J Endocrinol Reprod 18 (2014) 2 : 75 - 86 **Original Article**

Neuropeptide Y (NPY) distribution in the forebrain of adult spiny eel, *Macrognathus pancalus*

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Summary

In the present study, the distribution of neuropeptide Y (NPY)-immunoreactive neurons and fibers in the forebrain of adult spiny eel, *Macrognathus pancalus,* which is a bottom-dwelling nocturnal fish, was investigated. Serial Nissl-stained brain sections were used to demarcate forebrain regions and neuronal structures. NPY peptide-containing cell bodies and fibers localized immunocytochemically were found widely distributed throughout the forebrain. The brain areas showing NPY distribution included predominant cell groups in the telencephalon (nucleus entopeduncularis, NE; nucleus of area ventralis telencephali, Vn), diencephalon (nucleus preopticus, pars parvocellularis, NPOp; nucleus preopticus, pars magnocellularis, NPOm; nucleus lateralis tuberis, NLT) and mesencephalon (midbrain tegmentum, MT). The important areas with only NPY-immunoreactive (-ir) fibers included olfactory bulb (OB), area dorsalis telencephali pars anterioris (Da), dorsal part of Dmd (Dmdd), ventral subdivision of Dl (Dlv), anterior subdivision of Dl (Dla), preoptic area (POA), optic tectum (OTec) and nucleus recessi lateralis (NRL). The pattern of NPY distribution in the forebrain of *M. pancalus* suggests its role in processing of many physiological functions (viz., feeding, daily activities, reproduction and other metabolic processes). The basic information on anatomical localization of NPY in eel will help to understand better the seasonal variations of NPY and its interaction with other reproductive hormones.

Key Words: Forebrain, Immunocytochemistry, NPY distribution, Spiny eel.

Abbreviations

A, anterior thalamic nucleus; AC, anterior commissure; BSA, bovine serum albumin; CPA, central pretectal area; CPAd, central pretectal area dorsalis; CPAv, central pretectal area ventralis; CSF, cerebrospinal fluid; D, area dorsalis telencephali; Da, D pars anterior; DAB, diaminobenzidine; Dc, D pars centralis; Dca, anterior subdivision of Dc; Dcad, dorsal part of Dca; Dcav ,ventral part of Dca; Dcd, dorsal subdivision of Dc, Dcl, lateral subdivision of Dc; Dcm medial subdivision of Dc; Dcp,posterior subdivision of Dc; Dd, D pars dorsalis; Dl, D pars lateralis; Dla, anterior subdivision of Dl; Dld, dorsal subdivision of Dl; Dlp, posterior subdivision of Dl; Dlv ventral subdivision of Dl; Dm, D pars medialis; Dma anterior subdivision of Dm; Dmd, dorsal subdivision of Dm; Dmdd, dorsal part of Dmd; Dmdv, ventral part of Dmd; Dmv,ventral subdivision of Dm; Dmvd, dorsal part of Dmv; Dmvv, ventral part of Dmv; Dp, D pars posterioris; DPX distrene plasticiser xylene; ECL, External cellular layer; GL, glomerular layer; HC, horizontal commissure; HG, habenular ganglion; Hyp, hypothalamus; ICL, Internal cellular layer; IL, inferior lobe ;LFB, lateral forebrain bundle; MT, midbrain tegmentum;NE, nucleus entopeduncularis; NLT,nucleus lateralis tuberis; NPGl, nucleus preglomerular lateralis; NPGMm, nucleus preglomerular medialis; NPO, nucleus preopticus; NPOm, NPO pars magnocellularis; NPOp, NPO pars parvocellularis; NPY neuropeptide Y; NPY-ir, NPY immunoreactive;NRL, nucleus recessi lateralis; NT, ganglia of nervus terminalis (nucleus olfactoretinalis); OB, olfactory bulb; OC, optic chiasma; ONL, olfactory nerve layer; Otec, optic tectum; PBS, phosphate buffered saline; PI, pars intermedia part of Pit; Pit, pituitary; POA, preoptic area; PPD, proximal pars distalis part of Pit; PVO, paraventricular organ; RL, recessus lateralis; RPD, rostral pars distalis part of Pit; SCN, suprachiasmatic nucleus ;SFGS, stratum griseum et album superficial; SGC, stratum griseum centrale; SM, stratum marginale; SPV, stratum periventriculare; TA, tuberal area; Tel, telencephalon; Tlo, torus longitudinalis; TS, torus semicircularis; V, area ventralis telencephali; Vd, V pars dorsalis; Ve,ventricle; Vl, V pars lateralis; Vm, ventromedial thalamic nucleus; VMN, ventro medial nucleus; Vn, nucleus of V; Vp, V pars posterioris; Vs,V pars supracommissuralis; Vv, V pars ventralis

Introduction

Neuropeptide Y (NPY), a 36 amino-acid peptide first isolated from porcine brain (Tatemoto et al., 1982), is probably the most studied neuropeptide found across all the vertebrates and is highly conserved among them (Cerda-Reverter & Larhammar, 2000). In brain, the NPY concentration is relatively higher than of any other neuropeptide (Allen et al., 1983).

NPY-immunoreactivity has been extensively studied in the brain of several fish species, e.g., white sturgeon (Chiba & Honma, 1994), modern

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elasmobranchs (Vallarino et al., 1988) and teleosts (Subhedar et al., 1996; Gaikwad et al., 2004). In fish the NPY-immunoreactivity has also been reported in olfactory bulb (Subhedar et al., 1996), olfactory receptors neurons (Gaikwad et al., 2004), neurons of nervus terminalis (Castro et al., 1999), basal telencephalon and nucleus entopeduncularis (Rodríguez-Gómez et al., 2001; Pirone et al., 2008). In hypothalamus, which is principally involved in several daily and seasonal functions, the NPY-immunoreactive fibers are conspicuous in the preoptic area, suprachiasmatic nucleus, tuberal hypothalamus, paraventricular thalamic regions and pituitary gland (Rodríguez-Gómez et al., 2001)

The involvement of NPY in daily and seasonal physiology of fish is well known that ranges from daily feeding mechanism to the seasonal reproduction. In reproduction, the role of NPY in stimulating the release of gonadotropin and growth hormones is well known (Sakharkar et al., 2005) as suggested by colocalization of NPY and GnRH in forebrain of catfish (Gaikwad et al., 2005). NPY stimulates the secretion of GnRH and LH, and is linked with reproductive cycle (Gaikwad et al., 2003; Mazumdar et al., 2007). NPY-ir fibers have been identified in the hypophysis of the sea bass and ayu (Moons et al., 1989; Chiba et al., 1996). Role of NPY in the regulation of food intake (Himick & Peter, 1995; Peng & Peter, 1997) and other associated behaviors in fish has already been investigated (Narnaware et al., 2000; Volkoff & Peter, 2001).

While the distribution of NPY has been studied in many teleost fish species, the freshwater eels have received very little attention. Therefore, in the present study we localized the distribution and organization of NPY-immunoreactivity in the forebrain of spiny eel, which is nocturnal and bottom-dwelling. This will help understand the role of NPY in regulation of seasonal reproduction.

Materials and Methods

Animal handling and housing conditions

The spiny eel (*Macrognathus pancalus),* collected from River Gomti, Lucknow, India (27ºN, 81º E), were supplied by the local fishermen. Fish (n = 5) were collected in August 2011 (the time when their ovaries were in spawning state) and transported live in water filled bucket to the fish laboratory at Department of Zoology, University of Lucknow, Lucknow. The fishes weighed 15-20 g, and the mean length was 12 cm. In the laboratory, initially they were maintained in well-aerated glass aquarium under natural light/dark conditions for about 24 h. They were fed *ad libitum* with tubifex and live *Chironomus*. Prior to perfusion, fishes were anaesthetized with 2-phenoxy ethanol (0.05% in water, Sigma, St. Louis, MO, USA). They were perfused transcardially with 50 ml ice-cold phosphate buffered saline (PBS, pH 7.4) followed by 100 ml ice cold 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for fixation. The brains were quickly dissected out and post-fixed overnight in the same fixative. Thereafter, brains were cryoprotected by immersing them sequentially in 10%, 20% (2 h each) and finally 30% sucrose solution (Merck, Mumbai, India) in 0.1 M phosphate buffer (PB) at 4°C until they sank in the solution. Brains were embedded in 15% PVP solution (polyvinylpyrrolidone; PVP40T, Sigma St. Louis, MO) and sectioned in the coronal plane on a cryostat CM 1850 (Leica, Germany) at 20 µm thickness. Every alternate section was directly taken on glass slides coated with poly L-Lysine (P8920, Sigma, St. Louis, MO), and thus we prepared two series of brain sections on slides. The first series of sections were dried at room temperature for several hours, and then processed for Nissl staining (Klüver & Barrera, 1953) to identify regions of interest with the help of available literature on fish (Mukuda & Ando, 2003; Sakharkar et al., 2005; Burmeister et al., 2009). The second series of brain sections was stored at -80ºC until they were processed for the NPY immunocytochemistry.

NPY immunocytochemistry

For NPY immunocytochemistry, we used the standard streptavidin-biotin-peroxidase protocol with minor modifications as described by Sakharkar et al. (2005). The sections were processed at one time to minimize the difference, if any, in the staining intensity. The procedure for immunocytochemistry started with the washing of sections with 10mM phosphate-

buffered saline (PBS, pH 7.4; 3 times, 10 minutes each). Then sections were treated with 0.3% hydrogen peroxide (H_2O_2) dissolved in methanol for 30 minutes to block the endogenous peroxidase activity, washed in PBS (3 times, 10 minutes each), treated with blocking solution (PBSBT, 1% normal bovine serum albumin, BSA, dissolved in PBS containing 0.3% Triton-X-100) for 30 minutes. The sections were incubated overnight at 4ºC with primary antibody for NPY (dilution 1:6000; anti-rabbit polyclonal, N 9528; Sigma, St. Louis, MO). After overnight incubation, sections were washed with PBS (3 times, 10 min each) followed by incubation with secondary antibody (dilution 1:200; biotinylated goat anti-rabbit, E8386; Sigma, St. Louis, MO) for 2 h at room temperature. The sections were again washed with PBS (3 times, 10 min each), processed for 2 h at room temperature with Extravidin-peroxidase (dilution 1:200; E8386; Sigma, St. Louis, MO). After rinsing in PBS (3 times, 10 min each), sections were treated with diaminobenzidine solution (DAB; D4293; Sigma, St. Louis, MO), prepared in 0.1M phosphate buffer (pH 7.4) for 2-3 minutes, to visualize the antigen-antibody reaction. The color reaction was stopped by adding the PBS, when the optimum color was visible with minimal background. The sections were rinsed in distilled water and dried overnight at room temperature. The slides were washed in PBS, dehydrated in ascending grades of alcohol, cleared in xylene, mounted in DPX (Merck, Mumbai, India) and observed under microscope for analysis.

Microscopy and photography

The sections were observed under Leica DM 3000 microscope equipped with Leica DFC 420C camera. The chosen fields from sections were photographed using standardized illumination for all sections, and images were adjusted for size and minor contrast and brightness, if required, using Adobe Photoshop (version 7.0). The selected images were paneled using Corel Draw (version 13.0). The cytoarchitectonic maps were prepared to show the distribution of NPY-ir cells and fibers (Fig. 1).

Specificity of the antibodies

Several control procedures were employed to verify the specificity of the NPY antibody used, such as omission of the primary antibody from the reaction, replacement of the antiserum against NPY with buffer, normal bovine serum albumin (BSA) and pre-adsorption of 1 ml diluted primary antibody with the NPY peptide (N 5017; Sigma, St. Louis, MO) at 10^5 M concentration for 24 h at 4°C prior to incubation. All these control procedures did not yield any positive immunoreaction for NPY.

Results

NPY-ir cells and fibers were carefully studied in the serial transverse sections and drawn in the schematically representative figure 1. In spiny eel, NPY immunoreactive fibers and cells were widely distributed in the forebrain.

Olfactory bulb

Olfactory bulb is sessile as in many other teleosts. The organization of the olfactory bulb in spiny eel is distinguishable into four layers (Fig. 1a) which are the internal cellular layer (ICL), the external cellular layer (ECL), the glomerular layer (GL) and olfactory nerve layer (ONL). ICL showed a reasonable amount of NPY-ir fibers, although ECL, GL and ONL were devoid of NPY immunoreactivity (Figs. 1a, b; 3a).

Telencephalon

The telencephalon (Tel) of the spiny eel is divided into left and right hemispheres, which are differentiated into dorsal and ventral telencephalic regions. NPY- immunoreactivity was moderate to intense in both regions. In different telencephalic structures, we found consistently high NPY-ir fibres in the areas, such as dorsal part of Dmd (Dmdd), ventral part of Dmv (Dmvv), area dorsalis telencephali pars anterioris (Da), area dorsalis telencephali pars dorsalis (Dd), anterior subdivision of Dl (Dla), and posterior subdivision of Dl (Dlp), whereas comparatively less NPY-ir fiber density was found in the adjoining areas like dorsal subdivision of Dl (Dld), dorsal part of Dca (Dcad), median subdision of Dc (Dcm), area ventralis telencephali pars supracommissuralis (Vs), area ventralis telencephali, pars ventralis (Vv) and area ventralis telencephali pars dorsalis (Vd; Figs. 1b-g; 3b-e). Several intense, round or oval NPY-ir cells were found in area ventralis telencephali pars lateralis

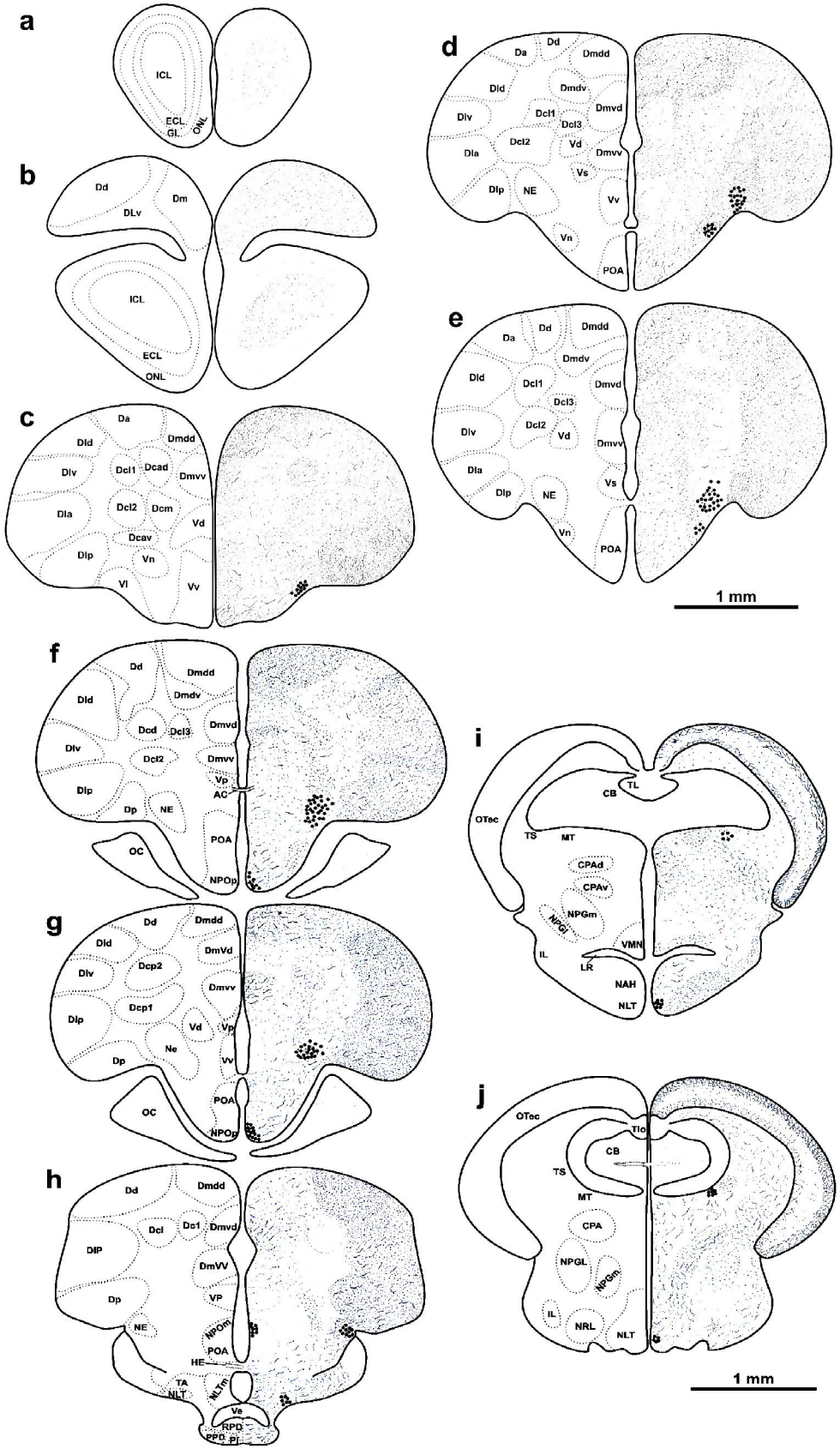

Fig.1 Schematic drawings of rostrocaudal series of ten representative transverse sections (**a-j**) at different levels in the brain of spiny eel, showing cytoarchitectonic areas on the left and distribution of NPY-immunoreactive (ir) cells (dark circles) and fibres on the right. Scale bar: 1mm.

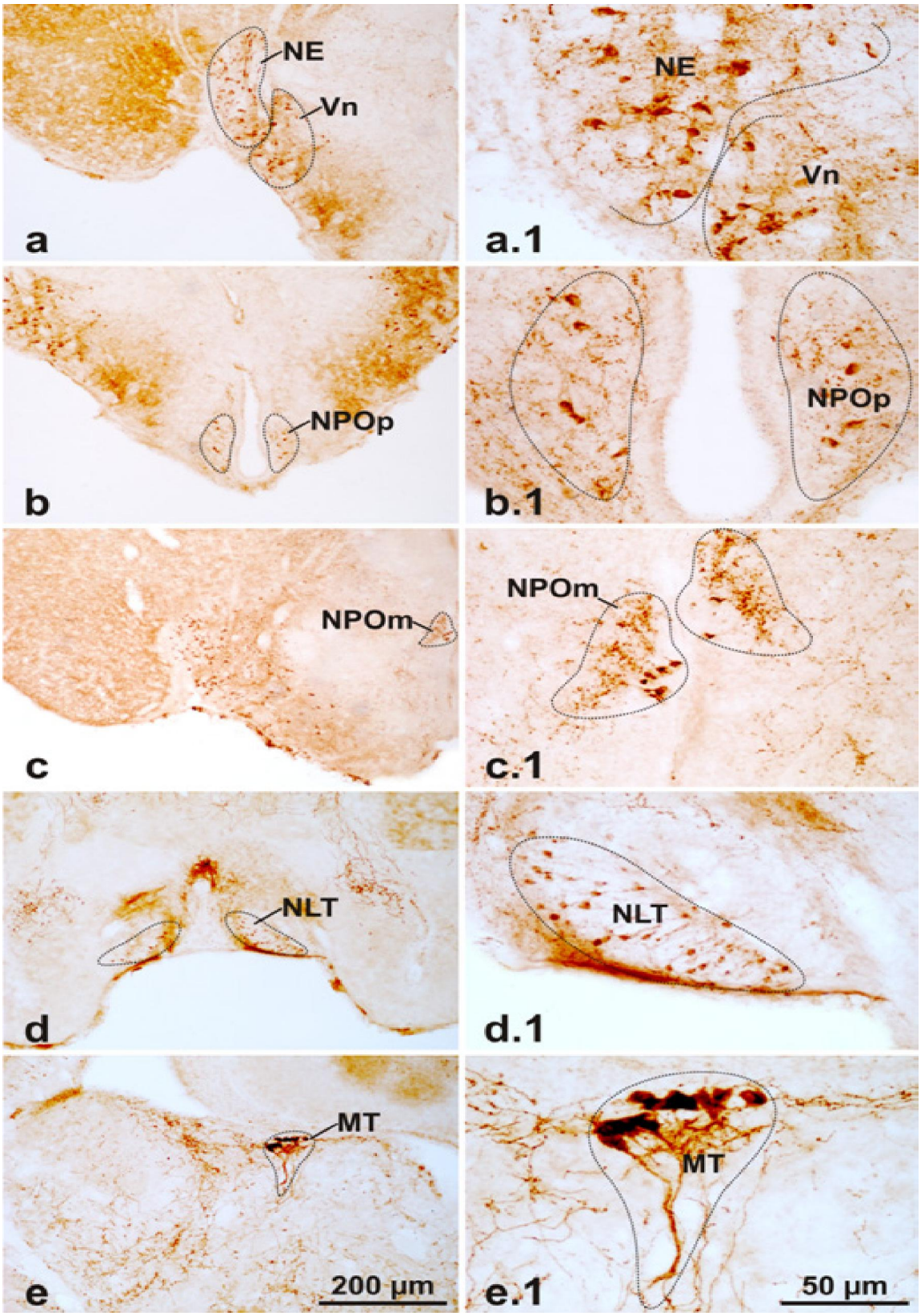

Fig.2 a-e: Photomicrographs showing NPY-ir cells and fibres in the different brain regions and nuclei of spiny eel.. **a.1- e.1**: Magnified views of respective brain region or nuclei. **a** and **a.1.** Nucleus entopeduncularis, NE; nucleus of area ventralis telencephali, Vn; **b** and **b. 1.** Nucleus preopticus pars parvocellularis, NPOp; **c** and **c.1.** Nucleus preopticus, pars magnocellularis, NPom; **d** and **d.1.** Nucleus lateralis tuberis, NLT; **e** and **e.1**. Midbrain tegmentum, MT. Scale bar a-e: 200 µm; in all magnified views (a.1- e.1): 50µm.

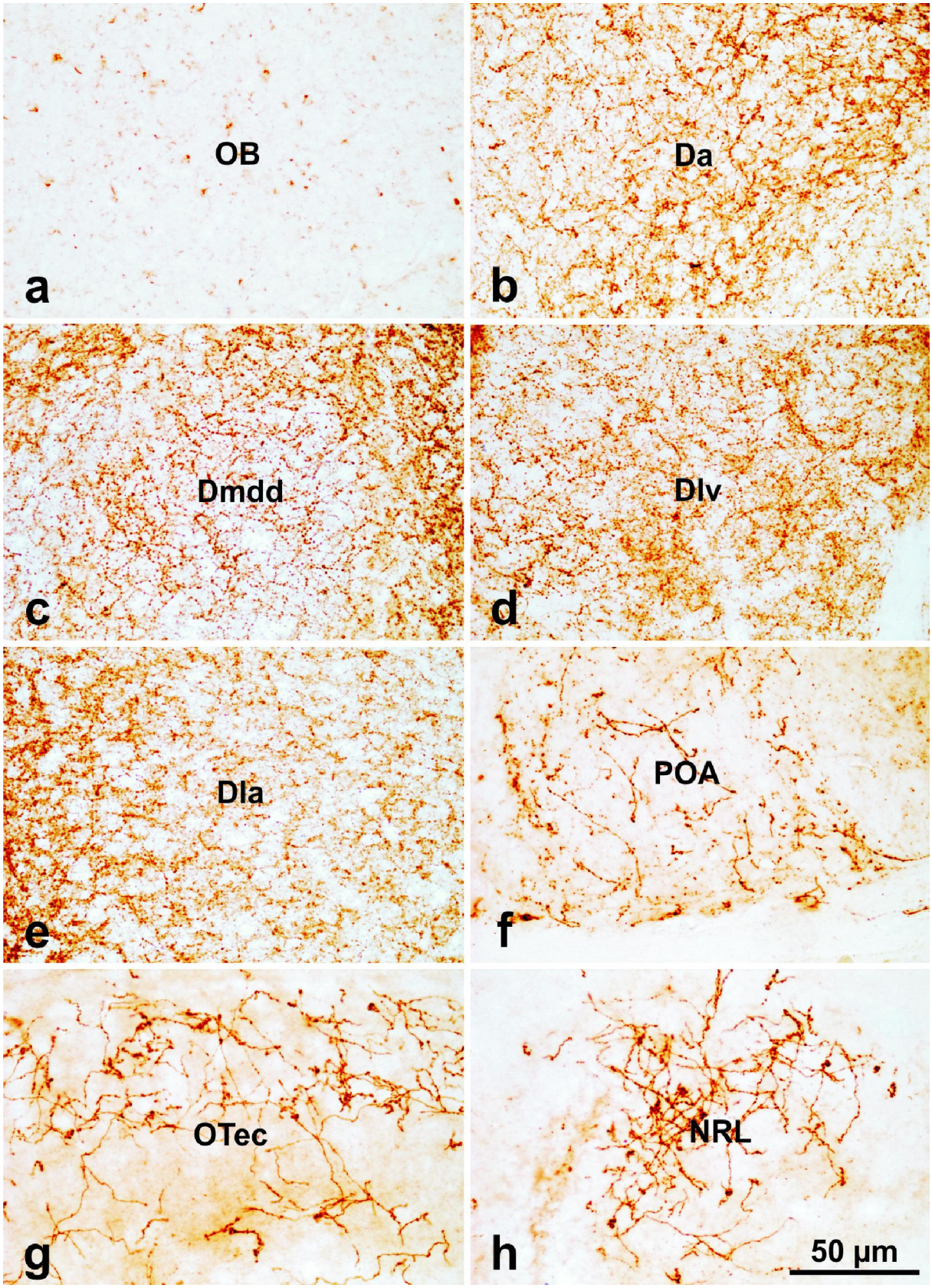

Fig.3. a-h: Photomicrographs showing NPY-ir fibres in the different brain regions of spiny eel. **a.** Olfactory bulb,OB; **b.** Area dorsalis telencephali pars anterioris, Da; **c**. Dorsal part of Dmd, Dmdd; **d.** Ventral subdivision of Dl, Dlv; **e.** Anterior subdivision of Dl, Dla; **f.** Preoptic area, POA; **g.** Optic tectum,OTec; **h.** Nucleus recessi lateralis, NRL. Scale bar: 50 µm (**a-h**)

(Vl; Fig. 1c). In the ventral telencephalic region, we found highest density of NPY containing neurons in nucleus entopeduncularis (NE; Figs. 1d-h; 2a-a.1). A few NPY-ir cells were also visible in the nucleus of area ventralis telencephali (Vn; Figs. 1d-e; 2a-a.1), which lay just adjacent to NE.

Diencephalon

The diencephalon (Die), located caudally to the Tel, is divided into six divisions: preoptic area (POA), epithalamus, thalamus, hypothalamus (Hyp), posterior tuberculum and pretectum. POA possesses strong and dense NPY-ir fibres (Figs. 1d-g; 3f). Moderate amount of NPY-ir cells have been found in the nucleus preopticus pars parvocellularis (NPOp; Figs. 1f-g; 2b-b.1) and in the nucleus preopticus pars magnocellularis (NPOm; Figs. 1h, 2c-c.1). NPY-ir fibres were also found in the median tuberal area (TA; Fig. 1h) of the hypothalamus and in different divisions of pituitary (Pit; Fig. 1h), viz., proximal pars distalis (PPD), rostral pars distalis (RPD) and pars intermedialis (PI; Fig. 1h). In the tuberal region, NPY-ir cells were clearly visible in nucleus lateralis tuberis (NLT; Figs. 1h-J; 2d-d.1).

Mesencephalon

The mesencephalon (mes) is dorsally expanded to OTec and the wide mesencephalic ventricle, and is divided into three major divisions from dorsal to ventral: OTec, TS and tegmentum. In the midbrain tegmentum (MT), very large NPY-immunoreactive cells bearing large ventrally directed processes were found (Figs. 1i-j; 2e-e.1). In the different divisions of the optic tectum (OTec; Fig. 1i-j; 3g), dense immunoreactive fibres were seen in the area of stratum marginale (SM) and stratum griseum et album superficiale (SFGS), although very less fibres were seen in stratum griseum centrale (SGC) and stratum periventriculare (SPV). In the torus semicircularis (TS), very less NPY-ir fibres were found (Figs. 1i-j), though in the inferior lobe of hypothalamus a dense NPY-ir fibre network was noticed in the nucleus recessi lateralis (NRL; Figs. 1j; 3h).

Discussion

This is the first study showing distribution of NPY immunoreactivity in the brain of spiny eel. The results showed that the NPY immunoreactivity is widely distributed in the brain similar to that observed in other teleosts (Subhedar et al., 1996; Chiba et al., 1996; Rodríguez-Gómez et al., 2001; Gaikwad et al., 2004; Pirone et al., 2008).

In spiny eel, the olfactory bulb is similar in anatomy to that in many other fish species, and is distinguishable into four concentric layers: ICL, ECL, GL and ONL. The NPY-ir fibres were visible in the olfactory bulb similar to other teleosts (Pontet et al., 1989; Chiba et al., 1996). Presence of NPY fibres in the olfactory bulb is a common feature of many other vertebrates, such as amphibians (Danger et al., 1985; Lazar et al., 1993), birds (Kuenzel & McMurtry, 1988) and mammals (Gall et al., 1986; Ohm et al., 1988). In spiny eel, presence of NPY-ir fibers in the olfactory bulb may indicate their role in olfaction, which is required for several daily and seasonal functions. Contrary to diurnal animals, in which vision plays major role in daily physiological activities, in eels being bottom-dweller and nocturnal, the olfaction seems to be more important than vision and plays a crucial role in regulating their daily activities, such as search for food, navigation, etc.

Our results, illustrating the distribution of NPY-ir cells and fibers in the telencephalon, are consistent with the previous studies done on other teleosts (Garcia-Fernandez et al., 1992; Marchetti et al., 2000; Traverso et al., 2003). Additionally, NPY-ir structures are also well described in the primitive bony fishes (Chiba and Honma, 1994), carps (Marchetti et al., 2000) and catfishes (Zandbergen et al., 1994; Gaikwad et al., 2004). In the nuclear entopeduncular (NE) region of the ventral telencephalon, a dense neuronal population of NPY-ir cells was evident. Presence of NPY-ir cells in NE has also been reported in several other fish species, such as *Clarias gariepinus* (Zandbergen et al., 1994), *Salmo trutta* (Castro et al., 1999), *Fundulus heteroclitus* (Subhedar et al., 1996), *Cyprinus*

carpio (Marchetti et al., 2000) and *C. batrachus* (Gaikwad et al., 2004). It is quite evident that presence of a rich NPY population is a common feature in the telencephalic area in fish (Rodríguez-Gómez et al., 2001). Moreover, NE region plays a role in reproduction. In tilapia, for example, the depletion of the NPY immunoreactivity in NE neurons was evident after castration, which was restored after testosterone treatment (Sakharkar et al., 2005). NPY mRNA was also reported in this telencephalic region in *Carassius auratus* (Peng et al., 1994; Vecino et al., 1994) and *Salmo trutta* (Silverstein et al., 1998). On the other hand, Reiner & Northcutt (1992) considered NE as homologue of the amygdala of mammals, which may suggest its role in learning and memory along with several other daily and social functions.

The diencephalon of spiny eel showed the richest presence of NPY-ir cells and fibres in several neuronal entities, such as preoptic area, tuberal area, inferior lobe of the hypothalamus and different divisions of pituitary gland. In several other teleosts, these areas are consistently reported to have a fair amount of NPY immunoreactivity (Reiner & Northcutt, 1992; Rodríguez-Gómez et al., 2001; Pirone et al., 2008). In teleosts, the role of preoptic area in the regulation of reproduction is well known (Kah et al., 1994; Parhar & Sim, 1994; Bushnik & Fernald, 1995). In our study also, the presence of NPY in the different divisions of preoptic area (NPop and NPom) suggest similar function. Additionally, in teleosts, neurons of nucleus preopticus periventricularis (NPop) of preoptic area (POA) send axonal projections to the pituitary (Rama Krishna & Subhedar, 1989; Holmqvist & Ekstrom, 1995), which raises the possibility of presence of NPY fibres in the pituitary. Gaikwad et al. (2004) and Sakharkar et al. (2005) reported the presence of NPY cells and fibres in the preoptic area and NLT of tilapia brain, and proposed the role of NPY in neuroendocrine regulation of the pituitary gland. Contrary to this, the absence of NPY-ir cells in POA probably suggests that its role is not well conserved in some teleosts, such as *Carassius auratus* (Pontet et al., 1989), *Salmo salar, Gambusia affinis* (Garcia-Fernandez et al., 1992) and *Acipenser transmontanus* (Chiba & Honma, 1994). Our results suggest that in the spiny eel, the POA, containing NPY cells and fibers, interact to process reproduction-related information and send output information to the pituitary gland via its NPY projections. Thus, it plays a major role in the regulation of reproduction in this lesser understood teleost.

In spiny eel, mesencephalon showed lesser NPY immunoreactivity as compared to telencephalon and diencephalon. In optic tectum (OTec), NPY fibres were fairly visible in different cellular layers including stratum marginale (SM) and stratum griseum et album superficiale (SFGS), although NPY-ir cells were absent in this region. In another nocturnal fish, *Tinca tinca* (Bonn, 1990), and a few diurnal teleosts *Odontesthes bonariensis*, (Traverso et al., 2003), *Poecilia latipinna* (Batten et al., 1990), and *Lethenteron japonica* (Chiba, 1999) moderate to intense NPY fibres were reported in the optic tectum. Besides, the NPY cells have also been reported in the optic tectum of many other diurnal teleosts such as *Carassius auratus* (Pickavance et al., 1992), *Salmo trutta fario* (Castro et al., 1999), *Cyprinus carpio* (Marchetti et al., 2000), and *Lepisosteus oculatus* (Chiba, 2005), which suggests its role in the processing of visual information (Meek & Nieuwenhuys, 1998) and, hence, in the proper functioning of daily activities. In addition, mesencepahalic torus semicircularis (TS) represents a complex neuronal structure, which receives inputs from the lateral line and other visual structures (Pritz, 1974; Kennedy & Browner, 1981). The TS possess NPY-ir fibers but not the NPY-ir cells as shown in *Cyprinus carpio* (Cuadrado & Covenas, 1993). In spiny eel the presence of only NPY fibres in the Otec and TS suggests that being nocturnal with bottom dwelling habit, there is lesser need of visual functioning and, therefore, other senses such as olfaction may play crucial role in them.

In the midbrain tegmentum (MT), NPY-ir cells were identified, which is consistent with the earlier studies in *Oreochromis mossambicus* (Sakharkar et al., 2005), *Salmo salar* and *Gambusia affinis* (Garcia-Fernandez et al., 1992). GnRH-II cells are also reported in this region (Parhar et al., 1996), which may suggest a role for midbrain tegmentum in the sexual behavior (pair bonding, copulation, etc).

In conclusion, the presence of NPY immunoreactivity in several brain regions of spiny eel suggests its role in regulation of daily and seasonal functions. The present study may serve as the first representative study done at neuronal level in this species. In addition, this preliminary information on the distribution of NPY-ir in the brain of spiny eel would provide useful data to study the regulation of NPY levels under different seasons with different sexual stages, and putative interactions of NPY with other endocrine factors in the regulation of metabolic and reproductive processes in this lesser understood species.

Acknowledgment

This work was supported by research grant from the Department of Science and Technology (DST), Government of India (SR/SO/AS–21/2008).

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