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# Calcium binding protein calretinin (29 kD) localization in the forebrain of the cichlid fish: An immunohistochemical study

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ABSTRACT

Ionic regulation is essential for the metabolism and cellular function. For many physiological processes, ionic calcium ($Ca^{+2}$) is important for example muscle contractions, nerve signaling, membrane permeability, cell division and hormone release. In nerve cells, the excess intracellular concentration of $Ca^{+2}$ causes cell death. It has been shown that certain calcium binding proteins (CaBPs) are essential for $Ca^{+2}$ homeostasis and protect neurons from excess $Ca^{+2}$ influx.

We are for the first time showing an unusual calretinin (CR) expression and significant differences in its occurrence in the forebrain of the cichlid fish (*Cynotilapia sp.*) compared to other teleosts. CR labeled neurons were seen in the dorsal and lateral part of the dorsal telencephalic area, entopeduncular nucleus (EN), nucleus preopticus (NPO), diffuse nucleus of lateral torus (NDTL), ventral hypothalamic nucleus (VH), preglomerular nucleus (NPG) and optic tectum. Surprisingly, large numbers of CR immunoreactive perikarya were noted in the optic chiasma (Oc). These neurons were oval with elongated processes and forming a huge fiber network in the Oc. Enormously CR stained fibers were seen in the lateral and medial olfactory tract. Widespread distributions of strongly CR labeled fibers were observed around the EN projecting dorsally into the telencephalon, Oc and optic nerve. Presence of CR in the NPO suggests that it may be involved in the hormonal regulation by the pituitary. As in vertebrates EN plays an important role in sensory functions, massive localization CR in the EN may suggests role of CR in sensory functions of the cichlid fish.



## 1. Introduction

In the last few decades, efforts have been made to understand the functional significance of the intracellular calcium binding proteins (CaBPs) present in the nerve cells. Some of the CaBPs are indirectly involved in the neurotransmission and also act as a neuroprotectant when intracellular calcium ion level increases in the neurons. Among 250 different CaBPs, only few important nerve cell specific CaBPs are calretinin (CR), calbindin and parvalbumin (Andressen et al., 1993). In recent years, these CaBPs are being used as selective markers to identify unknown neuronal populations and pathways in CNS (Andressen et al., 1993; Jadhao and Malz, 2007). Not only the CaBPs but also substances like NADPH-d has been used by researchers as an excellent neuroanatomical marker for the identification of unknown nuclei and their connectivities in the CNS (Jadhao et al., 1999; Jadhao and Malz, 2004; Schober et al., 1994a,b).

The localization of CR in the CNS of various vertebrate animals including fish has been shown by many workers however, only few studies have reported CR in the brain of teleosts (Arévalo et al., 1995; Castro et al., 2003, 2006a,b; Crespo et al., 1998; Díaz-Regueira and Anadón, 2000; Friedman and Kawasaki, 1997; Jadhao and Malz, 2007; Porteros et al.,1998; Wong, 1997).

Role of nucleus preopticus (NPO) in the pituitary hormonal regulation is well known (Peter and Fryer, 1983) and recent studies have shown inter-sexual and intra-sexual dimorphisms in the NPO of the fish (Jadhao et al., 2001; Jadhao, 2013). In fishes, so far nobody has shown the presence of CR in the NPO however, in the catfish pituitary gland CR has been reported (Jadhao and Malz, 2007). In view of less information on CR expression in the teleosts, the present study was aimed to see the distribution of CR in the forebrain of cichlid fish.

## 2. Materials and methods

In the present study, total 9 adult cichlid fishes (*Cynotilapia sp.*) 7–9 cm of body length of either sex procured from the local supplier were used. They were fully matured with ripened gonads.

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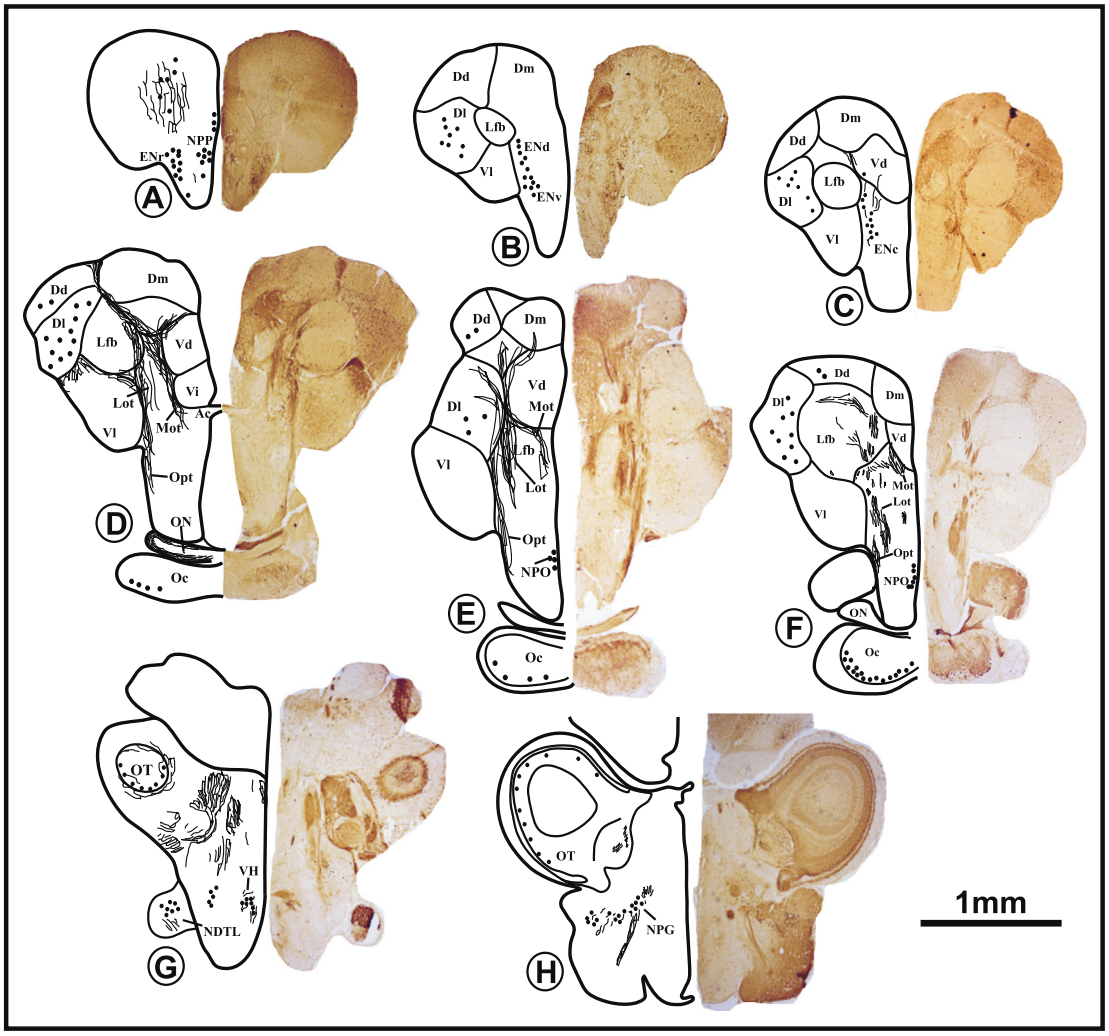


**Fig. 1.** (A–H) Schematic representation (left side) of the transverse sections of the forebrain (right side) showing distribution of CR stained somata (circles) and fibers (lines) in the forebrain of cichlid fish, *Cynotilapia sp.* (Dd, dorsal part of dorsal telencephalic area; Dl, lateral part of the dorsal telencephalic area; Dm, medial part of the dorsal telencephalic area; EN, entopeduncular nucleus; ENc, EN caudal part; ENd, EN dorsal part; ENr, EN rostral part; ENv, ventral part; Lfb, lateral forebrain bundle; Lot, lateral olfactory tract; Mot, medial olfactory tract; NDTL, diffuse nucleus of lateral torus; NPG, preglomerular nucleus; NPO, nucleus preopticus; NPP, nucleus periventricularis; Oc, optic chiasma; ON, optic nerve; Opt, optic tracts; OT, optic tectum; V, third ventricle; Vd, dorsal part of the ventral telencephalic area; VH, ventral hypothalamic nucleus, Vi, intermediate part of the ventral telencephalic area; Vl, lateral part of the ventral telencephalic area).

The fishes were anaesthetized using 2,4,6-Trimethyl-1,3,5 trioxane (paraldehyde LR). Fish brains were dissected out and kept in freshly prepared bouin's fixative for 24 h at 4 °C. The brains were transferred in 30% sucrose for 24 h at 4 °C. The brains were cryosectioned at 10 μm and alternate sections were taken on each slide.

### 2.1. Immunohistochemistry

The sections were air dried at room temperature and then they were hydrated for 10 min, followed by a wash of TBS (pH, 7.4) for 10 min. To remove endogenous peroxidase the sections were immersed in a 0.3% Triton X-100 and 30% hydrogen peroxide ($H_2O_2$) solution for 30 min. This was followed by 2 washes of TBS of 5 min each. Subsequently, non specific binding of the antibody was prevented by a 1 h treatment with 1% bovine serum albumin (BSA) in TBS. Without washing, the sections were then transferred into a solution of polyclonal rabbit antibody raised against calretinin (Swant, Bellinzona, Switzerland) at 1:1000 dilution in TBS and incubated at 26 °C for 18 h. After giving three changes of TBS for 5 min each, the sections were incubated for 1 h with goat antirabbit IgG at 1:200 dilution at 26 °C. The avidin biotin complex was prepared in TBS (pH = 7.4) and kept at 4 °C, half an hour before use. After incubation with secondary antibody for 1 h, avidin biotin complex (Vectastain ABC kit) was added on to the sections for 1 h. Finally the immune complex was visualized by 3′ 3′-diaminobenzidine (DAB) and 30% $H_2O_2$ in TBS for 10 min. The sections were then rinsed with 2 washes of TBS, dehydrated, mounted in D.P.X. and cover slipped.

The primary antibody used has previously been characterized by Schwaller et al. (1993) and has been documented to react specifically in teleost brains (Friedman and Kawasaki, 1997; Díaz-Regueira and Anadón, 2000; Jadhao and Malz, 2007). However, for the specificity of immunoreactions control procedures were adopted as (i) omission of the primary antibody from the reaction mixture, (ii) omission of secondary antibody from the reaction mixture, (iii) replacement of the antiserum by normal rabbit serum.

### 2.2. Western blot analysis

Immunoblot analysis was carried using brain homogenate of adult cichlid (*Cynotilapia sp.*). The fresh brains were homogenized with equal volume of homogenization buffer (1 M Tris buffer (pH 6.8) containing 20% glycerol and 10% SDS with PMSF as protease inhibitor. The homogenate obtained was then centrifuged at