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Research paper

Neuropeptide Y in the brain of *Euphlyctis cyanophlyctis* tadpoles responds to hypoxic stress

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ABSTRACT

Neuropeptide Y (NPY) has emerged as a novel peptide to antagonize various physiological consequences of stress within a mammalian brain. Hypoxia induced neuropeptide Y release in mammalian systems is well established. However, the possible role of NPY in regulating the effects of oxygen variation in lower vertebrates has not been investigated. We have studied the distribution and neuro-anatomical expression of NPY in the brain of *Euphlyctis cyanophlyctis* tadpoles, exposed to normal and reduced oxygen levels using immunohistochemistry. Animals exposed to hypoxia (<2 mg/ml) exhibited a significant amplification of NPY-immunoreactivity throughout the brain. Increased NPY-ir perikarya appeared in all the sub-divisions of pallium, septum and preoptic area of telencephalon; suprachiasmatic nucleus, central and lateral thalamus, infundibulum and habenular regions of diencephalon; and nucleus cerebella and medulla of rhombencephalon. Most of these regions form the stress and anxiety regulating centers of a vertebrate brain and some of the parallel regions also respond to respiratory reflexes in mammals. Hence, our results suggest NPY induced modulation of hypoxia in *Euphlyctis cyanophlyctis* tadpoles.

1. Introduction

Neuropeptide Y (NPY) is the most abundant neuropeptide reported in the central nervous system of vertebrates (Cerdá-Reverter and Larhammar, 2000) and is shown to regulate a wide range of physiological processes (Alldredge, 2010; Redrobe et al., 1999; Gehlert, 1999). Depending on its anatomical location, NPY is known to process mood in amygdala (Wu et al., 2011), memory in hippocampus (Borbély et al., 2013), learning and cognition (Redrobe et al., 1999). There are also consistent reports on its role as a sedative to antagonize various behavioural and physiological consequences of stress within the mammalian brain (Morales-Medina et al., 2011; Heilig, 2004). The versatile character of the NPY molecule allows it to integrate various body responses, which are fundamental to survival and adaptation. NPY, in addition to this, has also been observed to influence cardiovascular and respiratory responses to hypoxia (Poncet et al., 1996; Duszczyk et al., 2009). However, the available literature on its role in respiratory regulation is much limited and remains confined to mammals only. In the mammalian brain, Hypoxia induced NPY expression has been observed in the medulla oblongata, striatum and hippocampus (Poncet et al., 1996; Duszczyk et al., 2009). Neuropeptide Y is further shown to modulate respiratory responses through nodose ganglia (Kaczyńska and Szereda-Przestaszewska, 2010). The majority of the work in mammals has focused on the systemic levels of NPY after a stressful exposure to low oxygen content (Kaijser et al., 1994; Cheng et al., 1992; Lee et al., 2003a,b; Derrick and Zukowska, 2000; Liu et al., 2007). There are only a few reports about the central responses which might be involved in triggering the mechanisms to induce tolerance in hypoxic conditions (Poncet et al., 1996).

There are also no reports on the possible involvement of NPY in the respiratory reflexes in aquatic animals, which unlike air breathers are likely to experience variations in the ambient oxygen

Abbreviations: Al, amygdala lateralis; Am, amygdala medialis; CRF, corticotrophin releasing factor; DO, dissolved oxygen; DP, dorsal pallium; HPA, hypothalamo-pituitary-adrenal axis; IR, infundibular recess; MP, medial pallium; NAS, nucleus accumbens septi; NCER, nucleus cerebella; NHD, nucleus habinularis dorsalis; NHV, nucleus habinularis ventralis; NID, nucleus infundibularis dorsalis; NIV, nucleus infundibularis ventralis; NID, nucleus infundibularis ventralis; NMS, nucleus medialis septi; NPC, nucleus posterocentralis thalami; NPL, nucleus posterolateralis thalami; NPO, nucleus preopticus; NT, motor nucleus of the trigeminal nerve; OT, optic tectum; PC, posterior commissure; Pdis, pars distalis; Pi, pars intermediate; PN, pars nervosa; PR, preoptic recess; PVN, paraventricular nucleus; RA, raphe nucleus; SGP, striatum griseum periventricularis tecti; SGS, striatum griseum superficial tecti; STd, striatum pars dorsalis; STv, striatum pars ventralis; V, ventricle.

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in their habitat (Bickler and Buck, 2007). In fishes, with an increase in hypoxia severity, a proportional increase in the forebrain levels of various peptides including corticotrophin releasing factor (CRF) was observed (Bernier and Craig, 2005). Although poorly understood, stress sensation in fishes is known to be perceived through a complex neurocircuitry converging in the hypothalamic paraventricular nuclei (PVN) (Herman et al., 2003). CRF release in the PVN, through HPA axis coordinates the autonomic and behavioural responses to stress (Turnbull and Rivier, 1997). We have been interested in understanding the possible involvement of endogenous NPY in the modulation of central responses to hypoxic stress in the amphibian larvae.

The distribution and localisation of NPY in amphibian brain has already been determined (D'Aniello et al., 1996; Lazar et al., 1993; Cailliez et al., 1987; Danger et al., 1985). NPY possesses a vast distribution in the amphibian brain, but its functional association is not fully understood. We have used the pre-metamorphic tadpoles (Stage 38–39) of *Euphlyctis cyanophlyctis*-Indian skipper frog to study the role of NPY in the respiratory regulation. *Euphlyctis cyanophlyctis* breeds in temporary freshwater ponds, which are susceptible to seasonal drying up exposing the developing larvae to seasonal (Burggren and West, 1982) and diurnal fluctuations (Jones, 1961) in the dissolved oxygen content of the water medium. *Euphlyctis cyanophlyctis* tadpoles, thus, represent a suitable model to probe the central adaptive mechanisms involved in the modulation of hypoxic stress.

Euphlyctis cyanophlyctis tadpoles (stage 38–39) were exposed to acute hypoxic stress (>2 mg/l) and the comparative neuroanatomical expression of NPY against the normoxic animals was investigated using immunohistochemistry.

2. Material and methods

2.1. Animal collection and tissue processing

Euphlyctis cyanophlyctis tadpoles of the lower developmental stages (Stage 35–37) were collected from the fields of Taleghar in Ghat regions of Sahyadri hills in Maharashtra India. Animals were reared in glass chambers of $30 \times 45 \times 10\ cm^3$ and were fed with *ad libitum* boiled spinach until stage 38–39 was achieved (Gosner, 1960). Pre-metamorphic stage (38–39) was used because at this stage, the frog larvae reach their full size and all the anatomical and physiological changes in this stage are stabilised, until the start of metamorphosis in stage 40 (Gosner, 1960). The experiments were done in accordance to the ethical guidelines for animal usage established by Savitribai Phule Pune University, Pune India. Animals were properly acclimatized to room temperature and 12 h day/night cycle prior to any experimentation. Five tadpoles (n = 5) each were exposed to normal (>6 mg O_2 l^{-1}) and hypoxic (<2 mg O_2 l^{-1}) medium for 4 h (Yao et al., 2004; Luo et al., 2000) to induce stress. The dissolved oxygen (DO) content of the medium was measured by Winkler's method of DO determination. Oxygen levels were reduced by bubbling nitrogen gas into the medium (Andrew and Alteri, 2006; Ishihara et al., 1994). After 4 h of exposure, tadpoles of each grade-normoxic and hypoxic-were anesthetized by using 2-phenoxyethanol. Brains of the animals were surgically removed and fixed in bouin's fixative (24 h) followed by dehydration with 10% (2 h), 20% (2 h) and 30% (overnight at 4 °C) sucrose solution in phosphate buffer saline (PBS, 0.01 M, pH 7.4) for cryostat sectioning. The tissues were embedded in Shandon cryomatrix and cut on a cryostat in the transverse plane (Leica CM1510) at the thickness of 20 μm. Sections were mounted on poly-L-lysine coated slides and stored in −20 °C until further use for immunohistochemistry.

2.2. Immunohistochemistry

Possible alterations in expression levels of NPY in normal and stressed animals were assessed by using standard immunohistochemical protocol (Gaupale et al., 2013; Shewale et al., 2014). After washing the sections with phosphate buffer saline (PBS), the sections were immersed in methanol with 0.3% hydrogen peroxide for 1 h, washed again with PBS and treated with BSA solution (bovine serum albumin and 0.5% gelatin in PBS) for 1 h. This was followed by 1 h incubation with normal goat serum (1:40 dilution, Vectastain). The excess goat serum was blotted out and the sections were immediately put in an overnight (16 h) incubation at 4 °C with rabbit monoclonal antibody against NPY (1:5000) containing 0.5% BSA and gelatin. The next day, Sections were treated with biotinylated goat serum (1:200, Vectastain) for 1 h, followed by 1 h incubation with ABC reagent (Vectastain, ABC Kit, 1:100). Sections were washed before and after the addition of biotinylated goat anti-mouse IgG antibody in order to remove any unbound antibody from the slides. Sections were now treated with the substrate solution 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. Following this, the slides were washed in distilled water, dehydrated in alcohol, cleared in xylene, mounted in distyrene plasticizer xylene (Merck, India) and photographed.

2.3. Characterization of antibody

The monoclonal primary antibodies against NPY used in the present study were procured from Sigma Aldrich. The anti-NPY antibody was previously raised in rabbit in response to the immunogen synthetic Neuropeptide Y (NPY, Porcine) (N9528) conjugated to KLH. The antibody was characterized for its specificity in the frog brain. The anti-NPY antibody was used at a dilution of 1:5000. Sections were incubated with diluted antibodies preadsorbed with the peptide to determine the specificity of the antibody. No reaction was observed when the primary or secondary antibody was omitted from the reaction mixture.

2.4. Morphometry

Digital images of NPY immunoreactivity were taken on a Zeiss Imager microscope, with an Anxiocam HRC camera provided with the software Anxiovision Ref-4.8. Sections through the parallel regions of the brain were identified using anatomic landmarks (Neary and Northcutt, 1983; Wada et al., 1980) and were used for a comparative analysis of NPY-ir at different oxygen contents. Cell bodies stained in the specific brain regions were counted manually (using Image J software). An average count of the number of cells in a particular region was taken from five brain slices and considered for the comparative analysis of control and stressed animals.

2.5. Statistical analysis

Cell counts were plotted as box plots for different brain regions. The comparative analysis in the cell counts of five brain slices in the normal and hypoxia treated groups 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 free ware PAST version 2.14 (Hammer et al., 2001).

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