The Journal of Experimental Biology 215, 3559-3565

doi:10.1242/jeb.073296

RESEARCH ARTICLE

Induced synthesis of P450 aromatase and 17β-estradiol by D-aspartate in frog brain

Lavinia Burrone, Alessandra Santillo, Claudia Pinelli, Gabriella Chieffi Baccari and Maria Maddalena Di Fiore*

Dipartimento di Scienze della Vita, Seconda Università degli Studi di Napoli, Via Vivaldi 43, 81100, Caserta, Italy

*Author for correspondence (mariam.difiore@unina2.it)

SUMMARY

D-Aspartic acid is an endogenous amino acid occurring in the endocrine glands as well as in the nervous system of various animal phyla. Our previous studies have provided evidence that D-aspartate plays a role in the induction of estradiol synthesis in gonads. Recently, we have also demonstrated that D-aspartic acid induces P450 aromatase mRNA expression in the frog (*Pelophylax esculentus*) testis. P450 aromatase is the key enzyme in the estrogen synthetic pathway and irreversibly converts testosterone into 17β-estradiol. In this study, we firstly investigated the immunolocalisation of P450 aromatase in the brain of *P. esculentus*, which has never previously been described in amphibians. Therefore, to test the hypothesis that D-aspartate mediates a local synthesis of P450 aromatase in the frog brain, we administered D-aspartate *in vivo* to male frogs and then assessed brain aromatase expression, sex hormone levels and sex hormone receptor expression. We found that D-aspartate enhances brain aromatase expression (mRNA and protein) through the CREB pathway. Then, P450 aromatase induces 17β-estradiol production from testosterone, with a consequent increase of its receptor. Therefore, the regulation of D-aspartate-mediated P450 aromatase expression could be an important step in the control of neuroendocrine regulation of the reproductive axis. Accordingly, we found that the sites of P450 aromatase immunoreactivity in the frog brain correspond to the areas known to be involved in neurosteroid synthesis.

Key words: P450 aromatase, D-aspartate, 17β-estradiol, estrogen receptor.

Received 4 April 2012; Accepted 25 June 2012

INTRODUCTION

D-Aspartic acid (D-Asp) is an endogenous amino acid occurring in the endocrine glands as well as in the nervous system of various animal phyla (for a review, see D'Aniello, 2007). Although its function as a neurotransmitter is well defined, less is known about its physiological role in the brain (D'Aniello et al., 2011). D-Asp seems to play an important role in the development of the nervous system in rat and chicken (D'Aniello, 2007). In the adult rat, D-Asp is implicated in different neuronal activities including the physiology of learning and memory processes (Topo et al., 2010). While there are no studies on the involvement of D-Asp in neurosteroid synthesis in the brain, our previous studies in amphibians (Di Fiore et al., 1998; Raucci et al., 2004), reptiles (Assisi et al., 2001; Raucci et al., 2005a), birds (Di Fiore et al., 2008) and mammals (Lamanna et al., 2006; Lamanna et al., 2007; D'Aniello et al., 2000) have provided evidence that D-Asp plays a role in steroid synthesis in gonads. Recently, it has also been demonstrated that D-Asp induces P450 aromatase (P450 aro) mRNA expression in the frog (*Pelophylax esculentus*) testis (Burrone et al., 2012).

Neurosteroids are defined as neuroactive steroids synthesised *de novo* in the central nervous system. Many studies have revealed important roles of these steroids in mediating several brain functions, such as in sexual differentiation and reproductive behavior (Balthazart et al., 2009; Do Rego et al., 2009a). Among the different enzymes involved in neurosteroid synthesis, a prominent role is dedicated to cytochrome P450 aro, the key enzyme in the estrogen synthetic pathway, which irreversibly converts testosterone into estradiol. Aromatase is a member of the P450 cytochrome family encoded by the gene *cyp19* (Simpson et al., 1994). In the brain of some vertebrates, aromatase has emerged as a potential factor contributing to brain sexual dimorphism underlying the activation of male-typical sexual behavior (for reviews, see Cornil, 2009; Forlano et al., 2006; Garcia-Segura, 2008; Roselli, 2007). In the quail, hypothalamic aromatase regulates testosterone-induced aggressiveness by regulating the quantity of estradiol available for receptor binding (Schlinger and Callard, 1990). In songbirds, estrogens can organise and activate masculine neural circuits, such as those involved in vocalisation (Forlano et al., 2006). However, it is now assumed that estrogens have functional properties of neuromodulators, coordinating a variety of morphological, physiological and behavioral traits needed for successful reproduction (Balthazart et al., 2009).

Among amphibians, the brain expression of P450 aro mRNA is already detectable in the early developmental stages of *Xenopus laevis* (Iwabuchi et al., 2007; Urbatzka et al., 2007) and *Pleurodeles waltl* (Kuntz et al., 2004); it remains at a high level until metamorphosis. However, no sex-specific expression of the P450 aro gene has previously been observed in the brain of amphibians (Kuntz et al., 2004; Urbatzka et al., 2007).

Against this background, we firstly studied the immunolocalisation of P450 aro in the brain of the frog *P. esculentus*, which has never been previously described in adult or larval amphibians. Furthermore, to test the hypothesis that D-Asp mediates a local synthesis of P450 aro in the frog brain, we administered D-Asp intraperitoneally (i.p.) to male frogs and then assessed brain aromatase expression (mRNA and protein), sex hormone levels and

sex hormone receptor expression. In addition, since D-Asp is known to stimulate an increase of brain cAMP (D'Aniello et al., 2011; Spinelli et al., 2006), we investigated whether D-Asp treatment could affect cAMP response element binding protein (CREB) activity. The cAMP/PKA/CREB pathway is considered to be one of the signaling cascades through which the aromatase gene (*cyp19*) promoter is regulated (Stocco, 2008).

MATERIALS AND METHODS

Animals

Adult males of the green frog, *Pelophylax esculentus* (Linnaeus 1758) (previously *Rana esculenta*), were collected in the surroundings of Naples during the month of March (reproductive period). Animals were maintained under natural conditions of temperature and light and fed *ad libitum* with worms.

Immunohistochemistry

For detection of P450 aromatase, brains (N=3) were rapidly immersed in Bouin's fluid (Sigma-Aldrich, Milan, Italy) and then embedded in paraffin; serial sections (10 μm thick) were incubated in 1% normal goat serum (Sigma Chemical Corporation, St Louis, MO, USA). Next, they were incubated overnight at 4°C with rabbit polyclonal antibody against P450 aromatase (1:500; Abcam, Cambridge, UK). After washing in phosphate-buffered saline (PBS), the sections were incubated for 1 h at room temperature with biotinylated goat anti-rabbit antibody (1:150; Pierce, Rockford, IL, USA), followed by incubation for 1 h with streptavidin (1:200; Pierce). Bound antibody was visualised using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) and 0.3% H_2O_2 in Tris buffer (0.05 mol l^{-1}, pH 7.6). For negative controls, the primary antibody was omitted.

Experimental setup

Sixty frogs were divided into two groups. Animals of the first group were injected i.p. with 2.0 μmol g^{-1} body mass D-Asp (Sigma-Aldrich) dissolved in amphibian saline (Krebs-Ringer solution) for 14 consecutive days. This dose was chosen on the basis of preliminary experimental tests. The animals of the second group, used as controls, received solvent alone. One day after the last injection, frogs were first anesthetized by immersion in a 1% solution of MS-222 (Sigma-Aldrich) and then decapitated. Brains were immediately excised and stored at –80°C for biochemical analyses. Guidelines, found within the 'Principles of Laboratory Animal Care' (NIH publication no. 86-23, revised 1985), were followed throughout the experiments.

Specific determination of D-Asp in frog brain

Brains from D-Asp-treated frogs (N=5) and from controls (N=5) were first homogenised (Ultra-Turrax T25 homogenizer) with 0.2 mol l^{-1} Tris-HCl, pH 8.2, in a ratio of 1:20. Tissue homogenate (100 μl) was supplemented with 20 μl of 0.5 mol l^{-1} trichloroacetic acid and centrifuged at 15,000 ***g*** for 10 min. The supernatants were neutralised (to pH 6–8) using 1 mol l^{-1} NaOH, and the resulting sample was analysed by high-performance liquid chromatography using the method described previously (D'Aniello et al., 2000) for analysis of D-Asp oxidase (EC 1.4.3.1). The areas of the peaks of amino acid standards were used to calculate the amounts of D-Asp contained in the brain of control and D-Asp-treated frogs.

RNA isolation and cDNA synthesis

Total RNA was extracted from brains of D-Asp-treated (N=5) and control (N=5) frogs using the TRIzol standard protocol (Invitrogen Life Technologies, Carlsbad, CA, USA) and then treated for 30 min at 37°C with DNase I (10 U/sample) (Amersham Biosciences, Amersham, UK) to eliminate any contamination of genomic DNA. Total RNA purity and integrity were determined by spectrophotometry at 260/280 nm and electrophoresis on a denaturizing formaldehyde agarose gel. One microgram of total RNA was reverse-transcribed using the SuperScriptTM First-Strand Synthesis System kit (code 11904-018; Invitrogen Life Technologies).

Quantitative real-time PCR

Specific primer sets were designed for qRT-PCR using Primer3 (http://frodo.wi.mit.edu/primer3). Primers had the following sequences: P450 aromatase sense, 5′-GCACAGCATCCAAA-GACTGA-3′, and P450 aromatase antisense, 5′-ATGACCAA-GCCTACCACAGG-3′ (GenBank accession no. DQ449025; product size 155 bp); AR sense, 5′-ACTCCTGGATGGGA-CTGATG-3′, and AR antisense, 5′-TTGTGAGAGGTGAC-GCATTC-3′ (GenBank accession no. EU350950; product size 161 bp); ERα sense, 5′-TGGTGTCTGGTCTTGTGAGG-3′, and ERα antisense, 5′-TCCCTTTCATCATTCCCACT-3′ (GenBank accession no. DQ398027; product size 170 bp).

As internal control, the same cDNAs were amplified using P1 oligonucleotide primers with the following sequences: P1 sense, 5′-TTGTGAAGCTAAGGCCTGGT-3′, and P1 antisense, 5′-TCTTGTCTTCCGTGATGGTG-3′ (GenBank accession no. AJ298875; product size 171 bp). Each reaction consisted of 12.5 μl iQSYBR green Supermix (code 170-8882; Bio-Rad Laboratories, Milan, Italy), 2 μl of cDNA template and 6 pmol μl^{-1} primers. The expression of individual gene targets was analysed using the MyiQ2 Real-Time PCR machine (Bio-Rad Laboratories). The thermocycle program included a step at 95°C (3 min), 40 cycles of 95°C (10 s) and 56–58°C (56°C for P1; 57°C for ERα; 58°C for P450 aro and AR) (30 s). The subsequent denaturation step consisted of 61 cycles starting at 54°C and increasing 1°C every 10 s to generate a dissociation curve to confirm the presence of a single amplicon. In every qRT-PCR assay, samples were run in duplicate along with a negative template control (RNase-free water instead of cDNA template) and a negative reverse transcriptase control (cDNA template for which water was added instead of Superscript II). iQ5 Optical System Software (version 2.1; Bio-Rad Laboratories) was used to analyse the data. Individual gene target expression levels were normalised with respect to P1.

Protein-extract preparations

Brains from D-Asp treated (N=5) and control (N=5) frogs were homogenised directly in lysis buffer containing 50 mmol l^{-1} Hepes, 150 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 10% glycerol, 1% Triton X-100 (1:2 w/v), 1 mmol l^{-1} phenylmethylsulphonyl fluoride (PMSF), 1 μg aprotinin, 0.5 mmol l^{-1} sodium orthovanadate and 20 mmol l^{-1} sodium pyrophosphate, pH 7.4 (Sigma Chemical Corporation), then clarified by centrifugation at 14,000 ***g*** for 10 min. Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories).

Western blot analysis

Fifty micrograms of total protein extract was boiled in Laemmli buffer for 5 min at 95°C before electrophoresis. Afterwards, the samples were subjected to SDS–PAGE (13% polyacrylamide). After electrophoresis, proteins were transferred onto a nitrocellulose membrane. The complete transfer was assessed using pre-stained protein standards (Bio-Rad Laboratories). The

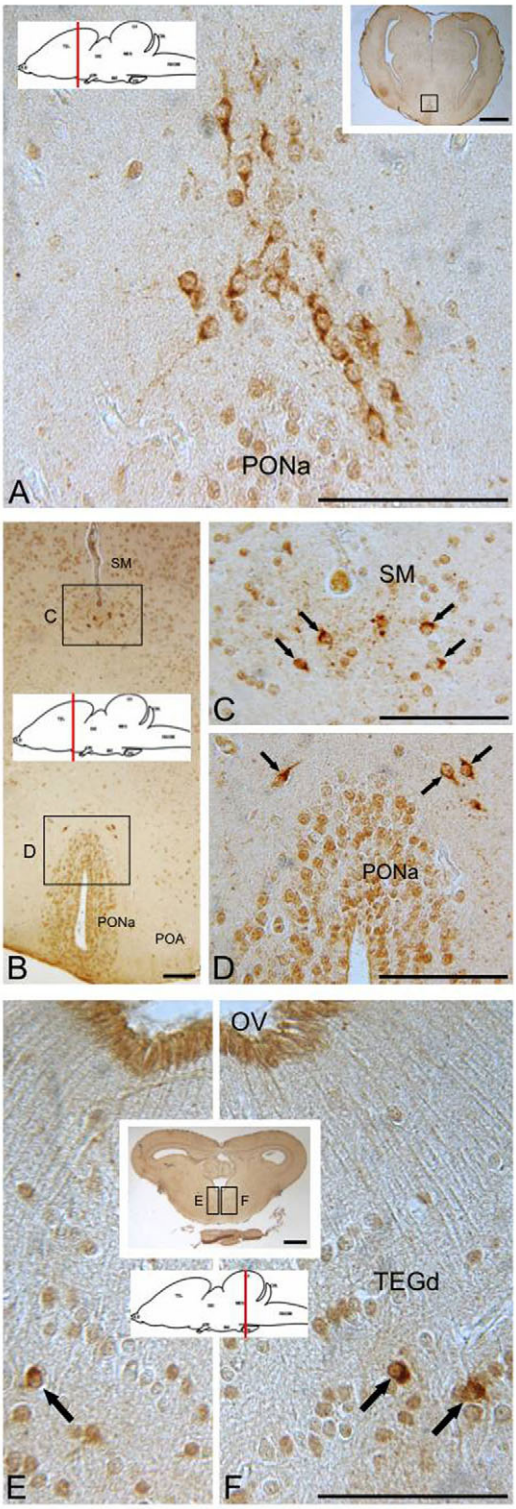

Fig. 1. Immunohistochemistry for P450 aromatase in male frog brain. (A–D) The rostro-caudal extent of the rostral aggregate of aromatase immunoreactive neurons (arrows), mainly in the anterior preoptic area. (E,F) The more caudal aggregate of immunoreactive neurons (arrows) in the mesencephalic tegmentum. The inset images represent a general view of the brain section at low magnification; each figure is an enlargement of the boxed area in the corresponding inset. The red lines show the brain area of represented sections. OV, optic vesicle; POA, preoptic area; PONa, anterior preoptic nucleus; SM, medial septum; TEGd, dorsal tegmentum. Scale bars, 100 μm.

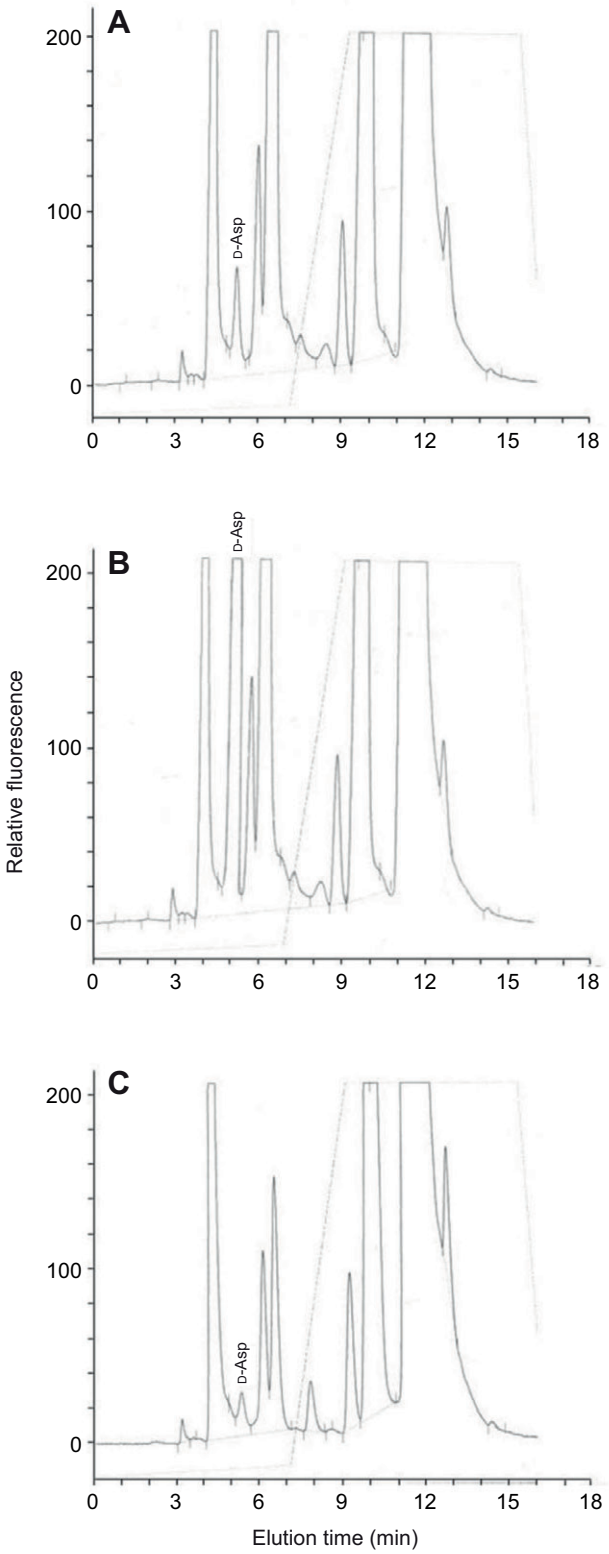

Fig. 2. Typical HPLC determination of D-Asp by the *o*-phthaldialdehyde-*N*-acetyl-L-cysteine (OPA-NAC) method in the frog brain. The peak in the chromatograms corresponds to D-Asp with a retention time of about 5 min. Broken lines represent the HPLC gradient program. (A) HPLC analysis of control frog brain. (B) HPLC analysis of D-Asp-treated frog brain. (C) HPLC analysis of D-Asp-treated frog brain after incubation with D-Asp oxidase. The marked decrease in the height of the D-Asp elution peak confirms the presence of D-Asp in the original sample.

membranes were first treated for 1 h with blocking solution (5% non-fat powdered milk in 25 mmol l^{-1} Tris, pH 7.4; 200 mmol l^{-1} NaCl; 0.5% TritonX-100, 1×TBS, 0.5% Tween) and then incubated overnight at 4°C with the following polyclonal primary antibodies: against P450 aro, diluted 1:500 (Abcam); against phospho-cAMP response element binding protein (P-CREB), diluted 1:500; against CREB, diluted 1:1000 (Cell Signaling Technology, Danvers, MA, USA) (all raised in rabbit); and against β-actin, raised in mouse, diluted 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with TBS/Tween, membranes were incubated with the horseradish-peroxidase-conjugated secondary antibody (1:2000 for P450 aro and P-CREB; 1:4000 for CREB and β-actin) for 1 h at room temperature. The reactions were detected using an enhanced chemiluminescence (ECL) system (Amersham Biosciences) (Chieffi et al., 2000).

Sex steroid assays in brain

Sex steroid determinations in the brain from D-Asp-treated frogs (N=6, three pools of two brains each) and from controls (N=6, three pools of two brains each) were conducted utilizing testosterone (Cayman Chemical Company, Michigan, MI, USA) and 17β-estradiol (DiaMetra, Milan, Italy) enzyme immunoassay kits. The sensitivities were 32 pg ml^{-1} for testosterone and 15 pg ml^{-1} for 17β-estradiol. The addition of D-Asp to the standard curve did not modify the assay sensitivity. Brains were homogenised 1:10 (w/v) with PBS 1×. The homogenate was then mixed vigorously with ethyl ether (1:10 v/v) and the ether phase was withdrawn after centrifugation at 3000 ***g*** for 10 min. The upper phase (ethyl ether) was transferred to 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 mol l^{-1} sodium phosphate buffer, pH 7.5, containing BSA at a concentration of 10 mg ml^{-1}, and then utilized for the assay (Di Fiore et al., 1998). Sex steroid recovery was 80% from brains. Steroid recovery was assessed by parallel processing of frog brain samples to which known amounts of steroids had been added prior to extraction and assay.

Statistical analysis

The values obtained were compared by Student's t-test for between-group comparisons. All data were expressed as the mean ± s.d. The levels of significance were set at P<0.01 and P<0.05.

RESULTS AND DISCUSSION

Distribution of P450 aro immunoreactivity in frog brain

To our knowledge, this is the first detailed description of the anatomical distribution of aromatase enzyme in the brain of an adult amphibian. Immunohistochemical analysis indicated that in male frog brain the P450 aro-ir neurons form two well-defined aggregates. The rostral subpopulation of ir neurons is a continuum, starting in the ventral medial septum and continuing dorsally up to the caudal-most portion of the septum in the posterior telencephalon, immediately rostral to the anterior commissure, and further caudal in the dorsal and lateral anterior preoptic area, overlying the rostral most anterior preoptic nucleus (Fig. 1A–D). The more caudal subpopulation of P450 aro-ir neurons is comparatively smaller in size and extends rostrocaudally from the not-so-well-defined diencephalon–mesencephalon boundary, roughly at the level of the posterior tubercle, up to the antero-dorsal and antero-ventral tegmental nuclei (Fig. 1E,F). It is noteworthy that the localisation of aromatase reported here corresponds to frog brain areas involved in neurosteroid synthesis (Do Rego et al., 2009a). Particularly, P450 scc-like-ir (CYP11a1) has been detected in neurons in the anterior preoptic area, but also in glial cells in the optic tectum in *P. esculentus* brain. 3β-HSD and CYP17-ir have been reported in different parts of the brain, including the preoptic area and the hypothalamus. $P450_{C17}$-ir cells are widely distributed in the telencephalon, diencephalon, mesencephalon and metencephalon (Do Rego et al., 2007; Do Rego et al., 2009b).

The distribution of aromatase in the brain has been described extensively in fishes, birds and mammals; aromatase expression has been detected in a variety of telencephalon and mesencephalon areas including the medial preoptic area, the ventro-medial nucleus of the hypothalamus and the amygdala, i.e. areas involved in sexual behavior and reproduction (Metzdorf et al., 1999; Roselli et al., 1985; Saldanha et al., 2000). In quails, morphological studies have revealed that the preoptic aromatase is specifically expressed in the sexually dimorphic medial preoptic nucleus (which is larger in males than in females) (Voigt et al., 2007), a structure where testosterone action is a priority for the activation of male sexual behavior (Panzica et al., 1996). In the lizard, aromatase-expressing cells in the male brain were more numerous in the preoptic areas whereas in the female they were more numerous in the ventromedial hypothalamus and amygdala (Cohen and Wade, 2011). Further studies will clarify a possible dimorphic distribution of aromatase in the frog brain.

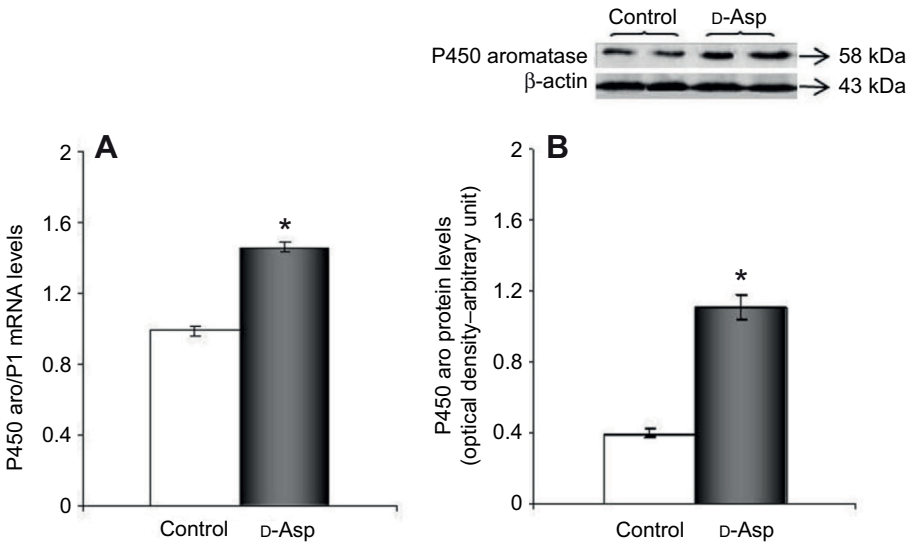

Fig. 3. (A) qRT-PCR-analysis of P450 aromatase mRNA in brains from control and D-Asp-treated frogs. The mRNA levels are expressed relative to the control group and are normalised to the ribosomal protein P1 mRNA levels. Values shown represent the means ± s.d. of five samples. (B) The upper panel shows western blot detection of P450 aro protein in brains from control and D-Asp-treated frogs. A specific band was observed at a size of 58 kDa (by comparison with co-migrating size markers; Bio-Rad, Melville, NY, USA). The lower panel shows the amount of P450 aro protein quantified using the Image J program and normalised with respect to β-actin (see upper panel). Values shown represent the means ± s.d. of five samples (two bands shown in upper panel). *P<0.05 *vs* controls.

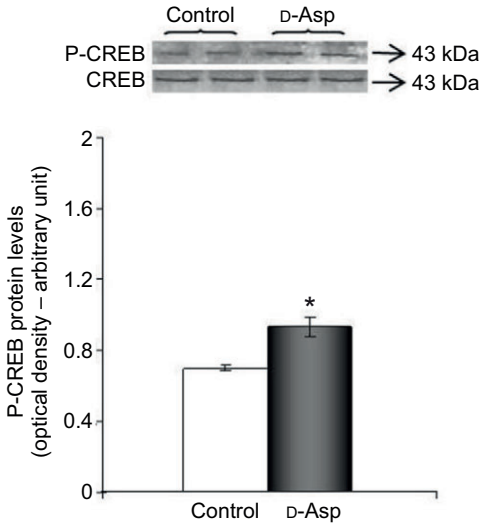

Fig. 4. The upper panel shows western blot detection of phospho-cAMP response element binding protein (P-CREB) in brains from control and D-Asp-treated frogs. A specific band was observed at a size of 43 kDa (by comparison with co-migrating size markers; Bio-Rad, Melville, NY, USA). The lower panel shows the amount of P-CREB quantified using the Image J program and normalised with respect to CREB protein (see upper panel). Values shown represent the means ± s.d. of five samples (two bands shown in upper panel). **P*<0.05 *vs* controls.

Brain D-Asp uptake and its effect on P450 aro expression

Fig. 2 reports typical HPLC brain profiles of a control sample (Fig. 2A), a D-Asp-treated sample (Fig. 2B) and a D-Asp-treated sample after incubation with D-Asp oxidase (Fig. 2C). The D-Asp levels in the brain of D-Asp-treated frogs (0.99±0.12 μmol g^{-1} tissue) were about 7-fold higher than those of the controls (0.18±0.03 μmol g^{-1} tissue), suggesting that exogenously administered D-Asp accumulates in frog brain (Fig. 2B). These results are consistent with previous studies demonstrating D-Asp accumulation in various organs of *P. esculentus* (Burrone et al., 2010; Di Fiore et al., 1998; Di Giovanni et al., 2010a; Raucci et al., 2004; Raucci et al., 2005b; Raucci and Di Fiore, 2011).

Our qRT-PCR and western blotting analyses showed that P450 aro mRNA (Fig. 3A) and protein (Fig. 3B) levels in the brain of D-Asp-treated frogs are significantly (*P*<0.05) higher than in the controls. Similarly, it was recently demonstrated that D-Asp treatment induces a significant increase of P450 aro mRNA expression in *P. esculentus* testis during the reproductive period (Burrone et al., 2012). Also in birds, *in vitro* studies have shown that neurotransmitters such as glutamate can trigger aromatase activation in the neurons (for a review, see Balthazart et al., 2009). It should be kept in mind that D-Asp can be taken up into neurons through high-affinity L-glutamate and L-aspartate transporters (D'Aniello et al., 2011; Fleck et al., 2001; Waagepetersen et al., 2001).

Regarding the regulation of aromatase activity in the vertebrate brain, in mammals this enzyme is reported to be regulated by androgens whereas in birds and fishes it is regulated by estrogens (Balthazart et al., 2003; Roselli and Resko, 1997). Little is known about the regulation of aromatase transcript in the amphibian brain. Aromatase mRNA levels decrease in response to high levels of triiodothyronine (T_3) in the brain of *Rana pipiens* tadpoles (Hogan et al., 2007) while they were observed to increase following 17α-ethinylestradiol exposure in *Rana catesbeiana* tadpoles (Gunderson et al., 2011).

Effects of D-Asp administration on brain CREB activity

D-Asp administration significantly (*P*<0.05) enhances brain CREB activity (Fig. 4). The cAMP/PKA/CREB pathway is considered to be the primary signaling cascade through which the P450 aromatase gene (*cyp19*) promoter is regulated (Stocco, 2008). Further evidence is gained from some previous studies carried out in rat and *Aplysia limacina* indicating that D-Asp induces an increase of brain cAMP *in vivo* and *in vitro* (D'Aniello et al., 2011; Spinelli et al., 2006). In parallel, studies in birds suggest that neurotransmitters such as dopamine can regulate aromatase activity trans-synaptically through second messenger systems (for a review, see Balthazart et al., 2009).

Sex steroid brain levels and androgen and estrogen receptor expressions after D-Asp administration

The brain testosterone levels in D-Asp-treated frogs were significantly (*P*<0.01) lower than those in controls (Fig. 5A). By contrast, the levels of 17β-estradiol were significantly higher (*P*<0.01) in brains of D-Asp-treated frogs than in the controls (Fig. 5A). These results strengthen our hypothesis that D-Asp administration induces not only an increase of transcript and protein P450 aro expression in the brain but also an increase of the enzymatic activity, indirectly evaluated through estradiol production. These results are in accordance with studies carried out in both frogs and boars, in which D-Asp treatment causes an increase of 17β-estradiol

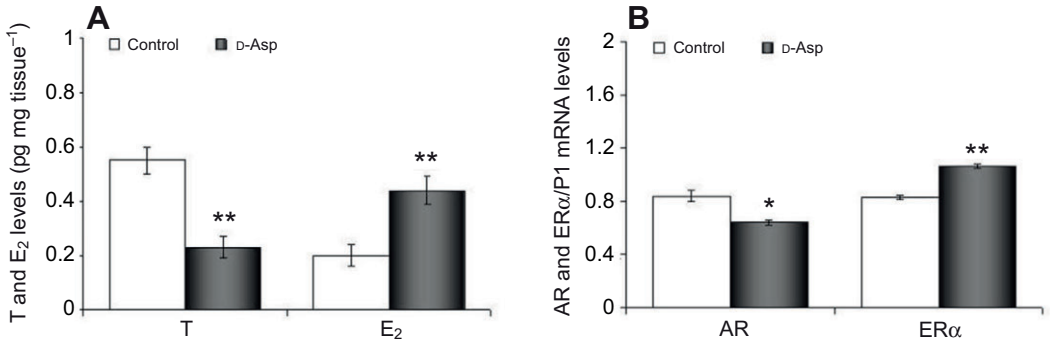

Fig. 5. (A) Testosterone (T) and 17β-estradiol (E_2) brain levels in control and in D-Asp-treated frogs. Each value represents the mean ± s.d. of three determinations. (B) qRT-PCR analysis of both androgen receptor (AR) and estrogen receptor (ERα) mRNA in brains from control and D-Asp-treated frogs. The mRNA levels are expressed relative to the control group and are normalised to the ribosomal protein P1 mRNA levels. Values shown represent the means ± s.d. of five samples. ***P*<0.01 and **P*<0.05 *vs* controls.

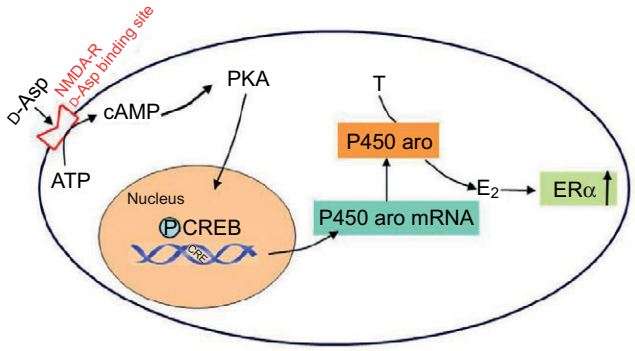

Fig. 6. Schematic representation of the putative mechanism of P450 aromatase activation induced by D-Asp in frog neurons. D-Asp is recognised by the receptor for *N*-methyl-D-aspartic acid (NMDA-R), whose activation increases cAMP. The resulting augmentation of cAMP concentration could then activate a variety of protein kinases (PKA), resulting in the phosphorylation of the CREB protein (P-CREB) to transform it into a transcriptional regulator, which would then lead to an increase in P450 aromatase synthesis. This enzyme induces 17β-estradiol (E_2) production from testosterone (T), with a consequent increase of its receptor (ERα).

(Lamanna et al., 2006; Lamanna et al., 2007; Raucci et al., 2004; Raucci and Di Fiore, 2011). Furthermore, *in vivo* and *in vitro* experiments on the ovary of the frog *P. esculentus* and the lizard *Podarcis s. sicula* demonstrated that D-Asp enhances aromatase activity, inducing testosterone conversion to 17β-estradiol (Assisi et al., 2001; Di Fiore et al., 1998).

Brain androgen receptor (AR) and estrogen receptor (ERα) mRNA levels were analysed using qRT-PCR from control and D-Asp-treated frogs (Fig. 5B). In D-Asp-treated brains, AR were significantly ($P<0.05$) lower than those in control brains whereas ERα mRNA expression levels were significantly ($P<0.01$) higher than controls (Fig. 5B). The results suggest a direct relationship between sex hormone levels and their receptor expressions. Therefore, the regulation of D-Asp-mediated P450 aro expression could be an important step in the control of ERα activity in the brain. On the other hand, several studies have clearly shown that brain aromatase expression coincides with the neuroendocrine regions expressing ERα, indicating that locally produced 17β-estradiol can affect the neuroendocrine regulations of the reproductive axis (for a review, see Balthazart et al., 2009).

CONCLUSIONS

The results of the present study indicate that the sites of P450 aro immunoreactivity in the frog brain correspond to the areas known to be involved in neurosteroid synthesis. More importantly, our results indicate that D-Asp enhances brain aromatase expression through the CREB pathway. Estradiol production from testosterone and the increase of its receptor are sequentially correlated. Thus, we propose a putative D-Asp-mediated mechanism in frog neurons (Fig. 6). D-Asp is known to be recognised by the *N*-methyl-D-aspartic acid-receptor (NMDA-R), whose activation increases cAMP (D'Aniello et al., 2011; Di Giovanni et al., 2010b; Spinelli et al., 2006). The resulting augmentation of cAMP concentration could then activate a variety of protein kinases, resulting in the phosphorylation of the CREB protein to transform it into a transcriptional regulator, which would then lead to an increase in aromatase synthesis. This enzyme induces estradiol production from testosterone, with a consequent increase of its receptor. Although much remains to be learned about the role of D-Asp in neurosteroidogenesis, this study has revealed a novel function of this amino acid in the brain, i.e. local modulation of aromatase expression.

LIST OF ABBREVIATIONS

AR	androgen receptor
CREB	cAMP response element binding protein
D-Asp	D-aspartic acid
E_2	17β-estradiol
ERα	estrogen receptor alpha
ir	immunoreactive
NMDA-R	*N*-methyl-D-aspartic acid-receptor
P1	ribosomal protein P1
P450 aro	P450 aromatase
qRT-PCR	quantitative real-time RT-PCR

FUNDING

This work was supported by the Second University of Naples, Italy.

REFERENCES

Assisi, L., Botte, V., D'Aniello, A. and Di Fiore, M. M. (2001). Enhancement of aromatase activity by D-aspartic acid in the ovary of the lizard *Podarcis s. sicula*. *Reproduction* **121**, 803-808.
Balthazart, J., Baillien, M., Charlier, T. D., Cornil, C. A. and Ball, G. F. (2003). Multiple mechanisms control brain aromatase activity at the genomic and non-genomic level. *J. Steroid Biochem. Mol. Biol.* **86**, 367-379.
Balthazart, J., Cornil, C. A., Charlier, T. D., Taziaux, M. and Ball, G. F. (2009). Estradiol, a key endocrine signal in the sexual differentiation and activation of reproductive behavior in quail. *J. Exp. Zool. A.* **311**, 323-345.
Burrone, L., Di Giovanni, M., Di Fiore, M. M., Baccari, G. C. and Santillo, A. (2010). Effects of D-aspartate treatment on D-aspartate oxidase, superoxide dismutase, and caspase 3 activities in frog (*Rana esculenta*) tissues. *Chem. Biodivers.* **7**, 1459-1466.
Burrone, L., Raucci, F. and Di Fiore, M. M. (2012). Steroidogenic gene expression following D-aspartate treatment in frog testis. *Gen. Comp. Endocrinol.* **175**, 109-117.
Chieffi, P., Colucci-D'Amato, G. L., Staibano, S., Franco, R. and Tramontano, D. (2000). Estradiol-induced mitogen-activated protein kinase (extracellular signal-regulated kinase 1 and 2) activity in the frog (Rana esculenta) testis. *J. Endocrinol.* **167**, 77-84.
Cohen, R. E. and Wade, J. (2011). Aromatase mRNA in the brain of adult green anole lizards: effects of sex and season. *J. Neuroendocrinol.* **23**, 254-260.
Cornil, C. A. (2009). Rapid regulation of brain oestrogen synthesis: the behavioural roles of oestrogens and their fates. *J. Neuroendocrinol.* **21**, 217-226.
D'Aniello, A. (2007). D-aspartic acid: an endogenous amino acid with an important neuroendocrine role. *Brain Res. Rev.* **53**, 215-234.
D'Aniello, A., Di Fiore, M. M., Fisher, G. H., Milone, A., Seleni, A., D'Aniello, S., Perna, A. F. and Ingrosso, D. (2000). 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**, 699-714.
D'Aniello, S., Somorjai, I., Garcia-Fernàndez, J., Topo, E. and D'Aniello, A. (2011). D-Aspartic acid is a novel endogenous neurotransmitter. *FASEB J.* **25**, 1014-1027.
Di Fiore, M. M., Assisi, L., Botte, V. and D'Aniello, A. (1998). D-Aspartic acid is implicated in the control of testosterone production by the vertebrate gonad. Studies on the female green frog, *Rana esculenta*. *J. Endocrinol.* **157**, 199-207.
Di Fiore, M. M., Lamanna, C., Assisi, L. and Botte, V. (2008). Opposing effects of D-aspartic acid and nitric oxide on tuning of testosterone production in mallard testis during the reproductive cycle. *Reprod. Biol. Endocrinol.* **6**, 28-36.
Di Giovanni, M., Burrone, L., Chieffi Baccari, G., Topo, E. and Santillo, A. (2010a). Distribution of free D-aspartic acid and D-aspartate oxidase in frog *Rana esculenta* tissues. *J. Exp. Zool. A* **313**, 137-143.
Di Giovanni, M., Topo, E., Santillo, A., D'Aniello, A. and Chieffi Baccari, G. (2010b). D-Aspartate binding sites in rat Harderian gland. *Amino Acids* **38**, 229-235.
Do Rego, J. L., Tremblay, Y., Luu-The, V., Repetto, E., Castel, H., Vallarino, M., Bélanger, A., Pelletier, G. and Vaudry, H. (2007). Immunohistochemical localization and biological activity of the steroidogenic enzyme cytochrome P450 17alpha-hydroxylase/C17, 20-lyase ($P450_{C17}$) in the frog brain and pituitary. *J. Neurochem.* **100**, 251-268.
Do Rego, J. L., Seong, J. Y., Burel, D., Leprince, J., Luu-The, V., Tsutsui, K., Tonon, M. C., Pelletier, G. and Vaudry, H. (2009a). Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine regulation by neurotransmitters and neuropeptides. *Front. Neuroendocrinol.* **30**, 259-301.
Do Rego, J. L., Seong, J. Y., Burel, D., Luu-The, V., Larhammar, D., Tsutsui, K., Pelletier, G., Tonon, M. C. and Vaudry, H. (2009b). Steroid biosynthesis within the frog brain: a model of neuroendocrine regulation. *Ann. N. Y. Acad. Sci.* **1163**, 83-92.
Fleck, M. W., Barrionuevo, G. and Palmer, A. M. (2001). Synaptosomal and vesicular accumulation of L-glutamate, L-aspartate and D-aspartate. *Neurochem. Int.* **39**, 217-225.
Forlano, P. M., Schlinger, B. A. and Bass, A. H. (2006). Brain aromatase: new lessons from non-mammalian model systems. *Front. Neuroendocrinol.* **27**, 247-274.
Garcia-Segura, L. M. (2008). Aromatase in the brain: not just for reproduction anymore. *J. Neuroendocrinol.* **20**, 705-712.

Gunderson, M. P., Veldhoen, N., Skirrow, R. C., Macnab, M. K., Ding, W., van Aggelen, G. and Helbing, C. C. (2011). Effect of low dose exposure to the herbicide atrazine and its metabolite on cytochrome P450 aromatase and steroidogenic factor-1 mRNA levels in the brain of premetamorphic bullfrog tadpoles (*Rana catesbeiana*). *Aquat. Toxicol.* **102**, 31-38.

Hogan, N. S., Crump, K. L., Duarte, P., Lean, D. R. and Trudeau, V. L. (2007). Hormone cross-regulation in the tadpole brain: developmental expression profiles and effect of T_3 exposure on thyroid hormone- and estrogen-responsive genes in *Rana pipiens*. *Gen. Comp. Endocrinol.* **154**, 5-15.

Iwabuchi, J., Wako, S., Tanaka, T., Ishikawa, A., Yoshida, Y. and Miyata, S. (2007). Analysis of the p450 aromatase gene expression in the *Xenopus* brain and gonad. *J. Steroid Biochem. Mol. Biol.* **107**, 149-155.

Kuntz, S., Chesnel, A., Flament, S. and Chardard, D. (2004). Cerebral and gonadal aromatase expressions are differently affected during sex differentiation of *Pleurodeles waltl*. *J. Mol. Endocrinol.* **33**, 717-727.

Lamanna, C., Assisi, L., Botte, V. and Di Fiore, M. M. (2006). Endogenous testicular D-aspartic acid regulates gonadal aromatase activity in boar. *J. Endocrinol. Invest.* **29**, 141-146.

Lamanna, C., Assisi, L., Botte, V. and Di Fiore, M. M. (2007). Involvement of D-Asp in P450 aromatase activity and estrogen receptors in boar testis. *Amino Acids* **32**, 45-51.

Metzdorf, R., Gahr, M. and Fusani, L. (1999). Distribution of aromatase, estrogen receptor, and androgen receptor mRNA in the forebrain of songbirds and nonsongbirds. *J. Comp. Neurol.* **407**, 115-129.

Panzica, G. C., Viglietti-Panzica, C. and Balthazart, J. (1996). The sexually dimorphic medial preoptic nucleus of quail: a key brain area mediating steroid action on male sexual behavior. *Front. Neuroendocrinol.* **17**, 51-125.

Raucci, F. and Di Fiore, M. M. (2011). D-Asp: a new player in reproductive endocrinology of the amphibian *Rana esculenta*. *J. Chromatogr. B* **879**, 3268-3276.

Raucci, F., Assisi, L., D'Aniello, S., Spinelli, P., Botte, V. and Di Fiore, M. M. (2004). Testicular endocrine activity is upregulated by D-aspartic acid in the green frog, *Rana esculenta*. *J. Endocrinol.* **182**, 365-376.

Raucci, F., D'Aniello, S. and Di Fiore, M. M. (2005a). Endocrine roles of D-aspartic acid in the testis of lizard *Podarcis s. sicula*. *J. Endocrinol.* **187**, 347-359.

Raucci, F., Santillo, A., D'Aniello, A., Chieffi, P. and Baccari, G. C. (2005b). D-Aspartate modulates transcriptional activity in Harderian gland of frog, *Rana esculenta*: Morphological and molecular evidence. *J. Cell. Physiol.* **204**, 445-454.

Roselli, C. E. (2007). Brain aromatase: roles in reproduction and neuroprotection. *J. Steroid Biochem. Mol. Biol.* **106**, 143-150.

Roselli, C. E. and Resko, J. A. (1997). Sex differences in androgen-regulated expression of cytochrome P450 aromatase in the rat brain. *J. Steroid Biochem. Mol. Biol.* **61**, 365-374.

Roselli, C. E., Horton, L. E. and Resko, J. A. (1985). Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system. *Endocrinology* **117**, 2471-2477.

Saldanha, C. J., Tuerk, M. J., Kim, Y. H., Fernandes, A. O., Arnold, A. P. and Schlinger, B. A. (2000). Distribution and regulation of telencephalic aromatase expression in the zebra finch revealed with a specific antibody. *J. Comp. Neurol.* **423**, 619-630.

Schlinger, B. A. and Callard, G. V. (1990). Aromatization mediates aggressive behavior in quail. *Gen. Comp. Endocrinol.* **79**, 39-53.

Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., Hinshelwood, M. M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C. R., Michael, M. D. et al. (1994). Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr. Rev.* **15**, 342-355.

Spinelli, P., Brown, E. R., Ferrandino, G., Branno, M., Montarolo, P. G., D'Aniello, E., Rastogi, R. K., D'Aniello, B., Baccari, G. C., Fisher, G. et al. (2006). D-Aspartic acid in the nervous system of *Aplysia limacina*: possible role in neurotransmission. *J. Cell. Physiol.* **206**, 672-681.

Stocco, C. (2008). Aromatase expression in the ovary: hormonal and molecular regulation. *Steroids* **73**, 473-487.

Topo, E., Soricelli, A., Di Maio, A., D'Aniello, E., Di Fiore, M. M. and D'Aniello, A. (2010). Evidence for the involvement of D-aspartic acid in learning and memory of rat. *Amino Acids* **38**, 1561-1569.

Urbatzka, R., Lutz, I. and Kloas, W. (2007). Aromatase, steroid-5-alpha-reductase type 1 and type 2 mRNA expression in gonads and in brain of *Xenopus laevis* during ontogeny. *Gen. Comp. Endocrinol.* **153**, 280-288.

Voigt, C., Ball, G. F. and Balthazart, J. (2007). Neuroanatomical specificity of sex differences in expression of aromatase mRNA in the quail brain. *J. Chem. Neuroanat.* **33**, 75-86.

Waagepetersen, H. S., Shimamoto, K. and Schousboe, A. (2001). Comparison of effects of DL-threo-beta-benzyloxyaspartate (DL-TBOA) and L-trans-pyrrolidine-2,4-dicarboxylate (t-2,4-PDC) on uptake and release of [3H]D-aspartate in astrocytes and glutamatergic neurons. *Neurochem. Res.* **26**, 661-666.