LOCALIZATION OF NITRIC OXIDE IN THE BRAIN OF THE *LABEO ROHITA* (HAM.)

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Abstract :

Nitric oxide is a novel gaseous paracrine signaling molecule. Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) is mainly used as a marker for the localization of nitric oxide synthase (NOS), an enzyme used in the production of nitric oxide (NO). NADPH-d positive neurons has been studied using the coenzyme NADPH as a histochemical localization. In contrast to other teleosts and mammals, the distribution pattern of L. rohita demonstrates species uniqueness. The location of nNOS in the L. rohita olfactory system reveals a positive response to NADPH-d in the nervus terminalis, mitral cells, and olfactory receptor neurons. NADPH-d positive neurons can be found in the telencephalon's nucleus entopeduncularis, nucleus preopticus pars parvocelluaris, and magnocellularis.

The tuberal area in the nucleus lateralis tuberis pars lateralis and medialis show NADPH-d positive neurons and fibers. The proximal pars distalis of the pituitary gland show NADPH-d positive cells. Localization of NADPH-d positive neurons and fiber in the nuclei in the brain related to reproduction and the presence in the pituitary gland also suggests its possible role in reproductive signaling.

Introduction :

A novel neurotransmitter, gaseous nitric oxide (NO) can be synthesized in the brain (Brann *et al.*, 1997). Different populations of neurons in the central and peripheral nervous systems are selectively stained by the NADPH-d histochemical method. The vertebrate brain uses nitric oxide (NO), a gaseous inter or intracellular messenger molecule, as a neurotransmitter and neuromodulator (Knowles *et al.*, 1989; Garthwaite, 1991; Bredt and Snyder, 1992). NO has a unique role in aquatic chordates, such as teleosts, and amphibians, so highly conserved (Locascio, *et al.*, 2023). NO producing systems have been described in the brains of different non-mammalian vertebrates such as fish (Holmquist, *et al.*, 1994; Bruning *et al*., 1995), frog in amphibians (Prasada Rao *et al*., 1997), turtle in reptiles (Bruning *et al*., 1994) and chicken in birds (Bruning, 1993). Nitric oxide synthase (NOS) is the enzyme that produces NO, and NADPH-diaphorase histochemistry shows that neurons have NOS (Hope *et al.*, 1991; Dawson *et al.*, 1991). NOS is localized by NADPH-d in the olfactory system of mammals (Hopkins *et al*., 1996), amphibians (Porteros, *et al*., 1996), and teleosts (Singru *et al*., 2003). Hypothalamic neuropeptide is controlled by the regulatory pathway through NO, such as GnRH (Wang *et al*., 1998; Prevot *et al*., 1999; 2000), Corticotropin-releasing factor (Prevot *et al*., 2000) and NPY (Bitran *et al*., 1999). NO is detected in the neurons of the preoptic area (Warembourg *et al*., 1999). Pituitary hormones such as GTH secretion can be modulated by NO (Yu *et al*., 1997). The cells expressing NADPH-d activity, located in the hypothalamus and pituitary are reported to be involved in hormone regulation (Jadhao *et al.* 1999). NO is needed for oocyte growth and egg stimulation in teleosts, much as it is in mammals (Rochon and Corti, 2020).

The role of NO in the brain and pituitary is still lacking in teleosts. The present work was therefore undertaken to study the possible role of NO in reproduction using histochemical localization of the Indian major carp, *Labeo rohita*.

Materials Anf Methods :

1. Collection and maintenance of fish :

Fish were gathered from the Nagpur region's natural habitats. After being transported to the lab, the fish were acclimated in an aquarium. Sexually matured fishes were selected with weights ranging between 1.25 kg to 1.75 kg and lengths 25 to 35 cm.

2. NADPH-diaphorase Histochemistry° :

The males of *Labeo rohita* (n = 6) were anesthetized with 2-phenoxyethanol and transcardial perfusion was performed with 600-750 ml ice-cold phosphate buffer (7.42 pH) followed by an equal volume of ice-cold Zamboni fixative. Tissue were fixed in Zamboni fixative for 24 hrs. The tissues were transferred to 10% and 30% sucrose solution as a cryoprotectant and cut on the Leica cryostat in transverse and sagittal planes at 15 m thickness. The sections were mounted on poly-2-lysine coated slides and processed for NADPH-diaphorase histochemistry. The histochemical process was carried out in complete darkness. The sections were rinsed in 0.1M PBS (pH 7.4) containing 0.3% triton X-100, followed by incubation in PBS containing nitroblue tetrazolium (NBT) and -NADP for 3-5 hrs at 37°C After being incubated and cleaned in PBS, the section slides were mounted in glycerol-jelly.

The same procedure was used for the controls, except NBT or NADPH were not added to the reaction mixture. Sections exhibited no staining, supporting the findings that NADPH-diaphorase activity was the reason for the staining.

Results :

In *Labeo rohita*, olfactory rosettes are present in a cavity enclosed by a skin flap having an inlet and outlet to allow water to flow. The Olfactory rosette consists of lamellae radiating from the central raphae. The olfactory receptor neurons (ORNs), basal cells, and supporting cells comprise the sensory portion of the olfactory epithelium.

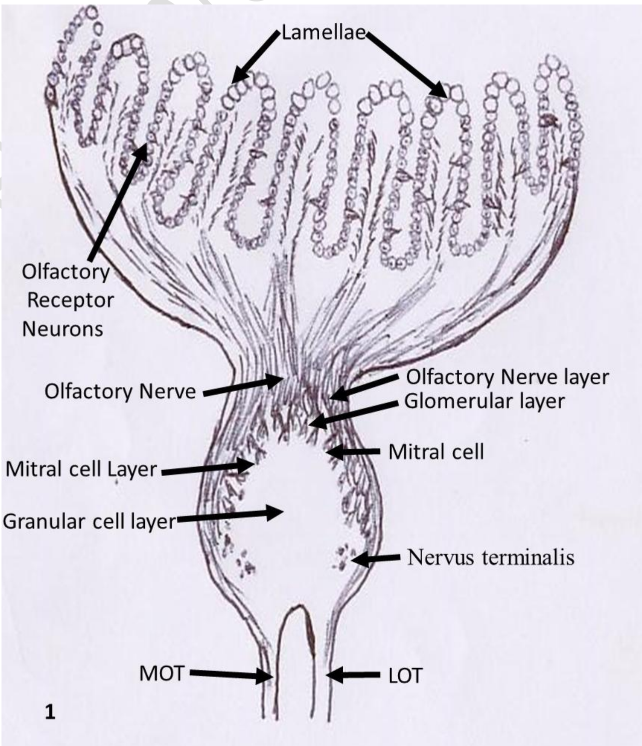

Figure 1. Diagrammatic representation of the Saggital section through olfactory organ and bulb showing the distribution of NADPH-d activity, granular cell layer; glomerular layer; mitral cell layer; MOT, medial olfactory tract; nervus terminalis; olfactory nerve; olfactory nerve layer.

The organization of NADPH-d activity shows wide distribution in the olfactory system and hypothalamo-hypophysial axis (Fig.1, 2). In sensory regions of the olfactory epithelium strong NADPH-d positive reaction is observed in ORNs (Fig.3A). NADPH-d-negative neurons are also seen in the sensory part of the olfactory epithelium (Fig.3A). The dendrites stained with NADPHd are long and thin (Fig. 3B). NADPH-d staining is also seen in a small number of basal cells next to the ORNs (Fig. 3A). The axons of the ORN's showed NADPH-d positive reaction (Fig.3B). These axons together formed fascicles in basal lamina (Fig.3B) and entered the olfactory nerve. The olfactory nerve's fascicles reached the olfactory nerve layer (ONL) of the olfactory bulb in a peripheral manner, displaying strong NADPH-d staining (Fig. 3C). The mitral cells in the glomerular layer were innervated by NADPH-d positive glomeruli (Fig. 3D). Mitral cells also show NADPH-d staining in the mitral cell layer (Fig.3C), but very few granular cells show NADPH-d positive reaction (Fig.3C). In the dorsal margin of the olfactory bulb, near the olfactory tract, giant cells of the Nucleus terminalis (NT) show moderate NADPH-d staining (Fig.3E). Lightly stained NADPH-d positive fibers are also seen in MOT.

In the hypothalamus, NADPHd positive cells are found in the Nucleus preopticus pars parvocellularis (NPOp) and Nucleus preopticus pars magnocellularis (NPOm) (Fig.3F, G). Both the perikarya and the axons of the NPOm exhibit NADPH-d activity (Fig. 3G). In the ventrolateral position of hypothalamus strong NADPHd positive activity is seen in the bigger sized neurons of the Nucleus lateralis tuberis lateral (NLTl) (Fig. 3 H). and few comparatively smaller sized neurons near ventricle exhibit NADPHd positive reaction in the Nucleus lateralis tuberis pars medialis (NLTm) (Fig. 3 I). In the pituitary gland, a number of moderately stained NADPHd positive cells are found in the proximal pars distalis (PPD) (Fig.3J).

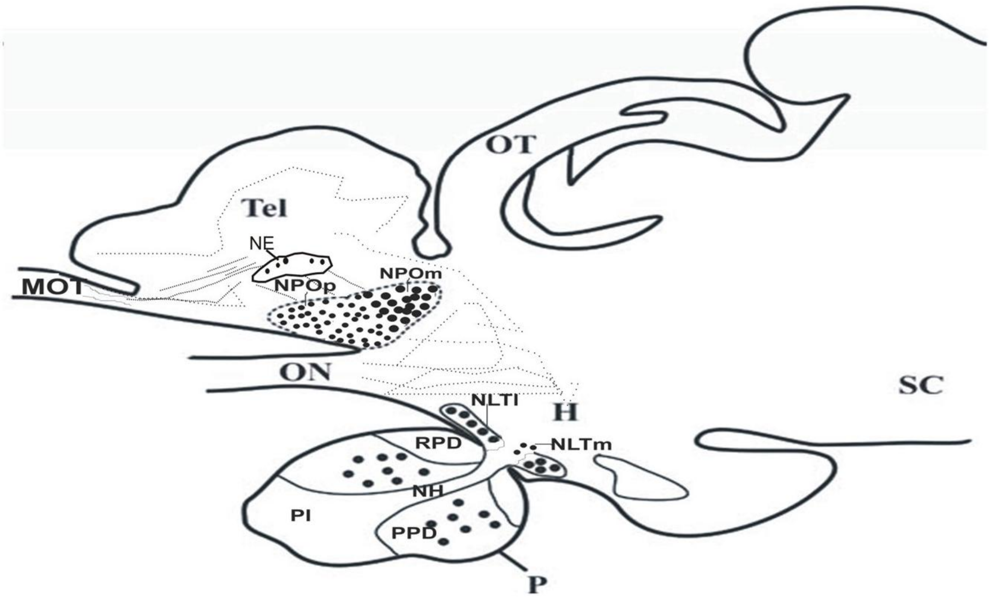

Figure 2. Diagrammatic representation of the saggital section through hypothalamus and pituitary gland showing the distribution of the NADPH-d activity, TEL, Telencephalon; MOT, medial olfactory tract ; NE, Nucleus entopeducularis; NH, Neurohypophysis; NPOm, Nucleus preopticus pars mangnocelularis; NPOp, nucleus preopticus pars parvocelaris; ON, optic nerve ;OT, optic tectum; P, pituitary gland; PI, pars intermedia; PPD, proximal pars distalis; RPD, rostral pars distalis; TEL, telecephalon; NLTl, neucleus lateralis tuberis pars lateralis; NLTm, nucleus lateralis tuberis pars medialis.

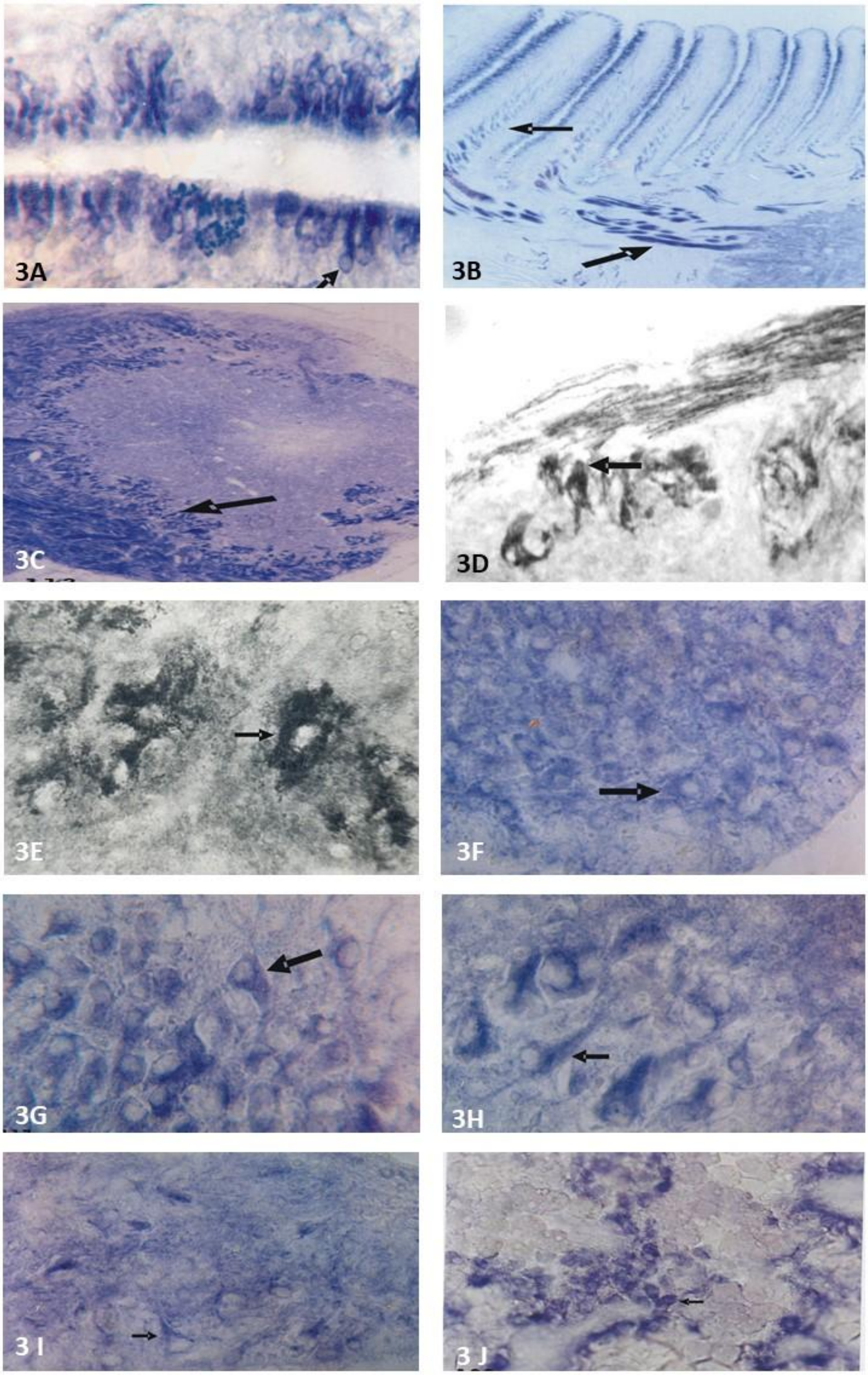

Figure 3. **A.** Saggital section of olfactory organ showing NADPH-d activity in the olfactory receptor neurons (arrow). 600X. **B**. Saggital section of olfactory organ showing NADPH-d positive fiber fascicle enter in the olfactory nerve (arrow). 60X. **C.** Saggital section of the olfactory bulb showing strong NADPH-d positive reaction in the olfactory nerve layer, glomerular layer and mitral cell layer (arrow). 25X. **D.** Saggital section of the olfactory bulb showing the NADPH-d fiber fascicles arborizes over the mitral cell (arrow). 250X. **E.** Saggital section of the olfactory

bulb showing strong NADPH-d reaction in the giant neurons of the nervus terminalis (arrow).400X.. **F.** Transverse section of the brain through hypothalamus showing strong NADPH-d reaction in the neurons of the nucleus preopticus pars parvocellulis (NPOp) (arrow). 400X**. G.** Transverse section of the brain through hypothalamus showing strong NADPH-d activity in the neurons of the nucleus preopticus pars mangnocellularis (NPOm) (arrow). 250X. **H.** Transverse section of the brain through hypothalamus showing strong NADPH-d positive reaction in the bigger neurons of the nucleus lateralis tuberis pars lateralis NLTl). 250X. **I.** Transverse section of the brain through hypothalamus showing moderate NADPH-d activity in the neurons of the nucleus lateralis tuberis pars medialis (NLTm). 250X. **J.** Saggital section of pituitary gland showing NADPH-d positive cell in the proximal pars distalis. 250X.

Discussion :

Distribution of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) for NO in the olfactory system and hypothalmo-hypophysial system in the Indian major carp, *Labeo rohita* is reported.

The vertebrate brain uses nitric oxide, a gaseous messenger molecule, as a neurotransmitter and neuromodulator (Garthwaite, 1991). Nitric oxide is synthesized in the brain by a single enzyme, nitric oxide synthase (NOS). Brain NADPH-diaphorase has been identified as NOS (Dawson *et al*., 1991: Hope *et al*., 1991). For the authenticity of results, controls were carried out such as incubation without substrate (NADPH) or without chromogen (nitro blue tetrazolium) which did not show any histochemical reaction.

The distribution of NADPH-diaphorase positive neurons has been histochemically investigated in mammals (Vincent and Kimura, 1992), birds (Bruning, 1993; Panzica *et al*., 1994), amphibians (Munoz *et al*., 1996) and teleosts (Anken and Rahmann, 1996). Size and staining intensity were used to separate three distinct populations of NADPH-diaphorase active cells. These differences have been similarly reported in previous classifications of NADPH-diaphorase positive cells in the central nervous system of mammals (Mizukawa *et al*., 1989).

In *L. rohita*, a wide distribution of NADPH-diaphorase activity in the olfactory system was seen. In the adult fish NADPH-diaphorase activity in olfactory receptor neurons (ORNs) is observed. Similarly, NADPH-diaphorase staining is reported in ORN's of *O. mossambicus* (Singru *et al*., 2003) and using nNOS antibody the results in the olfactory system were confirmed but NADPH-diaphorase reactivity is not reported in the ORN's of adult vertebrates (Sanchez-Islas and Leon-Olea, 2001; Schmachtenberg *et al*., 2001). The presence of NADPH-diaphorase in the ORNs of *L. rohita* suggests that Nitric oxide plays an important role in chemosensory signal transduction (Zhao *et al*., 1994; Singru *et al*., 2003) and its axons enter into the olfactory bulb through the olfactory nerve and innervate mitral cells in the glomeruler layer (Satou,1990). In olfactory epithelium, the presence of NADPH-diaphorase in the basal cell of *L. rohita* suggests that Nitric oxide may be involved in regeneration and development (Broillet and Firestein, 1996; Arnhold *et al*., 1997), as a renewing ORN arises from a basal cell division to form an immature bipolar neuron. The dendrites of the immature sensory neurons grow towards the surface of the olfactory epithelium and develop cilia or microvilli on it and a single axon extends towards the olfactory bulb. In this way, ORN regenerates and is exposed to odors dissolved within the olfactory mucous (Zippel *et al*., 1997).

In *L. rohita,* the mitral cells in the olfactory exhibit strong NADPH-diaphorase staining. Similarly, it is known that nNOS is present in the mitral cells of the sheep's olfactory bulb (Kendrick *et al.*, 1997). In *L. rohita* and other teleosts, such as *Rhodeus amarus*, the glomerular layer of the olfactory bulb (Baby *et al.*, 2000) and *Oreochromis mossambicus* (Singru *et al.*, 2003), has a high NADPH-diaphorase positive reaction. Granular cells in the olfactory bulb exhibit NADPH-diaphorase positive activity in the current investigation. It is known that the granular cells in the olfactory bulb have NOS immunoreactive activity in the anuran and urodele (Porteros *et al*., 1996) and in the sheep (Kendrick *et* *al*., 1997) and NADPH-diaphorase positive activity in the cichlid (Singru et al., 2003). Since granular cell dendrites have been shown to reach the glomerular layer and form reciprocal synapses with the mitral cell dendrites, it is possible that granular cells in the bulb will spread their dendrites to the GL (Satou, 1990).

Neurons in the nervus terminalis (NT) show moderate NADPH-diaphorase staining in *L. rohita*. As compared to other animals, NADPH-diaphorase staining is reported in the NT neurons of the cichlid fish (Singru *et al*., 2003), lungfish (Schober *et al*., 1994) and frog (Bruning and Mayer, 1996). GnRH and NADPH-diaphorase containing neurons in the olfactory bulb of *Rhodeus amarus (*Baby *et al*., 2000) have been reported. It has been suggested that NO influences the biosynthesis and release of GnRH (Wang *et al*., 1998). Therefore, it may be inferred that No may modulate the function of GnRH neurons in the olfactory system and NT and influence pheromone regulated reproduction (Dulka and Stacey 1991).

The median olfactory tract (MOT) exhibits weak fibres of NADPH-diaphorase staining in the current investigation. Similarly, in *Rhodeus amarus* NADPH-diaphorase positive granules are reported in the MOT (Baby *et al*., 2000). The presence of NADPH-diaphorase positive fibers in the MOT suggests that it is involved in sending reproductive signals to the brain. It has been mentioned, that the presence of GnRH neurons and NADPH-diaphorase positive granules in the MOT indicate that NO may play a role in olfactory transduction in *Rhodeus amarus* (Baby *et al*., 2000) and in rodents (Hopkins *et al*., 1996).

In the hypothalamo-hypophysial system, NADPH-diaphorase positive neurons are observed in the important areas of the hypothalamus and pituitary gland which are concerned with reproduction.

In the present study, NADPH-diaphorase positive cells are observed in the Nucleus pars parvocellularis (NPOp). Bony fishes' NPOp has been shown to include reactive NADPH-diaphorase (Bruning *et al.*, 1995; Jadhav *et al.*, 1999). It has already been reported that NPO neurons project to the pituitary gland in teleost (Angalade *et al*., 1993; Prasad Rao *et al*., 1993) and therefore, it may be inferred that they might play a role in various hypothalamo hypophysial regulatory systems in teleost as is also revealed in several higher vertebrates including mammals (Brann *et al*., 1997).

Intense staining of NADPH-diaphorase is seen in the neurons and axons of the Nucleus preopticus pars magnocellularis (NPOm) of *L. rohita*. Similar areas are also known to contain NADPH-diaphorase positive cells in the teleosts (Anken and Rahmann, 1996; Villani and Guarnieri, 1995). NPO neurons in the brain of *Tilapia* are shown to express GnRH (Parhar, 1999). GnRH and NADPH-diaphorase have been shown to co-localize in *Rhodeus amarus* (Baby *et al.*, 2000). In the present study, NPY fibers innervate NPO as mentioned earlier in the distribution of NPY. Therefore, these results suggest that GnRH secretion in the NPO may be stimulated by NPY and modulated by NO.

It is shown that *L. rohita* contains intense NADPH-diaphorase positive neurons of the Nucleus lateralis tuberis pars lateralis (NLTl) and nucleus lateralis tuberis pars medialis (NLTm). Bruning *et al.*, (1995) demonstrated the presence of NADPH-diaphorase staining in NLT neurons. The presence of GnRH in NLT neurons is demonstrated (Kah *et al*., 1984; Anderson *et al*., 1995) and the involvement of NLT in reproduction is also reported (Peter and Fryer, 1983). NLT neurons innervate the pituitary and are indirectly involved in the regulation of reproduction (Peter and Fryer, 1983). With their pituitary innervations, NO and GnRH in the NLT unquestionably demonstrate the function of nitric oxide in reproduction.

NADPH-diaphorase staining is encountered in the cells of the proximal pars distalis (PPD) area of the pituitary gland of *L. rohita*. Similar results are reported in *Ictalurus nebulosus* (Jadhao *et al.*, 1999). According to Gobetti and Zerani (1998), NADPH-diaphorase in the pituitary further implies that NO mediates basal and GnRH-induced gonadotropin production.

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