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Brain structure and ultrastructure of gonadotropic cell in female grunting toadfish, *Allenbatrachus grunniens* (Linnaeus 1758)

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Abstract

Importance of the work: There is little known of the neurobiology of *Allenbatrachus grunniens* (Linnaeus 1758), an estuarine toadfish inhabiting mangrove areas of the Indo-West Pacific, including Thailand.

Objectives: To identify the histomorphology of the brain and ultrastructure of the gonadotropic cells (GTH cells) in *A. grunniens*.

Materials & Methods: Mature female fish were collected from the Pranburi River estuary. Whole brains were dissected to examine their anatomical characteristics; the ultrastructure of the GTH cells was established.

Results: The large telencephalic regions and medium cerebellum likely aid in the survival of the species in structurally complex estuarine habitats. Histologically, two sub-regions of the telencephalon were similarly structured and composed of neuroglia, neurons and blood vessels. The optic lobe of the mesencephalon had six histologically distinct layers. GTH cells were identified in the adrenohypophysis of the pituitary gland. Based on electron micrography, the GTH II cells had an oval, eccentric nucleus, while the GTH I cell had a large, central nucleus and secretory granules. The secretory granules in the active GTH II cells indicated that synthetic activity might be related to final oocyte growth and ovulation.

Main finding: Basic knowledge about the neurobiology of *A. grunniens* was revealed based on histomorphological examination that should aid in understanding the role of GTH cells in the reproduction of the grunting toadfish. This study provided the first neurobiological evidence for further plans in aquaculture.

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Introduction

The grunting toadfish (*Allenbatrachus grunniens*), also known as the freshwater lionfish, belongs to the Family Batrachoididae (Order Batrachoidiformes) and is commonly found throughout the muddy beds of the coastal water and estuarine areas of the Indo-West Pacific, particularly Thailand (David et al., 2008). This toadfish is important as it is a euryhaline species and is generally accepted as an estuarine fish in Thailand. Over recent decades, naturalized populations of *A. grunniens* have been decreasing in the Pranburi River estuary, Thailand, due to increased consumption and reduced health status (Mitparian, 2018), and it is assumed that the conservation issues related to the food insecurity of this fish will become a major issue shortly. One important way to solve this problem is commercial aquaculture, which is particularly suited and recommended (Zohar and Mylonas 2001; Zohar et al., 2010). Nevertheless, scientific understanding of the neurobiology of this species, such as the basic neuroanatomy or neurophysiology relating to reproductive activity throughout the spawning season, has not been documented and is warranted for the development of appropriate aquaculture techniques.

Kotrschal et al. (1998) suggested that characterizations of the brain morphology might aid in interpreting the ecological context and adaptive significance, as well as help in understanding the behavior and habits of fish. A major portion of the large cerebral hemispheres and optic tectum of fish is involved in the regulation of learning, sensory integration and spatial navigation (Gonzalez-Voyer and Kolm 2010; Park and Bell, 2010) and in vision and orienting responses (Huber et al., 1997; Pollen et al., 2007). Additionally, it is well accepted that the brain is a key organ in controlling the reproduction of teleosts, which is regulated by various hormones in the hypothalamic-pituitary-gonadal axis (Nagahama, 2000). Hypothalamic substances are involved in the production and secretion of reproductive hormones from the gonadotropic cells of the pituitary gland. One important hypothalamic hormone is the gonadotropin releasing hormone (GnRH), which facilitates pituitary production and secretion of the other two distinct gonadotropic hormones (gonadotropins, GTHs): GTH I (FSH-like) and GTH II (LH-like). It is well known that these two hormones finally control gonadal development and maturation in fish (King and Millar 1992; Sherwood et al., 1993; Silverstein and Small, 2004).

The gonadotropic (GTH) cells are found in the adenohypophysis of the pituitary gland, especially in the ventral region of the proximal pars distalis and the rostral pars distalis (Leatherland and Sonstegard, 1980; Hibiya, 1982). Other works have demonstrated that the GTH cell of *Channa striata* is round or angular in shape and dispersed throughout the proximal pars distalis (Leatherland, 1970; Chinabut et al., 1993). With regard to function, the number of GHT cells and their secretory profile/activity in the proximal pars distalis are known to be associated with reproductive activity/status, such as the reproductive cycle and gonadal development in many fish species, including *Poecillia latipinna* (Sage and Bromage, 1970; Yong and Ball, 1982), *Oncorhypchus kisutch* (Leatherland and Sonstegard, 1980), *Sparus aurata* (Gothilf et al., 1997), *Pagrus major* (Kagawa et al., 1998), *Dicentrachus labrax* (Forniés et al., 2003) and *Scomber japonicus* (Nyuji et al., 2011).

As mentioned, basic knowledge about the neurobiology of *A. grunniens* is lacking. To shed light on this matter, the current study explored the morphology and histology of the brain of the female *A. grunniens*. In addition, the precise ultrastructure was identified of the GTH cell of this toadfish. The obtained results are the first neurobiological evidence for this species and could be valuable in the development of aquaculture.

Materials and Methods

Fish sampling and study area

Sexually mature *A. grunniens* females (total length approximately 16.5 cm, $n = 10$) were collected from October 2016 to September 2017 from the Pranburi River estuary, Thailand (N12º 24.314’ E099º 58.597’). The experimental protocol was approved by the Animal Care and Use Committee of the Faculty of Science, Chulalongkorn University in accordance with the Guide for the Care and the Use of Laboratory Animals prepared by Chulalongkorn University (Protocol Review No. 1723004).

Brain gross anatomy and histological analysis

All fish were euthanized using a rapid cooling method (Wilson et al., 2009) and measured for total length (in centimeters). The whole brains were dissected to observe their anatomical structure using stereomicroscopy (SZX12; Olympus; Japan). Photographs were taken with a digital camera (DP 11; Olympus; Japan) and the scientific drawings were created using Adobe Illustrator CS5 (Adobe Inc.; California, CA, USA). Each region of the brain was measured (n = 10). Then, all brains were fixed in Davidson’s fixative for 48 hr (Dietrich and Krieger, 2009) at room temperature. Subsequently, they were processed using a standard histological technique, as described by Presnell and Schreibman (2013) and Suvarna and Layton (2013). Paraffin sections were cut at a thickness of 4 μm and mounted on microscope slides. Then, they were stained with either Harris's hematoxylin and eosin to study the basic structure of the central nervous system (CNS) or Masson's trichrome, aniline blue and periodic acid-Schiff to identify the histochemical characteristics of the brain (Presnell and Schreibman, 2013; Suvarna and Layton, 2013). Histochemical sections were observed under light microscopy and photographed (TE750-Ua; Leica, Germany).

Ultrastructural analysis

Small pieces of the fresh pituitary gland (n = 5) were dissected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C. Tissues were washed three times with 0.1 M phosphate buffer (pH 7.4) and post-fixed in 1% osmium tetraoxide (OsO4). Afterward, they were processed using standard ultrastructural techniques (Rowden and Lewis, 1974); semi-thin sections (500 nm) were cut, stained with 0.5% toluidine blue and viewed using light microscopy. For the transmission electron microscopy study, ultra-thin sections were cut (about 90 nm), stained with uranyl acetate and lead citrate and viewed using electron microscopy (JEM-2100; Jeol; Japan) at 200 kV. Classification used the size of secretory granules measured for 10 GTH cells. The schematic illustrations of the GTH cell were created using Adobe Illustrator CS5.

Results and Discussion

Gross anatomy of brain section heading

The stereomicroscopy revealed that the CNS of *A. grunniens* was composed of the brain connected to the spinal cord and lateral eyes (Figs. 1A–1C and 2A–2C). Anatomical organization of the brain pattern was viewed from three aspects: dorsal, lateral and ventral (Figs. 2A–2C). Integrated data from all views confirmed that five anatomical areas—the telencephalon, mesencephalon, diencephalon, metencephalon and myelencephalon, with the cranial nerves distributed among them—were present within the cranial cavity or skull (Fig. 1C), similar to the general teleostean pattern (Turkmen et al., 2007; Genten et al., 2008; Sharareh et al., 2013). In the dorsal view, two telencephalic hemispheres were the largest part of the toadfish brain, being well developed and situated anteriorly in the snout (Fig. 1C) and composed of pairs of olfactory lobes (about 1.3 ± 0.56 mm in diameter) and large cerebral hemispheres (about 4.2 ± 0.97 mm in diameter) (Fig. 1C). The olfactory tract was connected to the olfactory lobe (Fig. 1C). The mesencephalon constituted the large optic tectum, which was almost the same size as the cerebral hemispheres (approximately 3.8 ± 1.4 mm in diameter) (Fig. 1C). The moderate-sized cerebellum (about 2.8 ± 1.13 mm) of the metencephalon was located between the optic tectum and the medulla oblongata of the myelencephalon (Fig. 1C). The myelencephalon, narrow medulla oblongata and vagal lobes were posteriorly located in the brain. The lateral view was similar to the dorsal view, except for the visualization of the diencephalon (Fig. 1D). In the ventral view, the cerebral hemispheres and the pituitary gland were anteriorly located to the brain (Fig. 1C). The largest part of the inferior lobe of the hypothalamus was centrally situated in the diencephalon (Fig. 1E).

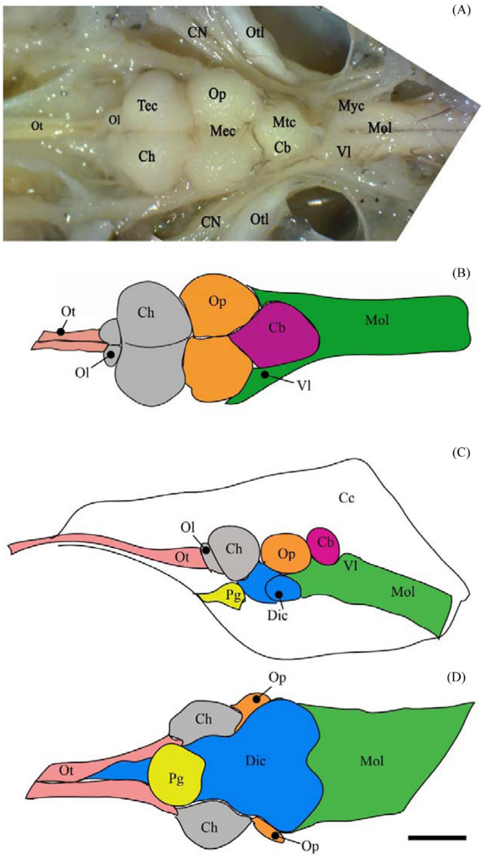

Fig. 1 Overall morphology of *Allenbatrachus grunniens* brain: (A). neuroanatomy of brain in dorsal view; (B–D) schematic diagrams of brain from different views: (B) dorsal view; (C) lateral view; (D) ventral view, where Cb = cerebellum, Cc = cranial cavity, Ch = cerebral hemisphere, CN = cranial nerve, Dic = diencephalon, Mec = mesencephalon, Mol = medullar of oblongata, Mtc = metencepahlon, Myc = myelencephalon, Ol = olfactory lobe, Op = optic lobe, Ot = olfactory tract, Otl = otolith, Pg = pituitary gland, Tec = telencephalon, Vl = vegal lobe. Scale bar = 2 mm

Brain histological organization

Histological and histochemical techniques confirmed the association of the telencephalon with the olfactory tract (Fig. 2). The two structures similarly contained neuronal fibers, blood vessels and neuroglia (Figs. 2A–2F).

The prominent feature of the optic tectum was its complex structure (Fig. 3A), comprising six distinct layers: stratum marginale, stratum opticum, stratum fibroetgricialem stratum album central, stratum griseum central and stratum periventriculae (Figs. 3B–3C). Beneath the optic tectum was the third ventricle, which was lined by ependymal cells (Fig. 3C).

The diencephalon of the brain was histologically divided into three zones from dorsal to ventral: the dorsal epithalamus, the middle thalamus and the ventral hypothalamus (Fig. 3A). The dorsal epithalamus comprised the pineal organ, the subcommissural organ (Fig. 3E) and the habenular ganglion containing several neurons (Figs. 3A–3F). The saccus vasculosus was located in the caudal-most parts of the inferior lobes of the hypothalamus and beneath the glomerular nucleus, referred to as the nucleus glomerulosus. It contained several neurons, as similarly reported in *Scyliorhinus canicula* (Turkmen et al., 2007) and *Epinephelus coioides* (Sharareh et al., 2013). The saccus vasculosus was histologically surrounded by a thin tunica albuginea, as well as prominent connective tissue. It was lined by a pseudostratified cuboidal epithelium on a basement membrane (Fig. 3G).

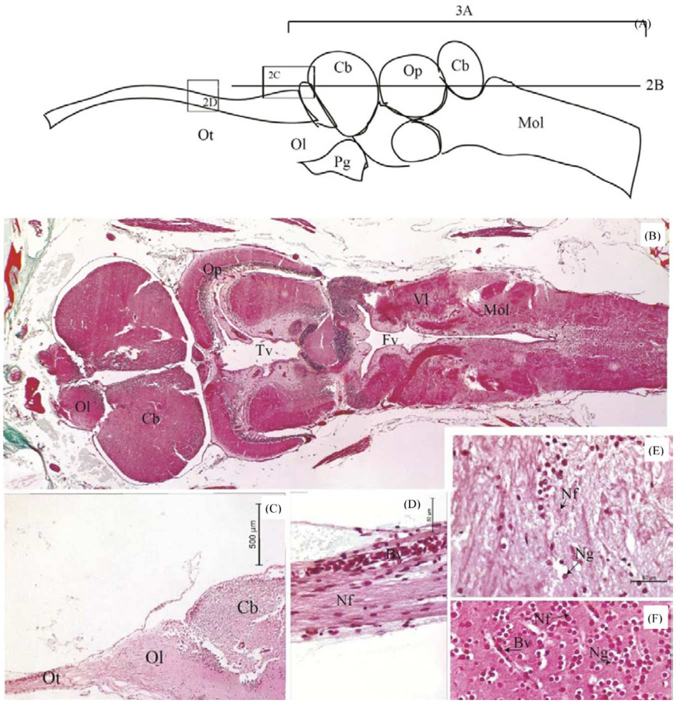

Fig. 2 *Allenbatrachus grunniens* brain in longitudinal sections: (A) schematic diagram; (B–F) light micrographs of: (B) overall histology of brain; (C–F) histological structure of olfactory tract; (D) telecephalon, including olfactory lobe (Ol) and cerebral hemisphere (Ch), where Bv = blood vessel, Cb = cerebellum, Fv = forth ventricle, Mol = medulla of oblongata, Nf = neuronal fiber, Ng = neuroglia, Ol = olfactory lobe, Op = olfactory lobe, Ot = olfactory tract, Pg = pituitary gland, Tv = third ventricle and Vl = vegal lobe, with all staining using Masson's trichrome

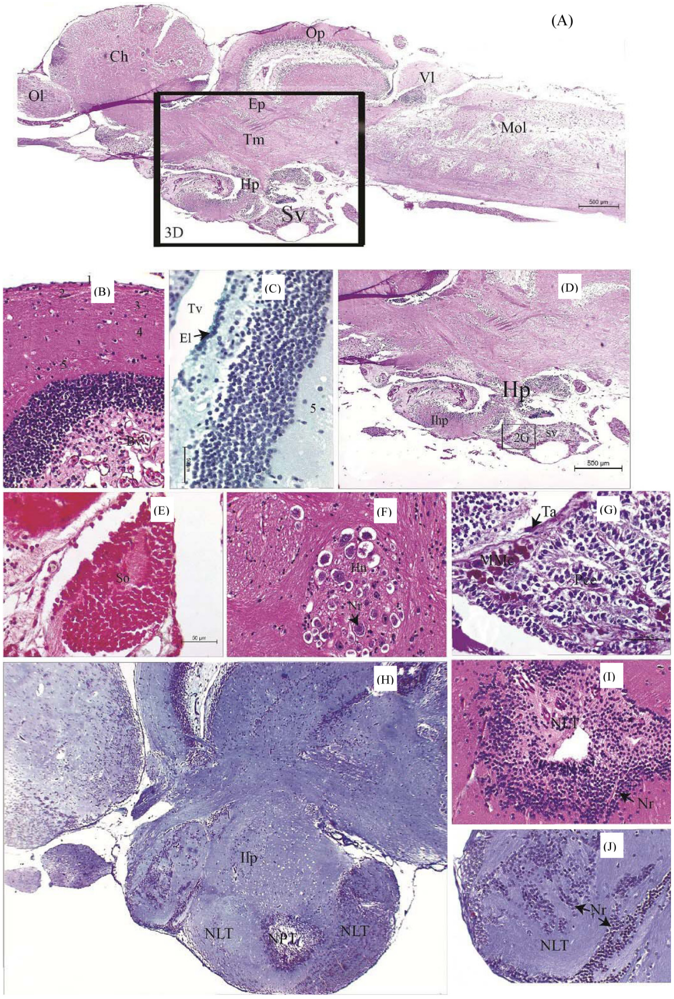

Fig. 3 Light micrograph of *Allenbatrachus grunniens* brain: (A) overall histology of brain in longitudinal section; (B–C) histological classification of opric lope, including 1 = menix, 2 = stratum marginale, 3 = stratum opticum, 4 = stratum fibroetgricialem, 5 = stratum album central, 6 = stratum griseum central and 7 = stratum periventriculae; (D) hypothalamus; (E) subcommissural organ; (F) habenula ganglion (Hn); (G) saccus vasculosus (SV); (H–J) localization of inferior lobe of hypothalamus (Ilp), including nucleus periventricularis (NPT) and nucleus lateralis tuberis (NLT), where El = ependymal lining, Ep = epithalamus, Ch = cerebral hemisphere, Hp hypothalamus, Ol = olfactory lobe, Op = optic lobe, MMc = melano-macrophage centre, Mol = medulla of oblongata, Nr = neurons, Ta = tunica albuginea, Tm = thalamus, Tv = third ventricle and Vl = vegal lobe

Melanomagcrophage center and red blood vessels were also found in this structure. The main role of the saccus vasculosus remains unknown, although another observation showed that it might be related with a sensor of seasonal changes in day length in fish (Nakane et al., 2013). Notably, the neurons of the hypothalamus in the diencephalon were observed as small dense clusters with a round nucleus. Three neuronal clusters (the nucleus preopticus-periventricularis) were identified (Fig. 3H): the nucleus periventricularis (NPT), nucleus preopticus (Np) and nucleus lateralis tuberis (NLT). The packed multilayers of the neuronal cells of the NPT were located dorso-posteriorly in the hypothalamus (Fig. 3I). The Np was located in the dorsal-anterior region of the hypothalamus and contained a small neuronal cluster (data not shown). The NLT was located in the caudal hypothalamus. Several neuron clusters were found in the horizontal plane, from the dorsal region to the rostral region of the hypothalamus (Fig. 3J).

The major component of the metencephalon was the cerebellum, which was located in the dorsal-posterior region between the optic tectum and the medulla oblongata (Fig. 4A). At the histological level, the cerebellum consisted of three different layers: the outer molecular layer, the Purkinje cell layer and the inner granular layer with specialized neuronal cells (Figs. 4B–4C).

The myelencephalon was formed by two sub-regions (medulla oblongata and the paired vagal lobes), as shown in Fig. 4A. The prominent paired vagal lobes were located in the dorsal medulla oblongata and contained only a few small-sized neurons, neuronal fibers and blood vessels (Fig. 4D). The fourth ventricle (fossa rhomboidea) was located in the middle of the caudal medulla oblongata (Figs. 4E–4F). In addition, various neuronal clusters and neuroglia were observed in the medulla oblongata (Figs. 4E–4F).

Another study suggested that the pattern of the brain morphology might be interpreted in an ecological context and have adaptive significance in some fish (Kotrschal et al., 1998). Phylogenetic relationship*s* and evolutionary processes in the brain pattern showed that it might be associated with lifestyle factors, such as feeding strategy and utilization of particular microhabitats (Huber et al., 1997; Kotrschal et al., 1998). The current data showed that the vast cerebral hemispheres and optic tectum were the largest observed and most developed parts in *A. grunniens*. These characteristics might reflect a higher degree of learning, sensory integration, spatial navigation (Gonzalez-Voyer and Kolm 2010; Park and Bell, 2010) and vision and orienting responses (Huber et al., 1997; Pollen et al., 2007). Although little attention has been paid to the biology of this benthic toadfish, it is known to be an active feeder that can survive in structurally complex estuarine habitats. The brain morphology of this toadfish may help it to catch prey and escape from predators. This hypothesis is supported by other discussion (Huber et al., 1997; Pollen et al., 2007) regarding *Gasterosteus aculeatus* (Park and Bell, 2010) and Tanganyikan cichlids (Gonzalez-Voyer and Kolm, 2010). Further investigations are needed of the relationship between behavior and the brain activity of *A. grunniens*.

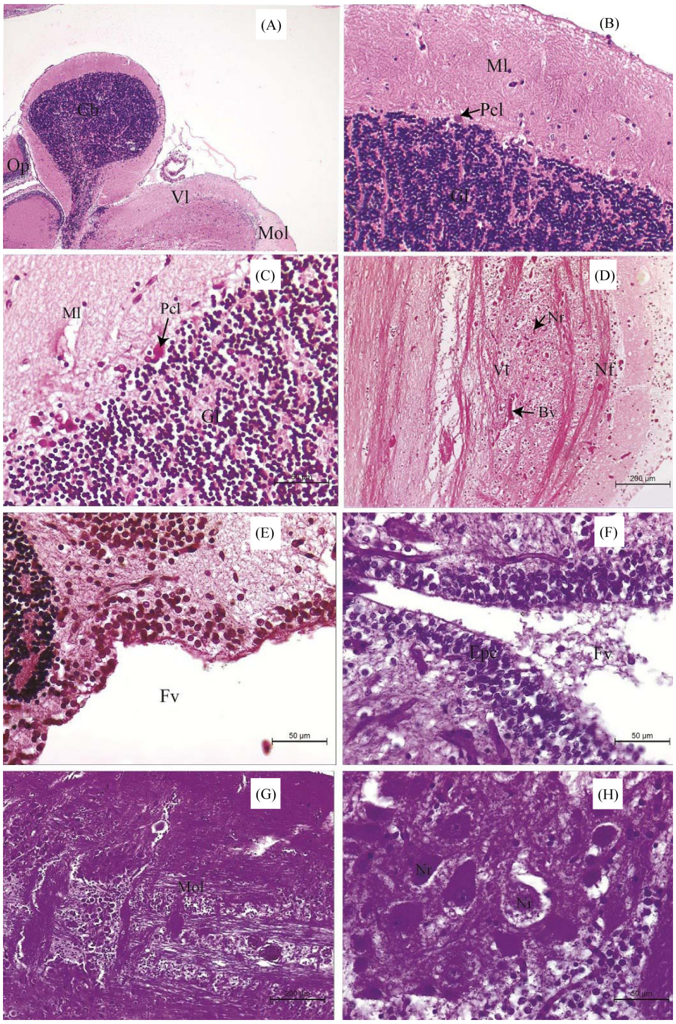

Fig. 4 Light micrographs of *Allenbatrachus grunniens* brain: (A) localization of cerebellum (Ch) between optic tectum (Op) and vagal lobe (VL); (B–C) high magnification showing cerebellum with three layers (molecular, Purkinje and granular layers); (D) neurofiber (Nf) and blood vessel (Bv) of vagal lobe clearly visible; (E–F) fourth ventricle (Fv) visible, extending along modular oblongata, which is lined by an ependymal cell (Epc); (G–H) structure of modular oblongata (Mol) containing several large neurons (Nr), with staining methods, (A–B)= Harris's hematoxylin and eosin; (C–E) = Masson's trichrome; (F–H) = periodic acid-Schiff

Histochemical staining identified the nucleus preopticus-periventricularis in the diencephalon, as also reported in other fish, such as, *Thunnus thynnus* (Palmieri et al., 2008) and *Rastrelliger brachysoma* (Senarat et al., 2019). This is believed to be the site of gonadotropin-releasing hormone (GnRH) production, also known as the "GnRH-positive neuronal cells" (Amano et al., 2004; Selvaraj et al., 2012). Investigation is required of the expression and immunohistochemical analyses of GnRH during the reproductive cycle, to determine their functions in *A. grunniens*.

Structure and ultrastructure of gonadotrophic cells in pituitary gland of mature females

Examination of the histological sections and semi-thin sections showed that the pituitary gland in this fish could be classified into two main areas based upon localization and cell composition: the adenohypophysis and the neurohypophysis (Figs. 5A–5B). In the semi-thin sections, basophilic cells were observed in the adrenohypophysis (Fig. 5C); these cells were reported to be homologous to thyrotropes, somatotropes and gonadotropes (Shimizu et al., 2003). The GTH cells were distinguished using electron micrography as sharing the characteristics of an oval nucleus and electron-dense secretory granules of varying sizes. Similar results have been reported in various fish species, including *Tridentiger obscurus* (Kaneko et al., 1986), *Clarias gariepinus* (Zandbergen et al., 1993) and *Thalassoma duperrey* (Parhar et al., 1998).

At the ultrastructural level, two types of GTH cells (GTH II and I) were observed in the adenohypophysis. The secretory granules in these cells could be divided into three types based on size: small (mean diameter of 120 ± 1.2 nm), medium (203 ± 2.34 nm) and large (1.5 ± 0.98 µm). The mean size of the GTH II cells was 12.7 ± 0.98 µm in diameter. These cells were oval in shape and had an oval-eccentrically nucleus. Abundant secretory granules with varying electron density, especially small granules, were observed throughout the cytoplasm. The GTH II cell was often found in contact with stellate cells (Figs. 5E and 5F–5G). A round or elongate Golgi complex and the rough endoplasmic reticulum (RER) were also well developed. In contrast, the GTH I cell had a large nucleus. It was polyhedral in shape and was about 10.09 ± 0.76 µm in diameter. Scant secretory granules (semi-dense/pale matrix) were scattered toward the periphery of the cell. Secretory granules of various sizes were observed, especially large and medium granules ranging from 200 nm to 0.8 µm in diameter. Notably, about 10–12 oval, electron-dense, large granules were also found in this cell (Figs. 5J–5K), some of which seemed to have originated by the fusion of the cisternae of the RER.

With the absence of previous descriptions about the pituitary of this toadfish, the current demonstrated for the first time that the toadfish has two types of GTH cells. This was similar to findings in other closely related teleosts (Leatherland 1970; Chinabut et al., 1993). Upon synthesis, GTH is packaged as granules and stored in secretory vesicles (Zohar et al., 2010). Therefore, the GTH production affects the number of secretory granules, which is associated with gonadal activity and the reproductive cycle (Zohar et al., 2010). In this regard, the abundant secretory granules in the GTH II cell may indicate LH production and oocyte growth in mature *A. grunniens*, as reported in other teleosts (Tyler et al., 1991; Planas et al., 1993;

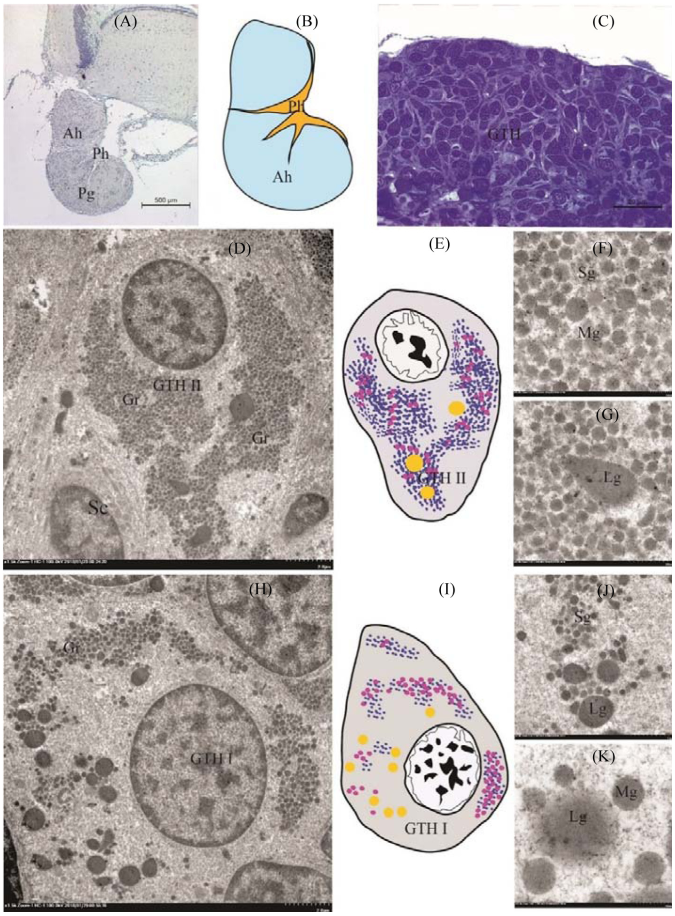

Fig. 5 (A) Light micrograph of *Allenbatrachus grunniens* pituitary gland; (B) ultrastructure of gonadotropic cell; (A–B) histology of pituitary gland (Pg) consisting of adenohypophysis (Ah) and neurohypophysis (Ph); (C) semi-thin section of gonadotropic cell (GTH); (D–G) ultrastructure and schematic diagram of GTH II; (H–K) ultrastructure and schematic diagram of GTH II, where Gr = granules, Sg = small-sized granules, Mg = medium-sized granules and Lg = large-sized granules

Nyuji et al., 2011). Further studies are warranted involving a combination of molecular biology and immunohistochemistry to identify the precise role of the GTH cells is *A. grunniens* reproduction.

In conclusion, the current study, describing the comprehensive morphology of the *A. grunniens* brain, provided a baseline to understand the neurobiology of this toadfish species. Integrated anatomical and histological characterization confirmed the large cerebral hemispheres and optic tectum of *A. grunniens*, which might imply a higher degree of learning, sensory integration, spatial navigation and vision. Importantly, the ultrastructural data regarding the pituitary GTH cell in toadfish have not been reported in reviews; hence the current study was the first to identify the two types of GTH cell. Because of the conserved function of GTH, the increase in pituitary GTH activity might indicate the final oocyte growth, as well as the beginning of the ovulation in *A. grunniens*.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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