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A Unique Expression Profile of Kisspeptin Receptor Genes During Final Oocyte Maturation in Female Chub Mackerel, *Scomber japonicus*

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Kisspeptin is a key regulator of not only pubertal onset but also the preovulatory luteinizing hormone (LH) surge in mammals. Our previous study demonstrated that both kiss1 and kiss2 mRNA levels increased during final oocyte maturation (FOM) and ovulation in the spawning cycle, and suggested their possible involvement in the regulation of these two processes. In this paper, we report the expression profiles of kisspeptin receptors (kissr1 and kissr2) in the brain of adult female chub mackerel during FOM and ovulation. Contrary to the expression of their ligands, the expression of both receptor genes significantly decreased during FOM. The rhythmic expression of kiss/kissr during the spawning cycle may be involved in regulation of the reproductive axis, especially the LH surge, and could be affected by the circadian clock or the circadian oscillation of an environmental factor.

Key words: circadian rhythm, final oocyte maturation, kisspeptin receptor, spawning cycle

INTRODUCTION

In mammals, the critical role of kisspeptin in maintaining normal pubertal onset is now widely recognized. Kisspeptin is the direct regulator of gonadotropin–releasing hormone (GnRH) release. *Kiss1* expression increases at puberty in rodents (Navarro *et al.*, 2004) and primates (Shahab *et al.*, 2005). In addition, the administration of exogenous kisspeptin was found to elicit a robust increase in the circulating level of GnRH (Messager *et al.*, 2005). Notably, this peptide should participate in activation of the gonadotropic axis in adulthood. Indeed, Navarro *et al.* (2004) showed that the expression of *Kiss1r* genes changes throughout the estrous cycle in the adult rat hypothalamus.

Ovulation is triggered by a surge of luteinizing hormone (LH) from the pituitary, which follows a surge of GnRH from the preoptic area. These surges are induced by the positive feedback activity of estrogen secreted by the ovary. However, GnRH neurons lack the estrogen receptor (ER α), and this missing link is filled by kisspeptin neurons. Kisspeptin neurons are localized at the anteroventral periventricular nucleus (AVPV) and express ER α their Kiss1 expression was shown to be reduced after ovariectomy, but increased by E2 treatment (Smith *et al.*, 2005). In addition, the rhythm of Kiss1 expression in the AVPV was shown to always be in phase with the LH surge in adult female rats (Smarr *et al.*, 2012).

Following discovery of the role of Kiss1/Kiss1r in the mammalian reproductive system, two paralogous kisspeptin genes (*kiss1* and *kiss2*) and associated receptors (*kissr1* and *kissr2*) were isolated in a few fish species (Biran *et al.*, 2008; Felip *et al.*, 2009; Felip *et al.*, 2015; Lee *et al.*, 2009; Li *et al.*, 2009; Zmora *et al.*, 2012). The marked increase in the expression of the receptor genes

at the time of puberty suggests that piscine kisspeptin also plays a pivotal role in pubertal onset (Biran *et al.*, 2008; Filby *et al.*, 2008; Martinez–Chavez *et al.*, 2008; Mechaly *et al.*, 2009; Mechaly *et al.*, 2010; Mohamed *et al.*, 2007; Nocillado *et al.*, 2007). In fact, kisspeptin antagonists were found to hinder sperm production in striped bass, *Morone saxatilis* (Zmora *et al.*, 2015). A large number of studies on kisspeptin in fish have focused on its role in puberty. However, little is known about its role in FOM and ovulation in fish.

The experimental scombroid fish chub mackerel (*Scomber japonicus*) is a multiple batch–spawning pelagic fish. Captive females in the spawning season have been shown to exhibit fully grown ovaries just prior to FOM, but fail to undergo FOM and ovulation spontaneously (Shiraishi *et al.*, 2005). To induce spawning in experimental tanks, an exogenous GnRH analogue (GnRHa) is typically injected, which induces an LH surge from the pituitary (Nyuji *et al.*, 2011). After GnRHa treatment, chub mackerel brood stock spawn daily for over a month, and the ovary shows a diurnal rhythm of FOM and ovulation in the spawning cycle.

The chub mackerel brain was found to express two kisspeptin and kisspeptin receptor genes, whose expression was shown to exhibit distinct variation in the female brain during pubertal transition and the seasonal gonadal cycle in adulthood (Ohga *et al.*, 2013; Ohga *et al.*, 2015; Selvaraj *et al.*, 2010). In addition, our previous study showed that both *kiss1* and *kiss2* expression dramatically increases during FOM and ovulation (Selvaraj *et al.*, 2012). This may reflect the acute shift of the endocrine system during the spawning cycle, indicating that the kisspeptin system may be involved in the regulation of FOM and ovulation in this species.

The present work is part of a series of studies aimed at understanding the molecular basis of chub mackerel reproduction, with the aim of correcting reproductive dysfunction in captivity. We report here the expression

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profiles of kissr1 and kissr2 mRNA in the brain of adult females during FOM and ovulation.

MATERIALS AND METHODS

Animals

In the present study, we used the same samples that have been previously reported on the expression changes of two kisspeptin forms (Selvaraj *et al.*, 2012). Adult chub mackerel were caught with a purse seine and reared in sea pens at a fish farm in Oita prefecture, Kyushu Island. The fish were reared under the natural photoperiod and temperature conditions and fed with commercial dry pellets (Higashimaru Co., Japan). Before the start of an experimental sampling, wild caught fish stocks were reared for 6 months. During the following spawning season (April–June), fish were transported to the Fishery Research Laboratory, Kyushu University, Fukuoka Prefecture, and stocked in a 3–m³ concrete outdoor tank with running sea water under natural photoperiod and temperature.

Spawning induction and tissue sampling

After 3 days of acclimation, four groups of chub mackerel were injected intramuscularly with GnRH agonist (D-Ala6, des-Gly10)-LHRH ethylamide (Sigma-Aldrich, St. Louis, USA) into molten coconut oil at $400 \,\mu g/$ kg body weight according to method of Scott et al. (1999). Before the GnRHa treatment, fish were anaesthetized with 2-phenoxyethanol (200 ppm) and female with late vitellogenic oocytes (LV: $600-650\,\mu\text{m}$ in diameter) were selected by ovarian biopsy using a plastic catheter tube (2 mm internal diameter), as described previously (Shiraishi et al., 2005; 2008). All injections were performed at 11:00 A.M.. Sampling times were fixed based on our previous data on time course of FOM and ovulation in chub mackerel induced by GnRHa (Shiraishi et al., 2008). The first spawning was observed 34-36 h post-injection, and performed every night between 22.00 and 24.00 h until sampling date. After 8 days from GnRHa treatment, sampling for each spawning stage fish (n = 7-8) was carried out in 13.00 h (germinal vesicle migration, GVM), 16.00 h (hydration, HY), 20.00 h (ovulation, OV) and next day 06.00 h (post-ovulation, POV). Gonadal stage was confirmed by histological analysis described below. In all sampling, the brains were removed following decapitation, snap-frozen in liquid nitrogen, and stored at -80°C until use. At the time of sampling, the fish were carefully treated and sacrificed following the guidelines for animal experiments in the Faculty of Agriculture and Graduate Course of Kyushu University.

Histological processing

Gonadal tissues were fixed overnight in Bouin's solution, dehydrated, and embedded in Technovit resin (Kulzer, Wehrheim, Germany) and sectioned at $4 \mu m$ using a Leica RM 2155 rotary microtome (Leica, Germany). Sections were stained with 1% toluidine blue.

Quantitative real-time PCR

Quantitative real-time PCR analysis was performed on an Mx 3000P quantitative PCR system (Stratagene). Total RNA was extracted from various gonadal stages of tissue using ISOGENE (Nippon Gene, Japan). Two microgram of total RNA from each tissue sample was digested with DNase I (Invitrogen) and used as template for reverse transcription using random hexamers (Takara). All transcripts were quantified using a standard curve method and a previously validated qRT-PCR for each gene, reported in detail previously (Ohga et al., 2013). Real-time PCR was performed using the Brilliant Ⅲ Ultra–Fast SYBR Green QPCR master mix (Agilent, CA) in a total volume of 10µl. PCR condition as follow: 95°C (5 min); 40 cycles at 95°C for 10 sec, 60°C for 30 sec. Melting curve analysis was also included at 1 cycle of 94°C for 1 min, 55°C for 30 sec, 95°C for 30 sec. The reference gene β -actin was also measured in each sample for relative quantitation. The primer sets for each gene are listed in Table 1.

Statistical analysis

Data were expressed as means±SEM (standard errors of the mean), and analyzed by one–way ANOVA followed by a Tukey's Multiple Comparison Test using Prism 4 (GraphPad Software, San Diego, CA).

RESULTS

The expression of brain *kissr1* and *kissr2* genes during the spawning cycle is shown in Figure 1. The levels of *kissr1* and *kissr2* significantly decreased from the LV

Table 1. Primer sequences used in present study

Primer	GenBank Accession		Sequence (5'-3')	Amplicon size (bp)	Tm (°C)	Efficiency (%)
kissr1	JX982322	Fw	CTACCTGCAGCAAGTGACTGC	146	60.6	98.4
		Rv	TCCAGATGGACACAGAAACAGC		62	
kissr2	JX982323	Fw	CCAGTTCATTGCTGCCTACC	164	60.1	86.8
		Rv	TCTTGGAGACCTTGCTCCTG		59.9	
β –actin	GU731674	Fw	ACCGGTATTGTCATGGACTC	127	56.6	96.6
		Rv	TCATGAGGTAGTCTGTGAGGTC		56.2	



Fig. 1. *kissr1* and *kissr2* expression during FOM.

Expression changes in mRNA levels of kissr1 and kissr2 in the brain of adult female chub mackerel during final oocyte maturation (spawning) process. β -actin is used as reference gene. Data are expressed as mean \pm SEM (n=5-6). Different letters represent significant difference (P<0.05) between stages. Representative sections of each maturational stages are presented at above the graph. Oocyte at late vitellogenic (LV) stage (A); oocyte at germinal vesicle migration (GVM) stage (B); oocyte at hydration (HY) stage (C); ovulated egg in the ovarian cavity (OV) (D); post-ovulatory follicles (POV) (E). Scale bars = 100 μ m.

stage to the FOM (GVM and HY) and ovulation (OV) periods. The expression of kissr1 was high in LV–stage brain, decreased significantly at the GVM stage, and was minimal at the HY stage. Subsequently, kissr1 expression significantly increased from the HY stage to the OV and POV periods. The level of kissr2 significantly decreased during the GVM stage, and then significantly increased from the HY to the POV stage.

DISCUSSION

In the present study, the expression of both kisspeptin receptors showed significant variation during the spawning cycle. This indicates that kissr1 and kissr2 mRNA is expressed in an ovarian stage-dependent manner in the female brain and suggests a relationship of the kisspeptin system with FOM and ovulation. On the other hand, in chub mackerel, high reproducibility was observed in terms of the appearance of each ovarian stage during the spawning cycle at particular times, namely 1300 h (GVM), 1600 h (HY), and 2000 h (OV) (Nyuji et al., 2011; Shiraishi et al., 2008), suggesting that the kisspeptin system is affected by the circadian clock or the circadian oscillation of an environmental factor. Daily oscillation in kisspeptin neuron activity has been reported, mainly in mammalian species. For example, in mice, AVPV Kiss1 expression showed circadian patterns under constant darkness and peaked coincident with the LH surge (Robertson et al., 2009). In addition, in a recent study that used the grass puffer *Takifugu niphobles*, both *kiss2* and *kissr2* showed clear diurnal and circadian variations in expression levels at the time of the spawning season (Ando *et al.*, 2014). This indicates that the *kiss2/kissr2* system may be involved in control of the precisely timed diurnal and spawning rhythm, possibly through the circadian clock (Ando *et al.*, 2014).

In breeding animals and healthy females, the circadian system plays a role in regulating ovulation. In rodents, the LH surge occurs in the evening, 2-3 h before the onset of nocturnal activity (Smarr et al., 2012). In women, the preovulatory LH surge begins between midnight and 0800 h (Cahill et al., 1998). In the case of chub mackerel, daily spawning was reported to occur between 2200 and 0000 h in captive breeding stock (Nyuji et al., 2011; Shiraishi et al., 2008) and wild fish in the sea (Yamada et al., 1998). At present, there is little information about circadian systems, such as clock genes or photoperiodic regulation, in chub mackerel. However, the fixed timing of spawning suggests that circadian regulation of the reproductive axis, especially the LH surge and the kisspeptin system, is involved in this event. Against this background, our recent study showed that the central injection of kisspeptin peptide upregulated the expression of pituitary $lh\beta$ subunit genes, suggesting their positive role in LH regulation (Ohga et al., 2014).

Our previous study showed that kiss1 and kiss2 mRNA expression in the brain peaked during the FOM and ovulation stages (Selvaraj et al., 2012). Notably, levels of GnRH1 peptide also coincided with an increase in the kisspeptin mRNA levels in the brain, and pituitary $LH\beta$ immunoreactivity was constantly high during FOM in the same fish samples (Nyuji et al., 2011; Selvaraj et al., 2012). In contrast to other reproductive factors, the levels of kisspeptin receptors decrease during the FOM and ovulation. This may be due to desensitization of the kisspeptin receptors. In monkeys, the continuous administration of human kisspeptin leads to desensitization of its receptor, Kiss1r (Ramaswamy et al., 2007; Seminara et al., 2006). In addition, in teleosts, chronic administration of Kiss2 reduced the expression of kissr2 genes in white bass, Morone chrysops, with gonadal recrudescence (Zmora et al., 2012). Based on these observations, it is possible that the decrease in kisspeptin receptor levels is caused by continuous kisspeptin stimulation during FOM.

In conclusion, significant variations in the levels of *kissr1* and *kissr2* mRNA in the brain during FOM and ovulation suggest the positive and important relationship of the kisspeptin system with the final phase of reproduction in female chub mackerel. From an aquacultural perspective, kisspeptin may unfortunately lack the ability to induce spawning. This is supported by our previous pharmacological trial showing that GnRHa, but not cognate kisspeptin synthetic peptide, induced FOM and ovulation in captive females (unpublished data). However, the present study raises the possibility that the kisspeptin system is involved in spawning and is affected by the circadian rhythm. The integrated kisspeptin system, including other reproductive or environmental fac-

tors, is an interesting focus for future research and will be important for understanding the cause of reproductive dysfunction in captive fish.

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