Summary of the UGC Major Research project

Project Title: Distribution and role of neuropeptide Y (NPY) and related peptides in the olfactory system and brain of anurans (File No. 41-156/ 2012)

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Period: 2012-2015

Workdone :

1. To study the distribution of NPY in the brain of *Microhyla ornata*

Neuropeptide Y (NPY) is found to be one of the most abundant neuropeptides within the brain of mammals (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; Hökfelt et al., 2008; Beck and Pourié, 2013; Loh et al., 2015, Gøtzsche et al., 2012; Gøtzsche-Woldbye., 2016; Tasan et al., 2016). It has been discovered that NPY enhances appetite in pigs (Tatemoto et al., 1992). Since then, various studies have shown that NPY is the most potent orexigenic peptide identified to date. Central administration of NPY results into the robust increase in food intake and body weight, and with chronic administration, can 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 inhibits 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 et al., 1993; Pierroz et al., 1995; 1996; Aubert et al., 1998). NPY suppresses sexual behaviour in rats (Clark et al., 1997). NPY agonists decrease 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 reduces courtship behaviour, indicating inhibitory role of NPY in reproductive behaviour in red sided garter snake (Morris and Crews, 1990).

Similar to mammals, several studies indicate that NPY regulates feeding and reproduction in teleost fishes (Campos et al., 2010; 2011). *In vitro* treatment of pituitary with NPY stimulates LH release in the goldfish (Peng et al., 1993). It also increases 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). Anatomical observations also support the role of NPY in LH secretion; NPY fibers make close appositions on LH cells in the pituitary of catfish (Gaikwad et al., 2003). Castration of cichlid fish results in significant reduction of NPY-immunoreactive cells in nucleus entopeduncularis (NE, Sakharkar et al., 2005). The administration of testosterone to juvenile male catfish results in a significant increase in NPY immunoreactivity in the NE cells (Mazumdar et al., 2007).

Sex specific differences in NPY expression in the central nervous system (CNS) 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 displays significantly higher expression levels in males than females (Urban et al., 1993). In the lizard, *Podaris hispanica*, NPY-immunoreactive cells in the lateral septum shows a clear sexual dimorphism, wherein the number of reactive cells are 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 et al., 1996; Shahjahan et al., 2014). In rats, it has been suggested that NPY neurons are activated by signals of reduced fuel availability, which increases 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 become 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-Ali et al., 2017; Ali et al., 2016), background adaptation (Galas et al., 2002), visual neurotransmission (Schwippert et al., 1998), antimicrobial activity (Karim et al., 2008) and feeding behaviour (Crespi et al., 2004), 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° 550N 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 hours and then cryoprotected in 10% (2 hours), 20% (2 hours) 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 collected 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, according to the manufacturer's instructions. The RT reaction used for the reverse transcription were 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 TM 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 sequence is as follows; NPY: F-5'-GAGACCCAGTCGCTGACAAA-3', and R-5'-ATATCTGGTGTTTCCGGGGC-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 seconds each (40 cycles); 95°C for 15 seconds. 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 seconds/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. 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*.

Dot blot:

A small circle was drawn with the help of pencil on nitrocellulose membrane to specify the area where protein sample spotted. Protein sample of 40µg was spotted on nitrocellulose membrane. The protein sample was allowed to dry. After drying the membrane was blocked with 3%BSA in tris buffered saline tween 20 (1x TBST) for 2 hours. 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 10 minutes each was given. Followed by incubation with secondary antibody conjugated with Horse Radish Peroxidase, HRP (1:500) for 1hour at room temperature. The membrane was washed with TBST thrice,10 minutes each. The blot was developed with ECL solution. After developing the blot was washed with TBST and then kept in distilled water.

2.4 Immunohistochemistry

Sections were used for the immunohistochemical localization of NPY as described previously (Ali et al., 2016). Briefly, the sections were washed with PBS thrice and treated with 0.3% hydrogen peroxide in methanol for 1 hour. 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 hour. After washing thrice, sections were incubated for 1 hour in normal goat serum (1:40, PK- 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 hour (1:200, PK-6101,Vectastain ABC Kit, Vector laboratories). Sections were washed twice and incubated with ABC reagent for 1 hour 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 minutes. Slides were washed in distilled water for 5 minutes, dehydrated, cleared in xylene for 30 minutes, 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, (Figure 1.B) (ii) omission of primary or secondary antibodies from the immunohistochemistry protocol, (Figure1.C), and (iii) preabsorption of 1 ml diluted primary antibody with the porcine NPY peptide (Sigma, N3266) at 10^{-5} M concentration for 24 hour at 4°C prior to incubation (Figure 1.D).

2.5 Morphometry

Digital images of NPY immunoreactivity were taken on a Carl Zeiss 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 (D'Aniello et al., 1996; Neary and Northcutt, 1983; Wada et al., 1980) 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 were 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.

2.6 Statistical Analysis

The differences between the two groups were tested for significance using Student t test, and the p-values less than 0.05 ($P < 0.05$) 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 (Figure 1.A), 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. Nucleus preopticus region of the frog brain showed positive immunoreaction (Figure 1.B). 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 immunoreaction (Figure 1.C, D).

3.2 Neuropeptide Y Distribution in the brain

NPY immunoreactivity was observed in various regions of adult brain including the telencephalon, diencephalon, mesencephalon and rhombencephalon. The neuroanatomical distribution of NPY immunoreactivity was studied in serial transverse sections and drawn in representative diagrams (Figure 2.A-I) and summarized in table 1. In the telencephalon, few NPY-ir perikarya interspersed in dense network of NPY beaded fibers were observed in the nucleus accumbens septi (NAS, Figure 3.A). Some NPY immunostained cells with moderate number of fibers were observed in the pallium mediale (PM) and pallium dorsale regions (PD, Figure 3.B, C). In diencephalon, strong NPY-ir cells with prominent nuclei and long axonal processes and fibers were observed in the nucleus preopticus (NPO, Figure 3.D). NPY-ir fibres with beaded granules were also seen in the NPO. NPO is the most conspicuous NPY containing cell group in the brain of *M. ornata.* A few, intensely stained NPY-ir cells were observed in the lateral forebrain bundle tract(LFB, Figure 3.E), whereas weakly stained, NPY positive cells were observed in the amygdala pars medialis region (AM, Figure 3.F). In the diencephalon, few NPY immunostained cells in the pallium laterale pars dorsalis (PLd) and pallium laterale pars ventralis (PLv) were observed (Figure 3.G, H). The intensity of staining varied from strong to weak and cells were oval to round in shape. In the thalamus, group of few NPY immunopositive cells were observed in the nucleus posterocentralis thalami (NPC, Figure 3.I). A group of some NPY immunostained cells were observed in the nucleus mesencephalicus nervi trigemini (NMNT, Figure 4.A). In NMNT, few somata had long processes and varying intensity of NPY immunoreactivity was observed. In hypothalamus, dense innervations of NPY containing cells, fibers, as well as granules were observed in nucleus infundibularis ventralis (NIV, Figure 4.B). The number of NPY-ir perikarya decreased from rostral to caudal end of NIV. NPY-ir neurons were located in

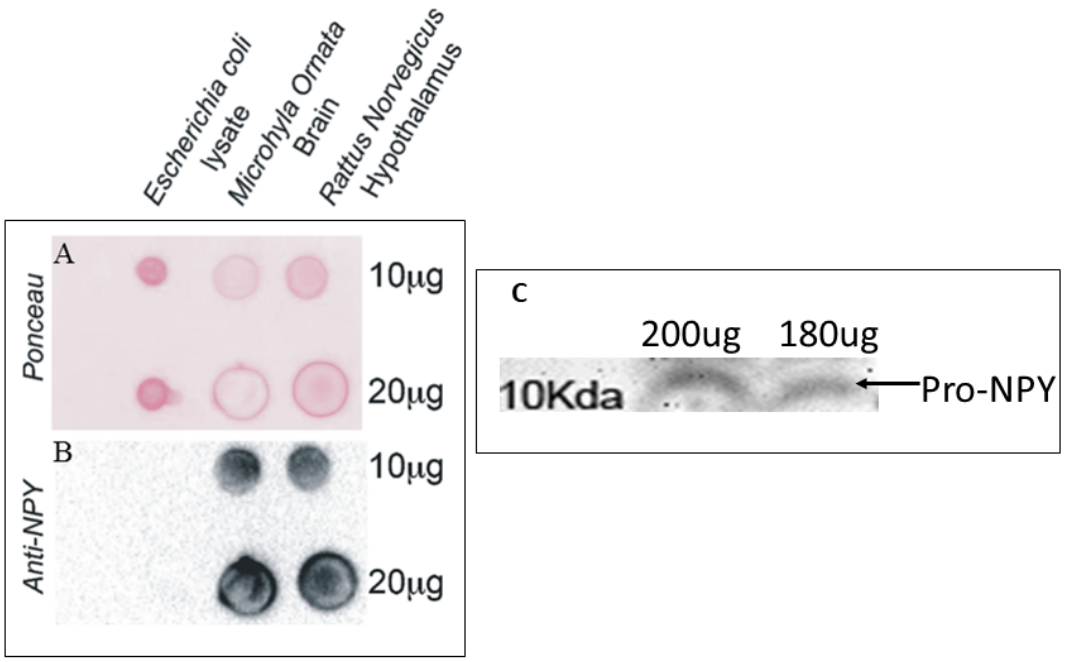


Figure 2

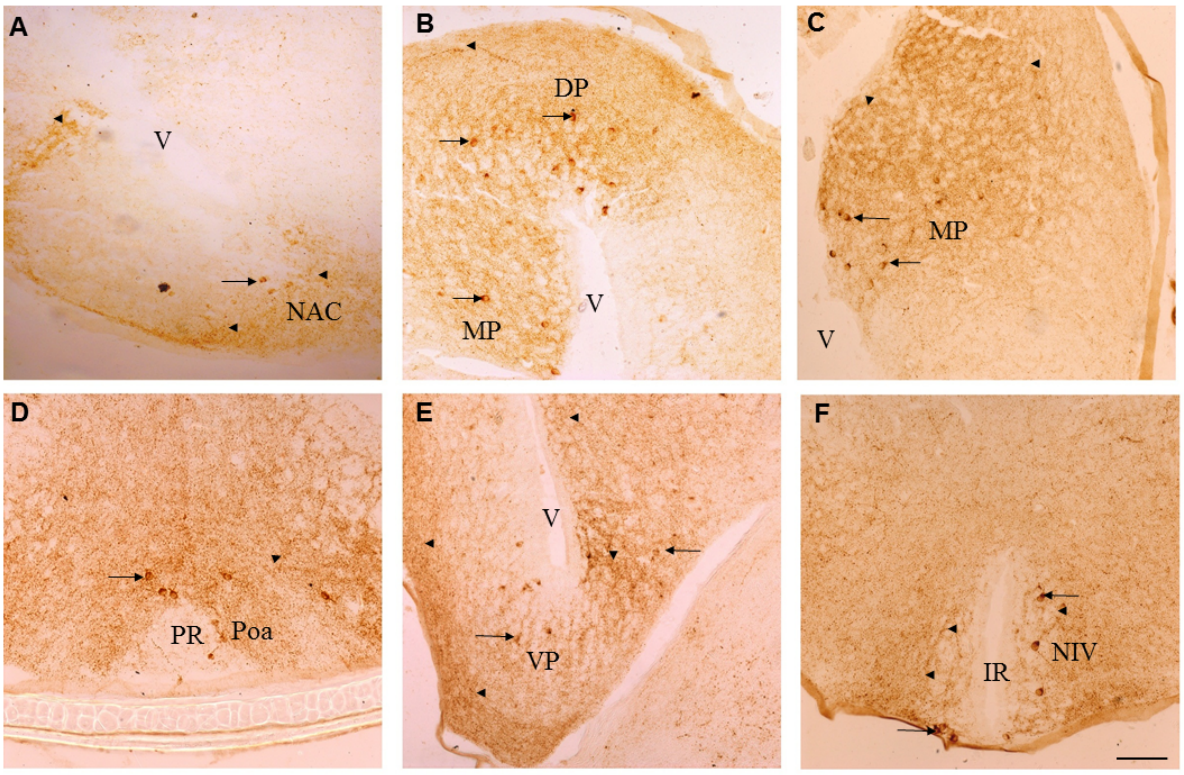


Figure 4.1

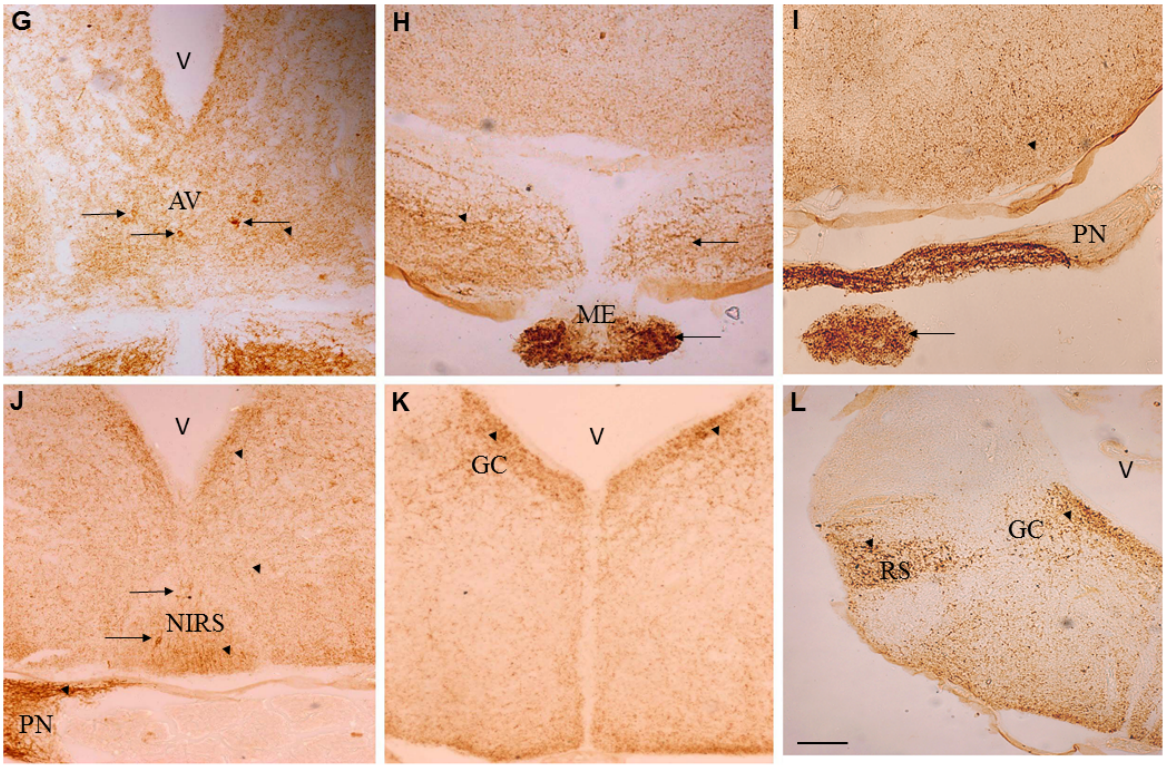
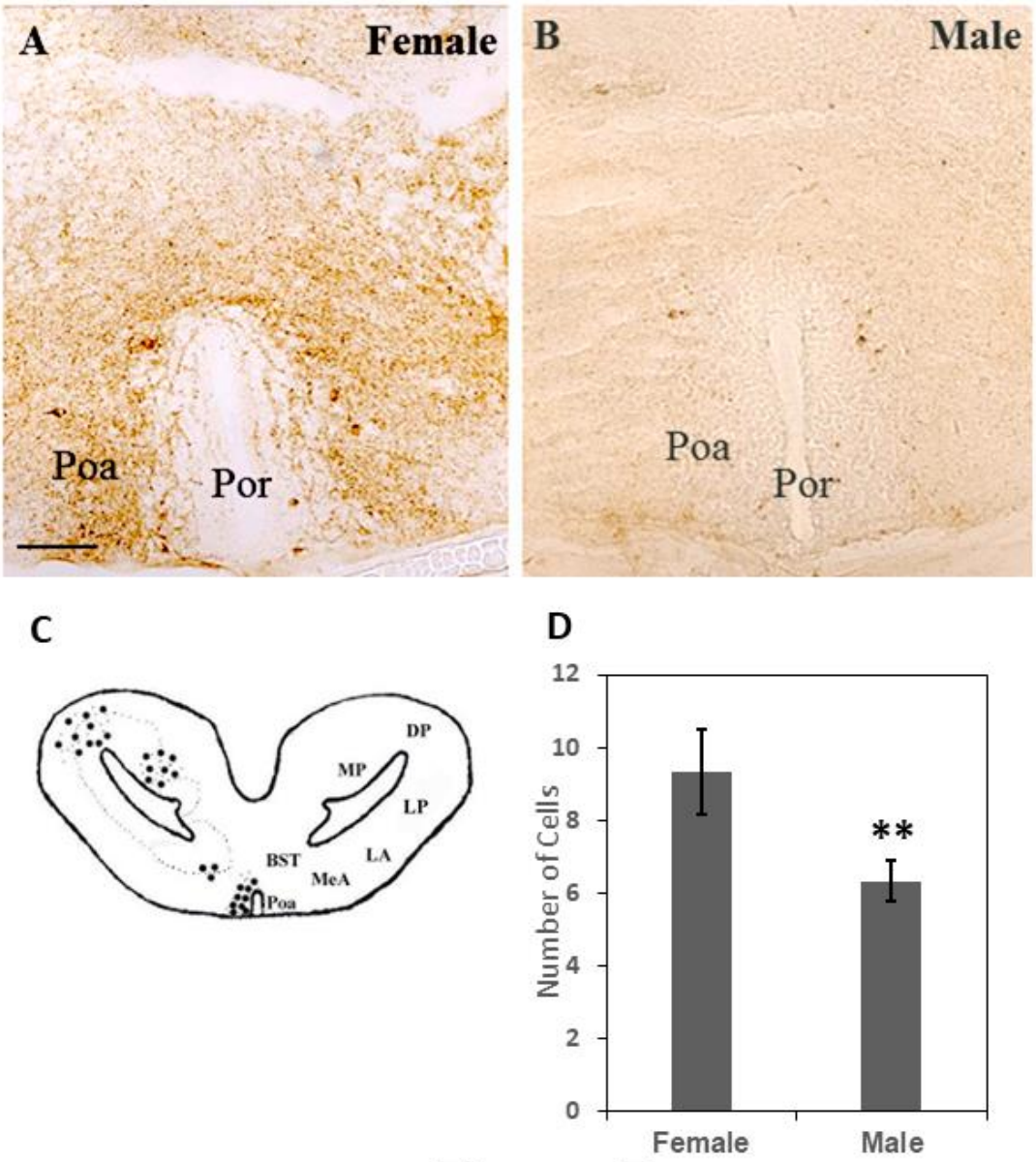


Figure 4.2


A
Female
Poa
Por
B
Male
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Por
C
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LP
LA
BST
MeA
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D
Number of Cells
12
10
8
6
4
2
0
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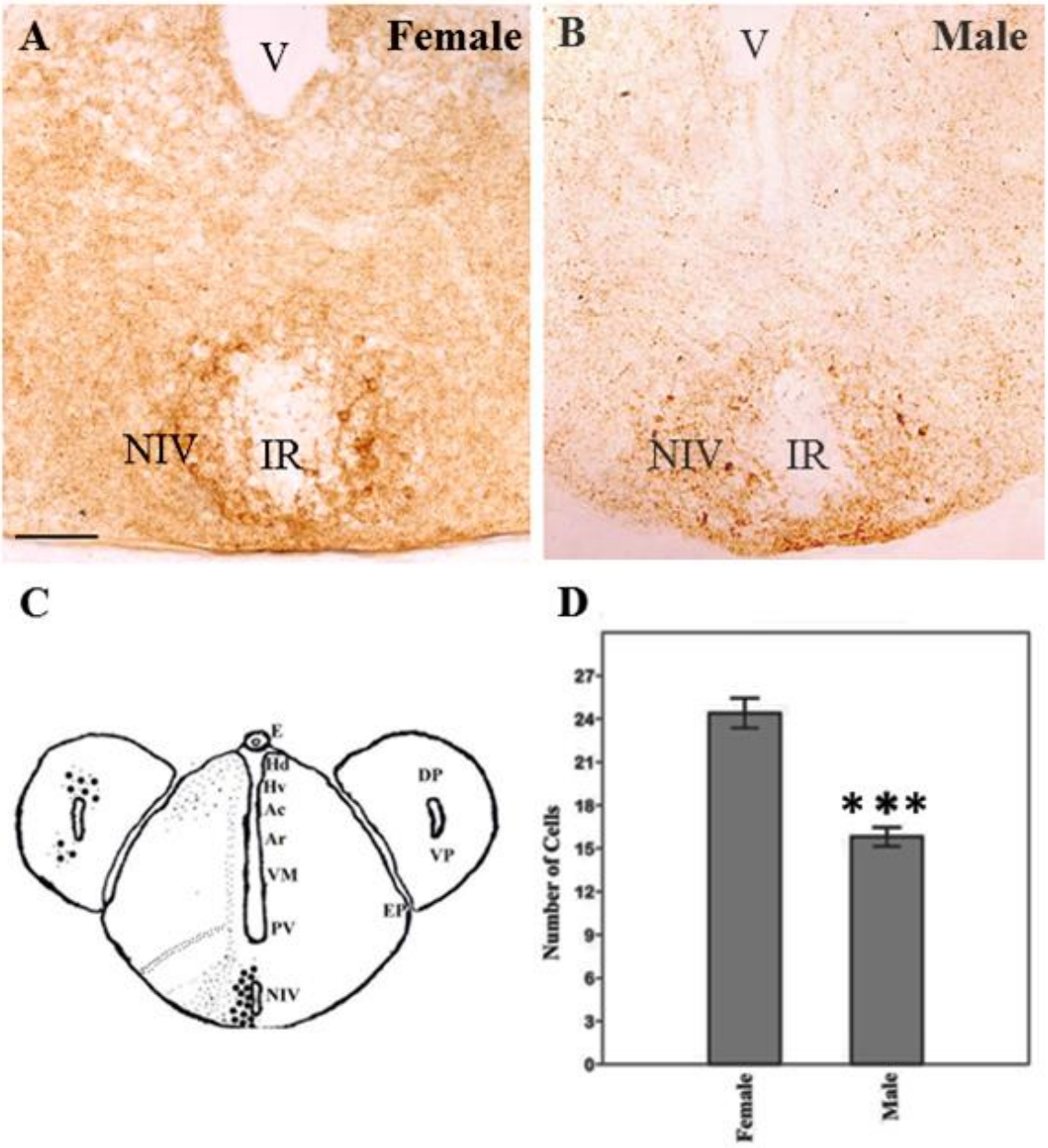
Female
Male

Figure 5


A
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Female
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27
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Figure 6

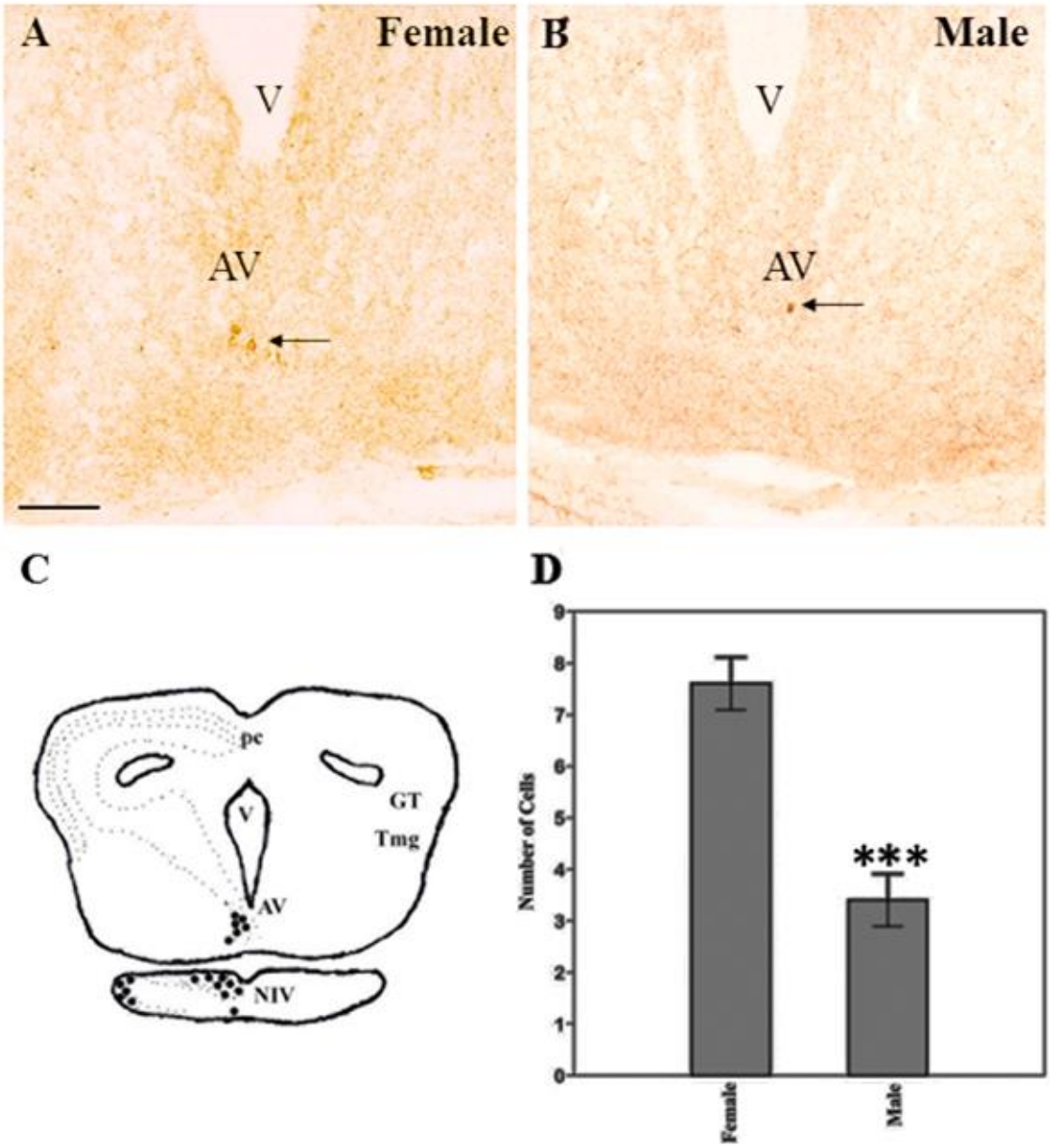


Figure 7

midbrain tegmentum, (nucleus anteroventralis ,NAV and Nucleus reticularis isthmi ,NRIS). In NAV, few NPY-ir perikarya were observed. NRIS located at the ventral side of NAV contained strong NPY immunoreactive fibers and granules (Figure 4.C). A very dense granular reaction of NPY was observed in median eminence (ME, Figure 4.D) and continued to the pituitary gland. Although pituitary was devoid of NPY-containing cells, moderate fibers and granules were seen in pars nervosa (PN, Figure 4.E). In rhombencephalon, raphe nucleus (RA) showed moderately stained granules and fibers (Figure 4.F). Some NPY-ir granules and fibers were seen in central grey of rostral rhombencephalon (GC, Figure 4.G).

3.3 Sex-specific differences of neuropeptide Y in hypothalamus

Although a wide distribution of NPY was observed throughout the brain of both male and female *M. ornata*, comparative analysis between them showed dimorphic changes in NPY immunoreactivity in some brain regions as shown in Figure 5A-C. A higher number of NPY cell bodies were observed in NPO ($p < 0.01$), NIV ($p < 0.001$) and NPC ($p < 0.001$) of female brain, as compared to that in the brain of the male frog (Figure 6.A). The NPY mRNA levels were quantified in three brain regions: rostral, middle and caudal by using qRT-PCR (Figure 6.B). The expression pattern was similar to that observed by immunohistochemistry; the NPY mRNA levels were higher in the middle brain of the female as compared to that in the male ($p<0.05$, Figure 6.C) No significant difference in NPY mRNA levels was observed in the rostral and caudal brain regions of the male and the female (Figure 6.C)..

4.Conclusion

The present study reveals the distribution and sex-specific differences in NPY-ergic system in the brain of the frog, *M. ornata.* 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 two additional nuclei, i.e. striatum dorsalis and lateral forebrain bundle.

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 NPO, NIV and NPC 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, while they were evident in the NPO of the diencephalon. The levels of the NPY peptide and mRNA expression in 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 NPO were observed in many mammals (De Vries and Boyle, 1998) and reptiles (Morris and Crews et al., 1990; Salom et al., 1994). More specifically, in amphibians, the overall volume of the NPO in *Bufo japonicus* male frog 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 NPO plays an important role in reproduction of anurans (Pinelli et al., 2014; Yoo et al., 2012) and (2) NPY exhibits dimorphic expression in *M. ornata*. These notions could be further supported by the strong role of NPO in the central regulation of reproduction. NPO 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). The NPO is already known to process feeding information through NPY secretion in mammals (Kalra et al., 1999). A higher expression of NPY in the NPO of the female brain than that of the male may be the result of the accelerated appetite of the 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 NPO (Peng et al., 1993). Similar type of steroidal regulation of NPY may be responsible for the sexual dimorphism of NPY in *M. 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). Arcuate nucleus in mammals, a homolog of 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 (Wilczynski et al., 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-cells may play a role in female-specific reproductive functions.

Sex-specific differences in the expression of NPY were also significant in NPC, where more expression of NPY was seen in the female than in the male. NPC 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 et al., 1983). Although we cannot offer explanation for the higher expression of NPY in this region of the 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 states 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.

2.*To study the effect of fasting and feeding on expression of NPY in the olfactory system and brain of *Euphlyctis cyanophlyctis.

Neuropeptide Y (NPY) has emerged as an important orexigenicagent in the vertebrate brain (Levine et al., 2004; Morton et al., 2006; Singru et al., 2008). While its role in food control is well established in mammals (Stanely, 1986; Schwartz et al., 2000, Zarjevski, 1993, Crespi, 2014, Kalra and Kalra., 2004), there are also increasing evidences of its in the food and feeding regulation of sub-mammalian vertebrates. NPY has been shown to stimulate appetite and food consumption in various fish species including goldfish, zebra fish, orange spotted grouper, red tilapia, rainbow trout and salmon (Aldegunde and Mancebo 2006; Matsuda et al., 2012; Yokobori et al., 2012; Wu et al., 2012; Volkoff et al., 1999). Intra cerebro-ventricular injections of NPY stimulate food intake in goldfish Carassius auratus and Channel fish Ictalaurus punctatus and NPY-antagonists have been seen to block this effect with no effect on the basal food intake (Silverstein JT et al., 2000; Narnaware YK, 2001). Recently, Li et al., reported involvement of NPY in increasing appetite and growth hormone expression in Olive flounder (Li et al., 2016). In amphibians, NPY induced food intake and its role in the modulation of feeding behaviour is well known. Crespi et al., 2004 reported fluctuations in the levels of NPY and its mRNA in the hypothalamus of X. laevis during different feeding conditions. Intracerebroventricular (icv) administration of NPY has been observed to stimulate food intake which could be blocked by the treatment of NPY Y1 receptor antagonist (Lopez-Patino et al., 1999; Shimizu et al., 2013). Along with its stimulatory role, NPY is also known to inhibit feeding in larval stage of western spade foot toad, while it continues to stimulate food intake in juveniles (Crespi and Denver., 2012). This opposite action has been associated with the differential expression of NPY receptors (Crespi and Denver 2012).For the central regulation of feeding, NPY in mammals and lower vertebrates (fishes) is known to engage various neuroanatomical regions of the brain including the olfactory nucleus, nucleus preoptics (NPO), arcuate nucleus (ARC), paraventricular nucleus (PVN), optic tectum and ventral telencephalic regions (Kohno and

Yada, 2012; Halford et al., 2004; Kalra et al., 1999; Yoo S et al., 2011). Increased levels of NPY mRNA in the forebrain in response to prolonged fasting are also reported (Silverstein JT, 1998; Qi I et al., 2016). Given that NPY is widely distributed in the brain of amphibians, (D'Aniello et al., 1996; Tuinhof R, et al., 1994, Ali et al., 2016) most of the reports in amphibians only discuss the physiological role of NPY in food and feeding and there is only one noteworthy study describing the neuroanatomical substrates exerting these physiological effects. Calle et al., (2006) reported an increase in the NPY expression in the supra chiasmatic nuclei in Xenopus laevis following a prolonged starvation. However, they did not find any significant difference in the rest of the food and feeding associated areas of the brain where NPY producing cells are present in abundance (D'Aniello et al., 1996). NPY neurons in the hypothalamus of mammals are known to reflect the prevailing energy state in mammals and also serve a glucosensory function (Mountjoy P et al., 2007; Polakof S et al., 2011; Bi et al., 2012; Kotagale N., 2014). The NPY system in fish is also known to respond to the manipulations of glucose levels in the brain (Polakof S et al., 2011; Polako S., 2012; RileyJr L et al., 2009). In the current study, we investigate the changes in the NPY system in the entire brain of tadpole exposed to energy rich as well as energy depleted states and to check whether the role of NPY in amphibians is conserved or not.

2. Materials and methods

2.1. Animals collection and procedure for intracranial treatments

The egg clutches of *Euphlyctis cyanophlyctis* were collected from the pond in Savitribai Phule Pune University campus and brought to the laboratory where these were kept in glass aquariu(70x40x20cm3)
containing aged declorinated tap water. The eggs hatched after 2–3 days. Tadpoles were provided with ad libitum boiled spinach that served as a food. Tadpoles of stage 38 (Gosner 1960; Shewale et al., 2015) were used for all the experiments. Stage 38 is characterized by
the increase in the length of individual toes and appearance of metatarsal tubercles (Gosner 1960). At this stage the larvae feeds voraciously and all the physiological processes are stabilized (Gosner 1960; Ali and Bhargava, 2016). All the experimental procedures were performed in accordance with the ethical guidelines established for animal usage by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and Savitribai Phule Pune University, Pune, India (Institutional committee for animal ethics, No. 538/CPCSEA). A group of tadpoles (n=5) were anesthetized with 2-phenoxyethanol (Sigma, St.Louis, Mo; 1:2000) and brains were dissected and fixed in Bouins fixative for 24 h. Additional tadpoles were divided into groups (n=5 in

each) and used as follows. The animals in group 2 were used as saline treated control (placebo controls), and groups 3 and 4 were used for 2 Deoxyglucose (16 μg/g body weight; SRL, India), glucose (16 μg/g body weight; Sigma, St. Louis; Mo) treatments respectively.
An additional group (n=5) were food deprived for 5 days which acted as the fasted group. All the above treatments were given dosage by intracranial route. The needle (#28) was glued into a 21G needle in a way that only 2mm of the needle tip remained exposed. The other end was connected to a 10 μL syringe. Conscious tadpole were immobilized in a wet cloth, the backwardly directed needle tip was positioned against the entry point and gently pushed through the skin and the solution was delivered into the cerebrospinal fluid (CSF)-filled space in the periphery of the brain. 1 μL of 0.9% saline was used as a vehicle in placebo control. The injection procedures were completed within 30–35 s and the tadpoles were returned to the tank. The stress due to injection procedure seems to be negligible since the tadpoles readily resumed their normal activity like swimming to the surface at regular intervals to gulp air. After an interval of 2 h post injection each animal was anesthetized with 2-phenoxyethanol (Sigma, Germany), brains dissected and fixed in Bouins fixative for 24 h at 4 °C, cryoprotected in 10% (2 h), 20% (2 h) and 30% (overnight at 4 °C) sucrose solution in Phosphate Buffer Saline (PBS, 0.0.1 M, pH 7.4). Tissues were then embedded in Shandon cryomatrix and serially cut on a cryostat (Leica CM1510) at 20 μm thickness in transverse plane. Sections were mounted on slides charged with poly-L-lysine (Sigma, USA) and stored in −20 °C until further use for immunohistochemical staining.

2.2. Immunohistochemistry

Sections were used for the immunohistochemical localization of NPY following the standard protocol (Shewale et al., 2015). 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 and incubated with blocking agent containing 0.5% Bovine Serum Albumin and 0.5% gelatin in PBS for 1 h. After washing thrice, sections were incubated for 1 h in normal goat serum (1:40 dilution, Vectastain). After incubation, excess goat serum was blotted and then sections were incubated with Rabbit monoclonal antibody against NPY (1:7000, Sigma) 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 (1:200, Vectastain).
Sections were washed and incubated with ABC reagent for 1 h at room temperature (Vectastain, ABC Kit, 1:100). After washing, sections were incubated with 3,3 diamino-benzidine 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, dehydrated, cleared in xylene, mounted

in distyrene plasticizer xylene (DPX, Merck, India) and photographed.

2.3. Morphometry

Digital images of NPY immunoreactivity were obtained on a Zeiss imager A2 microscope equipped with a ProgRes C3 digital camera run on ProgRes C3 software. For analysis, care was taken to match sections through the same region of the brain and at the same level using anatomic landmarks with the aid of frog stereotaxic atlas (Neary and Northcutt 1983; Wada et al., 1980). For cell counting, all visible cell bodies stained within the defined brain region were counted manually keeping the same counting area for all the treated groups. Data from each brain region in an animal was calculated by taking the average counts from five brain slices. Data from each slice was calculated by taking the average counts from the left and right sides of brain region of interest. Alternate sections exhibited robust staining in the same neuroanatomical regions. The size of the areas analyzed was kept the same for all experimental groups.

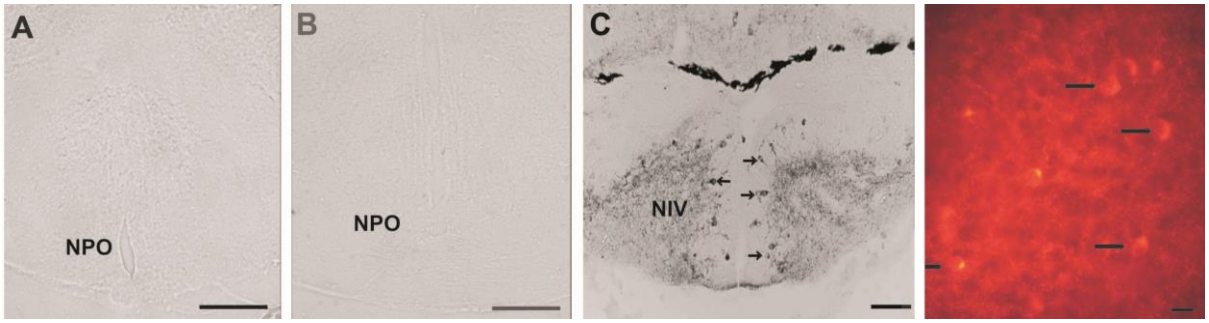
2.4. Statistical analysis

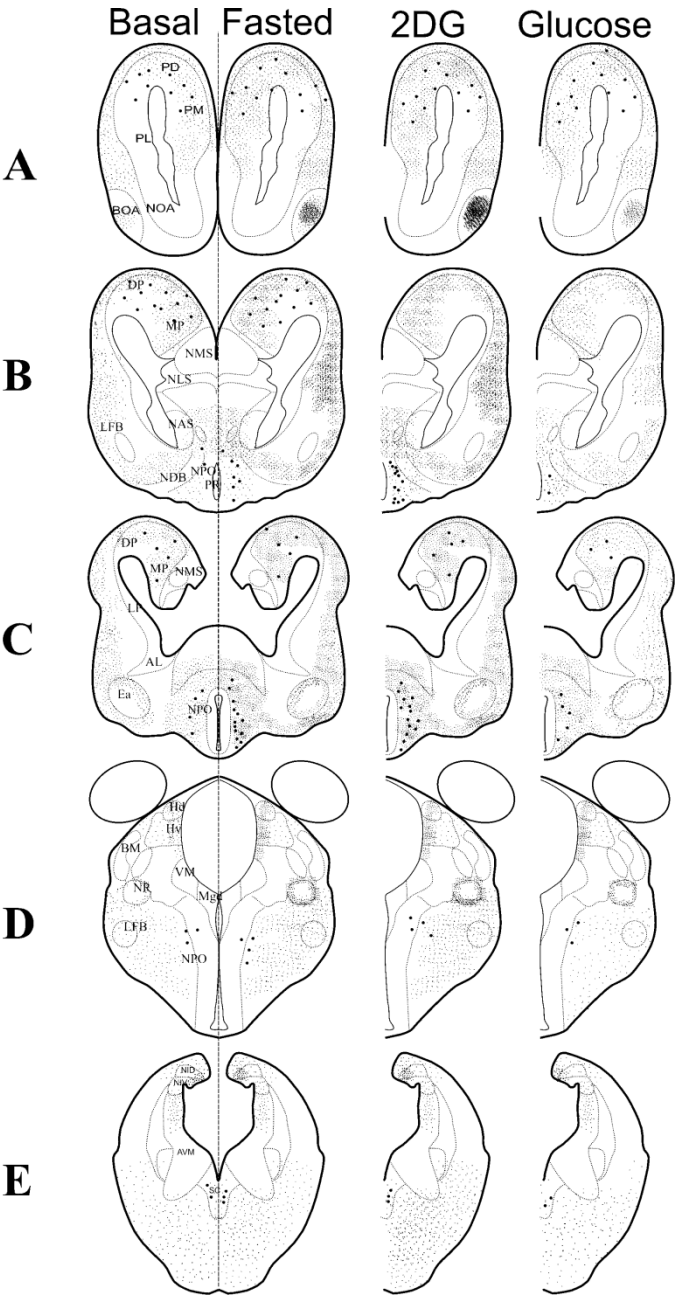
For each treatment in different areas of the brain, cell counts were plotted as box plots. The hypothesis that there was no significant difference in the cell counts of all the treated groups and was tested using Kruskal-Wallis test. A post hoc analysis using Mann-Whitney U test with Bonferroni correction was performed to check the difference between a pair treatment at a time. All statistical analysis was done in freeware PAST version 2.14 (Hammer et al., 2001). Total count of the number of S. Shewale et al. *Neuropeptides 71 (2018) 1–10*2 immunoreactive cells is compared with the respective treatment group and is expressed in terms of percentage in the result section.

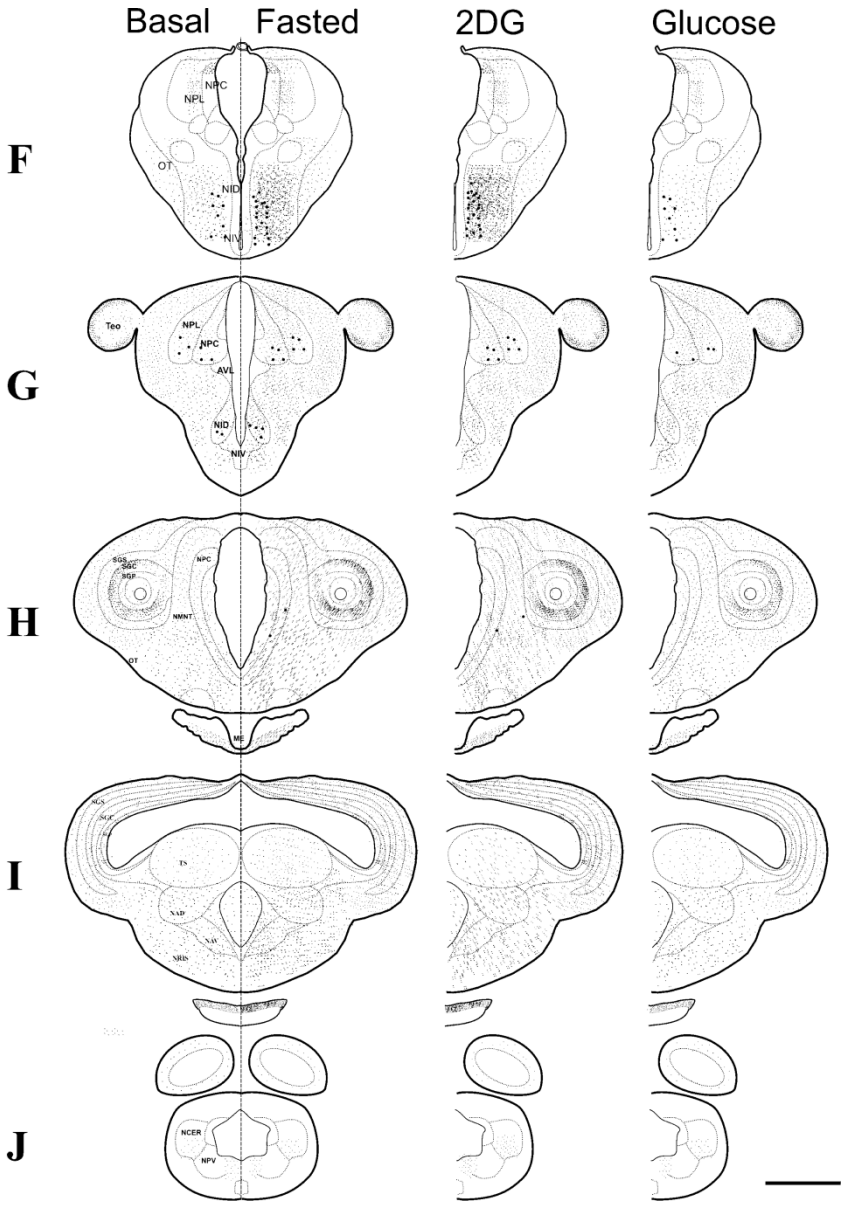
3. Results

Serial sections of the tadpole brain were observed for differential immunostaining of Neuropeptide Y. Antisera against NPY was checked for its specificity before any immunoreactions. The NPY immunoreactivity seems to be specific as omission of primary or secondary antibody from reaction mixture resulted in absolute loss of immunoreactivity (Fig. 1A). Furthermore, pre-adsorption of primary antibody with pure NPY peptide completely abolished all immunostaining (Fig. 1B). Also positive control section shows NPY cells (arrows) of nucleus infundibularis ventralis (NIV) region in bright field (Fig.1C) and immunofluorescent (Fig. 1D) preparations. Specificity of this antibody in the frog has been shown earlier in our laboratory (Ali et al., 2016; Heigrujam et al., 2017). NPY is one of the most conserved peptide in the vertebrates with a striking similarity from cartilaginous fishes

to mammals (90%). NPY in Torpedo and mice differ by only three amino acids (Blomqvist et al.,1992).






Basal
Fasted
2DG
Glucose
F
G
H
I
J
NPC
NPL
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AVL
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SGC
SGP
NMNT
ME
TS
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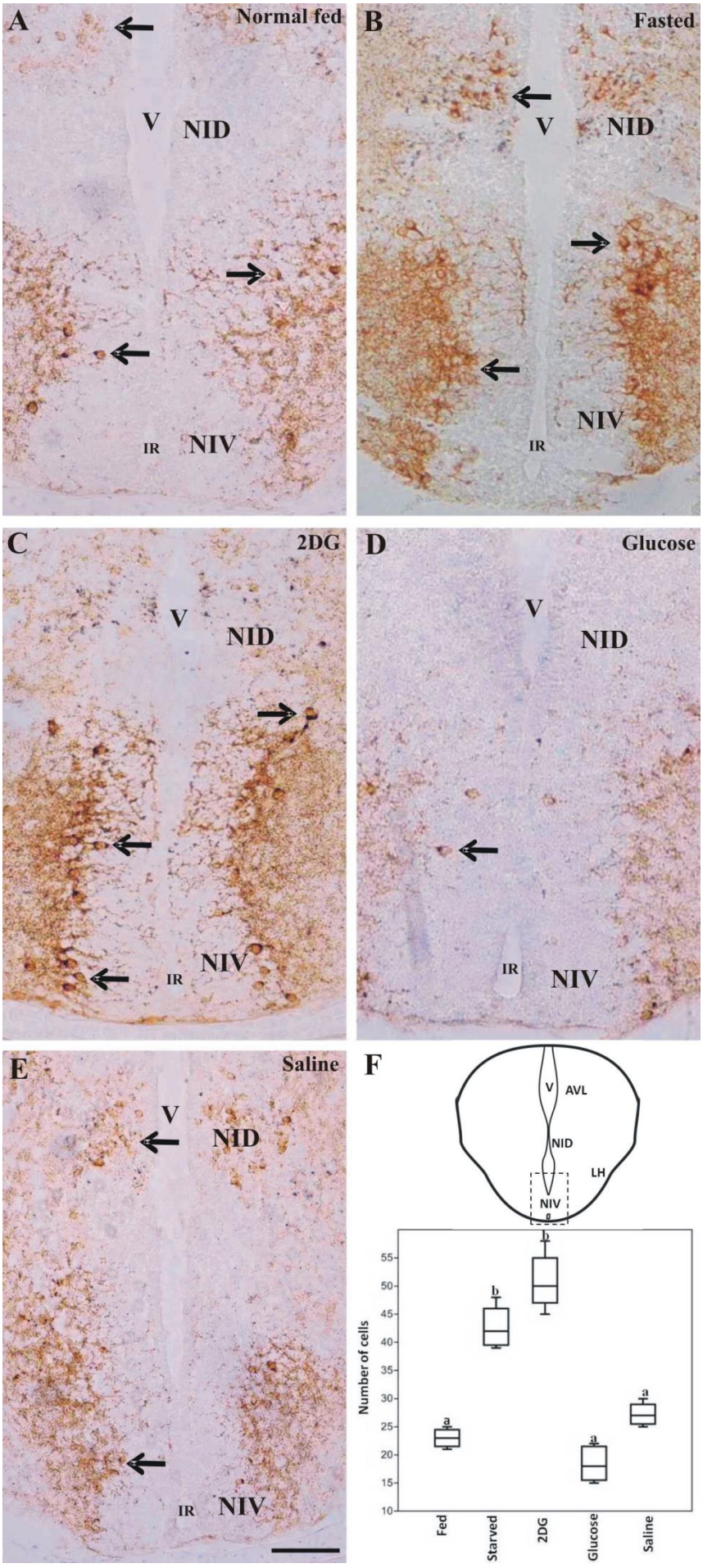
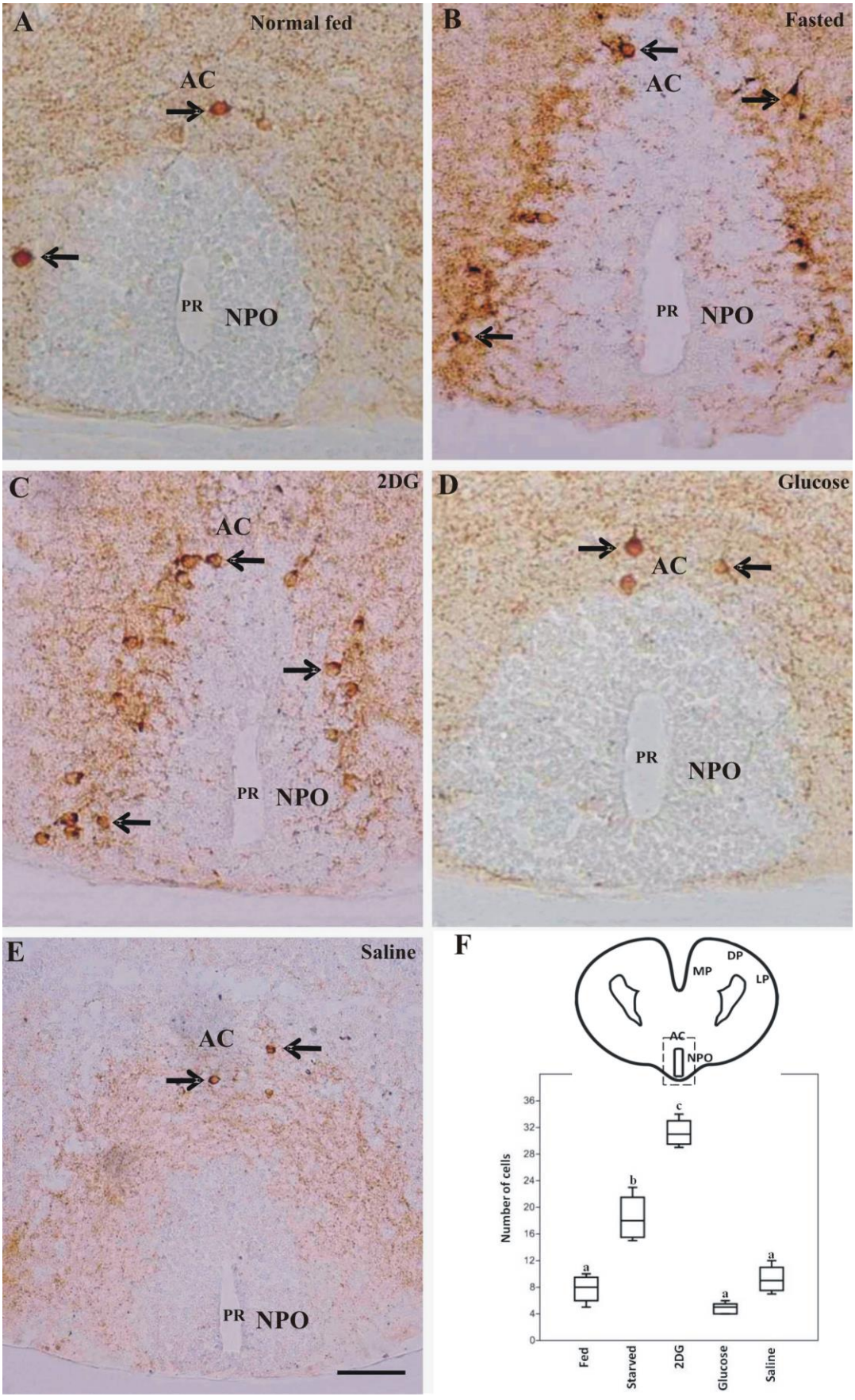
NAI
NRD
NCER
NPV


A
Normal fed
V
NID
IR
NIV
B
Fasted
V
NID
NIV
IR
C
2DG
V
NID
IR
NIV
D
Glucose
V
NID
IR
NIV
E
Saline
V
NID
IR
NIV
F
V
AVL
NID
LH
NIV
Number of cells
55
50
45
40
35
30
25
20
15
10
a
b
b
a
a
Fed
Starved
2DG
Glucose
Saline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PR
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AC
PR
NPO
E
Saline
AC
PR
NPO
F
DP
MP
LP
AC
NPO
Number of cells
36
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8
4
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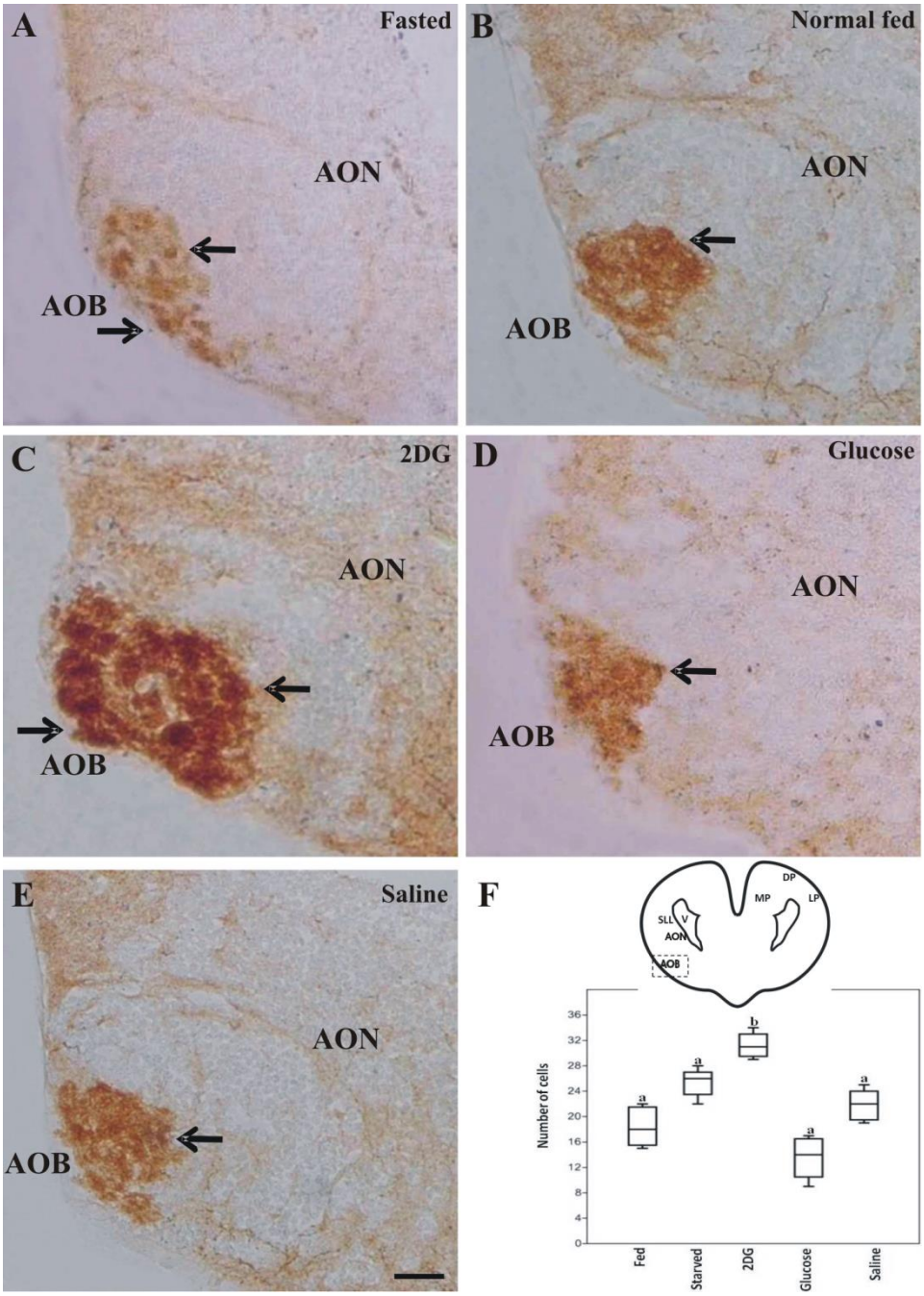
b
a
a
a
Fed
Starved
2DG
Glucose
Saline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AON
AOB
B
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AON
AOB
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2DG
AON
AOB
D
Glucose
AON
AOB
E
Saline
AON
AOB
F
DP
MP
LP
SLL
V
AON
AOB
Number of cells
36
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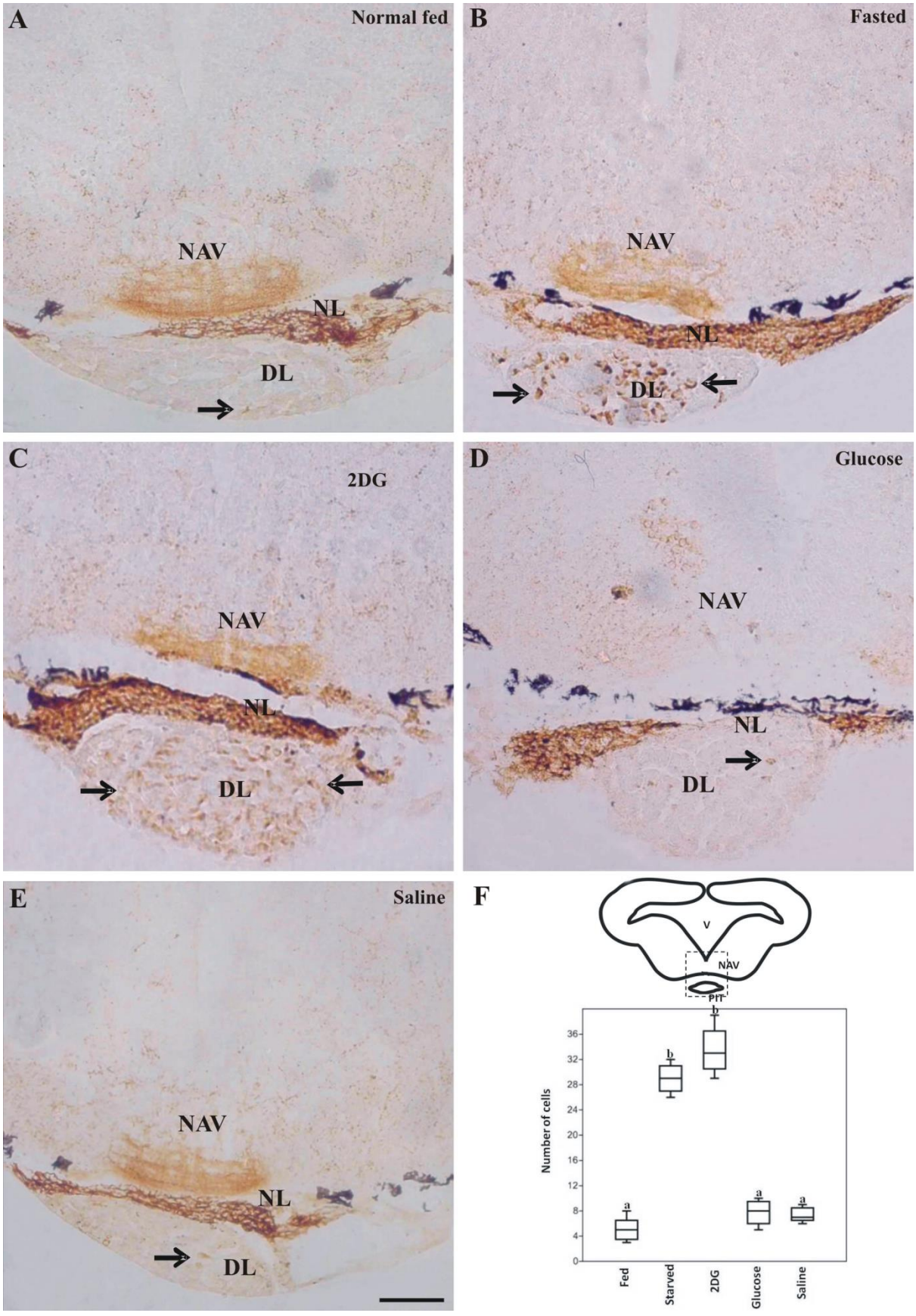
Glucose
Saline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NL
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NAV
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DL
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2DG
NAV
NL
DL
D
Glucose
NAV
NL
DL
E
Saline
NAV
NL
DL
F
V
NAV
Prr
Number of cells
0
4
8
12
16
20
24
28
32
36
a
b
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a
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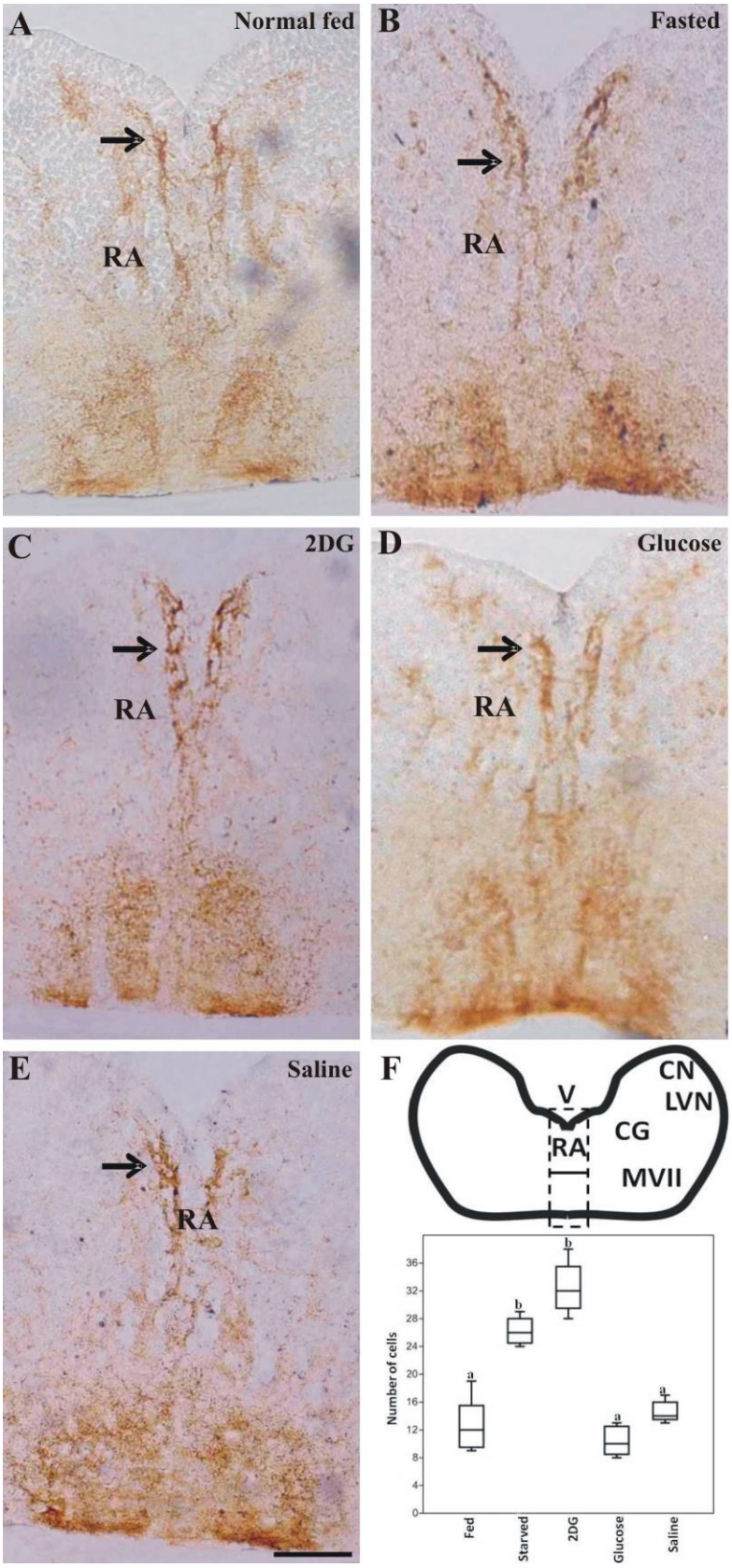
Fed
Starved
2DG
Glucose
Saline


A
Normal fed
RA
B
Fasted
RA
C
2DG
RA
D
Glucose
RA
E
Saline
RA
F
V
CN
LVN
CG
RA
MVII
Number of cells
0
4
8
12
16
20
24
28
32
36
a
b
b
a
a
Fed
Starved
2DG
Glucose
Saline

Neuroanatomical distribution and relative expression of NPY peptide in the tadpole brain of the frog Euphlyctis cyanophlyctis was studied in the response to the food deprivation and intracranial administration of 2-deoxyglucose (2DG) and glucose. NPY immunoreactive cell and fibers were seen throughout the brain and the pituitary gland. Brain areas were characterized by using stereotaxic atlas for frog brain provided by Wada et al., (1980) and "nuclear organization of bullfrog diencephalon" by Neary and Northcutt, (1983). The distribution of NPY peptide in the brain and pituitary gland of tadpoles in response to different treatments are described below. The basal distribution of NPY immunoreactivity remained the same as reported earlier in Euphlyctis cyanophlyctis (Ali et al., 2016), Rana esculenta (D'Aniello et al., 1996), Rana catesbeiana (Cailliez et al., 1987), Xenopus laevis (Tuinhof et al., 1994). NPY-ir cells was found in the pallium, septum and accessory olfactory bulb of telencephalon, Nucleus preopticus, superchiasmatic nucleus, epithalamus, thalamus and hypothalamus of diencephalon, and tegumentum region, raphe nucleus of mesencephalon (Fig. 2). Given below is the detailed description of the brain areas which displayed a significant response to feeding stimulation.

3.1. Telencephalon

In the telencephalon, although, the reaction was present in the pallium and septum regions, NPY immunoreactive cells and fibers differed markedly in the accessory olfactory bulb (AOB) and anterior olfactory nucleus (AON) region in different groups (Fig. 3A-F). There seems to be an increased number of NPY immunoreactive cells and fibers in the fasted animals (18%) than the normal fed groups. In addition, a differential increase in the number of immunoreactive cells of animals injected with 16 ppm of 2DG (17%) as compared with the saline treated and glucose treated groups ($P < .05$). Immunoreactive cells with fibers were scattered and denser in the AON region of the fasted group. The immunoreactive cells were small having round to oval nuclei. Profound immunoreactivity was seen in the 2DG injected animals (Fig. 3C) as compared with the glucose injected group.

3.2. Diencephalon

In the Diencephalon, there seems to be a robust increase in the NPY immunoreactive cells in the nucleus preopticus (NPO) of fasted groups (44%) as compared with normal fed control group (Fig. 4A-B). The perikaryon appears to be oval in shape with small process. The axonal processes of these neurons were projecting away from the ventricle. Isolated cells with beaded fiber system were present in the NPO of glucose and saline injected group. The group injected with 2DG exhibited a robust increase in the number of intensely stained immunoreactive cells (68%) than the glucose treated group (Fig. 4F; $P < .05$). The

immunoreactive cells were present on the lateral sides of the pre-optic recess. The cells were oval in shape with their axons projecting away from the center. Strong and dense immunoreactive fibers were also observed in all the groups. In the hypothalamus, the extensive distribution of NPY-ir neurons was seen in the Infundibulum. In nucleus infundibularis dorsalis (NID) intense immunoreactivity was seen in the fasted group as compared to the normal fed group (Fig. 5A-B). The neuronal axons were projecting away from the ventricle. A similar pattern of immunoreactivity with highly pronounced changes was observed in the nucleus infundibularis ventralis (NIV). The cells were round to oval in shape with their axons projecting towards the infundibular recess. Also there seems to be a sort of communication and intermingling between the axonal fibers of the neurons (Fig. 5C). In the 2DG injected group profound immunoreactivity and increased number of communicating cells (34%) were seen in comparable with the glucose injected group (Fig. 5C-D; $P < .05$). NPY immunoreactive fiber system was seen in all the treated groups with comparable intensity in 2DG treated group (Fig. 5C).

3.3. Rhombencephalon

In the raphe nucleus (RA), differential immunoreactivity is seen in all the treatments. A robust increase in the immunoreactive cells is observed in the fasted group (36%) when compared with the normal fed group (Fig. 6A-F). The cells were round to oval in shape with axonal endings away from the ventricle. Pronounced number of NPY immunoreactive cells was observed in the 2DG injected group (43%) as compared with the glucose and saline controls (Fig. 6F; $P < .05$). Also a remarkable increase in the beaded fiber content was observed in the RA of 2DG injected and the fasted animals.

3.4. Pituitary gland

Remarkable changes were observed in the pituitary gland of all the groups. The Distal lobe (DL) of the pituitary gland of fasted animals showed increased number of NPY immunoreactive cells (60%) as compared to a smaller amount of immunoreactive cells in the control group (Fig. 7 A-F). The cells were round to oval in shape with intense staining. Drastic increase in immunoreactive cells was clearly seen in the 2DG injected group (55%) and the food deprived group as compared to very few cells in the glucose treated group. NPY-ir fiber system was also observed in the nucleus anteroventralis tegmenti mesencephali (NAV) region of all the groups.

4.Conclusion

Neuropeptide Y possesses a widespread distribution in the amphibian brain. The peptide is produced in the dorsal, medial and lateral pallium; medial septum; medioventral

telencephalon; anterior pre-optic area of telencephalon, ventromedial, central and posterior thalamic nuclei, suprachiasmatic nuclei, infundibulum of diencephalon, anteroventral mesencephalic tegumentum of mesencephalon and central gray; cerebellar area; vestibular nuclei of rhombencephalon of larval forms (D'Aniello et al., 1996; Ali et al., 2016). However the distribution becomes much restricted in adults where the peptide could only be localised in the medial pallium, basal forebrain, preoptic area, ventral and dorsal nuclei of the infundibulum, tegmentum and trigeminal nerves (Ebersolea et al., 2001; Danger et al., 1985). The distribution of NPY in our studies corresponds to the distribution pattern of NPY in the developing larvae of Rana esculenta and Euphlyctis cyanophlyctis (Heigrujam et al., 2017, Ali et al., 2016, D'Aniello et al., 1996). In our model, NPY producing cells were seen in pallium, septum medialis, pars lateralis and pars medialis regions of amygdala and anterior pre-optic area in telencephalon; epithalamus, thalamus and hypothalamic nuclei of diencephalon, tegmentum area of mesencephalon and the raphe nucleus of rhombencephalon. By studying the expression of NPY in different nutritional conditions, herein we have tried to investigate the role of NPY-system in energy homeostasis in the brain of E. cyanophlyctus tadpoles. NPY modulated feeding behaviour is already known in mammals (Stanely, 1986; Schwartz et al., 2000, Zarjevski, 1993, Crespi, 2014, Kalra and Kalra., 2004) as well as non-mammalian vertebrates (Aldegunde and Mancebo 2006; Matsuda et al., 2012; Yokobori et al., 2012; Wu et al., 2012; Volkoff et al., 1999). In mammals ICV injection of NPY into cerebral ventricles stimulates food consumption and decreases energy expenditure (Stanley, B. G. 1986; Schwartz MW et al., 1999). Also, continuous or repeated central administration of NPY leads readily to obesity (Stanley, B. G et al., 1986; Zarjevski, N. et al., 1993) and NPY secretion reduces leptin/insulin signalling to the brain acting as an anabolic signalling molecule (Wilding, J. P. H. et al., 1993). NPY has been shown to stimulate appetite and food consumption in various fish species including goldfish, zebra fish, orange spotted grouper, red tilapia, rainbow trout and salmon. Intra cerebro-ventricular injections of NPY stimulate food intake in goldfish (Carassius auratus) and Channel fish (Ictalaurus punctatus) and NPY-antagonists have been seen to block this effect with no effect on the basal food intake (Silverstein JT et al., 2000; Narnaware YK, 2001). Much recently, Li et al., reported NPY to increase appetite and growth hormone expression in Olive flounder (Li et al., 2016).

Brain regions responding to the altered glucose levels included accessory olfactory bulb (AOB), anterior olfactory nucleus (AON) of forebrain, Nucleus pre-opticus (NPO) and hypothalamus (NIV, NID) of midbrain and raphe nucleus (RA) of hindbrain. Further, the distal lobe of pituitary (DL) and Nucleus anteroventralis tegmenti mesencephalic (NAV) also

responded to a similar increase and decrease of NPY against positive and negative nutritional states respectively. Increased levels of NPY-ir in the fasted and 2-deoxy-glucose (2DG) injected tadpoles against the normally fed and glucose injected tadpoles suggests its involvement in feeding regulation as reported earlier for mammals and sub-mammalian vertebrates (Shinji et al., 1999; Silverstein et al., 2000; Narnaware 2001). In our study, bulbus olfactorious accessorious (AOB) and nucleus olfactorious anterior (AON) in the forebrain presented a much conspicuous increase in NPY immunoreactive cells and fibers in glucose deprived state against the normally fed tadpoles. There was a two-fold increase in the fasted and 2DG injected animals. To our knowledge, this is the first report on the presence of NPY-ir material in these regions which becomes significant during food deprivation. AOB and AON are known to receive sensory neurons from the olfactory nerve to processthe brain (Mori et al., 1999; Morton et al., 2006). Fasting enhances olfactory sensing by the expression of appetite-stimulating peptides which increase the ability to locate food from an infinite array of environmental constituents (Tong et al., 2011). Increase in the NPY immunoreactive fibers during glucose deficient state may suggest its role as an appetizer and a possibility that NPY might be enhancing the sensory input of olfactory system to ensure efficient foraging.

Calle et al., 2006, while studying feeding in Xenopus laevis adults, reported an increase of NPY-ir in supra chiasmatic nucleus during starvation. However, they did not find any significant change of NPY expression in any other brain regions like olfactory nucleus, nucleus pre-opticus, infundibular nuclei which is surprising because these regions contain huge clusters of NPY immunoreactive cells in amphibians (Ali et al., 2016) and are also reported to be involved in the NPY mediated orexigenic responses in fishes and mammals (Stanley, B. G. 1986; Schwartz MW et al., 1999; Zarjevski, N., 1993; Kalra 1991). Nucleus pre-opticus, in our studies, also seems to respond to different nutritional status of the brain. This nucleus is already known to process feeding information through NPY secretion in mammals (Kalra et al., 1999). Furthermore, there was a significant difference in the NPY immunoreactivity of the distal lobe of pituitary between animals of different nutritional conditions. Earlier, Hanson and Dallman, 1995 have suggested an integrative role of NPY to process the feeding related information through the hypothalamo-pituitary-adrenal (HPA) axis. Strongly stained immunoreactive fibers in the distal lobe of pituitary during glucose deficient conditions points towards a similar functioning of neuropeptide Y in our model. Changes in the NPY immunoreactivity in the hypothalamic regions in our study are consistent with the findings

the olfactory information and communicate it to the higher centers of

of Calle et al., and also draws strong support from the known involvement of NPY-ir cells in hypothalamic feeding-center model in mammals and fishes (Shinji et al., 1999; Silverstein et al., 2000; Narnaware 2001; Schwartz et al., 1999; Zarjevski, 1993; Kalra 1999, Wang et al., 2015). Although the effect of NPY on food intake and feeding behaviour is well known (Muroyya et al., 1999, Silverstein et al., 2000; Narnaware 2001; Calle et al., in 2006; Suyama et al., 2017) but how the glucose levels in brain effect the expression of NPY is not understood. Burdakov et al., (2005) reported an inhibition in appetite promoting NPY neurons in the hypothalamic arcuate nucleus of mice brain. Arcuate nucleus in mammals is homologous to the infundibular regions of the frog brain. Glucose administration, in our study, decreased the expression of NPY in the nucleus infundibularis (NIV, NID). ICV administration of 2- deoxy-glucose exhibited an upregulation of NPY in the parallel regions of tadpole brain. Increase in the expression levels of NPY during glucose deprivation hints towards a possible regulation of NPY expression through glucose sensing neurons in the hypothalamus.

The expression of NPY in the olfactory bulb, nucleus preopticus, nucleus infundibularis ventralis, nucleus infundibularis dorsalis, raphe nuclei and pituitary was up regulated following energy depletion and reduced during energy enriched states. We conclude that the NPY system in these areas may be sensitive to glucose and play a role in regulating the feeding behaviour and energy homeostasis. The current study on amphibians suggests that this function of the NPY system may be a feature highly conserved across the entire vertebrate lineage.

3.To study the seasonal variation of NPY in the brain of *Microhyla ornata*

Seasonal transition modulates resource availability and environmental conditions that drives adaptation to distinct life stages of the animals. Amphibians are evolutionary significant as they are the connecting link between aquatic and terrestrial's life forms. They exhibit two distinct life stages breeding and non-breeding. Based on the breeding pattern they are classified as tropical and temperate. Tropical species have breeding pattern independent of season whereas temperate species exhibit season specific breeding cycle (Prado et al., 2005). The seasonal breeders are reported to sport drastic changes in physiology and appetitive behaviour mediated by neuroendocrine axis. The seasonal variations are sensed by the brain via neurotransmitters and neuropeptides that in turn, regulate the hypothalamus–pituitary–gonadal (HPG) axis (Joshi et al., 2018). Several neuropeptides involved in this process are gonadotropin releasing hormone (GnRH), gonadotropin inhibiting hormone (GnIH), Kisspeptin and Neuropeptide Y(NPY).

NPY is one of the most important orexigen which act as the mediator between energy state and reproductive behavior (Inaba et al., 2016; Muroi and Ishii, 2015). It is known to be involved in reproductive behaviour of mammals specifically intraventricular (IVT) administration of NPY peptide and fasting suppresses sexual behaviour in male rats (Clark et al., 1985; Inaba et al., 2016). In addition, NPY mRNA expression in the arcuate nucleus decreases with castration and is compensated by testosterone treatment (Urban et al., 1993). Like mammals, NPY has been linked to the reproductive behaviour in reptiles as well as in fishes. Intracerebroventricular (ICV) administration of NPY significantly reduced courtship behaviour in red sided garter snake (Morris and Crews, 1990). Castration of cichlid fish reduces NPY-immunoreactive cells in the nucleus entopeduncularis (Sakharkar et al., 2005). However, administration of testosterone increases NPY immunoreactivity in the nucleus entopeduncularis (Mazumdar et al., 2007). These studies suggest that region specific changes in the NPY levels in the brain regulate homeostasis between feeding and reproductive behaviour. Although role of NPY in co-ordinating energy homeostasis and reproduction is very well established in mammals. Some studies in fish and reptiles also suggest the same. Studies in amphibians that will confirm the evolutionary conserved role of NPY, needs to be investigated.

Temperate anurans exhibit pronounced seasonal cycle of reproductive activity. Interestingly, breeding cycle often coincide with appetite cycle, suggesting a link between energy homeostasis and the reproduction (Chavadej et al., 2000; Kumbar et al., 2001). *M. ornata* is a seasonal breeder with distinct reproductive phases :1) Pre-breeding phase 2) Breeding phase 3) Post-breeding phase (Kanamadi and Hiremath, 1993).This make *M. ornata* as an excellent model species for investigation of neuroendocrine factors involved in the regulation of seasonal changes in reproduction and appetite. Recently, we have reported sex specific differences in the expression of NPY in the brain of *M. ornata* in the breeding season (Halawale et al., 2019).Hence we may speculate that NPY may be involved in the energy dependent reproductive behaviour of aurans. In present study, we have investigated the seasonal variation of NPY using immunohistochemistry as well as quantitative real time-PCR in the brain of the frog, *M. ornata.*

2.Materials and Methods:

2.1Animal collection and tissue processing:

Five adult male frogs *M. ornata*, each from different reproductive phases were collected from the local perennial ponds over a period of 24 months from February 2016, to February 2018, which encompassed two consecutive reproductive cycles. The data pertaining

to the monthly rainfall, mean monthly temperature, and day length during each phase of reproductive cycle were collected from India Meteorological Department, Pune. All animal experiments were performed following the institutional animal ethics guidelines established by the Savitribai Phule Pune University. Frogs were anesthetized using chloroform and the brains were dissected out. For molecular studies, brain tissues were stored at -80°C. For immunohistochemistry, the brains were fixed in Bouin's fixative for 24 h. The tissues were 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 cut on a cryostat at 20 µm thickness. Sections were mounted on poly-L-lysine coated slides, and stored in -20°C for immunohistochemistry.

2.2Quantitative 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 in Hadawale et al., 2019.Using RNA isolation kit (Qiagen, USA),the total RNA was isolated from all the brain tissues. DNA-free™ DNA Removal Kit (Life Technologies, USA) was used to remove DNA contaminants. The RNA quantification done using Bio spec Nano spectrophotometer (Shimdzu, Kyoto, Japan). The reverse transcription of total RNA (100 ng) was done in duplicate using random hexamers and MultiScribe™ MuLV (Applied Biosystems, USA) with a final volume of 20 µl. The reaction of reverse transcriptase was 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. The qRT-PCR was run using duplicates of the cDNA on a StepOne^{TM} RT-PCR System (Applied Biosystems, USA) with 25 pmol of each primer and SYBR green qPCR master mix (ThermoFisher Scientific, USA), which includes purified AmpliTaq Gold® DNA Polymerase, a proprietary version of ROX™ dye, an internal passive reference and a blend of dTTP/dUTP. The mRNA sequences of NPY and HPRT used are published in our previous report Hadawale et al., 2019. The reference gene HPRT was measured in parallel as an internal control. The thermal cycle of the qRT-PCR had three stages: 95°C for 3 min (1 cycle); 95°C, 57°C, and 72°C for 30 seconds each (40 cycles); 95°C for 15 seconds. 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 seconds/step. The fold change for NPY mRNA was determined after normalization to HPRT using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.4Immunohistochemistry:

The immunohistochemistry protocol was performed as described previously (Hadawale et al., 2019). Briefly, the sections were washed with PBS three times and treated with 0.3% hydrogen peroxide in methanol for 1 h. Sections were then washed in PBS and incubated with blocking agent containing 0.5% BSA (bovine serum albumin) and 0.5% gelatin in PBS for 1 h. After washing with PBS, sections were incubated with normal goat serum for 1 h (1:40 dilution, Vectastain). After incubation, excess goat serum was blotted out and sections were incubated with rabbit monoclonal antibodies against NPY (N9528; Sigma) at 1:3500 dilutions containing 0.5% BSA and gelatin for overnight at 4 °C. Sections were then washed in PBS thrice and incubated with biotinylated goat anti- rabbit IgG antibody at room temperature (1:200, Vectastain). Sections were washed and incubated with ABC reagent for 1 h at room temperature (Vectastain, ABC Kit, 1:100). After washing, 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, dehydrated, cleared in xylene, mounted in distyrene plasticizer xylene (Merck, India).

2.5 Morphometry:

Digital images of NPY immunoreactivity were taken on a Carl Zeiss Imager M2 microscope, 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., 2014) and were used for a comparative analysis of NPY-immunoreactive (-ir) in respective brain regions. For the calculation of percent immunoreactive area within the defined brain regions, Image J software was used keeping the same counting area for control.

2.6 Statistical Analysis: The differences between the two groups were tested for significance using Two-way analysis of variance (ANOVA) , and the *p*-values less than 0.05 ($P < 0.05$) were considered to be significant.

3.Result:

3.1Seasonal changes of environmental factors:

The changes in the environmental factors (temperature, day length and rainfall) are summarized in Figure1. While the environmental temperatures as well as day length are at their minimum during post-breeding phase, the gonads are small and the Gonosomatic index (GSI) is low. When the temperature and day length increased to the highest point during their pre-breeding phase, the gonads showed a steep increase in the GSI. This suggests that the day length and rise in temperature seem to provide the cue for increase in the weight of gonads, which results into the peak of GSI during breeding phase. During the breeding season (July-

August) flooding of water bodies take place which triggers spawning activity of frogs. Thereafter, as the water bodies get dried, the frog pass into the hibernation i.e. post-breeding phase, during which the gonads regress and GSI falls rapidly.

3.2Seasonal variation of NPY:

a)Immunohistochemistry:

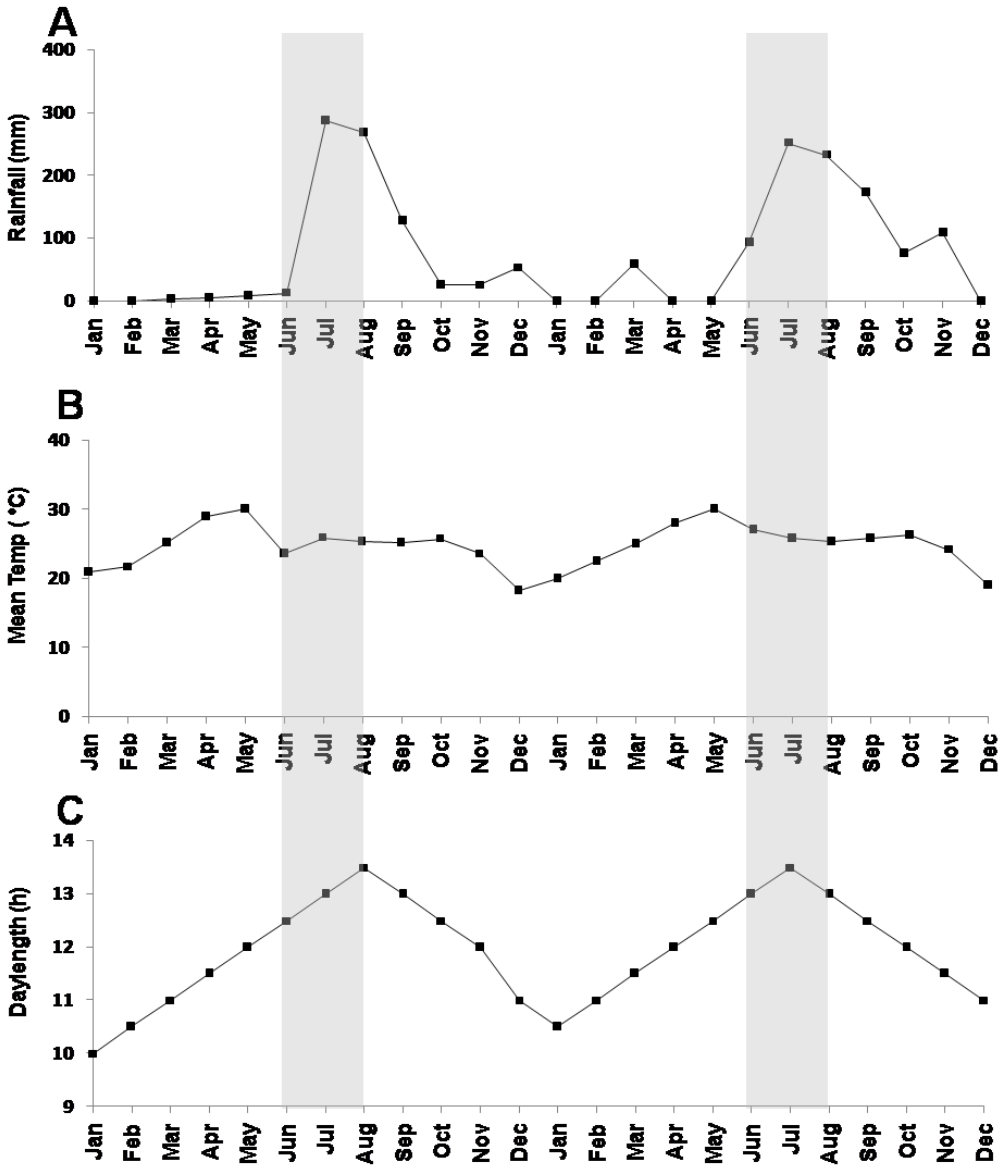
The present study confirms our previous observation on the occurrence of NPY immunoreactivity in the neurons of preoptic nucleus (Poa) and nucleus infundibularis ventralis (NIV) in the hypothalamus of *M. ornata.* The NPY- immunoreactive profile of the neurons of the Poa showed conspicuous changes during different phases of the reproductive cycle (Figure 4.). A pronounced increase in the NPY percentage immunoreactive area of the neurons was noticed ($p<0.5$) during breeding season. The immunoreactivity decreased slightly in post-breeding season ($p< 0.5$). Dramatic reduction ($p<0.5$) in NPY immunoreactivity was observed during the pre-breeding season (Figure 5.).

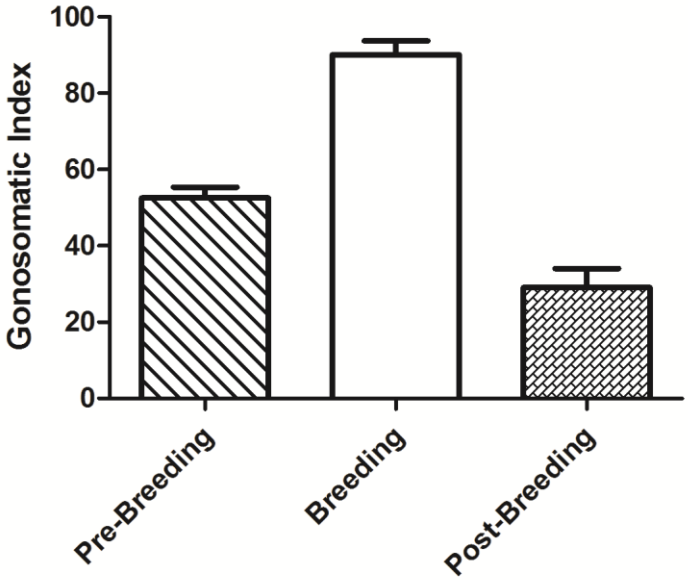
The consistent pattern of change was also detected in the neurons of NIV like Poa (Figure5). A few weak to moderate NPY-immunoreactive cells and granules were detected in the NIV region during pre-breeding season (Figure4). The number of NPY containing cells, were at its peak during the breeding season and it decreased in post-breeding season.(Figure 4.) The reduction in NPY-immunoreactivity was pronounced ($P<0.5$) in pre-breeding season.

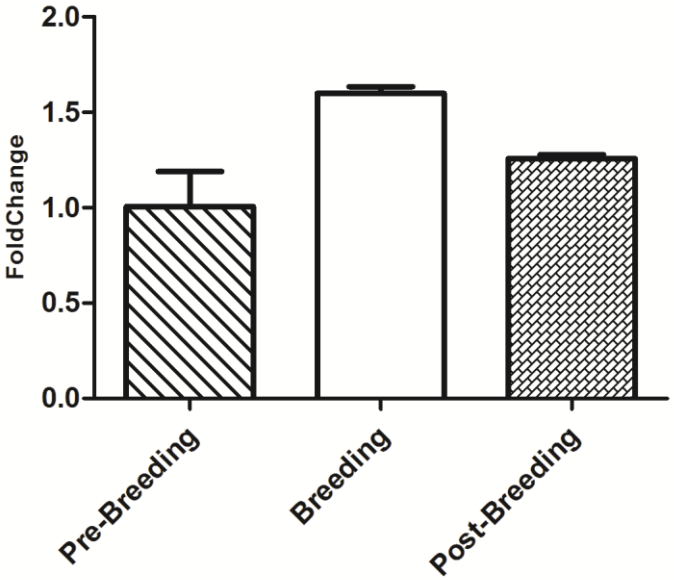
Seasonal changes in the NPY-immunoreactivity were also observed in the NRIS region of mesencephalon. The pattern for the seasonal differences in the peptide in the NRIS was similar to that observed in above two mentioned regions. The intensity of NPY-immunoreactivity was moderate as compared to that observed in POA and NIV region of hypothalamus. NPY-ir cells, fibres and granules in NRIS were at it maximum during breeding season($P<0.5$), which decreased gradually during post-breeding season and decreased dramatically in pre-breeding season ($P<0.5$). The other components of the brain such as telencephalon did not show a significant seasonal changes in the expression of NPY.

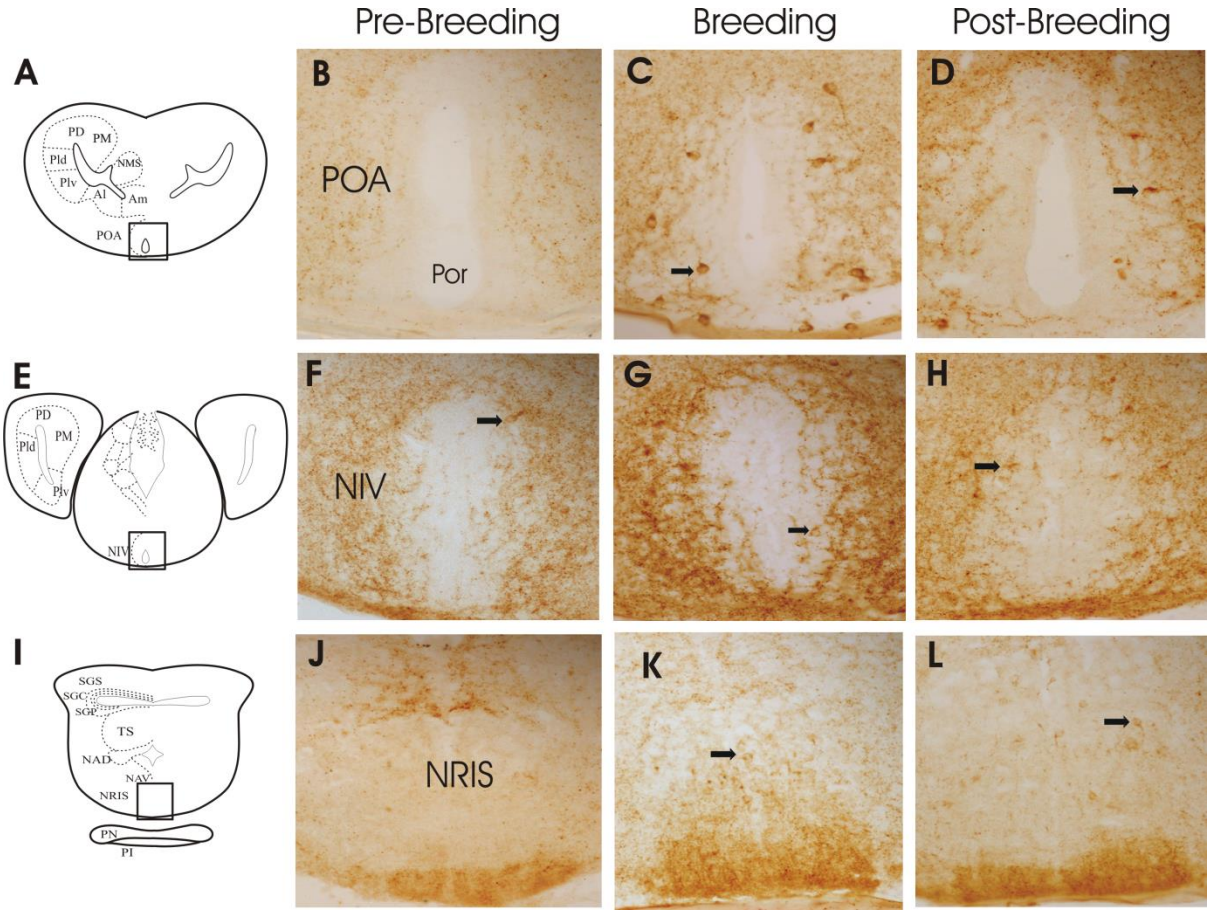
mRNA Quantification:

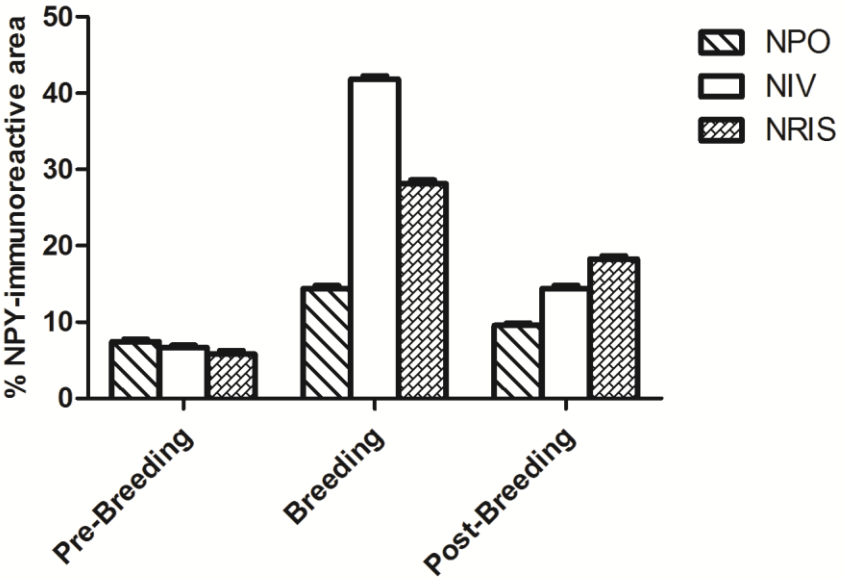
The middle part of the brain mainly contains hypothalamus where higher NPY mRNA levels were detected as compared to rostral and caudal part of the brain. Therefore middle part of the brain was used for the mRNA quantification. The results of mRNA quantification are in line with the immunohistochemistry results. The least expression of NPY mRNA levels were observed in the pre-breeding season followed by a dramatic increase in the breeding season which again falls in the post-breeding season(Figure 3).


A
Rainfall (mm)
400
300
200
100
0
B
Mean Temp (°C)
40
30
20
10
0
C
Daylength (h)
14
13
12
11
10
9
Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec

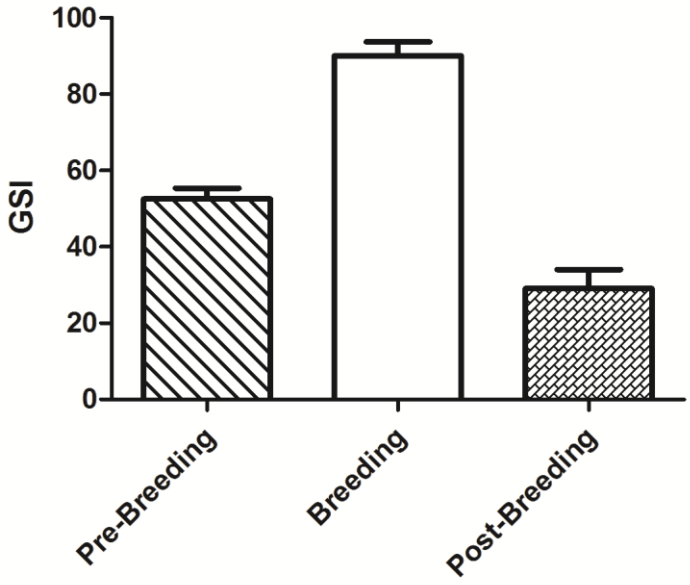

Gonosomatic Index
100
80
60
40
20
0
Pre-Breeding
Breeding
Post-Breeding


FoldChange
2.0
1.5
1.0
0.5
0.0
Pre-Breeding
Breeding
Post-Breeding


Pre-Breeding
Breeding
Post-Breeding
A
B
C
D
POA
Por
PD
PM
PId
PIv
NMS
AI
Am
POA
E
F
G
H
NIV
PD
PM
PId
PIv
NIV
I
J
K
L
NRIS
SGS
SGC
SGP
TS
NAD
NAV
NRIS
PN
PI


% NPY-immunoreactive area
50
40
30
20
10
0
NPO
NIV
NRIS
Pre-Breeding
Breeding
Post-Breeding


LC219936 Microhyla ornata
AB761985 Rana catesbeiana
XM_018568034 Nanorana parkeri
XM_018568033 Nanorana parkeri
XM_012964911 Xenopus tropicalis
BC121985 Xenopus tropicalis
BC135387 Xenopus tropicalis
KT202317 Xenopus tropicalis
XM_018242144 Xenopus laevis
L11296 Xenopus laevis
BC080407 Xenopus laevis
AY660754 Ambystoma mexicanum
AY626561 Epinephelus coioides
M87297 Carassius auratus
KC297502 Schizothorax prenanti
AF287347 Cyprinus carpio
NM_205473 Gallus gallus
M20373 Rattus norvegicus
NM_023456 Mus musculus
NM_001009452 Ovis aries
NM_000905 Homo sapiens
L22867 Lampetra fluviatilis
67/0.60
70/0.95
50/0.50
45/0.58
81/0.73
66/0.77
96/0.95
62/0.74
56/0.83
95/1.00
93/0.95
90/0.99
57/_
53/_
0.2


GSI
100
80
60
40
20
0
Pre-Breeding
Breeding
Post-Breeding

4. Conclusion:

The present results on the distribution of NPY in the brain of *M. ornata* confirm our earlier observations on the same frog. The present study reveals the seasonal variation of NPY peptide as well as mRNA levels in the brain of new Phylogenetic taxa, frog, *M. ornata*. The results show a marked correlation between NPY immunoreactivity and seasonal behaviour. A comparatively higher number of NPY-ir cells as well as mRNA levels were observed in the brain of frog, *M. ornata* during the breeding season, as compared to that in non-breeding season i.e. the pre-breeding and the post-breeding season. The seasonal alterations of NPY immunoreactivity were observed in the POA, NIV and NRIS, the regions which are important for reproductive physiology of anurans. The phylogenetic analysis of nucleotide sequences that encode NPY demonstrate that *M. ornata* is more similar to ranids than that of pipids. Mammals, aves and fishes were grouped in different clusters.

The role of NPY in regulation of reproduction through GnRH-LH axis has been extensively studied in mammals (Kalra et al., 1997). Some information on the profile of NPY-immunoreactivity in relation to reproduction in seasonal mammals is available (Gruenewald et al., 1994; Dobbins et al., 2004). The seasonal variation in NPY immunoreactive fibers was investigated in the suprachiasmatic nucleus (SCN) of both male and female jerboas (Lakhdar-Ghazal et al., 1995). The seasonal changes of NPY were also observed in lateral septum and periventricular preoptic nucleus of lizard, *Podarcis hispanica* (Salom et al., 1994).

The monsoon is the breeding season for the frogs, when they are in actively feeding state. Given that the NPY is an orexigenic peptide in frog, *Xenopus laevis* and *Euphlyctis cyanophlyctis* as reported in other animals(Crespi et al., 2004; Shewale et al., 2018), this may also contribute to the highest NPY mRNA and peptide levels in the brain of *M. ornata* in breeding season. Higher level of NPY was observed in the arcuate nucleus of male deer as well as in ewes in breeding season, might serve as a primary stimulus to trigger the sexual activity in the breeding season (Barrell et al., 2016; Barker-Gibb and Clarke, 2000; Clarke et al., 2000). We have observed a decreased expression of NPY during post-breeding and pre-breeding season. This is the hibernation period, when food intake is reduced/basal metabolic activities slows down and loss of bodyweight occurs during short-day photoperiod, which is also the time when the animals are sexually inactive (Wade and Bartness 1985; Bartness *et al.* 1989). NPY levels are known to decrease during hibernation (Doherty et al., 2014; Christoph et al 1990; Schwartz et al., in 2013

It is interesting to note that while our study is in complete agreement with the NPY triggered sexual behaviour, it does not follow a significant amount of literature where NPY levels are actually found to be reduced during the breeding season and elevate during the period of sexual quiescence (autumn, post-BS). The amount of NPY immunoreactive fibers in the SCN of both male and female jerboas was higher than in the period of sexual activity (spring-middle of summer) (Lakhdar-Ghazal et al., 1995).In addition to its role as an orexigen, NPY also suppresses reproductive function in ewes (Barker-Gibb et al., 1995; Clarke et al., 2005). NPY inhibits sexual behavior in rodents (Ammar et al., 2000; Clark et al., 1985; Inaba et al., 2016) and reptiles (Morris and Crews, 1990). All these studies suggests that in present study, NPY may be involved in the regulation of feeding behaviour and sexual behaviour or both of anuran *M. ornata*.. Further study is warranted to confirm these deductions. Christoph et al (1990) has reported that the expression of NPY decreased in the hibernating ground squirrels as compared to euthermic animals. The observation is also supported by Schwartz et al., in 2013.These results align with orexigenic activity of NPY. These studies support our observations and also give clue regarding the role of NPY in appetite cycle/pattern of frog. In contrast to this, the NPY expression remained elevated throughout hibernation of little brown bats (Laemle and Cotter 1992), hibernating jerboas (Ouezzani et al. 2016) and the American black bears (Bradford 2010). No changes in the expression of NPY were observed in the brains of hibernating and active bears (Gardi et al. 2011). These observations suggests that, NPY may have different functions in different hibernating species including *M. ornata* and needs to be further investigated.

Apart from reproductive and feeding related consequences, other factors may also contribute to the elevation of NPY expression in the brain of *M. ornata* in breeding season. The first possibility is the seasonal neurogenesis, which has been observed in some birds (Nottebohm, 2004; Balthazart et al., 2010; Barnea and Pravosudov, 2011; Brenowitz and Larson, 2015; Guigueno et al., 2016) and rodents (Walton et al., 2012; Galea and McEwen., 1999; Burger et al., 2014) and also amphibians (Bernocchi et al. 1990; Cerri et al., 2009). OR

Additionally, seasonal change in NPY expression could be triggered by the surge of steroid hormones occurring during the different seasons, or increase in the NPY expression may be the cause not the consequences of elevated levels of steroid hormones, concomitant to monsoon sexual awakening. The action of NPY may also differ with season in animals such as the ewes which experience a seasonal anoestrus. A stimulatory action on GnRH release was seen after in vivo perfusion of NPY into the median eminence (ME) in intact ewes (Advis et al. 1990) but this was only observed during the follicular phase of the oestrous

cycle in the breeding season and not at other times of the cycle or during the non-breeding season. In male rats, NPY gene expression is modulated by testosterone (T) throughout the arcuate nucleus (Urban et al., 1993). Estrogens up-regulate responsiveness to NPY to stimulate preovulatory GnRH and gonadotropin surges by increasing Y_1 Receptor gene expression both in the hypothalamus and the pituitary of mouse(Eva et al., 2006). In goldfish, the stimulatory action of NPY on gonadotropin release from anterior pituitary tissue is greater in sexually mature fish than in seasonally, sexually-regressed fish (Peng et al. 1993) and the effect also differs with oestrogen treatment in this species (Barker-Gibb et al., 1995). Based on this information, similar type of regulation of NPY expression may be speculated here. Further investigation is required in amphibians.] It has been argued that seasonal modulation of the NPY expression may contribute to annual cycles of food intake in mammals (Boswell et al., 1993; Ormseth et al., 1996)**.** Clarke et al (2001), has reported that the expression of NPY is closely linked to appetite cycle and not to the seasonal breeding pattern of sheep. Expression of NPY was low in August (when voluntary food intake, VFI was low), but high in December (when VFI had increased). Since NPY expression was high in December (anoestrus) and in March (breeding season), and VFI was high during both times, this further suggests that the expression of NPY was linked to VFI and not to the seasonal breeding pattern. Similar to this, the decrease in the expression of NPY in the brain of *M. ornata*. may be the reason for no food intake in post breeding as well as pre breeding season.

In lower vertebrates, NPY mRNA expressions were significantly lower in both the hypothalamus and the telencephalon in winter torpor fish than in summer fed fish. In contrast, in another seasonal fish, the winter flounder, higher expression of hypothalamic NPY was observed in the winter compared to the summer (MacDonald and Vokoff, 2009).The differences in response in transcript expressions between the two species may be explained by different survival strategies (Babichuk and Volkoff, 2013).

Other environmental/ abiotic factors such as photoperiod, temperature or any other environmental change could be the cause/ consequences for the seasonal alterations of NPY expression in the brain of *M. ornata*. In Atlantic cod ,brain NPY mRNA expression was not affected by various range of temperatures (Kehoe and Vokoff., 2008).Although there is lack of information regarding effect of temperature on expression of NPY in the brain of anurans.

Gene expression of NPY within the arcuate nucleus (ARC) of mammals has proved consistently unaffected by photoperiod across a number of studies (Reddy et al., 1999; Adam et al., 2000; Mercer et al., 1995; 2000, 2001; Peacock et al., 2003; Archer et al., 2004; Adam and Mercer., 2004; Barrett and Bolborea, 2011). However there are several reports about the

effect of photoperiod on the expression of NPY in mammals (Rousseau et al., 2002; Clarke et al.,2003; Archer et al., 2004; Ross et al., 2009; Clarke et al., 2006; Dobbins et al.,2004). The information on the effect of photoperiod on NPY expression in the brain of amphibians is lacking. Therefore, further study is required to check if photoperiod has any role in the seasonal change in expression of NPY in the brain of *M. ornata.*

The results of the present study also support our previous study regarding the role of NPY in the sexual behaviour of the anuran, *M. ornata* (Hadawale et al., 2019). These findings suggest that NPY is involved in the regulation of seasonal appetite pattern and seasonal breeding cycle or both of anurans. In order to understand whether changes in central appetite drive are a cause or consequence of state of reproductive function and how NPY is involved in this regulatory mechanism of energy homeostasis and reproduction of anurans, further study is needed.

Publications from the Project:

1) Kavita N. Hadawale, Nitin S. Sawant, Sneha Sagarkar, Amul J. Sakharkar, Shobha Y. Bhargava, Sex-specific distribution of neuropeptide Y (NPY) in the brain of *Microhyla ornata*. Neuropeptides 74 (2019) 1–10.

2) Swapnil Shewale, Ishfaq Ali, Kavita Hadawale, Shobha Bhargava. Neuropeptide Y in the brain of tadpole: response to positive or negative energy states.Neuropeptides 71 (2018) 1–10

3) Manuscript under preparation:
   Title: Seasonal variation of NPY in the brain of frog, *Microhyla ornata*