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Intrinsic innervation in the intestine of the lizard *Podarcis hispanica*

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Summary. The aim of this study was the description of the morphology and distribution of nerve structure elements in the intestine of the lizard *Podarcis hispanica* using different histochemical methods; namely acetylcholinesterase (AChE), formol-induced fluorescence for catecholamines (FIF), nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), and immunohistochemistry for vasoactive intestinal peptide (VIP), as well as substance P (SP) and electron microscopy. The AChE method showed fibres in the myenteric and submucosal plexus, with a higher fibre density in the large intestine. The highest number of related neurons was located in the myenteric plexus ganglia. Noradrenergic innervation was distributed through the myenteric and submucosal plexus, and also around blood vessels, with the highest fibre density in the large intestine. VIP immunohistochemistry showed a wide distribution of positive fibres throughout the intestine, although the highest density was again detected in the large intestine. Small positive cells for VIP were located at internodal segments in the plexus. SP labeling, although subtle, was present all along the intestine. It showed delicate varicose nets and few fibres innervating blood vessels. Small positive cells for SP were located in the large intestine. The indirect method to detect nitric oxide (NO)-producing system showed neural cells in the myenteric plexus ganglia of the large intestine. Electron microscopy showed ganglion neurons with scattered chromatin condensations, glial cells with higher electron density, and axons with varicosities occupied by different vesicles. We also identified certain cells as interstitial cells of Cajal due to their ultrastructural features. They were mostly located in the region of the myenteric plexus.

Key words: Histochemistry, Immunohistochemistry, Ultrastructure, Enteric nervous system, Reptilia

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Introduction

The enteric nervous system (ENS) can be defined as a tertiary division in the autonomic nervous system, because of its anatomic and functional independence from the central nervous system. The crucial role of the ENS in the regulation of digestive functions has been frequently stressed (Gershon, 1990; Gershon and Wade, 1993). Unfortunately, ENS-regulated behavior is highly complex, and its microcircuits have not been elicited (Costa and Brookes, 1994; Gershon and Wade, 1994). Considering that phylogenetic studies may help us understand it and following our ENS studies in amphibians (Junquera et al., 1986, 1987a,b, 1988), we focused on the reptilian ENS.

References to intestinal extrinsic innervation in reptiles are scarce. The vagus nerve innervates the anterior region of the digestive system, but we do not even know if it spreads further than the stomach. In *Trachydosaurus rugosus* (Lacertidae), splachnic nerve stimulation reached the intestine to originate a contraction that was not inhibited by adrenergic and cholinergic antagonists. This supported a non-adrenergic, non-cholinergic (NANC) innervation (Burnstock, 1972; Sneddon et al., 1973; Berger and Burnstock, 1979).

Studies on the intestinal ENS in reptilian species are incomplete. There was no detailed morphological description of the reptilian intestinal plexus until 1991. This was performed in a chelonian species using antiserum raised against neuron-specific enolase (NSE) (Timmermans et al.,1991). Intestinal adrenergic innervation was previously studied in the reptile *Trachydosaurus rugosus* by Read and Burnstock (1968). Yung et al. (1965) studied peristalsis in the turtle intestine, and concluded that this reflex would be mostly myogenic, as distension alone would not be a sufficient stimulus to initiate peristalsis. When considering peptidergic innervation, available data are partial or even contradictory. Reinecke et al. (1981) described VIP immunoreactivity in enteric nerves and endocrine cells of *Lacerta viridis*. Buchan et al. (1983) described fourteen neuropeptides in the digestive system of

Alligator mississipiensis, within endocrine cells, neural bodies, and neural fibres. Böttcher et al. (1985) found PYY-like peptides in the central and peripheral nervous system of the turtle. Masini (1986) located the neuropeptide VIP only in the small intestine in vipers, within nerve terminals and body cells. Holmgren et al. (1989) found bombesin-like immunoreactivity in the crocodile ileum and colon, within neural fibres and cell bodies. Ohtani et al. (1989) showed positive immunoreactivity for calcitonin gene-related-peptide (CGRP) in the reptilian small intestine In the midgut of the turtle (*Pseudemys scripta elegans*), Gabriel et al. (1990) described the presence of neuropeptide Y (NPY), mostly in the myenteric plexus. The same species has been investigated for the occurrence of immunoreactivity to nine neuropeptides of the gastrointestinal tract (Scheuermann et al., 1991). Lamanna et al. (1999b) showed the distribution of galanin immunoreactive neurons (GAL/IR) in the gastrointestinal tract of the lizard *Podarcis s. sicula*.

Few references focus on neuronal NO studies in non-mammal vertebrates, such as fishes (Li and Furness, 1993; Green and Campbell, 1994; Olsson and Karila, 1995; Brüning et al., 1996), amphibian (Li et al., 1992, 1993; Murphy et al., 1994; Williams and Pearson, 1997), reptiles (Brüning and Wehrenfenning, 1997; Lamanna et al., 1999a; Olsson and Gibbins, 1999), and birds (Boros et al., 1994; Li et al., 1994; Balaskas et al., 1995). Finally, there is a surprising absence of detailed electron microscopy descriptions of the intestine ENS in reptiles.

The aim of this study was to ascertain the neurochemical and ultrastructural pattern of gastrointestinal innervation in Class Reptilia, specifically in the lizard *Podarcis hispanica*. This might further our understanding of ENS evolution in the phylogenetic scale. In this study, we describe the intrinsic innervation of small and large intestines.

Materials and methods

We used twenty adult lizards *Podarcis hispanica* (Reptilia). The animals (12 males and 8 females) were collected in spring, summer, and autumn. They were sacrificed by cervical dislocation, under ether anesthesia. We studied the basic histology of the small and large intestine wall, in sections stained with haematoxylin-eosin or semi-thin sections with methylene blue.

Acetylcholinesterase method (AChE)

We adapted the method of El-Badawi and Schenk (1967), and Qayyum and Fatani (1985). Intestine samples of about 5 mm in length were longitudinally dissected, pinned on cork sheets and immersed in a 2% glyoxylic acid (pH 7) solution, for 5 to 30 minutes at room temperature. The tissue was then delaminated, to expose both the myenteric and the submucosal plexus, and postfixed in a 10% solution of formaldehyde in phosphate-buffered saline (PBS) at pH 7, for 30 minutes. After washing in distilled water, samples were incubated in AchE-specific medium. Optimal cholinesterase activity was obtained after incubating periods of 1-2 hours. Tissue samples were then dehydrated through a graded ethanol series, cleared in xylene, and mounted with DPX.

Formol-induced fluorescence (FIF) for catecholamines

We applied the method of Furness and Costa (1975), adapted for wholemount preparations. Tissue samples were prepared as described above. Once the intestine (large or small) wall was delaminated, both tissue sheets were stretched on a slide and exposed to para-formaldehyde vapors (1-3 hours at 80 °C). Samples were visualized through a Leitz-Orthoplan Fluorescence microscope, and checked for apple green (noradrenaline) or yellow (histamine and serotonine) fluorescence.

Immunohistochemistry for vasoactive intestinal peptide (VIP) and substance P (SP)

We followed the method of Costa et al. (1980) with minor modifications, to visualize the plexus three-dimensionally within "in toto" samples. Specimens were stretched and pinned on fine cork sheets. Fixation was made by immersion for 18 hours in a 15% picric acid-saturated solution with 2% formaldehyde in 0.1M PBS (pH 7.3), at 4 °C. After 30 minutes washing in 80% ethanol, samples were dehydrated with graded ethanol, and cleared in xylene. Samples were then rehydrated back to PBS and delaminated. We applied rabbit antiserum to both exposed surfaces, and incubated the tissue in a humid chamber for 16 hours at room temperature. After 3 washes in PBS, samples were incubated for 1 hour with a secondary antibody: goat anti-rabbit IgG conjugated to fluorescein-isothiocyanate (GAR-FITC) (Sigma), then washed in PBS for 15 minutes, mounted in glycerine at a 3:1 dilution in PBS, and kept under pressure overnight. Rabbit antiserum (INCSTAR, Stillwater, MN, USA) dilutions in PBS (pH 7.2-7.4) were 1:200 (VIP), and 1:800 (SP). Controls were performed by absorbing the anti-VIP and anti-SP antibodies with an excess of the respective antigens with PBS in the specific step. Controls were negative.

Nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemistry

We applied a method adapted from Scherer-Singler et al. (1983). Portions of both small and large intestine were obtained and incised longitudinally. Tissue samples were stretched and pinned on fine cork sheets, prior to fixation. Immersion fixation was performed for 1 hour in a solution of 4% paraformaldehyde in 0.1M PBS, pH 7.4, at 4 °C. At this stage, delamination was performed. We removed the mucosa, submucosa and inner muscular layer, exposing contact surfaces between circular and longitudinal muscle coating. We then treated specimens

for NADPH-diaphorase histochemistry for 30 minutes, using 0.25 mgr/ml Nitroblue tetrazolium (Sigma), 1 mgr/ml ß-NADPH (Sigma), and 0.5% Triton X-100 in 0.1M Tris buffer (pH 7.6), at 37 ºC. The reaction was stopped by rinsing the tissue in 0.1M PBS. The tissue was dehydrated and then mounted with DPX. In control experiments, the substrate ß-NADPH was omitted from the reaction mixture and no positive staining was observed. The reaction product of NADPH-diaphorase appeared as a blue/violet staining of different intensities.

Electron microscopy (EM)

We applied standard electron microscopy methods, including fixation in 2.5% glutaradehyde in Milloning buffer (pH 7.3), postfixation in 2% osmium tetroxide, staining with 7% uranyl acetate, dehydration and araldite embedding. Ultrathin sections were contrast-stained following conventional methods.

Results

AChE reaction

We found a wide distribution of AchE-positive elements throughout the intestine. The large intestine showed the highest density of labelled fibres. The myenteric plexus was arranged in regular networks of elongated, polygonal shape. The long axis of these polygons followed the craniocaudal direction of the intestine. Ganglia of pyramidal or trapezoidal shape were located in the nodes of this network (Fig. 1a,b). In the largest ganglia, the number of neurons ranged from 6 to 12. Scattered neurons were frequently seen within interconnection tracts. These neurons were pyramidal and of variable size. The nucleus was oval. It was prominent and centrally located, within a moderate or even scarce cytoplasm.

Networks forming the submucosal plexus were parallel to the vascular tree, and were particularly dense along the ileum (Fig. 1c). AchE-positive fibre bundles were thinner than those in the myenteric plexus. Small ganglia containing 3 to 4 AchE-positive neurons (Fig. 1d) were located at fibre intersections. Isolated neurons were also frequent, with similar morphology to that described above for myenteric plexus neurons. A second type of AchE-positive cells was observed in the submucosal plexus. These were bipolar, elongated cells, with a narrow, oval nucleus. These morphological features identified them as interstitial cells of Cajal (ICC) (Fig. 1e).

FIF reaction

The myenteric plexus showed a wide catecholamine-positive reaction, especially in the large intestine. Polygonal networks of intensely fluorescing varicose nerve fibres were found in the circular muscle layer (Fig 2a). Ganglia in the myenteric plexus were surrounded by multiple fluorescent fibres. We did not observe any positive cell bodies in these myenteric ganglia.

Innervation around blood vessels was profuse, arranged in their own plexus. Although close, this was separate from the above-mentioned myenteric primary plexus (Fig. 2b). A rich perivascular catecholaminergic innervation was present in the submucosa and mucosa. This was particularly remarkable in the basal zone of the mucosa, where the vascular network was highly innervated (Fig. 2c). Networks in the submucosal plexus included thin varicose fibres that progressed across the submucosa to innervate glandular bases within the mucosa.

VIP Immunoreactivity

We were able to observe a wide VIP-positive reaction in every intestinal segment of *Podarcis hispanica*. The large intestine again showed a higher density of VIP-ergic fibres. The primary myenteric plexus was formed by wide networks of varicose fibres (Fig. 3a). We observed VIP-positive immunoreactivity in the ganglia located at the primary plexus junctions. Nerve fascicles that contained immunoreactive fibres, originated at those ganglia. Small VIP-positive cells could be seen at internodal segments of the plexus network. Thin parallel fibrils followed the direction of muscle fibres in the inner muscle layer, and formed a delicate tertiary plexus (Fig. 3b). These fibres emerged perpendicular to the primary plexus, in a different focus plane. We did not observe VIP-ergic innervation surrounding blood vessels.

SP Immunoreactivity

SP immunoreactivity was quantitatively less than VIP immunoreactivity. However, positive reaction was present in every intestinal segment. Positive fibres were arranged in slender varicose networks. These formed the main plexus, shaped in elongated polygonal networkss (Fig. 4a). Ganglia showing scarce immunoreactivity were found at the vertex of network polygons (Fig. 4b). We only observed SP-positive neurons in the large intestine, where small neurons with scarce cytoplasm were located among the myenteric plexus. Thin varicose fibres, that originated in this plexus were in the circular muscle layer. Some positive fibres innervated blood vessels.

NADPH-d reaction

We were able to observe a wide array of NADPH-d-positive structures, including epithelial cells, neural elements, and blood vessels.

Isolated positive neuronal bodies were frequent at the myenteric plexus of the small intestine. However, in the large intestine, positive neurons were located in ganglia (8-10 cells). Positive neurons were also present along the plexus interconnection tracts (Fig. 5a).

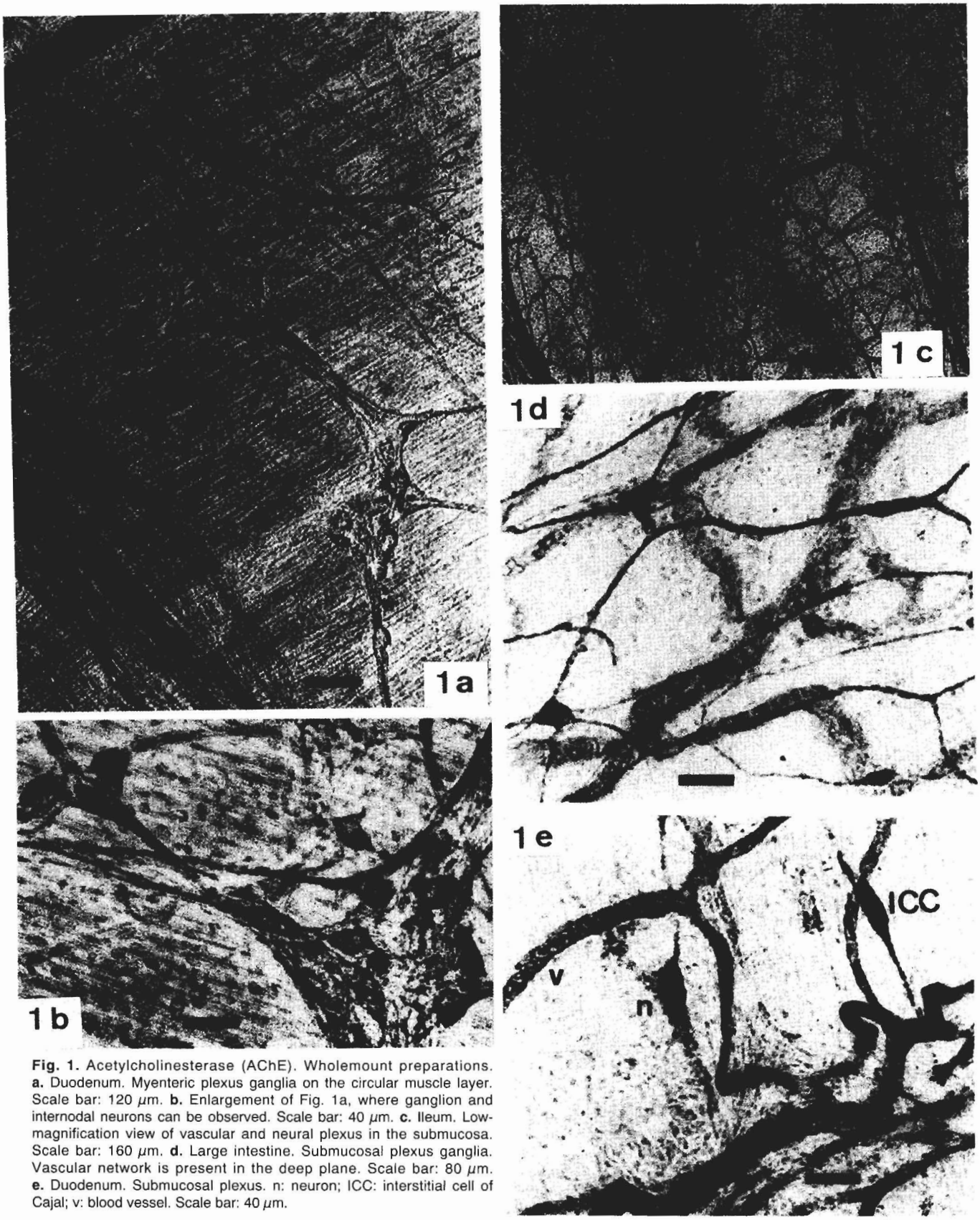
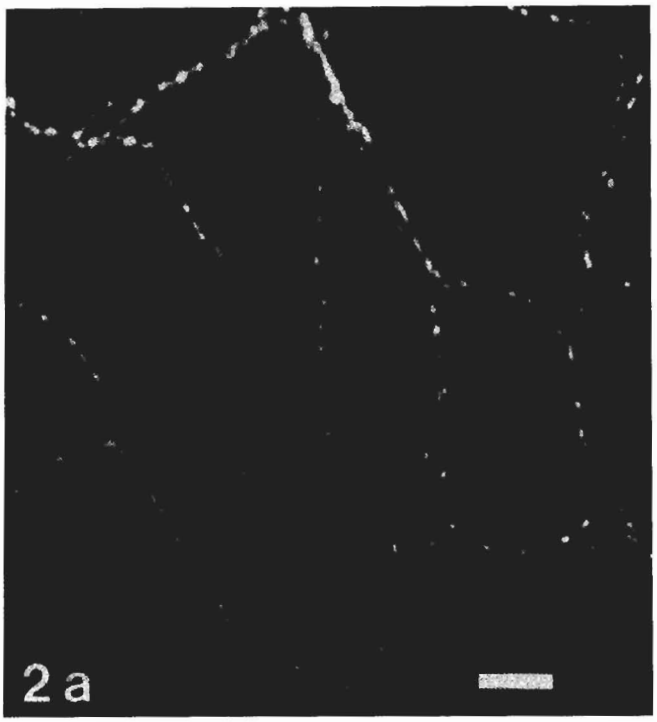


Fig. 1. Acetylcholinesterase (AChE). Wholemount preparations. **a.** Duodenum. Myenteric plexus ganglia on the circular muscle layer. Scale bar: 120 μm. **b.** Enlargement of Fig. 1a, where ganglion and internodal neurons can be observed. Scale bar: 40 μm. **c.** Ileum. Low-magnification view of vascular and neural plexus in the submucosa. Scale bar: 160 μm. **d.** Large intestine. Submucosal plexus ganglia. Vascular network is present in the deep plane. Scale bar: 80 μm. **e.** Duodenum. Submucosal plexus. n: neuron; ICC: interstitial cell of Cajal; v: blood vessel. Scale bar: 40 μm.



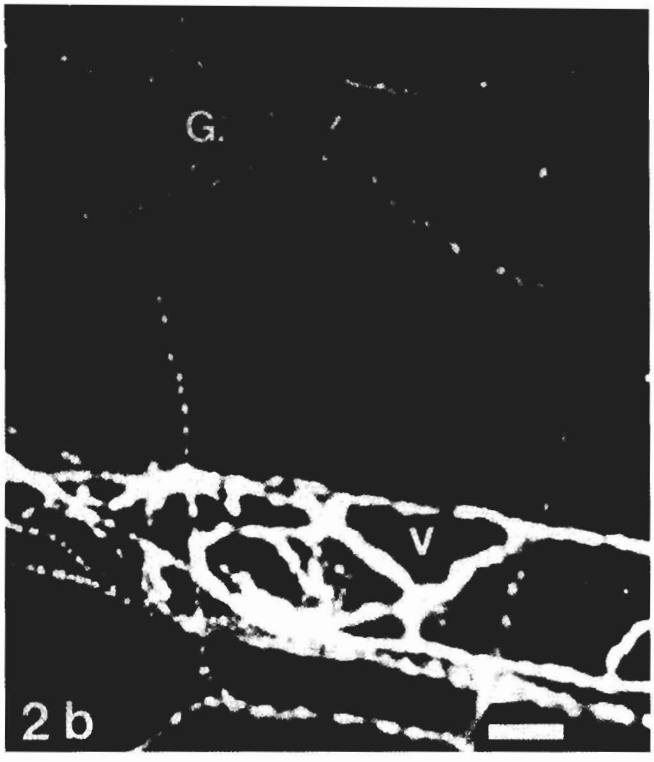


Fig. 2. Formol-induced fluorescence (FIF). Wholemount preparations. **a.** Small intestine. Varicose nets at the myenteric plexus on the circular muscle. Scale bar: 40μm. **b.** Small intestine. Myenteric plexus fibres surrounding a ganglion (G). Dense network of fluorescent fibres around a blood vessel (v). Scale bar: 40 μm. **c.** Large intestine. Submucosal plexus (arrows) and fluorescent fibres surrounding blood capillaries. Scale bar: 40 μm.

At the submucosal plexus, NADPH-d-positive cells were scattered along the network. Submucosal blood vessels showed an intense NADPH-d-positive reaction. Isolated positive cells could be observed parallel to the vascular distribution, and clusters of two or three positive cells could be observed close to these blood vessels (Fig. 5b).

EM observations

Thick nervous trunks crossed the muscle layer of the reptilian intestine at the sites of extrinsic innervation supply. This caused a non-homogeneous innervation density in transverse sections.

The longitudinal muscle was extremely thin, sometimes formed by just one or two rows of smooth muscle cells. Myenteric plexus ganglia were located between the circular muscle layer and these smooth muscle cells. ICC were frequently near nerve axon bundles in this region (Fig. 6a). Ganglion neurons showed wide nuclei with marginal chromatin, narrow but definite nucleoli, and scattered chromatin condensations that produced a typical spotting. Glial cells showed much smaller but more elongated nuclei, with higher electron density. These glial cells surrounded packed axons of variable thickness (Fig. 6b). Most axons were presented with varicosities filled with a mixed of vesicles: small electron-clear vesicles and a mixed population of vesicles (small diameter electron-clear vesicles and electron dense vesicles of different sizes).

Frequently, varicosities and neuronal somata were very close. However, it was difficult to distinguish synaptic specialization (Fig. 6c,d). Groups of axons were found, close to smooth muscle cells of the circular muscle layer (Fig. 7a). These axons contained small electron-clear vesicles and large electrondense vesicles. Just underneath the circular muscle, thick nervous bundles with some isolated neurons could be observed. Again, varicosities with electrondense or mixed vesicles were predominant (Fig. 7b).

A second submucosal plexus could be observed, parallel to the muscularis mucosae. At this plexus, neural

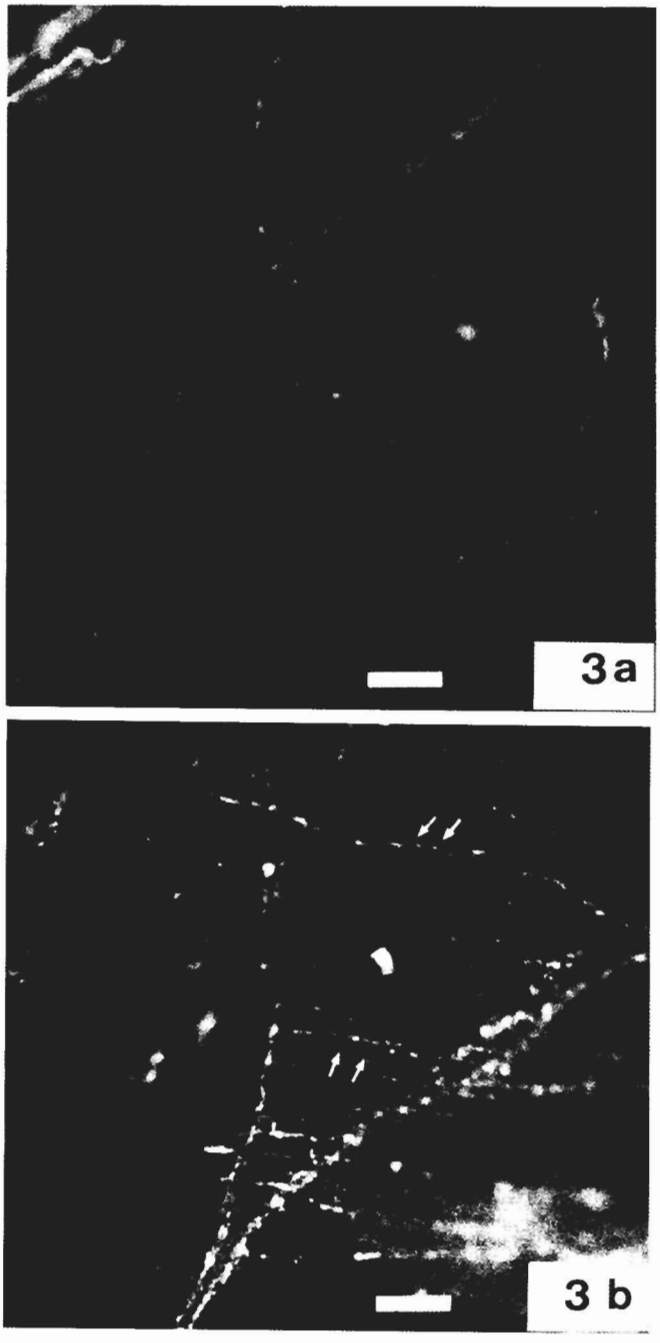


Fig. 3. Vasoactive intestinal peptide (VIP). Wholemount preparations. **a.** Large intestine. Myenteric plexus fibres. Scale bar: 110 μm. **b.** Small intestine. Thin varicose fibres in the tertiary plexus (arrow). Scale bar: 110 μm.

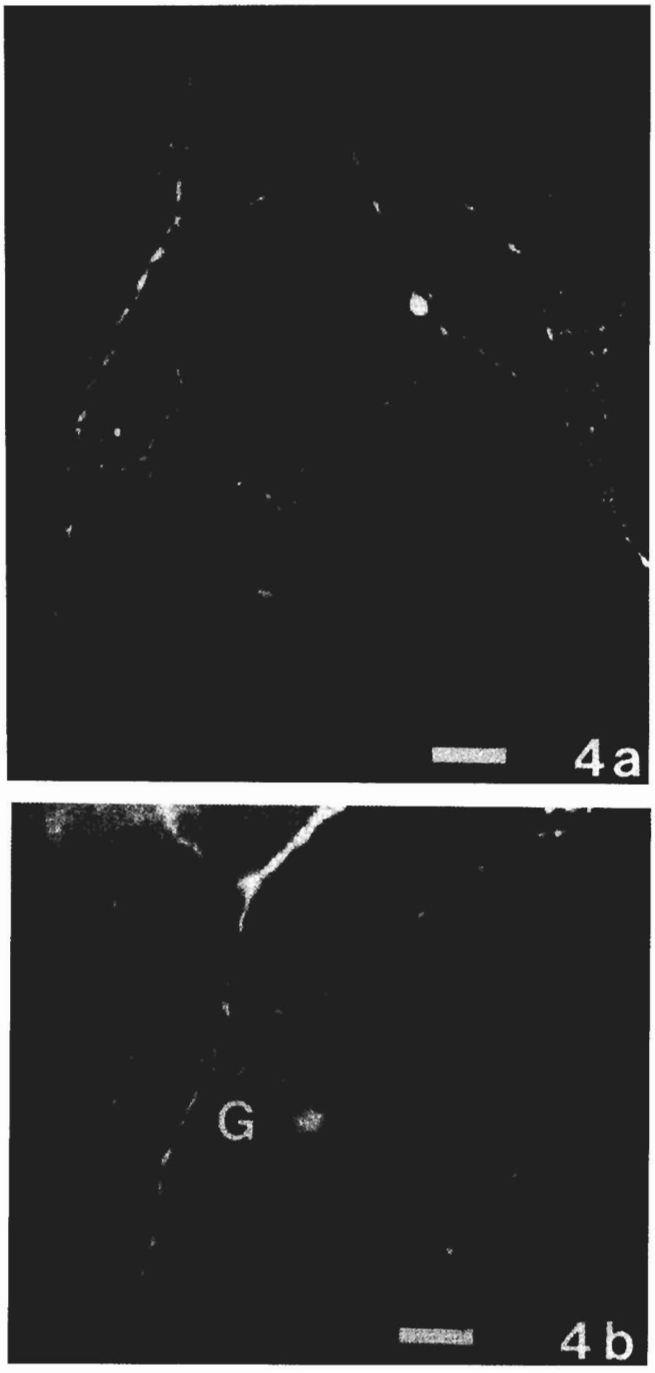


Fig. 4. Substance P (SP). Wholemount preparations. **a.** Large intestine. SP-positive fibres in the myenteric plexus, forming polygonal networks. Scale bar: 110 μm. **b.** Large intestine. Immunoreactive fibres surrounding a ganglion (G). Scale bar: 110 μm.

bundles showed a lower number of axons and, among them, isolated neuronal somata (Fig. 7c). In the lamina propria, we could observe a new cell type, its ultrastructural features (elongated nucleus, narrow cytoplasm with long processes) suggesting the ICC (Fig. 7c). Thinner nervous bundles, composed of 15-20 axons, approached the epithelium basal membrane (Fig. 7d).

Discussion

In spite of being non-specific, the use of AChE histochemical technique provided a well-defined pattern of intestine plexus morphology and distribution. The myenteric plexus in the intestine of *Podarcis hispanica* showed similar morphology to that in a chelonian reptile, *Pseudemys scripta elegans* (Timmermans et al., 1991). Our results differed from earlier descriptions in reptiles by Gabriel et al. (1988), when looking at cell density per ganglion. These authors only found solitary cells in the myenteric plexus at the intestine of *Lacerta agilis*. We were able to find 6 to 12 cells per ganglion, plus scattered neurons surrounding ganglia connections. Regional differences at the myenteric plexus along the intestine were minor, and the number of ganglion cells could vary from region to region, but not significantly.

Early studies in the reptilian ENS pleaded for a well-developed, aganglionic submucosal plexus (Gunn, 1951; Read and Burnstock, 1968; Nilsson and Holmgren, 1989). However, later studies in the alligator (Holmgren et al., 1989), chelonian (Timmermans et al., 1991), and lizard (Junquera et al., 1998), definitely confirmed that this plexus was ganglionic all along the gastrointestinal tract. As in the esophagus (Junquera et al., 1998), two well-defined submucosal plexuses were found in the intestine, with smaller ganglia and lower neuronal density than the myenteric plexus. The profuse AChE-positive vascular innervation suggests a partially extrinsic origin. In the myenteric and submucosal plexus, we observed fusiform cells that could be considered as ICC. The finding of ICC is original, as these are not usually located in the lamina propria in other species. This finding was verified and supported by EM observations.

Based on AChE observations, we concluded that, although morphology of reptilian enteric plexus was very similar to that of amphibians (Junquera et al., 1987a), reptilian plexus were more complex and the number of cells per ganglia was higher than in amphibians. Density of AChE-positive fibres is so high, compared to the number of positive neurons, that an extrinsic origin can be suspected.

The ratio adrenaline/noradrenaline was different in the different vertebrates. Classically, adrenaline acts as a

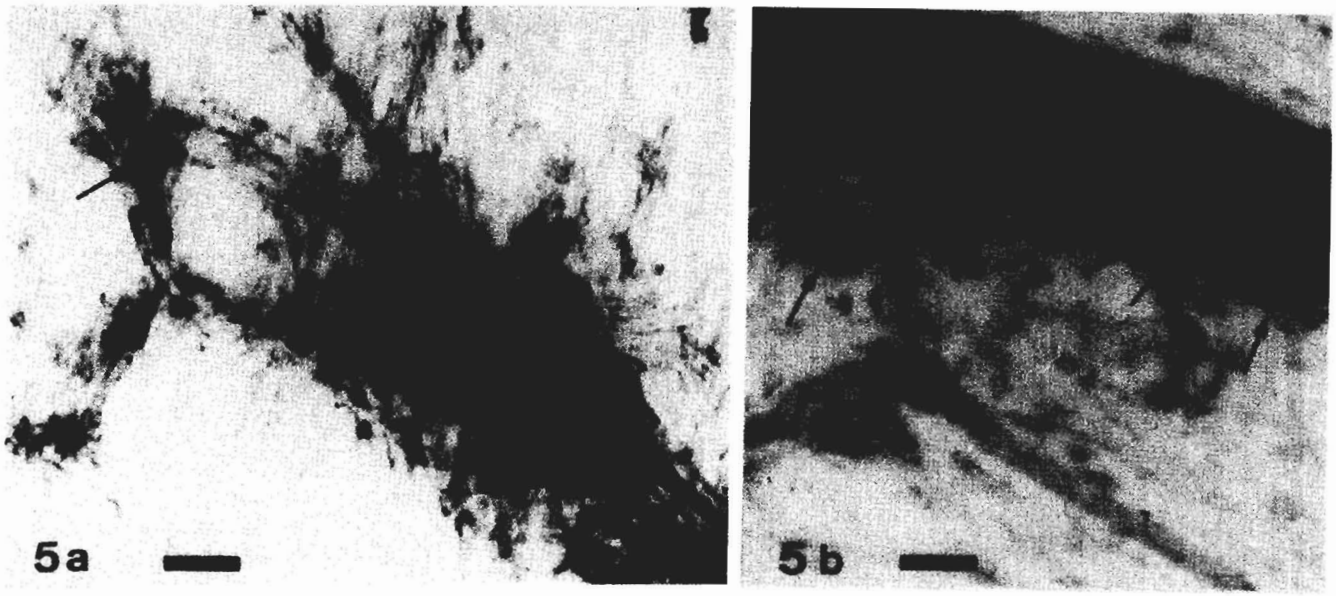
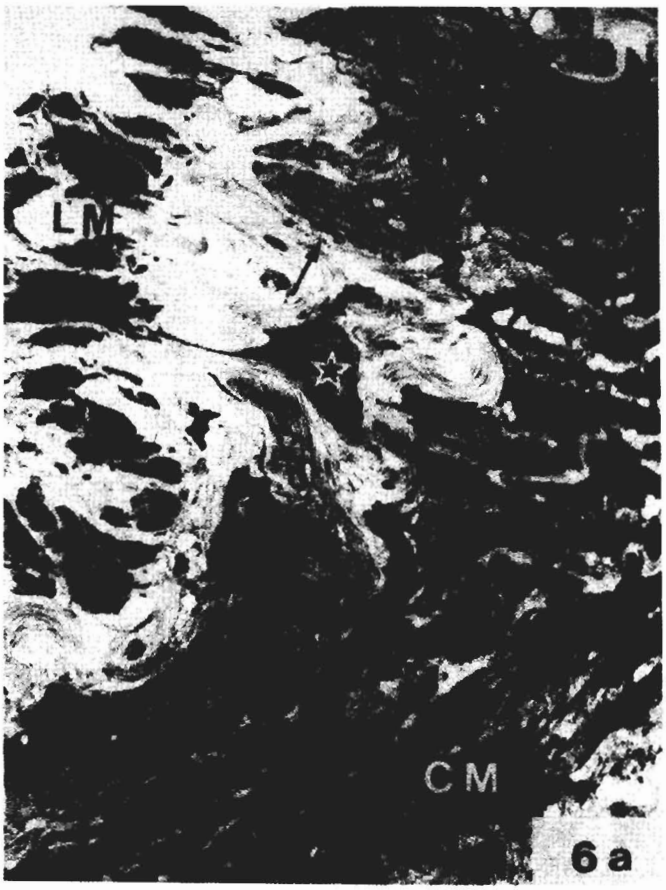


Fig. 5. NADPH-diaphorase histochemistry. Wholemount preparations. **a.** Large intestine. Positive neurons within a myenteric plexus ganglion and interconnection strands (arrow). Scale bar: 50 μm. **b.** Large intestine. Blood vessel in the submucosa. Positive cells in the nearby (arrows). Scale bar: 50 μm.

Fig. 6. Electron microscopy. **a.** Duodenum. Myenteric plexus ganglion. Three different neurons (N) can be observed, where nervous trunks approach (arrows). An interstitial cell of Cajal (ICC) (asterisk) gives out a long projection towards a muscle cell of the longitudinal layer (LM). Circular muscle (CM). Scale bar: 7 μm. **b.** Ileum. Myenteric ganglion neuron (N). A glial cell (GC) surrounds axons close to the neuron. Circular muscle (CM). Scale bar: 1.8 μm. **c.** Large intestine. Axons close to a myenteric plexus neuron. Scale bar: 2 μm. **d.** Enlargement of the previous figure that shows a mixed terminal synapting with the neuronal membrane (double arrow). Scale bar: 0.33 μm.



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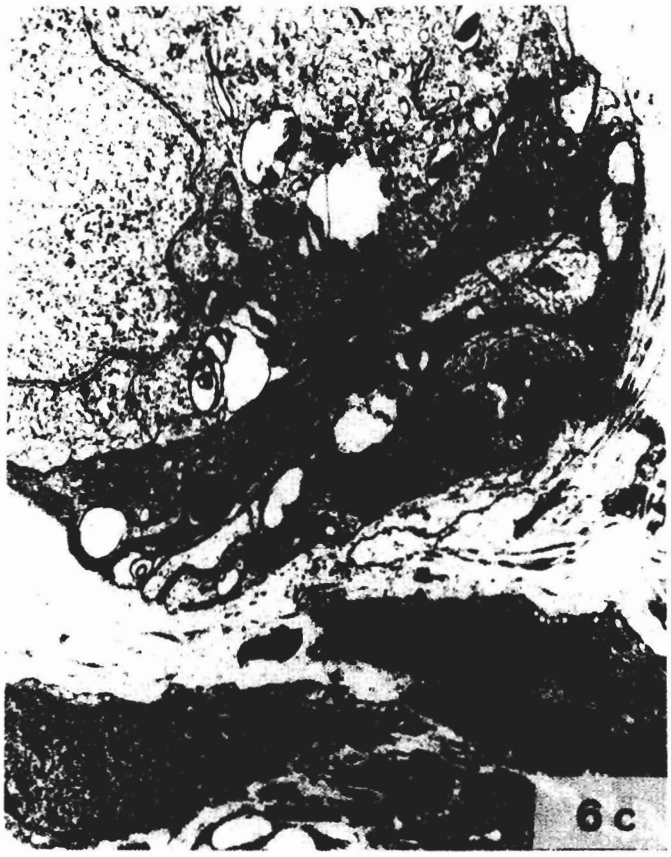

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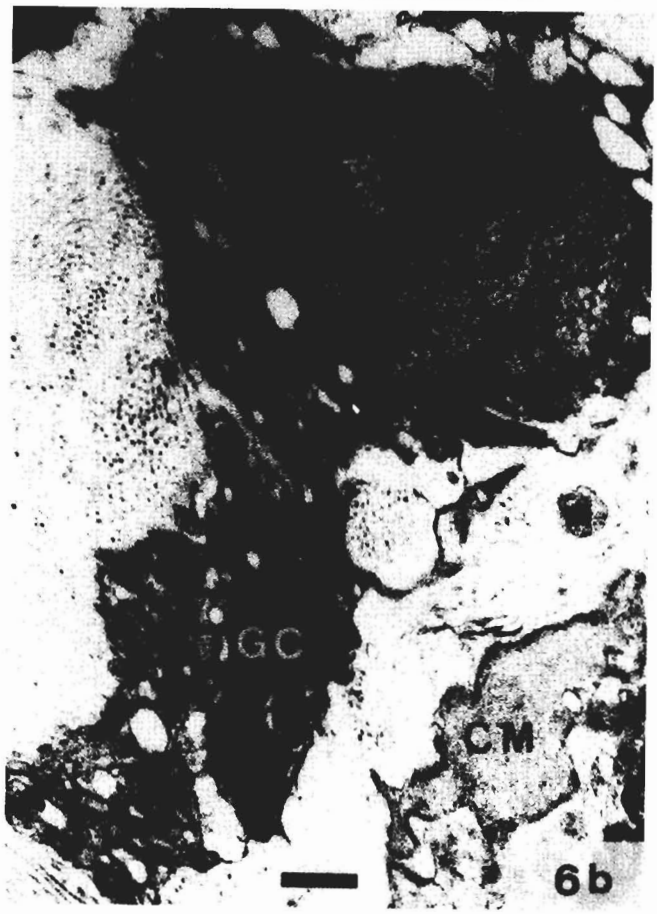

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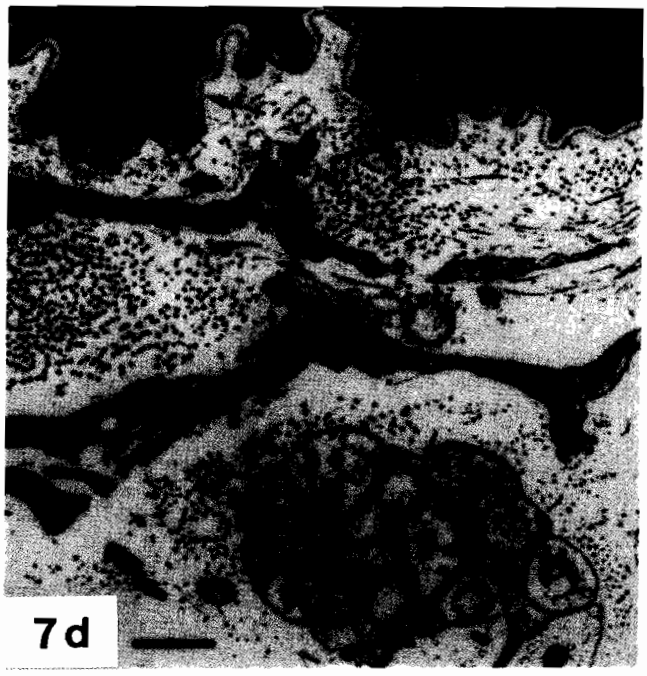

7d

Fig. 7. Electron microscopy. **a.** Ileum. Circular muscle. Axons in close contact with smooth muscle cells. Two types of vesicles can be differentiated: electron-clear vesicles and vesicles with an electrondense nucleus. Scale bar: 0.7 μm. **b.** Ileum. Submucosal nervous bundles, under the circular muscle. Electrondense vesicles (asterisk), mixed varicosity (arrow). Scale bar: 1 μm. **c.** Ileum. Deep submucosal plexus. Neuron (N), muscularis mucosae (MM). An interstitial cell (asterisk) sends a long projection towards a close group of axons. Scale bar: 7 μm. **d.** Large intestine. Axons forming bundles in the lamina propria. The epithelium basal membrane is clearly outlined (arrow). Scale bar: 1.4 μm.

catecholaminergic neurotransmitter mostly in fishes and amphibians, while noradrenaline does so in reptiles (Azuma et al., 1965), birds and mammals (Read and Burnstock, 1968). FIF technique should be helpful to determine which of these catecholamines are formed or held in a particular neuron. However, results in lower vertebrates were often inconclusive (Campbell, 1988). Our results proved a wide catecholaminergic innervation at the intestine of *Podarcis hispanica*, especially in the large intestine. A wide distribution was found in both myenteric and submucosal plexus, and also surrounding blood vessels. This innervation pattern in *Podarcis hispanica* clearly differed from the digestive innervation pattern in *Trachydosaurus rugosus*. Read and Burnstock (1969) noted that catecholaminergic fibres in this reptilian were sparsely scattered, isolated or in small bundles in the circular muscle layer of the large intestine, but not in the small intestine or the stomach. These differences could be related to seasonal variations in neural activity, an adaptive mechanism in poikilotherms, as suggested by others (Sigh, 1964; Gabriel and Budai, 1992). As Read and Burstock (1969) did, we were able to observe fluorescent terminal fibres surrounding ganglion cells. We did not observe positive neurons in the myenteric plexus. We suggest that these were sympathetic fibres.

We confirmed an increase in the catecholaminergic innervation at the reptilian intestine, if compared with amphibians (Junquera et al., 1987a). This was also observed in the esophagus of *Podarcis hispanica* (Junquera et al., 1998) and *Rana ridibunda* (Junquera et al., 1988). All these results support the proposal of Berger and Burnstock (1979) of a progressive increase in catecholaminergic nerves from lower to higher vertebrates.

Rabbit VIP antiserum was highly satisfactory for "in toto" reptilian specimens. We were able to observe a wide network of VIP-LI immunoreactive fibres in the myenteric plexus. There were also VIP-positive neurons, isolated among the fibres or grouped in small ganglia. These results found in *Podarcis hispanica* do not agree with descriptions in other reptiles, such as *Alligator mississipiensis* (Buchan et al., 1983), or *Viper aspis* (Masini, 1986). Buchan et al. (1983) described numerous neural fibres and cell bodies that concentrated at the submucosal plexus in fundus, antrum, and intestine, in *Alligator mississipiensis*. These authors found no neurons at the myenteric plexus. Masini (1986) observed that fibres in *Viper aspis* were located underneath the epithelium, at the lamina propria. Immunoreactive cells were found in the epithelium that lined mucosa foldings. These results were not observed in two different water snakes (*Natrix natrix* and *Natrix maura*). VIP was totally absent in the gut of the turtle (Scheuermann et al., 1991), whereas it is ubiquitous in that of the lizard, *Lacerta viridis* (Reinecke et al., 1981). Lamanna et al. (1999a) described VIP neurons in the stomach of the lizard, *Podarcis s. sicula*. Although we did not observe VIP-ergic innervation surrounding blood vessels in *Podarcis hispanica*, Morescalchi et al. (1997) described VIP-immunoreactive nerve fibres surrounding intestinal blood vessels in the sauride *Chalcides chalcides*. Wide VIP-ergic networks in the myenteric plexus were similar to those found in fish (Holmgren et al., 1982; Holmgren and Nilsson, 1983a,b; El-Sally, 1984) and amphibians (Junquera et al., 1986, 1987a,b; Gibbins et al., 1987; Gabriel et al., 1992; Murphy and Campbell, 1993). VIP-LI neurons projecting to the gut and the lung were described in the vagus nerve of *Bufo marinus* (Gibbins et al., 1987). Therefore, the extrinsic origin of some reported fibres in *Podarcis hispanica* cannot be dismissed.

SP-like immunoreactivity (SP-LI) was observed in delicate varicose networks, within the myenteric plexus along the whole intestine in *Podarcis hispanica*. SP-positive fibre density in the myenteric plexus was higher than in amphibians (Junquera et al., 1987; Gabriel, 1990; Gabriel et al., 1992; Murphy and Campbell, 1993). We could also observe SP-LI fibres that formed a modest perivascular plexus around some submucous blood vessels, as described in *Bufo marinus* (Osborne and Campbell, 1986; Murphy and Campbell, 1993).

Intrinsic SP-LI neurons have been described in the ENS of the fishes (Karila et al., 1998). In amphibians, SP-LI immunoreactive cells have been reported in *Necturus maculosus* (Holmgren et al., 1985), in the large intestine of *Rana ridibunda* (Junquera et al., 1987a), in the small intestine of *Bufo marinus* (Osborne and Gibbins, 1988), in the midgut of *Rana esculenta* (Gabriel, 1990), and to a lesser extent, in the stomach of *Ambystoma mexicanum* (Gabriel et al., 1992). However, no SP-LI cell bodies were present either in the hindgut of *Rana esculenta* (Gabriel, 1990), or in the intestine of *Hydromantes ambrosii* (Faraldi et al., 1993). In birds, these have been described in the digestive system (Brodin et al., 1981), and particularly in the anterior gut of the chicken (Aisa et al., 1987). In reptiles, Buchan et al. (1983) described these cells in the myenteric and submucosal plexus along the intestine of *Alligator mississipiensis*.

There was a heterogeneous distribution of SP-LI cells along the digestive system of the reptile in our study, and of other amphibian species that were revised. We could not identify this in the esophagus of *Podarcis*

hispanica, in a previous study (Junquera et al., 1998). We could only occasionally observe SP-positive immunoreactive cells in its caudal region. Immunoreactivity in these cells could be due to the presence of the peptide in the cytoplasm, or in adjacent axonal varicosities. According to Campbell (1988), it is more difficult to visualize SP-LI in somata than in varicose fibres. The low number of SP-positive neurons could be explained by the following reasons. First, given that different SP molecular configurations have been found in amphibians and birds (Creagh et al., 1980), an unrelated SP configuration in reptiles could have been undetected by our methods. An SP anti-rabbit antiserum may not be the best configuration to produce a sufficiently strong reaction to reveal positive neuronal bodies in reptiles. Secondly, changes in SP liberation due to seasonal variations in poikilotherm animals have been described (Singh, 1964). This could also affect our results. Finally, neural fibres projecting into every intestinal layer could originate in cell bodies located in dorsal root ganglia (Murphy and Campbell, 1993). This reasoning would support the hypothesis that SP-ergic fibres would be mostly sensory nerve fibres (Osborne and Campbell, 1986).

Different papers have shown the presence of NO all along the gastrointestinal tract in non-mammalian vertebrates. In fishes, Li et al. (1993) performed a study on the distribution of fibres and neural somata in the rainbow trout. Our results were similar to theirs regarding the widespread presence of these neural elements in the large intestine, verifying the progressive increase of gastrointestinal neural elements caudally. As in our findings, Brüning et al. (1996) described solitary neurons that were also located in the submucosal layer, often situated near blood vessels, in the large intestine. In amphibians, Li et al. (1992) performed a study on the wide distribution of NADPH-d-positive neural elements. In the toad, the number of neural cells was higher in the large intestine submucosa, sometimes lying close to blood vessels. Lamanna et al. (1999a) described the distribution of NADPH-d-reactive neuron in gastrointestinal tract of the lizard. They reported a moderate number of positive neurons in the myenteric plexus of the small and large intestine. In the submucous plexus, the neurons were not present. Olsson and Gibbins (1999) observed the distribution of nitric oxide synthase (NOS) in the gastrointestinal tract of the *Crocodylus porosus*. They found NOS-positive nerve cell bodies and fibres in all regions of the gut. In addition, small blood vessels in the submucosa and on the serosal surface of the gut were innervated by NOS-immunoreactive fibres. The positive reaction to NADPH-diaphorase in myenteric plexus neurons, allowed us to suggest that NO also plays an important role in reptiles as a signal molecule at the ENS.

Our ultrastructural observations agree with the results obtained with different techniques for optical microscopy. Due to the rarity of reptilian intestine electron microscopy studies in the literature, we cannot further discuss our findings with other authors. We have been able to show the presence of two well-defined submucosal plexuses, and ultrastructural features of three types of cell which are present in the reptilian intestinal plexus: neurons, glial cells, and interstitial cells. The abundance of electrondense varicosities in nerve trunks in the reptilian intestine leads us to suggest a significant evolutionary role of neuropeptides. The existence of frequent mixed-type varicosities supports the relevance of neuromodulation in the complex neurochemical pathways of reptilian intestinal plexus. The innervation of the reptilian intestine has proved to be complex in the studied lizard, and is far from the simple model that could be assumed phylogenetically.

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