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Research paper

Arginine vasotocin neuronal development and its projection in the adult brain of the medaka

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HIGHLIGHTS

- We established the transgenic medaka that enable us to observe AVT neurons by EGFP.
- Onset of AVT neurons occurred in two regions of embryonic brain at different stages.
- AVT somata were shown in preoptic area and the ventral hypothalamus in adult brain.
- AVT fibers projected into pituitary in main, but some into other brain regions.
- This fish is an intriguing model to explore AVT neuronal development and function.

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ABSTRACT

The neurohypophysial peptide arginine vasotocin (AVT) and its mammalian ortholog arginine vasopressin function in a wide range of physiological and behavioral events. Here, we generated a new line of transgenic medaka (*Oryzias latipes*), which allowed us to monitor AVT neurons by enhanced green fluorescent protein (EGFP) and demonstrate AVT neuronal development in the embryo and the projection of AVT neurons in the adult brain of *avt-egfp* transgenic medaka. The onset of AVT expression manifested at 2 days postfertilization (dpf) as a pair of signals in the telencephalon of the brain. The telencephalic AVT neurons migrated and converged on the preoptic area (POA) by 4 dpf. At the same stage, another onset of AVT expression manifested in the central optic tectum (OT), and they migrated to the ventral part of the hypothalamus (VH) by 6 dpf. In the adult brain, the AVT somata with EGFP signals existed in the gigantocellular POA (gPOA), magnocellular POA (mPOA), and parvocellular POA (pPOA) and in the VH. Whereas the major projection of AVT fibers was found from the pPOA and VH to the posterior pituitary, it was also found that AVT neurons in the three POAs send their fibers into wide regions of the brain such as the telencephalon, mesencephalon and diencephalon. This study suggests that the *avt-egfp* transgenic medaka is a useful model to explore AVT neuronal development and function.

1. Introduction

Arginine vasopressin (AVP) and its teleost ortholog, arginine vasotocin (AVT), are neuropeptides that have roles in regulating various physiological and behavioral events such as osmoregulation, stress response, metabolism, blood pressure, and aggressive and reproductive behaviors in vertebrates [1–3]. In mammals, AVP is primarily synthesized in the magnocellular and parvocellular neurons localized in three regions of the hypothalamus: paraventricular nuclei (PVN), supraoptic nuclei (SON), and suprachiasmatic nuclei (SCN) [4–6]. Arginine vasopressin produced by magnocellular neurons in the SON and PVN is transported through their axons, which project to the posterior pituitary, and is subsequently released into the systemic circulation and exerts its effect in the peripheral tissues [5–7]. Arginine vasopressin synthesized by parvocellular neurons in the SCN is not released into circulation *via* pituitary, and is thought to have a role in modulating circadian rhythms [9,10]. In the parvocellular neurons in the PVN, AVP is coexpressed with corticotropin-releasing factor (CRF) and

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synergistically works with CRF to stimulate stress response *via* hypothalamus–pituitary–adrenal gland axis of hormones [10,11].

In many teleost, AVT neurons have been identified by soma size in three populations in the POA: gigantocellular nuclei (gPOA), magnocellular nuclei (mPOA), and parvocellular nuclei (pPOA), and they have different physiological and behavioral functions [12–14]. For example, the AVT level in the pPOA is involved in the hormonal stress response *via* the hypothalamus–pituitary–interrenal axis in the European eel (*Anguilla anguilla*) and the rainbow trout (*Oncorhynchus mykiss*) [12,15]. Furthermore, previous reports show that AVT expression in the mPOA and gPOA somata have a role in osmoregulation and in the mediation of aggressive behavior, respectively [1,13]. On the other hand, AVT fibers are present in wide regions of the brain (in addition to the pituitary) such as the telencephalon, hypothalamus, mesencephalon, and spinal cord [14,16,17]. The AVT neurons in the mPOA project their fibers into the pituitary in rainbow trout [18]; however it is less known which regions of the brain or pituitary that AVT neurons in the other POAs project into. The knowledge of AVT neuron projections will provide valuable information concerning the multiple functions of AVT localized in several regions of the brain.

To clarify the projection of the AVT neurons from the three POAs in this study, we first established a transgenic medaka that expressed enhanced green fluorescent protein (EGFP) under the control of *avt* gene transcription. Using the transgenic medaka, we investigated AVT neuronal development in the embryonic brain and AVT neuron localization and projection in the adult brain.

2. Materials and methods

2.1. Animals

Medaka fish of the T5 strain (strain ID, MT827) were used to establish the transgenic line. These strain were supplied by the National BioResource Project (NBRP) Medaka (Okazaki, Japan). The T5 strain lacks pigments in the melanophores, leucophores and ridocytes, which results in less auto-fluorescence, especially from the leucophores. Thus EGFP signals can be observed more clearly in this strain than in other strains. All fish and embryos were maintained at 27 °C in a light:dark cycle of 14 h:10 h (light phase from 7 am to 9 pm). Fish were fed *Artemia* spp. (Miyako Kagaku Corporation, Tokyo, Japan) twice a day (at 10 am and 5 pm). Embryo and juvenile ages are expressed as days post fertilization (dpf) and days post hatching (dph), respectively.

2.2. Construction of the avt-egfp vector

A fosmid clone (GOLWFno393_p20) containing the medaka *avt* locus was obtained from NBRP Medaka (Okazaki, Japan). The targeting DNA fragment was prepared by polymerase chain reaction (PCR) amplification using the EGFP-poly(A)-Km vector [19], which was kindly gifted to us by Shin-ichi Higashijima of the National Institute for Physiological Sciences (NIPS, Okazaki, Japan) and by Minoru Tanaka of the National Institute for Basic Biology (NIBB, Okazaki, Japan). The primers used were 5′-ATGAGCGGGCTGTCCGTCAGACGTCCACACCGACAGCCTGCAGCG ATGCATCCACCGGTCGCCACCATGG-3′ and 5′-GCAGTTCTGGATGTAACAGGCGGAGGACAGAGCGAGGAATCCCAGG GCGCGTCGACCAGTTGGTGATTTTG-3′.

The target fragment was inserted at the translation initiation site of the *avt* gene in the fosmid clone (Fig. 1) by using a method of homologous recombination in *Escherichia coli* (DY380) [20]. The modified fosmid clone was purified by using the Qiagen Large-Construct Kit (Qiagen, Venlo, Netherlands), and used for microinjection.

2.3. Generation of transgenic lines

The construct was microinjected into the cytoplasm of one-cell stage embryos, which were collected within 30 min after spawning. The microinjection was performed in accordance with the method described by Kinoshita et al. [21]. The fluorescence of EGFP was monitored under a fluorescent microscope (BX-51; Olympus Corporation, Tokyo, Japan) equipped with a GFP filter. Only embryos showing EGFP fluorescence in the brain were allowed to grow into adulthood as the founder candidates. Adults of the founder candidates were pair-mated with wild-type adults of the T5 strain to obtain the F1 embryos. The F1 embryos were screened to identify the germ line-transmitting founders (F0) by the acquisition of EGFP fluorescence in the brain. To obtain homozygous transgenic offspring, heterozygous transgene carriers in F1 were intercrossed. The F5 and F6 heterozygous progeny were mainly used in the present study.

2.4. Observation of the embryos, juveniles, and adult brains

Live embryos (1, 2, 4 and 6 dpf) and juvenile (1 dph) were observed under a fluorescent microscope (BX-51; Olympus). For observation, juveniles were embedded in 2% methylcellulose with Yamamoto's Ringer solution (i.e., 128.3 mM sodium chloride [NaCl], 2.7 mM potassium chloride [KCl], 1.8 mM calcium chloride [$CaCl_2$],

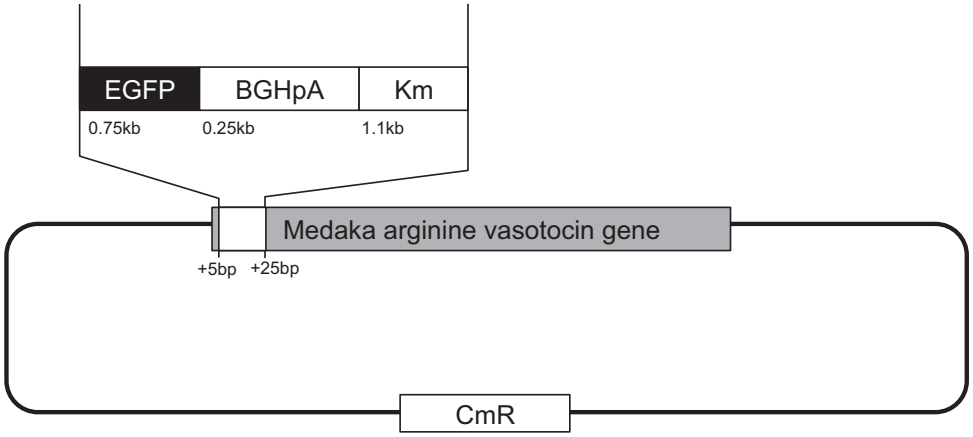

Fig. 1. The construct used to generate transgenic medaka expressing EGFP under the control of the *avt* promoter. Exon 1 of the *avt* gene in the fosmid clone (GOLWFno393_p20; NBRP Medaka, Okazaki, Japan) was modified with an EGFP fragment.