Projections of Chemically-Specified Neurons in the Guinea-pig Colon

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Summary. The arrangement of the enteric nerve plexuses in the colon of the guinea-pig and the distributions and projections of chemically specified neurons in this organ have been studied. Immunoreactivity for neuron specific enolase was used to examine the total population of neurons and individual subpopulations were studied using antibodies raised against calbindin, calcitonin gene-related peptide (CGRP), leu-enkephalin, gastrin releasing peptide (GRP), galanin, gamma aminobutyric acid, neuropeptide Y (NPY), somatostatin, substance P, tyrosine hydroxylase and vasoactive intestinal peptide (VIP). Neuronal pathways within the colon were lesioned using myotomy and myectomy operations and extrinsic pathways running between the inferior mesenteric ganglia and the colon were also severed. Each of the antibodies revealed nerve cells and nerve fibres or only nerve fibres within the wall of the colon. VIP, galanin and GRP were in anally projecting pathways in the myenteric plexus, as they are in other species. In contrast, there are differences in the projection directions of enkephalin, substance P, NPY and somatostatin nerve fibres between regions and species. Surprisingly, somatostatin and NPY fibres have opposite projections in the small intestine and colon of the guinea-pig. The majority of nerve fibres that innervate the circular muscle, including fibres with immunoreactivity for VIP, enkephalin, substance P, NPY, galanin and GRP come from the myenteric ganglia. The mucosa is innervated by fibres from both the myenteric and submucous ganglia. The present results suggest that the guinea-pig distal colon is a suitable place in which to determine relations between structure, neurochemistry and functions of enteric neural circuits.

Several hundred papers in the last 15 years have described the distributions of nerve cells and fibres that can be located in the gut wall on the basis of their chemistry (FURNESS et al., 1988). These publications have shown that more than 20 chemical features can be used to distinguish groups of enteric neurons. In a few regions, projections of chemically distinguished neurons have also been studied, these being the small intestine of the guinea-pig (see FURNESS and COSTA, 1987, for review), the small intestine and colon of the rat (EKBLAD et al., 1987, 1988; TRUDRUNG et al., 1990), the small intestine and colon of the dog (DANIEL et al., 1987; FURNESS et al., 1990a) and the human colon (DOMOTO et al., 1990). Comparison of results obtained indicate that considerable differences occur between species and regions in the distribution of chemical marker substances. Differences are even more apparent when projections of neurons are compared, because fibres that have similar distributions are then often found to have different origins. For example, substance P immunoreactive nerve fibres in the myenteric plexus project anally in the rat small intestine, but orally in the rat large intestine (EKBLAD et al., 1987, 1988). On the other hand, enkephalin, galanin, somatostatin and VIP/NPY fibres project in the same directions in the two regions of rat intestine.

If specific chemical markers are associated with specific functionally-defined neurons, then it would be expected that these markers would be in neurons with the same projections, independent of region or species. For example, it has been proposed that VIP is a transmitter of inhibitory muscle motor neurons that are the final neurons in descending inhibitory reflexes in all gut regions (MAKHLOUF et al., 1989). It is therefore consistent with the hypothesis of VIP's role that VIP is found in anally directed myenteric neurons in the guinea-pig small intestine (FURNESS and COSTA, 1979), in the rat small intestine and colon.

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(Ekblad et al., 1987, 1988), in the dog small intestine and colon (Daniel et al., 1987; Furness et al., 1990a, b) and in the human colon (Domoto et al., 1990). Additional comparisons of different regions are desirable so that further associations between neurochemistry and neural function might be made. At the moment the most thorough investigations of the correlations between chemistry and function have been for neurons in the guinea-pig small intestine. In the present work, the projections of neurons in the guinea-pig colon have been examined, so that comparisons can be made with the guinea-pig small intestine and the colons of dog and rat.

MATERIALS AND METHODS

In all experiments adult guinea-pig of both sexes, 200–250 g in weight, were used.

Surgical procedures

The animals were fasted for approximately 18 h prior to the operation. This facilitated operations by reducing the gas and food content of the caecum. Guinea-pigs were anaesthetized with a subcutaneous injection of sodium pentobarbitone, 15 mg/kg, and an intramuscular injection of fentanyl, 0.16 mg/kg, and fluanisone, 5.0 mg/kg. The animals were opened through a midline abdominal incision, and part of the distal colon was exteriorized. A large portion of the caecum also had to be exteriorized to gain access to the colon.

Myotomy

In 21 animals a circumferential cut was made through the external musculature of the distal colon, to the depth of the submucous plexus. The neuronal pathways in the myenteric plexus which project in the longitudinal axis of the colon were therefore interrupted. A loose loop of surgical silk was knotted around the nearest mesenteric blood vessel to mark the operation site.

Myectomy

In 14 animals, two circumferential cuts were made through the external musculature of the distal colon, approximately 5 mm apart. The longitudinal muscle plus myenteric plexus between the two lesions was carefully peeled off. Care was taken to ensure that none of the myenteric plexus remained in the operated region. This operation interrupted neuronal pathways projecting into the inner layers of the wall of the colon, as well as the longitudinal projections. The site was marked as for the myotomy.

Myectomy plus extrinsic denervation

After myectomy operations in a further four animals, the nerves supplying that area of the colon were crushed. The distal colon receives sympathetic and sensory input via the colonic nerves, which emerge from the inferior mesenteric ganglia and closely follow the arteries. A local denervation was achieved by crushing, with watchmaker’s forceps, those blood vessels which supplied the operated area in no less than three points along their course.

Extrinsic denervation

In 8 animals the colonic nerves were cut as they emerged from the inferior mesenteric ganglion, denervating the entire distal colon of sympathetic input and of those sensory fibres that follow the sympathetic nerves. The surrounding mesentery was also cut. To ensure that no fibres in close proximity to the inferior mesenteric artery remained intact, the artery was lightly swabbed around its circumference with cotton soaked in 80% phenol.

At the completion of all surgical procedures the peritoneum and abdominal muscle were sewn together with surgical silk, and the wound was lightly covered with antibiotic power (Cicatrin, Wellcome, NSW). The skin was stapled and an intramuscular injection (0.1 ml) of broad spectrum antibiotic, terramycin (Pfizer, New York), was administered.

Tissue specimens were taken 4–7 days after operation. All animals were killed by a blow to the head and bled via the carotid arteries. The segment of colon taken from operated animals extended about four centimetres either side of the operation. Control tissue was taken within 2 to 20 cm of the rectum from 18 unoperated animals. The segments were placed into one of two solutions, either phosphate-buffered saline, PBS (0.9% NaCl in 0.01 M sodium phosphate buffer, pH 7.0), prior to fixation for immunohistochemistry or into modified oxygenated Krebs solution, prior to incubation in culture medium (modified Krebs solution; (mM) Na+ 151; K+ 4.7; Ca2+ 2.8; Mg2+ 0.6; Cl− 143.7; H2PO4− 1.3; HCO3− 16.3; SO42− 0.6; dextrose 7.7).

Tissue preparation

Wholemounts

Fresh tissue was immediately placed in PBS. The segment of colon was cut open along the mesenteric
The tissue was laid out flat, mucosal side down, on a piece of balsa wood that had been marked to indicate the proximal end of the tissue. The tissue was then pinned out tautly with office pins.

The balsa wood was placed tissue side down in a Petri dish containing Zamboni's fixative (2% formaldehyde and 0.2% picric acid; STEFANINI et al., 1967), for 24 h at 4°C. Tissue was cleared of fixative by three 10 min washes in dimethylsulphoxide (DMSO). The DMSO was cleared from the tissue by three 10 min washes in PBS. Fixed tissue was stored in PBS containing 0.1% sodium azide and kept at 4°C.

For immunohistochemistry, the tissue was dissected into three layers using watchmaker's forceps. First, the mucosa was gently scraped off with a scalpel blade. The submucosal layer was gently teased from the circular muscle, and removed as a whole sheet. Strips of circular muscle were then carefully peeled from the longitudinal muscle layer. The remaining myenteric plexus/longitudinal muscle layer was kept as one.

Sections
For tissue to be examined in longitudinal section, the segment of colon was slit open and laid down on the balsa wood as normal, but was not stretched when pinned to the board. For tissue to be examined in transverse section the pellets were gently squeezed out of the colon and the intact tissue was immersed in Zamboni's fixative. Fixation and removal of the fixative were the same as above, but the tissue was stored in PBS-azide containing 30% sucrose as a cryoprotectant, for at least 24 h at 4°C.

Longitudinal sections, parallel to the long axis of the colon, were taken through the operation area. The area to be sectioned was cut from the fixed tissue, dabbed dry of excess moisture, and immersed in a cryomould containing OCT compound (Lab-Tek products, Nashville, Ill., USA). Orientation of the tissue was noted and the cryomould frozen in liquid nitrogen. Ten sections of 12 μm thickness were then cut at −20°C on a cryostat and picked up on poly-L-ornithine coated slides (0.1% poly-L-ornithine in distilled water). The sections were dehydrated for 30 min in a vacuum chamber containing P₂O₅, and stored at 4°C until used.

Immunohistochemical techniques
Prior to exposure to the primary antiserum, the tissue was incubated in PBS with 10% normal serum (from the species in which the secondary antiserum was raised) for 30 min in a humid chamber. Excess normal serum was removed and the tissue was covered with 25-50 μl of the primary antiserum, suitably diluted with a buffered PBS-azide solution, pH 7.0. The details of all primary antibodies are given below (Table 1). Incubation time for the primary serum was 18 to 24 h. The next day excess primary antiserum

<table>
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<th>Staining dilution</th>
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<tr>
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was removed in three 10 min PBS washes (wholemounts) or a 15 min PBS bath (sections). The tissue was then exposed to a secondary antiserum which contained antibodies conjugated with fluorescein-isothiocyanate (FITC). Incubation time for the secondary antibodies was 1 h. Excess secondary antibody was then washed away with PBS. Tissue sections were covered with a drop of buffered glycerol (pH 8.6) and coverslipped, while wholemount tissue was placed on a microscope slide in buffered glycerol and coverslipped.

Colchicine/veratridine treatment

Freshly obtained colon was placed in modified Krebs solution and opened along the mesenteric border. The pellets were removed and the tissue pinned out loosely on sterilized balsa wood. The segments were placed in culture medium, consisting of Dulbecco's modified Eagle's medium with Ham's nutrient mixture (medium DME-F12; Sigma Chemical Company, St Louis, MO, USA) containing 50 μM colchicine (also from Sigma, USA) for 24 h in a 37°C water bath, and buffered with medical air containing 5% CO₂. Next day the tissue was re-pinned on fresh balsa wood and fixed in Zamboni's solution as previously described for wholemounts.

In a further series of experiments the tissue was prepared as described above, but placed in culture medium containing initially only veratridine (Sigma, USA; 50 μM). Colchicine was added to the medium 2 h later to make a final concentration of 100 μM. After a further 5 h additional veratridine (50 μM) was added to the medium, and left overnight for 20 h. The next day the tissue was re-pinned and fixed in the standard manner.

Gamma-aminobutyric acid localization

Localization of gamma-aminobutyric acid (GABA) was achieved using a modified protocol. Fresh tissue was opened and pinned tautly onto a balsa wood frame, so that both tissue surfaces were exposed. The tissue was fixed in Zamboni's fixative plus 0.05% glutaraldehyde, for 4 h at room temperature. The fixative was cleared in three 10 min DMSO washes, three 10 min PBS washes and then a 1 h PBS wash. The tissue was stored in PBS overnight at 4°C. The next day the tissue was dissected apart and incubated with the anti-GABA antiserum for 48 h. The tissue was then washed and incubated with biotinylated horse anti-mouse secondary antiserum for 2 h, followed by a 1 h incubation with Streptavidin Texas Red complex. Normal horse serum (20% dilution) was added to all solutions to reduce background fluorescence.

Some tissue was loaded with GABA, in vitro, prior to immunohistochemical staining with the anti-GABA antiserum. This has been found to enhance the immunohistochemical localization of GABA (Furness et al., 1989a). Fresh tissue was immediately placed in culture medium, buffered with medical air containing 5% CO₂ and kept at 37°C. The culture medium also contained: nicardapine (3 × 10⁻⁵ M) to prevent muscle contraction, amino-oxyacetic acid (2 × 10⁻⁵ M) to block GABA transaminase activity; and β-alanine (10⁻³ M) to prevent GABA uptake by glial cells. The segment of colon was loosely pinned out on silicone elastomer (Sylgard, Dow Corning, Michigan, USA) in a Petri dish, and maintained in the culture medium for 20 min. GABA (5 × 10⁻⁴ M) was then added and the tissue incubated for a further 60 min. The preparations were re-pinned onto sterile balsa wood frames and fixed in Zamboni's solution, with 0.05% glutaraldehyde, for a further 4 h. The tissue was unpinned and washed in three 10 min changes of DMSO, followed by three 10 min washes with PBS. It was then treated with 0.1% NaCNBH₃ in 0.1 M PBS for 30 min, to reduce unreacted aldehyde groups, and washed in PBS for 5 h. The preparations were stored in PBS containing 0.1% NaN₃ (for wholemounts) or 0.1% NaN₃ plus 30% sucrose (for sections) at 4°C. Immunohistochemical localization of GABA was carried out as described above.
Fig. 1. Legend on the opposite page.
RESULTS

Arrangement of nerve plexuses

The organization of the ganglionated and non-ganglionated plexuses of the colon were examined in wholemount preparations and sections stained for the immunohistochemical localization of neuron specific enolase. The myenteric plexus consisted of large ganglia, most of which were elongated in the circumferential direction (Fig. 1), but not as precisely as in the small intestine of this species. The ganglia were connected by internodal strands of various calibres to form a continuous plexus. Secondary strands, that arose frequently from ganglia and internodal strands, ran in the circumferential direction (Fig. 1d). In preparations consisting only of the longitudinal muscle and myenteric plexus, the secondary strands were broken off after running up to about 1 mm. When the circular muscle was present, some secondary strands were traced into this layer. The ganglia, internodal strands and secondary strands gave rise to fine nerve fibre bundles that ran into the longitudinal muscle. Some of these fine strands took irregular courses similar to the fibres of the tertiary plexus of the small intestine, but the majority ran parallel to the longitudinal muscle (Fig. 1b). In sectioned material, nerve fibre bundles could be seen through the thickness of the longitudinal muscle (Fig. 1a). Thus the arrangement of fibres in the colon contrasts with that in the small intestine of the guinea-pig, where there is no plexus of fibres in the longitudinal muscle, but where the tertiary plexus is more prominent than in the colon. Sections revealed nerve fibres through the thickness of the circular muscle; unlike the small intestine, there was no deep muscular plexus in the colon. There was, however, a submucosal plexus of nerve fibres against the inner surface of the circular muscle, as has been reported in the colon of several species (Stach, 1972; Faussone Pellegrini, 1985; Christensen and Rick, 1987; Furness et al., 1990a).

The submucosa contained a three-dimensional plexus of interconnected ganglia (Fig. 1e, f). This gave the appearance of two plexuses of ganglia with numerous cross-connections, an outer plexus with mainly large ganglia that lay towards the inner surface of the circular muscle and an inner plexus of smaller ganglia, most of which were closer to the mucosa (Figs. 1f, 9e). The larger ganglia tended to have irregular, polygonal shapes, whereas the small ganglia were round or oval. Internodal strands of the two plexuses often crossed without connecting (Fig. 1f). Paravascular and perivascular nerve bundles were associated with arterioles in the submucosa and at the level of the myenteric plexus. The muscularis mucosae was innervated by nerve fibres running parallel to the muscle bundles that were mostly aligned in the long axis of the intestine (Fig. 1a). Numerous nerve fibre bundles were in the lamina propria mucosae.

Vasoactive intestinal peptide (VIP)

Distribution

VIP-immunoreactivity was found in nerve cell bodies of the myenteric and submucous plexuses, and in nerve fibres in the longitudinal muscle, circular muscle, myenteric plexus, submucous plexus, around submucosal arterioles, and in the mucosa (Fig. 2), as previously reported for guinea-pig colon by Schultzberg et al. (1980).

Nerve cell bodies showed only weak VIP-immunoreactivity in the myenteric ganglia of control tissue. However, cell bodies were strongly reactive for about 2 mm oral to myotomy or myectomy operations and had enhanced reactivity up to 12-15 mm oral. Cell bodies with stronger than usual immunoreactivity were also seen in the ganglia just anal to the operation site. Incubation of the tissue with colchicine (50 ,uM), or colchicine (50 ,uM) plus veratridine (50 ,uM), in vitro for 24 h, prior to immunohistochemical staining, greatly enhanced the reaction intensity of cell bodies (Fig. 2d). All the reactive cells appeared to be monoaxonal, and when the axon could be traced it went anally in the myenteric plexus. Two shapes of cell bodies were observed, small cells that had smooth outlines and were oval or tear shaped, and medium sized cells with Dogiel type I morphology (Fig. 2e-g). The smooth cells were almost always at the edges of ganglia. Each smooth cell gave rise to several faint, fine tapering processes that could be followed only for short distances, usually no more than a cell diameter. Each cell also had a more strongly reactive process that could often be followed for several hundred microns and in some cases for more than 1 mm anally within the myenteric plexus. The VIP-immunoreactive nerve cell bodies with Dogiel type I morphology had broad, flat dendrites and single, anally-directed axons. The distinctive shapes of VIP-immunoreactive cell bodies were little altered by colchicine treatment, but colchicine and veratridine together altered cell morphology considerably. Processes became shortened and dilated, and the cells appeared distorted. VIP-immunoreactive cells were most commonly found within the main body of a ganglion, although many cells were also
Fig. 2. Vasoactive intestinal peptide (VIP) immunoreactivity in nerve fibres and cell bodies of the guinea-pig distal colon. a. Wholemount of a myenteric ganglion. Fibres are densely arranged in the ganglia and are also in secondary strands and the tertiary plexus (arrows). b and c. Dense perisomatic fibre networks. Arrows point to cell bodies surrounded by immunoreactive fibres. d-g. VIP-immunoreactive nerve cell bodies in myenteric ganglia. d. After colchicine treatment a high proportion of nerve cell bodies are immunoreactive. e and f. Cell bodies with type I morphology. g. Cell with simple shape. h. Nerve fibres in the circular muscle. i. VIP immunoreactivity occurring in a high proportion of cell bodies in a submucous ganglion. j. A single immunoreactive nerve cell in a small submucous ganglion. k. Fine fibre network around a branching submucous arteriole. l. VIP immunoreactive fibres in the lamina propria of the glandular epithelium. Calibrations: a–c; d; f–i; k, l 50 μm; e, j 25 μm.
located in the internodal strands.

The myenteric ganglia were densely innervated by two types of varicose VIP-immunoreactive fibres which both contributed to a dense network throughout each ganglion. Fine varicose fibres formed a network throughout the ganglia and appeared to come close to all nerve cells (Fig. 2a, b). Fibres with larger varicosities formed baskets around a minority of nerve cell bodies in most ganglia (Fig. 2b, c). Non-varicose fibres also ran through the ganglia, internodal strands, secondary and tertiary plexuses.

Reactive fibres were found in the tertiary and longitudinal muscle plexuses (Fig. 2a). The fibres were varicose and could often be seen connecting with the myenteric plexus in whole mounts and sectioned tissue. The circular muscle was densely innervated with varicose fibres evenly distributed throughout its thickness. Some fibres were against the inner surface of the muscle. The fibres usually ran as small groups or in bundles, approximately parallel to the muscle bundles (Fig. 2h). In many transverse and longitudinal sections of the tissue, groups of fibres could be followed from myenteric ganglia into the circular muscle layer. VIP fibres formed a perivascular plexus around arterioles that ran in the plane of the myenteric plexus.

**Fig. 3.** Effects of myotomy and myectomy operations on the distribution of fibres with immunoreactivity for VIP. Myenteric ganglia 1 mm oral (a), at the oral margin (b) and 1 mm (c), 3 mm (d) and 8 mm (e) anal to a circumferential myotomy operation. Oral to the operation the innervation was normal and at the oral margin (arrows in b) accumulation occurred. Anally, there was a loss of innervation that gradually restored over distances of 8–10 mm. f–h. Longitudinal sections through the wall of the colon 2 mm oral, beneath a circumferential myectomy, and 2 mm anal to the edge of the myectomy. Oral and anal to the operation, the innervation of the circular muscle (cm) is normal, whereas there is a substantial loss in the region where the myenteric plexus was removed (g). Comparison of f and h also shows the reduction of fibre density in myenteric ganglia (arrows). Ganglia of the submucosa can be seen at the bottom of each micrograph. i and j. VIP immunoreactive fibres in the lamina propria of the mucosa in a normal region and in a region from which the overlying myenteric plexus was removed. Calibrations: a–e; f–h; i, j 50 µm.
Submucous ganglia of both the outer and inner plexuses contained many nerve cell bodies and a dense plexus of varicose fibres (Fig. 2i, j). Nerve cells with VIP-immunoreactivity in the submucous plexus were oval in shape, and had smooth surfaces with several long processes. Pre-incubation of the tissue with colchicine was necessary to visualize the VIP-immunoreactive cells in this layer. Varicose fibres were observed in both the ganglia and nerve strands in non-ganglionated plexuses of the submucosa; they were often seen alongside the submucosal arterioles (Fig. 2k).

The muscularis mucosae contained many reactive fibres, some of which could be traced from the submucous plexus. The lamina propria was abundantly innervated with VIP-immunoreactive fibres, particularly around the bases of the mucosal glands, and at the basal surfaces of the endothelial cells along the

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**Fig. 4.** Immunoreactivity for NPY in nerve fibres and cell bodies. **a.** Low power view of immunoreactivity in myenteric ganglia. Note the lack of reactive fibres in the tertiary plexus and background longitudinal muscle. **b and c.** Myenteric ganglia showing the scattering of reactive varicosities throughout the ganglia, non-varicose nerve fibres that pass through the ganglia (arrowheads in b), and an immunoreactive nerve cell (arrow in c). **d.** Section showing myenteric ganglia (arrows) and nerve fibres in the circular muscle (cm), that are primarily towards the inner aspect of the muscle layer. **e.** Section through the mucosa, showing nerve fibres between the glands and some immunoreactive mucosal endocrine cells (arrows). **f-i.** Submucous ganglia. In some ganglia there were numerous nerve fibres and some reactive cell bodies (f), whereas fibres were more rare in other ganglia (g). Nerve cells were strongly reactive, with numerous fine processes (h, i). **j.** Innervation of arterioles in the submucosa. Calibrations: a, j 100 μm; b-g 50 μm; h, i 25 μm.
length of the glands (Fig. 21).

**Projections**

Following a myotomy operation, VIP-immunoreactivity accumulated in cut nerve ends on the oral side of the lesion, and terminals were lost from myenteric ganglia for a limited distance on the anal side (Fig. 3a–e). The accumulations appeared as series of swellings within the individual fibres, these swellings extended back along the fibres as far as 1 mm oral to the lesion. The accumulations were seen in both varicose and non-varicose fibres. In some ganglia of the first two rows anal, fibres with large varicosities were seen, whereas other ganglia were denervated. Fibres with large varicosities increased in number, to become approximately like normal at about 3–5 mm. At these distances there was a part return of the finer varicose fibres; these came back to normal levels at about 8–10 mm. At 7 days after operation, regrowth of fibres was seen, but only on the oral edge of the lesion.

The removal of an area of myenteric plexus (myectomy) resulted in a substantial, and in most experiments complete, loss of immunoreactive fibres in the circular muscle and a decreased density of innervation was apparent for 1–2 mm anal to the lesion (Fig. 3f–h). A substantial loss of fibres occurred in the submucosa directly below the operation, and a small loss was observed in the muscularis mucosae and lamina propria of the mucosal layer (Fig. 3i, j).

Extrinsic denervation, by severing the colonic nerves, had no effect on the distribution of VIP-immunoreactive fibres in any layer of the distal colon.

**Neuropeptide Y (NPY)**

**Distribution**

NPY immunoreactivity was found in nerve cell bodies in the myenteric and submucous plexuses, and in nerve fibres in the circular muscle, the myenteric plexus, the submucous plexus and around submucosal arterioles, and in the muscularis mucosae and lamina propria of the mucosal layer (Fig. 4).

Few nerve cell bodies of the myenteric plexus were visualized, even with colchicine and veratridine pretreatment of the tissue. In contrast, cell bodies were commonly observed up to 1 mm either side of a myotomy lesion. The NPY-immunoreactive cell bodies were distinctive; the outlines were smooth and they were generally of an elongated oval shape with several long, tapering filamentous processes and one prominent axon that generally ran orally. The axons could always be traced for at least 1–2 mm; however, as they progressively became varicose in nature they also became indistinguishable from other fibres. The cells were only seen within the ganglia; unlike other classes of neurons, they were not found in the internodal strands.

Both varicose and non-varicose immunoreactive fibres of the myenteric plexus were brightly fluorescent. The varicosities of NPY-immunoreactive fibres were larger and further apart than those of other peptide-containing neurons. The non-varicose fibres were also comparatively thick in diameter, and could be traced throughout the preparation as they wound through the ganglia and internodal strands (Fig. 4b). The varicose fibres formed a loose network of fibres that meandered through the ganglia. Some of the nerve cells of the ganglia appeared to be surrounded by baskets of immunoreactive fibres. The non-varicose fibres ran in groups of $8.7 \pm 0.5$ (mean $\pm$ SEM, $n=57$) in internodal strands, coursing from one ganglion to another along the longitudinal axis of the colon. Reactive fibres were also in the secondary strands of the myenteric plexus.

The circular muscle contained a moderate density of NPY-immunoreactive varicose fibres, preferentially located at the base of the muscle layer, towards the submucous plexus (Fig. 4d). No fibres were seen in the tertiary plexus or longitudinal muscle layer.

The submucous plexus contained many immunoreactive nerve cell bodies and fibres (Fig. 4f–i). NPY-immunoreactive cell bodies were generally found in small groups of 1–4, and had fine tapering processes (Fig. 4h, i). They were strongly immunoreactive in normally prepared tissue. The ganglia contained the varicose and non-varicose processes of neurons located in this layer; the processes could be traced for distances of about 100 µm before terminating at non-immunoreactive targets or entering the mucosa. The non-ganglionated plexus contained many varicose fibres, some of which could be traced back to cell bodies in the submucous ganglia. A striking feature of the submucosal layer was the network of perivascular fibres, which formed an extremely dense plexus around all the arterial blood vessels (Fig. 4j). Simultaneous staining showed that perivascular NPY fibres were also reactive for the catecholamine synthesizing enzyme, tyrosine hydroxylase.

The muscularis mucosae contained many varicose NPY-immunoreactive fibres, as did the lamina propria. Fibres were especially prevalent around the bases of the colonic glands. NPY-immunoreactive enterochromaffin cells were commonly observed (Fig. 4e).
Projections
Myotomy operations in the myenteric plexus caused a massive accumulation of immunoreactive material in the cut fibres on the anal side of the lesion after five days. Accumulations were seen as large swollen beads, the accumulated immunoreactivity extending for 2 mm along the fibres. On the oral side, immunoreactive fibres were completely absent from ganglia in the first 2-4 mm and only returned to normal density in myenteric ganglia at a distance of 24 mm from the lesion (Fig. 5). The process of axonal regrowth at the anal edge of the operation was quite striking, with prominent, profuse outgrowths from the cut fibre strands (Fig. 5d).

A consequence of the myectomy operation was a total loss of immunoreactive fibres in the circular muscle beneath the operation area, and a partial loss of fibres for about 2 mm oral to the operation (Fig. 5f-h). There was also a partial loss of fibres in the submucous plexus below the myectomy. The muscularis mucosae and the lamina propria lost a substantial portion of NPY-immunoreactive fibres, again in the region directly below the myectomy.

Extrinsic denervation caused total loss of the perivascular fibres in the submucous plexus and a partial loss of fibres in the submucous ganglia and the mucosa.

Substance P and neurokinin A
Antibodies raised against substance P and against neurokinin A, which are structurally similar peptides derived from the same gene, were used. The pattern of immunoreactivity that was revealed by antisera against either peptide appeared identical. Unfortu-
Fig. 6. Distribution of substance P immunoreactivity in the wall of the distal colon. 

a. The dense pattern of nerve fibres in the ganglia of the myenteric plexus. 
b. The dense array of nerve terminals in a myenteric ganglion and nerve fibres in a secondary strand (arrows). 
c and d. Reactive cell bodies in a control (e) and a colchicine treated (d) myenteric ganglion. 
e. Nerve fibres in the circular muscle. 
f-h. Immunoreactivity in submucous ganglia of the inner (f) and outer (g, h) submucous ganglionated plexuses. Faintly reactive nerve cell bodies, surrounded by dense networks of terminals, occur in h (arrows). i and j. Sections at right angles (i) and parallel (j) to the circular muscle. In j a penetrating fibre bundle (asterisk) crosses the circular muscle coat from a myenteric ganglion (arrow) to the submucosa. k and l. Simultaneous localization of substance P (SP) and calcitonin gene-related peptide (CGRP), in fibres associated with a submucous arteriole. The majority of fibres were reactive for both peptides. 
m. Fibres in the colonic mucosa. Note the accumulation of fibres near the lumenal surface of the mucosa (arrows). Fibres associated with the muscularis mucosae are indicated by arrowheads.

Calibrations: a, b, f 100 μm; d, e, g-m 50 μm; c 25 μm.
nately, each of the antisera cross-reacted with the related peptide, so it could not be determined whether these peptides were differentially expressed in nerve fibres in the colon.

**Distribution**

Immunoreactivity for substance P and neurokinin A was found in nerve cell bodies of both the myenteric and submucosal plexuses, and in nerve fibres in the longitudinal and circular muscle plexuses, the tertiary plexus, the myenteric ganglia, submucous plexus, around submucosal arterioles, and in the muscularis mucosae and lamina propria of the mucosa (Fig. 6).

In the myenteric ganglia, immunoreactive nerve cell bodies had either Dogiel type I morphology or were large, oval cells. Type I cells had an irregular shape and many short, broad, lamellar processes. It was not possible to follow the long processes of either type of cell within the myenteric plexus because these fibres were obscured by numerous other fibres. In normal tissue, cell bodies were only faintly immunoreactive and were therefore best visualized in tissue that had been treated with colchicine or colchicine and veratridine (Fig. 6c, d). Cell bodies were not confined to the ganglia and were often found within internodal strands.

Both varicose and non-varicose immunoreactive fibres were found in the myenteric plexus. They formed networks around many myenteric cell bodies that were denser than those formed by any other immunoreactive fibres studied in this work; the density was similar to that seen for substance P fibres in the myenteric ganglia of the small intestine (COSTA et al., 1980a). Numerous reactive fibres ran in the internodal strands, and the varicose fibres also ran in the secondary and tertiary plexuses (Fig. 6a, b).

Both the longitudinal and circular muscle layers contained a rich supply of substance P- and neurokinin A-immunoreactive fibres. In sections of the tissue, immunoreactive fibres could be seen emerging from the myenteric plexus to ramify in the muscle layers. Circular muscle layer innervation was dense and evenly distributed throughout its thickness; fibres were varicose and ran in groups parallel to the muscle bundles (Fig. 6e, i). Substance P immunoreactivity was seen in the fibre bundles of the submucosal plexus against the inner surface of the circular muscle. Substance P immunoreactivity clearly revealed penetrating fibre bundles that connected the myenteric and submucous plexuses (Fig. 6j).

The submucous plexus contained a dense innervation of immunoreactive fibres, all of which were varicose and ramified throughout both the ganglia and in the nerve strands (Fig. 6f–h). In the ganglia they typically formed dense baskets around non-immunoreactive cell bodies, but individual fibres could not be followed. Only with colchicine pretreatment could cell bodies be visualized in this layer, and this treatment revealed that they were all oval with smooth outlines. Combined colchicine and veratridine treatment did not reveal a larger population of immunoreactive cell bodies and it slightly distorted cell morphology. A network of paravascular and perivascular nerve fibres formed a dense plexus around submucosal arteries (Fig. 6k). Myenteric arterioles were similarly innervated.

In the mucosa, both the muscularis mucosae and the lamina propria received a rich innervation of substance P- and neurokinin A-immunoreactive fibres. In the lamina propria, fibres were most frequent around the superficial parts of the colonic glands (Fig. 6m).

**Co-localization of substance P and calcitonin gene-related peptide**

Simultaneous localization of substance P and CGRP revealed coincident reactivity in numerous fibres around arterioles (Fig. 6k, l). However, the substantial majority of substance P immunoreactive fibres in the ganglia and muscle did not react with anti-CGRP antibodies.

**Projections**

Myotomy operations produced accumulations of immunoreactivity within the myenteric plexus, both oral and anal to the lesions. Accumulations in the varicose fibres took the form of large swollen varicosities, whereas long swollen ends were seen in non-varicose fibres. A minimal loss of fibres was detectable in the first ganglia on the oral side of the lesion, but innervation was normal from 0.5 mm oral and in ganglia anal to the lesions (Fig. 7). The circular muscle layer suffered a slight loss of immunoreactive fibres on the oral side of a myotomy, extending for approximately 1 mm. Fibre distribution in other layers of the gut was not affected by the myotomy.

After myectomy operations the circular muscle lost almost 100% of substance P- and neurokinin A-immunoreactive fibres directly below and there was partial loss for approximately 1 mm oral to the operation (Fig. 7c–e). Many fibres in the submucous plexus also degenerated in the region below the operation, but a small population of varicose fibres remained. A substantial loss of fibres occurred in the muscularis mucosae and lamina propria of the
mucosa, again only in the region below the operation.
Cutting the colonic nerves resulted in a total loss of immunoreactive fibres in the perivascular plexus around submucous and myenteric arterioles. In addition, some submucosal varicose fibres in the ganglionated plexus disappeared.

**Enkephalin**

**Distribution**
Enkephalin-like immunoreactivity was found in nerve cell bodies in the myenteric plexus, in nerve fibres in the myenteric ganglia, in fibres of the tertiary plexus, in the longitudinal muscle and in the circular muscle layer (Fig. 8). On occasion, immunoreactive cell bodies and fibres were seen in the submucous plexus, and very rarely immunoreactive fibres were seen in the mucosal layer at the bases of the glands.

The immunoreactive cell bodies of the myenteric plexus were Dogiel type I in shape as they displayed many short, lamellar processes and a single long process that could often be traced a short distance in the oral direction. As was the case with VIP-immunoreactive and substance P immunoreactive cells, the enkephalin-immunoreactive cells were readily visualized after the tissue had been incubated with colchicine or colchicine and veratridine (Fig. 8c). Colchicine and veratridine treatment distorted cell morphology. However, cell bodies with enhanced immunoreactivity, but without distortion, were seen close to lesions, both on the oral and anal sides. Enkephalin-immunoreactive cell bodies were not confined to the myenteric ganglia, but were commonly observed lying within the internodal strands.

Enkephalin-immunoreactive fibres were distributed throughout the myenteric plexus. They formed a dense network within the ganglia, although not as dense as that formed by substance P-immunoreactive fibres. Fibres were varicose and ran in the internodal strands, secondary strands and in the tertiary plexus (Fig. 8a, b).

The longitudinal muscle plexus contained varicose enkephalin-immunoreactive fibres. The circular muscle layer was richly supplied with immunoreactive fibres throughout its thickness. In both muscle layers

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**Fig. 7.** Effects of myectomy on substance P fibres of the myenteric plexus and circular muscle. a and b. Whole mounts of the myenteric plexus oral and anal to the myectomy. The dotted lines indicate where the plexus was interrupted by the operation. c-e. Sections taken 1 mm oral, at the site of the myectomy and 1 mm anal to the operation. Note the loss of fibres from the circular muscle in the region where the myenteric plexus had been removed. The thickness of the circular muscle is indicated by brackets. Calibrations: a, b 100 μm; c–e 50 μm.
Fig. 8. Enkephalin immunoreactivity in the distal colon. 

(a) and (b): Whole mounts of the myenteric plexus, showing the typical appearance of a myenteric ganglion and fibres in a secondary strand (arrow in (a)) and fibres in the tertiary plexus (arrows in (b)).

c: Nerve cell bodies in a myenteric ganglion in a preparation treated with colchicine and veratridine. The cells have Dogiel type I morphology.

d: A reactive nerve cell in a submucous ganglion.

e: An enkephalin reactive fibre running between mucosal glands; such fibres were rare.

f-i: Effects of a myotomy operation, made 4 days before the tissue was taken, on fibre distributions in the myenteric plexus. Depleted immunoreactivity was observed oral to the lesion (g) and fibre outgrowth was observed from cut strands on the anal side (arrows in (h)). Reactivity in nerve cell bodies is more prominent after operations (arrows in (g) and (i)).

j-l: Sections through the circular muscle taken 6 days after a myectomy show the loss of fibres under the myectomy (k) and slight depeletion of the innervation of the circular muscle in the first 1 mm oral (j).

Calibrations: a, b; c, d; f-l 50 μm; e 25 μm.
the fibre groups ran parallel to the muscle bundles.

In the submucosa, enkephalin-immunoreactive cell bodies and fibres were rarely seen, but when present they were brightly immunoreactive (Fig. 8d). The rare immunoreactive cell bodies that occurred in submucous ganglia were Dogiel type I and the reactive fibres were fine and varicose. In the muscularis mucosae and basal area of the lamina propria, fine varicose fibres were occasionally observed. If the diluted anti-leu-enkephalin antiserum was pre-equilibrated with leu-enkephalin (10^-3 M) no reactive cell bodies or fibres were found.

**Projections**

After the myotomy operation, cut nerve strands in the myenteric plexus showed accumulations in many fibres on the anal side of the lesion, and in very few fibres on the oral side (Fig. 8f-i). Loss of enkephalin-immunoreactive fibres from the ganglia was marked on the oral side of the lesion. In some ganglia adjacent to the lesion, no fibres remained; there was a steady increase in fibre numbers further oral from the lesions and normal innervation returned at 5-8 mm oral to operations. The number of fibres in the circular muscular was depleted on the oral side of the operation for a distance of about 1 mm.

The myectomy operation resulted in a total loss of immunoreactive fibres in the circular muscle directly below the operated region, and a depletion of fibres for 1-2 mm on the oral side of the operation (Fig. 8j-l). The few fibres present in the submucosa remained intact, as did the few fibres in the muscularis mucosae and lamina propria of the mucosa.

Extrinsic denervation had no effect on the distribution of enkephalin-immunoreactive fibres in any layer of the distal colon.

**Somatostatin**

**Distribution**

Immunoreactivity for somatostatin was found in nerve cell bodies in the myenteric plexus and the submucous plexus. Somatostatin-immunoreactive fibres were found in the myenteric and submucous plexuses, the circular muscle, the muscularis mucosae, and the lamina propria of the mucosa (Fig. 9).

In the myenteric ganglia, somatostatin-immunoreactive cell bodies could only be visualized after colchicine and veratridine treatment or after myotomy or myectomy operations. The cells were round or oval, but visualization of their processes was always difficult due to other brightly immunoreactive fibres. The majority of somatostatin-immunoreactive fibres were varicose, but there was a population of non-varicose fibres which ramified within the plexus. Varicose fibres formed a dense network throughout the myenteric ganglia; fibres also travelled in the internodal strands. A common feature of somatostatin-immunoreactivity in the myenteric plexus was clusters of terminals protruding from fibres within the myenteric ganglia, apparently innervating non-immunoreactive nerve cell bodies. This feature was also typical of both CGRP-immunoreactive and TH-immunoreactive fibres. The non-varicose fibres ran in groups of 11.4 ± 0.5 (mean ± SEM; n=54) in the internodal strands, coursing along the longitudinal axis of the gut.

Immunoreactive fibres were not seen in the longitudinal muscle layer. The circular muscle layer contained a low density of evenly distributed varicose fibres, usually grouped together in small bundles and running parallel with the muscle bundles (Fig. 9h). This observation is in contrast to the distribution of somatostatin-immunoreactivity in the circular muscle of the guinea-pig ileum where reactive fibres are absent or rare (COSTA et al., 1980b; SCHULTZBERG et al., 1980).

The submucosal layer contained many immunoreactive nerve cell bodies, and a sparse network of both varicose and non-varicose fibres. Nerve cell bodies were clearly visible in normal tissue. They were usually found alone or as groups of 2 to 4 (Fig. 9e). Their long filamentous processes could often be followed a short distance before they terminated on a non-immunoreactive target, or before they broke off entering the mucosa. Somatostatin-immunoreactive fibres did not innervate the vascular component of the submucous layer.

Within the mucosa, the muscularis mucosae contained a sparse innervation of somatostatin-immunoreactive fibres and the lamina propria received only a slightly denser innervation (Fig. 9f).

**Projections**

In the myenteric plexus after a myotomy operation, accumulations were seen on both sides of the lesion after five days, although the swellings were larger and more brightly immunoreactive on the anal side of the operation (Fig. 9a-d). Axonal loss was only obvious in ganglia on the oral side of the lesion, and a normal density of innervation was reached at a distance of 10 mm oral to the lesion. In the circular muscle, however, there was a distinct loss of immunoreactive fibres on the anal side of the operation. There was no alteration in fibre distribution in the other layers of the gut.
The myectomy operation caused a total loss of fibres in the circular muscle directly below, and up to 2 mm anal to the operation area (Fig. 9h–j). Distributions of fibres in the submucosa and mucosa were also affected in the area directly below the myectomy, both layers suffering a slight decrease in fibre density (Fig. 9f, g).

Combination of the myectomy with an extrinsic denervation caused an additional loss of fibres in the submucous plexus and in the mucosa, but a small population of fibres in both these layers remained.

Extrinsic denervation resulted in a small loss of somatostatin-immunoreactive fibres from the submucous plexus and the mucosa. There was no loss of fibres from the circular muscle.

Fig. 9. Distribution of immunoreactivity for somatostatin and effect of lesions. a–d. Control myenteric ganglia and myenteric ganglia 5 days after a myotomy operation. b shows a neuroma at the oral margin (arrows) and c shows prominent accumulation in a cut nerve strand (arrows) anal to the operation. e. Submucous ganglia. In focus is a ganglion of the outer plexus which contains 3 immunoreactive nerve cell bodies and out of focus is a ganglion of the inner plexus (asterisk). Immunoreactive fibres are in both ganglia. f and g. Sections through a control region of mucosa and under a myectomy, where fibre numbers are reduced. h–j. Sections through the circular muscle. The innervation 1 mm oral to the lesion is normal (h), fibres are lost beneath the myectomy (i) and 1 mm anal to the myectomy there is a slight depletion of innervation (j). Calibrations: a–e; f–j 50 μm
Gastrin releasing peptide (GRP)

Distribution

Immunoreactivity for GRP, the mammalian form of bombesin, was found in nerve cell bodies of the myenteric plexus and in nerve fibres in the longitudinal and circular muscle, in the muscularis mucosae, and the lamina propria of the mucosa (Fig. 10).

In the myenteric ganglia, immunoreactive cell bodies were occasionally visualized in normal tissue, but best visualization was achieved by pre-treating the tissue with colchicine and veratridine. GRP-immunoreactive cells were most commonly located on the outer margins of a ganglion or the outer margins of internodal strands (Fig. 10b). The GRP-immunoreactive cells had shapes corresponding to Dogiel type I and II neurons. The cytoplasm was granular in appearance.

GRP-immunoreactive fibres in the myenteric plexus formed dense networks throughout the ganglia (Fig. 10a). Fibres were also found in the internodal strands and secondary plexus and a small population was present in the tertiary plexus. Most fibres were varicose, but many non-varicose fibres ramified within the plexus. Baskets of varicose GRP-immunoreactive fibres were commonly found around non-immunoreactive cell bodies.

Immunoreactive fibres were occasionally found in the longitudinal muscle layer. They were typically fine and varicose. The circular muscle contained a substantial innervation with GRP-immunoreactive fibres which were evenly distributed throughout its thickness (Fig. 10i). They were also fine and varicose, and ran in small bundles parallel to the muscle bundles.

The submucosal layer did not contain any immunoreactive nerve cell bodies but varicose fibres ramified through the submucosal layer, and formed baskets around non-immunoreactive nerve cell bodies. The arterioles in this layer often had two or three perivascular varicose fibres with GRP-immunoreactivity (Fig. 10d). The mucosa was only sparsely innervated with GRP-immunoreactive fibres; the fine varicose fibres were more frequent in the muscularis mucosae than in the lamina propria.

Projections

After myotomy operations, the cut fibres in the myenteric plexus accumulated GRP-immunoreactivity on the oral side of the lesion, and GRP-immunoreactive fibres degenerated on the anal side of the lesion. Beaded swellings on the oral edge were only obvious in the varicose fibres, while non-varicose fibres became thicker and more brightly immunoreactive. A loss of GRP-immunoreactive fibres occurred anal to the lesion up to a distance of 14 mm, where a normal density of innervation finally returned (Fig. 10e–h).

Myectomy operations resulted in total loss of GRP-immunoreactive fibres from the underlying circular muscle (Fig. 10j). GRP-immunoreactive fibres were also lost from the circular muscle for up to 2.5 or 3 mm anal to the operated region (Fig. 10k). All GRP-immunoreactive fibres in the muscularis mucosae and lamina propria disappeared as a consequence of the myectomy, and there was a partial loss of immunoreactive fibres from the submucosa.

Extrinsic denervation had no effect on the GRP-immunoreactive fibre distribution in any layer of this region of the colon.

Galanin

Distribution

Galanin-like immunoreactivity was found in nerve cell bodies in the myenteric plexus and the submucous plexus. Reactive fibres were found in the longitudinal and circular muscle, in the myenteric plexus, submucous plexus and alongside submucosal arterioles, and in the muscularis mucosae and lamina propria of the mucosa (Fig. 11).

In the myenteric plexus, infrequent immunoreactive nerve cell bodies with oval profiles and a few fine processes were seen. Pre-treatment of the tissue with colchicine and veratridine resulted in more cell bodies of similar morphology becoming immunoreactive. The processes emerging from these cells could not be followed because they were too faintly immunoreactive. Galanin-immunoreactive fibres in the myenteric plexus were varicose and formed a loose network within the ganglia (Fig. 11a). Occasionally small baskets of fibres surrounded non-immunoreactive cells. Commonly only 3 or 4, and sometimes fewer fibres would run in an internodal strand. Few fibres were seen in the tertiary plexus.

Immunoreactive fibres were occasionally found in the longitudinal muscle layer when it was viewed in section, and more frequently in the circular muscle layer. They were varicose and fine in structure, and were oriented parallel to the long axes of the muscle bundles.

The submucous ganglia contained immunoreactive nerve cell bodies with round or oval cell bodies and tapering processes (Fig. 11c). They were infrequent, but more often encountered in tissue pre-treated with
colchicine and veratridine. On many occasions it was possible to follow long processes from a cell body as they wound through the ganglionated and non-ganglionated plexuses, until they were broken off entering the mucosa. Also present in the submucosal layer was a population of varicose fibres which ran through the ganglia and in the nerve strands. Submucosal arterioles could often be seen with 2 or 3 varicose fibres running alongside them (Fig. 11e).

The mucosa was densely innervated with galanin-

**Fig. 10.** Immunoreactivity for GRP. a, In the myenteric plexus fibres are in the ganglia and in the tertiary plexus (arrow). b, Reactive nerve cells in a myenteric ganglion, seen after colchicine treatment. c, Fibres in a submucosal ganglion. d, Fibres around an arteriole in the submucosa. e-h, Myenteric ganglia oral (e) and progressively more anal (f-h) to a myotomy operation performed 5 days previously. No changes were observed orally, whereas a graded loss occurred anal to such lesions. i-k, Sections through the circular muscle, 1 mm oral (i), beneath a myectomy (j) and 1 mm anal to a myectomy (k). Calibrations: a, b; c-h; i-k 50 μm
immunoreactive fibres in the lamina propria, particularly around the bases of the colonic glands (Fig. 11d). A sparse distribution of fibres with galanin-immunoreactivity was found in the muscularis mucosae.

**Projections**

An accumulation of immunoreactive peptide occurred in the cut strands on the oral side of myotomy operations. Regrowth of fibres from this site was observed after 6 days (Fig. 11f). Immediately on the anal side of the lesion there was a total loss of immunoreactive fibres from the ganglia and the internodal strands, and the return to a normal density of innervation occurred at a distance of 20 mm anal to the lesion (Fig. 11f-i). In the circular muscle layer there was a loss of immunoreactive fibres for 2-3 mm on the anal side of the lesion. Fibre distribution in the submucous plexus and mucosal layer was not affected by the myotomy.

Following a myectomy, the circular muscle directly beneath the operation lost all galanin-immunoreactive fibres. This loss of immunoreactive fibres extended a further 3 mm anal to the operation. In the submucous plexus there was a small loss of fibres in the ganglionated and non-ganglionated plexuses, and no loss of fibres from around the arterioles. In the mucosa there was also a decrease in the numbers of immunoreactive fibres. The muscularis mucosae and the lamina propria were affected to similar extents.

Extrinsic denervation had no effect on the distribution of galanin-immunoreactive fibres in any layer of the distal colon.

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**Fig. 11.** Galanin immunoreactive fibres in the distal colon. a. Reactive fibres form a network around non-reactive nerve cells in myenteric ganglia. b and c. Submucous ganglia showing reactive fibres and a reactive nerve cell. d. Section showing the dense innervation of colonic glands. The muscularis mucosae (mm) is sparsely innervated. e. Innervation of an arteriole in the submucosa. f. Neuroma formed at the site of lesion of a myenteric internodal strand oral to a myotomy, at 6 days. g-j. Loss of immunoreactive fibres 5 days after a myotomy seen in myenteric ganglia anal to the lesion. Calibrations: a, b, d-f 50 μm; c 25 μm; g-i 50 μm.
Calcitonin gene-related peptide (CGRP)

Distribution

CGRP-like immunoreactivity was found in nerve cell bodies of the myenteric and submucous ganglia. Immunoreactive fibres were in the circular muscle, myenteric plexus, submucous plexus, around submucosal arterioles, and in the muscularis mucosae and lamina propria (Fig. 12).

In the myenteric ganglia, nerve cell bodies had smooth outlines and were approximately oval, with several long tapering processes (Fig. 12b). In Dogiel's classification they correspond to type II neurons. They were frequently encountered and visualization was not significantly enhanced with colchicine and veratridine treatment. The non-varicose processes could often be followed for long distances (1-2 mm) but as the processes became varicose it became impossible to follow individual fibres.

Strongly reactive varicose and non-varicose immunoreactive fibres were found in the myenteric plexus. Varicosities were large and the fibres were of large diameter. Non-varicose fibres were also thick. Together they formed a loose network throughout individual ganglia and connected the ganglia via internodal strands. The non-varicose fibre distribution differed from the distinct pattern of NPY-immunoreactive and somatostatin-immunoreactive non-varicose fibres in that the CGRP-immunoreactive fibres typically ran alone or with one other fibre. They also tended to double back on themselves, branch frequently, and travel long distances circumferentially before continuing along the longitudinal axis of the colon. Typically they would travel through the centre of a ganglion, alter course to meander around an often immunoreactive cell, and continue on to the next ganglion. At other times they would travel directly over the outer margins of a ganglion, from one internodal strand to another. Another feature of both varicose and non-varicose CGRP-immunoreactive fibres in the myenteric plexus was the formation of small clusters of terminals which would protrude from internodal strands, or from the surfaces of ganglia, innervating non-immunoreactive targets, presumably nerve cell bodies (Fig. 12c, d). Most non-varicose fibres were also immunoreactive for substance P.

The circular muscle contained a small number of fine varicose CGRP-immunoreactive fibres. They were evenly distributed throughout the thickness of the muscle, and oriented in parallel to the muscle bundles. Reactive fibres ran in the penetrating fibre bundles between the myenteric and submucous plexuses (Fig. 12a).

The submucous plexus contained many immunoreactive nerve cell bodies, which had one or several long non-varicose processes (Fig. 12j, k). These were brightly immunoreactive and could be followed within the ganglionated plexus, however, they would eventually break off, suggesting a target in the mucosal layer. Also within the submucous plexus were brightly immunoreactive, thick varicose fibres. They could be followed within the ganglionated plexus and were frequently seen to surround other immunoreactive cell bodies. The submucous arterioles received a dense para- and perivascular innervation from CGRP-immunoreactive fibres. Paravascular fibres were generally non-varicose, and the perivascular fibres were generally varicose. Simultaneous staining revealed that the perivascular fibres with CGRP-immunoreactivity were also immunoreactive for SP (Fig. 6k, l).

Both the muscularis mucosae and the lamina propria of the mucosa contained CGRP-immunoreactive fibres. The fibres were varicose and formed a dense network around the basal aspects of the epithelial cells.

Projections

Large accumulations of immunoreactivity were evident in the cut strands on the oral edges of lesions through the myenteric plexus and there was a total loss of immunoreactive fibres from many ganglia of the first few millimetres on the anal side of the lesion. A normal density of immunoreactive fibres did not return for a distance of 20 mm anal to the lesion (Fig. 12e–g). In the circular muscle there was a loss of immunoreactive fibres in the region 2–3 mm anal to a myotomy.

When the overlying myenteric plexus was removed in a myectomy operation, all the CGRP-immunoreactive fibres within the underlying circular muscle degenerated. In the submucosal ganglia a large population of varicose fibres was lost. In the mucosa, most fibres in both the muscularis mucosae and the lamina propria consequently degenerated (Fig. 12h, i). Paravascular and perivascular fibres were not affected by the myectomy.

Extrinsic denervation resulted in a complete loss of the para- and peri-vascular nerves of the submucous plexus and also the non-varicose fibres of the submucous plexus (Fig. 12n, o). Furthermore, most of the prominent non-varicose fibres within the myenteric plexus degenerated (Fig. 12l, m). The mucosal layer was unaffected.
Fig. 12. Legend on the opposite page.
When an extrinsic denervation was combined with a myectomy, a small population of fibres remained in the underlying submucous plexus, in the muscularis mucosae, and in the lamina propria of the mucosa.

**Calbindin**  
**Distribution**  
Calbindin-like immunoreactivity was found in nerve cell bodies in the myenteric and submucous plexuses, and in nerve fibres in the circular muscle, myenteric and submucous plexuses, and the muscularis mucosae and lamina propria of the mucosa (Fig. 13). Calbindin-immunoreactivity was occasionally found in mucosal endocrine cells.

Immunoreactive nerve cell bodies in the myenteric plexus were either irregularly shaped, similar to Dogiel type I neurons or had Dogiel type II morphology (Fig. 13a–c). The strength of reaction varied, many cells being dull and grainy in appearance while others were strongly reactive. The cells often had a brightly stained nucleus and dark spots where, presumably, the nucleoli were located. In many instances it was possible to follow the main axonal process as it emerged from the cell body and travelled through the ganglion or internodal strand, however they could rarely be followed far because immunoreactivity was typically dull in the processes. Calbindin-immunoreactive cells were mainly found in groups of about 4, and up to 10, within the ganglia, or in smaller groups in the internodal strands.

Immunoreactive fibres within the myenteric plexus, although only weakly reactive, were fine and varicose, and formed a dense network throughout the plexus. The fibres commonly formed basket type arrangements around both immunoreactive and non-immunoreactive cell bodies. They travelled only in the internodal strands and secondary plexus.

Fibres in the circular muscle were faintly immunoreactive, and were fine and varicose. They were present in small groups, oriented in parallel with the long axes of the muscle bundles (Fig. 13f, h).

In the submucous plexus there were large numbers of type II immunoreactive nerve cell bodies, usually clustered in groups of 2 to 6 (Fig. 13d). Immunoreactivity in the neurons was variable and consequently tracing the paths of the main processes was difficult. Other calbindin-immunoreactive fibres were also observed in the ganglionated plexus and they too were fine and varicose in structure.

The lamina propria of the mucosa contained a small population of immunoreactive varicose fibres, typically around the bases of the colonic glands and along the bases of the mucosal epithelial cells (Fig. 13e).

**Projections**  
Myotomy operations caused small accumulations of immunoreactivity at both edges of the lesion. There was no evidence of axonal loss beyond the first row of ganglia (0.5–1 mm).

The myectomy operation resulted in a total loss of immunoreactive fibres in the circular muscle directly below the operation (Fig. 13g). There was also some loss of fibres in the submucous plexus. Furthermore, there was a slight loss in the lamina propria of the mucosa.

Extrinsic denervation had no effect on the calbindin-immunoreactive fibre distribution in the distal colon.

**Gamma-aminobutyric acid (GABA)**  
**Distribution**  
GABA-like immunoreactivity was found in nerve cell bodies in the myentericplexus only, and in nerve fibres in the circular muscle (Fig. 14a) and submucous plexus. Tissue which had been pre-loaded with GABA gave consistently stronger immunostaining than did unloaded tissue. Nerve cell body and fibre

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**Fig. 12.** Immunoreactivity for CGRP in nerve fibres and nerve cells in the distal colon. a. Section through the longitudinal muscle (lm), a myenteric ganglion (mg) and the circular muscle (cm). The muscle is very sparsely innervated and there is only a small number of fibres in the ganglia. A varicose fibre can be seen in a penetrating fibre bundle traversing the circular muscle (arrow). b. Reactive nerve cells seen in a myenteric ganglion. c and d. A small side branch from a fibre running through a myenteric ganglion (e) and a group of fibres (arrow) that appear to arise from a passing fibre bundle, also in a myenteric ganglion (d). e–g. Myenteric ganglia 1 mm oral (e) and 1 mm (f) and 20 mm (g) anal to a myotomy operation performed 5 days previously. h–i. Sections through the control mucosa (h) and through the mucosa beneath a myectomy (i). j and k. Examples of immunoreactive nerve cell bodies in submucous ganglia. l and m. Non-varicose axons that are typically observed in myenteric ganglia (l) contrasted to a ganglion after extrinsic denervation (m) which causes a substantial loss of such axons. n and o. Submucous arterioles (art) from control (n) and 6 days after extrinsic denervation (o). Calibrations: a, d; e–g; h, i; j, l, m; n, o 50 μm; b, c; k 25 μm.
Fig. 13. Calbindin immunoreactivity in nerve cells and fibres. a–c. Brightly reactive nerve cells in myenteric ganglia. Most cell bodies had Dogiel type II morphology (a), but some had irregular shapes (b). The type II cells gave rise to numerous fine processes (arrows in c). Numerous fine nerve fibres were also reactive (a). d. There were frequent oval immunoreactive cells in submucous ganglia, which also contained fine reactive nerve fibres. e. Nerve fibres in the lamina propria between colonic glands. f–h. Sections through the circular muscle, showing normal innervation 1 mm oral (f) and 1 mm anal (h) and loss of fibres beneath the lesion (g). Calibrations: a, d; f–h 50 μm; b, c; e 25 μm.

Fig. 14. Immunoreactivity for gamma-aminobutyric acid in the colon. a. Section which shows a reactive cell body in a myenteric ganglion (arrow) and reactive fibres in the circular muscle (cm). Fibres were very rare in the longitudinal muscle (lm). b. Wholemount showing the typical appearance of reactive myenteric nerve cells. c. Nerve fibres running across a ganglion of the myenteric plexus. Calibration: a; b, c 50 μm.
distribution pattern was not changed with the loading procedure.

In the myenteric ganglia, immunoreactive nerve cell bodies had a very distinct shape which corresponded to the type I neurons of Dogiel's classification (Fig. 14b), but were a shape not characteristic of any other intrinsic neuron examined in this study. They were relatively large, 20-30 μm in diameter, with many short, broad, lamellar processes. A single long process could be followed from the cell body for considerable distances (100-200 μm) in the plexus. The processes emerged as non-varicose axons but often became varicose further from the cell body. A common feature of these neurons was a number of protuberances, or spines, on the proximal portion of the axon. There were approximately 1 to 3 immunoreactive cell bodies in the majority of the larger myenteric ganglia, and occasionally they were located in the internodal strands. In almost every case they were located on the outer surfaces of the ganglion (Fig. 14a).

Immunoreactive fibres in the myenteric plexus were both varicose and non-varicose, and comparatively thick in diameter. They formed a loose network throughout the plexus and ran mainly in the internodal strands and secondary plexus. Very few fibres ran in the tertiary plexus. The GABA-immunoreactive fibres formed loose baskets around non-immunoreactive cells. They would also typically double back on themselves and run past their own cell body. There were only 1-3 fibres travelling in any one internodal strand (Fig. 14c).

In the circular muscle there was a moderately dense distribution of immunoreactive fibres throughout the thickness of the layer. They were mainly varicose, and ran in small groups parallel to the long axis of the circular muscle bundles. The longitudinal muscle did not contain any GABA-immunoreactive (Fig. 14a).

Immunoreactive fibres in the submucosal layer were infrequent. They possibly originate from myenteric neurons as there are no GABA-immunoreactive cells in the submucous plexus. No immunoreactive fibres were observed in the mucosa.

### DISCUSSION

#### Arrangement of plexuses

While there is general similarity of arrangement between the enteric plexuses of small intestine and colon in the guinea-pig, some pertinent differences occur. In the small intestine, the longitudinal muscle is innervated by nerve fibres of the tertiary plexus and no plexus of parallel nerve fibre bundles within the longitudinal muscle layers (a longitudinal muscle plexus) is found. In contrast, the present work revealed a longitudinal muscle plexus in the colon. A tertiary plexus was also found, but it is less well

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<tr>
<th>Table 2. Comparison of the directions that chemically-specified neurons project in the myenteric plexus</th>
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<td><strong>Guinea-pig</strong></td>
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<td>Small intestine</td>
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<td>VIP</td>
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<td>Galanin</td>
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<td>GRP</td>
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<td>Substance P</td>
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→: anally projecting, ←: orally projecting, o: myenteric fibres of extrinsic origin, -: not determined.

References: a) FURNESS and COSTA, 1987; b) FURNESS et al., 1987; c) FURNESS et al., 1990b; d) FURNESS et al., 1989c; e) this paper; f) EKBLAD et al., 1987; g) EKBLAD et al., 1985; h) EKBLAD et al., 1984a; i) BALLESTA et al., 1988; j) DANIEL et al., 1987; k) FURNESS et al., 1990a; l) DOMOTO et al., 1990; m) EKBLAD et al., 1988.
developed than that of the small intestine. In the colon, unlike the small intestine of the guinea-pig, but similar to some other species (Scheuermann et al., 1989), the submucosa contains two interconnected sets of ganglia forming inner and outer submucous plexuses. In the colon there is no deep muscular plexus such as occurs in the small intestine, but a sub-muscular plexus against the inner surface of the circular muscle was seen.

**Distributions of chemically-defined neurons**

The results show that in the small and large intestines of one species, the guinea-pig, the distributions and projections of some chemically-specified subgroups of enteric neurons are similar, while others are quite different. A similar phenomenon is found when the small and large intestines of rat are compared (Ekblad et al., 1987, 1988). In Table 2, one feature of enteric projections, their directions in the myenteric plexus, has been compared between regions and species. Differences between regions in the one species seem as great as differences between species. Nevertheless, some interesting common features are seen. Nerve fibres with VIP-immunoreactivity run anally in the myenteric plexus of the small and large intestines of guinea-pig, rat and dog. They also run anally in the human colon (Domoto et al., 1990). Some of the VIP fibres supply myenteric ganglia and some supply the circular muscle. The presence of anally directed VIP fibres to the circular muscle is consistent with VIP being a probable transmitter released from enteric inhibitory neurons (Makhlouf et al., 1989). Similarly, the directions of projection of neurons with galanin and GRP immunoreactivity in the myenteric plexus are similar, and these substances are thus possibly contained in functionally equivalent neurons in different regions and species. On the other hand, NPY-like immunoreactivity is unpredictable in its projections, both in relation to region and in relation to species. Projections of somatostatin and substance P fibres also show striking variations. One obvious conclusion is that it is not possible to extrapolate projections of chemically specific neuronal subgroups between species with any confidence. The results strongly imply that neuropeptides are not necessarily in functionally equivalent neurons in different regions of the gut, or in different species.

In the enteric nervous system, as in other parts of the nervous system, many neurons contain more than one potential neurotransmitter. An extreme case is the cholinergic secretomotor neurons of the guinea-pig small intestine; in addition to the principal transmitter, acetylcholine, these neurons have immunoreactivity for CCK, CGRP, galanin, neuromedin U, NPY and somatostatin (Bornstein and Furness, 1988; Furness et al., 1989). Associations of neuronal markers differ between species, for example in the rat small intestine NPY and VIP are in the same submucous neurons (Ekblad et al., 1984b; Pataky et al., 1990), whereas in the guinea-pig these two peptides are in separate submucous neurons (Furness et al., 1984). Many other interspecies and inter-region differences of association are known (Furness et al., 1989a; Hokfelt et al., 1987; Gibbins, 1989). One possible explanation for the differences could be that certain substances are principal transmitters of neurons whereas others are auxiliary substances, performing less critical roles in the transmission process or perhaps having non-transmitter functions. For example, excitatory motor neurons to the muscle of the gastrointestinal tract use acetylcholine as a neurotransmitter (Furness and Costa, 1987) but there are also non-cholinergic excitatory components of transmission, which in some parts of the intestine appear to be due to the release of substance P or a closely related substance (e.g., Leander et al., 1981; Björkroth, 1983; Niel et al., 1983). The presence of ascending pathways to the muscle in several areas of intestine (see Table 2) is consistent with this role of substance P. However, in the rat small intestine substance P is present in descending pathways (Ekblad et al., 1987). On the other hand, in a few places non-cholinergic excitatory transmission appears to be mediated by an opiate (Edin et al., 1980, Reynolds et al., 1984, 1985). Another substance which might be a principal transmitter is VIP, in enteric inhibitory neurons. VIP is found in descending pathways within the myenteric plexus and to the circular muscle in all regions and species studied. In human enteric neurons, NPY and galanin immunoreactivity is contained in many of the VIP nerve fibres innervating the muscle (Wattchow et al., 1988; Burleigh and Furness, 1990). Surprisingly, galanin contracts the muscle, whereas, as expected, VIP causes relaxation. In the guinea-pig small intestine, most of the VIP fibres contain enkephalin-immunoreactivity, but enkephalin seems to have no direct action on the muscle (Llewellyn-Smith et al., 1988).

In order to resolve the many questions raised by the differences revealed by this and other studies of the projections of chemically specified enteric neurons, correlations between the projections, the actions of neuropeptides and transmission from functionally identified neurons in circumscribed areas of
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