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RESEARCH PAPER

Anatomical identification of the neuroendocrine system in the *Nothobranchius furzeri* brain

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Abstract. The hypophysis functions as a central gland of the neuroendocrine system for regulating fundamental body physiology. Upon aging, several hormones produced by the endocrine system are dramatically altered. Recently, *Nothobranchius furzeri* (the turquoise killifish) has become a popular model for aging studies because of its short lifespan and highly conserved aging phenotypes. However, the anatomical details of the major neuroendocrine system of the killifish have not been investigated so far. In this study, we have identified the pituitary and pineal glands of the turquoise killifish, which are critical components of the brain endocrine system. These two neuroendocrine glands were weakly attached to the main body of the killifish brain. The pineal gland was located on the dorsal part of the brain, while the pituitary gland was located on the ventral part. Brain sections revealed that cells in the pituitary and pineal glands were more densely situated than in other regions of the brain. Further, three-dimensional images of the pineal and pituitary glands demonstrated their distinctive cellular arrangements. Vasopressin intestinal peptide (VIP) was strongly expressed in the neurohypophysis of the pituitary gland. Glial cells were found inside the pineal gland, while astrocytes covered the outside. These findings illustrate basic features of the neuroendocrine system of *N. furzeri*.

Key words: turquoise killifish, pineal gland, pituitary gland, endocrine glands

Introduction

The endocrine system consists of multiple glands and organs that secrete hormones essential for organismal development, reproduction, or homeostatic regulation. The hypothalamo-hypophysis axis, also called the neuroendocrine system, is a master link between the central nervous system and the endocrine system. The pituitary gland governs the secretion of major hormones that maintain body homeostasis, such as thyroid-stimulating hormone (TSH), prolactin (PRL), somatolactin (SL), growth hormone (GH), α-melanocyte-stimulating hormone (MSH), β-endorphin, adrenocorticotropic hormone (ACTH), follicle-stimulating hormone, luteinizing hormone, somatolacotropes (Sl), and somatolactin (SL). Based on Green's nomenclature (Green 1951), the pituitary gland consists of two major parts, the adenohypophysis (AH, consisting of rostral pars distalis (RPD), proximal pars distalis (PPD), and pars intermedia (PI)) and neurohypophysis (pars nervosa (PN)). The pineal gland is another critical endocrine system in the brain. The pineal gland primarily secretes melatonin, which is involved in the control of the circadian rhythm (Wurtman et al. 1963). It is known that some of the pineal and pituitary gland hormones are dysregulated during

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the aging process (Frohman 1994, Seraphin et al. 2008, Bartke & Darcy 2017, Muller-Fielitz et al. 2017, Uenoyama et al. 2018).

Nothobranchius furzeri (the turquoise killifish) is a teleost fish originating from temporal pools in Mozambique and Zimbabwe. The turquoise killifish is a useful animal model because it is breeds readily in captivity and has a relatively short life span (median life span 9-26 weeks; Terzibasi et al. 2008, Kim et al. 2016). Despite their short lifespan, the turquoise killifish has a highly conserved aging physiology relating to the neuroendocrine system, including neurodegeneration, decreased fecundity, and cognitive impairment. While the brain anatomy of the turquoise killifish has been previously reported, the neuroendocrine system has not yet been characterized (D'Angelo 2013). The anatomy of the endocrine system has been well-described for other teleost fish models, such as zebrafish and medaka (Wullimann et al. 1996, Ralph Anken 1998). In this study, we identified two major neuroendocrine organs in the turquoise killifish brain, which were compared with the zebrafish and medaka endocrine systems as a model for the endocrinology of aging.

Material and Methods

Fish husbandry and sampling

The short-lived turquoise killifish strain (GRZ-AD) was used. Fish were maintained as described previously (Dodzian et al. 2018). The fish room was kept on a 12/12 h light/dark cycle. Fish were fed twice each day, one and eight hours after lights on every day.

Every fish in this study was euthanized by supplying 1.5 g/L of ethyl 3-aminobenzoate methanesulfonate (MS-222, E10521, Sigma-Aldrich) to the fish tank water. When fish gill movement ceased, dissections were performed.

The animal husbandry and experiments in this study were carried out in accordance with the animal care and use protocol that is reviewed and approved by the Institutional Animal Care and Use Committee at Daegu Gyeongbuk Institute of Science and Technology, Republic of Korea (approval number: DGIST-IACUC-17103001-00).

Gross anatomy of the turquoise killifish brain

The whole fish body of young adult fish (8-10 weeks old, fourteen female and male fish) were fixed in 4% paraformaldehyde (PFA) overnight. Fish heads were carefully dissected under a stereomicroscope. Brain images were acquired in a 4% PFA solution using a stereomicroscope (M80, Leica Microsystems).

Nissl staining of killifish brain sections

For gross anatomy analysis, fixed brains were embedded in 2.5% agarose and sectioned with a Vibratome (VT1200S, Leica) at a thickness of 100 μm. Sections were transferred onto slide glasses and dried for 10 minutes. Sections were stained for five minutes in Nissl stain solution (0.1% cresyl violet (Sigma C-1971), 0.1% glacial acetic acid) and rinsed in distilled water for five minutes. Subsequently, sections were dehydrated

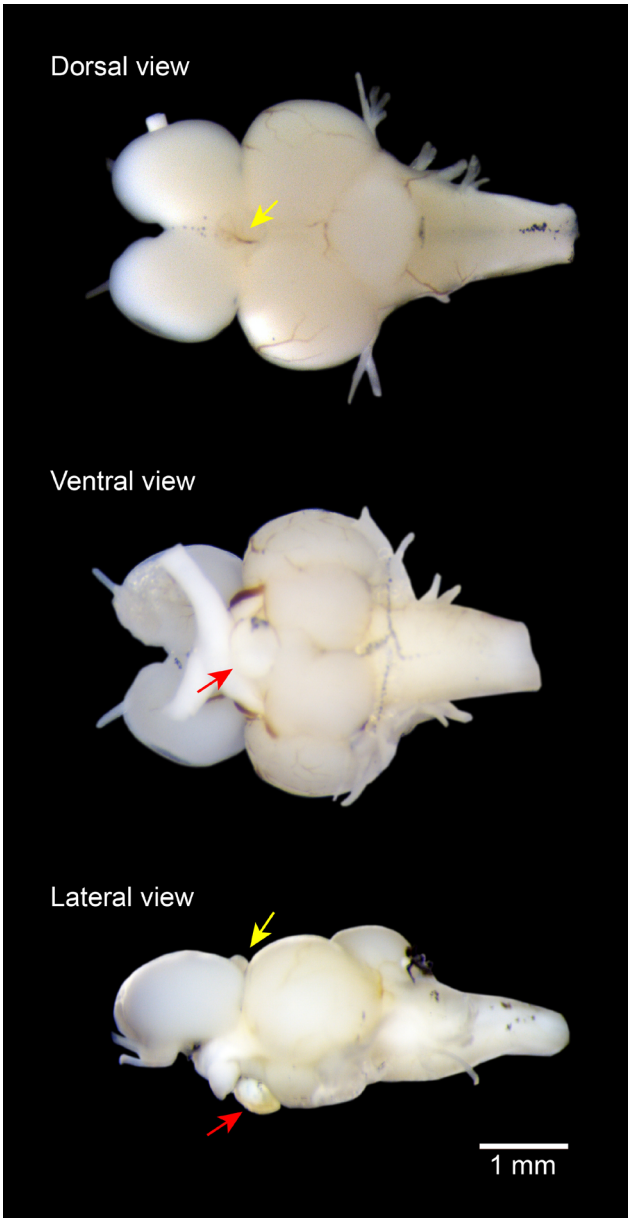

Fig. 1. Gross anatomy of the turquoise killifish. Yellow and red arrows indicate pineal and pituitary glands, respectively.

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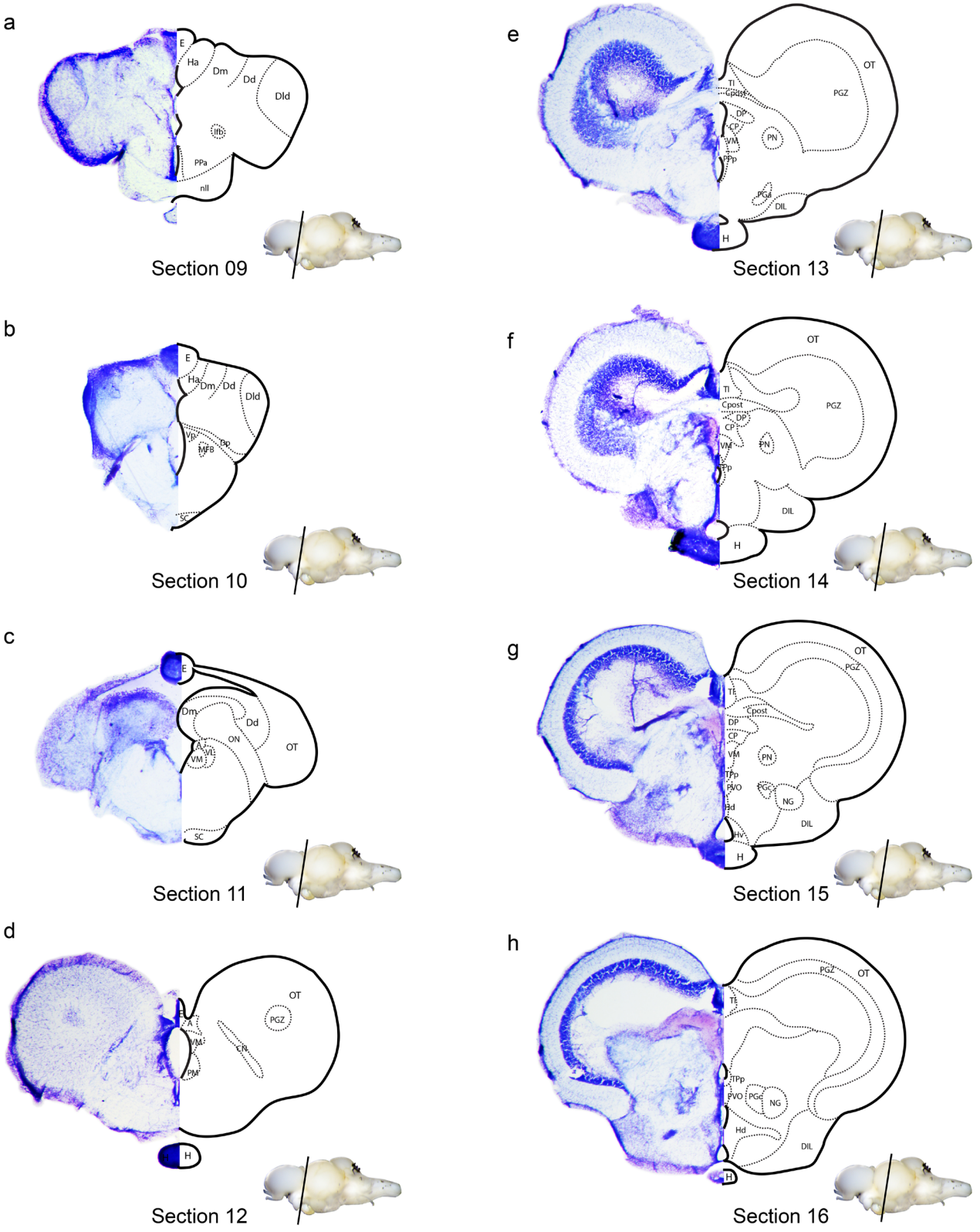

Fig. 2. Cross-section of the brain containing the pineal and pituitary glands: a-d) sections depict the epiphysis on the dorsal side of the brain; e-h) sections depict the hypophysis on the ventral side of the brain. Section numbers are linked with supplemental figures. Abbreviations of each figure can be found in the glossary of brain sections in Table S1.

in an ethanol series (50%→70%→95%) and de-stained in 100% ethanol until the sections exhibited the desired amount of staining. Stained sections were mounted with mounting medium (Fisher Chemical™ Permount™ Mounting Medium, SP15-100; Fisher Scientific). Section images were

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acquired using a stereomicroscope (M80, Leica Microsystems).

Whole brain immunostaining

The brains of nine six-week-old, sexually mature males were carefully dissected under a microscope and then fixed in 4% PFA overnight. Whole brains were cleared using a tissue immunostaining solution (Binaree Immuno Staining™ Kit for Brain, BINAREE), according to the manufacturer's protocol. Cleared brains were stained with DAPI (D9542, Sigma-Aldrich) and GFAP-Alexa 647 (ab194325, abcam), Vimentin-Alexa 647 (ab195878, abcam), or VIP-Alexa 647 (bs-0077R-A647, Bioss) antibodies. The killifish glial fibrillary acidic protein (GFAP), Vimentin, and vasopressin intestinal peptide (VIP) proteins showed 69%, 68%, and 46% of amino acid sequence identity, respectively, with those of human. Antibody specificity was tested using gel blot analysis (Fig. S1). Cleared and stained brains were embedded in 2% low-melting-point agarose in capillaries with an inner diameter of 2 mm. The embedded brains were tiled into 9-30 regions to cover the whole brain and imaged using a 20 × objective lens (W Plan-APOCHROMAT 20, Zeiss) on a Light Sheet microscope (Lightsheet Z.1, Zeiss). Stacked images were converted (Imaris File Converter × 64.9.2, Bitplane) and displayed using Imaris (Imaris × 64 7.6.0, Bitplane).

Results

The gross brain anatomy of the turquoise killifish revealing weakly-attached subregions in the dorsal and ventral parts of the brain

The gross brain anatomy of the turquoise killifish brain has been reported previously (D'Angelo 2013). The turquoise killifish brain comprises the 1) olfactory bulbs, 2) telencephalon, 3) diencephalon, 4) optic tectum, 5) cerebellum, and 6) rhombencephalon. In addition to the six main parts, we found two additional bulging structures in the ventral and dorsal regions of the turquoise killifish brain (Fig. 1). The two bulging organs, in particular the ventral one, were weakly attached to the main body of the brain and were thus easy to lose during dissection and further processing. One of the bulging organs was located in the dorsal brain nearest to the telencephalon and between the optic tectum, measuring approximately 0.4 mm in diameter. The other bulging organ was located in the ventral brain at the anterior part of the hypothalamus and caudal to the optic nerves, measuring approximately 0.7 mm in diameter. In comparison with the brain anatomy of the zebrafish and medaka, these additional bulging organs appeared to correlate with the pineal and pituitary glands. Similar to the dorsal bulging organ of the killifish, the pineal gland of the zebrafish and medaka is located between the telencephalic area and the optic tectum of the dorsal brain. The pituitary gland of the zebrafish and medaka is attached to the ventral brain near the hypothalamus, similar to the second bulging organ observed in the killifish ventral brain. Therefore, the location of the killifish brain hypophysis is similar to the position of that in the medaka.

Characterization of the neuroendocrine organs using Nissl staining

To further characterize the pineal and pituitary glands, a whole killifish brain was sectioned and stained with Cresyl violet (Nissl staining). The internal structures of the brain sections were re-identified based on previous descriptions of *N. furzeri* (D'Angelo 2013), medaka (Ralph Anken 1998), and zebrafish (Wullimann et al. 1996) brain anatomies (Table S1). GRZ-AD, a strain showing the shortest lifespan (median lifespan under our laboratory conditions, 16 weeks), was used for coronal sectioning of the brain (in rostral to caudal order). Intensive staining revealed that the pineal gland was located at the end of the telencephalon and extended to the beginning of the optic tectum. Sections of the pineal gland showed that it had a rounded triangular shape at its anterior end (Fig. 2a, b) and that it became more circular (Fig. 2c) and rod-shaped toward its posterior end (Fig. 2c). At the location of the distal pineal gland, the pituitary started to emerge and was observed as a separate, flat, and rounded shape. Similar to the pineal gland, the pituitary gland was more strongly stained with Cresyl violet than other regions of the brain (Fig. 2c). The pituitary bridged the left and right parts of the anterior hypothalamic regions in the diencephalon (Fig. 2d-h). The pineal and pituitary glands of the killifish brain could be viewed in one section depending on the sectioning angle (Fig. S2). The other parts of the killifish brain, including olfactory bulbs, telencephalon, the remaining optic tectum, cerebellum, and rhombencephalon, are annotated in an illustration (Fig. S3, S4; Table S1).

Anatomy of the turquoise killifish pituitary gland

A 3D imaging method was used to further analyse the detailed structures of the two neuroendocrine glands in the killifish brain. After increasing the transparency of the whole brain tissue, it

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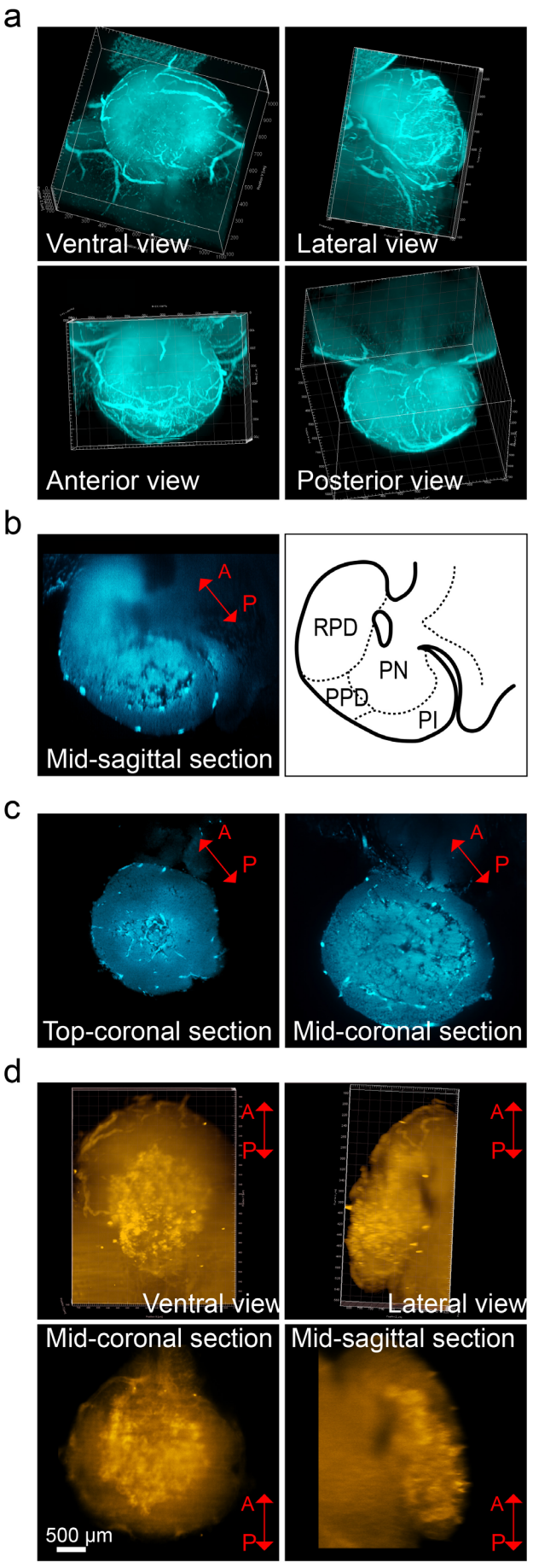

was stained with DAPI to observe its intact cell arrangements and the internal structures of the pineal and pituitary glands. As a master gland of the endocrine system, the pituitary gland resembled a flattened sphere covered with blood vessels at the ventral part of the brain (Fig. 3a, Movie S1). To compare the internal anatomy of the killifish pituitary gland with those of other teleost fish, cell densities were compared from mid-sagittal optical sections. The pituitary gland of turquoise killifish exhibited asymmetric differences in cell density, with the anterior region being more compact than the posterior region. The anterior pituitary of the killifish correlated with the RPD, whereas the posterior hypophysis correlated with the PPD, PN, and PI (Fig. 3b). The RPD occupied approximately one third of the volume of the killifish pituitary, and cells in this area were arranged compactly. The PPD was located in the ventral pituitary near to the RPD, PN, and PI. The PN was located in the dorsal pituitary and was surrounded by the RPD, PPD, and PI. Cells in the PN formed clusters surrounded by blood vessels. Additionally, the PN was connected to the hypothalamus as a "Y-shaped" structure in the turquoise killifish brain (Fig. 3c), and a small rounded cavity was observed between the PN and hypothalamus. The PI was positioned in the ventral pituitary. The surface of the pituitary was covered by blood vessels, especially the posterior part of the pituitary gland (Fig. 3a). We conducted further analysis by staining with a vasopressin intestinal peptide (VIP) antibody because VIP regulates the release of PRL and growth hormone (Sherwood et al. 2000, Kulick et al. 2005). Interestingly, the prolactin-releasing cells are known to be located in the RPD of the medaka (Aoki & Umeura 1970). VIP staining was mostly detected in the PN. Blood vessels partly covered the RPD of the hypophysis (Fig. 3d, Movie S2).

Anatomy of the turquoise killifish pineal gland

The other important neuroendocrine organ in the brain is the pineal gland. The killifish pineal gland

Fig. 3. Anatomy of the turquoise killifish pituitary gland. a) Three-dimensional features of the killifish hypophysis. The transparent killifish brain was stained with DAPI, a nuclear dye. b) A mid-sagittal section and illustration defining the internal structure of the killifish hypophysis. c) A coronal section of the killifish hypophysis. Rostral pars distalis (RPD), proximal pars distalis (PPD), and pars intermedia (PI), and pars nervosa (PN). d) Mid-sagittal and coronal sections of the VIP-stained hypophysis. Red arrows indicate the position of the brain; the letters A and P are abbreviations for anterior and posterior, respectively.

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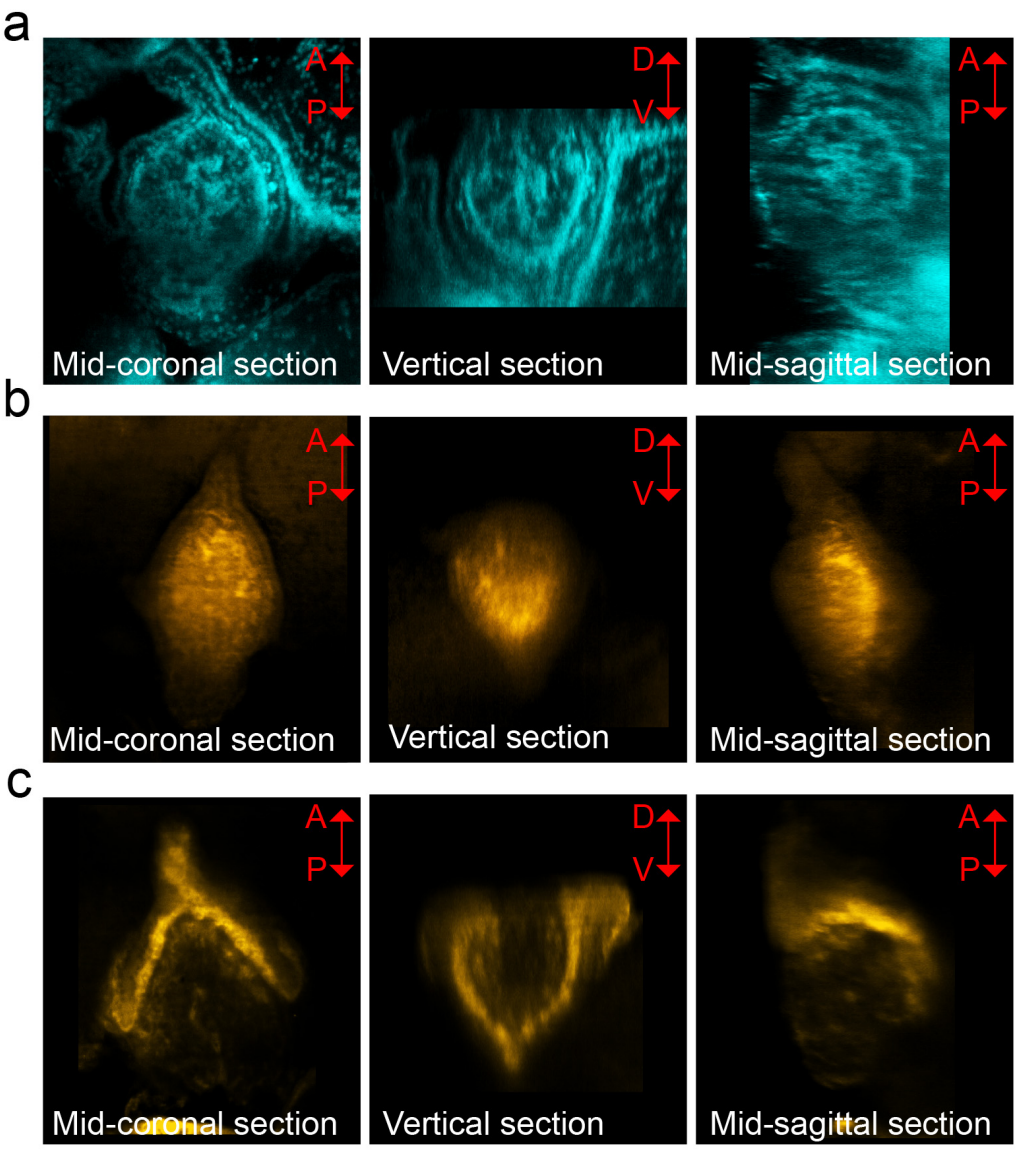

Fig. 4. Anatomy of the turquoise killifish epiphysis. a) Sections of the killifish epiphysis stained with DAPI; b) Sections of the killifish epiphysis stained with Vimentin; c) Sections of the killifish epiphysis stained with GFAP. Red arrows indicate position of the brain; the capital letters A, P, D, and V are abbreviations for anterior, posterior, dorsal, and ventral, respectively.

Table 1. Comparison of anatomical characteristics of the neuroendocrine glands of turquoise killifish, medaka, and zebrafish.

	Turquoise killifish	Medaka	Zebrafish
Pituitary gland (Aoki & Umeura 1970, Schmidt & Braunbeck 2011)	Weakly attached to the ventral brain		
	Located between the optic nerves and hypothalamus		Attached to the ventral hypothalamus
	Similar internal anatomy in sagittal section of the pituitary gland		Wider and separated PPD, smaller portion of PRD, and wider PN than those of the turquoise killifish and medaka
Pineal gland (Kuroyanagi et al. 2010, Menke et al. 2011)	Positioned between the telencephalon and the optic tectum of the dorsal brain		
	Ventral pineal gland is covered with single cell layers	Posterior pineal gland is partly covered with habenula	Crescent shape without extra covering

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protruded out from the brain and had a spherical shape, and its tail extended into the middle of the telencephalon (Fig. 4a, Movie S3). A coronal section of the pineal gland contained dozens of elliptically-shaped cell arrays (Fig. 4a). These globular cell arrays were covered by another layer of cells that were involuted inwards to surround the cell arrays, starting from about the middle of the pineal gland and forming an inverted and tailed pocket shape. This outer cell layer section of the pineal gland was located nearest to the telencephalon and the head of the pineal gland located with optic tectum and habenula (Fig. 4a).

The mammalian pineal gland consists mainly of pinealocytes, as well as astrocytes and microglia (Moller & Baeres 2002, Jiang-Shieh et al. 2003, Ibanez Rodriguez et al. 2016). To identify microglia and astrocytes, we used the characteristic markers vimentin and glial fibrillary acidic protein (GFAP), respectively (Graeber et al. 1988, Wohl et al. 2011, Zhang et al. 2019). Both vimentin and GFAP were expressed in the killifish pineal gland (Fig. 4b, 4c). Vimentin was detected specifically in the proximal region of the pineal gland (Fig. 4b, Movie S4), whereas GFAP localized preferentially to the outer cell layer of the pineal gland, covering half of the globular cell arrays (Fig. 4c, Movie S5).

Discussion

The neuroendocrine system plays a critical role in harmonizing physiology with behaviour in response to external stimuli. This system governs a wide range of biological processes including growth, reproduction, and metabolism. It is well known that these biological processes dramatically decline with aging. Due to the ethical issues surrounding the use of humans for experimental studies, as well as the long lifespan of mammalian model organisms, a new vertebrate model of aging is warranted to satisfy these requirements. The turquoise killifish is a novel model organism for studying the aging process because of its short life span and highly conserved aging phenotypes, including both visual and molecular alterations (Kim et al. 2016). In this study, the neuroendocrine system was described in the turquoise killifish brain. The pituitary gland is the master organ of the neuroendocrine system in the killifish pituitary, which is located in the ventral brain between the optic nerves and hypothalamus. Another important neuroendocrine organ, the pineal gland, is located in the dorsal brain between the telencephalon and optic tectum. Their localizations are consistent with other teleost fish species (Trudeau & Somoza 2020). Structural differences between the pineal and pituitary glands of turquoise killifish, medaka and zebrafish are listed in Table 1. As we mentioned above, the pituitary glands of turquoise killifish and medaka have similar anatomical structures, but their pineal glands are slightly different. Thus, with its short lifespan and conserved neuroendocrine system, the turquoise killifish is a good model for elucidating an age-dependent dysregulation of the vertebrate neuroendocrine system.

In this study, the neuropeptide VIP was specifically and strongly expressed in the PN of the killifish pituitary gland. VIP is known to be secreted by the RPD (Lam 1991). After synthesis by the hypothalamus, VIP is hypothesized to travel via the blood stream through the RPD, and is then stored in the PN. VIP is also known to play crucial roles in regulating the circadian clock (Colwell et al. 2003, Vosko et al. 2007, 2015, Hamnett et al. 2019), because it is strongly localized in the suprachiasmatic nucleus of the mammalian brain. Interestingly, VIP had accumulated in the pituitary, rather than the SCN, of the turquoise killifish brain. There is mounting evidence that the circadian clock system also governs diverse physiological functions in the turquoise killifish (Lucas-Sanchez et al. 2011, 2013, 2015). Furthermore, the pineal gland is the main organ that secretes melatonin, which is critical for the rhythmic behaviour of fish, such as sleep-wake cycles, and other circadian-clock-related activities. Taken together, this study elucidated the key structures of the neuroendocrine system in the turquoise killifish brain, which may be the key site of the circadian regulatory network.

Acknowledgements

We thank Koichi Kawakami (NIG, Japan) for his helpful suggestion of staining protocols and experimental design discussion. This work was supported by the Institute for Basic Science (IBS-R013-D1). Author contributions: Y. Kim conceived the project. E. Do, S. Lee and Y. Kim performed the experiments. Y. Kim wrote the manuscript. All authors read and made corrections to the manuscript.

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Supplementary online material

Table S1. Glossary for brain sections.

Fig. S1. Antibody specificities of anti-VIP, anti-vimentin, and anti-GFAP antibodies tested using whole protein extracts of brain. The specificities of anti-VIP-Alexa 647 (bs-0077R-A647, Bioss Inc.), anti-Vimentin-Alexa 647 (ab195878, Abcam plc), and anti-GFAP-Alexa 647 (ab194325, Abcam plc) were tested. Red arrows indicate the expected sizes of VIP, vimentin, and GFAP.

Fig. S2. Cross-sections of the telencephalic region of the turquoise killifish brain: a-e) sections depicting the olfactory bulbs of the ventral telencephalic region; f-h) sections depicting the hypophysis on the ventral brain.

Fig. S3. A turquoise killifish brain section showing both the hypophysis and the epiphysis.

Fig. S4. Cross-sections of the killifish brain: a-e) sections depicting the optic tectum and diencephalic regions; f-k) sections containing the cerebellum; l-r) sections of the rhombencephalic area.

(https://www.ivb.cz/wp-content/uploads/JVB-vol.-70-2-2021-DoE.-KimY.-Tables-S1-Fig.-S1-S4.pdf)

Movie S1. Three-dimensional structure of the killifish hypophysis stained with DAPI (https://www.ivb.cz/wp-content/uploads/JVB-vol.-70-2-2021-DoE.-KimY.-Movie-S1.avi).

Movie S2. Three-dimensional structure of the killifish hypophysis stained with VIP (https://www.ivb.cz/wp-content/uploads/JVB-vol.-70-2-2021-DoE.-KimY.-Movie-S2.avi).

Movie S3. Movie of the killifish epiphysis optical sections from the dorsal brain and nuclei stained with DAPI (https://www.ivb.cz/wp-content/uploads/JVB-vol.-70-2-2021-DoE.-KimY.-Movie-S3.avi).

Movie S4. Three-dimensional structure of the killifish epiphysis stained with Vimentin (https://www.ivb.cz/wp-content/uploads/JVB-vol.-70-2-2021-DoE.-KimY.-Movie-S4.avi).

Movie S5. Three-dimensional structure of the killifish epiphysis stained with GFAP (https://www.ivb.cz/wp-content/uploads/JVB-vol.-70-2-2021-DoE.-KimY.-Movie-S5.avi).

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