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The supramedullary cells of the teleost *Coris julis* (L.): a noradrenergic neuronal system

L. Mola, D. Sassi, and B. Cuoghi

Dipartimento di Biologia Animale, University of Modena and Reggio Emilia, Via Campi 213/D, I-41100 Modena, Italy

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SUMMARY

This study, carried out on *Coris julis* (Labridae), is a contribution to the immunocytochemical characterization of fish supramedullary neurons. The significance of these giant cells has been debated since the beginning of the twentieth century. Our research provides the first evidence for a noradrenergic feature of this neuronal system. The possible role of supramedullary neurons as components of the autonomic nervous system is discussed. Moreover, the present results, taken together with our previous studies, surmise that this the first known case of colocalization of a neuropeptide (gastrin/CCK-like) and noradrenaline in the nervous system of teleosts.

INTRODUCTION

Many species of teleosts belonging to different orders exhibit large neurons lying on the dorsal midline surface of the spinal cord named supramedullary neurons or supramedullary cells. In several species, they are aligned along the spinal cord, while in others they are clustered rostrally in the first part of the spinal cord. Several hypotheses have been advanced for the function of this neuron system. For many years, these neurons were considered persistent Rohon-Beard cells. That hypothesis has been excluded because the supramedullary neuron differentiation begins at advanced stages of development and is completed in juveniles, and because these neurons are present in juveniles and adults of *Hippocampus* and *Syngnathus*, species lacking Rohon-Beard cells during development (Marini and Benedetti, 1992).

On the grounds of electrophysiological research, Bennett *et al*. (1959 a,b,c) put forward the idea that supramedullary cells are motoneurons of the autonomic nervous system, even though their axons emerge from the spinal cord through the dorsal roots. Recent neuroanatomical studies in *Takifugu niphobles* demonstrate that the processes of the clustered supramedullary cells project peripherally through the trigeminal, vagal and spinal nerves (Funakoshi *et al*., 1995).

Our previous immunohistochemical research on the detection of neuropeptides show that aligned supramedullary neurons exhibit positive immunoreactivity to CCK-8, CCK-39 and gastrin (18-34), both in Perciformes (*Coris julis*) (Benedetti and Mola, 1988) and in Scorpaeniformes (*Trigla lucerna* and *Scorpaena porcus*) (Benedetti *et al*., 1993). Funakoshi *et al*. (1998) confirm gastrin/CCK-immunoreactivity in *T. niphobles* clustered supramedullary neurons. The same immunopositivity is

Correspondence to: L. Mola
E-mail: mola.lucrezia@unimo.it

also found in nerve terminals surrounding the cutaneous mucous glands, as well as in some free nerve endings in the epidermal layer near the mucous glands. The authors suggest a role as main autonomic elements of a sensory-motor reflex system for these neurons.

With the aim of clarifying if the supramedullary cells are components of the autonomic nervous system, we undertook the detection of catecholamines in the aligned supramedullary neurons of *Coris julis* (L.). We have chosen this species as a very suitable material for our neurobiological studies (Benedetti and Mola, 1988; Mola *et al*., 1992; Mola and Barozzi, 1998) because *C. julis* offers many advantages, among which are its availability all year round and the ease with which it can be kept in an aquarium. In this species, there are about one hundred supramedullary cells (cell diameter 60-65 μm) of different shapes and scattered below the external limiting membrane for about the first half (i. e. ca. 3 cm) of the spinal cord (Fig. 1).

MATERIALS AND METHODS

We examined twenty specimens (9-18 cm in length) of *Coris julis* (L.) captured in the Tirrenian Sea (Italy) and reared for seven days in oxygenated sea water tanks.

For monoamine detection, in ten of these animals axonal transport was blocked by the intraperitoneal injection of colchicine (Sigma, USA), 50 μg/g body weight in isotonic saline solution (340mosm), 24 h before the dissection (Batten *et al*., 1990). The other ten animals were used for enzyme detection.

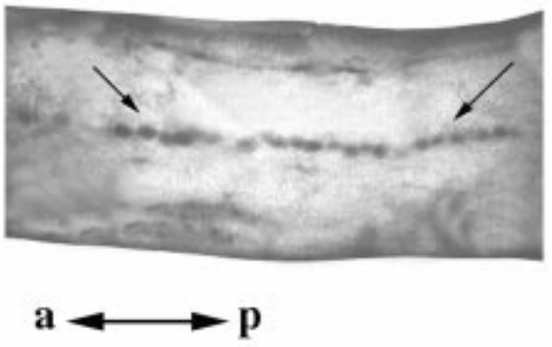

Fig. 1 - Picture at the stereomicroscope of a *Coris julis* spinal cord segment (ca. 1 cm long), *in toto*. Arrows indicate the supramedullary cells lying on the dorsal surface (frontal view). a p = antero-posterior axis. Staining with Trypan blue.

All twenty animals were sacrificed by decapitation; their spinal cord was rapidly removed and fixed in 4% freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer saline (PBS), pH 7.4, for 24 h at 4°C. Thereafter,, the material was cryoprotected for 24-48 h in PBS containing 20% sucrose at 4°C. Fixed tissues were embedded in O.C.T. compound (BDH, UK), frozen and cut in a cryocut (Micron). Transversal sections (16 μm) were picked up on gelatin-coated glass slides.

After inhibition of endogenous peroxidases by incubation for 30 min in 100% methanol containing 0.3% H_2O_2, sections were incubated overnight at 4°C in a moist chamber with primary antisera. We tested the following polyclonal antibodies: anti-tyrosine hydroxylase (TH) (Sigma, from mouse, 1:750 titer); anti-dopamine β-hydroxylase (DβH) (Chemicon, USA, from rabbit, 1:400 titer); anti-dopamine (DA) (Instar, USA, from rabbit, 1:2500 titer) and anti-noradrenaline (NA) (Chemicon, from rabbit, 1:250 titer). All antisera were dissolved in 0.01 M PBS**,** pH 7.4, containing 0.3% Triton X-100. The antigen-antibody complexes were visualized using biotinylated goat anti-rabbit or goat anti-mouse secondary serum (Dako, Italy) (diluted 1: 250 in 0.01 M PBS pH 7.4, containing 0.3% Triton X-100) for 30 min at room temperature, followed by the Biotin-Avidin Complex/HRP (Vectastain ABC system, Vector Labs., USA) in TRIS, pH 7.6, for 45 min at room temperature. 3,3'Diaminobenzidine tetrahydrochloride (DAB, Sigma) was employed as a chromogen.

Only for monoamine detection, before immunohistochemical reactions, the slides were incubated in a reducing solution containing 0.05 M sodium hydroborate for 3 min and rinsed 6 times for 5 min at 4°C in 0.01 M PBS, pH 7.4, containing 0.85% sodium metabisulphite. Sodium metabisulphite at the same concentration was added to the normal serum (incubation for 60-90 min) and to the serum containing antibodies.

The specificity of immunostainings was always checked by incubating sections with normal serum instead of specific antisera or incubating sections with antiserum preabsorbed with the respective antigen (10-100 μg/ml). The preabsorbtion procedures were carried out overnight at 4°C.

The slides were mounted with Eukitt and examined under a Leitz Diaplan microscope equipped with WILD photoautomat MPS 45. Some photos were made with Nomarski interference.

RESULTS

The present immunohistochemical study, aimed at identifying classical neurotransmitters in the supramedullary neurons of *C. julis*, pointed out immunopositivity to antibodies against some enzymes and reaction products involved in the biosynthesis of catecholamines. Indeed, cytoplasmic immunopositivity was found in all supramedullary neurons with anti-TH antibodies (Fig. 2A), anti-DβH antibodies and anti-NA antibodies (Fig. 2B). Immunoreactivity to anti-NA was almost of the same intensity in all supramedullary cells, probably due to axonal transport blockade, which, as is known, prevents neurotransmitter transit from the neural soma. The morphological pattern showed neurons with a process running ventrally (Fig. 2A, 2B), the course of which was not easily detectable. No immunoreactivity was observed for anti-DA antibodies.

The control procedures resulted in the absence of reactions.

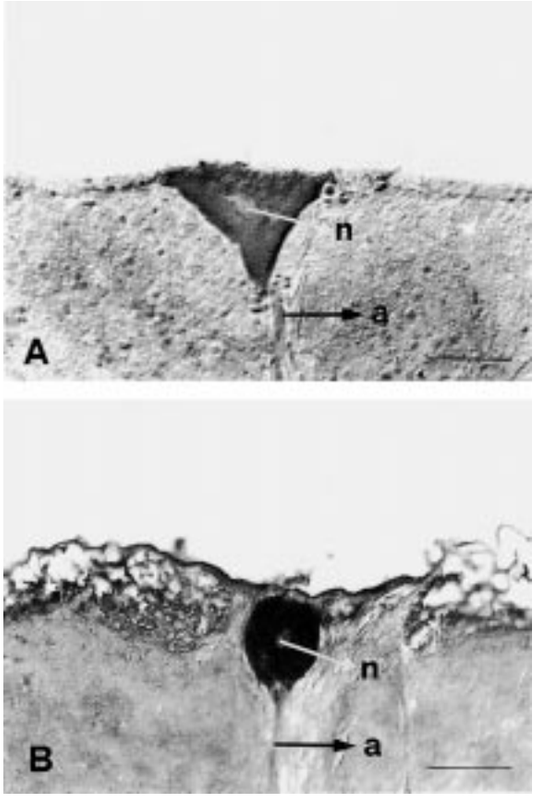

Fig. 2 – A) Transversal section of *Coris julis* spinal cord showing a supramedullary neuron (s. n.) immunoreactive to TH (1:750 dilution). Photo with Nomarski interference. n = nucleus, a = axon. Scale bar = 50 μm. **B)** Transversal section of *C. julis* spinal cord showing another supramedullary neuron immunoreactive to NA (1:250 dilution) after colchicine treatment. n = nucleus, a = axon. Scale bar = 50 μm.

DISCUSSION

As is known, several molecules are produced during biosynthesis of catecholamines (L-DOPA, DA, NA and adrenaline); some of them may be the final neurotransmitter, or represent an intermediate reaction product in the synthesis of other functional catecholamines. Thus, to identify which of these substances plays the role of neurotransmitter, it is important to precisely establish where the biosynthesis process is interrupted by testing the presence or absence of the required enzyme. Positivity toward anti-TH and anti-DβH antibodies indicates an enzymatic activity linked to catecholamine synthesis. In particular, the presence of DβH suggests that supramedullary neurons are not dopaminergic but rather noradrenergic or adrenergic; this is confirmed by the lack of immunoreactivity towards anti-DA antibodies and by the marked positivity to anti-NA antibodies. The lack of immunoreactivity towards anti-DA antibodies can be explained by supposing a rapid conversion of DA to NA by the enzyme DβH; thus, DA is present in amounts too small to be detected by usual immunohistochemical methods. Immunopositivity towards anti-NA antibodies, detectable in all supramedullary neurons, suggests that in these cells NA does not represent an intermediate step in the synthesis of adrenaline, but plays a role in neurotransmission.

The supramedullary neurons of *T. niphobles* turn out not to be immunoreactive for ChAT or TH (Funakoshi *et al.*, 1998). The authors state that these cells constitute a class of non-adrenergic/non-cholinergic autonomic neurons, which project

peripheral processes to both the cutaneous mucous glands and epidermis. On the contrary, our results showed immunoreactivity not only for TH but also for DβH and NA. The different immunoreactivity found for the first enzyme of catecholamines biosynthesis may be due to different specificities of tested antibodies or to other methodological differences. It is improbable that species differences can account for the diversity in immunoreactivities to TH between *C. julis* and *T. niphobles.* Indeed, in all species that have been studied by different authors, the supramedullary cells, both aligned and clustered, share the same main morphological (Marini and Benedetti, 1992; Cuoghi and Marini, 2001) and immunohistochemical (Benedetti and Mola, 1988; Benedetti *et al.*, 1993; Funakoshi *et al*., 1998) characteristics. Moreover, immunopositivity to anti-NA was also found in the clustered supramedullary neurons of the pufferfish *Tetraodon fluviatilis* (Cuoghi, unpublished data). For these reasons, we think that supramedullary neurons of fish constitute a noradrenergic system.

In addition, considering that cutaneous mucous glands in amphibians are innervated by the sympathetic adrenergic nerves, the hypothesis on the autonomic nature of supramedullary neurons (Bennett *et al*., 1959 a,b,c; Funakoshi *et al.*, 1998) appears strengthened by the present data.

Our previous immunocytochemical studies have shown that all supramedullary neurons of *C. julis* are immunoreactive towards a gastrin/CCK-like peptide (Benedetti and Mola, 1988). This seems to be the first known case of co-localization of a neuropeptide and monoamine in the nervous system of teleosts. At the moment, we can speculate that the neuropeptide acts in this noradrenergic neuronal system as a neuromodulator. Some support for this hypothesis comes from mammalian neuronal systems that use CCK in order to modulate monoamine action. For example, it is known that systemic administration of CCK releases noradrenaline in the dorsal region of the rat supraoptic nucleus (Kendrick *et al*., 1991; Onaka *et al*., 1995).

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