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Sexually dimorphic distribution of calcium-binding protein, calretinin in the preoptic area of the freshwater catfish, *Clarias batrachus* (Linn.)

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HIGHLIGHTS

- Calretinin (CR) is localized in the preoptic areas (POA) of *C. batrachus*.
- Sexually dimorphic distribution of CR is noted in the catfish brain.
- Presence of CR in the POA may suggest its role in the hormonal regulation.
- Sexually dimorphic distribution suggests gender-specific role of CR in fishes.

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ABSTRACT

Preoptic area (POA) plays an important role in the hormonal regulation of the pituitary gland in vertebrates. In this study we report the sexually dimorphic distribution of calcium-binding proteins calretinin (CR) in the POA in the freshwater catfish, *Clarias batrachus*. Nissl staining highlighted the presence of the nucleus praeopticus periventricularis (NPP) and other subdivisions of the nucleus praeopticus (NPO), including supraoptic (NPOs), paraventricular (NPOp) and magnocellular (NPOm) divisions. In NPO, CR immunoreactivity was noted only in females but not in males. In both sexes, CR stained perikarya were found in the NPP. Sexually dimorphic localization of CR in the POA supports the notion that CR may play a gender-specific role and may be involved in hormonal regulation in fishes.

1. Introduction

Structural variations among the brains of different species are common [1] and sexual differentiation of the vertebrate nervous system has been well studied [2]. In addition to the sexual differences, an intra-sexual dimorphism in the CNS has also been reported [3].

The hypothalamus is a sexually dimorphic region of the brain and many studies have shown sexual dimorphism in the preoptic area (POA) of vertebrates. Sexual dimorphism of avian species is found in the nuclei related to song control [4]. Gender specific differences in amphibians and reptiles have also been reported. In toads, mate calling regions of the brain, POA and the amygdala pars medialis, are significantly larger in males than in females [5]. Inter-sexual variations in the POA are commonly reported in different groups of fish. In both inter-sexual as well as intra-sexual dimorphisms, the nucleus preopticus (NPO) is a prime target for studies on the variability of CNS structures [6]. The NPO contains neurosecretory cells [7] and is involved in the control of hormonal regulation by the pituitary [8].

Morphological sex differences have been demonstrated in the brains of many fish [3,9–22] but it sometimes difficult to interpret because of multiple reproductive morphs of one sex or the ability to change sexes over the lifespan [21]. Nevertheless, a variety of sex differences have been reported such as in the GnRH/LHRH neurons [12,15,16,21], GABA and glutamic acid decarboxylase [10], galanin neurons [9,17,20], preprotachykinin [23] and aromatase [24]. Moreover, sex differences in the position of the POA and other brain regions are also shown [3]. Analysis of dendrite characteristics of killifish brain demonstrated that sex differences in hypothalamic neuronal morphology exist in teleosts [25].

Besides the presence of neuropeptides and neurochemicals in the brain, certain calcium-binding proteins (CaBPs) have been specifically identified in the CNS, which help indirectly in the neurotransmission [26]. Calretinin (CR) was identified by analysis of cDNA from chick retina consisting of 29-kDa and mainly present

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in the nervous system [27,28]. In teleosts, presence of CR has been reported in the brain [29–38] and pituitary gland [38]. Although we have recently shown enormous presence of CR in the NPO of cichlid fish [30], reports on its localization are fragmentary. In view of the paucity of information of CR distribution in the NPO of fish, this study was undertaken to analyze the distribution of CR in the catfish, *Clarias batrachus*.

2. Materials and methods

2.1. Animal collection and tissue preparation

Five males (body length: 36–45 cm) and six females (body length: 36–45 cm) of freshwater catfish, *C. batrachus* collected from the local supplier in Nagpur were used. Fish were anesthetized with tricaine methane sulfonate (0.01%, MS-222, Sigma), then transcardially perfused with 0.1 M phosphate buffered saline (PBS, pH 7.4) and, subsequently, with 4% paraformaldehyde (PFA) in PBS. The brains were removed and immersed in 4% PFA and post-fixed at room temperature for 24 h. Thereafter, they were dehydrated in ethanol and embedded in paraffin. In addition, after perfusion two male and two female brains were post-fixed in PFA at 4 °C for 4 h. Before freezing, the brains were cryoprotected in 30% sucrose buffer for 12 h at 4 °C and subsequently placed in embedding medium (15% Polyvinylpyrrolidone, SRL, India) for cryostat sectioning. The frozen and paraffin wax serial section were cut in transverse plane at 10 μm thickness in a cryostat and microtome, respectively. The sections were placed on alternate sets of slides. Paraffin embedded sections were used for Nissl staining and CR staining.

2.2. Nissl staining

Initially, paraffin sections were dewaxed by passing through xylene and graded dilutions of ethanol up to 90%. The sections were transferred in to 0.5% luxol fast (Sigma) solution prepared in 90% ethanol for 6 h at 57 °C. Then sections were processed through 90%, 70% ethanol, distilled water (5 min in each) and dipped in 0.5% lithium carbonate solution for 1–2 min. The sections were differentiated in 70% ethanol and kept in distilled water for 5 min. Thereafter, they were transferred in 0.2% cresyl violet (Sigma) solution for 20 min, dehydrated, cleared in xylene and mounted with DPX.

2.3. Immunocytochemistry

The paraffin sections were dewaxed and rehydrated before immunohistochemistry. After three washes in Tris-buffered saline (TBS; 0.1 M; pH 7.4), endogenous peroxidase was eliminated by immersion in a 0.3% Triton X-100 and 1% H_2O_2 solution for 30 min. This was followed by five washes in TBS. Subsequently, unspecific binding of the antibody was prevented by a 2 h treatment with 1% bovine serum albumin (BSA) in TBS. Without having been washed, the sections were then transferred into a solution of polyclonal rabbit antibody raised against calretinin (Swant, Bellinzona, Switzerland) at 1:1000 dilutions in TBS and incubated at room temperature (RT) for 24 h. After rinsing in TBS, the sections were incubated for 1 h at RT with goat anti-rabbit IgG (1:30; DAKO, Hamburg, Germany), washed twice (10 min each) in TBS and incubated for 1 h at RT with peroxidase anti-peroxidase complex diluted in TBS (1:200; PAP, Dako). Finally, the immune complex was visualized by incubation with 0.06% 3,3-diaminobenzidine (DAB, Sigma) and 0.005% H_2O_2 in TBS for 10 min. The sections were then rinsed in TBS, dehydrated counterstained with neutral red and cover slipped.

The primary antibody has been used previously and documented to react specifically in teleost brains [31,34,38] and also confirmed with western blot analysis [30]. For the specificity of immunostaining standard procedures were adopted. No immunoreactivity occurred in any of the tests.

2.4. Statistical analysis

To determine diameters, the mean of the long and the short axes was calculated and to avoid double counting in adjacent sections, only neurons with clearly visible nuclei were considered. Mean ± SEM were calculated. Means represent an average of the averages determined for each sample.

2.5. Images and photo plates

Images were taken on Axioplan 2 (Carl Zeiss) microscope using a Q Imaging digital camera and Image-Pro Discovery (Image-Pro Plus 5.0 version) software. Photographs were adjusted for brightness and contrast with Adobe Photoshop. Photo plates and line drawings were made using Corel Draw X4 version.

3. Results

3.1. Nissl staining (NS) analysis of preoptic area (POA)

We referred earlier reports on catfish brain [39,40] for the identification and demarcation of the nuclear boundaries within the POA. However, no sexually dimorphic organization of POA was noticed after NS.

NS revealed the nucleus praeopticus periventricularis (NPP) (Fig. 1a and a_1) and three subdivisions of the nucleus praeopticus (NPO) in the POA (Fig. 1b and b_1, c and c_1 and d and d_1) as depicted on the left side of Fig. 2a and d. The NPP is positioned lateral to the third ventricle and situated posteroventral to the anterior commissure (Figs. 1a and a_1 and 2a). The somata were small in size, mostly round or oval and 8 ± 0.4–10 ± 0.6 μm in diameter. Further caudal to NPP, the three subdivisions of the NPO were noted. The neurons located far lateral to the third ventricle above the optic tract constitutes the supraoptic division of the NPO (NPOs) (Figs. 1b and b_1 and c and c_1 and 2b and c). The cells were many, mostly round or oval in shape and measured 12 ± 0.7–18 ± 0.9 μm in diameter (Table 1). Many large perikarya were seen on the either side of the third ventricle above the optic chaisma to form the paraventricular division of the NPO (NPOp) (Figs. 1b and b_1 and c and c_1 and 2b and c). The somata were oval or round in shape and the neuronal diameter was 12 ± 0.8–22 ± 1.2 μm. In between NPOs and NPOp subdivisions some small cells were noted referred as the bridge cells (Figs. 1b and 2b). Further caudal few neurons were noted dorsally on the either side of the ventricle constitutes the magnocellular part of NPO (NPOm) (Figs. 1d and d_1 and 2d). The somata were mostly round or oval in shape with a size of 22 ± 1.1–28 ± 1.5 μm in diameter (Table 1).

3.2. Calretinin (CR) immunoreactivity (CR-ir) in POA

In POA of *C. batrachus*, CR-ir was noted only in females but not in males, except in the NPP (Fig. 2a and d). In both genders, NPP neurons were found intensely labeled with CR, located ventral to the anterior commissure (Figs. 2a and 3a and a_1 and b, b_1 and b_2). The neurons were oval with long processes. A few CR stained fibers were also seen in POA (Fig. 3b_2).

Only in females, in all three subdivisions of NPO, i.e., NPOs, NPOp and NPOm a strong CR-ir was noted (Figs. 2b and d and 4a and a_1, b, b_1 and b_2 and c, c_1 and c_2). In the NPOs, few CR positive neurons were located far lateral to the third ventricle (Figs. 2b and c and 4a and a_1). The perikarya were mostly oval or round and they were large in size compared to NPP and NPOp neurons. Further caudal in