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Possible role of the leptin system in controlling puberty in the male chub mackerel, *Scomber japonicas*

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ABSTRACT

Leptin directly regulates kisspeptin neurons in the hypothalamus and gonadotropin secretion from the pituitary, making it a central player in the onset of mammalian puberty. Recently, we identified two leptin genes (*lepa* and *lepb*) and a single leptin receptor (*lepr*) in the marine perciform fish chub mackerel; however, the expression of these genes did not correlate with the expression of important reproductive genes or ovarian stage during female puberty. Here, we expand upon these initial observations by evaluating the expression of *lepa*, *lepb*, and *lepr* during pubertal transition and under differential feeding conditions in the male chub mackerel. Reverse transcription-polymerase chain reaction (RT-PCR) showed that *lepa* was primarily expressed in the liver of pubertal and gonadal recrudescence adults, as well as in the brain of adult fish; *lepb* was primarily expressed in the brain of all fish tested; and *lepr* was widely expressed in a variety of tissues. qRT-PCR analyses revealed significant increases in the hepatic expression of *lepa* in accordance with testicular stage, whereas pituitary follicle-stimulating hormone (*fshβ*) expression increased in unison with hepatic *lepa*. In contrast, expression of both brain *lepa* and *lepb* dramatically decreased during pubertal transition, with brain kisspeptin 1 (*kiss1*) expression strongly correlating with leptin expression patterns. In pre-pubertal males, *lepa*, *lepb*, and *lper* gene expression in the brain, pituitary gland, and liver decreased in fish given a high feed diet, relative to the controlled feeding group. Taken together, these results indicate high sexual specificity of leptin expression, suggesting a possible role for leptin signaling in endocrine and neuroendocrine functions during spermatogenesis in the pubertal male chub mackerel.

1. Introduction

Pubertal timing is strongly associated with the magnitude of energy storage in mammals (Roa and Tena-Sempere, 2010). One such regulator underlying this association is the adipose hormone leptin, which helps regulate food intake and fat storage, and plays an essential role in the regulation of puberty (Sanchez-Garrido and Tena-Sempere, 2013). Leptin is secreted in proportion to fat stores, and acts within the brain to signal adequate energy reserves and satiety required for puberty onset (Halaas et al., 1995). The absence of this signal due to processes indicative of a negative energy balance, such as that seen in response to acute fasting, has been associated with decreased gonadotropin levels in female rats (True et al., 2011).

Kisspeptin encoded by *Kiss1* gene acts as upstream endogenous regulators of the reproductive axis in mammals (Irwig et al., 2004; Messager et al., 2005). Gonadotropin secretions produced in response to leptin are mediated by kisspeptin signaling in the neurons of the hypothalamus, which express the leptin receptor (Smith et al., 2006). Male leptin-deficient mice have significantly reduced expression of *Kiss1* mRNA in the hypothalamus, which is rescued by peripheral administration of exogenous leptin (Smith et al., 2006). However, despite this connection, leptin acts directly at the pituitary level, thereby bypassing some of the regulatory activity of *Kiss1* (Yu et al., 1997; Ogura et al., 2001; Tezuka et al., 2002).

In fish, several teleosts express multiple leptin genes (*lepa* and *lepb*) as a result of genome duplication (Gorissen et al., 2009; Kurokawa and Murashita, 2009; Zhang et al., 2013; Shpilman et al., 2014). Despite their orthologous relationship, these leptins are poorly conserved between mammals and fish, with most fish primarily expressing leptin in the liver (Gorissen et al., 2009; Huising et al., 2006; Kurokawa et al., 2005; Rønnestad et al., 2010; He et al., 2013). While organ-specific expression has been observed in some species, the role of leptin in the reproductive functions of teleost fish is extremely limited. Some evidence regarding its role in reproduction has been observed in male and female ayu (*Plecoglossus altivelis*), with circulating leptin levels found to be

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significantly higher during spawning compared to pre-spawning levels (Nagasaka et al., 2006). More recently, the gene expression levels of hepatic *lepa1* and *lepa2* were elevated during spermatogenesis in pubertal male Atlantic salmon (*Salmo salar*) (Trombley and Schmitz, 2013; Trombley et al., 2014). These observations suggest the possible involvement of leptin in teleost reproduction; however, the exact function of these proteins remains largely unknown. In addition, there remains a contentious debate regarding the function of piscine leptin in metabolic control. For example, some studies have presented evidence of increased serum leptin levels after a period of long-term calorie restriction (Kling et al., 2009; Trombley et al., 2012; Fuentes et al., 2012), whereas another study showed reduced hepatic leptin mRNA levels in striped bass (*Morone saxatilis*) in response to long-term fasting (Won et al., 2012).

In our previous study, we determined whether leptin plays an important role in the pubertal onset of female chub mackerel (*Scomber japonicus*) (Ohga et al., 2015a). In this study, we found that hepatic *lepa* expression was significantly lower in fish with high-energy reserves, and was unchanged between pre-pubertal and pubertal fish. However, similar expression may not exist in male fish due to the strong sexual specificity of this hormone. In humans, circulating leptin levels are higher in female subjects than in males (Casabiell et al., 2001). Similarly, increases in serum leptin levels have also been observed during the pubertal transition in female monkeys, with no effects seen in males (Garcia-Mayor et al., 1997; Plant and Durrant, 1997). Taken together, it is possible that pubertal leptin expression may exhibit significant differences between male and female fish.

The chub mackerel is considered an excellent candidate for marine aquaculture due to its high consumer demand and capacity for rapid growth. In support of this goal, our team recently developed standardized methods to support the full life cycle of this species in aquaculture. Of particular interest is the period of pubertal transition, which is important for the establishment of efficient aquaculture of any fish species. We previously identified several key molecular elements in the chub mackerel reproductive axis, namely, kisspeptins (*kiss1* and *kiss2*) (Selvaraj et al., 2010), kisspeptin receptors (*kissr1* and *kissr2*) (Ohga et al., 2013), three types of gonadotropin-releasing hormones (GnRHs) (Selvaraj et al., 2012), gonadotropins (GtHs) (Nyuji et al., 2012; Ohga et al., 2012), and GtH receptors (Nyuji et al., 2013).

In this study, we assessed leptin expression levels in the male chub mackerel, which were then compared to our previous analyses in females (Ohga et al., 2015a). We examined the mRNA levels of leptins, leptin receptor, kisspeptins, and GtHs in the brain, pituitary and liver of male chub mackerel under different pubertal stages and feeding conditions. Our results suggest a possible role for hepatic leptin in spermatogenesis in the male chub mackerel. In addition, we show a positive correlation between leptin and kisspeptin levels in a teleost species.

2. Materials and methods

2.1. Experimental fish

From two year ago, we started the marketing of full-life cycle reared chub mackerel. Pilot sampling was conducted for a few times due to the confirmation of fish growth. The female fish were used for previous analysis reported in Ohga et al. (2015a) and in this study, we used male samples. All experimental fish were cultured from fertilized eggs and reared under the natural photoperiod and temperature in Saga prefecture, Kyushu Island with two different feeding conditions: control feed group (1 time/day: 1.5% of the body weight) and high feed group (3 times/day: 3% of the body weight). Fish were fed a commercial diet (Kaneko Sangyo Co., Ltd., Japan, Hayashikane Sangyo Co., LTD., Japan and Higashimaru Co., LTD., Japan). The tissue sampling of male fish was conducted on November (23 weeks post hatching: wph), December (30 wph) 2013, and March (40 wph) 2014. Testicular stage was confirmed by hematoxylin-eosin staining of paraffin section. Food was withheld for 24 h prior to experiment and sampling was started between 13:00 and 14:00 h every time. 10–15 fish (mixed sex) were sampled at each period and fork length (FL), body weight (BW), viscera weight, and gonad weight (GW) were recorded. Gonadosomatic index (GSI) was calculated with GW/BW (except internal organ) × 100; condition factor (CF) was determined according to BW (except internal organ)/FL^3. Tissues were removed following decapitation and stored in RNAlater (QIAGEN). Only the adipose tissues were snapped-frozen in liquid nitrogen and stored at −80 °C until use. The tissue distribution analysis of adult (2+ years old) fish were measured using the same samples used from the previous study (Ohga et al., 2013). 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.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from the whole brain, pituitary, and other tissue sample (about 40 mg) was extracted using ISOGENE (Nippon Gene, Japan). The RNA concentrations and purity were estimated using Ultrospec™ 3000 pro spectrophotometer at 260 and 280 nm of wave length. Two microgram of total RNA was treated with DNase I (Invitrogen, USA) and used as template for reverse transcription. The cDNA systhesis was performed using Superscript III (Invitrogen, USA) in a 20 μl reaction mixture containing 2.5 mM dNTP mixture (Takara, Japan), 200 ng/μl random hexamer primers (Takara, Japan), 5× First-strand buffer, 0.1 M DTT, and RNaseH (1 unit). PCRs were performed in a final volume of 10 μl containing 5 μl 2× Amplitaq Gold PCR master mix (Applied Biosystems, CA, USA), 0.5 μl of each 10 μM sense and antisense primers, 3.0 μl PCR-grade water and 1.0. μl of synthesized cDNA. Thermal cycling consisted of initial denaturation at 95 °C (5 min); followed by 30 cycles at 95 °C for 15 s, 60 °C for 15 s, 72 °C for 30 s. The PCR products were analyzed in 3.0% agarose gels stained by ethidium bromide. For negative control, cDNA sample was replaced with PCR-grade water and confirmed for all primers and water were not contaminated. The quality of the mRNA was confirmed by parallel amplification of elongation factor 1 alpha (*ef1α*).

2.3. Quantitative real-time PCR

Quantitative real-time PCR analysis was performed on an Mx 3000P quantitative PCR system (Stratagene, USA). Template cDNA of whole brain, pituitary, and liver were synthesized by the same method as for RT-PCR analysis. For individual brain region, brain tissue was divided into seven parts. 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., 2015a, 2015b). PCR mixture (10 μl) contained 1.5 μl of sample or standard cDNA, 0.1 μM of primer sets, 3.75 μl of PCR-grade water, 0.05 μl of ROX dye and 5 μl of Brilliant III Ultra-Fast SYBR Green QPCR master mix (Agilent, CA). For negative control, cDNA sample was replaced with PCR-grade water. Duplicate reactions were performed for standards, target and reference genes. PCR condition were set as follow: 95 °C (5 min); 40 cycles at 95 °C for 10 s, 60 °C for 30 s. Melting curve analysis was also included at 1 cycle of 94 °C for 1 min, 55 °C for 30 s, 95 °C for 30 s. These data were analyzed using MaxPro-Mx3000P software version 3.00 (Stratagene, USA). The ribosomal protein L8 (*rpl8*) is used for brain and pituitary and β-actin is used liver as reference gene for relative quantitation. The primer sets for each gene are listed in Table 1.

2.4. Statistical analysis

Data were expressed as mean ± SEM (standard errors of the mean), and analyzed by unpaired two-tailed Student's *t*-test or Mann Whitney test followed by f-test or one-way ANOVA followed by a Tukey's

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