Enteric neural circuits underlying propulsion of content in the large intestine of mammals

By

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ENTERIC NEURAL CIRCUITS UNDERLYING PROPULSION OF CONTENT IN THE LARGE INTESTINE OF MAMMALS

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Summary

The enteric nervous system (ENS) plays a central role in mammalian gut physiology and is essential for normal function. Individual nerve cells contribute to the formation of complex functional circuits; their projections travel within and across the layers of the gut wall to form close associations with other neurons, smooth muscle cells and cells of the immune and endocrine systems. The circuits of the ENS take part in the initiation and control of gut motor patterns, they influence mucosal secretion, blood vessel tone and function of neuro-endocrine cells. They provide sensory feedback to the central nervous system and in turn are influenced by input from spinal efferent nerves. Over the last few decades, multiple distinct populations of ENS nerve cells have been described according to characteristics such as morphology, electrophysiology and immunoreactive content. However, our understanding of how these neurons are connected, to form physical and functional circuits, is far from complete.

The work presented in this thesis advances the detailed knowledge of connectivity of distinct neural circuits in the enteric nervous system of the mammalian colon. Chapter 1 is a review of current literature relevant to this project. In Chapter 2, we describe a novel circuit that involves the intrinsic sensory neurons of the gut preferentially directing their synaptic output onto populations of calretinin-containing neurons within the myenteric plexus of the guinea pig colon. We expand on this finding in Chapter 3, where neuronal tracing confirms our initial hypotheses and expands on previous knowledge of neuronal projections in the colon.

Chapter 4 explores the connectivity of intrinsic sensory neurons using immunohistochemistry and tissue culture in the mouse colon. Given the advancing genetic manipulation techniques

in this animal, it is of great interest to understand the connectivity within its ENS more fully and compare with already described systems.

Chapters 2-4 are primary research chapters based on articles published in peer-reviewed journals during the PhD candidature. These chapters contain the original published material formatted to match the rest of this thesis. Dr David Smolilo was the first author for each of these publications. Every chapter contains an outline of the contribution of individual joint authors to the work and a Co-Authorship Approval Form has been submitted in the appendix together with a PDF copy of all published material.

The references are listed at the end of each respective chapter.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed:

Dr David Smolilo

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Publications

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Chapter 1

Review of Literature

1.1. Structure of the mammalian gastrointestinal tract

The focus of this review is on principles shared across the enteric nervous system of all mammalian species studied, including human. The guinea pig has been the most studied animal in enteric neuroscience over the last few decades, therefore experimental data in this thesis relates to the guinea pig ENS unless otherwise stated.

1.1.1. Embryology of the gastrointestinal tract

The mammalian gastrointestinal tract (GIT) is a long muscular tube whose lumen is continuous with the outside environment. During early development, three flat layers of the embryonic disc undergo a complex series of folding manoeuvres to form enclosed body cavities [1]. The gut tube passes through these cavities but is open at the oral and anal end. It retains its connection to the body wall by a mesentery (a double layer of peritoneum) which allows passage of blood vessels, lymphatics and nerves (Figure 1.1). As the gut folds and grows in length, a population of neural crest cells starts their migration from the site of the developing spinal cord. They continue to divide and differentiate during their long journey and eventually populate the entire gut to become neurons of the enteric nervous system. Problems with this stage of development may result in sections of gut that lack a nervous system and do not function normally e.g. Hirschsprung disease.

The most basic division of the GIT is into a foregut, midgut and hindgut. These regions are associated with accessory organs and glands, derived from common tissue lineages. They obtain most of their blood supply from three major branches of the ventral aorta; the coeliac, superior mesenteric and inferior mesenteric arteries travel within the mesentery to supply the foregut, midgut and hindgut, respectively. They are closely accompanied by nerve fibres and lymphatics.

Structures proximal to the oesophagus are grouped together and classified as the pharyngeal gut, they are particularly important for the development of the head and neck. The oral cavity and associated structures arise from several discrete regions of the cephalad embryo with a contribution from migratory crest cells.

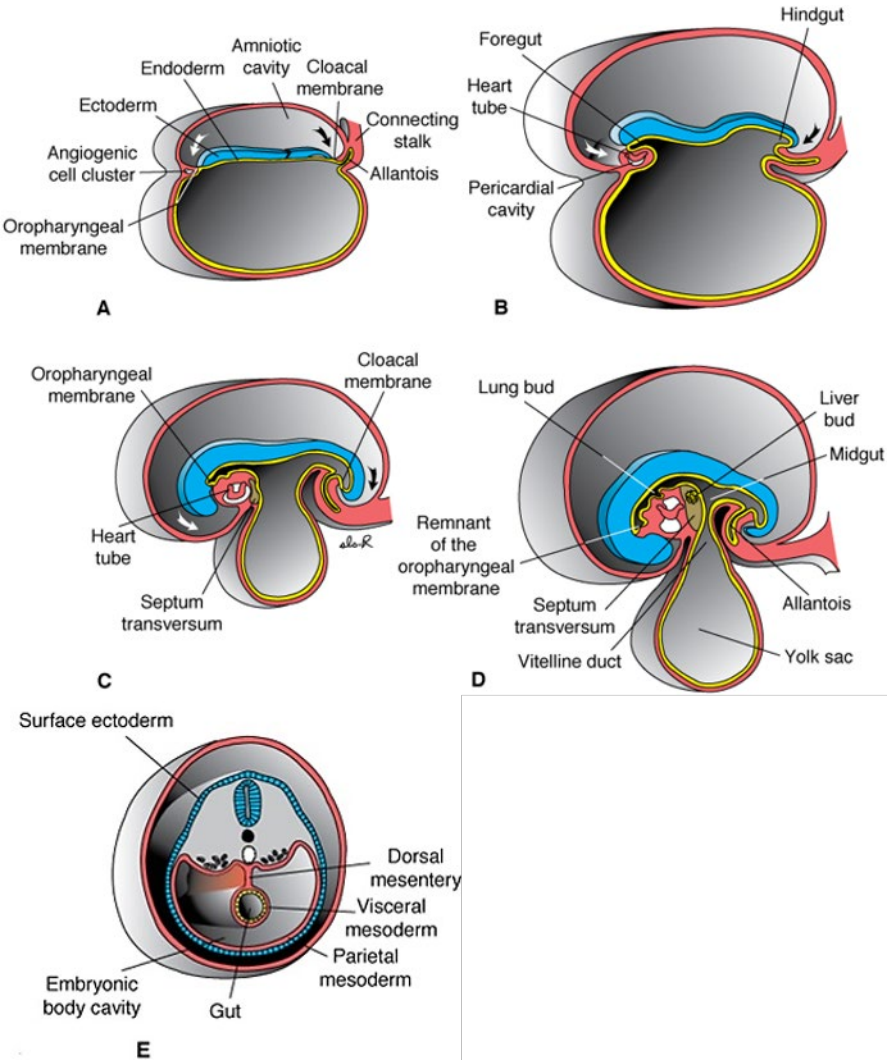

Figure 1.1 Mid-sagittal (A-D) and transverse (E) sections through a developing embryo illustrating the sequence of cephalocaudal folding, resulting in the formation of the gut tube and mesentery. Figure adapted from Langman's Medical Embryology 13th Ed 2014 [1].

1.1.2. Foregut

In the mature animal, between mouth and anus, we find several regions that are structurally and functionally distinct. The foregut encompasses the oesophagus, stomach and proximal duodenum. The liver and pancreas, organs that play vital roles in digestion and metabolism, also derive from this region and drain bile and pancreatic enzymes into the duodenum. In addition, the respiratory system starts as an outgrowth of the ventral foregut wall, which initially forms the trachea and lung buds.

1.1.3. Midgut

Midgut structures include the part of duodenum distal to the opening of the bile duct and the entire small intestine, divided into a proximal jejunum and distal ileum. The large intestine is also a midgut derivative, but only up to the splenic flexure, where we find the confluence of the superior and inferior mesenteric artery territories.

1.1.4. Hindgut

The hindgut contributes to formation of the descending colon, sigmoid, rectum and upper part of anal canal. It also forms part of the urogenital system via its ventral component. Each of the above sections are further anatomically subdivided. The duodenum for example has four distinct parts, followed by the jejunum and ileum of the small bowel. The large bowel includes the caecum, appendix, ascending, transverse, descending and sigmoid colon. The rectum is also usually described as having an upper, mid and lower section, and the anal canal is divided into an inner and outer muscular sphincter complex. The structural divisions do not always coincide with functional differences, which tend to be more gradual in nature.

1.1.5. Layers of gut wall

The wall of the gut has clearly defined tissue layers (Figure 1.2). The outside of the tube is covered by a thin layer of visceral peritoneum, known as the serosa. The muscular component of the gut is found in two layers, namely the longitudinal and circular muscle layers [2]. The nomenclature is consistent with the direction of muscle fibres relative to the axis of gut lumen i.e. longitudinal muscle fibres run parallel to the lumen whereas circular muscle fibres are oriented in a circumferential (perpendicular) plane.

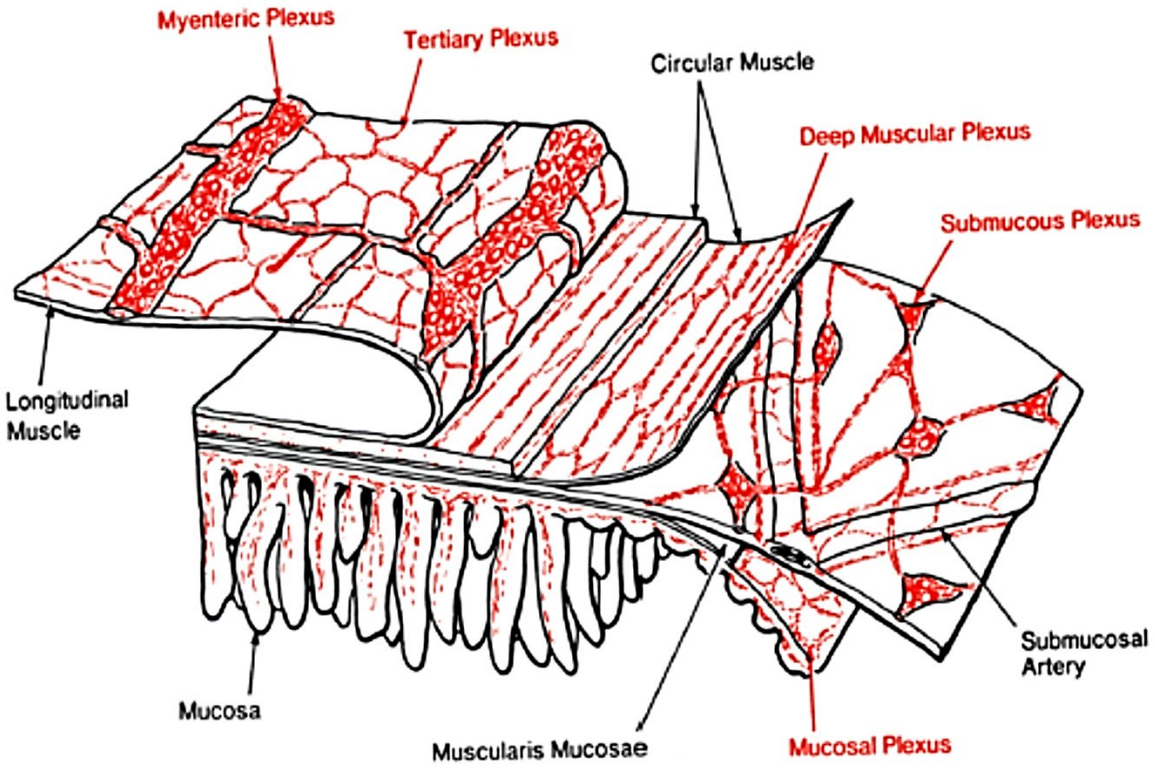

Figure 1.2 Figure illustrating the distinct layers of the gut wall with all of the neural elements coloured red. Adapted from Furness and Costa 'Types of nerves in the enteric nervous system' [3]

The mucosa forms the innermost layer of the gut, representing a physical barrier between the external world and the inner environment of the organism. It comprises the epithelium, lamina propria and muscularis mucosae. This barrier is at times only one cell layer thick. The mucosa folds to form villi and crypts, with some cells also characterised by micro-projections (microvilli). Both these features increase surface area greatly. Many different cell types make up the mucosal epithelial layer with functional adaptations that include a combination of secretion, absorption and protection.

The lamina propria supports the mucosa and contains enteric nerves (submucosal plexus), lymphatics and blood vessels, embedded in loose connective tissue. The muscularis mucosa consists of a thin layer of smooth muscle whose likely function is to allow the mucosa to fold and move.

The majority of enteric neurons are found in two separate layers. The myenteric plexus (Auerbach's plexus) lies between the longitudinal and circular muscle, while the submucosal plexus (Meissner's plexus) lies between the circular muscle and the muscularis mucosa [2]. Neuronal projections travel extensively between the two plexuses as they do to all of the layers of the gut wall.

1.2 Overview of the enteric nervous system

1.2.1 Organisation of the enteric nervous system

Within the wall of the gastrointestinal tract, a complex network of neurons make up the Enteric Nervous System (ENS). An estimated 200 to 600 million neurons comprise the ENS in the human gut, a number which approximates the population of neurons in the spinal cord [4]. The

ENS has been considered for a long time a part of the autonomic nervous system together with the sympathetic and parasympathetic divisions [5]. Separate from the brain and the spinal cord (the central nervous system, CNS) and confined to the periphery. In fact, if we step back and look at this from an evolutionary perspective, we see from the fossil record that the ENS likely developed independently, and preceded the appearance of the central nervous system, making it our ‘first brain’ so to speak [6].

Each ganglionated enteric plexus is a dense web of internodal strands speckled with thousands of ganglia. Each ganglion contains multiple neurons and each neuron gives off projections carrying message to local and distant targets. The myenteric plexus extends from the upper oesophagus to the internal anal sphincter and is mostly responsible for coordinating gut motility. The submucous plexus does not appear in the oesophagus and stomach, it contains less neurons then the myenteric plexus and is mainly involved in the regulation of secretory and vascular functions of the mucosa. Not surprisingly, accessory organs including the gallbladder, biliary tree and pancreas also contain ganglia, which are considered part of the ENS.

There is bidirectional flow of information between the ENS and CNS and their respective roles vary considerably along the GIT. The CNS is vital in controlling the striated muscle of the upper oesophagus and oropharynx and plays a major role in controlling defecation at the other end of the gut. The functioning of the small intestine and colon on the other hand is fully reliant on the ENS, with extrinsic input having a modulatory role. The importance of the ENS to normal gut function is highlighted by pathological states where the ENS is congenitally absent or undergoes degeneration through an acquired disease. Hirschsprung’s disease is characterised by segments of colon with no ENS and the result is a functional large bowel obstruction that may be lethal in the newborn if not surgically treated [7]. Chagas’ disease,

due to a protozoan infection, causes degeneration within the ENS of an adult, with a failure of colonic motility resulting in megacolon as one of the most common clinical presentations [8, 9]. In contrast, disrupting the extrinsic innervation of the gut has far less dramatic consequences and in fact, the intestine displays multiple reflex motility patterns after complete separation from the rest of the body.

1.2.2 Classification of enteric neurons

Enteric neurons show significant variation in morphology, neurochemical content, axonal projections and electrophysiology. Grouping nerve cells based on shared characteristics creates a multitude of distinct populations. In the guinea pig distal colon, for example, there are at least 17 types of neurons described [10].

Consistent with the gut's capacity for autonomous coordination of complex behaviours [11], enteric neurons can also be classified into three classic functional groups of sensory neurons, interneurons and motor neurons. Together they form circuits, which control intestinal functions. Sensory neurons (also known as intrinsic primary afferent neurons, IPANs) respond to various stimuli and initiate a patterned response. Interneurons have either ascending or descending projections, which propagate signals along the length and circumference of the bowel wall. Motor neurons translate the sum of the ENS activity into physical movement by driving the smooth muscle of the gut wall. As discussed in later sections of this thesis, we are increasingly starting to appreciate that a large proportion of enteric neurons have more than one functional role [12].

Based on the above information we can assume multiple subclasses of sensory, interneurons and motor neurons, forming distinct circuits, each responsible for a specific enteric function or

perhaps an aspect of a function. Inter-regional and inter-species variation in neurochemical markers of functionally homologous groups adds to the complexity of this topic.

Morphology

Dogiel provided one of the earliest and most influential morphological studies of enteric neurons and a classification system we still use today (See [13] for a review). His original classification system is commonly reduced to contain two main classes of neurons, particularly in small mammals; Dogiel type I neurons have a small cell body with multiple dendrites protruding from along its lateral edge and one single long process that is assumed to be an axon. Dogiel type II neurons have large round or oval cell bodies that give rise to multiple long and short processes in various arrangements.

Rather than being purely descriptive, his observations sought to deduce possible functional roles from the structural features observed. Based on his studies he was the first to suggest that type II neurons have projection patterns that could be consistent with a sensory function. He also described type I neurons whose long projections terminated in smooth muscle, proposing them to be motor neurons.

Projections

Neuroanatomical tracing has been widely used to characterise neuronal pathways, including in the enteric nervous system [14]. Techniques such as electrophysiological mapping, retrograde and anterograde tracing, in combination with immunohistochemistry have revealed in detail the organised nature of the ENS. From these studies, we know the expected polarity and length of projections of many classes of enteric neurons and the expected target. These data have transformed what was initially an entangled mess of nerve cells and projections into

a reliable map, which is particularly important when planning or interpreting data from functional experiments.

Chemical content

Enteric neurons within a specific species and regions of gut express a combination of chemicals and proteins, some of them neurotransmitters. The progression from simple stains through to histochemical and immunohistochemical methods has allowed cataloguing of enteric neurons according to the combinations of these markers within them. Markers found in the soma of a neuron usually correspond to the markers in the varicose terminals of the same cell, but this is not always the case [15]. Certain markers correspond to neurotransmitters with predictable physiological actions, this makes it possible to make tentative assumptions about the functional nature of neurons that contain them. A good example is acetylcholine (ACh) and nitric oxide synthase (NOS), which are markers of excitatory and inhibitory neurons respectively. However, since markers in the cell body are not always found in its varicose projections, functional inferences based on cell body content alone should be interpreted with caution.

1.3 Neurotransmitters in the ENS

The majority of neurochemicals contained within enteric neurons do not appear to function as neurotransmitters, however there are several neurotransmitters that have been characterised.

1.3.1 Inhibitory neurotransmitters

Evidence for inhibitory neurotransmission in the gut came about in the 1960s, when Burnstock et al measured inhibitory responses in colonic taenia using intracellular and sucrose gap recording techniques [16, 17]. The observed hyperpolarisation was resistant both to

acetylcholine and noradrenaline receptor antagonists and became known as non-cholinergic, non-adrenergic (NANC) neurotransmission. This initiated a search for inhibitory neurotransmitters within the ENS with several potential agents identified over the years.

What has emerged so far is that the inhibitory response in the gut is multi-factorial. When stimulated, enteric motor neurons likely co-release at least three different inhibitory mediators, although this does depend on the stimulus duration and magnitude. Nitric Oxide (NO), peptides (likely VIP) and purines are each responsible for a part of the post-junctional enteric inhibitory response. The relative contribution of each transmitter to this response tends to differ between regions of gut and species, but the concept of co-transmission is the same. As a general rule, in transmural nerve stimulation experiments, VIP release requires higher stimulus frequencies (5Hz or more) than NO and peptide release in order to elicit a peptide-mediated inhibitory response. Purines cause a fast, large amplitude depolarisation that last only for several hundred miliseconds, the response to NO is slower and can last for several seconds and VIP appears to cause the slowest response of the three agents [18]

Nitric Oxide (NO)

This is a major inhibitory neurotransmitter within the ENS [19]. Unlike other known neurotransmitters, NO is synthesised on demand and released as a gas, being produced by three different types of nitric oxide synthase (NOS) enzymes, namely neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), and has many known physiological roles apart from neurotransmission [20-22]. Since the initial experiments by Gillespie et al [23] on rat pelvic smooth muscle and Bult et al with experiments on canine gut [24], a great deal of data has shown that in the enteric nervous system NO is synthesized by neuronal nitric oxide synthase (nNOS), released by Dogiel type I enteric neurons [25, 26] and causes hyperpolarisation and relaxation of enteric smooth muscle [27]. Genetic knockout

experiments have confirmed that NO is synthesised on demand rather than being stored in vesicles and its synthesis is triggered by an increase in cellular Ca^{2+} [28]. The post-junctional effects of NO are dependent on cytoplasmic soluble guanylate cyclase (sGC) which activates the cGMP intracellular signalling pathway [29]. However, the exact mechanism of nitrergic gut muscle relaxation is still under investigation; smooth muscle forms a functional syncytium with interstitial cells, which makes it harder to separate the role of any single cell type (the section further on in this thesis entitled '*Role of Interstitial cells in the neural control of smooth muscle'* has a review of this topic).

Vasoactive intestinal polypeptide (VIP)

VIP was originally isolated from porcine small bowel and shown to have multiple biological actions, including that of potent vasodilatation [30]. Its discovery in neurons of the ENS that have descending and circular muscle projections prompted fairly early on the suggestion of an inhibitory neurotransmitter role [31]. VIP and NOS co-localize in a significant proportion of enteric neurons and circular muscle varicosities; in guinea pig distal colon 90% of myenteric VIP immunoreactive (VIP-IR) neurons also contained NOS and 65% of NOS-IR neurons contained VIP [10], in mouse small intestine almost 100% of NOS-IR neurons contain VIP and 75% of VIP-IR neurons contain NOS, the overlap between the two markers was similar in mouse colon [32]. Therefore, VIP is almost always present at the site of inhibitory (NOS) neurotransmission and is likely released at the same time when nerves are activated.

VIP is derived from a 170 amino acid precursor (prepro-VIP) together with peptide histidine isoleucine (PHI), both of them are stored in large dense-core vesicles in the ENS and released in response to a sustained increase in intracellular Ca^{2+} [33, 34], this usually requires multiple stimuli and is the reason that neuropeptides in peripheral organs are commonly associated with 'slow neurotransmission' [35].

In functional experiments vagal nerve stimulation caused VIP release, accompanied by relaxation of the stomach [36], exogenous VIP inhibited contraction of smooth muscle [37] and antibody-mediated VIP neutralisation caused a decrease in nerve-evoked relaxation by VIP and by electrical field stimulation [38]. More recently, using VIP knockout mice, Keef et al confirmed that VIP was responsible for an ultraslow component of inhibitory neuromuscular transmission in mouse internal anal sphincter [34].

Purines

Purinergic NANC neurotransmission is mediated by P2. P2 receptors are divided into ion-gated P2X and G-protein coupled P2Y receptors and further subdivided into multiple subtypes within each group ($P2X_{1\text{-}7}$ and $P2Y_{1,2,4,6,11,12,13}$) [39-41]. There is good evidence that small conductance Ca^{2+} sensitive K^{+} channels are involved [42, 43], and these are blocked by apamin [44].

ATP was put forward as a NANC inhibitory transmitter in the 1970's based on several muscle preparation experiments [45] and has had a lot of support for that role [46, 47]. More recent experiments have cast doubt on ATP as the purine inhibitory transmitter in the ENS. Mutafova-Yambolieva et al [48] have shown that β-Nicotinamide adenine dinucleotide (β-NAD) fulfils the classical criteria for a neurotransmitter better than NOS in mouse colon preparations. After blocking nitrergic neurotransmission, electrical field stimulation caused a frequency-dependent release of β-NAD from intrinsic neurons, which was blocked by tetrodotoxin (Na channel blocker) or ω-conotoxin GVIA (synthetic blocker of N-Type Ca^{2} channels); the response to β-NAD was apamin-sensitive and blocked by P2Y1 and P2Y2 receptor antagonists.

ATP release was not proportional to stimulation frequency and was significantly less sensitive to ω-conotoxin, which suggest that a significant proportion comes from sources other than inhibitory nerve terminals, furthermore the effects of exogenous ATP were sensitive to apamin but not to P2Y receptor antagonists [48]. Almost identical findings were reported in monkey and human colon [49], furthermore stimulation of myenteric neurons with ACh and Serotonin caused a disparate release of the two substances; β-NAD release, but not ATP, was inhibited by tetrodotoxin or ω-conotoxin GVIA [50]. The results strengthen the argument for β-NAD as the inhibitory purine neurotransmitter and suggest ATP is released from nerve cell bodies rather than motor nerve terminals. Two other substances have also been proposed as neurotransmitters in GIT muscles of human, monkey and mouse; ADP-ribose and uridine adenosine tetraphosphate (Up4A) satisfy both pre and post junctional requirements for a neurotransmitter [51, 52]. Like with most new theories, this one is not without its controversies, with suggestions of β-NAD being a pre-junctional modulator of neurotransmitter release rather than a neurotransmitter [53, 54]. More experiments are required to tease this out.

1.3.2 Excitatory neurotransmitters

There are two main mechanisms of excitatory synaptic transmission within the ENS; rapid neurotransmission via fast EPSPs mediated by neuro-transmitters that act on ligand-gated post-synaptic channels and slow EPSPs via G-protein-coupled receptors and second messenger pathways.

Acetylcholine (ACh)

Acetylcholine is the main excitatory neurotransmitter at the enteric neuro-neuronal synaptic junction and causes fast EPSPs in postsynaptic neurons via nicotinic receptors [55, 56]. Nicotinic receptors have been localized to ascending and circumferential myenteric pathways [57], and in functional experiments, blocking nicotinic receptors with hexamethonium caused

significant inhibition of reflex-activated ascending pathways while the descending inhibitory signal was mostly resistant [58]. This data indicated that ACh acting on nicotinic receptors is the predominant mechanism in ascending excitatory ENS pathways and provides a minor contribution to excitatory descending ENS pathways. There is also evidence for a minor role of muscarinic receptors in slow neuro-neuronal transmission [59, 60].

Serotonin (5-HT)

Serotonin mediates fast EPSPs by acting on 5-HT_3 receptors, which are ligand-gated ion channels [61]. Myenteric 5-HT neurons have long descending projections that provide input to other descending interneurons [62], they are found in excitatory descending pathways to circular muscle [63] and possibly participate in slow synaptic transmission to myenteric IPANs [64]. 5-HT receptors also locate to mucosal terminals of myenteric IPANs, where they possibly initiate reflex activity in response to 5-HT release by enterochromaffin cells [65], although the physiological role of serotonin in enteric circuits is far from established [66].

Purines

Purines (such as ATP) mediate fast EPSPs in enteric neurons by acting on P2X receptors [61]. These are not specific to any one sub-group of enteric neurons in the guinea pig gut, unless one examines the distribution of purine receptor subtypes. P2X(2) and P2X(3) are expressed in distinctive functional group of neurons [67, 68]; P2X(2) receptors seem to be specifically associated with fast synaptic transmission in descending motor pathways when one combines data from immunohistochemical and functional studies [67, 69, 70] and P2Y(1) (metabotropic) receptors likely contribute to slow synaptic transmission in descending pathways [71].

Tachykinins

Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are tachykinin peptides found in the GIT [72], although NKB is relatively sparse [73]. Three tachykinin receptors have currently been well characterised; NK1-3 receptors couple to GTP-binding proteins that use the phospholipase C/phosphoinositide signalling pathway [74] and mediated slow EPSPs. Tachykinins (particularly SP) are widespread throughout the ENS, not specific to any functional groups of neurons and usually co-localize with the synthetic enzyme for ACh, choline acetyltransferase (ChAT) [75-77]. Having said that, tachykinins play an important and specific role in slow synaptic transmission between IPANs of the guinea pig ileum [78-80].

1.3.3 *Other potential neurotransmitters within the ENS*

Glutamate is a major excitatory neurotransmitter within the central nervous system [81] and there is evidence for expression of glutamate, glutamate receptors and other proteins required for glutamate neurotransmission within the ENS [82, 83]. Despite this, there is not a lot of data that supports glutamate as a neurotransmitter within the ENS [84], although Swaminathan et al recently showed that glutamate may contribute to slow synaptic transmission in mouse distal colon [85].

γ-Aminobutyric Acid (GABA) is another central neurotransmitter that is easily detected in the ENS but whose function is elusive [86, 87], it may act more as a neuromodulator rather than a neurotransmitter as demonstrated in mouse ileum [88].

Cannabinoids, whether endogenous (anandamine, 2-AG), plant-derived or synthetic, have significant effects on the gastrointestinal system [89]. They are synthesized on-demand and appear to function as ‘retrograde synaptic signal molecules’ [90]. The CB1 receptor

associates with a range of cholinergic myenteric neurons in the guinea pig and rat [91] and functional studies have shown that cannabinoids inhibit intestinal motility, possibly by modulating cholinergic neurotransmission via pre- and post-synaptic mechanisms [92, 93]. The CB1 receptor also colocalises with all VIP and most of NPY-containing submucosal neurons and there is evidence that cannabinoid agonists inhibit mucosal secretion by modulating cholinergic secretomotor pathways [94]. The expression of the CB2 receptor in the gut is upregulated in states of inflammation, including inflammatory bowel diseases [95]. There is evidence for the endogenous cannabinoid system having a protective and regenerative role during inflammation of the gut, involving intrinsic and extrinsic signalling mechanisms [96].

1.4 Functional classification of enteric neurons

1.4.1 Sensory neurons

Neurons that detect changes in the physical or chemical environment of the tissue they innervate are essential to the function of any nervous system, including the ENS. They transduce and encode this information into a signal conveyed to integrative centres, where a response may be triggered based on the information received. Afferent neurons carry information towards central processing areas, such as reflex centres within the spinal cord or the myenteric plexus of the gut, where integration of multiple afferent signals takes place. Efferent neurons carry signals away from integrating circuits to effectors such as smooth muscle or secretory glands.

Only a subset of afferent neurons in the viscera are thought to underlie the conscious sensation of pain. It has been argued that only these neurons are correctly termed ‘sensory’ neurons, to distinguish them from the rest of the visceral afferents which lack direct

connections to the CNS, being confined to the viscera [97]. Despite this distinction, the term 'sensory' is often used more loosely (including in this thesis) to mean any component of the afferent pathway.

1.4.2 Intrinsic sensory neurons

Neurons located in the vagal, trigeminal and dorsal root ganglia of the central nervous system (CNS) provide sole sensory innervation for the whole body. The gut is an exception to this arrangement; it has a dual sensory innervation. In addition to extrinsic afferents, it contains an intrinsic population of afferent neurons located in the wall of the gastrointestinal tract. They are not strictly sensory neurons; being the first neuron of a reflex loop, their main role is to carry information to integrating circuits of the ENS. Although the presence of primary sensory neurons within the gut has been assumed for a long time, the proof of their existence has come much more recently. Furness et al demonstrated that motility reflexes may be initiated in guinea pig small intestine that has been isolated in organotypic culture long enough to allowing the complete degeneration of all extrinsic nerve fibres, ruling out the possibility of extrinsic circuits being involved in reflex initiation [98]. The heart is another organ that contains an intrinsic ganglionated neuronal network [99], possibly with its own intrinsic primary afferent neurons [100] however, there is not as much experimental data to date to support this.

Intrinsic Primary Afferent Neurons (IPANs)

IPANs are the first sensory neurons to be characterised in the guinea pig ileum [101]. They are Dogiel type II neurons that comprise an estimated 20% of the ENS neuron population [102, 103]. In the myenteric plexus, they have short local processes that ramify extensively, surrounding neurons in the same ganglion (including other IPANs). They also project to neighbouring myenteric ganglia, circular muscle, submucosal plexus and lamina propria of mucosa [104-106]. Although the majority of myenteric IPANs project locally or

circumferentially [107, 108], there is a sub-population with long descending projections, seen in both the small intestine and colon of the guinea pig [109, 110]. These cells with long descending projections also tend to have multiple short filamentous processes, and therefore have previously been described as ‘dendritic’ IPANs [104]. IPANs in the guinea pig and mouse colon interact with select groups of myenteric neurons via varicose ‘baskets’ which likely function to direct synaptic output and possibly form monosynaptic reflex arcs with circular muscle motor neurons [110, 111].

IPANs have AH type electrophysiological properties (AH-afterhyperpolarisation; a persistent membrane hyperpolarisation after an action potential) and respond to chemical stimulation of the mucosa [65, 101, 112, 113], stretch and compression [114-116], even when synaptic transmission is abolished. Furthermore, the magnitude of their response appears to be graded to strength of applied stimulus [117]. Stimulation of synaptic inputs to IPANs most commonly produces a slow excitatory postsynaptic potential (EPSP) characterised by depolarisation of cell membrane, blockade of the late AH and increase in somatic excitability [116, 118], this phenomenon peaks at about 15s and continues for as long as 4 minutes [119], although under certain condition a sustained slow EPSP may last for over 3 hours [118]. Slow EPSPs seem to be a consistant feature of AH cells, they are also present in mouse [116, 120] guinea pig [121], rat [122, 123] and even one human colon AH cell recording [60]. This slow, sustained change in membrane potential may function to make an IPAN more responsive to a sensory stimulus and possibly promote the transmission of the resultant signal to second order neurons in the network.

An interesting observation comes from a set of intracellular recordings from two myenteric neurons simultaneously [78]. It follows on from the study of synaptic inputs and demonstrates that varicosities of IPAN axons do in fact make functional synaptic connections with other

myenteric neurons, including other IPANS, and their synaptic transmission appears to be through slow EPSPs mediated by NK1 and NK3 dependent mechanisms [80, 124]. Combined with morphological and pharmacological studies, this suggests neighbouring IPANS form a self-reinforcing network in the myenteric plexus and rely predominantly on non-nicotinic neurotransmission.

Fast excitatory postsynaptic potentials (EPSP) are usually absent during intracellular recording from IPANs of mouse gut and occur rarely (<5%) in guinea pig [103, 120, 121, 125]. This finding is compatible with a sensory role, because it suggests IPANs are not driven by fast, synaptically mediated transmission. This is however, not quite consistent with other published observations; nicotinic acetylcholine and purinergic P2X receptors are known to mediate fast synaptic transmission [126] and these receptors are commonly found on IPANs in guinea pig intestine [67, 79, 127, 128]. More recently, calcium imaging of spontaneous and evoked myenteric neuronal activity in intact mouse colon demonstrated short latency calcium transients in 90% of IPANs examined [129]. A significant proportion of these would have been likely due to fast EPSPs. Another study used a voltage-sensitive dye in guinea pig ileum where all mechanosensitive calbindin-immunoreactive myenteric neurons received fast EPSPs in response to electrical stimulation of internodal strands [130]. In both the imaging studies, using hexamethonium (nicotinic receptor agonist) abolished the fast EPSPs, and in the mouse colon, application of DMPP (nicotinic receptor agonist) during complete synaptic blockade activated all IPANs, a finding that is consistent with the presence of nicotinic receptors on the neurons observed.

The reason for the discrepancy between functional imaging and microelectrode recordings is unknown. Interestingly, in a study looking at the pig small intestine, 77% of myenteric IPANS displayed fast EPSPs on intracellular recording [131]. Perhaps Dogiel type II cells in smaller

animals are more sensitive to microelectrode impalement compared to bigger species, although patch-clamp recordings, where there is no impalement of the cell, have not recorded fast EPSPs in mouse small intestine either [116]. The nature of synaptic input to IPANs is certainly an area where further investigation is required as we gather more data that points to functional polymodality of most ENS neurons.

Dogiel type II neurons are also found in the submucous plexus, but only in certain species and not others. This may relate to a greater complexity of the submucosal ENS in large mammals compared to the mouse or guinea pig. The submucous plexus of the mouse intestine consists of one layer of ganglia containing three types of neurons, all of them Dogiel type I [132]. In the Guinea pig, we find one layer of ganglia with four types of neurons, including Dogiel type II cells [133]. The pig has two distinct networks and 14 different types of neurons, including IPANS [134] and in the human intestine we find a third intermediate layer and possibly Dogiel type II cells as well [135-138]. When examined in the guinea pig intestine, submucosal Dogiel type II neurons send projections both to neurons within the submucosal network and the myenteric plexus [132, 133]. Using techniques that allow visualisation of calcium transients in enterocytes, Filzmayer et al [139] demonstrated that a significant proportion of submucosal neurons (regardless of their morphology) in the human and porcine colon are mechanosensitive (up to 24% in human colon).

The presence of classical putative IPANs and functionally mechanosensitive neurons in both plexuses of the larger animals raises the question of their respective roles. We know there is a functional division between the plexuses, with the myenteric plexus controlling gut motility and the submucosal plexus likely involved with mucosal secretion and absorption [140-144]. It is interesting to speculate whether the more complex submucosal network requires its own set of IPANs to drive the circuitry, whereas in the smaller species the myenteric IPANs suffice.

Mechanosensitive Dogiel type I neurons

It was not until recently that certain Dogiel type I neurons were shown to have mechano-sensory properties, and since then we have seen this group of neurons reported across several species and gut regions [12]. Mechanosensitive enteric neurons (MEN) may be grouped according to the type of mechanical stimulus they respond to (compression, tension or shear) and their firing pattern (rapid, slow or ultra-slow adaptation). Although Dogiel type II IPANs have certainly been shown to respond to mechanical stimulation [115, 116], the majority of mechanosensitive myenteric neurons are in fact a different population [12]. In both guinea pig and mouse intestine for example, interneurons and motor neurons responded in equal proportion to compression [130, 145]. Unlike Dogiel type II IPANs, these sensory neurons do not require muscle tension to fire [146], suggesting different populations of sensory neurons detect tension versus length in gut wall.

Spencer and Smith demonstrated tension-sensitive neurons in the guinea pig distal colon, which were all S (S – synaptic; receive synaptic input as seen by fast EPSPs on electrophysiological recordings) type cells [106]. Interestingly, two different populations of neurons are preferentially activated during circumferential versus longitudinal stretch [146]. Circumferential stretch activated ascending and descending cholinergic (excitatory) pathways, which in turn produced ascending muscle contraction and descending muscle inhibition. In contrast, longitudinal stretch triggered NOS containing (inhibitory) descending interneurons, which may lead to inhibition of muscle contraction. Tension-sensitive myenteric neurons have also been demonstrated in guinea pig ileum, stomach and oesophagus [130, 147] as well as in the sub-mucous plexus of the pig and human [139]. Several groups of mechanosensitive enteric neurons respond to both compression and tension, producing distinct firing patterns for the two different types of mechanical stress [12].

Mechanosensitive enteric neurons have not been a major consideration in our study of enteric circuits so far and their functional role is not fully understood. Given that contraction and distension of gut muscle causes deformation of myenteric ganglia and large changes in neuronal shape [130, 148], they are likely to be involved in controlling motility in some way. One possibility is that they are involved in local control of muscle tension; the tension-sensitive ultra-slowly-adapting mechanosensitive neurons in the stomach are predominantly inhibitory in nature and may be in part responsible for the phenomenon of adaptive volume accommodation [12]. The combined activity of compression and tension-sensitive motor neurons in the intestine could theoretically contribute to propulsive motor patterns that are independent of synaptic transmission. Studies have previously demonstrated resistance to hexamethonium of distension-induced reflexes in guinea pig ileum [149, 150] and colon [151, 152], giving credence to this idea.

Other types of mechanosensitive neurons associated with the ENS include intestinofugal neurons in the guinea pig and mouse colon [153, 154] and extrinsic neurons with afferent fibres in guinea pig oesophagus, stomach and rectum [155-157]. These likely form long-range reflex loops involving components of the autonomic nervous system.

The question of what mechanosensitive receptors or channels are found in these neurons is one that is a good starting point when trying to decipher their function within the ENS. Possible candidates so far include the large-conductance potassium channels (BK channels) [115] and the Piezo proteins. Quite interestingly, Piezo 1 has recently been localised in enterocytes of the human, mouse and guinea pig GI tract [158]. Expressed mostly (60%-100%) in inhibitory myenteric neurons, it was also seen in up to 76% of myenteric mechanosensitive neurons.

However, pharmacological experiments in that study, using activators and blockers of Piezo channels did not show any significant functional effect. Further experiments are warranted.

Intestinofugal neurons

Intestinofugal (viscerofugal) neurons have cell bodies within the wall of the gut and project to prevertebral ganglia, acting as the afferent limb of extrinsic reflex motor and secretomotor pathways. They are directly mechanosensitive, responding to changes in muscle length independent of muscle tension [153] and cause nicotinic-dependent fast EPSPs in postganglionic sympathetic neurons [159]. Sympathetic axons project back to the gut, running with the vascular bundle, completing the reflex arc [160].

Intestinofugal neurons are also activated and receive fast nicotinic signals from multiple other myenteric pathways [161, 162] giving them a dual role of sensory and inter-neurons. Quite interestingly, Hibberd et al recently demonstrated that large numbers of, if not all, sympathetic neurons in prevertebral ganglia become temporarily entrained to the same firing rate as myenteric circuits driving the colonic motor complexes in mouse colon [163]. This suggests sympathetic reflexes to the gut may be driven primarily by the sum of ENS activity, rather than discrete stimuli such as gut volume changes.

Rectospinal neurons

Rectospinal neurons belong to a singular class of afferent neurons, identified in the rat rectum, with their cell bodies mostly in the myenteric plexus they project directly to L6/S1 spinal cord segments [164, 165]. There is no functional data available, however these represent a possible direct viscerofugal conduit from the ENS to the CNS.

1.4.3 Extrinsic sensory neurons

The gastrointestinal tract is innervated by a combination of intrinsic and extrinsic systems and neural control of gut function is reliant on their interactions. Extrinsic afferent nerves transduce mechanical and chemical signals in the gut and carry them to the central nervous system for integration; they may lead to the activation of supraspinal centres as well as reflex pathways in the spinal cord that allow control of bowel function over larger distances and different regions of the GIT. Most of these signals do not cause conscious sensation but rather act on central neuroendocrine or autonomic centres. The signal they carry also undergoes significant modulation; descending spinal pathways from higher cognitive centres can influence central processing significantly [166]. Extrinsic sensory neurons also interact with many other cellular populations in the periphery; there is evidence that enteroendocrine cells, immune cells and gut microbiota are capable of altering mood and sensation, possibly through extrinsic sensory pathways [167].

Three major extrinsic afferent pathways exist: lumbosacral, thoracolumbar and vagal, with significant overlap, such that every region of the gut has a dual innervation. The upper gut, from the oesophagus through to proximal colon is innervated by vagal and thoracolumbar afferents, while thoracolumbar and lumbosacral pathways innervate the distal colon and rectum. In the rat, there is evidence that the vagal innervation extends as far distally as the descending colon although with decreasing density of innervation [168].

Vagal innervation

The vagus nerve is one of 12 paired cranial nerves. Vagus means ‘wanderer’ in Latin and is a very appropriate name for this nerve; it is the longest cranial nerve, coursing from brainstem, through the neck, thorax and into the abdominal cavity. It contains both afferent and efferent nerve fibres, although afferent fibres predominate as much as 9:1 [169]. Its afferent fibres innervate most of the organs in the chest cavity, including the heart, lower airways, aorta and

oesophagus and via its abdominal branches the GIT (up to proximal colon), liver, biliary system, pancreatic islets, uterus and adipose tissue vasculature [170]. It is therefore a key element of the body-brain connection that monitors and controls the cardio-pulmonary and digestive systems.

Vagal afferent fibres have nerve cell bodies in the nodose, jugular and petrosal ganglia, located at base of skull close to the bifurcation of the common carotid artery. The nodose and jugular ganglia differ in their embryological origin, coming from the epibranchial placode and neural crest respectively [171, 172]. Not surprisingly, the vagal neuronal populations within them differ too; jugular afferents are quite similar to spinal afferents (they both originate from neural crest) and likely play a role in nociception, nodose afferents on the other hand transmit innocuous signals from physiological stimuli that are vital for maintaining body homeostasis by activating the autonomic nervous system [171, 173]. As shown in the DRG afferent population, by analysing the transcription patterns of single cells, sensory neurons can be classified by their molecular 'fingerprint' into functional groups [174]. Just like neurons in the dorsal root ganglia, vagal afferents are actually quite specialized; there are distinct neurons sensitive to nutrients and stretch in the gut [175], the lung is innervated by two different vagal afferents with completely opposite physiological effects when activated [176], and recently Kapuri et al characterised 18 distinct neuronal populations within the mouse nodose ganglion based on their transcription profile [177]. This heterogeneity amongst vagal afferents suggests a greater range of sensory modalities and a wider field of sensory discrimination then previously suspected.

Central projections of vagal afferents are mainly to the nucleus tractus solitarius (NTS); areas outside the NTS that receive vagal afferent projections include, the spinal trigeminal nuclei, the area postrema (controls emesis and mediates the emetic reflex), the dorsal motor nucleus

and nucleus ambiguus [178-180]. Second order neurons from vagal brainstem nuclei project to autonomic ganglia and higher brain centres that are involved in hormonal, affective and behavioural responses.

A portion of vagal afferents make direct monosynaptic connections to parasympathetic efferents and both limbs of this reflex pathway travel in the same peripheral nerve trunks [181, 182]. This concept is the same for the sacral afferent fibres, they share nerve trunks with parasympathetic efferent projections and the neurons that give rise to these projections are synaptically connected with the afferents in the spinal cord [183]. It makes functional sense that nerve fibres responsible for a reflex pathway share common peripheral nerves.

Vagal sensory terminals

Intraganglionic laminar endings (IGLEs)

IGLEs are flat branching terminals of peripheral vagal axons, embedded in the connective tissue of myenteric ganglia. They are particularly concentrated in the oesophagus and stomach, becoming increasingly less dense in small bowel through to proximal colon [184]. The best immunohistochemical marker for IGLEs is the vesicular glutamate transporter 2 (VGluT2), which has been shown to label all IGLEs in mouse oesophagus [185]; other markers which are specific for IGLEs include purinergic receptors P2X2 and P2X3 in rat and mouse [185-187] as well as calbindin and calretinin in rat oesophagus [188, 189].

Functionally they act as low-threshold, tension-sensitive mechanoreceptors, reacting to both distension and contraction, independent of chemical neurotransmission [190, 191]. These properties, together with their abundance in the stomach has led to speculation about the

possible role of vagal IGLEs in appetite and feeding behaviour regulation. Vagal afferents are activated by increasing stomach volume [192], gastric load suppresses meal size [193] and both of these effects are dependent on an intact abdominal vagus [194]. More recently, genetic knockout mice with depleted IGLEs have been shown to have increased meal duration and increased volume of water per meal [195]; conversely, mice with abnormally high concentrations of IGLEs had smaller meals with greater intervals between feeding [196]. Despite the meal variations, the total daily food intake and body weight was the same between experimental groups, suggesting that vagal IGLEs only play a part in short-term satiety.

Intramuscular afferents

Vagal intramuscular afferents, also called intramuscular arrays (IMAs), are fine varicose terminals of vagal afferents. They are found in longitudinal and circular muscle, from oesophagus to the colon, oriented in parallel to each other and surrounding muscle fibres [197] and very closely associated with interstitial cells of Cajal [198]. Although currently no functional data exists, structurally they are well suited to being muscle stretch detectors [199].

Mucosal afferents

Vagal afferents within the mucosa have several region-specific morphologies. Afferents in the small intestine may be villus afferents, that supply varicose endings to the apical tips of villi or crypt afferents, which encircle the crypts of Lieberkuhn without entering the villi [198]. In the upper oesophagus, very fine ‘finger-like’ endings and ‘complex laminar’ mucosal terminals occur [200, 201]. Vagal mucosal afferents are sensitive to both mechanical and chemical stimuli. Although not activated by gut distension or contraction, they are sensitive to light mucosal stroking and compression [198, 202]. They are chemically activated by bile salts and a multitude of mediators released by the large population of enteroendocrine cells of the gut [203] in response to luminal content. They play an important role in satiety signalling;

Cholecystokinin (CCK) for example activates vagal mucosal endings [204] and influences food intake [205, 206], a phenomenon which requires an intact vagus nerve [207]. Vagal afferents also react to luminal amino acids, glucose, lipids and osmotic loads [208].

Muscular-mucosal afferents

There is possibly another group of vagal afferent terminals innervating the mucosa and the muscularis externa, demonstrated in oesophagus of ferret [209], *muscular-mucosal* afferents respond to mucosal distortion (particularly shear forces), distension and contraction of gut wall and may be well-suited to detecting movement of luminal content over the surface of the GIT [210]. A similar study in mice failed to identify this type of receptor, which could be due to the difficulty of separating mucosal stimulation from muscle distension in such thin tissue [211].

Spinal afferent gut innervation

Spinal afferents in the GIT are involved in long-range reflexes but are also responsible for sensations such as discomfort, bloating and pain [212, 213]. Their cell bodies are located in the dorsal root ganglia (DRG) in thoracic, lumbar and sacral dorsal nerve roots. Transecting dorsal nerve roots results in a loss of pain perception and much of non-painful sensation from the innervated area.

DRG neurons have pseudo-unipolar morphology with projections to both the viscera and spinal cord; their central projections terminate within the dorsal horn of the thoracic, lumbar and sacral spine where they synapse with second order neurons or travel via distinct spinal tracts to the brainstem [214]. Central projections of visceral spinal afferents are wide-spread and may travel several vertebral levels after entering the spinal cord; their input to second order neurons is often convergent with somatic afferents [215] or spinal afferents from other

viscera [216], this probably explains why visceral pain is difficult to localise and the phenomenon of referral to somatic structures like skin. Adding to this, the same DRG nerve cell bodies have been identified as projecting axons to different parts of the same organ or to two different organs [217-219]. Visceral afferent signals that reach the brain are processed in at least three regions; cingulate gyrus, insula and the somatosensory cortex, where they may result in visceral sensation and influence affect [220].

The peripheral processes to the gut project in splanchnic and pelvic nerves that, unlike the vagus nerve, carry mostly efferent fibres. Splanchnic nerves innervate most of the gut from distal oesophagus to rectum, contain mostly high-threshold mechanoreceptors [221] and are an important pathway in visceral nociception [222]. Pelvic nerves supply distal colon to internal anal sphincter, they mostly carry mechanoreceptors with lower threshold profiles and their main role is sensation during normal gut functions. However, chemosensitivity to known noxious stimuli is present in both pelvic and splanchnic nerves [223] and so are afferents with high threshold profiles [224, 225], indicating that pelvic nerves also carry nociceptive information underlying the sensation of pain from pelvic organs.

Thoracolumbar spinal afferents share peripheral nerve trunks with sympathetic projections to the gut, however there appears to be very little direct synaptic connectivity between the two pathways [226]; postganglionic sympathetic neurons receive multi-synaptic inputs from sympathetic preganglionic neurons located along multiple vertebral levels, forming a complex network within the thoracolumbar spine [227, 228].

Spinal afferent terminals

Anterograde tracing experiments from mouse dorsal root ganglia reveal a great variety of spinal afferent endings; 13 different morphological types can be distinguished with some individual axons that can supply three layer of gut and give rise to several hundred terminals [213, 229]. If one combines anatomical data with functional recordings, there are currently 5 types of afferent terminals recognised.

Vascular afferents

Spinal vascular afferents are mechano-nociceptors that innervate intramural and mesenteric blood vessels with a fine branching network of axons that run along mesenteric arterioles and follow them into the submucosa [230]. They make up the majority of afferent fibres in the thoraco-lumbar pathway and approximately one third of the sacral afferents [231]. They are capsaicin sensitive and respond to localised pressure on blood vessel walls [232], large contractions or distension of the gut, traction of the mesentery and increases in intravascular pressure of mesenteric vessels [233, 234]. They give off axon collaterals that project to all the layers of gut wall, including the myenteric plexus, where they can initiate motor activity by stimulating myenteric circuits [233, 235].

They express receptors for many endogenous mediators including, but not limited to 5HT, bradykinin, histamine, ATP and glutamate [210]. Vascular afferents also have efferent-like effects; by releasing CGRP and SP from their terminals, they cause vasodilatation and increased vascular permeability, resulting in localised oedema [236, 237]. They also cause mast cells to degranulate, releasing histamine and protease II, which stimulate the spinal afferent terminals in a positive feedback loop [238]. This phenomenon has been termed ‘neurogenic inflammation’ and may occur throughout the body [239, 240]. In parallel to other inflammatory mechanisms, its role is likely to increase blood flow to an area of damage or infection.

Muscular afferents

Intramuscular arrays (IMA) similar to ones present in vagal pathways have been described in the colon, rectum and internal anal sphincter [241]. There is little functional data apart from IMAs in the guinea pig internal anal sphincter and mouse rectum [242-244], where they seem to function as low-threshold mechanoreceptors.

Rectal intraganglionic laminar endings (IGLEs)

Rectal IGLEs are morphologically similar to their vagal counterparts, they are broad, flat terminals in myenteric ganglia, perhaps with less branching [156]. They act as slowly adapting low-threshold mechanoreceptors within the rectum of several species examined [156, 221, 245] and are activated by physical distortion or compression most likely mediated by stretch sensitive ion channels [246].

Mucosal afferents

Spinal mucosal afferents are quite similar to their vagal counterparts; they are remarkably sensitive to light stroking or compression of the mucosa but not to contraction or distension of the gut wall [221]. There is a greater concentration of mucosal afferents in the distal colon and rectum, they represent 1% and 15% of all afferents recorded from the mouse splanchnic and pelvic nerves respectively [221, 247]. Traced anterogradely from the DRG in mice, these afferents have fine varicose axons that ramify extensively in the mucosa and over 90% of them contain CGRP [229]. It is likely that pelvic mucosal afferents are involved in the control of defecation by providing conscious sensation of the passage of stool [231, 245]. Spinal mucosal afferents are also indirectly sensitive to chemical changes within the colorectal lumen;

Intraluminal irritants and microbial metabolites stimulate enterochromaffin cells to release 5-HT and activate mucosal afferents via synaptic-like connections [248].

Muscular-mucosal afferents

A population of spinal afferents with low-threshold sensitivity to both muscle distension and mucosal stroking has been described in the mouse pelvic spinal afferent pathway [221, 249]. These muscular-mucosal receptors have receptive fields in the distal 1cm of the rectum and become active in-sync with contractions of the gut wall [249]. Anterograde tracing from lumbosacral DRG has revealed a great variety within pelvic afferent terminals in the colorectum of the mouse [229], some of them innervate the crypts of Lieburkuhn and submucous ganglia and could anatomically correspond to the muscular-mucosal functional class of afferents. Just like spinal mucosal afferents, muscular-mucosal afferents may contribute to defecation mechanisms and conscious sensation. The transient receptor potential vanilloid 1 (TRPV1) channel and acid-sensing ion channel 3 (ASIC3) are needed for the proper functioning of muscular-mucosal afferents. Knockout mice lacking either of these channels have decreased mechanosensory function and pain response to colorectal distension [250], they also have a partial loss to the sensitising action of intra-colonic zymosan [251], suggesting that these afferents may also play a role in nociceptive mechanisms by contributing to peripheral sensitization.

1.4.4 Interneurons

Within the ENS, myenteric interneurons form a central processing network that integrates sensory input as well as hormonal and neural inputs from extrinsic sources. Interneurons are classified according to polarity of their projections; ascending and descending interneurons are further profiled according to the combination of neurochemicals present in their cell bodies. Classically, they are all Dogiel type I neurons. Their output is directed at motor neurons, intestinofugal neurons and interneurons [252]. What happens to the information converging

on this system in order to produce an output is largely unknown. It is likely that differential activity within interneuron groups allows a selection of gut motility patterns. Interneuron classes have the most variability in their neurochemical coding between gut regions and species [10, 32, 253], supporting this theory.

Interneurons have either ascending or descending projections, whose functional length is extended by synapsing to other interneurons of the same class, forming interneuron 'chains' [254-257]. Additional projections include ones to the submucous plexus, connecting the two networks [258, 259]. Large mammals such as human and pig have a more complex and multilayered sub-mucous plexus compared to small laboratory animals, not surprisingly, submucosal interneurons can be found in the former and not the latter groups [260, 261].

1.4.5 Motor neurons

Motor neurons innervate and control the muscle layers of the gut, including the longitudinal, circular and muscularis mucosae layers. Classically, they are Dogiel type I neurons with S-type electrophysiology that receive synaptic input from interneurons and IPANs via fast and slow EPSPs respectively. Motor neurons are divided into excitatory and inhibitory classes based their effect on smooth muscle tissue.

Excitatory motor neurons cause smooth muscle contraction; they contain the combination of choline acetyltransferase and tachykinin as immunohistochemical markers in all regions of the GIT, across all species examined. Other markers include enkephalin (ENK) and calretinin [10]. Inhibitory motor neurons cause smooth muscle relaxation; in all regions of the gut and all species examined, they may be consistently identified by the combination of NOS and vasoactive intestinal peptide (VIP). Other markers are region and species dependent and

may include gastrin-releasing peptide, neurofilament protein (NFP) triplet, neuropeptide Y, gamma-aminobutyric acid (GABA), galanin (GAL) and somatostatin [10]. Of course, we cannot be certain that all of the neurochemicals identified within a class of motor neurons have physiological significance.

In guinea pig small intestine all of the circular muscle motor neurons (CMMN) have their cell bodies in the myenteric plexus, where they comprise approximately 30% of the neuron population [262]. The axons of the myenteric CMMN form the deep muscular plexus before innervating the smooth muscle tissue with varicose terminals. In larger mammals, there is evidence that CMMN are also in the submucous plexus [137, 263]. Physiological and tracing experiments have shown inhibitory CMMN generally project aborally for longer distances then the excitatory CMMN, which tend to project in the oral direction [110, 264-269]. The majority of input received by CMMNs comes from pathways that project in the same direction as the motor neuron, with some minor exceptions [270]. This is congruent with the polarised nature of excitatory and inhibitory CMMN projections.

The innervation of the longitudinal muscle layer is surprisingly different to that of circular muscle and more variable. In guinea pig small intestine LMMN comprise approximately 24% of the myenteric neurons, their axons branch extensively in the tertiary plexus, located immediately deep to the longitudinal muscle wall [271] and are mostly excitatory in nature (97%) with no polarisation of their projections [272]. This arrangement is very similar in guinea pig proximal colon [269] but not the stomach, where the inhibitory LMMN comprise 40% of the longitudinal muscle population and also have a distinctive aboral polarity [273]. In human small intestine the majority of LMMN neurons are located oral to the site of innervation [274]; in the colon the longitudinal muscle is supplied by excitatory motor neurons with predominantly ascending projections and inhibitory neurons that are not polarised [275].

Vasomotor / Secretomotor neurons

Several distinct neuronal populations innervate the mucosal epithelium and interact with the hormonal / inflammatory systems to control local blood flow and mucosal secretion. Up to 9 litres of fluid is secreted into the gut lumen on a daily basis and most of this is reabsorbed before it reaches the distal colon [276]. It is therefore not surprising that movement of fluid and electrolytes across the gut epithelium is under tight regulation. It is highly likely that secretory and vasodilatory functions are directly linked to blood flow and motility *in vivo* [277, 278].

Mucosal secretion is determined by membrane channels and transport proteins, which actively regulate the movement of electrolytes and therefore water across the gut epithelium. Chloride and sodium ions are the main electrolytes with bicarbonate having a more important role in the duodenum and colon. Neurotransmitters released by enteric motor neurons act on epithelial membrane receptors to activate second messengers such as cAMP and Ca^{2+} which in turn stimulate the secretion of ions (in particular Cl^-) into the gut lumen [279, 280]. Water follows the osmotic gradient, passing through tight junctions between epithelial cells or aquaporin water channels [281]. The majority of enteric neurons involved in controlling mucosal function are found in the submucous plexus and are classified as motor (effector) neurons and further subdivided into cholinergic secretomotor\vasodilator neurons, cholinergic secretomotor (non-vasodilator) neurons and non-cholinergic secretomotor/vasodilator neurons [76].

Cholinergic neurons that control secretion and vascular tone make up 12% of submucous neurons [282, 283]. They are Dogiel type I neurons with filamentous dendrites and contain calretinin [133, 284]. Cholinergic neurons that only project to mucosa (secretomotor / non-

vasomotor) contain NPY as a marker and contribute 33% and 1% to the submucous and myenteric neuronal populations respectively [133, 282, 285].

Non-cholinergic secretomotor / vasodilator neurons make up 43% of submucosal neurons and 1% of myenteric neurons [282, 286]. They colocalise VIP as a marker, and are the only group of neurons in the submucous plexus that receive inhibitory postsynaptic potentials in addition to excitatory signals [287].

Secretory and vasodilatory reflexes may be activated by mechanical and chemical stimulation of the mucosa even when the myenteric plexus is surgically removed and extrinsic nerves ablated [288-290], suggesting that the submucous plexus contains all of the essential elements for this reflex. Vasodilatory reflexes may also be activated by myenteric neurons, whose projections can travel for several centimetres before stimulating motor neurons within the submucous plexus [291, 292]. Therefore, it is likely that the myenteric plexus input extends the length of gut over which reflexes occur and possibly coordinates mucosal function with motility patterns. There is sound evidence that extrinsic capsaicin-sensitive (sensory) afferents can stimulate mucosal secretion and vasodilatation by activating submucosal motor neurons [293, 294], however the exact mechanism of endogenous activation and circuit details are still to be determined.

1.4.6 Enteric glial cells

The ENS contains a large and very diverse population of glial cells; they are found in all layers of the gut wall and are closely associated with enteric neuronal structures. They outnumber enteric neurons as much as 7 to 1 [295], although this ratio is different between the myenteric and submucosal plexus as well as between species [296]. Although they are non-neuronal cells, found in the central as well as peripheral nervous system, a multitude of important functions within the ENS has recently been attributed to them, so they are worth mentioning

in this review. The greatest challenge to the study of enteric glial physiology has been the difficulty in selective manipulation of glial cells in an intact ENS and their functional sensitivity to being isolated, such as in cell culture for example. Recent developments in molecular biology and calcium imaging technology have advanced this field considerably. A growing body of data suggests the possibility of glial cells not only supporting the metabolic health of enteric neurons, but also taking part in enteric neuronal circuit activity. This has certainly added another layer of complexity to our attempts at understanding the physiology of the ENS.

Enteric glial cells can be divided into at least four distinct morphological groups in the mouse [295, 297]. They express several markers including Sox-10, GFAP and S100β, which are not specific for any of the distinct morphological groups and may be absent in the majority of glial cells outside of myenteric ganglia [297]. Furthermore, expression of these markers appears to be dynamic and significant phenotypic plasticity occurs with changes in their environment [297] and states of inflammation [298]. This is highlighted by experiments that show enteric glia can form enteric neurons in culture [299], they can also take on the function of astrocytes and oligodendrocytes when transplanted into the CNS [300, 301].

The exact role of enteric glia in gut function is still unknown. Enteric glia express receptors for, and respond to, all the major classes of neurotransmitters found in the ENS [302, 303]. They synthesise and release neurotransmitters such as purines and NO, inflammatory cytokines and several other factors [295, 304, 305] and they receive input from extrinsic neural pathways, such as the TH containing sympathetic fibres in guinea pig distal colon [306]. They are excitable cells, becoming active during specific neural patterns such as colonic migrating motor complexes in mouse colon [307]. The significance of such glial activation is not known, however recent experiments using chemogenetic mice have shown that selective activation of glial Ca^{2+} signalling is capable of causing neurogenic contractions in ileum and colon, with

no effect on neurogenic relaxation [308]. Furthermore, the propagation of Ca^{2+} signalling through the glial myenteric network depends on the activity of the connexin-43 hemichannel. Selective ablation of this protein causes constipation in the live mouse and impairment of colonic excitatory and inhibitory neuromuscular transmission in ex-vivo experiments [309].

Enteric glia may also influence non-neuronal cells within the gut; they are very closely associated with entero-endocrine cells [310] and glia-derived ATP could potentially signal surrounding interstitial cells or even smooth muscle cells directly [311]. Submucosal glia develop after birth and require the presence of gut bacteria in order to populate the lamina propria [312], which suggests an important interaction with gut microbiota. Selective activation of glia stimulates mucosal secretion while inhibiting glial function reduces secretion [313], implying a role in regulation of mucosal secretomotor function. Enteric glial cells also contribute to mucosal host defence by interacting with innate mucosal lymphoid cells and stimulating their immune response with neurotrophic factor signals [314].

Colonic transit slows down significantly with advanced age in humans, rats and mice [309, 315, 316]. Quite interestingly, ageing in mice is associated with reduced glial responsiveness to stimulation and dysregulated connexin-43 expression [309], suggesting the possibility of glial dysfunction playing a part in age-related colonic motility problems. Other pathological states where glial dysfunction is thought to be a causative or contributing factor include slow-transit constipation, postoperative ileus and chronic intestinal pseudo-obstruction [317-319].

Current data suggest that enteric glial cells are more than just an inert support structure for nerve cells, they potentially have roles in a wide spectrum of gut functions including motility, mucosal secretion and host defence. It is clear that a lot more research is needed to expand our understanding of enteric glial physiology.

1.5 Functional aspects of the gastrointestinal tract

Gut functions, such as motility, require coordination of cellular activity across a large number of cell types, within and outside the gut wall. Distinct cell populations contribute to the makeup of multiple tissue types, which in turn form the hollow organ. The structural complexity of this organ, combined with the overlapping expression of many signalling molecules, makes it very challenging at times to determine the contribution of individual cell types to a function of interest. Not surprisingly, there is still vigorous debate on the exact role of many cell classes across the GIT.

The gut has digestive, secretory, immune and endocrine functions. Ingested food and water are sequentially processed along the length of the tract. Mechanical and chemical digestion releases substances, which are absorbed, assimilated and used for growth and function of the animal. At the same time, unusable or harmful metabolic by-products are secreted and eliminated. Accessory organs including salivary glands, pancreas, liver, gallbladder and bile ducts closely associate with the GIT along its length to aid these functions.

The gut hosts the largest population of immune cells in the body. The various cell types are diffusely scattered close to the mucosal surface but also form specialised aggregations such as Peyer's patches in the terminal ileum or Waldeyer's ring in the oropharynx [320]. The immune system of the host animal relies on the lining of the intestine, both cellular and secretory, to control its exposure to antigens. It needs to strike a very delicate balance between tolerating the resident microbiome and mounting an offensive against invasive pathogens. Crohn's disease is a good example of what happens when this system fails, resulting in autoimmune inflammation, likely caused by an abnormal reaction of gut immune cells to harmless microbiota [321].

The gut is also the largest endocrine organ in the body. Enteroendocrine cells in luminal epithelium synthesise at least 20 different hormones with local and systemic effects. These hormones play a vital part in influencing the feeding behaviour of an animal, they regulate energy metabolism, likely modulate some motility patterns and control local secretion of digestive enzymes [322].

1.5.1 Muscular apparatus

Digestion of food requires grinding, mixing and propulsion along the GI tract, at the optimal rate and in the correct direction. The muscle of the gut provides the mechanical forces these motor patterns require for propulsion to take place. With the exception of proximal oesophagus, where striated muscle provides a degree of voluntary control, smooth muscle cells are the sole contractile element in the gut wall. Highly coordinated episodes of contraction and relaxation in the circular and longitudinal muscle layers underlie the required motility patterns. Smooth muscle also facilitates the function of multiple sphincters along the gastrointestinal tract. The lower oesophageal sphincter, the pylorus, the ileo-caecal valve and the internal anal sphincter function to control the rate and direction of movement of luminal contents. The heapato-pancreato-biliary system contains smooth muscle that controls storage and release of digestive substances into the duodenum.

The longitudinal muscle layer is distributed evenly around the circumference of the gut tube. However, in the colon of certain larger mammals, this layer condenses to form several longitudinal bands known as taenia coli. This pattern is quite easy to appreciate in the human colon for example. Contraction of the longitudinal muscle layer causes a shortening of the gut tube, a direct result of the orientation of muscle cells in this layer. In comparison, the circular muscle layer is thicker and causes a localised circumferential constriction of gut lumen when it contracts.

Excitation-contraction coupling

Smooth muscle cells of the gut are 'excitable' and therefore, like other cells of this kind, have a resting electrical potential across their cell membrane. The activity of Ion pumps located in the cell membrane creates a significant concentration gradient, such that there are greater amounts of cations (mainly Na^+ and Ca^{2+}) outside the cell compared to inside, resulting in a negative electrical potential across the membrane. An influx of positive charges causes this membrane potential to diminish or even reverse in polarity, a phenomenon known as membrane depolarisation. Depolarisation of smooth muscle membrane past a 'threshold potential' is the physiological trigger for smooth muscle contraction [323].

The contraction of a smooth muscle cell is due to the interaction of structural proteins actin and myosin, generating force by either changing the length of the cell in its long axis (isotonic contraction) or increasing tension without a change in length (isometric contraction). An increase in the cytoplasmic calcium concentration activates a biochemical pathway allowing actin and myosin to interact, with the net effect of converting stored chemical energy in the form of ATP into mechanical energy.

Depolarisation activates voltage dependent calcium channels in smooth muscle membrane and the resultant Ca influx down its concentration gradient causes an increase in cytoplasmic calcium concentration and muscle contraction. To a lesser extent, release of calcium from intracellular (sarcoplasmic reticulum) stores can contribute to cytoplasmic calcium concentration increases. This mechanism depends on activation of IP3 receptors on sarcoplasmic reticulum membrane [324].

1.5.2 Interstitial cells of Cajal and generation of slow waves

Smooth muscle of the gut is a complex tissue. Apart from smooth muscle cells (SMC), it also contains other cell types, known as interstitial cells. Each cell type displays intrinsic electrophysiological properties and expresses a combination of receptors for neurotransmitters, inflammatory mediators, hormones and paracrine agents. Interstitial cells of Cajal (ICC) and Platelet-derived growth factor receptor-α positive (PDGFRα+) cells are electrically coupled to SMCs and to each other, via gap junctions, forming a functional syncytium [325, 326]. This allows interstitial cells to modulate SMC excitability, which is therefore dependent on a complex interplay between intrinsic mechanisms across several cell types and extrinsic signals.

Smooth muscle of the gut displays an intrinsic rhythmicity, undergoing a cyclical oscillation of membrane potential. Commonly known as 'slow waves', this phenomenon was first reported in cat intestine [327] and likely underlies the phasic contractile behaviour quite commonly seen across the GIT. Recorded in the stomach, small intestine and colon of several species, including human, slow waves differ in frequency as well as morphology between species and between regions of gut [328]. They do not require the activity of the nervous system, hormones or paracrine agents. In fact, they can persist for days in isolated smooth muscle tissue kept in organotypic culture [329] [330].

Interstitial cells of Cajal (ICC) account for less than 10% of cells within smooth muscle tissue [331]. There are several types of ICC, based on their location within the layers of the gut. The myenteric ICC appear to be the source of slow waves, which regenerate from cell to cell and drive the oscillation of membrane potential in smooth muscle [332-334]. First described by Cajal in 1893 [335], their function was only more recently elucidated when looking at the role of the tyrosine kinase Kit receptor in mice. This protein was shown to be expressed in smooth muscle of developing mouse gut; blocking its function in normal young mice using a

monoclonal antibody resulted in abnormalities of gut motility and c-Kit gene mutation mice lacking the gene product developed a lethal paralytic ileus [336]. Mice lacking the c-kit tyrosine kinase activity also lacked any slow waves on recordings from intestinal muscle strips, which were present in wild type animals [333, 337].

Since the probability of smooth muscle contracting increases with membrane depolarisation and decreases with membrane repolarisation, slow waves effectively modulate smooth muscle excitability in a cyclical pattern and may drive phasic contractile activity. Slow waves can generate depolarisations large enough to cause muscle contraction, however in most cases other excitatory (depolarising) signals are required to depolarise the membrane potential past that threshold [338, 339].

The frequency of slow waves varies across GIT regions, an adaptation that is likely function-specific. In mouse internal anal sphincter, where resting tone is significant, slow wave frequency is quite high (about 70 cycles per minute, cpm), this initiates phasic contractions which summate to produce anal tone [340]. Mouse small intestine and proximal colon has slow waves at about 45 cpm and 15cpm, respectively [341, 342]. This variation reflects the maximum frequency of contractile activity likely to be seen within these regions. Interestingly, it appears that ICC are mechanosensitive and can alter the frequency of their slow wave in response to stretch [343].

Evidence suggests that a segment of gut may receive simultaneous input from more than one pacemaker system. In the rat colon for example, there are two distinct, superimposed patterns of cyclical activity in circular muscle, which originate in the myenteric and submucosal ICC networks independently [344]. There is evidence for a similar arrangement in dog and human colon, where it is possible to record the typical slow waves from circular muscle adjacent to

the submucous ICC network and a higher frequency pacemaker potential in circular muscle close to the myenteric ICC network [345, 346]. Very similar recordings have been obtained in pig colon when recording from circular and longitudinal muscle [347].

Our understanding of ICC physiology is still quite limited. There are numerous membrane channels and receptors (including neurotransmitter receptors) expressed by ICC, all of which potentially modulate their function; the presence of serotonin receptors for example hints at the possibility of a brain-gut connection. Further research could potentially uncover novel therapeutic targets that may extend beyond gut motility disorders and include other organs that have ICC-like cells with pacemaker roles, such as the uterus [348] or urinary tract [349].

Role of Interstitial cells in the neural control of smooth muscle

The mechanisms by which motor neurons control the smooth muscle of the gut are still not fully understood. The traditional model is that motor neurons release neurotransmitters, which act directly on smooth muscle cell receptors to cause either a contraction or an active relaxation (direct neurotransmission). This has recently been challenged by the idea that neurotransmitters act on interstitial cells to cause changes in their activity which in turn are passed on to electrically coupled smooth muscle cells (indirect neurotransmission).

Ultrastructural studies have demonstrated close contact between enteric nerve terminals and intramuscular ICCs in the stomach [350-352], colon [353] and deep muscular plexus ICCs of the small intestine [354-356]. Organised junctions with pre and post junctional synapse-associated proteins have been described between ICCs and motor neuron varicosities in the mouse stomach [357]. The same type of connectivity does occur between enteric nerve terminals and smooth muscle cells [352, 358], however this seems to occur with a lower

frequency [351, 359]. All thee cell types that make up gut smooth muscle tissue express receptors specific to neurotransmitters released by motor neuron projections [356, 360, 361]. They also express membrane channels and components of intracellular messenger systems that mediate post-junctional responses to these signalling molecules [362, 363].

Functional experiments suggest that ICC appear to transduce signals from enteric neurons, although the concept is still not without controversy [364, 365]. Excitatory cholinergic and inhibitory nitrergic responses in smooth muscle can be elicited in mouse intestine only after the development and maturation of ICC-DMP; blocking Kit receptors causes a loss of ICC and a loss of excitatory and inhibitory responses [366]. Mutant mice lacking ICC-IM in the stomach had reduced or absent smooth muscle responses to cholinergic and nitrergic neurotransmission [350, 367, 368].

Klein et al genetically engineered mice with an inducible knock-in Cre allele at the c-KIT locus, allowing them to target ICC in adult mice and perform loss of function experiments [369]. They demonstrated what had previously been reported only in germline mutant c-KIT mice and using monoclonal antibodies; acute depletion of ICC causes significant increases in GIT transit times and smooth muscle with depleted ICC lacks slow wave activity. Furthermore, excitatory junction potentials (and muscle contraction) could not be induced in ICC-depleted circular muscle of the colon and small bowel using electrical field stimulation, suggesting that ICC mediate excitatory signals from motor neurons. The effects of signals from inhibitory enteric neurons on ICC-depleted bowel were more difficult to interpret, ICC depletion had no effect on inhibitory junction potentials (IJP) but disrupting the intracellular Nitric Oxide (NO) signalling pathway (by deleting the Prkg1 gene) within ICC, abolished the NO-dependent slow component of the IJP (sIJP).

Dynamic intracellular Ca^{2+} changes (calcium transients), due to release of Ca^{2+} from intracellular stores, seem to play an important role in generating electrical activity patterns within ICC [370-373]. The increase in intracellular Ca^{2+} in turn activates Ca^{2+} sensitive membrane Cl^- channels coded by Ano 1, which has an identical distribution to c-KIT along the GIT and is strongly expressed in ICC [370, 374].

Baker et al used high-resolution imaging of calcium transients within ICC-DM of mouse intestine with ICC-specific expression of a genetically encoded fluorescent calcium marker (GCaMP3). Using electrical stimulation, pharmacological stimulation and inhibition, they demonstrated significant effects to Ca signalling within the ICC-DM in response to excitatory (NK1 and Muscarinic dependent) and inhibitory (NO and VIP) neurotransmission [375] [376], strengthening the argument for ICC as transducers of motor neurotransmission.

PDGFRα+ cells are found within the muscular layers of the gut, very closely intertwined with ICC to form part of the smooth muscle tissue [377]. They express P2Y1 (Purine) receptors and small-conductance Ca^{2+}– activated K^+ channels (SK3 channels) in high concentrations [378]. Exogenous ATP and P2Y1 agonists cause a significant hyperpolarisation of PDGFR α+ cells, which are partially blocked by P2Y1 antagonists [379]. Purinergic neurotransmission causes fast inhibitory junction potentials and inhibition of smooth muscle contractions in the gut [41, 380]; increasing amount of data suggests that PDGFR α+ cells are mainly responsible for this response [381, 382]. Activation of α1 adrenoceptors on PDGFRα+ cells in the mouse colon causes a SK-current-mediated hyperpolarisation of PDGFRα+ cells and inhibition of contractile activity [383]. This suggests PDGFRα+ cells may also be important effectors of sympathetic neural regulation of colonic motility.

It is likely that physiological smooth muscle responses come about through a complex interaction of motor and extrinsic autonomic neurons and the three types of cells that make up smooth muscle tissue. The exact contribution from each cell type is still to be resolved.

1.5.3 Extrinsic neural modulation of gut function

The autonomic system exerts neural and hormonal influence over body systems in order to maintain homeostasis. The sympathetic and parasympathetic divisions have distinct central and peripheral pathways and generally have antagonistic effects on dually innervated tissue, including the GIT. The stomach and oesophagus in particular rely heavily on extrinsic neural inputs for their proper function compared to the rest of the GIT, where the ENS displays a higher degree of independent neural control [75, 384].

1.5.4 Sympathetic nervous system

The sympathetic nervous system (SNS) has wide-ranging effects on the body, from simple spinal reflexes to very complex affective responses.

Sympathetic preganglionic neurons (SPNs)

SPNs originate within the thoracolumbar spinal cord (C8 to L4 in guinea pig). Most SPN cell bodies are located in the intermediolateral column of the lateral horn, in clusters within several autonomic nuclei [385, 386]. They supply para- and prevertebral sympathetic ganglia where they synapse with sympathetic postganglionic neurons. A subpopulation of SPNs project directly to the adrenal gland where they control the release of catecholamines by chromaffin cells [387].

SPNs receive direct synaptic input from supraspinal and intraspinal neurons but not sensory (DRG) neurons. Supraspinal input comes from five main regions of the brain, namely the rostral ventrolateral and ventromedial medulla, the caudal raphe nuclei, paraventricular hypothalamus nucleus and the region containing the A5 noradrenergic neurons [388, 389]. In addition to descending signals, SPNs also receive input from spinal interneurons, which are active even when descending pathways have been interrupted [390, 391].

There is evidence for several neurotransmitters being involved in transmission to SPNs. Glutamate causes fast excitatory post synaptic potentials (fEPSPs) while GABA and glycine both cause fast inhibitory post synaptic potentials (fIPSPs) [392, 393]. Exogenous catecholamines cause excitation and inhibition acting on α1 and α2 receptors, respectively [394], while serotonin is excitatory [395, 396]. There is a veritable palette of neuropeptides located in the region of spine where SPN have their cell bodies, the ones that have been tested have an excitatory effect on SPN (for a review, see [397]).

Multiple functional classes of SPNs exist, always associated with postganglionic neurons that receive their synaptic output and innervate target tissue. Examples of these functional classes include but are not limited to sudomotor, pilomotor, inspiratory, vasodilators, vasoconstrictors and motility-regulators; some of these functional classes can be identified by a unique combination of neurochemicals, which tends to be region and species specific [398-402].

Sympathetic postganglionic neurons

Sympathetic postganglionic cell bodies locate to sympathetic pre- and paravertebral ganglia, receive synaptic input mostly from SPN and project as the ‘final motor neurons’ to organs throughout the body. **Para**vertebral ganglia are paired structures, seen on either side of the

spinal cord, forming a ‘sympathetic chain’ from the cervical to the lumbar region. **Pre**vertebral ganglia associate with the three major ventral branches of the abdominal aorta and share the same terminology: coeliac, superior mesenteric and inferior mesenteric [403].

Within sympathetic ganglia, neurotransmission from SPNs to sympathetic postganglionic neurons is mediated by ACh acting on nicotinic receptors [404]. There is a sub-population of SPNs that also contain NOS, and are the likely source of nitrergic neurotransmission within sympathetic ganglia, and the adrenal medulla [405]. Numerous other substances that may have a role in neurotransmission have been identified in subpopulations of SPN including, GABA, ENK, corticotropin-releasing factor (CRF), somatostatin, substance P and VIP [406-408].

Prevertebral sympathetic ganglia receive preganglionic input from the spine via thoracic and lumbar splanchnic nerves; the hypogastric nerve has preganglionic neuronal projections that supply the pelvic plexus. **Para**vertebral sympathetic ganglia receive preganglionic input from SPN that project out of the spine through the ventral roots and white rami of spinal nerves [403]. Apart from preganglionic input, certain populations of postganglionic sympathetic neurons in the prevertebral ganglia also receive synaptic input from viscerofugal neurons (VFN) and collaterals of spinal sensory neurons. VFNs located in stomach through to rectum have been shown to project to sympathetic postganglionic neurons in the coeliac ganglion [409]; the superior and inferior mesenteric ganglia receive VFN projections from distal colon and rectum [410].

Nearly 100% of sympathetic fibres in guinea pig small intestine and stomach contain TH and β-Hydroxylase (markers of noradrenaline synthesis) [411], this is reflected by the dominance of noradrenergic neurons in mouse and guinea pig prevertebral ganglia [412, 413] and

supports the notion that noradrenaline is the main neurotransmitter used by sympathetic postganglionic neurons in the periphery.

Sympathetic terminals within the gut

Retrograde tracing studies in guinea pig suggest that the majority of the sympathetic supply to the GIT comes from prevertebral ganglia, with a smaller contribution from the paravertebral chain [414]. This is not consistent throughout the length of the gut as there is a large increase in concentration of paravertebral fibres in the distal gut; in the rectum and internal anal sphincter there may be more paravertebral than prevertebral sympathetic fibres [415, 416].

The output is organotopically arranged; foregut structures receive input mostly from the coeliac ganglion and hindgut structures are favoured by projections from more caudally located ganglia. Similarly, within a ganglion, there is a topography evident along the coronal plane [414]. This arrangement is logical, nerve fibres from the three prevertebral ganglia tend to travel with arteries of the same name, which in turn supply the GIT in a segmental fashion. Tracing studies in the rat and guinea pig reveal that the most distal part of the colon receives a significant sympathetic supply (up to one-third) from postganglionic sympathetic nerves located in the pelvic ganglia [417, 418].

Anterograde tracing from mesenteric nerve trunks shows that the myenteric plexus of the gut receives a very dense sympathetic innervation, with a high concentration of varicose fibres and many pericellular baskets evident [419]. There is also innervation of smooth muscle, including that of sphincters and blood vessels, as well as mucosa [420-423].

Regulation of gut motility

The sympathetic nervous system is capable of highly selective regulation of its output. For example, stimulating a known afferent pathway can elicit both excitation and inhibition of sympathetic effects [424, 425]. There are separate sympathetic pathways, functionally defined by their target cells, each with a unique discharge pattern that is appropriate for a given stimulus [389, 426]. Not surprisingly, separate populations of sympathetic neurons control motility, blood flow and mucosal secretion in the gut.

Experimental sympathetic nerve stimulation inhibits ascending and descending cholinergic interneurons in the colon, and causes relaxation of smooth muscle [427]. The likely mechanism involves sympathetic nerve terminals releasing noradrenaline, which inhibits excitatory neurotransmission in the myenteric plexus, via presynaptic α2 receptor dependent suppression of ACh release [428, 429].

Reflex pathways that activate sympathetic gut projections include a class of motility-regulating sympathetic preganglionic nerves. There may be at least two different populations, based on different responses to bladder and colonic stimulation [430], they do not require supraspinal inputs to function [430] and are likely to be controlled exclusively by visceral reflex circuits under normal physiological conditions [431]. Recordings from motility-regulating postganglionic sympathetic neurons indicate that the majority can be divided into the same functional groups, and have the same proportions, as the preganglionic neurons projecting to their ganglia [432]. This suggests that within this circuit, central patterns are transmitted to target organs without significant modulation along the way.

Regulation of gastrointestinal blood flow

Arteries, and to a lesser extent veins in the mesentery and bowel wall are densely innervated by vasoconstrictor sympathetic neurons that come from prevertebral and paravertebral ganglia [420]. Contraction of vascular smooth muscle causes a decrease in vascular diameter, increasing the resistance to blood flow and the intraluminal blood pressure. Vascular smooth muscle contraction in the gut caused by activation of sympathetic fibres is mediated by both α1 and P2X receptors, the relative contribution of each seems to be dependent on stimulus characteristics [433, 434]. A significant proportion of NPY-containing postganglionic neurons in the paravertebral ganglia function as vasoconstrictors to the mesenteric and submucosal circulation, likely mediating changes to intravascular volume (and thereby systemic blood pressure) by changing the amount of blood in the visceral circulation [420, 435, 436].

Regulation of secretory function

Although mucosal secretion is mainly controlled by neurons within the submucosal plexus, there is evidence that sympathetic input to submucous secretomotor neurons may indirectly inhibit it. Presynaptic inhibition and inhibitory postsynaptic potentials in submucous neurons are caused by sympathetic neurons releasing noradrenaline and somatostatin, which act on SST and α2 receptors [437-439]. Furthermore, this may be part of a reflex pathway involving the viscerofugal neurons providing direct input to postganglionic sympathetic neurons [440].

1.5.5 Parasympathetic nervous system

The parasympathetic nervous system contributes to the regulation of viscera, generally opposing the effects of sympathetic activation. Peripheral parasympathetic pathways come from two distinct regions. Neurons that innervate the head, chest and upper abdomen are part of the cranial nerve pathway, with the Vagus nerve providing the majority of parasympathetic input to the gut. The pelvis (including the distal GIT) is innervated by parasympathetic neurons that come from the sacral spinal cord. Afferent Vagal pathways, receptors and central nuclei

have already been discussed earlier in this thesis (under the section of ‘Extrinsic sensory neurons’); they will not be considered again.

Although similar in functional arrangement to the SNS, capable of selective activation of independent circuits in response to stimuli, there are some basic differences in anatomical considerations. Unlike in the SNS, the parasympathetic preganglionic neurons synapse onto postganglionic neurons in ganglia that are very close to or even within the target organs. In the gut, all enteric neurons and interstitial cells of Cajal that receive vagal innervation are in effect ‘postganglionic’ parasympathetic neurons. The two systems also differ in the neurotransmitters used.

Vagal efferent innervation of the GIT

The vagus nerve contains functionally mixed populations of parasympathetic preganglionic nerves in addition to its afferent fibres. Vagal preganglionic efferent fibres that project to the gut come from two regions within the medulla, namely the nucleus ambiguus and the dorsal motor nucleus of the vagus (DMNX). The pharynx and larynx are innervated by preganglionic fibres from the nucleus ambiguus, the stomach small intestine and proximal colon by neurons from the DMNX [168, 441, 442]. The source of innervation for the oesophagus varies between the two nuclei, in the species examined, due to differing composition of muscle (smooth v striated) making up the organ between species [443].

Preganglionic myenteric innervation does not appear to be specific for any enteric neuronal classes but is rather extensive, particularly in the stomach, duodenum and caecum [168, 444, 445]. Vagal effects therefore depend on the type of (postganglionic) enteric neuron being

stimulated at any one time and the same preganglionic vagal projections are capable of opposing effects in target tissue.

The DMNX is a paired nucleus in the medulla. Vagal parasympathetic neurons within the DMNX are organised in rostro-caudally oriented columns which innervate the gut through five subdiaphragmatic vagal branches, namely anterior and posterior gastric, hepatic, coeliac and accessory coeliac [446]. They are also arranged viscerotopically, the medial and lateral groups of cells projecting to proximal and distal regions of the GIT, respectively [443, 446]. This level of organisation is not maintained in the periphery; the stomach for example is innervated by both gastric branches and the hepatic vagal branch, the duodenum gets supplied by all of the branches and the colon is dependent on the coeliac and accessory coeliac branches [168, 442, 447].

The vast majority (>95%) of preganglionic vagal efferents are cholinergic [448], and release ACh onto nicotinic receptors found on postganglionic neurons of target tissue [443]. Although NO and catecholamine neurotransmitters are present in sub-populations of vagal preganglionic neurons [449, 450], the application of nicotinic receptor antagonists terminates vagal neurotransmission [451], suggesting a modulatory function for non-cholinergic substances neurotransmitters.

Vagal motor control of the gut

Coordinating the motility of the oropharynx and proximal oesophagus during swallowing is an important role of vagal pathways. Motor neurons involved in this function, are located in the nucleus ambiguus (NA), more specifically, vagal motor neurons that control all of the striated muscle of the pharynx and oesophagus locate to the rostral part of the NA called the nucleus

retrofacialis [452]. Experimental stimulation of oesophageal vagal motor neurons causes generalized contractions of striated muscle [453]. The coordinated motility pattern that creates peristalsis requires input from 'swallowing neurons' located in the nucleus tractus solitarius; they display a highly organised firing pattern which is transmitted to the vagal motor neurons and ultimately results in coordinated waves of striated muscle contractions in the oesophagus [452]. Once the food bolus passes into the smooth muscle portion of the oesophagus, reflex peristalsis is mediated by vagal preganglionic neurons located in the DMNX [452].

Vagal efferents provide parallel inhibitory and excitatory stimuli to smooth muscle of gut. Activating vagal efferent fibres can cause both contraction and relaxation of gastric smooth muscle [454-457] and acute vagotomy causes both an increase in tone in the fundus of stomach and a decrease in motility in its antrum [458]. It stands to reason that both inhibitory and excitatory mediators have to be released by postganglionic enteric neurons for that to occur. There is evidence that the excitatory pathway is mediated by ACh acting on muscarinic receptors and the inhibitory pathway likely depends on NO and/or VIP neurotransmission [458-461]. Furthermore, there appears to be tonic activity within the excitatory cholinergic pathway and neurally mediated relaxation of the stomach can happen by either inhibiting that tonic activity or activating the inhibitory pathway (reviewed in [462]). Vagal efferent input also plays a part in gastric acid secretion, particularly in the cephalic phase of food ingestion, once the food bolus enters the stomach however, both vagal and spinal reflex pathways as well as mucosal receptors regulate acid secretion (reviewed in [463]).

Vagal efferents are also involved in: controlling the tone of the lower oesophageal sphincter, pylorus and ileo-caecal valve [464-466], receptive relaxation of stomach [458] and modulation of contractile patterns in distal stomach, small bowel and proximal colon [467-469]. The extrahepatic biliary system, including the gallbladder and sphincter of Oddi, receive a dense

supply of vagal efferents that arise from the DMNX [470]. The functional significance is not fully understood, there is conflicting data from several studies (reviewed in [452]), however a excitatory role in bile evacuation seems likely.

Sacral parasympathetic innervation of distal gut

The majority of cholinergic parasympathetic projections to the distal colon, rectum, bladder and reproductive organs come from preganglionic neurons whose cell bodies locate to the sacral spinal cord (S1-S4 levels) [183]. Sacral preganglionic projections can take either of two pathways to the gut; they can either project directly to the bowel wall itself or alternatively, synapse with postganglionic neurons in pelvic ganglia (hypogastric / pelvic plexus). Postganglionic neurons from the pelvic ganglia then travel in rectal nerves to the distal colon and rectum to innervate enteric neurons [471-473]. Within the rectum, the myenteric plexus is most densely supplied by parasympathetic fibres, with the submucosal plexus and smooth muscle layers receiving a less-dense innervation [241]. One functional study showed that 50% of myenteric neurons in guinea pig rectum receive parasympathetic pelvic nerve input and most of them have ascending projections [474]. Nicotinic receptor blockade abolished fast EPSPs, however approximately 14% of neurons continued to receive slow EPSPs, suggesting non-cholinergic transmission plays a minor role in this pathway [474]. A population of preganglionic parasympathetic enkephalinergic (ENK) neurons has also been reported in the sacral spinal cord of rat and cat [475, 476]. Although there is currently no data on their functional significance, opioids have been shown to modulate ACh release from preganglionic sympathetic neurons that project to the colon [477], raising the possibility of enkephalins modulating cholinergic transmission in that system.

Pelvic ganglia are complex and variable structures, containing approximately equal proportions of parasympathetic and sympathetic neuronal cell bodies [478, 479]. Interestingly,

based on immunohistochemical studies, a proportion of postganglionic neurons within pelvic ganglia, including the neurons that project to the gut, receive both sympathetic and parasympathetic input [478]. Apart from the preganglionic parasympathetic input, pelvic ganglia also receive a minor contribution from viscerofugal neurons of the distal colon and rectum [480, 481]. There is no data on the function of this input, however this circuit would be well suited for mediating inhibitory colo-colonic or perhaps excitatory recto-colonic reflexes [482, 483].

Electrical stimulation of pelvic nerves evokes significant contractions from mid colon to rectum [484]. Transection of pelvic nerves in rats causes an acute disruption in the parasympathetic preganglionic input to the pelvic ganglia and results in decreased rectal motility and increased colonic transit time [485]. Surgical interruption of rectal branches from the pelvic plexus in a canine model results in disruption of the defecation reflex, loose stools and increased colonic transit [486]. Based on such studies it appears that sacral parasympathetic pathways modulate distal colorectal motility and play an important part in the process of defecation.

Aims of this PhD project

Over the last few decades, the guinea pig has been our animal of choice for studying the structure and function of the ENS. Guinea pig ileum in particular has been extensively studied and provided us with many insights [77], although a significant amount of data on gut motility has come from in vitro experiments on the colon [487-490]. Currently there are up to 13 distinct groups of myenteric neurons within the guinea pig colon containing the same neurochemical markers as found in the ileum, but in different combinations for each functional class [10, 105, 491, 492].

Identification of distinct circuits within the ENS is quite challenging, mainly because axons, varicosities and cell bodies are densely packed into myenteric ganglia making it hard to follow specific connections. Several studies have used techniques such as electron microscopy, high-resolution laser scanning confocal microscopy and immunohistochemistry to analyse structure and connectivity within the ENS. These studies have described inhibitory interneurons with axo-somatic connections to like cells, forming descending 'chains' of neurons from the same functional group [257]. In the same fashion, ascending calretinin-containing interneurons have also been described [254] and intrinsic primary afferent neurons were shown to provide input to interneurons and inhibitory motor neurons, forming mono- and poly-synaptic reflex arcs [493-496]. An interesting feature of some of these circuits were dense pericellular varicose 'baskets', surrounding a neuron or a group of neurons, visible at light microscopy level and containing synaptic connections when examined using scanning electron microscopy [495, 497], likely representing a functional connection.

Calbindin is a calcium binding protein that is a good marker of intrinsic primary afferent neurons in the guinea pig ileum [107, 498] but is not as specific in the colon [10]. Calbindin-immunoreactive dogiel type II neurons in the colon share enough properties with their small bowel counterparts to assume that they have the same sensory function [121, 125, 499-501] and they have previously been noted to contribute to calbindin varicose baskets in the myenteric plexus of guinea pig ileum and stomach [104, 502]. In the first part of the project, using immunohistochemistry and high resolution confocal microscopy, we wanted to expand on this finding and determine if calbindin baskets in the guinea pig distal colon associate with any specific group of myenteric neurons. If this was the case, we could further our knowledge of the connectivity within the distal colon, in particular circuits involving intrinsic sensory neurons. As it turned out, we discovered evidence for a novel circuit, involving putative IPANs that formed calbindin baskets clustered around calretinin neurons and based on examination

of varicosities within the calbindin baskets and circular muscle, we proposed that colonic IPANs form monosynaptic reflex arcs with excitatory motor neurons and interneurons [503].

In the second part of the project we set out to further this hypothesis; using retrograde tracing, immunohistochemistry and confocal microscopy we confirmed that calbindin baskets associate with calretinin ascending interneurons and calretinin excitatory motor neurons to circular muscle, we also identified unique classes of myenteric neurons based on their morphology, neuronal markers and polarity of projection [110].

With ongoing advances in genetic manipulation technology, the mouse is becoming increasingly important for ENS research. Yet, relatively little is known about mouse colonic circuits that underlie motility. We decided to investigate the connectivity of mouse colonic IPANs and compare to our findings in guinea pig. CGRP in mouse colon is specific to IPANS and to extrinsic (spinal) afferents and their varicosities [102, 504]. Using organotypic culture allowed us to isolate myenteric CGRP baskets without contamination from extrinsic sources. We described the populations of neurons within these baskets and analysed the neurochemical coding of CGRP basket and circular muscle varicosities. The most significant finding was that colonic IPANs in mouse colon direct their synaptic output to both excitatory and inhibitory neurons within their myenteric CGRP baskets [111].

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Chapter 2

Characterisation of calbindin-immunoreactive Dogiel type II neurons and their connections in the guinea pig distal colon

Statement of Authorship

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Contribution to paper: Performance of all of the experimental steps, Analysis of data, Writing of manuscript, including preparation of 6/9 figures and all tables.

Estimated overall percentage contribution: 80%

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David Wattchow: Reviewed manuscript and provided expert advice.

Nick Spencer: Reviewed manuscript and provided expert advice.

Introduction

Embedded within the wall of the gastrointestinal tract, the enteric nervous system (ENS) is formed by continuous networks that extend from distal oesophagus to proximal anal canal and contain neural circuits responsible for controlling most gut functions [75, 102, 505, 506]. The ENS acts on intestinal smooth muscle in concert with the pacemaker system [507, 508] to produce a wide range of coordinated and adaptable movements, it also acts on a variety of cells involved in secretory, immune, endocrine and paracrine functions. While autonomous in many ways [509], the ENS receives modulatory input from the sympathetic and parasympathetic divisions of the autonomic nervous system [510]. In small experimental animals, the motor functions involve mostly neurons of the myenteric plexus [142, 511], while neurons in the submucosal plexus have been shown to control mucosal secretion and vascular tone [56, 133, 512]. The importance of the ENS for normal human gut function is exemplified by Hirschsprung's disease, where a segment of large bowel is congenitally deficient in enteric neurons, commonly leading to impaired transit that may result in bowel obstruction.

Neurons within the ENS may be classified functionally as sensory neurons (intrinsic primary afferent neurons, IPANs), interneurons or motor neurons. However, based on morphology, projections, immunohistochemistry and electrophysiology, many more distinct neuronal populations have been identified [10, 77, 125, 286, 501]. This implies multiple subclasses of sensory, interneurons and motor neurons, forming distinct circuits, responsible for a specific enteric function or perhaps an aspect of a function. To add to this complexity, there is significant inter-regional and inter-species variation in neurochemical markers of functionally homologous neurons [75, 125, 501, 513]. Variability in neurochemical coding appears to be greatest in interneurons and least in excitatory and inhibitory motor neurons, which are essentially the common effector neurons of any motor circuit. This could explain in part how functionally distinct regions of the gut share similar neural circuitry and yet produce motor patterns that are very different.

The guinea-pig colon has been widely used in vitro to study colonic motor patterns and function [487-490]. Although the basic neuroanatomy is known, it has not been described to the same extent as guinea-pig small intestine [77]. The current classification of myenteric neurons in the guinea pig colon reveals up to 13 distinct groups [10, 491, 501, 514]. Here, the neurochemical markers present are similar to those described in the small intestine of the same species. However, the combinations of markers for the different functional classes of myenteric neurons differs significantly, with only the neurochemical coding of the inhibitory and excitatory motor neurons being conserved. This is perhaps not surprising since the two regions of gut differ in the composition of their luminal content, motor patterns and function.

A major difficulty in identifying circuits within the ENS is that myenteric ganglia have a dense concentration of projections and varicosities surrounding nerve cell bodies and therefore, any specific connections are hard to identify. Techniques such as high-resolution laser scanning confocal microscopy, electron microscopy and immunohistochemistry have allowed reliable analysis of structure and connectivity between enteric neurons. Several studies have used these techniques to detail neuronal connections in the myenteric plexus of the guinea-pig small intestine. On the assumption that neurochemical markers in cell bodies match the ones in their terminals, these studies describe descending inhibitory interneurons with axo-somatic connections to like cells, forming ‘chains’ of neurons from the same functional group [257]. Furthermore, IPANS (Dogiel type II calbindin neurons) were shown to provide input to these interneurons and to directly synapse onto inhibitory motor neurons, forming mono- and poly-synaptic reflex arcs [493-496]. Ascending chains of calretinin interneurons were also described, some of them providing input to longitudinal muscle motor neurons [254].

In this paper, we present evidence of a novel morphological arrangement in the guinea pig distal colon involving an important class of neurons, putative IPANs, and selective classes of

ascending interneurons and excitatory motor neurons. This hard-wired circuit has likely evolved over millions of years as a primeval reflex, to elicit local polarised neuronal reflexes.

Methods

Tissue collection, preparation and immunohistochemistry

All animal procedures were approved by the Flinders University Animal Welfare Committee. Colonic tissue was taken from guinea pigs that were killed with a blow to the back of the head followed by transection of the carotid vessels and spinal cord. Both males and females were used, within a weight range of 300-500g. The abdominal cavity was opened with a ventral midline incision and segments of distal colon up to 6 cm from the pelvic brim were removed and placed into a Krebs solution (118 mM NaCl, 4.75 mM KCl, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 11.1 mM D-glucose, 2.5 mM $CaCl_2$, gassed with 5% CO_2 in 95% O_2, pH 7.4). Segments were flushed of luminal content, opened along the mesenteric border and pinned flat, mucosal side up, to a Sylgard-lined dish (Dow Corning, Midland, MI). Preparations were then fixed in Zamboni's fixative (2% formaldehyde, 15% saturated picric acid in 0.1M phosphate buffer, pH 7) overnight. Whole-mounts of myenteric plexus and circular muscle were prepared by removing mucosa, submucosa and longitudinal muscle using sharp dissection. Dissected tissue was cleared by washing firstly in dimethylsulphoxide (DMSO, 10min x 3) and then phosphate buffered saline (PBS, 0.15M NaCl with 0.01M phosphate buffer, pH 7).

Preparations were incubated in a blocking solution of 10% normal horse serum in antibody diluent (0.1M PBS, 0.3M NaCl, 0.1% sodium azide) for 60 min at room temperature. Incubation in primary antibody was performed on a rocking tray at room temperature for two nights, followed by 3 x 10 min washes in PBS and incubation in secondary antibody for 4 hours at room temperature prior to mounting on a slide in 100% carbonate-buffered glycerol (pH 8.6).

The primary and secondary antibodies used in this study are listed in Table 1.1 and Table 1.2 respectively.

Table 1.1. *Primary antibodies used in study*

Primary antibody	**Raised**	**Immunogen**	**Source/catalog#**	**Dilution**
Calbindin	Rabbit	Recombinant rat calbindin D-28k	Swant/CB 38	1:2000
Calretinin	Goat	Human recombinant calretinin	Swant/CG-1	1:1000
NOS	Sheep	Recombinant rat brain neuronal NOS	Emson/K205	1:1000

NOS – nitric oxide synthase

Table 1.2. *Secondary antibodies used in study*

Secondary antibody	**Fluorophore**	**Source/catalog#**	**Dilution**
Donkey anti-rabbit IgG	Cy3	Jackson / 711165152	1:200
Donkey anti-goat IgG	Cy5	Jackson / 705175147	1:100
Donkey anti-sheep IgG	AMCA	Jackson / 713155147	1:100

IgG – immunoglobulin G; Cy3 – indocarbocyanine; Cy5 – indodicarbocyanine; AMCA – aminomethylcoumarin.

Antibody Characterization

Calbindin

Calbindin polyclonal antibody (Swant, Belinzona, Switzerland, Cat# CB 38, RRID:AB_10000340) was raised in rabbit against recombinant rat calbindin D-28k. Western blot of guinea-pig brain homogenate results in a single band at 28KDa (equivalent to protein's molecular weight). No staining was detected with this antibody in CNS tissue of knockout mice for D-28k calbindin (Swant calbindin data sheet). Furthermore, all staining was prevented on preincubation with recombinant rat calbindin D-28k [515].

Calretinin

Calretinin (Swant, Belinzona, Switzerland, Cat# CG1, RRID:AB_10000342) is a polyclonal antibody raised in goat against human recombinant calretinin. It does not stain brain tissue from calretinin knockout mice (manufacturer's data sheet). Pre-incubation with purified calretinin protein eliminated immunoreactivity in mouse brain tissue and on western blotting it produces a single band at 29-30k which is equivalent to the protein's molecular weight [516].

Neuronal Nitric Oxide Synthase (nNOS)

The neuronal nitric oxide synthase antibody (nNOS, Emson, Cat# K205, RRID:AB_2314957 – generously gifted by Dr. P. Emson) is polyconal and raised in sheep against recombinant rat brain nNOS. On Western blots of guinea pig inferior mesenteric ganglion it labels a strong band at 160kDa and a faint band at 40kDA [415].

Neuronal structures that were labelled with any of the 3 primary markers (antibodies) were regarded as calretinin-, calbinding- or NOS-immunoreactive neurons, varicosities or baskets. In the text, the terms calretinin, calbindin or NOS neuronal structures are used as equivalent to being immunoreactive.

Image acquisition and analysis

Fluorescence microscopy

Preparations were viewed using an epifluorescence microscope (Olympus IX71, Japan) with the appropriate filters for the fluorophores used. Using a 20x or 40x objective water immersion lens, fluorescent images were captured by a Roper Scientific camera and AnalySIS Imager 5.0 software (Olympus-SIS, Munster, Germany). Images were stored as TIFF files (1392 * 1080 pixels) and optimized for contrast and brightness using Adobe Photoshop (2015 Adobe Systems Software Ireland Ltd) prior to further analysis. ImageJ (NIH, Bethesda) was used for analysis of immunofluorescence intensity (grey value measurements) as well as nerve cell body size.

Myenteric calbindin-immunoreactive varicosities forming basket-like structures

Preparations of myenteric plexus labelled with calbindin antibodies revealed specialized dense clusters of varicosities that enveloped subsets of specific myenteric nerve cell bodies (Fig 1.1-1.2). We refer to these structures as ‘calbindin baskets’ and define them as the presence of intensely calbindin-immunoreactive varicose endings surrounding one or more myenteric neurons. More than one calbindin basket was termed a ‘basket cluster’.

Analysis of calbindin basket relationship to calretinin and NOS myenteric neurons

To determine the relationship between calbindin baskets and NOS nerve cell bodies, 6 ganglia from 6 animals (36 myenteric ganglia in total), immunolabelled for the two markers, were randomly selected and photographed. Three observers independently identified baskets in each photomicrograph of calbindin-immunofluorescence by drawing a digital outline and saving the image as a TIFF file. Observers did not have access to the matched ganglia NOS photomicrographs prior to the calbindin basket selection. To improve objectivity, only correlated calbindin baskets (where two or more observers selected the same basket within a ganglion) were used in the analysis. Baskets selected by only one observer were ignored.

Following basket identification, matching calbindin and NOS photomicrographs were superimposed using ImageJ software and calbindin baskets were scored for the presence or absence of NOS nerve cell bodies within them. A similar analysis was then performed by the same observers, using the same images of matched ganglia (in random sequence) but selecting firstly the NOS neurons and scoring them according to their relation to calbindin varicosities and baskets. To determine the relationship between calbindin baskets and calretinin neurons, a similar analysis was performed, using photomicrographs of 36 randomly selected ganglia stained for calbindin and calretinin (n = 6). Calbindin- and calretinin-immunoreactive nerve cell body size (area) and immunofluorescence intensity was also assessed in ImageJ. Only nerve cell bodies whose values were > 2 x S.D. above mean background intensity were considered immunoreactive and included in analyses.

Analysis of calbindin and calretinin colocalisation in circular muscle varicosities

Ten circular muscle regions within a preparation were randomly selected, and imaged using a 40x objective lens (n = 4), making sure no overlap occurred. Five regions were selected by using the calbindin filter, and the other five using the calretinin filter, in an alternating manner.

Within randomly selected regions, rapidly switching between two filters while keeping constant focus allowed the observer to determine whether a varicosity contained one or more markers. Since the immunoreactive fibres are parallel to the muscle and are relatively sparse, all fibres in each field were assessed and thus the relative proportions of coexistence refer to the total number of labelled fibres seen in each field of view.

Analysis of calbindin and calretinin colocalisation in myenteric varicosities

Within myenteric ganglia, a similar method was used to determine calbindin / calretinin colocalization within varicosities that were not associated with calbindin baskets. Due to the large number of varicosities seen, a random sample was selected from each field of view. One hundred varicosities chosen for the first marker were tested for the presence of the second marker, then 100 varicosities of the second marker from the same field were tested for the presence of the first marker. As the sample is reasonably large, the relative proportion of varicosities of each marker could be evaluated.

Analysis of calbindin Dogiel type II neuron relationship to calbindin baskets within ganglia

66 ganglia from four animals were examined using the fluorescence microscope by a single observer. Calbindin baskets and calbindin Dogiel type II cells were each counted and note was made of whether they were in contact with each other or located at some distance. . In this paper we defined Dogiel type II neurons on a morphological basis as previously described [501] i.e. larger, round or oval, smooth bodied cells with more than one long process.

Confocal microscopy

To examine colocalisation of calbindin and calretinin within calbindin baskets, as well as determine the relationship between calbindin Dogiel type II neurons and calbindin baskets,

whole-mount preparations were viewed with a Leica TCS SP5 scanning confocal microscope (Leica Microsystems, Wetzlar, Germany). Calbindin baskets were imaged in randomly selected ganglia with a 40x objective oil lens at 3x optical zoom. The pinhole was set to 1 AU, scan area at 1024 x 1024 pixels, scan speed of 400Hz with three line averages. Images were exported as TIFF files for analysis. Only varicosities making up the inside layer of a calbindin basket, all the way around, were analysed, as these are most likely to provide synaptic input to the cell within the basket.

Z-projections of select myenteric ganglia containing Dogiel type II cells and calbindin baskets, were created using a 60x objective oil lens at 1-2 optical zoom with all other settings as described above. Sequential scans were used to image ganglia labelled with two fluorophores, the images were acquired at 0.59 to 0.84 µM z-steps. Three dimensional projections were created using Imaris 8.4.1 software (Bitplane, Oxford Instruments) and exported as TIFF images or AVI video files.

Statistics

Statistical analysis was performed by ANOVA, Chi-squared test, or Student's two-tailed t-test for paired or unpaired data using IBM SPSS Statistics 20 for Microsoft Windows (release 20.0.0, IBM Corporation, USA). Differences between data sets were considered significant if $P < 0.05$. Results are expressed as mean ± standard error except where otherwise stated. Lower case "n" always indicates the number of animals used in a set of experiments.

Results

Calbindin baskets and calbindin Dogiel type II nerve cell bodies (putative IPANS)

Calbindin immunolabelling of guinea pig distal colonic myenteric plexus (n = 4) revealed intense cytoplasmic and less intense axonal/dendritic labelling of large, smooth nerve cell bodies, consistent with Dogiel type II morphological characteristics. Smaller, Dogiel type I nerve cell bodies were less frequently observed. Calbindin immunolabelling also revealed a dense supply of varicose axons within myenteric ganglia and internodal strands. Strikingly, calbindin varicosities appeared densely concentrated at particular sites in myenteric ganglia, giving the appearance of basket-like structures completely enveloping myenteric nerve cell bodies (Figure 2.1). We refer to these structures as calbindin baskets. Calbindin baskets had a tendency to form clusters, suggestive of selective connectivity within myenteric ganglia. Thus, we sought to study calbindin baskets further and elucidate their relationship with specific enteric neuronal classes.

Of 68 myenteric ganglia analysed, the vast majority (66/68, 97%, n = 4) had at least one calbindin basket. Each ganglion contained an average 5.4 calbindin baskets (range 1 – 12; n = 4). There was a very close association of Dogiel type II calbindin neurons (presumed IPANS) to calbindin baskets. Out of the 66 ganglia that contained baskets, 51 (77.3%) baskets had at least one IPAN cell adjacent to them i.e. directly in contact with basket or neuropil associated with basket cluster. Three ganglia with baskets had a calbindin IPAN in the same ganglion but not in contact; only eleven (16.7%) basket-containing ganglia lacked Dogiel type II calbindin neurons.

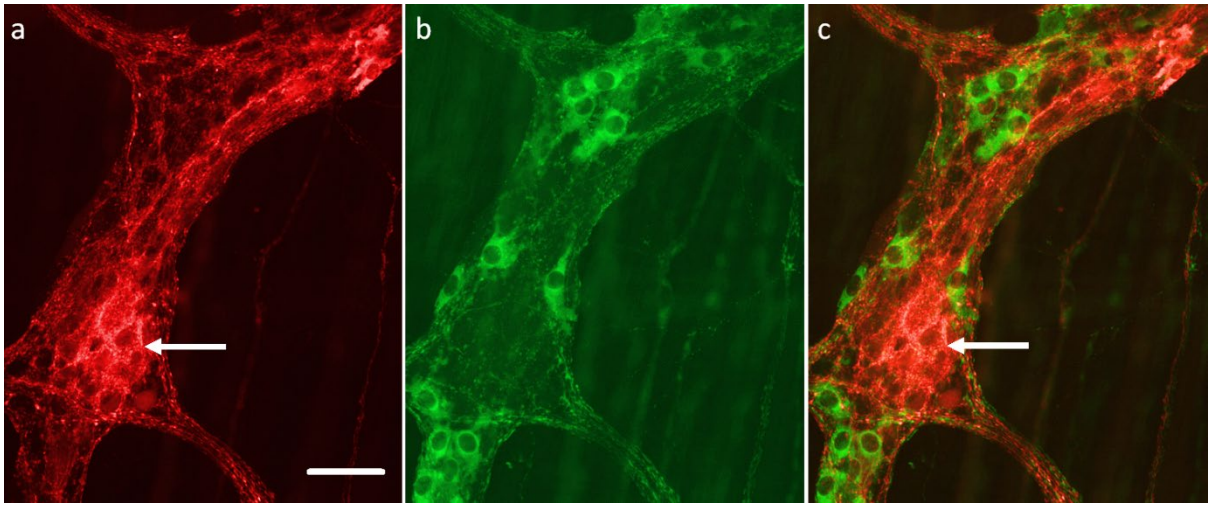

Figure 2.1 Matched fluorescence micrographs of guinea-pig myenteric ganglion immunolabelled with calbindin (a) and nitric oxide synthase (NOS - b). Note the cluster ofcalbindin baskets (arrow). Nerve cell bodies containing NOS do not occur within calbindin baskets. Scale bar = 50 µm.

Confocal microscopy revealed several notable features of calbindin baskets. The intensely calbindin-immunoreactive varicosities around the soma of individual neurons (basket cells) were most dense at the equator of the cell (horizontal axis of ganglion) and tapered off in density towards either pole (vertical axis of ganglion). Usually one of the poles, away from the cell equator, would be completely surrounded by a cap of varicosities with the opposite pole having very few or none, forming a basket or a calyx like structure (Figure 2.2). When several baskets clustered together, varicosities from multiple baskets combined to form a very dense neuropil centrally, with the baskets located around the periphery (Figure 2.3).

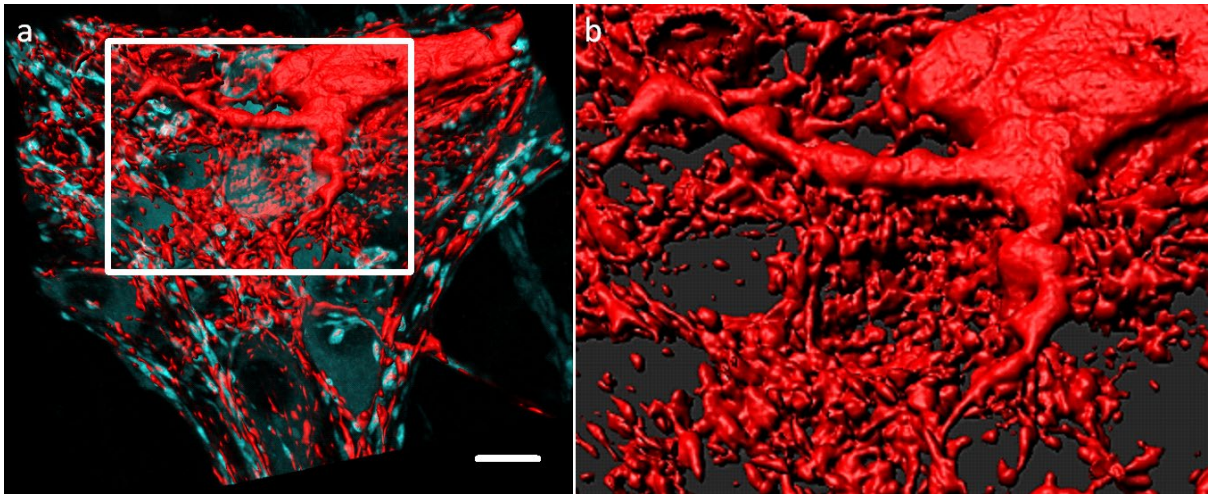

Figure 2.2. Three dimensional reconstruction of a confocal z-series showing a large calbindin Dogiel type II neuron (a,red) forming a basket around a calretinin-immunoreactive nerve cell body (a,cyan). b - Higher magnification view of the calbindin basket. Note that the terminals forming the basket can be traced directly to the Dogiel type II neuron. Scale bar = 50 µm.

Several z-projections were constructed to examine the relationship between calbindin Dogiel type II cells located adjacent to basket clusters. A fascinating observation was that baskets in close proximity were seen to arise directly from the varicose processes of the Dogiel type II cell (Figure 2.2). Z-projections from several ganglia with calbindin Dogiel type II cells at some distance from basket clusters, but within the same ganglion, show their projections directed towards the clusters and joining their neuropil. However, due to the high density of calbindin varicosities, it was difficult to identify baskets from any single such projection. There were also calbindin projections that originated outside of the ganglion examined, seen targeting basket clusters. This suggests that calbindin Dogiel type II cells contribute to basket formation both locally within the same ganglion, and at some distance in other ganglia.

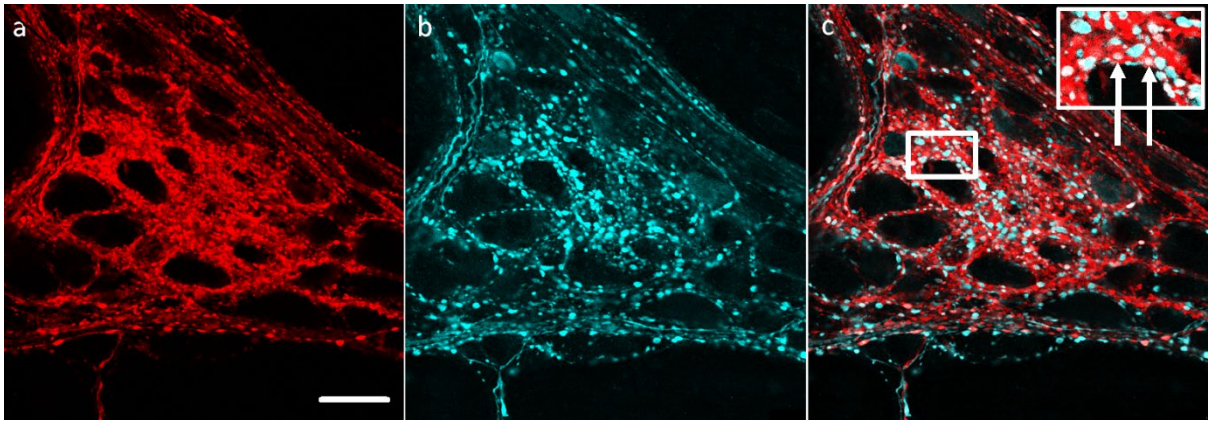

Figure 2.3. Matched confocal (single slice) micrographs of a single calbindin basket cluster. A dense neuropil is formed within the basket cluster, comprised of calbindin (red,a) and calretinin varicosities (cyan,b). A proportion of varicosities (~9%, see results) contained both calbindin and calretinin (see arrows in magnified section,c). Scale bar = 20 µm.

Calbindin baskets and NOS myenteric neurons

Six preparations of guinea pig distal colon were double immunolabelled for calbindin and NOS (n = 6). NOS immunolabelling revealed cytoplasmic fluorescence of myenteric nerve cell bodies and numerous varicosities within myenteric ganglia (Figure 2.1).

To analyse the relationship between calbindin baskets and nitrergic myenteric neurons, calbindin baskets were identified first and subsequently assessed for the presence of NOS nerve cell bodies within them. Three observers examined 36 ganglia (n = 6) after incubation with antisera to calbindin and NOS. An average of 170 calbindin baskets were identified per observer (range 151 to 192). There was a high level of agreement between observers, with 149 (88%) baskets selected by multiple observers and thus included in further analysis. An average of 4.1 ± 0.5 (range 0 to 13) calbindin baskets occurred within a ganglion and the

majority of ganglia had a cluster of baskets. Remarkably, only one (0.7%) NOS neuron occurred within 149 calbindin baskets.

Analysed in the reverse order, NOS nerve cell bodies were identified first and then calbindin immunoreactivity in the same location of the matched micrograph was assessed. An average of 452 NOS myenteric nerve cell bodies were independently identified (1355 total; n = 6). Overall, 7/1355 (0.5%) NOS neurons occurred within a calbindin basket (all seven were scored by the same observer) and the majority (66.1%) lacked surrounding calbindin fibres or varicosities.

Taken together, these data suggest a strong negative association between calbindin baskets and NOS myenteric neurons.

Relationship between calbindin baskets and myenteric calretinin neurons

Calretinin neurons comprise another major population of myenteric neurons in the guinea pig distal colon. Calretinin neurons tend to cluster within myenteric ganglia [10, 517], similar to the distribution of calbindin baskets identified in the present study. Thus, six preparations of guinea pig distal colon were double immunolabelled for calbindin and calretinin (n = 6). Calretinin immunolabelling revealed cytoplasmic labelling of myenteric nerve cell bodies of different intensities (see below), and numerous varicosities within myenteric ganglia (Figure 2.4).

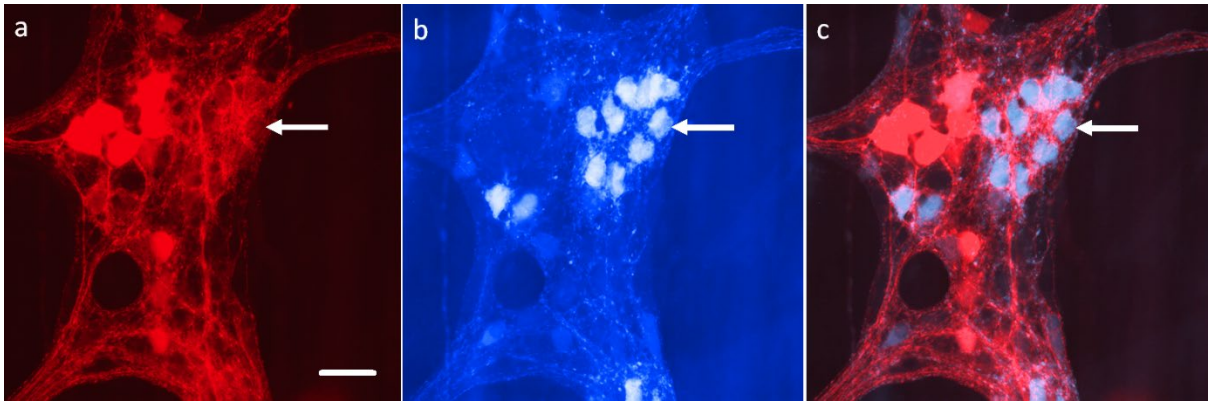

Figure 2.4. Matched fluorescence micrographs of a myenteric ganglion showing a calbindin basker cluster (a, arrow) and a cluster of calretinin-immunoreactive nerve cell bodies occurring inside them (b,c,arrow). Scale bar = 50 µm.

Similar analysis was performed as described above for NOS neurons by independent observers on 36 ganglia labelled by calbindin and calretinin antibodies (n = 6). An average of 217 calbindin baskets were first identified per observer with 90% observer agreement (195 baskets; n = 6). Of the 195 calbindin baskets, 159 (81.5%) contained a calretinin nerve cell body. In the converse analysis, 913 calretinin cells were identified first, and subsequently assessed for calbindin varicosities and baskets in matched micrographs. In total, 559/913 (61.2%) calretinin myenteric nerve cell bodies occurred within calbindin baskets while 76 (8.3%) calretinin cells lacked any associated calbindin varicosities. Taken together, these data suggest that calbindin baskets are selectively associated with calretinin myenteric neurons – a rare demonstration of circuit connectivity observable at the light microscopy level.

Calbindin and calretinin colocalization in myenteric nerve cell bodies

A subset of calretinin nerve cell bodies also contained calbindin immunoreactivity. However, the high intensity of calbindin immunofluorescence within basket clusters occasionally made

these cells difficult to discriminate using conventional fluorescence microscopy. Thus, in a separate series of experiments. Confocal analysis was used to determine the composition of basket cells with regards to the two immunohistochemical markers, see Figure 2.5. A total of 102 calbindin baskets from 19 ganglia were examined (n = 4). Out of the total population of 102 baskets, 42/102 (41.2%) contained CALR/- nerve cell bodies, 41/102 (40.2%) contained CALR/CALB nerve cell bodies, and 19/102 (18.6%) contained nerve cell bodies that expressed neither marker. No CALB/- nerve cell bodies occurred within calbindin baskets.

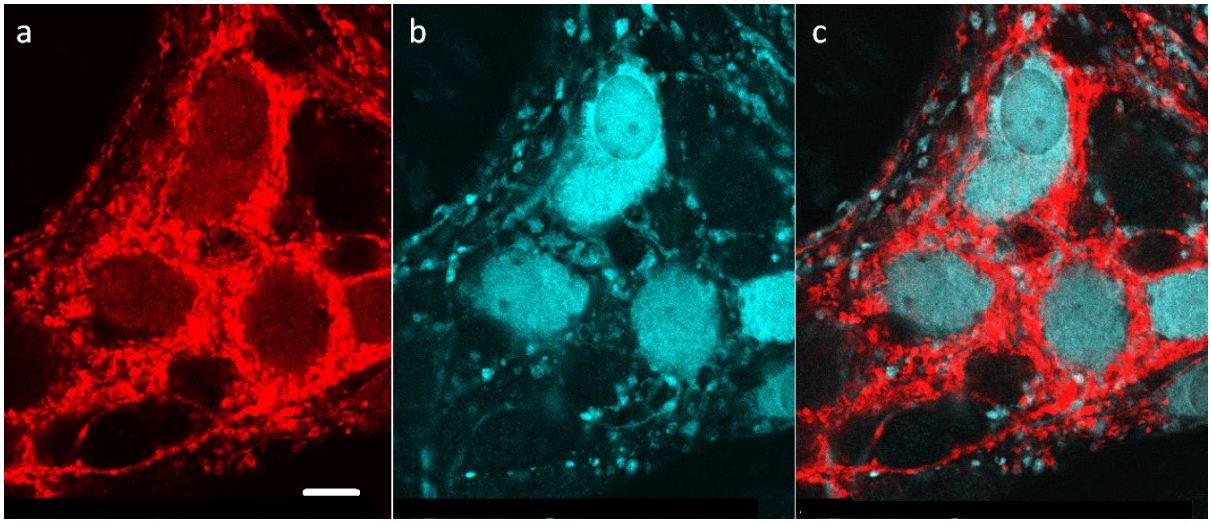

Figure 2.5. Matched confocal micrographs (single slice) of calbindin baskets (red,a) surrounding calretinin immunoreactive nerve cell bodies (cyan, a and c). Note also the colocalization of calbindin in calretinin immunoreactive nerve cell bodies. Scale bar = 10 µm.

The existence of CALR/CALB and CALR/- populations is suggestive of different functional classes. Thus, the populations of myenteric nerve cell bodies containing these markers were characterised in a further series of experiments. In total, 660 calretinin nerve cell bodies were identified in 36 ganglia (n = 6). 305 (46.2%) nerve cell bodies were CALR/-, 239 (36.2%) were CALR/CALB and 116 (17.6%) were CALB/-. Within each ganglion there were an average of

8.5 ± 2.3 CALR/- nerve cell bodies, 6.6 ± 1.2 CALR/CALB nerve cell bodies and 3.2 ± 1.3 CALB/- nerve cell bodies. The CALB/- profile was significantly associated with Dogiel type II morphological characteristics ($\chi 2 = 431.6$, $P < 0.001$, adjusted standardized residual = 20.8, n = 6). Eighty-four of 116 (72.4%) CALB/- nerve cell bodies were Dogiel type II, but a significant proportion had Dogiel type I characteristics (27.6%, 32/116 nerve cell bodies). The vast majority of calretinin neurons were Dogiel type I (305/305 and 236/239 of CALR/- and CALR/CALB nerve cell bodies, respectively). As expected, CALB/- nerve cell bodies were significantly larger than CALR/- and CALR/CALB nerve cell bodies (579 ± 54μm^2 vs 242 ± 14 μm^2 and 248 ± 16 μm^2, respectively, $P < 0.001$, Bonferroni post-test, 1-way ANOVA, n = 6). However, there was no significant difference in nerve cell body size between CALR/- and CALR/CALB neurons (Figure 2.6). Interestingly, although similar proportions of calbindin baskets contained CALR/- (40.2%, above) and CALR/CALB (41.2%, above) nerve cell bodies, CALR/CALB nerve cell bodies were significantly more likely to be surrounded by calbindin baskets, compared to CALR/- and CALB/- neurons ($\chi 2 = 140.9$, $P < 0.001$, adjusted standardized residual = 9.9, n = 6). In total, 154/239 (65%) of CALR/CALB nerve cell bodies occurred within calbindin baskets, compared to 106/305 (35%) of CALR/- nerve cell bodies and 0/116 CALB/- nerve cell bodies.

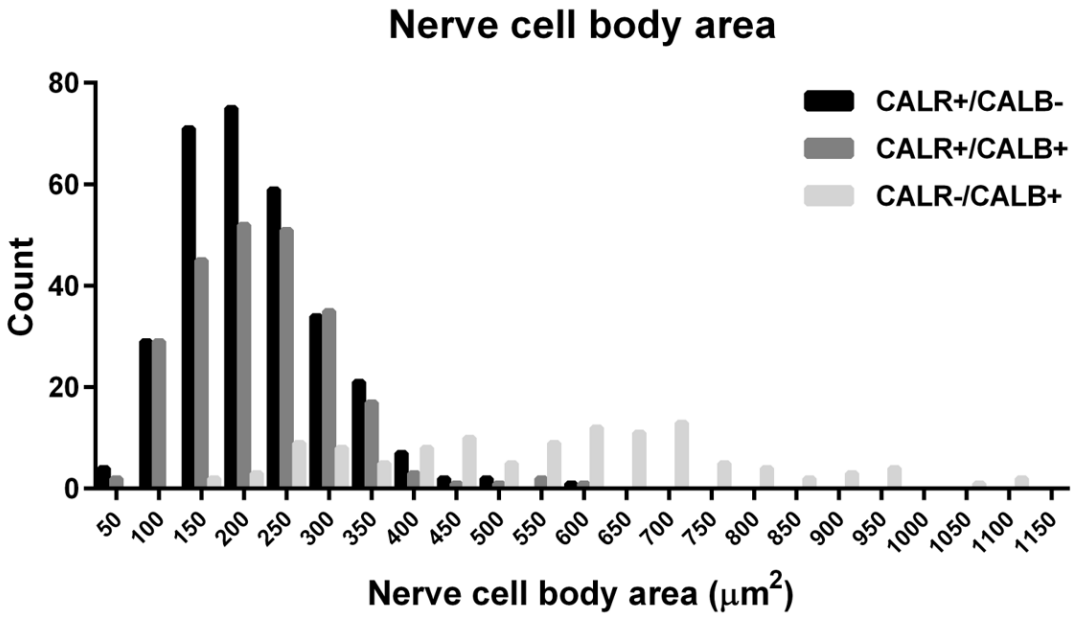

Figure 2.6. Frequency histogram showing nerve cell body sizes of calbindin- and calretinin immunoreactive myenteric neurons. Cells containing calbindin alone were predominantly Dogiel type II and significantly larger than cells containing either calretinin alone or both calbindin and calretinin together.

Myenteric nerve cell bodies that contained calretinin had different immunofluorescence intensities. Indeed, there appeared to be two different populations of calretinin-immunoreactive neurons based on strength of immunofluorescence intensity, with the strongly labelled neurons more often found in calbindin baskets. Measurement of calretinin immunofluorescence intensities revealed a bimodal distribution. However, the distribution did not appear to be explained by the two neurochemical profiles analysed (CALR/- and CALR/CALB): both CALR/- and CALR/CALB neurons showed similar bimodal distributions (Figure 2.7). Nevertheless, CALR/CALB nerve cell bodies showed significantly more intense immunofluorescence compared to CALR/- nerve cell bodies on average (245 ± 4% vs 209 ± 3% background fluorescence, respectively, $P < 0.001$, independent samples t-test, n = 6).

Also, CALR/- nerve cell bodies in calbindin baskets had significantly greater immunofluorescence intensity compared to nerve cell bodies outside of baskets, 191 ± 2.2% background intensity v 145 ± 2.3% ($P < 0.001$, independent samples t-test, n = 6), see Figure 2.8.

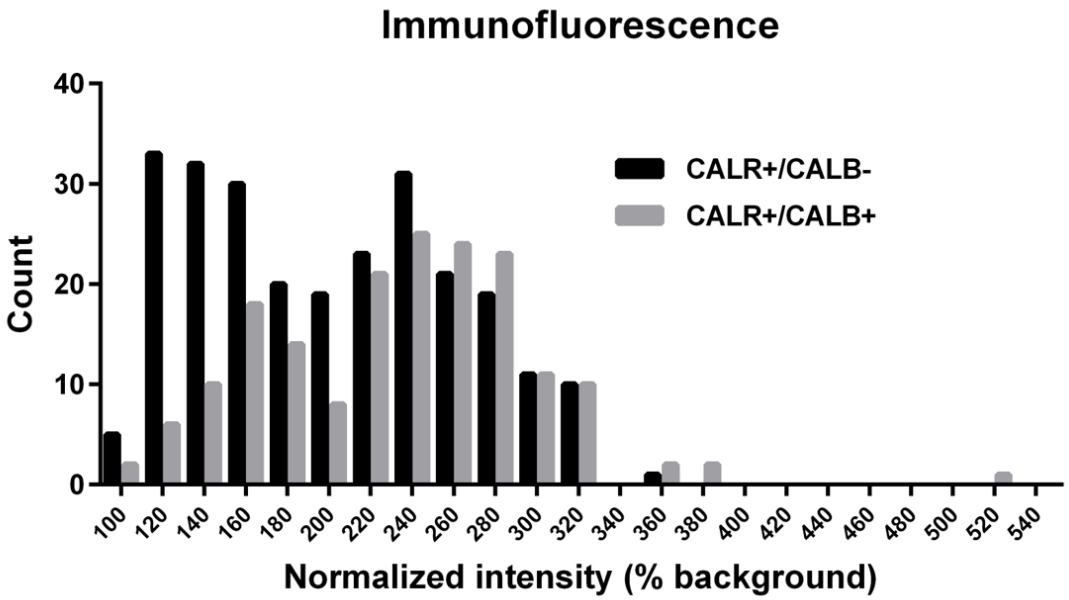

Figure 2.7. Frequency histrogram showing the distribution of calretinin immunofluorescence intensity (normalized to background intensity) among cell containing calretinin alone or both calretinin and calbindin. On average, cell containing both markers had higher calretinin fluorescence intensity (245 ± 4% vs 209 ± 3% background fluorescence).

Calbindin/calretinin colocalization in circular muscle varicosities

To assess the colocalization of calbindin and calretinin immunofluorescence in varicosities projecting into the circular muscle, a total of 40 randomly selected circular muscle fields containing 458 varicosities (n = 4) were scored for calbindin and calretinin immunoreactivity

using a fluorescence microscope. There were 141 CALB/- and 33 CALR/- varicosities, but the majority contained both markers (284/458, 62.0% CALB/CALR varicosities).

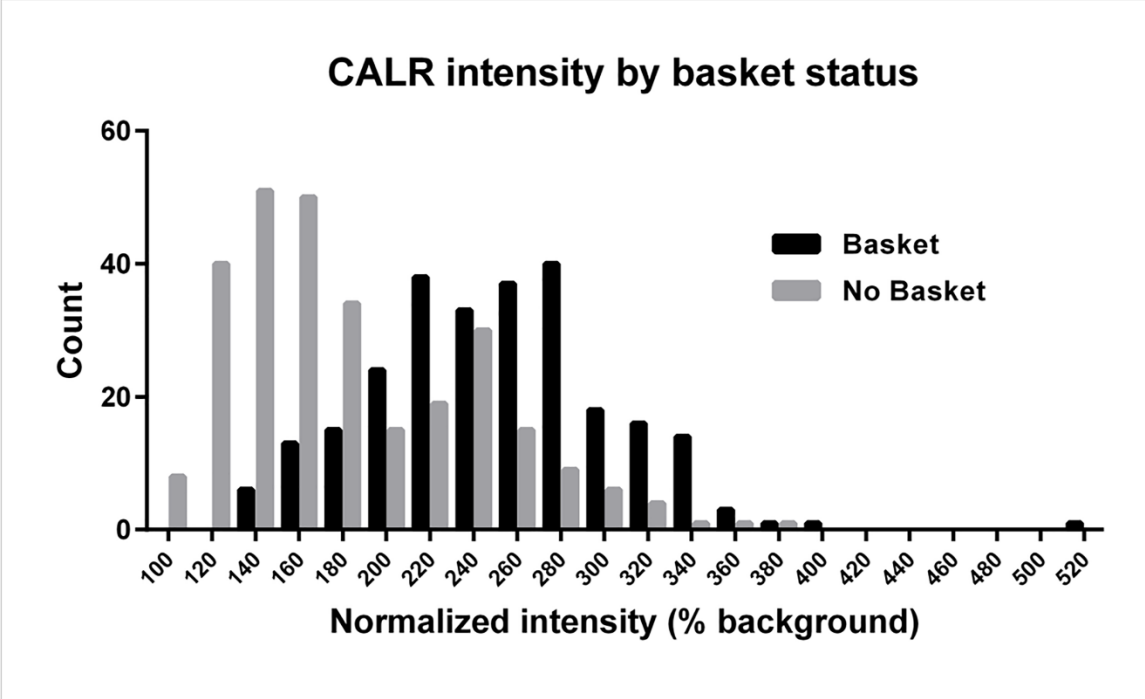

Figure 2.8. Frequency histrogram showing the distribution of normalized calretinin immunofluorescence in myenteric nerve cell bodies that occurred, or did not occur, within calbindin baskets. Overall, calretinin-immunoreactive neurons that occurred within calbindin baskets showed greater calretinin immunofluorescence in their nerve cell bodies.

The same preparations were then used to determine co-localisation in randomly selected myenteric plexus varicosities that were not associated with calbindin baskets. A total of 2309 varicose terminals were examined, 1054 were CALB+ out of which 120 (11.4%) colocalized with calretinin. Out of 1045 CALR+ varicosities examined, 90 (8.6%) were colocalized with

calbindin. Finally, the colocalization of calbindin and calretinin immunofluorescence in varicosities of calbindin baskets was assessed. The high density of innervation necessitated confocal microscopy. Thus, 54 calbindin baskets from 16 ganglia (n = 4) were examined using a confocal microscope. Within the calbindin baskets, 2603 varicosities surrounding and immediately adjacent to cell bodies were scored for calbindin and calretinin immunoreactivity. 1623 (62.4%) varicosities were CALB/-, 748 (28.7%) CALR/- and 232 (8.9%) were CALB/CALR i.e. colocalised.

This data suggests that the baskets are formed from varicosities of calbindin Dogiel type II IPAN's and receive the major contribution from putative sensory neurons, followed by calretinin ascending interneurons and a minor contribution from CALR/CALB neurons of unknown functional class.

Discussion

This work reveals a never before described morphological architecture in the ENS that suggests a specialized functional link between putative colonic IPAN's and histochemically identified ascending interneurons and excitatory motor neurons. The existence of different specialised circuits within the enteric nervous system has been surmised by the observation that several subsets of myenteric neurons exist, based on their neurochemistry, polarity and electrophysiology. This has been supported by a large body of functional and structural research that includes pharmacological studies, intracellular recording, lesion studies, tracing dyes and electron microscopy. Studies of these subclasses of myenteric neurons have begun to reveal some of their connectivity in guinea-pig small intestine; however, this work is the first indication in the guinea-pig colon that, as predicted, subsets of myenteric primary sensory, interneurons and motor neurons are connected in very specific ways to underlie motor function.

Calbindin-containing neurons in the guinea-pig small intestine comprise only one population, namely a larger subclass of Dogiel type II neurons (IPANS) which project locally, to submucosa and mucosa but not muscle [107, 498]. They are the first neurons in motor reflex circuits of the gut, therefore called intrinsic primary afferent neurons (IPANS). Shown to respond upon stimulation of mucosa or muscle stretch, they drive interneurons and motor neurons to initiate a response [101, 518, 519]. By contrast, in the guinea-pig colon, calbindin is present in both Dogiel type I and type II neurons that branch locally [121] and also have ascending and descending projections within the myenteric plexus, projections directed at underlying circular muscle, mucosa and submucosal plexus [499]. Calbindin Dogiel type II neurons in the proximal colon myenteric plexus have been shown to project to mucosa and also display AH (after-hyperpolarization) type characteristics on electrophysiological studies [500], therefore matching the properties of IPANS in the small intestine and presumably having similar function.

In the present work, we describe for the first time a remarkable arrangement of calbindin terminals within myenteric ganglia forming dense arrays of pericellular varicosities best described as baskets. Calbindin baskets were found in the majority of myenteric ganglia. They were easily identified in whole mount preparations using conventional fluorescence microscopy and typically, they were found in clusters of up to thirteen baskets. The most striking feature of these structures, as confirmed by confocal microscopy, is that they appear to arise directly from Dogiel type II calbindin myenteric neurons, which are very often found in contact with basket clusters or at least in the same ganglion. Another interesting observation is the mutual avoidance between calbindin baskets and NOS neurons. Given that NOS is generally a marker of inhibitory neurons, one can assume cells within the baskets to be excitatory neurons. NOS neurons are known to be activated by mechanical stimulation and take part in enteric reflexes, therefore the apparent lack of strong calbindin input to them suggests that in the colon they may rely on interneurons for this to occur.

Dense pericellular baskets formed from varicosities containing somatostatin and bombesin have previously been described in the guinea pig small intestine myenteric plexus [255, 494] and VIP immunoreactive baskets in guinea pig proximal colon [514]. Furthermore, baskets identified on light microscopy have been shown to provide synaptic input to the neurons they surround [495, 497] and close apposition of varicosities to cell bodies identified on confocal microscopy correlates well to structural evidence of synaptic input seen with electron microscopy [257, 493-495]. Although it remains to be confirmed at the level of electron microscopy, our work suggests strong synaptic input from Dogiel type II calbindin neurons to neurons that lie within the calbindin basket clusters. Such a direct connection between putative IPANs and an identifiable cluster of enteric neurons has not been observed before.

Calretinin is a 29-kD calcium binding neuronal protein that has 58% homology to 28kD Calbindin and was initially isolated from chick retina [520]. Calretinin cells in the myenteric plexus of the guinea pig colon have previously been observed to form ‘clumps’, most often at the edge of a ganglion or close to intermodal strands [10, 517]. The present work confirms this distribution but also for the first time, shows a good correlation of calretinin neurons to calbindin baskets with 39.4% of all CALR/- and 65% of CALR/CALB neurons located within a calbindin basket.

Neurons within calbindin baskets showed significantly greater intensity of calretinin fluorescence than neurons outside of baskets and morphologically all exhibited Dogiel type I dendritic and filamentous characteristics. In the myenteric plexus of guinea-pig distal colon this type of neuron has oral or local/circumferential projections [125] and all ascending filamentous interneurons have previously been shown to be calretinin immunoreactive [501]. Lesion studies have also shown calretinin neurons projecting to underlying circular muscle

[517], a finding which our data confirms with the observation of calretinin and calretinin/calbindin containing varicosities in the circular muscle layer. Based on the above, we can conclude that basket cells are likely to be composed of excitatory ascending interneurons as well as excitatory motor neurons to circular muscle. Also, our finding that a good proportion of nerve terminal varicosities (28.7%) within baskets are labelled by calretinin alone, suggests that calretinin neurons within clusters, form ascending chains of excitatory interneurons.

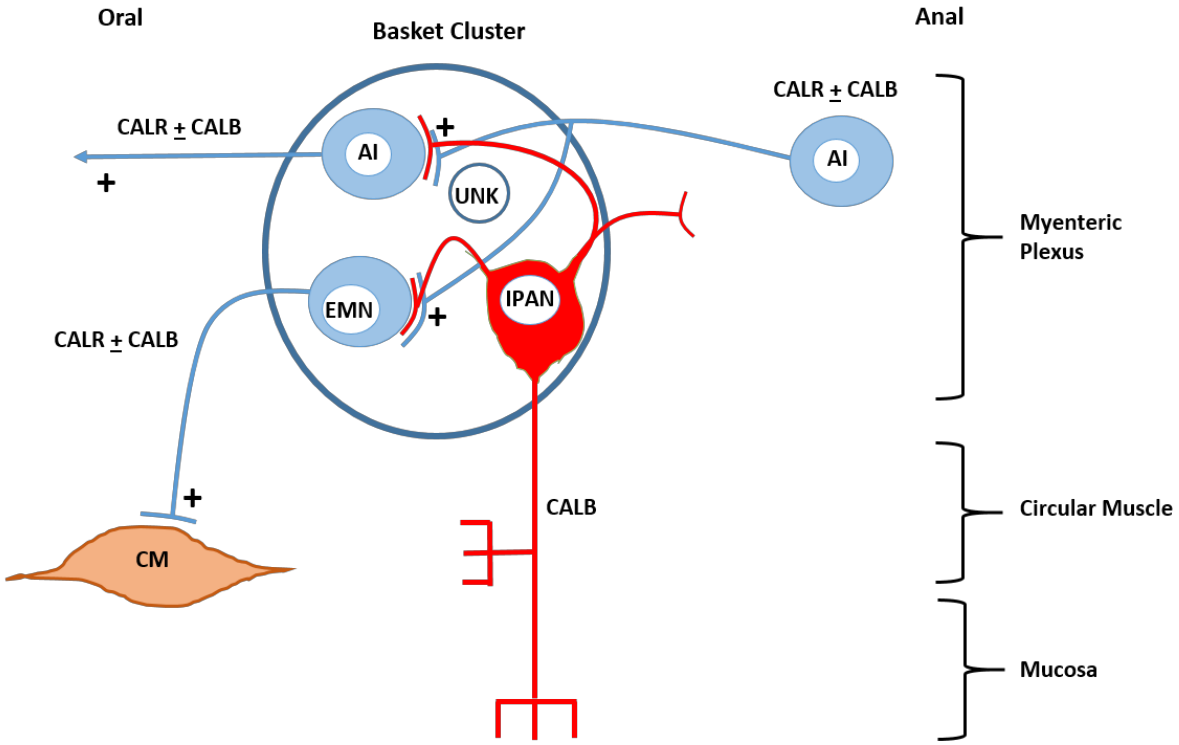

Figure 2.9. A schematic diagram showing the proposed myenteric neural circuits that involve calbindin baskets and three different populations of neurons. CM – Circular muscle cell, EMN – Excitatory motor neuron, AI – Ascending excitatory interneuron, IPAN – Intrinsic primary afferent neuron, UNK - unknown.

We are tempted to describe the preferential arrangement of calbindin baskets within myenteric ganglia as indication of a hard-wired “microcircuit” within the other ENS circuits. Specifically, we refer to Dogiel type II cells that give rise to calbindin baskets within colonic ganglia that contain both calretinin excitatory interneurons and excitatory motor neurons to circular muscle. Their major input appears to be from putative IPAN’s and they are linked by chains of ascending interneurons, forming excitatory reflex circuits (see Figure 2.9). How far this ascending chain would be effective is yet to be determined by functional studies. Therefore, this neural arrangement consists of a monosynaptic circuit where IPANS directly synapse onto excitatory motor neurons to circular muscle.

Excitatory circular muscle motor neurons in the guinea-pig colon have local or short ascending projections [269] and Dogiel type II neurons tend to project for less than 2mm [125], therefore this circuit is well poised to underlie a motor reflex pathway responsible for circular muscle contraction at the site of stimulation or a short distance orally. As the clusters of neurons within the calbindin baskets also contain calretinin interneurons, and these are known to project for several millimetres [125], the microcircuit revealed in this work includes a combination of short and long ascending excitatory reflex pathways. Interestingly, Dogiel type II neurons in the colon project to the mucosa, but the mucosal projections of these nerve endings are not required for intrinsic stretch-activated polarized reflexes. This was demonstrated in the guinea-pig distal colon, when the mucosa was removed and circumferential stretch-activated neuronal pathways to the smooth muscles were still robustly activated [489, 506, 521].

In conclusion, this work provides neurochemical and morphological evidence for a specific intrinsic neural circuit in the colon. The most likely functional role of this circuit is to underlie

ascending excitation and muscle contraction, triggered directly by the IPAN's. Although it is possible that the neural pathway revealed in this work may be involved in the formation and propulsion of the faecal pellet, it is not possible yet to attribute any specific motor pattern to this circuit, based on current data. To correlate this unique neuroanatomical arrangement with motor function, further studies are needed, focusing on projections and activity of cells within the baskets. It is possible that this circuit takes part in more than one motor pattern. Given the variety of motor patterns observed in the colon as well as evidence for extensive cross connectivity between neurons, ENS circuits must be capable of greater subtlety, redundancy and variety than previously suspected.

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Chapter 3

Retrograde tracing of neurons within the myenteric plexus and circular muscle of guinea pig distal colon

Statement of Authorship

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Principal Author Contribution:

David Smolilo

Contribution to paper: Performance of all of the experimental steps, Analysis of data, Writing of manuscript, including preparation of 12/22 figures and all tables.

Estimated overall percentage contribution: 80%

Joint Author Contributions

Marcello Costa: Contributed to data analysis, Reviewed manuscript.

Timothy Hibberd: Contributed to data analysis, generated 10/22 figures.

Simon Brookes: Reviewed manuscript, provided expert advice and taught the author neuronal tracing method used in study.

David Wattchow: Reviewed manuscript and provided expert advice.

Nick Spencer: Reviewed manuscript and provided expert advice.

Introduction

The enteric nervous system (ENS) contains polarised neural circuits responsible for controlling a wide variety of gastrointestinal functions Costa, Brookes [75]. Neurons that form ENS circuits can be functionally classified as sensory neurons (intrinsic primary afferent neurons, IPANS), interneurons and motor neurons. They can also be divided into several groups, based on the shape of their cell body and the number of long processes originating from it (See Figures 3.1-3.8) [522]. Dogiel Type I neurons have one long process originating from their cell body (monopolar neurons) and include many motor neurons and interneurons. Dogiel type II neurons have several long processes (multipolar neurons) and have a presumed role as sensory neurons. Based on morphology, chemical content and electrophysiology, 17 distinct populations of neurons have been described [10, 523]. This suggests the existence of multiple classes within each of the three functional groups.

Retrograde neuronal tracing ex vivo, using carbocyanine dyes, is a powerful tool for characterising enteric neural projections [14]. This method has been used extensively in guinea-pig gut to identify myenteric neurons that project to circular and longitudinal muscle in the stomach [524-526], small intestine [264, 267] and proximal colon [269]. Retrograde tracing from a target tissue, such as a muscle layer or the mucosa identifies nerve cell bodies with axonal projections within that target. Application of tracer directly to the myenteric plexus identifies neurons whose axons pass through the point of tracer application. This allows determination of polarity, length of projection and distribution pattern of neurons forming that plexus. This technique has also been used to map pathways in the human colon [253, 274].

Recently, we described a novel arrangement of myenteric neurons in guinea pig distal colon, in which calbindin-containing Dogiel type II neurons (presumed IPANS) formed distinct basket-like varicose structures around clusters of calretinin-containing nerve cell bodies [503]. Based

on immunohistochemical data and confocal microscopy this suggests specific connectivity between IPANS, excitatory motor neurons to circular muscle and excitatory ascending interneurons. Such a circuit is likely to form the basis of the polarised ascending excitatory intestinal reflex first described over a hundred years ago [11, 527, 528]. Similar basket-like varicose structures immunoreactive for somatostatin and bombesin have been previously described in the guinea pig small intestine [255, 494] and VIP immunoreactive baskets in the guinea pig proximal colon [514]. Using electron microscopy, these basket-like varicose structures have been shown to provide synaptic input to the cells they surround [495, 497].

In the present work, we combined immunohistochemistry with retrograde tracing from both the circular muscle and myenteric plexus layers to characterise projections of neurons immunoreactive for calbindin, calretinin and nitric oxide synthase (NOS), with particular attention to neurons found in calbindin-immunoreactive baskets that are involved in this distinctive circuit.

Methods

Tissue collection

DiI tracing was analysed in five (n=5) circular muscle and four (n=4) myenteric plexus preparations from nine animals out of a total of 36 preparations from 9 animals. All animal procedures were approved by the Flinders University Animal Welfare Committee. Adult guinea-pig of both sexes (360 – 480 g), were euthanised by stunning and exsanguination. Using aseptic technique, the abdomen was opened through the ventral midline followed by the removal of distal colon starting at the pelvic brim. Tissue was immediately placed in sterile Krebs solution (118 mM NaCl, 4.75 mM KCl, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM

$MgSO_4$, 11.1 mM D-glucose, 2.5 mM $CaCl_2$, aerated with 5% CO_2 in 95% O_2, pH 7.4), flushed of luminal content, and opened along the mesenteric border. Preparations were then pinned flat, mucosa uppermost, in a sterilised Sylgard-lined dish (Dow Corning, Midland, MI) and dissected to expose the region of interest. Preparations (ranging from 4-6 cm in length) were washed several times using sterile Krebs solution and then transferred to a sterilised organ culture dish, pinned flat with the serosa facing down.

Retrograde tracing and tissue culture

The dye 1, 1'-didodecyl-3,3,3',3'- tetra-methyl-indo-carbo-cyanine perchlorate (DiI, Molecular Probes, Eugene, OR) was evaporated from an ethanolic solution onto glass beads (Sigma Chemicals; 100 - 200 µm diameter). To trace projections to circular muscle, the mucosa and submucosa were gently peeled off, exposing the underlying circular muscle. A single DiI-coated glass bead was lightly pressed onto an area of circular muscle not visibly overlying a myenteric ganglion or internodal strands. To trace projections within the myenteric plexus, a short segment of circular muscle was stripped off to expose 2-3 rows of myenteric ganglia adhering to longitudinal muscle. A DiI-coated bead was carefully placed onto a single inter-nodal strand except in one preparation, where three beads were placed on adjacent inter-nodal strands (this preparation was excluded from quantitative analysis of circumferential projections).

After placement of DiI-coated beads, preparations were left for 10 minutes to allow the bead to adhere to the target tissue, preventing displacement during exchange of Krebs solution for culture medium (DME/F12; Sigma Chemical Co.) supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, 100IU/mL penicillin, 2.5µg/mL amphotericin and 20 µg/mL gentamicin (Cytosystems, Castle Hill, NSW, Australia), pH adjusted to 7.4. The tissue was cultured for 3 days in a humidified incubator (37°C, 5%CO_2 in air), with daily exchange of culture medium and constant agitation on a rocking tray.

Immunohistochemistry

Cultured tissue was fixed in modified Zamboni's fixative (2% formaldehyde, 15% saturated picric acid in 0.1 M phosphate buffer, pH 7) overnight. Fixed tissue was washed repeatedly in phosphate buffered saline (PBS, 0.15 M NaCl with 0.01 M phosphate buffer, pH 7.4) and mounted on a slide in bicarbonate-buffered glycerol (70% glycerol in 0.5 M sodium carbonate buffer, pH 8.6). To determine adequacy and specificity of DiI labelling, preparations were examined using an epifluorescence microscope with the appropriate filter (Olympus IX71, Japan) to visualise DiI-filled neurons. The criteria used for specificity of CM motor neuron labelling have previously been described and were based on punctate labelling that surrounded an unlabelled nucleus [264, 524]. Preparations with poor quality DiI uptake or with off-target filling were discarded. In total, 4/9 myenteric plexus and 5/27 circular muscle preparations from 9 animals were judged suitable for immunohistochemistry.

Whole-mount preparations selected for immunohistochemistry had remaining circular muscle removed to improve antibody penetration and visualisation. Tissue was permeabilised at room temperature in solutions of increasing carbonate-buffered glycerol concentration (60 minutes in 70%, 60 to 120 minutes in 100% glycerol, pH 8.6). Preparations were then washed in PBS and incubated in a blocking solution of 10% normal donkey serum in antibody diluent (0.1 M PBS, 0.3 M NaCl, 0.1% sodium azide) for 60 minutes at room temperature. Incubation with primary antibodies (Table 3.1) was performed on a rocking tray at room temperature for 48 hours, followed by a PBS wash and incubation with secondary antibodies (Table 3.2) for 4 hours. Preparations were then mounted on glass slides in carbonate-buffered glycerol.

Antibody Characterisation

Calbindin (CALB)

Calbindin polyclonal antibody (Swant, Belinzona, Switzerland, Cat# CB 38, RRID:AB_10000340) was raised in rabbit against recombinant rat calbindin D-28k. Western blot of guinea-pig brain homogenate results in a single band at 28KDa (equivalent to protein's molecular weight) and no staining was detected with this antibody in CNS tissue of knockout mice for D-28k calbindin [529]. Furthermore, all staining was prevented by pre-incubation with recombinant rat calbindin D-28k [515].

Calretinin (CALR)

Calretinin (Swant, Belinzona, Switzerland, Cat# CG1, RRID:AB_10000342) is a polyclonal antibody raised in goat against human recombinant calretinin. It does not stain brain tissue from calretinin knockout mice. Pre-incubation with purified calretinin protein eliminated immunoreactivity in mouse brain tissue and on western blotting it produced a single band at 29-30k which is equivalent to the protein's molecular weight [516].

Neuronal Nitric Oxide Synthase (NOS)

The neuronal nitric oxide synthase antibody (nNOS, Transduction Laboratories, Cat# N31020) is a monoclonal antibody raised in mouse against a 22.3 KDa protein fragment corresponding to amino acids 1095-1289 of human brain NOS. On Western blots of rat brain homogenate it labels a single band which corresponds to the expected molecular weight [530], manufacturer's data sheet.

Analysis of preparations

Whole-mount preparations were viewed using an epifluorescence microscope (Olympus IX71, Japan) equipped with fluorophore-specific filters and a computerised stage -mapping system which allowed accurate measurement of distance in both x and y axes with 1µm resolution [531]. Two independent observers were equally involved with mapping of preparations, each mapping approximately half of the preparations from each group. Locations of all DiI-filled nerve cell bodies relative to the application site and their neurochemical content were recorded. Maps of filled nerve cell bodies were generated based on x and y-axes, where x was parallel to the long axis of the specimen and coordinates were recorded as millimetres from application site. The tissue outline in each map-containing figure is representative of a typical preparation and contains coordinates for cells mapped from several animals. Maps and histograms of DiI-filled cells were generated using Prism 6 (GraphPad Software, Inc, La Jolla, CA, USA) software. In this paper we use the terms NOS, calretinin (CALR) and calbindin (CALB) neurons to mean neurons which have NOS, calretinin and calbindin immunoreactive content respectively. Different populations of retrogradely traced enteric neurons were distinguished based on their combination of neurochemical content and nerve cell-body morphology.

Statistical analysis

Statistical analysis was performed by Chi-squared test, or Student's two-tailed t-test for paired or unpaired data using IBM SPSS Statistics 23 for Microsoft Windows (release 23.0.3, IBM Corp., USA). Statistical differences were considered significant if $P < 0.05$. All data are presented as mean ± SD unless otherwise stated. Lower case "n" always indicates the number of animals.

Results

All raw data acquired in this study, including nerve cell body mapping coordinates and neurochemical and morphological classifications are available at http://dx.doi.org/10.25957/5b4c2a573e682

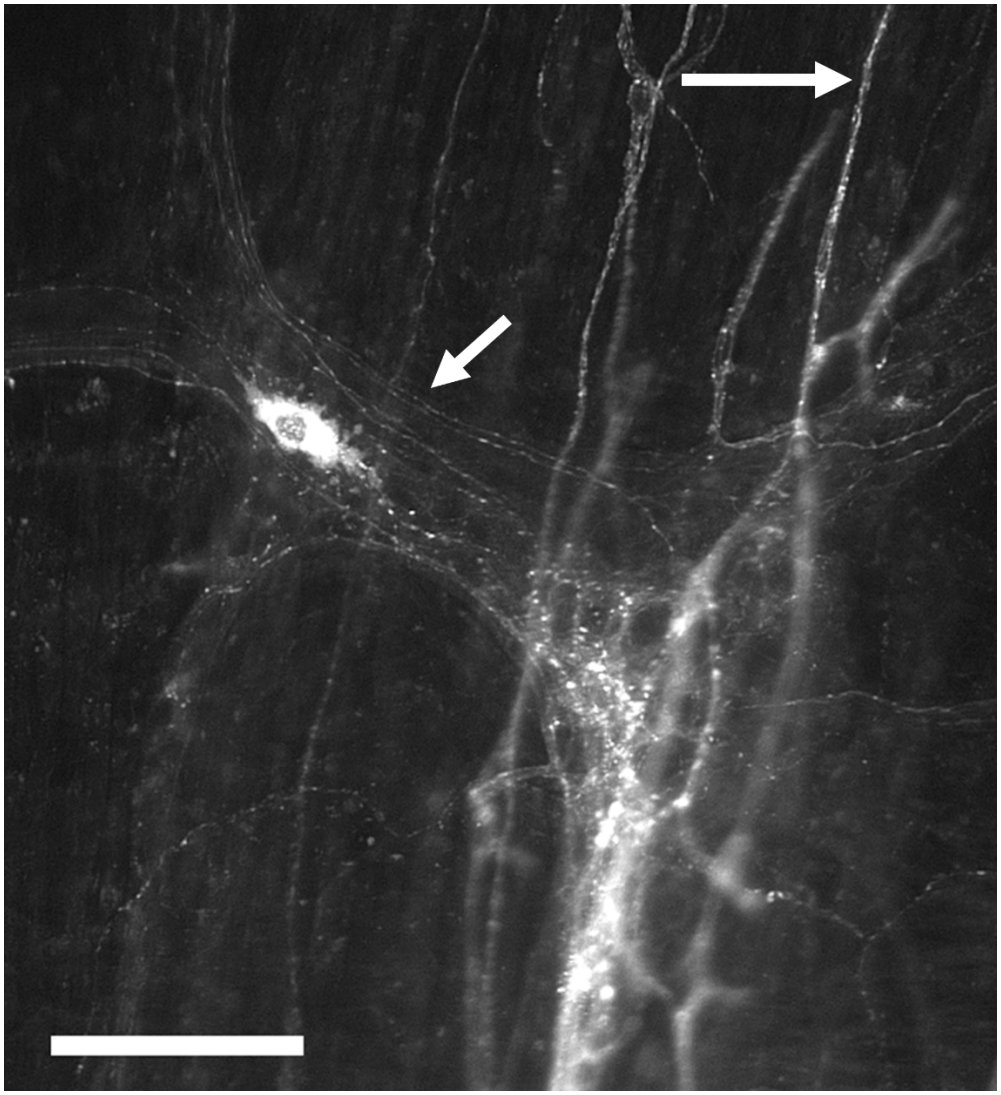

Figure 3.1. Fluorescence micrograph of guinea-pig myenteric ganglia demonstrating a DiI-filled Dogiel type I cell traced from circular muscle (a). Note the dye filled projections running within an intermodal strand (short arrow) as well as fibres running parallel to circular muscle (long arrow). Scale bar = 50 µm.

Filling from circular muscle (CM) and from myenteric plexus (MP)

Myenteric neurons projecting to circular muscle

A total of 1267 myenteric nerve cell bodies were retrogradely filled by DiI from the circular muscle (CM) layer (average 253 ± 180 cells per preparation, n = 5, see Figure 3.9). Of these, ascending circular muscle neurons comprised 54 ± 4% (709/1267 cells total) and descending circular muscle neurons comprised 46 ± 4% (558/1267 cells) of all filled cells. The vast majority of CM-projecting nerve cell bodies had Dogiel type I morphology (1188/1267 cells, 94 ± 4%, n = 5); 6% had Dogiel type II morphological characteristics (79/1267 cells).

Dogiel type I neurons filled from circular muscle

Most cells labelled from the circular muscle (624/1188) lacked all three neurochemical markers (calbindin, calretinin and NOS). Most of these triple-negative cells were located aboral to the DiI application site (563/624 cells) and represented the majority (79%, 563/709 cells) of ascending neurons labelled from circular muscle (Figure 3.9 - 3.10).

NOS neurons comprised the second most numerous group of cells traced from circular muscle (447/1267 cells). The vast majority had descending projections (435/447 cells, ~ 97%). Indeed NOS neurons comprised 95% of all neurons with descending projections to circular muscle and made up virtually all neurons with descending projections longer than 2mm. NOS-immunoreactive neurons consistently lacked both CALR and CALB immunoreactivity (Figure 3.10).

Two smaller populations of Dogiel type I neurons with calretinin immunoreactivity were observed; one contained CALR alone (CALR+/CALB- ; 43/1267 cells) and the other had both CALR and CALB (CALR+/CALB+; 72/1267 cells). Both populations had primarily ascending projections to circular muscle (Figure 3.11). Only two Dogiel type I cells (of 1267, n=5) traced from circular muscle contained CALB alone (CALR-/CALB+).

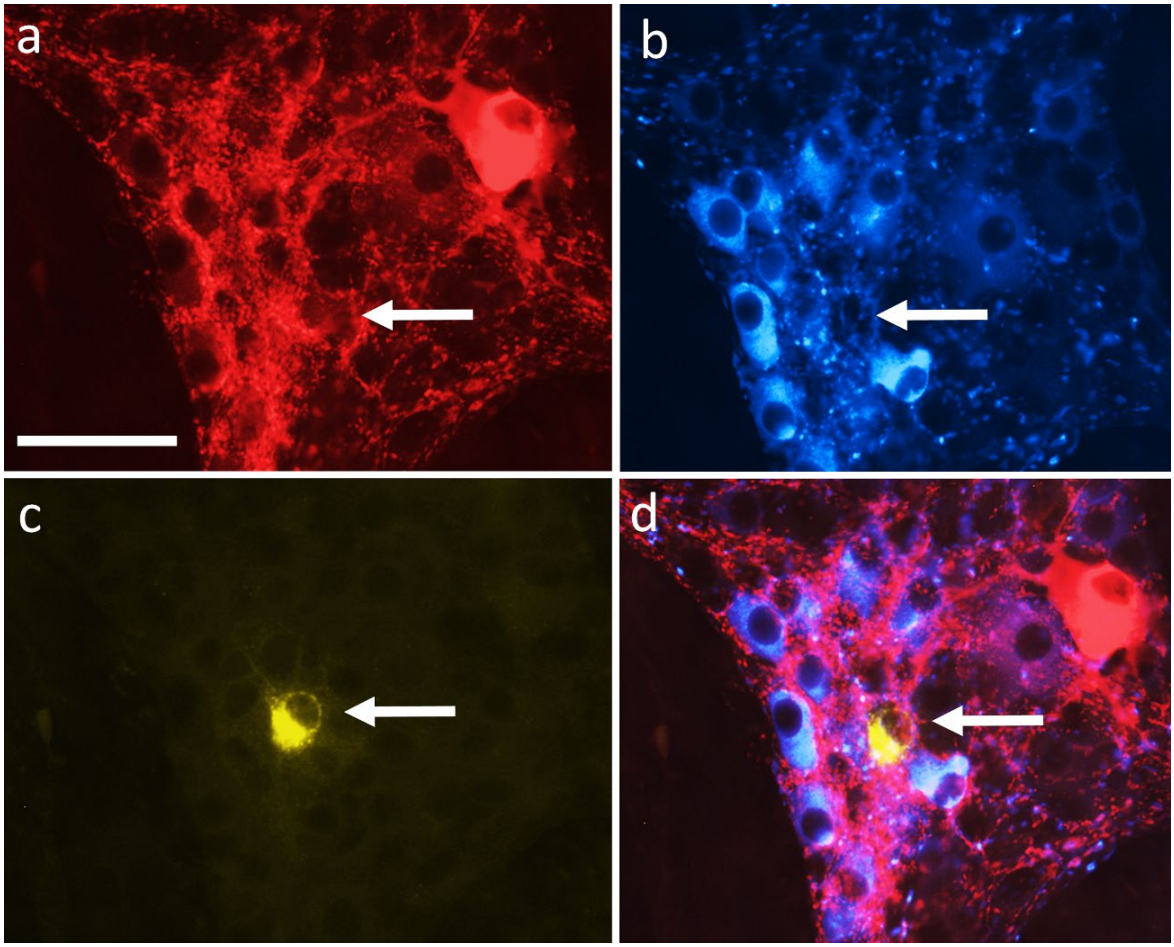

Figure 3.2. Matched fluorescence micrographs of a myenteric ganglion immunolabelled with calbindin (a) and calretinin (b), showing a DiI-filled Dogiel type I neuron (c, arrow) traced from circular muscle, which is CALB-/CALR-. Scale bar = 50 μm.

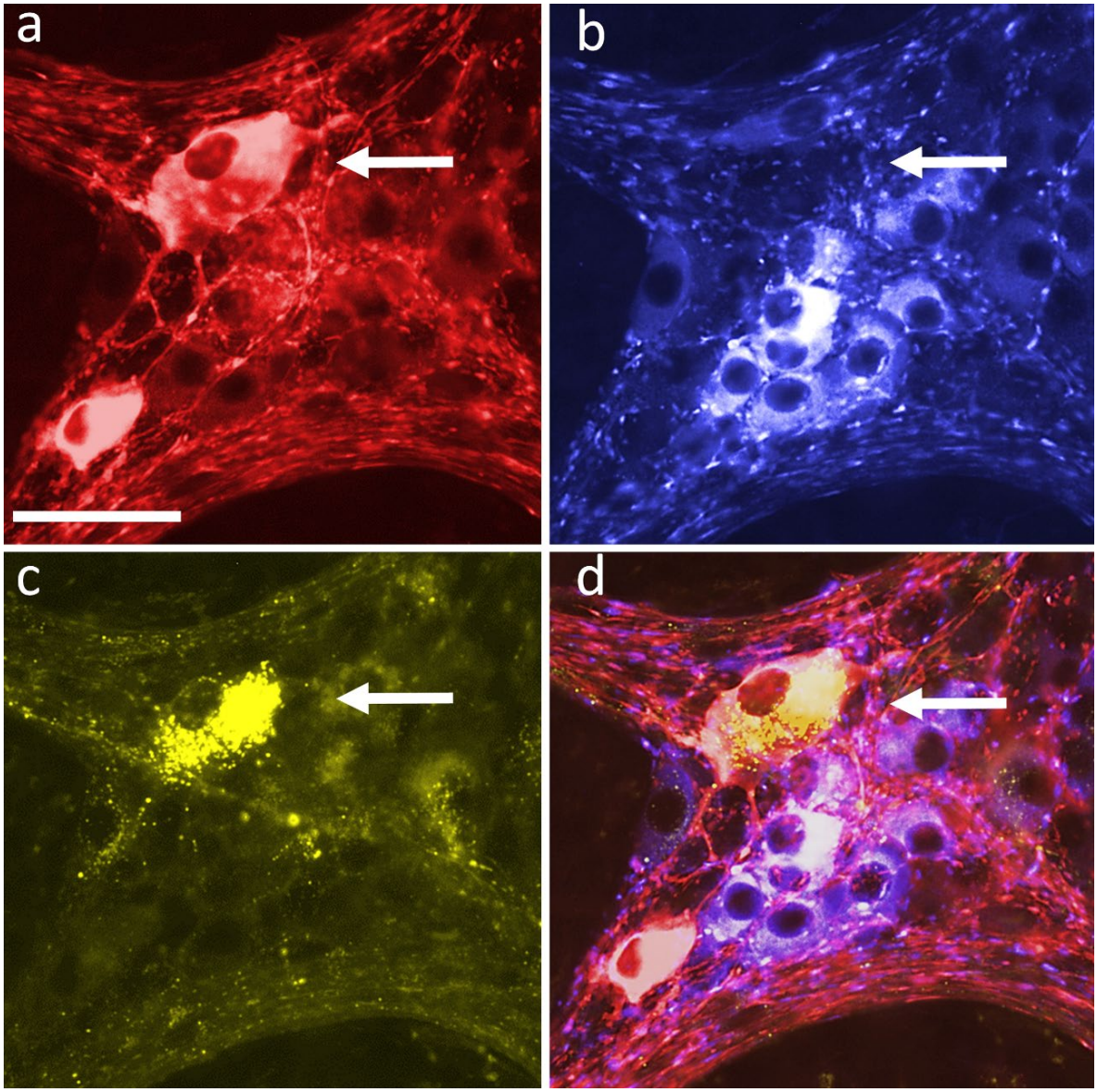

Figure 3.3. Matched fluorescence micrographs of a myenteric ganglion immunolabelled with calbindin (a) and calretinin (b), showing a DiI-filled Dogiel type II neuron (c, arrow) traced from circular muscle, which is CALB+/CALR-. Scale bar = 50 μm.

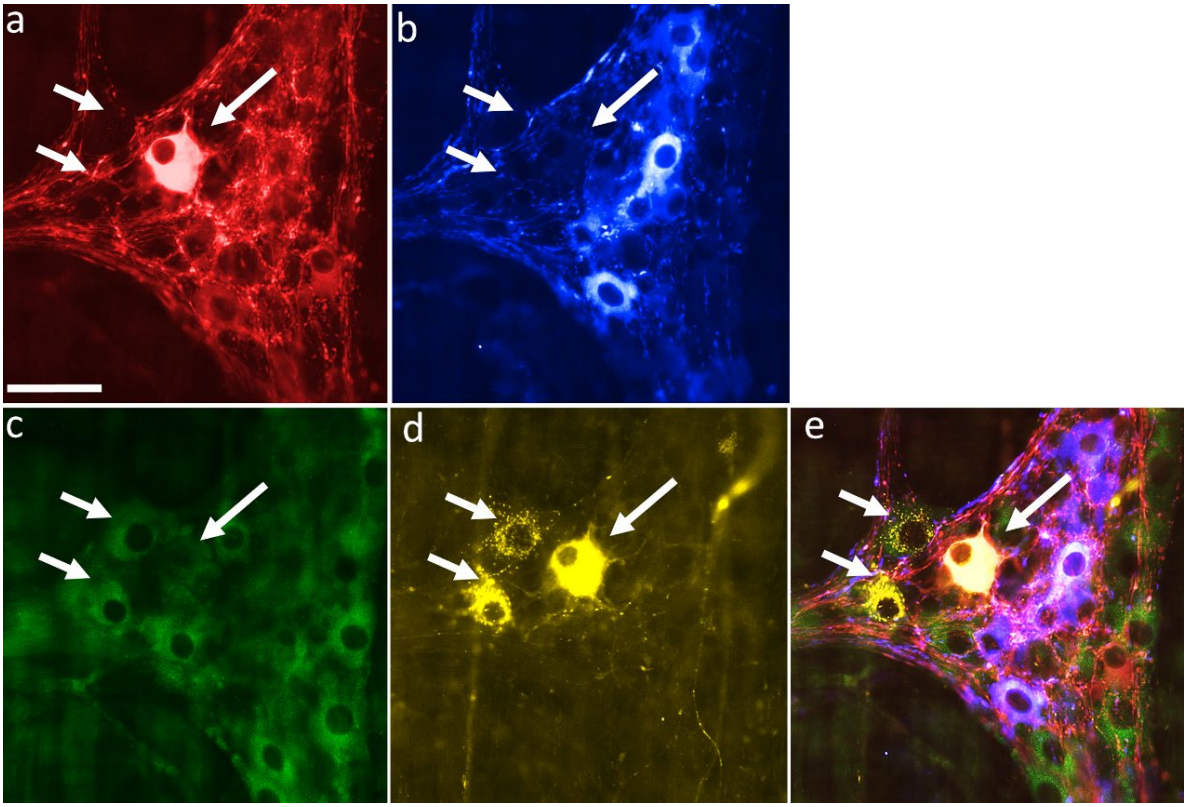

Figure 3.4. Matched fluorescence micrographs of a myenteric ganglion immunolabelled with calbindin (a), calretinin (b) and nitric oxide synthase (NOS – c), showing a calbindin-immunoreactive Dogiel type II cell (CALB+/CALR-/NOS-, long arrow) filled by DiI (d) which was applied to circular muscle. Note its close association to a cluster of calbindin baskets. Note also two DiI-filled, NOS-immunoreactive Dogiel type I cells (CALB-/CALR-/NOS+, short arrows). Scale bar = 50 μm.

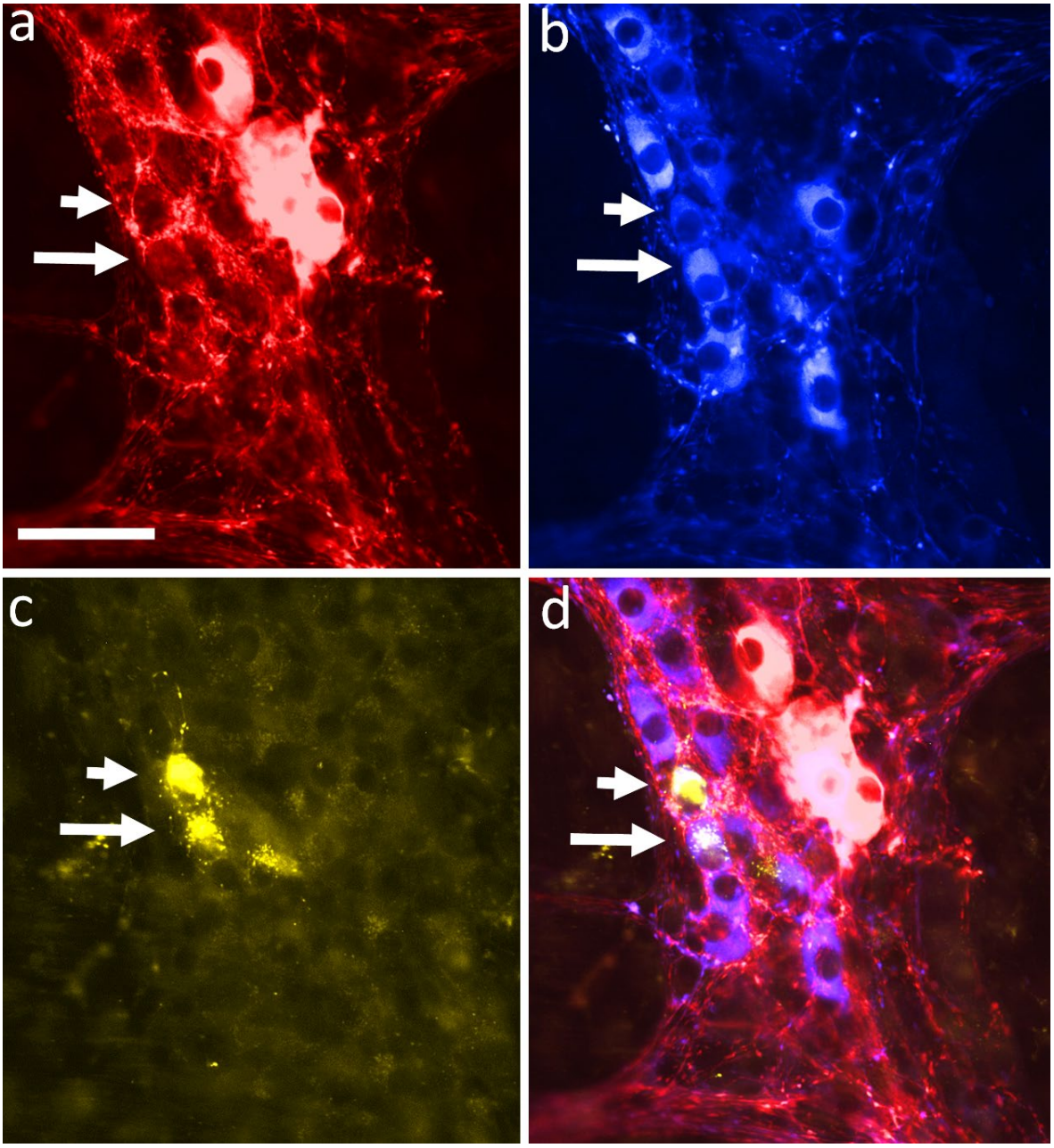

Figure 3.5. Matched fluorescence micrographs of a myenteric ganglion immunolabelled with calbindin (a) and calretinin (b) showing two DiI-filled cells (traced from circular muscle) located in calbindin baskets. Note one of the cells is CALB+/CALR+ (long arrow) and the other one is CALB-/CALR+ (short arrow). Scale bar = 50 μm.

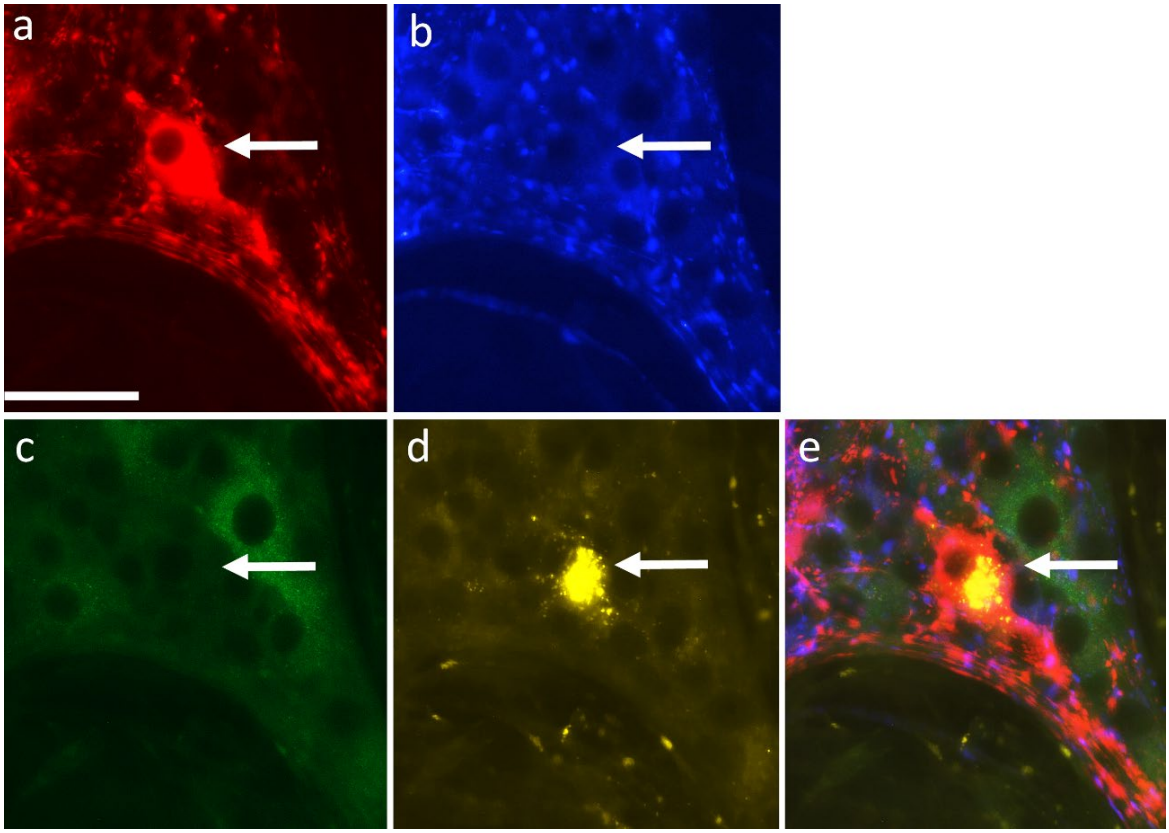

Figure 3.6. Matched fluorescence micrographs of a myenteric ganglion immunolabelled with calbindin (a), calretinin (b) and nitric oxide synthase (NOS – c), showing a calbindin-immunoreactive Dogiel type I neuron (CALB+/CALR-/NOS-, arrow) filled by DiI (d) which was applied to a myenteric internodal strand. Scale bar = 25 μm.

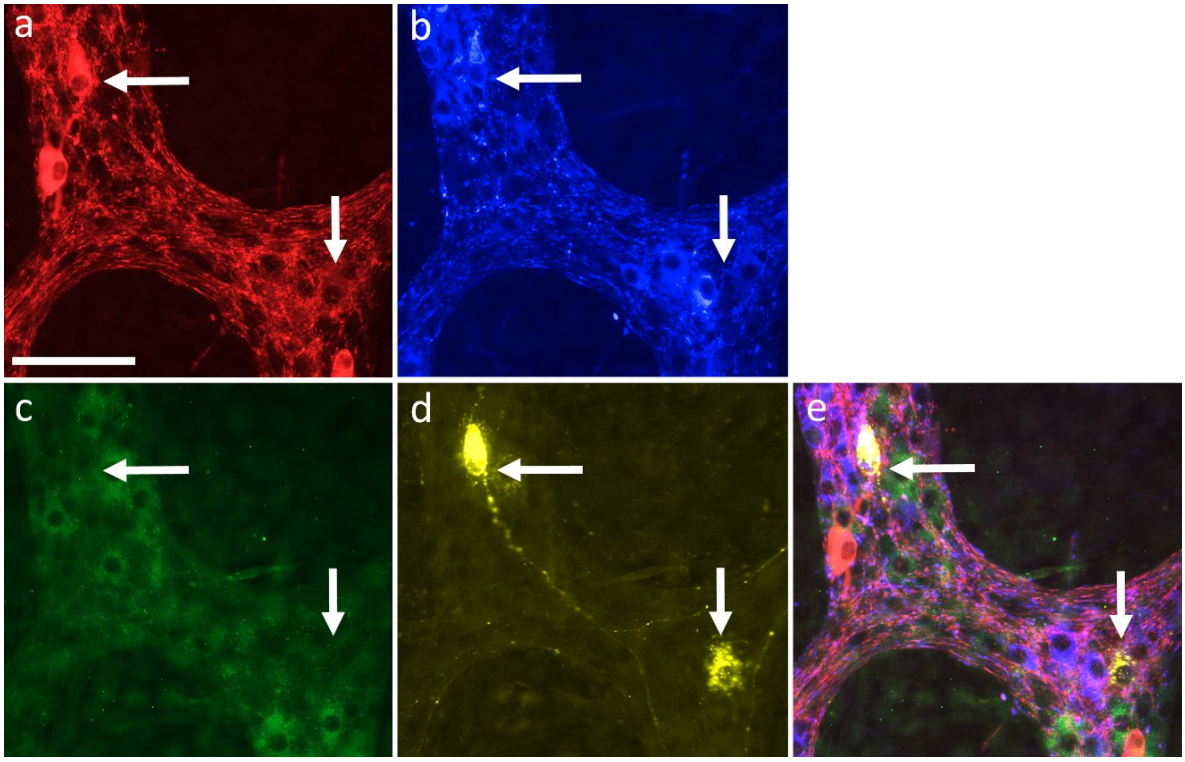

Figure 3.7. Matched fluorescence micrographs of a myenteric ganglion immunolabelled with calbindin (a), calretinin (b) and nitric oxide synthase (NOS – c), showing two DiI filled cells traced from the myenteric plexus (d). One of the cells is CALB+/CALR+ (horizontal arrow) and the other one is CALB+/CALR-/NOS- (vertical arrow). Note that both of these cells are located in a calbindin basket cluster. Scale bar = 50 μm.

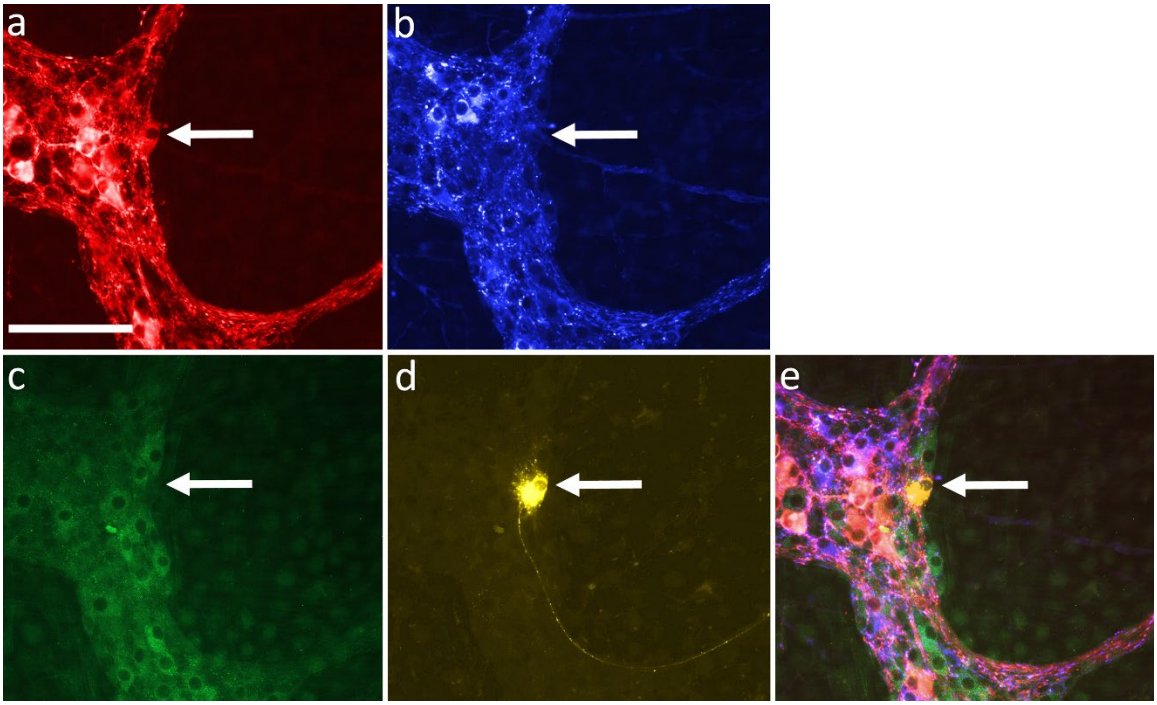

Figure 3.8. Matched fluorescence micrographs of a myenteric ganglion immunolabelled with calbindin (a), calretinin (b) and nitric oxide synthase (NOS – c), showing a CALB+/CALR-/NOS- Dogiel type I DiI-filled neuron (arrow, d) traced from the myenteric plexus. This class of neuron comprised a significant proportion of neurons with long descending projections. Scale bar = 50 μm.

Dogiel type II neurons filled from circular muscle

Of the 79 Dogiel type II neurons labelled from circular muscle, most were located close to the DiI application site (Figure 3.10), slightly favoring the oral side (46/79 cells). All of the Dogiel type II neurons were calbindin-immunoreactive and none of these were either CALR or NOS immunoreactive.

Populations of myenteric neurons projecting within the myenteric plexus

DiI applied directly to the myenteric plexus filled a total of 2757 myenteric nerve cell bodies (average 514 ± 493 cells per preparation, n = 4, see Figure 3.12). Myenteric neurons with descending projections comprised 60 ± 7% (1735/2757 cells total); the remaining 40 ± 7% (1022/2757 cells total) had orally-directed projections. Of the myenteric neurons filled from the myenteric plexus, 88% had a Dogiel type I morphology (2427/2757 cells); the remaining 12 % had Dogiel type II morphology (330/2757 cells; n = 4).

Seven populations of myenteric neurons traced from the myenteric plexus could be distinguished by their combinations of nerve cell body morphology and neurochemical content.

Dogiel type I neurons

Dogiel type I neurons lacking all three markers (NOS-/ CALR-/ CALB-) accounted for 27% of all neurons filled from the myenteric plexus (733/2757); Most of these (89%; 649/733) projected orally (Figure 3.13).

NOS+ neurons made up just under half (44%) of the Dogiel type I nerve cell bodies (1071/2427) filled from the myenteric plexus. Of these, nearly all (97%; 1044/1071 total cells)

cells were located oral to the dye application site. NOS-immunoreactive neurons accounted for about 60% of all descending neurons traced from the myenteric plexus (Figure 3.14, 3.20).

The next largest population of Dogiel type I neurons was immunoreactive for both calretinin and calbindin; they accounted for about 10% of all neurons filled from the myenteric plexus (285 of 2575). They had either descending (174 cells) or ascending (111 cells) projections (Figure 3.15). Another population contained calretinin-immunoreactivity without either of the other markers. These amounted to ~6% of neurons filled from the myenteric plexus (154/2757 cells). The majority of this type of cell had ascending projections (75%, 116/154 cells; Figure 3.13).

The last significant population of Dogiel type I neurons consisted of cells immunoreactive for CALB without either CALR or NOS (NOS-/CALR-/CALB+, Figure 3.15). These accounted for ~7% of filled neurons (184/2575 total cells) and were abundant in descending pathways (Figure 3.20), having some of the longest projections seen, up to the full length of preparations (35 mm). This type of neuron was rarely filled from the circular muscle.

Six cells retrogradely traced from the myenteric plexus had Dogiel type I morphology with CALR and NOS (NOS+/ CALR+/ CALB-, n = 4). Two had ascending projections and all had relatively short projections in the longitudinal axis (<7 mm oral or aboral to the DiI application site).

Dogiel type II neurons

Dogiel type II neurons traced from the myenteric plexus were all calbindin immunoreactive and comprised ~12% of all cells traced (330/2757 cells). The majority of these (67%; 220/330 cells; Figure 3.14) had descending projections.

Myenteric neurons within calbindin baskets projecting to circular muscle

About 9% of all DiI-filled myenteric neurons filled from circular muscle, were localized within calbindin baskets (113/1267); all of these had Dogiel type I morphology and were almost exclusively located aboral to the DiI application site (ie: had ascending projections). Considering just ascending neurons projecting to circular muscle, 16% were surrounded by calbindin baskets. (Figure 3.16). None were NOS immunoreactive.

Calbindin-basket neurons that project to circular muscle could be classified into three different populations based on their neurochemical code (Figure 3.17); CALR+/CALB+ (65/113 cells), CALR+ (24/113 cells) and cells with none of the markers (24/113 cells). Of all DiI-filled CALR+/CALB+ myenteric neurons projecting to the circular muscle, 90% were surrounded by baskets (65 of 72 cells). For DiI-filled CALR+/CALB- CM-projecting neurons, the proportion in baskets was 56% and only 3% for cells that lacked any of the 3 markers (NOS-/ CALR-/ CALB). Thus, neurons with CALR+/CALB+ neurochemistry that project to the circular muscle were largely exclusive to calbindin baskets.

Neurons within calbindin baskets traced from other myenteric ganglia

Myenteric neurons in calbindin baskets comprised about 7% of all neurons traced from the myenteric plexus (190/2757 cells). The majority of these neurons had ascending projections (185/190 cells), all had Dogiel type I morphology and shared the same mixture of

neurochemical codes as the circular muscle-projecting neurons in baskets (ie CALR+/CALB+ or CALR+ alone or lacking all 3 markers - see Figure 3.18).

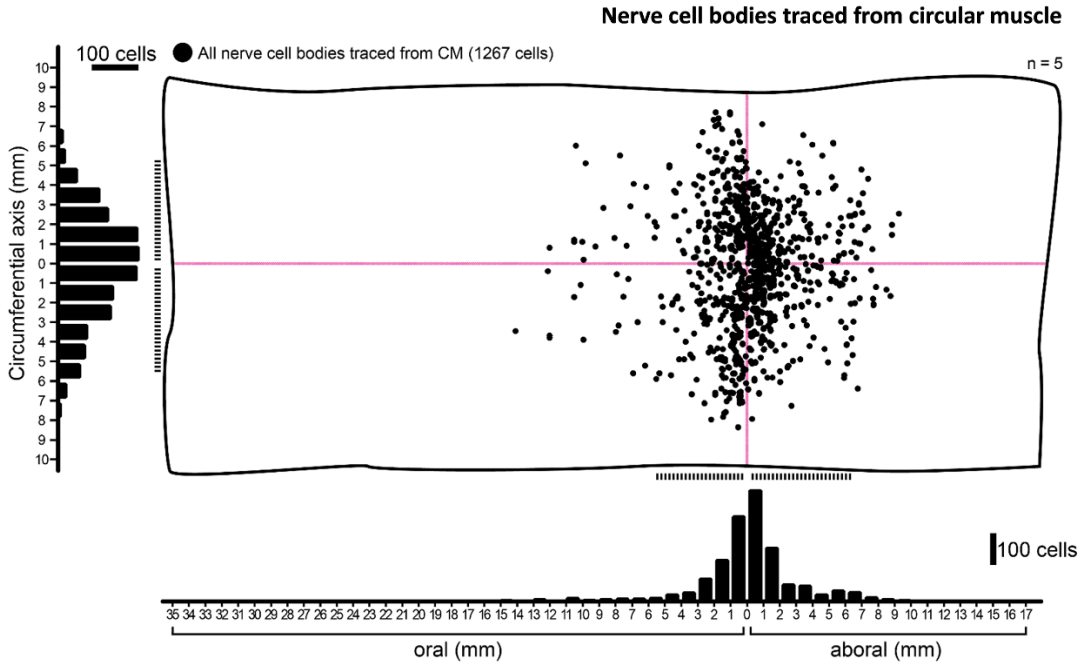

Figure 3.9. Composite map showing the distribution of myenteric nerve cell bodies traced from circular muscle (n = 5). Neurons traced from circular muscle had significantly shorter longitudinal projections than neurons traced from myenteric plexus. Here, each black circle represents a single myenteric nerve cell body retrogradely-labelled with DiI. Nerve cell bodies were traced from the DiI-application site, located at the intersection of the X and Y axes (pink solid lines); the X-axis corresponds to longitudinal axis of colon. Data represents all nerve cell bodies traced in 5 preparations (n = 5). The data is plotted within the outline of an example preparation for illustration purposes, this does not imply all data was acquired from a single preparation (see methods). The black dashed lined located outside the preparation outline indicate the ranges within which 95**%** of nerve cell bodies were located, either side of the DiI-application site.

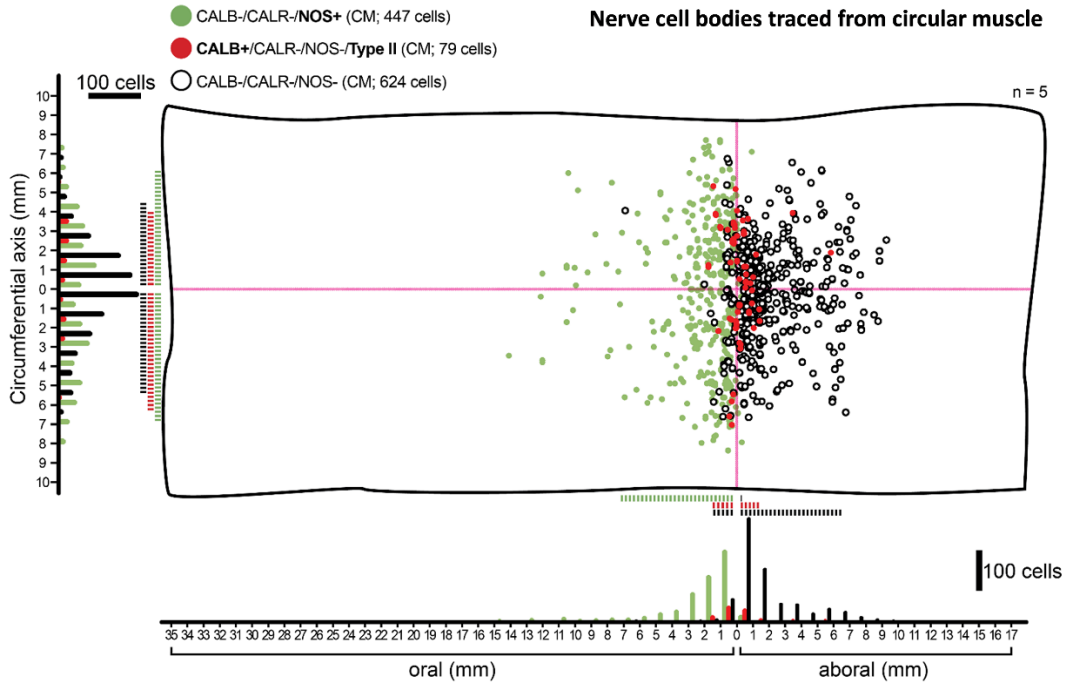

Figure 3.10. Composite map showing the distributions of three classes of myenteric neurons traced from circular muscle from 5 preparations (n = 5); Calbindin Dogiel type II neurons, NOS neurons and neurons lacking all three markers (CALB-/CALR-/NOS-). Note the CALB-/CALR-/NOS+ neurons had a well defined polarity, representing the vast majority of descending myenteric neurons to the circular muscle. Conversely, most ascending neurons were CALB-/CALR-/NOS-.

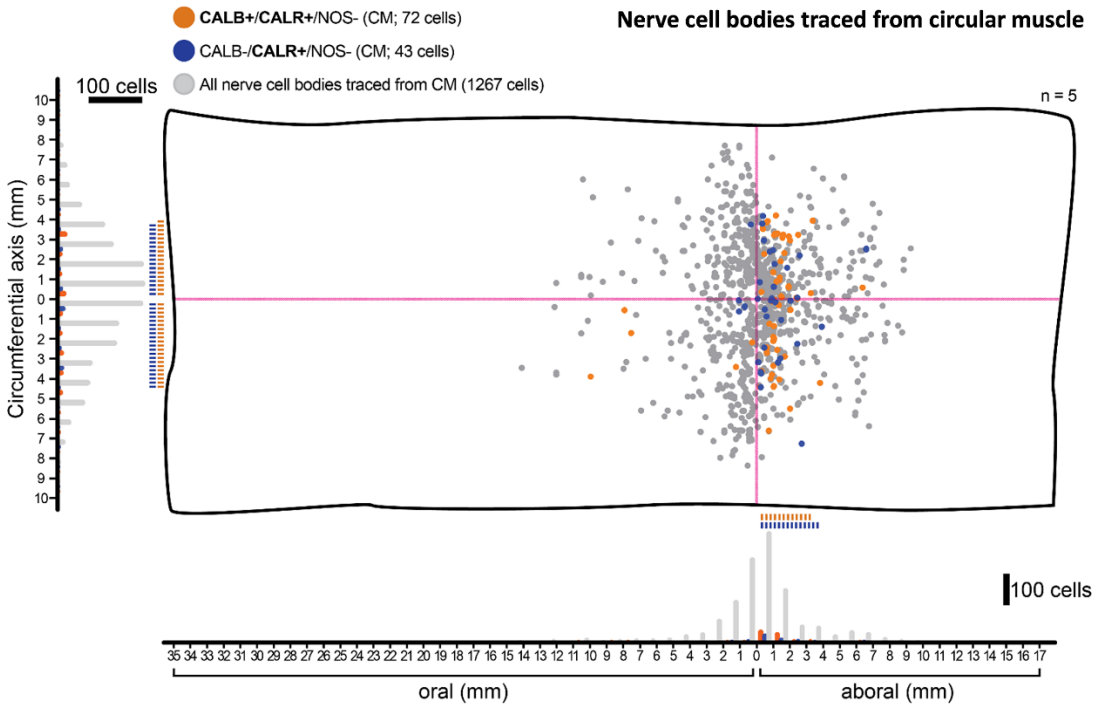

Figure 3.11. Composite map showing the distributions of calretinin-containing myenteric neurons traced from circular muscle in 5 preparations (n = 5). The majority of both classes of CALR+ neurons had ascending projections. Grey circles represent all nerve cell bodies traced from circular muscle, for comparison (1267 cells; n = 5).

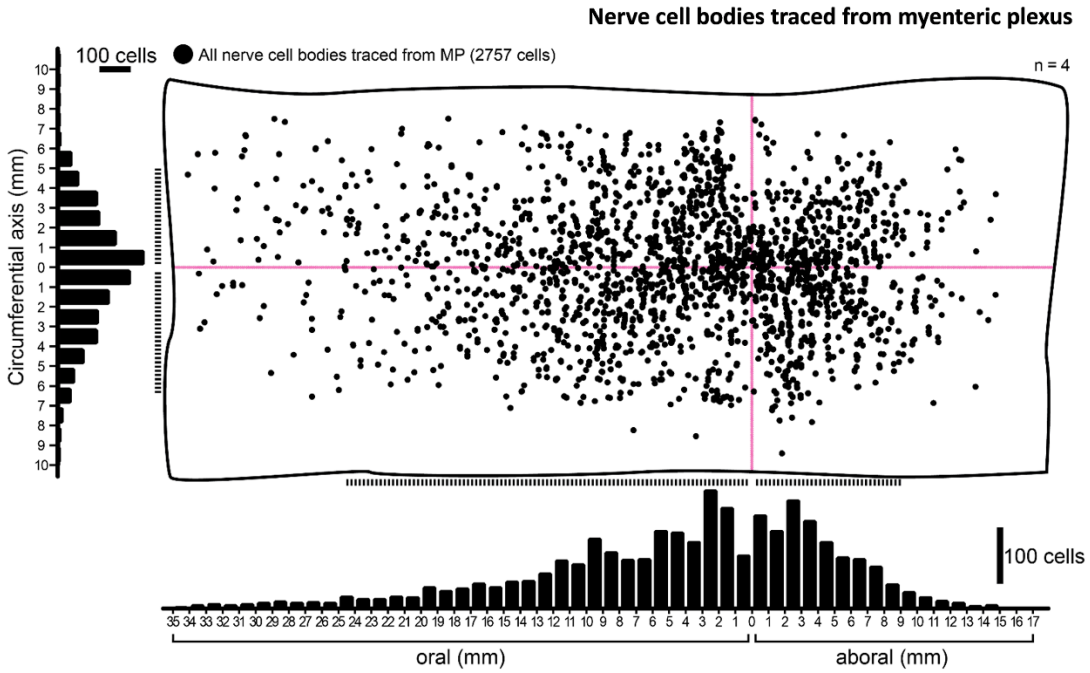

Figure 3.12. Composite map showing the distribution of myenteric nerve cell bodies traced from myenteric plexus in 4 preparations (n = 4). X-axis corresponds to longitudinal axis of colon. Note the greater range of longitudinal projections among neurons with descending projections, compared to ascending neurons.

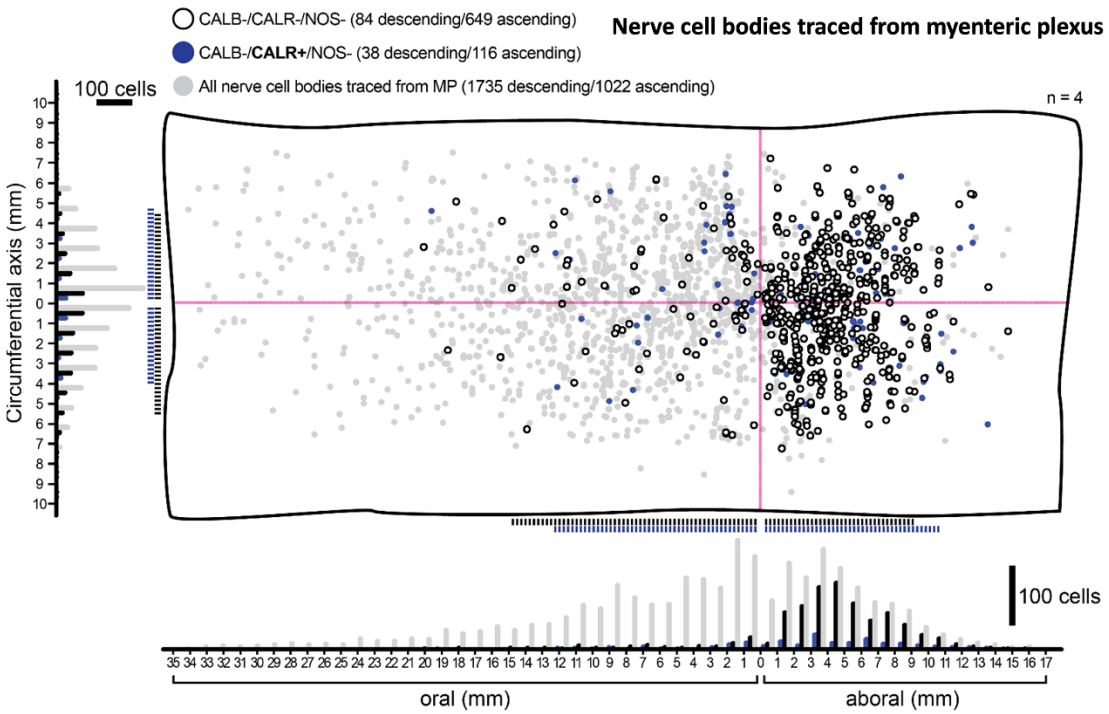

Figure 3.13. Composite map showing the distributions of two classes of predominantly ascending myenteric neurons traced from myenteric plexus: those containing calretinin and those lacking any of the three markers (n = 4).

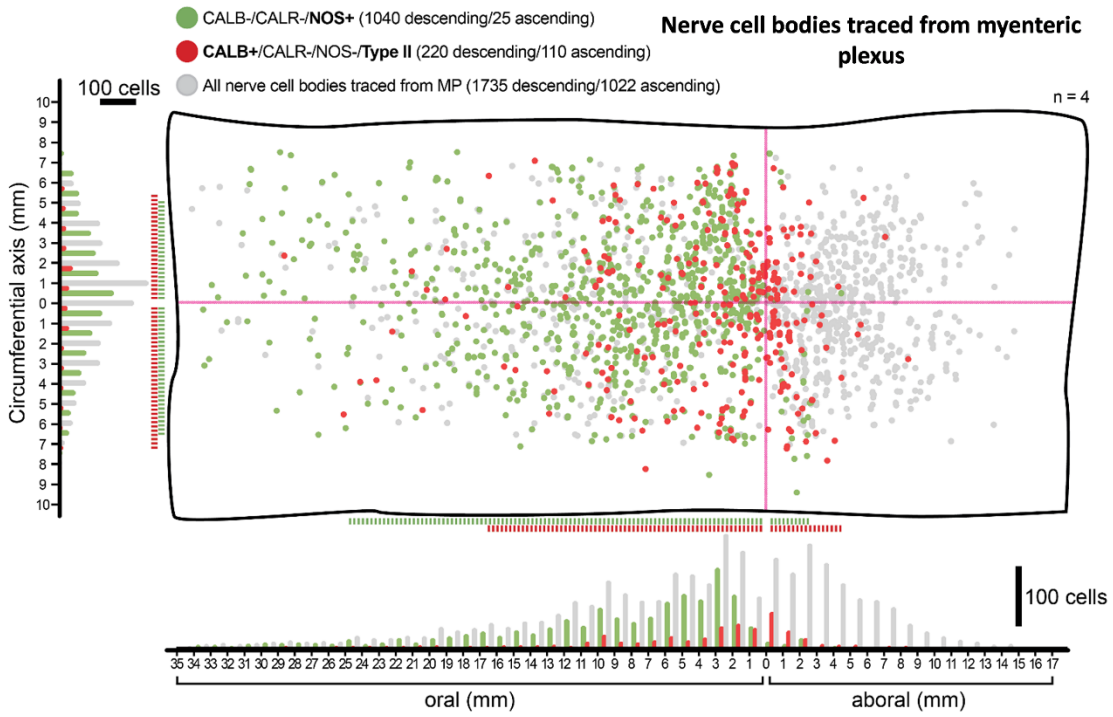

Figure 3.14. Composite map showing the distributions of NOS+ and Dogiel type II myenteric neurons traced from the myenteric plexus (n = 4). Long descending projections characterised both NOS+ and CALB+ Dogiel type II neurons.

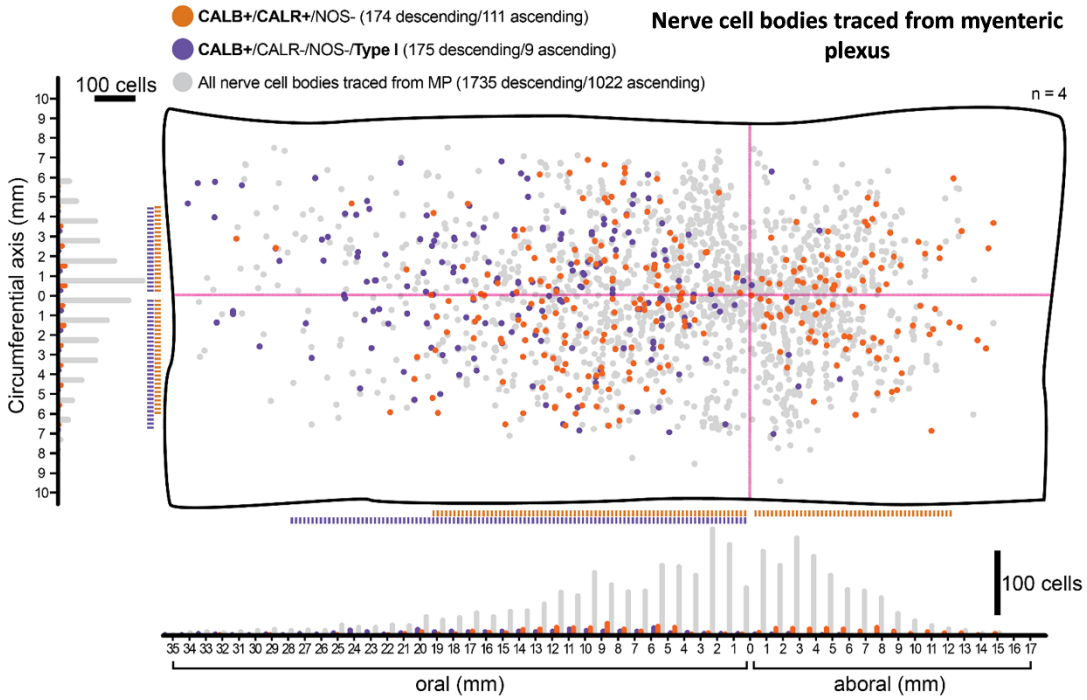

Figure 3.15. Composite map showing the distributions of calbindin-containing Dogiel type I myenteric neurons, traced from the myenteric plexus (n = 4). The majority of Dogiel type I neurons which contained calbindin alone had descending projections (174 descending vs. 9 ascending) whereas the population of CALB+/CALR+ neurons comprised more equal numbers of ascending and descending neurons (174 vs. 111).

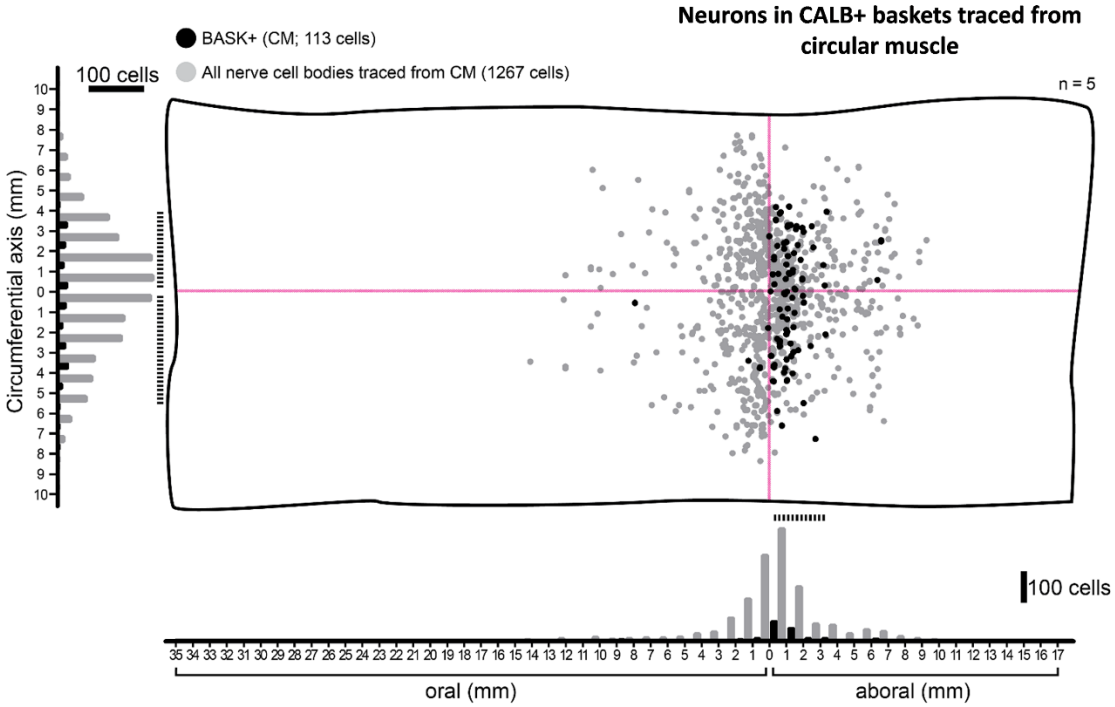

Figure 3.16. Composite map showing the distribution of all myenteric neurons traced from circular muscle whose nerve cell bodies were located within calbindin baskets (n = 5). Neurons within baskets were almost exclusively ascending.

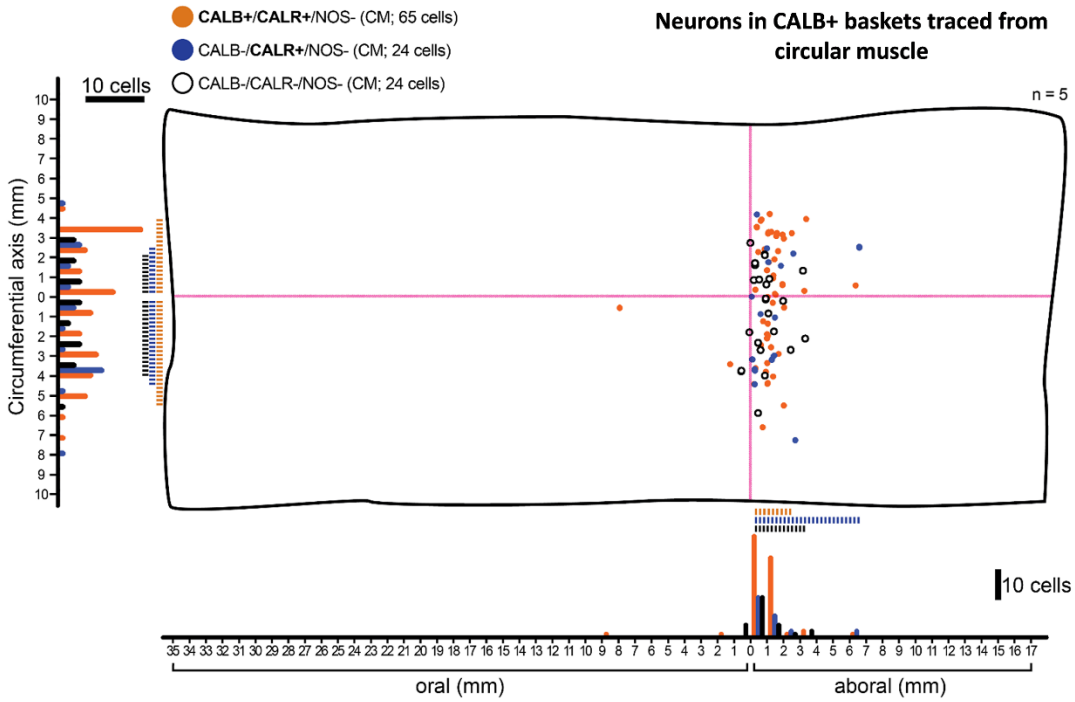

Figure 3.17. The distributions of myenteric neurons traced from circular muscle and located in calbindin baskets, based on calretinin and calbindin immunoreactivity (n = 5).

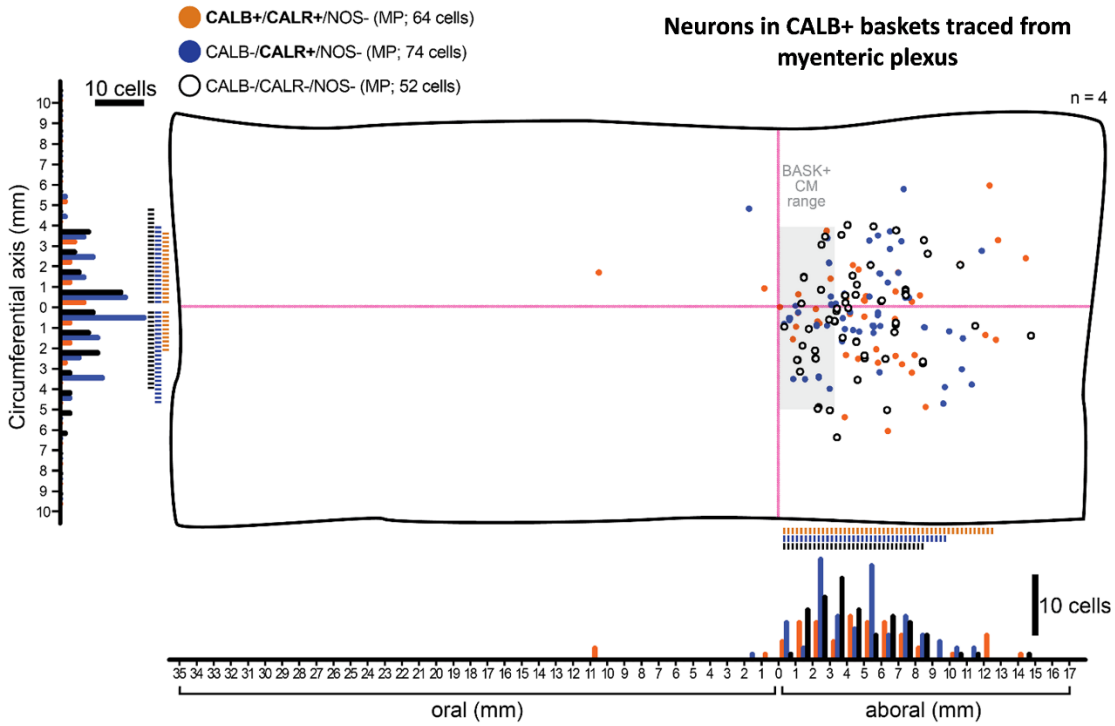

Figure 3.18. Distribution of myenteric neurons traced from the myenteric plexus and located in calbindin baskets, based on calretinin and calbindin immunoreactivity (n = 4). The shaded rectangle represents the 95% projection range of neurons in calbindin baskets traced from circular muscle. The occurrence of neurons outside this area suggests that neurons in calbindin baskets comprise populations of ascending interneurons as well as ascending neurons to circular muscle.

Comparison between projections to circular muscle and within the myenteric plexus

It was instructive to compare distributions of neurons labelled from circular muscle and myenteric plexus. A small majority of DiI-filled neurons traced from the myenteric plexus (60 ± 7%) had descending projections; for neurons projecting to circular muscle, the corresponding figure was less than half (46 ± 4%). Projections traced within the myenteric plexus were longer on average than those traced from the circular muscle (ascending: 3.7 ± 0.8 mm vs 1.7 ± 0.7 mm, descending: 7.4 ± 2.2 mm vs 1.6 ± 0.4 mm; P = 0.004 and <0.001, respectively, independent samples t-test, n = 4 and 5, respectively). Furthermore the longest ascending and descending projections within the myenteric plexus were greater than those to the circular muscle (14.68 mm vs 9.54 mm ascending MP- vs CM-traced neurons, respectively; 34.18 mm vs 14.17 mm, descending MP- vs CM-traced neurons, respectively; see also Figures 3.9 and 3.12 for comparison).

Fills from the circular muscle revealed DiI-filled axons running within the myenteric plexus. This means that DiI applied to myenteric ganglia (or internodal strands) will fill both circular muscle motor neurons and other populations of cells (interneurons and sensory neurons). Logically, DiI-filled neurons that project further in the myenteric plexus than the longest CM-projecting neuron are likely to belong to mixed populations of inter- and sensory neurons. We refer to these as "long ascending" or "long descending" neurons and defined them as lying beyond the region containing 95% of CM-projecting neurons for the same neurochemical class. By this definition, DiI applied to the myenteric plexus labelled 349 long ascending neurons and 994 long descending neurons (out of 2757 neurons in total; n = 4). Using this method, it is possible that we have failed to identify a population of short interneurons with projection lengths equal to or shorter than those of motor neurons. Indeed, using intracellular dye filling, interneurons have been shown to give off synaptic outputs in the first row of ganglia from the dye-filled cell body, on its way to the circular muscle layer [106]. This suggest that 'functionally' short interneurons do exist.

The largest population of long ascending neurons had Dogiel type I morphology and lacked CALB, CALR and NOS immunoreactivity (135/349 cells). In comparison, relatively few long descending neurons (80 of 994) lacked all 3 markers NOS-immunoreactive neurons comprised a small number of long ascending neurons (23 of 349 cells), but were the largest population of long descending neurons (493/994). CALB-immunoreactive Dogiel type I neurons were also abundant among the long descending neurons filled from the myenteric plexus. They represented 16% (160/994) of these cells but were sparse in long ascending pathways (9/349 cells, n = 4). CALR+/CALB+ Dogiel type I neurons comprised a higher proportion of long ascending neurons, (23%, 80/334 cells) than long descending neurons (8%, 79/994 cells; Figure 3.19 – 3.20).

Dogiel type I neurons which contained CALR-immunoreactivity without CALB or NOS were mostly ascending (64/96 cells). They made up 18% of long ascending neurons (64/334) but only ~3% of long descending neurons (32/994 cells; Figure 3.19 – 3.20).

Lastly, CALB-immunoreactive Dogiel type II neurons made up a significant proportion of neurons with long descending projections (18%, 165/994 cells; Figure 3.14) and a much smaller proportion of long ascending pathways (38/349 cells).

Neurons in calbindin baskets projecting to circular muscle or to other myenteric ganglia

Cells in calbindin baskets traced from circular muscle and from myenteric plexus included the same three neurochemical populations, although in different proportions.

Of all cells filled by DiI applied to the circular muscle (1267cells) CALR+/CALB+, CALR+/CALB- and CALR-/CALB-/NOS- cells in baskets accounted for 58%, 21% and 21% of

this subtype respectively. Of all cells filled by DiI applied to myenteric ganglia, the same classes of basket cells respectively made up 28%, 35% and 37% ($\chi 2$ test, $p < 0.001$, adjusted standardized residuals > 2, n = 5 and 4). Thus cells in calbindin baskets coded CALR+/CALB+ were more likely to project to the circular muscle, compared to the other groups (Figure 3.17).

One hundred and seventeen nerve cell bodies in calbindin baskets traced from the myenteric plexus, representing all three neurochemical classes, had oral projections longer than any neurons traced from circular muscle. Thus, all three immunohistochechemical classes surrounded by calbindin baskets must include both ascending motor neurons and ascending interneurons (Figure 3.18).

Circumferential projections

DiI-filled axons running in the myenteric plexus over several millimetres frequently show considerable sideways (circumferential) drift. Unsurprisingly then, the circumferential distributions of myenteric neurons traced from the circular muscle and from myenteric plexus (averaged over the length of the preparation) were very similar (5.38 mm orally and 6.20 mm aborally vs 4.99mm orally and 6.29 mm aborally, circular muscle and myenteric plexus, respectively, see all Figures). However, DiI applied to circular muscle filled extensive circumferential projections in secondary branches contacting the DiI bead in the circular muscle layer. These secondary branches were substantially disrupted in fills from the myenteric plexus (due to local removal of the circular muscle). This is evident when comparing Figures 3.9 and 3.12 close to the DiI application site. NOS+ neurons traced from circular muscle had longer circumferential distributions than other classes (average circumferential distance from DiI-application site: 3.2 ± 0.34 mm; $P < 0.05$, versus cells containing no marker and CALR+ neurons, Bonferonni post-test, 1-way ANOVA, n = 4)

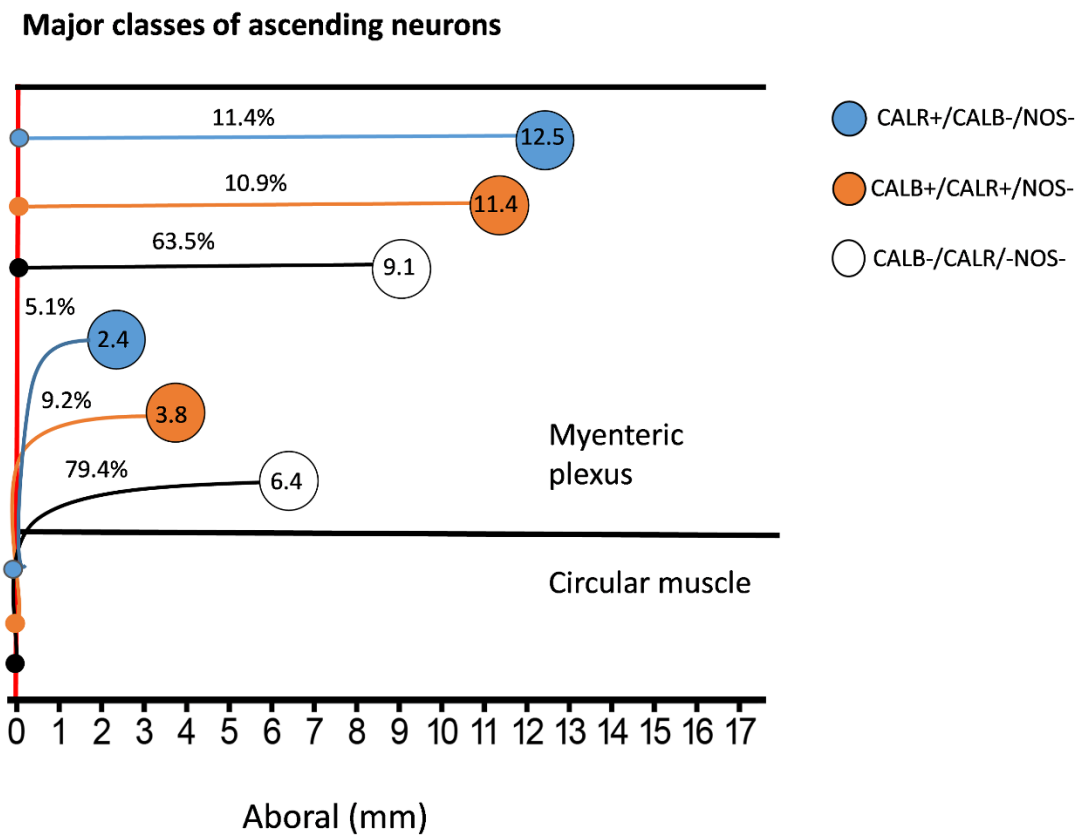

Figure 3.19. The major classes of ascending neurons identified in the present study. Here, projection lengths and proportions of Dogiel type I myenteric neurons **ascending projections** are shown. These cells **were not** associated with calbindin baskets. The length of neurons shown in this figure represents the maximum distance from the DiI application site within which 95% of neurons of that class occurred (the exact value in mm is given within each neuron outline). % refers to contribution of neuron class to total ascending projections within myenteric plexus or circular muscle respectively.

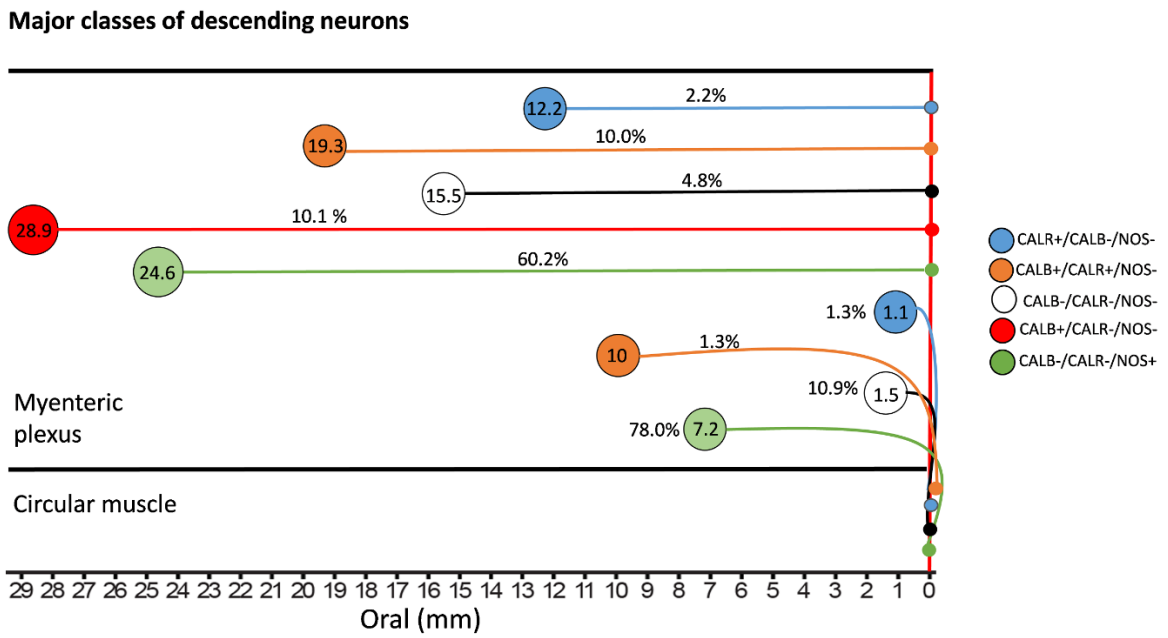

Figure 3.20. The major classes of descending neurons identified in the present study. Here, projection lengths and proportions of Dogiel type I myenteric neurons with **descending projections** are shown. These cells **were not** associated with calbindin baskets. Maximum distance away from DiI application site containing 95% of neurons shown for each class of neuron (number within each neuron outline in mm). % refers to contribution of neuron class to total descending projections within myenteric plexus or circular muscle respectively.

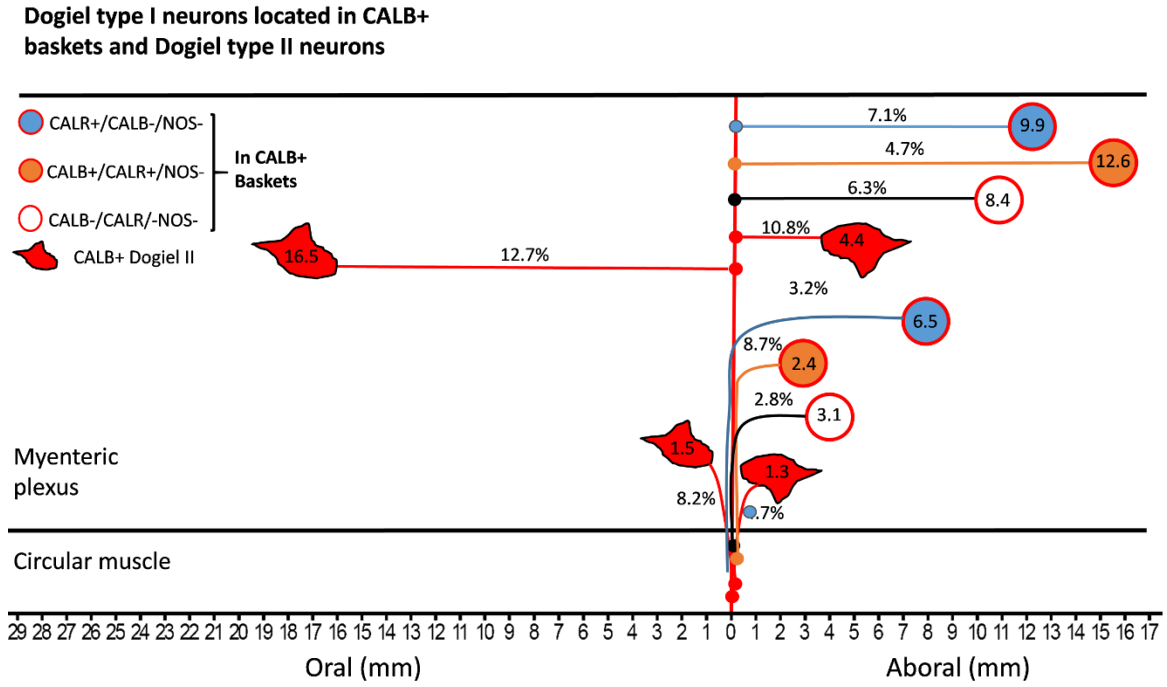

Figure 3.21. Schematic diagram showing projection lengths and proportions of Dogiel type I myenteric neurons located in calbindin baskets and Dogiel type II calbindin neurons, traced from myenteric plexus and circular muscle. Maximum distance away from DiI application site containing 95% of neurons shown for each class of neuron (number within each neuron outline in mm). % refers to contribution of neuron class to total ascending or descending projections within myenteric plexus or circular muscle respectively.

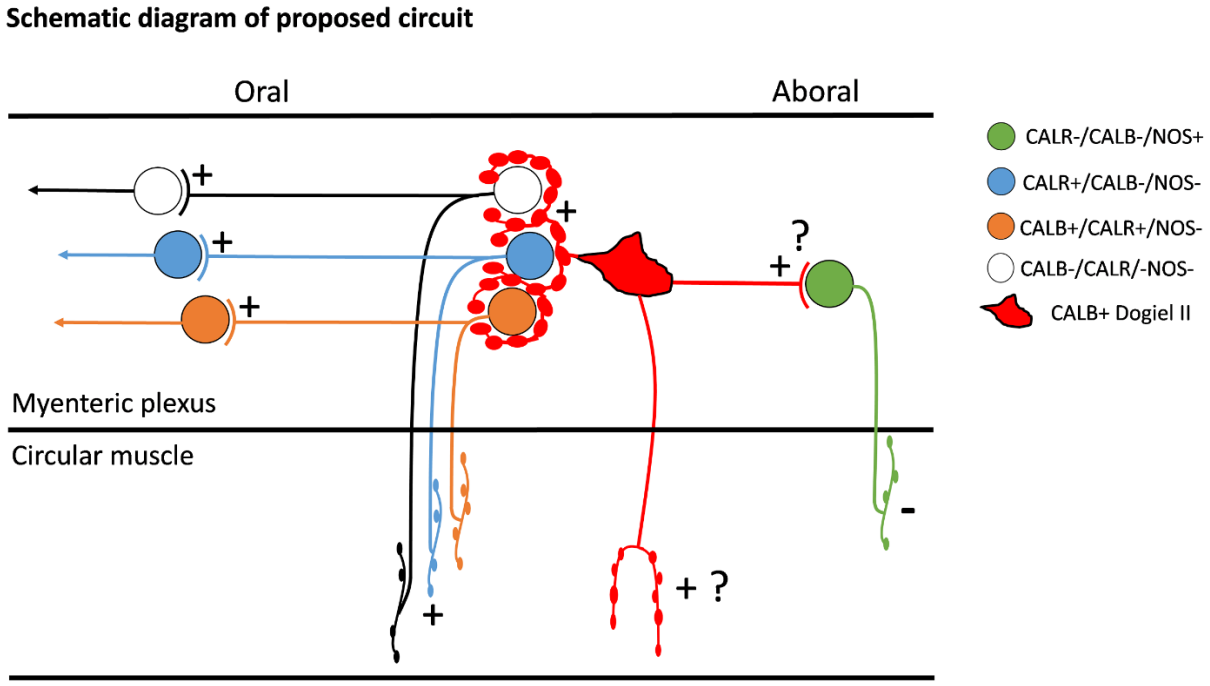

Figure 3.22. Schematic diagram showing a model of IPAN connections to specific classes of Dogiel type I myenteric neurons located in calbindin baskets and possible connections to descending (NOS) pathways, which remain to be determined.

Discussion

Our study is the first analysis of motor neurons in the guinea-pig distal colon; in addition, we have also described projections of neurons within the myenteric plexus. We were able to distinguish seven populations of myenteric neurons based on calbindin, calretinin and NOS immunoreactivity, cell morphology and projections. Furthermore, we were able to characterise myenteric neurons which receive highly selective input from calbindin-immunoreactive IPANS, via calbindin synaptic baskets [503].

Our work, summarised in Figure 3.22, builds on previously published data from studies based on immunohistochemistry and lesion experiments, to support the idea of multiple distinct enteric neural circuits. Our results also confirm some of the general principles of ENS organization; we saw a significant polarity of myenteric neuron projections, with the aborally-projecting neurons being more numerous and having longer projections than the orally-projecting neurons, an arrangement that was first described in the guinea-pig guinea pig small intestine [109, 492, 532] and the human colon [253, 274]. We have also shown that neurons running within the myenteric plexus have longer projections than neurons projecting to circular muscle and therefore include populations of non-motor neurons, likely to be interneurons or sensory neurons with long projections.

Neurons projecting to the circular muscle

Neurons that supply the circular muscle have axons in the deep muscular plexus and can be labelled by DiI with a high degree of selectivity. DiI applied to the surface of circular muscle, away from any underlying myenteric plexus ganglia or internodal strands, was clearly seen to label circular muscle fibres in a circumferential orientation to the application site (See Figure

3.1). Individual nerve fibres were seen running parallel to these muscle fibres for significant distances, within the deep muscular plexus, before reaching the myenteric plexus layer. Any preparations in which there was DiI labelling the tertiary plexus nerve fibres (supplying longitudinal muscle) or directly the axons of the myenteric plexus, were discarded.

Nerve fibres that are labelled by DiI, as they pass through the area of dye application to submucosa or mucosa, may 'contaminate' the pool of labelled motor neurons, however this is unlikely to represent significant numbers. This is supported by the different distribution patterns and different neuronal populations obtained when DiI is applied to mucosa directly [105].

Dogiel type I neurons with descending projections were largely NOS immunoreactive and therefore likely to be inhibitory motor neurons, which is consistent with a remarkably well preserved polarity of inhibitory motor neurons observed across all gut regions and species examined [268, 525, 526, 533].

While nearly all descending motor neurons with projections longer than 2mm contained NOS, there was a population (22%) of short descending motor neurons that did not contain NOS. It is possible that this represents a population of purinergic inhibitory motor neurons that contain immunoreactivity for the vesicular nucleotide transporter VNuT [534], a marker for vesicular ATP which is one of the other inhibitory transmitters of motor neurons [46, 535]. This may be evidence for 2 classes of inhibitory motor neurons, some of which may also contain the peptide VIP [10]. Alternatively, it is possible that some of the short descending motor neurons that lack NOS immunoreactivity may be excitatory motor neurons with local projections to the circular muscle, as described in the guinea pig small intestine [264, 267].

Our work also suggests the existence of at least three populations of Dogiel type I ascending motor neurons. The most numerous group lacks all three markers used in this study; the other two contained either CALR alone or CALR/CALB. It is likely that all three classes are excitatory motor neurons and would be expected to contain ChAT and possibly a tachykinin [10].

Many of these excitatory motor neurons are surrounded by dense, calbindin-immunoreactive nerve endings, previously described as “calbindin baskets”. These baskets arise, at least in part, from nearby calbindin Dogiel type II neurons [503], suggesting a very strong, direct connection between sensory neurons and some excitatory motor neurons.

Our finding that most of the CALR+/CALB+ motor neurons (90%) were in baskets suggests specificity to this connection, since a smaller proportion of CALR+/CALB- neurons were surrounded by baskets. These findings challenge previous assumptions that there is only one common final excitatory motor neuron pathway underlying intestinal motility. It also establishes the principle that functional classes of neurons may be distinguished by the types of synaptic inputs that they receive, as well as by their morphological and immunohistochemical coding.

When DiI was applied to circular muscle, a small proportion (6%) of the DiI-filled neurons were Dogiel type II with calbindin-immunoreactive content. These cells are likely to be IPAN’s similar to those in the small intestine that project extensively within the myenteric plexus and to the mucosa but apparently not to muscle [107, 498]. Dogiel type II neurons have been retrogradely labelled from circular muscle in guinea-pig and human proximal colon [269, 274] as well as guinea-pig ileum [264, 267], in similar small proportions to our data. This may

represent IPANS whose projections are filled at the circular muscle layer en route to the mucosa. Another possibility is that some Dogiel type II cells may have dedicated projections to circular muscle.

Calbindin-immunoreactive varicose axons are present in the circular muscle layer [503], and they disappear after the overlying myenteric plexus is surgically removed [514]. Also, some neurobiotin-filled Dogiel type II cells have projections to circular muscle with varicose endings [106] and a functional study of IPANS in guinea-pig ileum showed that they respond to circumferential tissue stretch and that this depends on smooth muscle tone [536]. Thus, a tantalising possibility is that varicose calbindin fibres in the circular muscle are efferent branches of IPANS, which form an axo-axonal reflex pathway capable of stimulating the circular muscle by releasing tachykinins, which have previously been shown to cause depolarisation and contraction of smooth muscle [537] via NK2 receptors. This could explain why pellet propulsion persists in colonic preparations during blockade of nicotinic, purinergic, 5HT3 and NK3 receptors [538].

Neurons traced from application of DiI to myenteric plexus

Application of tracer directly onto an internodal strand of the myenteric plexus filled more neurons with descending projections than ascending projections and projection lengths were significantly longer than for neurons innervating the circular muscle.

NOS-containing Dogiel type I neurons were the most numerous type of cell traced from myenteric ganglia; they and represented 39% of the DiI-filled myenteric cell population. Many of these neurons had longer projections than the longest neurons projecting to the circular muscle suggesting the presence of descending NOS interneurons, consistent with lesion

studies in guinea-pig colon and small intestine [539, 540]. NOS-containing cells accounted for 41% to the pool of long descending neurons (with projections longer than motor neurons), indicating that other populations of long descending interneurons must exist [10].

Calbindin-immunoreactive Dogiel type II neurons represented only 12% of the myenteric neurons retrogradely filled from the myenteric plexus which is lower than the 38% reported in guinea-pig small intestine [492]. Whether our data is an underestimate of the actual population size for methodological reasons, or a true difference in the roles played by IPANs in the small and large intestine remains to be established.

Calbindin immunoreactivity is present in only 12% of all myenteric neurons in the guinea pig colon (Lomax 2000) and these include both Dogiel type I and Dogiel type II neurons. In comparison, Calbindin is present in 24% of all myenteric neurons in the small intestine (Costa et al 1996) all of which are Dogiel type II. This suggests that Calbindin immunoreactivity, per se, does not determine or identify the function of enteric neurons.

Calretinin was present in two populations of Dogiel type I neurons; CALRET+/CALB- and CALRET+/CALB+. Initial studies of calretinin based on lesion experiments concluded that calretinin immunoreactivity is not specific to any functional class of neurons in the guinea-pig distal colon, localising in ascending and descending interneurons as well as motor neurons [517, 541]. In subsequent studies, these calretinin-containing classes were distinguished by colocalisation with other neurochemical markers [10]. Our results support these findings.

CALR+/CALB- nerve cell bodies filled from the myenteric plexus were mostly aboral to the DiI application site and many had projections longer than the longest circular muscle motor

neurons (Figure 3.13). This implies that CALR+/CALB- neurons include both ascending interneurons and motor neurons. Whether the same neuron can project to both the circular muscle and to other ganglia is not clear but seems unlikely based on the lengths of projection. Based on their neurochemical coding, CALR+/CALB- neurons are likely to contain ACh and TK as co-transmitters [10] and be excitatory.

In contrast, CALRET+/CALB+ neurons had preferentially aboral projections within the myenteric plexus (Figure 3.15) with significantly longer projections than neurons projecting to the circular muscle. Again this suggests that some of these neurons are likely to be descending interneurons. These neurons are also likely to be excitatory [10].

Dogiel type I neurons with calbindin alone (CALR-/CALB+) formed another significant class with long descending projections, accounting for 10% of all descending neurons (Figures 3.15, 20). Most of these cells are likely to be interneurons, however stretch sensitive neurons in guinea pig distal colon with long descending projections have been demonstrated using pharmacological methods [542] and Dogiel type I mechanosensitive S-Type neurons were also demonstrated using intracellular recordings [106]. Therefore, this group may also contain neurons that are sensory in function.

Lesion studies in the distal colon showed accumulation of calbindin-immunoreactive content on both sides of a myectomy, suggesting both ascending and descending projections [499]. However, subsequent studies based on colocalisation of markers suggested that calbindin interneurons in the distal colon are all descending [10]. Our work represents the first direct evidence for the existence of this group of descending interneurons in the guinea-pig distal colon. Dogiel type I calbindin interneurons have been shown to have descending projections in the proximal colon too [514].

Neurons in calbindin baskets

In our previous work, we revealed a specialised arrangement of calbindin-immunoreactive nerve endings forming dense, basket-like structures in most myenteric ganglia. Although previously described [499], we discovered the source of these structures to be adjacent Dogiel type II neurons (IPANS) and revealed their strong association with calretinin neurons [503]. In the present work, we have shown that the majority of cells in calbindin baskets have ascending projections (Figures 3.16, 3.18, 3.21) to either circular muscle or myenteric ganglia.

Approximately 9% of myenteric neurons that innervate circular muscle were surrounded by calbindin baskets and thus are likely to receive direct synaptic input from IPANS. All had orally directed projections and 79% of them were calretinin-immunoreactive. Thus many are likely to be excitatory motor neurons as we previously speculated [503]. This is a significant result because it suggests two different populations of circular muscle excitatory motor neurons; those with baskets and those without. The functional implications of this are that final excitatory motor pathways in the colon can be selectively activated by distinct circuits, which may responsible for different motor patterns.

Based on lengths of projections, some ascending interneurons were also surrounded by calbindin baskets. This also allowed comparison of neurochemical content between calretinin-immunoreative motor neurons and interneurons. The result of this comparison suggests that CALRET+/CALB+ neurons within baskets are likely to be excitatory motor neurons and conversely, motor neurons to circular muscle with that coding are almost exclusively found in calbindin baskets. This is a remarkable observation, in that it may provide a chemical code for a new class of motor neuron in the colon, characterised by strong input from sensory neurons (IPANS) and taking part in excitatory ascending reflex pathways. Several motor patterns have

been distinguished in the guinea-pig distal colon [490, 543]; establishing the functional role of this specific pattern of connectivity is an exciting prospect.

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Chapter 4

Characterisation of CGRP-immunoreactive Dogiel type II neurons and their connections in the mouse distal colon

Statement of Authorship

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Principal Author Contribution:

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Contribution to paper: Performance of all of the experimental steps, Analysis of data, Writing of manuscript, including preparation of all figures and 2/4 tables.

Estimated overall percentage contribution: 80%

Joint Author Contributions

Timothy Hibberd: Contributed to data analysis, generated 2/4 tables.

Marcello Costa: Contributed to data analysis, Reviewed manuscript.

David Wattchow: Reviewed manuscript and provided expert advice.

Dayan De Fontgalland: Reviewed manuscript and provided expert advice.

Nick Spencer: Reviewed manuscript and provided expert advice.

Introduction

The enteric nervous system (ENS) is essential for normal function of the mammalian gastrointestinal tract [544, 545]. Although capable of autonomous activity, there is a significant anatomical and functional association with the rest of the body systems. The ENS is comprised of a highly organised network of nerve cells, including its own population of intrinsic sensory neurons (intrinsic primary afferent neurons, IPANs). These have been best characterised in the guinea-pig ileum [102, 518], however analogous neurons have been demonstrated in small and large bowel of other animals [546, 547]. This suggests IPANs may be a common feature of the ENS. Data from structural and physiological experiments is consistent with them being the 'first neuron' of a circuit, likely to play an important role in initiating motor activity by activating both ascending and descending pathways in response to either chemical or physical stimuli [518]. The morphological characteristics shared by IPANs include a large, oval cell body with multiple axonal processes (Dogiel type II classification) that travel extensively within the myenteric plexus and project to the circular muscle and mucosa [108-110, 500, 548, 549]. Electrophysiological studies consistently correlate the presence of a long-lasting after-hyperpolarisation (AH) phase following a broad action potential with Dogiel type II morphology [103, 121, 125]. However, there is now a more complex picture emerging, with recent experiments challenging the concept of neurons with clearly defined and mutually exclusive functional roles. Dogiel type II cells receive fast synaptic inputs from other neurons, suggesting an active role in integrative activities of neural circuits [129, 130, 550, 551]. Conversely, neurons with other morphologies have been shown to be directly mechanosensitive and therefore sensory in their function [106].

We recently described a neural circuit involving calbindin-immunoreactive Dogiel type II cells (CALB+; presumed IPANs) in the guinea-pig distal colon. Here, they make strong morphological connections with CALR+ excitatory motor neurons to circular muscle and excitatory ascending interneurons via CALB+ varicose baskets [110, 552]. This circuit could

well represent the underlying neuroanatomy of the ascending excitation seen on local mechanical stimulation [506, 528] and also suggests a monosynaptic reflex arc to circular muscle. While the guinea pig ENS is the most extensively characterised, it is the mouse that is becoming an increasingly important mammalian species for enteric neuroscience research as advances in molecular biology techniques allow for gene manipulation and mice with specific genetic deletions. Yet, relatively little is known about the circuits underlying mouse colonic motor patterns. Whether colonic IPANs make similar connections in mouse as in guinea pig remains to be established. This is important since we know that IPANs (and other types of enteric neurons) show variations in neurochemical and electrophysiological characteristics, with differences being apparent between species and between different regions of the gut within the same animal [553].

In the mouse colon, CGRP occurs in intrinsic Dogiel type II cell bodies and processes [554], as well as in extrinsic (spinal) nerves and their varicose terminals [229, 504, 555-557]. In this study, isolated mouse colon was maintained in organotypic culture, leading to degeneration of extrinsic CGRP+ processes, leaving intrinsic nerve structures intact [558-560]. This allowed the use of CGRP as an exclusive marker for IPANs and their processes, making possible a study of their connectivity with NOS and CALR containing cells. We described baskets of CGRP+ varicose fibres in the ganglia of the myenteric plexus and characterised the neurons located within them. We also examined nerve terminals in circular muscle, to establish a connection to the cells in CGRP+ baskets, based on shared combination of immunochemical markers.

Methods

Tissue collection

Animal procedures were approved by the Animal Welfare Committee of Flinders University. In total, 8 C57BL/6 mice were killed by isofluorane inhalation, followed by the removal of the distal colon through a ventral midline incision. The colon was placed in a Sylgard-lined dissection dish, containing sterile Kreb's solution (118 mM NaCl, 4.75 mM KCl, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 11.1 mM D-glucose, 2.5 mM $CaCl_2$, aerated with 5% CO_2 in 95% O_2, pH 7.4).

After flushing luminal content, the terminal 20 mm of distal colon was incised longitudinally along the mesenteric border and pinned tightly to the dish surface, mucosa uppermost. Four control preparations were fixed overnight in Zamboni's fixative (2% formaldehyde, 15% saturated picric acid in 0.1 M phosphate buffer, pH 7). The other 4 preparations were subject to 3 washes in sterile Krebs solution (each instance in separate 10 ml specimen cups). All washes in Krebs solution or phosphate buffered saline (PBS) were performed on a rocking tray. Preparations were then transferred to a sterile Sylgard-lined culture dish, pinned flat with mucosa uppermost.

Organotypic culture

Sterile Krebs solution was replaced with culture medium (DME/F12; Sigma Chemical Co.) supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, 100 IU/mL penicillin, 2.5 µg/mL amphotericin and 20 µg/mL gentamicin (Cytosystems, Castle Hill, NSW, Australia), pH adjusted to 7.4. Preparations were cultured for 4 or 5 days in a humidified incubator (37°C, 5% CO_2 in air), with daily exchange of culture medium and constant agitation on a rocking tray. Cultured preparations were fixed as described above.

Immunohistochemistry and image acquisition

Cultured and control preparations were treated identically after fixation. Preparations were washed in phosphate buffered saline (PBS) to remove fixative (10 min x 3; 0.15 M NaCl with 0.01 M phosphate buffer, pH 7.4). After removing the mucosa by sharp dissection, tissue was cleared in dimethylsulphoxide (10 min x 3) followed by PBS washing (10 min x 3). Preparations were incubated with blocking solution for 60 min at room temperature (10% normal horse serum in antibody diluent; 0.1 M PBS, 0.3 M NaCl, 0.1% sodium azide). Incubation in primary antibody was performed on a rocking tray at room temperature for two nights, followed by 3 x 10 min washes in PBS and incubation in secondary antibody for 4 hours at room temperature prior to mounting on a slide in 100% carbonate-buffered glycerol (pH 8.6). The primary and secondary antibodies used in this study are listed in Table 4.1 and Table 4.2, respectively.

Preparations were viewed and imaged using an epifluorescence microscope (Olympus IX71, Japan) equipped with discriminating filters to match the fluorophores used (Chroma Technology Co., Battledore, VT). Images were captured by a Roper Scientific camera and AnalySIS Imager 5.0 software (Olympus-SIS, Munster, Germany) via 20x or 40x water immersion lenses. Images were stored as TIFF files (1392 * 1080 pixels) and optimized for contrast and brightness using Adobe Photoshop (2015 Adobe Systems Software Ireland Ltd) prior to further analysis.

Antibody characterisation

CGRP (Rabbit)

The CGRP antibody (Peninsula Laboratories, Cat# T-4032, RRID:AB_2313775) is a polyclonal antibody collected from rabbits immunised with a synthetic rat alpha-CGRP peptide. It produces the same staining pattern as reported in multiple studies using several other CGRP

antibodies and staining is reduced or completely prevented by preabsorption with rat CGRP [561].

Calretinin (Mouse)

The calretinin antibody used (CALR, Swant, Cat# 6B3, RRID:AB_10000320) is a monoclonal antibody raised in mice by immunisation with recombinant human calretinin – 22k [562]. It labels a band at 29KDa in brain homogenate of various animal species, including mouse, and there is no staining seen in the cerebellum of calretinin knockout mice [516]. In our study, the specificity of the calretinin immunohistochemical reaction was determined by omission of the primary antibody incubation step; we saw no staining when colonic mouse tissue was incubated in secondary anti-mouse antibody alone.

Neuronal Nitric Oxide Synthase (nNOS;Sheep)

The neuronal nitric oxide synthase antibody (nNOS, Emson, Cat# K205, RRID:AB_2314957 – generously gifted by Dr. P. Emson) is polyconal and raised in sheep against recombinant rat brain neuronal NOS. On Western blots of guinea pig inferior mesenteric ganglion it labels a strong band at 160 kDa and a faint band at 40 kDA [415]

Data analysis

Analysis of cell populations within CGRP baskets

Myenteric plexus preparations labelled with CGRP antibody revealed clusters of intensely CGRP-immunoreactive varicosities that formed a basket-like shape around one or more myenteric nerve cell bodies (Figure 4.1). In this paper, we refer to these structures as ‘CGRP+

baskets'. Myenteric nerve cell bodies within CGRP+ baskets were assessed for NOS and CALR immunoreactivity in 10 randomly selected and photographed ganglia from each preparation. To do this, CGRP baskets were identified in photomicrographs of CGRP immunofluorescence and a digital outline drawn around them using ImageJ software (NIH, Bethesda).

Matching photomicrographs of NOS and CALR immunofluorescence were superimposed and then analysed by rapidly switching between the images. Each identified CGRP+ basket was scored for the presence or absence of the two markers within a nerve cell body. Analysis of myenteric nerve cell bodies inside CGRP+ baskets was performed without prior incubation with colchicine and therefore nerve cell bodies were not scored for CGRP immunofluorescence (see results / discussion).

Colocalisation analysis of circular muscle nerve fibre varicosities

In the same preparations, CGRP, NOS and CALR immunofluorescence was imaged in 10 randomly selected fields of view within the circular muscle (40x, water immersion lens). Since NOS+ terminals were most numerous, the filter used to visualize NOS was used to determine focal plane depth for each field of view before imaging CGRP and CALR immunofluorescence. Focal depth was kept constant while switching filters and no overlap of photographed regions occurred. The photomicrographs of the three markers were matched and superimposed using ImageJ. CGRP+ varicosities were selected in the field of view by random movement of cursor (up to a maximum of 10) and a digital outline drawn around them. By switching between the other superimposed images, colocalisation with the other two markers in varicosities was scored. This protocol was repeated for the other two markers to obtain proportions of varicose fibres containing each marker alone and the other possible combinations of markers (Figure 4.2, Table 4.4).

Colocalisation analysis of CGRP basket varicosities

To examine colocalisation of immunoreactivity for other markers within CGRP basket varicosities, 10 myenteric ganglia from 4 cultured preparations were randomly selected and photographed using the 40x lens. All distinguishable varicosities making up the inner-most layer of selected CGRP baskets were examined as they are most likely to provide synaptic input to a neuron within the basket. The immunoreactive content of every varicosity was scored, and then the presence or absence of the other two markers determined using matched and superimposed photomicrographs as described above (Figure 4.3, Table 4.4).

Statistical analysis

Statistical analysis was performed by ANOVA, or Student's two-tailed t-test for paired or unpaired data using Prism 8 (GraphPad Software, Inc, La Jolla, CA, USA). Differences between data sets were considered significant if $P < 0.05$ and all P values are reported as exact values to three decimal places, except where $P < 0.001$. Results are expressed as mean ± standard error except where otherwise stated. Lower case "n" always indicates the number of animals used in a set of experiments.

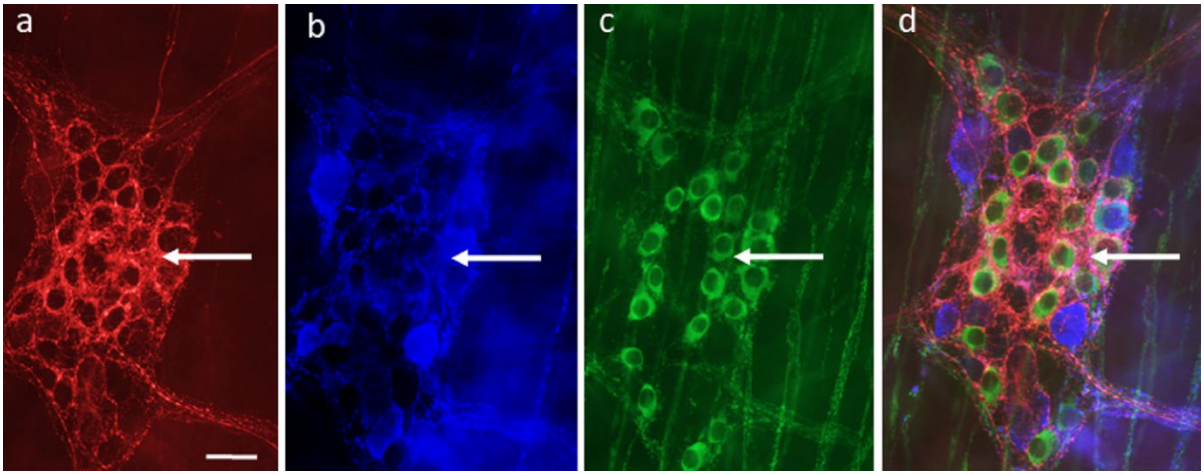

Figure 4.1. Matched fluorescence micrographs of a myenteric ganglion immunolabelled with CGRP (a), calretinin (b) and NOS (c). Note in the overlay (d) the CGRP+ baskets (arrow) clustered around a group of CALR+ and NOS+ neurons. Scale bar = 20 µm. Fresh-fixed tissue.

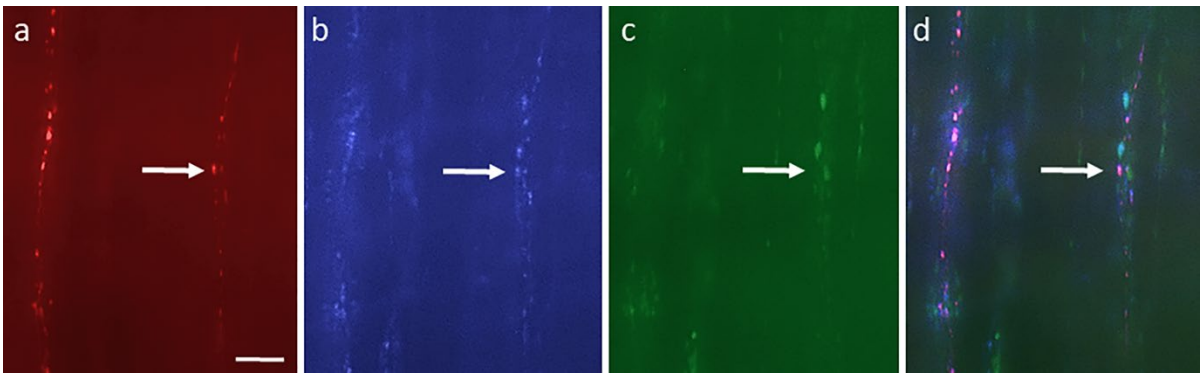

Figure 4.2. Matched fluorescence micrographs of circular muscle layer immunolabelled with CGRP (a), calretinin (b) and NOS (c). Note varicose nerve fibres running in the direction of muscle fibres. White arrow highlights a CGRP+CALR+ varicosity. Scale bar = 20 µm. Tissue from organotypic culture.

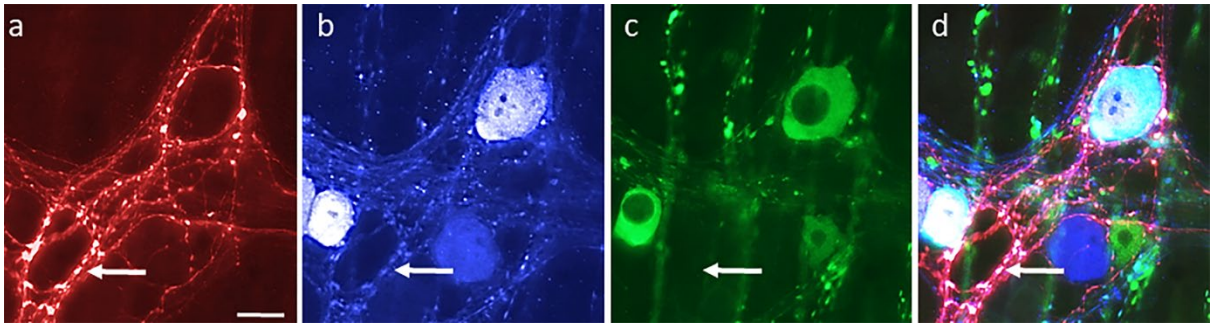

Figure 4.3. Matched fluorescence micrographs of myenteric plexus ganglion immunolabelled with CGRP (a), calretinin (b) and NOS (c). Arrow highlights a CGRP+CALR+ varicosity contributing to a CGRP basket. Scale bar = 20 µm. Tissue from organotypic culture.

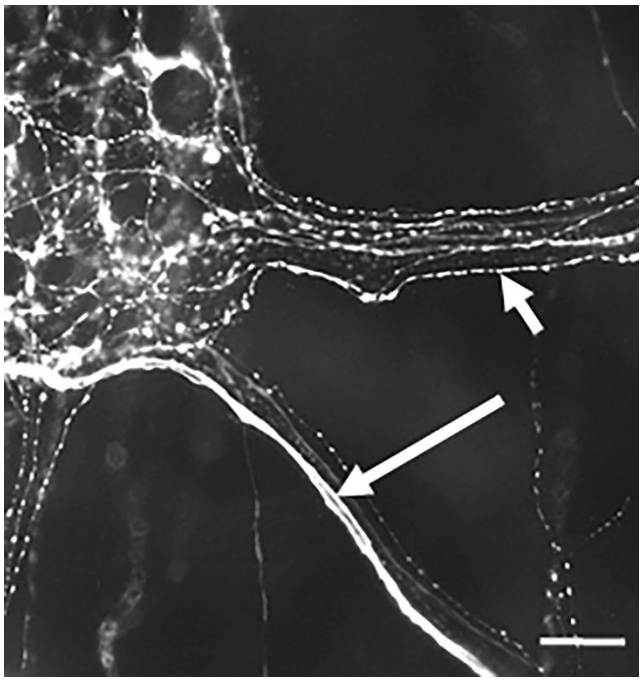

Figure 4.4. Fluorescence micrograph of a myenteric ganglion and internodal strands immunolabelled for CGRP. Note the two morphological subtypes of CGRP+ fibres; major smooth fibres (long arrow) and minor varicose fibres (short arrow). Scale bar = 20 µm. Tissue from organotypic culture.

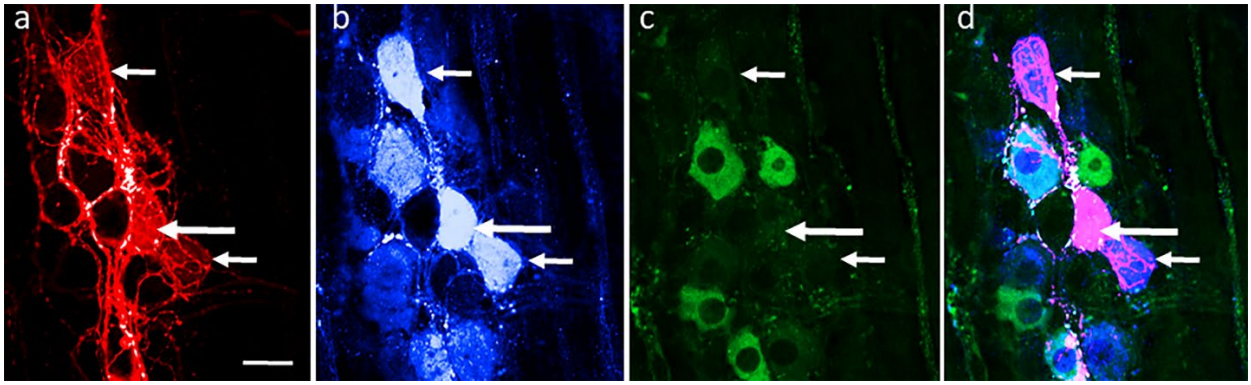

Figure 4.5. Matched confocal fluorescence micrographs of a myenteric ganglion immunolabelled with CGRP (a), calretinin (b) and NOS (c). Note the CGRP+/CALR+, Dogiel type II neuron (large arrow) which is contributing to local varicose baskets and making close contact with CALR+ neurons (small arrows). Scale bar = 20 µm. Tissue from organotypic culture.

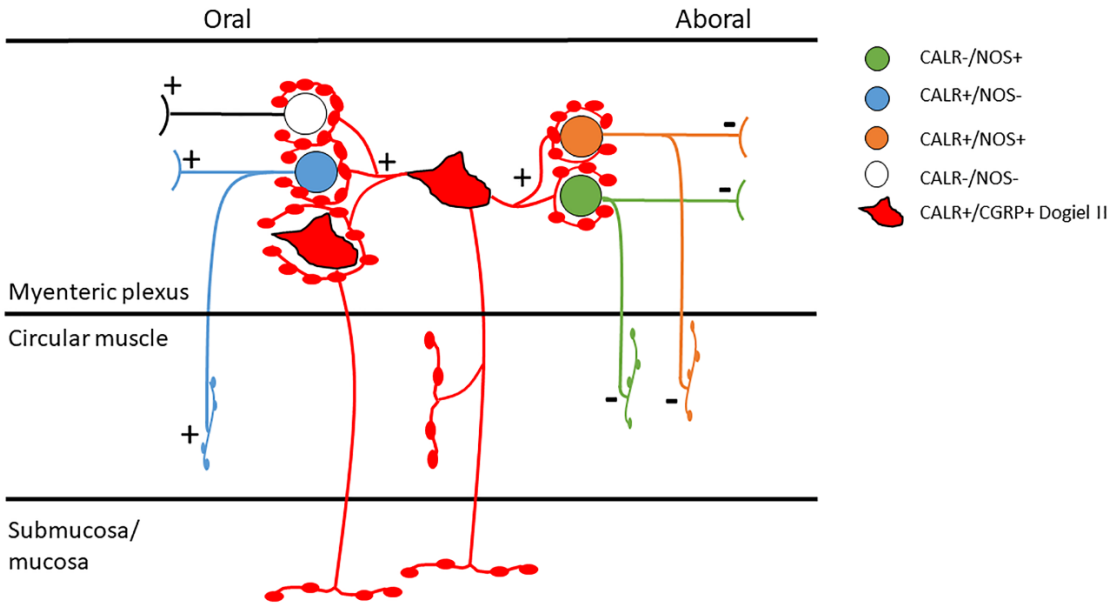

Figure 4.6. Schematic diagram showing a potential model of IPAN connections to specific classes of myenteric neurons located in CGRP baskets.

Table 4.1. *Primary antibodies used in study*

Primary antibody	Raised	Immunogen	Source/catalog#	Dilution
CGRP	Rabbit	Synthetic rat alpha-CGRP peptide	Peninsula/T-4032	1:1000
Calretinin	Mouse	Human recombinant calretinin - 22k	Swant/6B3	1:1000
NOS	Sheep	Recombinant rat brain neuronal NOS	Emson/K205	1:1000

NOS – nitric oxide synthase

Table 4.2. *Secondary antibodies used in study*

Secondary antibody	Fluorophore	Source/catalog#	Dilution
Donkey anti-rabbit IgG	Cy3	Jackson / 711165152	1:200
Donkey anti-mouse IgG	Cy5	Jackson / 136608	1:200
Donkey anti-sheep IgG	AMCA	Jackson / 713155147	1:200

IgG – immunoglobulin G; Cy3 – indocarbocyanine; Cy5 – indodicarbocyanine; AMCA – aminomethylcoumarin.

Results

Degeneration of extrinsic CGRP in organotypic culture

In fresh fixed preparations (n = 4), numerous CGRP+ axons and varicosities occurred in both myenteric plexus and circular muscle layers. Within myenteric ganglia dense clusters of CGRP+ varicosities formed basket-like structures (CGRP+ baskets) which surrounded nerve cell bodies. Relatively thick, smooth axons of intense CGRP-immunoreactivity passed through myenteric plexus and circular muscle. Such axons are characteristic of extrinsic spinal afferents [229], which degenerate in organotypic culture of isolated colon [560]. We refer to these axons here as “major axons”, while varicose fibres with relatively weak CGRP immunoreactivity were called “minor” axons (Figure 4.4).

Both axon types were quantified in myenteric internodal strands of freshly-fixed preparations (n = 4) and organ cultured preparations (n = 4). After organ culture, major axons decreased on average to less than 20% of levels in fresh fixed preparations (1.3 ± 0.3 to 0.2 ± 0.1 per internodal strand, P = 0.004, independent samples t-test, n = 4 control, 4 cultured). There was no significant change in the minor axon count in organ-culture preparations compared to fresh fixed preparations (4.1 ± 0.5 vs 4.1 ± 0.4 fibres per internodal strand, P = 0.977, independent samples t-test, n = 4 control, 4 cultured). Myenteric CGRP+ baskets were also quantified, to determine whether they were formed by neurons that were extrinsic or intrinsic to the gut. There were on average 12.3 ± 0.4 CGRP+ baskets per myenteric ganglion after organ culture, compared to 11 ± 1.3 CGRP+ baskets in fresh-fixed tissue (P = 0.337, independent samples t-test, n = 4 control, 4 cultured). Taken together, these results suggest that major fibres have predominantly extrinsic origins, while both minor fibres and CGRP+ baskets arise from intrinsic enteric neurons.

CALR and NOS content in myenteric nerve cell bodies

Analyses were performed on CGRP+ baskets and varicosities in cultured preparations, free of extrinsic CGRP sources (n = 4). Triple immunohistochemical labelling revealed NOS+ and CALR+ myenteric nerve cell bodies. On average there were 12.8 ± 0.5 NOS+ nerve cell bodies and 14.9 ± 2.6 CALR+ nerve cell bodies per myenteric ganglion (n = 4). Myenteric nerve cell bodies with the combination CALR+/NOS+ comprised 38 ± 5 % (4.9 ± 0.8 per ganglion; n = 4) of all NOS+ neurons and conversely, 33 ± 2 % (n = 4) of all CALR+ neurons.

CGRP+ myenteric nerve cell bodies were also detectable (Figure 4.5), including some located within CGRP+ baskets. However their staining was weak and inconsistent, therefore they were not taken into account during analysis. CGRP immunoreactivity of nerve cell bodies improved significantly after overnight incubation in colchicine, at the expense of immunoreactivity within CGRP varicosities, including those making up CGRP baskets.

CALR and NOS content in nerve cell bodies within the CGRP+ baskets

Most nerve cell bodies surrounded by CGRP+ baskets lacked both CALR and NOS immunoreactivity (CALR-/NOS-; 5.8 ± 0.2 per ganglion; 48 ± 3 %, n = 4; $P < 0.05$ compared to any other group; one-way ANOVA, Tukey post-test). CALR-/NOS+ (2.6 ± 0.6 per ganglion; 21 ± 4 %, n = 4) and CALR+/NOS- (2.4 ± 0.5 per ganglion; 19 ± 4 %, n = 4) were similarly proportioned with CALR+/NOS+ (1.6 ± 0.3 per ganglion; 13 ± 2 %, n = 4) representing the smallest group. These results are listed in Table 4.3.

Immunohistochemical characterisation of varicosities in CGRP+ baskets and circular muscle.

To study CGRP baskets in more detail, the CGRP basket varicosities were quantitatively analysed for the presence and colocalisation of calretinin and NOS immunoreactivity and compared to varicosities in the circular muscle (n = 4).

CGRP+ baskets

CGRP+ varicosities represented the greatest number of varicosities in CGRP+ baskets. CALR+ varicosities were also abundant, representing on average 80 ± 9 % of the number of CGRP+ varicosities, and there was significant co-localization of the two markers in the varicosity population (see below; n = 4). NOS+ varicosities were sparse in comparison; they averaged 12 ± 5 % the numbers of CGRP+ varicosities.

Most CGRP+ varicosities colocalized with CALR (59 ± 11 %, n = 4), or occurred alone (41 ± 11 %). None colocalized with NOS. Among CALR+ varicosities, the vast majority colocalized with CGRP (75 ± 5 %), and a small proportion occurred alone (22 ± 5 %); few contained NOS (3 ± 2 %) and none contained the combination of both CGRP+ and NOS+. Of the few varicosities in baskets that were NOS+, most contained NOS alone (63 ± 17 %) or colocalized with CALR (34 ± 16 %). Few contained both CGRP and CALR (2 ± 2 %), while none colocalized with CGRP alone. Thus, baskets were comprised predominantly of CGRP+ varicosities with or without CALR. A small proportion were CALR+ without CGRP, while NOS+ varicosities were sparse. These results are summarised in Table 4.4.

Circular muscle

The circular muscle layer contained nerve fibres with all three markers used in this study, with NOS being the most abundant and CGRP being relatively sparse. Of the CGRP+ varicosities examined, the most common contained CGRP alone (65 ± 9 %), and a smaller proportion also

contained CALR (27 ± 6 %). Few CGRP+ varicosities contained NOS (5 ± 4 %) or both CALR and NOS (2 ± 3 %). Among CALR+ circular muscle varicosities, most colocalized with NOS (52 ± 8 %) or occurred alone without CGRP (35 ± 3 %). A small proportion occurred with CGRP (13 ± 6 %), while virtually none (0 ± 1 %) occurred with both CGRP and NOS. Most NOS+ varicosities occurred without CGRP or CALR in circular muscle (80 ± 5 %). A small proportion contained CALR (19 ± 5 %). Few NOS+ varicosities occurred with CGRP (1 ± 1 %) or both CGRP and CALR (1 ± 1 %). These results are summarised in Table 4.4.

Differences between baskets and circular muscle

There were significant statistical interactions between chemical code and location (baskets vs circular muscle) for CGRP ($P = 0.005$) and CALR ($P < 0.001$), but not NOS varicosities ($P = 0.357$) by two-way ANOVA (Table 4.4).

Among the CGRP+ varicosities, CALR- / **CGRP+** / NOS- and **CALR+** / **CGRP+** / NOS- accounted for most varicosities in both baskets and circular muscle. However, CALR- / **CGRP+** / NOS- varicosities represented significantly greater proportions of all CGRP+ varicosities in the circular muscle (65 ± 9 %), compared to those in baskets (41 ± 11 %, $P = 0.027$, $n = 4$), while proportions of **CALR+** / **CGRP+** / NOS- varicosities were significantly greater in baskets (59 ± 11 %) than in the circular muscle (27 ± 6 %, $P = 0.003$, $n = 4$).

Amongst the CALR varicosities, those coded **CALR+** / **CGRP+** / NOS- were a significantly higher proportion of varicosities in baskets than in circular muscle, representing 75 ± 5 % and 13 ± 6 %, respectively ($P = 0.001$). Conversely, varicosities coded **CALR+** / CGRP- / **NOS+** represented higher proportions in circular muscle varicosities, 52 ± 8 %, compared to only 3 ± 2 % in baskets ($P = 0.018$). Varicosities containing CALR alone comprised modest proportions

in both circular muscle and baskets (35 ± 3 % and 22 ± 5 %, respectively), but they were not significantly different (P = 0.142). There were no statistically significant differences in the proportions of varicosities between these regions where NOS was the reference marker.

Table 4.3 – Proportions of nerve cell bodies inside CGRP baskets by neurochemical profile

Chemical code	**% CGRP baskets**	**Ave per ganglion**	**P (Tukey)***
CALR-/NOS-	48 ± 3	5.8 ± 0.2	-
CALR-/NOS+	21 ± 4	2.6 ± 0.6	0.023
CALR+/NOS-	19 ± 4	2.4 ± 0.5	0.030
CALR+/NOS+	13 ± 2	1.6 ± 0.3	0.002

*Compared to CALR-/NOS- group (one-way ANOVA, Tukey post-test, n = 4). No other comparisons between groups showed significant differences.

Table 4.4 – Proportions of varicosities by chemical code, compared between locations

Reference	Chemical code	Basket (%)	Circ. Muscle (%)	Adjusted P
CGRP	CALR- / **CGRP+** / NOS-	41 ± 11	65 ± 9	**0.027**
	CALR+ / **CGRP+** / NOS-	59 ± 11	27 ± 6	**0.003**
	CALR+ / **CGRP+** / **NOS+**	0 ± 0	2 ± 3	0.998
	CALR- / **CGRP+** / **NOS+**	0 ± 0	5 ± 4	0.955
CALR	**CALR+** / CGRP- / NOS-	22 ± 5	35 ± 3	0.142
	CALR+ / **CGRP+** / NOS-	75 ± 5	13 ± 6	**0.001**
	CALR+ / **CGRP+** / **NOS+**	0 ± 0	0 ± 1	0.862
	CALR+ / CGRP- / **NOS+**	3 ± 2	52 ± 8	**0.018**
NOS	CALR- / CGRP- / **NOS+**	63 ± 17	80 ± 5	0.825
	CALR+ / CGRP- / **NOS+**	34 ± 16	19 ± 5	0.810
	CALR+ / **CGRP+** / **NOS+**	2 ± 2	1 ± 1	0.875
	CALR- / **CGRP+** / **NOS+**	0 ± 0	1 ± 1	0.862

Percentages refer to the total reference marker population; P values refer to two-way ANOVA, Sidak post-tests (n = 4 in each group).

Discussion

In this study, we describe a neural circuit in mouse distal colon in which IPANs connect with sub-populations of myenteric neurons via specialised pericellular baskets of CGRP-immunoreactive varicose nerve fibres (Figure 4.6). Electron microscopy studies have demonstrated the presence of synaptic connections between varicose pericellular baskets and neurons that they surround [495, 497]. It is therefore likely that the connections described here represent functional synaptic connections.

Source of varicosities in myenteric CGRP+ baskets

Neurons coded CALR- / **CGRP+** / NOS- and **CALR+ / CGRP+** / NOS- were the major contributors to CGRP+ baskets in the myenteric plexus. CGRP in mouse colon is present in Dogiel type II neurons, which are putative IPANs of the ENS. In colchicine treated tissue, all CGRP-immunoreactive cells have Dogiel type II morphology and 99% of them also contain calretinin [554]. Conversely, the vast majority of CALR+ Dogiel type II cells contain CGRP [554]. Furthermore, the use of organotypic culture in our study eliminated any contamination by extrinsic CGRP containing nerve fibres. This makes it highly likely that the varicose CGRP+ nerve fibres of the pericellular baskets described in our work arise from IPANs.

The only other substantial contributor to baskets were varicosities that contain calretinin alone. Myenteric interneurons containing calretinin represent a potential source of these varicosities [563]. Additionally, small proportions of Dogiel type II neurons contain calretinin without CGRP [554]. Interestingly, the proportions of varicosities coded **CALR+ / CGRP+** / NOS- favoured baskets over circular muscle among both CGRP and CALR varicosities, but this was not the case for those coded CALR- / **CGRP+** / NOS-. This may reflect different branching patterns of the two populations or some degree of target specificity in differently coded Dogiel type II neurons.

Sources of varicosities in the circular muscle

Most CALR+ varicosities in the circular muscle occurred alone or with NOS. These results are compatible with those of Sang and Young (1996) [32], who also identified calretinin nerve terminals in the circular muscle, with and without colocalized NOS. These are likely to be projections of inhibitory and excitatory motor neurons, some of which likely originate from cells in CGRP+ baskets.

CGRP+ varicosities in the circular muscle layer were sparse. Very small proportions of these colocalised with either calretinin or NOS. Assuming all of them are from Dogiel type II cells, they either indicate direct circular muscle innervation by IPANs or possibly represent Dogiel type II projections that are passing through the circular muscle layer on their way to mucosa. The former possibility is supported by data from direct microelectrode recordings and neurobiotin injection into Dogiel type II cells, showing varicose terminals in the circular muscle layer of distal mouse colon [106].

Myenteric neurons within CGRP baskets

The largest proportion (48%) of neurons within CGRP+ baskets contained none of the markers used in our study (CALR-/CGRP-/NOS-), and therefore represent an opportunity for future investigation. The rest of the baskets housed neurons with a combination of NOS, CALR and CGRP immunoreactive content.

We found that approximately 33% of CGRP baskets were occupied by a NOS-containing neuron. NOS is a marker consistently found in inhibitory neurons of the gut, the majority of which project in the aboral direction and include motor as well as inter-neurons [563-565]. ChAT is a marker of excitatory neurons [532, 563, 566, 567], and generally most (>95%)

myenteric neurons either contain NOS or ChAT [568] i.e. they are either inhibitory or excitatory in nature. Based on this, it follows the other 67% of neurons in CGRP baskets are likely to be excitatory, ChAT-containing cells.

Calretinin is not specific to any functional group of cells, however in our study we can divide CALR+ cells into inhibitory or excitatory groups based on the presence or absence of NOS respectively. Both of these group of CALR+ cells are present in CGRP+ baskets.

Our finding of CGRP+ neurons in CGRP+ baskets strongly implies connectivity and possibly communication between IPANs of the myenteric plexus. Data that shows IPANs in mouse receive slow synaptic inputs upon electrical stimulation of circumferentially orientated internodal strands supports this idea [116]. And so does the finding of IPANs in guinea pig, which project to other IPANs around the gut circumference, thereby forming a recurrent network [78, 569]. It is likely that IPANs form similar networks in the mouse colon.

Proposed circuit

One of the earliest observations in gut physiology is that a focal stimulus triggers ascending excitation and descending inhibition [570]. This causes gut contraction oral to the stimulus and relaxation aboral, a mechanism likely promoting movement of luminal content in the appropriate direction. Based on the findings of this paper, we propose a neural circuit (Figure 6) that has all the elements necessary to underlie the above mechanism. This comprises IPANs, which respond to a stimulus, and in turn activate both excitatory (ascending) and inhibitory (descending) neurons in the CGRP baskets. We have detailed a similar circuit based on calbindin baskets in guinea pig distal colon, with one fundamental difference; the IPAN pericellular baskets did not contain NOS+ neurons [552]. This suggests a lack of direct input

from IPANs to inhibitory (NOS-containing) neurons in guinea pig colon and it must be assumed that the descending (inhibitory) circuit is activated via interneurons, or possibly that a different sensory neuron is involved.

Our study highlights fundamental similarities in neural connectivity of the enteric nervous system between species and gut regions, where intrinsic sensory neurons activate ascending and descending chains of interneurons and possibly drive motor neurons directly via monosynaptic pathways.

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Chapter 5

General Discussion

“Brevity is the soul of wit”

(Polonius in Hamlet, William Shakespeare)

The gut is the only hollow organ with its own intrinsic nervous system. The enteric nervous system (ENS) probably evolved before the central nervous system [6] and controls many (if not all) of the functions of the gastrointestinal tract, including motility. It is essential for normal life, as highlighted by the morbidity and mortality associated with maladies of its dysfunction [571]. The simple yet profound observation of reflex activity in a segment of gut separated from the host animal, noted as early as 1755 by the Swiss physiologist Albrecht von Haller [572], sparked a scientific investigation that is still ongoing today.

Much progress has been made in our understanding of the many types of enteric neurons that make up the ENS [10, 32, 76, 77, 101, 113, 121, 133, 136, 274, 286, 563]. The circuits they form [252, 268, 275, 493, 499, 500], the substances required for neurotransmission [18, 63, 150, 506, 573], the role of interstitial cells and glia [295, 337], the extrinsic systems that modulate ENS activity [153, 163, 397, 453, 473, 482, 574-577] and the motility patterns that are the result of intrinsic smooth muscle rhythms and enteric circuit activity [152, 487, 578-582]. Great technical advances in fields such as genetic manipulation and bioelectronics medicine rely on the ongoing study of neural circuits to provide the roadmap for their application.

The aim of this PhD project was to further our knowledge of neural circuits underlying colonic motility in mammals and in particular, how the intrinsic primary afferent neurons (IPANs) relate to other functional neuronal groups. We approached this aim from several angles, using techniques such as immunohistochemistry, fluorescence microscopy, organotypic culture and retrograde tracing.

Initially we explored the idea that calbindin–containing varicose ‘baskets’ in the myenteric plexus of guinea pig colon represent specialised structures that allow a concentration of

synaptic output onto a neuronal cell body contained within. We based this idea on previous observations of Somatostatin- and bombesin - containing pericellular baskets in guinea pig ileum [255, 494], VIP containing baskets in guinea pig proximal colon [514] and evidence of synaptic connections within these structures [495, 497], suggestive of a functional connection. Furthermore, we postulated that these calbindin baskets are formed from a significant contribution by IPANs of the colon, and if that was indeed the case, we could determine what other neurons receive their synaptic output.

We characterised myenteric calbindin baskets in guinea pig colon both quantitatively and qualitatively. One of the most striking features we noticed was that calbindin-immunoreactive Dogiel type II neurons were often closely associated to clusters of calbindin baskets, and on 3-dimensional reconstructions, it was clearly identified that some of these baskets arose directly from projections of these presumed IPANs. This was a very exciting detail, not previously reported. We were motivated to examine in detail the chemical coding of neurons within the myenteric baskets, the basket varicosities and varicosities within circular muscle, to characterise the neuronal populations within calbindin baskets and determine if they supply the circular muscle. Our decision to focus on NOS- and calretinin-immunoreactive neurons was based on previously described neurochemical profiles of different myenteric neuronal classes within the guinea pig distal colon [10] and our assumptions that many of these neurons likely represent second order neurons within myenteric circuits.

Analysing our data from the immunohistochemistry experiments, we made several interesting observations. NOS-immunoreactive neurons are very rarely found in calbindin baskets, given that NOS is a marker of inhibitory enteric neurons, this suggests that calbindin baskets contain excitatory neurons and therefore inhibitory neurons are not primarily activated by IPANs. This was certainly an unexpected finding because we know that during reflex activity, both

excitatory and inhibitory pathways are active simultaneously [528]. We speculate that inhibitory neurons in guinea pig distal colon are triggered by one or a combination of several possible inputs; NOS neurons are mechanosensitive and in fact respond to longitudinal stretch by inhibiting circular muscle activity [583], therefore inhibitory pathways may be activated directly by stretch. NOS neurons could also be activated via interneurons, which themselves could be mechanosensitive or receive excitatory input from IPANs.

We carried out further experiments using a retrograde tracing technique to address our hypotheses. We traced myenteric pathways and myenteric projections supplying the circular muscle, something that has not been done in guinea pig distal colon before. We found that there was indeed an association between calbindin baskets and excitatory myenteric neurons; these neurons were further shown to be ascending interneurons and circular muscle motor neurons. One of our key findings was that we could classify motor neurons into at least three groups, based on their immunoreactivity and association with calbindin baskets i.e their synaptic input. This was exceptionally interesting to us and suggests there are different populations of motor neurons, rather than a 'common final motor pathway'. Although the functional relevance of these findings remains to be established, we can speculate which known motor patterns could rely on these circuits, with a view to testing this in the future with functional experiments.

Propulsive motor patterns in the colon are the result of complex interplay between neurogenic (nerve-cell mediated) and myogenic (spontaneous muscle activity) mechanisms [152, 584]. Myogenic activity, such as 'ripples' and slow waves may be observed after blocking all nerve function [488, 585]; in rat and rabbit colon, myogenic activity alone is capable of generating anally-directed propulsion in vitro, which is not the case in guinea pig [584, 586, 587]. However, these motor patterns only occur after chemical stimulation using carbachol (ACh-

mimetic drug), their magnitude is significantly lower compared to normal controls and they lack directional polarity, suggesting that under physiological conditions, myogenic activity is not sufficient for colonic propulsion. Myogenic and neurogenic mechanisms work together through a self-sustaining neuro-mechanical loop, whereby luminal content progresses along the length of the gut by the sequential activation of polarised reflexes [584]. This mechanism is adaptable, the size and consistency of luminal content affects the speed of propulsion [487].

Several distinct neurogenic motor patterns occur in guinea pig colon devoid of any mobile content; transient neural events (TNEs), cyclic motor complexes (CMCs) and distal colon migrating motor complexes (DCMMC) have been recorded simultaneously in the isolated guinea pig colon [588]. It is very interesting to note that these motor patterns can occur in the same preparation either alone or in any combination, at the same time. Data presented in the study by Costa et al strongly suggests that specific neural circuits generate each of these patterns independently. CMCs and DCMMCs but not TNEs were blocked by smooth muscle relaxants (L-type calcium channel blockers), modulation of IPAN activity using TRAM-34 (antagonist of the calcium-activated potassium channel) disrupted CMCs but not TNEs or DCMMCs, only CMCs were sensitive to inhibition of nNOS activity and cholinergic neurotransmission via nicotinic receptors was required for CMCs and TNEs but not DCMMCs [588]. The circuit we have characterised involves IPANs and ascending excitatory interneurons, given the above observations on colonic motor patterns, it is possible that this circuit is involved in the generation of DCMMCs.

We were particularly interested to determine if a similar intrinsic neural circuit has evolved in the mouse distal colon. Hence, we performed a series of experiments designed to identify CGRP-containing intrinsic pathways that belong to intrinsic sensory neurons. Our findings in that study both confirm similarities in ENS structure between species but also highlight some

major differences. Arguably, the most remarkable observation was that IPANs in mouse distal colon associate with both inhibitory (NOS-containing) and excitatory neurons via the myenteric CGRP-immunoreactive varicose baskets. This is of course, in direct contrast to our findings in guinea pig colon, where calbindin baskets and NOS neurons were mutually exclusive. This potentially highlights a major difference in the activation of inhibitory pathways between the two species. The caveat with our two studies based on analysis and comparison of neurochemical coding in cell bodies and varicosities is that the neurochemical profile of a cell body does not always match the one in its varicose projections; therefore, it is vital to have functional data for a more complete picture. Colonic motor complexes occur in mouse colon, depend on a functional ENS and involve the coordination of synchronised activity of large populations of excitatory and inhibitory motor neurons [580, 589, 590]. Cyclic colonic motor patterns have also been recorded in several other species [582], including the isolated human colon [591]. The enteric circuit composed of a sensory neuron activating excitatory and inhibitory myenteric neurons could certainly underlie such motor activity.

A random mixture of both male and female animals was used in all of the above-mentioned studies. Although we have to consider the possibility of differences in ENS neuroanatomy and neurochemical profiles between the two sexes, we have not analysed our data based on sex. This is following the precedent of the majority of studies on guinea pig ENS as referenced in this thesis, and taking into account the greater number of animals that would have to be used in order to power the studies for such a question.

Studies that examine neural connectivity within the ENS provide the basis for understanding functional observations and guide further experiments. Correlating neural circuits with function helps to translate experimental data into a broader understanding of gut physiology, and sets up a foundation for the development of possible interventions in gut pathology.

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Appendix