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Molecular characterization and expression profiles of LPXRFa at the brain-pituitary-gonad axis of half-smooth tongue sole (*Cynoglossus semilaevis*) during ovarian maturation

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ABSTRACT

Gonadotropin-inhibitory hormone (GnIH) has been characterized by its ability to inhibit either basal or gonadotropin-releasing hormone (GnRH)-induced gonadotropin synthesis and release in birds and mammals. However, the physiological role of GnIH on the reproductive axis in fish remains inconclusive, with most studies focusing on the orders Cypriniformes and Perciformes. To gain insight into the role of GnIH in the regulation of reproduction in the order Pleuronectiformes, we first cloned the LPXRFa gene, the piscine ortholog of GnIH, in the half-smooth tongue sole. The full-length cDNA of LPXRFa was 918 bp in size with an open reading frame (ORF) of 585 bp that encoded a 194 amino acids preprohormone with a calculated molecular mass and isoelectric point of 21.73 kDa and 6.52, respectively. The LPXRFa precursor encoded two putative peptide sequences that included –MPMRF or –MPQRF motifs at the C-terminal. Tissue distribution analysis showed that LPXRFa transcripts could be detected at high levels in the brains of both sexes and to a lesser extent in the ovary, heart and stomach of females, while a noteworthy expression was observed in the kidney and muscle of males. Furthermore, the expression patterns of LPXRFa mRNA during ovarian maturation were also investigated. In the brain, the mRNA expression of LPXRFa increased significantly at stage III, declined at stage V and reached a maximum at stage VI. In the pituitary, the levels of LPXRFa mRNA remained stable during ovarian maturation and increased significantly to the top level at stage V and then declined back to basal levels. In contrast, the ovarian LPXRFa mRNA levels declined sharply at stage III and remained depressed over the course of ovarian maturation. Taken together, our results provide further evidence for the existence of LPXRFa in the order Pleuronectiformes and suggest its possible involvement in the regulation of reproduction in the female tongue sole.

1. Introduction

The neuroendocrine regulation of reproduction in vertebrates, including fish, is primarily controlled by the brain-pituitary-gonadal (BPG) axis, with each component secreting specific neuropeptides or hormones. The classical view of the BPG system holds that the neuropeptide control of gonadotropin secretion from the anterior pituitary gland is mainly through the stimulatory action of the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH); gonadotropins, in turn, act on the gonads to induce the production of sex steroids and stimulate gamete development. Sex steroids and inhibin suppress gonadotropin secretion *via* feedback from the gonads. However, an inhibitory neuropeptide of gonadotropin release was, until 2000, unidentified in any vertebrate.

In 2000, a novel hypothalamic dodecapeptide (SIKPSAYLPLRFamide) with RFamide at the C-terminus was isolated from the brain of the Japanese quail and termed gonadotropin-inhibitory hormone (GnIH) based on its ability to directly inhibit gonadotropin release from cultured anterior pituitary tissue (Tsutsui et al., 2000). Subsequently, a cDNA that encoded the GnIH precursor polypeptide was identified in the quail brain (Satake et al., 2001). To date, GnIH homologs have been detected in a variety of vertebrates from fish to humans (Munoz-Cueto et al., 2017; Ogawa and Parhar, 2014; Tsutsui et al., 2015; Ubuka et al., 2016). These GnIH genes encode a

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polypeptide that is possibly cleaved into three mature peptides in birds (GnIH, GnIH-related peptides [GnIH-RP-1 and -2]), reptiles (turtle GnIH, GnIH-RP-1 and -2) and some teleosts (LPXRFa-1, -2 and -3); two in mammals (RFamide-related peptides [RFRP-1 and -3]) and other teleosts (LPXRFa-1 and -2); and four in amphibians (frog GH-releasing peptide [fGRP] and fGRP-related peptides [fGRP-RP-1, -2, and -3]) (Munoz-Cueto et al., 2017; Ogawa and Parhar, 2014; Tsutsui et al., 2015; Ubuka et al., 2016). These peptides possess a characteristic -LPXRFa (X = L or Q) motif at the C-terminus and are designated LPXRFa peptides from a structural perspective; these peptides form a new group of the RFamide peptide family (Tsutsui, 2009).

It is generally accepted that GnIH/RFRP acts on GnRH neurons in the hypothalamus to inhibit gonadotropin synthesis and release in addition to affecting the function of the pituitary in birds and mammals (Clarke and Parkington, 2014; Kriegsfeld et al., 2010; Ogawa and Parhar, 2014; Ubuka et al., 2016). However, the exact physiological role of LPXRFa is still uncertain in fish. Goldfish LPXRFa peptides (gfLPXRFa-1, -2 and -3) stimulated the release of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and growth hormone (GH) from cultured pituitary cells of sockeye salmon (Amano et al., 2006). Similarly, tilapia LPXRFa-2 positively increased LH and FSH release both *in vivo* and *in vitro* (Biran et al., 2014). In addition, gfLPXRFa-1 stimulated the expression of LHβ, FSHβ, GH and prolactin mRNAs in the pituitary of grass puffer (Shahjahan et al., 2016; Shahjahan et al., 2011). On the other hand, intraperitoneal injection of zebrafish LPXRFa-3 reduced the plasma LH levels in goldfish (Zhang et al., 2010). Intraperitoneal administration of gfLPXRFa-2 and gfLPXRFa-3 significantly decreased FSHβ and/or LHβ mRNA levels in goldfish (Qi et al., 2013). Similarly, the inhibitory effects of LPXRFa peptides on LHβ transcript levels were also observed in orange-spotted grouper (Wang et al., 2015), common carp (Peng et al., 2016), and European sea bass (Paullada-Salmeron et al., 2016c). Interestingly, gfLPXRFa-3 exerted both stimulatory and inhibitory effects on pituitary LH release and gonadotropin subunit mRNA levels depending on maturational status and administration route (Moussavi et al., 2012, 2013). It should be noted that LPXRFa-1 inhibited LH and FSH release and stimulated GH release in intact pituitary cultures of *Cichlasoma dimerus* and that LPXRF-2 could act as an FSH-releasing factor in this fish species, which is the first report showing opposite effects between two LPXRFa peptides on FSH secretion in the same species (Di Yorio et al., 2016). Altogether, these LPXRFa peptides appear to regulate pituitary function across vertebrates, but the hypophysiotropic activities are divergent in various species, particularly in fish.

Half-smooth tongue sole is an economically important marine flatfish that is widely cultured in China. In nature, the body length of mature females is twice as long and the body weight is over six times greater than those of mature males, exhibiting sexual dimorphism of growth (Wang et al., 2017a). This flatfish exhibits an asynchronous ovarian maturation, and oocytes at different developmental stages are simultaneously present in a single ovary. The weight or volume of the mature testis is approximately 1/200–1/900 of the mature ovary (Ji et al., 2011). Recently, the genomic characterization of the tongue sole was completed, and the draft sequence is now available online (Chen et al., 2014). Thus, the tongue sole is an excellent animal model for studying the neuroendocrine regulation of reproduction in fish. To provide initial insight into the physiological role of LPXRFa in the neuroendocrine regulation of reproduction in Pleuronectiformes, an order that has not been examined until now, the aims of this study were (1) to obtain the full-length cDNA sequence of LPXRFa, (2) to study LPXRFa mRNA levels in various tissues, and (3) to examine the expression profiles of LPXRFa mRNA at the BPG axis during tongue sole ovarian maturation.

2. Materials and methods

2.1. Animals

All of the animal experiments were approved by the Animal Care and Use Committee of the Chinese Academy of Fishery Sciences. Approximately 2-year-old tongue sole specimens were purchased from a local fishery in Qingdao, China. The fish were reared in an indoor concrete tank with recirculating seawater at room temperature under a cyclical light–dark photoperiod (12 h:12 h). The fish were fed to satiation twice daily with a commercially available dry diet (Shengsuo Aquafeed Co., Ltd., Yantai, China).

2.2. Bioinformatics analysis and molecular cloning

To search for the putative LPXRFa gene sequence in the tongue sole, we used the European sea bass (*Dicentrarchus labrax*) LPXRFa peptide sequence (GenBank accession no. CEK03537) as a template. Using the tblastn program (http://blast.ncbi.nlm.nih.gov), we identified a potential homologous sequence for tongue sole LPXRFa, which was further experimentally confirmed by subsequent cDNA cloning and phylogenetic analysis. The full-length cDNA sequence of tongue sole LPXRFa was obtained by nested PCR coupled to 3′/5′ rapid amplification of cDNA ends (RACE) as previously described (Shi et al., 2015). In brief, total RNA was extracted from the brain of tongue sole using the RNAiso Plus reagent (Takara, Dalian, China), and one microgram of RNA was used as a template for cDNA synthesis using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instructions. Using gene-specific primers designed based on the putative tongue sole LPXRFa sequence (Table 1), PCR amplification was performed with the Recombinant Taq DNA Polymerase mix (Takara, Dalian, China). PCR conditions were as follows: denaturation at 95 °C for 5 min; followed by 35 cycles of 95 °C for 30 s,

Table 1
Primers used in this study.

Primer name	Primer sequence(5′-3′)	Purpose
LPXRFa-F1	TGAGCACCGTGTTCCTGTCAG	Partial CDS, 1st PCR
LPXRFa-R1	CCTTAAAGGACGTTCGCTCCAT	
LPXRFa-F2	TGTTCCTGTCAGTGCTCCTGATG	Partial CDS, 2nd PCR
LPXRFa-R2	CGCTCCATTTCCAACTCTTCTG	
LPXRFa-F3	CGAGCAGCGAGGAGGGGAGACACACGGT	3′RACE, 1st PCR
LPXRFa-F4	TGTGACCCCGACCGCCAGCAAAAGCA	3′RACE, 2nd PCR
LPXRFa-R3	GCCGTCAAGTCAAAATCTTCAGCCCAGTGC	5′RACE, 1st PCR
LPXRFa-R4	ATGTTGGCGTGTATGTGGGGGTTGACTGT	5′RACE, 2nd PCR
UPM (long)	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	Universal primers
UPM (short)	CTAATACGAC TCACTATAGGGC	
LPXRFa-F5	GGAAATCAGCCTACAGTGACAAAA	qPCR
LPXRFa-R5	GCCTCTCCAAGTCCAAACTCC	
18S-F	GGTCTGTGATGCCCTTAGATGTC	
18S-R	AGTGGGGTTCAGCGGGTTAC	