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Histochemistry

The innervation of the gastrointestinal tract of a chelonian reptile, *Pseudemys scripta elegans*

I. Structure and topography of the enteric nerve plexuses using neuron-specific enolase immunohistochemistry*

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Summary. The general morphological features of the intramural enteric nervous system of a chelonian species, i.e. the red-eared turtle, *Pseudemys scripta elegans*, has been studied in whole-mounts and cryosections by means of neuron-specific enolase immunohistochemistry. A clear visualization of both neuronal cell bodies and nerve fibres allows the recognition of a myenteric plexus as well as a submucous plexus in several gut regions, namely the stomach, midgut and hindgut. The highest innervation density was found in the midgut portion. In contrast to other lower vertebrates, such as amphibians and other reptilian groups, the present study clearly demonstrates the occurrence of neuronal cell bodies in the submucous plexus of all regions investigated. The neurons stained for neuron-specific enolase harboured smooth-contoured perikarya from which one or more processes emerge, as demonstrated for the mammalian enteric nervous system.

Introduction

Heretofore, the knowledge of the general morphology of the enteric nervous system (ENS) is mainly deduced from studies on mammals. These are small laboratory animals (for review see Gabella 1979; Furness and Costa 1987), larger mammals (Gunn 1968; Stach 1977a, b, 1989; Mannl et al. 1986; Scheuermann et al. 1989a; Timmermans et al. 1989, 1990), and man (Hoyle and Burnstock 1989). In 1933 Kondratjew briefly commented on the presence of a submucosal nerve network in a turtle. Other, more recent, studies on the intramural innervation of the reptilian gastrointestinal tract dealt exclusively with such restricted topics as the demonstration of adrenergic nerve fibres in the large intestine of a lizard (Read and Burnstock 1968), of bombesin immunoreactivity in the gut of a caiman (Holmgren et al. 1989), of VIP-immunoreactivity in the small intestine of a viper (Masini 1986) and of a number of regulatory peptides in the gastrointestinal tract of an alligator (Buchan et al. 1983). None of these investigations, however, discuss the general morphological features of the different enteric nerve networks in the reptiles studied.

In the last decade, the study of the architecture and the structure of the ENS of lower vertebrates (for review see Nilsson and Holmgren 1989) and of some invertebrates (Gabriel et al. 1988) has proved to be valid from an evolutionary and functional point of view because of the considerably lower number of intrinsic neurons involved. From a comparative morphological viewpoint, it is rather surprising that the reptiles, and in particular the chelonians, have been ignored so far. The present study offers the first extensive description of the architecture and the topographical organization of the ENS of a chelonian species, i.e. the red-eared turtle, *Pseudemys scripta elegans*, by means of immunocytochemistry using an antiserum raised against neuron-specific enolase (NSE).

Materials and methods

Specimens of both sexes of the red-eared turtle, *Pseudemys scripta elegans*, weighing 10–400 g, were obtained from the Antwerp Zoological Garden and from commercial dealers. The animals were fed ad libitum. Anesthesia was performed by an intraperitoneal injection of Nembutal (30 mg/kg body weight).

Segments from several regions of the gastrointestinal tract, i.e. the stomach, midgut and hindgut, were prelevated, ligated and filled with phosphate buffered saline (PBS). The samples were then immersed in 4% paraformaldehyde containing 0.2% picric acid in phosphate buffer (pH = 7.2; 0.01 *M*) for 2 h at room temperature and subsequently processed as described earlier (Scheuermann et al. 1987). Accordingly, the whole-mount preparations contained either (*1.*) the tela submucosa or (*2.*) the tela submucosa with the circular muscle layer and the adhering myenteric plexus or (*3.*) the longitudinal muscle layer with the adhering myenteric plexus. The tissues were preincubated in 10% normal donkey serum (017-000-121, Jackson Immunoresearch Laboratories) for 30 min, fol-

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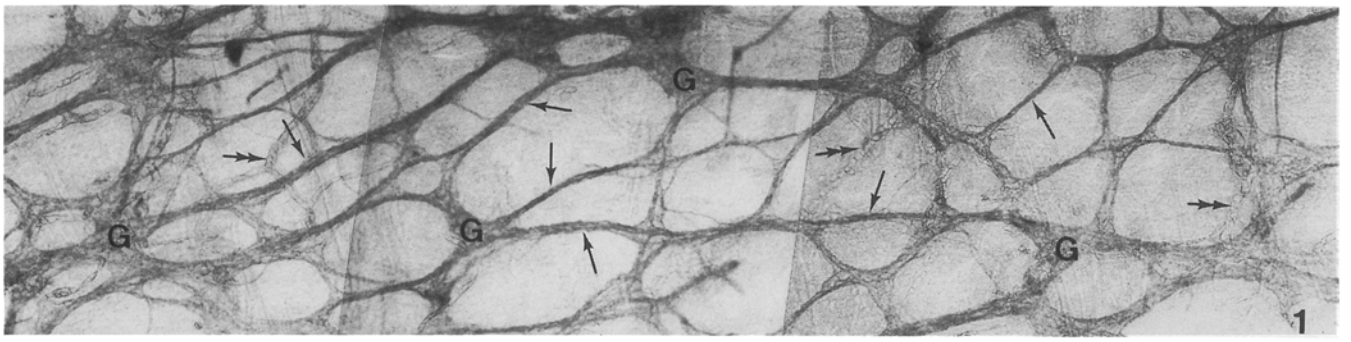
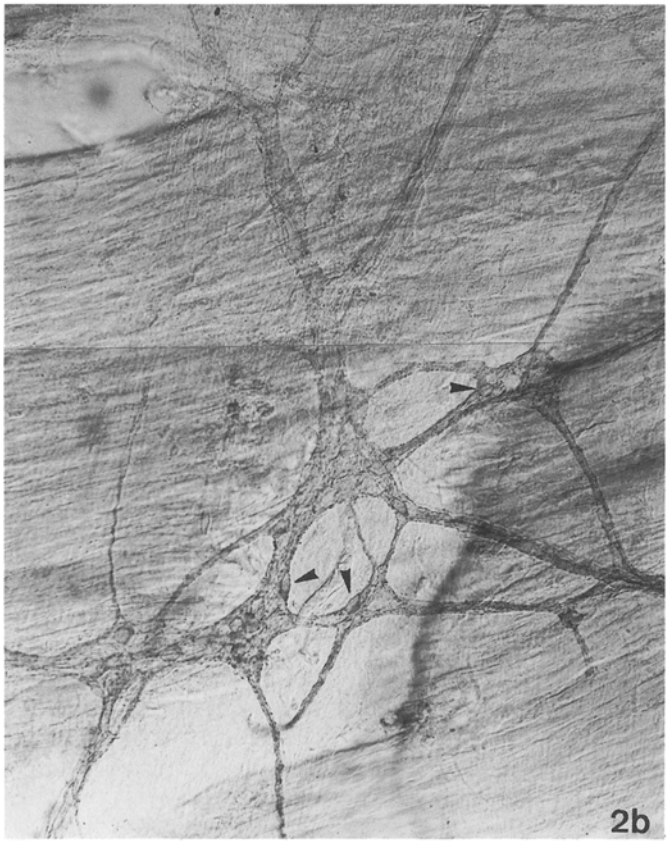
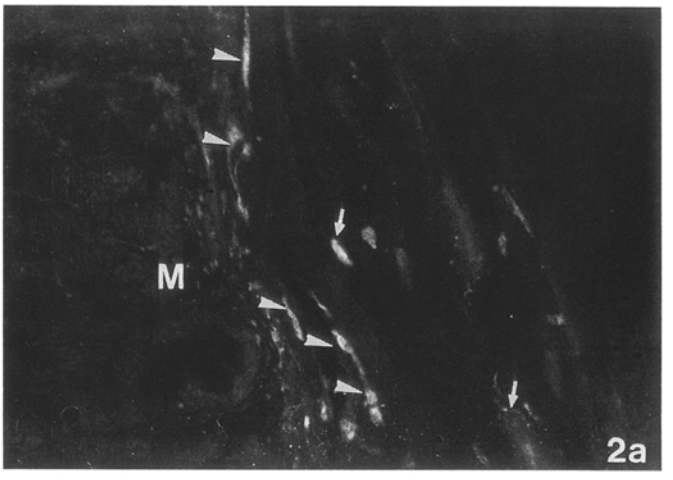






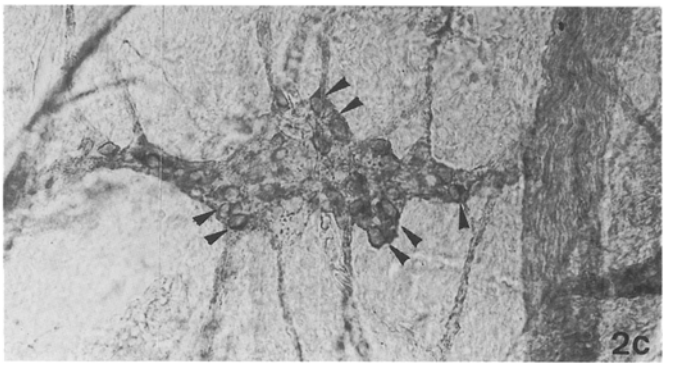


Fig. 1. Low power micrograph of the myenteric plexus in the pyloric region of the stomach of the red-eared turtle after immunolabelling for NSE. The ganglia (*G*) interconnected by relatively thick nerve bundles (*arrows*) form a dense nerve network. Capillaries (*double arrows*). ×85

Fig. 2a–c. Immunoreactivity for NSE in the pyloric region. **a** Cryostat section revealing the ganglia of the submucosal nerve network (*arrowheads*) and the myenteric plexus located between the obliquely running muscle bundles (*arrows*). (*M*) mucosal epithelium. **b, c** Whole-mounts containing the submucous plexus. Note the presence of immunoreactive neuronal perikarya (*arrowheads*). **a** ×70; **b** (*Nomarski optics*) ×185; **c** ×225

lowed by incubation in a primary antiserum raised against NSE (A589, Dakopatts, diluted 1:400) for 17–48 h. Donkey anti-rabbit biotinylated IgG (RPN.1004, Amersham, diluted 1:100) was used for 6 h at room temperature as the secondary antiserum, followed by incubation in streptavidin-biotinylated peroxidase complex (RPN.1051, Amersham, diluted 1:100) for another 17 h at room temperature. The immunoreaction was visualized using 4-Cl-1-naphtol.

For cryosectioning, the tissues were similarly fixed as for the whole-mounts. Excessive fixative was removed by three short rinses in dimethylsulphoxide, followed by immersion in 10%, 20% and 30% glycerol for 30 min each. Segments of the stomach, midgut and hindgut were then cut into small pieces, embedded in Tissue TEK II and frozen with CO_2. 15–25-µm-thick sections were made on an SLEE-cryostat at −20° C and collected on chrome-alum gelatin-coated slides. After pretreatment in normal serum for 30 min at room temperature, the sections were incubated in the primary antiserum overnight in a humid chamber at room temperature. FITC-conjugated goat anti-rabbit IgG (GAR/FITC, Nordic Immunological Laboratories, diluted 1:20) was used as the secondary antiserum for 2 h at room temperature. The sections were studied with a Leitz Orthoplan microscope equipped with a Leitz I2/3-filter combination (excitation filter BP 450–490; dichroic mirror TK 510; barrier filter K515).

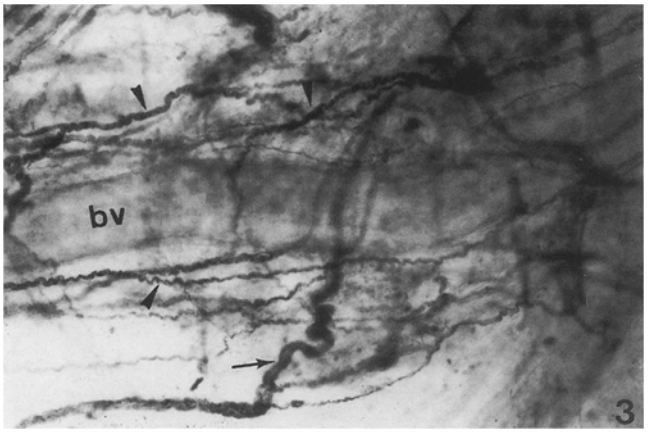


Fig. 3. Nerve bundles (*arrowheads*) of extrinsic origin run along the larger blood vessels (*bv*) and give off branches (*arrows*) to the intrinsic nerve meshwork formed by the myenteric plexus. × 65

Results

Stomach

At the macroscopic level the wall of the pyloric part is much thicker than that of the cardiac region. However, the density and topographical pattern of the intramural innervation (Fig. 1) do not differ conspicuously. The extrinsic nerves, with branches that run along the larger blood vessels, connect with the intrinsic meshwork of the myenteric plexus (Fig. 3). In the stomach of the red-eared turtle the myenteric plexus is composed of ganglia, built up of neurons that range in number from a few to several dozens (Fig. 1). The ganglia are interconnected through relatively thick nerve bundles. Thinner nerve strands can be found to run in the direction of the circular smooth muscle layer. Only few fibres are found to innervate the longitudinal smooth muscle layer. The latter is poorly developed in all gut regions of the species investigated. Some of the nerve bundles directed toward the submucous layer proceed in a submucous ganglionic nerve network which also contains a considerable number of neuronal cell bodies (Fig. 2a–c). The lamina muscularis mucosae seems scarcely innervated and only few fibres were found to run into the mucosal layer.

Midgut

From the nerves, running along the mesenteric border, smaller bundles branch off into the myenteric plexus (Fig. 4a). The ganglia are smaller and less elongated than those of the stomach. There is a number of NSE-immunoreactive perikarya and pericellular baskets, the latter indicating the occurrence of non-stained nerve cells. The number of neurons per ganglion does not exceed 15 (Fig. 4b, c). Most of the ganglionic cells stained for NSE have smooth contours, from which one or more nerve processes emerge (Fig. 4c). A dense aganglionic nerve network was observed in the circular smooth muscle layer (Fig. 4d), whereas only a few fibres could be demonstrated in the longitudinal muscle layer. In the midgut portion the submucous plexus is located at different levels and consists of a very dense network of both neuronal somata and intermingling nerve fibres (Fig. 4e).

Hindgut

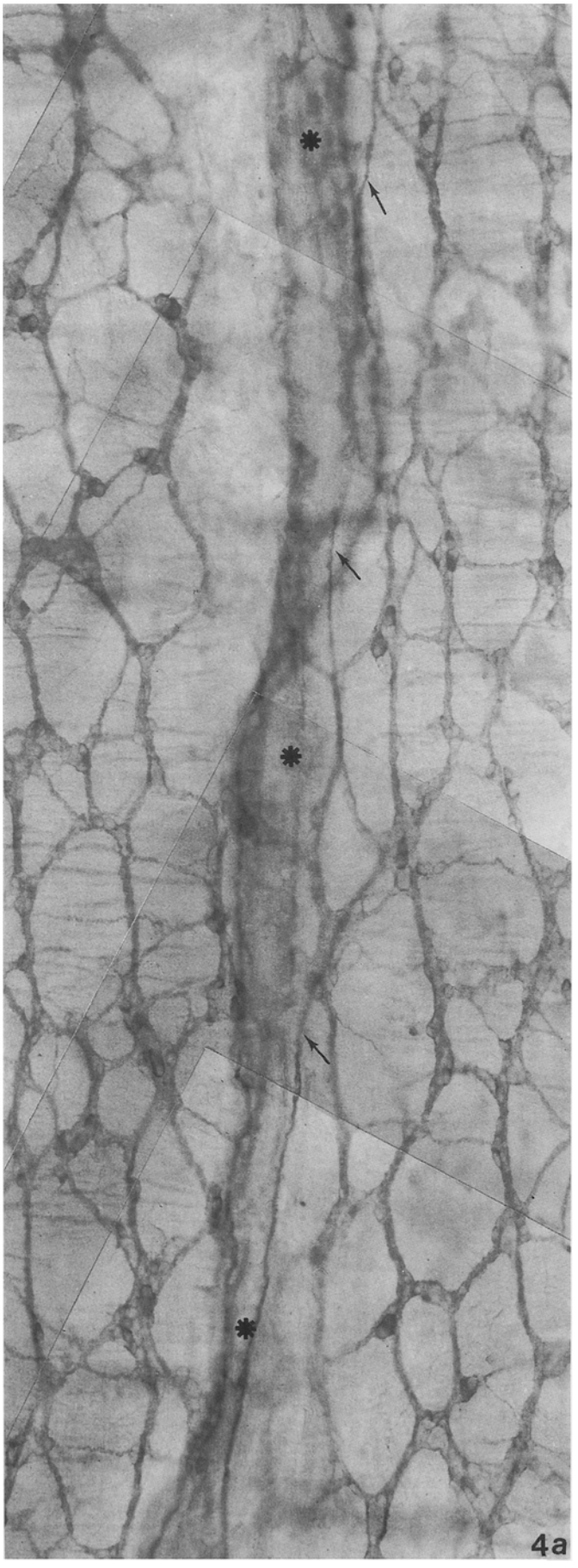
Compared to the midgut and, to a lesser extent, to the stomach, the intrinsic innervation pattern of the hindgut consists of a less dense nerve fibre network, both with respect to the myenteric and the submucous plexus (Fig. 5a–c). It also differs from the above-mentioned regions in that both ganglionic plexuses have less neuronal cell bodies (Fig. 5a, b). It seems that there are no anatomical arguments to propose the presence of a real muscular sphincter at the border between the midgut and hindgut portion. The innervation of the external circular muscle layer is, however, very pronounced.

Discussion

The primary antisera used in this study were raised against mammalian antigens. Obviously, they were most suited to recognize the characteristic morphology of the ENS of lower vertebrates. As in the mammalian ENS (Scheuermann et al. 1989b), immunostaining for NSE does not visualize all the neuronal cell bodies that occur in the intrinsic plexuses. Hence, this method is less suitable than e.g. the histochemical NADH-dependent dehydrogenase method, performed by Gabella (1969), to evaluate the number of neurons and to compare them between the different regions of the gastrointestinal tract (Gabella 1971, 1987). The applied immunocytochemical method may, however, be considered an excellent alternative to the methylene blue staining and the silver impregnation technique to investigate the general innervation pattern and the course of the nerve fibres because the method can easily be carried out and reproduced.

Similar to the amphibian ENS (Gunn 1951; Nilsson 1983), both a submucosal and myenteric plexus can be distinguished in the gut of the red-eared turtle. But in contrast to the amphibian ENS (Gunn 1951; Nilsson 1983; Junquera et al. 1986) and to some reptilian families, such as the *Lacertidia* (Read and Burnstock 1968), the red-eared turtle harbours a considerable, in some regions even high number of submucous neuronal cell bodies. In this respect, the submucosal plexus of the turtle gut resembles more closely that of the caiman, which, unlike other lower vertebrates, can be considered as a well-developed ganglionated nerve network (Holmgren et al. 1989).

In conclusion, immunohistochemistry of the ENS of the red-eared turtle at the light-microscopic level by means of antibodies against NSE on whole-mounts demonstrates greater similarities with the morphological fea-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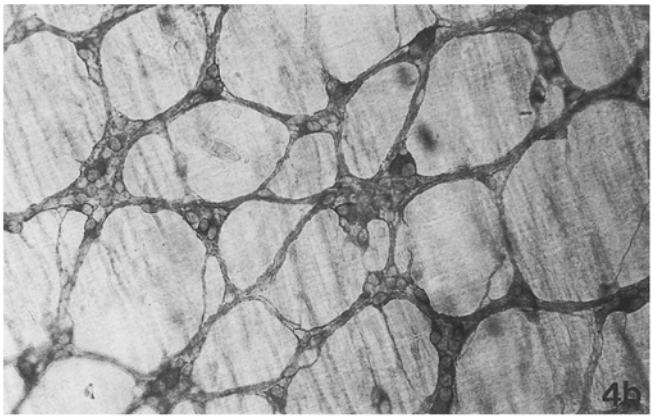
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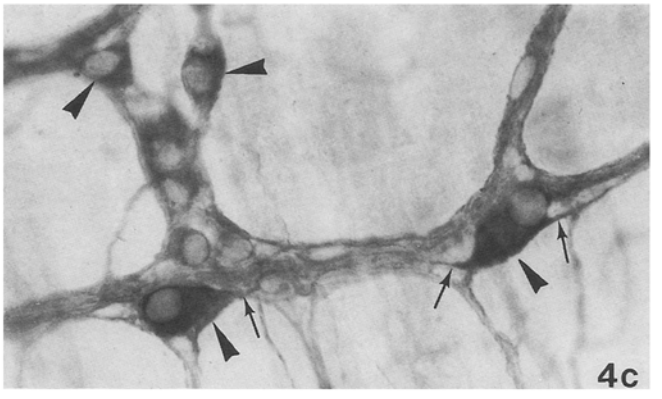
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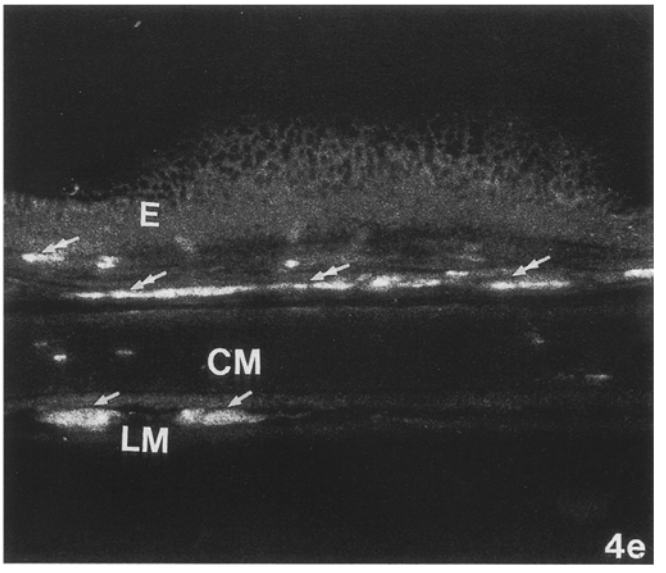
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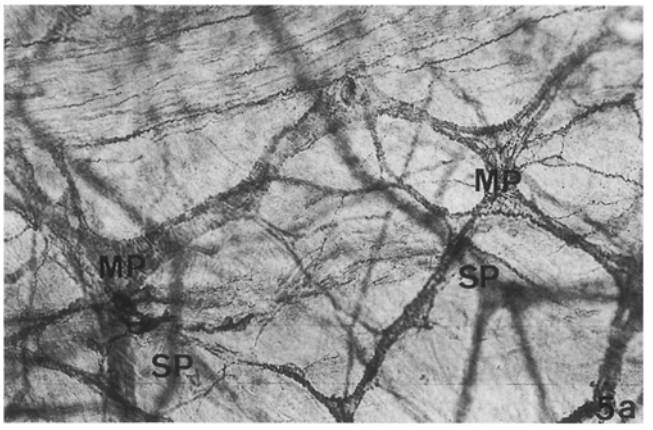

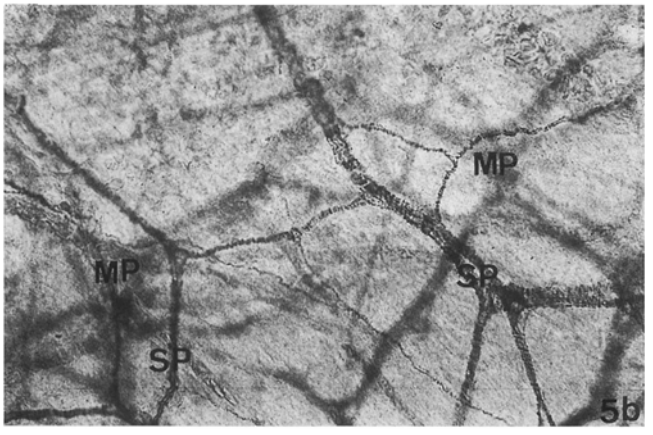

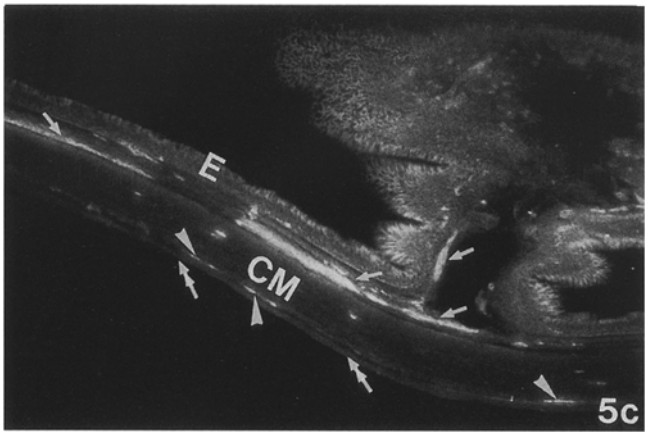

Fig. 5a–c. Enteric nerve networks in the hindgut immunolabelled for NSE. **a, b** Different planes of focus of the same site. In **a** the focal plane is at the level of the myenteric plexus (*MP*), while in **b** the focal plane is at the level of the submucous plexus (*SP*). **c** Cryostat section. (*Arrows*) submucous plexus; (*arrowheads*) myenteric plexus; (*CM*) circular muscle layer; (*double arrow*) longitudinal muscle layer; (*E*) epithelium. **a, b** ×160; **c** ×70

tures of the different ENS parts of small mammals than of amphibians or other reptilian groups as e.g. lizards. Moreover, this study provides a basis for an immunohistochemical investigation of the peptidergic component of the ENS in the red-eared turtle.

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Fig. 4a–e. Micrographs illustrating the immunoreactivity for NSE in the midgut of the red-eared turtle. **a** Low magnification of part of the mesenteric nerve (*asterisk*) and underlying myenteric plexus. Note the presence of nerve strands branching off from the mesenteric nerves to the myenteric plexus (*arrows*). **b** Detail of the dense meshwork of the myenteric plexus. **c** Most of the neurons (*arrowheads*) stained for NSE are characterized by a smooth contour from which one or more processes emerge (*arrows*). **d** Aganglionic innervation of the circular muscle layer. Nerve fibres run parallel to the length axis of the smooth muscle fibres. Note the faint contours of the underlying myenteric plexus. **e** Cryostat section showing both the myenteric (*arrows*) and submucous plexuses (*double arrows*). Note the high density of the submucosal ganglionic network which seems to be organized at different levels within the submucosal region. (*E*) Epithelium; (*CM*) circular muscle layer; (*LM*) longitudinal muscle layer. **a** ×165; **b** ×160; **c** ×410; **d** ×160; **e** ×150

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