Occurrence and neuroendocrine role of D-aspartic acid and *N*-methyl-D-aspartic acid in *Ciona intestinalis*

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Abstract Probes for the occurrence of endogenous D-aspartic acid (D-Asp) and *N*-methyl-D-aspartic acid (NMDA) in the neural complex and gonads of a protochordate, the ascidian *Ciona intestinalis*, have confirmed the presence of these two excitatory amino acids and their involvement in hormonal activity. A hormonal pathway similar to that which occurs in vertebrates has been discovered. In the cerebral ganglion D-Asp is synthesized from L-Asp by an aspartate racemase. Then, D-Asp is transferred through the blood stream into the neural gland where it gives rise to NMDA by means of an NMDA synthase. NMDA, in turn, passes from the neuronal gland into the gonads where it induces the synthesis and release of a gonadotropin-releasing hormone (GnRH). The GnRH in turn modulates the release and synthesis of testosterone and progesterone in the gonads, which are implicated in reproduction.

Key words: D-Aspartic acid; *N*-Methyl-D-aspartic acid; Gonadotropin-releasing hormone; Protochordate; Evolution; Reproduction; Testosterone; Progesterone; *Ciona intestinalis*

1. Introduction

Ciona intestinalis is a marine protochordate belonging to the subphylum Urochordata (Tunicata), class Ascidiaceae. This species is considered to be phylogenetically about 500 million years distant from the highest vertebrate group, and it is postulated that the ascidians share common ancestry with vertebrates. The bag-like body of *C. intestinalis* is surrounded by a tough tunic and is attached to the solid surface in the adult stage. The tadpole-like free-swimming larvae have pharyngeal slits and a dorsal hollow nerve cord plus a notochord revealing its evolutionary position as a chordate [1]. Metamorphosed *C. intestinalis* has several similarities with vertebrates. Since the endostyle, a region of the perforated pharynx in *C. intestinalis* and other ascidians, is able to accumulate iodine and contains free thyroxine, triiodothyronine and thyroglobulin, it has been postulated to be a homologue of the vertebrate thyroid gland [2,3]. In addition, *Ciona* gonads possess steroid hormones such as progesterone, testosterone, 17β-estradiol [4], a human-like thyroid-stimulating hormone [5], a porcine-like placental hormone relaxin [6], and two different forms of gonadotropin-releasing hormone (GnRH) with amino acid compositions similar to that occurring in birds and mammals [7]. Recent studies have also indicated the presence of D-aspartic acid (D-Asp) in the gametes as well as in the whole body homogenate of larvae and adults of *C. intestinalis* [8–10].

Endogenous D-Asp has been reported to occur in neuroendocrine tissues of various animal phyla: mollusks [11–13], crustaceans [14], amphibians [15], reptiles [16], fish [10], chicken [17], rat [18–24], and man [25–27]. Besides the biochemical determination of D-Asp in tissue homogenates, several immunohistochemical studies have suggested the presence of these amino acids in several tissues, such as the brain [22,28], adrenal gland [29], pineal gland [30], pituitary gland [31], and testis [32]. A gene expressing D-Asp in rat Leydig cells has also been described [33].

As far as the physiological correlates of D-Asp in the animal kingdom are concerned, important biological roles for this D-amino acid have now been postulated for many invertebrates, and particularly in higher vertebrates [20–24,34–36]. Well-known for its neuroexcitatory activity and because of its widespread localization, D-Asp is supposed to play important neurotransmission and neurosecretion roles within the central nervous system as well as in the biosynthesis and/or secretion of hormones in peripheral endocrine glands. On account of significant changes in its tissue concentration during ontogenesis, a potentially important ontogenetic role for D-Asp may be assumed [22].

The natural occurrence of *N*-methyl-D-aspartate (NMDA), a methylated form of D-Asp, has also been found in the nervous, endocrine and muscular tissues of invertebrate and vertebrate animals [10,20,21,37]. In nervous and endocrine tissues of the rat, it has been shown that NMDA is biosynthesized from D-Asp by an NMDA synthase, which catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to D-Asp [19]. NMDA is a potent agonist of the

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NMDA-type mammalian excitatory amino acid glutamate receptors [38,39], and has been shown to induce the release of hypothalamic and pituitary hormones in mammals [20,21,40].

Very little evidence is available on the natural occurrence of these amino acids in the neuroendocrine system(s) of lower chordates and virtually nothing is known about their possible physiological role(s). Because of a dynamic developmental pattern and a simplified anatomical structure, the ascidians represent an excellent experimental model for developmental and phylogenetic studies. The study reported here was designed to gather biochemical and physiological evidence for the occurrence of D-Asp and NMDA in neuroendocrine tissues, and their physiological role(s) in the ascidian *C. intestinalis*. The endogenous occurrence of D-Asp and NMDA in the neural complex and the gonads is unambiguously ascertained here. It is shown that D-Asp constitutes the precursor for the biosynthesis of NMDA in these tissues. In the gonads, NMDA stimulates the synthesis of GnRH, which in turn regulates the synthesis and release of the sex steroid hormones progesterone and testosterone.

2. Materials and methods

2.1. Tissue collection and chemicals

Adult *C. intestinalis* were collected from the Gulf of Naples, Italy, during a period when they show maximal reproductive activity (June–July). The tissues and blood samples taken from the animals were stored at −80°C until used, except for the in vitro experiments in which fresh ovaries were used. NMDA, D-Asp and other amino acids, *o*-phthaldialdehyde, methylamine (CH_3-NH_2), α-oxaloacetate, SAM, and mammalian GnRH (mGnRH) were from Sigma Chemical Co. (St. Louis, MO, USA). Sep-Pak cartridges or columns of the octadecyl-silyl resin, ODS-C_{18}, were from Waters, Millipore (Milford, MA, USA). The ultrafree-centrifugal filter membranes with a cut-off of 30 000 Da were from Millipore Corporation (Milford, MA, USA). Sephacryl S-200 resin (Pharmacia), and all other chemicals used in this work were analytical grade products.

2.2. Sample preparation

Tissues were homogenized 1:10 (w/v) in 0.2 M perchloric acid (PCA). In order to obtain enough tissue homogenate, the cerebral ganglia, neural glands (neural gland–dorsal strand plexus), and gonads were collected from 200–400 animals. The homogenate was centrifuged at 30 000×*g* for 30 min, and the supernatant was brought to pH 5–6 with 2 M KOH and left on ice for 20–30 min to obtain maximum precipitation of potassium perchlorate (obtained from the reaction between PCA and KOH). Then, the sample was centrifuged at 30 000×*g* for 10 min and the supernatant was divided into two portions. The first portion was used for the determination of D-Asp and NMDA by high performance liquid chromatography (HPLC), and for other D-amino acids by an enzymatic method; the second portion was used for the determination of GnRH. For this latter purpose, this fraction was purified as follows: the sample was loaded onto an ODS-C_{18} cartridge containing 0.8 g resin (Waters) (which had been previously activated with 100% methanol and equilibrated with distilled water followed by a wash of 5 ml H_2O). Then, the cartridge was eluted with 5 ml methanol and this eluate fraction (which contained GnRH) was dried in a Petri dish at room temperature, under a hood with aspiration, and the residue was dissolved in distilled water in proportion of 500 μl water for an amount of sample derived from 1 g tissue.

2.3. D-Aspartate oxidase, D-amino acid oxidase and their specificity

These two enzymes were used for the determination of D-amino acids. D-Aspartate oxidase (D-AspO; EC 1.4.3.1) is an oxidative enzyme which oxidizes only the dicarboxylic amino acids and their derivatives (NMDA, D-Asp, D-Glu, D-Asn and D-Gln) [41,42]. D-Amino acid oxidase (D-AAO; EC 1.4.3.3) is an oxidative enzyme of the same class as D-AspO, but it oxidizes all other amino acids in D-form except the above acidic amino acids [43]. D-AspO was obtained by overexpression and purified according to the described procedure [37]. D-AAO was purchased in purified form from Boehringer or another company.

2.4. Determination of D-Asp, NMDA, D-Glu, D-Asn and D-Gln

While total D-Asp, NMDA, D-Glu, D-Asn and D-Gln were determined by an enzymatic colorimetric method based on the use of D-AspO [41,42], D-Asp and NMDA were also determined individually by HPLC [19,20].

2.5. Biosynthesis of D-Asp and NMDA

In order to verify if D-Asp is synthesized from L-Asp and in which tissue, 200 mg of a pool of cerebral ganglia, or neural glands and gonads, were homogenized with 1 ml of 0.05 M sodium phosphate buffer, pH 7.5, containing 10 mM of sodium EDTA and centrifuged at 30 000×*g* for 30 min. The supernatant was then extensively dialyzed against the same buffer at 2–4°C to free totally the amino acids from the sample. Then 200 μl of the dialyzed sample was incubated with 20 μl of 0.1 M L-Asp (or with other L-amino acids) at 37°C for 60 min followed by the determination of D-Asp via HPLC. To verify the synthesis of NMDA, 200 μl of the dialyzed sample was incubated with 10 μl of 0.1 M SAM for 60 min at 37°C following by the determination of NMDA via HPLC.

2.6. Effect of NMDA on the release and synthesis of GnRH

In order to investigate if NMDA in *Ciona* induces the biosynthesis and release of a GnRH, 200 mg tissue (cerebral ganglia, neural glands and gonads) was cut into smaller pieces and washed three times with 2 ml of seawater. Then, the pieces of tissues were incubated in 1 ml of seawater containing NMDA or other amino acids at concentrations of 1.0 μmol/ml for 2 h at 25°C under moderate agitation. After incubation, the medium was separated from the tissue by centrifugation at 1000×*g* for 10 min and the supernatant was used for the determination of the GnRH. The precipitate was homogenized with 200 μl of 0.1 M trichloroacetic acid and centrifuged at 15 000×*g* for 10 min and the supernatant was used for the determination of GnRH by HPLC according to the earlier procedure [7].

2.7. Effect of the GnRH on the release and synthesis of testosterone and progesterone from the gonads of C. intestinalis

The gonads were cut into 400 μm slices using a tissue cutter. The slices (0.5 g) were washed three times in 2 ml of seawater and then incubated with 2 ml of seawater containing 5 mg of bovine serum albumin and 100 μg of synthetic mGnRH (Sigma) under moderate agitation for 2 h at 25°C. After incubation, to determine the amount of the hormones released, the seawater in which the gonads were incubated was extracted with 100 ml of ethyl ether and the ether phase was collected and left to evaporate at room temperature. The residue was dissolved in 500 μl of bovine serum albumin (1 mg/ml) and analyzed for the content of progesterone and testosterone using radioimmunoassay hormone kits from Biochem Immunosystem, Italia (Bologna, Italy). To determine the amount of hormone synthesized, the tissues were homogenized with 2 ml of seawater containing 5 mg/ml bovine serum albumin and centrifuged at 13 000×*g* for 10 min. The supernatant was used for the determination of the hormones, as described above.

3. Results

3.1. Occurrence and biosynthesis of D-Asp and NMDA in neuroendocrine tissues of C. intestinalis

The cerebral ganglion is the tissue that contains comparatively the highest concentration of D-Asp (365.5 ± 28.5 nmol/g tissue), followed in succession by the neural gland and gonads (Table 1). Interestingly, we also found that the blood of this ascidian contains D-Asp, in measurable quantities, though much lower than the other tissues tested (Table 1). In contrast, the highest concentration of NMDA was observed in the neural gland, with a value of 120.5 ± 13.0 nmol/g tissue, followed by the gonads and the cerebral ganglion (Table 1). It is interesting to observe that NMDA is also present in the blood, in a low but significant concentration (Table 1). Using the D-AspO enzymatic method [41,42] we found that D-Glu,

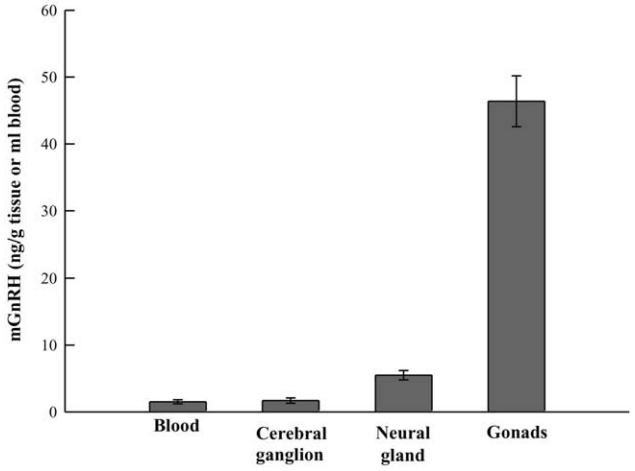

Fig. 1. Occurrence of GnRH in *C. intestinalis*. Results are the mean ± S.D. obtained from three experiments, where each experiment was carried out on a pool of tissues from 200 *C. intestinalis*. Blood was drawn from the heart with a syringe.

D-Asn and D-Gln are present as less than 2–3% of total D-Asp and NMDA (data not shown). Furthermore, using the hog kidney D-AAO we did not find any other measurable D-amino acids.

In order to determine the biosynthesis of D-Asp from L-Asp, via an aspartate racemase, cerebral ganglion, neural gland and gonad tissue homogenates were incubated with L-Asp, or other amino acids. The greatest D-Asp biosynthesis was found in the cerebral ganglion when these tissues were incubated with L-Asp (Table 2). However, incubation with L-Glu also moderately induced D-Asp biosynthesis which may be accounted for by the strong structural similarity between L-Asp and L-Glu. The other L-amino acids tested and Gly did not induce any significant production of D-Asp. This indicates that the only substrate for D-Asp synthesis was L-Asp and the cerebral ganglion contains a higher concentration of aspartate racemase. The other two tissues contain a lower concentration of this enzyme as is indicated by the comparatively much lower yield of D-Asp (Table 2).

Concerning the biosynthesis of NMDA, a highly significant increase of NMDA (165.5 ± 18.5 nmol/g of tissue) was observed in the neural gland, only under incubation with D-Asp and SAM (Table 2). Incubation with other amino acids did not modify the NMDA content of the neural gland homogenate. Furthermore, the production of NMDA, under D-Asp incubation, was comparatively much less marked in the cerebral ganglion and gonads (Table 2).

Table 1
Occurrence of D-Asp and NMDA in neuroendocrine tissues of *C. intestinalis*

	D-Asp	NMDA
Cerebral ganglion	365.5 ± 28.5	25.5 ± 4.5
Neural gland	160.0 ± 14.5	120.5 ± 13.0
Gonads	103.4 ± 10.5	32.5 ± 5.5
Blood	7.5 ± 1.5	2.5 ± 0.4

Results are in nmol/g tissue or ml blood and represent the mean ± S.D. obtained from three experiments. Each experiment was carried out on a pool of tissues from 200 *C. intestinalis*. Blood was drawn from the heart with a syringe.

Table 2
Biosynthesis of D-Asp and NMDA in *C. intestinalis*

	D-Asp	NMDA
Cerebral ganglion incubated with:		
D-Asp	–	10.5 ± 1.6
L-Asp	1050.5 ± 55.2	5.2 ± 0.5
L-Glu	68.5 ± 10.5	5.3 ± 0.4
L-Ala	65.0 ± 8.5	5.5 ± 0.6
Gly	50.2 ± 3.5	4.9 ± 0.4
NaCl	45.2 ± 4.4	5.0 ± 0.5
Neural gland incubated with:		
D-Asp	–	165.5 ± 18.5
L-Asp	88.5 ± 8.0	10.9 ± 1.5
L-Glu	48.8 ± 4.5	11.4 ± 2.6
L-Ala	34.5 ± 2.5	10.5 ± 1.8
Gly	30.5 ± 3.0	11.5 ± 2.1
NaCl	26.0 ± 2.6	10.2 ± 1.5
Gonads incubated with:		
D-Asp	–	24.4 ± 3.4
L-Asp	78.5 ± 5.4	15.6 ± 1.9
L-Glu	21.2 ± 3.5	15.2 ± 1.6
L-Ala	25.0 ± 4.2	13.4 ± 1.2
Gly	23.6 ± 3.8	14.5 ± 2.1
NaCl	20.2 ± 2.8	14.5 ± 1.6

The results are in nmol/g tissue and represent the mean ± S.D. obtained from three experiments. Each experiment consisted of incubating a tissue homogenate with 1 mM SAM and one of the amino acids listed in the table each at a concentration of 1 mM followed by measuring the D-Asp and NMDA synthesized. The controls consisted of incubation with NaCl (1 mM).

3.2. *Occurrence of GnRH in* C. intestinalis *tissues*

Previously we found that the gonads of *C. intestinalis* contain a form of GnRH whose biochemical composition and biological activity is similar to that found in mammals [7]. In this study, we have extended this observation to other tissues of *C. intestinalis* in order to discover whether, in addition to the gonads, this peptide hormone is also present in other tissues and if NMDA induces release and synthesis of GnRH in *C. intestinalis*, as it does in mammals. Besides the gonads, it was observed that GnRH is also found in the cerebral ganglion, neural gland and blood. The gonads contained a much higher concentration of this decapeptide hormone than the cerebral ganglion and neural gland (Fig. 1).

Table 3
Effect of NMDA and other amino acids on the release and synthesis of GnRH in the gonads of *C. intestinalis*

	GnRH released (ng/10 ml seawater)	GnRH synthesized (ng/100 mg gonads)
NMDA	9.6 ± 0.8*	28.0 ± 3.0*
NMLA	1.2 ± 0.4**	4.5 ± 0.8**
D-Asp	0.4 ± 0.2	3.0 ± 0.5
L-Asp	0.3 ± 0.1	2.4 ± 0.3
L-Glu	0.2 ± 0.1	2.8 ± 0.4
L-Ser	0.3 ± 0.1	3.1 ± 0.5
L-Ala	0.3 ± 0.1	3.3 ± 0.4
L-Met	0.3 ± 0.2	3.3 ± 0.2
L-Leu	0.3 ± 0.1	3.1 ± 0.4
Seawater	0.3 ± 0.1	3.3 ± 0.4

Results are the mean ± S.D. obtained from three experiments. Each experiment was carried out on 100 mg of glands washed in 10 ml of seawater and placed in 1 ml of seawater containing NMDA or the other amino acids listed in the table at the concentration of 1.0 mM. The control consisted of gonads incubated with seawater to which no amino acids had been added. *$P < 0.01$ versus control; **$P < 0.05$ versus control.

Interestingly, this peptide hormone is also present in the blood of this tunicate in a low but significant concentration (Fig. 1).

3.3. Effects of NMDA on the release and synthesis of GnRH in the gonads

The gonads were incubated in seawater containing NMDA or D-Asp or L-amino acids each at a concentration of 1.0 mM, and the release into the medium and synthesis in the tissues of GnRH were measured. This study was carried out only on gonads based on the observation that the gonads contain approximately 10–40 times the tissue concentration of GnRH in either the cerebral ganglion or the neural complex (Fig. 1). The results showed that 1 mM NMDA was able to induce a release of GnRH into the medium nearly 32-fold higher than the control (9.6 ± 0.8 ng versus 0.3 ± 0.1) (Table 3). Other amino acids did not cause any significant increase of GnRH concentration into the medium as compared with the release of GnRH evoked by NMDA. *N*-Methyl-L-aspartic acid (NMLA) at a concentration of 1 mM also gave a positive response, but was much less efficient than NMDA (Table 3). Regarding the synthesis, we also observed that only NMDA significantly elicits the synthesis of GnRH. In fact, when the gonads of *C. intestinalis* were incubated with NMDA at a concentration of 1.0 mM, the concentration of GnRH in the tissues rose from a value of 3.3 ± 0.4 ng/100 mg tissue (control) to 28.0 ± 3.0 ng/100 mg gonads, which corresponds to 8.5 times that of the control. Also in this case, as observed for the release, NMLA significantly stimulated the synthesis of GnRH, but it was a much less pronounced response than that obtained with NMDA (Table 3). Other amino acids had no effect on GnRH synthesis.

3.4. Effects of GnRH on the release and synthesis of testosterone and progesterone from the gonads of C. intestinalis

The results indicated that the gonads of *C. intestinalis* were significantly stimulated by GnRH (Figs. 2 and 3). In fact, after incubation with GnRH, both release and synthesis of

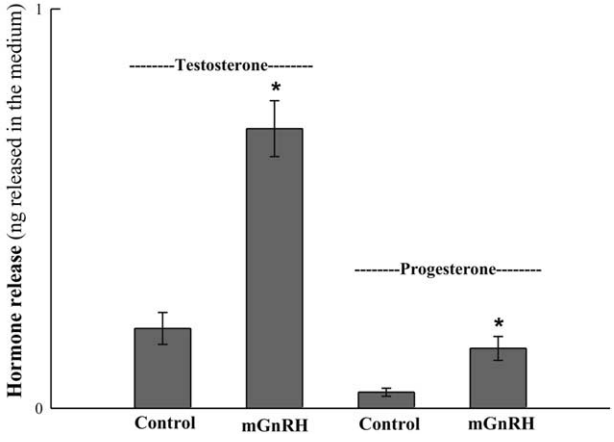

Fig. 2. Effects of mGnRH on the release of testosterone and progesterone from the gonads of *C. intestinalis*. Results are expressed in ng released in the medium or ng/g tissue and were obtained from three experiments. In each experiment the tissues were incubated for 2 h at 25°C in 10 ml of seawater. Release represents the amount of hormones found in seawater where slices from 1 g of tissue were incubated with 100 μg of mGnRH. Control consisted of slices incubated in seawater without mGnRH. $*P < 0.01$.

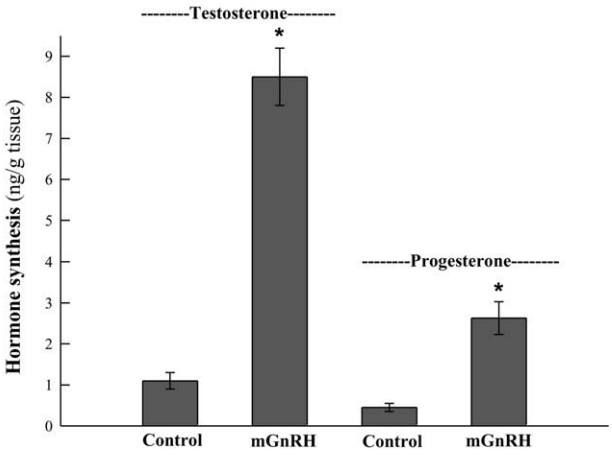

Fig. 3. Effects of mGnRH on the synthesis of testosterone and progesterone from the gonads of *C. intestinalis*. Results are expressed in ng released in the medium or ng/g tissue and were obtained from three experiments. In each experiment the tissues were incubated for 2 h at 25°C in 10 ml of seawater. Release represents the amount of hormones found in seawater where slices from 1 g of tissue were incubated with 100 μg of mGnRH. Control consisted of slices incubated in seawater without mGnRH. $*P < 0.01$.

the two sex hormones were significantly increased compared to the controls. Total testosterone content released in the medium in the presence of GnRH was found to be 0.7 ± 0.04 ng whereas total testosterone released without GnRH was 0.2 ± 0.04 ng, that is 3.5 times higher than the control (Fig. 2). The same situation was also observed for progesterone. The spontaneous release of progesterone in the medium from the gonads was 0.04 ± 0.01 ng, whereas when the gonads were stimulated with GnRH, the total content of progesterone released in the medium was found to be 0.15 ± 0.03 ng, that is 2.63 times increased (Fig. 2).

In addition to the release, we also found that GnRH elicits an increased production of testosterone and progesterone. Testosterone was found to be increased by 7.7 times compared to the control (8.5 ± 0.7 ng/g tissue in the sample stimulated by GnRH vs. 1.1 ± 0.2 ng/g tissue found in the control) (Fig. 3). Progesterone was found to be increased by 5.7 times compared to the control: 2.63 ± 0.4 ng/g tissue was found in the sample stimulated by the mGnRH and 0.45 ± 0.1 in the control (Fig. 3).

4. Discussion

In the present study, we have described the occurrence of endogenous D-Asp and NMDA in the cerebral ganglion, the neural gland and the gonads of this protochordate. The data showed that the cerebral ganglion concentration of D-Asp was 2.3-fold that of the neural gland and 3.5 times that of the gonads, whereas the NMDA concentration was highest in the neural gland, being 4.7 times higher than in the cerebral ganglion and 3.7 times higher than in the gonads. Moreover, we have observed that D-Asp acts as a precursor for NMDA synthesis. This study also provides evidence for a role for NMDA in regulation of GnRH secretion and synthesis by the gonads and confirms an earlier report on the GnRH induction of steroidogenic activity of the gonads in this species [7].

Only a few studies have been conducted to examine the

neuroendocrinological implications of these amino acids in lower vertebrates [9,15,16], with most attention being devoted to mammals. D-Asp and NMDA have important physiological roles in mammalian neuroendocrine–endocrine systems. Each of the two amino acids may act differentially in different tissues of the same species. In mammals, D-Asp stimulates the secretion of GnRH, α-melanocyte-stimulating hormone, γ-aminobutyric acid, prolactin, and growth hormone, but inhibits dopamine and, under certain physiological conditions, also prolactin [20,21,35]. In the male rat, while NMDA does not modify hypothalamic oxytocin release, it is stimulated by D-Asp, and both act negatively on posterior pituitary oxytocinergic activity [44].

On the other hand, it has been observed that NMDA is also implicated in endocrine roles, inducing GnRH and luteinizing hormone release [45]. Furthermore, like in the rat [20], the GnRH neurosecretory system in the rhesus monkey is responsive to NMDA and this responsiveness undergoes developmental changes [40].

In our investigation into the source of NMDA in the cerebral ganglion, neural gland and gonads of *C. intestinalis*, we found that of the five amino acids employed, D-Asp is selectively used as the precursor molecule for NMDA biosynthesis. It appears that L-Asp, and not L-Glu, L-Ala or Gly, is the favored precursor for D-Asp biosynthesis in all tissues examined. In mammals too, D-Asp is the selective precursor for the biosynthesis of NMDA. In mammals, the highest tissue concentration of D-Asp and NMDA has been observed in the adenohypophysis followed in succession by that in the gonads and hypothalamus [9,20,21]. The concentration of D-Asp in any area of the rat or human brain, however, was never more than one third of that of the ascidian cerebral ganglion. While the highest adenohypophyseal concentration of NMDA reported in a mammal was at the most one tenth of the NMDA concentration in the ascidian neural gland, NMDA was 8–10 times higher in the ascidian gonads, and the D-Asp concentration was nearly equal to that of mammalian gonads [9,20,21].

In the sea squirt, a strikingly high D-Asp synthesis occurs (using L-Asp as substrate) within the cerebral ganglion, and an even higher rate of NMDA synthesis is observed in the neural gland. Thus, we are inclined to argue that a part of the D-Asp formed in the cerebral ganglion must pass into the neural gland where it can contribute to the formation of the large amount of NMDA found there. The NMDA, in turn, passes into the gonads to induce GnRH synthesis and consequently steroidogenesis. Since D-Asp and NMDA are also measurable in the blood, it is possible that their transfer from the cerebral ganglion to the neural gland and thence into the gonads occurs through the blood stream. We are inclined to suggest that in the protochordates the neuroendocrine regulation (D-Asp–NMDA–GnRH) of gonad activity (reproduction) occurs without an adenohypophyseal component. Otherwise, there seems to exist a strong analogy with what we know now to occur in mammals. For example, D-Asp is synthesized from L-Asp by an aspartate racemase in the cerebral ganglion most likely as it does in the telencephalon of a mammal; it is then transferred into the neural gland through the blood stream in the protochordate and directly into the hypothalamus by selective uptake in the mammal. Here, i.e. in the protochordate neural gland and in the mammalian hypothalamus, D-Asp constitutes the preferred substrate for the synthesis of NMDA through the enzyme NMDA synthase. In the mammalian hypothalamus, it induces GnRH synthesis/secretion in order to stimulate pituitary gonadotropin synthesis and secretion which then act upon the peripheral target, the gonads. In the protochordate, instead, NMDA is transferred to the gonads where it induces the synthesis and release of a GnRH molecule which exerts a direct gonadotropic action upon the gonads. Like the hypothalamo-hypophyseal-gonad axis in mammals and other vertebrates, the tunicate neural complex is tied (via an activation on the part of excitatory amino acids, D-Asp and NMDA) to the gonad production of GnRH and consequently to reproduction.

All evidence now at hand seems to imply that the D-Asp- and NMDA-mediated neuroendocrine roles remain largely unchanged along the entire chordate lineage. To put it in other words, on a phylogenetic basis, this study is a contribution to the evolutionary history of D-Asp/NMDA-mediated neuroendocrine modulation in chordates, from protochordates to mammals.

References

[1] Burighel, P. and Cloney, R.A. (1997) in: Microscopic Anatomy of Invertebrates, Vol. 15 (Harrison, F.W. and Ruppert, E.E., Eds.), pp. 221–347, Wiley-Liss, New York.
[2] Barrington, E.J.W. and Thorpe, A. (1965) Gen. Comp. Endocrinol. 5, 373–385.
[3] Thorndyke, M.C. (1978) Nature 271, 61–62.
[4] Delrio, G., d'Istria, M., Milone, M. and Chieffi, G. (1971) Experientia 27, 1348–1350.
[5] Di Fiore, M.M., Perrone, L. and D'Aniello, A. (1997) Life Sci. 61, 623–629.
[6] George, D. and Schwabe, C. (1999) FASEB J. 13, 1269–1275.
[7] Di Fiore, M.M., Rastogi, R.K., Ceciliani, F., Messi, E., Botte, V., Botte, L., Pinelli, C., D'Aniello, B. and D'Aniello, A. (2000) Proc. Natl. Acad. Sci. USA 97, 2343–2348.
[8] D'Aniello, A., Vetere, A. and Padula, L. (1992) Comp. Biochem. Physiol. 102B, 795–797.
[9] D'Aniello, A., Di Fiore, M.M. and Fisher, G. (1998) Trends Comp. Biochem. Phsyiol. 4, 1–21.
[10] D'Aniello, A., De Simone, A., Spinelli, P., D'Aniello, S., Branno, M., Aniello, F., Rios, J., Tsesarskaja, M. and Fisher, G. (2002) Anal. Biochem. 308, 42–51.
[11] D'Aniello, A. and Giuditta, A. (1977) J. Neurochem. 29, 1053–1057.
[12] D'Aniello, A. and Giuditta, A. (1978) J. Neurochem. 31, 1107–1108.
[13] D'Aniello, A., Nardi, G., Vetere, A. and Ferguson, G.P. (1992) Life Sci. 52, 733–736.
[14] Okuma, E., Fujita, F., Amano, H., Noda, H. and Abe, H. (1995) Fish. Sci. 61, 157–160.
[15] Di Fiore, M.M., Assisi, L., Botte, V. and D'Aniello, A. (1998) J. Endocrinol. 156, 199–207.
[16] Assisi, L., Botte, V., D'Aniello, A. and Di Fiore, M.M. (2001) Reproduction 121, 803–808.
[17] Neidle, A. and Dunlop, D.S. (1990) Life Sci. 46, 1517–1522.
[18] Dunlop, D.S., Neidle, A., McHale, D., Dunlop, D.M. and Lajtha, A. (1986) Biochem. Biophys. Res. Commun. 142, 27–32.
[19] D'Aniello, A., Di Fiore, M.M., D'Aniello, G., Colin, F.E., Lewis, G. and Setchell, B.P. (1998) FEBS Lett. 436, 23–27.
[20] D'Aniello, A., Di Fiore, M.M., Fisher, G.H., Milone, A., Seleni, A., D'Aniello, S., Perna, A. and Ingrosso, D. (2000) FASEB J. 14, 699–714.
[21] D'Aniello, G., Tolino, A., D'Aniello, A., Fisher, G.H. and Di Fiore, M.M. (2000) Endocrinology 141, 3862–3870.
[22] Wolosker, H., D'Aniello, A. and Snyder, S.H. (2000) Neuroscience 100, 183–189.
[23] Wang, H., Wolosker, H., Pevsner, J., Snyder, S.H. and Selkoe, D.J. (2000) J. Endocrinol. 167, 247–252.
[24] Wang, H., Wolosker, H., Morris, J.F., Pevsner, J., Snyder, S.H. and Selkoe, D.J. (2002) Neuroscience 109, 1–4.

[25] Fisher, G.H., D'Aniello, A., Vetere, A., Padula, L., Cusano, G. and Man, E.H. (1991) Brain Res. Bull. 26, 983–985.
[26] Hashimoto, A., Kumashiro, S., Nishikawa, T., Oka, T., Takahashi, K., Mito, T., Takashima, S., Doi, N., Mizutani, Y., Kaneco, T. and Ootomo, E. (1993) J. Neurochem. 61, 348–351.
[27] Zaar, K., Kost, H.P., Schad, A., Volkl, A., Baumgart, E. and Fahimi, H.D. (2002) J. Comp. Neurol. 450, 272–282.
[28] Sakai, K., Homma, H., Lee, J.A., Fukushima, T., Santa, T., Tashiro, K., Iwatsubo, T. and Imai, K. (1998) Brain Res. 808, 65–71.
[29] Sakai, K., Homma, H., Lee, J.A., Fukushima, T., Santa, T., Tashiro, K., Iwatsubo, T. and Imai, K. (1997) Biochem. Biophys. Res. Commun. 235, 433–436.
[30] Lee, J.A., Homma, H., Sakai, K., Fukushima, T., Tashiro, T., Iwatsubo, T., Yoshikawa, M. and Imai, K. (1997) Biochem. Biophys. Res. Commun. 231, 505–508.
[31] Lee, J.A., Homma, H., Tashiro, T., Iwatsubo, T. and Imai, K. (1999) Brain Res. 838, 193–199.
[32] Sakai, K., Homma, H., Lee, J.A., Fukushima, T., Santa, T., Tashiro, K., Iwatsubo, T. and Imai, K. (1998) Biochim. Biophys. Acta 351, 96–105.
[33] Nagata, Y., Homma, H., Matsumoto, M. and Imai, K. (1999) FEBS Lett. 454, 317–320.
[34] Schell, M.J., Cooper, O.B. and Snyder, S.H. (1997) Proc. Natl. Acad. Sci. USA 94, 2013–2018.
[35] Pampillo, M., Scimonelli, T., Bottino, M.C., Duvilanski, B.H., Rettori, V., Seilicovich, A. and Lasaga, M. (2002) NeuroReport 13, 2341–2344.
[36] Nagata, Y., Homma, H., Lee, J.A. and Imai, K. (1999) FEBS Lett. 444, 160–164.
[37] Todoroki, N., Shibata, K., Yamada, T., Kera, Y. and Yamada, R. (1999) J. Chromatogr. B Biomed. Sci. Appl. 728, 41–47.
[38] Foster, A. and Roberts, P.J. (1978) J. Neurochem. 31, 1467–1477.
[39] Monaghan, D.T. and Cotman, C.W. (1986) Proc. Natl. Acad. Sci. USA 83, 7532–7536.
[40] Claypool, L.E., Kasuya, E., Saitoh, Y., Marzban, F. and Terasawa, E. (2000) Endocrinology 141, 219–228.
[41] Tedeschi, G., Negri, A., Ceciliani, F., Ronchi, S., Vetere, A., D'Aniello, G. and D'Aniello, A. (1994) Biochim. Biophys. Acta 1207, 217–222.
[42] Negri, A., Tedeschi, G., Ceciliani, F. and Ronchi, S. (1999) Biochim. Biophys. Acta 1431, 212–222.
[43] D'Aniello, A., Vetere, A. and Petrucelli, L. (1993) Comp. Biochem. Physiol. 105B, 731–734.
[44] Pampillo, M., de Carmen Diaz, M., Duvilanski, B.H., Rettori, V., Seilicovich, A. and Lasaga, M. (2001) Endocrine 15, 309–315.
[45] Arias, P., Carbone, S., Szwarcfarb, B., Fedeler, C., Rodriguez, M., Scacchi, P. and Moguilevsky, J.A. (1996) Brain Res. 740, 234–238.