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USE OF CALRETININ (CR) AND PARVABUMIN (PV) AS MAUTHNER CELL MARKERS IN SEA BASS LARVAE (*DICENTRARCHUS LABRAX*)

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Introduction

Studies on Mauthner neurons have provided fundamental information on neural biochemistry, development, synaptic morphology and physiology, and control of behaviour as fast-escape motor response after the reception of unexpected vibrational and/or visual stimuli (Eaton and Bombardieri, 1978) that can be generalized to many central neurons throughout vertebrates (Faber and Korn, 1978a; Nissanov and Eaton, 1989; Korn et al., 1990). Different studies using immunohistochemistry have described the calretinin (CR) and parvalbumin (PV) positive Mauthner cells (Crespo et al., 1998), revealing that their presence indicates these neurons need complex calcium-buffering system. Description of Mauthner cells in fish larvae constitutes a powerful tool to study fish larval behaviour. In the present study, we analyze the presence of these antibodies in the Mauthner cells of European Sea Bass larvae (*Dicentrarchus labrax*).

Material and methods

European Sea Bass larvae were obtained from natural spawning from France (Ecloserie Marine de Gravelines, Nord-Pas-de-Calais). The experiment was carried out in the Grupo de Investigación en Acuicultura facilities (Las Palmas de Gran Canaria, Canary Islands, Spain). Larvae were distributed into 2 tanks ($2m^3$) and fed with rotifers enriched with EFA (Selco, DHA Protein Selco, INVE, Dendermonde, Belgium) until they reached 15dah. At 7dah, larvae were fed with *Artemia* enriched with EFA followed by a commercial microdiet until they reached 50dah. All tanks were supplied with filtered sea water ($34g.l^{-1}$ salinity). Water temperature and dissolved oxygen during the experimental period ranged between 16.5-21ºC and 5.04-8.32ppm, respectively.

Sixty larvae were collected daily and fixed in 10% buffered formalin, dehydrated through graded alcohols, xylene, and finally embedded in paraffin wax.

Paraffin-embedded complete larvae sections were serially cut on Leica microtome at 3µm, stained with haematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970), Nissl (Raimundo García del Moral, 1993), and histologically evaluated using immunohistochemistry techniques.

For immunohistochemistry, some slides for each paraffin sections were collected on Poly-L coated slides. Sea bass larvae were processed for the demonstration of calretinin (CR) and parvalbumin (PV) immunoreactivity. The slides were dewaxed in xylene, rehydrated through graded alcohols, and incubated with 3% hydrogen peroxidase in methanol for 30min on a moving platform to block endogenous peroxidase activity. Enzymatic treatment, protease, was applied according to the used primary antibody. The enzymatic treatment applied in both primary antibodies (Swant, Bellinzona, Switzerland; CR, 1:700; PV, 1:700) was pronase 0.1% in PBS for 3min at room temperature. After that, slices were covered with 10% goat serum (for both polyclonal antibodies, PV and CR) in PBS for 30min before incubation with the primary antibody for 18h at 4ºC. When primary polyclonal antibody was used, a biotinylated pig anti-rabbit immunoglobulin G diluted 1 in 250 in PBS was applied for 30min as secondary reagent. An avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Burlingame, CA) diluted 1 in 50 in PBS was applied for 1h at room temperature to detect the different substrates. Slides were then incubated with DAB (3,3-diaminobenzidina tetraclorhidrato), diluted in Tris 0.1M containing hydrogen peroxide 3%, and checked microscopically for adequate chromogen development. Finally, sections were rinsed in tap water, counterstained with Harris´ Hematoxylin, dehydrated and mounted. Negative controls were performed replacing each primary antibody by PBS.

Results and discussion

The Mauthner cells appeared dorsally to the medial reticular nucleus and lateral to medial longitudinal fascicle nucleus, coinciding with the emergence of the magnocellular nucleus of the *Area octavolateralis* in sea bass. Our recent work has shown that CR antibody was only detected in Mauthner axons that coursed within the dorsal part of the medial longitudinal fascicle in larvae with more 32dah (Fig. 1a) in agreement with the result found in tench (*Tinca tinca*) by Crespo et al. (1998). 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 (Crespo et al., 1998). It has been demonstrated that the ultrastructural localization of calcium ions in the Mauthner cells changes under normal conditions and after prolonged stimulation (Moshkov et al., 1995).

Results showed that larvae at 6 and 10dah showed PV immunonegative in the Mauthner soma, dendrites and axon. By contrast, the first PV-reactive neurons

appeared in larvae from 13dah. PV immunolabeling was found in dendrites and axon (Fig. 1b). In larvae from 17dah, PV immunoreactive positive neurons were clearly distinguishable in all structures as dendrites, soma, and axons. In this study from 19dah, Mauthner soma, dendrites and axon were found to show a strong positive immunoreaction for a PV-like protein. In addition, there was still a marked PV immunonegative in the Mauthner soma membrane. The CR and PV activity found in agreement with previous reports in the Mauthner cell of lamprey (*Lampetra planeri*) (Schober et al., 1994), swordtail fish (*Xiphophorus helleri*) (Anken et al., 1996) and tench (Crespo et al., 1998), suggesting a role of nitric oxide in the circuits of fast escape responses. The presence of nitric oxide synthase in Mauthner cells could indicate an involvement of nitric oxide, as retrograde messenger, in long-term potentiation events taking place in these neurons (Anken et al., 1996).

The present study described a first report of the sea bass Mauthner cells development which will constitute an important tool in the understanding of the alternations in behavior found in fish larvae along development. Besides, it will further allow us to undertake tracing studies focusing the description of several sensorial systems.

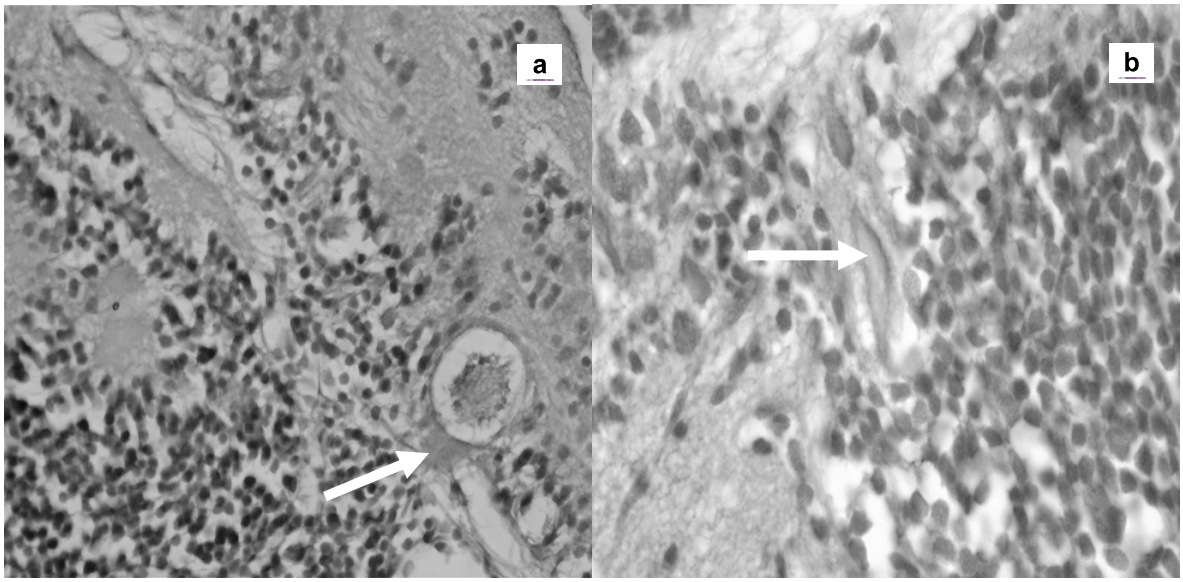

Fig. 1. (a) CR-immunositivity in Mauthner axon that coursed within the dorsal part of the medial longitudinal fascicle. (b) PV-immunopositivity in the Mauthner dendrites and axon and PV immunonegativity in soma. Scale bar: 100μm.

References

Bligh E.G. and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37:911-917.

Anken R.H., I. Sorger, D. Bremen and H. Rahmann. 1996. NADPH diaphorase reactivity in the Mauthner cells of the swordtail fish, Xiphophorus helleri, Neuroscience Letters 206:49–52.

Crespo C., R. Arévalo, J.G. Briñón, A. Porteros, J. Aijón and J.R. Alonso. 1998. Co-localization of calretinin and parvalbumin with nicotinamide adenine dinucleotide phosphate-diaphorase in tench Mauthner cells. Neuroscience Letters 250:107-110.

Eaton R.C. and R.A. Bombardier. 1978. Behavioral functions of the Mauthner neuron. p.221–244 In: Neurobiology of the Mauthner Cell, D.S. Faber and H. Korn (Eds.), Raven Press, New York.

Faber D.S. and H. Korn. 1978. Electrophysiology of the Mauthner cell: basic properties, synaptic mechanisms, and associated networks. p.47–131 In: Neurobiology of the Mauthner Cell, D. Faber, H. Korn (Eds.), Raven Press, New York.

Korn H., D.S., Faber and A. Triller. 1990. Convergence of morphological, physiological, and immunocytochemical techniques for the study of single Mauthner cells. p. 403–480p In: Handbook of Chemical Neuroanatomy, Vol. 8, A. Björklund, T. Hökfelt, F.G. Wouterlood and A.N. van den Pol (Eds.), Elsevier, Amsterdam.

Martoja R. and M. Martoja-Pierson. 1970. Técnicas de Histología Animal. Toray-Masson S.A., Barcelona, 350p.

Moshkov D.A. and I.M. Santalova. 1995. Distribution of calcium pyroantimonate precipitates in Xenotoca Mauthner cells at normal and increased functional activity. Neuroscience, 65:917–925.

Nissanov J., and R. C. Eaton. 1989. Reticulospinal control of rapid escape turning in fishes. Brain, Behaviour and Evolution. 37:272-285.

Raimundo García del Moral, 1993. Laboratorio de Anatomía Patológica. McGraw Hill Interamericana de España. Spain. 210p.

Schober A., C. Malz, W. Schober and D. Meyer. 1994. NADPH diaphorase in the central nervous system of the larval lamprey (Lampetra planeri), Journal of Comparative Neurology 345:94–104.