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Neuropeptides and the microcircuitry of the enteric nervous system

by Ida J. Llewellyn-Smith

Department of Anatomy and Histology and Centre for Neuroscience, School of Medicine, Flinders University, Bedford Park, S.A. 5042 (Australia)

Summary. The discovery of neuropeptides in enteric neurons has revolutionized the study of the microcircuitry of the enteric nervous system. From immunohistochemistry, it is now clear that some individual enteric neurons contain several different neuropeptides with or without other transmitter-specific markers and that these markers occur in various combinations. There is evidence from experiments in which nerve pathways are interrupted that populations of enteric neurons with different combinations of markers have different projection patterns, sending their processes to distinct targets using different routes. Correlations between the neurochemistry of enteric neurons and the types of synaptic inputs they receive are also beginning to emerge from electrophysiological studies. These findings imply that enteric neurons are chemically coded by the combinations of peptides and other transmitter-related substances they contain and that the coding of each population correlates with its role in the neuronal pathways that control gastrointestinal function.
Key words. Neuropeptides; enteric nervous system; intestine.

The mammalian gastrointestinal tract contains about as many neurons as the spinal cord[28]. These intrinsic (enteric) neurons, along with the processes of sympathetic, parasympathetic and sensory neurons supplying the gut, and enteric glial cells, make up the enteric nervous system (ENS), which is now generally classified as a third division of the autonomic nervous system. The cell bodies of the enteric neurons are grouped into ganglia in two main plexuses: the submucous plexus, discovered by Meissner in 1857[52], in the loose connective tissue of the submucosa, and the myenteric plexus, described by Auerbach in 1864[1], which lies between the longitudinal and circular layers of the mucularis externa. Non-ganglionated plexuses, which are continuous with the main ganglionated plexuses, supply the muscle layers, the mucosa and blood vessels.

The ENS influences or controls a variety of functions, including movement of digesta along the gastrointestinal tract, gastric acid secretion, transport of water and electrolytes, release of gastrointestinal hormones, and blood flow[29, 30]. Enteric reflexes persist even when the gut is disconnected from the central nervous system (e.g., Bayliss and Starling[2, 3]). Hence, the ENS must contain several different functional types of neurons, namely motor neurons to the muscle, secretomotor and vasomotor neurons, interneurons and sensory neurons. Futhermore, these neurons must be arranged in an orderly fashion to form circuits that govern the different enteric reflexes. The need to unravel the internal circuitry of the ENS was recognized early, but this analysis was frustrated until the past decade by the inadequacy of neurohistological and pathway tracing techniques. Since 1975, however, our knowledge of neuronal pathways in the ENS has increased significantly because of the discovery of neuropeptides in enteric neurons and because of technical advances, including light and electron microscopical methods for immunohistochemistry on whole mount preparations of separated gut layers, techniques for lesioning enteric nerve pathways, and correlated physiological, pharmacological and electrophysiological studies. The ways in which these devel-

opments have improved our understanding of the microcircuitry of the ENS are outlined below. It should be noted that most of the information provided here comes from experiments on the guinea pig small intestine, which has been the main model for studying enteric nerve pathways.

Histological and histochemical studies before 1975

Early investigators, using methylene blue or silver or osmium impregnation, stained either all enteric neurons and their processes or variable subsets that did not appear to correspond to any single anatomical or functional class (for review see Costa et al.[14]). These studies were useful for establishing the arrangements of the enteric plexuses, their interconnections and the shapes of enteric neurons in various regions of the gut and various species (for review see Gabella[37] and Furness and Costa[29]) but were not helpful for defining neuronal circuitry within the ENS. A notable series of observations on methylene blue stained enteric ganglia from several species was made by Dogiel at the end of last century[17]. Dogiel was able to define three types of enteric neurons on the basis of their shapes: type I neurons had many short irregular processes and one long process; type II neurons had many long processes, whereas type III cells had intermediate length branching processes[29]. Dogiel believed that his different neuron types corresponded to functional classes, with type I cells being motor and type II cells being sensory. Debate continued for many years about the validity of this proposal and even about the validity of Dogiel's classification system. However, recent experiments have shown that most enteric neurons have shapes that allow them to be fitted into a classification scheme similar to Dogiel's and that there are correlations between cell shape and function (see below).

The development and application of histochemical techniques in the 1950's and 1960's[14] brought only a few advances in understanding the neuronal circuitry of the ENS. Reactions to localize the neurotransmitter degrading enzymes, acetylcholinesterase and monoamine oxidase, began to be used to examine the ENS in the 1950's, but unfortunately these enyzmes were not confined to a single type of enteric neuron. In the 1960's it became possible to demonstrate catecholamine-containing neurons with fluorescence histochemistry. Noradrenergic fibers arising from postganglionic sympathetic neurons of prevertebral ganglia were found to supply primarily the enteric ganglia in non-sphincter regions of the gastrointestinal tract[26], but catecholamine nerve cell bodies were absent from the ENS in most regions of the gut in most species. These histochemical findings and information from physiological and pharmacological studies provided an understanding of how sympathetic nerves controlled motility and blood flow, which has seen little refinement since[29]. However, no information was gained from catecholamine fluorescence histochemistry about intrinsic reflex pathways involving the enteric neurons.

The presence of neuropeptides in enteric nerves

The finding that enteric neurons contained neuropeptides was a major breakthrough for the study of the ENS. Using immunohistochemical techniques, Pearse and Polak[55] and Nilsson and colleagues[52a] showed in 1975 that some enteric neurons were immunoreactive for substance P, and in the same year Hökfelt and colleagues[42] found that some enteric neurons contained somatostatin-like immunoreactivity. Since these discoveries little more than a decade ago, many neuropeptides have been localized immunohistochemically within nerve cell bodies and varicose nerve fibers in the ENS[36]. The substances detected immunohistochemically include calcitonin gene-related peptide (CGRP), cholecystokinin (CCK), dynorphin (DYN), enkephalin (ENK), galanin (GAL), gastrin-releasing peptide (GRP; also known as mammalian bombesin), neuropeptide Y (NPY), neurotensin, peptide HI, somatostatin (SOM), substance P (SP), neorokinin A (substance K), and vasoactive intestinal peptide (VIP). For many of these peptides, the molecular form of the antigen that is localized immunohistochemically has been determined[16]. Thus, immunohistochemistry has allowed many different populations of enteric neurons to be defined on the basis of their neuropeptide content. As well as being present in enteric neurons, neuropeptide immunoreactivity is also found in the processes of extrinsic neurons supplying the gut. In guinea pigs noradrenergic nerves to gastrointestinal blood vessels contain NPY[31], and those to submucous ganglia of the small intestine contain SOM[11]; capsaicin-sensitive, presumably sensory, fibers of extrinsic origin contain SP and CGRP[38].

From whole mount preparations of myenteric and submucous plexuses of guinea pig ileum immunohistochemically labelled for single neuropeptides (see Costa et al.[9]) it became clear that there was a correlation between cell shape and neuropeptide content, at least for some neuropeptides. In control preparations that had not been treated with colchicine, neuropeptide immunoreactivity was intense in some enteric neurons so that their shapes could be easily described. Thus, intensely ENK-positive myenteric neurons had a typical Dogiel type I morphology with a single long axon and many short dendrites[35], whereas NPY-immunoreactive myenteric neurons were Dogiel type III with a long process and many intermediate length dendrites[31]. For other peptides, such as substance P, the shapes of the nerve cell bodies could not be ascertained because processes did not contain enough immunoreactivity to be clearly visualized. The whole mount technique for immunohistochemistry also made it possible to count the number of immunoreactive neurons in the ganglionated plexuses so that the proportion of neurons immunoreactive for a particular neuropeptide in either myenteric or submucous ganglia could be calculated[14].

The different types of neuropeptide-immunoreactive nerves in the ENS have different distributions[36]. Conversely, many different types of peptide-containing nerves are present in each of the enteric plexuses. For example, in the guinea pig deep muscular plexus there are dense networks of varicose fibers immunoreactive for ENK, GAL, GRP, DYN, NPY, SP, or VIP. In the small intestine the mucosa is densely supplied with nerve fibers immunoreactive for SP, VIP and NPY. This suggested that many different populations of neurons, as defined by their content of a single peptide, may participate in the control of an effector such as the muscle or the mucosal epithelium. However, some types of peptide nerves are absent from some of the enteric plexuses. For instance, in most species, ENK neurons are absent from submucous ganglia and ENK fibers do not occur in the mucosal plexuses. SOM fibers, on the other hand, do not usually occur in the muscle plexuses. These differences in distribution suggested differences in function. Hence, ENK neurons are unlikely to be secretomotor since they are not associated with the mucosal epithelium. On the other hand, some are probably motor neurons to the muscle since their processes are found in the circular and deep muscular plexuses. SOM myenteric neurons do not appear to be motor neurons which affect the muscle directly but are probably interneurons for pathways travelling through the myenteric plexus since they have processes that supply other myenteric ganglia and form baskets around other myenteric neurons. Neuropeptide immunohistochemistry suggested that there were multiple types of enteric neurons and that many of them supplied the same effectors. The complex array of enteric reflexes appeared to be mirrored by an equally complex array of different neurochemical types of enteric neurons. How-

ever, the finding of multiple neurochemical messengers in the same neuron (see the article by Hökfelt in this issue) suggested that the complexity of the ENS might be overestimated by examining material that was immunohistochemically treated to reveal only a single peptide.

Co-localization of neuropeptides in enteric nerves

The first evidence that enteric neurons contained more than one neuropeptide was presented in 1980 by Schultzberg et al.[57], who showed that in the proximal colon of guinea pig SOM-immunoreactive submucous neurons also contained CCK. However, the extent of neuropeptide coexistence in submucous neurons became clear only when double labelling techniques with a variety of antisera were applied to guinea pig small intestine. Four populations of submucous neurons were identified on the basis of their immunoreactivity for VIP, NPY, CCK, SOM, SP and cholineacetyltransferase (ChAT), the acetylcholine-synthesizing enzyme[34]. About 45% of submucous neurons were immunoreactive for VIP and the remainder (about 55%) for ChAT, a finding which correlated well with the physiological observations that there were cholinergic and non-cholinergic secretomotor neurons[44]. The ChAT neurons were subdivided into 3 classes: those that contained NPY, CCK and SOM (29% of total submucous neurons), those that contained SP (11% of neurons) and those that were not immunoreactive for any of the peptides studied (14% of neurons). Subsequently, the ChAT/NPY/SOM/CCK submucous neurons have been shown to contain CGRP[33] and the VIP neurons to contain DYN[14]. The different types of submucous neurons were topographically organized: in smaller submucous ganglia the VIP/DYN neurons occurred in groups and in larger ganglia the groups were usually located centrally with ChAT neurons surrounding them.

Individual myenteric neurons also contain several neurochemical markers. Colchicine has been important for demonstrating this in guinea pig myenteric neurons because colchicine treatment in vitro raises neuropeptide immunoreactivity to a detectable level of detectability in many myenteric nerve cell bodies[12]. In guinea pig small intestine, the percentages of nerve cell bodies that are immunoreactive for individual neuropeptides after colchicine implies extensive coexistence[14]. The patterns of neuropeptide coexistence, as shown by double immunofluorescent staining[13] or by localization of individual neuropeptides in serial semithin section (fig. 1), are more complex in myenteric than in submucous neurons[29]. Consequently, myenteric neurons can be divided into many more than four types on the basis of their peptide content. Much of this complexity arises because several neurochemically distinct populations of myenteric neurons contain the same neuropeptide. For example, in guinea pig small intestine there are four populations of myenteric neurons that contain VIP and DYN with or without other neuropeptides, VIP/DYN, VIP/DYN/GRP, VIP/DYN/ENK/NPY and VIP/DYN/ENK/GRP/CCK neurons and at least two populations of SP neurons, SP and SP/ENK neurons. The different neurochemical types of neurons are probably also topographically organized within myenteric ganglia. VIP/ENK neurons with Dogiel type I morphology, for example, usually lie on the surfaces of the ganglia, mainly facing the circular muscle. Small type I SP/ENK neurons often have their cell bodies where internodal strands join the ganglia.

The link between the neurochemistry of guinea pig myenteric neurons and the shapes of their somas, which was apparent for some neuropeptide-containing neurons in untreated tissue, has been strengthened by observations on colchicine-treated tissue double-labelled to reveal more than one neuropeptide. The majority of myenteric neurons stained for neuropeptides after colchicine fall into one of Dogiel's morphological categories[29]. For instance, the VIP/DYN/ENK/NPY myenteric neurons have a typical Dogiel type I morphology with many short stubby dendrites and one long axon. The VIP/DYN/ENK/GRP/CCK neurons and the VIP/DYN/GRP neurons are also Dogiel type I. There are ChAT/NPY/CCK/SOM/CGRP neurons in the myenteric plexus as well

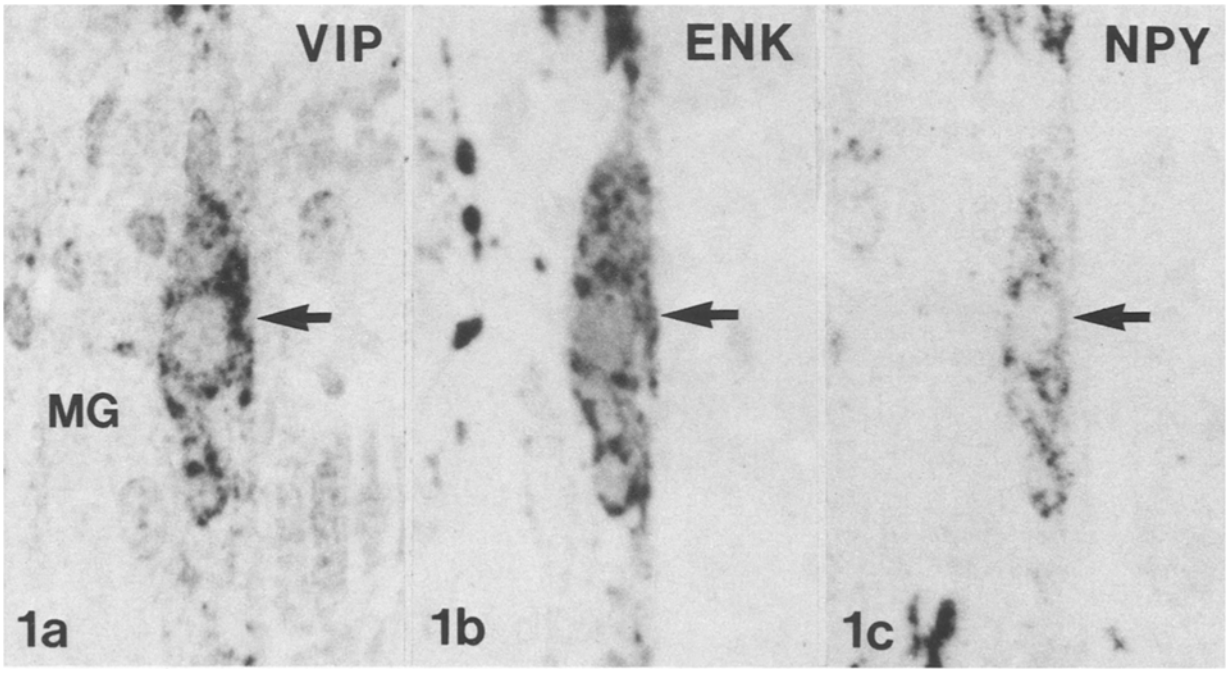

Figure 1. Serial semithin (1.5-μm) sections through a myenteric ganglion (MG) form guinea pig small intestine that was treated with colchicine in vitro. The sections were stained with antiserum to vasoactive intestinal peptide (VIP, 1a), enkephalin (ENK, 1b) or neuropeptide Y (NPY, 1c). The arrowed myenteric neuron, which lies at the edge of the ganglion, is immunoreactive for all three neuropeptides. × 920.

as in the submucous plexus[33] and these have a Dogiel type III morphology with intermediate length processes. The VIP/DYN myenteric neurons are also Dogiel type III.

Although most of the evidence for co-existence of neuropeptides in enteric neurons comes from studies on guinea pig ileum, there is also evidence for co-existence of neuropeptides in enteric neurons from other species[14]. However, the combination of peptides that occur together are not always the same as in guinea pig small intestine. In rats and pigs, for example, NPY in present in submucous neurons that contain VIP[18] whereas VIP-containing and NPY-containing submucous neurons form separate populations in the guinea pig small intestine.

The ultrastructure of neuropeptide-immunoreactive enteric nerves

Electron microscopic immunocytochemistry is a very useful tool for studying the connectivity of neuropeptide-containing neurons in the ENS. It provides two important kinds of information that light microscopic methods cannot. Since non-immunoreactive structures can be seen as well as immunoreactive ones by electron microscopy, it is possible to account for total populations of nerve fibers. Since synapses or close contacts can be observed with the electron microscope, it is possible to distinguish pre- and post-synaptic connections. Information on the ultrastructure of neuropeptide nerves, particularly in myenteric ganglia, is increasing[14] but most workers have examined enteric nerve cell bodies and nerve profiles that were immunoreactive for only a single neuropeptide. Since it is clear from light microscopic immunohistochemistry that enteric neurons contain multiple neuropeptides and that the same peptide can be present in several neurochemically distinct populations of neurons, any ultrastructural study that deals with tissue labeled for a single neuropeptide will provide information on several different populations of enteric nerves.

The first electron microscopic immunocytochemical observations on enteric nerves were published by Larsson in 1977[46], who demonstrated VIP-immunoreactive vesicles in nerve profiles in cat colon with a post-embedding staining technique. Since that time, there have been a number of ultrastructural investigations with pre- and post-embedding staining methods on VIP-, SP-, and ENK-positive nerve fiber profiles and nerve cell bodies in the enteric plexuses of various gut regions in several species[14]. SOM-[21a,56], CGRP-[24] and NPY-containing[23] enteric nerves have also been examined at the electron microscope level. These studies describe the occurrence, location and appearance of immunoreactive nerve cell bodies and nerve fiber profiles, the distribution of immunoreactivity within the fibers and cell bodies and the types of vesicles the immunoreactive fibers contain. In two papers neuropeptides have been shown by ultrastructural techniques to co-exist in the same nerve fiber profile, in one case by indirect methods[47], and in the other directly[61]. Neuropeptide-immunoreactive nerve profiles have sometimes been shown to form synaptic contacts on the cell bodies or processes of enteric neurons (e.g. fig. 2). In most cases, synapses were found in random ultrathin sections through immunocytochemically stained tissue. With this technology, NPY-immunoreactive[23] and SOM-immunoreactive[21a] synapses have been detected in guinea pig myenteric ganglia[23], VIP-immunoreactive synapses have been found in rat myenteric ganglia[25] and SP-immunoreactive synapses have been observed in human myenteric ganglia[48]. A more informative approach in terms of understanding ENS circuitry is to combine light and electron microscopy on the same tissue so that the location of synapses can be correlated with the distribution of immunoreactive fibers within a ganglion. In two studies baskets of immunoreactive fibers that were identified by light microscopy have been studied ultrastructurally. Synapses were found to occur where VIP-immunoreactive nerve fibers formed baskets around immunoreactive and non-immunoreactive nerve cell bodies in whole mount prep-

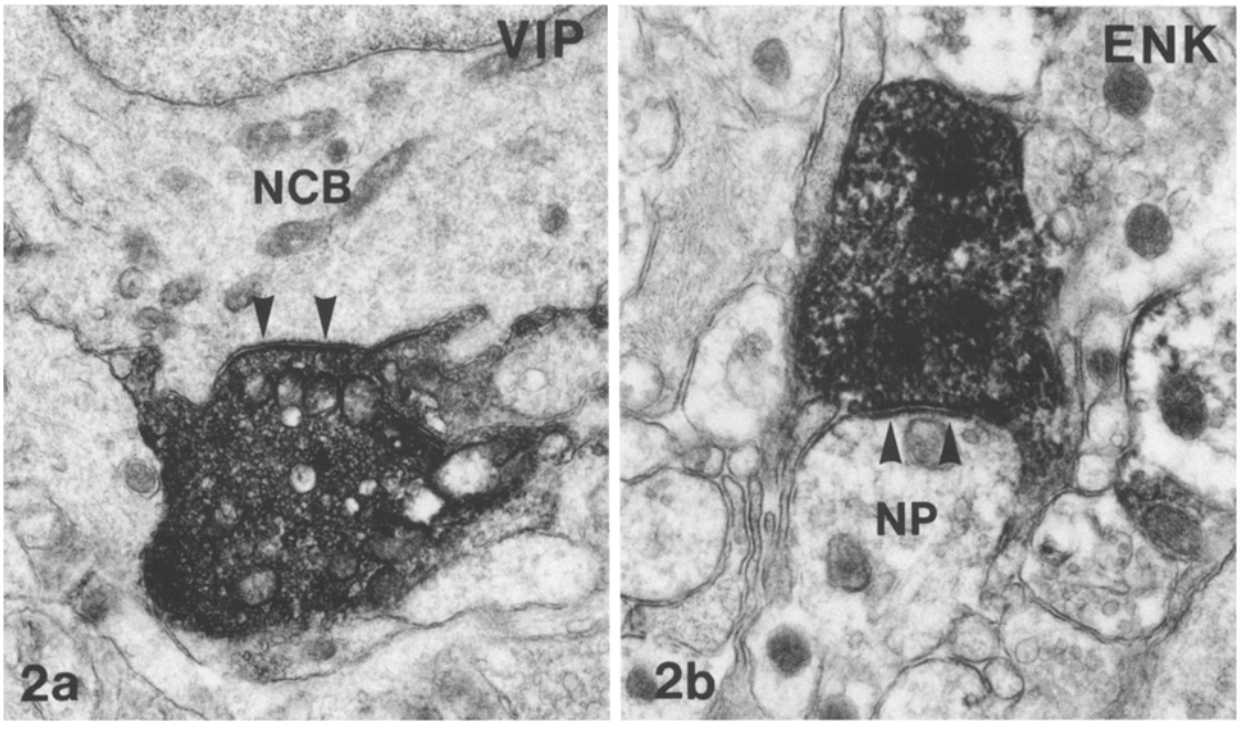

Figure 2. Electron micrographs showing synapses by neuropeptide-immunoreactive nerve fiber profiles in guinea pig myenteric ganglia. Figure 2a shows a VIP-immunoreactive nerve fiber profile forming a synapse (arrowheads) on a nerve cell body (NCB). × 24,000. Figure 2b shows an ENK-immunoreactive synapse (arrowheads) on a nerve process (NP). × 36,000.

arations of guinea pig myenteric ganglia[49] and when VIP-immunoreactive fibers formed baskets around submucous neurons in sections through rat submucous ganglia[51]. In guinea pig myenteric ganglia, ENK-immunoreactive nerve fibers in baskets also make synapses (own unpublished observations). Unfortunately, no studies have yet provided a systematic examination of the synaptology of enteric nerves immunoreactive for a single neuropeptide, or targeted the neuropeptide-containing synaptic inputs to a particular type of enteric neuron for fine structural investigation.

Projections of neuropeptide-immunoreactive enteric nerves

The development of lesioning techniques that allowed intrinsic nerve pathways to be analyzed was another significant step towards understanding the microcirucuitry of the ENS[27,32]. To define the projections of myenteric neurons, cuts are made around the circumference of the intestine through the longitudinal muscle and myenteric plexus (myotomy). If there are two cuts, the myenteric plexus can be left intact between them (double myotomy) or removed (myectomy). After these operations, immunoreactive material accumulates in the cut stumps of nerve fibers that are still attached to their cell bodies whereas nerve fibers severed from their cell bodies degenerate. In whole mount preparations of separated gut layers from operated animals processed for immunohistochemistry, the accumulations of immunoreactivity and the disappearance of fibers allow the polarity, lengths and minimum and maximum areas of innervation of myenteric neurons to be established. To aid in the study of the projections of submucous neurons, a short segment of gut is completely severed from and then rejoined to the rest of the intestine (homotopic autotransplant) and, after a few days, processed for immunohistochemistry[45]. The projections of enteric neurons outside the gastrointestinal tract are studied after disruption of pathways travelling to and from the small intestine through the mesenteric nerves (extrinsic denervation) or by retrograde transport.

These lesioning methods have been used most extensively for investigating the projections of chemically-identified neurons in the guinea pig ileum, Similar studies are now beginning to appear on neuropeptide-immunoreactive enteric neurons in rats[19–21], and dogs[15]. In guinea pig small intestine, immunoreactivity to a single neuropeptide is observed in several neuronal projections (see table)[14,29]. For example, VIP immunoreactivity seems to occur in 8 different types of enteric neuron[10,14,27,29]. Myenteric VIP neurons have three projections to ganglia: to other myenteric ganglia located more anally, to submucous ganglia after travelling anally through the myenteric plexus for several millimetres and to prevertebral ganglia. There are two projections of myenteric VIP neurons to the circular muscle: directly to circular muscle near the ganglion of origin or to circular muscle after travelling anally for several millimetres through the myenteric plexus. VIP-immunoreactive submucous neurons send processes to the mucosa, to other submucous ganglia and to submucosal arterioles. Of the other neuropeptide-containing neurons in the ENS, DYN-immunoreactive enteric neurons have been shown to have 6 different projections within the guinea pig small intestine; CCK neurons, ENK neurons, SOM neurons and SP neurons each have 5 different projections, GRP neurons and NPY neurons each have 4 different projections; and CGRP neurons have two projections. Since in lesion experiments the connections between cell bodies and terminals are deduced from patterns of degeneration, it cannot always be ascertained whether a particular nerve cell supplies a single target or whether some types of neuron could send collaterals to several different targets. This question is beginning to be answered through lesion experiments in which results from double immunofluorescent labelling are cross-correlated. For all the cases studied so far, it has been found that enteric neurons containing a specific combination of neuropeptides have a well-defined projection, sending their axons in a specific direction for a specific distance to a specific target[13]. For example, the VIP/DYN/ENK/NPY myenteric neurons of the guinea pig small intestine are the VIP neurons that project directly to the circular muscle supplying varicose axons to the circular muscle and deep muscular plexuses. The VIP/DYN/ENK/GRP/CCK myenteric neurons are the population that send their axons to the prevertebral ganglia where baskets are formed around some sympathetic neurons[50]. The VIP/DYN/GRP neurons have processes that travel anally through the myenteric plexus before supplying the circular muscle. To

Projections of enteric neurons immunoreactive for single neuropeptides in guinea pig small intestine

Neuropeptide	Cell body location	Projection
VIP	Myenteric ganglia	Anally to other myenteric ganglia
		Anally through the myenteric plexus and then to submucous ganglia
		To prevertebral ganglia
		Anally through the myenteric plexus and then to circular muscle
		Directly to circular muscle
	Submucous ganglia	To other submucous ganglia
		To the mucosa
		To submucous blood vessels
DYN	Myenteric ganglia	Anally to other myenteric ganglia
		Anally through the myenteric plexus and then to submucous ganglia
		To prevertebral ganglia
		Anally through the myenteric plexus and then to circular muscle
		Directly to circular muscle
	Submucous ganglia	To the mucosa
CCK	Myenteric ganglia	Anally to other myenteric ganglia
		Directly to submucous ganglia
		To prevertebral ganglia
		Directly to the mucosa
	Submucous ganglia	Directly to the mucosa
ENK	Myenteric ganglia	Orally to other myenteric ganglia
		Directly to submucous ganglia
		To prevertebral ganglia
		Orally to the circular muscle
		Anally to the circular muscle
SOM	Myenteric ganglia	To local myenteric ganglia
		Anally to other myenteric ganglia
		Directly to submucous ganglia
		Directly to the mucosa
	Submucous ganglia	To the mucosa
SP	Myenteric ganglia	To local myenteric ganglia
		Directly to submucous ganglia
		Directly to circular muscle
		Directly to the mucosa
	Submucous ganglia	To the mucosa
GRP	Myenteric ganglia	Anally to other myenteric ganglia
		Anally through the myenteric plexus and then to submucous ganglia
		To prevertebral ganglia
		Anally through the myenteric plexus and then to circular muscle
NPY	Myenteric ganglia	To ganglion of origin and more anal myenteric ganglia
		Directly to circular muscle
		Directly to the mucosa
	Submucous ganglia	To the mucosa
CGRP	Myenteric ganglia	Directly to the mucosa
	Submucous ganglia	To the mucosa

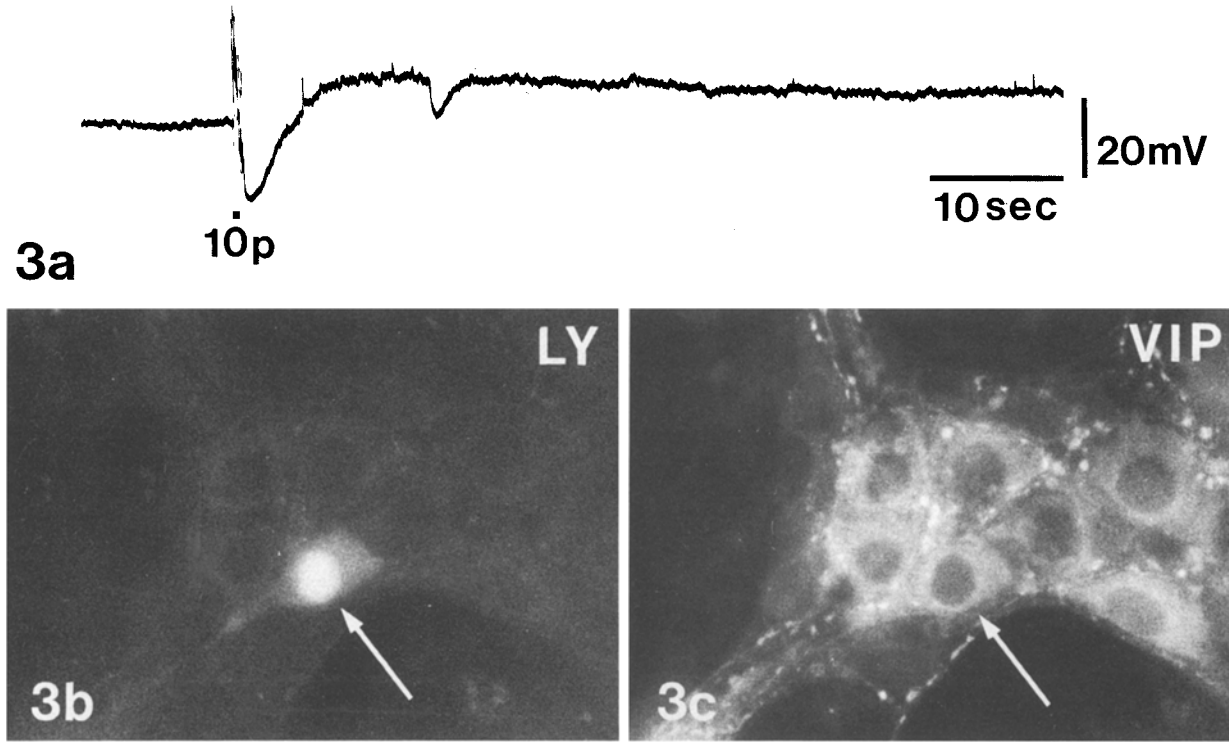

Figure 3. Correlation of electrophysiology and immunohistochemistry in a submucous neuron from guinea pig small intestine. Figure 3a shows an electrophysiological recording of an inhibitory synaptic potential and a slow excitatory synaptic potential in a submucous neuron after a stimulus of 10 pulses. The recording electrode contained Lucifer Yellow (LY) and 0.5 M KCl. The neuron was filled with LY after electrophysiological characterization and processed for immunohistochemistry to reveal VIP. The fluorescence micrographs show that the LY-filled neuron (arrow in fig. 3b) is immunoreactive for VIP (arrow in fig. 3c). The trace and micrographs were kindly supplied by Dr Joel Bornstein.

date, the branching pattern of only one type of neuropeptide-immunoreactive enteric neuron, the ChAT/NPY/CCK/SOM/CGRP neurons with cell bodies in both myenteric and submucous ganglia, has been visualized in its entirety[33]. This neuron type supplies terminals to only a single target, the mucosal epithelium.

Lesion experiments on guinea pig small intestine have also been valuable for defining the locations of the cell bodies of motor neurons. The motor neurons to the circular muscle must have their cell bodies in myenteric ganglia since no nerve fibers are present in the cirucular muscle of myectomized, extrinsically denervated animals on ultrastructural examination[62]. The cell bodies of the secretomotor neurons lie primarily in the submucous ganglia since the density of terminals in the mucosal plexus is not significantly altered after homotopic autotransplant, myectomy or extrinsic denervation[45].

Neuropeptide content and electrophysiology

A necessary step in the analysis of enteric circuitry is to define the functions of neurochemically-distinct populations of enteric neurons. One method for doing this is to combine electrophysiology with immunohistochemistry (fig. 3). Intracellular microelectrodes are used to record the properties of enteric neurons, which are then filled with dye[41] and processed to reveal their neuropeptide content[6,43]. This approach has shown that in both myenteric and submucous plexuses the peptide content of neurons correlates with the synaptic inputs they receive.

Myenteric neurons from guinea pig small intestine can be classified into two types on the basis of their responses to stimulation. S (for synaptic) neurons receive inputs through which fast excitatory synaptic potentials (ESP) are mediated; AH (for after-hyperpolarizing) neurons rarely have fast ESP but show hyperpolarizations that last for many seconds following action potentials in their somas[39,54]. After electrophysiological characterization and injection of fluorescent dye[6] or horseradish peroxidase[22], most S cells have been found to have Dogiel type I morphologies whereas AH neurons were found to be Dogiel type II. Furthermore, about half of the S cells in an electrophysiologically-identified sample of myenteric neurons were found to be immunoreactive for ENK whereas AH cells were always ENK-negative[6]. VIP-immunoreactivity is also absent from AH cells but some S cells contain this peptide[43]. Since VIP and ENK co-exist in many Dogiel type I myenteric neurons, it seems likely that these two studies were examining overlapping populations of S cells.

Almost all submucous neurons have fast ESP and a proportion also show inhibitory synaptic potentials (ISP) and slow ESP[40,60]. ISP were found almost exclusively in VIP-immunoreactive neurons (which are non-cholinergic – see above) when dye-filled, characterized submucous neurons were processed to reveal neuropeptide immunoreactivity[4]. VIP neurons also had fast ESP and usually slow ESP as well. The source of the ISP-producing fibers has been unclear until recently. The fibers producing the ISP were originally thought to arise from cell bodies in the enteric ganglia because some submucous neurons showed ISP after extrinsic denervation[40]. However, ISP in submucous neurons could be blocked pharmacologically by guanethidine or alpha adrenoreceptor antagonists[40,53], suggesting that the ISP were initiated by the extrinsic sympathetic nerves. It is now known from experiments after myectomy and extrinsic denervation that both intrinsic fibers and extrinsic noradrenergic fibers can cause ISP in VIP-immunoreactive submucous neurons[5]. Unlike VIP neurons, NPY submucous neurons (which are

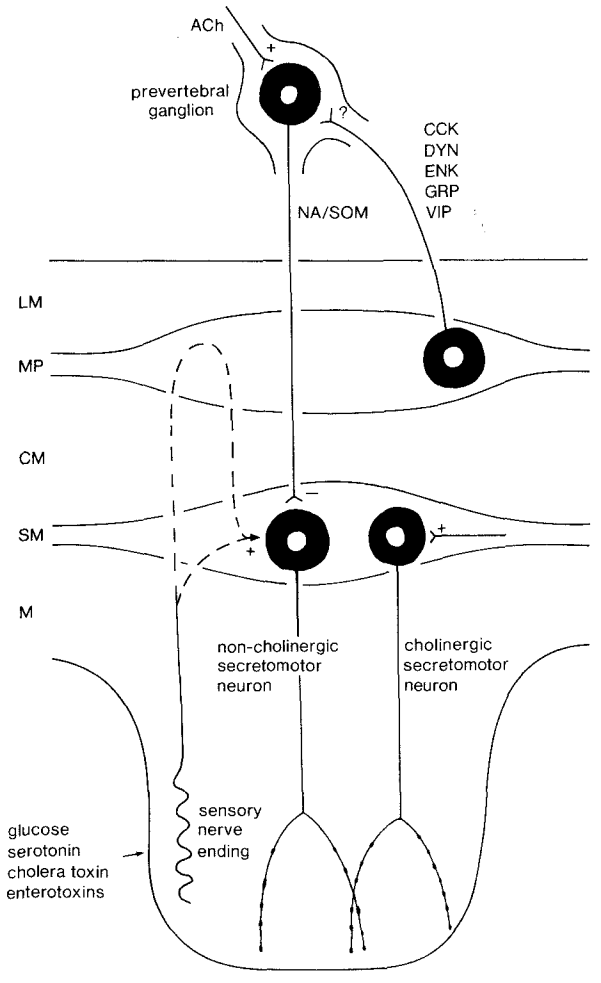

Figure 4. Secretomotor pathways in the intestine deduced from physiological, pharmacological, electrophysiological and immunohistochemical evidence. The chemical coding of the noradrenergic and intestinofugal neurons applies to the guinea pig small intestine. Exposure of the mucosa (M) to glucose, serotonin, cholera toxin or enterotoxins stimulates sensory nerve endings. A reflex is initiated that activates primarily the non-cholinergic secretomotor neurons, whose cell bodies, along with those of the cholinergic secretomotor neurons, are concentrated in the submucous plexus (SM). The intrinsic neurons that are involved in the transmission of the signal from the mucosa to the secretomotor neurons have not been identified. Noradrenergic neurons in prevertebral ganglia tonically suppress the non-cholinergic secretomotor neurons. Neurons in the myenteric plexus (MP) project to the prevertebral ganglia where they are presumed to excite the noradrenergic neurons. LM, longitudinal muscle; CM, circular muscle; ACh, acetylcholine, CCK, cholecystokinin; DYN, dynorphin; ENK, enkephalin; GRP, gastrin-releasing peptide; NA, noradrenaline; SOM, somatostatin; VIP, vasoactive intestinal peptide. Reproduced with permission from Furness and Costa (1987) 'The Enteric Nervous System', published by Churchill Livingstone.

cholinergic – see above) do not have inhibitory inputs but do show fast ESP[4]. After removal of the myenteric plexus some of the fast inputs to submucous neurons persist[7], indicating that some of the fast ESP on submucous neurons arise from myenteric neurons and some come from other submucous neurons.

There is now good physiological evidence that intrinsic secretomotor reflexes control water and electrolyte transport across the mucosal epithelium[8, 58, 59]. VIP neurons, which enhance secretion, are involved and there is an inhibition of secretion by sympathetic nerves. These findings in conjunction with the anatomical and electrophysiological results summarized above allow a partial circuit diagram to be drawn for the neuronal control of secretomotor reflexes (fig. 4)[29, 44].

Concluding remarks

Analysis of the microcircuitry of the enteric nervous system is at an exciting stage. Light microscopical immunohistochemistry for neurotransmitter-specific markers, including neuropeptides, has revealed the existence of many chemically-distinct populations of enteric neurons. The synaptology of neuropeptide-immunoreactive nerves selected by light microscopy in whole mount preparations can be defined by electron microscopy. Lesioning studies have indicated that each of the neurochemically-distinct populations of enteric nerves has a precise projection to a specific target following a specific path. Immunohistochemistry is beginning to be used in conjunction with electrophysiology to show the relationship between the neurochemistry of an enteric neuron and its synaptic input. The data gained from all these techniques make it clear that enteric neurons are chemically coded and indicate that the neurochemical coding of an enteric neuron may correlate with its position in the intrinsic circuitry of the ENS. By combining the information gained from the kinds of anatomical and electrophysiological studies discussed in this article with physiological data, it is becoming possible to establish the positions of the different populations of enteric neurons within neuronal circuits in the ENS and thereby define their roles in the pathways that control gastrointestinal function.

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Peptides in the mammalian cardiovascular system

by J. Wharton and S. Gulbenkian

Department of Histochemistry, Royal Postgraduate Medical School, Du Cane Road, London W12 OHS (England)

Summary. Ample immunocytochemical evidence is now available demonstrating that several peptides are present in the mammalian cardiovascular system where they are localised to nerve fibres and myocardial cells. The neuropeptides (neuropeptide Y, calcitonin gene-related peptide, tachykinins and vasoactive intestinal polypeptide) are localised to large secretory vesicles in subpopulations of afferent or efferent nerves supplying the heart and vasculature of several mammals, including man. Although they often exert potent pharmacological effects on the tissues in which they occur their physiological significance has still to be established. They may act directly via specific receptors and/or indirectly by influencing the release and action of other cardiovascular transmitters. In marked contrast, atrial natriuretic peptide is produced by cardiac myocytes and considered to act as a circulating hormone.
Key words. Peptides; cardiovascular system; immunocytochemistry; neuropeptide Y; calcitonin gene-related peptide; tachykinins; substance P; vasoactive intestinal polypeptide; atrial natriuretic peptide.

Introduction

It is now recognised that in addition to classical sympathetic (noradrenaline) and parasympathetic (acetylcholine) transmitters, the subpopulations of nerve fibres supplying the cardiovascular system also contain other putative transmitters including several so-called regulatory peptides. Considerable advances have been made in our knowledge of cardiovascular innervation following the application of histochemical and ultrastructural methods[26, 95] but it is the recent use of immunocytochemical techniques which has allowed us to demonstrate the presence of peptides and transmitter synthesising enzymes in cardiovascular nerves and thus distinguish between different autonomic nerve types. In the future several other immunocytochemical markers maybe of value in studies of cardiovascular innervation. These include two membrane proteins, synapsin and synaptophysin, specifically associated with the small secretory vesicles that store classical transmitters in nerve terminals[146, 153, 154, 218]; and the neuronal cytoplasmic protein, protein gene product 9.5 (PGP 9.5), which was originally extracted from human brain[107] and is present throughout the cardiovascular innervation[95].

In this article we review the immunocytochemical and pharmacological evidence concerning the localisation and actions of regulatory peptides in the mammalian heart and blood vessels. Of the peptides identified to date in cardiovascular nerves the most widely distributed are neuropeptide Y, calcitonin gene-related peptide, the tachykinins and vasoactive intestinal polypeptide.

Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid peptide, originally extracted from porcine brain and chemically characterised as having a C-terminal tyrosine amide group[192]. It belongs to a group of peptides which have a high degree of sequence homology, including pancreatic polypeptide (PP) and peptide YY (PYY)[68]. Sequence analysis of the cDNA encoding human NPY has revealed that the prepro-NPY molecule consists of 97 amino acids and its predicted post-translational processing yields three peptides corresponding to the signal peptide (28 amino acids), NPY (36 amino acids) and the C-flanking peptide of NPY (CPON, 30 amino acids)[148]. CPON immunoreactivity occurs naturally in mammalian tissues[7] and has an identical distribution to NPY in both the nervous system and adrenal medulla[93]. NPY/CPON-immunoreactive nerve fibres appear to be the most abundant of all the peptide-containing nerve populations identified to date in the mammalian cardiovascular system. High concentrations of both peptide sequences are found in the heart[7, 8, 92] where they occur in nerve fibres associated with the endocardium, myocardium, and coronary vessels and in epicardial nerves. The number of immunoreactive fibres tends to be greater in the atria than the ventricles and higher in the right atrium than the left. NPY/CPON-immunoreactive nerve fibres are also distributed around arteries (elastic and muscular) throughout the vascular system, forming an outer network of nerve bundles containing preterminal axons, running mainly parallel to the vessel and a perivascular plexus of fine, mainly varicose fibres and fascicles running around the vessel at the adventitial-medial border[61, 62, 67, 93, 149, 202]. The density of the perivascular plexus varies in different species, as well as with vessel size and site. The immunostained nerve fibres are usually confined to the adventitial-medial border of systemic vessels, however, nerve fibres are known to penetrate the media of some large arteries in a number of species[26, 85]. We have observed NPY/CPON-immunoreactive nerve fibres in the outer media of the pig elastic pulmonary artery, running in a circular direction, in association with both the vasa vasorum and smooth muscle cells between the elastic laminae.

Most of the published studies concerning the distribution of NPY-immunoreactive nerves in the vascular system have used rats, guinea pig and cat tissues[61, 62, 67, 91, 93, 134, 143, 149], but the presence of NPY-immunoreactivity has also been noted in human omental[54], mesenteric[67], skin[108] and cerebral vessels[6]. We have localised NPY/CPON immunoreactivity to nerves around human spinal (fig. 2), coronary, pulmonary (fig. 5), renal, gastric, splenic and mesenteric blood vessels. These immunoreactive nerves occur in a perivascular plexus around both arteries and veins, the plexus being less dense in the latter, and represent a subpopulation of the total innervation which displays PGP 9.5-immunoreactivity (fig. 1).

Combined immunocytochemical and denervation studies have demonstrated that the distribution of NPY-immuno-