RESEARCH PAPER

Neuroanatomical study of Forebrain of spotted snake head fish *Ophiocephalus Punctatus* (Bloch)

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Abstract

Neuroanatomical studies on forebrain of spotted snake head fish *Ophiocephalus punctatus* is carried out by taking serial transverse sections and using Niss'l staining (Kluver and Barrera staining). The olfactory bulbs were found to be sessile and characterized by four concentric layers arrange centripetally, the olfactory nerve layer, glomerular layer, mitral cell layer, and granular cell layer. The telencephalon was divided into dorsal and ventral telencephalic regions, exhibiting distinct neuronal populations based on cellular size, morphology, staining intensity, and spatial distribution. The diencephalon is differentiated into preoptic area, hypothalamus, thalamus, and epithalamus, with several distinct neuronal nuclei showing characteristic cytoarchitectonic features. This investigation demonstrates distinct regional differentiation in neuronal organization throughout the forebrain of *O. punctatus*. The reported study in this fish will be useful in tracing the neuronal system of *O. punctatus* and subsequent studies of the functional aspects of these nuclei in the regulation of reproductive physiology and feeding behaviour of this species.

Keywords: *Ophiocephalus punctatus*, spotted snakehead, forebrain, Nissl staining, telencephalon, diencephalon
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Introduction

In the vertebrates, brain stands in the centre of nervous system which represents one of the most complex physiological systems in the body, showing notable structural and functional diversity while retaining a fundamentally conserved developmental organization throughout evolution. Various neuroanatomical studies have proven that the central nervous system of all vertebrates develops from three primary embryonic parts: the forebrain, midbrain and hindbrain. Further, the forebrain has undergone the greatest evolutionary diversification and it plays key role in the various functions including sensory integration, learning and memory, spatial navigation, neuroendocrine regulation, emotional behaviour, and reproductive activities (Nieuwenhuys *et al.,* 1998; Butler and Hodos, 2005; Northcutt *et al.*, 2008).

Consequently, studies to understand the structural organization of the forebrain has become a very important objective of neurobiology because it gives important insights into the evolution and functional insights of vertebrate nervous systems. Among the living vertebrate's teleost's represents the largest and most diverse group. There are more than 35,000 reported species spanning remarkable range of aquatic habitats, thus such diversity has made teleost's unmatchable experimental model organism for studying developmental neurobiology, behavioural neuroscience, and comparative anatomy (Nelson *et al.,* 2016; Striedter, 2005). Among the different parts of the teleost brain forebrain due to its unique developmental organisation and functional complexities has attracted significant academic interest. Compare to tetrapod teleost shows completely different forebrain development process. The telencephalon in the teleost is develops through the process called eversion which is fundamentally different from tetrapod evaginated telencephalon (Folgueira *et al.,* 2012). The pallial organization of teleost fishes demonstrates considerable structural diversity, and its subdivisions can be divided on the basis of their neuroanatomical position position, and neuronal connectivity (Yamamoto *et al.,* 2007). However, recent investigations confirmed many unique structures of teleost forebrain corresponds to homologous pallial and subpallial regions found in other vertebrates (Mueller and Wullimann, 2009).

The term Cytoarchitecture refers to study of microscopic organisation of neuron depending up on their morphology size, staining affinity, orientation, and spatial distribution. Since the pioneering exploration of Brodmann and the classical studies of Nieuwenhuys *et., al* on fish neuroanatomy, cytoarchitectonic analysis using Nissl staining has served as the principal method for delineating anatomical boundaries within the brain of vertebrates. (Nieuwenhuys *et al.,* 1998).

The forebrain in the teleost is consist of the telencephalon and diencephalon, each comprising numerous structurally distinct nuclei that involved e in sensory processing, motor coordination, neuroendocrinology, learning, memory, and behavioural biology. The telencephalon is divided into the dorsal telencephalic area (area dorsalis telencephali) and the ventral telencephalic area (area ventralis telencephali). The dorsal telencephalon includes the

medial (Dm), lateral (Dl), dorsal (Dd), central (Dc), and posterior (Dp) subdivisions, and the ventral telencephalon comprises the ventral (Vv), dorsal (Vd), lateral (Vl), supra commissural (Vs), and intermediate (Vi) nuclei. Different evolutionary and developmental studies have shown suggested that these regions exhibit homologies with the mammalian forebrain region like hippocampus, amygdala, basal ganglia, septum, and olfactory cortex despite their telencephalic eversion (Mueller and Wullimann, 2009; Northcutt, 2008).

The spotted snakehead fish, *Ophiocephalus punctatus*, belongs to the order perciformes and is distributed widely over the freshwater aquatic water bodies of South and Southeast Asia. The fish is characterised by presence of accessory respiratory organ and hence it shows remarkable tolerance to oxygen deficient conditions. Other characteristics include territorial behaviour, parental care and ecological plasticity. Owing to these features *O. punctatus* is widely used in studies related to fish physiology, neuro-endocrinology, toxicology, and environmental biology. Although, extensive investigations have been carried on the physiological, behavioural and endocrine aspects, studies focused on detailed descriptions of neuronal organization and nuclear architecture are scarce. Therefore, lack of neuroanatomical baseline literature has restricted the comparative interpretation of neurochemical and functional studies.

In depth understanding of the cytoarchitectonic features of the forebrain of *O. punctatus* is crucial for understanding structural organization of its telencephalic and diencephalic nuclei and for comparing the same with other teleost. This will help further in investigations in neuropeptide localization, developmental neurobiology and in studying evolution of neuropeptide system in vertebrates.

Material and methods

Adult spotted snakehead fish, *Ophiocephalus punctatus* (Bloch, 1793), were procured from water bodies situated in and around Bhandara District, Maharashtra, India. Fishes measuring approximately 15–20 cm in length and of 250–350 g weight were used for the study. Three experimental sets (N = 6 per set) of fishes organised to ensure reproducibility of the observations. Fishes were brought to the laboratory alive and acclimatised in laboratory condition. The fishes were anesthetized with 2-phenoxy ethanol prior to dissection. Immediately after dissection, brain with intact pituitary cleaned with appropriate saline solution and fixed in freshly prepared Bouin's fixative for 24 hours at room temperature.

After fixation, dehydration of the brains was carried out by using graded ethanol series, cleared in xylene, and paraffin block were prepared using molten paraffin. The tissues were subsequently embedded in paraffin blocks with proper orientation to obtain serial sections through the entire rostro-caudal extent of the brain. Paraffin sections of the brain of thickness of 10µm were cut using rotary microtome, sections were spread and dried before histological processing. The sections of brain with pituitary were deparaffinised in xylene and passed through different grades of ethanol. They were stained in 0.1% solution of Laxol fast blue in 0.5% ethanol for 6 to 8 hrs., at 57°C. Sections were rinsed in 9% ethanol to wash off the excess stain and brought down to distilled water through descending grades of ethanol Differentiated quickly in 0,1% lithium carbonate solution and passed through several changes of 70% ethanol till there was sharp contrast between the greenish blue white matter and colourless grey matter, Sections were washed thoroughly in distilled water and stained in 0,1% cresyl violet solution for 2 hrs., then differentiated in several changes of 95% ethanol, absolute ethanol, cleared in xylene and mounted in DPX and cover slipped. The stained sections were examined under a compound light microscope at different magnifications to identify various neuronal nuclei and anatomical subdivisions of the forebrain.

Identification and nomenclature of forebrain nuclei were based on comparative cytoarchitectonic characteristics described in standard teleost neuroanatomical atlases and previous investigations on teleost brains (Wullimann and Rink, 2002; Magalhães-Horn *et al.,* 2018). Study has been carried out with permission of Institutional animal ethical committee (IAEC), PGTD of Zoology RTM Nagpur University Nagpur.

Results

Like in teleost, brain of *O. punctatus* is consisting of three parts forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon). Further, forebrain is partitioned into telencephalon and diencephalon (Fig. 1). In *O. punctatus* pair of Olfactory organ is located in the snout region involved in the olfaction, is connected to the olfactory bulbs via olfactory nerve. These bulbs in turn are caudally attached to the telencephalic hemisphere without any neuronal track, and thus olfactory bulb is sessile. Olfactory bulbs found to consist of four layers organised in the concentric way. Outermost is primary olfactory nerve layer (ONL) followed by glomerular layer (GL), mitral cell layer (MCL), and innermost is granular cell layer (GCL). Caudally the concentric arrangement in the bulb is less noticed, and the cells of the internal cell layer penetrate in the ventral telencephalon.

Telencephalon

Telencephalon is divided into area dorsalis telencephali and area ventralis telencephali. The area dorsalis telencephali is subdivided into pars medialis (Dm), pars centralis (Dc), pars lateralis dorsal (Dld) and pars lateralis ventral (Dlv). Area ventralis telencephali is divided into pars dorsalis (Vd), pars lateralis (VI) and pars ventralis (Vv) (Fig.2 A-C and Fig. 4 a-i). Area dorsalis telencephali pars medialis (Dm) occupies dorsal medial part of dorsal telencephalon (Fig.2 A-C and Fig. 4 a-i).

Fig.1 Broad diagrammatic representation of sagittal section of the forebrain and pituitary gland of the *O. punctatus* showing- **OB**, Olfactory bulb; **TEL,** Telencephalon; **POA**, Preoptic area; **ET**, Epithalamus; **T,** Thalamus; **HT**, Hypothalamus; **Pit**, Pituitary gland.

Fig.2 Diagrammatic representation of transverse section of the forebrain and pituitary gland of the *O. punctatus* showing **A-C**. Telencephalon is divided into area dorsalis telencephali and area ventralis telencephali. The area dorsalis telencephali is subdivided into pars medialis **(Dm),** pars centralis **(Dc),** pars lateralis dorsal **(Dld)** and pars lateralis ventral **(Dlv).** Area ventralis telencephali is divided into pars dorsalis **(Vd),** pars lateralis (**VI)** and pars ventralis **(Vv); ON**, Optic nerve; **OC**, Optic chiasmata; **OVLT**, organum vasculosum laminae terminalis; **LFB,** Lateral forebrain bundle; **NAC**, nucleus anterior commissure.

Fig.3 Diagrammatic representation of transverse section of the forebrain and pituitary gland of the *O. punctatus* showing **D-G**. Diencephalon is composed of various prominent nucleuses which include preopticus periventricularis dorsal **(NPPd),** nucleus preopticus periventricularis ventral **(NPPv),** nucleus preopticus magnocellularis **(NPOm),** nucleus preopticus parvocellularis **(NPOp)**, Nucleus hypothalamicus ventralis **(NHv);** nucleus hypothalamicus dorsalis **(NHd);** Nucleus lateralis tuberis **(NLT);** NLT pars lateralis **(NLTl);** NLT pars medialis **(NLTm);** nucleus recesses lateralis inferior **(NRLi);** nucleus recesses lateralis superior **(NRLs);** Ventro medial thalamnic nucleus **(VMN);** Ventro lateral thalamic nucleus **(VLN);** Dorsal habenular nuclei **(DHN);** Ventral habenular nuclei **(VHN);** Nucleus inferior lobi pars dorsalis **(NILd);** Nucleus inferior lobi pars ventralis **(NILv).**

Fig. 4 (a-i) T. S. of dorsal and ventral telencephalon. **a.** Dorsal telencephalon shows Dld, Dlv, Dc, Dm, Vv, VI, Vd and NE, (100X).**b.**Dm showing compactly arranged and intensely stained cells, Niss'l staining. (400X). **c.** Dorsal telencephalon showing Dc Niss'l staining, (400X). **d-e.** Dorsal telencephalon showing Dld and Dlv showing bigger and intensely stained perikarya, Nissl staining, (400X). **f-h.** Ventral telencephali showing intensely stained perkarya of Vd, Vv and Vl, (400X). **i.** Ventral Telencephalon showing NE, Niss'l staining, (400X).

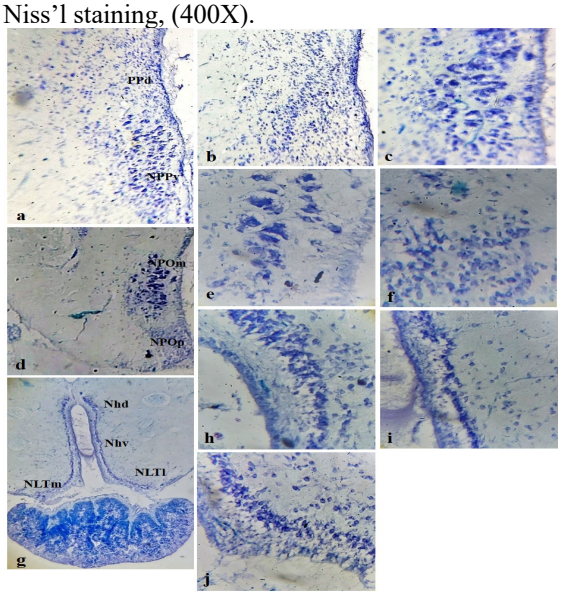
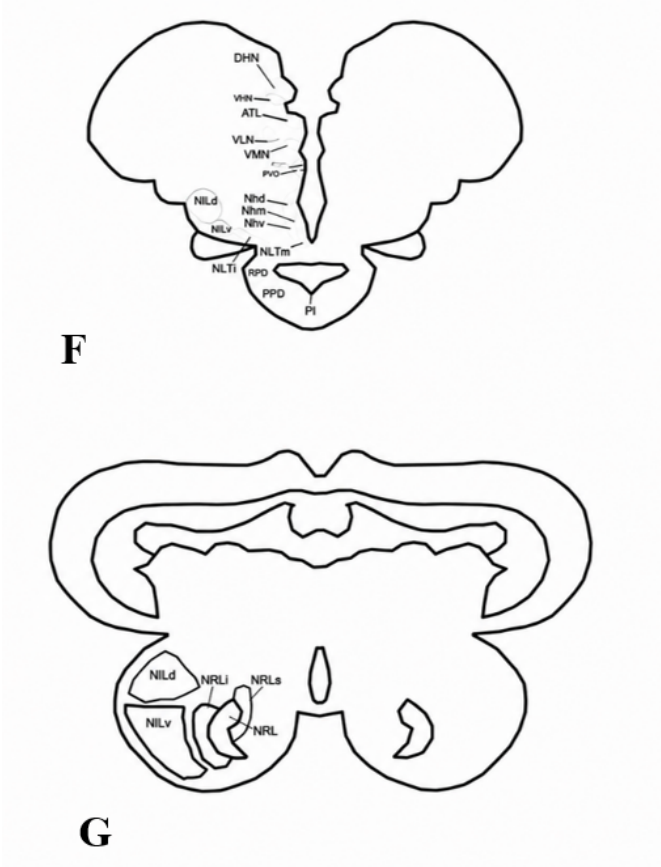


Fig. 5, **a,** T. S. of forebrain and pituitary in Periventricular region showing nucleus Periventricularis pars distal (NPPd), Periventricularis pars ventral (NPPv) **(100X);b-c,** Periventricular region showing nucleus periventricularis pars dorsal (NPPd), Periventricularis pars ventral (NPPv) Nissl's staining **(400X);** d, Preopticus region showing



nucleus NPOm and NPOpNiss'l staining **(100X); e-f,** Preopticus region showing larger and intensely stained perikarya of NPOm, Niss,l staining **(400X); g,** Hypothalamus showing Nucleus lateral tuberis medial (NLTm)and nucleus lateralis tuberis lateral (NLTl), Nucleus hypothalmicus dorsal (NHd), Nucleus hypothalmicus ventral (NHv), Niss'l staining **(100X). h-j,** Region showing Nucleus lateral tuberis medial (NLTm)and), Nucleus hypothalmicus dorsal (NHd), Nucleus hypothalmicus Ventral (NHv), Niss'l staining **(400X**)

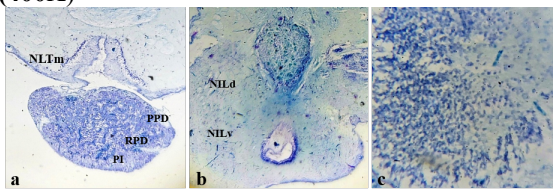


Fig. 6, a. T. S. of forebrain and pituitary gland showing PPD, RPD, PI in pituitary gland and NLTm, Niss'l staining (**100X)**; **b**, Inferior lobe showing cells of NILd and NILv, Niss'l staining in forebrain **(100X); c,** rostral forebrain showing cells of NILd and NILv, Niss'l staining **(400X);**

Dm has small sized cells which show moderate Niss'l stain. Towards lateral side of dorsal telecephali lies area dorsalis telencephali pars lateralis. Area dorsalis telencephali pars lateralis further divided into two regions, area dorsalis telencephali pars lateralis dorsal (Dld) and area dorsalis telencephali pars lateralis ventral (Dlv). Dld is towards the lateral dorsal side and consists of rounded moderately stained cells (Fig.2 A-C and Fig. 4 a-i). Dlv is located towards the lateral ventral side of area dorsalis telencephali with sized moderately stained cells. Dc occupies almost central part of area dorsalis telencephali and comprises intensely stained perikarya (Fig.4 a-i).

Area ventralis telencephali which occupies ventral medial portion of telencephalon (Fig) in turn is divided into area ventralis telencephali pars ventralis (Vv), pars dorsalis (Vd) and pars lateralis (VI) (Fig.4 a-i). Smaller, sparsely arranged cells are confined to Vv (Fig.4 a-i). Bigger compactly arranged cells which show intense Niss'l staining are distributed in Vd(Fig.4 a-i). They show moderate Niss"1 staining. VI in turn consists of a few but large sized cells with intense Niss'1 staining ((Fig.4 a-i). Telencephalon also consists of medial and lateral forebrain bundles in ventro lateral position. Close to the forebrain bundle, nucleus entopeduncularis (NE) (Fig.4 a-i) with bigger sized cells showing intense Niss'l staining is located.

Diencephalon

Diencephalon in *O. punctatus* consists of four regions- Preoptic areas. hypothalamus, thalamus and epithalamus (Fig. 5 a-j). Diencephalon is composed of various prominent nucleus which include preopticus periventricularis dorsal (NPPd), nucleus preopticus periventricularis ventral (NPPv), nucleus preopticus magnocellularis (NPOm), nucleus preopticus parvocellularis (NPOp), nucleus lateral tuberis (NLT), dorsal habenullar nucleus (DHN), ventralhabenullar nucleus (VHN) etc.

Preoptic area

In *O. punctatus*, preoptic area occupies region anterior to hypothalamus, thalamus, epithalamus (Fig. 4 d-f). Preoptic area is anteriorly covered by anterior commissure, dorsally by the telencephalon and ventrally by the optic chiasma. It encircles the preoptic recess of the third ventricle. Both AF positive and AF negative groups are present in the preoptic area. Among the neuronal groups of preoptic area, nucleus preopticus (NPO) has the distinction of being AF positive. Other neuronal groups are AF negative. In the paraventricular region which encircle third ventricle, neuronal complexes are paired and are AF- negative. Nucleus preopticus periventricularis (NPP) is present in paraventricular region (Fig. 5 a-c) It is present close to the preoptic recess on both the sides. NPP is divided into nucleus preopticus periventricularis dorsal (NPPd) and nucleus preopticus periventricularis ventral (NPPv). Neurons of NPPd are comparatively less in number and show moderate Nissl's stain (Fig. 5 a-c). Cells of NPPv are small, aggregated and show intense Nissl's staining. On either side of the preoptic recess, neurons of nucleus preopticus (NPO) are present. Based on the size, NPO is divided into NPO pars paravocellularis (NPOp) and NPO pars magnocellularis (NPOm). The neurons of NPOm are located dorsally around the third ventricle while NPOp lies above the optic chiasma (Fig. 5d). Cells of NPOm are larger and intensely stained with Niss'1 staining (Fig. 5e). Cells of NPOp are comparatively smaller but these perikaryon are also intensely stained with Niss'l staining (Fig. 5f). Perikarya of both, NPOm and NPOp are AF positive. Neurons of NPOm and NPOp collectively form the hypothalamo-hypophysial tract which innervates the pituitary.

Hypothalamus

In hypothalamus, rostral to the horizontal commissure (HC) lies tuberal area. It consists of neurons located on either side of the third ventricle and its lateral recess. In the tuberal area, lateral side of the third ventricle shows prominent nuclei which are nucleus hypothalamicus ventralis (NHv) and nucleus hypothalamicus dorsalis (NHd) (Fig. 5g). NHd cells are located toward dorsal side and are intensely stained by Nies'l stain. NHv has small cells with moderate Nissl's staining, Nucleus lateralis tuberis (NLT) is present near the infundibulum (Fig. 5g). It lies ventral to the horizontal commissure and runs along the base of hypothalamus. Depending upon the position and size of neurons, NLT is divisible into NLT pars

lateralis (NLTl) and NLT pars medialis (NLTm). NLTm are formed by larger, fewer cells which show intense Niss'l staining. Large, rounded cells form NLTI which are present laterally and show intense Niss'l staining. Behind the tuberal area, third ventricle on lateral side gives lateral recesses. Circumventing the lateral recess is nucleus recess lateralis (NRL). Based on the position, NRL is divisible into nucleus recesses lateralis inferior (NRLi) and nucleus recesses lateralis superior (NRLs). NRLi bears smaller, compactly arranged cells. They show intense Niss1 staining abundant cells showing moderate Niss'l staining are confined to NRLs.

Posterior to the tuberal area, inferior lobe is present. Inferior lobe bears nucleus inferior lobi (NIL). NIL is grouped into nucleus inferior lobi pars dorsalis (NILd) and nucleus inferior lobi pars ventralis (NILV). NILd has spindle shaped cells showing intense Niss'l staining. Rounded cells showing moderate Niss'l staining form NILv (Fig. 6b, 6c). Infundibulum or stalk formed axonal bunch connect hypothalamus to pituitary gland. Pituitary gland of *O. punctatus* is of platybasic type as neurohypophysis form plate like structure (Fig. 6a). The pituitary gland is divided into adenohypophysial region and neurohypophysis. Adenohypophysis is further Subdivided into rostral pars distalis (RPD), proximal pars distalis (PPD) and pars intermedia (PI) (Fig. 6a). Cells of RPD show intense staining of Niss'l. cells of PPD show moderate staining, AF positive cells are seen in PPD.

Thalamus

Thalamus is divisible into dorsal and ventral thalamus. Ventral thalamus also bears ventromedial thalamic nucleus (VMN) and ventrolateral thalamic nucleus (VLN). The neurons of VMN are spindle shaped and show intense Niss'l staining, whereas smaller, numerous cells showing moderate Niss'1 staining form VLN.

Epithalamus

Epithalamus is marked by paired habenular ganglia. Dorsal habenular nuclei (DHN) and ventral habenular nuclei (VHN) are present in each habenular ganglion. Cells of VHN show intense Niss'l staining and that of DHN show moderate Niss'l staining.

Discussion

The present investigation mainly focuses on detailed cytoarchitectonic description of the forebrain of *Ophiocephalus punctatus* (Bloch). The neuronal arrangements observed in the study demonstrates that forebrain is structurally consist of discreet neuronal populations and nuclear groups which can be differentiated on the basis of neuronal size, shape, staining intensity, density, and spatial distribution. This cytoarchitectonic criteria has been used to detect and delineate neuronal regions in the forebrain of teleost and provide an important neuroanatomical atlas for comparative neuroanatomical studies. (Nieuwenhuys *et al.,* 1998; Butler and Hodos, 2005).

In this study, the olfactory bulbs were found to consist of four layers organised in the concentric way. outermost is primary olfactory nerve layer (ONL) followed by glomerular layer (GL), mitral cell layer (MCL), and innermost is granular cell layer (GCL). A comparable four-layered organization and sessile condition of the olfactory bulbs has been observed in the dwarf snakehead *Channa gachua* by Baile and Patle (2011), which shows considerable degree of neuroanatomical structural similarity within genus *Channa*. Similar organization of olfactory bulb has also been documented in channel catfish *Ictalurus punctatus,* although the size, number, arrangement, and relative prominence of cellular layers may vary among species (Bass, 1981; Nieuwenhuys *et al.,* 1998).

The telencephalon of *O. punctatus* found to be clearly differentiated into dorsal and ventral telencephalic regions. The dorsal telencephalon consists of the medial (Dm), central (Dc), and lateral divisions, with the lateral region further divided into dorsal (Dld) and ventral (Dlv) regions. Further the ventral telencephalon consisted of Vv, Vd, and Vl, along with nucleus entopeduncularis. These findings from the study are broadly in a line with the general organization of the teleost telencephalon and with previous investigations in snakeheads, cichlids and in other ray-finned fishes (Bass, 1981; Baile and Patle, 2011; Burmeister *et al.,* 2009). These observations are very important in drawing the evolutionary interpretation if teleost telencephalon. Although, there is difference in the developmental organization of telencephalon and the tetrapod's, comparative studies reveal correspondence between teleost pallial and subpallial territories and regions of the forebrain of tetrapods. (Northcutt, 2008; Mueller and Wullimann, 2009).

The diencephalon of *O. punctatus* into preoptic, hypothalamic, thalamic, and epithalamus. This organizational finding is consistent with the broad structural pattern described for the teleost diencephalon (Nieuwenhuys *et al.,* 1998; Northcutt, 2008). The preoptic area was located around the preoptic recess of the third ventricle and contained the nucleus preopticus periventricularis, marked by dorsal and ventral subdivisions, along with magnocellular and parvocellular components of the nucleus preopticus. Earlier studies have also shown the crucial role of the preoptic area and nucleus preopticus in reproductive and endocrine regulation in *Ophiocephalus punctatus* (Belsare, 1967). More advance studies have demonstrated that various neuroendocrine signalling systems are distributed across the telencephalic and diencephalic regions of *O. punctatus*, further emphasizing indispensable

role of accurate anatomical identification of these nuclei (Tripathi and Prakash, 2022; Bakshi *et al.,* 2023).

Hypothalamus in the fishes contains several anatomically and functionally distinct neuronal cell groups that composes of the neuroendocrine system and regulate diverse physiological functions (González *et al.,* 2009). The thalamic region is represented by dorsal and ventral part, with the ventral thalamus containing ventromedial and ventrolateral nuclei. The ventromedial thalamic nucleus possesses spindle-shaped, darkly stained neurons, whereas the ventrolateral nucleus consisted of numerous smaller neurons showing moderate staining.

The epithalamus composed of paired habenular ganglia containing dorsal and ventral habenular nuclei. The ventral habenular neurons showed intense Nissl staining compared with the dorsal habenular neurons. The habenular apparatus is evolutionary conserved in the vertebrates. Thus The presence of distinct dorsal and ventral habenular regions in C. punctatus is consistent with the arrangements described in other teleosts and supports the conserved nature of epithalamus (Nieuwenhuys *et al.,* 1998; Northcutt, 2008; Bianco and Wilson, 2009). The organization of these neuronal populations gives insights into the crucial anatomical framework for analysing the neuroendocrine regulation mediated by the forebrain components in the teleost forebrain (Herget *et al.,* 2014).

Overall, the cytoarchitectonic organization observed in *O. punctatus* reveals clearly differentiated forebrain consisting of structurally distinct telencephalic and diencephalic neuronal cellular architecture. The combination of serial transverse section analysis and Nissl staining found to be useful for identifying and distinguishing these regions. Current findings establish a morphological baseline for upcoming investigation involving neuropeptide distribution, neurotransmitter systems, reproductive neuroendocrinology, and comparative evolution of the teleost nervous system.

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