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Research report

Calretinin-like immunoreactivity in the optic tectum of the tench (*Tinca tinca* L.)

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Abstract

The distribution of calretinin-like immunopositive cells and fibers in the optic tectum of the tench (*Tinca tinca*) was studied by using a polyclonal antibody and the avidin-biotin-peroxidase technique. A clear laminated pattern of calretinin-like immunoreactivity was observed. The stratum periventriculare demonstrated a large number of strongly labeled cells whereas in the strata album centrale and griseum centrale, and at the boundary between the strata griseum centrale and fibrosum et griseum superficiale, some scarce, weakly immunostained cells were observed. No immunoreactive cells were seen in the strata fibrosum et griseum superficiale, opticum and marginale. Cells belonging to neuronal types X and XIV, previously characterized using Golgi impregnation, were found to be calretinin-like immunoreactive. Most calretinin-like immunopositive fibers were found in the strata fibrosum et griseum superficiale and opticum with a distribution pattern similar to retinotectal axons in these layers. In agreement with previous biochemical studies, our data suggest that, by contrast to all other classes of vertebrates, instead of calretinin and calbindin D-28k, only one protein is present in teleosts. Nevertheless, the calretinin-like immunostaining pattern in the teleost optic tectum was more complex than that previously described for calbindin D-28k. When compared to the calretinin-immunostaining in the rat superior colliculus, it is evident the presence in both amniotes and anamniotes of calretinin-immunopositive retinotectal axons. However, the distribution patterns of intrinsic calretinin-immunoreactive cells were different. Immunolabeled cells have been described in all layers of the superior colliculus, whereas the cells containing calretinin were restricted to the three deep strata of the tench optic tectum, a more similar distribution to what has been reported in the chick optic tectum.

Keywords: Calbindin D-28k; Calcium-binding protein; Calretinin; Optic tectum; Teleost

1. Introduction

Calretinin (CR) is a 29 kDa calcium-binding protein that belongs to the 'EF-hand' family, a group of proteins which bind calcium with dissociation constants in the micromolar range [13]. It was initially identified from a cDNA clone of chicken retina [18] and later detected in diverse types of cells, ranging from local inhibitory neurons to sensory projective neurons [17]. CR is closely related to calbindin D-28k (CaBP) with a 60% coincidence in their primary amino acid sequence in both birds and mammals [11,18]. Due to this high homology, some polyclonal antisera against CaBP cross-react with CR in both mammalian and avian brains [15,18]. The genes coding for both calcium binding proteins derive from a common ancestor by gene duplication but both genes were separated on different chromosomes during the genome evolution [11]. Despite their high homology, both calcium-binding proteins have been separately conserved throughout tetrapod evolution [20].

While both CR and CaBP have been demonstrated in all vertebrate groups from amphibians to mammals, biochemical data indicate that only one band of 28 kDa

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is present in the fish brain [10]. There are no immunohistochemical data available on the presence of CR in the nervous system of teleosts. Therefore, it is interesting to know whether CR or an immunologically related protein can be detected with available anti-CR antibodies in the fish brain and to compare the positive elements with those observed after CaBP-immunostaining.

The distributions of CR and CaBP in the chick and rat brains have been compared using in situ hybridization and immunohistochemistry. The distribution patterns of CR and CaBP are very different in several brain regions [21,26], but coexistence of both proteins has been reported in neurons of the chick dorsal root ganglia, in the inner ear, and in the retina [19]. The CR-immunoreactivity pattern overlaps that of CaBP in some regions of the rat brain such as the superior and inferior colliculi, the substantia nigra, the cochlear nucleus and the nucleus of the solitary tract [1]. Therefore, it seems that the neuronal systems expressing both calcium-binding proteins in mammals are relatively independent, including neurons where both proteins are expressed and other cells only positive for one of them.

The optic tectum is a highly differentiated part of the mesencephalon of teleosts. In these animals, it is the main integration center for visual, lateral line, and tactile information [7]. The distribution of CaBP in the optic tectum of teleosts is known, with a characteristic pattern of cell and fiber labeling [8]. In the present study, we analyze the distribution of CR in the optic tectum of a teleost, the tench (*Tinca tinca* L.) using a polyclonal antibody against CR which in mammals does not cross-react with CaBP [12,19]. We compare these results to previous data on the distribution of CaBP in the teleost optic tectum [8]. Our results provide a more complex distribution pattern than what has been previously reported using anti-CaBP antibodies.

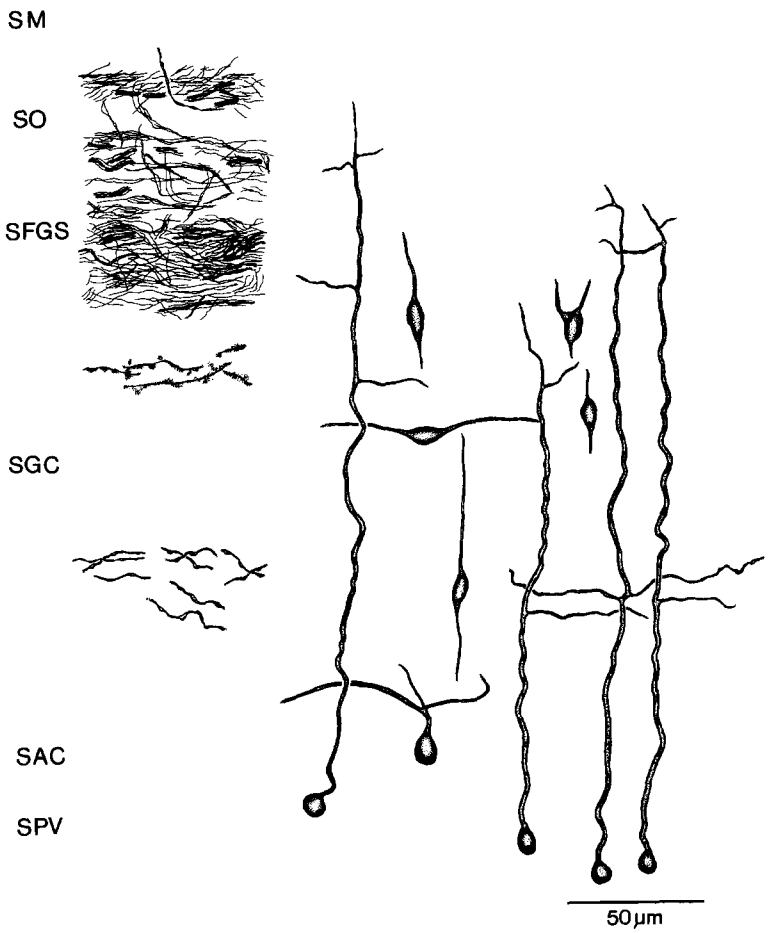
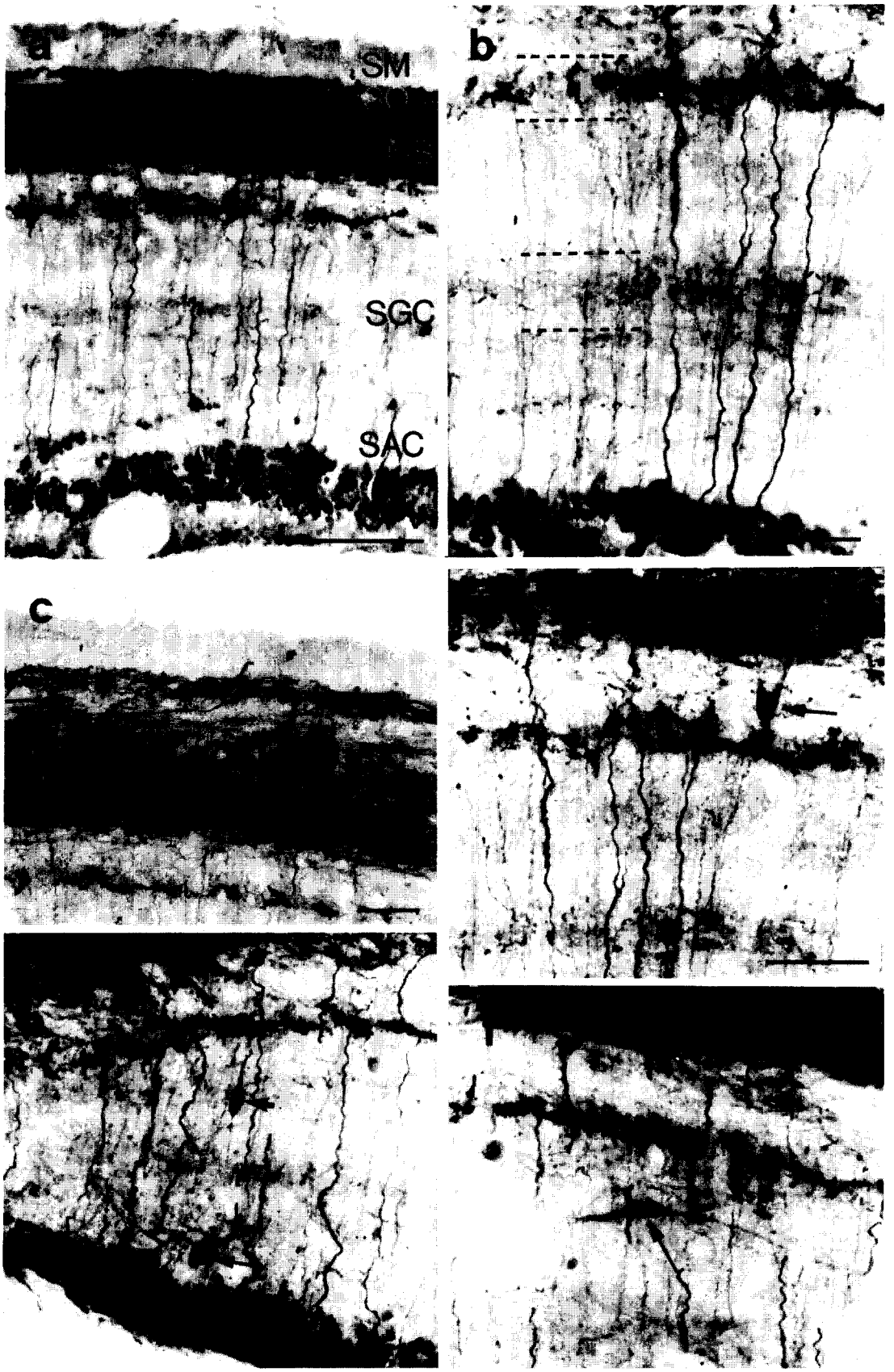


Fig. 1. Schematic drawing illustrating the distribution of CR-like immunoreactive cell types and fibers in the optic tectum of the tench. Labeled fibers can be observed on the left side and immunopositive cell types on the right one. SPV, stratum periventriculare; SAC, stratum album centrale; SGC, stratum griseum centrale; SFGS, stratum fibrosum et griseum superficiale; SO, stratum opticum; SM, stratum marginale; bar = 50 μm.


a
SM
SGC
SAC
b
c

2. Materials and methods

Ten adult tench, *Tinca tinca* L. (Cyprinidae, Teleostei) weighing 150–200 g obtained from a local breeder (Ipescon, Salamanca) were used in the present study. The animals were anesthetized in a solution of 0.03% tricaine methanesulphonate (MS-222, Sigma) and perfused transcardially with 50 ml of 0.63% saline solution followed by 200 ml of a fixative containing 4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.4 (PB).

The brains were removed from the skulls, postfixed in the same fixative for 6 h and immersed in PB containing 30% sucrose (v/v) for cryoprotection. Thirty μm thick transverse sections were cut with a cryostat and processed for immunocytochemistry.

The free-floating sections were processed following the avidin-biotin-peroxidase method. The sections were preincubated with 5% normal goat serum and 0.1% Triton X-100 in PB for 1 h at 4°C. Thereafter, they were incubated with anti-CR serum (1:1000 in PB containing 5% normal goat serum) for 48 h at 4°C. The antibody used has been exhaustively characterized [18,19] and previously used in different regions of the rat brain [17]. The sections were washed in PB and incubated with biotinylated goat anti-rabbit immunoglobulin G (Vector Labs., Burlingame USA; 1:200 in PB) for 1 h at room temperature and then in avidin-peroxidase complex (Vector Labs; 1:225 in PB) for 2 h. Tissue-bound peroxidase was visualized with 0.07% 3,3'-diaminobenzidine and 0.003% H_2O_2 in 0.1 M Tris-HCl buffer (pH 7.6) under visual control. Finally, the sections were mounted on slides, dehydrated in an increasing ethanol series, cleared with xylene, and coverslipped using Entellan (Merck).

The specificity of immunostaining was controlled by omitting the antibody (first or second) in each incubation step. No residual immunoreactivity was observed.

3. Results

From the tectal ventricle to the pial surface, six layers can be differentiated in the teleost optic tectum: stratum periventriculare (SPV), album centrale (SAC), griseum centrale (SGC), fibrosum et griseum superficiale (SFGS), opticum (SO) and marginale (SM) [23].

Light-microscopic examination of CR-like stained sections of the tench optic tectum demonstrated positive staining in all layers except for the SM (Figs. 1,2a). The CR-like immunoreactivity showed a laminated distribution pattern which was similar at rostral, medial and caudal levels of the optic tectum.

Most CR-like immunoreactive cells were present in the SPV. Only a few immunostained neurons were detected in the SAC and SGC, specially located in the ventrolateral area of the optic tectum, close to the optic tract (Fig. 2e). No CR-like immunolabeled cells were found in the SFGS, SO, and SM.

CR-like immunoreactive neurons in the SPV were strongly labeled and were distributed uniformly in the whole extent of the layer but only a subpopulation of periventricular cells was immunoreactive (Fig. 2a,b,e). These immunostained cells were piriform or rounded, with a large prolongation arising from the dorsal pole of the soma and running perpendicularly to the surface of the optic tectum toward the superficial strata (SFGS and SO) (Figs. 1 and 2b). This main prolongation gave rise to lateral branches at different levels in the SGC and SAC. The branches in the SFGS and SO were hard to follow because of the high density of CR-like immunoreactive fibers in the retinorecipient layers. The ramifications in the SGC showed varicosities along their courses and they formed two immunoreactive bands, one in the middle of the stratum and another one in the superficial part (Fig. 2b). Some of these prolongations resembled the axon-like processes of similar neurons described in Golgi studies. The morphological characteristics of the CR-like immunostained cells of the SPV corresponded with type XIV neurons as described by Meek and Schellart [6] using Golgi impregnation. Scarce CR-like immunolabeled neurons with similar morphological characteristics to those described in the SPV were detected in the SAC and SGC.

The remaining CR-like immunolabeled cells were weakly stained and located in the SAC and SGC. A small number of round monopolar CR-like immuno-

Fig. 2. a: photomicrograph of a transverse section showing the distribution of CR-like immunoreactivity in the optic tectum of the tench. Note the high density of fibers in the SFGS and SO and the abundant positive cells located in the SPV. Abbreviations as in Fig. 1. b: CR-like immunoreactive cells in the SPV. Their prolongations extend radially toward more superficial strata and they branch at different levels in the SGC (both levels marked with dashed lines). c: high magnification of SFGS and SO. Note the strong immunoreactivity associated with the retinorecipient strata and the laminated distribution of CR-like immunostaining within the SO. Abundant CR-like immunoreactive fibers can be seen in the superficial and deep regions of the SO (arrowheads), whereas only a few immunopositive fibers are observed in the middle region of the stratum (arrow). d: CR-like immunoreactive triangular neuron (arrow) in the SGC-SFGS boundary with two prolongations reaching the SFGS and another prolongation descending towards deeper strata. e: photomicrograph of the ventrolateral area of the optic tectum. One CR-like immunopositive cell located in the SAC (arrow) and another one in the SGC (arrowhead) can be observed. f: horizontal fusiform neuron located in the SGC (arrow). Two prolongations arising from both poles of the soma are labeled. Bars: a = 100 μm; b,e = 50 μm; c = 50 μm; d,f = 50 μm.

reactive cells could be seen in the SAC. These cells were similar in size to the stained cells located in the SPV and had a main dendritic trunk oriented parallel to the optic tectum lamination (Fig. 1). Their dendrites branched, giving rise to secondary dendrites which coursed vertically for a short distance in the SAC and SGC.

Scattered fusiform neurons with two unbranched proximal prolongations which arose from opposite poles of the soma and ran for relatively short distances were observed in the SAC and SGC. Both vertical and horizontal oriented cells were observed. Some vertical developed fusiform neurons were located at the boundary between the SGC and the SFGS and their apical prolongations reached the SFGS (Fig. 2f).

There was another population of CR-like immunopositive cells located close to the SGC-SFGS boundary. These were pyramidal neurons with two ascending prolongations and another prolongation which descended towards deeper strata (Fig. 2d). The morphological characteristics of these cells coincide with those classified as type X neurons by Meek and Schellart [6].

The tectal layers receiving the retinal input (SFGS and SO) displayed the highest density of CR-like immunoreactive fibers (Fig. 2a,c). They were observed throughout all the extension of the SFGS. These CR-like labeled fibers may correspond to axons and terminals coming from the retina. The distribution pattern of CR-like immunopositive fibers in the SO showed a clear trilaminated appearance. Thus, abundant CR-like immunoreactive fibers were seen in the superficial and deep regions, whereas only a few immunopositive fibers were detected in the middle of the stratum (Fig. 2c). This laminated distribution of CR-like immunostaining within the SO was less distinct in the ventrolateral area of the optic tectum.

4. Discussion

The distribution pattern of CR-like immunoreactivity in the teleost optic tectum is more complex than that previously reported for CaBP [8]. We consider that these differences are due to variations in the immunocytochemical procedure. Thus, bundles of fibers in the SFGS and SO, and cells in the SPV are positive after immunostaining with antibodies raised against both calcium binding proteins, whereas the CR-like positive cells located in the SAC and SGC have not been previously described after CaBP-immunostaining.

The distribution of CR-like positive cells along the SPV is similar to that of CaBP-stained neurons as shown by Miguel-Hidalgo et al. [8]. Only a subgroup of periventricular cells is immunoreactive to CR and CaBP. Although the morphological characteristics of all periventricular neurons are similar, different groups of cells belonging to this neuronal type may be involved in different functions and may contain different neuronal markers. Other immunocytochemical studies confirm the chemical heterogeneity of these neurons. Thus, in addition to CR and CaBP, subpopulations of periventricular neurons are positive to choline acetyltransferase [3,27] and neuropeptide Y [16,24].

In addition to periventricular neurons, several CR-like immunopositive neurons located in the SAC and SGC, not previously described with CaBP immunocytochemistry, were observed. They showed a weak immunostaining and their dendritic fields were only partially stained, therefore the correspondence with the cell types described by Meek and Schellart [6] using silver impregnation is unclear. The only exception are the pyramidal neurons located in the SGC-SFGS boundary that demonstrated similar morphological characteristics to the type X neurons [6]. The present results provide for the first time an immunocytochemical marker for neurons located in the SAC and SGC. These neurons were negative to choline acetyltransferase [3], neuropeptide Y, enkephalin, substance P, serotonin and FMRFamide [24].

The distribution of CR-like labeled fibers in the SO and SFGS was similar to that of CaBP and coincided with that of retinotectal axons as observed in degeneration and autoradiographic studies [14]. In addition, after eye enucleation a reduction of CaBP-immunoreactivity has been detected in the SO and SFGS [8]. These results suggest that ganglion cells in the teleost retina should be positive for CaBP/CR [25]. CR-like immunopositive ganglion cells have been detected in other species such as monkey, pig, sheep, rat, cat, pigeon and salamander [12].

The optic tectum of teleosts is considered a comparable structure to the superior colliculus of mammals. Comparing our observations in the teleost optic tectum with previous data on the distribution of CR-like immunoreactivity in the rat superior colliculus both similarities and differences can be observed. The main coincidence is detected in the distribution of CR-like positive fibers. In the rat, the optic tract contains a dense bundle of CR-like immunoreactive fibers [1,22]. The retinorecipient layers of the superior colliculus, the target of these retinal axons, demonstrated a high density of CR-like immunostained fibers and terminals [4] as we have reported in the tench optic tectum.

Concerning the distribution of CR-like immunopositive intrinsic neurons in the rat superior colliculus, previous results are contradictory: Résibois and Rogers [17] have found CR-like immunopositive neurons restrictedly located in the deep layers, whereas Arai et al. [1] described CR-like immunoreactive cells in all layers of the rat superior colliculus. Rogers and

Résibois [22] described that CaBP-positive and CR-positive neurons are distributed in separate layers with no colocalization in the same cells of both calcium-binding proteins. In the chick optic tectum, CR is detected in many fibers in layers 1 and 2, in many weakly labeled neurons in layer 10, in some multipolar cells in layer 12, and in sparse, giant multipolar cells of layer 13 [19]. This distribution is more similar to our observations in the tench optic tectum, with a segregated distribution of CR-immunopositive fibers and cell bodies, with the immunolabeled cells located in the deep layers, and the fibers in more superficial strata.

It is hitherto unknown why only some neurons express one of these calcium-binding proteins. Since calmodulin is expressed in all eukaryotic cells [5], the simultaneous presence of other neuronal calcium-binding proteins, such as CaBP, CR, parvalbumin, hippocalcin, neurocalcin, and others in the same neurons may indicate a finer handling of calcium. These cells would be able to use different calcium-binding proteins for different calcium-related events.

Dechesne et al. [2] have proposed that CR plays a particular role in the sensory pathways. Our results suggest that CR or a related protein may be directly involved in the transmission of visual information reaching the optic tectum from the retina, and an additional effect through the presence in the tectal circuitry of intrinsic immunopositive cells located in the SPV, SAC and SGC.

This immunohistochemical study reveals the existence of CR or an immunologically related protein in the Central Nervous System of a teleost. On the other hand, Western blot analysis indicates that only one band corresponding to a protein of 28 kDa is found in the fish brain, whereas in the brain of tetrapod vertebrates there are two proteins, one of 28 kDa (CaBP) and another of 29 kDa (CR) [10]. The previous description of CaBP-immunopositive neurons in the tench optic tectum has been done using a polyclonal antibody raised in rabbits against CaBP isolated from chick [8]. CR and CaBP seem to be extraordinarily and separately conserved through vertebrate evolution [11], but the teleosts including the species used in the present study are a lateral line on vertebrate evolution and cannot be directly compared with species belonging to other classes of vertebrates [9]. Contrary to all other vertebrate classes, it seems that there is only one protein (CR/CaBP) in teleosts, corresponding to the single band observed in Western blots, and the antibodies raised against both calcium-binding proteins (obtained using proteins purified from mammals and birds) recognized this single protein. Since the CR-like immunostaining demonstrates new elements, always weakly labeled, in the teleost optic tectum, it indicates that our method using an anti-CR serum provides a more sensitive method to demonstrate elements containing the CR/CaBP calcium-binding protein existent in the teleost central nervous system.

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