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Localization of neurotransmitters, peptides and nNOS in the pseudobranchial neurosecretory cell system and associated carotid labyrinth of the catfish, *Clarias batrachus*

Daniele Zaccone[a,*], Anita Gopesh[b], Giuseppe Anastasi[c], Angelo Favaloro[c], Alessandra Sfacteria[d], Fabio Marino[d]

[a] *Department of Animal Biology and Marine Ecology, Faculty of Science, University of Messina, I- 98166 Messina, Italy*
[b] *Department of Zoology, University of Allahabad, Allahabad, India*
[c] *Department of Biomorphology and Biotechnologies, Faculty of Medicine, University of Messina, Italy*
[d] *Unit of Veterinary Pathology, Dept. Sanità Pubblica Veterinaria, University of Messina, Polo Universitario dell'Annunziata, I-98168 Messina, Italy*

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ABSTRACT

The carotid labyrinth is an enigmatic endocrine structure of unknown chemosensory function lying in the gill region of the catfishes. The carotid body is found at the carotid bifurcation of amphibians and all mammalian vertebrates on the evolutionary tree. It is a vascular expansion comprised of a cluster of glomus cells with associated (afferent and efferent) innervations. In the catfish species studied (*Clarias batrachus*) a neurosecretory cell system consisting of pseudobranchial neurosecretory cells connect the carotid labyrinth or large vessels (both the efferent branchial artery and dorsal aorta), and is likely akin to the glomus cells, but comparing these structures in widely divergent vertebrate species, the conclusion is that the structural components are more elaborate than those of terrestrial vertebrates. However, these cells reveal both an endocrine phenotype (such as the association with capillaries and large vessels) and the presence of regulatory substances such as neurotransmitters and neuropeptides producing good evidence for high levels of conservation of these substances that are present in the glomus cells of mammalian vertebrates. VIP-immunopositive neuronal cell bodies are detected in the periphery of the carotid labyrinth. They are presumptive local neurons that differ from pseudobranchial neurosecretory cells, the latter failing to express VIP in their soma.

Introduction

Evolution has devised several specialized types or receptor organs to monitor closely changes in oxygen levels, or the corresponding carbon dioxide and pH levels (Van Lommel, 2009). These include arterial, lung vascular, and brain stem respiratory center receptors. The arterial chemoreceptors (i.e. the carotid bodies and aortic bodies) are composed of highly vascularized and densely innervated clusters of sustentacular and glomus cells (see review by Kummer, 1996). The glomus cells, revealing an endocrine phenotype, are closely associated with capillaries derived from the body's main arteries, the carotid artery and aorta. There is a long standing debate about a possible endocrine role for the carotid body, which also functions as a structure that detects changes in the chemical composition, tonicity and temperature of its environment (reviewed by Acker, 1989).

There is now considerable evidence regarding the peripheral control of ventilation by the carotid body, which receives afferent innervations from the carotid sinus nerve (CSN), a branch of the glossopharyngeal nerve (Gonzales et al., 1994). In addition to this afferent pathway, different sources of efferent innervation to the carotid body have been proposed that include: (a) a postganglionic sympathetic supply arising from the superior cervical sympathetic ganglion (reviewed by Kummer, 1996); (b) autonomic neurons that are directly attached to the carotid body in most species (reviewed by Kummer, 1996) and (c) individual ganglion cells or small groups of neurons that are located along the course of the glossopharyngeal and CSN (Campanucci et al., 2006). Campanucci et al. (2006) have further demonstrated a key role of nitric oxide (NO) in mediating the efferent inhibition of rat carotid body chemoreception via NO release by neurons located at the site of branching of the CSN and glossopharyngeal nerve. The carotid body is regarded as a source of

Abbreviations: ATP, adenosine triphosphate; ChAT, choline acetyltransferase; CSN, carotid sinus nerve; NEC, neuroepithelial cell; eNOS, endothelial nitric oxide synthase; EBA, efferent branchial artery; nNOS, neuronal nitric oxide; NO, nitric oxide; PACAP, pituitary adenylate cyclase activating polypeptide; SP, substance P; TH, tyrosine hydroxylase; VIP, vasoactive intestinal polypeptide.

* Corresponding author.
E-mail address: zacconed@unime.it (D. Zaccone).

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both nNOS and eNOS synthase isoforms and NO has an inhibitory effect on carotid body function (Prabhakar, 1999).

The amphibians have a pair of small vascular expansions at the bifurcation of the common carotid artery into the internal and external carotid arteries. These vascular expansions have been called the carotid labyrinths, and are composed of glomus cells (type 1 cells) showing afferent, efferent and reciprocal synapses on these cells (reviewed by Kusakabe, 2009). The amphibian carotid labyrinths function as peripheral arterial chemoreceptors and baroreceptors sensitive to changes in arterial O_2 and CO_2 tension levels, as well as hydrogen ion concentrations, and levels of blood pressure (Ishii et al., 1966), thus sustaining an important role in the regulation of respiratory and cardiovascular systems. Kusakabe (2002) indicated the importance of the carotid labyrinths in the maintenance of homeostasis and blood pressure and blood supply to cephalic regions.

While analogy of amphibian carotid labyrinths and the mammalian carotid body has been considered, the ontogeny of the carotid labyrinths in catfishes is still questioned. In amphibians, the carotid labyrinths are classified into two groups according to the origin of the external and internal carotid arteries. Carotid labyrinths of catfishes have a topographical relationship with the principal head arteries (Srivastava and Singh, 1980), but an alternative view about the origin of the carotid labyrinth in these fishes is concerned with the corpus cavernosum and the pseudobranch (Hughes and Munshi, 1979). Moreover, in catfishes a peculiar pseudobranchial neurosecretory cell system is found in close proximity to the efferent branchial artery (EBA) and carotid labyrinth (reviewed by Gopesh, 2009).

Using immunohistochemical markers of mammalian carotid bodies, the aim of the study was to demonstrate the presence of these markers in the pseudobranchial cells and associated carotid labyrinth of the catfish, *C. batrachus*, to show high levels of conservation in the components of the autonomic innervation of these structures.

Materials and methods

Tissue preparation

Ten adult specimens (100 g body weight) of Indian male catfish, *C. batrachus* Linnaeus 1758, were collected locally in ponds at Hallahabad (Uttar Pradesh State, India) in July, ambient temperature 28 °C and transferred to the Neurobiology Division of the University of Allahabad, India. They were maintained in circulating aquaria at 28 °C for a brief period of time (one week) since this fish consumes more oxygen from air (58%) than from normoxic water (42%). Fishes were anesthetized in 0.016% MS222 (tricaine methanesulphonate, Sandoz, Hanover, NJ, USA) prior to the fixation of the tissues. The carotid labyrinths with adjacent tissues were dissected on site. Tissues after fixation were dehydrated in a graded series of ethanols and stored in 70% ethanol.

Histology

Freshly dissected neurosecretory cell masses, carotid labyrinths, surrounding tissues, and attached nerves were removed and immediately fixed in 4% paraformaldehyde (PFA) in 0.1 phosphate buffered saline (PBS), pH 7.4 for 2–4 h, dehydrated in an ascending series of ethanols and tissues were routinely processed for embedding in Paraplast wax (McCormick Scientific, St. Louis, MO, USA). 8 μm sections were cut with a rotary microtome. Sections were stained with hematoxylin-eosin for general assessment of tissue structure. Sections for immunohistochemistry were mounted with Vectashield (see below).

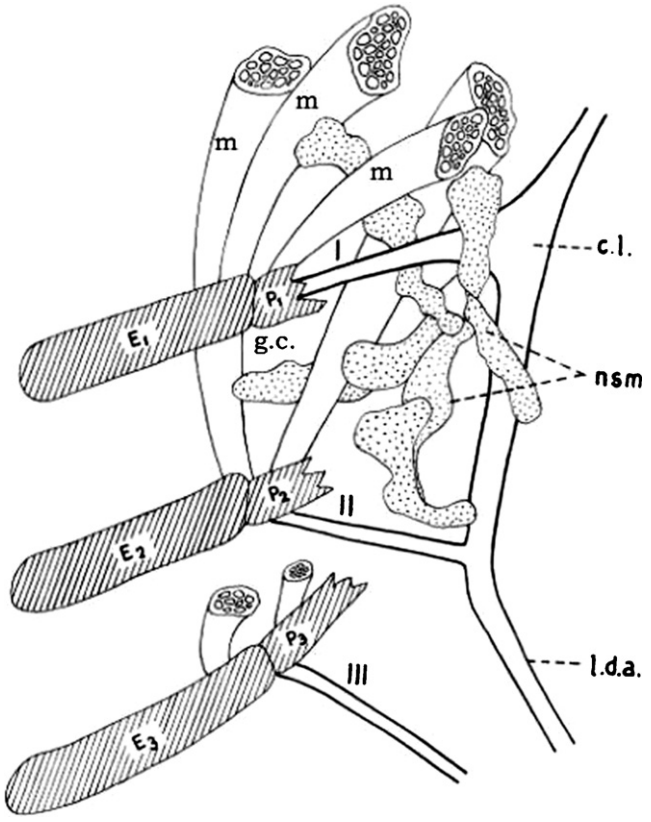

Fig. 1. Schematic sketch of dissected palate of the catfish, *Clarias batrachus*, showing position of the pseudobranchial neurosecretory cell masses (NSM) in relation to the gill cavity, muscle fascicles (M), aortic arches (I–III) and carotid labyrinth (CL). E1, first epibranchial arch; P1, first pharyngobranchial arch; GC, gill cavity; E2, second epibranchial arch; P2, second pharyngobranchial arch; E3, third epibranchial arch; P3, third pharynobranchial arch; LDA, lateral dorsal aorta.

Confocal immunofluorescence

For immunohistochemistry, sections were deparaffinized and rehydrated, rinsed several times in PBS and blocked in 10% normal goat serum for 1 h. Primary antibodies (used individually or in combination, Table 1) were diluted in a permeabilizing solution (PBS, 0.2% Triton x-100, 0.1% sodium azide) according to the optimal dilutions (detailed in Table 1) and placed on the slides to incubate overnight at room temperature. Sections were then treated with fluorescently labelled secondary antibodies diluted in PBS (goat-anti-rabbit secondary antibodies conjugated with fluorescein isothiocyanate, FITC), 1:50 and goat-anti-mouse secondary antibodies conjugated with the red fluorophore, Alexa 58, 1:100 and left to incubate at room temperature for 2 h in the dark. After washing, the sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) to prevent photobleaching, and cover-slipped.

Control experiments

In control experiments, either preincubation with blocking peptide or omission of the primary antibody resulted in complete abolition of immunostaining. Blocking peptides for ChAT, TH, nNOS, VIP, PACAP and SP (Table 2) were preincubated with primary antibodies (3 μg/l per μg peptide) overnight at 4 °C before application.

Samples were viewed with a Zeiss LSM DUO confocal laser scanning microscope with META module (Carl Zeiss AG, Oberkochen, Germany). All images were digitized at a resolution of 8 bits into an array of 2048 × 2048 pixels. Optical sections of fluorescent speci-