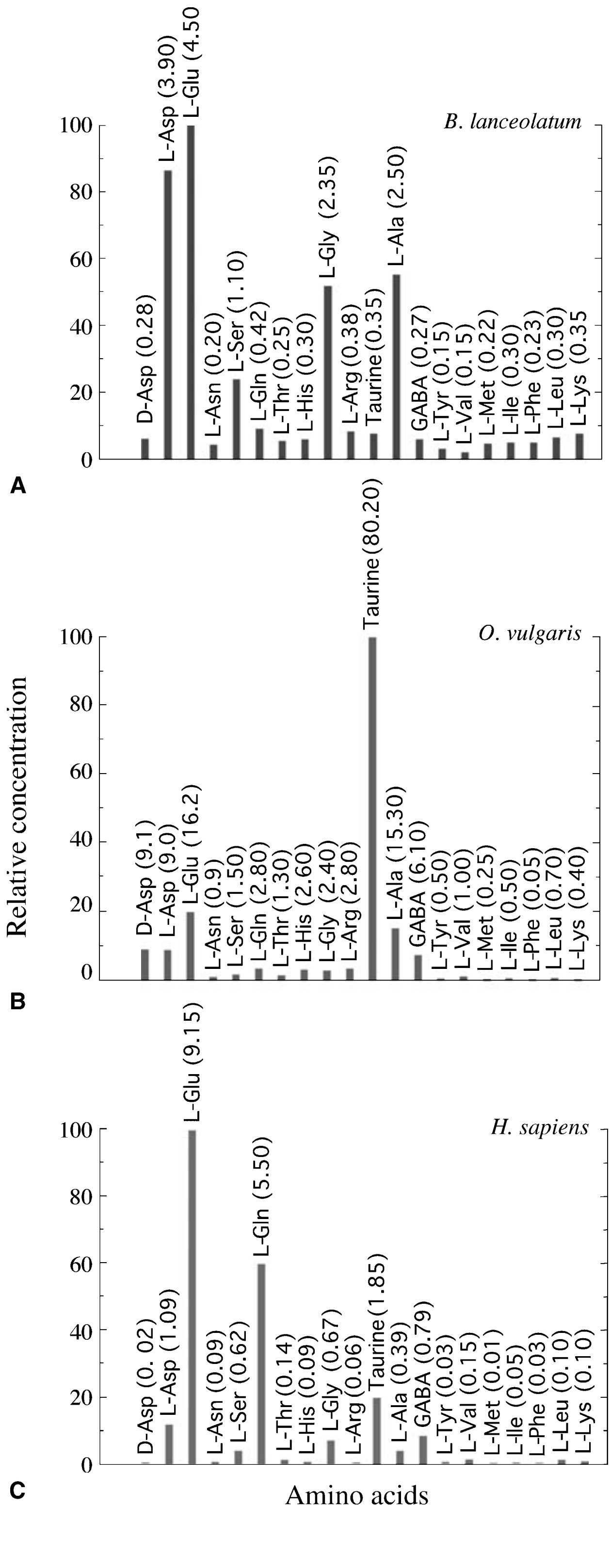

Fig. 4. Pattern of free amino acids in the neural tube of *Branchiostoma lanceolatum* (**A**), brain of *Octopus vulgaris* (**B**), and human brain (**C**). 100% value in (**A**) is given to L-Glu, since it represents the highest amino acid concentration (4.5 ± 0.6 μmol/g tissue). L-Asp, L-Ala, L-Gly and L-Ser are the amino acids present at significant concentrations. All other amino acids are lower than 2–10% compared to L-Glu (**B**). 100% is given to taurine since it represents the highest concentration amino acid (80.2 ± 7.1 μmol/g tissue). L-Glu, D-Asp, L-Asp and L-Ala are the other four amino acids at significant concentrations. All other amino acids are lower than 2–10% compared to taurine. **C** 100% is given to L-Glu (9.15 μmol/g tissue). L-Gln is the other amino acid at significant concentration. Except for L-Asp and taurine, all other amino acids are lower than 2–10% compared to L-Glu

differs greatly from the lower invertebrates, proving more similar to the vertebrates.

Figure 4 shows a schematic graphic in which the concentrations of the amino acids of each animal phylum are presented in terms of the percentage concentration of each amino acid compared to the concentration of the most abundant amino acid, which is taken as 100. In this figure, differences in free amino acid composition between the three groups of animals are highlighted: in *O. vulgaris*, taurine alone represents more than 50% of the sum of all free amino acids (Fig. 4B). In the human brain, the L-glutamic acid represents about 50% of the sum of all the free amino acids listed in Table 1, and together with L-glutamine, reaches almost 80% of all free amino acids (Fig. 4C). In the neural tube of amphioxus, L-Glu and L-Asp together represent 50% of the sum of all free amino acids (Fig. 4A) and together with L-Gly and L-Ala, they reach almost 85% of the total free amino acids.

Interestingly, among other neuroactive substances we found that the amino acid derivative GABA, the main inhibitory neurotransmitter of vertebrates, is present in amphioxus in quite low concentrations compared to the two other animal species analysed. Accordingly, an immunohistochemical study was performed demonstrating that GABA is distributed in the amphioxus central and peripheral nervous systems (Anadón et al., 1998).

Discussion

In conclusion, the results obtained in this study on amphioxus nervous tissues reflect in some way the evolution of the nervous system from invertebrates to vertebrates. In fact, in molluscs and mammals, L-aspartic acid, and above all L-glutamic acid, remain the two most important amino acids involved in neurotransmission. We also show that in the nervous tissues of amphioxus, L-Asp and L-Glu are present in high concentrations. Hence, our data confirm the prevalence of L-Asp and L-Glu in the cephalochordate nervous system, which from an evolutionary point of view, is situated between molluscs and mammals. In addition, as shown in Table 1, the nervous system of the mollusc *O. vulgaris* possesses two other non-excitatory neutral amino acids, taurine and L-alanine. The present study thus suggests that there is a relationship between steps in the evolution of the nervous systems and concentrations of L-Asp and L-Glu. The cephalochordates are phylogenetically closer to mammals than molluscs, and our biochemical analyses seem to be in agreement with the intermediate position amphioxus holds in evolution. The role of D-aspartic acid in the amphioxus neural system remains unknown. It has been shown that D-Asp plays a neurotransmitter or neuromodulator role in the mollusc Opistobranchia *Aplysia limacine* (Spinelli et al., 2006). In addition, it has been demonstrated that during the embryonic life of mammals and birds, a transient high concentration of D-aspartic acid occurs in the brain (200–300 nmol/g tissues) (Neidle and Dunlop, 1990; Hashimoto et al., 1993, 1995). It then returns to lower levels (20–30 nmol/g) after birth (Neidle and Dunlop, 1990; Hashimoto et al., 1993) and increases with age (Hashimoto et al., 1995; Dunlop et al., 1986). These results indicate that in vertebrates, D-Asp is involved in the development of the nervous system during embryonic life and in the endocrine system during adult life (Dunlop et al., 1986; D'Aniello et al., 2000). In fact, it has been shown that in the adult, D-Asp is involved in endocrine functions since it can induce the release of GnRH (Gonadotropin Releasing Hormone), LH (luteinizing hormone), GH (growth hormone) (D'Aniello et al., 2000) and testosterone (D'Aniello et al., 1996). Thus, as in mammals D-aspartic acid may have a role in either the development of amphioxus' nervous system or the functioning of its endocrine system. Thus, it would be of great interest to perform further investigations to verify whether D-aspartic acid is involved in specific neuronal and hormonal activity in amphioxus, as a living ancestor of vertebrates.

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