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D-Aspartic acid implication in the modulation of frog brain sex steroid levels

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ABSTRACT

There is evidence that D-aspartate (D-Asp) modulates sex hormone levels in frog testis by regulating the activity of P450 aromatase (P450 aro), the key enzyme which converts Testosterone (T) in 17ß-Estradiol (E2). Here we report, for the first time, that there is a direct correlation among brain levels of D-Asp, P450 aro, E2 and Estradiol Receptor (ERα) in the male frogs during the reproductive as well as the post-reproductive phases of the breeding cycle, with highest levels being observed in the post-reproductive period. D-Asp i.p. administration to frogs ready for reproduction, induced an increase of brain P450 aro protein expression with concomitant enhancement of both E2 levels and ERα expression; at the same time, brain T levels and Androgen receptor expression decreased. In contrast, in the post-reproductive frogs, D-Asp treatment did not modify any of these parameters. Taken together, these results imply that the regulation of P450 aro expression by D-Asp could be an important step in the control of E2 levels in the frog brain.

1. Introduction

D-Aspartate (D-Asp) is an enantiomer occurring in the free form in several tissues of vertebrates and invertebrates [8]. This amino acid has been found in endocrine glands, particularly in those associated with the reproductive system of several vertebrates [2,9,11,12,19,25–27,31–34,44] and many studies have shown that it is implicated in the biosynthesis and/or secretion of sex hormones. The presence of D-Asp has also been described in the vertebrate nervous system [14,18,28,38,41,45]. Whereas during early ontogenesis of rat and chicken, relatively high brain levels of D-Asp have been taken to indicate that it plays an important role in the development of the nervous system [14,28], in the adult animal, there are indications that D-Asp plays a role in neurotransmission, either as a neurotransmitter or as a neuromodulator [41,42].

It is known that brain sex steroids modulate the male sexual behavior in a wide variety of species [7]. Estrogens are synthesized from Testosterone (T) via P450 aromatase (P450 aro), the key enzyme in the estrogen synthetic pathway, which irreversibly converts T into 17ß-Estradiol (E2). Aromatase is a member of the P450 cytochrome family encoded by the *cyp19* gene [40]. 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 [7,15,16,37]. In the quail, hypothalamic aromatase regulates testosterone-induced aggressiveness by regulating the quantity of E2 available for receptor binding [39]. Among amphibians, the brain expression of P450 aro mRNA is already detectable in the early developmental stages of *Xenopus laevis* [20,43] and *Pleurodeles waltl* [23]; it remains at a high level until metamorphosis.

Putting together these data as well as a recent study [6], in which it was demonstrated that D-Asp in the frog testis modulates T and E2 levels by regulating P450 aro activity, we considered worthwhile to investigate the involvement of D-Asp in modulating sex hormone levels in the brain tissue of the frog. *Pelophylax esculentus* (commonly known as the green frog or the edible frog; formerly *Rana esculenta*) is the anuran amphibian model of choice because of our over 40-year long familiarity with its reproductive biology [36]. It has a seasonal reproductive cycle and it is an excellent experimental model to study the basic mechanisms of reproductive processes. This seasonally breeding frog lives in the Mediterranean area and show a reproductive cycle characterized by two main phases: the reproductive (March–June) and the post-reproductive (September–January). Adult males collected in these two main periods were subjected to D-Asp treatment in order to analyze its effects on the brain amino acid uptake, P450 aro expression, T and E2 levels and the expression of relative receptors.

2. Materials and methods

2.1. Animals

Sexually mature males of green frog, *P. esculentus* (ca. 25 g b.w.), were collected in the surroundings of Naples during March (reproductive period) and November (post-reproductive period). During D-Asp treatment frogs were maintained under natural conditions of temperature and light and fed *ad libitum* with worms. Frogs were acclimatized 4 days prior to D-Asp injections. Captivity of a

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few weeks, under such "natural" conditions do not generate any hormonal unbalance, especially of sex steroids [see [36], review].

2.2. Experiments

For both phases of reproductive cycle, 42 frogs were divided into two groups. Animals of the first group were injected i.p. with 2.0 μmol/g b.w. D-Asp (Sigma–Aldrich, Milan, Italy) dissolved in amphibian saline (Krebs–Ringer's 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 anesthetised by immersion in a 1% solution of MS-222 (Sigma–Aldrich, Milan, Italy) and then decapitated. Brains were immediately excised and stored at −80 °C for biochemical analyses. The care and treatment of animals used in this study were in accordance with local and national guidelines governing animal experiments (M. D. No. 77-2003A; M. D. No. 103-2005A).

2.3. Specific determination of D-Asp in frog brain

Brains from D-Asp-treated frogs ($N = 5$) and from controls ($N = 5$) 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 and centrifuged at 15,000*g* for 10 min. The supernatants were neutralizedfor 10 (to pH 6–8) using 1 M NaOH, and the resulting sample was analyzed by high-performance liquid chromatography using the method described previously [35] 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.

2.4. Protein extraction and Western blot analysis

In both phases of reproductive cycle, brains from D-Asp-treated ($N = 5$) and from control ($N = 5$) frogs were homogenized directly in lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100 (1:2 weight/volume), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg aprotinin, 0.5 mM sodium orthovanadate, and 20 mM sodium pyrophosphate, pH 7.4 (Sigma Chemical Corporation, St. Louis, MO), then clarified by centrifugation at 14,000*g* for 10 min. Protein concentration was determined by Bradford assay (Bio-Rad, Melville, NY). Fifty microgram of total protein extracts were 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 prestained protein standards (Bio-Rad, Melville, NY). The membranes were first treated for 1 h with blocking solution (5% non-fat powdered milk in 25 mM Tris, pH 7.4; 200 mM NaCl; 0.5% TritonX-100, TBS/Tween) and then incubated overnight at 4 °C with the following polyclonal primary antibodies: against P450 aro diluted 1:500 (Abcam, Cambridge, UK) and against β-actin, raised in mouse, diluted 1:2000 (Santa Cruz Biotechnology, Inc., CA). After washing with TBS/Tween, membranes were incubated with the horseradish-peroxidase-conjugated secondary antibody (1:2000 for P450 aro; 1:4000 for β-actin) for 1 h at room temperature. The reactions were detected using an enhanced chemiluminescence (ECL) system (Amersham Bioscience, UK).

2.5. RNA isolation and cDNA synthesis

In both reproductive and post-reproductive periods, total RNA was extracted from brains of D-Asp-treated ($N = 5$) and control ($N = 5$) frogs, using TRIzol standard protocol (Invitrogen Life Technologies, Carisbad, CA) and then samples were treated for 30 min at 37 °C with DNase I (10 U/sample) (Amersham Bioscience, UK) to eliminate any contaminations 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, Carisbad, CA).

2.6. Quantitative Real Time-PCR

Specific primer sets were designed for quantitative Real Time-PCR (qRT-PCR) using Primer 3 (http://frodo.wi.mit.edu/primer3). Primers had the following sequences: AR sense, 5′-ACTCCTGGATGGGACTGATG-3′ and AR antisense, 5′-TTGTGAGAGGTGACGCATTC-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 primers. The expression of individual gene targets was analyzed using the MyiQ2 Real-Time PCR machine (Bio-Rad Laboratories, Milan, Italy). 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 AR) (30 s). Then, there was a denaturation step consisted by 61 cycles starting at 54 °C and increasing 1 °C/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, Milan, Italy) was used to analyze the data. Individual gene target expression levels were normalized with respect to P1.

2.7. Sex steroid assays

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 17ß-Estradiol (DiaMetra, Milan, Italy) enzyme immunoassay kits. The sensitivities were 32 pg/ml for testosterone and 15 pg/ml for 17ß-Estradiol. The addition of D-Asp to the standard curve did not modify the assay sensitivity. Brains were homogenized 1:10 (w/v) with PBS 1X. 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 M sodium phosphate buffer, pH 7.5, containing BSA at a concentration of 10 mg/ml, and then utilized for the assay [11]. 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.