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Local differences in calretinin immunoreactivity in the optic tectum of the ocellated dragonet

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ABSTRACT

The optic tectum of the ocellated dragonet (*Synchiropus ocellatus*) was studied with immunohistochemistry. Antibodies raised against the calcium binding protein calretinin (CR) revealed a lamination similar to that already reported for other ray finned fish. Most immunoreactive fibers could be observed in those layers receiving retinal afferents and most immunoreactive cells occur in the stratum periventriculare. However, there are marked differences in the presence of other calretinin-positive cell types and immunoreactive lamina between the dorsomedial and ventrolateral parts of the tectum. *Synchiropus* is a bottom dwelling fish with strong functional subdivisions of the visual system into dorsal and lateral visual fields. The differences in calretinin-positive cell bodies and fibers may be a sensitive indicator of functional differences of tectal circuitry.

1. Introduction

The optic tectum is one of the best investigated nervous structure in ray finned fish. It is homologous to the mammalian superior colliculus and the main recipient of retinal afferents. In most fish, it forms two prominent lobes which encompasses the midbrain ventricle and is ventrally bound to the midbrain torus semicircularis and tegmentum. In cross-sections, the optic tectum displays a distinct pattern of lamination. These laminae are, from the pial surface to the ependyma, the stratum marginale (SM), the stratum opticum (SO), the stratum fibrosum et griseum superficiale (SFGS), the stratum griseum centrale (SGC), the stratum album centrale (SAC) and the stratum periventriculare (SPV) (Vanegas et al., 1974). Although the most prominent afferents are retinal fibers, the optic tectum also receives input from a number of other areas including the torus longitudinalis, the torus semicircularis, the superficial pretectum, the thalamus, and the telencephalic area dorsalis pars centralis. Tectal efferents include the nucleus corticalis, pretectal nuclei, the thalamus, the torus semicircularis, and the rhombencephalic reticular formation. There are a number of reviews dealing with the abundance of work about tectal connections, e.g. Vanegas and Ito (1983), Meek (1983), Meek and Nieuwenhuys (1998).

The optic tectum is known to be immunoreactive to calcium-binding proteins. In the tench, the distribution of calbindinin (CB), calretinin (CR) and parvalbumin (PV) reveals a characteristic pattern of cell and fiber labeling (Miguel-Hidalgo et al., 1991; Arévalo et al., 1995; Crespo et al., 1999). CR is abundant in the retinorecipient laminae and most immunoreactive cells occur in the SPV. These cells possess an apical dendrite which runs to the upper tectal layers. On its way, it gives rise to several secondary dendrites (Arévalo et al., 1995). To date, the studies on the tench remain the only detailed description on the distribution of tectal calcium binding proteins.

In the present study, we investigate the distribution of CR in the optic tectum of the ocellated dragonet (*Synchiropus ocellatus).* Dragonets are a group of benthic, marine fish. Most of them are native to the indo-pacific region. *Synchiropus* is a bottom dwelling fish with strong functional subdivisions of the visual system into dorsal and lateral visual fields. Our research shows that its optic tectum is divided into a dorsomedial and a ventrolateral part. The corresponding differences in calretinin distribution in the tectum suggests that it reflects these functional differences.

2. Materials & methods

For this study, seven specimen of *Synchiropus ocellatus* were acquired from local dealers. Five specimen were used for immunohistochemistry, one specimen was used for nissl staining and one specimen was used for SDS-Page. All experiments were conducted under the guidelines of the German animal welfare act.

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After deep anesthesia with Ethyl 3-aminobenzoate methanesulfonate (1:10000, MS-222, Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) the dorsal portion of the skull was removed and the specimens were immersed in 4% phosphate buffered paraformaldehyde. Following overnight fixation, brains were removed from the cranial cavity and placed in the same fixative for 24 h. Prior to cryo sectioning with a Leica CM 1850 (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) specimens were cryoprotected overnight in phosphatbuffer (PB – pH 7.4, 0.1 M) containing 30% sucrose. One specimen was cut at 25 μm and sections were mounted on gelatin coated glass slides, stained with 1% cresyl violet, dehydrated and coverslipped with Roti®-Histokitt (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Five specimen were cut at 35 μm and placed free floating in phosphate buffered saline (PBS – pH 7.4, 0.01 M). For deactivation of endogenous peroxidase activity sections were placed for 10 min in PBS containing 1% H_2O_2 and subsequently washed three times for ten minutes in PBS. Sections were then incubated for 24 h in a monoclonal mouse anti-calretinin antibody (1:5000 in PBS; swant® Swiss antibodies, Marly FR, Switzerland) containing 2% Donor Horse Serum (Biowest, Kansas City, MO, USA) and again washed three times in PBS. The secondary biotin conjugated antibody (Biotin-SP-conjugated AffiniPure Donkey Anti-Mouse IgG (H + L), Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was used at a dilution of 1:500 and sections were incubated for 1 h. After incubation, sections were treated with the VECTASTAIN Elite ABC Kit (VECTOR LABORATORIES, INC., Burlingame, CA, USA) for 1 h and again washed three times in PBS. Finally, sections were placed in 10 ml PBS containing 0.025% DAB (3,3′-Diaminobenzidine, Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), 200 μl Nickel ammonium sulfate (1 g on 100 ml H_2O_{dest}) and 12 μl 30% H_2O_2 (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). The reaction resulted in a dark brown reaction product. After the desired level of staining was reached, reaction was stopped in PBS and following three more washing steps, sections were mounted on gelatin covered glass slides, dehydrated and coverslipped. Slides were observed and microphotographs were taken with a Canon EOS 60D (Canon Deutschland GmbH, Krefeld, Germany) mounted on a Zeiss Axio Lab.A1 (Carl Zeiss Microscopy GmbH, Jena, Germany).

To test the specificity of the calretinin antibody, brain extracts of *Synchiropus* were separated by SDS-Page gel (15% acrylamide) at a constant current of 25 mA for 1.5 h. For comparison, brain extracts of mouse and a protein marker (Roti®-Mark 10–150, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were also applied to the gel. Proteins were then transferred by western blot onto nitrocellulose paper at 40 mA overnight. To block non-specific binding sites the nitrocellulose paper was incubated in 4% non-fat milk powder in PBS (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) prior to staining according to the procedure above. Blots were then scanned with an Epson Perfection V37 (EPSON Deutschland GmbH, Meerbusch, Germany).

The brightness and sharpness of all microphotographs were adjusted with GIMP 2.8 (The GNU Image Manipulation Program, www.gimp.org) and figures were arranged with Inkscape 0.91 (www.inkscape.org).

3. Results

The antibody we used was tested by the manufacturer for several species including mouse and zebrafish for its specificity. It does not cross-react with calbindinin-28k or other related CaBPs and binds to a protein at 29 kDa. Our western blots of brain extracts from dragonet and mouse stained with the monoclonal CR-antibody resulted in a single band close to 29 kDa (Fig. 1). Since there was only a single band stained it suggests that the antibody we used binds specifically to calretinin.

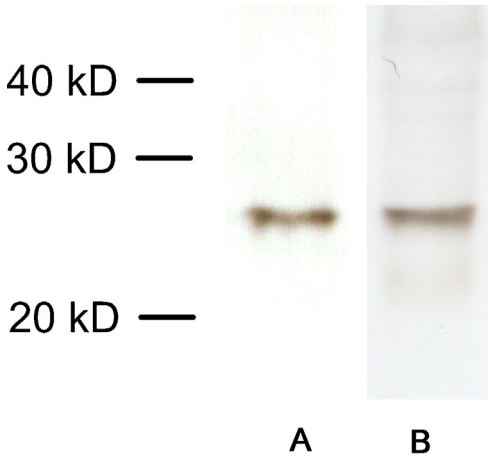

Fig. 1. Western blot from SDS-Page gel of (A) *Synchiropus ocellatus* (B) *Mus musculus*. There is a single band stained close to 29 kDa.

The optic tectum of *Synchiropus ocellatus* is similar to that of other teleosts. It consists of paired lobes that encompasses the midbrain ventricle with the six laminae described by Vanegas et al. (1974). However, both tectal hemispheres are divided into a dorsomedial and a ventrolateral part through a lateral sulcus. This sulcus begins at the most rostral point of the tectum but becomes flatter into caudal direction (Fig. 2). There are large differences in calretinin immunoreactivity between the two parts. The ventrolateral part is more differentiated than its dorsomedial counterpart and the density of CR-ir cells is much higher. In addition, both parts differ in the type of cell immunoreactive for calretinin. The dorsomedial part seems to have its CR-ir concentrated in the upper regions of the tectum (Fig. 2B,D).

The ventrolateral part of the tectum exhibits most CR-positive fibers in the stratum opticum (SO), the stratum fibrosum et griseum superficiale (SFGS) and the stratum griseum centrale (SGC) (Fig. 3A). The SO is intensely stained and is separated from the SFGS by a thin unstained layer. Lamination in the SFGS is diffuse, but there is a small band of fibers in its upper region close to the SO which is stronger stained than the rest of the SFGS (Fig. 2A). A thick, intensely stained fiber layer is also present in the middle part of the SGC (Fig. 2A). There is also a weakly stained band of CR-ir in the stratum album centrale (SAC). There are however, no terminals or fibers visible. Most CR-positive cells occur in the stratum periventriculare (SPV) (Fig. 3A). Two different cells types can be distinguished. Cell type 1 is most abundant. They are small round cells (Fig. 3A,C) with a cell body around 2–3 μm in diameter. A cell process could not be observed. Cell type 2, which is less common possess a larger cell body (around 5–7 μm) and is found at the upper margin of the SPV (Fig. 3C). This cell type is characterized by a thick dendrite which runs straight upwards into the middle fiber layer of the SGC. However, there is no dendritic arborization visible, probably due to the intense background staining of this layer. There are some other scattered cells labeled, mostly in the deeper layer of the SGC and in the SAC (Fig. 3A). They are few in numbers and weakly stained. They sometimes appear to have a bipolar morphology and referred here as type 3 cells.

In the dorsomedial part of the tectum, the upper region exhibits strongest immunoreactivity while most immunoreactive cells occur in the deeper layers (Fig. 3B). The immunoreactivity in the SGC is strongly reduced and there is only a faint fibrous layer stained (Figs. 2 B,C; 3 B). This layer borders directly to the SFGS. The strongest label occurs in the SO and the SFGS which form a dense