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Projections of chemically identified myenteric neurons of the small and large intestine of the mouse

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ABSTRACT

The projections of different subpopulations of myenteric neurons in the mouse small and large intestine were examined by combining immunohistological techniques with myotomy and myectomy operations. The myotomies were used to examine the polarity of neurons projecting within the myenteric plexus and showed that neurons containing immunoreactivity for nitric oxide synthase (NOS), vasoactive intestinal peptide (VIP), calbindin and 5-HT projected anally, while neurons with substance P (SP)-immunoreactivity projected orally, in both the small and large intestine. Neurons containing neuropeptide Y (NPY)- and calretinin-immunoreactivity projected locally. In the large intestine, GABA-immunoreactive neurons projected both orally and anally, with more axons tending to project anally. Myectomy operations revealed that circular muscle motor neurons containing NOS/VIP/$\pm$NPY and calretinin neurons projected anally both in the small and large intestine, while SP-immunoreactive circular muscle motor neurons projected orally. In the large intestine, GABA-IR circular muscle motor neurons projected both orally and anally. This study showed that although some neurons, such as the NOS/VP inhibitory motor neurons and interneurons, SP excitatory motor neurons and 5-HT interneurons had similar projections to those in other species, the projections of other chemical classes of neurons in the mouse intestine differed from those reported in other species.

Key words: Enteric nervous system; calcium binding protein; neuropeptides; nitric oxide synthase.

INTRODUCTION

The mouse presents great advantages for the study of the enteric nervous system because of the relative ease with which genetic manipulations can be made in this species and because of the large screening for genetic mutants that has been undertaken. This has already led to a number of fruitful studies of the murine enteric nervous system. For example, Kapur et al. (1992) used a transgene coupled to the dopamine β hydroxylase promoter to study the development of the enteric nervous system in normal mice and in mice with a Hirschprung's disease-like aganglionosis (*ls*/*ls* mice) and were able to document a defective migration of vagal neuroblasts in the *ls*/*ls* mutant. Gershon et al. (1993) used the *ls*/*ls* mutant to identify that an abnormality of laminin contributes to the mutant phenotype. Further insight into the enteric nervous system development was obtained by Hosoda et al. (1994), who showed that disruption of the gene for the endothelin B receptor caused colonic aganglionosis in the mutant mice. The same group showed that disruption of the endothelin B receptor also occurs in human Hirschsprung's disease (Puffenberger et al. 1994). Another example of a genetic manipulation in mice occurs in the W/W^v mutant where an abnormality of the *c-kit* protein causes loss of myenteric interstitial cells and abnormalities of electrical activity in intestinal muscle (Ward et al. 1994*a*; Huizinga et al. 1995).

Mice also present advantages in developmental studies because of their short, uniform and well documented gestation, which has been exploited in several studies of the developing enteric nervous system (Gershon et al. 1993).

Despite the great potential of mice for analysis of enteric nervous system organisation and function by gene targeting, and for studying the development of

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this system, baseline data on the chemistry of enteric neurons have been restricted to scattered reports. It is only recently that a comprehensive account of the chemistries of murine enteric neurons has been completed (Sang & Young, 1996). This work detailed the distributions and patterns of colocalisation of calbindin, calretinin, gamma-aminobutyric acid (GABA), 5-hydroxytryptamine (5-HT), nitric oxide synthase (NOS), neuropeptide Y (NPY), substance P (SP) and vasoactive intestinal peptide (VIP) in murine enteric neurons. It confirmed conclusions from other studies that some aspects of enteric neurochemistry are well preserved between species, whereas others show significant variation (Messenger & Furness, 1990; Ekblad et al. 1991; Barbiers et al. 1995; Furness et al. 1995).

A further way to relate the chemistries of enteric neurons to their functions is to determine their projections within the gut wall. Studies of this type have been undertaken in guinea pigs (Furness & Costa, 1987; Furness et al. 1989; Costa et al. 1992*b*) rats (Ekblad et al. 1987, 1988), dogs (Daniel et al. 1987; Furness et al. 1990*a*), pigs (Timmermans et al. 1994; Barbiers et al. 1995), humans (Domoto et al. 1990: Wattchow et al. 1995) and even toads (Murphy et al. 1994), but not until now in mice.

MATERIALS AND METHODS

A total of 57 Balb/C or BL/6 × DBA adult mice of both sexes, 17–32 g in weight, were used in this study. The mice were anaesthetised with a subcutaneous injection of pentobarbitone sodium, 60 mg/kg (Boehringer Ingelheim, Sydney, Australia), 30 min prior to microsurgery, and then a midline abdominal incision was made through the skin, body wall and peritoneum. A small region either of the small or large intestine was exteriorised and then a myotomy or myectomy operation was performed (see below). Following the operations, the animal was given an intramuscular injection of 0.05 ml of the antibiotic, Terramycin (0.5 mg/ml oxytetracycline base, Pfizer Agricare, Sydney, Australia).

Myotomy

In 39 animals, a single circumferential cut was made through the external musculature to the depth of the submucous plexus. This procedure severs longitudinal nerve pathways running in the myenteric plexus (Furness & Costa, 1979). The operation site was marked by tying a small piece of cotton thread around a blood vessel in the adjacent mesentery. Seventeen operations were performed in the middle small intestine (2–4 cm orally from the ileocaecal junction) and 22 in the proximal colon (about 2 cm aboral to the ileocaecal junction). The animals were left from 2–10 d before being killed.

Myectomy

In 18 animals, 2 circumferential cuts, ~ 3 mm apart, were made through the longitudinal and circular muscle layers and the longitudinal muscle layer and myenteric plexus between the cuts were peeled away. The operation site was marked as described above. Nine myectomies were performed in the middle small intestine and 9 in the proximal colon, about 2 cm from the caecum. Animals were left from 7–10 d before tissues were taken.

Tissue preparation

The mice were killed by cervical dislocation. Segments of operated intestine were relocated and collected in sodium phosphate buffered saline (PBS, 0.9% NaCl in 0.01 M sodium phosphate buffer, pH 7.0) containing the muscle relaxant, nicardipine (10^{-6} M; Sigma, St Louis, MO, USA). The intestine was opened along the mesenteric border after oral and anal ends were marked for orientation.

Wholemounts. Tissues were pinned to balsa wood with the mucosal side down. Apart from tissue to be processed for GABA or 5-HT immunohistochemistry (see below), the tissue was fixed in Zamboni's fixative (2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0) overnight at 4 °C. The fixative was removed by washing in dimethylsulphoxide (DMSO, 3 × 10 min) and then PBS (3 × 10 min). The external musculature and submucosa/mucosa were dissected apart and the circular muscle removed, leaving the longitudinal muscle with attached myenteric plexus.

Sections. Tissues were pinned to balsa wood without stretching and fixed as described for wholemounts. After the fixative was removed, the tissues were stored in PBS containing 0.1% sodium azide plus 30% sucrose (PBS-suc) for 24 h at 4 °C and then transferred into a 50:50 mixture of PBS-suc:OCT (Lab Tek products, Nashville, IL, USA) for another 24 h at 4 °C before being transferred to pure OCT to be sectioned. Segments of tissue that included the operated area, and at least 10 mm of tissue on both sides of the operation, were oriented in cryomolds that contained OCT and were frozen in isopentane

Table 1. *Primary antisera used in the study*

Antibody	Species	Dilution for wholemounts	Diluton for sections	Source or reference
Calbindin	Sheep	1:800	—	Kind gift of Dr P. C. Emson
Calretinin	Rabbit	1:1000	1:1000	Rogers, 1989
GABA	Rabbit	1:1600	1:3200	Maley & Newton, 1985
5-HT	Rabbit	1:1000	—	Kind gift of Dr R. P. Elde
NOS	Sheep	1:2000	1:2000	Kind gifts of Drs I. Charles and P. C. Emson
NPY	Rabbit	1:800	1:1600	Maccarrone & Jarrott, 1985
SP	Rat	1:200	1:800	Cuello et al. 1979
VIP	Rabbit	1:800	1:1000	Kind gift of Dr M. Epstein

Table 2. *Biotinylated secondary antisera used in wholemounts*

Species in which primary antibody was raised	Biotinylated secondary antisera
Rabbit	Biotinylated donkey antirabbit (1:200, Amersham, Melbourne, Australia)
Rat	Biotinylated sheep antirat (1:200, Amersham)
Sheep	Biotinylated donkey antisheep (1:100, Jackson ImmunoResearch, West Grove, PA, USA)

Table 3. *Secondary antisera used with different antisera in immunohistochemical experiments in sections*

Primary antibodies	Secondary antisera
Rat	Donkey antirat fluorescein isothiocyanate (FITC 1:100, Jackson ImmunoResearch)
Rabbit	Donkey antirabbit indocarbocyanine (Cy3, 1:1600, Jackson ImmunoResearch)
Sheep	Donkey antisheep FITC, 1L:100, Jackson ImmunoResearch)

which had been cooled in liquid nitrogen. Longitudinal sections of 10 μm thickness were cut on a cryostat and picked up on slides coated with amino propyl triethoxy-silane (APTS, Sigma). The sections were air-dried on the slides for 60 min at room temperature and then processed for immunohistochemical staining.

Immunohistochemistry

Wholemount preparations were incubated in the primary antibodies listed in Table 1 overnight at room temperature. After removal of the primary antibodies by rinsing in PBS (3×10 min), the tissue was incubated in biotinylated antisera (Table 2) for 2 h at room temperature, and then in avidin-biotin-horseradish-peroxidase (Vectastain ABC Kit; Vecta, Burlingame, CA, USA) for another 2 h. The immunorectivity was developed by the diaminobenzidine (DAB) method. The reactive tissues were dehydrated through graded ethanols and xylene and permanently mounted with Depex.

Sections were incubated overnight in primary antibodies against the following antigens: NOS, VIP, NPY, GABA, SP or calretinin, at the dilutions given in Table 1. The sections were then washed and incubated in the secondary antisera indicated in Table 3 for 90 min at room temperature. The unbound secondary antisera were washed away with PBS (3×10 min). The sections were mounted in bicarbonate-buffered glycerol.

Preincubation for 5-HT localisation. Tissues to be processed for 5-HT immunohistochemistry were preloaded with 5-HT in vitro. After the animal was killed, tissues were pinned on balsa wood and incubated in DME F-12 tissue culture medium (Sigma) containing nicardipine (10^{-5} M) and pargyline (Sigma, a monoamine oxidase inhibitor, 5×10^{-5} M) with 5% CO_2 for 30 min at 37 °C. 5-HT was then added to give a final concentration of 10^{-7} M and tissues were incubated for another 30 min. Tissues were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.0) for 90 min at 4 °C. The fixative was removed by washing in 80% ethanol (10×10 min) and PBS (3×10 min).

Preincubation for GABA localisation. Tissues to be processed for GABA immunohistochemistry were preloaded with GABA in vitro as described by Furness et al. (1989). In summary, operated segments of proximal colon were dissected from animals and placed in warmed culture medium (DME medium

from Sigma) containing nicardipine (3×10^{-5} M). Segments of tissue were cut open along the mesenteric border and pinned onto balsa-wood board with the mucosa side down. Those from myotomy operations were pinned stretched for wholemounts and those from myectomy operations pinned unstretched for sections. The preparations were placed into vials in a 37 °C hot water bath containing tissue culture medium (preincubation medium) which comprised nicardipine (3×10^{-5} M), amino-oxyacetic acid (AOAA, 2×10^{-5} M; Sigma), an enzyme inhibitor used to block GABA transaminase activity, and β-alanine (1×10^{-3} M; Sigma), used to prevent uptake of GABA into glial cells. Preincubation was for 30 min. Each preparation was then transferred into a clean pot containing preincubation medium plus GABA (5×10^{-9} M; Sigma) and incubated for 1 h. The preparations were fixed in Zamboni's fixative containing 0.05% glutaraldehyde (added fresh) for 4 h at room temperature. Fixative was removed from the tissue by 3 washes in DMSO. The tissue was then washed in PBS before being treated with sodium borohydride (1% solution in 0.1 M phosphate buffer) on a rocker for 30 min. Following this, each preparation was dissected and incubated in primary antisera as described above.

Estimation of projection distance of nerve fibres in the circular muscle

The length of the projection of circular muscle motor neurons was established by counting the number of immunoreactive nerve fibres in the circular muscle layer in photographs taken from sections. One section from each animal was photographed and counted. For each animal, immunoreactive fibres were counted in a region of tissue about 10 mm from the operation site (to determine control density), and at 100 μm intervals from the operation site to the region where the density of innervation returned to control levels.

RESULTS

Myotomy was used to examine the polarity of myenteric neurons that project to the myenteric ganglia and myectomy to examine the polarity of myenteric neurons projecting to the circular muscle or to the myenteric ganglia. Myotomy specimens were prepared only as wholemounts and myectomy specimens as wholemounts or sections. The results are summarised in Tables 4 and 5.

NOS

Myenteric plexus. Following myotomy and myectomy, accumulations of NOS-immunoreactive (IR) material were observed in nerve fibres in the myenteric plexus only on the oral side of the cut and outgrowths of NOS-IR axons extended into the cut from the oral edge (Fig. 1*a*, *b*). In some animals, a deficit of NOS-IR terminals was noticeable in the first 2–3 rows of myenteric ganglia anal to the cut.

Circular muscle. Following myectomy, a deficit of fibres in the circular muscle was apparent for approximately 0.8 mm anal to the operation site in frozen sections of small intestine (Fig. 3*b*) and 1 mm anal to the operation site in the large intestine. On the anal side of the operation, NOS-IR neurons and fibres were observed in the myenteric plexus before NOS fibres appeared in the circular muscle in both the small and large intestine. In the region of tissue from which the external muscle was removed, there was a complete loss of NOS-IR fibres from the submucous plexus in the small intestine, and substantial loss of NOS-IR fibres was detected in the submucous plexus in the large intestine.

VIP

Myenteric plexus. Following myotomy, accumulations of VIP-IR were observed in cut nerve endings on the oral edge of the lesion in wholemounts of small and large intestine. There were also prominent outgrowths of VIP-IR fibres from the oral side. There was a noticeable loss of terminals from myenteric ganglia on the anal side, in the first 3 rows in the small intestine (Fig. 4*a*, *b*) and first 4–6 rows of myenteric ganglia in the large intestine. VIP-IR cell bodies were observed on the anal side of the operation site (Fig. 4*b*), but were not present on the oral side or in control tissue (Fig. 4*a*).

Circular muscle. After myectomy, there was an absence of VIP-IR fibres in the circular muscle on the anal side of the cut. The deficit of VIP-IR fibres in the circular muscle extended for about 0.8 mm anal to the lesion in the small intestine (Fig. 2*e*, *f*) and 1.4 mm in the large intestine. There was no obvious loss of VIP fibres in the submucous plexus and mucosa in either the small or large intestine.

5-HT

Myenteric plexus. Myotomy resulted in accumulation of 5-HT-IR material in nerve terminals in the myenteric plexus on the oral side and outgrowths of 5-

Table 4. *Results of myotomy and myectomy in wholemounts of myenteric ganglia*

Region	Marker	Number of operations	Number of experiments in which fibre outgrowth was observed		Deficit of fibres in myenteric ganglia	
			Oral	Anal	Oral	Anal
Small intestine	NOS	7	5/7	0/7	0/7	3/7
	VIP	2	2/2	0/2	0/2	0/2
	NPY	2	2/2	2/2	0/2	0/2
	5-HT	2	2/2	0/2	0/2	0/2
	Calbindin	2	2/2	1/2	0/2	0/2
	Calretinin	6	4/6	4/6	0/6	0/6
	SP	2	2/2	2/2	1/2	0/2
Large intestine	NOS	8	8/8	0/8	0/6	4/6
	VIP	2	2/2	0/2	0/2	2/2
	5-HT	2	2/2	0/2	0/2	2/2
	Calbindin	2	2/2	0/2	0/2	2/2
	Calretinin	7	7/7	7/7	0/7	0/7
	SP	4	1/4	4/4	2/4	0/4
	GABA	6	6/6	6/6	2/6	4/6

Table 5. *Results of myectomy on sections*

Region	Marker	Number of operations	Length of circular muscle showing a deficit in the number of nerve fibres (μm: mean ± S.E.M.)	
			Oral	Anal
Small intestine	NOS	4	0	760 ± 260
	VIP	4	0	825 ± 250
	NPY	4	0	750 ± 50
	Calretinin	4	150 ± 50	600 ± 100
	SP	4	430 ± 90	200 ± 80
Large intestine	NOS	3	0	1050 ± 50
	VIP	3	0	1400 ± 380
	Calretinin	3	0	720 ± 20
	SP	3	850 ± 150	150 ± 50
	GABA	4	1600 ± 460	2270 ± 730

HT fibres from the severed stumps in both the small and large intestine. On the anal side, there was a significant loss of 5-HT-IR terminals from approximately the first 5 mm anal to the operation in the small intestine (Fig. 4*c*, *d*) and 4 mm in the large intestine (2 preparations). The density of 5-HT-IR terminals in the myenteric plexus appeared to return to normal after 10–12 mm in the small intestine and 8–10 mm in the large intestine. Prominent nerve cell bodies were seen in the first few rows of myenteric ganglia on the anal side of the operation in the small intestine (Fig. 4*d*) and large intestine.

Circular muscle. Because there are no 5-HT-IR nerve fibres in the circular muscle either in the small or large intestine of the mouse (Sang & Young, 1996), no myectomy specimens were processed for 5-HT immunohistochemistry.

SP

Myenteric plexus. After myotomy and myectomy, sprouting SP-IR nerve fibres were mainly observed on the anal side of the operation site in wholemounts of small and large intestine (Fig. 1*c*, *d*). However, there were also a small number of outgrowths on the oral side of the operation site. Following some operations, there appeared to be a loss of SP-IR terminals in the first 2 rows of myenteric ganglia on the oral side of the operation site.

Circular muscle. There was some variation between animals in the effects of myectomy on the distribution of SP-IR nerve fibres in the circular muscle layer. In the small intestine of all operated animals examined ($n = 4$), there was a deficit of SP-IR nerve fibres in the circular muscle for about 0.4 mm on the oral side of

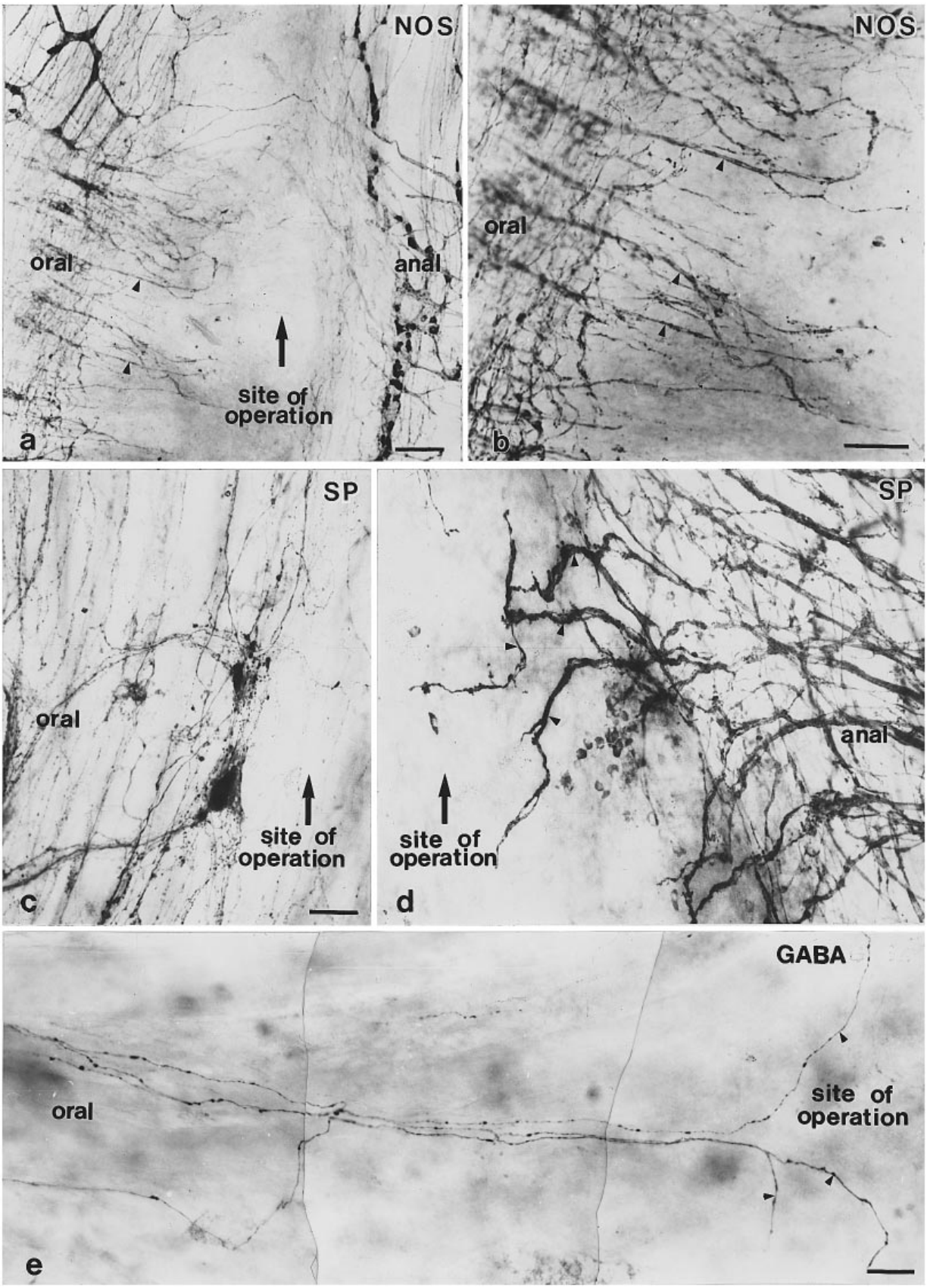

Fig. 1. Wholemounts of intestine 10 d following myotomy, showing fibre outgrowths from the oral or anal sides of the lesion. (*a*) Low power micrograph showing immunoreactivity for NOS in the large intestine. NOS-IR is present in outgrowths of nerve fibres on the oral side of the lesion (arrowheads). There was no NOS-IR fibre regrowth from myenteric ganglia on the anal side of the operation site. (*b*) High power micrograph of the oral side of an operation site showing many NOS-IR nerve fibre outgrowths (arrowheads). (*c*, *d*) Pattern of SP

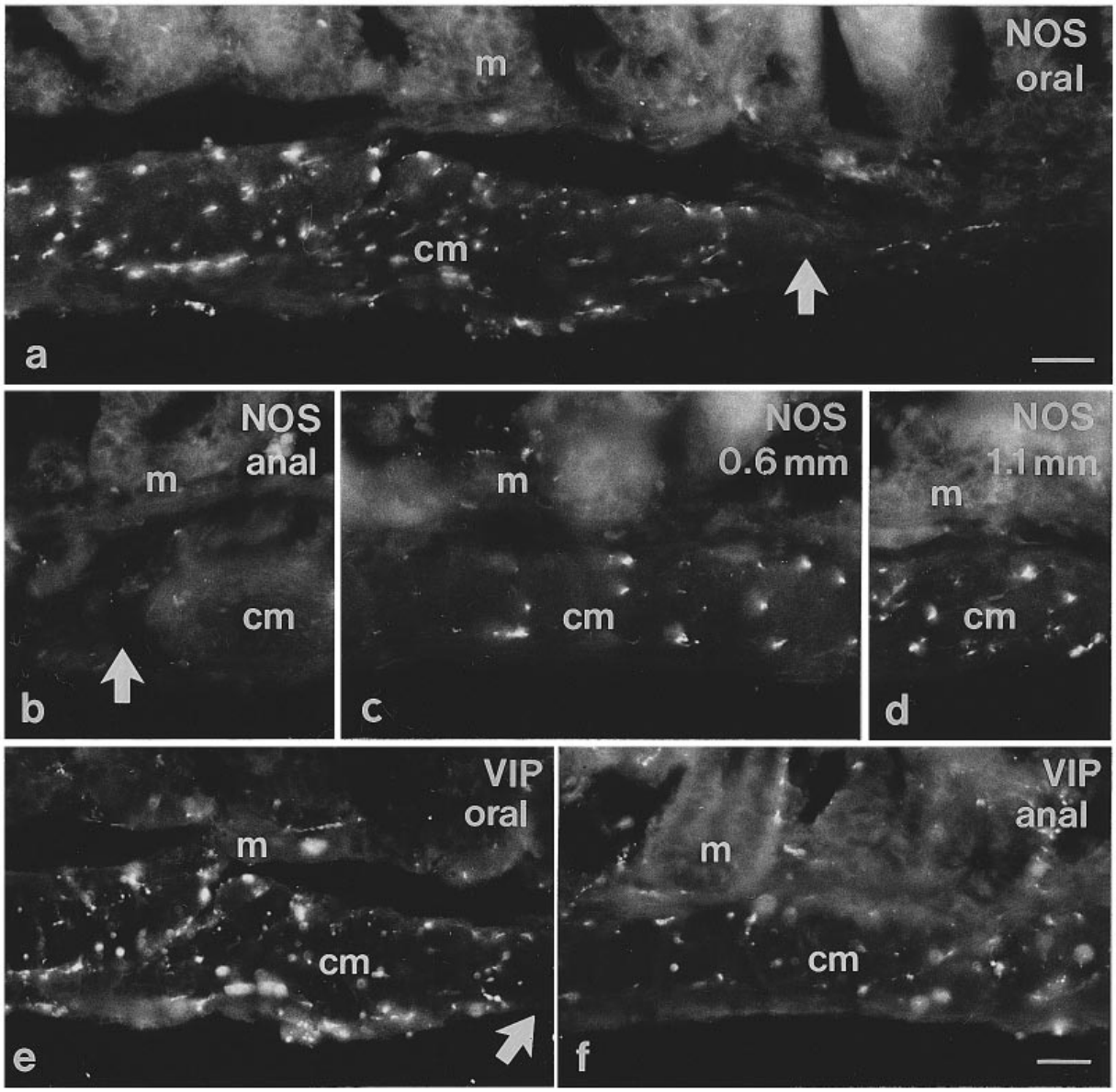

Fig. 2. Fluorescence micrographs of longitudinal frozen sections showing the consequences of myectomy on the distribution NOS-IR nerve fibres (*a–d*) and VIP-IR nerve fibres (*e*, *f*) in the small intestine. In all of the micrographs, the oral end of the section is towards the left. (*a*) On the oral side of the operation site, there is no decrease in the number of NOS-IR nerve fibres in the circular muscle (cm). The operation site is indicated by the large arrow. m, mucosa. (*b*) Immediately anal to the operation site (large arrow), there is a complete loss of NOS-IR nerve fibres from the circular muscle (cm). m, mucosa. (*c*) The middle of this micrograph is approximately 600 μm anal to the edge of the operation site. A small number of NOS-IR nerve fibres are present in the circular muscle (cm), particularly at the anal (right) side. (*d*) 1.1 mm anal to the operation site, the density of NOS-IR nerve fibres in the circular muscle (cm) has returned to control levels. m, mucosa. (*e*) On the oral side of the operation site (large arrow), there is no change in the number of VIP-IR nerve fibres in the circular muscle (cm). m, mucosa. (*f*) The middle of this micrograph is approximately 700 μm anal to the operation site. A small number of VIP-IR nerve fibres are present in the circular muscle (cm), particularly at the anal (right) side. m, mucosa. Bars: 25 μm in *a* (applies also to *b*, *c*, *d*); 25 μm in *f* (applies also to *e*).

immunoreactivity on the oral (*c*) and anal (*d*) side of a myotomy in the large intestine. (*c*) There are no SP-IR nerve fibres growing from myenteric ganglia on the oral side towards the operation site. (*d*) On the anal side of the operation site, there are many SP-IR nerve fibres (arrowhead) growing in an oral direction towards the lesion. (*e*) Long GABA-IR nerve fibres (arrowheads) growing from the oral side of a myotomy in the large intestine towards the operation site. The GABA-IR nerve fibre outgrowths were sparse compared with the NOS-IR fibres on the oral side and the SP-IR fibres on the anal side of lesion. Bars: 100 μm in *a*; 50 μm in *b*; 50 μm in *c* (applies also to *d*); 25 μm in *e*.

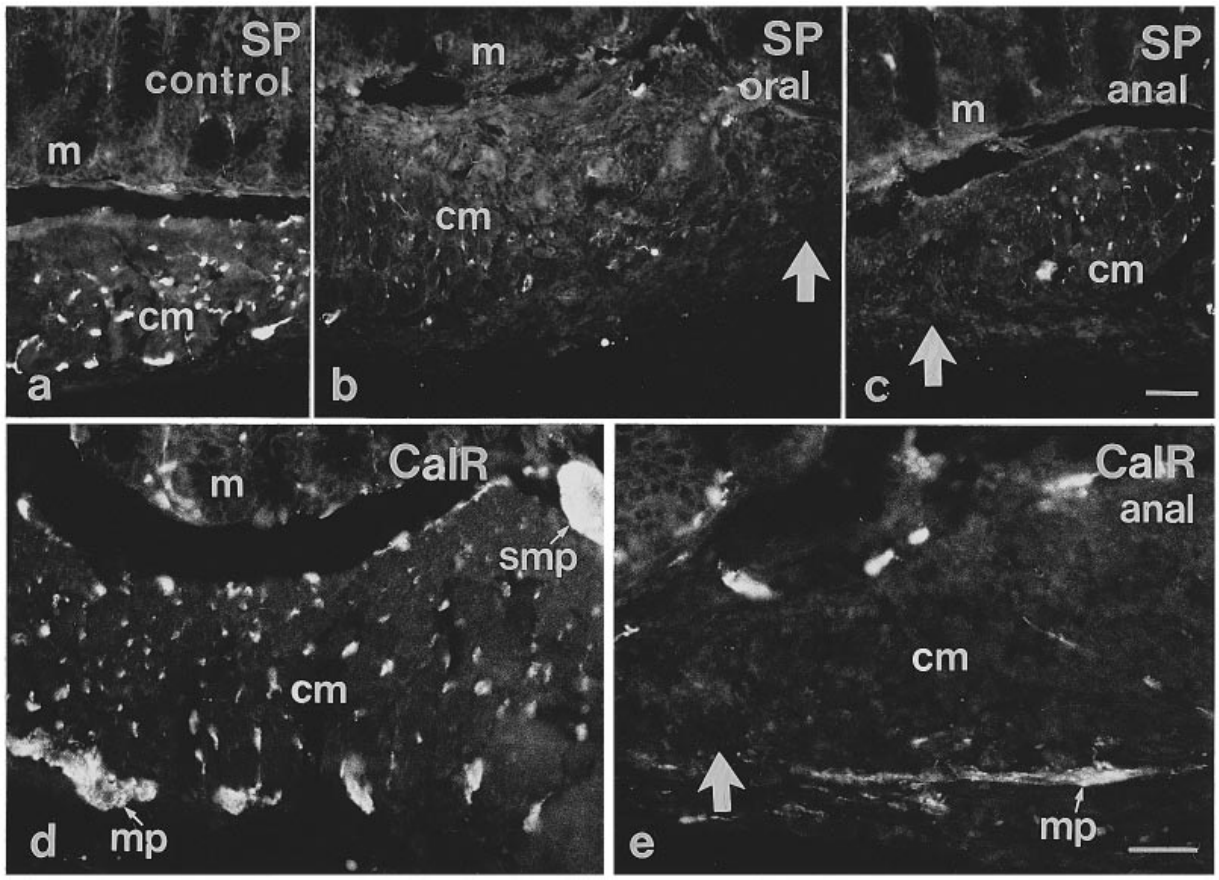

Fig. 3. Fluorescence micrographs of longitudinal frozen sections showing the consequences of myectomy on the distribution of substance P-IR nerve fibres (*a–c*) and calretinin-IR nerve fibres (*d*, *e*) in the large intestine. In all of the micrographs, the oral end of the section is towards the left. (*a*) There is a high density of substance P-IR nerve fibres in the circular muscle (cm) several mm oral to the operation site. m, mucosa. (*b*) Immediately oral to the operation site (large arrow), there is a large reduction in the number of substance P-IRnerve fibres present in the circular muscle (cm). m, mucosa. (*c*) There is also a reduction in the number of substance P-IR nerve fibres in the circular muscle (cm) immediately anal to the operation site (large arrow). m, mucosa. (*d*) Pattern of calretinin immunoreactivity oral to an operation site. The right-hand side of this micrograph is 150 μm oral to the operation site. There is a high density of calretinin-IR nerve fibres in the circular muscle (cm). m, mucosa; mp, myenteric plexus; smp, submucous plexus. (*e*) Immediately anal to the operation site (large arrow), there is a large reduction in the number of calretinin-IR nerve fibres in the circular muscle (cm), although there is some immunoreactivity in the myenteric plexus (mp). Bars: 50 μm in *c* (applies also to *a*, *b*); 50 μm in *e* (applies also to *d*).

the operation site. However, in 1 animal there was also a deficit of SP-IR fibres in the circular muscle on the anal side of the lesion for 0.2 mm. In the large intestine, there was a loss of SP-IR nerve terminals from the circular muscle in both the oral and anal sides of the operation site in 2 animals (Fig. 3*a–c*). However, in 1 animal there was no obvious change in the number of SP-IR fibres in the circular muscle layer at either edge of the lesion. The density of SP-IR fibres in both the submucous plexus and mucosa appeared unchanged at the operation site.

Calretinin

Myenteric plexus. Following myotomy and myectomy, outgrowths of calretinin-IR nerve fibres were detected on both sides of the operation site in the small intestine, but there was no obvious change in the number of calretinin-IR fibres present in myenteric ganglia on either the oral or anal sides. In the large intestine, outgrowths of calretinin-IR fibres were also observed at both the oral and anal sides of the operation site. However, there appeared to be more fibres on the oral side than the anal side, and there was also some loss of calretinin terminals in the first row of myenteric ganglia on the anal side.

Circular muscle. Myectomy resulted in losses of calretinin-IR fibres in the circular muscle on both sides of the lesion in the small intestine, but for a greater distance on the anal side (0.6 mm) than on the oral side (0.15 mm). In the large intestine, a loss of calretinin fibres from the circular muscle was observed on the anal edge of the lesion for about 0.7 mm (Fig. 3*d*, *e*). No deficit of calretinin-IR fibres was observed in the submucous plexus and mucosa at the operation sites.

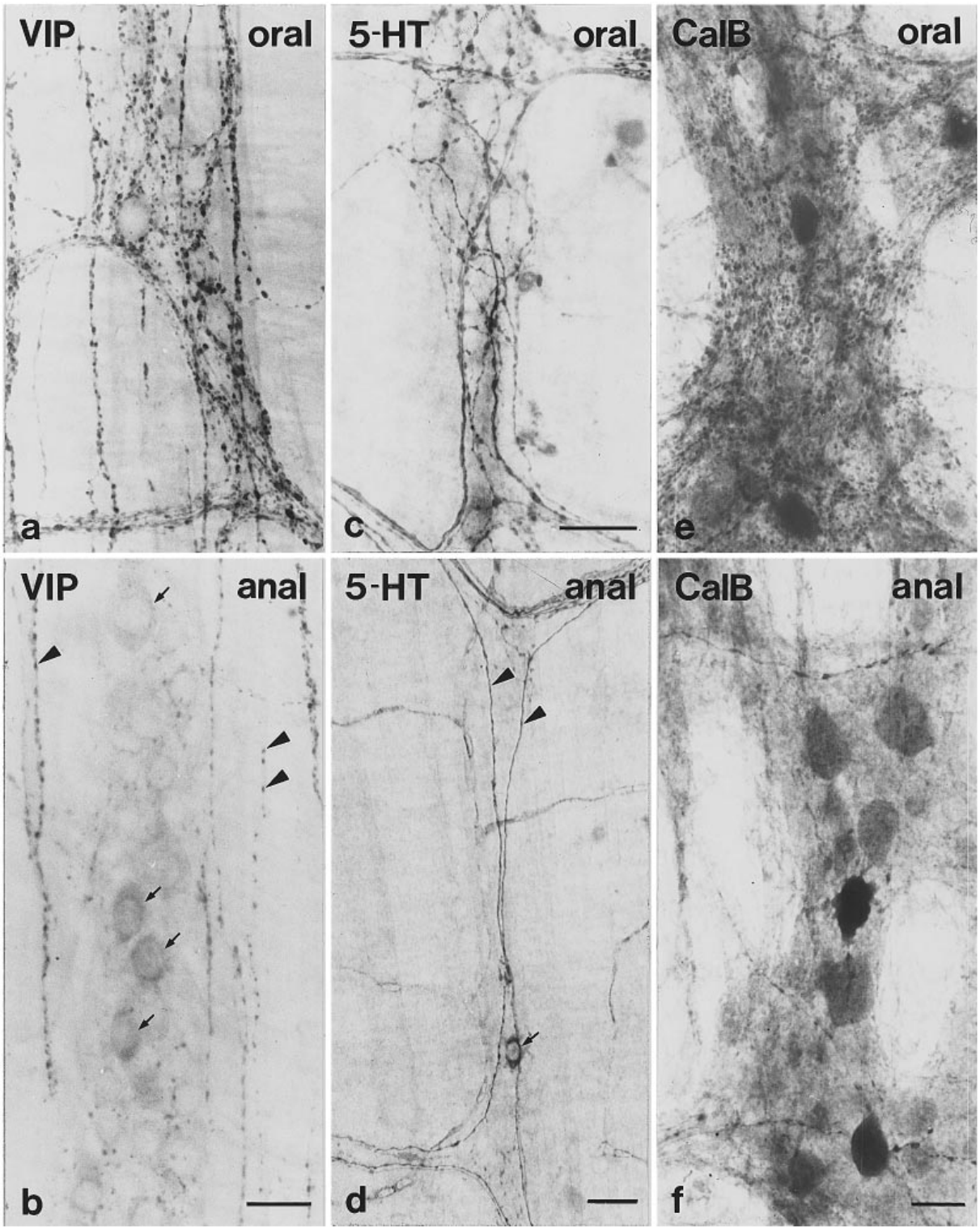

Fig. 4. Effects of nerve lesions on the density of VIP-IR (*a*, *b*), 5-HT-IR (*c*, *d*) and calbindin-IR (*e*, *f*) nerve terminals in myenteric ganglia in wholemounts 10 d after myotomy. In ganglia from control animals and in the ganglia oral to an operation site, the density of VIP-IR nerve terminals was high. (*a*) A myenteric ganglion 2 rows of ganglia oral to a myotomy in the small intestine. There are many VIP-IR nerve terminals forming pericellular baskets around a large proportion of the myenteric ganglion cells. (*b*) A myenteric ganglion 2 rows of ganglia anal to an operation site. There are very few VIP-IR nerve terminals in the ganglion; however VIP-IR nerve terminals (arrowheads) are present in the overlying circular muscle. Faint VIP-IR cell bodies are present within the ganglion (small arrows). (*c*) A myenteric ganglion 3 rows of ganglia oral to a myotomy in the small intestine. 5-HT-IR nerve fibres surround many of the cell bodies in the ganglion. (*d*) A myenteric ganglion 4 rows of ganglia anal to a myotomy. 5-HT-IR nerve terminals are extremely sparse in the ganglion, although 2 nonterminal axons (arrowheads) are present coursing through the ganglion. A strongly stained, 5-HT-IR cell body (small arrow) is also present in the ganglion. (*e*) Pattern of calbindin immunoeactivity in a myenteric ganglion immediately oral to a myotomy in the large intestine. A very dense plexus of calbindin-IR nerve terminals and some calbindin-IR cell bodies are present in the ganglion. (*f*) Pattern of calbindin

Calbindin

Myenteric plexus. Following myotomy, outgrowths of calbindin-IR nerve fibres occurred mainly on the oral side of the operation site in the small intestine, although there were also small outgrowths of calbindin-IR nerve fibres on the anal side. A loss of calbindin-IR terminals was observed on the anal side in the first 1–2 rows of the myenteric ganglia. In the large intestine, outgrowths of calbindin-IR nerve fibres were only detected on the oral side of the operation site. There was a deficit of terminals in the first 3–6 rows of myenteric ganglia on the anal side (Fig. 4*e*, *f*).

Circular muscle. Since no calbindin-IR nerve fibres are present in the circular muscle (Sang & Young, 1996), no myectomy preparations were examined for calbindin immunohistochemistry.

NPY

Because NPY-IR neurons are sparse in the large intestine (Sang & Young, 1996), projections of NPY-IR neurons were only examined in the small intestine.

Myenteric plexus. After myotomy, outgrowths of NPY-IR nerve fibres were observed on both the oral and anal sides of the operation site (Fig. 5); however, there were more outgrowths on the anal than on the oral side. The density of terminals in the myenteric ganglia appeared unchanged on both sides.

Circular muscle. Myectomy resulted in a deficit of NPY-IR nerve fibres in the circular muscle on the anal side for about 0.8 mm. The number of NPY-IR nerve terminals on the oral side appeared unchanged.

GABA

Because GABA-IR neurons are sparse in the small intestine (Sang & Young, 1996) projections of GABA-IR neurons were only examined in the large intestine.

Myenteric plexus. GABA-IR was present in nerve fibre regrowth from the myenteric plexus on both sides of the operation site. Nerve fibre regrowth often occurred as single fibres that extended into the operated area (Fig. 1*e*). Regrowth of fibres on the oral edge of the cut was more prolific, and often extended longer distances, than on the anal side. A noticeable loss of nerve terminals occurred in the first 2–3 rows of myenteric ganglia on the anal side and in the first row of ganglia on the oral side of the lesion in some animals.

Circular muscle. Following myectomy, there was a deficit of GABA-IR fibres in the circular muscle which extended for approximately 1–2 mm on both sides of the operation site. In wholemounts, GABA-IR nerve fibres projected both orally and anally towards the lesion and some could be followed directly to the circular muscle.

DISCUSSION

This is the first study to examine anatomically the projections of myenteric neurons in the mouse small and large intestine and has demonstrated that myotomy and myectomy that have been used in larger mammals can also be performed in mice. By combining the results of this study with the results of a previous study, which examined the colocalisation of substances in myenteric neurons of mice (Sang & Young, 1996), and with observations of the projections of different classes of myenteric neurons in other species, certain deductions that relate chemistry, projection and function can be made. Thus myenteric neurons in the mouse intestine could be divided into inhibitory motor neurons, excitatory motor neurons, motor neurons of unknown physiological role, and interneurons.

Putative inhibitory circular muscle motor neurons

Pharmacological analysis of transmission from inhibitory motor neurons indicates that they utilise several transmitters, often in combination, and that the combinations of transmitters vary between different regions of the gastrointestinal tract, and between different species (see Furness et al. 1995 for review). The transmitters that pharmacological studies suggest participate in transmission include NO, VIP, PACAP and ATP (Hoyle et al. 1990; Sanders & Ward, 1992; Stark & Szurszewski, 1992; Jin et al. 1994). In most species, NOS and VIP are found colocalised in inhibitory motor neurons (Costa et al. 1992*b*; Ekblad et al. 1994; Ward et al. 1994*b*; Barbiers et al. 1995). We have previously shown that NOS- and VIP-IR are also colocalised in fibres innervating the circular muscle of the mouse small and large intestine (Sang & Young, 1996), and the present work shows that these fibres arise from cells in the myenteric plexus and run anally to supply the muscle. Physiological studies in other species indicate that the inhibitory motor neurons project anally to the circular muscle. It is thus reasonable to conclude that neurons

immunoreactivity in a myenteric ganglion 2 rows anal to a myotomy. Although many calbindin-IR cell bodies are present, calbindin-IR nerve terminals are sparse. Bars: 25 μm in *b* (applies also to *a*); 25 μm in *c*, *d*; 25 μm in *f* (applies also to *e*).

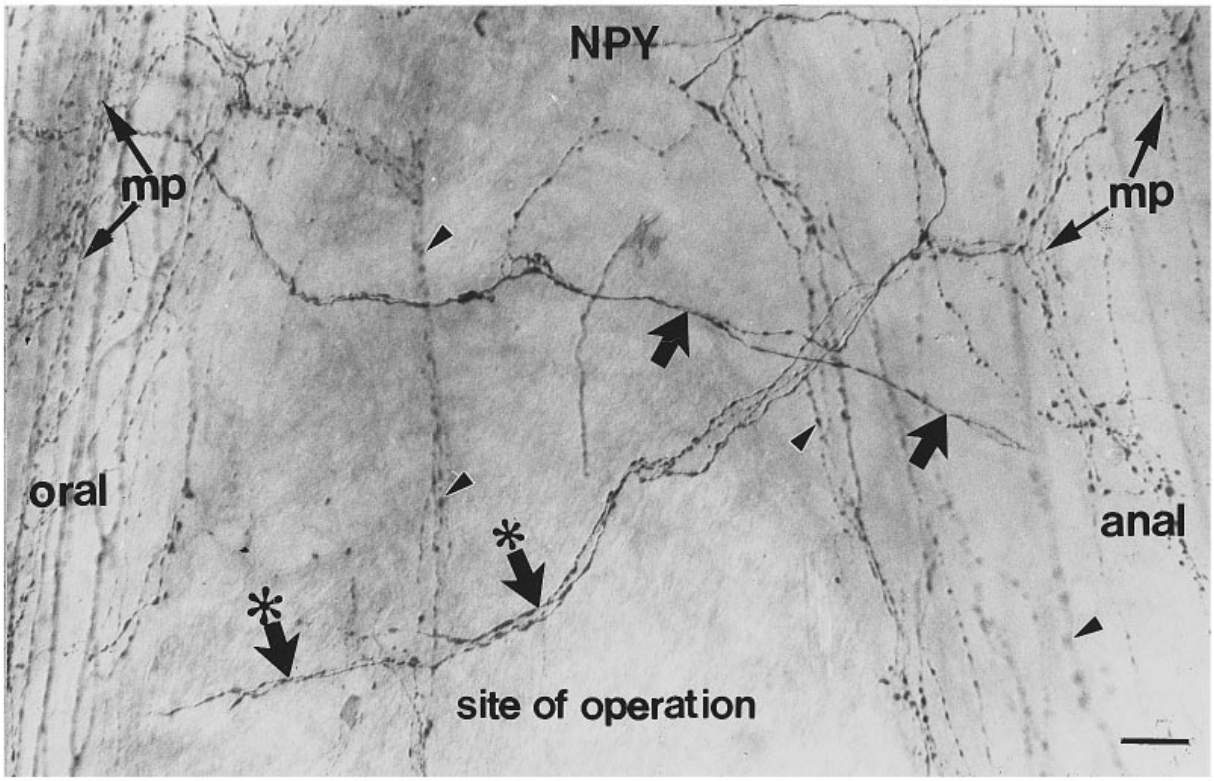

Fig. 5. Wholemount preparation of small intestine showing the pattern of NPY immunoreactive 10 d following a myotomy operation. Immunoreactive outgrowths of fibres, that arise from the myenteric plexus, (mp) are present growing from the oral side towards the anal side (arrows) and from the anal side growing towards the oral side (arrows with asterisks). Immunoreactive fibres (arrowheads), which are slightly out of focus, are present in the muscle overlying the preparation. Bar, 25 μm.

with cell bodies in the myenteric plexus that are NOS/VIP-IR, and project anally to the muscle, are inhibitory motor neurons in the mouse small and large intestine. In some intestinal regions of some species, NPY is found colocalised with NOS and/or VIP in a subpopulation of anally projecting inhibitory motor neurons (Ekblad et al. 1984, 1988; Sundler et al. 1989; Timmermans et al. 1994; Uemura et al. 1995), but there does not appear to be any evidence for a role of NPY as a primary transmitter. NPY was also found in a subpopulation of anally-projecting, putative inhibitory motor neurons in the mouse small intestine.

Putative excitatory circular muscle motor neurons

In the mouse intestine, as in other species, acetylcholine is the primary transmitter of excitatory motor neurons (Fontaine et al. 1984; Okasora et al. 1986; Unekwe & Savage, 1991). Pharmacological studies in other species have shown that tachykinins, including SP, are released along with acetylcholine from excitatory motor neurons (see Bartho & Holzer, 1985), and in all species that have been examined, SP-IR is present in nerve fibres supplying the muscle layers (Costa et al. 1981; Daniel et al. 1987; Ekblad et al. 1988; Sang & Young, 1996). The colocalisation of SP with choline acetyltransferase (ChAT, a marker of cholinergic neurons) has yet to be examined in the mouse. As in the guinea pig small and large intestine (Messenger & Furness, 1990; Brookes et al. 1991*b*), canine small intestine (Furness et al. 1990*a*) and rat colon (Ekblad et al. 1988), the SP-IR circular muscle motor neurons in the mouse small and large intestine projected mainly orally, and are therefore likely to be excitatory motor neurons. The SP-IR excitatory motor neurons in the large intestine projected up to 0.8–0.9 mm orally whereas those in the small intestine projected only 0.4–0.5 mm orally. A previous electrophysiological study by Okasora et al. (1986) examined the projections of motor neurons in the mouse large intestine by recording, in vitro, the membrane potential in the circular muscle at varying distances from a stimulation site. Their results showed that cholinergic excitatory junction potentials could be recorded up to 2 mm from the stimulation site. The results of the current study, combined with the results of the electrophysiological study of Okasora et al.

(1986), suggests that there may be some orally projecting cholinergic excitatory motor neurons in the mouse large intestine that do not contain SP-IR. SP-IR circular muscle motor neurons do not project orally in all regions of all species. For example, in the rat small intestine the SP-IR motor neurons project mainly anally (Ekblad et al. 1987). The present study showed that in the mouse intestine, some of the SP-IR neurons may also project locally and/or a short distance anally. In the guinea pig small intestine it has also been deduced that GABA is in both inhibitory and excitatory motor neurons (Williamson et al. 1996).

GABA-IR and calretinin-IR circular muscle motor neurons

GABA. In the mouse large intestine, some of the GABA-IR motor neurons were found to project anally. Of the GABA-IR nerve terminals in the circular muscle of the large intestine of the mouse, only a small proportion also show NOS-IR, and none show VIP-IR (Sang & Young, 1996). This study has shown that the GABA-IR (presumably inhibitory) circular muscle motor neurons project greater distances anally than either the VIP or NOS circular muscle motor neurons. GABA is also present in orally projecting motor neurons to the circular muscle in the mouse large intestine, suggesting that GABA may be present in a subset of excitatory motor neurons.

Calretinin. Calretinin-IR motor neurons innervate different muscle layers in different species and in different regions of the same species. In the guinea pig small intestine, calretinin-IR motor neurons only innervate the longitudinal muscle (Brookes et al. 1991*a*), whereas in the guinea pig colon (McConalogue et al. 1994) and in mouse intestine (Sang & Young, 1996), calretinin-IR motor neurons innervate both the circular and longitudinal muscle. The projection patterns of calretinin-IR motor neurons appear to differ between species and between different regions of the same species.

The present study showed that in the mouse small and large intestine, the calretinin-IR motor neurons projected predominantly anally. This was surprising because our previous colocalisation study had shown almost no colocalisation between calretinin and NOS or VIP. It is unknown whether the anally projecting calretinin-IR motor neurons are a subpopulation of inhibitory motor neurons that do not contain NOS or VIP, or whether they form part of a descending excitatory reflex pathway. Excitatory circular muscle motor neurons with short descending projections have been described in the guinea pig small intestine (Brookes et al. 1991*b*; Williamson et al. 1996), although it is unknown if a similar pathway is present in the mouse small intestine. This study also showed that some calretinin-IR circular muscle motor neurons in the small intestine projected locally and/or a short distance orally.

Interneurons

This study investigated the projections of the interneurons in the mouse intestine by performing myotomy operations and then determining whether there were fibre outgrowths from myenteric ganglia on the oral or anal side of the operation site, and whether there was a deficit of nerve fibres in myenteric ganglia on either side of the lesion. Both in the small and large intestine, the NOS-, VIP-, calbindin- and 5-HT-IR neurons projected predominantly or exclusively anally. In the large intestine, most of the GABA-IR neurons also projected anally. Thus the majority of identified interneurons projected anally. In both regions, the SP-IR neurons projected orally and locally. In addition, the calretinin-IR neurons in both regions projected locally and the NPY-IR neurons in the small intestine also projected locally.

As in the mouse intestine, in the guinea pig small intestine, there are more subpopulations of descending interneurons than there are of ascending interneurons (Costa et al. 1992*a*). This probably reflects the necessity of transmitting a greater diversity of information anally than orally during peristalsis or other migratory complexes.

5-HT. As in the guinea pig small intestine and colon (Furness & Costa, 1982; Wardell et al. 1994) and the porcine colon (Barbiers et al. 1995), in the mouse small and large intestine, 5-HT-IR interneurons project anally for a long distance. Thus, although the role of the 5-HT descending interneurons is not clear (Young & Furness, 1995), they appear to be well conserved across different mammalian species.

NOS/VIP. This study revealed that interneurons containing VIP and/or NOS in the mouse small and large intestine project anally for a short distance. Hence the polarity of NOS/VIP-IR interneurons in the mouse intestine is similar to that in the guinea pig (Costa & Furness, 1983; Messenger & Furness, 1990; Costa et al. 1992*b*; McConalogue & Furness, 1993; Messenger, 1993), rat (Ekblad et al. 1987, 1988, 1994; Sundler et al. 1989), dog (Daniel et al. 1987), pig (Barbiers et al. 1995) and human (Domoto et al. 1990). The absence of NOS-IR from the submucous plexus above myectomy sites in the mouse small

intestine indicates that some of the myenteric NOS interneurons project to submucous ganglia.

Calretinin and calbindin. Neurons containing calbindin-IR projected anally both in the small and large intestine, whereas the calretinin-IR neurons projected locally. From a limited number of studies of the projection patterns of calbindin- and calretinin-IR neurons in the guinea pig (Furness et al. 1990*b*; Messenger & Furness, 1990; Brookes et al. 1991*a*, 1995; McConalogue et al. 1994) and mouse intestine, it seems that the projection patterns of calretinin- and calbindin-IR interneurons are variable.

GABA. The small number of GABA-IR nerve fibre outgrowths on the oral side compared with the anal side following myotomy suggests that most of the GABA-IR interneurons in the large intestine of the mouse project anally, although a small subpopulation also appears to project orally.

SP and NPY. As in the guinea pig intestine (Costa et al. 1981; Messenger & Furness, 1990) and rat colon (Ekblad et al. 1988), SP-IR neurons that give rise to terminals in the myenteric plexus of the mouse projected both orally and locally. The projections of NPY-IR neurons show variation between species and also between different regions of the same species. In the guinea pig small intestine (Uemura et al. 1995) and rat intestine (Ekblad et al. 1987, 1988), NPY-IR interneurons project anally. In contrast, in the canine ileum (Daniel et al. 1987), they project orally. In the mouse small intestine, the NPY-IR interneurons or sensory neurons distribute locally with an oral bias.

Conclusions

The present study reinforces the concept that certain neurotransmitters preserve their characteristics despite being present in different species and different regions of the gastrointestinal tract. For example, NOS/VIP-IR circular muscle motor neurons and interneurons and 5-HT interneurons project anally in all species examined, while SP-IR excitatory muscle motor neurons project mainly orally. Nevertheless, there are also some chemically coded neurons, such as calretinin-IR, calbindin-IR, NPY-IR and GABA-IR interneurons or motor neurons that issue different projection patterns in different species and in different regions of the same species.

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