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Profiles of gonadotropin-inhibitory hormone and melatonin during the sex change and maturation of cinnamon clownfish, *Amphiprion melanopus*

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ABSTRACT

The present study aimed to determine the relationship between melatonin and gonadotropin-inhibitory hormone (GnIH) and their effect on reproduction in cinnamon clownfish, *Amphiprion melanopus*. Accordingly, we investigated the expression pattern of GnIH, GnIH receptor (GnIH-R), and melatonin receptor (MT-R1) mRNA and protein, as well as the plasma levels of melatonin, during sex change in cinnamon clownfish. We found that GnIH and MT-R1 mRNA and melatonin activity were higher in fish with mature brain than in fish with developing gonads, and using double immunofluorescence staining, we found that both GnIH and MT-R1 proteins were co-expressed in the hypothalamus of cinnamon clownfish. These findings support the hypothesis that melatonin plays an important role in the negative regulation of maturation and GnIH regulation during reproduction.

1. Introduction

Until recently, gonadotropin-releasing hormone (GnRH) has been the only known hormone to control the synthesis and release of gonadotropin hormones (GTHs) from neuropeptides present in the hypothalamus. However, Tsutsui et al. [1] discovered gonadotropin-inhibitory hormone (GnIH) in the brain of Japanese quail, *Coturnix japonica*, and a subsequent study reported that GnRH is not the only hormone that controls vertebrate reproduction, since it interacts with GnIH [2,3]. Studies in birds have also demonstrated that GnIH acts on the anterior pituitary gland through a specific GnIH receptor (GnIH-R or GPR147) and inhibits the synthesis and release of GTHs, thereby influencing the hypothalamic-pituitary-gonadal (HPG) axis and controlling the reproductive cycle [3–6].

The regulation of GTH synthesis and its secretion is also affected by light exposure, including photoperiod [1], and photoperiod-mediated changes in reproductive hormone production are mainly mediated by melatonin, which is released from the pineal gland and retina [7]. Melatonin levels increase during the night and decrease during the day, thereby functioning as a neuroendocrine signal that is closely associated with the regulation of circadian rhythms [7]. The effect of melatonin is mediated by melatonin receptors (MT-Rs), which promote growth and inhibit sexual maturation by reducing the release of both follicle stimulating hormone (FSH) and luteinizing hormone (LH) [8,9]. Teleost fish have three subtypes of MTs: MT-R1, MT-R2, and MT-R3 [10,11]. Previous study reported the function of MT-R1 that modulate arterial vasoconstriction, cell proliferation in cancer cells, and reproductive and metabolic functions [12]. In addition, melatonin also affects the neurogenic function of GnIH, interacts with other hypothalamic peptides in the reproduction control system, *via* its responses to light levels and photoperiod, and stimulates the synthesis and secretion of GnIH [9,13].

Therefore, we examined the effect of GnIH and melatonin during the sex change and sexual maturation of cinnamon clownfish, *Amphiprion melanopus*. This species of clownfish typically lives as adult mated pairs or as an adult pair with an immature individual, and social ranking in the group determines the sexes of the fish. In general, the female is the larger and dominant individual. However, if a dominant female dies or becomes absent, the male partner will undergo a sex change to become female, and the immature individual will undergo a sex change to become male [14].

Abbreviations: GnIH, gonadotropin-inhibitory hormone; GnIH-R, gonadotropin-inhibitory hormone receptor; MT, melatonin; MT-R, melatonin receptor.

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In the present study, we aimed to investigate the relationship of GnIH and melatonin during sex change; for this purpose, we measured the mRNA expression of GnIH, GnIH-R, and MT-R1, as well as plasma levels of melatonin, using double staining for GnIH and GnIH-R and immunohistochemistry techniques for MT-R1 and plasma melatonin.

2. Materials and methods

2.1. Fish husbandry

The present study was conducted with male (10.5 ± 1.2 g), males at 90 days after removing female (15.2 ± 0.9 g) and female (22.2 ± 2.1 g) cinnamon clownfish, which were purchased from the Corea Center of Ornamental Reef and Aquarium (Jeju, Korea). Sexual maturity was determined by the presence of mature ova and sperm, and mated pairs (male and dominant female) were established prior to the experiments in 100-L tanks with circular filtration at 27 ± 1 °C and with a photoperiod of 12 h light:12 h dark (lights on 07:00–19:00 h). In addition, the fish were fed twice daily (09:00 h and 17:00 h) with a commercial marine aquarium fish feed (Jeilfeed Company, Kyoungnam, Korea).

The technique for inducing sex change was modified from the methods described by An et al. [15]. After mated pairs (male and dominant female) were established, sex change was induced in male fish by removing the female from each group and adding an immature individual. At 90 days after female removal, male fish underwent sex changes from males to females. We divided the sex change process into three developmental stages (i.e., maturity stages: I, mature male; II, male at 90 days after female removal; III, mature female) and sampled fish from each stage. All fish were anesthetized in 2-phenoxyethanol (Sigma, St. Louis, MO, USA) and decapitated prior to tissue collection, during which brain tissue was removed, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. The plasma samples were separated from blood using centrifugation (4 °C, 10,000 × *g*, 5 min) and stored at −80 °C until analysis.

2.2. Quantitative PCR (qPCR)

QPCR was conducted to determine the relative expression levels of GnIH, GnIH-R, and MT-R1 mRNA using cDNA reverse-transcribed from the total RNA extracted from the brains during the sex change. Total RNA was extracted from each sample using the TRI reagent® (Molecular Research Center, Inc., Ohio, USA) according to the manufacturer's instructions using DNase treated total RNA. Total RNA (2 μg) was reverse-transcribed in a total volume of 20 μL, using an oligo-d(T)$_{15}$ anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at 4 °C for use in qPCR. The following qPCR primers were designed with reference to the known sequences of the cinnamon clownfish (GenBank accession numbers: GnIH, KT455505; GnIH-R, KT455506; MT-R1, HM107821; β-actin, JF273495): GnIH forward (5′-CCC TCT TCG CTT CGG GCG GGA TG-3′) and reverse (5′-GAA TCG CTG AGG GAG GTT GAT A-3′) primers; GnIH-R forward (5′-AAC CAC AGC GGC TCA GTG TGT CC-3′) and reverse (5′-ACC AGA CAG AGG AAG ACA AA-3′) primers; MT-R1 forward (5′-CTG CTG GTG GTG ATG ATG-3′) and reverse (5′-GGT CTC TCT TCC CTC CTG-3′) primers; β-actin forward (5′-GGA CCT GTA TGC CAA CAC TG-3′) and reverse (5′-TGA TCT CCT TCT GCA TCC TG-3′) primers. qPCR amplification was conducted, using a Bio-Rad CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions, with initial denaturation at 95 °C for 5 min, then followed by 35 cycles of denaturation (95 °C for 20 s), annealing (55 °C for 20 s), and extension (72 °C for 10 s), followed a final extension at 72 °C for 10 min. Specific amplification was confirmed by melting curve analysis. Each experimental group was run in triplicate in 5 different experiments, and β-actin was used as an internal control. The efficiencies of the reactions were determined by analyzing the amplification curves and all data were expressed as changes, with respect to the corresponding β-actin-calculated cycle threshold (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and the internal control (β-actin) was calculated using: $\Delta\Delta Ct = 2^{\wedge}-(\Delta Ct_{sample} - \Delta Ct_{internal\ control})$.

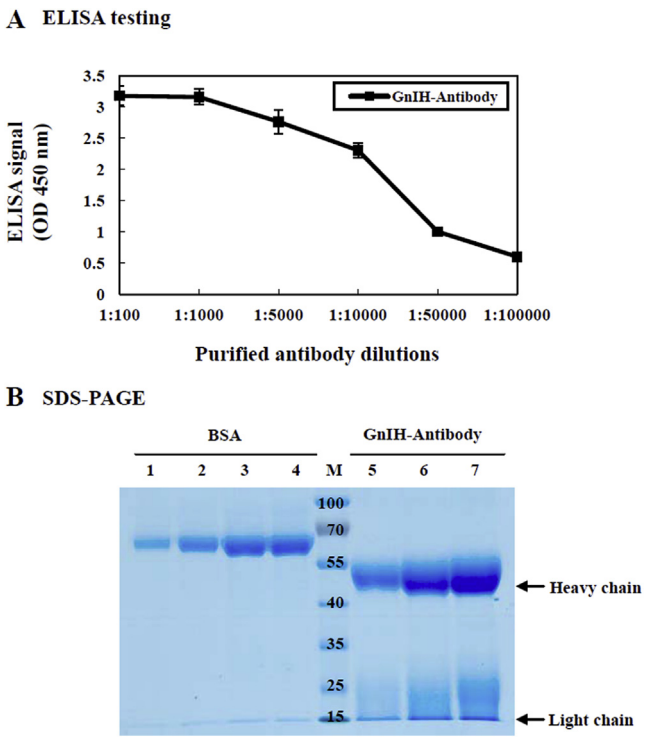

Fig. 1. Titration of the purified anti-GnIH antibody with ELISA and SDS-PAGE. (A) The ELISA curve exhibits a hyperbolic shape in the range of 1:100–1:100,000 dilution on the logarithmic dilution scale. (B) Analysis of purified GnIH polyclonal antibody using SDS-PAGE and Coomassie Brilliant Blue staining. Lane 1: 1 mg/mL bovine serum albumin (BSA), Lane 2: 2 mg/mL BSA, Lane 3: 3 mg/mL BSA, Lane 4: 4 mg/mL BSA, M: molecular weight marker, Lane 5: 1 μL of purified GnIH antibody, Lane 6: 2 μL of purified GnIH antibody, Lane 7: 3 μL of purified GnIH antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Production of GnIH polyclonal antibody

To obtain the antigen of the cinnamon clownfish GnIH antibody, a synthetic peptide was designed from highly conserved regions of GnIH amino acid sequences of cinnamon clownfish and other teleosts (N-ter-TLNVAPTSGRVSSPTILRLH-C-ter), synthesized by Cosmo Genetech (Seoul, Korea), and coupled to BSA. A rabbit was injected with 100 μg of the BSA-conjugated synthetic peptide in Freund's complete adjuvant and boosted at 2-week intervals using subcutaneous injections of 200 μg BSA-conjugated synthetic peptide in Freund's incomplete adjuvant. The rabbit was bled at 1 week after the fifth injection, and antiserum was purified *via* affinity peptide column coupling using the BSA-conjugated peptide. The purified antibody recognized full-length (23 kDa) cinnamon clownfish GnIH.

The serum antibody titer and purity of the purified antibody were determined using enzyme-linked immunosorbent assay