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Distribution and localization of 3β-hydroxysteroid dehydrogenase (3β-HSD) in the brain and its regions of the catfish *Heteropneustes fossilis*

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ABSTRACT

In vertebrates, steroids are synthesized *de novo* in the central and peripheral nervous system, independent of peripheral steroidogenic glands, such as the adrenal, gonads and placenta. 3β-Hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD) is a key steroidogenic enzyme in vertebrate gonads, placenta and adrenal. It mediates the oxidation and isomerization reactions of progesterone from pregnenolone, 17-hydroxyprogesterone from 17-hydroxypregnenolone and androstenedione from dehydroepiandrosterone. In the present study, we examined the expression of 3β-HSD cDNA by real time-PCR and localization of the mRNA by *in situ* hybridization in the brain and its regions during the different phases of the reproductive cycle of the catfish *Heteropneustes fossilis*. Further, 3β-HSD activity was assayed biochemically to show seasonal variations. We showed significant seasonal and sexual dimorphic changes in the levels of transcript abundance in the whole brain and its regions. In whole brain, level was the highest in post-spawning phase and lowest in spawning phase in males. In females, there was a progressive increase through resting phase to pre-spawning phase, a decline in the spawning phase and increase in the post-spawning phase. In the preparatory phase, the highest transcript level was seen in medulla oblongata and the lowest in pituitary in males. In females, the level was the highest in the hypothalamus and lowest in olfactory bulb and pituitary. However, in the pre-spawning phase, in males it was the highest in telencephalon and hypothalamus and lowest in pituitary. In females, the highest transcript level was in olfactory bulb and lowest in pituitary. 3β-HSD enzyme activity showed significant seasonal variation in the brain, the highest in the resting phase and lowest in the preparatory and spawning phases. *In situ* hybridization showed the presence of 3β-HSD transcript was especially high in the cerebellum region. The presence of 3β-HSD in the brain may indicate steroidogenesis in the catfish brain.

1. Introduction

Earlier it was thought that steroidogenesis in vertebrates was restricted to gonadal and adrenocortical tissues. Neurosteroidogenesis from cholesterol is well established in the vertebrate brain (Baulieu, 1997; Tsutsui et al., 1999; Diotel et al., 2011; Do Rego and Vaudry, 2016). The neurosteroids are important to neural development and function in a number of vertebrate species. Recently, a growing body of research in teleost brain indicates that the locally-produced steroids are involve in a variety of physiological functions including proliferation, differentiation and development of brain during embryonic life. It has been well documented that neurosteroids regulate various physiological and behavioural processes in adult brain controlling locomotion, feeding, sexual behaviour, and learning and memory (Majewska, 1992; Kavaliers and Kinsella, 1995; Baulieu, 1998; Do Rego et al., 2000; Schlinger et al., 2008; Do Rego et al., 2009, 2012; Chaube et al., 2015).

The ability of neurons to produce steroids was demonstrated by various researchers (Baulieu, 1981; Corpéchot et al., 1983; Baulieu, 1997). In several neuroanatomical studies have demonstrated the presence of key steroidogenic enzymes in the central nervous system (CNS) in different vertebrates, including fishes (Pasmanik and Callard, 1985; Pasmanik et al., 1988; Mathieu et al., 2001; Vallarino et al., 2005). 3β-Hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD) is a key enzyme of steroidogenesis in all peripheral steroidogenic glands (gonads, adrenal cortex and placenta) and is involved in oxidation and isomerization of 3β-hydroxy-Δ^5-steroids (pregnenolone and dehydroepiandrosterone (DHEA) into Δ^4ketosteroids (progesterone and androstenedione), (Hanke and Chester Jones, 1966; Vanson et al., 1996; Ukena et al., 1999a; Payne and Hales, 2004; Schlinger et al., 2008; Raghuveer and

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Senthilkumaran, 2012). Therefore, its presence in the brain is an important feature of steroidogenesis. The expression/activity of 3β-HSD has been demonstrated in the mammalian brain (Weidenfeld et al., 1980; Jung-Testas et al., 1989; Dupont et al., 1994; Guennoun et al., 1995; Sanne and Krueger, 1995; Kohchi et al., 1998; Robert et al., 2001; Yu et al., 2002). In non-mammalian vertebrates, 3β-HSD expression/activity has been reported mainly in birds (Vanson et al., 1996; Ukena et al., 1999a; Tsutsui and Yamazaki, 1995; Usui et al., 1995; Matsunaga et al., 2001; London et al., 2006; Schlinger et al., 2008) and amphibians (Mensah-Nyagan et al., 1994; Do Rego et al., 1998, 2000, 2001, 2007; Takase et al., 1999; Inai et al., 2003; Bruzzone et al., 2010). In fish, 3β-HSD-expressing cells have been demonstrated in the brain of dipnoans and teleosts (Mathieu et al., 2001; Sakamoto et al., 2001a,b; Diotel et al., 2011). In order to elucidate the physiological significance of neurosteroids in the brain, it is necessary to have a detailed study on sex, seasonal and brain regional differences in the biosynthesis of neurosteroids. The catfish *Heteropneustes fossilis* (*H. fossilis*), popularly, 'stinging catfish', belongs to a group of fish having characters close to primitive bony fishes (Bruton, 1996). The catfish is an economically important edible fish. Its flesh is high on protein and low on fat content. It is the lone member of the family Heteropneustidae or Saccobranchidae and is therefore important for phylogenetic/molecular studies. It is an air-breathing fish with great aquaculture potentials especially for in derelict and waste water culture. The catfish has been investigated extensively for reproductive endocrinology and endocrinology research in India (Sundararaj, 1981). Thus, in the present investigation, an attempt was made to study the seasonal and regional distribution of 3β-HSD in the siluroid catfish *H. fossilis* during the annual reproductive cycle. We report 3β-HSD mRNA expression, *in situ* hybridization of the transcript localization and biochemical analysis of enzyme activity in the catfish brain to indicate neurosteroid formation.

2. Material and methods

2.1. Animal collection and acclimation

H. fossilis is a freshwater, air breathing catfish whose reproductive cycle is divided into five phases: resting (November–January, 10.5L:13.5D, 18 ± 2 °C), preparatory – (February–April, 11.5 L:12.5 D, 22 ± 2 °C), prespawning (May–June, 13.5 L:10.5D, 28 ± 2 °C), spawning (July–August, 13.0L:11.0D, 29 ± 2 °C) and post spawning (September-October, 11.5L:12.5, 22 ± 2 °C) phases. Adult catfish (40–60 g) were purchased from a local fish market from Varanasi during all the above reproductive phases and were maintained in tanks (1 × 1 × 0.2 M) with circulating water under natural conditions for 48 hours after arrival to overcome stress due to transportation and were fed daily with goat liver *ad libitum*. After acclimatization, both male and female catfish in each reproductive phase were killed by decapitation between 9.00 and 11.00 h (local time). The whole brain and its regions such as olfactory bulb, telencephalon (minus olfactory tract and bulb), hypothalamus, pituitary and medulla oblongata were dissected (Chaube and Joy, 2002) and were stored at −80 °C. The thawed tissues were used for various investigations of 3β-HSD assay (mRNA, biochemical and *in situ* hybridization).

All experiments were performed in accordance with the guidelines of the Animal Ethics Committee of Banaras Hindu University, Varanasi and all care was taken to prevent cruelty of any kind.

2.2. Chemicals and reagents

In the present study, the following molecular biology kits and reagents were used: Revert-Aid Hminus first-strand cDNA synthesis kit (Fermentas, Hanover, MD, USA), DNase I, RNase-free (Ambion Inc., Austin, TX, USA), RNA later (Ambion Inc., Austin, TX, USA), RNeasy lipid tissue mini kit (Qiagen, Hilden, GmBH, Germany), 2X PCR master mix (Fermentas, Hanover, MD, USA). Pregnenolone (5-pregne-3β-ol-2one), DHEA (5-androsten-3β-ol-17 one) and other chemicals such as phenol, chloroform, agarose, tris base, glacial acetic acid, EDTA-Na_2, alcohol, methanol were of molecular biology grade and purchased from Sigma-Aldrich Ltd., St. Louis, MO, USA. The primers used in the present study were synthesized by integrated DNA Technologies (Coralville, IA, USA). Nicotinamideadenine dinucleotide sodium salt (NAD), nicotinamideadenine dinucleotide reduced form (NADH), iodonitrate trazoliumchloride (INT), nicotinamide adenine dinucleotide phosphate (NADP), and phenazenemethosulfate (PMS) were purchased from Sisco Research Laboratory, Mumbai, India.

2.2.1. Reagents for biochemical assay

(a) Phthalate in-buffer (50 mM, pH 3.0): 2.55 g of potassium hydrogen phthalate dissolved in a mixture of 51 mL N/10 HCL and 2.5 mL Tween 20; pH was adjusted to 3.0 and the volume made up to 250 mL with distilled water; (b) Tris–HCL buffer (0.1 M, pH 7.8) (c) NAD (5 mM) and (d) colour reagent: 40 mg INT, 10 mg PMS, and 0.5 mL-Tween 20 were dissolved in 50 mL distilled water for standard curve. For the enzyme assay, PMS was omitted from the reagent. The reagent containing PMS was stored in a dark bottle. (e) The substrate (Pregnenolone or DHEA) was first dissolved in 0.3–0.5 ml of dimethyl formamide (DMF) and the stock solution (1 mM) prepared in 50 or 100 Tris–HCl buffer (0.1 M, pH 7.8).

2.3. Total RNA isolation and cDNA synthesis

Total RNA was extracted from brain (70–80 mg). Tissue was homogenized using a T10 basic ULTRA-TURRAX homogenizer (IKA, Steaufen, Germany) in 1 mL QIAZOL (Qiagen) buffer and total RNA was isolated with RNeasy Lipid Tissue Mini Kit (Qiagen) and treated with 2U DNase I RNase-free (Ambion) for 30 min at 37 °C, following the manufacturer's protocol. The total RNA yield was determined in a Nano Drop (ND-1000 Spectrophotometer, Nano Drop Technologies, Rockland, DE, USA) and quality assessed by formamide RNA gel electrophoresis. The RNA samples with A260/A280 ratio of 1.8–2.0 were used in the cDNA synthesis reaction.

For Reverse transcription (RT), 5 μg of total RNA of each sample was reverse transcribed in a 20 μl reaction with the Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania, EU), following the manufacturer's protocol. Briefly, in a sterile nuclease – free tube on ice, the following reagents were added: total RNA template (5 μg), 1 μL of random hexamer primer (100 pM) and nuclease – free water to 12 μL. After mixing gently and centrifuging briefly, the mixture was incubated at 65 °C for 5 min. Then it was chilled on ice, centrifuged and the vial placed back on ice to add 4 μL of 5X reaction buffer, 1.0 μL of RiboLock RNase Inhibitor (20U/μl), 2ulof 10 mM dNTP Mix and 1.0 μL of Revert Aid H Minus M-MuLV Reverse Transcriptase (200U/μL). The mixture was incubated for 5 min at 25 °C, followed by 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min. cDNA was stored at −80 °C. Negative control reactions were performed without the addition of the reverse transcriptase for a subset of the RNA sample. Determinations of optimal template concentration and annealing temperature were validated routinely.

2.4. Sequencing of 3β-HSD gene from H. fossilis

A gene specific primer of 3β-HSD was designed from mRNA sequence of 3β-HSD of closely related species *Clarias batrachus*

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