0013-7227/00/$03.00/0
Endocrinology

Vol. 141, No. 10
Printed in U.S.A.

The Role of D-Aspartic Acid and *N*-Methyl-D-Aspartic Acid in the Regulation of Prolactin Release*

GEMMA D'ANIELLO, ACHILLE TOLINO, ANTIMO D'ANIELLO, FRANCESCO ERRICO, GEORGE H. FISHER, AND M. MADDALENA DI FIORE

Laboratory of Neurobiology (A.D.'A., F.E., M.M.D.), Zoological Station of Naples, Villa Comunale, 80121 Naples, Italy; Institute of Gynaecology and Obstetrics (G.D.'A., A.T.), School of Medicine, University Federico II, Via Pansini 5, 80131, Naples, Italy; Department of Chemistry (G.F.), Barry University, Miami Shores, Florida 33161; and Department of Scienze della Vita (M.M.D.), Second University of Naples, Via Vivaldi, 81100 Caserta, Italy

ABSTRACT

In this study, using an enzymatic HPLC method in combination with D-aspartate oxidase, we show that *N*-methyl-D-aspartate (NMDA) is present at nanomolar levels in rat nervous system and endocrine glands as a natural compound, and it is biosynthesized *in vivo* and *in vitro*. D-aspartate (D-Asp) is its natural precursor and also occurs as an endogenous compound. Among the endocrine glands, the highest quantities of D-Asp (78 ± 12 nmol/g) and NMDA (8.4 ± 1.2 nmol/g) occur in the adenohypophysis, whereas the hypothalamus represents the area of the nervous system where these amino acids are most abundant (55 ± 9 and 5.6 ± 1.1 nmol/g for D-Asp and NMDA, respectively). When D-Asp is administered to rats by ip injection, there is a significant uptake of D-Asp into the adenohypophysis and a significant increase in the concentration of NMDA in the adenohypophysis, hypothalamus and hippocampus, suggesting that D-Asp is an endogenous precursor for NMDA biosynthesis. Experiments conducted on tissue homogenates confirm that D-Asp is the precursor of the NMDA and that the enzyme catalyzing this reaction is a methyltransferase. S-adenosyl-L-methionine (SAM) is the methyl group donor. *In vivo* experiments consisting of ip injections of sodium D-aspartate show that this amino acid induced a significant serum PRL elevation and this effect is dose and time dependent. *In vitro* experiments conducted on isolated adenohypophysis or adenohypophysis coincubated with the hypothalamus, showed that the release of PRL is caused by a direct action of D-Asp on the pituitary gland and also mediated by the indirect action of NMDA on the hypothalamus. Then, the latter induces the release of a putative factor that in turn stimulates the adenohypophysis reinforcing the PRL release. In conclusion, our data suggest that D-Asp and NMDA are present endogenously in the rat and are involved in the modulation of PRL release. (*Endocrinology* **141:** 3862–3870, 2000)

D-ASPARTIC ACID (D-ASP) is an endogenous amino acid present in nervous tissues and endocrine glands of invertebrates and vertebrates. This amino acid was found for the first time in the brain, stellate ganglia and axoplasmic fluid of the cephalopods *Octopus vulgaris*, *Loligo vulgaris*, and *Sepia officinalis* (1–2). Later, it was found in many other invertebrates (3–5) and vertebrates. In vertebrates, D-Asp occurs in the nervous system of chicken (6), rat (7–9), and man (10–11). In humans, it is present in the brain of embryos (10) and adults (11), as well as in the cerebrospinal fluid (12). D-Asp occurs at high levels in embryos nervous system, whereas in adult animals it nearly disappears, but increases in endocrine glands, particularly in the pituitary (7, 13), in the adrenal (8) and pineal gland, where it has been hypothesized to play an important role as a novel messenger molecule (14). Recently, we found that D-Asp levels increase in the testes during the two phases of testosterone synthesis: immediately before birth and during sexual maturity (13). In the rat it is localized in Leydig and Sertoli cells of the testes (13), and in *Octopus vulgaris* it is localized in the reproductive glands (5). These data suggest that D-Asp is implicated in hormonal processes and in steroidogenesis because Leydig cells are the source of testosterone synthesis (15). In support of this hypothesis is the discovery that D-Asp occurs in the ovary of *Rana esculenta*, where it is involved in the control of testosterone release during the sexual cycle (16) and in spermatogenesis in the rat testis (17).

Many studies have shown that the excitatory amino acid *N*-methyl-D-aspartic acid (NMDA) is able to stimulate the release of several hormones from adenohypophysis (18–34) and from pig cultured pituitary cells (35). In addition, an immunohistochemical study revealed that NMDA receptors are colocalized in specific hormone-secreting cells of the anterior pituitary (36). The primary site of action of NMDA has been suggested to be at the level of the hypothalamus via the control of hypothalamic releasing factors (37–42). Other studies revealed the presence of NMDA receptors in hypothalamic neurons (43) and their association with GnRH hormone neurons (44). Because NMDA is biochemically the methylated form of D-Asp, we have hypothesized that 1) NMDA could be an endogenous compound and D-Asp is the natural precursor for its biosynthesis, and 2) both D-Asp and NMDA are implicated in hormonal release regulation. To give support to this hypothesis, we have conducted *in vivo* and *in vitro* experiments to know the role of D-Asp and NMDA in the regulation of hormonal release on the hypothalamus-hypophysis axis. PRL was chosen as a typical ad-

Received February 24, 2000.
Address all correspondence and requests for reprints to: Antimo D'Aniello, Stazione Zoologica di Napoli, A. Dohrn, Villa Communale 1, Department of Neurobiology, Naples 80121, Italy. E-mail: daniello@alpha.szn.it.
* This work was principally supported by and carried out at the Zoological Station, Naples, Italy. G.H.F. gratefully acknowledges support from NIH Grant NIGMS-MBRS/SCORE SO6GM45455.

enohypophysial hormone because pilot experiments indicated that it was one of the most reliable indicators of hormone release induced by D-Asp and NMDA.

Materials and Methods

Materials

D-amino acid oxidase (EC 1.4.3.3, D-AAO) purified from hog kidney (15 U/mg; 5 mg/ml suspension in 3.2 M ammonium sulfate) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). All D- and L- amino acids including D-aspartic acid and *N*-methyl-D-aspartic acid, BSA, o-phthaldialdehyde (OPA), *N*-acetyl-L-cysteine (NAC), β-mercaptoethanol, methylamine (CH_3-NH_2), S-adenosyl-L-methionine (SAM or AdoMet) and Tris (Tris-hydroxymethyl aminomethane) were purchased from Sigma (St. Louis, MO). The kits for RIA (^{125} I) determination of PRL and *N*-[^{3}H]methyl-D-Aspartic acid (60–85 Ci/mmol) were purchased from Amersham International, Inc. (Buckinghamshire, UK). All solvents for HPLC were reagent grade and purchased from Merck or C. Erba (Milan, Italy). Cation exchange resin (AG 50W-×8, H^+ form, 100–200 mesh, 60–150 μm size) was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA).

Preparation of D-aspartate oxidase

D-aspartate oxidase (D-AspO, EC 1.4.3.1) (45–47) was obtained in purified form from beef kidney at the concentration of 5 mg/ml; 25 U/ml (48, 49).

Animals

Wistar male rats of 50 days old were purchased from Charles River Laboratories, Inc. (Como, Italy) and were housed, 2 per cage, in a controlled environment animal facility at 24 C that was on a 12-h light, 12-h dark cycle (lights on from 0700–1900 h). The animals were fed standard laboratory food pellets and water *ad libitum*. Care of animals was in accordance with institutional guidelines. Rats were killed by decapitation.

Sample purification.

To detect reliably D-Asp and NMDA, the tissue sample must be purified before being subjected to analyses for these amino acids. In particular, because NMDA occurs at very low concentration (1/1,000–1/10,000 than the common amino acids) it was necessary to purify and concentrate NMDA from the other cellular components. The devised procedure was the following:

The tissue (20–1000 mg) taken from the animal as soon as killed was homogenized in a ratio of 1:10 with 0.1 M trichloroacetic acid (TCA). Because some tissue weights were too small, *e.g.* hypophysis, hypothalamus etc., pools of tissue from several animals were combined to obtain almost 20 mg. Then, to calculate at the end of the purification the recovery of the NMDA, the homogenate was mixed with 10 μl of [^{3}H]-NMDA (0.1 μCi/ml, 0.012 pmol, 11,000 DPM) and centrifuged at 40,000 × *g* for 20 min. The sample was purified by cation exchange column chromatography (AG 50W X-8 resin) as described by Di Fiore *et al.* (16). An aliquot of the sample was used for the determination of free D-Asp (see below), whereas the remaining portion was subjected to further purification of NMDA as follows: The sample was mixed with 4 ml of borate buffer (0.02 M, pH 8.0) and with a solution of 1.0 M of OPA reagent in methanol using in proportion: 500 μl of OPA reagent for an amount of sample coming from about 1 g of original tissue. The pH of the solution was brought to 8.0–8.5 with 1 M NaOH and left 30 min at room temperature. The mixture was acidified to pH 2.0–2.5 with 1 M HCl and left at room temperature for 10 min. Then the sample was centrifuged for 10 min at 20,000 × *g* and the supernatant was purified on an Octadecylsilyl-C_{18} (ODS-C_{18}) cartridge (2 g packed weight of the ODS-C_{18}) (Waters Co., Allentown, PA). After absorption of the sample, the cartridge was washed with 4 ml of 0.01 M HCl, and the eluents were combined and again purified on a small column (1 × 2 cm) of cation exchange resin (AG50W X-8) using the same procedure as above (16). The residue was dissolved in 200 μl of distilled water and finally used for the determination of NMDA.

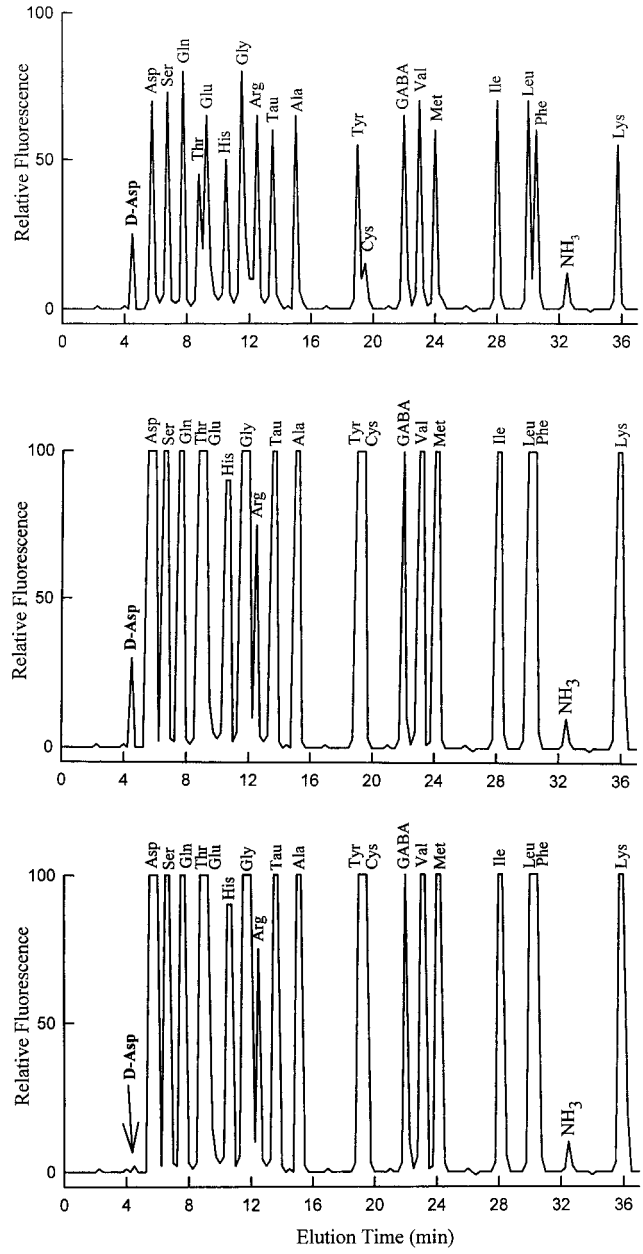


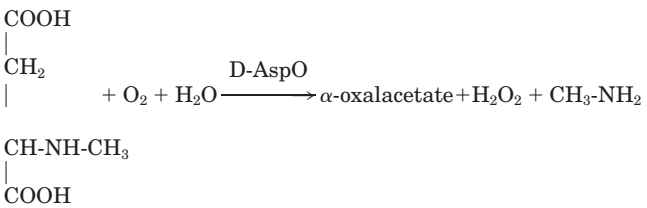
FIG. 1. Typical HPLC determination of D-Asp by the OPA-NAC method. *Upper panel*, HPLC separation of a standard mixture of amino acids. 10 μl of mixture containing each L-amino acid at the concentration of 0.1 μmol/ml and D-Asp at the concentration of 0.02 μmol/ml are mixed with 90 μl of borate buffer (0.02 M, pH 9.0), then derivatized with 5 μl of OPA-NAC, injected into the HPLC, and detected the fluorescence. *Middle panel*, Analysis of a rat brain cortex extract obtained after purification by cation exchange resin: 20 μl of the sample are mixed with 80 μl of borate buffer (0.02 M, pH 9.0) and 5 μl of OPA-NAC reagent. *Lower panel*, The same sample as used in the *middle panel* was previously treated with D-AspO to oxidize the D-Asp and then derivatized with OPA-NAC. The *arrow* shows the disappearance of the peak corresponding to the elution of D-Asp.

Determination of D-aspartic acid

D-Aspartic acid was determined by an HPLC method combined with the use of D-aspartate oxidase (see Fig. 1). The method is that described by Aswad (50) and modified by Di Fiore *et al.* (16).

Determination of NMDA

NMDA was determined using an enzymatic HPLC method based on the measurement of the CH_3-NH_2 (methylamine) generated by the oxidation of NMDA by D-AspO according the following reaction:



The CH_3-NH_2 was determined by the HPLC after derivatization with OPA-mercaptoethanol as follows: 40 μl of sample (previously brought to pH 8–2-8.4) was mixed with 5 μl of H_2O and 1 μl of D-AspO and incubated at 37 C for 15 min. After that, 5 μl of OPA-mercaptoethanol reagent (prepared by dissolving 10 mg of OPA in 2 ml methanol 50% and 20 μl of mercaptoethanol) were added and mixed. After 2 min, 20 μl of this mixture was injected onto a C_{18} Supelcosil HPLC column (0.45 × 25 cm, Supelco, Inc., Belafonte, PA) using the Beckman Coulter, Inc.-Gold HPLC System. The column was eluted using a gradient consisting of solvent A (5% acetonitrile in 30 mM sodium acetate buffer, pH 5.5) and solvent B (70% acetonitrile in 30 mM sodium acetate buffer, pH 5.5) as follows: 0–40% B over 5 min, then 100% B in 12 min, staying at 100% B for 2 min, and back to 0% B in 1 min. The flow rate was 1.2 ml/min. The CH_3-NH_2 (and the amino acids if still present in the sample) were detected fluorometrically at an excitation wavelength of 330 nm and an emission wavelength of 450 nm. The CH_3-NH_2 elutes as a sharp peak at the retention time of 13.1–13.2 min, well separated from ammonia and other amino acids (Fig. 2). The same procedure was carried out for a blank sample and an internal standard. The blank consisted of the sample plus 5 μl of H_2O, but no D-AspO was added during the incubation. The internal standard consisted of the sample, but 5 μl of NMDA at the concentration of 0.1 μmol/ml (0.5 nmol) was added to the sample instead of H_2O. The amount of NMDA in the sample was determined as follows:

$$\text{nmoles of NMDA in 20 } \mu\text{l of injected sample} = \frac{(\text{area of } CH_3\text{-}NH_2 \text{ peak of the sample}) - (\text{area of } CH_3\text{-}NH_2 \text{ peak of the blank sample})}{(\text{area of } CH_3\text{-}NH_2 \text{ peak of the internal standard}) - (\text{area of } CH_3\text{-}NH_2 \text{ peak of the sample})} \times 0.2$$

The method is specific for the determination of NMDA because the oxidation of NMDA with D-AspO produces CH_3-NH_2. The sensitivity of this method is such to detect a minimal amount of 10 - 20 pmol/assay.

Determination of other D- amino acids and L-amino acids

The determination of the other D-amino acids is carried out using a fluorometric method based on the use of the D-amino acid oxidase, according to the procedure of D'Aniello *et al.* (9) and Okuma and Abe (51). The determination of L-amino acids is carried out using the method of Godel *et al.* (52).

Biosynthesis of NMDA: in vivo and in vitro studies

Because NMDA is biochemically the methylated form of D-Asp (NMDA containing a CH_3 group substituted for a hydrogen in the α amino group of D-Asp), we hypothesized that NMDA could be biosynthesized from D-Asp. To validate this hypothesis, *in vivo* and *in vitro* experiments were carried out. The *in vivo* experiments consisted of injecting ip into rats, a solution of 0.5 M D-Asp at a dose to obtain 0.2–2.0 μmol/g body weight of animal. Thirty minutes to 5 h later, the rats were killed, and tissues were processed for purification and determination of NMDA, as described above. The *in vitro* experiments were performed on 200 mg of tissue homogenized (1:10) in PBS and dialyzed for 4 h to eliminate the endogenous NMDA. The tissue homogenate was incubated with shaking at 37 C for 60 min with 1 ml of PBS solution containing 10 mg/ml of BSA, 20 mM D-Asp, 10 mM EDTA (metalloprotease inhibitor), 50 mM sodium or potassium tartrate (inhibitor for mammalian D-AspO), and 5 mM SAM (methyl group donor). After incubation, 0.2 ml of 1.0 M TCA was added to the assay mixture and centrifuged at 30,000 × *g*. The supernatant was subjected to the purification and analysis of NMDA as described above.

In vivo effects of D-Asp on PRL release

To study the effects of D-Asp on PRL release, 50-day-old male rats were injected by ip with a solution of 0.5 M D-Asp, using an appropriate volume to inject 0.5 to 4.0 μmol/g body weight. Thirty minutes to 2 h after injection, the animals were killed by decapitation. Blood was collected, incubated at 37 C for 30 min, and centrifuged for 30 min at 3,000 × *g*. Serum was separated from the red cells and used for PRL determination by RIA method (see below). To detect the total occurrence and the synthesis of PRL in the pituitary gland, this gland was removed and homogenized in a solution of PBS containing 10 mg/ml of BSA (pH 7.4) in proportion of 1 mg of gland with 1 ml of solution (1:1,000). Then this homogenate was centrifuged for 5 min at 10,000 rpm, and the supernatant was again diluted 1:10, 1:100 and 1:1,000 in PBS-Albumin and used for the determination of PRL by RIA method. Parallel experiments were also conducted using other D- and L- amino acids instead of D-Asp. Each amino acid was injected ip to rats at the concentration of 2.0 μmol/g animal body weight, and PRL levels were measured 60 min after injection.

In vitro studies on the effects of D-Asp and NMDA on the PRL secretion from the adenohypophysis

These experiments were carried out to know the specific target at which D-Asp and NMDA act in stimulating PRL release. The experiments consisted of incubating rat adenohypophysis alone or in combination with the hypothalamus in a medium containing alternatively D-Asp or NMDA. Determinations of PRL released in the medium were performed at different times. In detail, the experiment was carried out as follows: from male rats of 50 days old, the pituitary gland and the hypothalamus were taken as soon as after decapitation. The adenohypophysis was separated from the hypothalamus and cut into four portions (making vertical and longitudinal cuts). The hypothalamus was also cut into four portions. After that, each of the four pieces of the adenohypophysis were transferred to a tube containing a nutrient mixture solution (Nutrient Mixture Ham's F-10; Life Technologies, Inc., Gaithersburg, MD) supplemented with BSA (10 mg/ml medium). The adenohypophysis was incubated alone or together the hypothalamus in the nutrient solution (1 mg of tissue with 1 ml of nutrient). To the medium was added D-Asp or NMDA (0.5 M) to obtain a final concentration between 0.02 to 2.0 mM and incubated at 25 C with gentle shaking for 240 min. At each fixed time, the shaking was stopped for 5 min to permit the sedimentation of the pieces of tissue and 200 μl of the medium were taken and stored at 0 C until analyzed for PRL release. In control experiments D-Asp or NMDA were omitted.

PRL determination

PRL was determined by a double antibody RIA method using a kit for the determination of rat PRL purchased from Amersham International (Buckinghamshire, UK). The assay was reliable in a range of 0.5–5 ng/tube. The serum from the *in vivo* experiments was examined undiluted and diluted 1:2 and 1:4 in PBS-Albumin reagent. The samples from the *in vitro* experiments were analyzed at the dilution as described in the assay section.

Statistical analyses

The results given in the text are expressed as the mean ± SD. Data were analyzed by one-way ANOVA followed by Duncan's multiple range test.

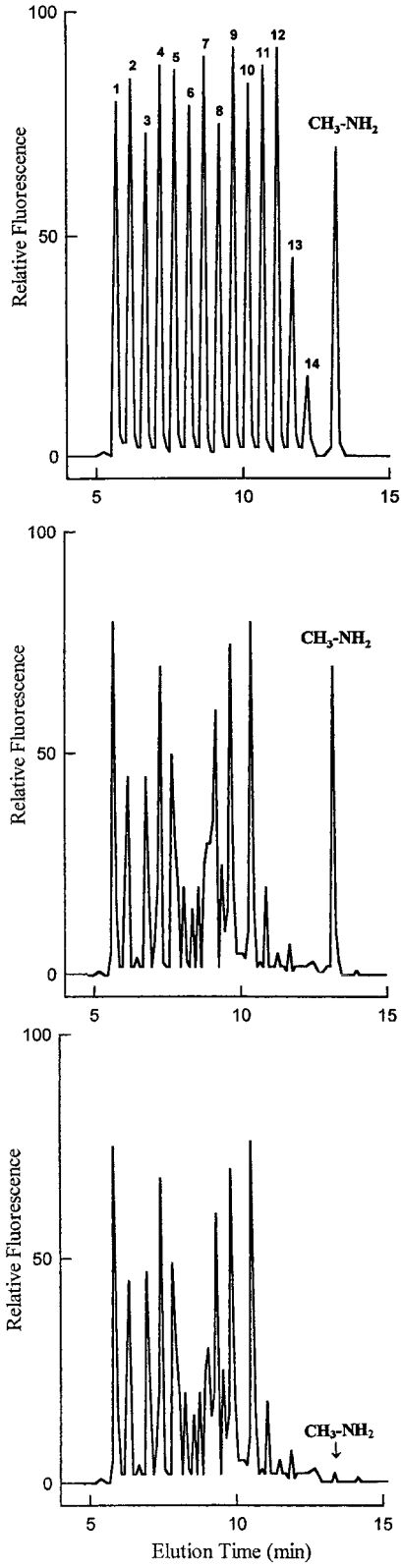


FIG. 2. Typical HPLC determination of methylamine (CH_3-NH_2) coming from the oxidation of NMDA with D-AspO. *Upper panel*, HPLC separation of a standard mixture of amino acids and CH_3-NH_2 (methylamine). Thirty microliters of mixture containing each L-amino acid and CH_3-NH_2 at the concentration of 0.01 μmol/ml are mixed with 20 μl of borate buffer (0.2 M, pH 8.2), derivatized with 10 μl of OPA-Mercaptoethanol, injected into the HPLC, and detected by fluorescence. The peak at elution times of 13.1–13.2 min corresponds to that of CH_3-NH_2. *Middle panel*, Analysis of a rat brain cortex extract obtained after the last step of purification (see: *Purification of the sample for NMDA determination* in *Material and Methods*). Thirty microliters of the sample are mixed with 20 μl of borate buffer (0.2 M, pH 8.2) and with 1 μl of D-AspO (25 U/ml). After incubation for 20 min at 37 C, the sample is derivatized with 10 μl of OPA-Mercaptoethanol reagent, injected into the HPLC, and detected by fluorescence. The peak at elution time of 13.1–13.2 min corresponds to CH_3-NH_2 came from the oxidation of NMDA with D-AspO. *Lower panel*, Analysis of the same sample shown in *middle panel*, but this time the sample was not subjected to the D-AspO treatment. The *arrow* shows the disappearance of the peak corresponding to the CH_3-NH_2 elution. The numbers on the top of the peaks are: 1 = Asp; 2= Glu; 3= Ser; 4= Thr+His; 5 = Gly, 6 = Arg, 7 = Ala, 8= Tyr+Cys; 9 = Val+Met; 10= Ile; 11 = Leu; 12 = Phe; 13= Lys; and 14 = NH_3.

Results

Endogenous occurrence of free D-Asp in rat tissues and its accumulation in response to acute D-Asp treatment

The results obtained in this work confirmed that rat tissues possess D-Asp, as had been previously reported (6–10, 13–14) specifically in neuroendocrine tissues. Here, we found that in male rats of 50 days old, D-Asp is mostly concentrated in the adenohypophysis and hypothalamus at a mean concentration of 78 ± 12 and 55 ± 9 nmol/g tissue, followed by the testes, hippocampus and total brain with values of 45 ± 7, 34 ± 8 and 18 ± 4 nmol/g tissue, respectively. The liver and blood possess only traces of D-Asp and muscle only an undetectable amount (Table 1). In addition to these findings, we have also observed that if rats received sodium D-aspartate via ip injection at a dose of 2.0 μmol/g body weight of animal, rat tissues have the capacity to significantly accumulate D-Asp. Among the various tissues analyzed, the adenohypophysis is the tissue with the highest ability for D-Asp accumulation. In fact, as is shown in the Table 1, 1 h after the rat received D-Asp the adenohypophysis accumulates an amount of D-Asp corresponding to 390 ± 60 nmol/g tissue (5.0 times more than the basal value). After 2 h, this increased to 990 ± 130 nmol/g (12.6 times) and after 5 h the accumulation rose to 1,350 ± 260 nmol/g (17.3 times). This increase also occurred in the other tissues analyzed (testes, total brain, hypothalamus, and hippocampus), but the accumulation was less evident than in the adenohypophysis. In general, D-Asp increased in these tissues about 2–3 times above the basal level after one hour of the injection, 4–5 times after 2 h, and 3.5–4.5 times after 5 h (Table 1). These results thus indicate that the adenohypophysis possesses a particular affinity in accumulating D-Asp and that this is a specific peculiarity for D-Asp, because other D- and L-amino acids (L-Asp, D- and L-Ala, D- and L-Glu) injected in the same way were not significantly taken up by the adenohypophysis or other neuroendocrine tissues (data not shown).

Occurrence of NMDA in rat tissues

In this work, using a specific HPLC enzymatic method associated with the use of the D-AspO, we were able specifically to detect NMDA in the sample. The method was based on HPLC determination of the methylamine coming from the oxidation of NMDA with D-AspO. As is shown in

TABLE 1. Occurrence of free D-Asp in rat tissues and its accumulation in response to acute D-Asp treatment

	Endogenous occurrence of D-Asp in rat tissues (nmol/g tissue)	D-Asp accumulation after ip injection[a] (nmol/g tissue) Time post treatment		
		1 h	2 h	5 h
Adenohypophysis	78 ± 12	390 ± 60	990 ± 130	1350 ± 260
	—	(5.0)	(4.7)	(17.3)
Testes	45 ± 7	130 ± 25	215 ± 35	158 ± 21
	—	(2.9)	(4.7)	(3.5)
Total brain	18 ± 4	45 ± 8	75 ± 15	68 ± 12
	—	(2.5)	(4.2)	(3.7)
Hypothalamus	55 ± 9	143 ± 19	245 ± 46	195 ± 32
	—	(2.6)	(4.4)	(3.5)
Hippocampus	34 ± 8	81 ± 13	138 ± 24	123 ± 21
	—	(2.3)	(4.0)	(3.6)
Liver	8 ± 2	—	—	—
Blood	2 ± 1	—	—	—
Hind leg muscle	<0.1	—	—	—

[a] This consisted of an ip injection of sodium D-aspartate solution, 0.5 M, pH 7.4, using an appropriate volume to administer 2.0 μmol/g body weight. The rats were injected in the morning and killed after the times indicated in the table. The results represent the mean ± SD, obtained from 5 separate experiments, each carried out on a pool from 3 animals of 50 days old. In *parentheses* are reported the number of times D-Asp accumulation is increased over the basal value.

TABLE 2. Occurrence of NMDA in rat tissues

	nmol/g tissue
Adenohypophysis	8.4 ± 1.2
Testes	1.6 ± 0.5
Total brain	1.7 ± 0.4
Hypothalamus	5.6 ± 1.1
Hippocampus	4.2 ± 0.8
Liver	0.5 ± 0.3
Hind leg muscle	0.2 ± 0.1

The results are the mean ± SD obtained from tissues of 5 male rats of 50 days old, using the enzymatic HPLC method.

Table 2, the highest NMDA occurrence was found in the adenohypophysis, which corresponded to a value of 8.4 ± 1.2 nmol/g tissue followed by the hypothalamus (5.6 ± 1.1 nmol/g), hippocampus (4.2 ± 0.8 nmol/g), total brain, and testes (1.7 ± 0.4 and 1.6 ± 0.5 nmol/g, respectively). NMDA is also present in the liver and in the muscle, but at very low concentrations compared with the other tissues (Table 2). In addition to the above results, it was noted that the ratio between the occurrence of NMDA and D-Asp is close to between 9–11 for all tissues examined except for the testes, where the ratio was 28. Thus, these results led us to think that there exists a specific enzymatic system which controls the endogenous synthesis of NMDA from D-Asp.

Biosynthesis of NMDA: in vivo and in vitro studies

When D-Asp was administered to male rats (ip 2.0 μmol/g body weight) a significant increase of NMDA was observed 2 h after the injection (Fig. 3). The hypothalamus had the highest activity in NMDA biosynthesis. In fact, from a basal level of 5.6 ± 1.1 nmol/g tissue, it rose to levels of 18.3 (ratio 3.26-fold increase). The adenohypophysis and the hippocampus were the other two tissues in which the biosynthesis was well evident. In these tissues the elevation of NMDA was about 2.8–3.0 times. In fact, from the value of 8.4 ± 1.2 and 4.2 ± 0.8 nmol/g tissue, NMDA rose to 23.5 ± 3.5 and 12.4 ± 2.5, respectively. In the total brain and liver NMDA biosynthesis was observed to a lower extent (Fig. 3). Because these

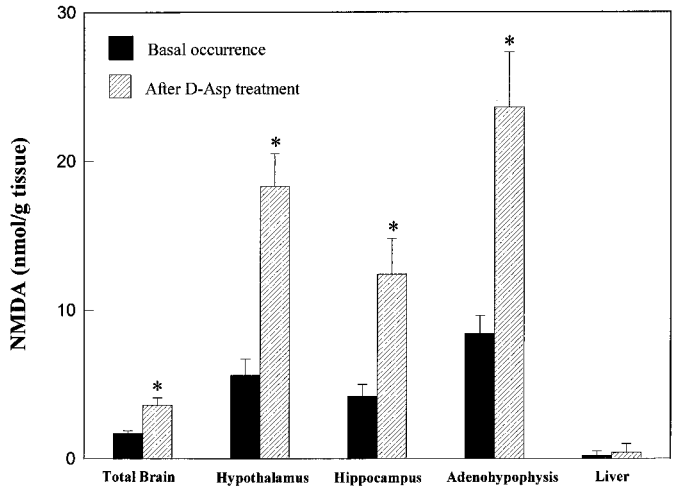


FIG. 3. Occurrence of NMDA in rat tissues and *in vivo* biosynthesis. The *black bars* represent the basal levels of NMDA. The *gray bars* represent the concentration of NMDA biosynthesized in rat tissues after injection of 0.5 M sodium D-aspartate, pH 7.4, at doses to obtain 2.0 μmol/g body weight. After 1 h, tissues were taken from the animals and processed for NMDA purification as detailed in *Materials and Methods* and then subjected to HPLC analysis for NMDA determination.

data show that NMDA is biosynthesized *in vivo* after rats have ingested D-Asp, we hypothesized that this amino acid could constitute the precursor for NMDA and that the enzyme that catalyzes this reaction could be a methyltransferase that specifically transports a methyl group from a donor to D-Asp. To support this hypothesis, we performed *in vitro* experiments where a tissue homogenate was incubated with D-Asp and SAM (the universal methyl donor in transmethylation reactions). One hour after incubation, the mixture was treated with TCA, purified as described above, and the NMDA generated was determined. The results obtained from this experiment demonstrated that the biosynthesis of NMDA also occurs *in vitro* (Table 3). In addition, it also demonstrated that D-Asp is the precursor for NMDA

synthesis and SAM is the substrate for the -CH_3 group donor (Table 3). The hypothalamus proved to be the tissue in which this biosynthesis occurred at the highest rate (30.5 ± 5.1 nmol/assay mixture) following by hippocampus, adenohypophysis, brain and liver (Table 3). When the tissue was incubated in the same conditions, but without D-Asp or SAM, this biosynthesis did not occur.

Effects of D-Asp on PRL secretion: in vivo experiments

In this experiment, we tested the effects of D-Asp on serum PRL release in 50-day-old male rats. The animals were injected (ip) with different doses of D-Asp between 0.5 and 4.0 μmol/g BW and after 30 min, 60 min, and 120 min the concentrations of serum PRL were measured. The results obtained from this study demonstrate that D-aspartate injection evokes a significant increase of PRL secretion that is dose and time dependent. In fact, when rats had ingested D-Asp at the doses of 0.5 μmol/g body weight, after 60 min from the ingestion, the levels of PRL increased 1.9 times compared with the PRL control levels (21.4 ± 3.6 *vs.* 10.9 ± 2.3 ng/ml serum; $P < 0.01$) (Fig. 4). At this dose, no significant increase was observed after 30 min or 120 min from the injection. At the dose of 1.0 μmol/g body weight, the PRL release was statistically significant after 30 min (1.8 times; $P < 0.01$) and the significant elevation persisted at 60 min (3.1 times; $P < 0.01$) and at 120 min (1.94 times; $P < 0.01$) from the injection. At the dose of 2.0 μmol/g body weight, the increase of PRL concentration was very significant at times of 30 min and 60 min (respectively, 2.4 and 2.65 times higher than the control, $P < 0.01$), but not at 120 min. Finally, at the dose of 4.0 μmol/g animal body weight, at 30 min from the injection the PRL level arose to 42.1 ± 7.1 ng/ml (3.74 times increased; $P < 0.01$) and to 25.4 ± 3.5 ng/ml after 60 min from the injection (2.22 times increased; $P < 0.01$). After 120 min, the concentration of PRL in the blood was not increased, and actually lower than the control (6.2 ± 1.2 ng/ml *vs.* the 11.4 of the control). In these experiments none of the following amino acids: L-Asp, L-Glu, D-Glu, L-Ala, D-Ala, show any significant ability in inducing the release of PRL (data not shown), thus indicating that D-Asp is the only amino acid which possesses such activity.

In vitro study on the effects of D-Asp and NMDA on PRL release

To clarify the role of D-Asp and NMDA on PRL secretion and at same time to establish the targets of these two molecules, sets of *in vitro* experiments were carried out. As is shown in Fig. 5, when the adenohypophysis was incubated with D-Asp (1 mM), the serum PRL concentrations rose significantly with respect to the control incubations. In fact, in the medium where the adenohypophysis was incubated with D-Asp, after 240 min of incubation, the PRL level was found be 94.5 ± 12.4 ng/mg *vs.* 22.3 ± 3.4 ng/mg of the control (4.2 times higher; $P < 0.01$) (Fig. 5, *left panel*). Interestingly, it was observed that NMDA (0.1 mM), contrary to D-Asp, stimulates the adenohypophysis to a lesser extent in inducing the PRL release. In fact PRL release at this time was only 32.4 ± 5.5 ng/mg adenhypophysis (Fig. 5, *left panel*). However, if the adenohypophysis was coincubated with the hypothalamus, we observed that PRL release was much more increased (Fig. 5, *right panel*). In fact, in the presence of D-Asp, PRL concentration in the medium rose to 180.1 ± 19.5 ng/mg of adenohypophysis (increased 5.2 times *vs.* control; $P < 0.01$). In addition, if NMDA instead of D-Asp was added to the medium, PRL release was further increased reaching the value of 210.2 ± 15.3 ng/mg of adenohypophysis (6.1 times *vs.* control; $P < 0.01$).

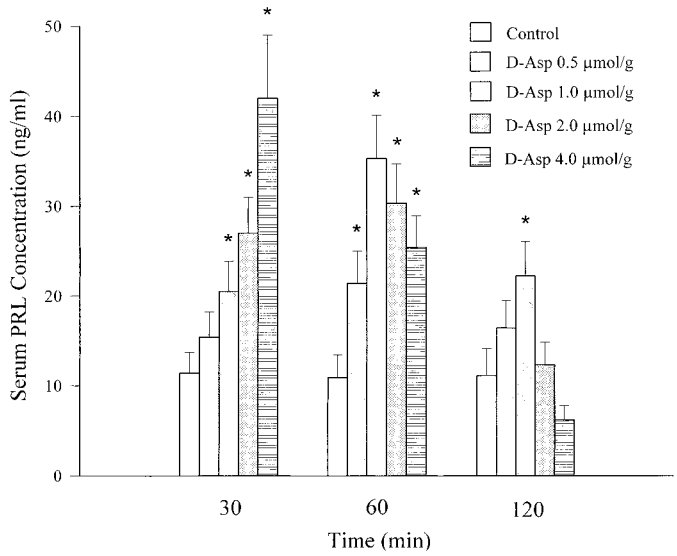


FIG. 4. Effect of D-Asp on the release of serum PRL. The values represent the mean ± SD of PRL levels in the rat blood of 50 days-old before and after rats received ip injection of D-Asp at doses between 0.5 to 4.0 μmol/g of body weight. Serum PRL concentrations were measured at 30, 60, and 120 min after D-Asp administration. The *asterisks* mean that the differences in serum levels of PRL were statistically significant *vs.* control ($P < 0.01$).

TABLE 3. *In vitro* biosynthesis of NMDA from rat tissue homogenate

	D-Asp + SAM	only D-Asp	only SAM
	nmol/ml assay mixture		
Adenohypophysis	18.2 ± 3.1	<0.2	<0.1
Total brain	14.3 ± 3.0	<0.3	<0.1
Hypothalamus	30.5 ± 5.1	<0.2	<0.1
Hippocampus	23.4 ± 3.5	<0.2	<0.1
Liver	12.2 ± 3.1	<0.2	<0.1
Hind leg muscle	<0.5	<0.1	<0.1

The results are the mean ± SD obtained from four experiments carried out on tissues 50-day-old rats as described in *Materials and Methods*. SAM is the abbreviation of S-Adenosyl-L-methionine.

Discussion

In the present study, we report the occurrence of endogenous D-aspartic acid and NMDA in the nervous system and endocrine glands of the rat and provide evidence for their neuroendocrine role in the regulation of PRL release. The adenohypophysis is the tissue in which these two amino acids occur at the highest levels of concentration (78 ± 12 nmol/g for D-Asp and 8.4 ± 1.2 nmol/g for NMDA). The hypothalamus, the hippocampus, and testes are other tissues in which these two amino acids are also present in considerable amounts (Tables 1 and 2). The pituitary gland pos-

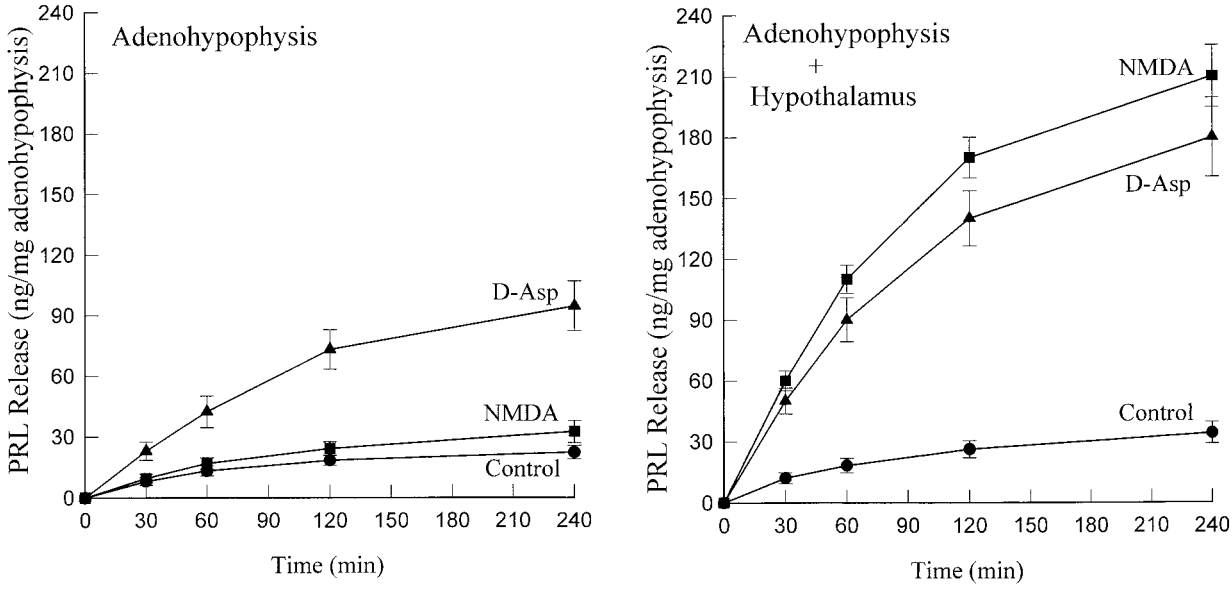


FIG. 5. Effects of D-Asp and NMDA on PRL release from isolated adenohypophysis and hypothalamus. The concentration of D-Asp and NMDA used in the medium were 1 mM and 0.1 mM, respectively. The results are expressed as ng of PRL released in the medium from each mg of adenohypophysis incubated. The values represent the mean ± SD of the results obtained from four different experiments. *Left panel*, PRL release induced from D-Asp, $P < 0.01$ *vs.* control and *vs.* NMDA. *Right panel*, PRL release induced from D-Asp and NMDA, $P < 0.01$ *vs.* control.

sesses a very high ability to accumulate D-Asp, when this amino acid is acutely administered to rats. In fact, after ip injection of D-Asp at a dose of 2 μmol/g body weight, this amino acid accumulates in the pituitary at the rate of 5.0, 12.6, and 17.3 times over the basal level after 1 h, 2 h, and 5 h, respectively (Table 1). Interestingly, this gland also contains the highest amount of NMDA (Table 2). Only one example of the occurrence of NMDA in living organisms has been reported until now, that is in muscle extract of the blood shell *Scapharca broughtonii* (53). However, it should be noted that our finding of NMDA in mammalian neuroendocrine tissues is a novel discovery and demonstrates the role of NMDA in hormonal regulation.

In our previous study (13), we demonstrated that D-Asp is implicated in the release of LH in adult male rats. Here, we demonstrate that this amino acid possesses the capacity to induce the release of PRL in rat blood. An important point was to know if the induction of the discharge of PRL was due to the specific action of D-Asp on the pituitary gland or if instead the release of PRL was mediated by another molecule whose target action could be the pituitary or also the hypothalamus. On this regard, various authors have demonstrated that synthetic NMDA (available commercially and obtained by chemical synthesis) is involved in adenohypophysial hormone secretion (18–35). In addition, it also stimulates some hypothalamic factors, including PRL factors (37–42). Because D-Asp and NMDA have a structural similarities (NMDA is the methylated form of D-Asp), we have hypothesized that also NMDA could be present in neuroendocrine tissues as an endogenous molecule and that D-Asp could be its natural precursor. Using a sensitive and specific enzymatic HPLC method devised here, we were able to demonstrate that NMDA is actually present in rat tissues. The concentration of NMDA in neuroendocrine tissues is at levels (nmol) comparable to those of many known hormones of the hypothalamus-hypophysis axis. NMDA is biosynthesized *in vivo* and *in vitro*, and D-Asp is its natural precursor (Tables 2 and 3, and Fig. 3). In fact, the enzyme implicated in this reaction utilizes D-Asp as substrate and SAM as a donor of the methyl group. It constitutes a novel methyltransferase enzyme, which we tentatively have termed: D-aspartate-*N*-methyl transferase or *N*-methyl-D-aspartate synthase.

In vivo experiments (Fig. 4) have demonstrated that when D-Asp was administered to rats via ip injection, it exerted an effect on PRL release that was dose-time dependent. At the dose of 0.5 mM and within the time between 30 min to 4 h, no PRL release was observed, probably because the concentration of D-Asp is not sufficient to be accumulated into the adenohypophysis or to reach the hypothalamus through the brain barrier. At times of 60 until 120 min, a significant increase of serum PRL concentrations was observed. This is due to the fact that in the adenohypophysis after this time the accumulation of D-Asp was 5.0–12.6 times higher than the basal levels (Table 1), and it is possible that this concentration is sufficient to stimulate an increase of PRL release. At D-Asp dose of 1.0–4.0 μmol/g animal body weight, PRL release is stimulated significantly already only 30 min after the injection. Probably this can happen because at this dose D-Asp is accumulated in high amount in the adenohypophysis and also in the hypothalamus (Table 1), where it induces the secretion of PRL at faster rates. However, if the dose is too elevated, *i.e.* 4.0 μmol/g, an immediate discharge in PRL release occurs in the blood and this amount is so strong that serum PRL levels are found below the basal level after 2 h after the injection of D-Asp.

In vitro experiments conducted on isolated adenohypophysis have indicated that D-Asp has a direct action on the pituitary gland in the induction of PRL release and that this action is dependent on incubation time and D-Asp concen-

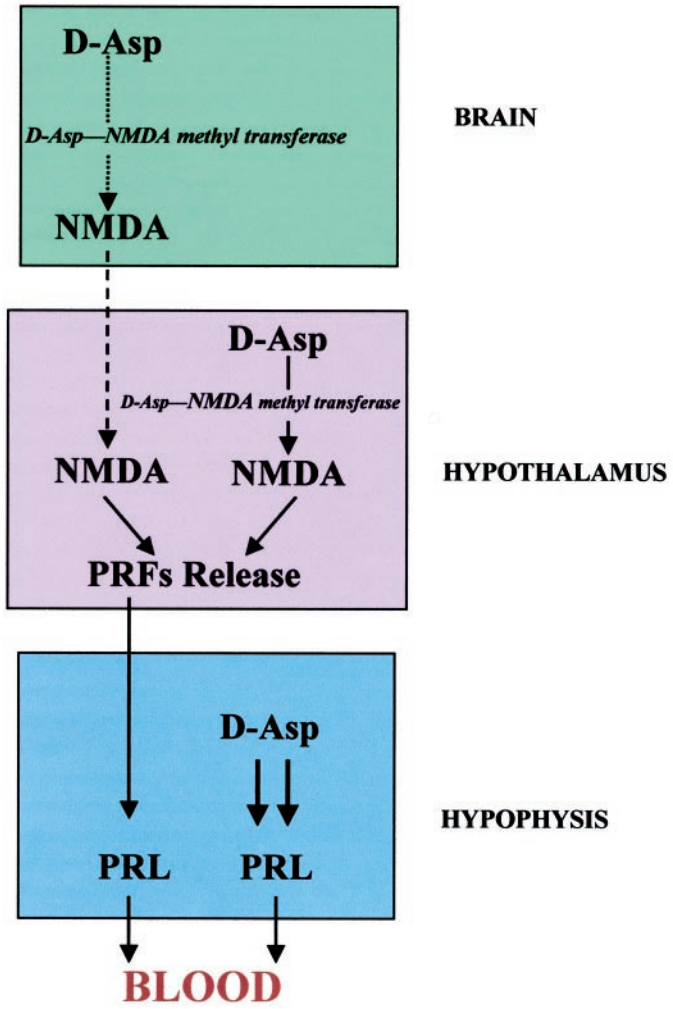


FIG. 6. Proposed pathways of the action of D-Asp and NMDA on the PRL release from rat pituitary gland. The *dashed arrow* indicates NMDA generated from D-Asp. The *double arrow* indicates the direct action of D-Asp on the pituitary gland in PRL release. The *single arrow* indicates the action of D-Asp on PRL release through the biosynthesis of NMDA. NMDA stimulates the release of PRFs by the hypothalamus, which in turn amplify the secretion of PRL at the hypophysis.

tration (Fig. 5, *left panel*). However, when the adenohypophysis was incubated together with the hypothalamus and D-Asp, a higher amount of PRL concentration in the medium was registered (Fig. 5, *right panel*). This could be due to the fact that during the incubation, an aliquot of D-Asp is transformed to NMDA, which induces an increase in the hypothalamus of some hypothalamic releasing factor/s in the medium, including PRL factors, which reinforce the release of PRL from the pituitary gland.

The consideration that the D-Asp and NMDA are implicated in the PRL release is further supported by the results of other authors who have demonstrated by immunohistochemical studies that receptors for NMDA have been localized in anterior pituitary hormone cell types, including PRL (36) as well as in the hypothalamus (43), that are associated with GnRH neurons (44). However, it is also reported that in some particular physiological conditions, NMDA can induce an inhibitory effect on PRL release and secretion, *i.e.* in female rats during lactation (54), in prepubertal female rats (55), in hypoprolactinaemic female rats (56), and in oestrogenized male rats (57).

In conclusion, the results obtained in this work provide evidence that D-Asp and NMDA are present in rat neuroendocrine tissues as endogenous compounds. D-Asp constitutes the natural precursor for the biosynthesis of NMDA and both D-Asp and NMDA play a role in the regulation of PRL release. D-Asp acts directly on the adenohypophysis, whereas NMDA on the hypothalamus promoting the release of some hypothalamic factor/s, which in turn reinforce/s the PRL release from the adenohypophysis. A proposed pathway of the involvement of D-Asp and NMDA in the PRL release is presented in Fig. 6.

Acknowledgments

We are grateful to Drs. Margherita Branno e Francesco Aniello, Department of Biochemistry and Molecular Biology of the Zoological Station, Naples, Italy for their help in the preparation of the D-aspartate oxidase obtained by molecular biology using messenger RNA from beef kidney.

References

1. **D'Aniello A, Giuditta A** 1977 Identification of D-aspartic acid in the brain of *Octopus vulgaris*. J Neurochem 29:1053–1057
2. **D'Aniello A, Giuditta A** 1978 Presence of D-aspartate in squid axoplasm and in other regions of the cephalopod nervous system. J Neurochem 31:1107–1108
3. **D'Aniello A, Nardi G, Vetere A, Ferguson GP** 1992 Occurrence of free D-aspartic acid in the circumsoesophageal ganglia of *Aplysia fasciata*. Life Sci 52:733–736
4. **D'Aniello A, Vetere A, Padula L** 1992 Occurrence of free D-amino acids in the gametes, embryos, larvae and adults of the sea-squirt *Ciona intestinalis*. Comp Biochem Physiol 102B:795–797
5. **D'Aniello A, Di Cosmo A, Di Cristo C, Fisher GH** 1995 D-aspartate in the male and female reproductive system of *Octopus vulgaris* Lam. Gen Comp Endocrinol 100:69–72
6. **Neidle A, Dunlop DS** 1990 Developmental changes in free D-aspartic acid in the chicken embryo and in the neonatal rat. Life Sci 46:1517–1522
7. **Dunlop DS, Neidle A, McHale D, Dunlop DM, Lajtha A** 1986 The presence of free D-aspartic acid in rodents and man. Biochem Biophys Res Commun 141:27–32
8. **Hashimoto A, Nishikawa T, Oka T, Takahashi K** 1993 Widespread distribution of free D-aspartate in rat periphery. FEBS Lett 331:4–8
9. **D'Aniello A, D'Onofrio G, Pischetola M, D'Aniello G, Vetere A, Petrucelli L, Fisher GH** 1993 Biological role of D-amino acid oxidase and D-aspartate oxidase: Effects of D-amino acids. J Biol Chem 268:26941–26949
10. **Hashimoto A, Kumashiro S, Nishikawa T, Oka T, Takahashi K, Mito T, Takashima S, Doi N, Mizutani Y, Kaneco T, Ootomo E** 1993 Embryonic development and postnatal changes in free D-aspartate and D-serine in the human prefrontal cortex. J Neurochem 61:348–351
11. **Fisher GH, D'Aniello A, Vetere A, Padula L, Cusano G, Man EH** 1991 Free D-aspartate and D-alanine in normal and Alzheimer brain. Brain Res Bull 26:983–985
12. **Fisher GH, Petrucelli L, Gardner C, Emory C, Frey WH, Amaducci L, Sorbi S, Sorrentino G, Borghi M, D'Aniello A** 1994 Free D-amino acids in human cerebrospinal fluid of Alzheimer disease, multiple sclerosis, and healthy control subjects. Mol Chem Neuropathol 23:115–124
13. **D'Aniello A, Di Cosmo A, Di Cristo C, Annunziato L, Petrucelli L, Fisher G** 1996 Involvement of D-aspartic acid in the synthesis of testosterone in rat testes. Life Sci 59:97–104
14. **Schell MJ, Cooper OB, Snyder SH** 1997 D-aspartate localizations imply neuronal and neuroendocrine roles. Proc Natl Acad Sci USA 94:2113–2018
15. **Ilpo T, Huhtaniemi, IT, Catt KJ** 1984 Functional maturation of rat testis Leydig cells. In: Dufau KJ, Dufau ML (eds) Hormone Action and Testicular Function. The New York Academy of Sciences, New York, vol 438:283–303
16. **Di Fiore MM, Assisi L, Botte V, D'Aniello A** 1998 D-aspartic acid is implicated in the control of testosterone production by vertebrate gonad. Studies on the female green frog, *Rana esculenta*. J Endocrinol 156:199–207
17. **D'Aniello A, Di Fiore MM, D'Aniello G, Colin FE, Lewis G, Setchell BP** 1998 Secretion of D-aspartate by the rat testis and its role in endocrinology of the testes and spermatogenesis. FEBS Lett 436:23–27
18. **Price MT, Olney JW, Cicero TJ** 1978 Acute elevations of serum luteinizing hormone induced by kainic acid, *N*-methyl aspartic acid, or homocysteic acid. Neuroendocrinology 26:352–358

19. **Price MT, Olney JW, Anglim M, Buchsbaum S** 1979 Reversible action of *N*-methyl aspartate on gonadotropin neuroregulation. Brain Res 176:165–168
20. **Price MT, Olney JW, Mitchell MV, Fuller T, Cicero TJ** 1978 Luteinizing hormone releasing action of *N*-methyl aspartate is blocked by GABA or taurine but not by dopamine antagonists. Brain Res 158:461–465
21. **Schainker BA, Cicero TJ** 1980 Acute central stimulation of luteinizing hormone by parenterally administered *N*-methyl-D,L-aspartic acid in the male rat. Brain Res 184:425–437
22. **Wilson RC, Knobil E** 1982 Acute effects of *N*-methyl-L-aspartate on the release of pituitary gonadotropins and prolactin in the adult female rhesus monkey. Brain Res 248:177–181
23. **Arslan M, Pohe CR, Smith MS, Plant JM** 1982 Studies in the role of the *N*-methyl-D-aspartate (NMDA) receptors in the hypothalamic control of prolactin secretion. Life Sci 50:295–300
24. **Urbanski HF, Ojeda SR** 1987 Activation of luteinizing hormone-releasing hormone release advances the onset of female puberty. Neuroendocrinology 46:273–276
25. **Estienne MJ, Schillo KK, Green MA, Hileman SM, Boling JA** 1989 *N*-methyl-D-L-aspartate stimulates growth hormone but not luteinizing hormone secretion in the sheep. Life Sci 44:1527–1533
26. **Brann DW, Mahesh VB** 1991 Endogenous excitatory amino acid involvement in the preovulatory and steroid-induced surge of gonadotropins in the female rat. Endocrinology 128:1541–1547
27. **Chang WJ, Barb CR, Kraeling RR, Rampacek GB, Asanovich KM** 1993 *N*-methyl-D,L-aspartate modulation of pituitary hormone secretion in the pig: role of opioid peptides. Domest Anim Endocrinol 10:305–313
28. **Kumar V, Lincoln GA, Tortonese DJ** 1993 Effects of excitatory amino acids receptor agonists and antagonists on the secretion of melatonin, luteinizing hormone and prolactin in the ram. J Neuroendocrinol 5:649–654
29. **Estienne MJ, Harter-Dennis JM, Barb CR, Hartsock TG, Campbell RM, Armstrong JD** 1996 *N*-methyl-D,L-aspartate-induced growth hormone secretion in barrows: possible mechanisms of action. J Anim Sci 74:597–602
30. **Downing JA, Joss J, Scaramuzzi RJ** 1996 The effects of *N*-methyl-D,L-aspartic acid and aspartic acid on the plasma concentration of gonadotropins, GH and prolactin in the ewe. J Endocrinol 149:63–72
31. **Fitzgerald BP, Davison LA** 1997 Comparison of the effects of *N*-methyl-D,L-aspartic acid on gonadotropin and prolactin secretion in anestrous mares and mares exhibiting estrous cycles during anestrus. Biol Reprod 57:36–42
32. **Halloway AC, Leatherland JF** 1997 The effects of *N*-methyl-D,L-aspartate and gonadotropin-releasing hormone on *in vitro* growth hormone release in steroid-primed immature rainbow trout, *Oncorhynchus mykiss*. Gen Comp Endocrinol 107:32–43
33. **Estienne MJ, Hurlock WF, Barb CR** 1998 Serum concentrations of luteinizing hormone, growth hormone, and cortisol in gilts treated with *N*-methyl-D,L-aspartate during the estrous cycle or after ovariectomy. J Anim Sci 76:2162–2168
34. **Pinilla L, Gonzalez L, Tena-Sempere M, Dieguez C, Aguilar E** 1999 Gonadal and age-related influences on NMDA-induced growth hormone secretion in male rats. Neuroendocrinology 69:11–19
35. **Barb CR, Barrett JB, Rampacek GB, Kraeling RR** 1993 *N*-methyl-D,L-aspartate modulation of luteinizing hormone and growth hormone secretion from pig pituitary cells in culture. Life Sci 53:1157–1164
36. **Bhat GH, Mahesh VB, Chu ZW, Chorich LP, Zamorano PL, Brann DW** 1995 Localization of the *N*-methyl-D-aspartate R1 receptor subunit in specific anterior pituitary hormone cell types of the female rat. Neuroendocrinology 62:178–186
37. **Gay VL, Plant TM** 1987 *N*-Methyl-D,L-aspartate elicits hypothalamic gonadotropin-releasing hormone release in prepubertal male rhesus monkeys (*Macaca mulatta*). Endocrinology 120:2289–2296
38. **Bourguignon JP, Gerard A, Franchimont P** 1989 Direct activation of gonadotropin-releasing hormone secretion through different receptors to neuroexcitatory amino acids. Neuroendocrinology 49:402–408
39. **Zanisi M, Messi E** 1991 Sex steroids and the control of LHRH secretion. J Steroid Biochem Mol Biol 40:155–163
40. **Arias P, Carbone S, Szwarcfarb B, Feleder C, Rodriguez M, Scacchi P, Moguilevsky JA** 1996 Effects of aging on *N*-methyl-D-aspartate (NMDA)-induced GnRH and LH release in female rats. Brain Res 740:234–238
41. **Bettendorf M, de Zegher F, Albers N, Hart CS, Kaplan SL, Grumbach MM** 1999 Acute *N*-methyl-D,L-aspartate administration stimulates the luteinizing hormone releasing hormone pulse generator in the ovine fetus. Horm Res 51:25–30
42. **Claypool LE, Kasuya E, Saitoh Y, Marzban F, Terasawa E** 2000 *N*-methyl-D,L-aspartate induces the release of luteinizing hormone-releasing hormone in the prepubertal and pubertal female rhesus monkey as measured by *in vivo* push-pull perfusion in the stalk-median eminence. Endocrinology 141:219–228
43. **Petralia RS, Yokotani N, Wenthold RJ** 1994 Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody. J Neurosci 14:667–696
44. **Bhat GK, Mahesh VB, Lamar CA, Ping L, Aguan K, Brann DW** 1995 Histochemical localization of nitric oxide neurons in the hypothalamus: association with gonadotropin-releasing hormone neurons and co-localization with *N*-methyl-D-aspartate receptors. Neuroendocrinology 62:187–197
45. **Tedeschi G, Negri A, Ceciliani F, Ronchi S, Vetere A, D'Aniello G, D'Aniello A** 1994 Properties of the flavoenzyme D-aspartate oxidase from *Octopus vulgaris*. Biochim Biophys Acta 1207:217–222
46. **D'Aniello A, Vetere A, Petrucelli L** 1993 Further study on the specificity of D-amino acid oxidase and of D-aspartate oxidase and time course for complete oxidation of D-amino acids. Comp Biochem Physiol 105B:731–734
47. **D'Aniello A, Rocca E** 1972 D-aspartate oxidase from the hepatopancreas of *Octopus vulgaris* Lam. Comp Biochem Physiol 41B:625–633
48. **Negri A, Massey V, Williams Jr CH** 1987 D-aspartate oxidase from beef kidney: purification, and properties. J Biol Chem 262:10026–10034
49. **Negri A, Tedeschi G, Ceciliani F, Ronchi S** 1999 Purification of beef kidney D-aspartate oxidase overexpressed in *Escherichia coli* and characterization of its redox potentials and oxidative activity towards agonists and antagonists of excitatory amino acid receptors. Biochim Biophys Acta 1431:212–222
50. **Aswad DW** 1984 Determination of D- and L-aspartate in amino acid mixtures by high performance liquid chromatography after derivatization with a chiral adduct of o-phthaldialdehyde. Anal Biochem 137:405–407
51. **Okuma E, Abe H** 1994 Simultaneous determination of D- and L-amino acids in the nervous system of crustaceans using precolumn derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate and reversed-phase ion-pair high performance liquid chromatography. J Chromatogr B 660:243–250
52. **Godel H, Graser T, Foldi P, Pfaender P, Furst P** 1984 Measurement of free amino acids in human biological fluids by high-performance liquid chromatography. J Chromatogr 297:49–61
53. **Sato M, Inoue F, Kanno N, Sato Y** 1987 The occurrence of *N*-methyl-D-aspartic acid in muscle extract of the blood shell, *Scapharca broughtonii*. Biochem J 241:309–311
54. **Abbud R, Smith MS** 1993 Altered luteinizing hormone and prolactin responses to excitatory amino acids during lactation. Neuroendocrinology 58:454–464
55. **Pinilla L, Gonzales D, Tena-Sempere M, Aguilar R, Aguilar E** 1996 Effects of *N*-methyl-D-aspartate and kainic acid on prolactin secretion in prepubertal female rats. Eur J Endocrinol 135:464–468
56. **Pinilla L, Tena-Sempere M, Aguilar R, Aguilar E** 1998 Effects of *N*-methyl-D-aspartic acid and kainic acid on prolactin secretion in hyper- and hypoprolactinaemic conditions. Eur J Endocrinol 138:460–466
57. **Pinilla L, Tena-Sempere M, Aguilar E** 1995 The role of excitatory amino acid pathways in the control of pituitary function in neonatally oestrogenized male rats. J Endocrinol 147:51–57