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Sex hormone levels in the brain of D-aspartate-treated rats

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ABSTRACT

D-Aspartate (D-Asp) is an endogenous amino acid present in the central nervous system and endocrine glands of various animal taxa. D-Asp is implicated in neurotransmission, physiology of learning, and memory processes. In gonads, it plays a crucial role in sex hormone synthesis. We have investigated the effects of chronic (30 days D-Asp drinking solution) and acute (i.p. injection of 2 μmol/g bw D-Asp) treatments on sex steroid synthesis in rat brain. Furthermore, to verify the direct effect of D-Asp on neurosteroidogenic enzyme activities, brain homogenates were incubated with different substrates (cholesterol, progesterone, or testosterone) with or without the addition of D-Asp. Enzyme activities were measured by evaluating the in vitro conversion rate of (i) cholesterol to progesterone, testosterone, and 17β-estradiol, (ii) progesterone to testosterone and 17β-estradiol, (iii) testosterone to 17β-estradiol. We found that D-Asp oral administration produced an increase of approximately 40% in progesterone, 110% in testosterone, and 35% in 17β-estradiol. Similarly, the results of the acute experiment showed that at 30 min after D-Asp treatment, the progesterone, testosterone, and 17β-estradiol levels increased by 29–35%, and at 8 h they further increased by a 100% increment. In vitro experiments demonstrate that the addition of D-Asp to brain homogenate + substrate induces a significant increase in progesterone, testosterone and 17β-estradiol suggesting that the amino acid upregulates the local activity of steroidogenic enzymes.

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1. Introduction

D-Aspartic acid (D-Asp) is an endogenous amino acid present in all animal taxa, including humans [1]. It was first observed in the brain of cephalopods [2], and subsequently detected in the nervous system of the frog [3–6], chicken, rat, mouse, and man [1,7,8]. When exogenous D-Asp was administered to rats, it accumulated in the brain as well as in the endocrine glands, especially in the adenohypophysis, testis, and adrenal gland [9]. Albeit its function as a neurotransmitter is well defined, less is known about the physiological role of this amino acid in the brain [10–12]. D-Asp seems to play an important role in the development of the nervous system [1]. In the adult rat, D-Asp is implicated in different neuronal activities including the physiology of learning and memory processes [13]. Characterization of genetic and pharmacological mouse models

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with abnormally higher levels of D-Asp has indicated that increased D-Asp enhances hippocampal NMDAR-dependent synaptic plasticity, dendritic morphology, and spatial memory [14].

Our previous studies have demonstrated that D-Asp is concentrated in rat endocrine glands, particularly in the pituitary gland and testes [15–17]. It can elicit the release of GnRH from the hypothalamus, the luteinizing hormone from the pituitary gland and testosterone from testis inducing spermatogenesis [9,18–22]. Following D-Asp administration to adult rats, an increase in the serum progesterone levels was also detected [9]. However, while a prominent role of D-Asp in sex hormone synthesis in the gonads is well defined, as well as another local factor, the Pituitary Adenylate Cyclase-Activating Peptide (PACAP) [23,24], studies are lacking regarding the involvement of D-Asp in the synthesis of these hormones in the brain. It is well known that in the central nervous system, biologically active steroids, also called neurosteroids, are synthesized de novo from cholesterol or from circulating inactive precursors [25–30]. Many studies have revealed the potential mediating role of these steroids in several brain functions, such as proliferation as well as activity and survival of nerve cells [27,29,31–33].

To test the ability of D-Asp to regulate sex hormone synthesis in the brain, we administered D-Asp solution to adult rats and determined progesterone, testosterone, and 17β-estradiol brain levels. Moreover, we carried out in vitro experiments to verify if D-Asp directly affects steroidogenic enzyme activities in the brain. Particularly, rat brain homogenates were incubated with different substrates: cholesterol, progesterone, and testosterone, with or without the addition of D-Asp. Thus, neurosteroidogenesis enzyme activities were measured by evaluating the in vitro conversion rate of:

- cholesterol to progesterone, testosterone, and 17β-estradiol;
- progesterone to testosterone and 17β-estradiol;
- testosterone to 17β-estradiol.

2. Materials and methods

2.1. Animals

Male Wistar rats, weighing 300–350 g, were kept under regulated conditions of temperature (24 ± 2 °C) and lighting (12 h light, 12 h dark cycles). They received commercial food pellets ad libitum. The experimental protocols described below, as well as the housing conditions, were in accordance with the Italian guidelines (D. Lvo 116/92) and authorized by the local Animal Care Committee (ASL 44, Prot. Vet. 22/95). All efforts were made to reduce animal suffering and the number of animals.

2.2. In vivo experiments

2.2.1. D-Asp oral administration

The rats were divided into two groups: the first group was allowed to drink a solution consisting of 20 mM D-Asp for 30 days; rats of the second group were given to drink fresh water. The dose was chosen based on our previous study, where we had established that this concentration was not dangerous to animals over the course of several months [21]. At the end of the treatment, rats were first anesthetized by an i.p. injection of chloral hydrate (40 mg/100 g body mass) and rapidly decapitated. The brains were dissected out, weighed, and rapidly analyzed for biochemical determinations.

2.2.2. D-Asp intraperitoneal administration

Rats were injected i.p. with 2.0 μmol/g body weight D-Asp dissolved in a saline solution. The dose was chosen based on preliminary experiments carried out with different doses (0.5–4.0 μmol/g body weight) of D-Asp [34,35]. Control rats received a saline injection. At different time points (30 min, 2 h, 8 h) after D-Asp or saline solution injections, the animals were anesthetized by an i.p. injection of chloral hydrate (40 mg/g body mass) and then decapitated. The brains were dissected out, weighed, and rapidly analyzed for biochemical determinations.

2.3. In vitro experiments

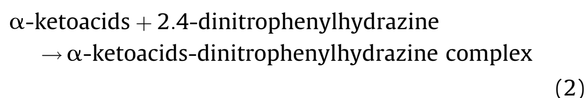
Brains from adult male rats were homogenized 1:10 in Tris-HCl 0.05 M pH 7.4. After centrifugation at 3000 g for 10 min, 3 mL of supernatant were separately incubated with 20 μL of 0.1 mg/mL substrate (cholesterol, progesterone, or testosterone), without or with 20 μL of D-Asp 1 M. Then, 1 mL of the assay mixture was taken from each sample and mixed with 9 mL methanol. The remaining samples were incubated at 37 °C under shaking. After 30 min was taken 1 mL of the assay mixture, which was mixed with 9 mL of methanol. After 120 min was taken 1 mL of the assay mixture, which was mixed with 9 mL of methanol. Then, the samples mixed with methanol were centrifuged at 13,000 rpm, and the supernatants were dried in appropriate glass Petri dishes at 40–50 °C under shacking. The residues were mixed with 0.2 mL of 10 mg/mL bovine serum albumin (BSA) in PBS to dissolve the hormones from the Petri dish in the BSA solution, and 5–20 μL were used for the hormonal determinations.

2.4. d-Aspartate determination

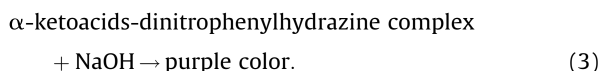
The brains from both oral- and intraperitoneal- treated rats ($n = 3$ from each experimental group) were first homogenized (Ultra-Turrax T25 homogenizer) with 0.2 M Tris–HCl, pH 8.2, in a ratio of 1:20. Tissue homogenate (100 μL) was supplemented with 20 μL of 0.5 M trichloroacetic acid (TCA) and centrifuged at 15,000 g for 10 min.

The supernatants were neutralized (to pH 6–8) using 1 M NaOH and the resulting sample was used for the determination of D-Asp by an enzymatic method based on the reaction between D-aspartate oxidase (D-AspO) and D-Asp, as follows:

$$\text{D-Asp} + O_2 + H_2O \xrightarrow{\text{D-AspO}} \alpha\text{-ketoacids} + H_2O_2 + NH_3 \quad (1)$$



$$\alpha\text{-ketoacids} + 2.4\text{-dinitrophenylhydrazine} \rightarrow \alpha\text{-ketoacids-dinitrophenylhydrazine complex} \quad (2)$$



$$\alpha\text{-ketoacids-dinitrophenylhydrazine complex} + \text{NaOH} \rightarrow \text{purple color.} \quad (3)$$

D-AspO, EC 1.4.3.1., was purified, and the assay procedure was as follows [36]. Into each of the two Eppendorf tubes were put 50 μL of sample (prepared as above) and 50 μL of 0.1 M Tris-HCl, pH 8.2. Then, to the first tube (sample) was added 5 μL of purified D-AspO (5 mg/mL) and to the second tube (blank sample) was added 5 μL of H_2O. All tubes were incubated for 30 min at 37°C. After that, 20 μL of 5 mM 2,4-dinitrophenylhydrazine (dissolved in 5 M HCl) were added to each tube, mixed, and left to incubate at room temperature for 10 min. Then, 300 μL of 1 M NaOH were added to each tube, and the absorbance of each sample was read at 445 nm against its blank.

To determine the concentration of D-Asp, a standard consisting of 50 μL of D-Asp 0.1 μmol/mL was used instead of the sample. This was read against a general blank consisting of 50 μL of distilled water instead of sample.

2.5. Sex steroid assays

Sex steroid levels were determined in the brains from each experimental group, using progesterone, testosterone, and 17β-estradiol enzyme immunoassay kits (Dia-Metra, Milan, Italy). The sensitivities were 50 pg/mL for progesterone, 70 pg/mL for testosterone, and 8.7 pg/mL for 17β-estradiol. The addition of D-Asp to the standard curve did not modify the assay sensitivity. Brains were homogenized to 1:10 (w/v) with PBS 1X. The homogenate was then mixed vigorously with methanol (1:10 v/v) and centrifuged at 3000 g for 10 min. The supernatant was transferred into a glass tube, and was left to evaporate on a hot plate at 40–50°C under a hood. The residue was dissolved in 0.25 mL of 0.05 M sodium phosphate buffer, pH 7.5, containing BSA at a concentration of 5 mg/mL, and then used for the assay [4,6]. The sex steroid recovery was 80% from brains. Steroid recovery was assessed by parallel processing of rat brain samples to which known amounts of steroids had been added prior to extraction and assay.

2.6. Statistical analyses

ANOVA followed by a Student–Newman–Keuls' test was used to evaluate differences between groups. Differences were considered statistically significant at $P < 0.05$. All data were expressed as the mean ± S.D.

3. Results

3.1. d-Asp oral administration increases brain d-Asp and sex hormone levels

Following chronic treatment (30-day D-Asp drinking solution) brain D-Asp levels (160 ± 10 nmol/g) were about twice those of controls (80 ± 7 nmol/g).

The levels of progesterone (P), testosterone (T), and 17β-estradiol (E_2) in the brain of D-Asp-treated rats were significantly increased with respect to controls (Fig. 1). Particularly, P and E_2 increased about 1.3-fold over basal levels, whereas T was increased by about 2-fold.

3.2. d-Asp intraperitoneal administration increases brain d-Asp and sex hormone levels

Following acute treatment (i.p. injection of 2 μmol/g bw) brain D-Asp levels were significantly higher at 30 min (167 ± 11 nmol/g), 2 h (168 ± 15 nmol/g) and 8 h (134 ± 20 nmol/g), than those of controls (43 ± 5 nmol/g).

As reported in Fig. 2, brain levels of the steroid hormones were significantly increased in response to D-Asp injection. Particularly, at 30 min P, T and E_2 concentrations increased about 1.3-fold over basal levels. After 2 h, P and T reached

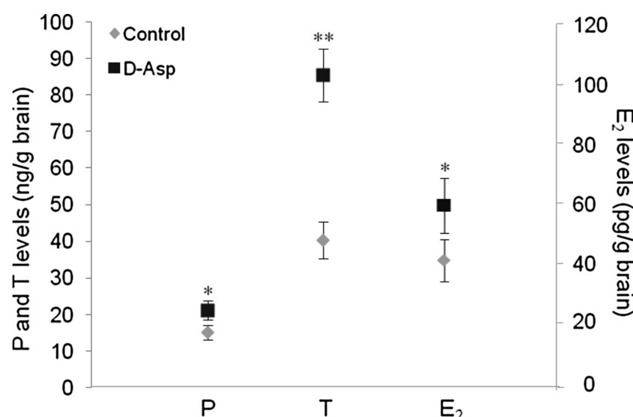


Fig. 1. Progesterone (P), testosterone (T) (ng/g), and 17β-estradiol (E_2) (pg/g) levels in the brain from control and D-Asp-treated rats. The experiment consisted of the oral administration of a 20 mM D-Asp drinking solution for 30 days; then the brain was taken from the animal and analyzed for hormone determination. The levels of P, T and E_2 in the brain of D-Asp-treated rats were significantly increased with respect to controls. Data are expressed as mean ± SD; $n = 5$ for each point; *: $P < 0.05$; **: $P < 0.01$ vs. control.

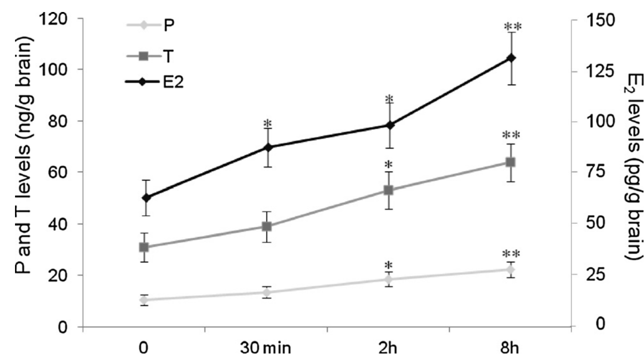


Fig. 2. Progesterone (P), testosterone (T) (ng/g), and 17β-estradiol (E_2) (pg/g) levels in the brain from control and D-Asp-treated rats. The experiment consisted of an i.p. injection of 2.0 μmol/g bw D-Asp dissolved in a saline solution; after 30 min, 2 h, and 8 h, the brain was taken from the animal and analyzed for hormone determination. The brain levels of the steroid hormones were significantly increased in response to D-Asp injection. Data are expressed as mean ± SD; $n = 5$ for each point; *: $P < 0.05$; **: $P < 0.01$ vs. control.

Table 1
Steroid hormone levels in brain homogenate + substrate with or without D-Asp.

	Progesterone (ng/g)	Testosterone (ng/g)	17β-estradiol (Pg/g)
Incubation with cholesterol			
A			
Zero time			
H + C	5.2 ± 1.1	15.0 ± 3.2	30.4 ± 5.0
H + C + D-Asp	5.5 ± 1.2	16.2 ± 4.2	33.4 ± 4.4
30 min			
H + C	7.4 ± 2.2	7.4 ± 2.2	33.5 ± 7.4
H + C + D-Asp % of increase	15.5 ± 3.1**	31.3 ± 4.2**	42.8 ± 6.5
	109.4%	322.9%	27.8%
2h			
H + C	12.5 ± 3.1	31.2 ± 4.2	55.4 ± 7.5
H + C + D-Asp % of increase	36.3 ± 4.5**	55.3 ± 6.4**	74.8 ± 9.6*
	190.4%	77.2%	35.0%
Incubation with progesterone			
B			
Zero time			
H + P	–	16.0 ± 2.8	31.6 ± 4.5
H + P + D-Asp	–	17.3 ± 2.5	33.2 ± 4.4
30 min			
H + P	–	32.1 + –4.5	38.4 ± 3.4
H + P + D-Asp % of increase	–	64.4 ± 6.2**	45.6 ± 2.1*
		100.6%	18.7%
2 h			
H + P	–	88.4 ± 8.6	45.1 ± 5.1
H + P + D-Asp % of increase	–	150.6 ± 9.4**	84.8 ± 9.6**
		70.3%	88.0%
Incubation with Testosterone			
C			
Zero time			
H + T	–	–	33.5 ± 5.1
H + T + D-Asp	–	–	34.5 ± 5.2
30 min			
H + T	–	–	38.3 ± 5.3
H + T + D-Asp % of increase	–	–	65.6 ± 7.1**
			71.3%
2h			
H + T	–	–	55.2 ± 6.2
H + T + D-Asp % of increase	–	–	95.8 ± 10.6**
			73.5%

The results represent the mean ± s.d. obtained from three individual determinations. H: brain homogenate; C: cholesterol; P: progesterone; T: testosterone.
* $P < 0.05$
** $P < 0.01$.

about 1.8-fold the basal level, and E_2 1.5-fold. After 8 h, P, T and E_2 increased about 2-fold over the basal level.

3.3. d-Asp upregulates brain steroidogenic enzyme activities

Rat brain homogenates were incubated with different substrates (cholesterol, P, or T) with or without the addition of D-Asp and, neurosteroidogenesis enzyme activities were measured by evaluating the in vitro conversion rate of cholesterol to P, T and E_2; P to T and E_2; T to E_2.

As reported in Table 1, significantly higher sex hormone levels were observed after 30 min and 2 h from the addition of D-Asp to brain homogenate + substrate as compared to those of the respective controls (brain homogenate + substrate). Specifically, after D-Asp addition to the homogenate + cholesterol mixture, P was enhanced approximately by 110% at 30 min and 190% at 2 h; T increased by about 320% at 30 min and 77% at 2 h; E_2 increased by about 28% at 30 min and 35% at 2 h (Table 1A).

The addition of D-Asp to the brain homogenate + progesterone mixture induced a T increase by approximately 100% at 30 min and 70% at 2 h; E_2 increased by 18% at 30 min and 88% at 2 h (Table 1B).

Finally, the addition of D-Asp to brain homogenate + - testosterone mixture induced an E_2 increase by about 70% either at 30 min or 2 h (Table 1 C).

4. Discussion

As previously demonstrated [13], we found that the rat brain possesses the capacity to take up and accumulate exogenously administered D-Asp. Interestingly, endogenous amino acid levels doubled following 30 days of oral D-Asp treatment. We observed that the intraperitoneal administration of D-Asp rapidly accumulated in the brain. Specifically, at 30 min, a 200% increase was reported, remaining high for 2 h and decreasing after 8 h.

Because of the increased brain D-Asp levels, we observed a rise in P, T, and E_2 levels. In particular, 30 days of D-Asp oral administration produced an increase by approximately 40% P, 110% T and 35% E_2. Accordingly, a recent study reports that in both sham- and neuropathic mice, chronic D-Asp treatment significantly increased the levels of P, T and E_2 in the prefrontal cortex and hippocampus [37]. In addition, previous studies have demonstrated that D-Asp chronic oral administration to adult rats induces an increase by 100% of both serum and testis T, but not of E_2 levels [21].

The results of the acute experiment showed that D-Asp induces a rapid increase of sex hormone levels in the brain. In particular, 30 min after D-Asp treatment brain, P, T, and E_2 levels increased by 29–35% with further increase at 2 h and a 100% increase at 8 h. It is reported that in rats, P and T increased in the blood 5 h after D-Asp treatment [9]. In particular, T was 3.3-fold higher than in the basal serum levels, whereas P increased 2.7-fold. Other hormones: 17β-estradiol, androstenedione, 17α-hydroxyprogesterone and cortisol, were not affected by D-Asp injection [9].

D-Asp specific receptors have not yet been identified, however numerous reports indicate that the NMDA receptor (NMDAR) possesses an affinity for D-Asp [21,38–40]. There is also evidence that D-Asp induces an increase in NMDAR expression in the rat brain [7,8]. Interestingly, previous findings indicate the crucial role of the brain NMDAR in the synthesis of neurosteroids [41,42]. In particular, in pyramidal neurons, brief administration of NMDA in the presence of extracellular mg is sufficient to facilitate neurosteroidogenesis, and low levels of NMDAR activation are sufficient to increase neurosteroid immunostaining.

There is no doubt that brain steroid levels depend on peripheral steroid levels as well as on local synthesis and

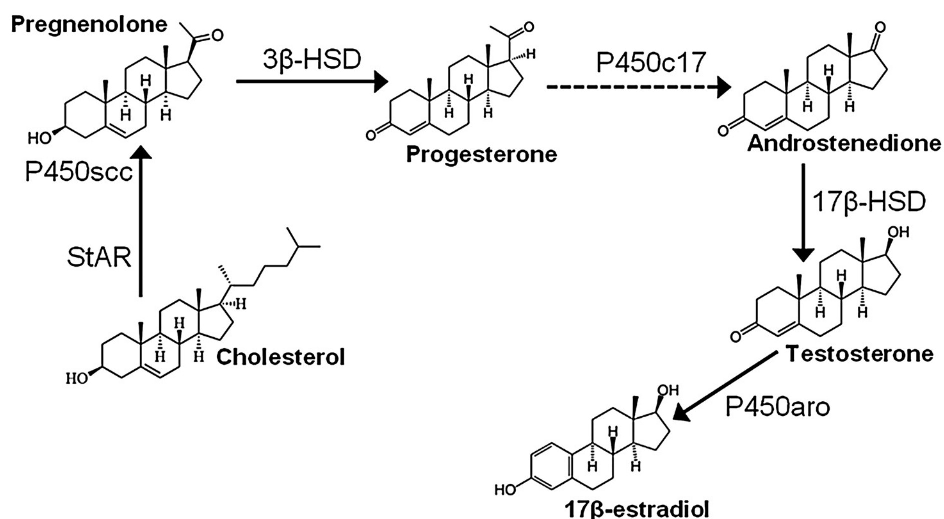


Fig. 3. Simplified scheme of sex steroid biosynthesis pathway. Cholesterol is translocated by StAR to the inner mitochondrial membrane, where it is converted into pregnenolone by P450 cholesterol side-chain cleavage (P450 scc). Next pregnenolone is converted into progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD), progesterone to androstenedione by steroid 17α-hydroxylase (P450c17). Then, 17β-hydroxysteroid dehydrogenase (17β-HSD) catalyzes the conversion of androstenedione to testosterone, which finally is converted into 17β-estradiol by cytochrome P450 aromatase (P450 aro).

metabolism [27,28]. As already mentioned, D-Asp influences the hypothalamus, pituitary, gonad axis and consequently sex steroid plasma levels [15–17]. The results of our in vivo experiments strongly suggest that D-Asp induces an increase of sex hormone levels in the brain, but we cannot exclude that increased levels may be due to an increase in circulating hormones. The addition of D-Asp to brain homogenates with substrate (cholesterol, progesterone, or testosterone) induced a significant increase in steroid hormone levels. As shown in Fig. 3, the steroidogenic pathway begins with a StAR-mediated translocation of cholesterol across the mitochondrial membrane where cholesterol is converted to pregnenolone by the P450 side-chain cleavage enzyme (P450scc); pregnenolone diffuses into the cytosol and is converted in the smooth endoplasmic reticulum to progesterone and then androstenedione by 3β-hydroxysteroid dehydrogenase (3β-HSD). Subsequently, 17β-hydroxysteroid dehydrogenase catalyzes the conversion of androstenedione to testosterone, which finally is converted into 17β-estradiol by cytochrome P450 aromatase (P450 aro) [43–48]. Therefore, our results demonstrate that D-Asp upregulates the activity of enzymes involved in brain P, T, E_2 synthesis. The different percentage of increase in P, T, E_2 levels suggests a different effect of D-Asp on the activity of the different enzymes.

These latter data confirm previous findings indicating that D-Asp induced synthesis of both mRNA and/or protein P450 aro in the frog's brain and testis [4,6,49]. Furthermore, an in vitro study performed on the boar testis revealed that D-Asp enhances aromatase activity [50,51]. Finally, in vitro experiments carried out on purified rat Leydig cells demonstrated that D-Asp increased gene and protein expression of StAR as well as P450scc and 3β-HSD transcripts [52,53].

In conclusion, our study demonstrates that D-Asp upregulates sex hormone levels in rat brain via the enhancement of steroidogenic enzyme activities and thus suggests a novel function of this amino acid in the brain, i.e. the local activation of steroid synthesis. Further studies are needed to clarify whether the D-Asp-induced neurosteroidogenesis could be mediated through NMDAR.

Disclosure of interest

The authors declare that they have no competing interest.

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References

[1] A. D'Aniello, D-Aspartic acid: an endogenous amino acid with an important neuroendocrine role, Brain Res. Rev. 53 (2007) 215–234.
[2] A. D'Aniello, A. Giuditta, Identification of D-aspartic acid in the brain of *Octopus vulgaris*, J. Neurochem. 29 (1977) 1053–1057.
[3] L. Burrone, M. Di Giovanni, M.M. Di Fiore, G. Chieffi Baccari, A. Santillo, Effects of D-aspartate treatment on D-aspartate oxidase, superoxide dismutase, and caspase 3 activities in frog (*Rana esculenta*) tissues, Chem. Biodivers. 7 (2010) 1459–1466.
[4] L. Burrone, A. Santillo, C. Pinelli, G. Chieffi Baccari, M.M. Di Fiore, Induced synthesis of P450 aromatase and 17β-estradiol by D-aspartate in frog brain, J. Exp. Biol. 215 (2012) 3559–3565.
[5] M. Di Giovanni, L. Burrone, G. Chieffi Baccari, E. Topo, A. Santillo, Distribution of free D-aspartic acid and D-aspartate oxidase in frog *Rana esculenta* tissues, J. Exp. Zool. A Ecol. Genet. Physiol. 313 (2010) 137–143.
[6] A. Santillo, C. Pinelli, L. Burrone, G. Chieffi Baccari, M.M. Di Fiore, D-Aspartic acid implication in the modulation of frog brain sex steroid levels, Gen. Comp. Endocrinol. 181 (2013) 72–76.
[7] F. Errico, F. Napolitano, R. Nisticò, A. Usiello, New insights on the role of free D-aspartate in the mammalian brain, Amino Acids 43 (2012) 1861–1871.
[8] N. Ota, T. Shi, J.V. Sweedler, D-Aspartate acts as a signaling molecule in nervous and neuroendocrine systems, Amino Acids 43 (2012) 1873–1886.
[9] A. D'Aniello, M.M. Di Fiore, G.H. Fisher, A. Milone, A. Seleni, S. D'Aniello, et al., Occurrence of D-aspartic acid and *N*-methyl-D-aspartic acid in rat neuroendocrine tissues and their role in the modulation of luteinizing hormone and growth hormone release, FASEB J. 14 (2000) 699–714.
[10] S. D'Aniello, I. Somorjai, J. Garcia-Fernàndez, E. Topo, A. D'Aniello, D-Aspartic acid is a novel endogenous neurotransmitter, FASEB J. 25 (2011) 1014–1027.
[11] P. Spinelli, E.R. Brown, G. Ferrandino, M. Branno, P.G. Montarolo, E. D'Aniello, et al., D-aspartic acid in the nervous system of *Aplysia limacina*: possible role in neurotransmission, J. Cell. Physiol. 206 (2006) 672–681.
[12] F. Errico, R. Nisticò, G. Palma, M. Federici, A. Affuso, E. Brilli, et al., Increased levels of d-aspartate in the hippocampus enhance LTP but do not facilitate cognitive flexibility, Mol. Cell. Neurosci. 37 (2008) 236–246.
[13] E. Topo, A. Soricelli, A. Di Maio, E. D'Aniello, M.M. Di Fiore, A. D'Aniello, Evidence for the involvement of D-aspartic acid in learning and memory of rat, Amino Acids 38 (2010) 1561–1569.
[14] F. Errico, R. Nisticò, A. Di Giorgio, M. Squillace, D. Vitucci, A. Galbusera, et al., Free D-aspartate regulates neuronal dendritic morphology, synaptic plasticity, gray matter volume and brain activity in mammals, Transl. Psychiatry 4 (2014) e417.
[15] M.M. Di Fiore, A. Santillo, G. Chieffi Baccari, Current knowledge of D-aspartate in glandular tissues, Amino Acids 46 (2014) 1805–1818.
[16] M.M. Di Fiore, A. Santillo, S. Falvo, S. Longobardi, G. Chieffi Baccari, Molecular mechanisms elicited by d-aspartate in leydig cells and spermatogonia, Int. J. Mol. Sci. 17 (2016) E1127.
[17] M.M. Di Fiore, L. Burrone, A. Santillo, G. Chieffi Baccari, Endocrine Activity of D-Aspartate in Nonmammalian Animals, in: T. Yoshimura, T. Nishikawa, H. Homma (Eds.), D-Amino Acids. Physiology, metabolism, and application, Springer, 2016, pp. 157–172.
[18] A. D'Aniello, M.M. Di Fiore, G. D'Aniello, F.E. Colin, G. Lewis, B.P. Setchell, Secretion of D-aspartic acid by the rat testis and its role in endocrinology of the testis and spermatogenesis, FEBS Lett. 436 (1998) 23–27.
[19] G. D'Aniello, A. Tolino, A. D'Aniello, F. Errico, G.H. Fisher, M.M. Di Fiore, The role of D-aspartic acid and *N*-methyl-D-aspartic acid in the regulation of prolactin release, Endocrinology 141 (2000) 3862–3870.
[20] E. Topo, A. Soricelli, A. D'Aniello, S. Ronsini, G. D'Aniello, The role and molecular mechanism of D-aspartic acid in the release and synthesis of LH and testosterone in humans and rats, Reprod. Biol. Endocrinol. 7 (2009) 120.
[21] A. Santillo, S. Falvo, P. Chieffi, L. Burrone, G. Chieffi Baccari, S. Longobardi, et al., D-aspartate affects NMDA receptor-extracellular signal-regulated kinase pathway and upregulates androgen receptor expression in the rat testis, Theriogenology 81 (2014) 744–751.
[22] A. Santillo, S. Falvo, P. Chieffi, M.M. Di Fiore, R. Senese, G. Chieffi Baccari, D-Aspartate induces proliferative pathways in spermatogonial GC-1 cells, J. Cell. Physiol. 231 (2016) 490–495.
[23] M. Agnese, S. Valiante, L. Rosati, P. Andreuccetti, M. Prisco, Pituitary Adenylate cyclase-Activating Peptide (PACAP) and PAC1 receptor in the testis of cartilaginous fish Torpedo marmorata: A molecular and phylogenetic study, Comp. Biochem. Physiol. B 191 (2016) 26–35.
[24] L. Rosati, M. Prisco, M.M. Di Fiore, A. Santillo, S. Valiante, P. Andreuccetti, et al., Role of PACAP on testosterone and 17β-estradiol production in the testis of wall lizard Podarcis sicula, Comp. Biochem. Physiol. A 191 (2016) 180–186.
[25] K. Tsutsui, K. Ukena, M. Takase, C. Kohchi, R.W. Lea, Neurosteroid biosynthesis in vertebrate brains, Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 124 (1999) 121–129.
[26] R.C. Agís-Balboa, G. Pinna, A. Zhubi, E. Maloku, M. Veldic, E. Costa, et al., Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis, Proc. Natl. Acad. Sci. USA 103 (2006) 14602–14607.

[27] J.L. do Rego, J.Y. Seong, D. Burel, J. Leprince, V. Luu-The, K. Tsutsui, et al., Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine regulation by neurotransmitters and neuropeptides, Front. Neuroendocrinol. 30 (2009) 259–301.
[28] J.L. do Rego, H. Vaudry, Comparative aspects of neurosteroidogenesis: From fish to mammals, Gen. Comp. Endocrinol. 227 (2016) 120–129.
[29] P. Porcu, A.M. Barron, C.A. Frye, A.A. Walf, S.Y. Yang, X.Y. He, et al., Neurosteroidogenesis today: novel targets for neuroactive steroid synthesis and action and their relevance for translational research, J. Neuroendocrinol. 28 (2016) 12351.
[30] A. Santillo, S. Falvo, M.M. Di Fiore, G. Chieffi Baccari, Seasonal changes and sexual dimorphism in gene expression of StAR protein, steroidogenic enzymes and sex hormone receptors in the frog brain, Gen. Comp. Endocrinol. 246 (2017) 226–232.
[31] E.E. Baulieu, Neurosteroids: a novel function of the brain, Psychoneuroendocrinology 23 (1998) 963–987.
[32] K.L. Schmidt, D.S. Pradhan, A.H. Shah, T.D. Charlier, E.H. Chin, K.K. Soma, Neurosteroids, immunosteroids, and the Balkanization of endocrinology, Gen. Comp. Endocrinol. 157 (2008) 2662–2674.
[33] T.D. Charlier, C.A. Cornil, C. Patte-Mensah, L. Meyer, A.G. Mensah-Nyagan, J. Balthazart, Local modulation of steroid action: rapid control of enzymatic activity, Front. Neurosci. 19 (2015) 9–83.
[34] R. Monteforte, A. Santillo, M. Di Giovanni, A. D'Aniello, A. Di Maro, G. Chieffi Baccari, D-Aspartate affects secretory activity in rat Harderian gland: molecular mechanism and functional significance, Amino Acids 37 (2009) 653–664.
[35] S. Falvo, M.M. Di Fiore, L. Burrone, G. Chieffi Baccari, S. Longobardi, A. Santillo, Androgen and oestrogen modulation by D-aspartate in rat epididymis, Reprod. Fertil. Dev. 28 (2016) 1865–1872.
[36] A. D'Aniello, G. D'Onofrio, M. Pischetola, G. D'Aniello, A. Vetere, L. Petrucelli, G.H. Fisher, Biological role of D-amino acid oxidase and D-aspartate oxidase. Effects of D-amino acids, J. Biol. Chem. 268 (1993) 26941–26949.
[37] A. D'Aniello, L. Luongo, R. Romano, M. Iannotta, I. Marabese, S. Boccella, et al., d-Aspartic acid ameliorates painful and neuropsychiatric changes and reduces β-amyloid Aβ1-42 peptide in a long lasting model of neuropathic pain, Neurosci. Lett. 651 (2017) 151–158.
[38] A.C. Foster, G.E. Fagg, Comparison of L-(^{3}H)glutamate, D-(^{3}H)aspartate, DL-(^{3}H)AP5 and (^{3}H)NMDA as ligands for NMDA receptors in crude postsynaptic densities from rat brain, Eur. J. Pharmacol. 133 (1987) 291–300.
[39] M. Di Giovanni, E. Topo, A. Santillo, A. D'Aniello, G. Chieffi Baccari, D-Aspartate binding sites in rat harderian gland, Amino Acids 38 (2010) 229–235.
[40] F.C. Doherty, C.D. Sladek, NMDA receptor subunit expression in the supraoptic nucleus of adult rats: dominance of NR2B and NR2D, Brain Res. 1388 (2011) 89–99.
[41] K. Tokuda, Y. Izumi, C.F. Zorumski, Ethanol enhances neurosteroidogenesis in hippocampal pyramidal neurons by paradoxical NMDA receptor activation, J. Neurosci. 31 (2011) 9905–9909.
[42] Y. Izumi, D.B. Clifford, C.F. Zorumski, Low concentrations of *N*-methyl-D-aspartate inhibit the induction of long-term potentiation in rat hippocampal slices, Neurosci. Lett. 137 (1992) 245–248.
[43] W.L. Miller, Molecular biology of steroid hormone synthesis, Endocr. Rev. 9 (1988) 295–318.
[44] D.M. Stocco, The role of the StAR protein in steroidogenesis: challenges for the future, J. Endocrinol. 164 (2000) 247–253.
[45] G. Moeller, J. Adamski, Integrated view on 17β-hydroxysteroid dehydrogenases, Mol. Cell. Endocrinol. 301 (2009) 7–19.
[46] W.L. Miller, StAR search-what we know about how the steroidogenic acute regulatory protein mediates mitochondrial cholesterol import, Mol. Endocrinol. 21 (2007) 589–601.
[47] S. Carreau, H. Bouraima-Lelong, C. Delalande, Estrogens: new players in spermatogenesis, Reprod. Biol. Endocrinol. 1 (2011) 90–94.
[48] V. Papadopoulos, W.L. Miller, Role of mitochondria in steroidogenesis, Best Pract Res Clin Endocrinol. Metab. 26 (2012) 771–790.
[49] L. Burrone, F. Raucci, M.M. Di Fiore, Steroidogenic gene expression following D-aspartate treatment in frog testis, Gen. Comp. Endocrinol. 175 (2012) 109–117.
[50] C. Lamanna, L. Assisi, V. Botte, M.M. Di Fiore, Endogenous testicular D-aspartic acid regulates gonadal aromatase activity in boar, J. Endocrinol. Invest. 29 (2006) 141–146.
[51] C. Lamanna, L. Assisi, V. Botte, M.M. Di Fiore, Involvement of D-Asp in P450 aromatase activity and estrogen receptors in boar testis, Amino Acids 32 (2007) 45–51.
[52] Y. Nagata, H. Homma, J.A. Lee, K. Imai, D-Aspartate stimulation of testosterone synthesis in rat Leydig cells, FEBS Lett. 444 (1999) 160–164.
[53] F. Raucci, A. D'Aniello, M.M. Di Fiore, Stimulation of androgen production by D-aspartate through the enhancement of StAR, P450scc and 3β-HSD mRNA levels in vivo rat testis and in culture of immature rat Leydig cells, Steroids 84 (2014) 103–110.