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REGULAR ARTICLE

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Neurochemical classification of enteric neurons in the guinea-pig distal colon

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Abstract Previous studies have identified the chemistries, shapes, projections and electrophysiological characteristics of several populations of neurons in the distal colon of the guinea-pig but it is unknown how these characteristics correlate to define the classes of neurons present. We have used double-label immunohistochemical techniques to identify neurochemically distinct subgroups of enteric neurons in this region. On the basis of colocalisation of neurochemical markers and knowledge gained from previous studies of neural projections, 17 classes of neurons were identified. The myenteric plexus contained the cell bodies of 13 distinct types of neurons. Four classes of descending interneurons and three classes of ascending interneurons were identified, together with inhibitory and excitatory motor neurons to both the circular and longitudinal muscle layers. Dogiel type II neurons, which are presumed to be intrinsic primary afferent neurons, were located in myenteric and submucosal ganglia; they were all immunoreactive for choline acetyltransferase and often calbindin and tachykinins. Three classes of secretomotor neurons with cell bodies in submucosal ganglia were defined. Two of these classes were immunoreactive for choline acetyltransferase and the other class was immunoreactive for both vasoactive intestinal peptide and nitric oxide synthase. Some of the secretomotor neurons probably also have a vasomotor function. The neural subtypes defined in the present study are similar in many respects to those found in the small intestine, although differences are evident, especially in populations of interneurons. These differences presumably reflect the differing physiological roles of the two intestinal regions.

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Introduction

Within the tubular portion of the mammalian gastrointestinal (GI) tract, ganglionated plexuses of the enteric nervous system are situated between the circular and longitudinal smooth muscle layers (the myenteric plexus) and amongst the connective tissue that lies between the mucosa and the circular muscle layer (the submucosal plexus). These plexuses contain the neural components of local reflex arcs (sensory neurons, interneurons and motor neurons) that are responsible for the moment to moment control of GI motor systems. Within the myenteric plexus, the neural circuitry is predominantly involved in the reflex regulation of the contractile activities of the external musculature, whereas motor neurons of the submucosal plexus regulate the secretomotor and vasomotor activities of the mucosa.

Within the past 30 years, the combination of immunohistochemical, electrophysiological, pharmacological, microsurgical and retrograde tracing techniques has produced a dramatic increase in the understanding of the neural circuitry and neurophysiology of the enteric nervous system (Wood 1994; Costa et al. 1996; Timmermans et al. 1997; Furness et al. 1999). One outcome of these studies is the discovery that enteric neurons each contain several chemical markers that provide a chemical code that relates to the functions and targets of the neurons (Furness et al. 1989a; Ekblad et al. 1991). Subsequently, multiple label immunohistochemical techniques have taken advantage of this chemical code to define seemingly all the neuron types in the small intestine of the guinea-pig (Furness et al. 1995, 2000; Costa et al. 1996). Fourteen different classes of enteric neurons have been identified on this basis. Less comprehensive neurochemical classifications have been deduced for neurons within most regions of the GI tract of guinea-pigs and other

species including mouse, rat, human, pig, dog and toad (Timmermans et al. 1990; Li et al. 1993; Murphy et al. 1994; Barbiers et al. 1995; Schemann et al. 1995; Sang and Young 1996; Porter et al. 1997; Wattchow et al. 1997; Pfannkuche et al. 1998a, 1998b; Wang et al. 1998; Mann et al. 1999; Vanden Berghe et al. 1999).

Although some neurochemicals are contained within homologous neurons within different regions of the GI tract of different species, there are also marked differences between species and between regions of the GI tract of the same species (see Furness et al. 1995). In the distal colon of the guinea-pig, a substantial amount of data has been accumulated, primarily by using single-label immunohistochemistry (Messenger and Furness 1990; McConalogue and Furness 1993, 1996; McConalogue et al. 1994; Wardell et al. 1994; Neunlist and Schemann 1998) and more recently by filling neurons with intracellular markers via microelectrodes (Lomax et al. 1999). However, it is not possible to deduce the populations of neurons present in the distal colon from these individual studies as only very limited colocalisation studies had been undertaken previously. The use of double-labelling immunohistochemical techniques in the present work enables a direct comparison of the organisation of enteric circuitry of the distal colon to be made with the small intestine of the guinea-pig, in order to detect differences in the organisation between these two regions of the GI tract that have differing motor activities.

Materials and methods

Tissue was obtained from guinea-pigs of both genders, in the weight range 200–400 g. Animals were stunned by a blow to the head and killed by severing the carotid arteries and spinal cord. All procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee. Segments of colon, between 2 and 5 cm from the pelvic brim, were removed, opened along the mesenteric border, pinned tautly on balsa board and immersed in Zamboni's fixative (2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0) at 4°C overnight.

In order to visualise cell-body immunoreactivity for neuropeptides, some preparations were incubated in colchicine, under sterile conditions, for 24 h prior to fixation, as described previously (Furness et al. 1989b). To enhance serotonin (5-HT) immunoreactivity in myenteric cell bodies, some preparations underwent a 5-HT loading protocol (see Young and Furness 1995) prior to fixation in 4% formaldehyde overnight. Localisation of gamma-aminobutyric acid (GABA) was improved by loading fresh tissue with GABA (5×10^{-9} M) prior to fixation. This was achieved by placing tissue in culture medium that was warmed to 37°C and that was buffered with medical air containing 5% carbon dioxide for 20 min. The culture medium also contained nicardipine (3×10^{-7} M) to suppress muscle movement, amino-oxyacetic acid (2×10^{-5} M) to block GABA transaminase activity and β-alanine (10^{-3} M) to prevent glial uptake of GABA. Following this loading protocol, tissues were pinned on balsa wood and fixed in Zamboni's fixative containing 0.05% glutaraldehyde for 4 h. Tissue that was fixed in Zamboni's solution was cleared of fixative with 3×10 min washes in dimethylsulphoxide, followed by 3×10 min washes in phosphate-buffered saline (PBS; pH 7.2). Tissue that was fixed in formaldehyde was cleared of fixative by 3×10 min washes in PBS.

Immunohistochemistry

Following fixation and clearing, three types of dissections were performed. The mucosa, submucosa and circular muscle were removed to produce whole-mounts of longitudinal muscle plus the myenteric plexus. In the second type of preparation, the circular muscle layer was left intact, together with the longitudinal muscle and myenteric plexus, and the third type of dissection removed the mucosa and muscularis externa to leave behind the intact submucosa. All preparations were incubated in a 10% solution of normal horse serum and 1% Triton X-100 in 0.1 M sodium phosphate buffer for 30 min at room temperature, prior to exposure to combinations of primary antisera (Table 1).

Following incubation for one or two nights at room temperature in combinations of antisera, tissue was washed in PBS and then incubated in a mixture of secondary antibodies (Table 2) comprising one antibody linked to biotin and one directly labelled with fluorescein isothiocyanate (FITC) for 2 h. The tissue was then washed for 30 min in PBS and incubated with streptavidin-Texas Red for 90 min. A final wash in PBS was made before tissue was mounted in glycerol buffered with 0.5 M sodium carbonate buffer (pH 8.6).

Preparations were examined on a Zeiss Axioskop microscope equipped with the appropriate filter cubes for discriminating between FITC and Texas Red fluorescence. Images were recorded by using an ImagePoint cooled charge-coupled device camera

Table 1 Characteristics of primary antibodies

Tissue antigen	Host	Dilution	Code (reference)
Calbindin	Rabbit	1:1600	DEMLR 1(Furness et al. 1988)
Calretinin	Rabbit	1:1000	7696 (Mann et al. 1997)
Bombesin	Mouse	1:100	2AII (Costa et al. 1984)
CGRP	Rabbit	1:1000	MacI (Morris et al. 1984)
Enkephalin	Mouse	1:200	Seralab, Crawley Down, Sussex, UK
GABA	Rabbit	1:1600	(Maley and Newton 1985)
VAChT	Goat	1:1000	1624 (Li and Furness 1998)
5-HT (serotonin)	Rat	1:500	3M55 (Wardell et al. 1994)
NCB antigen	Rabbit	1:200	CBII (Young et al. 1993)
Neural NOS	Rabbit	1:200	N74 (Anderson et al. 1995)
Neural NOS	Sheep	1:2000	H05 (Williamson et al. 1996)
NPY	Rabbit	1:1600	JM263 (Maccarrone and Jarrott 1985)
Somatostatin	Mouse	1:400	S895 (Buchan et al. 1985)
TK	Rat	1:200	NCI/34HL (Cuello et al. 1979)
TH	Mouse	1:400	T341, Boehringer Mannhein
VIP	Rabbit	1:200	7913 (Furness et al. 1981)

Table 2 Secondary antibodies or streptavidin complexes used

Antibody or streptavidin label	Dilution	Source[a]
Biotinylated donkey anti-rabbit IgG	1:100	Jackson
Biotinylated donkey anti-sheep IgG	1:100	Jackson
Biotinylated horse anti-mouse IgG	1:100	Vector
Biotinylated horse anti-rabbit IgG	1:100	Jackson
Donkey anti-rabbit IgG FITC	1:50	Amersham
Donkey anti-sheep IgG FITC	1:50	Jackson
Donkey anti-rat IgG FITC	1:100	Jackson
Streptavidin-Texas Red	1:100	Amersham

[a] Supply companies: Amersham, Melbourne Australia; Jackson Immunosearch Lab., Pa., USA; Vector Lab., Burlingame, Calif., USA

(Photometrics, Tucson, Ariz., USA) and V for Windows imaging software (Digital Optics, Auckland, New Zealand).

Nomenclature

In order to simplify the presentation, we have designated neurons with immunoreactivity for the cholinergic markers choline acetyltransferase (ChAT) and/or the vesicular acetylcholine transporter (VAChT) as ACh neurons.

Quantitative analysis

The proportions of neurons that were immunoreactive for a particular neurochemical and that were also immunoreactive for other neurochemicals was estimated by examining double-stained preparations. In each case, double-stained preparations from several animals were used. For statistical purposes, the number of animals from which preparations were taken was used as the value of *n*. The percentage of neurons that were immunoreactive for a particular marker and that were also immunoreactive for another neurochemical was calculated and expressed as mean ± the standard error of the mean (SEM).

Results and discussion

Myenteric plexus

Anti-nerve-cell-body (NCB) antiserum labelled numerous neuronal perikarya within ganglia of both submucosal and myenteric plexuses of the guinea-pig distal colon. When these preparations were carefully examined, no nerve cells that were unstained could be found. When antisera that stained fewer than all nerve cells were used, unstained nerve cells could be recognised by faint protein fluorescence and by the space that they left amongst stained cells and axons. Staining of all neuron somas with NCB antiserum was consistent with the observations of Young et al. (1993) in the small intestine and McConalogue et al. (1994) in the colon. Nerve cells that were immunoreactive for nitric oxide synthase (NOS) had very strong staining and it was decided to use this immunoreactivity in double-labelling experiments to determine the proportions of nerve cells immunoreactive for other neurochemicals.

All NCB-immunoreactive (NCB-IR) and NOS-IR nerve cell bodies were counted in preparations double-stained by anti-NOS and NCB antisera. From counts of myenteric plexus preparations taken from six animals, 2710 NCB-IR cell bodies were encountered, whereas in the same preparations, 1057 NOS-IR cells were counted. Total numbers of neurons were counted, rather than neurons per ganglion, because it was often difficult in the distal colon to define where one ganglion ended and another began. The counts indicated that 39±2.5% (*n*=6) of the total population of myenteric neurons were NOS-IR; this proportion was higher than previously obtained by Furness et al. (1994b), who reported that 25% of neurons in the myenteric plexus of the guinea-pig distal colon were NOS-IR. NOS-IR nerve cells in the myenteric plexus were uniaxonal; many had Dogiel type I morphology (Dogiel 1899) and a range of cell body sizes, as was observed by McConalogue and Furness (1993).

Table 3 Sizes of chemically defined classes of neurons. This table lists some of the major groups of neurons that occur in the guinea-pig distal colon and that can be defined by combinations of immunohistochemical markers. Some of these can be divided into subgroups for which precise proportions have not been determined. However, they can be identified by their projections in the colon (Fig. 7)

Myenteric ganglia		
Ganglion type	Immunostaining pattern	Percentage
NOS-IR cell bodies	NOS/VIP	25%
	NOS/ChAT	2%
	Other NOS	15%
ChAT-IR cell bodies	ChAT/calbindin/TK (Dogiel type II)	8%
	ChAT/TK/ENK	17%
	ChAT/calretinin	18%
	ChAT/5-HT	2%
	ChAT/NOS	2%
	ChAT/VIP	3%
Submucosal ganglia	ChAT/TK/calbindin	22%
	NOS/VIP/±calbindin	41%
	ChAT/calretinin	11%
	ChAT/NPY/CGRP/SOM	26%

The proportions of myenteric neurons immunoreactive for other neurochemical markers were calculated by counts undertaken in preparations double-stained for NOS and a second marker (Table 3). As NOS immunoreactivity was contained in 39% of the total population of myenteric neurons, we used this factor to convert our data to the proportion of the total population of myenteric neurons that each marker represented. On this basis, 57±4.5% (*n*=5) of myenteric neurons were ChAT-IR, 25±0.5% (*n*=4) were vasoactive intestinal peptide (VIP)-IR, 19±1% (*n*=4) were calretinin-IR, 17±3% (*n*=3) were enkephalin (ENK)-IR and 10±1% were calbindin-IR (*n*=4). Of neurons that were ChAT-IR, 3±1% (*n*=3) were immunoreactive for NOS, whereas 2±0.4% (*n*=3) of calretinin-IR cell bodies (Fig. 1B, B') and 11±4% (*n*=3) of calbindin-IR cell bodies were also NOS-IR. All tachykinin (TK)-IR cell bodies were ChAT-IR, whereas no colocalisation was seen between TK immunoreactivity and NOS immunoreactivity.

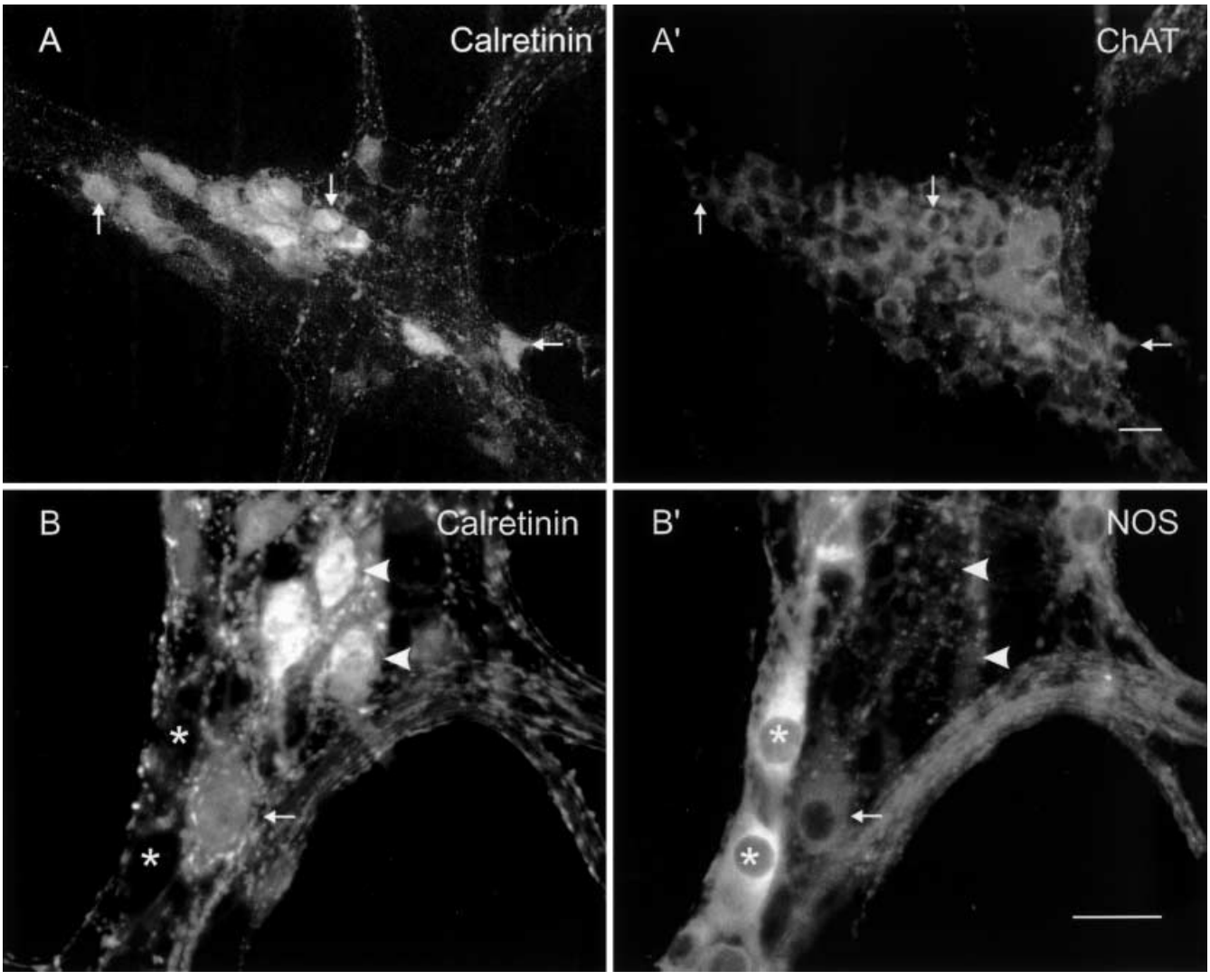

Fig. 1A–B Whole-mounts of myenteric ganglia demonstrating colocalisation of calretinin-IR with ChAT-IR (**A, A'**) and NOS-IR (**B, B'**). **A, A'** All calretinin-IR cell bodies within this ganglion are ChAT-IR (e.g. *arrows*). **B, B'** A rare calretinin-IR cell body that is also NOS-IR (*arrow*). This calretinin-IR cell body is surrounded by a basket-like formation of calretinin-IR varicosities. The majority of calretinin-IR cell bodies are not NOS-IR (e.g. *arrowheads*) and most NOS-IR neurons are not calretinin-IR (*asterisks*). *Bars* 25 μm

Antiserum raised against VIP was used on colchicine-treated preparations of myenteric plexus. Although somal morphology was often distorted by this treatment, immunoreactive cells were always uniaxonal. Around 90% of VIP-IR cell bodies were NOS-IR and 65% of NOS-IR cell bodies were VIP-IR (Fig. 2C, C'), whereas in preparations from the same animals, 13% of VIP-IR neurons were ChAT-IR.

Calretinin immunoreactivity was localised in neurons that often lay in clumps within myenteric ganglia. These cells were surrounded by a dense layer of immunoreactive varicosities, which made it difficult to define the morphologies of immunoreactive cell bodies. Nonetheless, all immunoreactive neurons appeared to be uniaxonal as previously reported (McConalogue et al. 1994). Of calretinin-IR cell bodies, 95±2.4% (*n*=3) were ChAT-IR (Fig. 1A, A').

All ENK-IR cell bodies, which had ascending axonal projections and Dogiel type I morphology (Messenger and Furness 1990), were ChAT-IR (Fig. 2B, B'). Around 16% of ENK-IR cell bodies were VIP-IR but no colocalisation between ENK and NOS immunoreactivity was seen. Sixty-five percent of ENK-IR cell bodies were TK-IR, although this might have been an underestimate because of the relatively low intensity of cell-body staining for TK. Seven percent of ENK-IR cell bodies were calretinin-IR.

Somatostatin (SOM)-IR neurons, many of which were deduced to be ascending interneurons (see below), were examined in colchicine-treated myenteric plexus preparations to determine the degree of overlap of SOM-IR with other neurochemical markers. All SOM-IR neurons were ChAT-IR and calretinin-IR, which is a marker of ascending interneurons that have filamentous dendrites (Lomax et al. 1999). Moreover, the majority (about 75%) of SOM-IR cell bodies were VIP-IR.

Calbindin antisera enabled the morphology of immunoreactive neurons to be defined. Of 200 calbindin-IR neurons examined, 79% had Dogiel type II morphology,

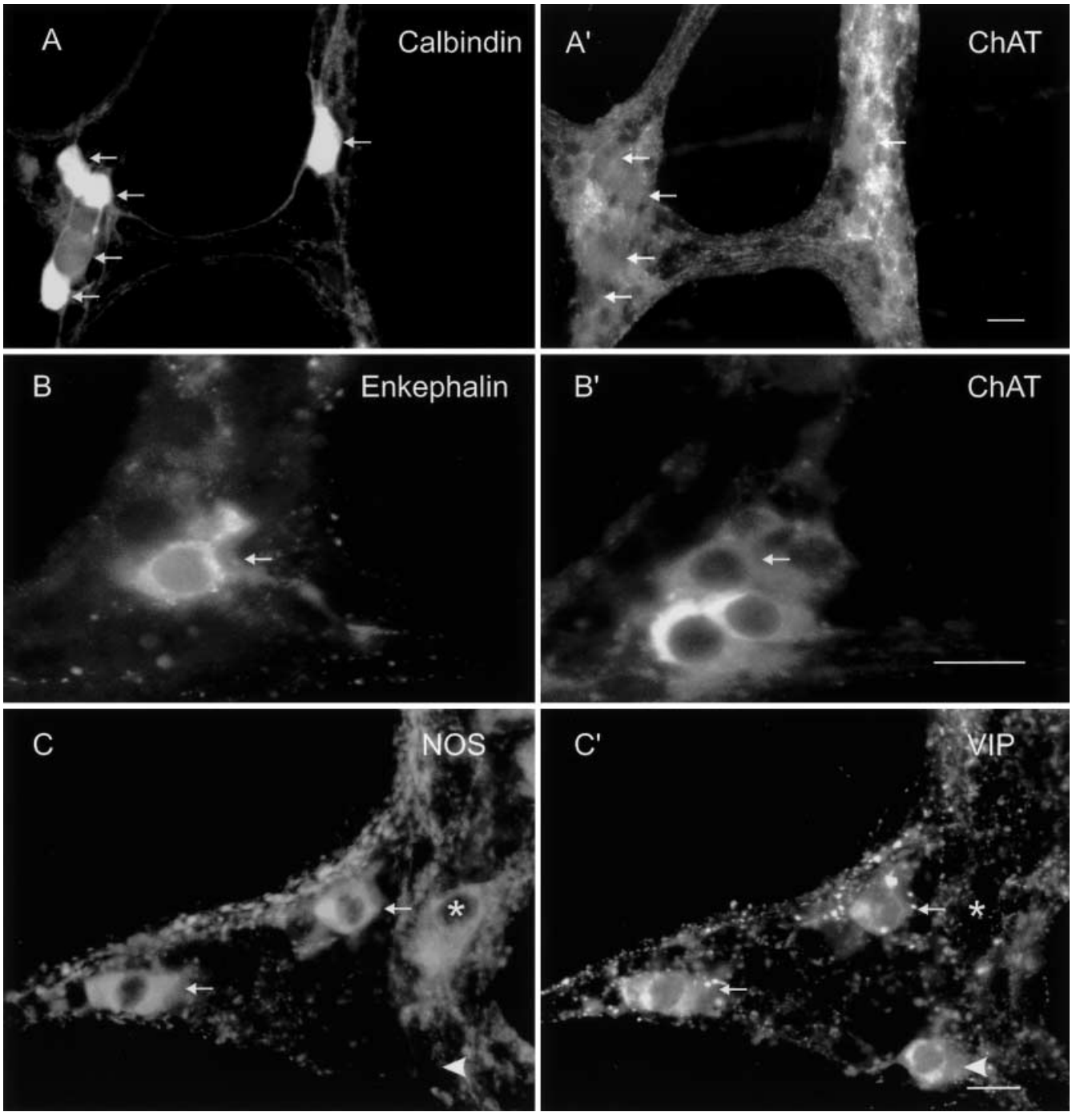

Fig. 2A–C Paired micrographs of whole-mount preparations of myenteric ganglia illustrating colocalisation of neurochemical markers. *Arrows* Colocalisation. **A, A'** All calbindin-IR neurons that have Dogiel type II morphology are ChAT-IR. **B, B'** An ENK-IR cell body that is ChAT-IR. All ENK-IR neurons in myenteric ganglia are ChAT-IR. **C, C'** A myenteric ganglion containing two NOS-IR cell bodies that are also VIP-IR. Note that not all NOS-IR neurons in this ganglion are VIP-IR (*asterisks*), nor are all VIP-IR neurons immunoreactive for NOS (*arrowheads*). *Bars* 25 μm

i.e. large oval cell bodies with several fine processes. The majority of the remaining calbindin-IR cell bodies displayed Dogiel type I morphology. Eighty-nine percent of calbindin-IR neurons in preparations from three animals were ChAT-IR (Fig. 2A, A'). As mentioned earlier, 11±4% (n=3) of calbindin-IR cell bodies were also NOS-IR, suggesting that all calbindin-IR neurons had immunoreactivity for ChAT or NOS.

Preparations of colchicine-treated myenteric plexus were examined to determine the degree of overlap between neuropeptide Y (NPY) immunoreactivity and NOS immunoreactivity. Eighty-five percent of NPY-IR cell bodies were NOS-IR.

A previous immunohistochemical study examined the shapes and projections of neurons immunoreactive for 5-HT (Wardell et al. 1994). 5-HT-IR was contained in a population of neurons that had Dogiel type I morphology

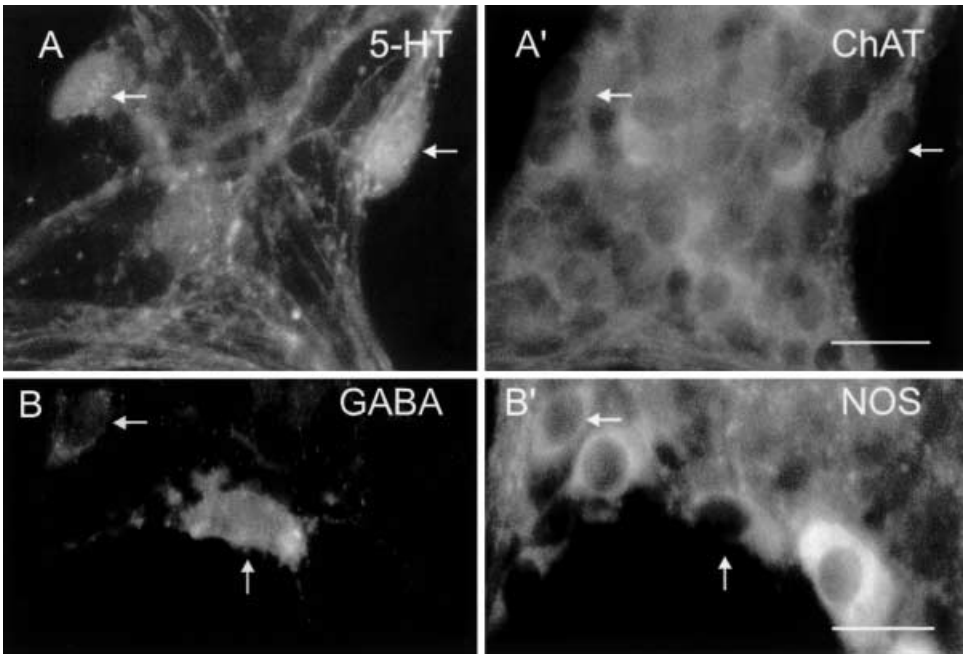

Fig. 3 Whole-mount preparations of myenteric plexus that had undergone 5-HT (**A, A'**) or GABA (**B, B'**) loading protocols. **A, A'** Colocalisation of 5-HT-IR with ChAT-IR in cell bodies (*arrows*). **B, B'** All GABA-IR cell bodies are also NOS-IR (*arrows*). Note that GABA-IR reveals the morphology of the cell body more completely than does NOS-IR. *Bars* 25 µm

and that comprised about 2% of the total population of myenteric neurons. These are the descending interneurons in the myenteric plexus that provide terminals in myenteric and submucosal ganglia. Kadowaki et al. (1999) have recently provided evidence that one of the targets of these neurons in the submucosal plexus is NOS-IR neurons. In the present work, it was found that all 5-HT-IR neurons were ChAT-IR (Fig. 3A, A'), calbindin-IR and calcitonin gene-related peptide (CGRP)-IR, whereas a small minority (15%) were calretinin-IR.

All GABA-IR cell bodies had Dogiel type I morphology (Messenger and Furness 1990) and NOS-IR (Fig. 3B, B') but none was ChAT-IR or calretinin-IR. The terminals of GABA-IR neurons innervate the circular muscle anal to their cell bodies (Messenger and Furness 1990).

Muscle motor neurons

Previous immunohistochemical studies in the guinea-pig distal colon indicate that the following neurochemical markers occur in varicose nerve fibres within the circular muscle layer: NOS, NPY, TK, VIP, GABA, SOM, ENK and calbindin (Messenger and Furness 1990; McConalogue and Furness 1993). By examining the colocalisation patterns of staining revealed by antisera raised against these markers, together with VAChT immunoreactivity in varicosities within the circular muscle, we have identified two classes of motor neurons. No VIP-IR fibres within the circular muscle are immunoreactive for TK or VAChT, but all VIP-IR fibres are NOS-IR and vice-versa. This implies that, as in other gut regions, there are separate populations of NOS/VIP and ACh/TK nerve fibres innervating the circular muscle. Moreover, NOS and VIP antisera stain anally projecting fibres in the circular muscle, whereas ACh/TK fibres project orally to the muscle (Messenger and Furness 1990; McConalogue and Furness 1993). We also found that all ENK-IR fibres were TK-IR and 40/43 TK-IR fibres were ENK-IR. No ENK-IR fibres were NPY-IR. Therefore, our results indicate that there is a population of anally directed neurons that innervate the circular muscle that has the chemical code NOS/VIP/±NPY and a population of orally directed fibres with the code ACh/TK/ENK. To this can be added the observation in the present work that all GABA-IR cell bodies are NOS-IR; GABA-IR neurons project anally to the circular muscle (Messenger and Furness 1990). SOM-IR fibres also project anally to the circular muscle but are less numerous than other fibre types. Thus, the chemical coding of anally projecting fibres can be extended to NOS/VIP/±NPY/±GABA/±SOM.

The direction of projection of these neurons and abundant pharmacological evidence indicate that they are the inhibitory motor neurons to the circular muscle (see Sanders and Ward 1992; Furness et al. 1995; Shuttleworth and Keef 1995). Indeed, neurons with the chemical code of NOS plus VIP are inhibitory muscle motor neurons in all regions of the GI tract of all species examined (e.g. Furness and Costa 1979; Brookes et al. 1991; Timmermans et al. 1994; Sang and Young 1996; Porter et al. 1997; Clerc et al. 1998b; Wang et al. 1998). In addition to pharmacological evidence for VIP and NO being inhibitory transmitters, there is evidence supporting a role for ATP or a related purine in inhibitory neuromuscular transmission in the GI tract (e.g. Crist et al. 1992) but ATP cannot be selectively localised to neurons.

Immunohistochemical studies indicate that TK-IR and ENK-IR fibres supply the circular muscle oral to their cell bodies (Messenger and Furness 1990). This observation, together with the present observation of colocalisa-

tion of VAChT and TK in fibres within the muscle, indicates that ACh/TK/±ENK is the chemical code for excitatory motor neurons innervating the circular muscle of the colon. Again, pharmacological studies support the role of ACh and TKs as co-transmitters at excitatory neuromuscular junctions throughout the GI tract of all species thus far examined (see Furness and Costa 1987; Holzer and Holzer Petsche 1997).

A small proportion of fibres in the circular muscle have calbindin immunoreactivity but colocalisation of markers in these axons has not been determined. These could be axons of motor neurons or, as in the duodenum, of intrinsic primary afferent neurons (Clerc et al. 1998b).

In the longitudinal muscle layer, immunoreactivity for the following neurochemicals was observed in nerve fibres: calretinin, NOS, VIP, TK, VAChT and ENK, which confirms and extends previous observations (Messenger and Furness 1990; McConalogue and Furness 1993). Examination of double-stained preparations of the distal colon in the present work identified two separate populations of longitudinal muscle neurons. There was no colocalisation of VIP immunoreactivity with either TK immunoreactivity or VAChT immunoreactivity in fibres in the longitudinal muscle. TK immunoreactivity was always colocalised within fibres immunoreactive for VAChT, and NOS-IR fibres were always VIP-IR and vice versa. This indicates that there are two populations of motor neurons to the longitudinal muscle: a cholinergic population that contains TK and a nitrergic population that also contains VIP. In addition, calretinin-IR fibres were always VAChT-IR, and ENK-IR fibres were always immunoreactive for both VIP and NOS. Thus, the chemical code of longitudinal muscle motor neurons is ACh/TK/±calretinin and NOS/VIP/±ENK. Again, the colocalisation of these neurochemical markers is consistent with NOS and VIP being contained in inhibitory motor neurons and ChAT, TK and calretinin being contained in excitatory motor neurons to the longitudinal muscle (Furness et al. 1995; Shuttleworth and Keef 1995; Holzer and Holzer Petsche 1997). In the guinea-pig ileum, the vast majority of longitudinal muscle motor neurons are immunoreactive for ChAT, indicating that the innervation of the longitudinal muscle is predominantly excitatory. Whereas it was not possible to determine the proportions of longitudinal muscle motor neurons that were ChAT-IR in the present study, the observation of inhibitory junction potentials in the longitudinal muscle of the colon suggests a significant inhibitory innervation (Furness 1969).

Dogiel type II, presumptive intrinsic primary afferent neurons

Neurons with Dogiel type II morphology were revealed by antisera raised against calbindin, although in contrast to the ileum, not all calbindin-IR neurons have Dogiel type II morphology (Messenger and Furness 1990; see above). All Dogiel type II calbindin-IR neurons were ChAT-IR and the majority were also immunoreactive for TK; by analogy with the small intestine, it is possible that some Dogiel type II neurons are not stained by calbindin antisera but are ChAT-IR and TK-IR (Furness et al. 1990b). Electrophysiological recordings, combined with injection of the intracellular marker biocytin, indicate that all Dogiel type II neurons in the myenteric plexus of the distal colon have AH type electrophysiological characteristics (Lomax et al. 1999), which is also the case for most Dogiel type II neurons in the ileum, where AH/Dogiel type II neurons have been shown to be intrinsic primary afferent neurons (IPANs) that are responsive to sensory stimuli of various modalities (see Furness et al. 1998). There is some lability in the occurrence of the late hyperpolarising potential (AHP) that follows the action potential and characterises AH neurons, depending on their state of excitation by slow transmitters (Furness et al. 1998). In the duodenum, where neurons tend to be in a more excited state than in the ileum, not all AH neurons display a late AHP (Clerc et al. 1998a). Calbindin-IR neurons provide dense innervation of myenteric ganglia with short oral or anal projections (Messenger and Furness 1990). A retrograde tracing study has revealed that calbindin-IR Dogiel type II neurons in the myenteric plexus also innervate the mucosa of this region and have AH type electrophysiological characteristics (Neunlist et al. 1999). Thus, the projections, electrophysiological characteristics and chemistries of ACh/calbindin/TK-IR Dogiel type II neurons in the distal colon are consistent with the projections of IPANs in the small intestine (Furness et al. 1990b; Song et al. 1994). The proportion of calbindin-IR Dogiel type II neurons in myenteric ganglia of the colon (8%) is less than half the proportion found in the ileum (Furness et al. 1990b).

Descending interneurons

Several neurochemicals are contained in varicosities of axons that project from oral to anal within the myenteric plexus. VIP, gastrin-releasing peptide (GRP), NOS, calbindin, 5-HT and calretinin have all been deduced to be in descending interneurons (Messenger and Furness 1990; McConalogue and Furness 1993; Wardell et al. 1994). By examining the colocalisation patterns of these neurochemical markers, together with ChAT and VAChT, in myenteric cell bodies and varicosities, four classes of descending interneurons have been identified in the present work. There is a class of cholinergic descending interneurons that is immunoreactive for VIP and often GRP, NOS and calbindin, as previously described (Lomax et al. 2000). On the basis of cell-body staining observed in the present study, another class of cholinergic descending interneuron is 5-HT-IR, ChAT-IR, CGRP-IR, calbindin-IR and sometimes calretinin-IR. The existence of the other two classes is deduced from the observations that all VIP-IR varicosities are also VAChT-IR (Fig. 4), that around 25% of NOS-IR varicos-

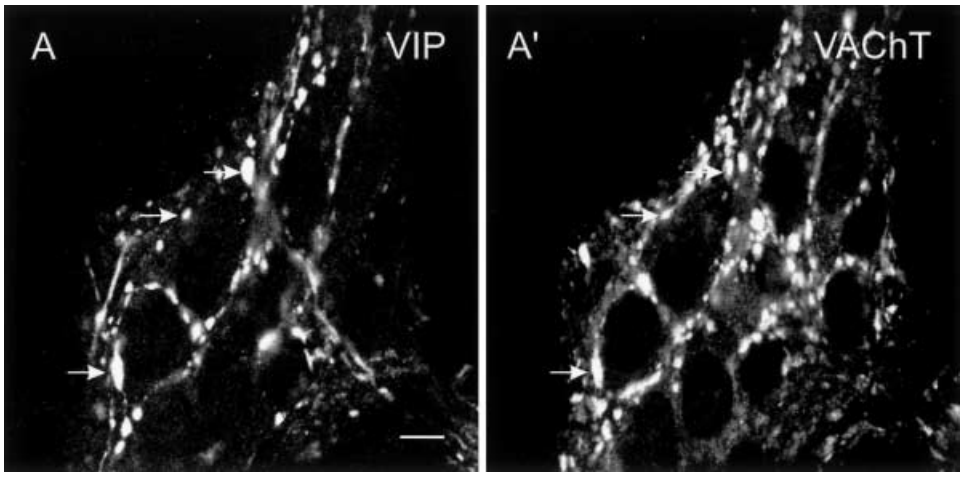

Fig. 4 Paired photomicrographs of a myenteric ganglion acquired by using a confocal microscope (optical thickness 0.7 µm), illustrating colocalisation of VIP-IR (**A**) and VAChT-IR (**A'**) within varicosities (examples at *arrows*). Note that not all VAChT-IR varicosities are VIP-IR. *Bar* 10 µm

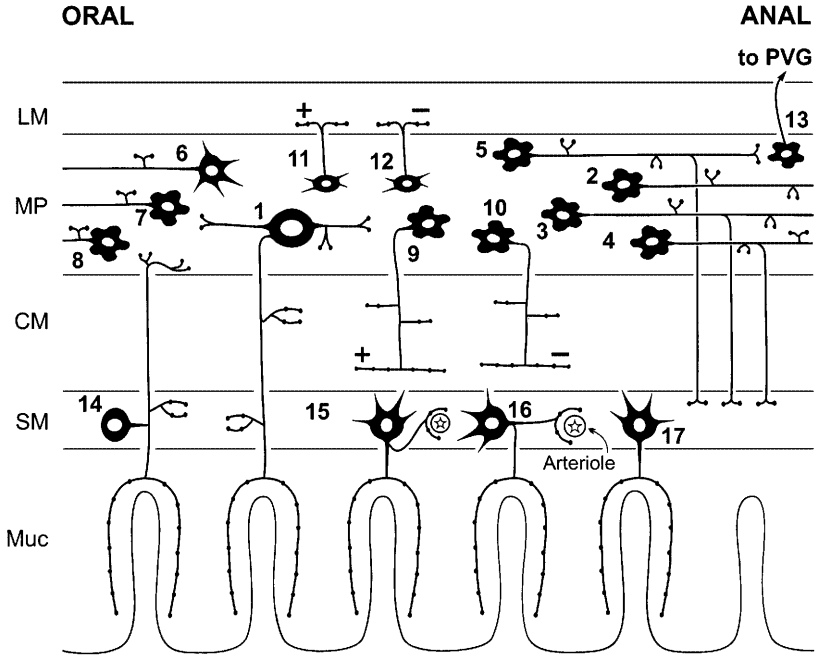

Fig. 5 Diagram of the types of neurons in the distal colon of the guinea-pig, defined by their chemistries, projections, cell body morphologies and presumed functions (*LM* longitudinal muscle layer, *MP* myenteric plexus, *CM* circular muscle layer, *SM* submucosa, *Muc* mucosa, *PVG* prevertebral ganglia). Myenteric neurons: *1* ACh/calbindin/TK, intrinsic primary afferent neuron, *2* ACh/VIP, descending interneuron, *3* NOS, descending interneuron, *4* ACh/5-HT/CGRP/calbindin/±calretinin, descending interneuron, *5* ACh/VIP/GRP/±NOS/±Calb, descending interneuron, *6* ACh/SOM/calretinin/VIP, filamentous ascending interneuron, *7* ACh/TK/ENK/±VIP±calretinin, ascending interneuron, *8* ACh/NPY/±TK/±SOM/±calretinin, ascending interneuron, *9* ACh/TK/±ENK, excitatory motor neuron to the circular muscle, *10* NOS/VIP/± GABA/±NPY, inhibitory motor neuron to the circular muscle, *11* ACh/TK/calretinin, excitatory motor neuron to the longitudinal muscle, *12* NOS/±VIP/±ENK, inhibitory motor neuron to the longitudinal muscle, *13* ACh/±NOS/±VIP/±GRP/±calbindin/±calretinin, intestinofugal neuron. Submucosal neurons: *14* ACh/TK/calbindin (22%), intrinsic primary afferent neuron, *15* NOS/VIP/± calbindin (41%), non-cholinergic secretomotor/vasodilator neuron, *16* ACh/calretinin (11%), cholinergic secretomotor/vasodilator neuron, *17* ACh/NPY/CGRP/SOM (26%), cholinergic secretomotor neuron

ities are not VAChT-IR and that only about 45% of VIP-IR varicosities are NOS-IR. This suggests that a class of descending interneuron is immunoreactive for VIP and ChAT and another class is immunoreactive for NOS but not for ChAT or VIP. These data indicate that the colon is similar to the small intestine in having ACh/5-HT, NOS and ACh/NOS/VIP descending interneurons but that it lacks the ACh/SOM neurons seen in the small intestine (Costa et al. 1996; Furness et al. 2000)

Ascending interneurons

Lomax et al. (1999) have described a class of myenteric ascending interneurons in the distal colon that have relatively long filamentous dendrites and are immunoreactive for calretinin. In the small intestine, myenteric neurons with this morphology are immunoreactive for SOM and ChAT but project anally (Portbury et al. 1995; Song

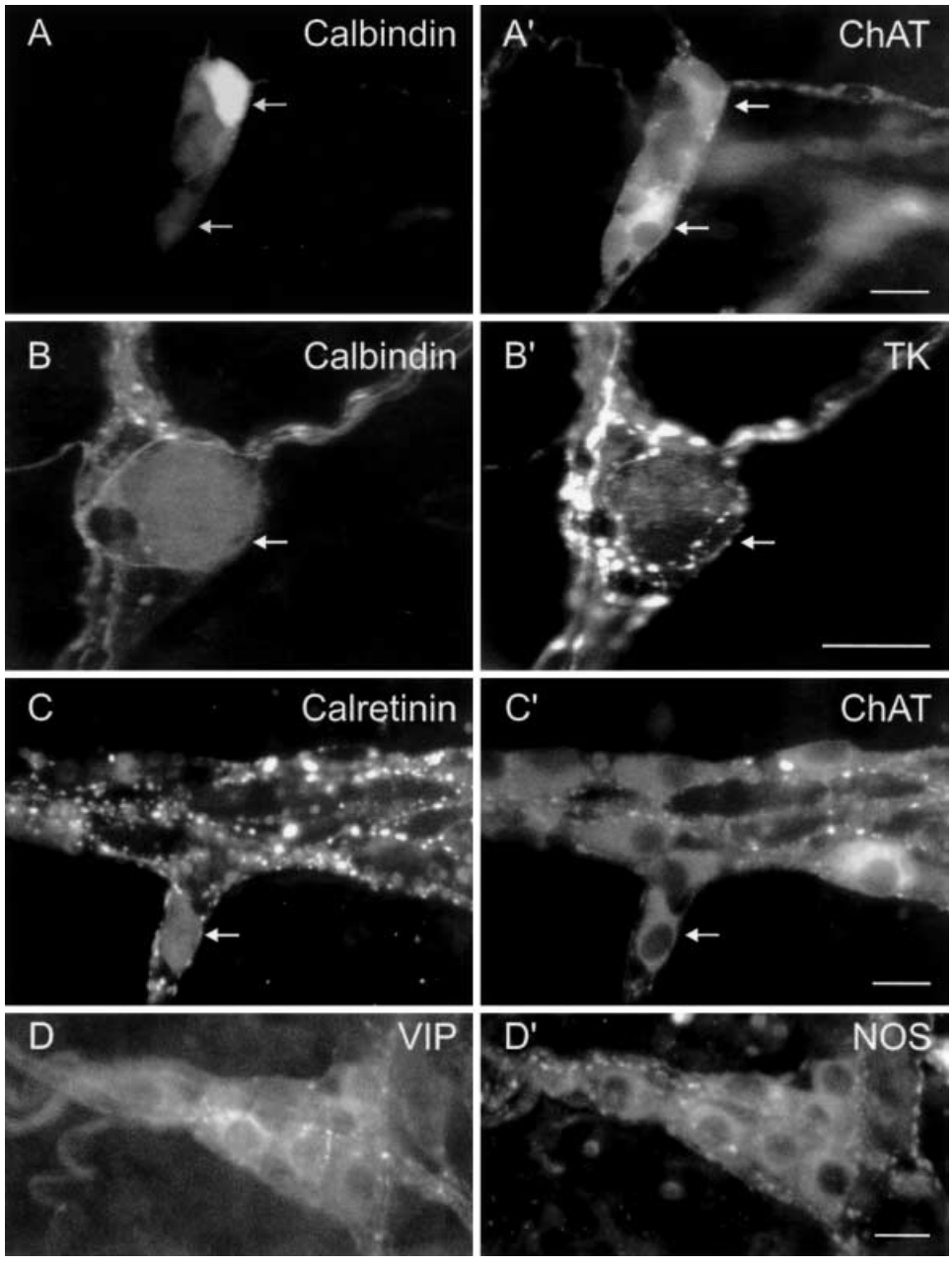

Fig. 6A–D Paired micrographs of nerve cells in the submucosal plexus demonstrating colocalisation of neurochemical markers. *Arrows* Colocalisation. **A, A'** Calbindin immunoreactivity is contained in cell bodies that, in this ganglion, are ChAT-IR; most calbindin-IR cell bodies were ChAT-IR but not all ChAT-IR cell bodies were calbindin-IR. **B, B'** A calbindin-IR neuron that is also TK-IR; all TK-IR neurons had calbindin-IR. Note that TK-IR varicosities surround this cell. **C** A calretinin-IR cell body in a ganglion containing a dense innervation by calretinin-IR varicose nerve fibres. The calretinin-IR cell body is also ChAT-IR (**C'**). **D, D'** Complete overlap between VIP-IR and NOS-IR cell bodies in a submucosal ganglion. *Bars* 25 µm

et al. 1997; Pompolo and Furness 1998). Filamentous ascending interneurons in the distal colon are also immunoreactive for SOM, ChAT and VIP. This conclusion has been arrived at by examining colocalisation patterns in SOM-IR cell bodies and varicosities, which are all calretinin-IR. Another class of cholinergic ascending interneuron exists that is immunoreactive for TK, ENK and often calretinin and VIP. A third class of cholinergic interneuron has been defined by examining the colocalisation patterns of markers within NPY-IR varicosities, because NPY-IR neurons have previously been shown to innervate myenteric ganglia oral to their cell bodies (Messenger and Furness 1990); the varicosities contain VAChT immunoreactivity and often immunoreactivity for TK, SOM and calretinin. Moreover, ChAT immunoreactivity has been observed in 40% of NPY-IR cell bodies examined, the non-ChAT-IR cell bodies being NOS-IR inhibitory motor neurons to the circular muscle. Thus, there are three types of ascending interneuron, each of which seems to be cholinergic (Fig. 5), compared with one type in the small intestine.

Intestinofugal neurons

The chemical coding of intestinofugal neurons has been defined in previous studies. Intestinofugal neurons are immunoreactive for ChAT and often VIP, NOS, calbindin, calretinin and GRP (Furness et al. 1990a; Messenger and Furness 1991; Anderson et al. 1995; Mann et al. 1995; Sharkey et al. 1998).

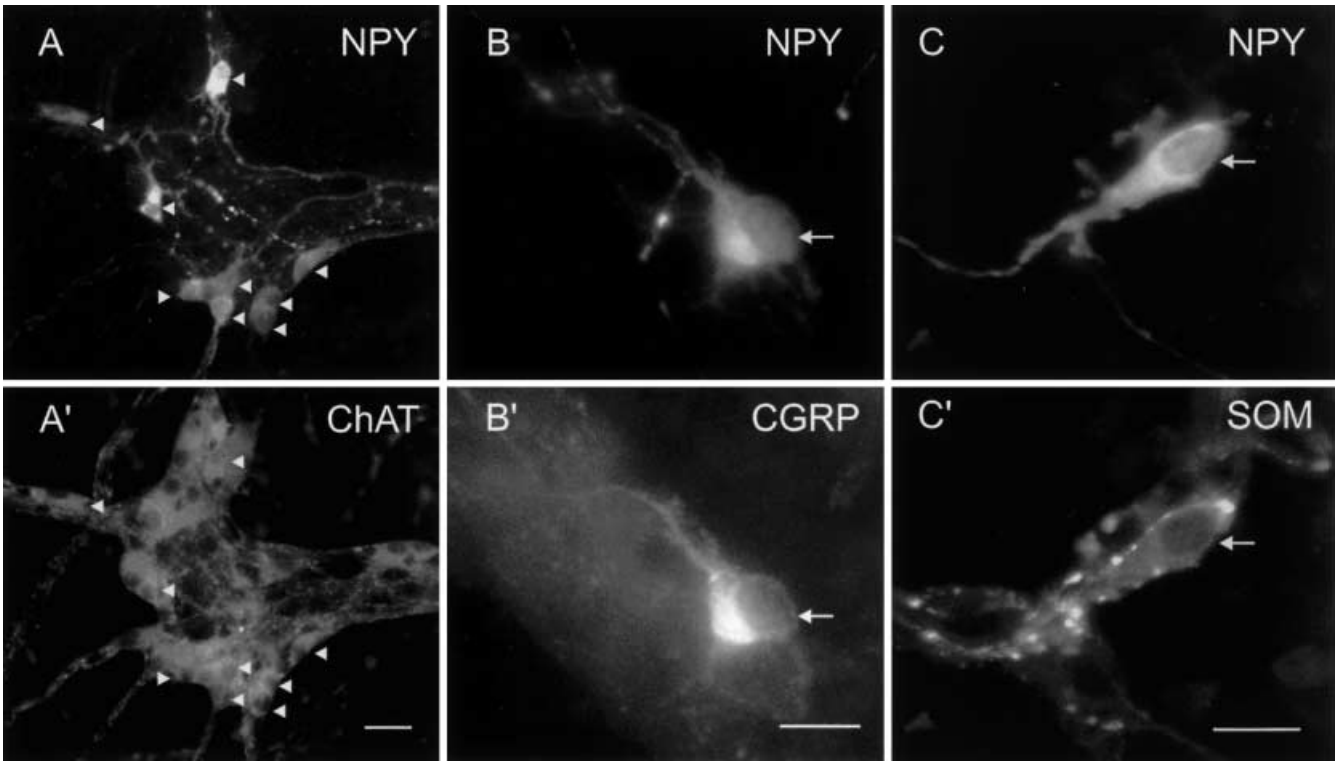

Fig. 7A–C Whole-mount preparations of submucosal plexus taken from preparations of distal colon that had been treated with colchicine. Paired micrographs showing the colocalisation of immunoreactivity for NPY (**A, B, C**) with immunoreactivity for ChAT (**A'**), CGRP (**B'**) and SOM (**C'**). **A, A'** All NPY-IR somata (*arrowheads*) are immunoreactive for ChAT, whereas not all ChAT-IR somata are NPY-IR. **B, B'** An NPY-IR neuron that is also CGRP-IR (*arrow*); there was complete overlap of immunoreactivity in submucosal cell bodies, although CGRP-IR is not contained in all NPY-IR terminals. **C, C'** An NPY-IR neuron that is also immunoreactive for SOM (*arrow*) as were all NPY-IR neurons. Note the SOM-IR varicosities surrounding the immunoreactive cell body. *Bars* 50 µm (**A, A'**), 25 µm (**B, B', C, C'**)

Submucosal plexus

In preparations double-labelled for NOS and NCB, NOS-IR cell bodies were calculated to comprise 41±2% (n=3) of the total population of submucosal nerve cell bodies. One hundred NOS-IR cell bodies in preparations from three animals were examined for colocalisation with ChAT immunoreactivity. NOS immunoreactivity and ChAT immunoreactivity were never colocalised but NOS immunoreactivity plus ChAT immunoreactivity appeared to account for all cell bodies. Therefore, ChAT immunoreactivity was contained in around 59% of the total population of submucosal plexus cell bodies. For the reasons mentioned above, the population of submucosal plexus neurons immunoreactive for NOS was set as the index (41%) for calculating the sizes of the populations immunoreactive for other markers. Calretinin immunoreactivity was contained in 11±2% of cell bodies, calbindin immunoreactivity in 31±1% and NPY immunoreactivity in 26±1.5% of submucosal cell bodies (n=3 in each case).

One hundred VIP-IR cell bodies were examined for colocalisation with NOS immunoreactivity in colchicine-treated submucosal preparations from three animals. All VIP-IR cell bodies were NOS-IR (Fig. 6D, D'). In three preparations taken from the same animals, no VIP-IR neurons were immunoreactive for ChAT. These data are in good agreement with that of Neunlist and Schemann (1998) who found that ChAT immunoreactivity and VIP immunoreactivity were contained in separate populations. They also found that VIP-IR neurons constituted 41% of all submucosal plexus nerve cells, the same proportion that was found in the present work.

ChAT immunoreactivity was colocalised in cell bodies immunoreactive for calretinin, CGRP, NPY, SOM and TK and in most calbindin-IR cell bodies. All CGRP-IR, NPY-IR (Fig. 7A, A') and SOM-IR cell bodies were ChAT-IR. In two preparations counted, 100% of CGRP-IR cell bodies were SOM-IR and NPY-IR (Fig. 7B, B'). All NPY-IR cell bodies were SOM-IR (Fig. 7C, C'). In three preparations, colocalisation of VIP and NPY immunoreactivity in cell bodies was never observed. Thus, we can deduce that 26% of submucosal neurons are ACh/CGRP/NPY/SOM. SOM immunoreactivity never surrounded submucosal arterioloes, whereas dense innervation of arterioles by NPY-IR and CGRP-IR varicose processes was evident, although these two markers were never colocalised in varicosities around arterioles. The NPY-IR periarterial fibres are probably sympathetic vasoconstrictor axons (Messenger and Furness 1990; Browning et al. 1999). NPY immunoreactivity was present in 85% of SOM-IR neurons, although all NPY-IR neurons were SOM-IR, which indicates that SOM-IR may be present in a subgroup of either ACh/calretinin or

ACh/TK/calbindin neurons (Fig. 5). McConalogue and Furness (1996) observed calretinin-IR periarterial fibres in the mucosa of the colon. As SOM-IR fibres never surround arterioles, it is likely that SOM immunoreactivity is contained in a subgroup of the ACh/TK/calbindin neurons.

In three preparations of colchicine-treated submucosal plexus, all TK-IR cell bodies were ChAT-IR. Eighty percent of calbindin-IR cell bodies were ChAT-IR (Fig. 6A, A'), whereas 68% of calbindin-IR cell bodies were immunoreactive for TK (Fig. 6B, B'). Three preparations were examined for colocalisation of calbindin and NOS immunoreactivity. Fifteen percent of calbindin-IR cell bodies were NOS-IR. Thus, there is a population with the chemical code ACh/calbindin/TK and another population of submucosal neurons that are NOS/VIP/±calbindin.

All calretinin-IR cells examined in three preparations were ChAT-IR (Fig. 6C, C'), whereas no colocalisation of NOS, VIP, TK or NPY immunoreactivity with calretinin was observed, indicating the existence of a fourth class of submucosal neuron with the code ACh/calretinin.

Therefore, there are four distinct classes of neurons within the submucosal plexus (see Fig. 5). The largest population (41%) encountered is a class of non-cholinergic secretomotor/vasomotor neurons, immunoreactive for NOS, VIP and often calbindin. A class of VIP-IR submucosal neurons is also present in the guinea-pig ileum (Furness et al. 1984, 2000; Costa et al. 1996). In this region, these neurons are in receipt of fast excitatory synaptic input from neurons in the myenteric and submucosal plexus and an inhibitory input from postganglionic sympathetic neurons (Bornstein et al. 1986). These neurons innervate the musosal epithelium, submucosal arterioles and neurons in the myenteric plexus (Furness and Costa 1979; Furness et al. 1987; Song et al. 1998; Porter et al. 1999).

The other three classes of submucosal neurons are each immunoreactive for ChAT. One class is immunoreactive for ChAT, calbindin and TK. In the submucosal plexus of the ileum, a similar class of neurons exists that has been postulated to consist of IPANs responsive to distortion of the mucosa (Kirchgessner et al. 1992; Furness et al. 1998). Antisera raised against calbindin label many neurons in the submucosal plexus of the distal colon that display Dogiel type II morphology (Messenger and Furness 1990; present study), which is the morphology that the IPANs within the ileum display.

Another neurochemically distinct class is comprised of ACh/NPY/CGRP/SOM neurons. These neurons also exist within the ileum and are cholinergic secretomotor neurons (Furness et al. 2000). Varicosities immunoreactive for NPY or CGRP surround many submucosal arterioles but this innervation is removed by extrinsic denervation, ruling out an intrinsic source for these varicosities (Messenger and Furness 1990).

The smallest population identified in the submucosal plexus is that which contains ChAT immunoreactivity and calretinin immunoreactivity (11%). These neurons exist in the ileum and are cholinergic secretomotor/vasomotor neurons (Costa et al. 1996; Furness et al. 2000).

Comparison with the guinea-pig small intestine

The guinea-pig small intestine has been the model preparation used for deciphering the neural circuitry of the enteric nervous system (Furness et al. 1994a, 1999; Wood 1994; Costa et al. 1996). Although the patterns of motor activity of the external musculature and the mucosa of the colon differ from those of the small intestine (Furness and Costa 1987; Christensen 1994; Cooke and Reddix 1994), the only marked difference in neural circuitry between the two regions appears to be that of the myenteric interneurons; both the IPANs and the motor neurons to the muscle and the mucosa appear to be homologous to those in the small intestine. In the small intestine, three classes of descending and one class of ascending interneuron have been identified (Costa et al. 1996; Furness et al. 2000), whereas in the colon, four classes of descending interneuron and three classes of ascending interneuron have been defined on the basis of chemical coding (Lomax et al. 2000; present work). Physiological, pharmacological and immunohistochemical studies have shown that the external musculature of every region of the GI tract of all species examined receives inhibitory and excitatory innervation from enteric motor neurons (see Furness et al. 1995). There is physiological evidence that motility reflex pathways in the intestine, regardless of the sensory modalities stimulated, act through common motor neurons (Smith et al. 1991, 1992). Thus, the present observation that the motor neurons identified in the colon do not differ from those in the small intestine might be expected. The activity of the motor neurons, however, is determined for the most part by the activity of the interneurons that synapse onto them. The differences in the classes of interneurons between the colon and the small intestine identified in this study may help explain the differing motor activities of the colon and small intestine. It is likely that examination of the connectivity patterns between IPANs and interneurons and between interneurons and motor neurons in different regions of the gut will yield valuable insights into the mechanisms of the generation of differing motility patterns in the GI tract. Conceiveable differences might be related to the presence of the interdigestive migrating motor complex (MMC) in the small but not the large intestine (e.g. Hasler 1995). Thus, in the small intestine, there may be interneurons involved in conducting the MMC along the gut; these are absent from the large intestine (Pompolo and Furness 1998). On the other hand, ascending interneurons that conduct traffic from the pelvic nerves and their intramural extensions (Furness and Costa 1987) are predicted in the colon but not in the small intestine.

The classes of neurons in the submucosal ganglia of the distal colon seem homologous with those in the ile-

um, being three classes of secretomotor/vasomotor neurons and a class of intrinsic primary afferent neuron (see above). One significant difference is that the non-cholinergic secretomotor neurons, which are VIP-IR in both places and are in a similar proportion (45% in ileum, 41% in distal colon), have NOS immunoreactivity in the colon but not in the ileum. Thus, the pharmacology of transmission from these neurons may differ between the regions.

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References

Anderson CR, Furness JB, Woodman HL, Edwards SL, Crack PJ, Smith AI (1995) Characterisation of neurons with nitric oxide synthase immunoreactivity that project to prevertebral ganglia. J Auton Nerv Syst 52:107–116

Barbiers M, Timmermans JP, Adriaensen D, De Groodt Lasseel MHA, Scheuermann DW (1995) Projections of neurochemically specified neurons in the porcine colon. Histochemistry 103:115–126

Bornstein JC, Costa M, Furness JB (1986) Synaptic inputs to immunohistochemically identified neurones in the submucous plexus of the guinea-pig small intestine. J Physiol (Lond) 381: 465–482

Brookes SJH, Steele PA, Costa M (1991) Identification and immunohistochemistry of cholinergic and non-cholinergic circular muscle motor neurons in the guinea-pig small intestine. Neuroscience 42:863–878

Browning KN, Cunningham SMC, Duncan L, Timmermans JP, Lees GM (1999) Regional differences in the sympathetic innervation of the guinea pig large intestine by neuropeptide Y- and tyrosine hydroxylase-immunoreactive nerves of divergent extrinsic origin. J Comp Neurol 410:515–530

Buchan AMJ, Sikora LKJ, Levy JG, McIntosh CHS, Dyck I, Brown JC (1985) An immunocytochemical investigation with monoclonal antibodies to somatostatin. Histochemistry 83: 175–180

Christensen J (1994) The motility of the colon. In: Johnson LR (ed) Physiology of the gastrointestinal tract. Raven, New York, pp 991–1024

Clerc N, Furness JB, Bornstein JC, Kunze WAA (1998a) Correlation of electrophysiological and morphological characteristics of myenteric neurons of the duodenum in the guinea-pig. Neuroscience 82:899–914

Clerc N, Furness JB, Li ZS, Bornstein JC, Kunze WAA (1998b) Morphological and immunohistochemical identification of neurons and their targets in the guinea-pig duodenum. Neuroscience 86:679–694

Cooke HJ, Reddix RA (1994) Neural regulation of intestinal electrolyte transport. In: Johnson LR (ed) Physiology of the gastrointestinal tract. Raven, New York, pp 2083–2132

Costa M, Furness JB, Yanaihara N, Yanaihara C, Moody TW (1984) Distribution and projections of neurons with immunoreactivity for both gastrin-releasing peptide and bombesin in the guinea-pig small intestine. Cell Tissue Res 235:285–293

Costa M, Brookes SJH, Steele PA, Gibbins I, Burcher E, Kandiah CJ (1996) Neurochemical classification of myenteric neurons in the guinea-pig ileum. Neuroscience 75:949–967

Crist JR, He XD, Goyal RK (1992) Both ATP and the peptide VIP are inhibitory neurotransmitters in guinea-pig ileum circular muscle. J Physiol (Lond) 447:119–131

Cuello AC, Galfre G, Milstein C (1979) Detection of substance P in the central nervous system by a monoclonal antibody. Proc Natl Acad Sci USA 76:3532–3536

Dogiel AS (1899) Über den Bau der Ganglien in den Geflechten des Darmes und der Gallenblase des Menschen und der Säugetiere. Arch Anat Physiol Leipzig Anat Abt Jg 1899: 130–158

Ekblad E, Håkanson R, Sundler F (1991) Microanatomy and chemical coding of peptide-containing neurons in the digestive tract. In: Daniel EE (ed) Neuropeptide function in the gastrointestinal tract. CRC Press, Boston, pp 131–179

Furness JB (1969) An electrophysiological study of the innervation of the smooth muscle of the colon. J Physiol (Lond) 205: 549–562

Furness JB, Costa M (1979) Projections of intestinal neurons showing immunoreactivity for vasoactive intestinal polypeptide are consistent with these neurons being the enteric inhibitory neurons. Neurosci Lett 15:199–204

Furness JB, Costa M (1987) The enteric nervous system. Churchill Livingstone, Edinburgh

Furness JB, Costa M, Walsh JH (1981) Evidence for and significance of the projection of VIP neurons from the myenteric plexus to the taenia coli in the guinea-pig. Gastroenterology 80:1557–1561

Furness JB, Costa M, Keast JR (1984) Choline acetyltransferase and peptide immunoreactivity of submucous neurons in the small intestine of the guinea-pig. Cell Tissue Res 237:329–336

Furness JB, Costa M, Rökaeus Å, McDonald TJ, Brooks BD (1987) Galanin-immunoreactive neurons in the guinea-pig small intestine: their projections and relationships to other enteric neurons. Cell Tissue Res 250:607–615

Furness JB, Keast JR, Pompolo S, Bornstein JC, Costa M, Emson PC, Lawson DEM (1988) Immunohistochemical evidence for the presence of calcium binding proteins in enteric neurons. Cell Tissue Res 252:79–87

Furness JB, Morris JL, Gibbins IL, Costa M (1989a) Chemical coding of neurons and plurichemical transmission. Annu Rev Pharmacol Toxicol 29:289–306

Furness JB, Pompolo S, Murphy R, Giraud A (1989b) Projections of neurons with neuromedin U-like immunoreactivity in the small intestine of the guinea-pig. Cell Tissue Res 257:415–422

Furness JB, Kuramoto H, Messenger JP (1990a) Morphological and chemical identification of neurons that project from the colon to the inferior mesenteric ganglia in the guinea-pig. J Auton Nerv Syst 31:203–210

Furness JB, Trussell DC, Pompolo S, Bornstein JC, Smith TK (1990b) Calbindin neurons of the guinea-pig small intestine: quantitative analysis of their numbers and projections. Cell Tissue Res 260:261–272

Furness JB, Bornstein JC, Pompolo S, Young HM, Kunze WAA, Kelly H (1994a) The circuitry of the enteric nervous system. Neurogastroenterol Motil 6:241–253

Furness JB, Li ZS, Young HM, Forstermann U (1994b) Nitric oxide synthase in the enteric nervous system of the guinea-pig: a quantitative description. Cell Tissue Res 277:139–149

Furness JB, Young HM, Pompolo S, Bornstein JC, Kunze WAA, McConalogue K (1995) Plurichemical transmission and chemical coding of neurons in the digestive tract. Gastroenterology 108:554–563

Furness JB, Kunze WAA, Bertrand PP, Clerc N, Bornstein JC (1998) Intrinsic primary afferent neurons of the intestine. Prog Neurobiol 54:1–18

Furness JB, Bornstein JC, Kunze WAA, Clerc N (1999) The enteric nervous system and its extrinsic connections. In: Yamada T, Alpers DH, Laine L, Owyang C, Powell DW (eds) Textbook of gastroenterology, vol 1. Lippincott, Williams and Wilkins, Philadelphia, pp 11–35

Furness JB, Clerc N, Gola M, Kunze WAA, Fletcher EL (2000) Identification of component neurons and organisation of enteric nerve circuits. In: H-J Krammer, Singer MV (eds) Neurogastroenterology – from the basics to the clinics. Kluwer, Dordrecht, pp 134–147

Hasler (1995) Motility of the small intestine. In: Yamada T (ed) Textbook of gastroenterology, vol 1. Lippincott, Williams and Wilkins, Philadelphia, pp 207–233

Holzer P, Holzer Petsche U (1997) Tachykinins in the gut. Part 1. Expression, release and motor function. Pharmacol Ther 73:173–217

Kadowaki M, Kuramoto H, Kuwahara A (1999) Morphological relationship between serotonergic neurons and nitrergic neurons for electrolyte secretion in the submucous plexus of the guinea pig distal colon. Brain Res 831:288–291

Kirchgessner AL, Tamir H, Gershon MD (1992) Identification and stimulation by serotonin of intrinsic sensory neurons of the submucosal plexus of the guinea pig gut: activity-induced expression of Fos immunoreactivity. J Neurosci 12:235–248

Li ZS, Furness JB (1998) Immunohistochemical localization of cholinergic markers in putative intrinsic primary afferent neurons of the guinea-pig small intestine. Cell Tissue Res 294:35–43

Li ZS, Murphy S, Furness JB, Young HM, Campbell G (1993) Relationships between nitric oxide synthase, vasoactive intestinal peptide and substance P immunoreactivities in neurons of the amphibian intestine. J Auton Nerv Syst 44:197–206

Lomax AE, Sharkey KA, Bertrand PP, Low AM, Bornstein JC, Furness JB (1999) Correlation of morphology, electrophysiology and chemistry of neurons in the myenteric plexus of the guinea-pig distal colon. J Auton Nerv Syst 76:45–61

Lomax AE, Zhang JY, Furness JB (2000) Origins of cholinergic inputs to the cell bodies of intestinofugal neurons in the guinea pig distal colon. J Comp Neurol 416:451–460

Maccarrone C, Jarrott B (1985) Differences in regional brain concentrations of neuropeptide Y in spontaneously hypertensive SH and Wistar Kyoto WKY rats. Brain Res 345:165–169

Maley B, Newton BW (1985) Immunohistochemistry of gamma-aminobutyric acid in the cat nucleus tractus solitarius. Brain Res 330:364–368

Mann PT, Furness JB, Pompolo S, Mäder M (1995) Chemical coding of neurons that project from different regions of intestine to the coeliac ganglion of the guinea pig. J Auton Nerv Syst 56:15–25

Mann PT, Southwell BR, Young HM, Furness JB (1997) Appositions made by axons of descending interneurons in the guinea-pig small intestine, investigated by confocal microscope. J Chem Neuroanat 12:151–164

Mann PT, Furness JB, Southwell BR (1999) Choline acetyltransferase immunoreactivity of putative intrinsic primary afferent neurons in the rat ileum. Cell Tissue Res 297:241–248

McConalogue K, Furness JB (1993) Projections of nitric oxide synthesizing neurons in the guinea-pig colon. Cell Tissue Res 271:545–553

McConalogue K, Furness JB (1996) Calretinin immunoreactivity of motor neurons in the guinea-pig distal colon and taenia coli. Cell Tissue Res 284:367–372

McConalogue K, Low AM, Williamson S, Bornstein JC, Furness JB (1994) Calretinin-immunoreactive neurons and their projections in the guinea-pig colon. Cell Tissue Res 276:359–365

Messenger JP, Furness JB (1990) Projections of chemically-specified neurons in the guinea-pig colon. Arch Histol Cytol 53:467–495

Messenger JP, Furness JB (1991) Calbindin-immunoreactive nerve terminals in the guinea pig coeliac ganglion originate from colonic nerve cells. J Auton Nerv Syst 35:133–142

Morris HR, Panico M, Etienne T, Tippins J, Girgis SI, MacIntyre I (1984) Isolation and characterization of human calcitonin gene-related peptide. Nature 308:746–748

Murphy S, Li ZS, Furness JB, Campbell G (1994) Projections of nitric oxide synthase- and peptide-containing neurons in the small and large intestine of the toad *Bufo marinus*. J Auton Nerv Syst 46:75–92

Neunlist M, Schemann M (1998) Polarised innervation pattern of the mucosa of the guinea-pig distal colon. Neurosci Lett 246:161–164

Neunlist M, Dobreva G, Schemann M (1999) Characteristics of mucosally projecting myenteric neurones in the guinea-pig proximal colon. J Physiol (Lond) 517:533–546

Pfannkuche H, Reiche D, Firzlaff U, Sann H, Schemann M (1998a) Enkephalin-immunoreactive subpopulations in the myenteric plexus of the guinea-pig fundus project primarily to the muscle and not to the mucosa. Cell Tissue Res 294:45–55

Pfannkuche H, Reiche D, Sann H, Schemann M (1998b) Different subpopulations of cholinergic and nitrergic myenteric neurones project to mucosa and circular muscle of the guinea-pig gastric fundus. Cell Tissue Res 292:463–475

Pompolo S, Furness JB (1998) Quantitative analysis of inputs to somatostatin immunoreactive descending interneurons in the myenteric plexus of the guinea-pig small intestine. Cell Tissue Res 294:219–226

Portbury AL, Pompolo S, Furness JB, Stebbing MJ, Kunze WAA, Bornstein JC, Hughes S (1995) Cholinergic, somatostatin-immunoreactive interneurons in the guinea pig intestine: morphology, ultrastructure, connections and projections. J Anat 187:303–321

Porter AJ, Wattchow DA, Brookes SJH, Costa M (1997) The neurochemical coding and projections of circular muscle motor neurons in the human colon. Gastroenterology 113:1916–1923

Porter AJ, Wattchow DA, Brookes SJH, Costa M (1999) Projections of nitric oxide synthase and vasoactive intestinal polypeptide-reactive submucosal neurons in the human colon. J Gastroenterol Hepatol 14:1180–1187

Sanders KM, Ward SM (1992) Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. Am J Physiol 262:G379–G392

Sang Q, Young HM (1996) Chemical coding of neurons in the myenteric plexus and external muscle of the small and large intestine of the mouse. Cell Tissue Res 284:39–53

Schemann M, Schaaf C, Mäder M (1995) Neurotransmitter coding of enteric neurones in the guinea pig stomach. J Comp Neurol 353:161–178

Sharkey KA, Lomax AE, Bertrand PP, Furness JB (1998) Electrophysiology, shape and chemistry of intestinofugal neurons projecting from guinea pig distal colon to inferior mesenteric ganglia. Gastroenterology 115:909–918

Shuttleworth CWR, Keef KD (1995) Roles of peptides in enteric neuromuscular transmission. Regul Pept 56:101–120

Smith TK, Bornstein JC, Furness JB (1991) Interaction between reflexes evoked by distention and mucosal stimulation: electrophysiological studies of guinea-pig ileum. J Auton Nerv Syst 34:69–76

Smith TK, Bornstein JC, Furness JB (1992) Convergence of reflex pathways excited by distension and mechanical stimulation of the mucosa onto the same myenteric neurons of the guinea pig small intestine. J Neurosci 12:1502–1510

Song ZM, Brookes SJH, Costa M (1994) All calbindin-immunoreactive myenteric neurons project to the mucosa of the guinea-pig small intestine. Neurosci Lett 180:219–222

Song ZM, Brookes SJH, Ramsay GA, Costa M (1997) Characterization of myenteric interneurons with somatostatin immunoreactivity in the guinea-pig small intestine. Neuroscience 80:907–923

Song ZM, Costa M, Brookes SJH (1998) Projections of submucous neurons to the myenteric plexus in the guinea pig small intestine. J Comp Neurol 399:255–265

Timmermans JP, Scheuermann DW, Stach W, Adriaensen D, De Groodt Lasseel MHA (1990) Distinct distribution of CGRP-, enkephalin-, galanin-, neuromedin U-, neuropeptide Y-, somatostatin-, substance P-, VIP- and serotonin-containing neurons in the two submucosal ganglionic neural networks of the porcine small intestine. Cell Tissue Res 260:367–379

Timmermans JP, Barbiers M, Scheuermann DW, Stach W, Adriaensen D, Mayer B, De Groodt Lasseel MHA (1994) Distribution pattern, neurochemical features and projections of nitrergic neurons in the pig small intestine. Ann Anat 176: 515–525

Timmermans JP, Adriaensen D, Cornelissen W, Scheuermann DW (1997) Structural organization and neuropeptide distribution in the mammalian enteric nervous system, with special attention to those components involved in mucosal reflexes. Comp Biochem Physiol 118A:331–340

Vanden Berghe P, Coulie B, Tack J, Mawe GM, Schemann M, Janssens J (1999) Neurochemical coding of myenteric neurons in the guinea-pig antrum. Cell Tissue Res 297:81–90

Wang YF, Mao YK, Fox Threlkeld JET, McDonald TJ, Daniel EE (1998) Colocalization of inhibitory mediators, NO, VIP and galanin, in canine enteric nerves. Peptides 19:99–112

Wardell CF, Bornstein JC, Furness JB (1994) Projections of 5-hydroxytryptamine-immunoreactive neurons in guinea-pig distal colon. Cell Tissue Res 278:379–387

Wattchow DA, Porter AJ, Brookes SJH, Costa M (1997) The polarity of neurochemically defined myenteric neurons in the human colon. Gastroenterology 113:497–506

Williamson S, Pompolo S, Furness JB (1996) GABA and nitric oxide synthase immunoreactivities are colocalized in a subset of inhibitory motor neurons of the guinea-pig small intestine. Cell Tissue Res 284:29–37

Wood JD (1994) Physiology of the enteric nervous system. In: Johnson LR (ed) Physiology of the gastrointestinal tract. Raven, New York, pp 423–482

Young HM, Furness JB (1995) An ultrastructural examination of the targets of serotonin-immunoreactive descending interneurons in the guinea-pig small intestine. J Comp Neurol 356: 101–114

Young HM, Furness JB, Sewell P, Burcher E, Kandiah CJ (1993) Total numbers of neurons in myenteric ganglia of the guinea-pig small intestine. Cell Tissue Res 272:197–200