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*

Sung-Pyo Hur [a], Yuki Takeuchi [a], Yuriko Esaka [a], Wambiji Nina [a], Yong-Ju Park [c], Hyeong-Cheol Kang [c], Hyung-Bok Jeong [b], Young-Don Lee [c], Se-Jae Kim [b], Akihiro Takemura [a,*]

[a] Department of Biology, Chemistry and Marine Sciences, Faculty of Science, University of the Ryukyu, Nishihara, Okinawa 903-0213, Japan
[b] Department of Biology, Jeju National University, 66 Jejudaehakno, Jeju Special Self-Governing Province 690-756, Republic of Korea
[c] Marine and Environmental Research Institute, Jeju National University, 3288 Hamduk, Jocheon, Jeju Special Self-Governing Province 695-814, Republic of Korea

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.
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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

* Corresponding author. Tel./fax: +81 98 895 8993.
E-mail address: takemura@sci.u-ryukyu.ac.jp (A. Takemura).

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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.

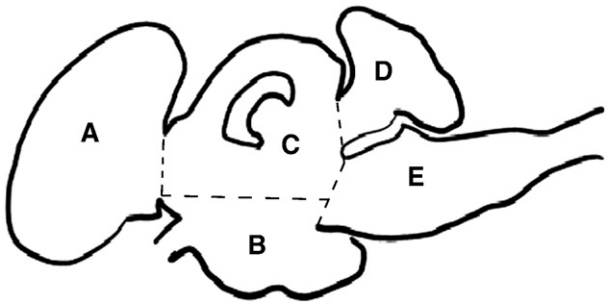

Fig. 1. Schematic diagram of the threespot wrasse brain (sagittal section) used for RT-PCR analysis of pro-AVT and pro-IT mRNA expressions. The brain was separated into five portions; part A; telencephalon, part B; hypothalamus including preoptic area, part C; optic tectum, part D; cerebellum, and part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

Table 1
Primer sets used in this study.

Primer	Sequence
Cloning primer for partial	
AVT-Forward	5′-GAC AGT GCA TGT CGT GYG-3′
AVT-Reverse	5′-ARC TCT CTG TGT TAC AGC AG-3′
IT-Forward	5′-ACA TCT CCA ACT GTC CCA TC-3′
IT-Reverse	5′-GCA GGA GCT TCA GGA TGA C-3′
Cloning primer for RACE PCR	
AVT-GSP1	5′-TCC CTC AGA TCC ACA GGC TCT ACC TC-3′
AVT-GSP2	5′-AGT GCA TGT CGT GTG GTC CCA GAG-3′
AVT-NGSP1	5′-GGG GTG AGC AGG TAG TTC TCC TCC A-3′
AVT-NGSP2	5′-CCC AAT ATC TGC TGT GGG GAA GGT C-3′
IT-GSP1	5′-TGG CAG GGG GTG AGC AGG TAG TTC T-3′
IT-GSP2	5′-GAG GTC AAT CAT GGA CGC ACC TCA G-3′
IT-NGSP1	5′-CAG CAG ATA CTG GGG CCA AAG CAG-3
IT-NGSP2	5′-GCT GCT TTG GCC CCA GTA TCT GCT-3′
RT-PCR primer	
AVT-PCR-Forward	5′-CAC TCC GTG AAC CCT CTG TG-3′
AVT-PCR-Reverse	5′-GTG GAA CAG GGA TGG TCT TC-3′
IT-PCR-Forward	5′-GTG TCC GTG TGC CTT CTT TT-3′
IT-PCR-Reverse	5′-TCA GCA TCA CAG CAG ACT CC-3′
β-actin-PCR-Forward	5′-ACT ACC TCA TGA AGA TCC TG-3′
β-actin-PCR-Reverse	5′-TTG CTG ATC CAC ATC TGC TG-3′
Real-time qPCR primer	
AVT-qPCR-Forward	5′-GAC AGG GAT CAG ACA GTG CAT-3′
AVT-qPCR-Reverse	5′-CTC CTC CAC ACA GTG AGC TG-3
IT-qPCR-Forward	5′-CAC AGC GCA AGT GCA TGT-3′
IT-qPCR-Reverse	5′-CCA AAC AGT GGG CTG TCT CT-3′
β-actin-qPCR-Forward	5′-GAG ATT GTG CGT GAC ATC AAG GAG-3′
β-actin-qPCR-Reverse	5′-CAT CAG GCA GCT CGT AGC TCT TC-3′

R (A, G); Y (C, T).

ncbi.nlm.nih.gov/gorf/gorf.html). Sequence identities were verified using BLAST, NCBI database. Multiple alignments for phylogenetic analysis were carried out using ClustalW (http://www.ebi.ac.uk/clustalw). A phylogenetic tree was constructed by the JTT method with the PRODIST program from the PHYLIP package (ver. 3.63, J. Felsenstein, University of Washington, Seattle, WA, USA). One thousand bootstrap trials were run using the neighbor-joining method. The CONSENSE program of PHYLIP was used to construct a strict consensus tree.

2.5. RT-PCR analysis of pro-AVT/IT mRNA abundance

The abundance of pro-AVT and -IT mRNA in the peripheral and neural tissues of threespot wrasse was assessed by RT-PCR. Primer sets are shown in Table 1. Amplicon length was maintained as close to 400–500 bp as possible, and the primer melting temperatures were set at 58 °C. Each PCR reaction mix contained 50% GoTaq Green master mix (Promega), 10 μM of each forward and reverse primer, and 50 ng of cDNA template. The RT-PCR cycling conditions were followed by 28 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 58 °C, and extension for 1 min at 72 °C.

2.6. Real-time quantitative PCR (qPCR)

Pro-AVT and -IT transcript levels were assayed using the CFX96™ Real Time System (Bio-Rad, Hercules, CA, USA) and a SYBR Green premix PCR kit (TaKaRa Bio). Primer sequences were designed using the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Table 1). The primer sets were selected to amplify a cDNA fragment that spanned two exon/intron boundaries of the pro-AVT and -IT gene, thus preventing amplification of genomic DNA. Amplicon length was maintained as close to 80–150 bp as possible and the primer melting temperatures set at 60 °C. Each PCR reaction mix contained 50% SYBR Premix Ex Taq, 10 μM of forward and reverse primer, and 20 ng of cDNA template. The initial 1 min denaturation was followed by 40 cycles of denaturation for 5 s at 95 °C, and annealing and extension for 1 min at 60 °C. To ensure specificity, a dissociation curve analysis was performed by slowly raising the temperature of the sample from 60 °C to 95 °C. Temperature curves showed a single amplified product with complete absence of primer–dimer formation (data not shown). Expression of the pro-AVT and -IT genes in each sample was normalized to the amount of the internal control *β-actin* gene.

2.7. Statistical analysis

All qPCR data are expressed as the mean ± standard error of the mean (SEM). For comparing pro-AVT and -IT abundance between the day (12:00 h) and night (24:00 h) groups, Student's *t*-test was used. Two-way analysis of variance (ANOVA) was performed to compare the effect of melatonin treatment on pro-AVT and pro-IT mRNA abundance, followed by Newman-Keuls test. A probability of $P<0.05$ was considered statistically significant.

3. Results

Full-length cDNAs of pro-AVT (GenBank Accession No. GU212657) and pro-IT (GU212656) from the threespot wrasse were 945 (Fig. 2A) and 755 bp (Fig. 3A) in length, respectively. Both pro-AVT and -IT contained a complete open reading frame (460 and 471 bp long, respectively). The deduced amino acid sequence consisted of 154 amino acids for pro-AVT and 156 amino acids for pro-IT, both of which were on the order of what would be expected for a signal peptide, the hormone, a Gly–Lys–Arg bridge, and neurophysin. The neurophysins of pro-AVT and pro-IT included a leucine-rich core segment that resembled copeptin of mammalian AVP and OXT families, respectively.

The deduced amino acid sequence of pro-AVT was compared with that of both nonmammals and mammals pro-AVPs. A phylogenetic analysis was performed to characterize the evolutionary relationships between nonmammals and mammals pro-AVPs. Pro-AVT of the threespot wrasse was most similar to that of other fish species (94–96%), followed by pro-AVTs of amphibians (51–57%) and mammals (48–53%) (Fig. 2B). The deduced amino acid sequence of pro-IT was also compared with that of the prohormones of IT, OXT, and mesotocin (MT). Threespot wrasse pro-IT showed a high sequence similarity to that of other fish species (95%), followed by pro-OXT of mammals (54–56%), and pro-MT of nonmammals (52–61%) (Fig. 3B).

Distribution of pro-AVT and -IT mRNA levels was analyzed by RT-PCR. When expression of both genes was checked in peripheral and neural tissues, they were detected only in the whole brain (Fig. 4A). In the portions of the brain, pro-AVT mRNA was expressed in the hypothalamus area (Fig. 4B). On the other hand, pro-IT mRNA was expressed in the telencephalon, hypothalamus area, optic tectum, cerebellum and medulla oblongata (Fig. 4B). The pro-AVT and pro-IT mRNA expression pattern did not differ between mature females and males (data not shown).

Fig. 5 shows the day–night difference in pro-AVT and -IT mRNA abundance in the brain. Pro-AVT and -IT mRNA abundance was significantly higher at 12:00 h than at 24:00 h. A similar pattern of pro-AVT and pro-IT mRNA abundance was observed in both sexes (data not shown).

The effect of a single i.p. melatonin injection was assessed. Pro-AVT mRNA levels in the whole brain decreased significantly within 1 h of melatonin administration. No significant difference in pro-AVT abundance was observed between the experiment and control groups at 2 h after administration (Fig. 6A). The abundance of pro-IT mRNA in the whole brain was not altered by melatonin administration (Fig. 6B).

A

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AGACATCCACACAGGAATCATCTCATCCAACACCAGCAACGATGCCGCACTCCGTGAACC    60
                                              M  P  H  S  V  N  P

CTCTGTGCGTCCTGGGACCCCTCGTCGCGCTGTCCTCGGCTTGCTACATTCAGAACTGTC   120
  L  C  V  L  G  P  L  V  A  L  S  S  A  C  Y  I  Q  N  C  P
 Signal peptide                                  Hormone

CCCGAGGAGGGAAACGCGCACTGCCGGAGACAGGGATCAGACAGTGCATGCCGTGTGGTC   180
  R  G  G  K  R  A  L  P  E  T  G  I  R  Q  C  M  P  C  G  P

CCAGAGACAAGGGCCGCTGCTTCGGCCCCAATATCTGCTGTGGGGAAGGTCTTGGCTGTT   240
  R  D  K  G  R  C  F  G  P  N  I  C  C  G  E  G  L  G  C  L
                                 Neurophysin

TGTTGGGCTCCCCAGAAACAGCTCACTGTGTGGAGGAGAACTACCTGCTCACCCCCTGCC   300
  L  G  S  P  E  T  A  H  C  V  E  E  N  Y  L  L  T  P  C  Q

AGGCAGGAGGTAGAGCCTGTGGATCTGAGGGAGGGCGCTGTGCTGCCTCAGGAGTCTGCT   360
  A  G  G  R  A  C  G  S  E  G  G  R  C  A  A  S  G  V  C  C

GTACCTCAGAGAGCTGTGCGGTGGACTCTGACTGCCTGGGGGAGATCGAGCCATCAGATC   420
  T  S  E  S  C  A  V  D  S  D  C  L  G  E  I  E  P  S  D  Q

AGGCCGACAGCTCTGCAGGGAGCTCGCCTGCAGAGCTGCTGCTGCGGCTGCTACATGTGG   480
  A  D  S  S  A  G  S  S  P  A  E  L  L  L  R  L  L  H  V  A
                                 Copeptine

CCACAAGAGGACAGACCGAGTACTGAATCAGACAAGAATGAAGACCATCCCTGTTCCACT   540
  T  R  G  Q  T  E  Y  *

TTCAGCCTAGAGATTTAAACCTTGAACCAATAAAAATCAAAATCCATGTCAAAGCTCAAC   600
TCTTTTTCCTAACTGCTGTGATCTGTCATATAGAGAGAACTATTTTCATTTTCAGAGGAA   660
AGAGAGGGACATAGATTATGTGTATGATAAGACTATAGAATACATGTTTATTTCCACATA   720
GAAATGTACATAATTTTGCACATACTGTATACAAAATTTATTCTTTATCATGCAAGAAAG   780
AAATTGTCTACCGAGTTTTGGAAACACTGTAAATAAACCAGTGAGTGGGGACGAAAGTTG   840
CAAGGAACATCCATGTAAAACCATGCTTGAGCAGTTGGTTCTTGGCTCTTAGAAATATTA   900
TTGTTTATTGAAAATGTGTTAAAAATACAGAGTGTATCACTACCA-(Poly A tail)    945
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B

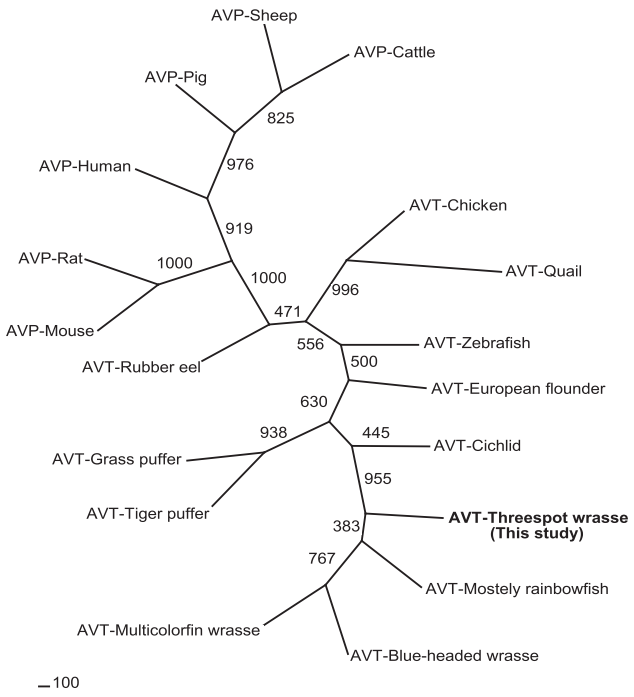

Fig. 2. (A) Nucleotide and deduced amino acids sequences of the ORF region in the *H. trimaculatus* AVT cDNA (GenBank accession NO. GU212657). Signal peptide, hormone, and neurophysin are indicated by solid lines, while copeptine by a dot line. Opened column suggests leucine-rich core segment. The stop codon is marked by an asterisk. (B) Phylogenetic tree of AVT. Lengths of horizontal lines indicated the genetic distance. One thousand bootstrap repetitions were performed, and values are shown at the inner nodes. Scientific name accession numbers for AVT and AVP protein used in the phylogenetic trees analysis are as follows: blue-headed wrasse (*T. bifasciatum*, AY167033), cattle (*Bos taurus*, NM_176854), chicken (*Gallus gallus*, X55130), cichild (*A. burtoni*, AF517935), European flounder (*Platichthys flesus*, AB036517), grass puffer (*T. niphobles*, AB297919), human (*Homo sapiens*, BC126224), mostely rainbowfish (*H. tenuispinis*, GU212654), mouse (*Mus musculus*, NM_009732), multicolorfin wrasse (*H. poecilopterus*, DQ073094), pig (*Sus scrofa*, NM_213952), quail (*Coturnix coturnix*, AY786510), rat (*Rattus norvegicus*, NM_016992), rubber eel (*Typhlonectes natans*, AF228336), sheep (*Ovis aries*, NM001126341), tiger puffer (*T. rubripes*, FRU90880), zebrafish (*Danio rerio*, AY168623) and threespot wrasse (this study).

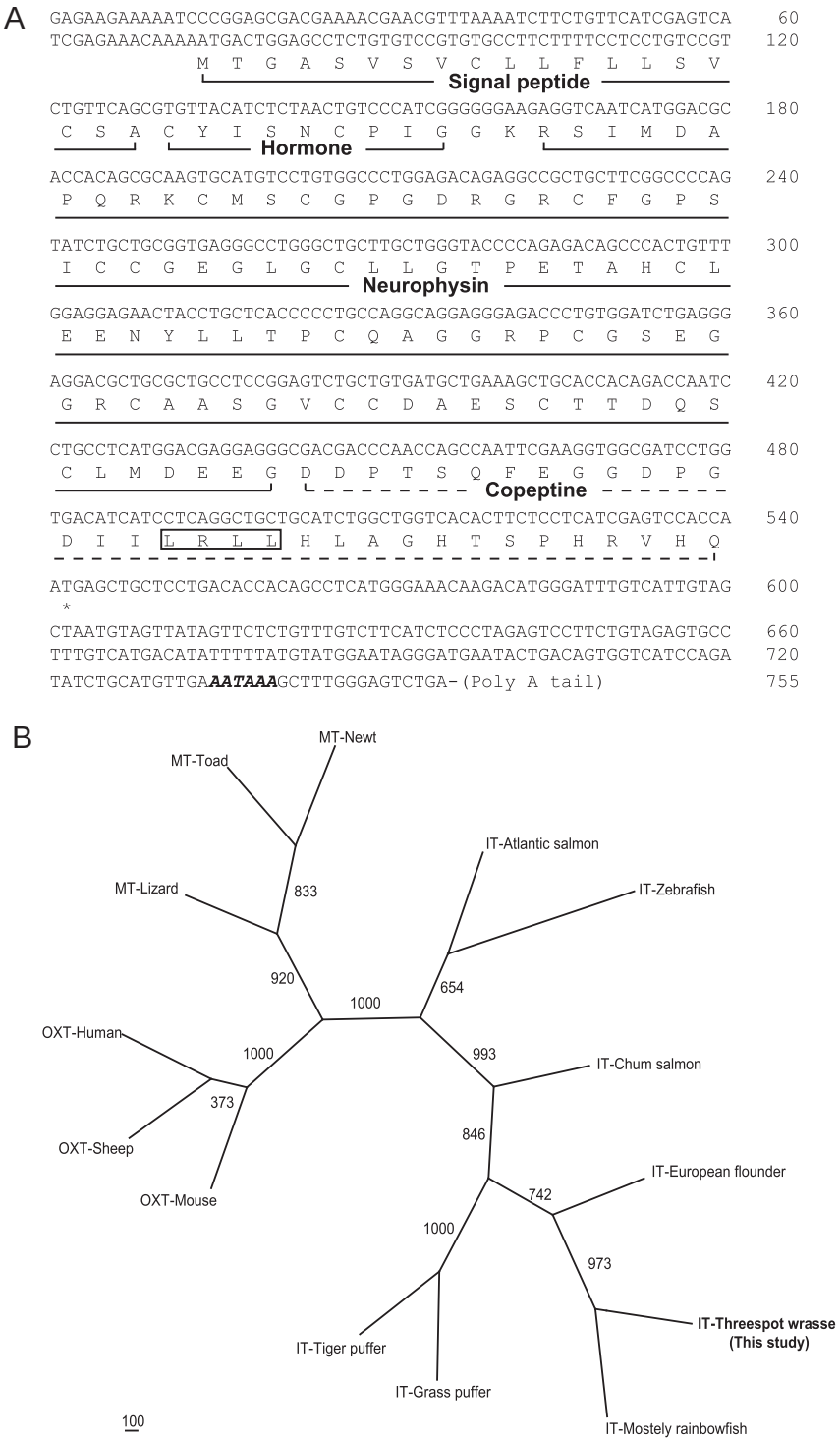

Fig. 3. (A) Nucleotide and deduced amino acids sequences of the ORF region in the *H. trimaculatus* IT cDNA (GenBank accession NO. GU212656). Signal peptide, hormone, and neurophysin are indicated by solid lines, while copeptine by a dot line. Opened column suggests leucine-rich core segment. (B) Phylogenetic tree of IT. Lengths of horizontal lines indicated the genetic distance. One thousand bootstrap repetitions were performed, and values are shown at the inner nodes. Scientific name accession numbers for IT, MT and OXT protein used in the phylogenetic trees analysis are as follows: Atlantic salmon (*Salmo salar*, EF558622), chum salmon (*Oncorhynchus keta*, D10940), European flounder (AB036518), grass puffer (AB297919), human (NM_000915), lizard (*Podarcis sicula*, AY052827), mostely rainbowfish (GU212655), mouse (NM_011025), newt (*Taricha grnulosa*, EF526213), sheep (NM001009801), tiger puffer (FRU90880), toad (*Bufo japonicas*, M16232), zebrafish (AY069956) and threespot wrasse (this study).

4. Discussion

We successfully cloned threespot wrasse pro-AVT and -IT cDNAs, which encoded putative proteins consisting of 154 and 156 amino acids, respectively. The sequences contained a signal peptide followed by the respective hormone and neurophysin linked by a Gly–Lys–Arg bridge. The structures of threespot wrasse pro-AVT and -IT were similar to those of the white sucker *Catostomus commersoni* (Heierhorst et al., 1989), the European flounder *P. flesus* (Warne et al., 2000), several salmonids (Hyodo and Urano, 1991; Hiraoka et al., 1997), and the grass puffer *T. rubripes* (Kato et al., 2005; Motohashi et al., 2008). Phylogenetic analysis revealed that pro-AVT of the threespot wrasse clustered with

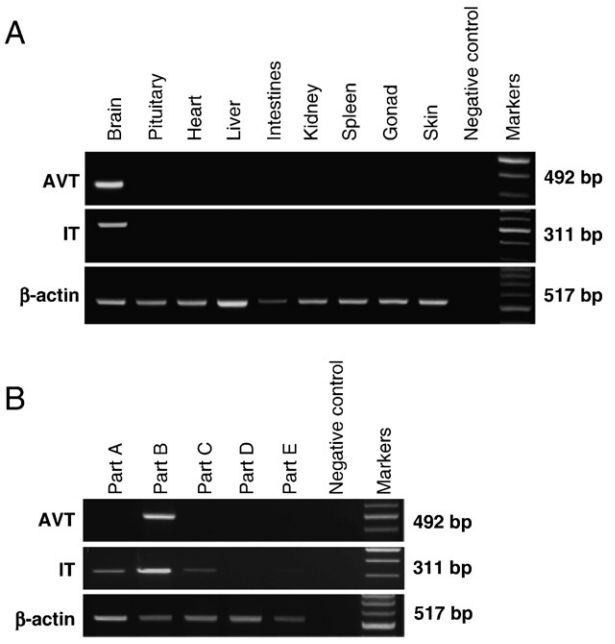

Fig. 4. Distribution of pro-AVT and pro-IT mRNA expressions in the organ and tissue (A) and portion of brain (B) of threespot wrasse. Organ and tissue samples ($n=7$) were collected from the fish at 12:00 h. The brain was further divided into five parts (see Fig. 1) They were analyzed with RT-PCR. The expression of β-actin mRNA was used as reference. Samples without cDNA templates were loaded as negative control. Marker; 100 bp DNA ladder.

nonmammals AVT and mammals AVP. Similarly, pro-IT of this species clustered with mammals OXT and several nonmammals MT. These results suggest that our clones contain the sequence of a neurohypophysial hormone.

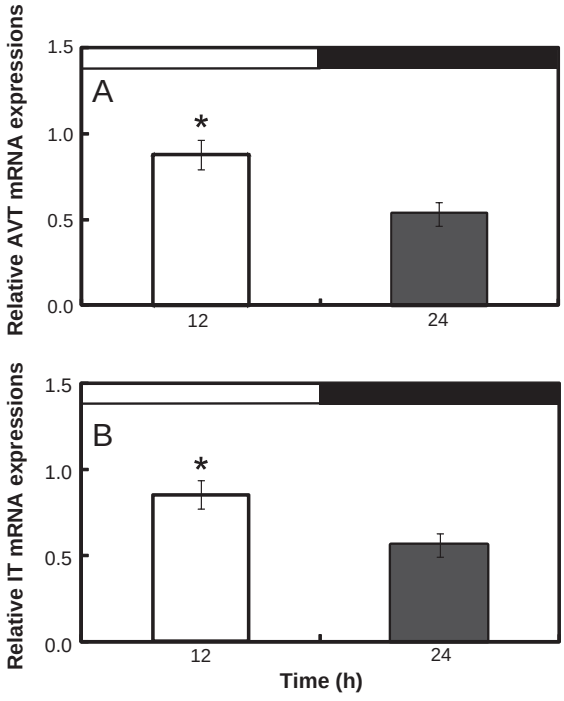

Fig. 5. Day–night variations of mRNA abundance of pro-AVT (A) and pro-IT (B) in the brain of threespot wrasse. After acclimatization, the fish were sampled at 12:00 h ($n=10$) and 24:00 h ($n=10$). The brain was subjected to measurement of mRNA abundance of pro-AVT and pro-IT with real-time quantitative PCR. The relative values of pro-AVT and pro-IT expression were normalized against β-actin and then averaged. Values are expressed as means ± SEM. Horizontal bar with white and black colors in the figures indicates light and dark phases, respectively. Asterisk represents statistical difference at $P<0.05$.

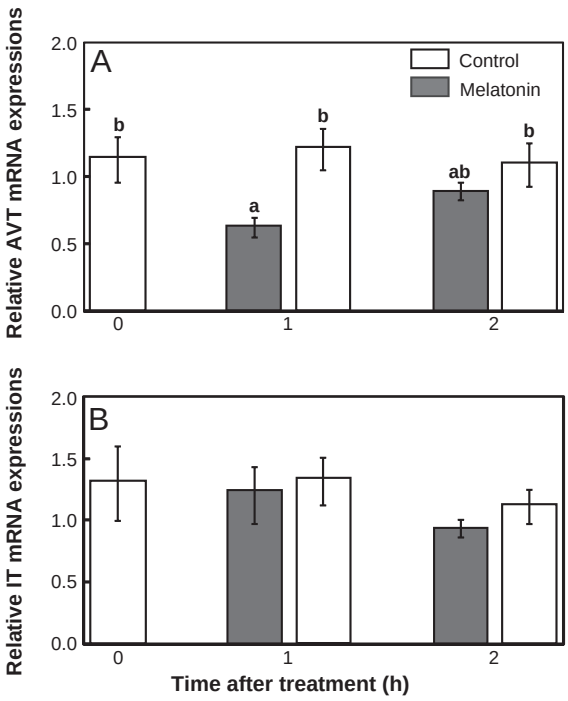

Fig. 6. Effect of a single melatonin injection on mRNA abundance of pro-AVT (A) and pro-IT (B) in the brain of threespot wrasse. Melatonin was administrated i.p. to the fish of experimental group ($n=6$) at concentration of 1 mg/kg body weight. The vehicle was injected to the fish of control group ($n=6$). The brain was collected at 0, 1 and 2 h after injection and subjected to measurement of mRNA abundance of pro-AVT and pro-IT with real-time quantitative PCR. The relative values of pro-AVT and pro-IT expression were normalized against β-actin and then averaged. Values are expressed as means ± SEM. The different letters indicate statistically different values ($P<0.05$).

Several studies on the distributions of AVT and IT in the fish brain have been published (Goossens et al., 1977; Van den Dungen et al., 1982; Goodson et al., 2003; Saito et al., 2004). Saito et al. (2004) observed by immunohistochemistry that AVT and IT fibers are distributed widely in the ventral telencephalic area, the ventral and dorsal thalami, the anterior and posterior tuberis, the torus semicircularis, and the optic tectum in the brain of rainbow trout. Molecular approaches have revealed that AVT and IT genes are expressed in the magnocellular and parvocellular neurons of the preoptic area (Gilchriest et al., 1998; Godwin et al., 2000). In this study, we detected pro-AVT and -IT transcripts in the brain. However, their expression patterns differed; AVT mRNA was expressed only in the hypothalamus, while IT mRNA was present in the telencephalon, hypothalamus including preoptic area, optic tectum, cerebellum, and medulla oblongata. These results indicate that the expression of AVT mRNA is more localized than that of IT mRNA. Goodson et al. (2003) reported that in contrast to AVT, IT-like immunoreactivity was widely distributed in the area of the vocal–acoustic system in the plainfin midshipman fish *Porichthys notatus* and indicated that IT is associated with the whole process of vocal–acoustic behavior in this fish. No experimental evidence exists on the differences in AVT and IT transcript expression patterns in the threespot wrasse. However, IT may be involved in various physiological processes that are themselves related to wrasse behavior. Alternatively, physiological changes relating to seasonal maturity and sex change may be involved in generating this difference in gene expression.

Pro-AVT mRNA abundance in whole-brain samples was highest during the day and decreased at night. Thus, this gene product may participate in daytime-based physiological processes in this species. Several studies have reported a similar phenomenon for both mammalian AVP (Burbach et al., 1988; Yamase et al., 1991; Kalsbeek et al., 1995) and fish AVT (Gilchriest et al., 1998; Kulczykowska, 1999;

Kulczykowska et al., 2001). Kulczykowska (1999) measured plasma AVT levels in rainbow trout that had been acclimatized to freshwater under natural photoperiodic conditions (LD16:8). AVT concentrations increased steadily during the light phase, peaked at the end of the light phase (16:00 h), and then decreased to the basal level at 3 h before sunrise (05:00 h). A similar daily variation in plasma AVT levels was observed in chronically cannulated European flounders maintained under natural photoperiodic conditions (LD16:8) (Kulczykowska et al., 2001). Moreover, a quantitative *in situ* hybridization method demonstrated that in rainbow trout reared under long photoperiodic conditions (LD16:8), AVT mRNA abundance in the parvocellular neurons, but not in the magnocellular neurons, increased during the light phase and decreased during the dark phase, although immunological reaction against anti-AVT antibody was observed in both neuronal populations (Gilchriest et al., 1998). Thus, AVT transcript levels in the hypothalamus and secretion into the blood circulation are likely activated during the day if the fish were reared under long photoperiodic conditions. Gilchriest et al. (1998) also noted that AVT mRNA abundance in the parvocellular neuron is related to diurnal activity of the hypothalamo–pituitary interrenal axis because of an inverse relationship between changes in AVT mRNA abundance and plasma cortisol levels. Therefore, secretion of pituitary hormones is partially regulated by the action of AVT. Daily variation in AVP levels is related to oscillation of the circadian system because AVP is synthesized in the suprachiasmatic nucleus (SCN), which is the center of the circadian system in mammals (Harmar, 2003). The contribution of AVT to the circadian system remains unknown in teleosts; existence of the master clock is not evidenced, although the pineal organ shares abilities of circadian system in certain teleosts.

Since plasma levels of melatonin and AVT fluctuate in opposite phases, studies have proposed that some interaction occurs between these hormones in fish (Kulczykowska, 1999; Kulczykowska et al., 2001). Indirect evidence supporting this interaction was obtained in rainbow trout, in which the plasma AVT level was highest at night (22:30 h) under continuous light conditions, whereas it was low during the day (11:30 h) under continuous dark conditions (Kulczykowska, 1999). In the case of salmonids, melatonin synthesis in the pineal organ is driven only by the environmental light–dark cycle, and not by the biological clock (Iigo et al., 2007a, b). In the European flounder, administration of melatonin at night failed to lower plasma AVT levels (Kulczykowska et al., 2001). This may be partially due to the time that the experiment was carried out or to endogenous melatonin that is itself influenced by a biological clock (review in Falcón et al., 2010). Our data suggest that daytime melatonin administration suppressed pro-AVT mRNA abundance in the brain within 1 h. Although plasma melatonin levels in labrid species have not been measured, ocular melatonin synthesis is regulated in a circadian manner (Iigo et al., 2006). Therefore, melatonin, in conjunction with the circadian system, may be responsible for the down-regulation of pro-AVT transcript production in the brain. This thought may be supported by *in vitro* experiments in rat, in which melatonin inhibited the release of both AVP and OXT in the cultured hypothalamus within 20 min after melatonin treatment (Yasin et al., 1993; Yasin and Forsling, 1998). It is suggested that melatonin has an effect of acute suppression, but not on phase-shifting, of neurophysins.

Pro-IT mRNA abundance in the whole brain of threespot wrasse showed a similar day–night change to that of pro-AVT mRNA. This was comparable to previous reports in juvenile rainbow trout (Kulczykowska, 1999) and Atlantic salmon (Gozdowska et al., 2006), in which plasma IT levels did not show daily fluctuations. In addition, little change in IT mRNA abundance in parvocellular neurons was observed in rainbow trout (Gilchriest et al., 1998). In this point, maturity level of the fish used may be important, as was the case in the masu salmon *Oncorhynchus masou*. Since there are evidence that sex steroid hormones influence IT mRNA abundance (Ota et al., 1999a, b; Saito et al., 2003). Since we used sexually mature individuals, it is likely that sex steroid hormones alter IT mRNA abundance in this wrasse species. Alternatively, the difference may be due to wrasse behavior. Unlike other teleosts, the activity of wrasses is completely arrested at night when these fish sleep under the sandy bottom (Lenke, 1988; Nishi, 1989, 1990, 1991; Gerkema et al., 2000), where they are exposed to extreme hypoxic conditions (Steffensen, Vijayan, and Takemura, unpublished data). This kind of stress may affect IT mRNA abundance. Studies have reported that in the gilthead sea bream *Sparus auratus* and three-spined stickleback, the IT response differs among stressors (Mancera et al., 2008; Kulczykowska et al., 2009). High-density (HD) stressors stimulated the release of AVT and IT from the pituitary to the circulation; although food deprivation does not influence AT and IT plasma levels, it is suggested that AVT and IT synthesis in the hypothalamus is affected in the gilthead sea bream (Mancera et al., 2008). HD stressors induced higher AVT concentrations in male brains but higher IT concentrations in female brains in the three-spined stickleback (Kulczykowska et al., 2009). In this study, melatonin administration did not affect pro-IT transcript levels after 2 h, which may indicate that the expression of pro-AVT and -IT mRNA in the brain is regulated by different mechanisms.

In conclusion, we cloned pro-AVT and -IT from, and demonstrated their expression patterns within the brain of a tropical wrasse. Motohashi et al. (2008) found an increase in the expression of AVT mRNA, but not IT mRNA, in the brain of pre-spawning female grass puffers *T. niphobles*, as well as higher expression in females. The suggestion was made that the difference in expression pattern between females and males could be attributable to sex differences in courtship behavior during the spawning season. Moreover, Black et al. (2004) noted that IT-immunoreactive cells in the forebrain of the blue-banded goby *Lythrypnus dalli* were present in greater numbers in both females and fish that had recently changed sex, suggesting that IT is related to social control of sex changes. Since the threespot wrasse is a hermaphroditic species (Warner, 1984), sexual status, including seasonal and social conditions, likely alters the levels of neuropeptides. Further studies would be needed to elucidate precisely how neuropeptides are involved in wrasse reproduction.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) to AT. And Grant-in-Aid for Scientific Research, and a Joint Research Project under the Japan-Korea Basic Scientific Cooperation Program from Japan Society for the Promotion of Science to A.T.

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