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Research paper

Dynamic expression of vasotocin and isotocin receptor genes in the marbled eel (*Anguilla marmorata*) following osmotic challenges

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ABSTRACT

To examine the physiological roles of arginine vasotocin receptor (AVTR) and isotocin receptor (ITR) in osmoregulation of a euryhaline teleost, the marbled eel (*Anguilla marmorata*), three different genes coding for AVTRV1a2, AVTRV2 and ITR were cloned by screening an *A. marmorata* cDNA library. These receptors were expressed differentially and ubiquitously in the eight tissues we examined. The changes in mRNA expression levels of AVTRV1a2, AVTRV2, and ITR were assessed in a time-course study following salinity transfer from fresh water (FW, 0‰) to fresh water (FW, 0‰), brackish water (BW, 10‰) or saline water (SW, 25‰). When eels were transferred to BW, mRNA levels underwent an adaptive period, from 0 to 24 h, and a chronic regulatory period, starting at 24 h after transfer. In the adaptive period, the relative mRNA expression of AVTRV1a2, AVTRV2, and ITR increased in BW. But after this adaptive period, the mRNA levels of the three genes were significantly decreased compared to FW (control group, 0 h). The mRNA expression levels of AVTRV1a2, AVTRV2 and ITR were low in SW. The protein level of AVTRV1a2, a key protein in the brain, was also investigated and found to be consistent with mRNA results. Our results indicated that the nonapeptide receptor system may play a role in the acute stress response induced by hyper-osmotic challenge in marbled eels.

1. Introduction

The neuropeptide arginine vasotocin (AVT), the representative hormone in the vasopressinergic family, is homologous to mammalian arginine vasopressin (AVP). AVP has been shown to be involved in several distinct processes, such as salt balance (Simon-Oppermann et al., 1988), smooth muscle contraction in the avian oviduct associated with oviposition (Sasaki et al., 1998), blood pressure regulation (Robinzon et al., 1988), sexual and aggressive behavior (Goodson et al., 2004), and external stress (Romero et al., 1998). Isotocin (IT), a teleostean neuropeptide synthesized in neurons and is homologous to mammalian oxytocin (OXY). It is located in the preoptic and lateral tuberal nuclei, from which it is transported to the neurohypophysis for storage and release into the systemic bloodstream (Schreibman and Halpern, 1980; Hm et al., 1982).

AVT is considered to be the ancestral peptide in the vertebrate neurohypophysial family whereas IT only occurs in teleost fish (Martos-Sitcha et al., 2013). A synthesis and secretion of AVT and IT into the circulatory system has been measured following exposure to different environmental salinities (Haruta et al., 1991; Hyodo and Urano, 1991). These hormones are thought to bind to their receptors, members of the G-protein coupled receptor family, which may influence physiological responses. Long-term acclimation of teleost fish to external salinity changes may be influenced by expression of arginine vasotocin receptor (AVTR) and isotocin receptor (ITR) via hormonal control (Bond et al., 2002; Warne et al., 2005; Cádiz et al., 2015). AVTRs regulate osmoregulatory processes in hyperosmotic environments through their antidiuretic role (Bond et al., 2002). The presence of AVTR in branchial pavement cells is associated to the ion exchange function in gills (Balment et al., 2006). Blood AVT indirectly influences the ion flux, such as transepithelial Cl^- transport (Olson, 2002). However, it has also been suggested that AVT could have a direct effect on water

Abbreviations: arginine vasotocin, (AVT); isotocin, (IT); arginine vasotocin receptor, (AVTR); isotocin receptor, (ITR); fresh water, (FW); brackish water, (BW); saline water, (SW)

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transport (Marshall, 2003). Na^+ secretion is mediated via an AVTRV1-type receptor in sea bass gill respiratory cells (Guibbolini and Avella, 2003).

AVTRV1a2, AVTRV2, and ITR were cloned in several fish species such as *Cyprinodon variegatus amargosae* (Lema, 2010), *Protopterus annectens* (Konno et al., 2009), *Sparus aurata* (Martossitcha et al., 2013; Martos-Sitcha et al., 2014), *Catostomus commersonii* (Mahlmann et al., 1994) and *Platichthys flesus* (Warne et al., 2005). Similar localization patterns were recorded regarding the V1a2 subtype in the brain of the rock hind *Epinephelus adscensionis* and the model cichlid *Astatotilapia burtoni* in situ hybridization and immunohistochemistry (Kline et al., 2011; Lin et al., 2012). V1a-type and V2-type AVT receptors were cloned and plasma AVT exerted a renal tubular antidiuretic effect in flounder (Warne et al., 2005). In *C. n. amargosae*, three AVT receptor subtypes were isolated and identified as V1a1, V1a2 and V2 receptors and their expressions were measured after 5 h and 20 h following an acute hyperosmotic challenge (Lema, 2010). In *S. aurata*, mRNA expression levels of AVTRV1a2, AVTRV2, and ITR changed when the fish were submitted to hyper and hypo-osmotic challenges (Martos-Sitcha et al., 2014). Although several studies have demonstrated the osmoregulatory role of AVTRV1a2, AVTRV2, and ITR, long-term acclimation to external salinity changes has not yet been demonstrated in teleost fish.

To examine the long-term effects of salinity on AVTRV1a2, AVTRV2, and ITR in teleost fish, we chose a catadromous fish, *A. marmorata*, as an experimental model. This species grows in rivers and breeds in the sea. This change in salinity during life cycle requires efficient osmoregulatory mechanisms to exhibit a remarkable ability to adjust the physiology when eels are transferred from marine (hyperosmotic) to freshwater (hypoosmotic) environments (Cao et al., 2018). In an attempt to explore the physiological mechanisms in catadromous eels, full-length cDNAs for AVTRV1a2, AVTRV2, and ITR from *A. marmorata* were cloned and the tissue distribution was studied. Changes in mRNA expression levels over multiple time points (0, 6, 12, 24, 72, 120, 240 and 360 h) were assessed following exposure to different environmental salinities in brain, gills, and intestine. We also investigated the protein expression of AVTRV1a2-type in the brain. Our data provide a basis for further studies of osmoregulation in eels.

2. Materials and methods

2.1. Fish and maintenance

Marbled eels (length: 23.1 ± 2.7 cm; weight: 21.1 ± 2.5 g) were collected from the Hainan Wenchang Jinshan Eel Technology Co., Ltd. in Hainan, China. The company has obtained an aquatic wild animal catching permit from the Ministry of Agriculture of the People's Republic of China since 2004. Eels were held in laboratory tanks (200 L) filled with filtered FW at 25 °C in a 12 h light: 12 h dark cycle for 1 month. The eels were starved for 2 days before sampling. All experiments were performed according to the Guideline for the Care and Use of Laboratory Animals in China. This study was approved by the Ethics Committee of Experimental Animals at Nanjing Normal University (SYXK2015-0028).

2.2. Experimental treatment

Eels were acclimated from FW to FW, BW, or SW for 15 days. BW and SW were prepared from aerated dechlorinated tap FW with the addition of standardized amounts of synthetic sea salt (Aquarium Systems, Mentor, OH, USA). The sampling was done at the same time of the day and randomized between groups. The water was continuously circulated through fabric-floss filters and partially refreshed every week. All treatments were conducted in triplicates. At sampling, eels were anaesthetized for 3 min, and tissues (brain, spleen, intestine, kidney, liver, gills, muscle, and heart) were collected and kept at −80 °C until use. Brain, gill, and intestine tissues were collected from each eel raised in the three salinity conditions (FW, BW, and SW) at 0, 6, 12, 24, 72, 120, 240, and 360 h (n = 9 at each time point) for RNA and protein isolations. Eels from FW (0 h) were used as the control (n = 9).

2.3. RNA extraction and cDNA synthesis

Total RNAs from above-mentioned tissues were extracted with high purity RNA fast extract reagent (BioTeke, Beijing, China). The integrity of the RNA was determined by 1% agarose gel electrophoresis with a Bioanalyzer (Agilent 2100, California, USA). A NanoDrop 2000 spectrophotometer (Thermo, Wilmington, DE, USA) was used to estimate the quantity and purity (A260/A280) of each sample. The RNA was only used when the Abs260 nm/Abs280 nm ratio was 1.9–2.05. One μg of isolated RNA was used to synthesize first-strand cDNA using HiScript QRT SuperMix for qRT-PCR and gDNA wiper was used to eliminate genomic DNA contamination (Vazyme, Nanjing, China). Samples were stored at −20 °C.

2.4. Cloning of AVTRV1a2, AVTRV2 and ITR from A. marmorata

AVTRV1a2, AVTRV2, and ITR were cloned based on the published fish cDNA sequences (*A. marmorata* GSE95803) with primers to amplify partial sequences. First-strand cDNA synthesis of AVTRV1a2, AVTRV2, and ITR was performed with gene specific primers (AVTRs -GSP1 and ITR -GSP1) using SuperScript II RT (Invitrogen, Carlsbad, USA) and the 3′-CDS Primer A (3′-RACE-Ready cDNA) following the manufactures' instructions. The full-length AVTRV1a2, AVTRV2, and ITR genes were cloned using the 3′ and 5′ Rapid Amplification cDNA End (RACE) methods. The full-length AVTRV1a2, AVTRV2, and ITR cDNAs were obtained from the overlapping 5′ and 3′ fragments. All primers are listed in Table S1.

2.5. Bioinformatic analysis

Homologous analysis was performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The open reading frame (ORF), molecular weight and translations of the deduced protein sequences were performed using DNAstar software. The Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/) was used to obtain signal sequence and domain prediction. Phylogenetic analysis was conducted with MEGA 5 using the Neighbor-Joining (NJ) method, and the reliability of the branching was tested by bootstrap resampling (1000 replicates).

2.6. Quantitative real time PCR (qRT-PCR)

The mRNA levels of AVTRV1a2, AVTRV2, and ITR were quantified with a real-time thermal cycler (Applied Biosystems, USA) using FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) according to the manufacturer's protocol. Quantitative RT-PCR primers for AVTRV1a2, AVTRV2, and ITR are listed in Table S1. The primers used in qRT-PCR were designed with Primer 5.0. The housekeeping gene β-actin (specific primers: Actin-F and Actin-R) was used as an internal control. All experiments were performed in triplicates. The amplification was carried out with a 20 μL reaction system containing 10 μL FastStart Universal SYBR Green Master (Roche, Basel, Switzerland), 4 μL cDNA template (500 ng, diluting 10 times after reverse transcription), and 3 μL of forward and reverse primers (2 mmol/L) in StepOnePlus Real-Time PCR System (ABI) (Applied Biosystems, USA). qRT-PCR was programmed at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 55 °C for 1 min. A melting curve analysis was performed after each reaction to confirm the efficiency of qRT-PCR and to verify that no primer-dimers or other nonspecific products were synthesized during the reaction. The expression levels of AVTRV1a2,