Neuropeptides 74 (2019) 1–10

Contents lists available at ScienceDirect

Neuropeptides

journal homepage: www.elsevier.com/locate/npep

Sex-specific distribution of Neuropeptide Y (NPY) in the brain of the frog, *Microhyla ornata*

Kavita N. Hadawale[a], Nitin S. Sawant[a], Sneha Sagarkar[b], Amul J. Sakharkar[b], Shobha Y. Bhargava[a,*]

[a] Department of Zoology, Savitribai Phule Pune University, Ganeshkhind Road, Pune 411 007, India
[b] Department of Biotechnology, Savitribai Phule Pune University, Ganeshkhind Road, Pune 411 007, India

ARTICLE INFO

Keywords:
Neuropeptide Y
Sex-specific distribution
Amphibians
Immunohistochemistry
mRNA
Hypothalamus

ABSTRACT

Neuropeptide Y (NPY) is involved in sex-specific behavioural processes in vertebrates. NPY integrates energy balance and reproduction in mammals. However, the relevance of NPY in reproduction of lower vertebrates is understudied. In the present study, we have investigated neuroanatomical distribution and sex-specific differences of NPY in the brain of *Microhyla ornata* using immunohistochemistry and quantitative real time PCR. NPY is widely distributed throughout the brain of *M. ornata*. We observed NPY immunoreactivity in the cells of the nucleus accumbens, striatum pars dorsalis, dorsal pallium, medial pallium, ventral pallium, bed nucleus of stria terminalis, preoptic nucleus, infundibular region, median eminence and pituitary gland of adult *M. ornata*. A higher number of NPY- immunoreactive cells were observed in the preoptic nucleus ($p < .01$), nucleus infundibularis ventralis ($p < .001$) and anteroventral tegmental nucleus ($p < .001$) of the female as compared to that of the male frog. Real-Time PCR revealed higher mRNA levels of NPY in the female as compared to male frogs in the mid-brain region that largely contains the hypothalamus. Sexual dimorphism of NPY expression in *M. ornata* suggests that NPY may be involved in the reproductive physiology of anurans.

1. Introduction

Neuropeptide Y (NPY) is one of the most abundant neuropeptides within the brain of mammals (Tatemoto et al., 1982, Reichmann and Holzer, 2016) and is involved in different physiological processes such as reproduction, feeding, circadian rhythm, processing of pain, neurogenesis and memory (Berglund et al., 2003; Magni, 2003; Sperk et al., 2007; Hokfelt et al., 2008; Beck and Pourié, 2013; Loh et al., 2015; Gotzsche et al., 2012; Gøtzsche and Woldbye, 2016; Tasan et al., 2016). Various studies have shown that NPY is the most potent orexigenic peptide identified to date (Tatemoto et al., 1982). Central administration of NPY resulted into the robust increase in food intake and body weight, and with chronic administration, could eventually produce obesity (Zarjevski et al., 1993; Corp et al., 2001; Yang et al., 2009).

NPY is known to regulate reproductive behaviour in mammals (Pierroz et al., 1995, 1996; Aubert et al., 1998). NPY stimulates luteinizing hormone (LH) secretion in the pituitary of sex steroid primed rats, whereas it could inhibit LH release in castrated rats (Kalra and Kalra, 1996). Also, elevated levels of NPY in the hypothalamus are reported to cause tonic inhibition of pulsatile gonadotropin releasing hormone (GnRH) secretion in nutritional deficiency paradigm (Catzeflis

Abbreviations: Ac, caudal subnucleus of the anterior nucleus; Ar, rostral subnucleus of the anterior nucleus; AV, anteroventral tegmental nucleus; BST, bed nucleus of the stria terminalis; DP, dorsal pallium; Ea, anterior entopeduncular nucleus; EP, posterior entopeduncular nucleus; GC, central gray; GT, griseum tectale, periventricular part; Hd, dorsal habenular nucleus; Hv, ventral habenular nucleus; IR, infundibular recess; LA, lateral amygdala; LH, lateral hypothalamic nucleus; Lpd, lateral posterodorsal nucleus; Lpv, lateral posteroventral nucleus; ME, median eminence; MeA, medial amygdala; Mg, magnocellular nucleus; MP, medial pallium; LP, lateral pallium; NAC, nucleus accumbens; NAD, nucleus antero dorsalis tegmenti mesencephalic; NCER, cerebellar nucleus; NID, nucleus infundibularis Dorsalis; NIV, nucleus infundibularis ventralis; NMLF, nucleus of the medial longitudinal fasciculus; NRIS, nucleus reticularis isthmi; pc, posterior commissure; PC, precommissural nucleus; PI, pars intermedia; PN, pars nervosa; Poa, preoptic nucleus; Por, preoptic recess; PR, principal nucleus of the torus semicircularis; Pv, paraventricular nucleus; RS, nucleus reticularis superior; SCN, suprachiasmatic nucleus; SGP, striatum griseum periventricularis; SGS, striatum griseum superficial tecti; Std, striatum pars dorsalis; Stv, striatum pars ventralis; Teo, optic tectum; Tmg, magnocellular nucleus of torussemicircularis; TP, nucleus of the tuberculum posterior; TS, torus semicircularis; V, ventricle; VM, ventromedial thalamic nucleus; VP, ventral pallium

* Corresponding author.
E-mail address: shobha@unipune.ac.in (S.Y. Bhargava).

https://doi.org/10.1016/j.npep.2019.01.004
Received 24 April 2018; Received in revised form 20 January 2019; Accepted 20 January 2019
Available online 21 January 2019
0143-4179/

et al., 1993; Pierroz et al., 1995, 1996; Aubert et al., 1998). NPY suppressed sexual behaviour in rats (Clark et al., 1997). NPY agonists decreased lordosis duration in ovariectomized (OVX) Syrian hamsters brought into oestrus with ovarian steroid treatment (Corp et al., 2001). Similar to mammals, NPY has been linked to the reproductive behaviour in reptiles. Intracerebroventricular (ICV) administration of NPY significantly reduced courtship behaviour, indicating inhibitory role of NPY in reproductive behaviour in red sided garter snake (Morris and Crews, 1990).

Several studies indicated that NPY regulates feeding and reproduction in teleost fishes (Campos et al., 2010, 2011). In vitro treatment of pituitary with NPY stimulated LH release in the goldfish (Peng et al., 1993). Anatomical observations also supported the role of NPY in LH secretion; NPY fibers made close appositions on LH cells in the pituitary of catfish (Gaikwad et al., 2003). It could increase LH beta subunit (LHb) mRNA levels, but not follicle stimulating hormone beta subunit (FSHb) mRNA levels in the pituitary of tilapia (Yaron et al., 2001). Castration of cichlid fish resulted in significant reduction of NPY-immunoreactive cells in the nucleus entopeduncularis (Sakharkar et al., 2005). The administration of testosterone to juvenile male catfish precipitated into a significant increase in NPY immunoreactivity in the nucleus entopeduncularis (Mazumdar et al., 2007).

Sex specific differences in NPY expression in the central nervous system have been reported in mammals and reptiles (Rugarn et al., 1998; Urban et al., 1993; Salom et al., 1994). Comparison of NPY gene expression throughout the rostrocaudal extent of the arcuate nucleus of rats displayed significantly higher expression levels in males than females (Urban et al., 1993). In the lizard, *Podaris hispanica*, NPY-immunoreactive cells in the lateral septum showed a clear sexual dimorphism, wherein the numbers of reactive cells were significantly higher in the periventricular preoptic nucleus of males, as compared to females (Salom et al., 1994). Although the sexual dimorphism of NPY in mammals and reptiles is reported, related information in lower vertebrates, including amphibians, is largely missing.

NPY integrates energy balance and reproduction at the cellular and molecular levels in the hypothalamus of mammals (Kalra and Kalra, 1996; Shahjahan et al., 2014). In rats, it has been suggested that NPY neurons are activated by signals of reduced fuel availability, which increased NPY expression in the paraventricular nucleus (PVN) and preoptic area (Poa) to stimulate feeding behavior and suppress GnRH release, respectively. The neuroendocrine 'crosstalk' between reproductive and bioenergetic systems controls GnRH release, and hence reproductive activity, until nutritional and environmental conditions becomes synchronous and more favourable (Acosta-Martinez et al., 2007).

Although widespread distribution of NPY in the amphibian brain (Danger et al., 1985, 1986; McKay et al., 1992) is known to serve various functions, including modulation of the central responses to stress (Heigrujam et al., 2017; Ali and Bhargava, 2016), background adaptation (Galas et al., 2002), visual neurotransmission (Schwippert, 1998), antimicrobial activity (Karim, 2008) and feeding behaviour (Crespi et al., 2004; Shewale et al., 2018), its relevance to reproduction in amphibians is not yet reported. Amphibians serve as an ideal model system for studying molecular, developmental and evolutionary biology (Brown and Cai, 2007). The frog, *M. ornata*, used in the present study, is an annual breeder and its reproductive phase corresponds to the monsoon of the Indian subcontinent. The sex-specific differences of NPY peptide and its mRNA levels in the brain of *M. ornata*, were investigated using immunohistochemistry and quantitative real time-PCR respectively.

2. Materials and methods

2.1. Animal collection and tissue processing

Male (n = 10) and female (n = 10) adult frogs, *M. ornata* (body weight: 0.60–1.65 g; snout-vent-length, SVL: 2–2.8 cm) were collected in the reproductively active season (July–September) from an ephemeral water body situated in the campus of Savitribai Phule Pune University (18° 550 N and 73° 820E). Five male and female frogs were anesthetized using chloroform and the brains were dissected out in rostral, middle and caudal, using dissecting microscope. These dissected brain tissues were further stored at −80 °C for molecular studies. All animal experiments were performed following the institutional animal ethics guidelines established by the Savitribai Phule Pune University. For immunohistochemistry, five complete brains of each male and female frogs were fixed in Bouin's fixative for 24 h and then cryoprotected in 10% (2 h), 20% (2 h) and 30% (overnight at 4 °C) sucrose solution in phosphate buffered saline (PBS; 0.01 M, pH 7.4). The tissues were embedded in Shandon Cryomatrix (Thermo Scientific, UK) and serially cut on a cryostat at 20 µm thickness in transverse planes. Sections were mounted on Poly-L-lysine coated slides, and stored at −20 °C till further analysis.

2.2. Quantitative real-time PCR for mRNA measurements

Quantitative real-time PCR (qRT-PCR) was performed for NPY and hypoxanthine phosphoribosyl transferase (HPRT) mRNA quantification of rostral, middle and caudal regions of brain, as described previously with specific modifications (Sagarkar et al., 2017). HPRT was chosen as the reference gene as an internal control. The total RNA was isolated from all the brain tissues using RNA isolation kit (Qiagen, USA). The DNA contaminants were removed using a DNA-free™ DNA Removal Kit (Life Technologies, USA), and the RNA was quantified using Biospec Nano spectrophotometer (Shimdzu, Kyoto, Japan). The total RNA (100 ng) was reverse transcribed in duplicate using random hexamers and MultiScribe™ MuLV (Applied Biosystems, USA) in a final volume of 20 µl. The reverse transcriptase reaction was 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. The duplicates of the cDNA were subjected to qRT-PCR on a StepOne™ RT-PCR System (Applied Biosystems, USA) using 25 pmol of each primer and SYBR green qPCR master mix (ThermoFisher Scientific, USA), which includes purified AmpliTaq Gold® DNA Polymerase, a blend of dTTP/dUTP and a proprietary version of ROX™ dye, an internal passive reference. The mRNA sequences of NPY and HPRT was derived from transcriptome sequence of *M. ornata* and submitted to DNA Databank of Japan (DDBJ). The accession numbers for NPY and HPRT are as follows: LC219936 and LC314150. Primers were designed using Primer Blast tool of National Center for Biotechnology Information (NCBI) and the sequences are as follows; NPY: F-5′-GAGACCCAGTCGCTGACAAA-3′, and R-5′-ATATCTGGTGTT TCCGGGGC-3′, HPRT: F-5′-TGGTGACCTCCCATGTCTCT-3′ and R-5′-TGTATCCCGAAGCACTACGC-3′. The reference gene HPRT was measured in parallel as an internal control. The thermal profile used for the qRT-PCR had three stages: 95 °C for 3 min (1 cycle); 95 °C, 57 °C, and 72 °C for 30 s each (40 cycles); 95 °C for 15 s. After the PCR amplification, melt curve analysis was performed in the temperature range of 60 to 95 °C with 0.5 °C increment at a rate of 5 s/step. The fold change for NPY mRNA was determined after normalization to HPRT using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.3. Characterization of antibody

The in silico translation of the mRNA sequence of *M. ornata* (LC219936) as derived from our transcriptomic analysis was carried out using Expasy translate tool to obtain the putative amino acid sequence of the NPY peptide. Protein sequence alignment was carried out using blastp (Fig. 1A). NPY antibody (N9528; Sigma) used in this study is raised against the porcine (*Sus scrofa*) NPY peptide, which showed 94% identity with the putative amino acid sequence of NPY in *M. ornata* (Fig. 1A). Same antibody (N9528; Sigma) was also employed to detect the NPY in the brain of another amphibian, *Ambystoma mexicanum* (*A. mexicanum*, Mousley et al., 2006). Interestingly, the NPY peptide in *A.*

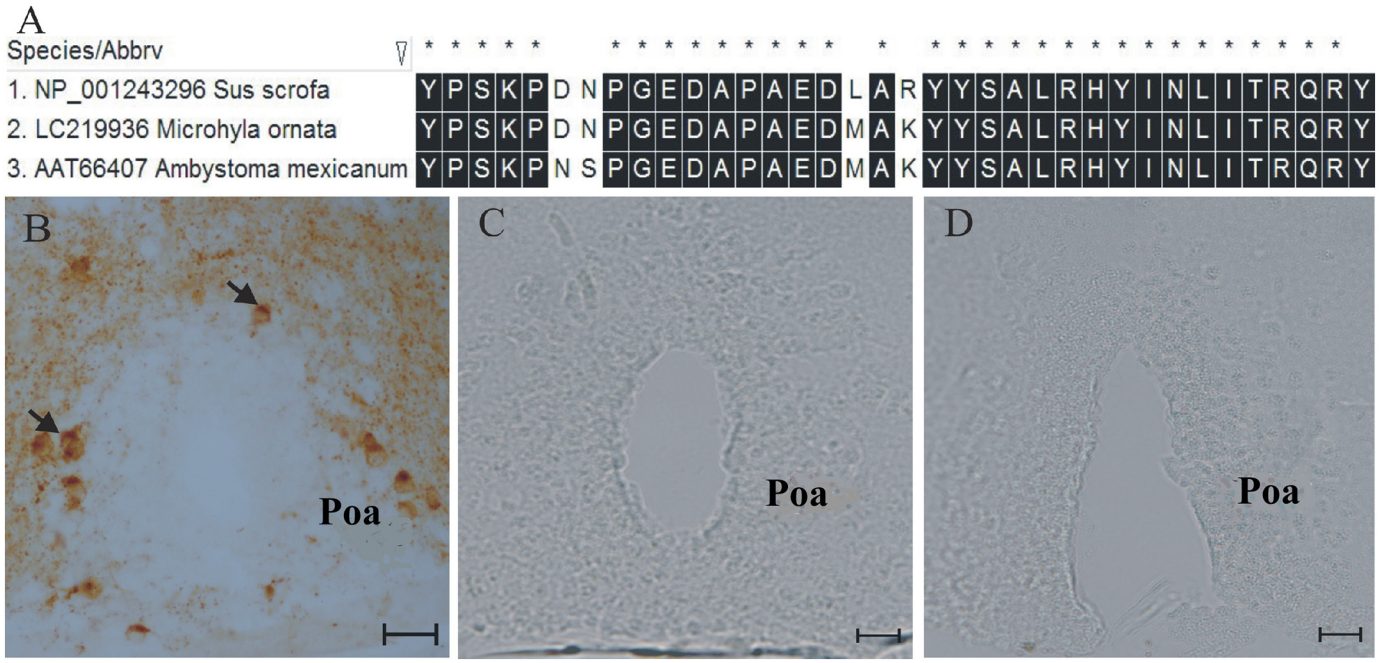

Fig. 1. Amino acid alignment of putative NPY peptide of the frog, *Microhyla ornata* with the amphibian, *Ambystoma mexicanum* and porcine, *Sus scrofa*. Highlighted sequences indicate the conserved amino acids in NPY peptides (A). Transverse sections through the preoptic nucleus (Poa) of the *M. ornata* showing NPY-ir cells after the application of NPY antibodies (B) after the primary antibody was omitted from the reaction mixture (C) loss of immunoreactions when the antisera were pre-adsorbed with NPY protein (D). Magnification: 40×, Arrows indicate cells, scale bar: 100 µm.

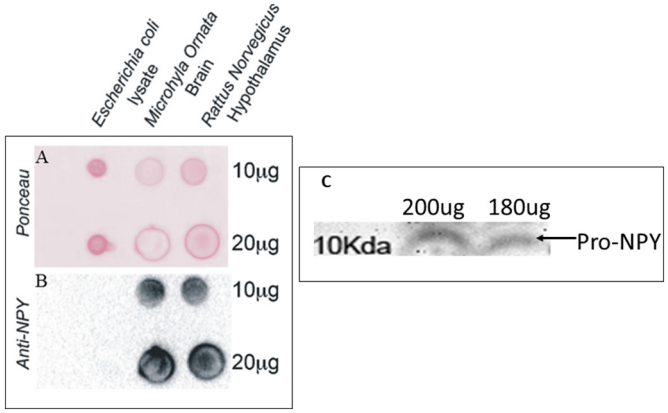

Fig. 2. A) Ponceau stained 20 µg and 40 µg protein samples from bacterial lysate (negative control), *M. ornata* brain and *Rattus norvegicus* hypothalamus (positive control) B) Dot blot stained with NPY antibody (N9528; Sigma) C) Western blot analysis of *M. ornata* total brain lysate (200 µg and 180 µg) showing specific immunolabeling of pro-NPY peptide ~10kd.

mexicanum exhibited 97% identity with that of the *M. ornata* (Fig. 1A). These observations support the strong specificity of the NPY antibody employed in this study to detect NPY peptide in *M. ornata*.

2.4. Dot blot analysis

The specificity of the NPY antibody was checked by performing a Dot blot with protein sample from brain of *M. ornata*, the hypothalamus of rat (positive control) and Bacterial cell lysate (negative control). Protein sample was spotted on nitrocellulose membrane and it was allowed to dry. After drying the membrane was blocked with 3%BSA in tris buffered saline tween 20 (1× TBST; 0.05% tween 20) for 2 h. Blot was incubated with rabbit monoclonal antibody against NPY (N9528; Sigma) at 1:500 dilution containing 3% BSA in TBST overnight at 4 °C. After incubation, three washes of TBST for 10 min each was given. Followed by incubation with secondary antibody conjugated with Horseradish Peroxidase (HRP; 1:5000) for 1 h at room temperature. The membrane was washed with TBST thrice, 10 min each. The blot was developed with ECL solution using Chemidoc (Thermofisher Scientific). The membrane was also stained with Ponceau to ensure equal loading protein.

2.5. Western blot

Western blot analysis of Neuropeptide Y from crude extracts of adult *M. ornata* brain was performed. The tissue was homogenized in homogenizing buffer containing 50 mM Tris–HCl (pH 8.0), 5 mM EDTA, Triton X100 (0.1% v/v), 1 mM PMSF and PI (protease inhibitor) cocktail. The homogenate was centrifuged at 10,000 rpm for 15 min to eliminate cell debris. Protein was estimated following Bradford method and resolved on 16% SDS-polyacrylamide gel by electrophoresis. Proteins were transferred to PVDF membrane by electro-blotting in transfer buffer (192 mM glycine, 25 mM Tris, 0.1% SDS, 20% methanol) for 2 h at 20 mV. Further, the membrane was incubated with blocking solution (5% BSA) in TBST (Tween 20–0.05%) pH 7.5 at room temperature and incubated overnight at 4 °C with NPY antibody (N9528; Sigma) at 1:200 dilution. Membrane was washed in TBST and incubated with HRP conjugated secondary antibody; goat anti-rabbit IgG (PK-6101) at 1:500 dilutions for 2 h. Blots were developed using enhanced chemiluminescence kit (Advansta) according to manufacturer's instructions using chemidoc (Thermofisher Scientific).

2.6. Immunohistochemistry

Sections were used for the immunohistochemical localization of NPY as described previously (Ali et al., 2016; Shewale et al., 2018). Briefly, the sections were washed with PBS thrice and treated with 0.3% hydrogen peroxide in methanol for 1 h. Sections were then washed in PBS thrice and incubated with blocking agent containing 0.5% bovine serum albumin (BSA) and 0.5% gelatine in PBS for 1 h. After washing thrice, sections were incubated for 1 h in normal goat serum (1:40, PK-

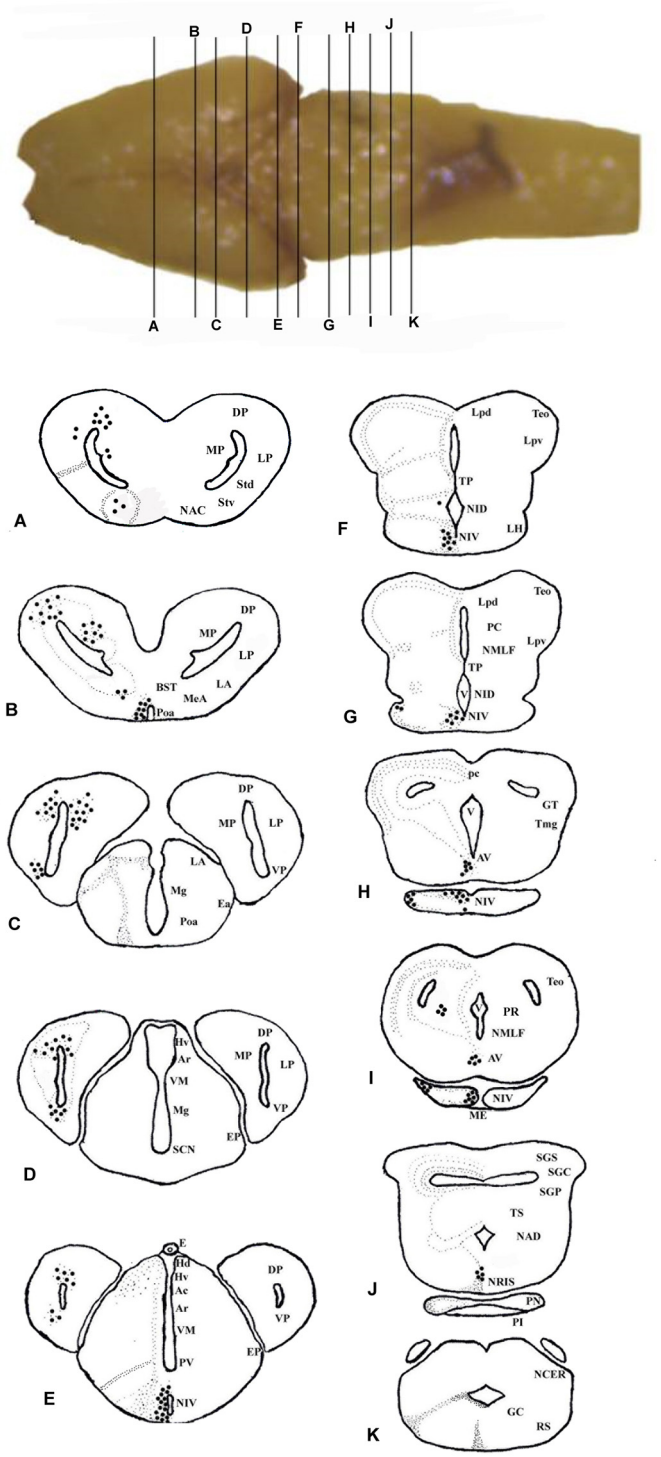

Fig. 3. Schematic drawings of rostrocaudal series of transverse sections (A–K) of the brain of frog *M. ornata* showing cytoarchitectonic areas on the right and distribution of NPY-ir cells (dark circles), granules (dots) and fibers on the left. At the top right shows a lateral view of the brain indicating levels of the corresponding transverse sections. Large dots are neurons. Wavy lines represent NPY-immunoreactive fibers and dots indicate their terminals. For abbreviations, see list.

6101, Vectastain ABC Kit, Vector laboratories). After incubation, excess goat serum was blotted out and sections were incubated with rabbit monoclonal antibodies against NPY (N9528; Sigma) at 1:3500 dilution containing 0.5% BSA and gelatin overnight at 4 °C. Sections were then washed in PBS thrice and incubated with biotinylated goat anti- rabbit IgG antibody at room temperature for 1 h (1:200, PK-6101, Vectastain ABC Kit, Vector laboratories). Sections were washed twice and incubated with ABC reagent for 1 h at room temperature (1:100, PK-6101, Vectastain ABC Kit, Vector Laboratories). After washing twice, sections were incubated with 3,3 diaminobenzidine tetra hydrochloride (DAB) in tris buffer (0.05 M, pH 7.2) containing 0.02% H_2O_2 for 8–10 min. Slides were washed in distilled water for 5 min, dehydrated, cleared in xylene for 30 min, mounted in distyrene plasticizer xylene (Merck, India) and photographed. The following control procedures were also adopted for verifying the specificity of the immunoreaction: (i) incubation of sections in NPY antibody as a positive control, (Fig. 1B) (ii) omission of primary or secondary antibodies from the immunohistochemistry protocol, (Fig. 1C), and (iii) preabsorption of 1 ml diluted primary antibody with the porcine NPY peptide (Sigma, N3266) at 10^{-5} M concentration for 24 h at 4 °C prior to incubation (Fig. 1D).

Table 1
Distribution of NPY-immunoreactive cells in the brain of frogs, *Microhyla ornata*, *Xenopus laevis* and *Rana esculenta*.

Areas	*M. ornate* (Current study)	*X. laevis* (Tuinhof et al., 1994)	*R. esculenta* (D'Aniello et al., 1996)
Olfactory Bulb			
Internal granular layer	−	+	−
Glomerular layer	−	+	−
Accessory olfactory bulb	+	−	+
Pallium			
Medial Pallium	+	+	+
Lateral Pallium	+	+	+
Dorsal Pallium	+	+	+
Subpallium			
Nucleus accumbens	+	+	−
Lateral amygdala	−	+	−
Medial amygdala	+	+	−
Striatum	+	−	−
Lateral Septum	−	+	−
Medial Septum	−	+	−
Thalamus			
Central Thalamic Nucleus	−	−	−
Ventromedial Thalamic Nucleus	−	+	+
Posterior Thalamic Nucleus	+	+	+
Preoptic area			
Preoptic Nucleus	+	+	+
Hypothalamus			
Suprachiasmatic Nucleus	+	+	+
Infundibulum	+	+	+
Median Eminence	+	+	+
Pituitary Gland			
Neural lobe	−	+	−
Intermediate lobe	+	+	+
Distal lobe	+	−	−
Tegmentum mesencephali			
Tegmental Nucleus	+	+	−
Anteroventral Nucleus	+	−	+
Torus semicircularis	−	+	−
Interpeduncular Nucleus	−	+	+
Isthmic Nucleus	+	+	−
Raphe Nucleus	+	+	−
Nucleus of solitary tract	−	+	−
Superior olivary Nucleus	−	+	−
Central grey	+	−	+
Cerebellar Nucleus	−	−	+

2.7. Morphometry

Digital images of NPY immunoreactivity were taken on a Carl Zeiss

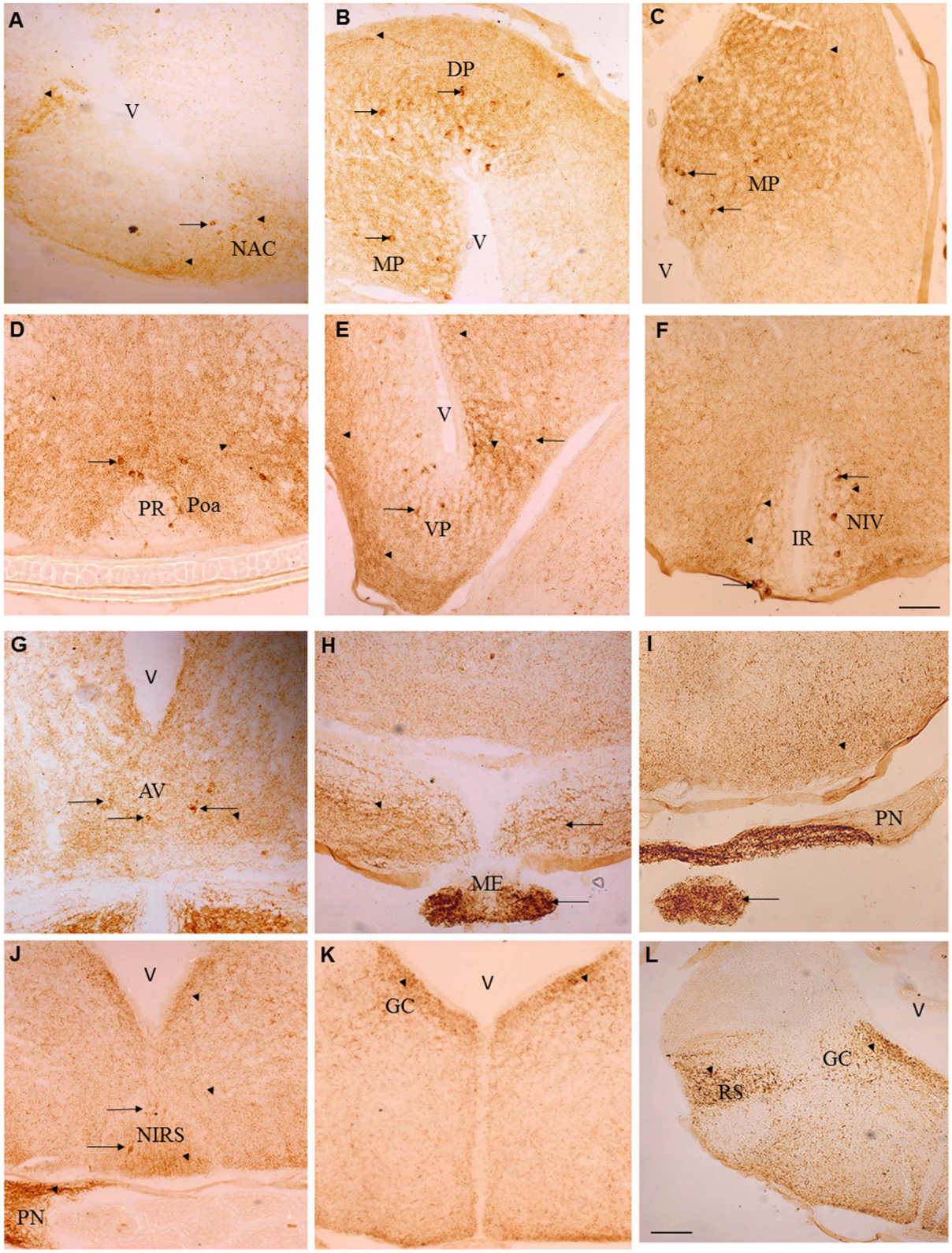

Fig. 4. (1) Sagittal section through the telencephalon showing NPY-ir cells (arrows) and granules in nucleus accumbens (NAC; A), NPY-ir neurons were seen in the dorsal pallium (DP, B) and the medial pallium (MP, C), Intense NPY-ir cells were observed in the preoptic nucleus (Poa, D). NPY-ir neurons were observed in the ventral pallium (VP, E). The nucleus infundibularis ventralis (NIV) region of the hypothalamus showed expression of strong NPY-ir neurons and granules (F). (2) Few NPY-ir cells were observed in the anteroventral tegmental nucleus (AV, G). The median eminence (ME) was densely stained with NPY-ir granules (H). In the pituitary gland, pars nervosa (PN) showed presence of some granules and fibers (I). Dense fibers and granules were observed in the nucleus reticularis isthmi (NRIS, J). In the rhombencephalon, central gray (GC) showed few granules (K) and the raphe nucleus (RS) showed dense granules (L). Arrows represents cells and arrowhead represents fibers. Scale bars: 200 μm, Image magnification: 20×.

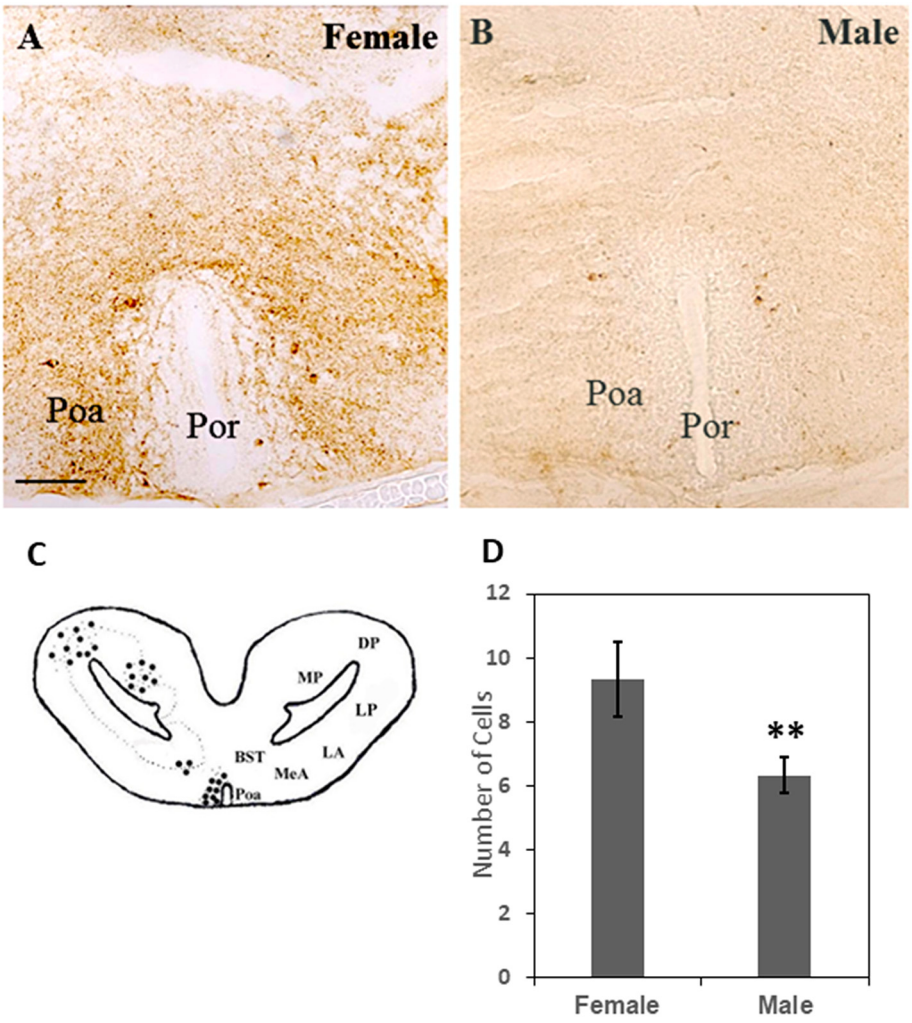

Fig. 5. Sex-specific distribution of NPY was observed in some regions of brain of *M. ornata*. The preoptic nucleus (Poa) showed differential expression of NPY, female brain showed more expression as compared to male brain A) female B) male. Arrows represents cells and arrowhead represents fibers, Scale bars: 200 µm, Image magnification:20×. C) Schematic representation of the region D) Graph showing number of NPY immunoreactive cells in the Poa. Error bar represents standard deviation. (*Indicated significant differences between male and female; **P < .01, one-way ANOVA followed by post hoc Tukey's test).

Imager M2 microscope, with an Axiocam 506 Colour camera provided with the Zen 2.3 Pro software. The five sections each, through anatomically matched region of the brain, were identified using anatomic landmarks (Morona and González, 2008, 2009; Hall et al., 2013; Pinelli et al., 2015) and were used for a comparative analysis of NPY-immunoreactive (-ir) in respective brain regions. For cell counting, all visible cell bodies stained within the defined brain regions was counted using Image J software, keeping the same counting area for control. Data from each brain region in an animal was calculated by taking the average counts from five brain slices.

2.8. Statistical analysis

The differences between the two groups were tested for significance using one-way analysis of variance (ANOVA) followed by post hoc Tukey's test, and the p-values < .05 (P < .05) were considered to be significant.

3. Results

3.1. Antibody specificity

Since a high degree of conservation was observed in NPY peptide sequences from pig and frog after alignment of their amino acid sequences (Fig. 1A), antiserum directed against the porcine NPY antigen was used in the present study. This antiserum was checked for its specificity prior to immunohistochemical localization of NPY in the frog brain. Preoptic nucleus of the frog brain showed positive immunoreaction (Fig. 1B). However, omission of primary or secondary antibodies and pre-adsorption of primary antibodies with synthetic porcine NPY peptide in the immunohistochemistry protocol did not produce any immunoreactions (Fig. 1C, D). Dot blot performed with *M. ornata* brain and the hypothalamus of rat (positive control) showed that the neuropeptide Y antibody binds to the protein in the mammalian and frog brain. However, the dot blot analysis produced no signal when antibody was tested with bacterial cell lysate (negative control) (Figure 2AB). Western blot analysis indicated specific immunolabeling of pro-NPY peptide (~10Kda) from the total brain lysate of *M. ornata* (Fig. 2C).

3.2. Neuropeptide Y distribution in the brain

NPY immunoreactivity was observed in various regions of the adult brain including the telencephalon, the diencephalon, the mesencephalon and the rhombencephalon. The neuroanatomical distribution of NPY immunoreactivity was studied in serial transverse sections, drawn in representative diagrams (Fig. 3A–K) and summarized in Table 1. In the telencephalon, few NPY-ir perikarya interspersed in dense network

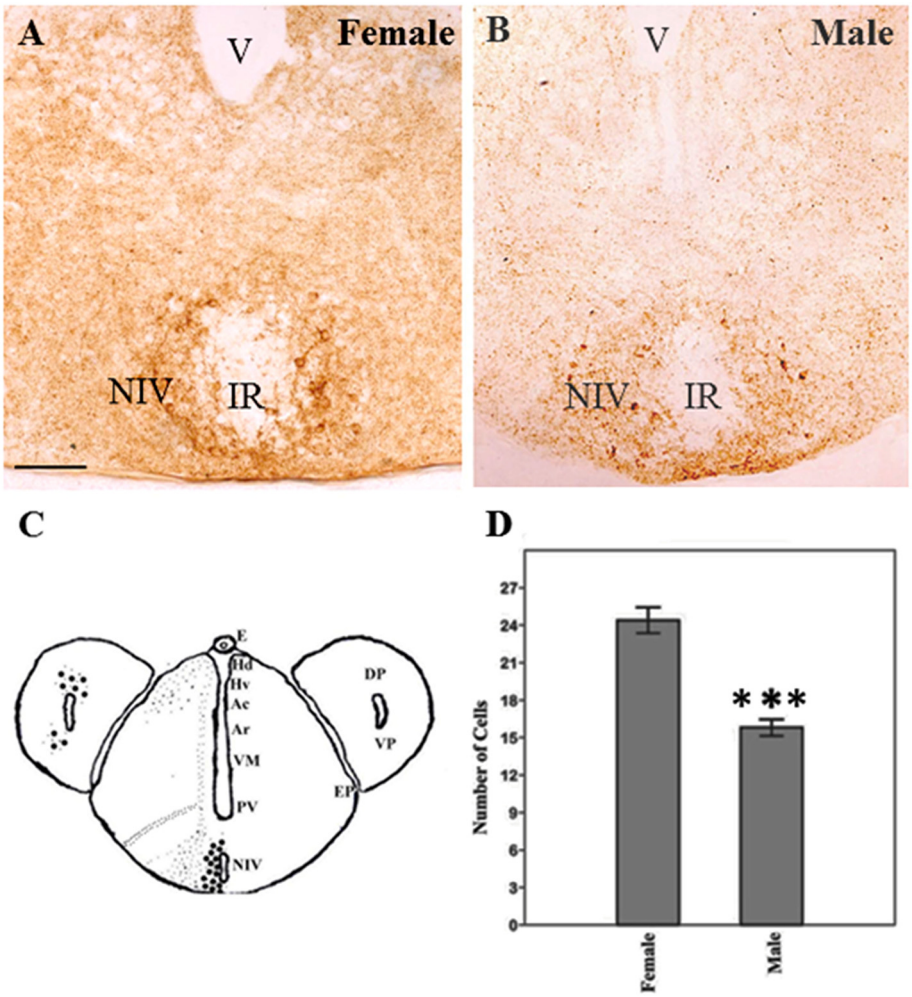

Fig. 6. Sex-specific distribution of NPY was observed in some regions of brain of *M. ornata*. In the hypothalamus, NIV showed significant differences in NPY-expression where it was more in female than male A) female B) male. Arrows represents cells and arrowhead represents fibers, Scale bars: 200 µm, Image magnification:20×. C) Schematic representation of the region D) Graph showing number of NPY immunoreactive cells in NIV. Error bar represents standard deviation. (*Indicated significant differences between male and female; ***P < .001, one-way ANOVA followed by post hoc Tukey's test).

of NPY-ir beaded fibers were observed in the nucleus accumbens (NAC; Fig. 4A). Some NPY immunostained cells with moderate number of fibers were observed in the dorsal pallium (DP) and the medial pallium (MP) regions (Fig. 4B, C). In the diencephalon, strong NPY-ir cells with prominent nuclei and long axonal processes and fibers with beaded granules were observed in the preoptic nucleus (Poa; Fig. 4D). It is the most conspicuous NPY containing cell group in the brain of *M. ornata*. NPY positive cells were also observed in the bed nucleus of the stria terminalis (BST) region. In the secondary prosencephalon, NPY immunostained cells were observed in the dorsal, medial and ventral pallium (DP, MP, LP; Fig. 4E). The intensity of staining varied from strong to weak and cells were oval to round in shape. In the hypothalamus, dense innervations of NPY containing cells, fibers, as well as granules were observed in the nucleus infundibularis ventralis (NIV, Fig. 4F). The number of NPY-ir perikarya decreased from rostral to caudal end of the NIV. A group of few NPY immunopositive cells were observed in the anteroventral tegmental nucleus (AV; Fig. 4G). A group of some NPY immunostained cells were observed in the principle nucleus of the torus semicircularis (PR). A very dense granular reaction of NPY was observed in the median eminence (ME, Fig. 4H) and continued to the pituitary gland. Although pituitary was devoid of NPY-containing cells, moderate fibers and granules were seen in the pars nervosa (PN, Fig. 4I). NPY-ir neurons were also located in the nucleus reticularis isthmi (NRIS). The NRIS located at the ventral side of the NAV contained some NPY-ir cells and strong NPY immunoreactive fibers as well as granules (Fig. 4J). In the rhombencephalon, some NPY-ir granules and fibers were seen in the central gray (GC; Fig. 4K). Moreover, the nucleus reticularis superior (RS) showed moderately stained granules and fibers (Fig. 4L).

3.3. Sex-specific differences of neuropeptide Y

Although a wide distribution of NPY was observed throughout the brain of both male and female *M. ornata*, comparative analysis showed dimorphic changes in NPY immunoreactivity in some brain regions (Figs. 5, 6, 7). A higher number of NPY cell bodies were observed in the Poa ($p < .01$), the NIV ($p < .001$) and the AV ($p < .001$) of female brain, as compared to that in the brain of the male frog (Figs. 5, 6, 7). The NPY mRNA levels were quantified in three brain regions: Rostral, middle and caudal by using qRT-PCR (Fig. 8). The middle part of the brain predominantly contains the hypothalamus. The mRNA expression pattern was similar to that observed by immunohistochemistry; the NPY mRNA levels were higher in the middle part of the brain of the female as compared to that in the male ($p < .05$, Fig. 8). No significant difference was observed in NPY mRNA levels in the rostral and caudal brain regions of the male and the female (Fig. 8).

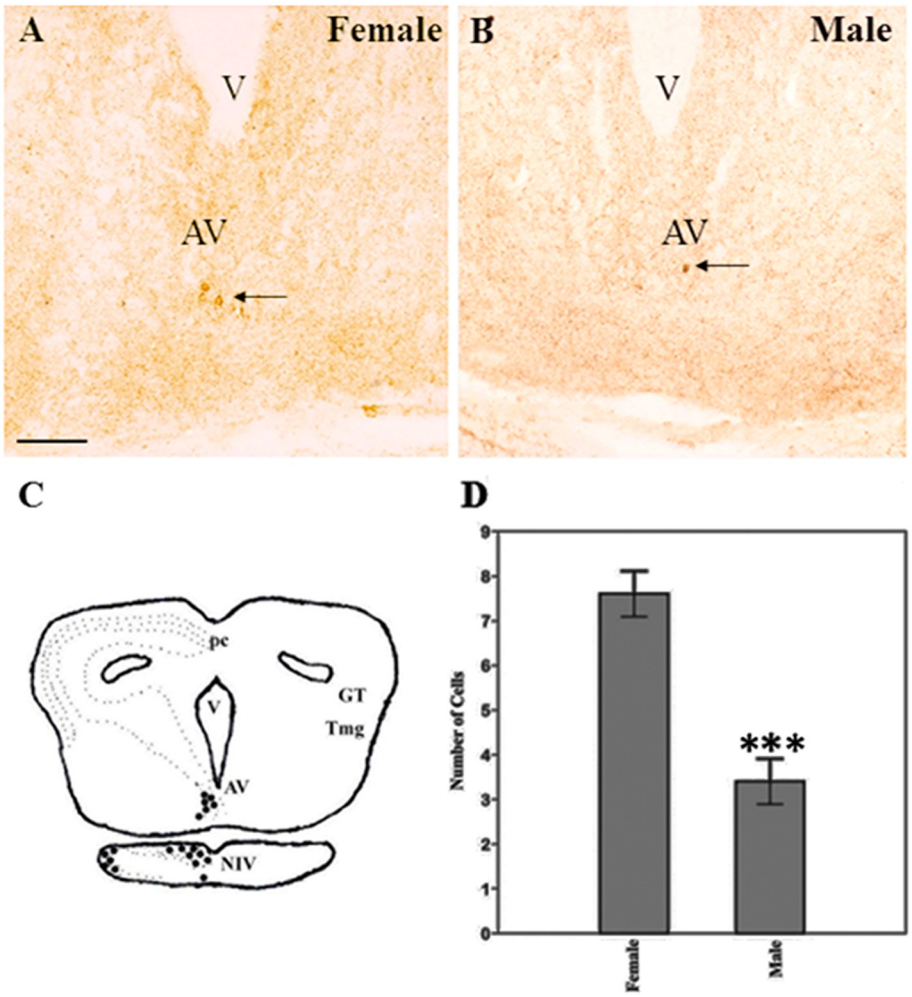

Fig. 7. Sex-specific distribution of NPY was observed in some regions of brain of *M. ornata*. The anteroventral tegmental nucleus (AV) region showed differences in NPY-expression where it was more in female than male A) Female B) Male. Arrows represents cells and arrowhead represents fibers, Scale bars: 200 µm, Image magnification: 20×. C) Schematic representation of the region D) Graph showing number of NPY immunoreactive cells in the AV. Error bar represents standard deviation. (*Indicated significant differences between male and female; ***P < .001, one-way ANOVA followed by post hoc Tukey's test).

4. Discussion

The distribution pattern of NPY-ir cells in the brain of *M. ornata* is similar to earlier reports with few variations (D'Aniello et al., 1996; Tuinhof et al., 1994) as shown in Table 1. In addition to the known areas, NPY-ir cells were also observed in striatum pars dorsalis.

These variations in the distribution of NPY immunoreactivity in the brain of *M. ornata* and other species could be attributed to the species-specific differences. Further, the current study, for the first time, reports the sex-specific distribution of NPY in the brain of *M. ornata*, whose breeding phase coincides with the onset of the monsoon. These sex-specific differences were observed in the Poa, NIV and AV regions of the brain, which play a major role in the regulation of reproduction. NPY expression was higher in these regions of the female brain as compared to that in the male brain.

In the telencephalon, no significant sex-specific differences of NPY were observed, except the Poa. The levels of the NPY peptide and mRNA expression in the Poa of females were higher than that in males; the observations support the previous findings showing this area as sexually dimorphic in different aspects in many vertebrates. For example, the morphological differences in organization of the Poa were observed in many mammals (De Vries and Boyle, 1998) and reptiles (Morris and Crews, 1990; Salom et al., 1994). More specifically, in amphibians, the overall volume of the preoptic nucleus in male *Bufo japonicus* is larger than its conspecific (Takami and Urano, 1984). Although such differences have not been reported in *M. ornata*, the NPY in *M. ornata* might be involved in the regulation of reproductive physiology of sexes in the light of the observations that (1) the Poa plays an important role in reproduction of anurans (Pinelli et al., 2014; Yoo and Jang, 2012) and (2) NPY exhibits dimorphic expression in *M. ornata*. These notions could be further supported by the strong role of the Poa in the central regulation of reproduction. The Poa responds to auditory stimulation in male and female frogs (Hoke et al., 2005), which results in the production of steroid hormones (Burmeister and Wilczynski, 2005; Lynch and Wilczynski, 2005). It is also known to process feeding information through NPY secretion in mammals (Kalra et al., 1999). The higher expression of NPY in the Poa of the female brain than that of the male may be the result of the accelerated appetite of female in the breeding season. In addition to this, sexual divergence in the diet of anurans has been observed in the tusked frog, *Adelotus brevis* (Katsikaros and Shine, 1997). Although the present study does not provide any clue to this notion, it paves the way for future studies in *M. ornata* to determine the relevance of sex-specific differences of NPY in energy demands, feeding behaviour and reproductive status during breeding season. In goldfish, it is also reported that ovarian steroids, testosterone (T) and estradiol (E2) stimulate the expression of NPY mRNA in the Poa (Peng et al., 1993). Similar type of steroidal regulation of NPY may be responsible for the sexual dimorphism of NPY in *M.*

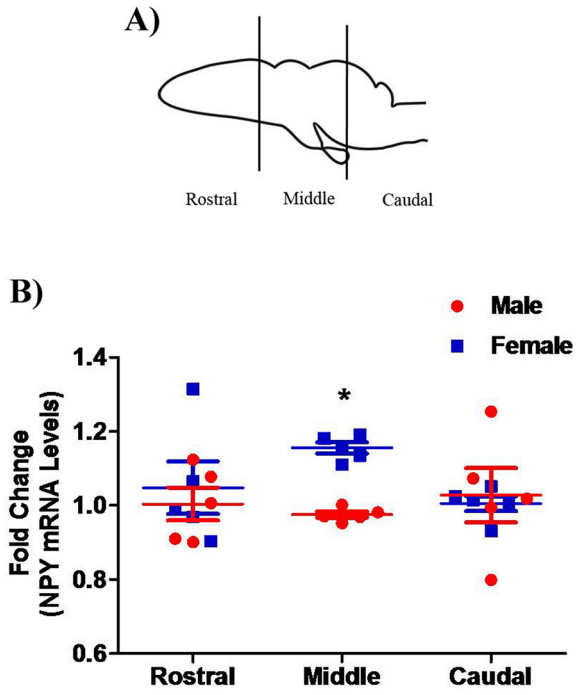

Fig. 8. A) Schematic representation of longitudinal section of *M. ornate* brain, the vertical lines represent demarcation of the rostral, middle and caudal tissue regions that were used for mRNA quantification. B) Graphs showing mRNA levels of neuropeptide Y (NPY) in rostral, middle and caudal regions of male and female *M. ornata* brain. Values (n = 5) are represented as means (± SEM) and are indicated as significantly different as compared to sham control group (*p < .05, one-way ANOVA followed by post hoc Tukey's test).

ornata.

Sexual dimorphism in NPY expression was observed in the NIV of the brain of *M. ornata*. The infundibulum has been identified as a target of steroids in anurans (DiMeglio et al., 1987; Kelley et al., 1975; Morrell et al., 1975). Additionally, the cells in this region also express GnRH (Rastogi et al., 1998). The arcuate nucleus in mammals, a homolog of the infundibulum in frog brain (Wada et al., 1980), is reported to express higher levels of NPY in male rats than in females (Rugarn et al., 1998). On the contrary, we have observed higher levels of NPY in the infundibular region of females than in males. This difference may be attributed to the differential sex steroid profile of the frog (Lynch and wilczynski, 2005) or the physiological status of the frog. In addition, the sexual dimorphism in NPY expression was observed to be female-dominant, suggesting that these NPY-ir cells may play a role in female-specific reproductive functions.

Sex-specific differences in the expression of NPY were also significant in the AV, where intense expression of NPY was seen in the female compared to the male. The tagmental area is known to act as a junction to receive neuronal information from various regions of the brain and process it through the hypothalamo-pituitary axis for further coordination (Eldred et al., 1980; Neary and Northcutt, 1983). Although we cannot offer explanation for the higher expression of NPY in the AV in female frog, we speculate its involvement in neuromodulation of sex-specific activities of amphibians during the breeding season.

The sex-specific differences in the NPY system, either due to gonadal sex, hormonal state and/or their interactions, could be significant for shaping sexual and other context-dependent differences in the reproductive physiology of anurans. Similar to mammals and reptiles, the findings suggest that NPY in amphibians is likely to be an important regulator of reproductive physiology of genders. Further studies are needed to understand the circuitries of NPY involved directly or indirectly in the regulation of energy balance and reproduction in the anuran brain.

Acknowledgements

SB acknowledges the research grant received from Scientific Engineering and Research Board, Government of India, New Delhi (SERB, F. GOI-A-719) and Department Research and Development Program (DRDP), Centre for Advanced Studies (CAS), Department of Science and Technology (DST-PURSE) of the Department of Zoology, Savitribai Phule Pune University (SPPU), Pune, India. AJS acknowledges the grants from the University Grants Commission (UGC), Government of India (F.4-5/151-FRP)/2014(BSR), University Research Grant Scheme (SPPU), DST-PURSE, and Department Research and Development Program (DRDP) of the Department of Biotechnology, SPPU, Pune, India. We acknowledge the kind help provided by Ms. Shilpy Sharma for dot blot analysis in the manuscript.

References

Acosta-Martinez, M., Horton, T., Levine, J.E., 2007. Estrogen receptors in neuropeptide Y neurons: at the crossroads of feeding and reproduction. Trends Endocrinol. Metab. 18, 48–50.
Ali, I., Bhargava, S., 2016. Neuropeptide Y in the brain of *Euphlyctis cyanophlyctis* tadpoles responds to hypoxic stress. Gen. Comp. Endocrinol. 251, 38–45.
Aubert, M.L., Pierroz, D.D., Gruaz, N.M., d'Alleves, V., Vuagnat, B.A., Pralong, F.P., Blum, W.F., Sizonenko, P.C., 1998. Metabolic control of sexual function and growth: role of neuropeptide Y and leptin. Mol. Cell. Endocrinol. 140, 107–113.
Beck, B., Pourié, G., 2013. Ghrelin, neuropeptide Y, and other feeding-regulatory peptides active in the hippocampus: role in learning and memory. Nutr. Rev. 71, 541–561.
Berglund, M.M., Schober, D.A., Statnick, M.A., McDonald, P.H., Gehlert, D.R., 2003. The use of bioluminescence resonance energy transfer 2 to study neuropeptide Y receptor agonist-induced beta-arrestin 2 interaction. J. Pharmacol. Exp. Ther. 306, 147–156.
Brown, D.D., Cai, L., 2007. Amphibian metamorphosis. Dev. Biol. 306, 20–33.
Burmeister, S.S., Wilczynski, W., 2005. Social signals regulate gonadotropin-releasing hormone neurons in the green treefrog. Brain Behav. Evol. 65, 26–32.
Campos, V.C., Collares, T., Deschamps, J.C., Seixas, F.K., Dellagostin, O.A., Lanes, C.F., Sandrini, J., Marins, M.F., Okamoto, M., Sampaio, L.A., Robaldo, R.B., 2010. Identification, tissue distribution and evaluation of brain neuropeptide Y gene expression in the Brazilian flounder *Paralichthys orbignyanus*. J. Biosci. 35, 405–413.
Campos, V.C., Collares, V.T., Seixasl, F.K., Deschamps, J.C., Fernandes, L.F., Marins, Okamoto, M.H., Sampaio, L.A.N., Robaldo, R.B., 2011. NPY and sbGnRH Gene Expression in Juvenile and Adult Male Brazilian Flounder *Paralichthys orbignyanus*. Ciência Rural, Santa Maria. 41. pp. 1927–1930.
Catzeflis, C., Pierroz, D.D., Rohner-Jeanrenaud, F., Rivier, J.E., Sizonenko, P.C., Aubert, M.L., 1993. Neuropeptide Y administered chronically into the lateral ventricle profoundly inhibits both the gonadotropic and the somatotropic axis in the intact adult female rats. Endocrinology 224–234.
Clark, J.T., Kalra, P.S., Kalra, S.P., 1997. Neuropeptide Y stimulates feeding but inhibits sexual behavior in rats. Obes. Res. 5 (3).
Corp, E.S., Gre'co, B.J., Powers, B., Carrie, L., Bivens, M., Wade, G.N., 2001. Neuropeptide Y inhibits estrous behavior and stimulates feeding via separate receptors in Syrian hamsters. Am. J. Physiol. Regul. Integr. Comp. Physiol. 280, 1061–1068.
Crespi, E.J., Vaudry, H., Denver, R.J., 2004. Roles of corticotropin releasing factor, neuropeptide Y and corticosterone in the regulation of food intake in *Xenopus laevis*. J. Neuroendocrinol. 16, 279–288.
Danger, J.M., Leboulenger, F., Guy, J., Benyamina, M., Tonon, M.C., Martel, J.C., Jegau, S., Pelletier, G., Vaundry, H., 1985. Localisation and identification of neuropeptide y (NPY)- like immunoreactivity in the frog brain. Peptides 6, 1225–1236.
Danger, J.M., Leboulenger, F., Guy, J., Benyamina, M., Tonon, M.C., Martel, J.C., Pierrie, S.S., Pelletier, G., Vaundry, H., 1986. Neuropeptide Y in the intermediate lobe of the frog pituitary acts as an alpha-MSH release inhibiting factor. Life Sci. 39, 1183–1192.
D'Aniello, B., Vallarino, M., Pinelli, C., Fiorentino, M., Rastogi, R., 1996. Neuropeptide Y: localization in the brain and pituitary of the developing frog (*Rana esculenta*). Cell Tissue Res. 285, 253–259.
De Vries, G.J., Boyle, P.A., 1998. Double duty for sex differences in the brain. Behav. Brain Res. 92, 205–213.
DiMeglio, M., Morrell, J.I., Pfaff, D.W., 1987. Localization of steroid-concentrating cells in the central nervous system of the frog *Rana esculenta*. Gen. Comp. Endocrinol. 67, 149–154.
Gaikwad, A., Biju, K.C., Subhedar, N., 2003. GnRH-LH secreting cells axis in the pituitary of the teleost *Clarias batrachus* responds to neuropeptide Y treatment: an immunocytochemical study. Gen. Comp. Endocrinol. 131, 126–133.
Eldred, W.D., Finger, T.E., Nolte, J., 1980. Central projections of the frontal organ of Rana pipiens, as demonstrated by the anterograde transport of horseradish peroxidase. Cell Tissue Res 211, 215–222.
Galas, L., Tonon, M., Beaujean, D., Fredriksson, R., Larhammar, A., Lihrmann, I., Jegou, S., Fournier, A., Chartrel, N., Vaudry, 2002. Neuropeptide Y inhibits spontaneous α-melanocyte-stimulating hormone (α-MSH) release via a Y5 receptor and suppresses thyrotropin-releasing hormone-induced α-MSH secretion via a Y1 receptor in frog melanotrope cells. Endocrinology 143 (5), 1686–1694.
Gøtzsche, C.R., Woldbye, D.P., 2016. The role of NPY in learning and memory. Neuropeptides 55, 79–89.

Gotzsche, C.R., Nikitidou, L., Sorensen, A.T., Olesen, M.V., Sorensen, G., Christiansen, S.H., Angehagen, M., Woldbye, D.P., Kokaia, M., 2012. Combined gene over-expression of neuropeptide Y and its receptor Y5 in the hippocampus suppresses seizures. Neurobiol. Dis. 45, 288–296.
Hall, I.C., Ballagh, I.H., Kelley, D.B., 2013. The Xenopus amygdala mediates socially appropriate vocal communication signals. J. Neurosci. 33, 14534–14548.
Heigrujam, E., Ali, I., Bhargava, S., 2017. NPY up-regulation in the tadpole brain of *Euphlyctis cyanophlyctis* during osmotic stress. Gen. Comp. Endocrinol. 251, 46–53.
Hoke, K.L., Ryan, M.J., Wilczynski, W., 2005. Social cues shift functional connectivity in the hypothalamus. Proc. Nat. Acad. Sci. USA 102, 10712–10717.
Hokfelt, T., Stanic, D., Sanford, S.D., Gatlin, J.C., Nilsson, I., Paratcha, G., 2008. NPY and its involvement in axon guidance, neurogenesis, and feeding. Nutrition 24, 860–868.
Kalra, S.P., Kalra, P.S., 1996. Nutritional infertility: the role of the interconnected hypothalamic Neuropeptide Y–galanin–opioid network. Front. Neuroendocrinol. 17, 371–401.
Kalra, S.P., Dube, M.G., Pu, S., Xu, B., Horvath, T.L., Kalra, P.S., 1999. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight 1. Endocr. Rev. 20, 68–100.
Karim, I.A., 2008. Antimicrobial activity of neuropeptides against a range of micro-organisms from skin, oral, respiratory and gastrointestinal tract sites. J. Neuroimmunol. 200, 11–16.
Katsikaros, K., Shine, R., 1997. Sexual dimorphism in the tusked frog, *Adelotus brevis* (Anura:Myobatrachidae): the roles of natural and sexual selection. Biol. J. Linnean Soc. 60, 39–51.
Kelley, D.B., Morrell, J.I., Pfaff, D.W., 1975. Auto radiographic localization of hormone-concentrating cells in the brain of an amphibian, *Xenopus laevis*. J. Comp. Neurol. 164, 4759.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta DeltaC(T)) Method. Methods 25, 402–408.
Loh, K., Herzog, H., Shi, Y.C., 2015. Regulation of energy homeostasis by the NPY system. Trends Endocrinol. Metab. 26, 125–135.
Lynch, K.S., Wilczynski, W., 2005. Gonadal steroid fluctuations in a tropically breeding female anuran. Gen. Comp. Endocrinol. 143, 51–56.
Magni, P., 2003. Hormonal control of the neuropeptide Y system. Curr. Protein Pept. Sci. 4, 45–57.
Mazumdar, M., Sakharkar, A., Singru, P., Subhedar, N., 2007. Reproduction phase-related variations in neuropeptide Y immunoreactivity in the olfactory system, forebrain, and pituitary of the female catfish, *Clarias batrachus* (Linn.). J. Comp. Neurol. 504, 450–469.
McKay, D.M., Shaw, C., Halton, D.W., Thim, L., Buchnan, K.D., 1992. The primary structure and tissue distribution of an neuropeptide Y. Regul. Pept. 37, 143–153.
Morona, R., González, A., 2008. Calbindin-D28k and calretinin expression in the forebrain of anuran and urodele amphibians: further support for newly identified subdivisions. J. Comp. Neurol. 511, 187–220.
Morona, R., González, A., 2009. Immunohistochemical localization of calbindin-D28k and calretinin in the brainstem of anuran and urodele amphibians. J. Comp. Neurol. 515, 503–537.
Morrell, J.I., Kelley, D.B., Pfaff, D.W., 1975. Autoradiography localization 42–48 of hormone-concentrating cells in the brain of an amphibian *Xenopus laevis*. Estradiol. J. Comp. Neurol. 164, 63–77.
Morris, Y.A., Crews, D., 1990. The effects of exogenous neuropeptide Y on feeding and sexual behavior in the red-sided garter snake (*Thamnophis sirtalis parietalis*). Brain Res. 530, 339–341.
Mousley, A., Polese, G., Marks, N., Eisthen, J., 2006. Terminal nerve-derived neuropeptide Y modulates physiological responses in the olfactory epithelium of hungry axolotls (*Ambystoma mexicanum*). J. Neurosci. 26, 7707–7717.
Neary, T.J., Northcutt, R.G., 1983. Nuclear organization of the bullfrog diencephalon. J. Comp. Neurol. 213, 262–278.
Peng, C., Humphries, S., Peter, R.E., Rivier, J.E., Blomqvist, A.G., Larhammar, D., 1993. Actions of goldfish neuropeptide Y on the secretion of growth hormone and gonadotropin-II in female goldfish. Gen. Comp. Endocrinol. 90, 306–317.
Pierroz, D.D., Gruaz, N.M., D'Allèves, V., Aubert, M.L., 1995. Chronic administration of neuropeptide Y into the lateral ventricle starting at 30 days of life delays sexual maturation in the female rat. Neuroendocrinology 61, 293–300.
Pierroz, D.D., Catzeflis, C., Aebi, A.C., Rivier, J.E., Aubert, M.L., 1996. Chronic administration of neuropeptide Y into the lateral ventricle inhibits both the pituitary-testicular axis and growth hormone and insulin-like growth factor I secretion in adult male rats. Neuroendocrinology 137, 3–12.
Pinelli, C., Rastogi, R.K., Scandurra, A., Jadhao, A.G., Aria, M., D'Aniello, B., 2014. A comparative cluster analysis of nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemistry in the brains of amphibians. J. Comp. Neurol. 522, 2980–3003.
Pinelli, C., Jadhao, A.G., Biswas, S.P., Tsutsui, K., 2015. Neuroanatomical organization of the brain gonadotropin-inhibitory hormone and gonadotropin-releasing hormone systems in the frog Pelophylaxesculentus. Brain Behav. Evol. 85, 15–28.
Rastogi, R.K., Meyer, D.L., Pinelli, C., Fiorentino, M., D'Aniello, B., 1998. Comparative analysis of GnRH neuronal systems in the amphibian brain. Gen. Comp. Endocrinol. 112, 330–345.
Reichmann, F., Holzer, F., 2016. Neuropeptide Y: a stressful review. Neuropeptides 55, 99–109.
Rugarn, O., Hammar, M., Theodorsson, A., Theodorsson, E., Stenfors, C., 1998. Sex differences in neuropeptide distribution in the rat brain. Peptides 20, 81–86.
Sagarkar, S., Mahajan, S., Choudhary, A.G., Borkar, C.D., Kokare, D.M., Sakharkar, A.J., 2017. Traumatic stress-induced persistent changes in DNA methylation regulate neuropeptide Y expression in rat jejunum. Neurogastroenterol. Motil. 29, 13074.
Sakharkar, A.J., Singru, P.S., Sarkar, K., Subhedar, N.K., 2005. Neuropeptide Y in the forebrain of the adult male cichlid fish *Oreochromis mossambicus*: distribution, effects of castration and testosterone replacement. J. Comp. Neurol. 489, 148–165.
Salom, S., Font, C., Gracia, F., 1994. Seasonal sexually dimorphic distribution of neuropeptide Y-like immunoreactive neurons in the forebrain of the lizard *Podarcis hispanica*. J. Chem. Neuroanat. 7, 217–225.
Schwippert, W.W., 1998. Neuropeptide Y (NPY) or fragment NPY 13–36, but not NPY 18–36, inhibit retinotectal transfer in cane toads *Bufo marinus*. Neurosci. Lett. 253, 33–36.
Shahjahan, M., Kitahashi, T., Parhar, I., 2014. Central pathways integrating metabolism and reproduction in teleosts. Front. Endocrinol. 36, 1–17.
Shewale, S., Ali, I., Hadawale, K., Bhargava, S., 2018. Response of NPY immunoreactivity in the tadpole brain exposed to energy rich and energy depleted states. Neuropeptides 71, 1–10.
Sperk, G., Hamilton, T., Colmers, W.F., 2007. Neuropeptide Y in the dentate gyrus. Prog. Brain Res. 163, 285–297.
Takami, S., Urano, A., 1984. The volume of the toad medial amygdala-anterior preoptic complex is sexually dimorphic and seasonally variable. Neurosci. Lett. 44, 253–258.
Tasan, R.O., Verma, D., Wood, J., Lach, G., Hörmer, B., de Lima, T.C.M., Herzog, H., Sperk, G., 2016. The role of neuropeptide Y in fear conditioning and extinction. Neuropeptides 55, 111–126.
Tatemoto, K., Carlquist, M., Mutt, V., 1982. Neuropeptide Y—a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature 296, 659–660.
Tuinhof, R., González, A., Smeets, A.J., Roubos, E.W., 1994. Neuropeptide Y in the developing and adult brain of the South African clawed toad *Xenopus laevis*. J. Chem. Neuroanat. 7, 271–283.
Urban, J.H., Bauer-Dantoin, A.C., Levine, J.E., 1993. Neuropeptide Y gene expression in the arcuate nucleus: sexual dimorphism and modulation by testosterone. Endocrinology 132, 117–121.
Wada, M., Urano, A., Gorbman, A., 1980. A stereotaxic atlas for diencephalon nuclei of the frog *Rana pipiens*. Archiv. Histol. Japonicum 43, 157–173.
Yang, L. Karen A. Scott, Hyun, Jayson, Tamashiro, Kellie L., Tray, Nancy, Moran, Timothy H., Bi, Sheng, 2009. Role of dorsomedial hypothalamic neuropeptide Y in modulating food intake and energy balance. J. Neurosci. 29, 179.
Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Levavi-Sivan, B., Elizur, A., 2001. Regulation of gonadotropin subunit genes in tilapia. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 129, 489–502.
Yoo, E., Jang, Y., 2012. Abiotic effects on calling phenology of three frog species in Korea. Animal Cells Syst. 16, 260–267.
Zarjevski, N., Cusin, I., Vettor, R., Rohner-Jeanrenaud, F., Jeanrenaud, B., 1993. Chronic intracerebroventricular Neuropeptide-Y administration to normal rats mimics hormonal and metabolic changes of obesity. Endocrinology 133, 1753–1775.