Comparative Biochemistry and Physiology, Part B 163 (2012) 59–64

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

Molecular cloning, tissue distribution, and ontogeny of gonadotropin-releasing hormone III gene (GnRH-III) in half-smooth tongue sole (*Cynoglossus semilaevis*)

Xiaosu Zhou [a], Qilin Yi [a], Qiwang Zhong [b], Chunmei Li [a], Shafi Muhammad [a], Xubo Wang [a], Zhigang Wang [a], Jie Qi [a], Haiyang Yu [a], Quanqi Zhang [a,*]

[a] College of Marine Life Science, Ocean University of China, Key Laboratory of Marine Genetics and Breeding, Ministry of Education, #5 Yushan Road, Qingdao 266003, PR China
[b] Department of Bioscience and Engineering, Jiangxi Agricultural University, Economic and Technological Development District, Nanchang 330045, PR China

ARTICLE INFO

Article history:
Received 16 January 2012
Received in revised form 14 March 2012
Accepted 28 April 2012
Available online 8 May 2012

Keywords:
Cynoglossus semilaevis
GnRH-III
Gene cloning
Promoter analysis

ABSTRACT

Gonadotropin-releasing hormone (GnRH) is a neuropeptide that plays a vital role in hypothalamus-pituitary-gonad (HPG) axis. In the present study, the GnRH-III gene was isolated from half-smooth tongue sole (*Cynoglossus semilaevis*). In the 1160 bp genomic sequence, four exons, three introns, and 5′-/3′-flanking sequences were identified. The putative peptide was 92 residues long, including a putative signal peptide containing 23 amino acids, the GnRH decapeptide, a proteolytic cleavage site of three amino acids and a GnRH associated peptide of 56 amino acids. The overall amino acid sequence of *C. semilaevis* GnRH-III (csGnRH-III) was highly conserved with other teleost GnRH-III genes. Phylogenetic analysis showed the evolutionary relationships of csGnRH-III with other known GnRH genes. A 320 bp promoter sequence of the csGnRH-III was also analyzed, and several potential regulatory motifs were identified which were conserved in the GnRH promoters of other teleosts. Quantitative real-time PCR analysis indicated csGnRH-III was expressed only in brain and gonads. In *C. semilaevis*, the csGnRH-III transcript was maternally deposited and appeared to be developmentally regulated during embryogenesis and early larval development. Comparing sequence and expression patterns of csGnRH-III with other teleosts GnRH-IIIs suggested that the main function of GnRH-III might be conserved in teleosts.

1. Introduction

The activation of hypothalamus–pituitary–gonad (HPG) axis is regarded as the prerequisite for the sexual maturation (Melamed et al., 1998; Okubo and Nagahama, 2008). Gonadotropin-releasing hormone (GnRH), a type of decapeptide neurohormone, is the key regulator that plays pivotal roles in vertebrate reproduction (Krieger et al., 1982). Its principal function is to stimulate the synthesis and release of pituitary gonadotropins (GTHs), follicle stimulating hormone (FSH) and luteinizing hormone (LH), which then transfer to gonads and regulate gametogenesis and steroidgenesis (Okuzawa and Kobayashi, 1999; Lethimonier et al., 2004; Tostivint, 2011). In addition, GnRH can also serve as a neuromodulator or a neurotransmitter (Eisthen et al., 2000). Indeed, GnRH is crucial for the onset of sexual development and the whole reproduction process.

Since its original isolation in porcine hypothalamus various subsequent studies regarding GnRH have been implemented in numerous species (Matsuo et al., 1971). To date, 25 distinct isoforms have been reported including 14 in vertebrates and 11 in invertebrates, all of them have similar molecular structures (Burgus et al., 1972; Kah et al., 2007; Kavanaugh et al., 2008; Okubo and Nagahama, 2008; Tostivint, 2011). Previous phylogenic and functional analysis in vertebrates suggested that GnRH genes fall into three distinct paralogous lineages, each of which shared not only a molecular signature but also characteristic expression sites in the brain (Fernald and White, 1999). According to the universal nomenclature, the three classified forms were described as GnRH-I, GnRH-II and GnRH-III, respectively (White et al., 1998; Fernald and White, 1999; Lethimonier et al., 2004; Kavanaugh et al., 2008). The majority of teleosts possessed all the three forms (Lethimonier et al., 2004). GnRH-I (seabream type GnRH, or other species-specific releasing form) was a form peculiar to each species, such as sbGnRH-I in seabream, hrGnRH-I in herring, and mdGnRH-I in medaka and so on. GnRH-II (chicken type GnRH) that was first found in chicken was well conserved from fish to mammals. GnRH-III (salmon type GnRH), identified in salmon first, was another specific form only present in fish (Lethimonier et al., 2004; Tostivint, 2011).

Although the systematic functional association and diversification among the three gene forms are not clear yet, some specific physiological functions of each form have already been elucidated according to the studies focused on the expression site located in the brain of teleosts (Pham et al., 2006; Palevitch et al., 2007; Ohkubo et al., 2010; Shahjahan et al., 2010). GnRH-I expressed primarily in preoptic-hypothalamic areas (Okubo and Aida, 2001). It is usually more abundant

Abbreviations: *C. semilaevis*, *Cynoglossus semilaevis*; csGnRH-III, *Cynoglossus semilaevis* GnRH-III; qPCR, quantitative real-time PCR; DPF, days post fertilization.

* Corresponding author. Tel./fax: +86 532 82031806.
E-mail address: qzhang@ouc.edu.cn (Q. Zhang).

1096-4959/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2012.04.010

in the brain compared to other forms and is considered as the hypothalamic form acting as the main regulator for the normal reproduction (Fernald and White, 1999; Okubo and Nagahama, 2008). As an isoform highly conserved among species, GnRH-II was mainly expressed in neuron of the midbrain tegmentum, and has been described in all jawed vertebrates studied (Steven et al., 2003). It has functions for modulating the sexual behavior and controlling appetite (Motohashi et al., 2008; Roch et al., 2011).

GnRH-III is mainly located in the ventral forebrain and dispersed in terminal nerve, and is thought to act as a neuromodulator which can regulates various behaviors including spawning migration and olfaction (Wirsig-Wiechmann, 2001; Dubois et al., 2002). In zebrafish, knockdown of GnRH-III in early larva development stages could result in defective brain and heart (Sherwood and Wu, 2005). Meanwhile, several reports proved that GnRH-III had partial overlapping expression locations with GnRH-I in certain regions of the olfactory bulb and pre-optic area, implying that GnRH-III might involved in the hypophysiotropic process. Thus, not surprisingly, in some fish which possessed only two forms of GnRH, the function of the absent form appeared to be compensated by either of two forms. In primitive fish eel and catfish that lacks GnRH-I, the hypophysiotropic functions would be implemented by GnRH-III (White and Fernald, 1998; Lethimonier et al., 2004; Hildahl et al., 2011; Tostivint, 2011).

Half-smooth tongue sole (*Cynoglossus semilaevis*) is an important cultured marine fish in China. However, its sexual maturation takes three years which restricts its reproduction (Li et al., 2010; Sun et al., 2010). GnRH-III is directly linked to the initiation of puberty and the control of the reproductive system. It is the fish, this specific form can be speculated to be indispensable to the fish reproduction system(Torgersen et al., 2002; Abraham et al., 2009. As such. the potential application of GnRH-III may help to improve the reproductive management in half-smooth tongue sole.

In this study, we first cloned the full-length of the fish specific *GnRH-III* from half-smooth tongue sole and quantified the expression in different tissues and early developmental stages. In addition, phylogenetic, gene structure and potential regulatory motifs of the promoter analysis were also implemented to provide some information for the possible application of GnRH-III in this species.

2. Materials and methods

2.1. Fish and tissue sampling

The experimental half-smooth tongue soles *C. semilaevis* (Pleuronectiformes, Cynoglossidae) were collected from the fish hatchery of Laizhou Mingbo Aquatic Co., Ltd., Shandong, China. Eight adults (four females and four males) were randomly sampled from the tank and anesthetized in 0.1% tricaine methanesulfonate (MS-222). Heart, liver, spleen, gill, kidney, whole brain, intestine, ovary, testis and skeletal muscle tissues were dissected and snapped frozen in liquid nitrogen, then stored at −80 °C. Artificially fertilized eggs of *C. semilaevis* were incubated at 22 °C in hatching tank with clean seawater and continuous aeration. Embryos or larvae of different developmental stages (including unfertilized eggs, morula, high blastula, gastrula, neurula, eye-bud, tail-bud, heart-beating, hatching, 2 days post fertilization (DPF) and 5 DPF were collected in liquid nitrogen and preserved at −80 °C. The muscle tissue (about 30 μg) for genomic DNA extraction was dissolved in 5 mL DNA extraction buffer containing 10 mM Tris–HCl pH 8.0, 125 mM NaCl, 10 mM EDTA pH 8.0, 0.5% SDS and 4 M Urea (TNES) and stored at room temperature.

2.2. Extraction of RNA and genomic DNA

Total RNAs were isolated from samples at different developmental stages and tissues of eight healthy adults (four females and four males) using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The removal of genomic DNA contamination was achieved by DNase I treatment and verified by PCR using β-actin gene specific primers and RNA template.

Genomic DNA was isolated from TNES preserved samples. In brief, 15 μL of preserved sample was diluted by 600 μL TNES buffer and digested overnight with proteinase K at the final concentration of 100 μg/mL at 50 °C. DNA was then extracted with phenol and chloroform/isoamyl alcohol, precipitated with isopropanol, washed with 70% ethanol and dissolved in ddH_2O.

2.3. Cloning and sequencing of C. semilaevis GnRH-III gene

Total RNA (about 1 μg) from the whole brain tissue was reverse transcribed using MMLV-Reverse-Transcriptase (RNase H^-) (TaKaRa, Dalian, China) and random primer to obtain the first-strand cDNA used for cloning the central part sequence of the gene. Degenerate PCR primers CS-Fw and CS-Rv (Table 1) for initial fragment of GnRH-III gene were designed from the conserved regions of teleost GnRH-III mRNA sequences identified by sequence alignment. PCR condition was as follows: 94 °C predenaturation for 4 min, 35 cycles of 94 °C denaturation for 30 s, 54 °C annealing for 30 s, 72 °C extension for 30 s, and then a final elongation at 72 °C for 10 min.

Gene specific primers CS-5′Rv and CS-3′Fw for 5′ and 3′ RACE respectively (Table 1) were designed from the partial sequence obtained above. SMART RACE PCR Amplification kit (Clontech, CA, USA) was used to obtain 5′ and 3′ unknown cDNA regions according to the manufacturer's protocol. 5′RACE was got using primers UPM (from the kit) and CS-5′Rv, while 3′RACE was got using primers NUP (from the kit) and CS-3′Fw. The RACE PCR conditions were: 94 °C for 5 min, 35 following cycles of 94 °C for 30 s, 61 °C for 30 s, 72 °C for 1 min, and then a final elongation of 72 °C for 10 min.

The full-length coding sequence and genomic sequence of *C. semilaevis* GnRH-III (csGnRH-III) were amplified with two gene specific primers (CS-w-Fw/CS-w-Rv) (Table 1). PCR conditions were: initial step of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final elongation of 72 °C for 10 min.

The promoter region of csGnRH-III was amplified by using the Genome Walking Kit (TaKaRa), and the primers CS-p-1/CS-p-2/CS-p-3 were designed according to the instruction (Table 1). PCR condition was followed the steps in the instruction book of the kit.

All PCR reactions were performed using a GeneAmp PCR System 9700 (Applied Biosystems, Forster City, CA, USA), and all PCR products were separated by 1.8% agarose gel electrophoresis. The target fragment was regenerated using EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc), ligated into the pMD-18 T vector (TaKaRa) and transformed to competent *Escherichia coli* (Trans5α). Positive

Table 1
Primers used in this study.

Name	Sequence (5′–3′)	TM (annealing temperature)	Usage
CS-Fw	ATGGAA/GGCAA/GA/GCAGCAGA/GGT	54 °C	Fragment PCR
CS-Rv	CGGTCGAAATATCTGGAGTC	54 °C	Fragment PCR
CS-5′Rv	ATCGTTGCTTCCAGCTCGCCTAC	61 °C	5′ RACE PCR
CS-3′Fw	GCCCAAACCCAAGAGAGGCTTAGAC	61 °C	3′ RACE PCR
CS-w-Fw	ATGTTAAGACGGCAAACACAG	55 °C	Full-length PCR
CS-w-Rv	CGGTCGAAATATCTGGAGTC	55 °C	Full-length PCR
CS-p-1	AGCCGCAGCCTCACTCACCT	64.4 °C	Genome walking
CS-p-2	CACTCTGCTGCCTGTTTCCATCAC	65.8 °C	Genome walking
CS-p-3	ATCGTTGCTTCCAGCTCGCCTAC	66.8 °C	Genome walking
CS-rt-Fw	AGGCAGCAGGGTGATCGTG	61 °C	qPCR
CS-rt-Rv	CACCTGGTAGCCATCCATAAGAC	61 °C	qPCR
18S-RT-Fw	GGTCTGTGATGCCCTTAGATGTC	61 °C	qPCR
18S-RT-Rv	AGTGGGGTTCAGCGGGTTAC	61 °C	qPCR