

INTESTINAL MOTILITY

Cholinergic and nitroergic interneurons in the myenteric plexus of the human colon

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Background: Myenteric interneurons are involved in the reflexes that control the motility of the human colon.

Aims: The distribution of choline acetyltransferase (ChAT) and nitric oxide synthase (NOS) immunoreactivity in myenteric interneurons was investigated in this study.

Methods: Dil (1,1'-didodecyl 3,3',3'-indocarbocyanine perchlorate) was applied to the myenteric plexus of the human colon followed by organotypic culture. Retrogradely labelled neurones, with projections longer than motor neurones (>10 mm), were studied to exclude motor neurone populations. ChAT and NOS immunoreactivity was then determined in the interneurons.

Results: We found that 90% of interneurons projecting orally contained ChAT and none contained NOS. Ninety five per cent of descending interneurons were labelled with ChAT and/or NOS antisera; 46% contained NOS immunoreactivity alone, 20% contained ChAT immunoreactivity alone, and 29% contained both ChAT and NOS. Anally directed interneurons had significantly longer projections than orally projecting interneurons.

Conclusions: Nearly all interneurons contain either NOS or ChAT immunoreactivity. Orally projecting interneurons are of two types: 90% contain ChAT alone and the remainder contain immunoreactivity for neither ChAT nor NOS. There are three main types of anally projecting interneurons: the largest, which contains NOS but not ChAT, and the two smaller classes which contain ChAT and NOS, and ChAT alone.

The enteric nervous system controls the function of the gastrointestinal tract.¹ Functional classes of neurones in the enteric plexuses include ascending and descending interneurons, motor neurones to the circular and longitudinal muscle, sensory neurones, and secretomotor neurones. It has recently become possible to identify these classes of neurones by retrograde labelling from the targets of their projections.²

In a previous study using retrograde tracing in the human colon, myenteric interneurons were shown to have projections up to 68 mm long while myenteric neurones innervating the circular and longitudinal muscle layers and the submucosa had shorter projections (almost all less than 10 mm).³ In a later study, we showed that there are myenteric interneurons that project anally which contain vasoactive intestinal peptide (VIP) or calcitonin and others that project orally which contain tachykinins (TK).⁴ However, only a small proportion of myenteric interneurons were immunoreactive for any of these neurochemicals. Thus the neurochemistry of the majority of anally and orally projecting interneurons has not been identified in the human colon.

Neurones and nerve fibres containing choline acetyltransferase (ChAT), the enzyme which synthesises acetylcholine, are abundant in the myenteric ganglia in the human colon.⁵ There are also many myenteric neurones and nerve fibres containing nitric oxide synthase (NOS), the enzyme which synthesises nitric oxide, in the human intestine.^{6,7} Nerve cell bodies which contain ChAT and NOS have been described in the myenteric plexus of the human colon and these neurones did not project to the circular muscle.⁸ The abundance of nerve cell bodies and nerve fibres in the myenteric ganglia which contain ChAT and/or NOS suggests that there are significant populations of interneurons containing these enzymes.

Cholinergic transmission has been demonstrated in human colonic tissue⁹ and is involved in both ascending and descending pathways.¹⁰ While the role of nitric oxide in inhibitory transmission to the smooth muscle of the human intestine is

well established,¹¹ its role in synaptic transmission is unclear although it may act as a neuromodulator at synapses in descending interneurons in the guinea pig small intestine.¹² The purpose of this study is to classify myenteric interneurons in the human colon using retrograde tracing combined with immunohistochemistry for ChAT and NOS to form the basis for the neurochemical classification of human myenteric interneurons.

METHODS

Tissue collection

Eight specimens of ascending colon were obtained with prior informed consent from patients (three men, five women) undergoing surgery for cancer of the colon (age range 46–78 years; median 72). The segment of intestine was taken from bowel not involving the tumour and had not been subject to obstruction. Patients with functional disorders such as slow transit constipation and irritable bowel syndrome were excluded from the study. Immediately after removal from the patient, a segment of colon, measuring 80×30 mm, was excised from the intertaenial region of the margin of the resected specimen, placed in oxygenated Krebs' solution, and transported to the laboratory. Frozen section histology was performed to confirm that the tumour did not involve the specimens. The use of human intestine for these experiments was approved by the Flinders Clinical Research Ethics Committee.

Retrograde labelling

The full thickness piece of colon was pinned out on a Sylgard lined petri dish (Dow Corning, Michigan, USA) and the

Abbreviations: ChAT, choline acetyltransferase; Dil, 1,1'-didodecyl 3,3',3'-indocarbocyanine perchlorate; NOS, nitric oxide synthase; VIP, vasoactive intestinal peptide; TK, tachykinins.

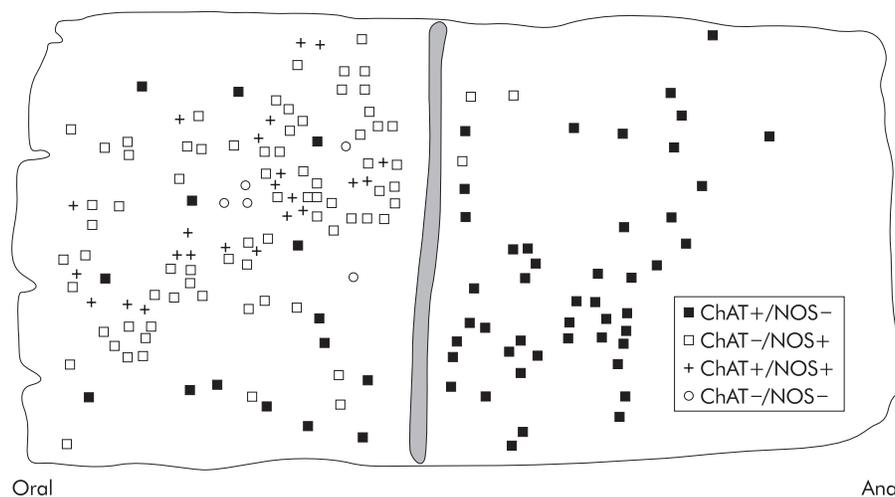


Figure 1 Scatterplot showing the location of 1,1'-didodecyl 3,3,3',3'-indocarbocyanine perchlorate (DiI) containing neurones with choline acetyltransferase (ChAT) or nitric oxide synthase (NOS) immunoreactivity in a single preparation. The shaded strip indicates the DiI application site. Almost all neurones with ascending projections contain ChAT whereas there are several subgroups with descending projections. Calibration bar, 10 mm.

mucosa and submucosa were removed by microdissection. A 5 mm wide strip of circular muscle was removed across the centre of the preparation and a transverse incision was made through the myenteric plexus. A line of glass beads (Sigma Chemicals, St Louis, Missouri, USA), 200 μ m in diameter, coated with the lipid soluble dye 1,1'-didodecyl 3,3,3',3'-indocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, Oregon, USA) were then placed along the incision, thus contacting nerve fibres running in the internodal strands (fig 1).^{3,4} Using this methodology, longitudinal but not circumferential projections could be determined.

After 10 minutes, DiI coated beads had adhered to the underlying tissue and remained in place for the duration of the organ culture. The preparations were covered with culture medium (DME/F12; Sigma Chemicals) supplemented with 10% heat inactivated fetal bovine serum, penicillin 100 IU/ml, streptomycin 100 μ g/ml, amphotericin B 2.5 μ g/ml, and gentamicin 20 μ g/ml (Cytosystems, NSW, Australia) and adjusted to pH 7.4. They were placed on a rocking tray in a humidified incubator containing 5% CO₂ in air at 37°C and the medium was changed daily during the culture period. After five days in organotypic culture, the preparations were fixed for 16–24 hours in modified Zamboni's fixative (0.2% saturated picric acid and 2% paraformaldehyde in phosphate buffer 0.1 M; pH 7.2) at 4°C. They were then washed repeatedly in phosphate buffered saline (0.15 M NaCl in 0.01 M sodium phosphate, pH 7.2) and the remaining circular muscle was dissected away to make a whole mount of the myenteric plexus attached to the thin longitudinal muscle layer of the intertaenial intestine. Preparations were then cleared in 100% bicarbonate buffered glycerol (pH 8.6) for three days.

Immunohistochemistry

DiI labelled preparations were incubated with a primary antiserum to ChAT and/or NOS. Two preparations were single labelled with antisera to ChAT (code PO3; rabbit polyclonal 1:1000; Yeboah, Germany¹³) and three were single labelled with antisera to NOS (code K205; sheep polyclonal 1:1000; donated by PC Emson¹⁴). Three other preparations were double labelled with both antisera for four days at room temperature. All primary antisera were diluted in 10% normal donkey serum. After 3×15 minute washes in phosphate buffered saline, secondary antisera were then added to the specimen for two days. For rabbit antisera, fluorescein conjugated donkey antirabbit IgG (Amersham, Little Chalfont, UK; code N 1034) was used at 1:50; for sheep antisera, Cy5 conjugated donkey antisheep IgG (Jackson Immunoresearch, West Grove, Pennsylvania, USA; code 25324) was used at 1:20.

The preparation was mounted in buffered glycerol (pH 8.6) and viewed under an AX70 epifluorescence microscope (Olympus Optical, Tokyo, Japan) fitted with appropriate filter blocks to discriminate DiI, fluorescein, and Cy5. The positions of labelled cell bodies relative to the centre of the DiI application site were recorded using a computerised stage mapping system² and reconstructed using graph plotting software (Sigmaplot; Jandel, Corte Madera, California, USA). Images were recorded using an image capturing program (NIH Image 1.59; NIH, Maryland, USA).

Control experiments were performed by omitting primary and secondary antisera to ensure that there was no non-specific labelling. Control experiments showed that there was no cross reactivity between the antisera. Neurone counts are expressed as means and comparisons of projection length

Table 1 Number of DiI containing neurones immunoreactive for each antiserum (number of neurones with projections longer than 10 mm in parentheses)

No of patients	ChAT+	NOS+	ChAT+/NOS+	Non-immunoreactive	Total No of DiI filled neurones
2	143 (42)	n/a	n/a	140 (48)	283 (90)
3	371 (147)	191 (97)	114 (66)	77 (37)	753 (347)
3	n/a	268 (133)	n/a	398 (171)	666 (304)

ChAT, choline acetyltransferase; DiI, 1,1'-didodecyl 3,3,3',3'-indocarbocyanine perchlorate; NOS, nitric oxide synthase; n/a, not applied.

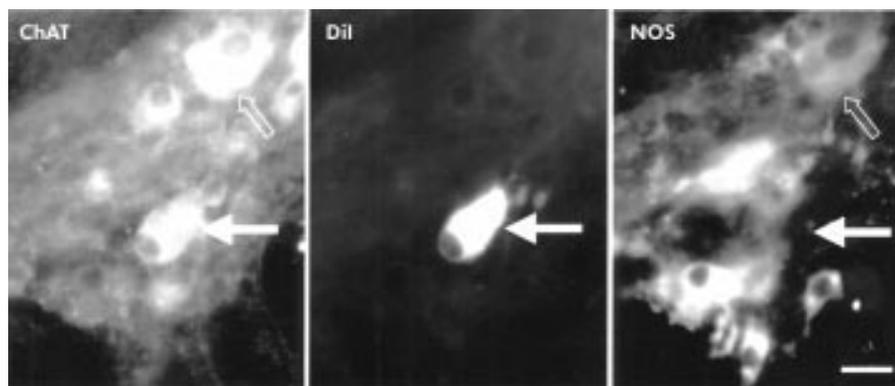


Figure 2 Myenteric neurone labelled 16 mm anal to the 1,1'-didodecyl 3,3',3'-indocarbocyanine perchlorate (DiI) application site on the myenteric plexus. The nerve cell body was immunoreactive for choline acetyltransferase (ChAT) but not nitric oxide synthase (NOS) (filled arrows). A neurone not labelled with DiI (open arrow) was immunoreactive for both ChAT and NOS. Calibration bar, 20 μ m.

were made using factorial ANOVA and expressed as a Scheffe F test with a significance level of 5%.

RESULTS

General

Myenteric neurones which had been retrogradely labelled from internodal strands were located up to 33 mm orally from the application site and up to 29 mm anally—that is, to the end of the preparation. In the eight preparations, a mean of 213 neurones were labelled with DiI (range 75–471; SEM 55). Significantly more neurones had descending projections (mean (SEM) 69 (3)%; range 59–84%) than ascending projections (31 (3)%; range 16–41%), as previously described.⁴

Immunohistochemistry

Eight preparations of myenteric plexus, which had been retrogradely labelled with DiI applied to the myenteric plexus, were successfully labelled with ChAT and/or NOS antisera (table 1, fig 1).

To be included in this study, immunohistochemical labelling had to be of a consistently high standard throughout the whole preparation to avoid false negative results. From a total of 16 preparations, eight were judged to reach this standard and the rest were discarded.

Orally projecting neurones

In preparations labelled with ChAT antiserum, 90% (SEM 4.9%; n=5) of neurones projecting orally for more than 10 mm contained ChAT immunoreactivity (fig 2).

In contrast, only 0.3% (SEM 0.3%; n=6) of neurones projecting more than 10 mm orally contained NOS immunoreactivity (table 2). These NOS immunoreactive orally projecting neurones were all located within 5 mm of the DiI application site, suggesting that they had short projections. In the three preparations labelled with both ChAT and NOS, 11 (7)% of orally projecting neurones with projections longer than 10 mm remained unlabelled by either antiserum and 0.7% contained both ChAT and NOS (fig 3).

Anally projecting neurones

There were more neurochemical types of myenteric neurones with descending projections than ascending projections (fig 3). The most abundant type contained NOS but not ChAT (table 3, fig 4).

In the preparations labelled with ChAT antiserum, 49% (SEM 8%; n=5) of neurones projecting more than 10 mm anally contained ChAT. In preparations labelled with NOS antiserum, 67% (SEM 6%; n=6) of neurones projecting more than 10 mm anally contained NOS. In the three preparations labelled with both ChAT and NOS, 29 (6)% of neurones with long aboral projections contained both ChAT and NOS (fig 5) and 4% were not labelled with either antiserum.

Length of projection

Neurones with anally directed axons had mean projections of 11.5 mm (SEM 0.4) whereas projections of those with orally directed axons were significantly shorter (mean 9.6 mm, SEM 0.4; $p=0.003$). There were no significant differences detected between the mean lengths of the projections of the different neurochemical types of neurones with descending or ascending projections. As the preparations had retrogradely labelled neurones extending to the limits of the tissue, not all myenteric interneurones were demonstrated in this study so these results do not give a definitive description of the lengths of all myenteric interneurones.

Morphology

Most of the neurones (99%) labelled from the myenteric plexus, which could be classified, had lamellar dendrites and a single process (that is, Dogiel type I morphology). Only 1% (14 of 1702 neurones) had a smooth cell body and several tapering processes (that is, Dogiel type II morphology). Of these Dogiel type II cells, none was NOS immunoreactive and 50% (7/14) were ChAT immunoreactive. The distributions of Dogiel type I and II neurones were not compared because of the small number of Dogiel type II neurones.

Table 2 Proportions of neurones with short and long oral projections labelled with ChAT and/or NOS antisera

Antibody applied	Short (<10 mm)			Long (>10 mm)		
	ChAT+	NOS+	ChAT+/ NOS+	ChAT+	NOS+	ChAT+/ NOS+
ChAT	73%	n/a	n/a	93%	n/a	n/a
NOS	n/a	11%	n/a	n/a	0%	n/a
ChAT and NOS	82%	4%	0%	89%	0%	1%

ChAT, choline acetyltransferase; NOS, nitric oxide synthase; n/a, not applied.

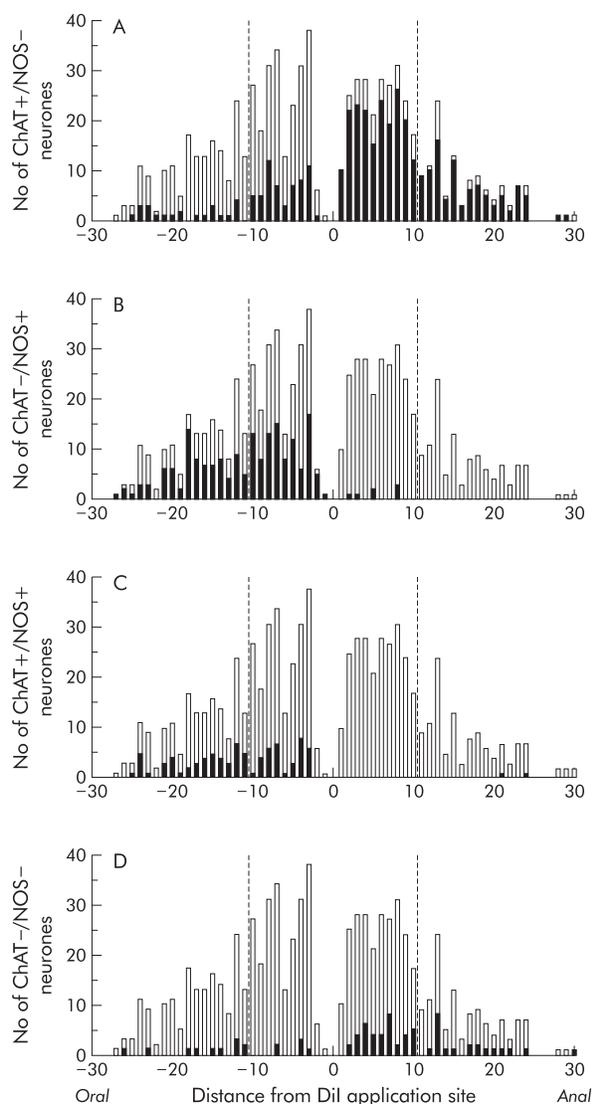


Figure 3 Histograms showing the distribution of (A) choline acetyltransferase (ChAT) +/nitric oxide synthase (NOS)⁻, (B) ChAT⁻/NOS⁺, (C) ChAT⁺/NOS⁺, and (D) ChAT⁻/NOS⁻ neurones as a proportion of the total number of 1,1'-didodecyl 3,3,3',3'-indocarbocyanine perchlorate (DiI) labelled neurone. Data were pooled from the three preparations labelled with both ChAT and NOS antisera. Dotted lines at -10/+10 indicate the projection limit of non-interneurons; hence neurones outside these limits are interneurons.

DISCUSSION

In this study, myenteric neurones projecting within the myenteric plexus were shown to fall into distinct neurochemical types. All functional classes of myenteric neurones are likely to project for some distance within the myenteric plexus and thus be labelled by DiI applied directly to the plexus, as

was carried out in the present study. However, it is possible to identify groups of labelled cells which are likely to be interneurons, based on data from previous studies. The main functional classes of myenteric neurones are ascending and descending interneurons, circular and longitudinal muscle motor neurones, and sensory neurones.¹⁵ Secretomotor, vasomotor, and intestinofugal neurones comprise fewer than 3% of myenteric neurones in the guinea pig ileum.¹⁵ In the human intestine, very few myenteric neurones project to the mucosa³ suggesting that secretomotor and vasomotor neurones are likely to be extremely few in number. Almost all circular and longitudinal muscle motor neurones in the human colon project for distances of less than 10 mm.³ Neurones with Dogiel type II morphology have been shown to be AH neurones in the guinea pig small intestine. They make a major contribution to circumferential pathways, although 10% also have long aboral projections in this small animal model.¹⁶⁻¹⁸ In the guinea pig colon, Dogiel type II cells project predominantly in the longitudinal axis of the gut for mean distances of 3-4 mm in the oral and aboral directions.¹⁹ In contrast, in the present study, Dogiel type II neurones were seldom identifiable more than 1-2 mm oral or anal to the DiI application site in the human colon, as reported previously.³ Thus it can safely be concluded that nearly all myenteric neurones with longitudinal projections of longer than 10 mm in the human colon are likely to be interneurons and will be considered as such for the purposes of this discussion. Neurones with short projections (that is, less than 10 mm) could belong to any functional class and are not discussed further.

Ascending interneurons

Ascending interneurons formed two neurochemical types: the largest (90%), which contained ChAT without NOS, and a smaller population (10%) which contained neither ChAT nor NOS. A previous study found that 22% of myenteric neurones in the human colon with ascending projections were TK immunoreactive but did not contain VIP or calretinin.⁴ It follows that there is a type of ascending interneurone which contains both ChAT and TK, and another containing ChAT without TK. There may also be one other small population which does not contain ChAT. In contrast, ascending interneurons in the guinea pig ileum form a single class, all having a chemical coding of ChAT/TK/enkephalin/calretinin/neurofilament protein triplet.¹⁵ This suggests that ascending pathways in the human colon may be rather more complex than those described in the guinea pig ileum. However, the predominance of ChAT immunoreactivity in ascending pathways correlates well with pharmacological evidence that ascending excitation, evoked by stretching human intestine, is abolished by hexamethonium, a nicotinic antagonist.¹⁰ The same is true for guinea pig and rat colon.²⁰

Descending interneurons

Ninety five per cent of descending interneurons were labelled with ChAT and/or NOS antisera. Three types of descending interneurons were described in this study. The largest (46%) was NOS immunoreactive but not ChAT immunoreactive. There were two less numerous types: one containing both

Table 3 Proportions of neurones with short and long anal projections labelled with ChAT and/or NOS antisera

Antibody applied	Short (<10 mm)			Long (>10 mm)		
	ChAT+	NOS+	ChAT+/ NOS+	ChAT+	NOS+	ChAT+/ NOS+
ChAT	44%	n/a	n/a	36%	n/a	n/a
NOS	n/a	59%	n/a	n/a	57%	n/a
ChAT and NOS	22%	47%	28%	20%	46%	29%

ChAT, choline acetyltransferase; NOS, nitric oxide synthase; n/a, not applied.

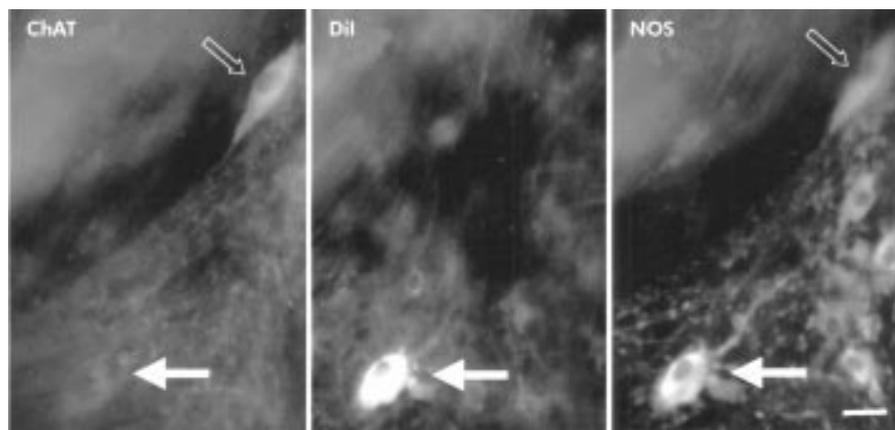


Figure 4 Anally projecting interneurone labelled 11 mm from the 1,1'-didodecyl 3,3,3',3'-indocarbocyanine perchlorate (Dil) application site. The nerve cell body was immunoreactive for nitric oxide synthase (NOS) but not choline acetyltransferase (ChAT) (filled arrows). A neurone not labelled with Dil (open arrow) was immunoreactive for both ChAT and NOS. Calibration bar, 20 μ m.

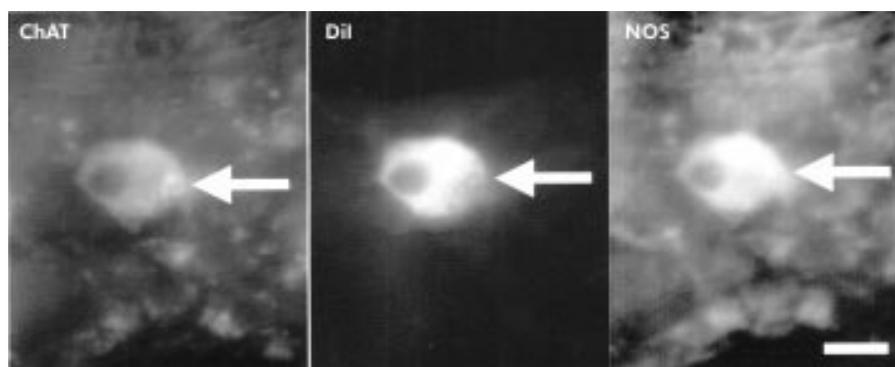


Figure 5 Descending interneurone containing both choline acetyltransferase (ChAT) and nitric oxide synthase (NOS) (arrows). It was located 20 mm oral to the 1,1'-didodecyl 3,3,3',3'-indocarbocyanine perchlorate (Dil) application site. Calibration bar, 20 μ m.

ChAT and NOS (29%) and another group containing ChAT but not NOS (20%). VIP and calretinin have been shown to be present in 51% and 23%, respectively, of descending interneurons in the human colon.⁴ VIP immunoreactivity has been demonstrated in a subset of NOS immunoreactive myenteric neurones in the human intestine^{21,22} but does not colocalise with calretinin immunoreactivity in myenteric neurones.²³ The precise combinations of NOS, ChAT, VIP, and calretinin present in each type of descending interneurone remain to be established.

The complexity of different classes of descending interneurons in the human colon relative to ascending interneurons has also been found in the guinea pig ileum where there are at least four functional classes of descending interneurons, three containing ChAT with other combinations of markers and one containing NOS with other markers.¹⁵ ChAT and NOS immunoreactivity coexist in some human myenteric neurones⁶ but as yet have not been directly demonstrated to coexist in guinea pig ileum, although the vesicular acetylcholine transporter is present in some VIP immunoreactive varicosities in myenteric ganglia²⁴ which are likely to be immunoreactive for NOS.²⁵ Their presence in the guinea pig gall bladder can be deduced from the observation that all myenteric neurones in that organ contain ChAT and there is a small population which contains NOS.²⁶

The description in this study that ChAT is present in many descending interneurons is supported by the finding that the descending inhibitory component of the peristaltic reflex in the human intestine is abolished by hexamethonium,¹⁰ indicating that cholinergic interneurons are involved in this pathway. The functional significance of NOS immunoreactive descending interneurons is not known as there is currently

no evidence that nitric oxide is involved in synaptic transmission in the human gastrointestinal tract. In guinea pig ileum, it has been suggested that nitric oxide, released from the cell bodies of descending interneurons, suppresses transmission from synaptic connections made with them by enteric sensory neurones.^{12,27} Our study raises the possibility that a group of descending interneurons release acetylcholine and nitric oxide simultaneously to effect their actions.

Correlation between neuroanatomy and motility patterns

The relative paucity and short projection length of ascending interneurons, observations which have been previously described in both guinea pig and human intestine,^{3,4,15} is likely to reflect the fact that there is only one ascending neural response, the ascending excitatory reflex, and there are several descending responses, including the descending excitatory reflex, the descending inhibitory reflex, and the migratory motor complex. The reason why there are more types of descending interneurons than ascending interneurons in both human and guinea pig intestine is not known although it is possible that different subclasses of descending interneurons are involved in different reflexes.

This study has demonstrated that ChAT and NOS are markers for almost all of the interneuronal populations and these need to be subdivided with other markers to have a description of all of the functional classes of interneurons. It is likely that there are more neurochemicals present in the myenteric interneurons than have been described in this and our previous study.⁴ Other markers which have been found to be present in cell bodies and nerve fibres in the myenteric ganglia of human intestine include calcitonin gene related

peptide, neuropeptide Y, galanin, somatostatin, and met-enkephalin.²¹⁻²⁸⁻³⁰ All of these neurochemicals are likely to be present in subpopulations of interneurons and further studies combining retrograde labelling with immunohistochemistry for these markers may establish the chemical coding of human myenteric interneurons. Determination of the transmitters contained in each class of interneurone would provide valuable information regarding the nature of reflex pathways involved in motility patterns in the human gastrointestinal tract. The finding that ChAT and/or NOS antibodies label the majority of ascending and descending interneurons is a significant first step in this direction as most other markers reveal only small populations of interneurons. This will provide the basis for more accurate characterisation of the neurochemical classes involved in both physiological and pathological processes.

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