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Research paper

Social rank-dependent expression of gonadotropin-releasing hormones and kisspeptin in the medaka brain

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ABSTRACT

Social interactions regulate the expression of several neuropeptides that have a central role in the reproductive system of mammals. Nonmammalian vertebrates also have these neuropeptides or paralogs, however, studies on the social regulation of reproductive physiology in nonmammalian species are limited. In this study, we examined whether the expression of gonadotropin-releasing hormones (GnRHs) and kisspeptin (Kiss1) is affected by social hierarchy resulting from the outcomes of male-male competition in medaka fish (*Oryzias latipes*). Four males were introduced to each other in an experimental tank, and classified as the most aggressive dominant or the most submissive subordinate fish, based on the frequency of their aggressive acts during a short-term competition. Dominant and subordinate males maintained their social rank during a long-term competition. Immediately after short-term competition, gonadotropin-releasing hormone-3 (GnRH3) level in the olfactory bulb was significantly higher in subordinate males than in dominant males. After long-term competition, dominant males had high level of gonadotropin-releasing hormone-1 (GnRH1) in the preoptic area, whereas subordinate males had lower Kiss1 level in the nucleus ventral tuberis. On the other hand, the levels of gonadotropin-releasing hormone-2 (GnRH2) in the nucleus lateralis valvulae and Kiss1 in the nucleus posterioris periventricularis, and plasma 11-ketotestosterone (11-KT) concentration did not differ between subordinate and dominant males after the short- and long-term competitions. These results suggest that social hierarchy regulates the expression of GnRH1, GnRH3, and Kiss1 without affecting 11-KT level in male medaka.

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1. Introduction

Social interactions influence reproductive physiology in many vertebrates from fish to rodents (Burmeister and Wilczynski, 2005; Stevenson et al., 2008; Lemaître et al., 2011; Oliveira, 2012). For example, dominant individuals have an active reproductive axis of hormones via hypothalamus-pituitary-gonad (HPG) axis, whereas the reproductive function of subordinate individuals is often suppressed (Cardwell and Liley, 1991; Francis et al., 1993; Cardwell et al., 1996; Maruska and Fernald, 2010). At the apex of the HPG axis, gonadotropin-releasing hormone-1 (GnRH1) has a key role in stimulating the release of gonadotropins into the bloodstream. In addition to the hypophysiotropic GnRH1, many animals have additional GnRHs (Kasten et al., 1996; Gestrin et al., 1999; Latimer et al., 2000; Okubo and Nagahama, 2008). One is gonadotropin-releasing hormone-2 (GnRH2), which is localized in the midbrain, and another is gonadotropin-releasing hormone-3 (GnRH3), produced by neurons whose somata lie along the forebrain terminal nerve (Okubo and Nagahama, 2008; Oka, 2009). There are many studies on the social rank-dependent expression of GnRH1, however, less is known about whether other types of GnRH are associated with social status. Recent studies have shown that GnRH2 and GnRH3 neurons projecting their axons widely in the brain have regular and pacemaker electrical activities, whereas GnRH1 neurons have irregular and episodic electrical activity (Abe and Oka, 2000; Kanda et al., 2010; Karigo and Oka, 2013). These facts led us to hypothesize that GnRH2 and GnRH3 are associated with competitive behaviors that determine social hierarchy. In the present study, we examined the expression of three types of GnRH in the brain of dominant and subordinate medaka fish (*Oryzias latipes*) after male-male competition.

Similar to other social fish species (Øverli et al., 1999; Clotfelter and Paolino, 2003; Burmeister and Wilczynski, 2005; Dahlbom et al., 2012), the medaka has competitive behaviors between males involved in establishing social hierarchy (Kawabata, 1954; Kagawa, 2013, 2014). In previous studies, males were classified as aggressive dominant and submissive subordinate medaka immediately after encountering their conspecifics, and their social ranks remained unchanged for several weeks (Kagawa, 2013, 2014).

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Furthermore, it has been reported that the expression of the neuropeptide arginine vasotocin (AVT) in distinct regions of the brain differs between dominant and subordinate individuals (Kagawa, 2013). These studies suggest that the medaka fish is an ideal model to observe the relationship between social hierarchy and neuropeptides, which has been reported for other animal species (Goodson and Thompson, 2010; Meyer-Lindenberg et al., 2011).

A recent study (Kanda et al., 2013) found that AVT neurons in the magnocellular preoptic area (mPOA) in the medaka brain express the kisspeptin receptor (GPR54). After competition, mPOA AVT expression increases in dominant male medaka but not in subordinate male medaka (Kagawa, 2013); therefore, we hypothesized that kisspeptin expression differs between dominant and subordinate medaka. In mammals, Kisspeptin (Kiss1) and GPR54 in the brain are essential for reproductive functions via GnRH neurons (Funes et al., 2003; Lapatto et al., 2007; Clarkson et al., 2008). On the other hand, studies of the reproductive function of Kiss1 in teleosts have been initiated in recent years (Kanda et al., 2013; Selvaraj et al., 2013; Espigares et al., 2015; Escobar et al., 2016). If social interactions between male medaka regulate their reproductive physiology, then Kiss1 and androgen levels might be affected by their social rank. In this study, in addition to GnRH, we examined Kiss1 levels in the brain and plasma androgen levels of dominant and subordinate male medaka.

2. Materials and methods

2.1. Animals

Oryzias latipes (d-rR strain) were hatched and grown in the laboratory and maintained at 23 °C–25 °C under a daily photoperiod cycle of 14 h light/10 h dark (lights on at 7:00 a.m.). They were fed *Artemia* spp. (A&A Marine, Salt Lake City, UT, USA) once daily. Approximately 10 adult males and 10 adult females (age, 5 months postfertilization) were maintained in four stock tanks ($30 \times 22 \times 8$ cm; 5 L). Behavioral observations were conducted during short- and long-term contests using an experimental tank ($20 \times 14 \times 14$ cm; 3 L) containing four males from four different stock tanks. To distinguish between individuals, the contestants were marked by an intramuscular injection with Elastomer Tag (Northwest Marine Technology Inc., Shaw Island, WA, USA) at least 1 week before using them for the short- and long-term contests.

2.2. Experimental procedure

For the short-term contests ($n = 8$), the experimental tank was divided into quarters using opaque gray dividers and an individual male was acclimated in each compartment for 1 day (Fig. 1A). At 9:00 on the following day, the dividers were gently removed. Aggressive behavior exhibited by each male was quantified through direct observation for 20 min. For the long-term contests ($n = 8$), males were allowed to interact continuously for 19 days. On eight randomly selected days, the 20-min observations were conducted twice daily (at 9:00 and 14:00).

The number of agonistic encounters was determined by the method modified from our previous study (Kagawa, 2013). In brief, four types of aggressive behaviors were recorded: (1) replace: approaching slowly towards, stopping nearby and displacing another individual; (2) attack: approaching rapidly towards, stopping nearby and displacing another individual; (3) chase: attacking twice or more towards the same fleeing individual; (4) contact: contact often including biting another individual (Fig. 1B).

In short-term contests ($n = 8$), the most and the least aggressive individuals in each group of four males were classified as dominant and subordinate, respectively, based on the number of aggressive behaviors observed in the contests. In long-term contests ($n = 8$), based on the number of aggressive acts averaged per 20 min throughout the contest, the most and the least aggressive fish in each group of four males were classified as dominant and subordinate, respectively.

Immediately after the contests, the fish were quickly anesthetized with 0.1% 2-phenoxyethanol and their body mass and standard length were measured. Blood samples were then quickly collected from the hemal arch in the caudal peduncle using heparinized capillary glass, and centrifuged for 15 min at 3000*g*, 4 °C to separate plasma. The plasma samples were kept at −80 °C until plasma 11-ketotestosterone (11-KT) was analyzed. After collecting the blood samples, the fish were decapitated and their whole brains were quickly removed.

2.3. Analyses for the expression of GnRHs and Kiss1

For the analyses of GnRHs and Kiss1 level, eight dominant and eight subordinate males obtained from groups subjected to short- or long-term contests were used. The brains were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.3) at 4 °C for 6 h. The brains were then rinsed in 70% ethanol at 4 °C, dehydrated through a graded ethanol series, and embedded in paraffin. Brain cross-sections (8 μm thick) were cut and analyzed using *in situ* hybridization, as described by Kobayashi et al. (2000). Briefly, the sections were washed with PBS and treated with 15 μg/ml protease K for 15 min at 37 °C and then incubated with 0.25% acetic anhydride in triethanolamine/HCl for 10 min. Finally, the sections were hybridized with 0.15 μg/ml digoxigenin (DIG)-labeled antisense ribonucleic acid (RNA) probes overnight at 58 °C. Probes were transcribed from *gnrh1*, *gnrh2 and gnrh3* complementary deoxyribonucleic acid (cDNA) of medaka (donated by Dr. K. Okubo, GenBank accession no. AB041333, AB041330 and AB04133, respectively) and from *kiss1* cDNA of medaka (donated by Dr. S. Kanda, GenBank accession no. AB272755) using an RNA labeling mix (Roche, Basel, Switzerland). A sense RNA probe was used as a negative control (data not shown). After hybridization, the sections were washed with 2x saline sodium citrate, and then immersed with 1.5% blocking reagent (Roche) for 1 h and incubated with an alkaline phosphatase-conjugated anti-DIG antibody (1:1500; Roche) overnight at 4 °C. The sections were washed and treated with a chromogen solution (1.0 mg/ml 4-nitroblue tetrazolium chloride (Roche), 0.2 mg/ml 5-bromo-4-chloro-3-indoyl-phosphate (Roche) in Tris-HCl buffer) until a visible signal was detected. Five regions of brain were examined under a microscope for probe signals: preoptic area (POA) for GnRH1, nucleus lateralis valvulae (NLV) for GnRH2, olfactory bulb (OB) for GnRH3, and nucleus posterioris periventricularis (NPPv) and nucleus ventral tuberis (NVT) for Kiss1. The area with each probe signal was measured in each region of all brain slices with Image J software, version 1.44 (National Institutes of Health, Bethesda, MD, USA). Animal experiments were approved by the Animal Care and Use Committee of the Faculty of Science and Technology, Kindai University (Higashiosaka City, Japan; approval no. KASE-20-008).

2.4. Enzyme immunoassay of 11-ketotestosterone

Plasma 11-ketotestosterone (11-KT) concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, Cat. No. 582751) in accordance with the manufacturer's instructions. Prior to assay, plasma samples were extracted three times with ethyl acetate/hexane in glass tubes, and then reconstituted in assay buffer. All samples were assayed in duplicate, plates were read at 415 nm using a microplate reader (MTP-450, Corona electrics), and hormone levels determined based on a standard curve. The minimum detectability of the assay was

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