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Co-localization of calretinin and parvalbumin with nicotinamide adenine dinucleotide phosphate-diaphorase in tench Mauthner cells

Carlos Crespo, Rosario Arévalo, Jesús G. Briñón, Angel Porteros, José Aijón, José R. Alonso*

Dpto. Biología Celular y Patología, Universidad de Salamanca, E-37007 Salamanca, Spain

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Abstract

The co-localization of calretinin (CR) and parvalbumin (PV) immunoreactivity with nicotinamide adenine dinucleotide phosphate-diaphorase (ND) activity was analyzed in the Mauthner cells of the tench. Mauthner cells were ND active, and ND staining was observed in the soma, axon cap region, and axon of these neurons. CR co-localized with ND in the axon of the Mauthner cells but not in the cell body or in the dendrites, whereas PV immunoreactivity co-localized with ND in the soma, axon and dendrites. The presence of two different calcium-binding proteins in the Mauthner cells indicates that these neurons need complex calcium-buffering systems. The co-localization of these calcium-binding proteins with ND might suggests their involvement in nitric oxide-related events. © 1998 Elsevier Science Ireland Ltd. All rights reserved

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The Mauthner cells have served, more than any other vertebrate neuron, as particular models in neuroscience. These neurons, exclusively present in the central nervous system of lampreys, fish and some amphibians, mediate fast-escape motor responses, important in predator avoidance after the reception of unexpected vibrational and/or visual stimuli [7]. The Mauthner cells receive numerous inputs from the eighth nerve, vestibular nuclei of the octavolateral area, reticular formation and tectobulbar and cerebellotegmental tracts [21] and project their axons through the medial longitudinal fascicle towards the spinal cord. Numerous reports have classified the different morphological and neurochemical types of afferences establishing synaptic contacts with Mauthner cells [9,11]. These studies have demonstrated that the different types of axonal ending display a topographically-specific distribution on the Mauthner cells surface, which may be therefore considered a 'miniature brain' with different 'topographical regions' [2].

Using nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry, it has been recently suggested that the Mauthner cells of teleosts contain nitric oxide synthase [2]. This enzyme produces nitric oxide that could be implicated in long-term potentiation (LTP) events taking place in the Mauthner cells at the level of both excitatory and inhibitory synaptic junctions [2]. Neurons producing nitric oxide require a high-sensitivity buffer system of the intracellular calcium levels, because neuronal nitric oxide synthase is dependent on calcium and calmodulin [19]. In addition, the LTP and short-term potentiation (STP) mechanisms, such as those reported in the Mauthner cells, require precise changes in the intracytoplasmic concentration of calcium at the postsynaptic level [15,18]. Finally, it has been recently reported in the Mauthner cells that their cellular coupling through gap-junctions requires a high postsynaptic increase in calcium concentrations [12]. Together, these previous data suggest that Mauthner cells need high-sensitivity calcium-buffer systems to regulate intracytoplasmatic calcium levels.

Calretinin (CR) and parvalbumin (PV) are two EF-hand calcium-binding proteins involved in calcium homeostasis [8]. These calcium-binding proteins appear in specific neu-

* Corresponding author. Tel.: +34 23 294400, ext. 1854; fax: +34 23 294549; e-mail: jralonso@gugu.usal.es

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ronal populations throughout the central nervous systems of vertebrates [1], but their specific functions in these neuronal populations remain, so far, poorly understood. In the present study, we analyze the presence of CR, PV and their co-localization with NADPH-diaphorase (ND) in the Mauthner cells of a cyprinid teleost, the tench.

Ten adult males and females of *Tinca tinca* (L., 1758), obtained from commercial sources (Fisheries 'Ipescon,' Salamanca, Spain), were deeply anesthetized using an aqueous solution of 0.03% tricaine methanesulfonate (MS-222, Sandoz, Basel, Switzerland), and perfused through the conus arteriosus with 50 ml of 0.63% saline solution containing 5 UI/ml heparin followed by 150 ml of a fixative solution composed of 4% (w./v.) paraformaldehyde and 2% (w./v.) picric acid in 0.1 M phosphate buffer, pH 7.4 (PB). After perfusion, the brains were removed and postfixed by immersion in the same fixative for an additional 4 h at 4°C. After rinsing in PB, tissue was cryoprotected with 30% (w./v.) sucrose in PB. Coronal and sagittal 20-μm thickness sections were serially cut on a Leica cryostat, and thaw-mounted on chrome alum-gelatinized slides. A one-in-three series from eight animals was processed for ND histochemistry as previously reported [3]. Briefly, sections were incubated at 37°C for 1–2 h in 1 mM β-NADPH, 0.8 mM nitro blue tetrazolium, and 0.06% Triton X-100 in 0.1 M phosphate buffer (pH 7.6). All chemicals were purchased from Sigma, St. Louis, MO. Second and third series from these eight specimens were processed for the demonstration of CR and PV immunoreactivity, respectively, as previously described [6]. Briefly, sections were incubated in primary antibody (Swant, Bellinzona, Switzerland; CR, 1:10 000 in PB; PV, 1:1000 in PB, 48 h at 4°C), biotinylated anti-mouse immunogammaglobulin (Vector, Burlingame, CA; 1:250 in PB, 2 h at room temperature) and Vectastain Elite ABC reagent (Vector; 1:250 in PB, 2 h at room temperature). The sections from the two other animals were used for ND/CR or ND/PV double labeling and for the specificity controls of the ND histochemistry and CR and PV immunocytochemistry [3,6]. In ND/CR and in ND/PV double-labeled sections, the CR or PV immunocytochemical staining was carried out after ND histochemistry, as previously reported [4]. No residual staining was found for controls.

The Mauthner cells of the tench were located dorsal to the superior reticular formation at the intermediate level between the trigeminal and the abducens nuclei. These neurons were easily identified by their particular morphology and gigantic size (around 130 μm in maximum diameter). After ND staining, the soma, the axon cap region and long portions of the axon displayed a strong ND activity, whereas only the initial portion of the dendrites were ND-labeled (Fig. 1a). In addition to the Mauthner axons, their myelin sheaths were ND-labeled after ND histochemistry (Fig. 1b). Occasionally, large ND-positive oligodendrocytes (even 25 μm in maximum diameter) were visualized with their processes ensheathing the Mauthner axons. After CR and PV immunocytochemistry both proteins appeared in the tench Mauthner cells. CR immunoreactivity was detected in the Mauthner axon that coursed within the dorsal part of the medial longitudinal fascicle (Fig. 1e), but it was not found in the soma or in the dendrites of these neurons (Fig. 1c,d). By contrast, PV immunolabeling was found in the Mauthner soma, dendrites and axon (Fig. 1f–h). In addition, the Mauthner cells were surrounded by a dense network of CR and PV immunopositive axon terminals. CR-containing axon terminals showed a topographically-specific distribution: small club-like endings were found innervating the soma and lateral dendrite, whereas the axon cap region was surrounded by large club-like terminals (Fig. 1c,d). PV immunopositive axon terminals were found innervating the lateral dendrite (Fig. 1g), whereas no PV immunopositive axon endings were found surrounding the soma, the ventral dendrite or the axon cap region.

This study describes for the first time the presence of buffer calcium-binding proteins, such as CR or PV, in the Mauthner cells, and their co-localization with ND activity in these neurons. The ND activity found in the Mauthner cells of the tench is in agreement with previous reports in the Mauthner cells of the lamprey and the swordtail fish [2,16] suggesting a role of nitric oxide in the circuitries of fast-escape responses. Neurons constitutively synthesizing nitric oxide require a high-sensitivity calcium-buffer system, since the synthesis of this neurotransmitter is calcium-dependent [19]. Although calmodulin is the only calcium-binding protein whose implication in the synthesis of nitric oxide has been demonstrated, it has been suggested that some buffer calcium-binding proteins such as CR or PV might also be implicated in the calcium buffering events needed for the synthesis of nitric oxide [14]. The presence of nitric oxide synthase in Mauthner cells could indicate an involvement of nitric oxide, as retrograde messenger, in LTP events taking place in these neurons [2]. The co-localization of ND with CR and PV in Mauthner cells may suggest a functional implication of these calcium-binding proteins in LTP. Both LTP and STP are calcium-dependent events [15,18] and the expression of CR has been demonstrated to contribute to the control of synaptic plasticity in LTP of mouse dentate gyrus [17]. A similar involvement of CR and/or PV in synaptic plasticity of Mauthner cells can be hypothesized. Moreover, the STP of electrotonic synapses in Mauthner cells requires a high increase in the postsynaptic concentration of calcium mediated via activation of postsynaptic NMDA receptors [12]. PV could constitute an effective calcium sensor to regulate the homeostasis of this cation after high increases in the Mauthner cells.

The heterogeneous distributions of CR and PV in the brain [1,8], and their different subcellular distributions in the Mauthner cells argue for different and independent roles for both proteins. The Mauthner cells are fast-firing neurons involved in the motor startle reflex [9], and PV may shorten the refractory phase of action potentials in the fast-firing events and thus protect the cells from calcium overload. A

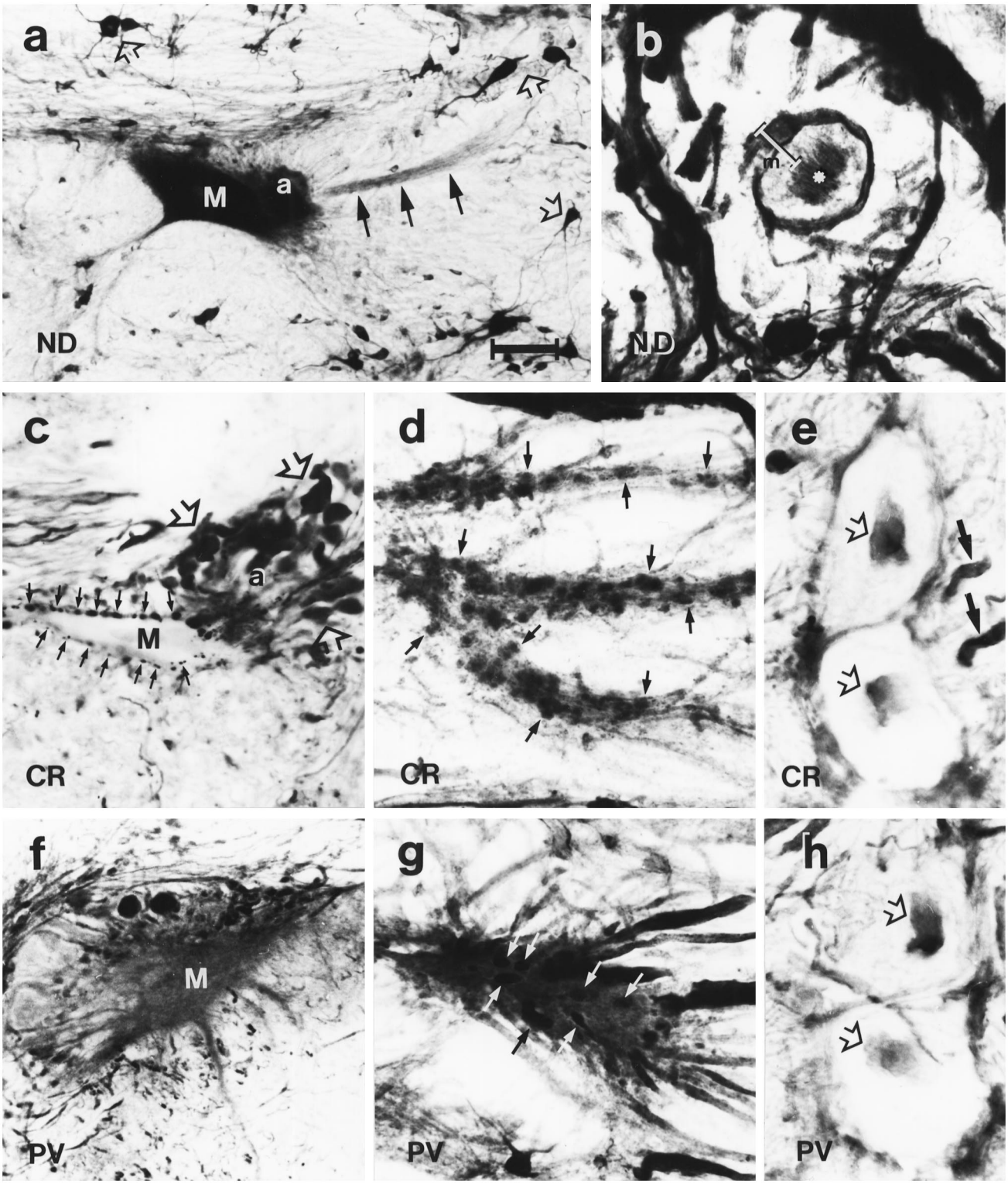

Fig. 1. (a) ND staining in the soma (M), axon cap region (a), and axon (arrows) of a Mauthner cell. Adjacent ND-active neurons can be observed (open arrows). (b) ND-active axon of a Mauthner cell (asterisk) coursing through the medial longitudinal fascicle. Note that the thick myelin sheath (m) displays ND activity, mainly in its outer portion. (c) The soma (M) and axon cap region (a) of Mauthner cells are CR immunonegative, but appear innervated by small (arrows) and large (open arrows) CR-containing axon endings respectively. (d) CR immunonegative lateral dendrite of a Mauthner cell surrounded by CR immunostained axon terminals (arrows). (e) CR immunopositive rostral Mauthner axons (open arrows). Adjacent CR immunolabeled axons of the medial longitudinal fascicle can be observed (arrows). (f) PV-containing soma of a Mauthner cell (M). (g) PV-immunopositive lateral dendrite of Mauthner cells innervated by PV-containing axon terminals (arrows). (h) PV-immunopositive rostral Mauthner axons (open arrows) in the medial longitudinal fascicle. Scale bar (a,c,f), 50 μm; (b,d,e,g,h) 25 μm.

similar role has been proposed for PV in fast-firing neurons of the mammalian cortex [5]. The axons of the Mauthner cells have been reported to contain the basic components of the protein synthesis [20]. This fact may account for the specific presence of CR immunoreactivity in the Mauthner axons but not in the somata or dendrites of these neurons. The specific distribution of CR in the axons suggests an involvement of CR-mediated calcium buffering mechanisms in functional aspects of the axonal physiology such as neurotransmitter release, or nervous signal transduction. It has been demonstrated that the ultrastructural localization of calcium ions in the Mauthner cells changes under normal conditions and after prolonged stimulation [10]. These authors suggest that membrane subsynaptic organelles are the primary structures which sequester, accumulate and retain calcium and in consequence control many physiological events of Mauthner cells. The presence of CR in the axons of Mauthner cells may constitute an additional calcium-sequestering system further modulating the levels of this cation. Finally, calcium-activated proteolysis of neurofilament proteins has been demonstrated in the goldfish Mauthner axons [13]. Calcium sequestration by calcium-binding proteins such as CR or PV in these axons could affect the ability to degrade neurofilament proteins and thus account for the delayed degradation of these proteins in distal segments of Mauthner axons maintained in vivo [13]. Our study indicates that the Mauthner cells constitute an interesting model to elucidate the roles of calcium-binding proteins in the neuronal physiology and their relation with nitric oxide.

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