Comparative Biochemistry and Physiology, Part A 158 (2011) 490–497

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Diurnal expression patterns of neurohypophysial hormone genes in the brain of the threespot wrasse *Halichoeres trimaculatus*

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ARTICLE INFO

Article history:
Received 11 October 2010
Received in revised form 6 December 2010
Accepted 8 December 2010
Available online 15 December 2010

Keywords:
Arginine vasotocin
Daily rhythm
Hypothalamus
Isotocin
Melatonin
Threespot wrasse

ABSTRACT

The aim of this study was to determine the involvement of neurohypophysial hormones in the diurnal patterns of the threespot wrasse *Halichoeres trimaculatus*, which is common in coral reefs and exhibits daily behavioral periodicity. Prohormone cDNAs of the neurohypophysial peptides, arginine vasotocin (AVT) and isotocin (IT), were cloned by 3′- and 5′-rapid amplification of cDNA ends (RACE). The distribution and expression patterns of pro-AVT and -IT mRNAs in the brain were determined using reverse-transcription polymerase chain reaction (RT-PCR) and real-time quantitative PCR, respectively. The respective full-length cDNAs of pro-AVT and -IT were 945 and 755 bp in length, respectively. The deduced amino acid sequences for pro-AVT and pro-IT were 154 and 156 residues in length, respectively. Both pro-peptides contained a signal sequence followed by the respective hormones and neurophysin connected by a Gly–Lys–Arg bridge. Pro-AVT mRNA was detected only in the hypothalamus area, while pro-IT mRNA in the whole part of the brain. The relative abundance of pro-AVT and -IT mRNA varied according to time of day; it was significantly greater at 12:00 h than at 24:00 h. Following intraperitoneal administration of melatonin, pro-AVT mRNA abundance in the brain decreased, while pro-IT mRNA abundance remained unchanged. These results demonstrate that daily fluctuations of pro-AVT and pro-IT levels in the brain of threespot wrasse are differentially regulated.

1. Introduction

Arginine vasotocin (AVT) and isotocin (IT) belong to the vasopressin (AVP) and oxytocin (OXT) families, respectively. These peptide hormones are produced in the hypothalamus and released at the neurohypophysis of ray-finned fishes of the infraclass Teleostei (Acher, 1993b) and synthesized as part of a larger precursor molecule with a neurophysin carrier protein. AVT and IT are stored in the neurohypophysis and released in response to appropriate stimuli (Warne et al., 2000). These neurohypophysial hormones play a role in physiological adaptation to internal and external changes. AVT is involved in osmoregulation and cardiovascular homeostasis (Acher, 1993a) and in reproductive and social behavior (Foran and Bass, 1999; Goodson and Bass, 2001). In contrast, knowledge regarding the physiological roles of IT is limited (Warne et al., 2000), although recent reports have suggested that IT plays a role in regulating social behavior (Goodson and Bass, 2000; Goodson et al., 2003) and/or stress responses (Mancera et al., 2008).

High-performance liquid chromatographic analyses have revealed that in the plasma of both the rainbow trout *Oncorhynchus mykiss* (Kulczykowska and Stolarski, 1996; Kulczykowska, 1999) and the European flounder *Platichthys flesus* (Kulczykowska et al., 2001), AVT, but not IT, levels were higher during daylight hours and decreased during the night. Additionally, IT levels were higher at midnight than during the day in the brains of juvenile Atlantic salmon *Salmo salar* (Gozdowska et al., 2006). Diurnal changes in AVT in the neural tissues of rainbow trout were also examined; AVT transcript abundance in parvocellular neurons remained high during the light phase and then decreased during the dark phase (Gilchriest et al., 1998), which suggests that the neurohypophysial hormones are involved in various physiological processes. Thus, these peptides may be secreted into the circulation and directly influence physiological processes in peripheral tissues. Alternatively, external stimuli may be conveyed from the hypothalamic region to the pituitary through the action of peptide hormones and finally to the peripheral tissues through fluctuations in pituitary hormone concentrations. This latter is supported by the fact that single AVT and IT neurons project toward both the pituitary and extra-hypothalamic regions in teleosts (Saito et al., 2004), but how external stimuli are transduced to daily changes in AVT and IT levels remains unclear. One possibility is that the circadian system in neurons directly regulates neurohypophysial hormone

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1095-6433/$ – see front matter
doi:10.1016/j.cbpa.2010.12.011

levels, although no evidence supporting this exists in the literature. Alternatively, melatonin may modulate AVT and IT levels, since this indoleamine hormone is synthesized by the pineal organ (a primary mediator of photoperiodic information) and fluctuates daily with a decrease during daytime and an increase during nighttime (review in Falcón et al., 2010). Indeed, melatonin has been reported to regulate AVP levels for mammals (Yasin et al., 1993) and AVT for fishes (Kulczykowska, 1999; Kulczykowska et al., 2001).

Wrasses (family Labridae) inhabit the rocky and coral reefs of tropical and temperate waters. Most wrasse species exhibit a day-active and night-inactive rhythm; the fish swim and show feeding and reproductive activities during daylight and rest under the sandy bottom during the night. Some wrasses repeat such rhythmic activity even under invariant illumination, suggesting a robust circadian regulation (Lenke, 1988; Nishi, 1989, 1990, 1991; Gerkema et al., 2000). Therefore, wrasse species are useful for studies on how the circadian system regulates daily activities in fish. The aim of this study was to examine the involvement of AVT and IT in the circadian system of the threespot wrasse *Halichoeres trimaculatus*, a species common in coral reefs. To attain our purpose, we first cloned and characterized the cDNA of pro-AVT and -IT using 3′- and 5′-rapid amplification of cDNA ends (RACE) and then examined diurnal variations in pro-AVT and -IT mRNA abundance in the brain using real-time quantitative PCR (qPCR). In addition, involvement of melatonin in the regulation of pro-AVT and -IT transcript abundance in the brain was evaluated.

2. Materials and methods

2.1. Animals

Mature fish were collected in July 2009 from coral reefs around Sesoko Island, Okinawa, Japan, during the daytime low tide by fishing with a hook and line. They were reared in outdoor polyethylene tanks (200 L) with aerated running seawater under natural photoperiod (LD14:10) and water temperature conditions (30 ± 1 °C) at Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Japan, and were fed with commercially available pellets (EP3; Marubeni Nisshin, Tokyo, Japan). Fish used in the present study had body mass and total length of 18.5 ± 2.7 g and 11.9 ± 0.7 cm, respectively.

After acclimation for at least 2 weeks, fish were removed from tanks, anesthetized in seawater containing 0.01% 2-phenoxyethanol (Kanto Chemicals, Tokyo, Japan), and then euthanized by decapitation. At 12:00 h, fish were weighed and organs and tissues (brain, pituitary liver, intestine, kidney, spleen, gonad, and skin) ($n = 7$) were collected to assess the organ and tissue distribution of AVT and IT mRNA. A portion of the brain samples was separated into five parts; part A, telencephalon; part B, hypothalamus including preoptic area; part C, optic tectum; part D, cerebellum; and part E, medulla oblongata (Fig. 1). For assessing day–night difference in AVT and IT mRNA levels, the whole brain was collected at 12:00 and 24:00 h ($n = 10$ each). Tissue collection at 24:00 h was carried out under dim light conditions. Samples were immediately frozen and stored at −80 °C until required.

To evaluate the effect of melatonin on AVT and IT mRNA abundance in the brain, fish were transferred to two aquaria (60 L) with running seawater at ambient temperature. After acclimatization for 1 week, melatonin (1 mg kg^{-1} in saline; Sigma, St. Louis, MO, USA) was injected i.p. into the fish ($n = 18$) in one aquarium at 11:00 h. The vehicle only was injected i.p. to fish ($n = 18$) in the other aquarium (control group). At 0, 1, and 2 h post-injection, the brain ($n = 6$ per group) was harvested and immediately frozen at −80 °C.

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

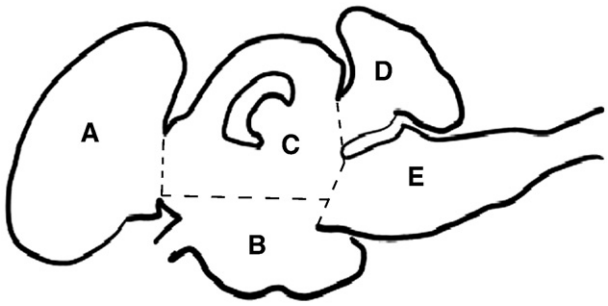

Fig. 1. Schematic diagram of the threespot wrasse brain (sagittal section) used for RT-PCR analysis of pro-AVT and pro-IT mRNA expressions. The brain was separated into five portions; part A; telencephalon, part B; hypothalamus including preoptic area, part C; optic tectum, part D; cerebellum, and part E; medulla oblongata.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from the frozen tissues using the TriPure Isolation reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. Genomic DNA contaminating the total RNA was digested with deoxyribonuclease (Wako, Tokyo, Japan). The quantity of total RNA was assayed spectrophotometrically at 260 and 280 nm, and samples with an A260/A280 ratio of 1.7–2.0 were used for cDNA synthesis. cDNA was synthesized from 1000 ng of total RNA using the PrimeScript™ RT Reagent Kit (TaKaRa Bio, Otsu, Japan) for molecular cloning and real-time quantitative PCR, respectively, according to the manufacturer's instructions.

2.3. Cloning of pro-AVT and -IT cDNA

Pro-AVT and -IT cDNA fragments were produced by reverse-transcription (RT)-PCR amplification using a degenerate primer set (AVT-Forward, AVT-Reverse for AVT and IT-Forward, IT-Reverse for IT; Table 1). Primers were designed based on the highly conserved regions of pro-AVT and -IT sequences from several fish species (GenBank Accession Numbers: *Platichthys flesus*, AB036517; *Takifugu niphobles*, AB297919; *Thalassoma bifasciatum*, AY167033 for AVT; *Takifugu rubripes*, AB297920; *Takifugu niphobles*, U90880; *Oncorhynchus keta*, D10941 for IT). PCR was performed using 30 cycles each of denaturation (45 s at 94 °C), annealing (45 s at 53 °C), and extension (1 min at 72 °C). PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and then sequenced using a PRISM 3730XL Analyzer (Applied Biosystems, Foster City, CA, USA).

After identity of the amplified DNA fragments had been confirmed by BLAST analysis, full-length cDNA was obtained by RACE using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. AVT- and IT-specific and nested primers for RACE were designed based on 277 and 217 bp partial cDNA fragment sequences (Table 1). The initial PCR was carried out using 5 cycles at 94 °C for 5 s, 72 °C for 3 min; 5 cycles at 94 °C for 5 s, 70 °C for 10 s, and 72 °C for 3 min; and 25 cycles at 94 °C for 5 s, 68 °C for 10 s, and 72 °C for 3 min. Nested PCR was performed using 28 cycles and the following conditions: 94 °C for 5 s, 68 °C for 10 s, and 72 °C for 2 min. cDNA fragments were cloned into the pGEM-T easy vector system and sequenced.

2.4. Sequence analysis

Nucleotide sequences were analyzed using BLASTN (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA) and the amino acid sequences of AVT and IT deduced using a translator program (ORF Finder, NCBI, http://www.