General and Comparative Endocrinology 197 (2014) 18–25

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Cloning and expression analysis of tyrosine hydroxylase and changes in catecholamine levels in brain during ontogeny and after sex steroid analogues exposure in the catfish, *Clarias batrachus* ☆

Sajwan Khatri Mamta [a], Kavarthapu Raghuveer [a,1], Cheni-Chery Sudhakumari [a], Anbazhagan Rajakumar [a], Yaraguntappa Basavaraju [b], Balasubramanian Senthilkumaran [a,*]

[a] Department of Animal Sciences, School of Life Sciences – Centre for Advanced Studies, University of Hyderabad, P.O. Central University, Hyderabad 500046, Andhra Pradesh, India
[b] Fisheries Research and Information Centre (Inland), Karnataka Veterinary, Animal and Fisheries Sciences University, Bengaluru 560089, Karnataka, India

ARTICLE INFO

Article history:
Received 17 May 2013
Revised 12 November 2013
Accepted 17 November 2013
Available online 6 December 2013

Keywords:
Tyrosine hydroxylase
Catecholamines
Sex differentiation
Hypothalamo-hypophyseal axis
Sex steroid analogues
Teleosts

ABSTRACT

Tyrosine hydroxylase (Th) is the rate-limiting enzyme for catecholamine (CA) biosynthesis and is considered to be a marker for CA-ergic neurons, which regulate the levels of gonadotropin-releasing hormone in brain and gonadotropins in the pituitary. In the present study, we cloned full-length cDNA of *Th* from the catfish brain and evaluated its expression pattern in the male and female brain during early development and after sex-steroid analogues treatment using quantitative real-time PCR. We measured the CA levels to compare our results on Th. Cloned *Th* from catfish brain is 1.591 kb, which encodes a putative protein of 458 amino acid residues and showed high homology with other teleosts. The tissue distribution of *Th* revealed ubiquitous expression in all the tissues analyzed with maximum expression in male and female brain. Copy number analysis showed two-fold more transcript abundance in the female brain when compared with the male brain. A differential expression pattern of *Th* was observed in which the mRNA levels were significantly higher in females compared with males, during early brain development. CAs, L-3,4-dihydroxyphenylalanine, dopamine, and norepinephrine levels measured using high-performance liquid chromatography with electrochemical detection in the developing male and female brain confirmed the prominence of the CA-ergic system in the female brain. Sex-steroid analogue treatment using methyltestosterone and ethinylestradiol confirmed our findings of the differential expression of *Th* related to CA levels.

1. Introduction

Catecholamines (CAs) are an important component of the monoaminergic system in the hypothalamus. CAs modulate the levels of gonadotropin-releasing hormone (GnRH), which subsequently regulates the synthesis and release of gonadotropins (GTHs), follicle stimulating hormone and luteinizing hormone in teleosts. The CAs include L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine (DA) and norepinephrine (NE), which plays a decisive role in certain physiological processes to control the events of reproduction through the hypothalamo-hypophyseal axis. Among the CAs, DA exerts an inhibitory control on GTH while NE stimulates GTH by modulating GnRH synthesis and pulsatile release in teleosts (Chang and Peter, 1983; Popesku et al., 2008; Senthilkumaran and Joy, 1996; Vidal et al., 2004).

CA biosynthesis begins with the hydroxylation of tyrosine to L-DOPA catalyzed by tyrosine hydroxylase (Th), a rate-limiting enzyme, which is further processed to DA by aromatic amino acid decarboxylase. Dopamine β-hydroxylase (DβH) converts DA into NE (Flatmark, 2000). CA biosynthesis is well conserved among vertebrates and is similar among various tissues such as neuronal and non-neuronal chromaffin cells (Reiner et al., 1994). Th belongs to the family of aromatic amino acid hydroxylases (AAAHs) that includes tryptophan hydroxylase (tph), which is phosphorylated by protein kinase A and cyclin-dependent kinases (Fitzpatrick, 1999; Ledley et al., 1987; Raghuveer et al., 2011; Sudhakumari et al., 2010). AAAHs exhibit sequence homology and similarity in structural, physical, and catalytic properties despite the differences in functional characteristics (Ledley et al., 1985, 1987). Th is highly specific, uses tetrahydrobiopterin as a cofactor, contains non-heme iron and has a catalytic C-terminal and regulatory N-terminal domains (Ledley et al., 1985). In vertebrates, Th is found in diverse

☆ This study was conducted at the University of Hyderabad.

* Corresponding author. Tel.: +91 40 23134562/23134662; fax: +91 40 23010307/23010120.
E-mail addresses: bsksl@uohyd.ernet.in, senthilkumaranb@yahoo.com (B. Senthilkumaran).
[1] Present address: Section on Molecular Endocrinology, Eunice Kennedy Shriver National Institutes of Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA.

0016-6480/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygcen.2013.11.022

tissues comprising the central and sympathetic nervous system, brain, adrenal medulla, and other peripheral sympathetic neurons (Flatmark et al., 2002; Miyajima et al., 2013). The regulation of *Th* and the phenotypic development of cells expressing Th have been studied in both larva and adult *Danio rerio* (Guo et al., 1999; Sallinen et al., 2009). However, only few studies relating Th to the GTH axis in the seasonal reproductive cycle or hypoxia have been reported (Chaube and Joy, 2003; Rahman and Thomas, 2009). Previous studies showed that catfish undergo an annual reproductive cycle in response to environmental cues most likely through changes in GnRH–GTH release (Goos et al., 1999). Variations in the GnRH content influence seasonal changes in the responsiveness of GTH to GnRH, which is partially influenced by a feedback mechanism exerted by gonadal steroids (Habibi et al., 1989) through the brain monoaminergic system (Goos et al., 1999). In addition to NE, serotonin (5-HT) stimulates the release of GnRH and GTHs by acting at the level of the hypothalamo-hypophyseal axis in teleosts (Goos et al., 1999; Hery et al., 1997; Peter et al., 1991; Senthilkumaran et al., 2001; Wong et al., 2004). Although monoamines (CAs and 5-HT) influence GnRH–GTH release, very few studies have indicated the role of these correlates during sexual development (see Prathibha et al., 2013). In this context, our previous reports showed variations in GnRH–GTH immunoreactivity in genetic XX and XY populations of the Nile tilapia (Sakai et al., 2005; Swapna et al., 2008). Consequently, the sexual dimorphic expression of *tph* was observed during early development in the Nile tilapia and catfish (Raghuveer et al., 2011; Sudhakumari et al., 2010). Subsequent analysis also revealed high levels of 5-HT in the brain of developing males undergoing gonadal sex differentiation (Raghuveer et al., 2011). Previous reports demonstrated that the treatment of parachlorophenylalanine (pCPA) during early sex development/differentiation in the Nile tilapia mimicked the effects of estradiol and resulted in a higher number of females (Tsai and Wang 1999; Tsai et al., 2000). Possibly, the serotonergic system may be involved in male 'brain sex differentiation' in teleosts. However, changes in the levels of 5-HT in the brain during early development may be a consequence rather than a cause of gonadal sex differentiation/development (Raghuveer et al., 2011). Earlier studies supported the fact that increased levels of 5-HT blocked sex reversal by blocking DA or NE (Larson et al., 2003). Considering this evidence, it will be interesting to analyze the levels of CAs and the expression of *Th* during male and female brain sex development. To enable this, *Th* was cloned and the ontogenic expression pattern was analyzed in the brain of the catfish, *Clarias batrachus,* during sexual development along with the measurement of CAs in the male and female brain. We also examined the *Th* expression and CA levels after treating with sex steroid analogues.

2. Materials and methods

2.1. Animal and sampling: Normal and sex steroids treatment

C. batrachus, which is commonly referred to as the air-breathing catfish or the Asian catfish, is an annual breeder that undergoes a seasonal pattern of reproductive cycle. Hatchlings at different age groups were obtained by *in vitro* fertilization and reared in the aquaculture facility at the University of Hyderabad as described previously (Rajakumar et al., 2012). Catfish at different age groups [0, 10, 20, 30, 40, 50, 75, 100, 150, 200, and 250 days post hatch (dph) and adult] were collected and brains were isolated except 0 dph were whole larva is used for total RNA extraction and quantification of different CAs. Morphological distinction of the gonads occurs in catfish around 50 dph (Raghuveer and Senthilkumaran, 2009) and hence these age group juveniles were chosen for hormone analogue studies using methyltestosterone (MT) and ethinylestradiol (EE_2). Juvenile catfish of 50 dph were divided into three groups of 50 larvae each and maintained in well-aerated aquarium tanks containing filtered water with or without treatment compounds of 1 μg/L MT or EE_2. The treatments were done continuously and replicates for each treatment group were maintained separately. Both MT and EE_2 used were first dissolved in absolute alcohol and air-dried to dissolve it in physiological saline. After 50 days of exposure, catfish (100 dph) were sacrificed by anesthetizing with 100 mg/L of MS 222 (Sigma, St. Louis, MO, USA) in mild ice-cold water by following general animal ethical guidelines.

2.2. Molecular cloning of full length Th from catfish brain

Total RNA was isolated from catfish brain using TRI-reagent (Sigma) and the concentration and quality was determined using a NanoDrop spectrophotometer (ND-2000; NanoDrop Technologies and Wilmington, Delaware, USA). The quality was further checked using formaldehyde agarose gel electrophoresis. Total RNA (5 μg) was reverse transcribed to first strand cDNA using Superscript III according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). A set of degenerate primers (Table 1) were designed based on the available *Th* nucleotide sequences from the DDBJ/EMBL/GenBank data base. RT-PCR was performed at 94 °C (1 min), 55 °C (30 s) and 72 °C (1 min) for 35 cycles using degenerate primers. The partial cDNA fragment obtained was cloned in pGEM®-T easy vector (Promega, Madison, WI, USA) and sequenced. Based on the sequence information of the partial cDNA, gene-specific primers (GSP) were designed and used for 5′ and 3′ RACE (Clontech, Mountain View, CA, USA). The 5′ and 3′ RACE cDNA templates from brain total RNA were prepared using the SMARTer™ RACE cDNA amplification kit (Clontech) according to the manufacturer's protocol. Primary and nested touchdown PCR were performed with the 5′ and 3′ GSP primers (Table 1), universal primer A mix, nested universal primer using the advantage polymerase kit (Clontech) according to the manufacturer's protocol. All the nested PCR amplified DNA fragments of *Th* were gel purified, cloned in pGEM®-T easy vector (Promega), sequenced, and analyzed using Lasergene software 7.1.0 (DNASTAR, Madison, WI, USA). The nucleotide and deduced amino acid sequences of *Th* cDNA were verified using the NCBI-BLAST search. The open reading frame (ORF) of *Th* was cloned from catfish brain using ORF Primers (Table 1) and the identity was confirmed using NCBI-BLAST and ClustalW.

2.3. Tissue distribution and copy number of Th in adult catfish

Quantitative real time PCR (qRT-PCR) was performed to analyze the tissue distribution of *Th* in adult male and female catfish. Total RNA was extracted from different tissues (brain, spleen, gill, heart, muscle, kidney, liver, and ovary of female and testis and brain of male) of adult catfish using the method described above. Reverse transcription was performed using 1 μg of total RNA,

Table 1
List of primers used for *Th* analysis.

Primers	nucleotide sequence (5′→3′)
*Th*dg Fr	GT(GT)TT(CT)CA(AG)TG(CT)AC(AGC) CAGTA
*Th*dg Rv	GACAG (AGC)AG(CT)CC (AG CT)GC(AGCT)CC(AG)TA(AGC)GC
Th 5′GSP1	CCAAGAGAAGCGAGCCCAATCTCCTG
Th 5′GSP2	GCAGTCTGGCTCTGGTGAGTGCAT
Th 3′GSP1	CAGGAGATTGGGCTCGCTTCTCTTGG
Th 3′GSP2	GGTTCACGGTTGAGTTTGGGCTCTGC
Th RT Fw	CCAGAGCCAGACTGCTGTCACGAG
Th RT Rv	CACCGTACGCCTTCACTGTTC
18s rRNA Fw	GCTACCACATCCAAGGAA
18s rRNA Rv	CGGCTGCTGGCACCAGACTTG