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Kisspeptin2 stimulates the HPG axis in immature Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

It has been suggested that kisspeptin influences reproduction and onset of puberty in fishes. Unlike mammals, which produce only one kisspeptin (Kiss1), some teleosts have two, Kiss1 and Kiss2, both thought to be involved in the stimulation of gonadotropin (GTH) secretion. In Nile tilapia (*Oreochromis niloticus*), however, only Kiss2 has been identified so far. The effect of Kiss2 on GTH release varies significantly depending on species and reproductive stage. Furthermore, its physiological function in this species is not clearly defined. In this study, *kiss2* gene expression profiles were examined using quantitative real-time PCR (qRT-PCR) in the brain, pituitary, and gonads of Nile tilapia at different reproductive stages (male: immature, pre-spermiation, post-spermiation; female: immature, pre-spawning, post-spawning). The *kiss2* mRNA expression profiles of the brain, pituitary, and gonads of both sexes shared a similar pattern their expression was significantly higher at the immature stage than at the mature or post-spawning stages, implying it is involved in early gonadal maturation in this species. To investigate the effect of kisspeptin on the hypothalamus-pituitary-gonad (HPG) axis in vivo, synthetic kisspeptin2 (FNYNPLSLRF) was injected into immature male and female tilapia intraperitoneally (i.p.) at a dose of 200 pmol/g body weight. The results showed that synthetic Kiss2 administration increased the expression of *GnRH I*, *fshβ* and *lhβ* mRNA in the brain and increased 17β-estradiol (E_2) and 11-ketotestosterone (11-KT) levels in the plasma. These results suggest that *Kiss2* stimulates the expression of *GnRH* and *GTH* genes in immature Nile tilapia.

1. Introduction

The hypothalamic-pituitary-gonadal (HPG) axis is of central importance to the reproductive processes of various vertebrate species, including fishes. Recently, it has been suggested that the peptide, Kisspeptin, is an essential component in the regulation of the HPG axis of many species of animals (Roa et al., 2008; Tena-Sempere et al., 2012). Multiple forms of kisspeptin exist, with their function and potency differing between species. Unlike mammals, which produce only one kisspeptin (Kiss1), teleosts synthesize two forms (Kiss1 and Kiss2). These include zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) (Kitahashi et al., 2009; van Aerle et al., 2008).

The administration of Kiss1 was shown to induce an increase in luteinizing hormone (LH) in female goldfish (*Carassius auratus*) (Li et al., 2009). In zebrafish, Kiss2 increased the expression of *GTH* subunits (Kitahashi et al., 2009). In the brain and pituitary of both sexes of grass puffer (*Takifugu niphobles*), the expression of *kiss2* mRNA was greater during the breeding season than it was during the non-breeding season (Shahjahan et al., 2010). In the male chub mackerel (*Scomber japonicus*) brain, the expression of *kiss2* mRNA gradually decreased as reproductive activity progressed (Selvaraj et al., 2010). Expression of the *kiss2* gene increased in male senegalese sole (*Solea senegalensis*) during spermatogenesis, but only increased in females during the breeding season (Mechaly et al., 2012). Variations of this kind have made it difficult to understand the mechanism of kisspeptin action in fish reproduction.

Our test species, Nile tilapia (*Oreochromis niloticus*), is one of the major aquaculture species worldwide. Its precocious-maturation and frequent spawning are, however, obstacles that hamper the producing of this species. Understanding neurohormonal control of its reproduction, and then developing methods for overcoming such problems, would be useful. To date, Kiss2 is the only form of kisspeptin identified in this species (Ogawa et al., 2013). It is not known, however, whether or not this form is physiologically potent enough to be a dominant form of kisspeptin in this species. To determine its potency, *kiss2* gene expression profiles were investigated in the brain, pituitary, and gonads of both male and female Nile tilapia at different reproductive activities, using quantitative real time-PCR (qRT-PCR). In second part of the study, synthetic Kiss2 was intraperitoneally (i.p.) injected into immature male and female Nile tilapia to determine whether or not Kiss2 induces sexual maturation. Its, in vivo effects on the HPG axis were also investigated

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using qRT-PCR, histological observations, and ELISA for sex steroid hormones.

2. Materials and methods

2.1. Expression profiles of the kiss2 gene in the brain, pituitary and gonads of Nile tilapia

2.1.1. Fish and tissue sampling

Nile tilapia were spawned and reared in rearing facilities at Sunmoon University (Asan, Chung Nam, Korea). They were reared in a closed recirculating culture system with water temperature at 27 ± 1 °C, and under a 10 h dark: 14 h light photoperiod, during which they were fed commercial dry pellets (Woosung Co., Korea) twice a day. Immature and mature individuals (Table 1) were grouped according to different reproductive stages, and were then sampled to investigate *kiss2* gene expression at the various stages. The mature group was reproductively active, exhibiting spawning or spermiation. For tissue collection, all fish were anesthetized with benzocaine (50 ppm), after which samples of their brains, pituitaries and gonads were taken. These were stored at −80 °C until total RNA extraction. Parts of the gonads were fixed in 10% formalin for histological sectioning. All animal maintenance and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Sunmoon.

2.1.2. Gonadal histology

To determine reproductive stages, formalin-fixed gonads sampled from immature and mature groups of fish (Table 1) were dehydrated in a series of increasing alcohol solutions up to 100%, after which they were embedded in paraffin. The paraffin-embedded tissues were sectioned at 5–6 μm and stained with hematoxylin and eosin. The stained sections were observed under a light microscope (DM500, Leica, Germany) and photographed using a Leica 300FX camera and LAS software (Leica Microsystems GmbH, Germany). The reproductive stages of male and female tilapia were divided into three groups (male: immature, pre-spermiation and post-spermiation; female: immature, pre-spawning and post-spawning), which was based on histological observations (Table 1).

2.1.3. Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from the brain, pituitary, and gonad samples using Trizol reagent (Invitrogen, USA). All reagents used in cDNA synthesis were purchased from Promega (USA). Each total RNA (1 μg) sample was treated with DNase I (RQ1 RNase-free DNase) to remove genomic DNA and, then, reverse transcribed according to the manufacturer's protocol using Oligo $(dT)_{15}$ primer, 10 mM dNTP mix, M-MLV (Moloney murine leukemia virus) reverse transcriptase and the buffer provided.

Expression of the *kiss2* gene was investigated by qRT-PCR using the cDNAs as templates. All primers for qRT-PCR were designed using the Beacon Designer software (Bio-Rad, Hercules, CA, USA) (Table 2). The qRT-PCR reactions were carried out using Topreal™ qPCR 2× PreMIX SYBR Green (Enzynomics, Korea) and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The conditions for qPCR reaction were as follows: initial denaturation at 95 °C for 15 min, 45 amplification cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s and elongation at 72 °C for 30 s. All samples were duplicated. Expression values of *kiss2* mRNA was obtained as a Ct (threshold cycles) value. Abundance levels of mRNA were normalized against the amount of *GAPDH* mRNA. Relative abundance was determined using the comparative threshold cycle method, $2^{-\Delta\Delta Ct}$, along with CFX Manager™ Software (Bio-Rad).

Table 2
List of Primers used for quantitative real-time PCR analysis.

Genes (GenBank accession No.)	Primer	Sequences
Kiss2 (NM_001279486)	Forward	5′-CTA CTG TTG GCT GTG GT-3′
	Reverse	5′-CTG CTC CTG TTG CAT GTG TT-3′
LHβ (AY294016)	Forward	5′-GCT GTC ACC CAG TAG AGA-3′
	Reverse	5′-TTG CTG AAT GGT ATC TTG ATG A-3′
FSHβ (AY294015)	Forward	5′-ACT TCA TTC ATA CTG ACG ACT G-3′
	Reverse	5′-TTG CTC TGT GTA TTT CAC CTC-3′
GnRHI(AB101665)	Forward	5′-CTC GCA GGG ACG GTG TTT-3′
	Reverse	5′-TCT TCC CTC CTG GGG TCA GT-3′
GAPDH (JN381952)	Forward	5′-TTA AGG AAG CCG TCA AGA AG-3′
	Reverse	5′-CAG CAC CAG CAT CAA AGA-3′

2.2. In vivo effects of synthetic kisspeptin (Kp-10) on the HPG axis in Nile tilapia

2.2.1. Fish and peptides

Juvenile Nile tilapia (body length: 6.9 ± 0.6 cm, body weight: 5.7 ± 1.8 g, GSI < 0.25%) were used to study the in vivo effects of the synthetic kisspeptin. Kiss2 peptide (Kp-10, FNYNPLSLRF-NH_2), which was synthesized and purified using high-performance liquid chromatography according to the manufacturer's procedure (Peptron, Korea). The purity of peptides used in study was >95%. The peptide was diluted in phosphate-buffered saline (PBS).

2.2.2. Administration of kisspeptin and sampling

Fish were injected with Kp-10 or PBS i.p. twice weekly for a total of 8 times, and at a dose of 200 pmol/g body weight ($n = 50$ per treatment). Dose was determined based on previous studies that looked at the effects of kisspeptins (Kiss1 and Kiss2) on sea bass (*Dicentrarchus labrax*) (Felip et al., 2009). Throughout the experimental period, photoperiod and water temperature were maintained at a 10 h dark: 14 h light cycle and at 27 ± 1 °C, respectively. The fish were fed twice daily with commercial dry pellets (Woosung Co., Korea).

Two days after the second injection of the first week, several fish (n = 5–6 for each sex) were removed from the experimental tank, and anesthetized with 4-aminobenzoic acid ethyl ester (50 ppm). Blood was withdrawn from the caudal vein of each fish using a syringe (1 mL) treated with heparin sodium to measure the concentration of 17β-estradiol (E_2) and 11-ketotestosterone (11-KT). The blood was centrifuged at 1000g for 30 min and at 4 °C to separate the plasma. The plasma was stored at −70 °C until it was analyzed. Fish were subsequently euthanized for sample collection by an overdose of anesthetic. The brains and pituitaries of these fish were surgically excised

Table 1
Information of experimental fish used for expression profiles of *Kiss2* gene in the brain, pituitary and gonads.

Group		Body length (cm)	Body height (cm)	Body weight (g)	No. of fish
Immature male		5.2 ± 0.1	1.5 ± 0.1	2.2 ± 0.2	8
Mature male	Pre-spermiation	23.9 ± 0.4	9.0 ± 0.4	282.4 ± 22.6	7
	Post-spermiation	25.7 ± 0.8	9.1 ± 0.4	367.0 ± 44.2	4
Immature female		5.2 ± 0.2	1.7 ± 0.1	2.5 ± 0.2	7
Mature female	Pre-spawning	20.9 ± 0.3	7.5 ± 0.3	196.9 ± 74.4	6
	Post-spawning	19.4 ± 1.2	6.7 ± 0.4	150.4 ± 26.2	4