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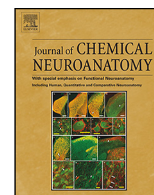

Neuroanatomical evidence for the involvement of β-endorphin during reproductive stress response in the fish *Oreochromis mossambicus*

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ABSTRACT

Although neuroendocrine regulation of stress response involving hypothalamo-pituitary-adrenal axis is well established in mammals, the mechanism of functioning of endocrine-stress axis is not completely elucidated in fish. Our previous studies suggested a possible role for the opioidergic mediation of reproductive stress response in fish. In the present investigation, by immunocytochemical approach, we studied the distribution of β-endorphin (β-EP) secreting neurons in the brain of the tilapia *Oreochromis mossambicus* exposed to aquacultural stressors. Intensely stained β-EP immunoreactive neurons were encountered in the nucleus lateralis tuberis (NLT) region during previtellogenic and vitellogenic phases in both controls and in fish exposed to aquacultural stressors. Furthermore, at the end of the prespawning phase in controls, weak staining in β-EP neurons was accompanied by intensely stained luteinizing hormone (LH) immunoreactive cells in the proximal pars distalis (PPD) of the pituitary gland and a significantly higher gonadosomatic and hepatosomatic indices, suggesting the attenuation of inhibitory effect of β-EP on reproductive axis prior to spawning. However, in fish exposed to stressors, several darkly stained β-EP immunoreactive cells with dense fibre projections towards the hypothalamo-hypophysial tract were concomitant with weakly immunoreactive LH content in the PPD of the pituitary gland and a significantly lower gonadosomatic and hepatosomatic indices compared to those of controls. These results suggest that stress-induced activation of β-EP secreting neurons in the NLT region might lead to the inhibition of LH secreting cells-ovary axis in fish.

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1. Introduction

β-endorphin (β-EP) is an endogenous opioid peptide resulting from processing of the precursor proopiomelanocortin (POMC). A role for inhibitory effect of β-EP on reproduction has been suggested in mammals (Akil et al., 1984; Skinner and Herbison, 1997; El Ouezzani et al., 2005), reptiles (Ganesh and Yajurvedi, 2003), amphibians (Facchinetti et al., 1993), and fish (Ganesh and Chabbi, 2013; Chabbi and Ganesh, 2013). β-EP-containing neuronal system has been described in the brain of fishes such as the bogue *Boops boops* (Vallarino, 1985), the rainbow trout *Salmo gairdneri* (Vallarino et al., 1989), the green molly *Poecilia latipinna* (Batten et al., 1990), and the catfish *Clarias batrachus* (Khan et al., 1999; Sarkar and Subhedar, 2000). However, very few studies have provided evidence for the involvement of β-EP in reproduction, but limited to seasonally breeding fish. For example, expression of β-EP-like immunoreactivity in the olfactory bulbs (Sarkar and Subhedar, 2001) and the brain (Sakharkar et al., 2006) are correlated with the phase of seasonal reproduction in the catfish *C. batrachus* and the carp *Cirrhinus mrigala* respectively. However, to date no studies have focused on the neuroanatomical relationship between β-EP secreting cells and pituitary-ovary axis in continuously breeding fish. Furthermore, release of β-EP in response to stressors is documented in mammals (Polkowska and Przekop, 1988; Herbert, 1995; Fazio et al., 2008), humans (Kofinas et al., 1985; Mirilas et al., 2010), the frog *Rana esculenta* (Mosconi et al., 1994), and in fishes such as the brown trout *S. trutta* (Sumpter et al., 1985), the gilthead seabream *Sparus aurata* (Mosconi et al., 1998; Arends et al., 1999; Rotllant et al., 2000) and the common carp *Cyprinus carpio* (Van den Burg et al., 2005). Previous studies in a continuously breeding fish *Oreochromis mossambicus* (Ganesh and Chabbi, 2013; Chabbi and Ganesh, 2013) have suggested a role for the involvement of β-EP in stress response along luteinizing hormone (LH) secreting cells–ovary axis. However, the neuroanatomical data to support its involvement during reproductive stress response is generally lacking. Therefore in the present investigation, we studied the distribution of β-EP secreting neurons in the brain of the tilapia *O. mossambicus* exposed to aquacultural stressors and corresponding alterations in the activity of LH secreting cells-ovary axis during the phase of the ovarian cycle.

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2. Materials and methods

2.1. Experimental animals

Adult *O. mossambicus* were collected in the month of February-March from perennial ponds in and around Dharwad District (longitude 75°01′E, latitude 15°27′N) and were acclimatized to small freshwater tanks measuring 3 × 3 × 3 ft. at a stocking density of 10 females + 5 males for a period of two months. The mean natural photoperiod was 11.27 ± 0.15L, 12.72 ± 0.15D, whereas the mean water temperature was 29.14 ± 0.16 °C during the period of acclimatization and experimentation. From this stock, mouth-brooding fish were selected and the eggs were manually removed by inverting their heads in a bucket containing water. The stripped fish weighing 25–35 g were used for experimentation. The fish were fed everyday with commercially available food pellets during the period of acclimatization and experimentation. Each aquarium containing aquatic plants was fitted with an aerator to ensure the supply of oxygen. The experimental procedures were carried out in accordance with guidelines by the Institutional animal ethics committee.

2.2. Experimental design

Five female fish were sacrificed on the day of egg removal and considered as initial controls. The remaining stripped fish (n = 30) were divided into two groups, controls and stress group at a sex ratio of five females and two males per 75-L outdoor glass aquaria (size, 36 × 12 × 18 inches; L × W × H). The controls were kept undisturbed, whereas those in stress group were subjected to a combination of different known aquaculture stressors. The detailed procedure for stress regime has been described earlier (Chabbi and Ganesh, 2012). Briefly, fish were exposed to four kinds of stressors, namely handling, chasing, frequent netting and low water levels, four times a day. These stressors have been shown to activate the stress axis through significant cortisol response in tilapia (Chabbi and Ganesh, 2012; Chabbi and Ganesh, 2014). The female fish in both controls and stress group were sacrificed on day 12 (previtellogenic phase), day 18 (vitellogenic phase) and day 23 (prespawning phase). At the time of autopsy the weights of the body, the ovary and the liver were recorded to calculate the gonadosomatic index (GSI; 100 × weight of the ovary/body weight) and hepatosomatic index (HSI; 100 × weight of the liver/body weight).

2.3. Aldehyde- fuchsin (AF) staining

Two brains of the female *O. mossambicus* (non-experimental) were fixed in Bouin's fluid fixative for 24 h, dehydrated in graded alcohols, and embedded in paraffin wax. Serial paraffin sections of 5 μm thickness were cut in transverse as well as sagittal planes and stained with AF. The nucleus lateralis tuberis (NLT) region in these sections was demarcated with the help of the brain map described previously for *O. mossambicus* (Pepels et al., 2002; Singru et al., 2007; Chabbi and Ganesh, 2015). The outlines of the brain areas were prepared using Corel draw graphics suite software version 15.0.

2.4. Immunocytochemistry

All female fish (n = 35) were anaesthetized using 2-phenoxy ethanol (1:1500) and perfused transcardially with 20 ml of ice-cold phosphate buffered saline (PBS, pH 7.4) followed by 20 ml of ice-cold Bouin's fluid. The brains along with the pituitary glands were dissected out and post-fixed in the same fixative for 12 h. The tissues were rinsed in PBS and transferred to ice cold 30% sucrose solution. TissueTek-embedded brains along with pituitaries were sectioned at 14 μm thickness in sagittal and transverse planes using a cryostat (Leica CM1510S, Germany) and thaw-mounted on Poly-L-Lysine coated slides. The sections kept in the moist chamber were processed for the immunocytochemical localization of β-EP in the brain and LH in the pituitary gland by streptavidin-biotin-peroxidase method. The sections were rinsed in PBS for 15 min. Endogenous peroxidase activity was neutralized by incubation of sections with hydrogen peroxide and 30% methanol (1:9) solution for 10 min. Sections were washed in PBS for 15 min and treated with blocking solution containing 2% bovine serum albumin (BSA) +0.4% Triton X-100 in PBS for 30 min followed by incubation overnight (12 h) at 4° C temperature with polyclonal human anti-β-EP antibody produced in rabbit (Immunostar, USA, 1:2000), for the labeling of β-EP immunoreactive cells in the NLT region of brain, and rabbit polyclonal human LHβ antiserum (National Hormone and Peptide Program, USA, 1:8000) for the detection of LH secreting cells in proximal pars distalis (PPD) of the pituitary gland. The sections were then washed in PBS and kept for 1 h incubation in biotinylated goat antirabbit IgG (Sigma, USA). The sections were again washed in PBS and incubated for 1 h with streptavidin peroxidase (Sigma, EXTRA 3). Subsequently, the sections were washed in PBS and stained with 3-Amino-9-ethyl carbazole (AEC) to visualize the antigen-antibody complex in the form of reddish brown precipitate, rinsed in distilled water (dw) and mounted with glycerol-gelatin. The following control procedures were performed to confirm specificity of the observed reaction: (a) Omission of the primary antibodies from the reaction or replacement of these antibodies with normal serum (2% BSA), (b) omission of the secondary antibody, and (c) preabsorption of diluted primary antibody with the rat and the human β-EP peptide (E1142 and E6261, Sigma, USA) and the salmon pituitary gland LH or the human pituitary gland LH (Sigma, USA) at 10^{-5} M for 24 h prior to incubation respectively for brain and pituitary gland containing sections. These control procedures invariably gave negative results. Same observer conducted the immunocytochemistry protocol throughout and the duration of exposure of sections

Table 1
Effect of stress on gonadosomatic and hepatosomatic indices during different phases of the ovarian cycle in the fish *O. mossambicus*.

Days of the ovarian cycle	GSI		HSI	
	Control	Stress	Control	Stress
(Day 1)	0.66 ± 0.06[a]	0.69 ± 0.05[a]	0.90 ± 0.03[a]	0.90 ± 0.08[a]
(Day 12)	1.18 ± 0.07[a]	0.75 ± 0.08[a]	0.94 ± 0.05[a]	0.78 ± 0.12[a]
(Day 18)	1.68 ± 0.13[a]	1.03 ± 0.12[a]	1.08 ± 0.15[a]	0.98 ± 0.24[a]
(Day 23)	3.92 ± 0.12[b]	0.96 ± 0.19[a,*]	1.85 ± 0.16[b]	1.03 ± 0.29[a]
	P < 0.05	*NS*	*P* < 0.05	*NS*

Note: Values are means ± SE; One way ANOVA and Tukey test: Groups with same superscripts are not significantly different, whereas different superscripts indicate significant ($P \prec 0.05$) difference; NS, not significant.

[*] Student *t*-test, indicates significant ($P < 0.05$) difference compared to controls.

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