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Molecular cloning of kisspeptin receptor genes (*gpr54-1* and *gpr54-2*) and their expression profiles in the brain of a tropical damselfish during different gonadal stages

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ABSTRACT

The kisspeptin receptor (GPR54) mediates neuroendocrine control of kisspeptin in the brain and acts as a gateway for a pulsatile release of hypothalamic gonadotropin-releasing hormone. This study aimed to clone two GPR54 genes (*gpr54-1* and *gpr54-2*) from the brain of the sapphire devil *Chrysiptera cyanea*, a tropical damselfish, and to study their involvement in reproduction. The partial sequences of the sapphire devil *gpr54-1* cDNA (1059 bp) and *gpr54-2* cDNA (1098 bp) each had an open reading frame encoding a protein of 353 and 366 amino acids, respectively, both of which had structural features of a G-protein-coupled receptor. The expression of *gpr54-1* mRNA was observed in the diencephalon and telencephalon, and *gpr54-2* mRNA was found in the optic tectum of sapphire devil. When *gpr54-1* and *gpr54-2* mRNA levels were examined in the brain of sapphire devil by real-time quantitative polymerase chain reaction (qPCR), they were found to increase during late vitellogenesis and post-spawning. Treatment of fish with estradiol-17β (E2) resulted in an increase in *gpr54-1* and *gpr54-2* expression in the brain of sapphire devil. Thus, kisspeptin receptors likely mediate the activity of kisspeptin in the brain and are involved in controlling reproductive events in a tropical damselfish.

1. Introduction

Kisspeptin is thought to be a possible gatekeeper of the hypothalamic–pituitary–gonadal (HPG) axis (Yaron and Levavi-Sivan, 2011), and may be involved in controlling the onset of puberty in mammals (de Roux et al., 2003; Seminara et al., 2003). When the HPG axis is activated during mammal reproduction, kisspeptin specifically binds to the kisspeptin receptor (GPR54), which belongs to the G-protein-coupled receptor family and is localized on GnRH neurons (Herbison et al., 2010). The physiological role and ligand specificity of kisspeptin in fish may be identical to those in mammals (Taranger et al., 2010). However, the kisspeptin system in fish may be complicated due to paralogous kisspeptin genes (*kiss1* and *kiss2*) that have been identified in several species. In addition, paralogous GPR54 genes (*gpr54-1* and *gpr54-2*) have been reported in certain teleosts (Parhar et al., 2004; Brian et al., 2008; Lee et al., 2009; Li et al., 2009; Zmora et al., 2012; Escobar et al., 2013; Ohga et al., 2013). Parhar et al. (2004) reported that GPR54-2 localizes in GnRH neurons in the brains of the Nile tilapia *Oreochromis niloticus*. On the other hand, Escobar et al. (2013) failed to observe a direct network between the kisspeptin system and GnRH neurons in the ventral telencephalon or preoptic area in the European sea bass *Dicentrarchus labrax*. In the European sea bass, *gpr54-1* mRNA expression is widely distributed in the brain, while *gpr54-2* mRNA expression is limited to the habenula, the ventral telencephalon, and the proximal pars distalis of the pituitary (Escobar et al., 2013). Therefore, the kisspeptin system is thought to have diverse functions in teleosts.

Alvarado et al. (2013) reported that the abundance of *gpr54-1* and *gpr54-2* mRNA increased from late vitellogenesis to post-spawning in European sea bass. A concomitant increase in the expression of *kiss2*, but not *kiss1*, was also observed during this time. A similar result was noted in the chub mackerel *Scomber japonicus* during early vitellogenesis (Ohga et al., 2013). Moreover, the expression of *kiss2* and *gpr54-2* in the brain of the Senegalese sole, *Solea senegalensis*, increased during spawning season (Mechaly et al., 2012). These results strongly indicate that the expression profiles of kisspeptins (*kiss1* and *kiss2*) and their receptors (*gpr54-1* and *gpr54-2*) are closely related to the rapid progression of gonadal development in fish. Regarding the kisspeptin system, most studies have been performed using temperate fish. Despite various reproductive strategies, little is known about the involvement of the kisspeptin system in the reproductive processes of tropical fish.

The sapphire devil *Chrysiptera cyanea* is a tropical damselfish that is widely found in shallow reefs in the Indo-West Pacific area. A

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previous study performed in subtropical waters (26°42′N, 127°52′E) revealed that vitellogenesis lasts for 6 months starting in March and that spawning repeats several times over a 4-month period beginning in May (Bapary et al., 2009). As the reproductive behavior of this species is regulated by photoperiod, water temperature, and food availability (Bapary et al., 2009, 2012; Bapary and Takemura, 2010), it is an ideal tropical species to study the involvement of environmental factors in HPG axis activation through the kisspeptin system. The aim of this study was to examine the involvement of GPR54 in the reproduction of this species. Two GPR54 genes (*gpr54-1* and *gpr54-2*) were cloned from the brain of the sapphire devil and characterized. Tissue distribution, as well as the expression profile of both genes in relation to ovarian development, was investigated using real-time quantitative polymerase chain reaction (qPCR). The effect of estradiol-17β (E2) treatment on *gpr54-1* and *gpr54-2* expression in the brain was also evaluated.

2. Materials and methods

2.1. Animals

Mature females ranging from 1.65 to 3.75 g in body mass were used. They were collected from coral reefs around Sesoko Island, Okinawa, Japan using a hand net during the daytime low tide and transported to Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Japan. They were reared in stock polystyrene tanks (500 l capacity) equipped with running seawater and an aeration system under natural photoperiod conditions and in ambient water temperature. They were fed daily at 1000 h with commercial pellets (EP1; Nisshin-Marubeni, Tokyo, Japan).

Fish were removed from tanks, immediately anesthetized with 0.01% 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan), and euthanized by decapitation. For molecular cloning ($n = 3$) and tissue distribution ($n = 4$), the whole brain was excised, immediately frozen in liquid nitrogen, and stored at −80 °C.

Mature females were also collected from Sesoko Island from February to September 2013 to study the expression profiles of *gpr54-1* and *gpr54-2* mRNA in relation to ovarian development. As previously described, the fish were anesthetized and the brains were stored at −80 °C. Pieces of ovary were fixed in Bouin's solution for subsequent histological testing.

All experiments were conducted in compliance with the guidelines of the Animal Care and Use Committee established by the University of the Ryukyus and the regulations for the care and use of laboratory animals in Japan.

2.2. Hormone treatment

This experiment was performed in December when the ovary of the sapphire devil is under repressed conditions and is occupied by immature oocytes. Individuals ranging from 0.69 to 1.15 g in body mass were randomly transferred to two glass aquaria (30 l capacity) with the ambient aeration system at 26 ± 1 °C under short-day conditions (LD = 10:14; light on at 0800 h and off at 1800 h). Each aquarium contained six individuals. E2 (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in absolute ethanol and diluted to 1 mg/ml. E2, at a final concentration of 1 μg/l, was added to one aquarium (the experimental group) while the other aquarium received the vehicle only (the control group). Water was replaced daily by running out with fresh seawater. Treatment was repeated for 7 days. At the end of the E2 exposure period, the brain was removed from each individual after terminal anesthesia by immersion in 0.01% 2-phenoxyethanol.

2.3. Histological procedures

Following dehydration using an ethanol series and permutation with xylene, ovaries were embedded in paraffin (m.p. 56–58 °C; Histologie, Merck, Darmstadt, Germany), serially sectioned at 5 μm, and then stained with hematoxylin and eosin for microscopic observation. Based on the oocyte staging of the white-spotted spinefoot *Siganus canaliculatus* (Hoque et al., 1998), oocytes were classified into the following six stages: the peri-nucleolus stage (PNS), oil droplet stage (ODS), yolk vesicle stage (YVS), primary yolk stage (PYS), secondary yolk stage (SYS), and tertiary yolk stage (TYS). Atretic oocytes (AO) were also subjected to histological observation (Rahman et al., 2000). Ovarian development was divided into four phases; *i.e.*, immature (IM), early vitellogenesis (EV), late vitellogenesis (LV), and post-spawning (PS).

2.4. Cloning of GPR54 genes

Total RNA was extracted from the whole brain sample using RNAiso plus (Takara Bio, Otsu, Japan) according to the manufacturer's protocols. The first strand of cDNA was reverse-transcribed from 1 μg of total RNA using reverse transcriptase in the PrimeScript RT reagent Kit (Takara Bio). Oligonucleotide primer sets (Table 1) for sapphire devil *gpr54-1* and *gpr54-2* were designed based on chub mackerel (GenBank accession no. **JX982322**) and striped sea bass (**GU351869**) sequences, respectively.

PCR conditions for partial cloning were as follows: 1 cycle at 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 56 °C (*gpr54-1*) or 58 °C (*gpr54-2*) for 30 s, and 72 °C for 1 min, followed by 1 cycle at 72 °C for 3 min. The predicted products from each PCR were checked by electrophoresis on 2% agarose gels (Takara Bio). The amplified products were subcloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) according to the manufacturer's protocols, transformed into JM109 competent cells (Takara Bio), and then sequenced.

The 5′ rapid amplification of cDNA ends (RACE) of sapphire devil *gpr54-1* and *gpr54-2* was conducted using the SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories, Mountain View, *CA*, USA). Gene-specific RACE primers for sapphire devil *gpr54-1* and *gpr54-2* were designed based on the partial cDNA fragment sequences. The first 5′ RACE PCR was performed as follows: 1 cycle at 94 °C for 4 min, 34 cycles at 94 °C for 30 s, 59 °C (*gpr54-1*) or 58 °C (*gpr54-2*) for 30 s, and 72 °C for 1 min, followed by 1 cycle at 72 °C for 3 min. The nested PCR of 5′ RACE was performed as follows: 1 cycle at 94 °C for 4 min, 25 cycles at 94 °C for 30 s, 60 °C (*gpr54-1* and *gpr54-2*) for 30 s, and 72 °C for 1 min, followed by 1 cycle at 72 °C for 4 min. The cDNA fragments were cloned into the pGEM-T Easy Vector system and sequenced.

Table 1
Forward and reverse primers sets used to determine gene expression of *gpr54-1* and *gpr54-2* from the sapphire devil.

Primer	Sequence
Cloning primer for partial	
gpr54-1-forward	5′-TCACCAAACACCAGCAGATG-3′
gpr54-1-reverse	5′-GCTTCTCCTTTGGGTGTCTG-3′
gpr54-2-forward-1	5′-CAGGCAGATGAGAACAGCAA-3′
gpr54-2-reverse-1	5′-GCGAAGAGGAGGACGATTAC-3′
gpr54-2-forward-2	5′-CCAGCCCACTGTAGAACCT-3′
gpr54-2-reverse-2	5′-TTATTGTTGCCTTCCTCTGC-3′
Cloning primer for RACE	
gpr54–1-GSP1	5′-AGGAACAGGACCACCATCAC-3′
gpr54–1-NGSP1	5′-CAGGGATGGACAGGAGTAAGG-3′
gpr54–2-GSP1	5′-GCGAAGAGGAGGACGATTAC-3′
gpr54–2-NGSP1	5′-GGGTGGACAGGATGAAGGA-3′
Real-time PCR primer	
gpr54-1-rt-forward	5′-GCCTCCGCAGTTACATTCTG-3′
gpr54-1-rt-reverse	5′-TGGAACGCCACAGAAACAC-3′
gpr54-2-rt-forward	5′-TGAGGATGAGGAGGGAGAAC-3′
gpr54-2-rt-reverse	5′-TGAAGGGGACACAGCACAC-3′
Ef1α-rt-forward	5′-ACGTGTCCGTCAAGGAAATC-3′
Ef1α-rt-forward	5′-GGGTGGTTCAGGATGATGAC-3′