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Valentina Patrizia Gallo [a]; Fiorenza Accordi [a]; Jan Ohlberger [a]; Annalena Civinini [a]
[a] Dipartimento di Biologia Animale e dell'Uomo, Università "La Sapienza", Roma, Italy

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# The chromaffin system of the beluga sturgeon *Huso huso* (Chondrostei): histological, immunohistochemical and ultrastructural study

VALENTINA PATRIZIA GALLO
FIORENZA ACCORDI
JAN OHLBERGER
ANNALENA CIVININI
Dipartimento di Biologia Animale e dell'Uomo,
Università "La Sapienza",
Viale dell'Università 32, I-00185 Roma (Italy)
E-mail: *valentina.gallo@uniroma1.it*

ABSTRACT

The histology and fine structure of the chromaffin cells were examined in the beluga sturgeon *Huso huso* Linnaeus, 1758. Chromaffin cells, identified by the chromaffin reaction and immunohistochemical reactions for tyrosine hydroxylase, dopamine-β-hydroxylase and phenylethanolamine-N-methyltransferase, were spread over the entire length of the kidney, mainly localized in the walls of the cardinal and caudal veins and their main branches. Ultrastructural analysis revealed two different types of catecholamine containing cells distinguished by their secretory granules, as in other vertebrate species. According to the different electron-densities of the granules, the cells could be distinguished as adrenaline and noradrenaline producing cells. The presence of nerve fibres and a few endings led us to hypothesize the neural control of catecholamine secretion. Moreover, the positivity of the immunohistochemical reaction for neuronal nitric oxide synthase, an enzyme that catalyzes the synthesis of nitric oxide (NO), in nerve elements close to the chromaffin cells suggested the involvement of NO in the regulation of cell function. The distribution, cytological aspects and regulation of the chromaffin system of the beluga sturgeon are compared with the same system in other bony fishes.

KEY WORDS: *Huso huso* - Chondrostei - Chromaffin system - Immunohistochemistry - Ultrastructure.

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## INTRODUCTION

In fishes, the adrenergic chromaffin cells and the interrenal steroidogenic cells form the so-called adrenal homolog. Most research on this topic has been performed on teleosts, the modern bony fishes, whereas few studies have dealt with non-teleost fishes. In teleosts, the chromaffin tissue is mainly localized in the anterior part of the kidney or head kidney associated with the posterior cardinal veins, although it can also be found in the posterior kidney in certain species; the interrenal tissue is confined to the head kidney. The two tissues can be separate, contiguous or intermingled, but do not form glandular capsules. The chromaffin tissue is easily identified by the chromaffin reaction (which gave the cells their name). The distribution of these cells in fishes has been studied by histological, immunohistochemical and cytological techniques and the adrenaline and noradrenaline content has been confirmed biochemically in some species (see reviews by Chester Jones & Mosley, 1980; Reid *et al.*, 1998; Gallo & Civinini, 2003).

The living chondrosteans – sturgeons, paddlefishes and bichirs – are primitive bony fishes, which separated in the Palaeozoic from the lines leading to holosteans and teleosts (Neopterygii). There is little information about the adrenal homolog of Chondrostei, most of it regarding species belonging to the order Acipenseriformes. A hundred years ago, Giacomini (1904) examined the kidney and the adrenal homolog of the Baltic sturgeon *Acipenser sturio*. Using the chromaffin reaction, he found chromaffin cells in the walls of the posterior cardinal veins, especially of the larger left one, and in the walls of the renal veins. Subsequent studies (Giacomini, 1933, 1934) were performed on *A. rutenus*, *Scaphirhynchus platyrhynchus* and *Polyodon spathula*. The adrenergic system of *Huso huso* was studied by fluorescence analysis and catecholamine content by Balashov *et al.* (1981). Green fluorescent chromaffin cells were found mainly in the walls of the posterior cardinal veins and also in the wall of the celiaco-mesenteric artery; fluorescent ganglion cells were also found along the splanchnic nerve following the celiaco-mesenteric artery. Large amounts of catecholamines, especially adrenaline, were found in the cardinal veins, and small amounts of both catecholamines were detected in the celiaco-mesenteric artery.

As regards the second order of living chondrosteans, the Polypteriformes, De Smet (1970) described the adrenal homolog of *Polypterus*. He was unable to clearly recognize chromaffin cells in specimens preserved with dichromate fixative; nevertheless he considered a few isolated, voluminous cells with a large, round nucleus and distinct granulation in the walls of the posterior cardinal veins to be "homologues of the adrenalinogenic cells of higher vertebrates".

Because of the interesting phylogenetic position of Chondrostei, we thought that it would be useful to ex-

amine other aspects of the chromaffin system in this taxon, as part of a comparative study of the adrenal homolog and its control in lower vertebrates. Therefore, we studied the histology and cytology of the chromaffin cells and the immunohistochemical localization of the enzymes involved in catecholamine synthesis in the beluga sturgeon *H. huso* Linnaeus, 1758. We also investigated the immunohistochemical localization of the enzyme neuronal nitric oxide synthase (nNOS), which catalyzes the synthesis of nitric oxide (NO). This molecule appears to be involved in the regulation of cellular activity of the adrenal gland. In fact, nNOS has been localized in mammalian chromaffin cells and nerve elements (Afework *et al.*, 1992, 1995; Holmberg *et al.*, 1998; Schwarz *et al.*, 1998) and in frog chromaffin cells (Cartier *et al.*, 2001); among fishes, it has been found to be sparsely localized in chromaffin cells and more frequently in nerve elements close to the adrenal homolog in the teleost *Oncorhynchus mykiss* (Gallo & Civinini, 2001).

## MATERIALS AND METHODS

### *Specimens*

The study was performed on six specimens of the beluga (or giant) sturgeon *H. huso* (70-100 cm long) obtained from a commercial supplier (Paolucci, La Doganella, Rome). Deep anaesthesia was induced by immersion in a solution of MS-222 (tricaine methane sulfonate, Aldrich, Milwaukee) at a concentration of 0.5 g/litre.

### *Histology and immunohistochemistry*

Fragments of tissue, some of them containing the cardinal and caudal veins, were taken from the anterior, intermediate and posterior kidney. They were fixed in Wood's (1963) fixative for normal histology or in cold Bouin for 6 h for immunohistochemistry, and then embedded in paraffin. Sections (6 µm thick) were stained with haematoxylin-eosin or processed for immunohistochemical reactions. The reactions were performed as formerly described (Gallo & Civinini, 2001) using the LSAB kit (Dako) based on the labelled streptavidin-biotin method. The sections were incubated in the following antisera: anti-tyrosine hydroxylase (anti-TH, Eugene Tech, 1:500, 24 h, 4 °C), anti-dopamine-β-hydroxylase (anti-DβH, Eugene Tech, 1:200, 24 h, 4 °C), anti-phenylethanolamine-N-methyltransferase (anti-PNMT, Eugene Tech, 1:1000, 3 h, 4 °C) and anti-neuronal nitric oxide synthase (anti-NOS-I, Santa Cruz Biotechnology, 1:800, 24 h, 4 °C). The reaction was developed using 3-amino-9-ethylcarbazole (AEC) as chromogen and resulted in a red-coloured precipitate at the antigen site. Omitting the primary antisera and replacing specific antiserum with normal rabbit serum controlled the specificity of the antibodies.

### *Electron microscopy*

Kidney fragments close to the veins were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 for 3 h at 4 °C and postfixed in 1% osmium tetroxide in Millonig buffer for 1 h. After dehydration in a graded alcohol series and xylene, the samples were embedded in Araldite. Semithin sections (2 µm) were stained with methylene blue to localize the chromaffin cells. Thin sections (800 Å) stained with uranyl acetate and lead citrate were examined with a Philips CM 10 transmission electron microscope.

## RESULTS

The kidney of the beluga sturgeon is situated between the peritoneum and vertebral column, from the heart to the cloaca. It is Y-shaped with the anterior part, or head kidney, divided into two halves and the posterior part fused in a single body. The anterior and posterior parts are connected by rather thin right and left intermediate parts (Fig. 1). The caudal vein is situated on the dorsal surface of the posterior kidney and divides into the two cardinal veins at the bifurcation point of the kidney. The histological study showed that the three parts of the kidney are composed of haematopoietic tissue, renal corpuscles and tubules; the renal tubules are prevalent in the posterior part of the kidney. The chromaffin cells, clearly recognizable by their yellow-orange colour in samples fixed in Wood's fixative and stained with haematoxylin-eosin, are localized in the walls of the caudal and cardinal veins (Figs 2, 3). There is no difference among the three parts of the kidney with regard to the presence and localization of the chromaffin cells. They can be isolated or grouped, deeply embedded in the vessel walls (Fig. 2) or projecting into the lumina of the blood vessels (Fig. 3); they are also present in the walls of renal veins and in nerve trunks and ganglia (Fig. 2). The immunohistochemical reactions for TH, DβH and PNMT confirmed the histological results: numerous red-coloured cells positive for both TH (Fig. 4) and DβH (Fig. 5) (noradrenaline + adrenaline cells) are found in the vein walls and in the nerve elements, while less numerous PNMT-positive cells (adrenaline cells) (Fig. 6) show the same distribution. The chromaffin cells show no positivity for nNOS, whereas positive ganglion cells and fibres are frequently observed (Fig. 7); they are evident in semithin sections stained with methylene blue, their cytoplasm is light or dark grey and is clearly distinguishable from the light blue cytoplasm of the renal or haematopoietic cells (Fig. 8). In electron

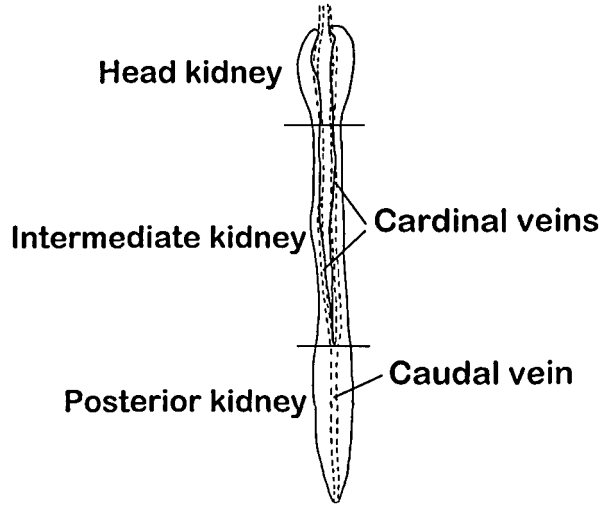


Fig. 1 - Drawing of the kidney of *Huso huso*. Cardinal and caudal veins are represented by dotted lines.

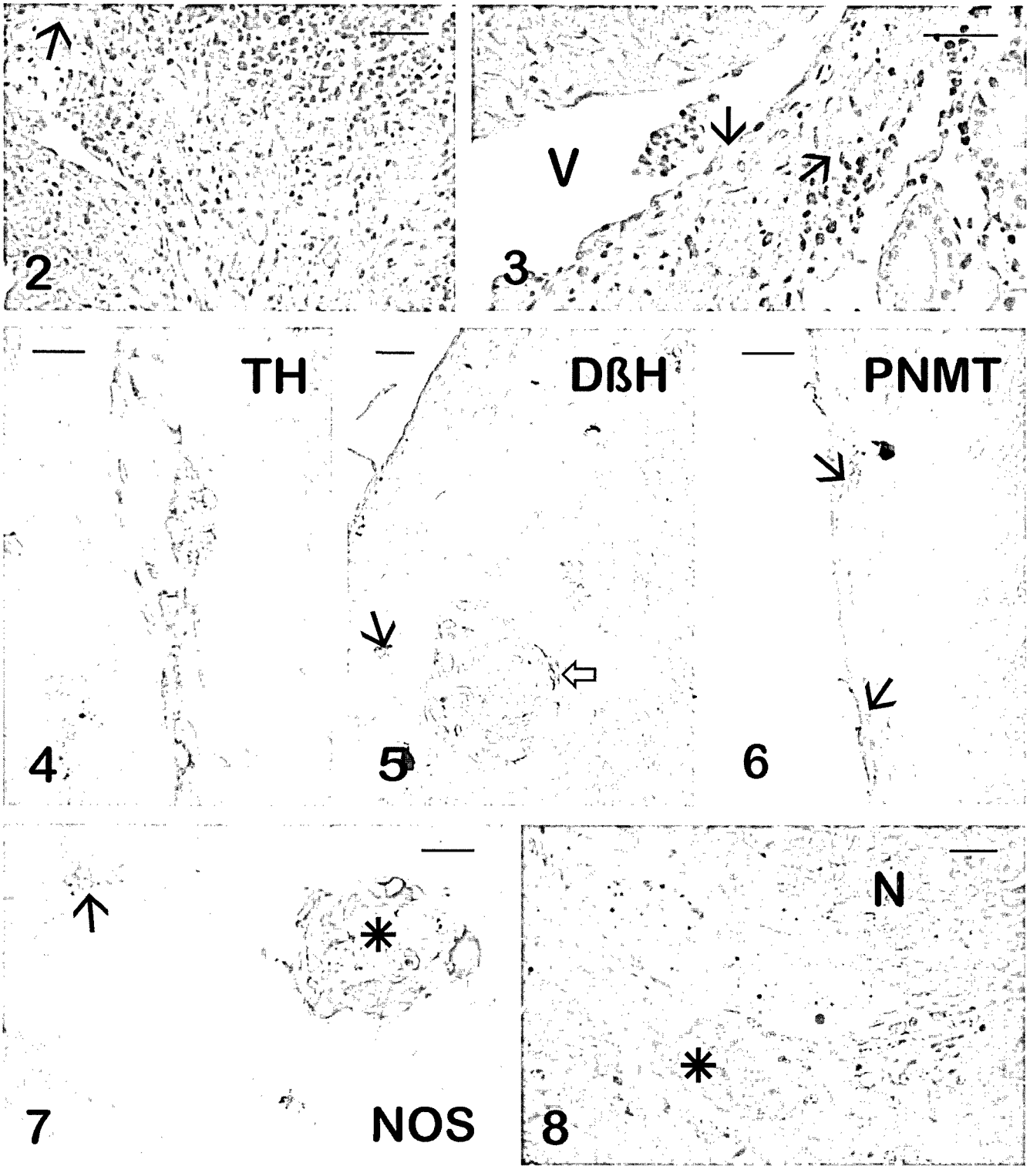


Fig. 2-8 - *Huso huso*. **2** - Chromaffin cells with yellow cytoplasm localized in the wall of a vein and in a ganglion (arrow) (Wood-haematoxylin-eosin; bar, 50 µ). **3** - Chromaffin cells (arrows) in the wall of a vein (V); some of them line the vein lumen (Wood-haematoxylin-eosin; bar, 50 µ). **4** - Immunohistochemical reaction for tyrosine hydroxylase (TH); some positive cells are localized in the vein wall (bar, 25 µ). **5** - Immunohistochemical reaction for dopamine-β-hydroxylase (DβH). The positive cells are localized in the vein wall (solid arrow) and in a ganglion (empty arrow) (bar, 50 µ). **6** - Immunohistochemical reaction for phenylethanolamine-N-methyltransferase (PNMT); some positive cells (arrows) are localized in the vein wall (bar, 50 µ). **7** - Immunohistochemical reaction for neuronal nitric oxide synthase (nNOS); positive neurons in a ganglion (asterisk); some thin nerve fibres appear positive as well (arrow) (bar, 50 µ). **8** - Semithin section of kidney; chromaffin cells with grey cytoplasm (asterisk) surrounded by haematopoietic cells are localized close to a big nerve trunk (N) (methylene blue; bar, 25 µ).

microscopy, the chromaffin cells appear polymorphic, often with prolongations; they are surrounded by a thick layer of collagen fibres and by supporting cells (Fig. 9). The nucleus can also be polymorphic, with zones of heterochromatin and an evident nucleolus. The endoplasmic reticulum and Golgi complex are poorly developed. Elongated mitochondria with lamellar cristae and dense matrix are scattered in the cytoplasm. Dense bodies and microtubules are sometimes present.

As regards the different types of chromaffin cells, it is not always easy to clearly differentiate between adrenaline and noradrenaline cells on the basis of the electron-density of the chromaffin granules, as observed in other vertebrate species (Coupland, 1965). Nevertheless, some differences are evident when the two types of cells are close to one another under the electron microscope (Fig. 9). The cells of one type contain rounded, homogenous and very electron-dense chromaffin granules (Figs 9 and 10) with an average diameter of 205 nm (max 290 nm); they are surrounded by a membrane that generally adheres to the content; in some granules, the membrane is separated from the content by a halo of variable width (Fig. 10, insert). The cells of the other type contain granules of very variable electron-density (Figs 9 and 11) with an average diameter of 150 nm (max 260 nm). Some granules are very electron-dense and homogeneous, others are less electron-dense and finely granular; the enclosing membrane is generally separated from the content by a narrow halo of constant width (Fig. 11, insert). Both types of cells sometimes show a heterochromatic nucleus with dilated perinuclear cisterna and highly indented outline, vacuolated endoplasmic reticulum and swollen rounded mitochondria with clear matrix and rarefied cristae (Fig. 12). The chromaffin cells are rarely adjacent to interrenal cells; the latter are recognizable by the presence of electron-dense or electron-lucent lipid droplets and mitochondria with tubulovesicular cristae typical of steroid secreting cells and they can show microvillous prolongations contacting the chromaffin cells (Fig. 13). Nerve trunks with myelinated and unmyelinated fibres run close to the chromaffin cells and sometimes contain prolongations of these cells (Fig. 14). Nerve fibres are found close to or even within chromaffin cells; however, nerve endings with the typical specializations are quite rare (Fig. 15).

## DISCUSSION

Our study is the first histological, immunohistochemical and ultrastructural description of the chromaffin system of the chondrostean fish *H. huso*. The results agree with the histological observations of Giacomini (1904, 1934) on other chondrostean species and with the biochemical results of Balashov *et al.* (1981) on the same species. In Chondrostei, the chromaffin cells are spread along the entire length of the kidney, localized in the walls of the cardinal, caudal and renal veins, whereas in the other bony fishes they tend to be concentrated in a more or less broad anterior zone of the kidney. In Teleostei, the chromaffin cells are localized mainly in the head kidney, being found in the posterior kidney (in smaller numbers) in only a few species (Gallo & Civinini, 2003). In Holostei, the chromaffin cells are localized mostly in the anterior half of the kidney (Giacomini, 1933; Youson *et al.*, 1976; Bhattacharyya *et al.*, 1981; Nilsson, 1981). In *Protopterus* (belonging to Dipnoi), the chromaffin cells are localized in the walls of the atrium, the most anterior part of the left cardinal vein and the intercostal arteries (Abrahamsson *et al.*, 1979). In *Huso*, the presence of chromaffin cells in the vessel walls along the entire length of the kidney can be considered a primitive condition, as in Cyclostomes the chromaffin tissue is broadly scattered and localized in the walls of the posterior cardinal and caudal veins, the aorta and other arteries, in the heart and in the spinal ganglia (Coupland, 1965; Perry *et al.*, 1993; Reid *et al.*, 1995; Nilsson & Holmgren, 1998). The results of the immunohistochemical reactions for TH, DβH and PNMT, suggesting the presence of two different catecholamine secreting cell types, agree with the results of Balashov *et al.* (1981) whose biochemical analyses demonstrated the presence of both adrenaline and noradrenaline in the region of the cardinal veins. Nevertheless, it is not always easy to identify adrenaline and noradrenaline containing cells under the electron microscope. They can only be distinguished by careful observation of the ultrastructural characteristics of the granules in sections in which the two cell types are close to one another. In most vertebrates, after fixation in glutaraldehyde and osmium tetroxide, the noradrenaline cells show strongly electron-dense chromaffin granules, as noradrenaline is fixed *in situ* and combines with proteins and other compounds to form a homogeneous and dense precipitate; the adrenaline cells show less dense chromaffin granules, as adrenaline is washed out and the moderately electron-dense granule is formed by proteins and other compounds (Coupland & Hopwood, 1966). In *Huso*, the granules of one cell type appear strongly electron-dense, suggesting that they are noradrenaline cells, whereas those of the other cell type show variable electron-density. We hypothesize that the latter are adrenaline cells containing granules that do not completely lose the hormone after fixation. The chromaffin granules of *Huso* appear different from those of other bony fishes, as the limiting membrane generally adheres closely to the content; in teleosts, the noradrenaline granules show strongly electron-dense content separated from the limiting membrane by a wide halo, while the adrenaline granules show a less dense core separated from the limiting membrane by a halo of constant width. The ultrastructural aspect of the chromaffin granules of *Huso*, i.e. the variable electron-density of the adrenaline granules and the absence of a wide halo between the core and the limiting membrane, is more similar to that of some amphibians (Accordi & Gallo, 1982; Grassi Milano & Accordi, 1983; Ac-

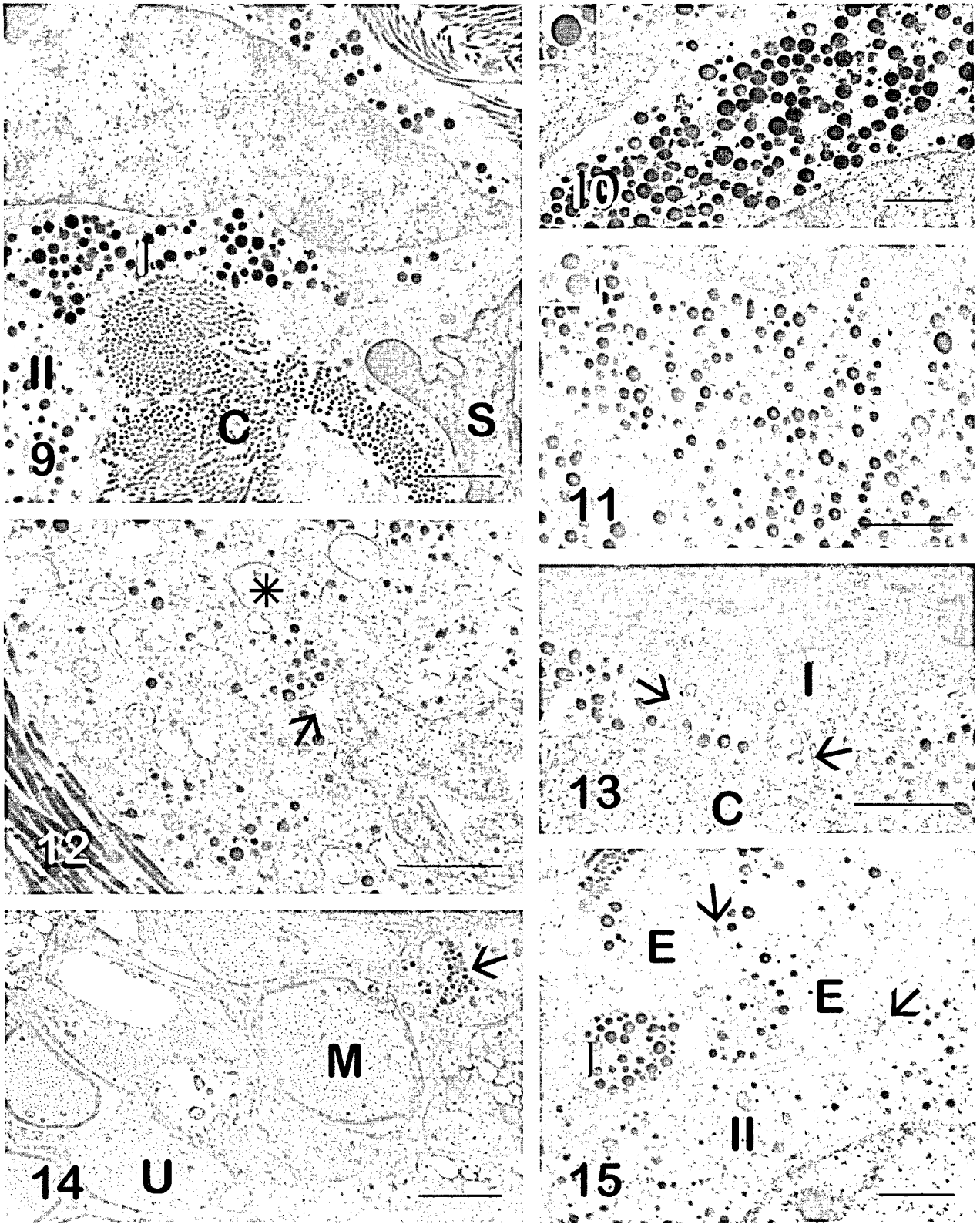


Fig. 9-15 - *Huso huso*. **9** - Chromaffin cells of type I and II surrounded by collagen fibres (C) and supporting cells (S) (bar, 1 μ). **10** - Chromaffin cell of type I, probably a noradrenaline cell, with strongly electron-dense granules (bar, 1 μ); in the insert, enlargement of a granule. **11** - Chromaffin cell of type II, probably an adrenaline cell, with granules of variable electron-density (bar, 1 μ); in the insert, enlargement of three granules. **12** - Chromaffin cell showing enlarged perinuclear cisterna and endoplasmic reticulum (arrow); the nucleus is highly indented; the mitochondria are rounded, swollen and with few cristae (asterisk) (bar, 1 μ). **13** - Chromaffin (C) and interrenal (I) cells projecting microvilli onto one another (bar, 1 μ). **14** - Nerve trunk containing myelinated (M) and unmyelinated (U) nerve fibres and the prolongation of a chromaffin cell (arrow) (bar, 2 μ). **15** - Chromaffin cells of types I and II; nerve endings (E) with synaptic specializations (arrows) are evident (bar, 1 μ).

cordi & Grassi Milano, 1990; Accordi, 1991). Ultrastructural studies on other non-teleost bony fishes are very scanty: the available data regard the chromaffin cells of the holostean *Amia calva* (Youson, 1976) and the dipnoans *Protopterus* and *Lepidosiren* (Scheuermann, 1993) and *Neoceratodus* (Chopin & Bennett, 1995). In these cases only one type of chromaffin cell containing granules with strongly electron-dense content was described. Chromaffin and interrenal cells in *Huso* are generally separated, but in some cases they can be contiguous or in tight contact, as demonstrated by the presence of microvilli protruding from the plasma membrane; this suggests a paracrine interaction, as observed in the adrenal gland of teleosts (Reid *et al.*, 1996, 1998) and other vertebrates (Ehrhart-Bornstein *et al.*, 1998; Mazzocchi *et al.*, 1998). The presence of chromaffin cells inside some ganglia and of nerve fibres and endings close to the chromaffin cells suggests neural control of catecholamine secretion; however, endings with typical synaptic thickenings are rather rare. In teleosts, the chromaffin cells are generally well innervated and the nerve endings show typical presynaptic specializations (Gallo *et al.*, 2001), whereas in Cyclostomes the chromaffin tissue appears to lack extrinsic innervation (Augustinsson *et al.*, 1956; Paiement & McMillan, 1975; Perry *et al.*, 1993; Bernier & Perry, 1996, 1998). As there are no other studies on the innervation of the adrenal homolog of chondrosteans, we do not know whether the sparse innervation of the chromaffin cells of *Huso* indicates a primitive condition or simply reflects species differences among chondrosteans. In any case, we cannot exclude non-synaptic paracrine control of the chromaffin system of *Huso*, as there is a close connection between the nerve elements and the chromaffin cells.

The immunohistochemical reaction for nNOS, an enzyme that catalyzes the synthesis of NO, revealed positivity in some ganglion cells and nerve fibres in blood vessel walls where chromaffin cells were localized, whereas the chromaffin cells were not positive. The research of Holmberg *et al.* (1998) on the role of NO in the rat, guinea pig and mouse adrenal indicated that the chromaffin cells (especially the noradrenergic ones) and blood vessels are targets for NO. In trout and in mammals, nitrergic nerves appear to be involved in the control of cellular activity of chromaffin and interrenal cells (Gallo & Civinini, 2001). We hypothesize that in *Huso*, as in other vertebrates, nitrergic nerves may be involved in the control of the blood vessels and the cellular activity of chromaffin and/or interrenal cells in a paracrine manner, as NO is a diffusible gas that is not stored in vesicles.

The results of our study indicate that the chromaffin system of *Huso* presents some primitive aspects, such as its wide distribution and perhaps sparse innervation, whereas the ultrastructural characteristics are more similar to those of amphibians than to those of other bony fishes. Further studies on other chondrostean species could provide more information about the chromaffin system of this interesting taxon.

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