

Some morphological and histochemical features of the midgut myenteric plexus of the common european frog, *Rana Esculenta*

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Summary. The neuron morphology and distribution of four putative transmitters were investigated in the myenteric plexus of frog (*Rana esculenta*) midgut. The gross morphology was revealed by NADH-diaphorase histochemistry, and the shape of the neurons by silver impregnation. Nerve cells had heterogeneous distribution: they either formed ganglia or placed as solitary neurons in the duodenum, while in the rest of the midgut only solitary neurons were observed. Three morphologically distinct cell types were revealed by silver impregnation: mainly type I and type II neurons cells were seen in the duodenum, while the rest of the intestine contained type II and III cells. Catecholamine fluorescence was revealed in nerve fibres in the duodenum, while few small nerve cells were observed in the small intestinal region. Acetylcholinesterase histochemistry showed strongly reactive nerve cells that were associated with the main fibre bundles in the duodenum. Only longitudinally oriented fibres and occasionally stained neurons were seen in the small intestine. Substance P immunocytochemistry revealed an extensive plexus, which contained a moderate number of stained perikarya in the full length of the midgut. Gamma-aminobutyric acid showed non-uniform distribution in the two parts of the midgut: a stronger and more regular fibre staining was found in the duodenum than in the rest of the intestine. Ultrastructural observations demonstrated that intrinsic neurons received synaptic inputs from the profiles contained agranular vesicles, while «P»-type profiles established close contacts with neurons. Both profile types formed close contacts with the smooth muscle cells. In spite of the several neuromorphological and ultrastructural similarities to the mammalian species, the transmitter distribution described here does not match in all respects to that found in the enteric nervous system of

those animals. This finding reflects the view, that the neurochemical coding system and the projection pattern within the plexus may vary considerably among different classes of vertebrates.

Key words: NADH-diaphorase, Silver impregnation, Acetylcholinesterase, Induced catecholamine fluorescence, GABA, Substance P, Histochemistry, Ultrastructure

Introduction

The anatomy of the enteric plexuses and the main morphological features of the enteric neurons have been described in amphibia (Gunn, 1951). She pointed out that the midgut myenteric plexus of frog contains solitary nerve cells which display mainly Dogiel type II and III morphology. Type II cells are small, round cells with several long processes, while the type III cells are even smaller, with short axon bearing no collaterals or they may have bipolar-like morphology. The presence of catecholamines (CAs) has been reported in enteric neurons in *Bufo marinus*, but the morphology of these cells were not described in detail (Read and Burnstock, 1968, 1969). Similarly, acetylcholinesterase (AChE)-positive neurons (classified as cholinergic cells) have been observed in the anuran myenteric plexus (Wong et al., 1971). The contractile effects of purines has also been demonstrated, however, no morphological analysis was undertaken (Burnstock, 1972; Burnstock et al., 1972; Sneddon et al., 1973). Several neuroactive peptides, like vasoactive intestinal peptide (VIP); (Buchan et al., 1981), substance P (SP) and somatostatin (SOM) (Junquera et al., 1987; Gábel, 1990) have been found in considerable amounts in the enteric nervous system of different amphibian species. The list of neuroactive substances has been recently broadened by the inclusion of γ -aminobutyric

acid (GABA), that is present in the myenteric neurons of frog stomach (Gábel and Eckert, 1989). The distribution pattern of these substances within the midgut and the possible cell morphology-transmitter correlations, however, have not been studied by any of the above investigators. In the present work we aimed at studying the neurochemistry and ultrastructure of the midgut myenteric plexus in the common European frog (*Rana esculenta* L.). We wished to establish possible links among the cell distribution and morphology and the transmitter content of these neurons. The functional aspects of the neurochemical and the ultrastructural findings are also discussed.

Materials and methods

A total of 22 adult *Rana esculenta* of both sexes were used. The animals were either decapitated (for silver impregnation, NADH-diaphorase (NADHd), AChE and fluorescent CA histochemistry) or anesthetized with urethane and perfused transcardially (for GABA and SP immunocytochemistry, and for EM).

Silver impregnation was carried out in frozen sections by the standard method of Cauna (1959).

Histochemical procedures

a. NADHd histochemistry. Both the duodenum and the small intestine were removed, rinsed and distended in Krebs solution. Tissues were incubated at room temperature for 30-60 min in the incubating medium contained 2.5 mg nitroblue tetrazolium and 10 mg NADH in 20 ml 0.1 M phosphate buffer (PB) (Gabella, 1969). Samples were then fixed in neutral formalin for at least 12 h at room temperature. Muscle layer whole mounts were prepared from the gut and mounted in neutral glycerol. Hundred neurons were selected for diameter measurements from each gut segment. Mean and s.d. was calculated.

b. AChE histochemistry. Tissue preparation was performed as described in a. Tissues were incubated for 1-2 h at 4° C in a medium contained 13mM acetylthiocholine iodide, 18mM copper sulphate, 33mM glycine, 25mM succinate and 0.2mM ethopropazine-HCl (Pearse, 1960). Fixation and preparation of the tissue were carried out as described in a.

c. Detection of CAs. The midgut was quickly dissected and incubated in a mixture of sucrose (6.8%), potassium dihydrogenphosphate (3.2%) and glyoxylic acid (1%) for 15 min at 4° C (de la Torre and Surgeon, 1976). After the incubation, the mucous and submucous layers of the gut wall were removed, and the muscle layer was stretched on slides and dried in a continuous air flow at room temperature. For the formation of the fluorophore, heat treatment was

performed at 95° C for 4 min. Specimens were then coverslipped in fluorescence-free paraffin oil, viewed and photographed in a Leitz Orthoplan fluorescence microscope equipped with an HBO-200 mercury lamp and an E-3 filter block.

d. Immunocytochemistry for GABA and SP. Animals were perfused first with physiological saline, followed by a fixative (for GABA: 100 ml aqueous saturated picric acid, 25 ml 25% glutaraldehyde and 1 ml glacial acetic acid; for SP: 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1M PB). The intestine was removed and opened at the mesenteric border. The muscle layer was separated from the other layers of the gut wall, postfixed in the same fixative for 4 h followed by an overnight washing in salinated PB (PBS). Preincubation was carried out in 20% normal goat serum. Antisera were raised in rabbits (anti-GABA: see Blechsmiedt et al., 1988, anti-SP: Amersham) and the samples were incubated with them for 24 h (dilutions: GABA 1:200, SP 1:1000). After washing in PBS, goat anti-rabbit secondary antibody was applied in a dilution of 1:20 for 1 h, followed by peroxidase-antiperoxidase complex for 3 h (diluted 1:60). After short rinses in PBS, specimens were transferred into TRIS-HCl buffer (0.05M, pH 7.6). Tissues were preincubated in 3,3'-diaminobenzidine-HCl (0.05%) for 15 min, in darkness, then 0.01% H₂O₂ was added to the incubation mixture and further reacted for 15 min. Specimens were then either mounted in Canada balsam or in glycerol.

For control, the primary antibody was omitted from the incubation steps, which resulted in no staining; preadsorption of the anti-GABA antibody with different amino acids (Füller et al., 1989) and of the anti-SP antibody with different peptides (Saffrey et al., 1982) did not affect the staining. Preadsorption of the GABA antibody with GABA-glutaraldehyde-bovine serum albumine complex (20 h at 4° C) and the SP antibody with porcine SP (10⁻⁵M, 20 h at 4° C) completely abolished the staining.

EM procedure

Animals were perfused transcardially as described in d. The fixative contained 2% paraformaldehyde and 2% glutaraldehyde in 0.1M PB (pH 7.4). Small tissue blocks were cut from the muscular layer and fixed further with immersion for 3 h. After washing in 0.1 M PB containing 7.5% sucrose, osmication was performed in 2% OsO₄ in PB for 1 h. Samples were dehydrated in an ascending series of ethanol, stained in 75% ethanol with saturated uranyl acetate and embedded in Durcupan ACM resin. Ultrathin sections were cut with Reichert OM U2 microtome, stained with lead citrate (Reynolds, 1963), viewed and photographed in a Jeol 100B or Tesla BS 540 electron microscope.

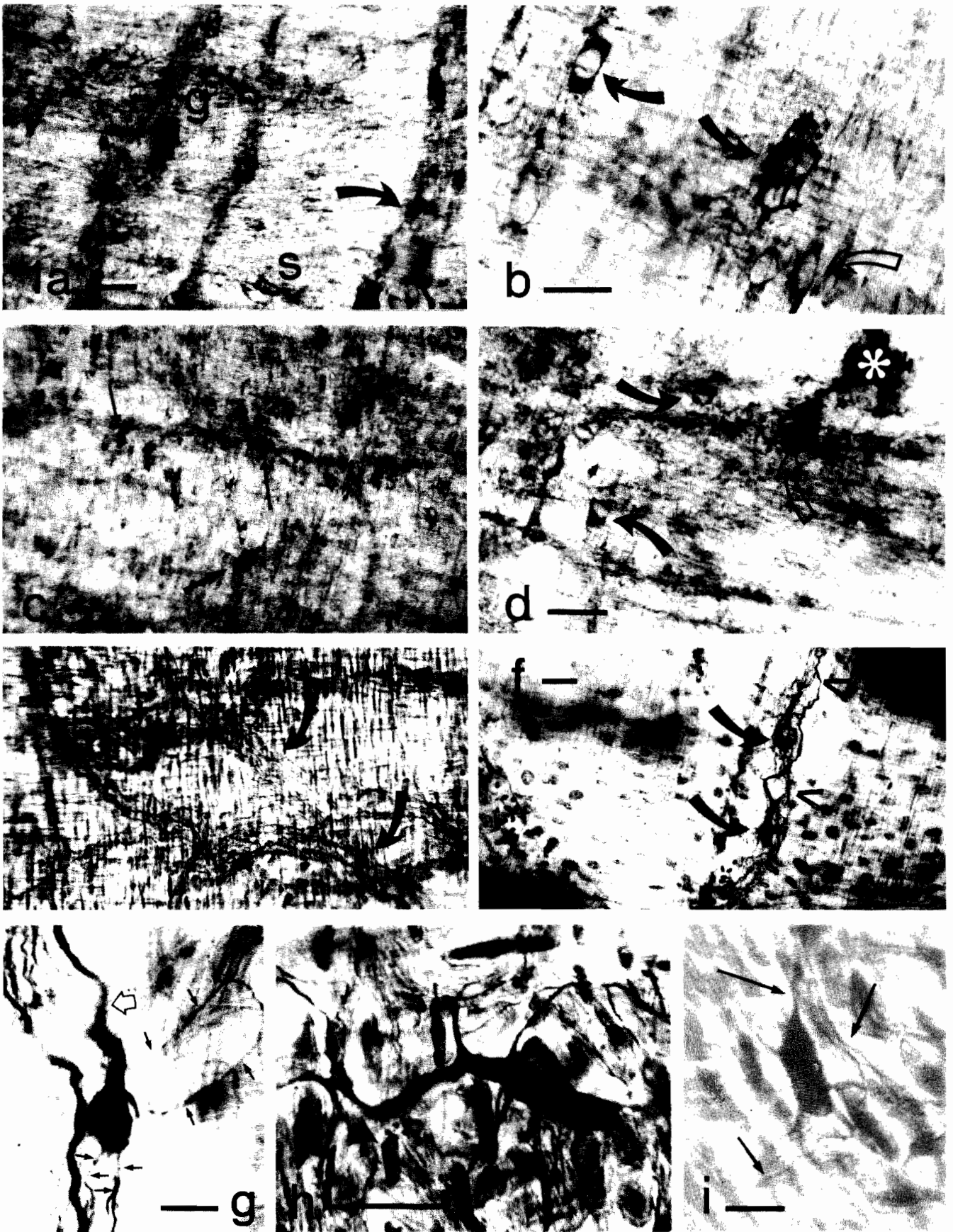


Fig. 1. Microscopic anatomy of frog midgut myenteric plexus. **a.** NADHd staining in the duodenal myenteric plexus. G: ganglia, S: solitary neurons. Arrow points to a large nerve bundle. Bar: 20 μ m. **b.** Perikarya from the duodenum. NADHd staining. Arrow: large multipolar neuron, open arrow: a small bipolar-like neuron. Bar: 20 μ m. **c.** NADHd-staining whole mount from the small intestine. Arrows: nerve cells. Bar: 30 μ m. **d.** Myenteric neurons (arrows) from the small intestine stained with NADHd. Asterisk: pigment cells. A large neuron (open arrow) can be seen in close proximity to the mesenteric border. Bar: 15 μ m. **e.** Silver-impregnated duodenal plexus. Arrows: nerve bundles; open arrow: neuron. Bar: 20 μ m. **f.** Silver-stained small intestine. A longitudinally oriented nerve bundle (arrowheads) with inserted nerve cells (arrows). Bar: 20 μ m. **g.** Type II neuron. Arrows: fine processes, open arrows: thick process. Bar: 12 μ m. **h.** Type I neuron. Note the considerable number of short processes of the cell. One process seems to project farther (indicated by an arrow). Bar: 20 μ m. **i.** The small nerve cell type from the small intestine. Few short processes can be seen clearly (arrows). Bar: 15 μ m.

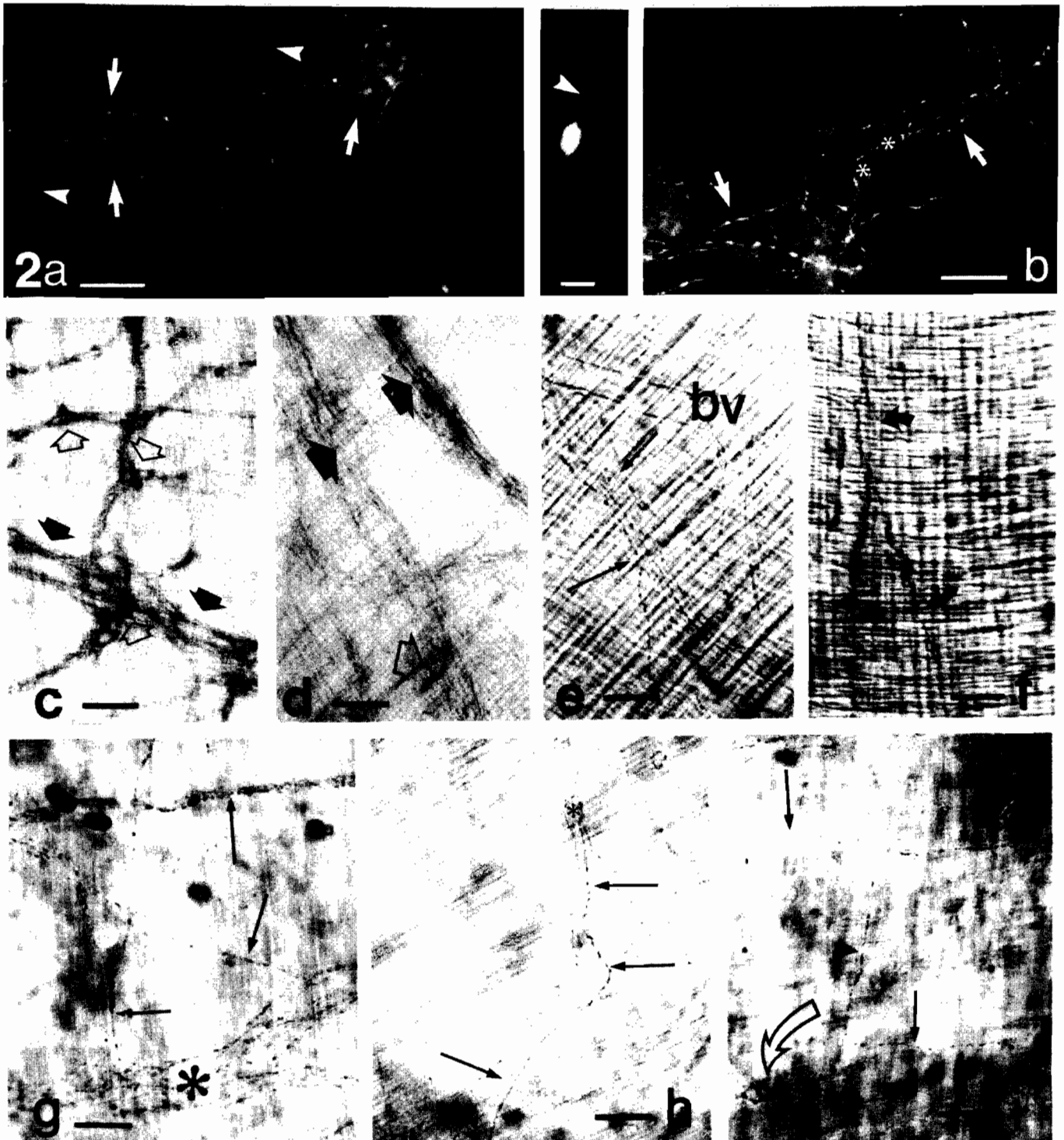


Fig. 2. Localization of the putative transmitters (CAs, GABA, SP) and AChE in the midgut myenteric plexus. **a.** CA-fluorescent fibres (arrows) in the duodenum follow a blood vessel. Arrowhead: single fibre penetrating into the circular muscle. Bar: 20 μ m. **b.** CA-specific fluorescence in the small intestine. Asterisks: pericellular baskets, arrows: main longitudinal branches of the plexus. Bar: 20 μ m. Insert: Stained neuron, arrowhead: axon. Bar: 10 μ m. **c.** AChE-reactivity in the duodenum. Arrows: nerve bundles, open arrows: stained cell bodies. Bar: 20 μ m. **d.** Faintly AChE-positive nerves (arrows) contain a small number of jointly stained perikarya (open arrow) in the small intestine. The secondary and tertiary plexuses are also slightly visible. Bar: 10 μ m. **e.** GABA-stained fibres (arrows) in the duodenum. The orientation of the fibres is longitudinal. bv: blood vessel. Bar: 20 μ m. **f.** GABA-immunoreactive single branching fibre (arrows) in the small intestine. Labelled cell bodies are absent. Bar: 15 μ m. **g.** SP-immunoreactive fibres in a ganglion (asterisk) and single fibres (arrows) in the duodenum. Bar: 20 μ m. **h.** SP-positive fibre system (arrows) in the small intestine. Asterisk: pericellular basket. Bar: 20 μ m. **i.** Longitudinal (arrows) and circumferential (arrowhead) nerve fibres inside the musculature. The silhouette of an SP-immunoreactive cell body (open arrow) is visible above the actual focus plane. Bar: 20 μ m.

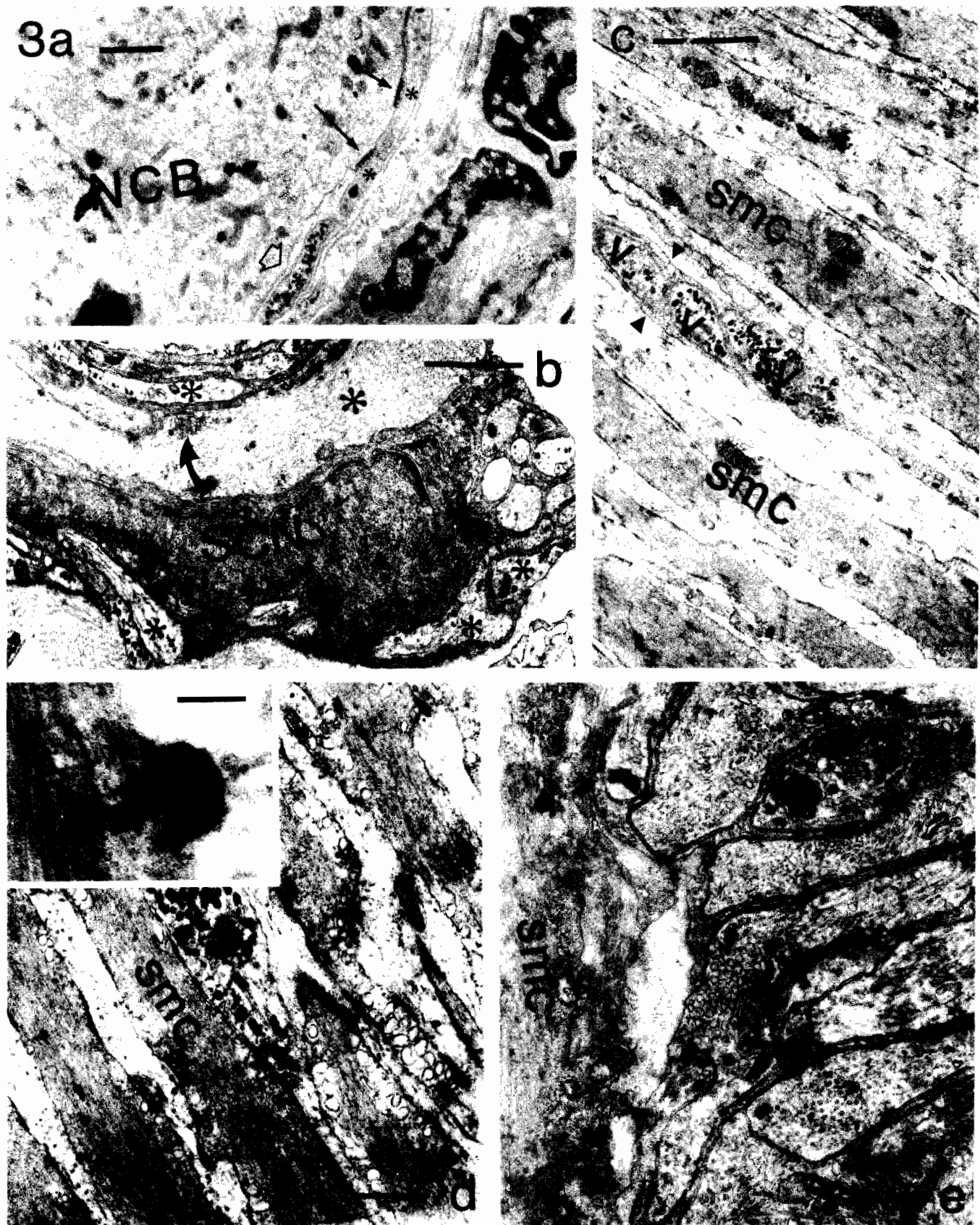


Fig. 3. Ultrastructure of the midgut myenteric plexus and the neuromuscular junctions. **a.** Synapsing (arrows) and non-synapsing (open arrow) profiles (asterisks) on a nerve cell body (NCB). Bar: 1 μ m. **b.** Schwann cell with a strongly heterochromatic nucleus (SCN) and the encapsulated axon profiles (asterisks). Arrow: vesicle accumulation within a large diameter axon. Bar: 2 μ m. **c.** Axon bundle within the muscular layer covered with Schwann-cell processes (arrowheads). smc: smooth muscle cells, v: varicosities. Bar: 3 μ m. **d.** LGV-containing profile in close contact with a smooth muscle cell (smc). Bar: 2 μ m. Insert: Exocytosis of a large granulated vesicle (arrow). smc: smooth muscle cell. Bar: 0.2 μ m. **e.** Agranular vesicle-containing profile (asterisk) in junctional apposition with a smooth muscle cell (smc). Bar: 0.8 μ m.

Results

NADHD staining revealed both ganglia and solitary neurons in the duodenal myenteric plexus (Fig. 1a), while only solitary neurons in the small intestine (Fig. 1c). In the duodenum, most of the perikarya were large and multipolar ($16 \pm 1.2 \mu\text{m} \times 11 \pm 0.8 \mu\text{m}$ in size), but some neurons had a smaller diameter ($12.2 \pm 1.0 \mu\text{m} \times 9.9 \pm 1.0 \mu\text{m}$) and bipolar-like morphology (Fig. 1b). In the rest of the small intestine, neurons were smaller ($10 \pm 0.9 \mu\text{m} \times 7 \pm 0.7 \mu\text{m}$), with a few large-diameter neuron ($16 \pm 1.0 \mu\text{m} \times 17 \pm 1.3 \mu\text{m}$), located always at the mesenteric border (Fig. 1d).

Silver impregnation displayed the same gross morphological differences as revealed by the NADHD method: the partly ganglionated duodenal plexus contained thick nerve bundles, but some thinner branches could also be seen (Fig. 1e). In the small intestine, thin, longitudinally oriented nerve bundles were visible (Fig. 1f). In the duodenum, two neuron types were observed: type I cells had an elongated soma, with several short and only one long processes (Fig. 1h) while type II neurons had a pear-shaped cell body and few long processes, one of which was thick and projected to other ganglia (Fig. 1g). The small intestine also contained two characteristic nerve cell types, one of which was classified as type II. The other (type III) was different from the duodenal cell types. These neurons had small, round or rectangular perikarya, and only a small number of processes all terminating in close proximity (10–50 μm) to the cell body (Fig. 1i).

Regional differences were observed in the distributions of different transmitter candidates. The glyoxylic acid-induced fluorescence revealed a network of CA-containing fibres around the blood vessels in the duodenum (Fig. 2a). A few varicose fibres left these plexus to penetrate into the circular muscle layer. CAergic fibres and small, unipolar-like nerve cells were also observed (3–5% of the perikarya) in the myenteric plexus of the small intestine (Fig. 2b). These fibres ran mainly parallel to the longitudinal axis of the gut. Pericellular baskets were present in low number only.

The AChE reaction showed prominent differences between the two segments of the midgut. The duodenal region possessed a strongly reactive fibre system, which included stained cell bodies (Fig. 2c). The stained cell population did not exceed 10% of the perikarya. Slightly labelled nerve bundles and very few AChE-positive perikarya (not more than 1–2% of the neurons) were characteristic in the small intestine (Fig. 2d).

In the duodenal and the small intestinal myenteric plexus sparse GABA-immunoreactive fibres were observed. These nerves exhibited a clear longitudinal orientation (Fig. 2e). The GABAergic fibres in the small intestine sometimes diverged (Fig. 2f). Immunoreactive perikarya were detected neither in the duodenum nor in the rest of the intestine.

Ganglia, connective strands and solitary fibres formed the SP-positive plexus in the duodenum (Fig. 2g), in contrast to the longitudinally oriented fibre tracts of the small intestine (Fig. 2h). The SP-positive neurons located in these regions were small, multipolar (Fig. 2i); or rarely had bipolar character. Immunoreactive fibres could be traced into the circular muscle layer, where fibres projected both longitudinally and circumferentially (Fig. 2i). The number of SP-positive neurons was small (2–3% of the perikarya) in both studied gut region.

Ultrastructural observations revealed that the nerve cells received both synaptic inputs from the terminals containing agranular vesicles (AGVs), and non-synaptic inputs from the axons containing large granulated vesicles (LGVs). These contact sites were found mostly of the somata of the myenteric neurons (Fig. 3a), whilst synapses were rare in the neuropil. Nerve bundles entered into the muscle layer (Fig. 3b), and ran as small groups of varicose nerve processes between the smooth muscle cells (Fig. 3c). Both processes with AGV and LGV content established junctional contacts with the smooth muscle cells (Figs. 3d, e). The junctional gap varied from 40 nm to 100 nm. Exocytotic profiles of LGVs were sometimes observed (Fig. 3d, insert).

Discussion

The results of the present study confirm Gunn's (1951) previous description in connection with the gross morphology of the myenteric plexus and the shape of the individual nerve cells. We extended Gunn's observations by describing the distribution of four putative transmitters within the midgut myenteric plexus and by correlating the cell morphology with the actual transmitters content. All the four investigated transmitters play a major role in the process of peristalsis. Acetylcholine (ACh) and SP are considered to be the main excitatory motoric transmitters of the mammalian gut (see Furness et al., 1988a), while CAS and GABA inhibit the peristalsis (Cherubini and North, 1984; Furness et al., 1988a). The same probably applies to the submammalian vertebrates, however, neurons may be organized in a different neurochemical coding system (Nilsson, 1983; Nilsson and Holmgren, 1989).

Gross morphology of the plexus and the cell types

Our results agree with the classical view (Gunn, 1951) that the partly ganglionated duodenal myenteric plexus contained Dogiel types I and II neurons. Indeed, a recent silver impregnation study has shown the same morphological cell types in the duodenum of adult *Rana catesbiana* (Torihashi, 1990) that were described in the present study in *Rana esculenta*. The non-ganglionated small intestine included only very few type I cells, mainly at the mesenteric border, in contrast to large number of type II, and mainly small type III cells. The type II cells in frog are substantially smaller than the type I cells, thus the situation is quite

different from the mammalian intestine (Furness et al., 1988b).

Neurochemical markers in the plexus

The CA-fluorescent neurons were small, unipolar-like cells, suggesting that they correspond to type III cells. These cells may have more than one processes, but the low CA-content does not allow their satisfactory identification. These cells were always located outside the longitudinal fibre bundles. The presence of CAergic cell bodies in the *Bufo hindgut* has been first described by (Read and Burnstock, 1968, 1969). The present investigation showed, that approximately 3-5% of the myenteric nerve cells exhibited CA-fluorescence in frog. Fluorescent fibres were less numerous and CAergic perikarya disappeared in the duodenum. This finding supports view, that the CAergic innervation diminishes toward the oral end of the alimentary canal (Junquera et al., 1986, 1988; Gábel et al., 1989). The CAergic fibres in the duodenum mostly seemed to be confined to the nerve plexuses supplying of blood vessels. Only a few fibres left this plexus to innervate the circular musculature.

Potentially cholinergic elements were identified by AChE histochemistry. Although the presence of AChE is not exclusive to the cholinergic nerve cells (Burt and Silver, 1973), this method is widely used to map certain neuronal populations of the gut plexuses in vertebrate species (Gunn, 1968; Wong et al., 1971; Gabella and Halasy, 1987; Halasy et al., 1989). AChE-positive cells in the duodenum may belong mainly to the type I category on the basis of their size. In the small intestine, the number of strongly AChE-positive nerve cells was substantially lower than in the duodenum. Since the cholinergic cells represent a large proportion of the enteric interneurons in the mammalian enteric nervous system (Furness and Costa, 1987), it is not clear whether these cells in lower vertebrates are simply not present in significant numbers in lower vertebrates or the strong AChE reactivity rather stains those cells which receive cholinergic inputs. Indeed, virtually all parts of the frog alimentary canal receives cholinergic innervation from different parts of the autonomic nervous system, the bulk arising from the vagus nerve (Taxi, 1958). Further studies are needed to clarify this issue.

GABA-immunoreactive fibres, but not nerve cells, were present in small number in the full length of the midgut. Recently we have reported GABA-immunoreactive cell bodies in the myenteric plexus of the stomach (Gábel et al., 1989) and fibres in the small intestine (Gábel et al., 1990). In contrast, the presence or the absence of GABA-immunoreactive somata could not be clearly demonstrated. Both our present and previous studies indicate the existence of a group of GABAergic nerve cells in the myenteric plexus of pyloric stomach from where fibres project to supply the rest of the

alimentary tract with GABA-positive fibres. The action of GABA on the peristalsis on submammalian vertebrate gut has not yet been studied. In mammals, this substance inhibits the myenteric neurons, including motoneurons, thus indirectly inhibits the peristalsis (Cherubini and North, 1984).

The presence of SP in nerve cells and fibres of the fore- and midgut myenteric plexus of *Rana ridibunda* has been demonstrated (Junquera et al., 1986, 1987, 1988). The morphology and the distribution of these cells have not been identified, since these studies were carried out in sections. In the midgut of *Rana esculenta*, an extensive fibre system was revealed in both duodenal and small intestinal myenteric plexuses. SP-positive nerve cells represented about 2-3% of the total neuron number. These cells may be responsible for the coordinated physiological activity of the muscle layer, since SP-neurons innervate a large proportion of the unlabelled nerve cells and the muscles (North, 1982; Wood, 1984; Smith et al., 1988). SP seems to be the most wide-spread and most potent non-cholinergic excitatory transmitter in the alimentary canal of the mammalian (Barthó and Holzer, 1985) as well as in the nonmammalian vertebrate species (Nilsson and Holmgren, 1989). The results of the EM study have indicated the presence of presumably cholinergic synaptic terminals on the somata of the myenteric neurons similarly to mammals (Smolen, 1988). The LGV profiles revealed in this study may store SP, VIP or other peptides (Sundler et al., 1980; Polak et al., 1982) like in the mammalian myenteric nerves («P» type profiles). Since these profiles were also in contact with the neuronal somata, both ACh and the peptides seem to be able to influence the peristalsis through their action on myenteric nerve cells. At the same time, junctional appositions of both profile types were found on the smooth muscle cells. While the mode of ACh release is not clear, the peptide release is by way of exocytosis from the LGVs (Buma et al., 1984; Benedeczy and Halasy, 1988).

Mismatches between frog and mammals in cell morphology, transmitter content and distribution

Although the neuron morphology and the ultrastructural features described in the present study show similarities to those of mammals (Baumgarten et al., 1970; Cook and Burnstock, 1976; Gabella, 1979; Gershon, 1981; Komuro et al., 1982; Furness et al., 1988b), numerous differences of the transmitter content established. In all examined mammalian species cholinergic cells correspond to the Dogiel type I category and they are present throughout the full length of the alimentary canal (Furness et al., 1988a). In the frog midgut, only the duodenal myenteric plexus contained these type I cells, the small intestine was free of them. In contrast to frog, in most of the mammalian myenteric plexuses, CAergic nerve cells were not found. CA-containing nerve endings in the guinea-pig myenteric ganglia have failed to show

immunoreactivity either for neuropeptide Y or for somatostatin, while the paravascular fibres contained neuropeptide Y (Costa and Furness, 1984). In the frog, these possible transmitter coexistences have not yet been studied. GABA-positive fibres were rare in the *Rana* midgut. In contrast, the midgut myenteric ganglia of some mammalian species contained GABAergic cell bodies (Jessen et al., 1966; Saito and Tanaka, 1986; Hills et al., 1987). GABAergic elements seem to be intrinsic to the myenteric plexus of lower vertebrates too (Baetge and Gershon, 1986; Fekete and Gábel, 1989; Gábel et al., 1990). The SP-positive nerve cells in the frog midgut shared mainly type III morphology, similarly to part of SP-immunoreactive neurons in the guinea-pig small intestine (Furness and Costa, 1987).

The chemically identified subsets of the *Rana esculenta* midgut myenteric nerve cells account for only 10% of the total nerve cell number: the neurochemical nature of the remaining 90% is unknown at present. Few of them may be serotonergic, like in *Bufo* (Anderson and Campbell, 1984) and some of the rest may contain peptides like in other anurans (e.g. VIP: Buchan et al., 1981; or SOM: Junquera et al., 1987). Further studies are required to clarify the relations of the different transmitter systems to each other and to establish the possible coexistence of classical and non-classical transmitters and modulators in unidentified cell populations of the frog enteric nervous system.

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