Chapter 1
Review of the literature

Introduction

Viscerofugal neurons are a specialized class of enteric neurons, being the only type of enteric neuron not confined to the wall of the gut. They form reflex circuits with prevertebral sympathetic neurons and some types of viscerofugal neurons with central projections probably interact directly with parasympathetic nerves that project to the gut. These reflex circuits exist in the context of many shorter and longer range feedback loops. Neural circuits in the enteric nervous systems are relatively short, typically a few millimetres in range. Viscerofugal neurons form circuits that may affect gut functions between different gut regions. On top of this are even broader autonomic regulatory circuits formed by visceral spinal and vagal afferent neurons, central interneurons and chains of autonomic motor neurons of the sympathetic and parasympathetic nervous systems which regulate normal digestive processes and responses to noxious stimuli in the gut. Finally, the sympathetic and parasympathetic nervous systems locally and globally regulate gut function in the context of the whole body to meet the demands of the environment. Thus, all three divisions of the autonomic nervous system and their afferent inputs are relevant to the study of enteric viscerofugal neurons. The following review generally focuses on the cellular components and connectivity of these systems in mammals, choosing not to detail all species differences but rather the architecture valid in principle in all species.

Sympathetic nervous system

The sympathetic nervous system (SNS) is one of three anatomically, developmentally, and functionally distinct divisions of the autonomic nervous system (Langley, 1921). The autonomic nervous system executes neural and hormonal

control over organs of the body to maintain homeostasis. The functional effects of SNS control of target tissues are often antagonistic to parasympathetic effects when organs are dually innervated. Major targets of the SNS include the heart, arteries, veins, gastrointestinal tract, spleen, kidney, bladder, reproductive organs, eye, airways, glands, piloerector muscles, liver, white and brown adipose tissue, pancreas, adrenal medulla and lymphoid tissue. SNS motor neurons are arranged in chains with two links: preganglionic and postganglionic neurons. Sympathetic motor pathways to different organs, and often within the same organ, are functionally distinct and differentially modulated to provide patterned responses to incoming sensory stimuli. Coordinated inputs from the brain and spinal circuits to the different subtypes of sympathetic neurons drive patterned behaviours. Thus, activities of the SNS range from localized spinal segmental reflexes that may involve a single functional class of neuron, such as when a drop in blood pressure evokes reflex activation of vasoconstrictor neurons to normalize pressure, to complex patterns like the expression of affective states (fear, pleasure etc.) involving changes in the activity of many sympathetic motor pathways throughout the body.

Preganglionic neurons

Neuroanatomical characteristics

Organization

Functionally distinct, “final common autonomic pathways” execute sympathetic control of target organs (Jänig, 1986). These pathways begin with sympathetic preganglionic neurons (SPNs) of the spinal cord. Along the rostrocaudal axis, SPNs are present from the final cervical vertebrae (C8), throughout all thoracic vertebral

segments, to the upper lumbar segments: L4 in guinea-pigs (McLachlan, 1985, McLachlan et al., 1985), or L3 in rats (Strack et al., 1988).

Within the spinal cord, SPN cell bodies are located bilaterally in 4 autonomic nuclei. Most SPNs occur in the intermediolateral column of the lateral horn (IML; identical to Rexed's lamina 7) in small interconnected clusters, or "nests", at the border of the grey and white matter (Petras and Cummings, 1972, Cabot, 1990). SPNs are also located in the central autonomic area (CAA, also known as the paraependymal part of the intercalated nucleus), located just dorsal of the central canal. Bridging the gap between IML and CAA are clusters of SPNs in the intercalated nucleus (IC). Finally, clusters of SPNs occur also in the dorsolateral funiculus of the white matter. Bundles of SPN dendrites project mediolaterally and rostrocaudally (Dembowsky et al., 1985, Pilowsky et al., 1994), connecting SPN clusters and forming a "ladder"-like appearance.

SPNs that supply paravertebral ganglia occur on the ipsilateral side of the spinal cord, while SPNs innervating prevertebral and pelvic ganglia are located bilaterally (Cabot, 1990). SPNs from several vertebral levels contribute inputs to a single ganglion. The SPN supply follows a rostrocaudal gradient that reflects the position of the target. The axons of SPNs exit the ventral root of the corresponding vertebral level.

Morphology of preganglionic nerve cell bodies

SPN have a diverse range of morphologies. SPN somata are significantly larger than dorsal horn interneurons but smaller than skeletal muscle motor neurons. Their major

and minor axes range 15-60μm and 10-40μm, respectively (Petras and Cummings, 1972, Chung et al., 1975, Oldfield and McLachlan, 1981, Barber et al., 1984, Dembowsky et al., 1985, Morgan et al., 1986a, Cabot and Bogan, 1987, Nadelhaft and McKenna, 1987, Forehand, 1990). Dendrites of SPN are expansive and constitute the majority of their surface area. SPN dendrites in the IML are predominantly oriented in the rostrocaudal plane; extending up to 2.5mm in the cat - well into and beyond adjacent clusters of SPN. Dendro-dendritic interactions between SPN may arise from this arrangement (Dembowsky et al., 1985), although ultrastructural studies argue against this possibility, because individual dendrites are largely ensheathed by glial cells except where synapses occur (Markham and Vaughn, 1990). Some SPNs in the IML have dendrites in the mediolateral plane which intermingle with laterally orientated dendrites of IC SPNs – bridging the nuclei (Oldfield and McLachlan, 1981, Barber et al., 1984, Baron et al., 1985, Krukoff et al., 1985, Bacon and Smith, 1988). Many IC SPN dendrites and the majority of their cell bodies are orientated in the mediolateral axis of the transverse plane. Their dendrites are more radially complex than those of the IML, but often extend medially into the CAA. The dendritic arbours of CAA SPNs apparently have no directional bias (Petras and Cummings, 1972, Petras and Faden, 1978, Dalsgaard and Elfvin, 1981, Oldfield and McLachlan, 1981, Barber et al., 1984). SPN of the dorsolateral funiculus also have rostrocaudally orientated dendritic arbours, while a few have been reported to have transverse orientations that extend into the white matter. Although some topographical correlations with morphology have been identified (Hinrichs and Llewellyn-Smith, 2009), functional relationships have not.

Neurochemical content

SPNs are immunoreactive for the enzyme required for acetylcholine synthesis, choline acetyltransferase (ChAT; Barber et al., 1984, Cabot and Bogan, 1987), and release acetylcholine onto postganglionic cells (Feldberg and Gaddum, 1934). Their terminals within ganglia contain the vesicular acetylcholine transporter (VAChT) for loading acetylcholine and proteins required for fast exocytotic release (Gibbins et al., 2003a). Some populations of SPN express the synthetic enzyme for nitric oxide, nitric oxide synthase (NOS; Anderson, 1992, Grkovic and Anderson, 1997, Hinrichs and Llewellyn-Smith, 2009) and are thought to be the predominant source of nitrergic transmission in ganglia and the adrenal medulla (Hinrichs and Llewellyn-Smith, 2009). Gamma amino butyric acid (GABA) has also been identified in a subpopulation of SPNs that project to the superior cervical ganglion (Ito et al., 2007). Numerous peptides that may function as neurotransmitters or neuromodulators have been identified in subsets of SPNs. These include enkephalin (ENK), corticotropin-releasing factor (CRF), leutinizing-hormone-releasing hormone, neurotensin (NT), secretoneurin, somatostatin (SOM), substance P (SP), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), calcitonin gene-related peptide (CGRP) and cocaine and amphetamine regulated transcript. However, these findings must be treated with care, since the use of colchicine to increase immunoreactivity in the cell body may alter gene expression (Ceccatelli et al., 1991, Rethelyi et al., 1991, Pu et al., 1999). Immunohistochemical identification of neuropeptide content in nerve terminals on the other hand, does not require colchicine (Gibbins, 1995).

Functional classes

Types of SPN include vasoconstrictors (skeletal muscle, skin and viscera), vasodilators (skeletal muscle and skin), sudomotor, secretomotor (salivary, thyroid, visceral), bronchomotor, cardiomotor, pilomotor, inspiratory, pupillo-motor, lipomotor, and motility-regulators (gut, urinary tract, reproductive organs). Other types of sympathetic neurons may innervate immune tissues and the pineal gland (Kappers, 1960).

Neurochemical coding and functional class

Neurons of a specific functional class may express unique combinations of proteins and neurotransmitters that can be detected immunohistochemically, termed "chemical coding". The chemical coding of a few types of SPN have been elucidated. This requires that the function and chemical code of the postganglionic neurons to which the SPNs project is known. While all SPNs contain acetylcholine, those which control postganglionic neurons that innervate salivary glands also contain the calcium binding protein, calretinin, or NOS (Grkovic and Anderson, 1995, Chiba and Tanaka, 1998). Those which control postganglionic neurons that innervate the heart contain both calretinin and NOS (Anderson, 1998, Richardson et al., 2006). SPNs that eventually control the musculature (tarsal muscle) of the upper eye lid have either ENK and calretinin, ENK and calbindin or NOS without ENK (Chanthaphavong et al., 2003). Sudomotor neurons contain CRF (Shafton et al., 1992), and those controlling adrenaline release from the adrenal medulla have calretinin (Edwards et al., 1996). Vasoconstrictors projecting to lumbar paravertebral ganglia contain CGRP (Gibbins, 1992). Like chemical coding of enteric neurons in the gut, the coding patterns may be specific to region and species studied.

Presympathetic neurons

Supraspinal presympathetic neurons

Anatomical studies of the descending inputs to SPNs show that the sympathetic outflow is predominantly supplied by five cell groups in the brain: the paraventricular hypothalamic nucleus (containing glutamate + oxytocin/CRF/arginine vasopressin [AVP]), A5 noradrenergic cell group, caudal raphe region (serotonin [5HT]), rostral ventrolateral medulla (RVLM; glutamate + ENK/SP/VIP/thyrotropin-releasing hormone [TRH]), and ventromedial medulla (Strack et al., 1989). Less prominent input may come from the locus coeruleus, arcuate nucleus, prefrontal cortex (Fritschy et al., 1987, Holets et al., 1988, Hurley et al., 1991, Bacon and Smith, 1993, Elias et al., 1998), and from orexin neurons of the lateral hypothalamus (co-transmitter with glutamate; Geerling et al., 2003, Llewellyn-Smith et al., 2003).

Intraspinal presympathetic neurons

Trans-synaptic viral labelling studies show that inputs to SPNs arise from neurons with cell bodies in the IML and spinal cord laminae 5, 7 and 10. These studies indicate that sensory neurons of the dorsal root ganglia do not make direct synaptic contacts onto SPNs, but rather, are involved in multisynaptic pathways. Spinal cord interneurons provide ongoing synaptic inputs to SPN in spinal cord slice preparations where descending supraspinal inputs have been severed (Dun and Mo, 1989, Spanswick et al., 1994, Krupp and Feltz, 1995, Spanswick et al., 1998).

Transmitter inputs

In electrophysiological studies, glutamate, GABA and glycine powerfully affect SPN firing. In SPN, glutamate evokes fast excitatory postsynaptic potentials (EPSPs) while GABA and glycine mediate fast inhibitory postsynaptic potentials (IPSPs). Axons that contain either glutamate or GABA constitute 95% of synaptic input to SPN (Llewellyn-Smith et al., 1992, Llewellyn-Smith et al., 1995, Llewellyn-Smith et al., 1998). These amino acid transmitters are supplied by both supraspinal and intraspinal neurons. Supraspinal inputs containing glutamate or GABA are thought to arise largely from the RVLM and caudal raphe nuclei (Minson et al., 1991, Stornetta et al., 2002, Stornetta et al., 2004).

The catecholamines adrenaline and noradrenaline are also likely involved in the physiological regulation of SPN firing. Excitation and inhibition mediated by α_1 and α_2 adrenergic receptors, respectively, is evoked by exogenous catecholamines (Inokuchi et al., 1992). Synaptic terminations containing catecholamine-synthesizing enzymes, tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase, occur on SPNs (Minson et al., 2002), which disappear after spinal cord transection (Llewellyn-Smith et al., 2006), suggesting they have a supraspinal origin. These inputs are likely to include those from the C1 adrenergic neurons in the RVLM and noradrenergic neurons of the A5 cell group (Jansen et al., 1995).

At least 20 neuropeptides exist in the lateral horn where SPNs occur (Llewellyn-Smith, 2009). Exogenous SP, TRH, oxytocin, AVP, PACAP and orexin are excitatory in SPNs, and occur in synapses apposing SPNs or in areas occupied by SPNs (Chiba and Masuko, 1987, Bacon and Smith, 1988, Vera et al., 1990,

Llewellyn-Smith et al., 1991, Pilowsky et al., 1992, Poulat et al., 1992a, Poulat et al., 1992b, Chiba et al., 1996, Dun et al., 2002, Llewellyn-Smith et al., 2003).

Serotoninergic varicosities also surround SPNs. These are likely to be excitatory since exogenous 5HT activates SPNs (Ma and Dun, 1986, Lewis and Coote, 1990, Lewis et al., 1993, Pickering et al., 1994, Madden and Morrison, 2006, Madden and Morrison, 2008). The principle source of 5HT is the caudal raphe nuclei, and minor inputs may be supplied by spinal interneurons (Newton et al., 1986).

Spinal preganglionic neuron reflexes

Investigations in normal and spinalized animals have characterized spinal reflexes in 5 functionally identified classes of SPN (cutaneous, muscle and visceral vasoconstrictor, sudomotor and motility-regulating) evoked by stimulation of known groups of afferent neurons. For example, noxious cutaneous stimulation causes reflex excitation of sudomotor and muscle vasoconstrictor SPNs and inhibition of cutaneous vasoconstrictor SPNs (Grosse and Jänig, 1976). Activation of Pacinian corpuscles inhibit cutaneous vasoconstrictor neurons while exciting sudomotor neurons (Kümmel, 1983). The above examples show that excitation and inhibition can arise from the same stimulus, suggestive of differential spinal interneuronal processing. When somatic or visceral afferent neurons are stimulated electrically, they evoke short and long latency reflex discharges in SPN (Dembowsky et al., 1985, Bahr et al., 1986a, Bahr et al., 1986b, c). Short latency reflex discharges to somatic or visceral afferent stimulation may be attributed to short spinal circuits, while long latency discharge involves both spinal and supraspinal circuitry (Dembowsky et al., 1985).

Visceral motility-regulating preganglionic reflexes

"Motility-regulating" SPNs affect motor functions of the viscera, including the gastrointestinal tract. Their effect on the gut was demonstrated in early studies of "viscero-inhibition" where the lumbar splanchnic nerves were transected or stimulated (Pflüger, 1857, cited in Furness 2006, Bayliss and Starling, 1899, Cannon, 1906, Garry, 1933). Electrical stimulation of the central cut end of dorsal roots was shown by Bayliss and Starling (1900) to evoke inhibition of the colon in spinal animals, which is now known to cause reflex modulation of SPN activity. Extracellular electrophysiological recordings of identified motility-regulating SPNs have been made (Bahr et al., 1986b, c, Bartel et al., 1986). There may be two types of motility-regulating SPN based on their spinal reflex behaviour (Bartel et al., 1986). These two types of neurons respond to bladder and colonic stimulation in opposite ways, however both types show strong reflex excitation to anal stimulation (Bartel et al., 1986). Excitation of motility-regulating SPNs by anal stimulation may be mediated by activation of low-threshold sacral afferents (Bahns et al., 1987, Janig and Koltzenburg, 1991). In addition, neither of the motility-regulating SPNs are affected by arterial baro- and chemo-receptor stimulation or cardiac activity (Bahr et al., 1986b). This suggests that motility-regulating SPNs are under specific control by visceral afferent reflex circuits under normal physiological circumstances. Interestingly, reflex modulation of motility-regulating SPNs is little affected by spinal cord transection. They remain functional in acutely spinalized animals, suggesting that these neurons are under little ongoing control by descending spinal pathways (Bartel et al., 1986). This is not true of other types of SPN, such as cutaneous vasoconstrictor SPNs, where spinal reflexes are severely depressed or absent hours to days after transection (Horeyseck and Jänig, 1974, Jänig, 1985).

Thus, motility-regulating SPNs may have relatively less reliance on descending input, but this does not preclude strong activation by supraspinal regions under particular physiological or behavioural states. Spinal reflex behaviour in other classes of SPN after transection may become sensitized after recovery from spinal transection, which probably underlies "autonomic dysreflexia" in humans with spinal injuries. Reflexes in other types of SPN are suspected based on clinical observations and functional studies of sympathetic effector organs (Jänig, 1985, Janig, 2006).

Postganglionic neurons

Cell bodies of postganglionic neurons form organized clusters in sympathetic ganglia, which also contain glial cells, mast cells, extra-adrenal chromaffin cells, Schwann cells, as well as axonal terminations of SPNs, viscerofugal neurons and spinal sensory neurons.

Prevertebral ganglia

The three prevertebral ganglia lie just ventral to the junctions of the aorta and the coeliac, superior mesenteric and inferior mesenteric arteries. These ganglia are unpaired, but may have distinct lobes fused together. They supply visceral organs, including the gut, liver, gallbladder, pancreas and spleen. In addition, the bilateral pelvic plexuses are a mixed group of neurons containing both sympathetic and parasympathetic motor neurons that supply the lower gut and pelvic viscera.

Sources of input

The prevertebral ganglia receive preganglionic inputs via thoracic and lumbar splanchnic nerves and sympathetic preganglionic neurons supplying the pelvic

plexus arrive via the hypogastric nerves. Retrograde labelling studies show the SPNs supplying the coeliac ganglia (CG) and superior mesenteric ganglia (SMG) emerge from the thoracic spinal cord (T_4-T_{12} and T_7-T_{13}, respectively), and those supplying the inferior mesenteric ganglia (IMG) and pelvic plexus largely derive from the lumbar spinal cord (T_{13}-L_3 and L_1-L_2, respectively; Hancock and Peveto, 1979, Strack et al., 1988). These findings are largely in agreement with Gaskell (1886) who identified the thoracolumbar distribution of visceroinhibitory nerves that emerged from the spinal cord using electrical stimulation. SPNs projecting to the CG and SMG are predominantly located in the intermediolateral column of the spinal cord (Strack et al., 1988). The IMG receives inputs from SPNs originating in the central autonomic nucleus (Strack et al., 1988). Certain types of prevertebral neurons receive additional inputs from viscerofugal neurons with cell bodies in the gut as well as collaterals of spinal sensory neurons. Retrograde labelling studies of viscerofugal neurons demonstrate that the coeliac ganglion receives inputs from viscerofugal neurons located in most of the length of the gut – from the stomach to the rectum, with most arising from the proximal colon (Messenger and Furness, 1992). The SMG and IMG receive viscerofugal inputs from the distal colon and rectum; most arise from the rectum (Messenger and Furness, 1993). Likewise, the pelvic ganglia receive viscerofugal neuron input from the distal colon and rectum, however the numbers are only a fraction (<10%) of those received by prevertebral ganglia (Luckensmeyer and Keast, 1995a). Prevertebral ganglia lie outside the blood brain barrier; therefore, paracrine and circulating endocrine signalling molecules in the blood that influence the excitability of sympathetic neurons may be considered another important source of input.

Prevertebral supply to the gut

Prevertebral ganglia are the principle source of sympathetic fibres in the gut, with a smaller contribution from paravertebral ganglia (Trudrung et al., 1994). The source of sympathetic fibres in the gut loosely follows a rostrocaudal gradient with respect to the ganglia (i.e. the upper gut is predominantly innervated by CG, while SMG, IMG and pelvic plexus supply increasingly distal regions of gut; Szurszewski and Miller, 2006). Consistent with the idea that the sympathetic nervous system yields specific and patterned output, gastrointestinal motility, secretions and blood flow are independently controlled by separate populations of sympathetic neurons, namely: motor, secretomotor and vasomotor neurons. In certain cases, these populations of neurons have been defined by their combination of synaptic inputs, morphology, chemical code and electrophysiological properties. In the following paragraphs, the properties of sympathetic prevertebral neurons will be related to the functional classes of neurons that innervate the gut.

Neurochemistry

The majority of neurons in mammalian prevertebral ganglia contain enzymes required for noradrenaline, including TH and dopamine β-hydroxylase (Szurszewski and Miller, 2006). Small populations (2-5% in guinea-pig IMG) of non-adrenergic sympathetic neurons exist in the prevertebral ganglia of rat, guinea-pig and pig (Szurszewski and Miller, 2006). Some of these neurons are cholinergic (Sann et al., 1995b). Several enzymes and neuropeptides associated with neurotransmission have been identified in the cell bodies of mammalian prevertebral sympathetic neurons, including SOM, neuropeptide Y (NPY), VIP, ENK, galanin (GAL), NOS, CGRP and AVP (Szurszewski and Miller, 2006). The physiological role of most peptides in

prevertebral neurons has not been clarified. In paravertebral neurons the role of a few of these have been studied at their peripheral targets. For example, NPY may act as a vasoconstrictor (Lundberg 1996) and may potentiate the effects of NA in the heart (Potter, 1987); and VIP released from cholinergic vasodilator, sudomotor and secretomotor neurons may be a vasodilator (Gibbins, 1995). In addition, purine transmitters adenosine triphosphate (ATP) or β-nicotinamide adenine dinucleotide are probably released with noradrenaline in vasoconstrictor neurons (Smyth et al., 2000, Smyth et al., 2009). However these transmitters are not amenable to immunohistochemical analysis.

Chemical coding and functional classification

In the guinea-pig coeliac ganglia, there is a robust relationship between peptide content and the functional role of sympathetic neurons. SOM- and NPY–immunoreactive (IR) cells form substantial populations of the total amount of cells in prevertebral ganglia. In the guinea-pig stomach and small intestine, most sympathetic fibres (99%) contain TH and dopamine β-hydroxylase, suggesting they use noradrenaline (NA) as their neurotransmitter (Macrae et al., 1986). Sympathetic fibres that supply secretomotor neurons of the submucous plexus also have SOM, those that innervate mesenteric vessels have NPY, and fibres in the myenteric plexus which surround motor neurons and interneurons have neither peptide. Thus secretomotor, vasomotor and motor sympathetic neurons are coded with noradrenaline NA/SOM, NA/NPY and NA-only, respectively (Costa and Furness, 1984, Macrae et al., 1986). NA-only and NA/SOM neurons are preferentially distributed in the medial CG and NA/NPY neurons occur in the lateral lobes (Gibbins, 1995). Chemical coding of functional subtypes is less well understood in

the IMG. Here, there is a much greater proportion of NA/SOM cells (about 75%), and NA/SOM fibres in the large intestine ramify throughout the myenteric plexus (Parr and Sharkey, 1996).

Morphology and synapses in prevertebral ganglia

Postganglionic neurons in mammalian prevertebral ganglia have single, unmyelinated axons with cell bodies varying in size from 17-40μm in their major axis. Numerous dendrites emanate from their cell bodies, and typically project within a single plane (Szurszewski and Linden, 2012). This organization gives rise to a layered arrangement of neurons within prevertebral ganglia which suggests physiological interactions between postganglionic neurons are more likely within than across layers. It is not known whether preganglionic or afferent inputs to prevertebral ganglia are differentiated between layers (Szurszewski and Linden, 2012). The great majority of synaptic appositions on postganglionic neurons occur on their dendrites which form the majority of postganglionic surface area. The density of preganglionic synaptic inputs received by each postganglionic neuron is approximately the same; however some additionally receive viscerofugal neuron inputs at about the same density as preganglionic inputs – effectively doubling the density of synapses on their cell membranes (Gibbins et al., 2003b). Overall however, synapses occur on only about 1-2% of the cell surface area of postganglionic neurons in sympathetic ganglia, and these are randomly distributed across the cell soma and dendrites (Forehand, 1985, Gibbins et al., 1998). In guinea pig CG, motor and secretomotor neurons have the largest cell bodies and dendritic arbours (Boyd et al., 1996, Anderson et al., 2001) and receive more synapses than vasomotor neurons (Gibbins et al., 2003b). Secretomotor and motor neurons, but not

vasomotor neurons, receive the inputs of viscerofugal neurons, which can be distinguished by immunoreactivity for VIP (Lindh et al., 1986, Macrae et al., 1986, Gibbins et al., 2003b). Anatomical and electrophysiological studies show NPY-IR neurons in the lateral lobes do not receive inputs from viscerofugal neurons; they also have much smaller dendritic arbours (Gibbins et al., 2003b).

Electrophysiological classification of sympathetic neurons

An electrophysiological classification scheme has been used to differentiate neurons in sympathetic ganglia based on their responses to sustained depolarizations (Cassell et al., 1986, Cassell and McLachlan, 1987). Three major types have been described – tonic, phasic and long after-hyperpolarization (LAH) neurons (although intermediate examples have been found; Jobling and Gibbins, 1999). Tonic neurons continuously and rhythmically discharge during depolarization. Phasic neurons discharge a short burst of action potentials at the onset of depolarization, followed by a refractory period. LAH neurons are a subset of phasic neurons which fire once at the onset of depolarization followed by a prolonged hyperpolarization. Differences in firing behaviours are attributable to potassium channel conductances that inactivate firing (Jänig and McLachlan, 1992). Phasic neurons have a prominent M current mediated by non-inactivating voltage-gated potassium channels, while LAH have two types of Ca^{2+}-activated potassium channels that are responsible for the long after-hyperpolarization. Neither of these channels is prominently expressed in tonic neurons, which more commonly express A- and D-type potassium channels. In guinea-pig, tonic and LAH sympathetic neurons are somewhat exclusive to prevertebral ganglia. Tonic neurons are either NA/SOM secretomotor or NA/- motor neurons (Keast et al., 1993). LAH neurons often have NPY (Gibbins et al., 1999).

Almost all paravertebral neurons are phasic neurons; they also constitute 15-25% of prevertebral neurons (49% of CG, 2% of IMG; Szurszewski and Miller, 2006). The lateral lobes of the CG contain mainly phasic neurons which are NPY-IR vasomotor neurons (Gibbins et al., 1999); these cells are probably located in the splanchnic ganglia in rats and mice, homologous to the lateral lobes of the guinea pig (Gibbins and Morris, 2006).

Synaptic input strength

Synaptic inputs to postganglionic sympathetic neurons vary in amplitude, even among inputs of the same origin. Stimulation of preganglionic inputs gives rise to EPSPs of different amplitudes in postganglionic neurons (McLachlan and Meckler, 1989). Variation in the amount of transmitter released by exocytosis, spatial density/composition of postganglionic membrane channels and the geometric location of synapses all likely contribute to the heterogeneity of EPSP amplitudes. Inputs that evoke large suprathreshold depolarizations with a high "safety factor" have been referred to as "strong" inputs (Crowcroft and Szurszewski, 1971, Gibbins and Morris, 2006). Postganglionic neurons in paravertebral and prevertebral ganglia typically receive a small number (1-3) of strong inputs from preganglionic neurons (Hirst and McLachlan, 1984, 1986). Conversely, postganglionic neurons receive more numerous inputs from preganglionic neurons that are "weak", which evoke smaller, subthreshold, EPSPs. Tonic neurons have fewer strong inputs than phasic or LAH neurons, instead receiving numerous weak inputs (Jänig and McLachlan, 1992). Indeed, peripheral (viscerofugal) inputs to tonic neurons (which tend to be "weak") outnumber preganglionic inputs (McLachlan and Meckler, 1989). Thus, the role of integration and summation of inputs are presumably more important in motor and secretomotor neurons than in other sympathetic neurons with prominent strong

inputs. In the latter, the physiological importance of weak inputs is unclear. In rat paravertebral ganglia, analysis of output discharges of reflex-evoked and electrically stimulated synaptic events suggested weak inputs play little role in determining output (McLachlan et al., 1997, McLachlan, 2003). However, direct analyses of weak synapses in paravertebral ganglia suggests several mechanisms by which weak inputs may amplify preganglionic activity (Horn and Kullmann, 2007). Convergent weak inputs activated within a sufficiently small time interval (<30ms) may summate to reach action potential threshold, depress or facilitate subsequent synaptic inputs, or shunt after-hyperpolarizing currents (Karila and Horn, 2000, Rimmer and Horn, 2010).

Paravertebral ganglia

Cell bodies of most final motor neurons (postganglionic neurons) in the SNS are grouped in paravertebral and prevertebral ganglia. The paravertebral ganglia run bilaterally adjacent the spinal cord spanning rostrocaudally from the cervical to sacral levels, interconnected by nerve trunks, resulting in a chain-like, linear network. A single pair of ganglia occurs per vertebral segment from the thoracic to sacral spinal cord. The most caudal lumbar or sacral ganglia may be fused together in some species. At the most rostral end of the paravertebral sympathetic chain are several cervical ganglia fused together which forms the superior cervical ganglion as well as the stellate ganglion which includes several fused cervical and thoracic ganglia. SPNs projecting out of the ventral roots project into the paravertebral ganglia through the white rami via the spinal nerves (**figure 1.01**). Paravertebral motor neurons leave the ganglia via the grey or white rami en route to somatic effector cells or via the splanchnic nerves en route to the viscera.

Sympathetic preganglionic and paravertebral neuron pathways

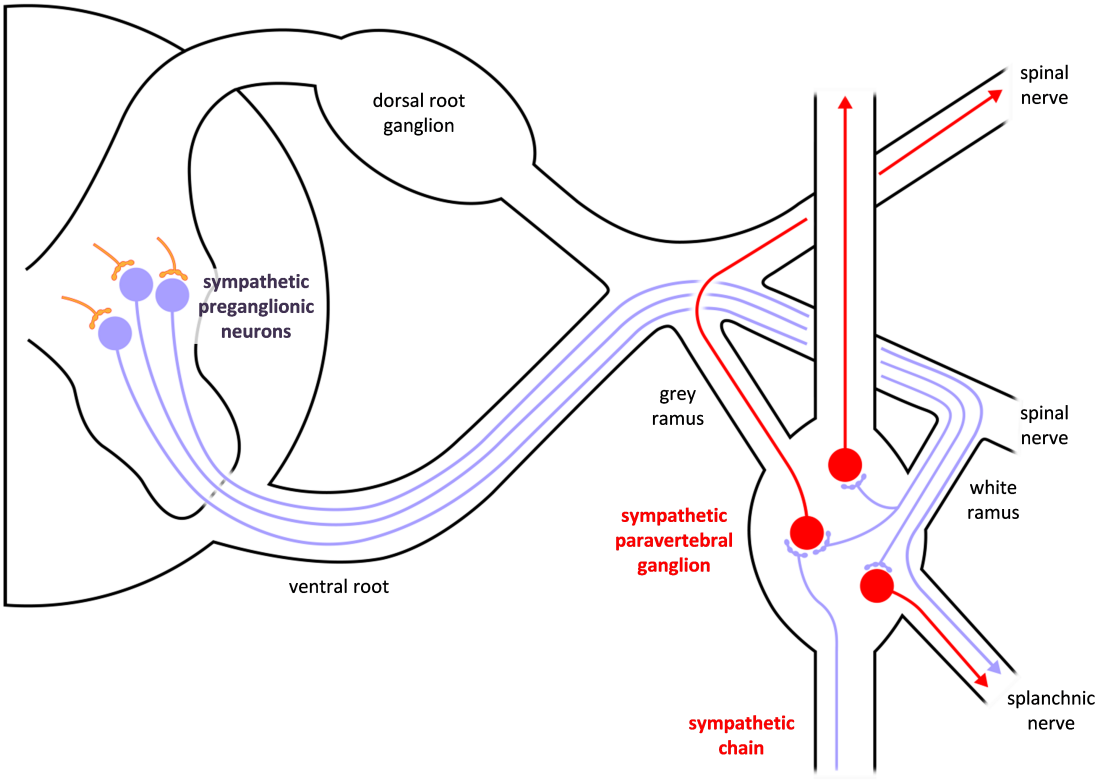

Figure 1.01

Sympathetic preganglionic and paravertebral neurons. This schematic diagram shows the pathways taken by sympathetic preganglionic neurons (purple) to postganglionic paravertebral neurons (red). Axons of sympathetic preganglionic neurons emerge from the spinal cord via the ventral root and enter paravertebral ganglia via the white rami and spinal nerves. They may also project along the sympathetic chain before synapsing on postganglionic neurons. Postganglionic paravertebral neurons targeting somatic structures project via grey rami and spinal nerves while paravertebral neurons targeting the viscera make projections through the splanchnic nerves. Figure by Ian Gibbins; used with permission.

Paravertebral supply to the gut

Postganglionic neurons of the paravertebral ganglia are the predominant source of sympathetic neurons in the body. However, they are less abundant in the gastrointestinal tract compared to the amount arising from prevertebral ganglia (Trudrung et al., 1994, Quinson et al., 2001). Axons from paravertebral sympathetic neurons may pass through a prevertebral ganglion on the way to the viscera, thereby running in the same nerve trunks as prevertebral sympathetic neurons, spinal sensory neurons and viscerofugal neurons (Messenger et al., 1994). Retrograde tracing studies show that the vertebral levels from which paravertebral neurons to the gut originate shifts according to the proximo-distal location of their targets in the gut. Most paravertebral neurons supplying the stomach to proximal colon in the rat are in the lower thoracic ganglia (Quinson et al., 2001). Studies in the guinea-pig distal gut show peak supplies originating from the lower lumbar and sacral ganglia (Olsson et al., 2006). A large increase in the number of paravertebral fibres occurs in the distal gut (Olsson et al., 2006). In the rectum, the sympathetic supply to the gut may switch from principally prevertebral to paravertebral fibres (Olsson et al., 2006). More distally, the internal anal sphincter sympathetic innervation is almost entirely derived from the sacral paravertebral ganglia and pelvic plexus (Costa and Furness, 1973a). Many paravertebral neurons to the gut are NPY-IR vasoconstrictor neurons that supply the vasculature of the mesentery and submucosa (Hill et al., 1987, Chevendra and Weaver, 1992). They participate in cardiac homeostasis, regulating resistance to blood flow and the amount of blood stored in the viscera (Furness and Costa, 1974). Paravertebral neurons to the gut are probably phasic neurons since almost all neurons in paravertebral ganglia are found to be phasic (Jänig and McLachlan, 1992).

Sympathetic terminations in the gut

The densest innervation of the gut by sympathetic nerves occurs in the enteric ganglia where most, if not all, enteric neurons are surrounded by fine noradrenergic varicose fibres (Tassicker et al., 1999a). There is also dense innervation of the smooth muscle of the sphincters and vasculature. Sparse innervation of non-sphincter smooth muscle and the mucosa also occurs (including villi; Furness and Costa, 1974, Thomas and Templeton, 1981).

Regulation of motility

Sympathetic nerves inhibit gastrointestinal motility by reducing neurogenic input to smooth muscle in non-sphincteric regions, as well as by activating constriction of the sphincters. Noradrenaline, released from sympathetic nerve terminals presynaptically inhibit myenteric neurons, suppressing acetylcholine release (Hirst and McKirdy, 1974). Intestinal motor activity being largely dependent on myenteric neurons is inhibited by the suppression of excitatory neurotransmission. Presynaptic inhibition is mediated by activation α_2 adrenergic receptors (Hirst and McKirdy, 1974). Whether sympathetic neurons exert an ongoing inhibition of gut motility under normal physiological conditions is not clear. This is partly due to the act of taking measurements, which can affect the results. For example, sympathetic nerve section releases inhibition of motility where the gut has been handled, exposed, or where measurement devices have been inserted (Cannon and Murphy, 1906, Olivecrona, 1927, cited in Furness and Costa 1974, Garry, 1934, Jansson, 1969). In these cases, acute inhibition of motility is probably mediated by input from spinal sensory and/or viscerofugal neurons sympathetic neurons. Where motility has been measured without gut handling, chronic splanchnic nerve section in the cat showed little effect

on the rate of gastric emptying (Cannon, 1906, M'Fadden et al., 1935). However, contrary results were in dogs (McCrea, 1926). In small intestine, motility was accelerated by splanchnic nerve section for meals containing predominantly protein, but not carbohydrate, suggesting the physical or chemical properties of digested material modulate sympathetic input to motility-controlling circuits in the intestine (Cannon, 1906).

Regulation of secretion

Chloride ion secretion into the gut lumen (carrying water by osmosis), is largely under the control of submucous enteric neurons. Inhibition of secretion by sympathetic neurons is indirect through inhibition of submucous secretomotor neurons which innervate enterocytes. Noradrenaline and somatostatin is released from sympathetic nerve terminals, causing presynaptic inhibition as well as inhibitory post-synaptic potentials in submucous neurons (North and Surprenant, 1985, Shen and Surprenant, 1990, Foong et al., 2010). Noradrenaline acts mainly through α_2-adrenergic receptors and somatostatin via SST_1 and SST_2 G-protein coupled receptors (GPCRs; North and Surprenant, 1985, Foong et al., 2010). Cutting the sympathetic nerves to the intestines causes a large and sustained increase in secretion, indicating that sympathetic neurons have an ongoing inhibitory effect (Brown and Miller, 1991). Sympathetic secretomotor neurons may be controlled locally from the gastrointestinal tract since they receive synaptic contacts from viscerofugal neurons as well as inputs from spinal sensory neurons (Gibbins and Morris, 2006). Functional evidence has been found to support this (Quinson and Furness, 2002). Perfusion of the large intestine with hypertonic solution, but not isotonic or hypotonic solutions, triggered increased secretion in segments of jejunum

that were separated from the rest of the gut by transection. It is hypothesized the hypertonic solution altered viscerofugal neuron inputs to sympathetic neurons in the coeliac ganglion, leading to the release of tonic inhibition on secretion (Quinson and Furness, 2002).

Regulation of blood flow

Both prevertebral and paravertebral sympathetic vasoconstrictor neurons project to the enteric/mesenteric vasculature. These neurons course along the adventitia of the blood vessels, forming a dense adrenergic plexus in mesenteric and enteric arteries, while veins are more sparsely innervated (Furness and Costa, 1974). Noradrenaline acting on α_1 adrenergic receptors, NPY acting on Y_2 receptors and ATP on P2X receptors evoke contraction of vascular smooth muscle cells thereby decreasing the calibre of the blood vessels and increasing resistance to blood flow. It is likely that the frequency of action potential discharge determines which combinations and relative quantities of stored neurotransmitter are released. For example, in rat small mesenteric arteries, high frequency discharges evoke noradrenaline release while low frequencies cause release of a non-adrenergic transmitter (Sjoblom-Widfeldt et al., 1990); in pig spleen, high frequency discharge evokes noradrenaline and NPY release, while low frequencies (<2Hz) favour NPY release only (Pernow et al., 1989). The pattern of action potential discharge is also likely to play a role in determining neurotransmitter output. Coordinated bursts of action potentials, compared with equally spaced discharge at the same overall frequency (approximately 6Hz) evoked significantly more noradrenaline release from sympathetic nerves and a 44% greater contractile response in rat caudal artery (Hardebo, 1992). Some prevertebral, but not paravertebral vasoconstrictor neurons

may receive input from spinal sensory neurons (Gibbins and Morris, 2006), indicating that sympathetic regulation of blood flow in the gut is predominantly controlled by the central nervous system, rather than by peripheral reflexes.

Peripheral reflexes

An historical account of studies that led to the discovery of sympathetic reflexes that may function independent of the central nervous system is described by Szurszewski and Miller (1994). Briefly, there were early observations that mechanical or chemical stimulation of the gut could evoke inhibition in distant regions which required intact spinal cord and splanchnic (and mesenteric) nerves. In bladder, stimulation of central cut ends of the hypogastric nerves to the inferior mesenteric ganglion caused activation of smooth muscle, suggesting that entirely peripheral autonomic reflexes may also exist (Sokownin, 1877, cited in Szurszewski and King, 1989). However, Langley (1894) showed this was probably antidromic activation of bifurcating efferent axons – a "pseudo-reflex". The possibility of a genuine peripheral reflex was revived by the work of Garry (1933) who showed, with *in vivo* manometry, that cutting the mesenteric nerves to the colon released inhibition of gut motility even after splanchnic nerves were cut. This was followed up by Kuntz (1940), who used a preparation with a lesion between two segments of colon, thus connected only by mesenteric nerves to a decentralised inferior mesenteric ganglion. He found that distension of one segment caused inhibition of motor activity in the other (Kuntz, 1940). The same experiment was carried out using chronically decentralized ganglia with the same result, ruling out any possibility that it was a pseudo-reflex (Kuntz and Saccomanno, 1944). It was established that peripheral reflexes occur in the

sympathetic nervous system, probably with afferent inputs coming from enteric neurons.

Contribution of the adrenal medulla to gut functions

Catecholamines released from the adrenal medulla into the blood primarily affect cellular metabolism (e.g. glycogen mobilization, lipolysis). However, circulating catecholamines may also contribute to inhibition of intestinal motility and blood flow (Furness and Burnstock, 1975). Overall, motility appears more sensitive to circulating catecholamines than the intestinal vasculature (Furness and Burnstock, 1975). Intestino-intestinal inhibitory reflexes were not affected by removing the adrenal medulla (Kock, 1959). However, circumstances of generalized sympathetic arousal (including loss of blood volume, exercise, and certain affective states) may increase circulating levels of catecholamines, contributing to inhibition of intestinal blood flow and motility (Furness and Burnstock, 1975). Pheochromocytoma increases blood catecholamines and may be associated with reduced gut motility and ischemia (Dibaise and Quigley, 1998). Removal of the adrenal medulla has been shown to reduce the inhibition of intestinal motility evoked by hypotension, blood loss, or afferent nerve stimulation (Youmans et al., 1940, Hamilton et al., 1944, Kock, 1959). In addition, blood immediately taken from cats that had been "excited" by barking dogs evoked relaxation of control gut tissue; relaxation was not evoked using blood from adrenalectomized cats (Cannon and de la Paz, 1911). These data support a role for the adrenal medulla in modulation of gut function. Other roles of acute changes in circulating catecholamines include the modulation of nociceptor thresholds (Khasar et al., 1998a, Khasar et al., 1998b, Khasar et al., 2003),

inflammatory responses (Miao et al., 2000, Miao et al., 2001) and memory acquisition (McGaugh and Roozendaal, 2002).

Parasympathetic nervous system

Innervating many of the same structures, and sharing many basic structural features with the sympathetic nervous system, the parasympathetic nervous system (PNS) contributes to the physiological regulation of visceral organs. In contrast to the general inhibitory effects of sympathetic activation in the gut, parasympathetic activation is in many cases "permissive", facilitating activation of enteric neural circuits that promote secretion, motility and sphincter relaxation. PNS motor neurons occur in chains of two arranged in series, like the SNS. The anatomical arrangement of the PNS is described as "cranio-sacral", owing to its two major outflows: the brainstem and the sacral spinal cord. PNS preganglionic neurons that control organs in the head, thorax and upper abdomen emerge from the midbrain and brainstem, while neurons that control the pelvic viscera, including the distal bowel, emerge from the sacral spinal cord. Preganglionic neurons synapse with postganglionic neurons that are within or close to the target organ in relatively small ganglia compared to the SNS. Like the SNS, the multiple circuits of the PNS may be independently recruited by incoming stimuli, giving rise to a nuanced role in homeostatic maintenance.

Cranial parasympathetic supply to the head

Cranial preganglionic neuron cell bodies are located in nuclei of the brainstem and midbrain, including the visceral efferent oculomotor nucleus, superior and inferior salivary nuclei, nucleus ambiguus, and the dorsal motor nucleus of the vagus

(DMNX; Janig, 2006). Preganglionic axons from these nuclei travel via the third (oculomotor), seventh (facial), ninth (glossopharyngeal) and tenth (vagus) cranial nerves. Four major pairs of parasympathetic ganglia supply target organs in the head: the ciliary ganglia supplies the iris and ciliary muscles of the eye; the sphenopalatine ganglia supplies vasodilator and secretomotor innervation to the lacrimal glands, nasal mucosa and mucosa of the palate; the submandibular ganglia supply vasodilator and secretomotor innervation to the submandibular and sublingual salivary glands; the otic ganglia supplies the parotid gland and blood vessels of the lower jaw with secretomotor and vasodilator neurons (Janig, 2006). Other minor parasympathetic ganglia are located at various points along the seventh and ninth cranial nerves that supply the local vasculature and glands.

Cranial parasympathetic supply to thorax and abdomen

Vagal parasympathetic preganglionic neurons innervate organs of the thorax and abdomen; including the gastrointestinal tract, heart, airways, thyroid, pancreas, liver, gallbladder and white adipose tissue (Kreier et al., 2002, Janig, 2006). Preganglionic and postganglionic neurons synapse in small ganglia near or within target organs, e.g. the cardiac plexus of the heart and enteric plexuses of the GIT. Functionally mixed populations of preganglionic neurons project to the target organs, which can mediate opposing effects. Gut-projecting preganglionic neurons, for example, may evoke gastric contractions or relaxations, via actions on excitatory and inhibitory enteric motor neurons, respectively. The vagal innervation to the gut, which is the main focus here, comes from two regions in the medulla: the nucleus ambiguus and the DMNX.

Nucleus ambiguus

The nucleus ambiguus is intimately involved in coordinating swallowing behaviour. Motor neurons to the soft palate, pharynx, larynx, heart, oesophagus, and upper oesophageal sphincter lie in the nucleus ambiguus - a paired column of cell bodies located within the medulla oblongata (Bieger and Hopkins, 1987, Chang et al., 2003, Rogers and Herman, 2012). Pharyngeal and oesophageal motor neurons occur in the rostral end of the dorsal nucleus ambiguus in a region called the compact formation, or nucleus retrofacialis (Bieger and Hopkins, 1987). These cell bodies contain choline acetyltransferase and most have myelinated axons (Cecio and Califano, 1967, Armstrong et al., 1983, Jones and Beaudet, 1987, Loewy and Spyer, 1990, Ruggiero et al., 1990). Axons reach the oesophagus via the vagus and the recurrent laryngeal branch of the vagus (Neuhuber et al., 2006). A minor proportion reach the oesophagus via the superior laryngeal nerves (Andrew, 1956). Terminations occur as motor endplates on striated muscle fibres (Neuhuber et al., 2006), where they release acetylcholine (Bartel et al., 1986, Storr et al., 2001). Indeed, stimulation of the dorsal nucleus ambiguus evokes strong contractions, which are abolished by nicotinic neuromuscular blockers but not muscarinic receptor antagonists (Bieger, 1984, Bieger, 1993). During swallowing, sequenced activation of pharyngeal and oesophageal motor neurons generate peristalsis. The patterns of activity required to produce peristalsis is the result of neural circuits occurring in the nucleus ambiguus, the nearby reticular formation, and the nucleus of the solitary tract (NTS; Goyal and Sivarao, 1999). These medullary structures are referred to as the swallowing pattern generator (Goyal and Sivarao, 1999, Lang, 2009). The central nucleus of the NTS contains the "pre-motor" neurons that evoke the serial activation of nucleus ambiguus motor neurons underlying peristalsis. The central nucleus of the NTS

receives glutamatergic input from vagal afferents that feed sensory information back into the swallowing pattern generator (Altschuler et al., 1989, Cunningham and Sawchenko, 1989). Sensory feedback from these afferent sources is required for complete execution of swallowing (Lang, 2009).

Dorsal motor nucleus of the vagus

Vagal parasympathetic neurons are responsible for coordinating many digestive activities. It has been known for over a century that the parasympathetic efferents which direct gut functions are in the DMNX in the medulla (Rogers et al., 1996, Rogers and Herman, 2012). These paired nuclei are fusiform shaped, run longitudinally adjacent the central canal, and contain around 20,000 efferent neurons (Blessing, 1997). These cells are ChAT-IR and organized in longitudinal columns viscerotopically (Houser et al., 1983, Fox and Powley, 1985, Hornby and Wade, 2011). Cells located in the medial regions of the nucleus supply more proximal gut regions. Those projecting to more distal gut regions are increasingly laterally located (Hornby and Wade, 2011). Some preganglionic neurons appear to be spatially organized in the nucleus according to their function: medial and rostral located cells produce contractions of the lower oesophageal sphincter and stomach; cells in the lateral and caudal DMNX evoke relaxations in both these regions (Abrahams et al., 2002, Zhou et al., 2008). Vagal efferent neurons to the gut have an ongoing, pacemaker-like, discharge rate of about 1-2Hz (Rogers and Herman, 2012). This property confers exquisite sensitivity to alterations of membrane potential, which may result in large differences in firing rate (Rogers and Herman, 2012).

Peripheral inputs to the dorsal motor nucleus of the vagus

Input to the DMNX and nucleus ambiguus arise peripherally from visceral afferent and humoral sources, and from the descending projections of higher brain structures. Most visceral afferent input is vagal, although functional evidence indicates splanchnic sources also make contributions (Grundy et al., 1981, Barber and Yuan, 1989, Renehan et al., 1995, Traub et al., 1996). Visceral afferent inputs are highly convergent numerically and spatially: afferent neurons outnumber efferent neurons about 10 to 1 (Prechtl and Powley, 1990), while electrophysiological studies indicate that a single DMNX neuron may receive afferent inputs from most of the length of the gut (Grundy et al., 1981). Although some afferents make direct contacts with DMNX neurons (see below), most visceral input to the DMNX is pre-processed by way of interneurons from the NTS (medullary projections). These inputs are excitatory or inhibitory in nature and most are glutamatergic, GABAergic or noradrenergic (Leslie, 1985, Maley, 1996). Polysynaptic brainstem circuits from vagal afferents to efferent neurons which underlie "vago-vagal" reflexes can exert long range control over digestive processes (Rogers et al., 1996). Several types of vagal reflexes are known to exist; including those controlling the reservoir function of the stomach, gastric motility, secretions, emesis, pancreatic and gallbladder secretion, as well as intestinal, colonic and sphincter functions. Vagal reflexes supplement the intrinsic neural circuits of the gut, which often exert similar effects (Hennig et al., 1997). The "latent functions" in enteric circuitry can compensate for the losses of function that occurs in the event of vagotomy, indicating a capacity for plasticity (Grundy et al., 1993, Wei et al., 1997). Immediate effects of vagotomy on gastric function include a depression of antral motility and reservoir function of the fundus. Currently, the best characterized vagal reflex is "receptive relaxation" of the

gastric musculature to accommodate incoming food, which may be triggered by swallow-induced oesophageal distension (Cannon and Lieb, 1911). The reflex increases the reservoir capacity of the stomach while keeping intragastric pressure low. Receptive relaxation occurs in concert with a second relaxation reflex that typically becomes active later in the digestive process called "adaptive relaxation", or "gastric accommodation" (De Schepper et al., 2004). The afferent arm of receptive relaxation involves oesophageal distension-sensitive vagal afferent neurons – probably the intraganglionic laminar endings (Zagorodnyuk and Brookes, 2000). These neurons terminate in the pars centralis region of the NTS (Rogers et al., 1999). Oesophageal distension excites second order NTS neurons which in turn project to, and activate, populations of parasympathetic neurons in the lateral and caudal DMNX, which evoke relaxation of the fundus (Rogers et al., 1999). Simultaneous inhibition of putatively excitatory medial and rostral DMNX neurons occurs (Rogers et al., 1999). This is consistent with the idea that reciprocal innervation is required for coherent control of both excitatory and inhibitory enteric neurons (Davison and Grundy, 1978). The identity of the second order pars centralis NTS neurons that project into the DMNX appears to be those which utilize noradrenaline (Rogers et al., 2003). The TH-IR NTS neurons form a "shell" around a "core" of NOS-IR NTS neurons; the latter appear not to have a role in the receptive relaxation reflex (Rogers et al., 2003). In support, receptive relaxation may be partially blocked by α_1 or α_2 adrenergic receptor antagonists in the brainstem (Rogers et al., 2003). "Adaptive relaxation" causes further gastric relaxation in response to distension and nutrients in the stomach and small intestine. The magnitude of relaxation is probably sensitive to nutrient composition and degree of mechanical stimulation (De Schepper et al., 2004). Gastric relaxation, whether evoked by vagal input or not, is dependent on

release of inhibitory transmitters from intrinsic enteric motor neurons to the smooth muscle of the gut, including nitric oxide (Desai et al., 1991, Desai et al., 1994, Takahashi and Owyang, 1997, Tack et al., 2002), and possibly also purines (Hennig et al., 1997).

Circulating nutrients, cytokines and gastrointestinal hormones in the blood may influence the DMNX given the NTS, area postrema, and possibly the dendrite processes of DMNX neurons themselves are outside the blood-brain barrier. The firing output of the DMNX is affected by blood glucose levels, with increased glucose decreasing gastric motility and pressure via actions on neurons in the NTS and DMNX (Mizuno and Oomura, 1984, Sakaguchi et al., 1985, Sakaguchi et al., 1994, Ferreira et al., 2001). Hormones released into circulation during or after digestive activity also act centrally to affect vagal output. Peptide YY for example, released into circulation by ileal enteroendocrine cells when fatty acids are present, gains access to the DMNX and NTS where it acts via neuronal Y_2 receptors, causing inhibition of gastric motility (Chen and Rogers, 1995, 1997, Chen et al., 1997). Another interesting example of how humoral factors influence vagal parasympathetic function is tumor necrosis factor alpha (TNFα), a cytokine released in abundance by macrophages during infection (Carswell et al., 1975, Waage, 1987). TNFα can evoke stereotypical "sickness behaviour", including symptoms of nausea, decreased appetite, hyperalgesia and slow gastric emptying, among others (Dantzer et al., 2008). Both NTS and DMNX neurons express receptors for TNFα that respond to exogenous and endogenous TNFα, causing inhibition of identified gastric excitatory DMNX neurons and subsequent hypomotility (Kinouchi et al., 1991, Hermann and Rogers, 1995, Hermann et al., 1999, Emch et al., 2000, 2002). As well, TNFα

increases vagal afferent glutamate release (Emch et al., 2000, 2001), probably via TNFα receptors located on their central processes (Hermann et al., 2004) in addition to sensitizing effects of TNFα at vagal (and spinal) peripheral terminals (Blatteis, 2000, Dantzer, 2001). Such effects likely underlie the altered sensitivity of vagal reflexes under conditions of infection (Hermann and Rogers, 1995, Hermann et al., 1999).

Central inputs to the dorsal motor nucleus of the vagus

Central descending inputs to DMNX are complex, arising from at least 12 brain regions, and involve several transmitters including, but not limited to: 5HT, dopamine, noradrenaline, glutamate, TRH, CRF, NPY, opioids, endocannabinoids, leptin, orexin and melanocortin (Loewy and Spyer, 1990, Rogers and Herman, 2012). The best characterised central inputs to the DMNX are those arriving from the raphe nuclei – the raphe obscurus and pallidus (Rogers et al., 1980, Rogers and Herman, 2012). Raphe inputs release the peptide thyrotropin releasing hormone (TRH), which potently excites parasympathetic efferents in the DMNX (Tache et al., 1980). Concomitant raphe TRH projections are sent to the NTS, inhibiting these neurons, and functionally uncoupling vago-vagal reflex circuits upon activation. TRH in the brainstem evokes strong parasympathetic nerve activation leading to profuse gastric acid secretion and vigorous motility. Raphe projections also contain and release 5HT (Iverfeldt et al., 1989), which interacts synergistically with TRH, evoking greater postsynaptic excitation (McCann et al., 1988, McTigue et al., 1992). Also terminating on DMNX neurons are the C1 neurons of the RVLM which release glutamate, and possibly also catecholamines, activating them (DePuy et al., 2013). Oxytocin-containing projections from the PVN terminate in NTS and DMNX; both

of which are excitatory (Swanson and Hartman, 1980, Rinaman, 1998). PVN inputs appear to play a role in regulating the general tone of vagal reflex activities: ablation of these projections diminishes the sensitivity of vagal reflexes to vagal afferent stimuli (Rogers and Hermann, 1985). CRF-containing projections also derive from the PVN as well as the pontine micturion centre (Barrington's nucleus) which may mediate effects of affective states on gut motility, such as stress-evoked inhibition of gastric emptying (Valentino et al., 1995, Coşkun et al., 1997).

Vagal anatomy

Axons of gastrointestinal parasympathetic preganglionic neurons and vagal afferent neurons run through the vagus nerve from the brainstem to the gut. The left and right vagus nerves run down either side of the oesophagus, merging with sympathetic nerve trunks forming an oesophageal plexus (Chamberlin and Winship, 1947, Peden et al., 1950). The most proximal (cervical) oesophagus is supplied by the superior laryngeal nerves. The thoracic and abdominal oesophagus receive the oesophageal nerves which branch off the left and right vagus (Chamberlin and Winship, 1947). Anterior and posterior vagal nerve trunks emerge from the oesophageal plexus, traverse the diaphragm, divide, then merge with paravascular sympathetic nerves. The anterior vagus divides into the hepatic, accessory celiac and gastric branches. The posterior vagus divides into celiac and gastric branches (Powley et al., 1983, Prechtl and Powley, 1985). The gastric and celiac branches innervate the stomach. All branches of the vagus provide innervation to the duodenum, while the celiac branches supply the remaining small intestine and proximal colon (Berthoud and Neuhuber, 2000). Several small paraganglia containing nerve cell bodies of unknown function have been identified at bifurcation points along the subdiaphragmatic vagus

(Prechtl and Powley, 1985, Kummer and Neuhuber, 1989, Dahlqvist et al., 1994). The anatomical descriptions of the vagal supply to the gut are consistent with a large body of research suggesting the vagus affects gut function throughout most of its length (Garry, 1934, Stavney et al., 1963). Consistently, anterograde labelling studies from the DMNX shows innervation from the oesophagus to proximal colon, with the stomach receiving the densest supply. Myenteric neurons are the principle targets of parasympathetic fibres, although there is also a sparse supply to submucosal neurons (Berthoud et al., 1990, Neuhuber et al., 1998, Rogers and Herman, 2012).

Vagal parasympathetic innervation of the gut

Vagal preganglionic neurons exert selective control over gastrointestinal functions, including secretions and muscle contractility, by modulating activity of enteric circuits. Enteric neurons constitute parasympathetic "postganglionic" neurons in parasympathetic pathways to the gut. These pathways can mediate either excitatory or inhibitory effects on gastrointestinal functions. A comprehensive characterization of the connectivity between neurons of the DMNX and enteric neurons is not complete. Early anterograde studies suggested preganglionic neurons exerted control through a specific set of enteric target neurons ("command" neurons; Kirchgessner and Gershon, 1989). However, subsequent work showed preganglionic innervation was widespread, particularly in the stomach, duodenum and caecum where the majority of enteric neurons appear to receive inputs, rather than a single neuronal class (Berthoud et al., 1990, Schemann and Grundy, 1992, Zheng and Berthoud, 2000). It is more likely that there are subsets of preganglionic neurons that selectively innervate classes or combinations of enteric neurons (Kuramoto et al., 2013). The observation that NOS-IR DMNX neurons preferentially project to areas

involved in vagal-mediated inhibition is suggestive of such a relationship between parasympathetic and enteric neurons (Krowicki et al., 1997, Guo et al., 2001, Rogers and Herman, 2012). Coherent functional responses are likely achieved by coordinated output from the DMNX. That excitatory and inhibitory enteric neurons both appear to be contacted by vagal efferent neurons suggests a reciprocal control arrangement, which would produce complementary rather than antagonistic effects (Davison and Grundy, 1978). Functional responses to preganglionic input may also depend on firing frequency. For example, gastric relaxation caused by vagal activation of enteric neurons was mediated by nitric oxide occurs with low frequency stimulations (2.5Hz), and higher input frequencies evoke peptide-mediated (VIP, 10Hz) relaxations (Takahashi and Owyang, 1995). The principle form of transmission between preganglionic and postganglionic neurons is neurochemical, mediated by acetylcholine released onto postganglionic nicotinic receptors (Schemann and Grundy, 1992). Vagal stimulation typically evokes multiple fast EPSPs in gastric enteric neurons, indicating they receive inputs from several vagal efferent fibres (Schemann and Grundy, 1992). Vagal efferents are also subject to modulation by sympathetic neurons which may presynaptically inhibit their transmitter release (Rogers and Herman, 2012). Enteric neurons integrate these extrinsic inputs with those from intrinsic enteric neurons (Schemann and Grundy, 1992). In the stomach, single vagal fibres often provide functional inputs to many neurons of the same ganglion (Schemann and Grundy, 1992). Consistently, anterograde tracing of parasympathetic axons in the gut shows extensive fibre branching (Berthoud et al., 1990). This may explain how it is possible that a relatively small number of parasympathetic efferents exert control over a large number of enteric neurons.

Sacral parasympathetic innervation

A substantial source of cholinergic parasympathetic innervation to the gut emerges from the sacral spinal cord. The cell bodies of these nerves are in the sacral parasympathetic nucleus of lamina 5 and 6 from segments S_1 to S_4 (de Groat et al., 1981). The major targets of sacral parasympathetic nerves include the distal gut, bladder and reproductive organs. Most gut-projecting axons emerging from the nucleus travel through pelvic and rectal nerve trunks. Synapses with postganglionic neurons occur in either the pelvic ganglia or in the gut wall itself. Electrical stimulation of the pelvic nerves potently activates contractions of the lower bowel (Garry and Gillespie, 1955). Conversely, sectioning the sacral nerves decreases spontaneous motility of the distal bowel in vivo, suggesting that motility-regulating enteric circuitry is tonically excited by parasympathetic inputs (Rostad, 1973). This population of parasympathetic neurons is intimately involved in defecation by controlling propulsive activity in the distal colon and rectum (Langley and Anderson, 1895).

Pelvic ganglia

The gross structure of pelvic ganglia (also referred to as "inferior hypogastric ganglia", "hypogastric plexus" or "pelvic plexus"; Keast, 1999), varies across species and gender. In many mammals including humans, the pelvic ganglia are several distinct cell clusters contained within connective tissue in the pelvic cavity (Mitchell, 1993, Keast, 1999). In the guinea pig, there are two major clusters of cell bodies (the anterior and posterior plexus) as well as several smaller accessory ganglia (Costa and Furness, 1973b). The innervation of the gut derives from each plexus, although their contributions differ depending on the region of gut (Costa and Furness, 1973b,

Olsson et al., 2006). The number of pelvic neurons supplying the gut increases distally from the colon to the rectum (Luckensmeyer and Keast, 1994, Olsson et al., 2006). In addition, males have more neurons in the pelvic ganglia than females; attributable mainly to an increased innervation of reproductive organs (Greenwood et al., 1985, Keast, 1999).

The pelvic ganglia contain cell bodies of both sympathetic and parasympathetic neurons in approximately equal numbers (Luckensmeyer and Keast, 1995b, Keast, 1999). Sympathetic preganglionic inputs arrive via the hypogastric nerves and parasympathetic inputs come from the pelvic nerve (Langley and Anderson, 1895). This includes some parasympathetic axons that pass through the ganglia without synapsing en route to the gut (Olsson et al., 2006). Hypogastric nerve stimulation causes inhibition of motility whereas stimulation of the pelvic nerves potently evokes contractility (Langley and Anderson, 1895, Ridolfi et al., 2009). A small number of neurons that project to the gut receive both sympathetic and parasympathetic terminals (Luckensmeyer and Keast, 1995b). Other pelvic neurons that have both sympathetic and parasympathetic inputs are vasodilator neurons to the uterine artery (Jobling et al., 2003). Like other autonomic ganglia, preganglionic input is principally nicotinic/cholinergic (Crowcroft and Szurszewski, 1971, de Groat and Krier, 1976, Krier and Hartman, 1984, Keast, 1999). Most inputs in smaller animals including rats, mice and guinea pigs are thought to be axosomatic due to the morphological simplicity of cells in pelvic ganglia (sympathetic and parasympathetic alike), which typically lack dendrites (Tabatabai et al., 1986). Pelvic ganglion cells receive a relatively small amount of preganglionic input that evokes large membrane depolarisations upon stimulation which ensure action potentials are transmitted from

the central nervous system to the periphery with a high safety factor (Crowcroft and Szurszewski, 1971). For this reason, neurons in the pelvic ganglia may be considered simple relays. Nevertheless, the pelvic ganglia receive a small amount of peripheral inputs from viscerofugal neurons in the distal colon and rectum that project via the rectal nerves and branches of the penile nerve (Luckensmeyer and Keast, 1995a, 1998). Whether these interact with sympathetic or parasympathetic cells is not clear, however they may play a role in intestinal reflexes that operate in a proximal to distal direction (Semba, 1954, Kreulen and Szurszewski, 1979b).

Neurochemical coding

The association between chemical coding and functional classes of neurons within pelvic ganglia is less well defined than in the sympathetic ganglia. Up to 11 different combinations of enzymes and neuropeptides occur in guinea pig paracervical ganglia (female equivalent of the male anterior major pelvic ganglia). Some of these neuronal classes are associated with distinctly coded presynaptic terminals, suggesting they are arranged in functionally distinct pathways (Morris and Gibbins, 1987). Pelvic sympathetic neurons that project into the gut resemble sympathetic neurons elsewhere, containing immunoreactivity for TH, DβH and NPY (Mitchell et al., 1993, Dhami and Mitchell, 1994, Keast, 1995, Olsson et al., 2006). However, some sympathetic neurons lacking TH-IR (25%) may be cholinergic and contain VIP (Keast, 1995, Keast et al., 1995). Some VIP-containing neurons receive both sympathetic and parasympathetic preganglionic inputs (Luckensmeyer and Keast, 1995b). Most pelvic neurons to the gut are ChAT-IR (58%), while a smaller proportion (<30%) are TH-IR (Keast and de Groat, 1989, Olsson et al., 2006). In

addition, most pelvic neurons to the gut contain NOS, which is present in both cholinergic and non-cholinergic neurons (Olsson et al., 2006).

Sacral parasympathetic innervation of the gut

The peripheral endings of sacral parasympathetic fibres ramify extensively in the myenteric plexus of the rectum after entering through rectal nerves (Olsson et al., 2004). Most fibres innervate myenteric neurons; however submucous plexus and smooth muscle layers also receive innervation. The range of parasympathetic influence over lower gut function in some species may be extended by way of nerve trunks running between the muscle layers of the gut called "shunt fascicles", which carry parasympathetic fibres orally from the rectum up through the colon (Christensen et al., 1984, Fukai and Fukuda, 1984). Dye-filling of rectal myenteric neurons that receive pelvic nerve input revealed that most had axons that projected orally (Tamura, 1997), suggesting that the parasympathetic nerves may connect with chains of ascending interneurons. The spatial range of functional effects that parasympathetic inputs exert on the distal gut is not yet well defined. Intracellular recordings reveal about half of the myenteric neurons in the rectum receive parasympathetic inputs. These synaptic inputs were excitatory nicotinic/cholinergic (Tamura, 1997). Some neurons (14%), receive an additional slow excitatory input, which was observed with repeated stimulation of the pelvic nerves (Tamura, 1997). However, the types of myenteric neurons which receive input have not been characterized. In the Tamura study, functional parasympathetic input was not associated with the electrophysiological classification of neurons (Tamura, 1997).

Extrinsic sensory innervation of the gastrointestinal tract

The gastrointestinal tract is endowed with dense sensory innervation that transduces mechanical and chemical events into neural signals for integration in the central nervous system, much of which does not reach consciousness. The predominant role of sensory signalling is to activate central autonomic and neuroendocrine systems. Sensory neurons are continuously modulated by immune, endocrine and microbial processes, many of which are not well understood. Three major sensory pathways from the gut exist, corresponding to, and interacting with, the 3 major sources of efferent inputs: vagal, thoracolumbar, and lumbo-sacral pathways. Studies of gastrointestinal sensory afferents have led to numerous descriptions of their anatomical, histological, neurochemical, mechanoreceptive, and chemoreceptive properties. Relatively recent advances in combined structure-function studies are now enabling the synthesis and clarification of this data culminating in a recent account that suggests that there are just 5 morphological types of extrinsic visceral sensory neuron (Brookes et al., 2013).

Gastrointestinal vagal afferents

The vagus nerve is predominantly comprised of afferent nerve fibres (Sengupta and Gebhart, 1994b). Besides the gut, vagal afferents innervate the lungs and airways, heart and aorta, liver, portal vein and bile ducts, thymus, pancreatic islets, glomus cells in vagal paraganglia, uterus, gallbladder and the vasculature of adipose tissue (Berthoud and Neuhuber, 2000, Gautron et al., 2011). The gastrointestinal innervation spans the oesophagus to proximal colon, paralleling the extent of, and occupying the same nerve trunks as the vagal efferent supply. A small population of

vagal afferents may take a spinal route via thoracic intercostal nerves (Harper et al., 1935). Most gastrointestinal vagal afferents are unmyelinated C-fibres and have cell bodies in the nodose or jugular ganglia, which are located bilaterally proximal to the carotid bifurcation at the base of the skull (Andrews, 1986). Nodose and jugular afferents differ in embryonic origin; nodose neurons derive from the epibranchial placodes while jugular afferents, like spinal sensory neurons, derive from the neural crest (Yu et al., 2005). The neurochemical and sensory properties of jugular afferents resemble those of spinal afferents which typically have higher thresholds to mechanical stimuli and are suspected to play a nociceptive role while nodose afferents are predominantly attuned to physiological stimuli (Yu et al., 2005, Nassenstein et al., 2010, Brierley et al., 2012, Hayakawa et al., 2012).

Central projections of vagal afferent neurons

The central processes of vagal afferents terminate in the nucleus tractus solitarius (Gwyn et al., 1979, Kalia and Sullivan, 1982, Leslie et al., 1982). Gastrointestinal vagal afferents enter most subnuclei within the NTS (Sawchenko, 1983). The parvocellular, medial, and commissural nuclei are the primary targets for gastrointestinal afferents (Sawchenko, 1983, Beyak et al., 2006). Outside the NTS, afferents also project into the spinal trigeminal nuclei (Altschuler et al., 1989). In species that have an emetic reflex, afferents project into the area postrema (Altschuler et al., 1989, Grundy and Scratcherd, 1989). Dendrites of vagal efferents may also enter the NTS (Shapiro and Miselis, 1985, Rinaman et al., 1989). In addition, some vagal afferent processes enter the DMNX, possibly forming direct connections with vagal efferents (Beckstead and Norgren, 1979, Kalia and Sullivan, 1982, Neuhuber and Sandoz, 1986). Second order neurons of the NTS project to

autonomic nuclei via medullary projections (including the nucleus ambiguus and DMNX) or ascend into higher regions via ascending, supramedullary projections. Supramedullary projections terminate in brain structures that mediate hormonal, affective, behavioural and cognitive responses to afferent input. Medullary projections include the vagal reflex circuits that regulate digestive functions (described in "inputs to the dorsal motor nucleus of the vagus", above).

Transmitters & Receptors

The putative transmitter used by vagal afferents for central transmission is glutamate (Andresen and Mendelowitz, 1996, Ritter, 2011); vesicular glutamate transporters can be detected immunohistochemically at their terminals (Sykes et al., 1997), and electrical stimulation evokes glutamate release (Allchin et al., 1994). Synaptically released glutamate acts upon ionotropic glutamate receptors (AMPA, NMDA and kainate) and metabotropic glutamate receptors postsynaptically and presynaptically (Hornby, 2001, Ritter, 2011). Postsynaptic excitation of second order neurons in the NTS is mediated mostly by AMPA or kainate receptors, with a smaller contribution by NMDA receptors (Hornby, 2001, Ritter, 2011). Immunohistochemical studies show 60-70% of gastric distension-activated second order neurons in the NTS express NMDA and AMPA receptors (Berthoud and Neuhuber, 2000, Berthoud et al., 2001a). Several other transmitter substances expressed by subsets of vagal afferents for which their functional role, if any, is currently unknown. These include substance P, cholecystokinin (CCK), gastrin, SOM, VIP, 5HT, dopamine, NPY, CGRP and cocaine and amphetamine regulated transcript (Leslie, 1985, Dockray and Sharkey, 1986, Kummer et al., 1993, Ichikawa and Helke, 1997, Zhuo et al., 1997, Broberger et al., 1999).

Vagal peripheral endings

Types of vagal afferent neurons in the gut

Intraganglionic laminar endings

"Intraganglionic laminar endings" (IGLEs) are the peripheral terminals of vagal low-threshold mechanoreceptors in the oesophagus, stomach and small intestine. Unlike most vagal afferents, IGLEs have lightly myelinated Aδ fibres, and become unmyelinated at various distances from the myenteric ganglia (Rodrigo et al., 1975, Yu et al., 2005, Neuhuber et al., 2006). They are flattened, leaf-like structures – several of which may branch from a parent axon. IGLEs are embedded within the connective tissue sheath of myenteric ganglia and have been identified in histochemical, immunohistochemical and neuronal tracing studies in several species (Lawrentjew, 1929, cited in Zagorodnyuk and Brookes, 2000, Nonidez, 1946, Rodrigo et al., 1975, Christensen et al., 1987, Neuhuber, 1987, Yamamoto et al., 1994, Sang and Young, 1998, Zagorodnyuk and Brookes, 2000). The vagal IGLEs are particularly prominent in the upper digestive tract, supplying almost every ganglion in the oesophagus (Neuhuber et al., 1998), and about half of the gastric enteric ganglia (Berthoud et al., 1997). Their density becomes sparse distal to the mid duodenum (Berthoud et al., 1997, Wang and Powley, 2000).

In rat oesophagus, the calcium-binding proteins calretinin and calbindin are specific neurochemical markers of IGLEs (Kuramoto and Kuwano, 1994, Kressel and Radespiel-Tröger, 1999); these calcium-binding proteins are also expressed in other rapidly adapting mechanoreceptors (Duc et al., 1994). Another calcium binding protein, neurocalcin, is immunochemically detectable in IGLEs, but it has also been

detected in vagal efferent fibres (Iino et al., 1998). Puringeric $P2X_2$ and $P2X_3$ receptors are specific markers of IGLEs in rat and mouse. However, these markers do not reveal the entire population of IGLEs since they occur in only half the population of IGLEs revealed by neuronal tracing (Castelucci et al., 2003, Wang and Neuhuber, 2003, Raab and Neuhuber, 2005). The most comprehensive specific marker for IGLEs appears to the vesicular glutamate transporter 2 (VGluT2), which appears to label all neuronally-traced IGLEs in mouse oesophagus (Raab and Neuhuber, 2005). VGluT1 is also detectable in IGLEs, as well as in intrinsic myenteric neurons in the rat (Ewald et al., 2006). In guinea-pig, VGluT1, which is also detectable in oesophageal motor end-plates, labelled IGLEs more strongly than VGluT2 (Zagorodnyuk et al., 2003). Other low-threshold mechanosensory afferents in skin and muscle also express VGluT1 (Oliveira et al., 2003, Todd et al., 2003).

Combined neuronal tracing and ultrastructural tracing studies of IGLEs revealed some properties suggestive of an efferent function, including close appositions to myenteric neurons as well as vesicle and mitochondrial clustering in the terminal endings (Neuhuber, 1987). Immunohistochemical techniques have revealed that IGLEs possess some of the synaptic machinery necessary for vesicle-loading and exocytosis, including synaptophysin, synaptotagmin, vesicular glutamate transporters, and some may contain SP-loaded vesicles (Kressel and Radespiel-Tröger, 1999, Raab and Neuhuber, 2003, Zagorodnyuk et al., 2003). Despite these indications, excitation mapping studies using Fos immunohistochemistry shows vagal afferent stimulation has no substantial excitatory effect on enteric neurons (Zheng et al., 1997a, Zheng and Berthoud, 2000).

Numerous electrophysiological recordings from low-threshold vagal mechanoreceptors had been made before morphological and electrophysiological techniques were combined to positively identify IGLEs as the low threshold mechanoreceptors (Zagorodnyuk and Brookes, 2000). This was achieved by use of rapid neuroanatomical tracing from nerve trunks from which vagal afferents had been recorded. These studies showed IGLEs corresponded to the mechanotransduction sites revealed by von Frey hair probing (Zagorodnyuk and Brookes, 2000, Zagorodnyuk et al., 2001). This was an unexpected result, as another morphologically specialized ending in the gut – the intramuscular array – was previously argued as the most likely candidate to be the low threshold mechanoreceptor based on anatomical considerations (Berthoud and Powley, 1992). The low-threshold vagal mechanoreceptors were the first to be physiologically characterized in extracellular recordings from vagal nerve trunks to the gut wall (Paintal, 1954, Iggo, 1955). Small, "physiological" amounts of gut distension, or focal compression of their transduction sites, were found to promptly increase their discharge rates (Paintal, 1954, Iggo, 1955). By analogy with Golgi tendon organs of skeletal muscles, they were described as behaving like slowly adapting "in-series" tension receptors given their robust activation by both passive distension and active contractions of the gut musculature (Iggo, 1955). Later, more tightly controlled in vitro studies confirmed a linear relationship between IGLE discharge and gut wall tension (Zagorodnyuk et al., 2003). This is interesting because the location of IGLEs is clearly parallel to the muscle layers. Furthermore, although IGLEs can be activated by chemical mediators like ATP (Page et al., 2002, Zagorodnyuk et al., 2003), they do not appear to be dependent upon chemical signalling from other cells for mechanotransduction (Zagorodnyuk et al., 2003). Thus, it is unknown exactly how

local physical forces relate to the mechanotransduction process in IGLEs. Proposed mechanisms include compression of the endings by the muscle layers, and shearing forces exerted by locally coupled muscle cells (Brookes et al., 2013). Also currently unknown is the identity of the mechanosensitive ion channels on IGLEs, although transient receptor potential (TRP) channels are a likely candidate (Zagorodnyuk et al., 2003). The low mechanical thresholds of IGLEs are suggestive of a role in signalling distension-evoked feelings of satiety, which is of major therapeutic interest in treating obesity, cachexia and other disorders that affect feeding behaviour. Neurotrophin-4 knock-out mice show massive reductions of IGLEs, and in support of a role for IGLEs in feeding behaviour, have larger meal sizes (Fox et al., 2001). Mice expressing the neurotrophin-4 coding sequence in place of the brain-derived neurotrophic factor sequence, or mice with reduced intestinal brain-derived neurotrophic factor expression, had substantially more IGLEs (Fan et al., 2000, Chi et al., 2004, Biddinger and Fox, 2014). Mice with more IGLEs had decreased meal sizes and were more sensitive to the satiating effects of CCK (Chi et al., 2004, Biddinger and Fox, 2014). Furthermore, body and diet composition affect IGLE signalling and mechanosensitivity through ghrelin and leptin release – probably another mechanism through which these hormones affect feeding in addition to their profound central and metabolic effects (Kentish et al., 2012, Kentish et al., 2013a, Kentish et al., 2013b).

Intramuscular afferents

As mentioned above, the intramuscular array (IMA) was proposed to be the vagal low threshold mechanoreceptor prior to the discovery it was in fact the IGLEs (Berthoud and Powley, 1992, Zagorodnyuk and Brookes, 2000). IMAs are terminal

arrangements of fine varicose fibres ("axonal telodendria") that run parallel to each other and adjacent muscle fibres (Berthoud and Powley, 1992). Their varicosities contain both small agranular and large granular vesicles (Powley et al., 2008). IMAs occur from oesophagus to the colon in the circular and longitudinal muscle layers, but are conspicuously concentrated in the fundus and around the major sphincters (Wang and Powley, 2000). An extensive association between IMAs with intramuscular interstitial cells of Cajal has been observed (ICC-IM; Berthoud and Powley, 1992); ultrastructural studies indicate synapse-like junctions occur between ICC and IMAs (Powley et al., 2008). On these grounds it is speculated that IMAs, ICC, smooth muscle cells and possibly extrinsic efferents form complexes that transduce mechanical stimuli (Powley and Phillips, 2011). However, there is currently no functional evidence that identifies the physiological role of vagal IMAs, but ICC have been shown to be mechanosensitive in stomach (Won et al., 2005). It is possible that the in vitro flat-sheet preparations employed to identify and characterize sensory neurons with punctate mechanotransduction sites (Zagorodnyuk and Brookes, 2000), may not be suited to the IMA given their relatively expansive endings, possible orientation preferences for activation and non-uniform distribution in the gut (Powley and Phillips, 2011). Studies of nodose and jugular sensory neurons were performed using tube preparations of the oesophagus (Yu et al., 2005). This work identified a functional class of vagal afferent that had a high distension threshold, lower firing rate than IGLEs, C-fibre conduction velocities, and a dynamic firing behaviour that spanned into the noxious range. Unlike IGLEs, these were capsaicin sensitive. Neurons were of either nodose or jugular origin and were dissociable by their SP content (present in jugular but not nodose afferents) and sensitivity to P2X receptor agonists (only nodose afferents were sensitive,

comparable to IGLEs; Yu et al., 2005). These nociceptor-like afferents have not yet been positively linked with a peripheral morphological structure but it is speculated they may have IMA endings (Brookes et al., 2013). Neuroanatomical work suggests the peptide-containing jugular afferents are largely restricted to the oesophagus (Hayakawa et al., 2011).

Web-like endings

Most recently, an anatomically distinct set of "web-like" endings traced from the nodose ganglia have been described in the subserosal layer of the gastric antrum, adjacent the gastric sling muscles (Powley et al., 2012, Powley et al., 2013). Morphologically, they appear to be expansive lamellar structures with looping or "honeycombed" terminal arborizations (individual afferents supplied an average $0.44mm^2$). No physiological data exists upon which to speculate a function for these putatively afferent neurons. Nevertheless, they have been speculated to be mechanoreceptors involved in reflex control of the lower oesophageal sphincter (Powley et al., 2012).

Mucosal afferents

Mucosal afferents are major chemical sensors in the gut and likely to play a prominent role in satiation, digestive regulation and emetic reflexes by sampling the nutrient composition, acid and osmotic load of ingested materials. Mucosal endings show mechanosensitivity to light stroking or probing of the mucosa but not to active contractions or gut distensions. Chemosensitivity among mucosal afferents may be activated via the 10-20 different classes of enteroendocrine cells that release a plethora of paracrine and endocrine mediators in response to luminal content.

Mucosal afferents have been morphologically identified in the rat oesophagus (Neuhuber, 1987, Dütsch et al., 1998, Kressel, 1998, Wank and Neuhuber, 2001), lower oesophageal sphincter (Clerc and Condamin, 1987), stomach (Neuhuber, 1987, Patterson et al., 2002, Patterson et al., 2003, Gautron et al., 2011, Powley et al., 2011), small intestine (Berthoud et al., 1995, Williams et al., 1997, Patterson et al., 2002, Patterson et al., 2003, Gautron et al., 2011, Powley et al., 2011), and distal colon (Wang and Powley, 2000). Endings of mucosal afferents ramify beneath epithelial cells, and may extend into the intestinal villi, but do not appear to enter the epithelial layer to directly sample contents in the lumen (Berthoud et al., 1995). Several distinct morphologies of mucosal endings have been identified. The cervical oesophagus is richly innervated by vagal mucosal afferent neurons (Neuhuber, 1987, Dütsch et al., 1998, Wank and Neuhuber, 2001). This corresponds to the uppermost 10mm of the oesophagus (Neuhuber et al., 2006). Here, mucosal finger-like endings occur, which label for calretinin and CGRP, or CGRP only (Kressel, 1998, Wank and Neuhuber, 2001). These small-calibre endings branch off a parent axon, giving a finger-like appearance. Several hundred of these endings were identified in the rat cervical oesophagus (Wank and Neuhuber, 2001). Exclusive to this region are "complex laminar" mucosal endings, which arise from the superior laryngeal nerve and have cell bodies in the jugular and petrosal ganglia. They have been identified with calretinin and calbindin immunohistochemistry (Kuramoto and Kuwano, 1994, Dütsch et al., 1998). Their central terminations in the NTS differ from IGLEs and IMAs, predominantly ending in the interstitial subnucleus (Wank and Neuhuber, 2001). Not unlike IGLEs in appearance, the endings have a spiny laminar morphology, spreading underneath epithelial cells with small varicose fibres that

project up in-between epithelial cells (Dütsch et al., 1998, Wank and Neuhuber, 2001). Vagal innervation of the mucosa significantly drops off in the thoracic oesophagus but is increased near the lower oesophageal sphincter (Wank and Neuhuber, 2001). Recently described vagal mucosal afferents in the small intestine include villus afferents, which supply plates of varicose endings to the apical tips of intestinal villi; crypt afferents, whose terminal varicose processes encircle intestinal glands or crypts; and antral gland afferents, which preferentially arborize along gastric antral glands (Powley et al., 2011). In the gastric mucosa, 30% of ghrelin cells were in close proximity to vagal afferents that express the voltage-gated sodium channel $NaV_{1.8}$; some of which appeared to make appositions (Gautron et al., 2011).

The first physiological investigations of vagal mucosal afferent neurons using electrophysiological methods were performed over 50 years ago (Iggo, 1957, Paintal, 1957). These vagal afferents signal the luminal presence of glucose, amino acids, lipids, bile salts and osmotic loads – much of which is detected at the duodenal mucosa (Mei, 1985, Blackshaw et al., 2007). The chemosensory cells of the mucosal epithelium directly sample the luminal environment with a vast array of receptors (many GPCRs) for nutrient sensing (Steinert and Beglinger, 2011). The functional role of these cells is reminiscent of primary sensory cells in taste buds of the oral epithelium and so has led to the common referral of these cells as “taste” cells (Blackshaw et al., 2007). Indeed many of the taste signalling mechanisms that operate in the oral epithelium also occur in the mucosal epithelium (Steinert and Beglinger, 2011). There are multiple types of chemosensory cells, differentially distributed along the gastrointestinal tract, each with their own receptive and

hormonal release capabilities. Nutrient reception evokes exocytotic release of signalling molecules from the basolateral membrane onto adjacent nerve endings.

Mediators (mostly peptides) released from the mucosal epithelium may act both locally on vagal mucosal afferent terminals and systemically as true hormones or neuroendocrine agents. Local concentrations at vagal afferent terminals are likely to be orders of magnitude higher than those in the blood. Some of the mediators include gastrin, ghrelin, histamine, gastrin releasing peptide, secretin, CCK, glucose-dependent insulinotropin polypeptide, neurotensin, glucagon-like peptides, peptide YY, somatostatin and 5HT (Steinert and Beglinger, 2011). Taking CCK as an example; CCK has been shown to activate vagal mucosal afferents (Blackshaw and Grundy, 1990). CCK is contained within I cells of the mucosal epithelium, which are located close to vagal mucosal afferents (Berthoud and Patterson, 1996). CCK release from epithelial cells is evoked by luminal amino acids and long-chain fatty acids, activating vagal mucosal afferents via the CCK_A receptor (Eastwood et al., 1998, Lal et al., 2001). CCK receptor agonists and antagonists, reduce and increase short term food intake, respectively, and suppression of food intake by CCK is blocked by ablating the vagal afferent neurons either surgically or by neonatal treatment with capsaicin (Gibbs et al., 1973, Smith et al., 1985, Ritter et al., 1986, Hewson et al., 1988). There appears to be differential sensitivity to chemosensory cell mediators among vagal mucosal afferents, indicating there may be different populations of vagal mucosal afferents (Brookes et al., 2013). If possible, paired morphological and physiological characterization of these afferents will be necessary to dissociate any functional subtypes of mucosal afferents. Given their role in satiation and digestive regulation, such a characterization may be of significant therapeutic interest across a range of pathologies.

Muscular-mucosal afferents

A single study has been published on ferret vagal muscular-mucosal afferents (Page and Blackshaw, 1998). The only defining feature of these afferents established so far is their dual tension-mucosal mechanosensitivity. They respond to contractions of the musculature, producing burst-like patterns when contractions are rhythmic. In addition, they are sensitive to mucosal stroking and probing (Brierley et al., 2012). One interpretation is that these afferents may have dual transduction sites in smooth muscle (possibly muscularis mucosae) as well as mucosa. However, the anatomical identity of these afferents has not been positively identified.

Spinal afferent neurons

Spinal sensory neurons convey information about the physical and chemical environment from the periphery to the central nervous system. The great majority of spinal sensory neurons innervate the rest of the body; the visceral sensory innervation probably accounts for fewer than 5% of the body's dorsal root ganglion (DRG) sensory neurons (Sengupta and Gebhart, 1994b). Spinal afferent neurons are pseudo-unipolar neurons with cell bodies located in the neural crest-derived DRG, which lie in the dorsal roots that enter each vertebral segment. Peripheral processes of spinal afferents to the gut travel in two major nerve pathways: splanchnic nerves supply most of the gut from lower oesophagus to the rectum; pelvic nerves innervate a more restricted region from distal colon to internal anal sphincter. The oesophagus also has a supply of spinal afferents arising from the thoracic spinal nerves. In contrast to the vagus, spinal nerves to the gut are mostly composed of efferent fibres. About 10-20% of the fibres in splanchnic nerve pathways are afferent while the

pelvic nerve has about 30-50% afferent fibres (Grundy and Scratcherd, 1989). The quality of the sensory information carried by these pathways is likely to be different due to the types of sensory neurons they carry. Splanchnic nerves carry high-threshold mechanoreceptors (Brierley et al., 2004, Song et al., 2009). Pelvic nerves contain mechanosensory neurons with lower thresholds than those in splanchnic pathways (Brierley et al., 2004). Splanchnic nerve section can abolish pain perception in humans, suggesting it is a major visceral pain pathway (Bentley and Smithwick, 1940). Pelvic nerves are somewhat similar to the vagus as they probably contribute to non-noxious sensation during normal gut processes, such as defecation (Lembo et al., 1994, Yamanouchi et al., 2002). Nevertheless, afferents with both low- and high mechanical thresholds occur in the pelvic nerves which suggest the pathway may also carry nociceptive information (White, 1943, Zagorodnyuk et al., 2011, Feng et al., 2012).

Peripheral pathways

Splanchnic and pelvic nerve pathways contain mixed populations of afferent and efferent fibres. Splanchnic afferents travel alongside sympathetic efferent fibres and traverse paravertebral and prevertebral ganglia en route to the gut, giving off collateral projections in the prevertebral ganglia. Distal to prevertebral ganglia are mesenteric nerves which contain spinal sensory, postganglionic sympathetic and enteric viscerofugal axons. Pelvic afferents traverse the pelvic ganglia en route to the gut and travel in the same nerve bundles as preganglionic and postganglionic parasympathetic nerves, as well as enteric rectospinal neurons. Distal to the pelvic ganglia are the rectal nerves which additionally contain some enteric viscerofugal axons. The extrinsic afferent pathways from the gut are summarized in **figure 1.02**.

Extrinsic afferent pathways from the gut

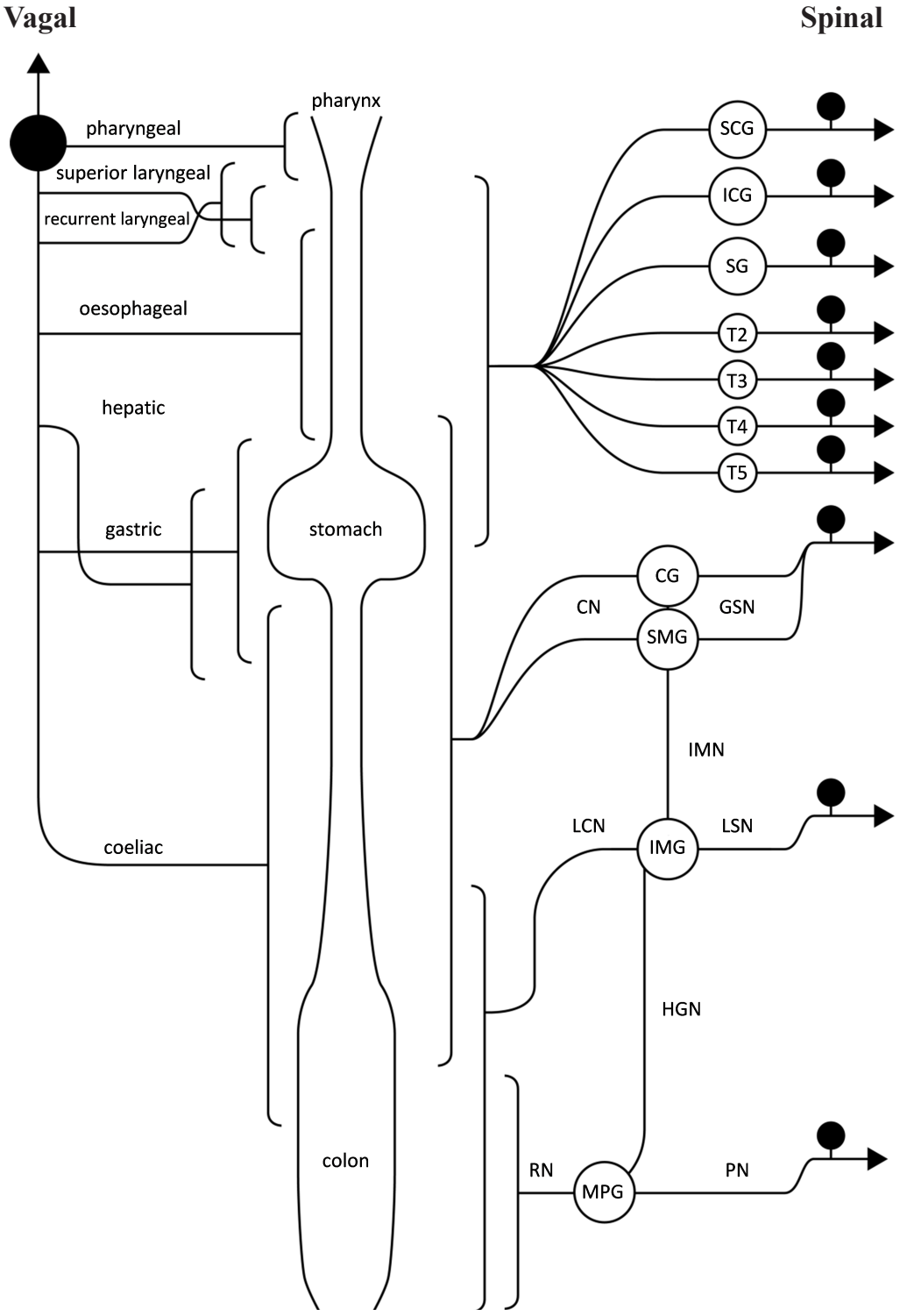

Figure 1.02 - see next page for legend

Figure 1.02

The extrinsic sensory innervation of the gastrointestinal tract. This schematic diagram shows the approximate range of spinal and vagal sensory afferent innervation along the gut. This figure approximates the arrangement of the extrinsic innervation in the most widely studied mammals, including rat, mouse, and guinea pig. The vagal afferent innervation, shown on the left, supplies the gut from the pharynx to the proximal colon. The spinal innervation ranges from the oesophagus to rectum. Note that most regions receive a dual afferent innervation. The upper gut receives both vagal and spinal innervation, while the distal colon and rectum receive a dual thoracolumbar (splanchnic nerve) supply and lumbosacral (pelvic nerve) supply. Abbreviations: CG – coeliac ganglion; CN – coeliac nerve; GSN – greater splanchnic nerve; HGN – hypogastric nerve; ICG – intermediate cervical ganglia; IMG – inferior mesenteric ganglia; IMN – intermesenteric nerve; LCN – lumbar colonic nerve; LSN – lesser splanchnic nerve; MPG – major pelvic ganglia; PN – pelvic nerve; RN – rectal nerve; SCG – superior cervical ganglia; SG – stellate ganglia; SMG – superior mesenteric ganglia. This figure adapted from Beyak and colleagues (2006).

Dorsal root ganglia

The spinal afferent supply to any given region of gut arises from the DRG of several segments. The range of segments overlap substantially such that a single DRG probably supplies sensory innervation to multiple regions of gut (in addition to other visceral organs and somatic structures; Brierley et al., 2012). The rostrocaudal range of DRG that supply the gut is loosely viscerotopic: rostral DRG tend to supply more proximal regions, whereas more caudal DRG supply distal regions. A region of gut typically has one or two "peaks" in the distribution of their spinal afferent supply. That is, it gets a preferential supply of nerves arising from one of a few DRG. Distal colon, for example, receives a spinal afferent innervation arising from mid thoracic to lower sacral DRG. However, neuroanatomical tracing demonstrates clearly two peaks in the distribution of spinal afferents; one arising from thoracolumbar DRG, and another from lumbosacral DRG (Vizzard et al., 2000, Robinson et al., 2004). These reflect the nerve supply from splanchnic and pelvic pathways, respectively.

Central projections of spinal afferent neurons

Axons of spinal afferents enter the spinal cord, synapsing on populations of second order neurons. The primary pathway is through the dorsal root to the ipsilateral dorsal horn of the spinal cord via Lissauer's tract (Cervero and Connell, 1984a, b, Morgan et al., 1986b), although some visceral afferents enter through the ventral roots (Grundy and Scratcherd, 1989). In the dorsal horn, spinal visceral afferent collaterals project medially and laterally, surrounding spinal neurons in laminae I and V, and to a lesser extent laminae VII and X (Cervero and Connell, 1984a, b, Morgan et al., 1986b, Sugiura et al., 1989). Some fibres cross the midline, terminating in the contralateral gray matter (Sugiura et al., 1989).

Consistent with their role in parasympathetic reflexes, pelvic afferents have prominent projections into the sacral parasympathetic nucleus, coursing laterally into laminae V and VI, and medially to the dorsal commissure and dorsal columns (de Groat et al., 1981, Morgan et al., 1981). Splanchnic visceral afferents provide input to short intraspinal and supraspinal circuits that modify sympathetic preganglionic output, forming the basis of spinal sympathetic reflexes (see "visceral motility-regulating preganglionic reflexes", above). Additionally, splanchnic inputs ascending to the brainstem may interact with the parasympathetic nervous system through projections into the dorsal motor nucleus of the vagus (Grundy and Scratcherd, 1989).

The central terminal arborizations formed by visceral afferents are diffuse in comparison with the relatively specific terminations of somatic afferents and may project rostrocaudally up to six vertebral segments upon entering the spinal cord (Sugiura et al., 1989). Many second order neurons receive converging inputs from both visceral and somatic afferents (Cervero and Tattersall, 1987), or multiple visceral organs (Brumovsky and Gebhart, 2010). These properties probably underlie the characteristic poor localization of visceral sensation, its common referral to somatic structures, and the phenomenon of cross-sensitization between organs. Nevertheless, some spinal neurons in lamina X appear to receive just visceral input (Honda, 1985).

Visceral afferent pathways ascending in the spinal cord via several contralateral pathways in the ventrolateral, lateral, and dorsolateral white matter, and via a

putatively nociceptive ipsilateral pathway in the dorsal column which decussates in medullary nuclei en route to the thalamus (Willis et al., 1999). The contralateral pathways include the spinothalamic, spinohypothalamic, spinoreticular, spinomesencephalic/parabrachial and spinosolitary tracts. Higher brain regions process visceral information, possibly evoking conscious sensations or modulating affect. These include somatosensory cortex, insula and cingulate gyrus (Van Oudenhove et al., 2007, Knowles and Aziz, 2009).

Glutamate

The most heavily implicated neurotransmitter candidate for spinal afferent neurons generally, including gastrointestinal afferent neurons in the dorsal horn, is glutamate. Glutamate is the most abundant neurotransmitter in the central nervous system (Broman et al., 2003) and depolarizes many spinal neurons (Curtis and Watkins, 1960). Glutamate is released into the spinal cord in response to primary afferent electrical stimulation (Willis Jr and Coggeshall, 2004). Electrical stimulation of primary afferents evoke glutamatergic excitatory post synaptic potentials in the dorsal horn, particularly in the superficial layers (Schneider and Perl, 1988). Likewise, glutamate immunoreactivity is abundant throughout the dorsal horn, especially in the superficial laminae (Broman et al., 1993, Valtschanoff et al., 1994), and often co-localizes with primary afferent neuron peptide markers CGRP and SP (Merighi et al., 1991). But glutamate is a ubiquitous molecule, involved in multiple cellular processes, and is also a precursor for GABA, which is also a prominent neurotransmitter in the spinal cord. More specific markers of glutamatergic neurons are the proteins required for loading glutamate into vesicles which includes vesicular VGluT1 (Ni et al., 1994), VGluT2 (Aihara et al., 2000), and VGluT3 (Gras et al.,

2002). These proteins appear differentially expressed among primary afferents. VGluT1 immunoreactivity is commonly found in larger diameter afferents and is distributed throughout the dorsal horn (Todd et al., 2003). VGluT2 immunoreactivity is prominent in small diameter (often peptidergic) afferents and is most abundant in the superficial laminae (Todd et al., 2003, Brumovsky et al., 2007). VGluT3 expression in the dorsal horn is relatively sparse (Morris et al., 2005), but it is expressed in DRG by a subset of unmyelinated primary afferents (Seal et al., 2009). Some primary afferents appear to express little or no detectable VGLUT proteins (Todd et al., 2003, Morris et al., 2005), indicating there may be non-glutamatergic primary afferent neurons or an unknown glutamate transporter proteins is employed. Additionally, the glutamatergic phenotype may be dynamically regulated by age, inflammation and injury (Brumovsky et al., 2007, El Mestikawy et al., 2011).

Glutamate and spinal afferent neurons to the gut

Neuroanatomical studies in mouse suggest that almost all colorectal afferents in pelvic and splanchnic pathways express VGluT2 mRNA and protein in DRG (Brumovsky et al., 2011). VGluT2 is also prominent in colorectal afferent peripheral endings (Spencer et al., 2008, Brumovsky et al., 2011), but this does not seem to be the case in guinea pig (Olsson et al., 2004). VGluT1 is also expressed by a small population of medium to large sized colorectal afferents in mouse (Brumovsky et al., 2011). Functional studies show that increasing synaptic clearance of glutamate by overexpression of glial excitatory amino acid transporter 2 produces substantial reductions in pain behaviours evoked by colorectal distensions (Lin et al., 2009). That glutamate is released from gastrointestinal afferents in the dorsal horn is supported by electrophysiology and activation-mapping studies using Fos

immunohistochemistry. These studies strongly implicate the ionotropic AMPA, NMDA, and kainate glutamate receptors in activation of dorsal horn neurons by innocuous and noxious distensions of the colorectum (Zhai and Traub, 1999, Kozlowski et al., 2000, Ji and Traub, 2001, Ji and Traub, 2002, Traub et al., 2002), and splanchnic nerve stimulation (Laird et al., 1995). NMDA receptors appear more important in signalling noxious distensions, and make a larger contribution during inflammation (Zhai and Traub, 1999), although capsaicin-induced colonic hyperalgesia also rapidly recruits new AMPA receptor subunits to spinal neuron cell membranes, potentiating their responses (Galan et al., 2004). Metabotropic glutamate receptors are present in the dorsal horn on spinal neurons and presynaptically on primary afferent terminals (Willis Jr and Coggeshall, 2004). All subtypes of metabotropic glutamate receptors occur in DRG (Carlton and Hargett, 2007). Metabotropic glutamate receptors may potentiate ionotropic glutamate receptor activation, and modulate somatic nociceptive processing at various levels in the central nervous system, including the dorsal horn, but a specific role in central gastrointestinal afferent signalling remains to be determined (Willis Jr and Coggeshall, 2004, Blackshaw et al., 2011). Functional evidence indicates a peripheral role for metabotropic glutamate receptor, mGluR5. Antagonists of this receptor substantially reduced colorectal distension-induced pain behaviour via reduced mechanosensory signalling of colonic afferent neurons, including pelvic muscular and muscular-mucosal afferents, as well as splanchnic vascular afferents (Lindstrom et al., 2008). Additional analgesic actions of mGluR5 antagonism in the central nervous system cannot be ruled out from these studies (Blackshaw et al., 2011). Overall, the available data strongly supports a role for glutamate in

gastrointestinal sensory afferent signalling in the spinal cord but the types of neurons involved in glutamatergic transmission are yet to be positively identified.

Neuropeptide content

Most visceral afferent neurons contain one or more neuropeptides (Molander et al., 1987). The most prevalent among the spinal afferents to the gut are CGRP and SP, which often co-exist in the putatively nociceptive vascular afferent neurons (described below). Both these peptides are implicated in visceral pain transmission in the spinal cord, in addition to neurotransmitter, inflammatory and vasodilator functions in the periphery. Other peptides identified in various types of primary afferent neurons include neurokinin A, neurokinin B, VIP, NT, SOM, GAL, bombesin, ENK, dynorphin, endomorphins, CCK, TRH, CRF, PACAP and amylin (Willis Jr and Coggeshall, 2004).

Substance P is loaded into dense core vesicles that are transported from the cell body to the central terminals of many small and medium sized spinal afferents. Exogenous SP evokes prolonged depolarization of nociceptive dorsal horn neurons (Salter and Henry, 1991). Endogenous SP release, evoked by dorsal root stimulation, elicits a neurokinin 1 receptor (NK_1R) mediated increase in Fos expression (Badie-Mahdavi et al., 2001). NK_1R antagonists acting centrally attenuate visceromotor pain responses to noxious colonic distension (Okano et al., 2002). NK1R gene knockout mice also have diminished pain behaviours to noxious colonic distensions and intracolonic capsaicin treatment, but normal pain responses to mustard oil (Laird et al., 2000). Additionally, NK_2Rs partially mediate rectal distension-evoked

visceromotor responses (Julia et al., 1994), and contribute to transmission of mechanical hyperalgesia after colonic inflammation (Laird et al., 2001).

CGRP is contained within small- and medium-sized spinal afferent neurons, often colocalizing with SP and loaded in the same dense core vesicles both centrally and peripherally (Gulbenkian et al., 1986, Merighi et al., 1988). In contrast to nodose vagal afferents, most spinal visceral afferent cell bodies contain CGRP (Sternini and Anderson, 1992, Dütsch et al., 1998, Robinson et al., 2004, Brumovsky et al., 2011). In the dorsal horn, CGRP is largely restricted to primary afferent terminals (Gibson et al., 1984, Chung et al., 1988, Traub et al., 1989a, Traub et al., 1989b), and its release can be evoked by capsaicin stimulation in vivo, and in spinal cord slices in vitro (Saria et al., 1986, Diez Guerra et al., 1988). Several neuromodulatory effects may be exerted by CGRP in the dorsal horn, including inhibition of SP degradation, increased presynaptic calcium influx, potentiation of SP release, potentiation of the postsynaptic depolarizing effects of SP and prolonged depolarization of dorsal horn neuron. Supporting a functional role for CGRP in visceral nociception, CGRP receptor antagonists in the spinal cord reversed inflammation-induced mechanical hyperalgesia (Plourde et al., 1997).

Interestingly, immunohistochemistry of peptide-containing afferent neurons in the dorsal horn shows that most lack the protein machinery required for fast exocytotic release of glutamate (Morris et al., 2005). Large, peptide-containing dense-cored vesicles may lack or have a lesser dependence upon soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins for exocytosis (Burgoyne and Morgan, 2003) and might therefore contribute the bulk of signalling

in these afferents. However, the relative contributions of peptide and glutamate release in peptidergic afferents is currently not known and is probably dynamically regulated. It is not currently possible to determine experimentally if glutamate is released specifically from the peptidergic neuron terminals into the dorsal horn. This is of considerable interest in understanding visceral pain, given most visceral afferents are peptidergic.

Types of spinal afferent neurons

Thoraco-lumbar & sacral vascular afferents

Vascular afferent neurons are a putatively nociceptive afferent neuron with a high threshold of activation to gut distension. In thoraco-lumbar pathways they comprise the majority (>85%) of afferents, and in sacral pathways about one third of afferents (Brierley et al., 2004). They have receptors to a wide array of endogenous mediators, and can exert efferent-like effects via peptides release from their peripheral endings onto the blood vessels, and onto neurons in the gut. These neurons have also been referred to as “serosal” or “mesenteric” afferent neurons, based on early suggestion about their anatomical location (Leek, 1977). Neuroanatomical tracing reveal that they are peri-vascular in location (Song et al., 2009), thus the terms “serosal” and “mesenteric” are misnomers. Initially described in recordings from splanchnic nerves to the mesentery, they were found to be sensitive to mechanical probing near the branch points of blood vessels, to large contractions or distensions of the gut, and to traction of the mesentery (Bessou and Perl, 1966). They have fine, branching axons that run along mesenteric arterioles taking similar pathways to sympathetic efferent neurons, with which they commonly overlap and intermingle (Westcott and Segal,

2013). Mesenteric arterioles continue into the gut wall in the submucosa, as do the vascular afferents which supply them. A single vascular afferent may have transduction sites on the blood vessels of both mesentery and the submucosa (Song et al., 2009). Splanchnic, but not pelvic, vascular afferents have endings in the mesentery (Brierley et al., 2004). This is most likely due to the absence of mesenteric vessels in the rectum. In the colon and rectum, the transduction sites of thoracolumbar vascular afferent tend to densely population the region near the mesenteric attachment. However, those originating from pelvic nerves are more evenly distributed (Berthoud et al., 2001b, Brierley et al., 2004). Axon collaterals of vascular afferents have been traced into the myenteric and submucosal ganglia, as well as the mucosa and outer muscle layers (Lynn et al., 2003, Song et al., 2009). Axon collaterals have not been implicated in mechanoreception but may initiate motor activity by the release of tachykinins onto enteric neurons (Barthó et al., 1982, Takaki and Nakayama, 1989, 1990). In general, the distribution of vascular afferents follows their sympathetic innervation. They are present in other visceral organs including the pancreas, spleen, kidneys, bladder and ovaries, and are probably ubiquitous throughout the body (Floyd and Morrison, 1974, Leek, 1977, Longhurst et al., 1984, Longhurst and Dittman, 1987, Song et al., 2009).

Electrophysiological recordings from vascular afferents have been made throughout the gastrointestinal tract and the mesenteries of several species (Brierley et al., 2012, Brookes et al., 2013). Compared to low-threshold afferents, they have a low ongoing firing rate (<1Hz) and increases in firing rate evoked by distension are modest (Song et al., 2009). Single neurons typically have multiple punctate receptive fields (Bessou and Perl, 1966, Morrison, 1973, Blumberg et al., 1983, Sengupta et al., 1990, Lynn

and Blackshaw, 1999, Berthoud et al., 2001b, Song et al., 2009). Large "noxious" distensions or probing with relatively stiff von Frey hairs are necessary for their activation. They are not sensitive to low levels of distension or mucosal stroking (Brierley et al., 2012). Their mechanosensitivity differs according to location; thresholds in the submucosa are lower than those in the mesentery (Song et al., 2009). In the relatively uncompliant mouse rectum, large forces (generated by loads over 10g) are required for activation (Brierley et al., 2008, Brierley et al., 2009, Hughes et al., 2009, Zagorodnyuk et al., 2012). In mouse, mechanotransduction may partly mediated or modulated by the TRPA1 channel; TRPA1 gene knockout or channel blockade significantly attenuates their mechanosensitivity, as well as bradykinin-evoked hypersensitivity (Brierley et al., 2009). Importantly, TRPA1 is implicated in behavioural measures of pain, such as the visceromotor response (Cattaruzza et al., 2010). Another TRP channel, TRPV4, is detectable on vascular afferents and knockout or antagonists reduce both mechanosensitivity and visceromotor responses to colorectal distension. It also reduces sensitization evoked by proteases (Brierley et al., 2008). TRPV1 is the best characterized of the TRP channels on sensory neurons and is expressed by vascular afferent neurons, conferring capsaicin sensitivity (Longhurst et al., 1984). Large doses of capsaicin dramatically attenuate mechanosensitivity in thoracolumbar vascular afferents (Brierley et al., 2005); the decreased mechanosensitivity caused by capsaicin apparently depended on concomitant TRPA1 expression by these neurons (Brierley et al., 2009). A plethora of other receptors for endogenous chemical mediators are expressed by vascular afferents. These mediators include but are not limited to ATP, 5HT, bradykinin, glutamate, histamine, nerve growth factor, proteases, cytokines, protons and prostaglandins (Brookes et al., 2013). Many of the immune-associated

mediators can evoke long-term changes in their excitability, particularly after "recovery" from gastrointestinal inflammation (Hughes et al., 2009).

Efferent functions & sympatho-sensory interactions of vascular afferents

Vascular afferent neurons synthesize and transport tachykinin peptides, including SP and CGRP, to their peripheral endings. Detected immunohistochemically, these peptides may be used to identify extrinsic afferent neurons (Gibbins et al., 1985, Brookes et al., 2013). CGRP acting on its cognate GPCRs causes relaxation of vascular smooth muscle cells, while SP acts on vascular endothelial cells to evoke nitric oxide release and alteration of endothelial cell structure, increasing vascular permeability (Westcott and Segal, 2013). Strong activation of vascular afferents evokes release of these peptides onto blood vessels, causing vasodilation and increased vascular permeability (Brunsden et al., 2002, Holzer, 2006). Localized oedema may result, and neurokinin receptors located on peripheral afferent terminals may be activated, further stimulating peptide release in a positive feedback cycle. This "neurogenic inflammation" mechanism occurs throughout the body to enhance blood perfusion of a potentially damaged region. This occurs in the gut; for example, reflux of gastric acid into unprotected regions causes local vasodilation via activation of TRPV1 receptors on vascular afferents (Holzer, 2006). The ability of vascular afferents to increase vascular permeability varies significantly between gut regions. For example, capsaicin-induced vascular permeability is more pronounced in vessels supplying the small intestine compared to stomach and colon (Sann et al., 1996). The functional effect on the vasculature by afferent neurons is clearly antagonistic to the vasoconstrictor role of sympathetic efferents. In addition to their indirect opposing effects, CGRP from afferents prejunctionally inhibits sympathetic neurons and

diminishes NA release, while common sympathetic transmitters NA, ATP or NPY can impair CGRP release and afferent-mediated vasodilation (Westcott and Segal, 2013). However, while NA may be inhibitory under normal conditions, in circumstances of acute and chronic tissue damage or inflammation, NA can gain an excitatory effect on afferent neurons and may underlie some acute and chronic pain conditions (Pertovaara, 2006).

Thoraco-lumbar & sacral mucosal afferents

As with vagal mucosal afferents, spinal mucosal afferents are defined by a lack of sensitivity to distension stimuli, but they may be activated by mucosal stroking or probing (Lynn and Blackshaw, 1999, Brierley et al., 2004). Spinal mucosal afferents are distributed throughout the gut. However, detailed accounts of their morphology and distribution are lacking. In the upper digestive tract, spinal afferents with cell bodies in the cervical and upper thoracic DRG innervate the oesophageal mucosa with a relatively even distribution along its length compared to the vagal mucosal innervation (Dütsch et al., 1998). Almost all of these contain CGRP, indeed about 90% of spinal afferents in rodent oesophagus contain CGRP (Green and Dockray, 1987, Uddman et al., 1995, Dütsch et al., 1998). Their peripheral endings were described as fine, branching, varicose fibres (Dütsch et al., 1998). In the lower gut, unpublished neuroanatomical tracing of rectal nerve trunks has revealed fine varicose branching afferents in the subepithelial plexus but positive identification in physiological mapping studies are pending (Brookes et al., 2013). More mucosal afferents exist in pelvic than splanchnic pathways (Brierley et al., 2004). Details of the chemosensory and mechanosensory roles of either population are sparse. In populations of splanchnic mucosal afferents, serotonin is a potent activator through a

combination of $5HT_3$ receptors and non-$5HT_3$ receptors (Hicks et al., 2002). Early electrophysiological studies also suggested some of these afferents may be glucose-sensitive (Sharma and Nasset, 1962, Perrin et al., 1981, Mei et al., 1984) and that blocking the transport of hexoses (including glucose) into epithelial cells abolishes the glucose-evoked firing (Hardcastle et al., 1978). The physiological role of spinal mucosal afferents (particularly pelvic afferents) is speculated to be involved in detection of consistency and/or shearing forces in the hindgut associated with stool passage (Brierley et al., 2012).

Rectal intraganglionic laminar endings

Electrophysiological studies of pelvic nerve pathways have indicated low-threshold tension receptors supply the rectum of the cat, guinea pig, rat and mouse with similar properties to those encountered in the upper digestive tract (Janig and Koltzenburg, 1991, Sengupta and Gebhart, 1994a, Lynn et al., 2003, Brierley et al., 2004). Combined physiological mapping of mechanical transduction sites and neuroanatomical tracing revealed the endings of afferent neurons as rectal intraganglionic laminar endings (Lynn et al., 2003). These endings are similar to those in vagal pathways in morphology with flattened leaf-like terminals in myenteric ganglia, but simpler with less terminal branching (Lynn et al., 2003). There is a rich supply of rectal IGLEs in the rectum, but the density of innervation diminishes substantially in the distal colon (Olsson et al., 2004). Rectal IGLEs are activated by focal mechanical probing, active contractions of both muscle layers and low levels of distension, but continue to encode distension into the noxious range (Lynn et al., 2005, Zagorodnyuk et al., 2011). Detailed studies of their mechanosensitivity indicate they directly transduce mechanical stimuli most likely

through TRP channels and are activated when their endings in myenteric ganglia are physically distorted or compressed by local muscle contractions (Zagorodnyuk et al., 2005).

Intramuscular arrays

Longitudinal and circumferential IMAs similar to those described in the vagus occur in colon, rectum and internal anal sphincter (Lynn et al., 2003, Olsson et al., 2004, Lynn and Brookes, 2011). So far only circumferentially-orientated IMAs in the internal anal sphincter originating from the pelvic nerve have been associated with a functional correlate. These were identified as slowly-adapting low threshold mechanoreceptors, sensitive to circumferential distension and focal tissue compression (Lynn and Brookes, 2011).

Muscular-mucosal afferent neurons

A population of muscular mucosal afferents travelling in pelvic pathways has been recorded in mouse (Brierley et al., 2004, Zagorodnyuk et al., 2011, Zagorodnyuk et al., 2012). Most have receptive fields distributed evenly throughout in the terminal 10mm of the rectum. In the absence of an anatomical identification, the term "muscular-mucosal" has been applied due to their combined mechanosensitivity to light mucosal stroking and distension of the muscle wall. In vitro studies of their activity during spontaneous and evoked propagating contractions through the colon and rectum show they are typically quiescent but discharge during contractions (Zagorodnyuk et al., 2012). Strong contractions of the gut wall by evoked bethanechol also activate these neurons, indicative of an in-series type behaviour typical of tension receptors (Zagorodnyuk et al., 2012). These afferents have been

referred to as “wide-dynamic range” to denote their ability to fire at low thresholds and continue firing more rapidly well into the range of noxious distensions, indeed, until the tissue tears (Sengupta et al., 1990, Zagorodnyuk et al., 2011, Zagorodnyuk et al., 2012). Interestingly, in the endothelin-3 knockout mouse, the discharge rate of this type of neuron is significantly lower without changes in activation threshold. This coincided with significantly reduced visceromotor response to colorectal distension, a putative indicator of pain (Zagorodnyuk et al., 2011).

The enteric nervous system

Structure

The gut is a muscular tube, contiguous with the external environment, which extends from mouth to anus with enlargements for storage. The wall of the gut has distinct tissue layers. Innermost is the mucosal lining which is a layer that is a single cell thick in parts and separates the external and internal environments. The mucosal surface may be arranged into crypts (large intestine) or crypts and villi (small intestine) which are lined with exocrine cells. These include enterocytes and goblet cells which secrete their contents (water, electrolytes, mucous) into the lumen, as well as endocrine cells that secrete hormones largely from their basolateral surfaces. The mucosal layer is characterized by a high rate of turnover, with new cells being continually generated from stem cells located at the base of the crypts. Overlying the mucosa is the loose connective tissue of the lamina propria, containing nerves (mucosal pleuxus, see **figure 1.03**), blood vessels, lymphatics, and the muscularis mucosa, a thin smooth muscle layer. A fibrous layer of connective tissue containing the well vascularised submucous plexus of enteric neurons (or Meissner’s plexus)

lies between muscularis mucosa and the thick circular muscle layer. Embedded between the circular muscle and outer longitudinal muscle layer is the myenteric plexus of enteric neurons (or Auerbach's plexus). Outermost is the thin serosal membrane that wraps around the gut wall. Many of the layers are illustrated in **figure 1.03**.

Layers of the gut wall

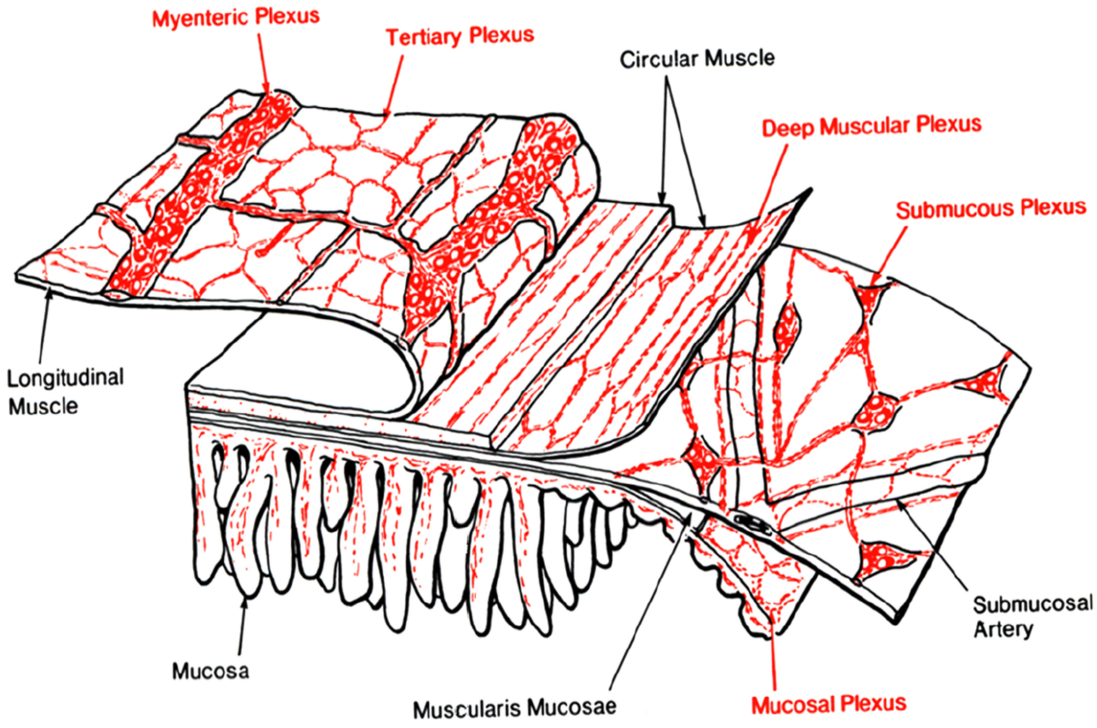

Figure 1.03

The layers of the gut wall. This figure shows the enteric nervous system within several distinct layers of the small intestine. Neural elements are labelled in red, non-neural structures are labelled in black. The myenteric plexus is located between the inner circular and outer longitudinal muscle layers. These layers are innervated via the deep muscular plexus and tertiary plexus, respectively. The submucous plexus is located on the inner side of the circular muscle and supplies much of the innervation to the mucosal plexus and submucosal vasculature. This figure refers specifically to the guinea pig and was adapted from Furness and Costa (1980).

Gut Functions

Digestion causes the physical degradation of ingested material mechanically by the muscle wall, and chemically through various secreted substances. Smooth muscle exerts force upon luminal contents intermittently mixing, grinding and propelling content from the oral cavity to the anal canal. Secretions catalyse cleavage of ingested molecules, protect the gut wall and maintain water and electrolyte homeostasis. Motility, secretions and absorption of nutrients are coordinated by enteric and extrinsic neural circuits. Besides digestive functions, the gut has several other critical physiological functions. The gut is large endocrine organ. Taking into account the extensive posttranslational processing of peptide hormones, over a hundred different hormones are secreted from the gut (Rehfeld, 2012). These hormones act locally and systemically, and serve other functions, including neurotransmission or neuromodulation (Rehfeld, 2012). The immune function of the gut is substantial, containing within it about 70% of the body's immune cells (Schenk and Mueller, 2008). Immune cells of the intestinal epithelia provide an array of ongoing protective antimicrobial defences that prevent pathogens, ever present in food and water, from breaching the mucosal lining and entering the bloodstream. The commensal microbial colonization of the gut is currently a particularly exciting area of research with widespread implications for bodily health. A microbial biomass of over 1kg is present in the human gut, comprising 10 times as many cells as the host's body, and over 2,000 known different species of bacteria and fungi (Neish, 2009). The composition and metabolic activities of the microbiome is modified by nutrition, ageing and stress, among other factors (Flint et al., 2012). Microbial metabolism supplies an array of mediators to the gut ranging from beneficial organic acids to potentially carcinogenic nitrogenous metabolites and proinflammatory

liposaccharides (Flint et al., 2012). Downstream functional effects have been identified on gut motility, sensory transduction, stress reactivity and other emotional behaviours, by mechanisms that include changes in the composition and excitability of enteric and central neural circuits (Bravo et al., 2011, Forsythe and Kunze, 2013, Foster and McVey-Neufeld, 2013).

Organization

The enteric nervous system (ENS) is the third division of the autonomic nervous system defined by Langley (Langley, 1921). Quantitatively comparable to the spinal cord, the ENS is the largest division of the autonomic nervous system, comprising an estimated 100-200 million neurons in the human. All enteric neurons and their processes, with the exception of the viscerofugal neurons, are entirely confined to the gut wall. Enteric neurons are organized in the web-like form of ganglionated plexuses; many clusters of neuron cell bodies (ranging 5-200 in guinea pig ileum) interconnected by axon bundles (Meissner, 1857, Billroth, 1858, Auerbach, 1862, cited in Furness 2006). The interconnected myenteric plexus and submucous plexuses contain most of the neurons in the ENS. Some minor ganglia have been described in the mucosa and sub-serosa (Drasch, 1881, Schabadasch, 1930, Stöhr, 1934, cited in Furness 2006). The myenteric plexus is the larger plexus and spans the entire gut length, principally coordinating gut motor behaviours; the smaller submucous plexus exists from the distal stomach to the rectum and regulates vascular, secretory, and mucosal functions. In large animals including humans, the submucosal plexus can form 2-3 distinct interconnected layers (Porter et al., 1999, Timmermans et al., 2001); small mammals, including guinea pig, have a single layer. Non-neuronal glial cells resembling astrocytes interact with enteric neurons,

outnumbering neurons in the gut by up to ten-fold (Jessen and Mirsky, 1983). The ratio of glial cells to neurons becomes larger in larger animals; human myenteric ganglia contain on average 3.5-4 times the ratio of glial cell to neurons (Hoff et al., 2008). Sensory neurons, interneurons, and motor neurons exist in the ENS, forming neural circuits that underlie gut functions. Extensive interactions occur between enteric and extrinsic nerves, but enteric neural circuits can independently sample the physical and chemical environment and send processed output to effector muscle and secretory cells. This functional autonomy of the ENS was inferred over a hundred years ago upon the observation that gut reflex behaviours persisted after complete isolation from the central nervous system (Bayliss and Starling, 1899).

Ways to classify enteric neurons

Functional classes of enteric neurons (sensory neurons, interneurons and motor neurons) have been defined by morphology, axonal projections, specific histochemical and immunohistochemical characteristics, and electrophysiological properties. Guinea pig small intestine is the most studied and comprehensively characterized preparation to date, for a variety of practical and historical reasons (Costa et al., 1996, Costa and Brookes, 2008). Unless otherwise stated, descriptions of enteric neurons refer this preparation. Recently, it has become apparent that enteric neurons do not necessarily fit discretely into functional classification schemes. For example, some interneurons and motor neurons show properties of sensory neurons (Mazzuoli and Schemann, 2009), but classifications are useful for descriptive purposes.

Soma-dendritic morphology and axonal projections

Golgi staining (silver impregnation), intracellular dye filling, neuronal tracing and transgenic expression of fluorescent proteins have been used to reveal the morphology of enteric neurons. Typically, enteric neurons are simpler in their soma-dendritic morphology than many other peripheral and central neurons, indicative of lesser synaptic convergence. Early studies by Dogiel (1899, cited in Furness 2006) established the morphological classification scheme that is still widely used to describe enteric neurons. Up to 8 morphologies of enteric neurons have been described, but most studies in small mammals and rodents have fit cells into 2-3 basic classes: "simple" cells, Dogiel type I, and Dogiel type II. Simple cells have smooth, small cell bodies, a single axon and few or no short processes. Dogiel type I neurons have small to medium sized cell bodies, a single axon and lamellar dendrites, or less frequently filamentous dendrites. Dogiel type II neurons have large smooth cell bodies, multiple axonal processes and long filamentous dendrites.

Projections of enteric neurons revealed by intracellular dye-filling, retrograde neuroanatomical tracing and electrophysiological mapping techniques indicate that the majority of all classes of neurons have short axonal projections of only a few millimetres. Thus, enteric circuits operate over short distances and reflex behaviours persist in very short, gut preparations, in vitro. However, some neurons have been described which project relatively long distances, up to 10cm, down the gut (Brookes et al., 1995, Song et al., 1997).

Chemical Coding

Enteric neurons reliably express combinations of proteins and transmitter molecules that may be detected using histochemical and immunohistochemical techniques.

Consistency occurs across preparations, provided species and gut region are the same. Used in combination with previously known characteristics of enteric neurons including morphology and axonal projections, a virtually complete account of the "chemical coding" of enteric neuron cell bodies has been generated for the guinea pig small intestine. This includes 14 classes of enteric neurons in the myenteric plexus (Costa et al., 1996), and 4 classes in the submucosal plexus (Furness et al., 1984, Bornstein and Furness, 1988, Steele and Costa, 1990, Song et al., 1992). Cell body chemical coding is a useful identification tool, and has clarified the organization of enteric neurons. Recently, the chemical coding of the varicose terminals of enteric and extrinsic neurons has been investigated (Sharrad et al., 2013b). Interestingly, while chemical codes of cell bodies are suggestive, they do not necessarily indicate varicosity coding, limiting assumptions that can be made about functional transmitters based on cell body coding (Sharrad et al., 2013a).

Electrophysiology

Extracellular and intracellular electrophysiological recordings of single enteric neurons demonstrated that 2-3 major classes of enteric neurons could be defined by their electrophysiological properties. The most widely investigated of these are 'S-neurons' and 'AH-neurons' (Hirst et al., 1974, Bornstein et al., 1994). The "S" in S-neuron meant "synaptic", referring to the ongoing or stimulus evoked fast EPSPs they received. The property is no longer class-defining, but the nomenclature has persisted. S-neurons often fire repetitive action potentials that increase in frequency upon a depolarizing stimulus. They have relatively small resting membrane potentials, higher input resistance and lack of a prominent long after-hyperpolarization. Their action potentials are typically blocked by tetrodotoxin.

AH-neurons (after-hyperpolarizing) have more negative resting membrane potentials and lower input resistance. They fire one or a few action potentials at the onset of depolarization. A prominent shoulder on repolarization phase owing to calcium influx often occurs, typically followed by a period of prolonged hyperpolarization (1-20 seconds) caused by the increased intracellular calcium evoking increased conductance through intermediated conductance potassium channels (IK channels; Wood, 1989). The contribution of calcium to the action potential confers a capacity to fire in the presence of sodium channel blocker tetrodotoxin. Calcium substitution with other divalent cations, or specific blockade of the IK channels, increases AH neuron excitability and the number of action potentials discharged upon depolarization. AH-neurons generally do not receive fast EPSPs, but occasional small amplitude fast EPSPs have been reported (Iyer et al., 1988, Tamura et al., 2001).

Electrophysiological properties are dynamically regulated by channel conductances that can be altered by synaptic inputs, necessitating assessment of several properties before assigning a classification. Slow EPSPs for example may evoke cyclic adenosine monophosphate-mediated closure of IK channels in AH-neurons, reducing potassium efflux. As above, this attenuates long after-hyperpolarizations, increasing the likelihood of repetitive spike discharge while other inputs and mediators may evoke the opposing effect, dramatically reducing cell excitability (Wood, 1989).

Types of enteric neurons

Sensory neurons

It has long been assumed that the gut has intrinsic sensory neurons (Bayliss and Starling, 1899). This was unequivocally established only relatively recently, where motility reflexes were shown to persist after chronic extrinsic denervation, ruling out the possibility of axon reflexes from surviving severed extrinsic neurons (Furness et al., 1995). Enteric neuron cell bodies are deformed by muscle movement (Gabella and Trigg, 1984), and the capacity to transduce physical forces into action potentials is widespread among enteric neurons (Mazzuoli and Schemann, 2009).

Intrinsic primary afferent neurons

The most comprehensively characterized enteric sensory neurons to date are named "intrinsic primary afferent neurons" (IPANs; Kunze et al., 1995). They occur in both submucous and myenteric plexuses, possessing distinct Dogiel type II morphology and AH-neuron electrophysiological characteristics. Quantitatively, IPANs comprise around 20% of enteric neurons (Young et al., 1993, Costa et al., 1996). About 80% of IPANs contain calbindin in the guinea pig small intestine (Iyer et al., 1988, Song et al., 1991). Many also contain SP and ChAT (Costa et al., 1996). IPAN processes project to the mucosa and within adjacent enteric ganglia (Song et al., 1994). Physical distortion of their processes evokes spike discharges that are graded with stimulus strength (Kunze et al., 1998) and a proportion additionally fire to mucosally applied chemical stimuli (Kirchgessner et al., 1992, Bertrand et al., 1998).

IPANs appear to be directly mechanosensitive, since their stretch responses persist during synaptic blockade (Kunze et al., 2000). They fire phasically to distension and to active contractions of the muscle in circumferential and longitudinal axes, appearing to lack direction selectivity (Kunze et al., 1999). Muscle paralysis or enzymatic destruction of the connectivity between smooth muscle cells and enteric neurons abolishes stretch-activated firing of IPANs (Kunze et al., 1999). Thus, IPANs show an "in-series"-type behaviour characteristic of tension receptors. The identity of the mechanotransducing ion channels on IPANs is unknown, but they do not appear to require calcium conductance (Kunze et al., 1999, Kunze et al., 2000, Mao et al., 2006).

IPAN axons typically project less than 1mm orally, and 2-3mm circumferentially around the gut wall, surrounding most, if not all, neuron cell bodies in their own and adjacent ganglia (Furness et al., 1990b, Bornstein et al., 1991b). About 10% of putative IPANs, suspected to mediate descending reflexes, make very long (up to 11cm) anally-directed axonal projections (Brookes et al., 1995). Synaptic contacts are made with multiple other types of enteric neurons, as well as other IPANs, (Pompolo and Furness, 1988) where they can evoke NK1 receptor- or NK_3 receptor-mediated slow EPSPs (Kunze et al., 1993, Neunlist et al., 1999, Alex et al., 2001, Johnson and Bornstein, 2004, Gwynne and Bornstein, 2009). Connectivity with other IPANs is probably self-reinforcing, such that local stimuli may activate a population of IPANs simultaneously (Bertrand et al., 1997). A widespread reduction in IK channel conductance in an IPAN network caused by slow EPSPs would be likely to evoke a large increase in burst discharge. This may be ideally suited to initiate excitation of enteric reflex circuits. This emergent property of their arrangement has

not been directly demonstrated to date. Outside the guinea pig small intestine, putative IPANs do not show the same correlations between morphology, coding and electrophysiology (Cornelissen et al., 2000, Nurgali et al., 2004); limiting the assumptions that can be made about other preparations. Furthermore, IPANs show plasticity in electrophysiological characteristics under conditions of inflammation (Lomax et al., 2005), and in response to the composition of the microbiome (McVey Neufeld et al., 2013).

Other sensory neurons

Direct mechanosensitivity also occurs among enteric neurons that are not Dogiel type II/AH neurons. Myenteric S-neurons have been described in distal colon which fire bursts of action potentials in stretched preparations, and these persist during synaptic blockade (Spencer and Smith, 2004). The morphology of these neurons was consistent with Dogiel type I ascending interneurons (Spencer and Smith, 2004). Interestingly, burst discharging mechanosensory units were described in early extracellular recordings from myenteric neurons (Wood, 1970), a proportion of which also persisted under synaptic blockade (Wood, 1975). Unlike IPANs, mechanosensory S-neurons fired during muscle paralysis, indicating separate populations of enteric neurons may detect tension and length in the gut wall (Smith et al., 2007). As is the case with IPANs, the mechanotransduction mechanisms in mechanosensory S-neurons have not been delineated. In addition, their firing responses to dynamic stretch has not been observed (Wood, 2008).

Use of voltage-sensitive dyes in gut preparations enables electrophysiological events in multiple enteric neurons to be recorded simultaneously (Obaid et al., 1992). Saline

injection- and von Frey probe-mediated mechanical deformation of enteric ganglia evoked stimulus-graded mechanosensitive firing in about 25% of all neurons recorded in guinea pig and mouse ileum, and mouse distal colon (Mazzuoli and Schemann, 2009, Mazzuoli and Schemann, 2012). Among the mechanosensitive neurons were interneurons and motor neurons, revealed by retrograde tracing (Mazzuoli and Schemann, 2009). Different populations of neurons show rapidly- and slowly-adapting responses to mechanical stimulation (Mazzuoli and Schemann, 2012). Thus, enteric neurons may fit into more than a single functional classification, complicating the conceptualization of the enteric nervous system.

Interneurons

Enteric interneurons receive synaptic inputs from sensory neurons, other interneurons and extrinsic sympathetic and parasympathetic neurons. In turn they supply output to other enteric interneurons and motor neurons. Interneurons ascend and descend along the gut, some traversing tissue layers, connecting the major plexuses (Song et al., 1998, Costa and Brookes, 2008). Their long axons and arrangement into chains of like-interneurons extend reflexes and motor patterns along the gut beyond the range formed by simple sensory neuron-motor neuron circuits. Polarized motility reflexes may be reinforced by cross-innervation among ascending and descending interneurons (Pompolo and Furness, 1995). Interneurons add processing capability to neural circuits and probably enable the behavioural repertoire and flexibility apparent in the gastrointestinal tract. It has been speculated that dominance of activation among interneuronal classes may dictate the emergence of complex motility patterns like segmentation, peristalsis and migrating motor complexes (Brookes and Costa, 2006). More than other functional classes of

neurons, types of interneurons vary across gut regions (Lomax and Furness, 2000, Brookes, 2001a, Schemann et al., 2001), which probably confers to a great degree the specific behavioural programs executed within those regions. As mentioned, interneurons may be multifunctional (Spencer and Smith, 2004, Mazzuoli and Schemann, 2009, Mazzuoli and Schemann, 2012); some are directly mechanosensory and may respond to the mechanical forces they regulate, adding complexity to understanding how enteric circuits work.

Four basic types of interneurons are present in the guinea pig small intestine where interneurons have been best characterized: 3 descending, 1 ascending (Costa et al., 1996). All contain detectable choline acetyltransferase in their cell bodies (Steele et al., 1991). They can be differentiated by their distinctive chemical codes in their cell bodies: descending classes contain 5HT, SOM or NOS/VIP; ascending neurons have calretinin (Costa et al., 1996). While all have Dogiel type I morphology, the 5HT and NOS/VIP interneurons have lamellar dendrites (Costa et al., 1982, Furness et al., 1994, Young and Furness, 1995), and SOM interneurons have filamentous dendrites (Song et al., 1997). Quantitatively, each population comprises 1-5% of myenteric neurons (Costa et al., 1996, Furness, 2000).

Fast excitatory neurotransmission between interneurons is principally nicotinic cholinergic, with contributions from fast excitatory purinergic and serotonergic signalling in descending pathways (Galligan and Bertrand, 1994, LePard et al., 1997, Galligan et al., 2000).

Submucosal interneurons do not occur in guinea pig, but myenteric interneurons make axonal projections into the submucosal ganglia. In large mammals such as pig and human where the submucosal plexus is greatly expanded, submucosal interneurons have been identified (Timmermans et al., 1997, Brehmer et al., 2010).

Ascending interneurons

Chains of ascending interneurons occur in the guinea pig ileum myenteric plexus (Brookes, 2001a). In addition to calretinin, their cell bodies label for ChAT, SP, ENK and neurofilament protein triplet, in addition to calretinin (Costa et al., 1996). Their uniaxonal projections occasionally bifurcate, spanning up to 14mm (Song et al., 1996). They form interneuronal chains by projections to other ascending interneurons, and also make contacts with excitatory motor neurons (Bornstein et al., 1991a, Smith et al., 1992, Pompolo and Furness, 1995, Brookes et al., 1997a). They have S-neuron electrophysiological characteristics, fire tonically and reveal a distinct transient outward current on depolarization (Brookes et al., 1997a). Nicotinic receptor agonists potently activate the ascending pathways and electrical stimulation evokes fast EPSPs (Brookes et al., 1997a). Inputs come from NOS-IR descending interneurons and at least two functionally distinct populations of IPANs, in addition to innervation supplied by other ascending interneurons (Smith et al., 1992, Pompolo and Furness, 1995). The ascending interneurons spread stimulus-evoked excitation to excitatory motor neurons that innervate circular muscle, which can evoke muscle contractions oral to a stimulus – a characteristic of the "law of the intestine" (Bayliss and Starling, 1899). Indeed, the observation that excitatory junction potentials in circular muscle occur up to 35mm oral to a mucosal stimulus (Smith and Furness, 1988), implies ascending neurons are critical to motility reflexes, given IPANs

project no more than 1mm orally and excitatory motor neurons less than 10mm. Using partitioned organ baths to study the pharmacology of ascending excitatory reflexes, transmission between ascending interneurons and from ascending interneurons to excitatory motor neurons of the circular muscle appears to be largely cholinergic via nicotinic receptors (Smith and Furness, 1988, Tonini and Costa, 1990, Johnson et al., 1996), although the latter may also include a component mediated by NK_3 receptors (Bornstein et al., 2004). In colon, there are 3 classes of ascending interneurons (Lomax and Furness, 2000). It has been speculated they may play roles in transmitting ascending excitation from pelvic parasympathetic inputs, which are not present in the small intestine (Furness, 2000). As mentioned previously, most enteric neurons that receive pelvic nerve inputs make oral projections (Tamura, 1997). In addition, at least one of these classes is likely to be directly mechanosensory (Spencer and Smith, 2004).

SOM-IR descending interneurons

One population of descending interneurons contain somatostatin (Costa et al., 1977, Costa et al., 1980b). Their cell bodies also label for ChAT and the population comprises around 4% of myenteric neurons (Costa et al., 1996). Possessing distinct smooth ovoid cell bodies with several long tapering filamentous dendrites, their single axons project up to 70mm to both myenteric and submucous ganglia (Portbury et al., 1995, Song et al., 1996, Song et al., 1997). They resemble S-neurons electrophysiologically, with relatively low resting membrane potentials, high input resistances and receive fast and slow EPSPs (Stebbing and Bornstein, 1996, Song et al., 1997). However, they show also some AH-neuron characteristics, including a "sag" in membrane potential during injected hyperpolarising currents (IH-like

cationic current), and some have long after-hyperpolarisations after repeated action potential discharge (Song et al., 1997). Circumferential inputs to SOM-IR interneurons from IPANs are sparse (Stebbing and Bornstein, 1996, Pompolo and Furness, 1998). It has been suggested SOM-IR neurones play little role in local descending reflexes (Pompolo and Furness, 1998). Most inputs to SOM-IR arise from other SOM-IR interneurons or from NOS-IR descending interneurons (Portbury et al., 1995, Mann et al., 1997). SOM-IR interneurons in turn supply NOS-IR interneurons, thus forming interconnecting chains (Mann et al., 1997). Despite the lack of direct input from IPANs, distension can activate SOM-IR neurons (Thornton and Bornstein, 2002). It is possible distension-evoked activation is mediated through NOS-IR interneurons which receive more prominent inputs from IPANs (Stebbing and Bornstein, 1996, Thornton and Bornstein, 2002). SOM-IR interneurons may play a role in motility since they innervate inhibitory motor neurons (Mann et al., 1997). A role in the passage of migrating myoelectric complexes (MMC) has been postulated on the basis of their relatively higher order position and their spatial distribution, which largely parallels the occurrence of the MMC (Pompolo and Furness, 1998). However, this possibility remains to be tested. Functional projections are also made in the submucosa to VIP-IR secretomotor neurons, suggesting they may contribute to the coordination of motility, blood flow and secretion (Shen and Surprenant, 1993). Here, they may evoke somatostatin-mediated IPSPs in conjunction with the SOM-IR terminals of extrinsic sympathetic neurons (Foong et al., 2010). SOM-IR interneurons may be involved in long descending secretomotor reflexes. These take a pathway through the myenteric plexus to VIP-IR submucosal neurons (Reed and Vanner, 2007).

5HT-IR descending interneurons

Serotonin-accumulating myenteric interneurons identified in early immunohistochemical studies (Costa et al., 1982) were demonstrated to project their axons anally into myenteric and submucous ganglia using degeneration techniques (Furness and Costa, 1982). 5HT-IR interneurons also label for ChAT and neurofilament protein triplet and comprise about 2% of myenteric neurons (Steele et al., 1991, Costa et al., 1996). Morphologically, they are uniaxonal Dogiel type I neurons, with two distinct soma-dendritic morphologies apparently dependent on their location within ganglia (Meedeniya et al., 1998). Axons extend up to 100mm down the gut (Meedeniya et al., 1998). Like other interneurons, their terminal endings preferentially target the same interneurons, forming chains of like-neurons down the gut (Young and Furness, 1995). They do not make contacts with inhibitory motor neurons (Young and Furness, 1995). However, pharmacological data suggest these neurons may activate excitatory motor neurons, mediating descending excitation (Jin et al., 1989, Monro et al., 2002) while their submucosal projections are suggested to mediate secretomotor reflexes (Furness, 2000). The 5HT interneuron is present also in stomach and distal colon where they have similar morphology and pathways (Wardell et al., 1994, Reiche et al., 2000). Release of 5HT, probably from interneurons, contributes to fast excitatory neurotransmission in 11% of myenteric neurons via the $5HT_3$ ionotropic receptor (Zhou and Galligan, 1999). In addition, 5HT evokes $5HT_{1A}$ receptor-mediated slow inhibitory neurotransmission in a subset of myenteric neurons (Johnson and Bornstein, 2004). The role of 5HT in neurotransmission is difficult to interpret due to several factors, including the ubiquity and variation of 5HT receptors, the prominence of 5HT

release from enterochromaffin cells, and uptake of 5HT and related indole-amines by neurons that may not synthesize 5HT de novo (Gershon et al., 1994).

NOS-IR descending interneurons

A third class of descending interneuron in the guinea pig contains combinations of NOS, VIP, gastrin-releasing peptide, GAL, NPY, dynorphin, neurofilament protein triplet, alkaline phosphatase and ChAT (Brookes, 2001a, Brookes and Costa, 2006, Furness, 2006). This less understood class constitutes about 5% of myenteric neurons. There is significantly variation and overlap among chemical coding within this group and they have been split into different subgroups previously (Costa et al., 1996), but there is no indication whether the distinction is physiologically significant. In any case, the NOS interneuron has been highly conserved through evolution and is present in nearly all vertebrate classes (Timmermans et al., 1997). They are uniaxonal Dogiel type I neurons that form descending chains (Young et al., 1995). They also make synaptic contacts with SOM-IR interneurons, calretinin interneurons, and inhibitory motor neurons (Young et al., 1995, Mann et al., 1997). Electrophysiologically they are S-neurons that receive fast excitatory synaptic input circumferentially from IPANs and longitudinally from other interneurons (Stebbing and Bornstein, 1996). NOS interneurons are likely to play a prominent role in descending inhibitory reflexes through their direct connections with both sensory neurons and inhibitory motor neurons. Pharmacological data implicates a role for nitric oxide in descending inhibitory pathways (Yuan et al., 1995). Purines may also be used by this class of neuron for fast synaptic transmission, acting postsynaptically on P2X receptors in the small intestine (LePard et al., 1997, Bian et al., 2000).

Equivalent descending pathways in the colon, however, may be cholinergic (Bian et al., 2004).

Motor neurons

Dense assemblies of motor neurons innervate the circular and longitudinal muscle layers, comprising around 60% of myenteric neurons. Excitatory and inhibitory neurons have distinct neurochemical phenotypes. After tracing from muscle, excitatory motor neurons may be identified by immunohistochemical detection of choline acetyltransferase and tachykinin peptides; inhibitory motor neurons are reliably identified by their VIP and NOS content.

Motor neuron axons branch extensively and have multiple varicose transmitter release sites that often (but not always) closely appose ICC and fibroblast-like cells through which signalling to smooth muscle may be mediated (Wang et al., 1999, Ward and Sanders, 2001, 2006, Kurahashi et al., 2011). In small intestine, circular muscle motor axons often run parallel to muscle fibres, concentrating in layer of the muscle, forming the "deep muscular plexus". The plexus separates a thin outer layer of muscle cells from the bulk of the circular muscle (Gabella, 1987). Axons supplying longitudinal muscle ramify in the tertiary plexus located on the inner face of the longitudinal muscle wall, from which varicosities release transmitter (Llewellyn-Smith et al., 1993, Furness et al., 2000a). In larger mammals the axons may penetrate the muscle layer and run between muscle fibres similar to circular muscle motor neurons.

Axonal projections of excitatory and inhibitory motor neurons polarise in the same way as the "law of the intestine". That is, excitatory motor neurons on average make oral projections and inhibitory motor neurons project anally. Polarity is more

prominent in motor neurons to circular muscle than longitudinal muscle (Brookes, 2001a). Throughout most of the gut, the majority of motor neurons are excitatory. Excitatory motor neurons are typically smaller in cell body size and in the length of their axons, compared to inhibitory motor neurons (Brookes, 2001a).

Motor neurons to the circular muscle

In guinea pig, all cell bodies of circular muscle motor neurons are in the myenteric plexus, comprising around 28% of myenteric neurons (Wilson et al., 1987, Furness, 2006). In larger mammals, some also occur in submucosal ganglia (Furness et al., 1990a, Porter et al., 1999). Retrograde tracing and immunohistochemistry reveals most excitatory motor neurons make projections between 5mm oral and 2mm anal to circular muscle; the longest project up to 8mm orally. A large subset of excitatory motor neurons contains SP, with stronger immunoreactivity reported in those with longer axonal projections (Brookes et al., 1991b). Most inhibitory motor neurons project 0 to 6mm anally; the longest up to 25mm (Brookes et al., 1991b).

Chemical coding differs between long and short-projecting neurons: long excitatory motor neurons tend to contain the opioid peptide ENK; shorter excitatory neurons lack ENK (Brookes et al., 1997a). Long descending inhibitory motor neurons contain VIP, NOS, gastrin-releasing peptide and neurofilament protein triplet; short inhibitory neurons contain VIP, NOS, ENK, NPY, GABA and GAL (Furness et al., 1987, Brookes et al., 1991b, Williamson et al., 1996). Immunohistochemical detection of ChAT, NOS, VIP and tachykinins in motor neurons is unquestionably related to neuromuscular transmission, but the physiological significance of other detected substances in motor neurons is less clear. Evidence suggests GABA and

ENK may presynaptically inhibit transmitter release (Waterfield et al., 1977, Kaplita et al., 1982), and it has been speculated gastrin-releasing peptide and NPY may act as neuromodulators (Brookes, 2001a). Electrophysiologically, motor neurons to circular muscle are S-neurons. Many receive fast EPSPs from pathways projecting in the same direction of their axonal projections, although some receive inputs from both ascending and descending pathways (Smith et al., 1992).

Motor neurons to the longitudinal muscle

Longitudinal muscle motor neurons form a population that is distinct to circular muscle motor neurons, and comprise around 24% of myenteric neurons (Brookes et al., 1992). They have small, simple cell bodies, few short thin or lamellar dendrites, and tend to cluster where internodal strands and myenteric ganglia intersect (Brookes et al., 1992, Smith et al., 1999). The axons of longitudinal muscle motor neurons are short, projecting less than 3mm. Most (>97%) in guinea pig small intestine are excitatory ChAT-IR neurons, most also have calretinin (Brookes et al., 1991a), and around half contain tachykinins (Brookes et al., 1992). Inhibitory motor neurons contain NOS and VIP (Costa et al., 1980a, Costa and Furness, 1983, Costa et al., 1992, Williamson et al., 1996). Subsets also have NPY (Uemura et al., 1995) and GABA in their varicosities (Jessen et al., 1986, Furness et al., 1989), which may modulate transmitter release in the tertiary plexus. Despite the relatively small population of inhibitory motor neurons, they appear to exert significant muscle relaxations upon electrical stimulation (Osthaus and Galligan, 1992, Yunker and J. Galligan, 1998). Electrophysiological studies of longitudinal muscle motor neurons indicate they are highly excitable S neurons attributable to their high membrane

resistance. They receive fast EPSPs from ascending and descending pathways, fire tonically during depolarization and often spontaneously (Smith et al., 1999).

Motor neurons to the muscularis mucosae

The intestinal muscularis mucosae is comprised of very thin inner circular and outer longitudinal muscle layers (Uchida and Kamikawa, 2007). This layer invades mucosal villi, facilitating vigorous movements of the mucosa and villi that are not associated with movements of the outer muscle layers (King and Arnold, 1922). In guinea pig colon, atropine partially abolishes electrically-stimulated contractions of the muscularis mucosae, suggesting cholinergic and non-cholinergic excitatory inputs are supplied by motor neurons (Uchida and Kamikawa, 2007). Consistent with this, a dense innervation is supplied by tachykinin-IR and CGRP-IR fibres (Ishikawa and Ozaki, 1997). Exogenous application of these peptides evoke potent activation and inhibition of muscle contractility, respectively (Ishikawa and Ozaki, 1997). Populations of VIP-IR inhibitory and SP-IR excitatory motor neurons in submucous plexus are a putative major source of innervation to the muscularis mucosae in canine intestine (Angel et al., 1983, Angel et al., 1984, Furness et al., 1990a). Some innervation in guinea pig ileum, however, appears to arise from myenteric neurons that contain SP (Schultzberg et al., 1980, Steele and Costa, 1990). In regions of gut where submucous ganglia are lacking (stomach and oesophagus for example), the motor innervation of the muscularis mucosae presumably originate in the myenteric plexus.

Excitatory neuromuscular transmission

Acetylcholine, the principle excitatory transmitter at smooth muscle, acts on muscarinic M_2 and M_3 GPCRs. Tachykinins act on G-protein coupled NK_1 and NK_2 receptors (Zagorodnyuk et al., 1994, Sternini et al., 1995, Zagorodnyuk et al., 1995, Grady et al., 1996, Harrington et al., 2010). Motor neuron firing activity modulates transmitter contributions to neuromuscular signalling. In longitudinal muscle, single electrical stimuli evoke principally cholinergic contractions (Paton, 1955) but repetitive stimuli can evoke non-cholinergic, SP-mediated contractions (Ambache and Freeman, 1968, Franco et al., 1979). Similar responses occur to different stimulation frequencies in circular muscle (Costa et al., 1985). Muscarinic receptor blockade in vivo significantly inhibits gut motility whereas tachykinin receptor blockade shows little effect, suggesting a greater relative importance of acetylcholine in neuromuscular transmission (Borody et al., 1985, Galligan et al., 1986, Furness, 2000). In addition to acetylcholine and tachykinins, there is evidence suggesting that purinergic input contributes to excitatory neuromuscular transmission (Zagorodnyuk et al., 1996, Zagorodnyuk and Maggi, 1998).

Inhibitory neuromuscular transmission

Inhibitory neuromuscular transmission plays a key role in peristalsis by causing smooth muscle relaxation ahead of propagating contractions. The major inhibitory transmitters to gastrointestinal smooth muscle include NO, ATP and VIP. These transmitters evoke smooth muscle relaxation through induction of cyclic guanosine monophosphate, $P2Y_1$ and VPAC receptors, respectively (Matsuda and Miller, 2010). VIP and NO are functionally linked in nerve terminals, such that each regulates the release of the other (Murthy, 2006). Nitrergic inhibitory neuromuscular

transmission may be mediated through intramuscular interstitial cells of Cajal, causing an amplification of signalling (Ward and Sanders, 2001). Fibroblast-like cells that express the platelet-derived growth factor receptor-α have been shown to be closely associated with intramuscular interstitial cells of Cajal (Cobine et al., 2011). These fibroblast-like cells may mediate inhibitory purinergic neurotransmission to smooth muscle via $P2Y_1$ receptors (Kurahashi et al., 2011, Gil et al., 2013). Other transmitters implicated in inhibitory signalling include the VIP-related peptides PACAP and peptide histidine isoleucine, β-nicotinamide adenine dinucleotide, carbon monoxide, hydrogen sulphide and NT (Matsuda and Miller, 2010).

Secretomotor/vasomotor neurons

Three types of secretomotor neurons are present in guinea pig small intestine, all of which innervate the mucosal epithelium, while two classes also send collateral axons to submucous arterioles. Most cell bodies of secretomotor neurons are in the submucous plexus, but a small proportion are in myenteric ganglia. Activation of secretomotor neurons generates an ionic current across the mucosal epithelium (Hubel, 1978, Carey et al., 1985). This current is predominantly due to active chloride ion secretion through the cystic fibrosis transmembrane conductance regulator channels into the lumen (Frizzell and Hanrahan, 2012). Chloride ion secretion is accompanied by sodium ions and water, passing between epithelial cells and through apical aquaporin channels (Frizzell and Hanrahan, 2012). Cholinergic and non-cholinergic populations of submucosal neurons regulate chloride secretion (Cooke, 1984). Both populations also regulate local blood flow by controlling submucosal arteriole diameter in conjunction with extrinsic neuronal and other, non-neuronal mechanisms (Vanner and Surprenant, 1996). Secretion and vasodilation

reflexes probably always occur in conjunction, given that secretion is largely dependent upon local blood flow to supply electrolytes.

VIP-IR secretomotor vasodilator neurons

Non-cholinergic secretomotor/vasodilator neurons comprise about 43% of all submucous neurons (Song et al., 1992). Some secretomotor neurons have cell bodies in myenteric ganglia (Costa et al., 1996). They have Dogiel type I morphology with lamellar or filamentous dendrites (Furness et al., 2003). Their axons project preferentially in a circumferential direction for short distances to mucosal villi with no apparent longitudinal polarity (Song et al., 1992). These neurons provide a dense supply of VIP to the mucosa, a potent stimulant of intestinal secretion (Barbezat and Grossman, 1971, Schwartz et al., 1974). VIP-IR secretomotor neurons resemble S-neurons, receiving prominent nicotinic fast EPSPs and tonically discharging with direct depolarizing current injection. They are the only neurons in the submucous plexus to receive inhibitory postsynaptic potentials (Bornstein et al., 1986, Evans et al., 1994). The IPSPs are mediated by extrinsic sympathetic inputs via α_2 adrenergic receptors (North and Surprenant, 1985), and by intrinsic inputs, most likely from descending myenteric interneurons acting via somatostatin SST_1 and SST_2 receptors, and probably also serotonin $5HT_{1A}$ receptors (Mihara et al., 1987, Bornstein et al., 1988, Foong et al., 2010).

ChAT-Calretinin secretomotor vasodilator neurons

Cholinergic secretomotor/vasodilator neurons comprise about 12% of submucous neurons (Song et al., 1992). They are uniaxonal and project to submucosal arterioles and the mucosal epithelium (Neild et al., 1990, Brookes et al., 1991a, Li and Furness,

1998). Their projections to the mucosa are shorter than those of VIP-IR submucosal neurons, and tend to be circumferentially directed without a longitudinal polarity (Song et al., 1992). Morphologically they are Dogiel type I neurons with filamentous dendrites, described as "stellate", and identifiable by their calretinin content in the submucous plexus (Brookes et al., 1991a, Furness et al., 2003). They discharge repetitively to direct depolarizing current injections and receive fast nicotinic EPSPs (Bornstein et al., 1986, Neild et al., 1990).

NPY-cholinergic secretomotor (non-vasodilator) neurons

Around 33% of submucosal neurons are cholinergic NPY-IR secretomotor neurons that make projections to the mucosal epithelium but do not innervate arterioles (Furness et al., 1984, Song et al., 1992). Their projections to the mucosa are not polarised in the oral or anal directions but may extend up to 2.9mm circumferentially (Song et al., 1992). A small proportion (<1%) have their nerve cell bodies located in the myenteric ganglia (Furness et al., 1985, Costa et al., 1996). All are uniaxonal neurons with filamentous dendrites and resemble S-neurons, receiving nicotinic excitatory fast EPSPs from descending myenteric pathways (Bornstein et al., 1986, Evans et al., 1994, Moore and Vanner, 2000).

VIP-IR interplexus interneurons

A small population of submucosal neurons immunoreactive for VIP project to the myenteric plexus without sending collateral axons to the mucosa or vasculature (Song et al., 1998). These may represent a class of "interplexus" interneurons. It has been speculated they could be "displaced" VIP/NOS myenteric interneurons, as a

similar proportion of NOS-IR neurons have been identified in the submucous plexus (Furness et al., 1994, Furness, 2006).

Viscerofugal neurons

Neuroanatomical characteristics of viscerofugal neurons

Distribution of viscerofugal nerve cell bodies

Layer distribution

Retrograde neuronal tracing studies have revealed the location of viscerofugal neuronal cell bodies within the layers of the gut wall. In small experimental animals, the majority of studies report they are located in the myenteric plexus only, while in larger animals viscerofugal neuron cell bodies have been identified in the myenteric and outer submucous plexuses. **Table 1.01** summarizes the published data reporting the locations of viscerofugal nerve cell bodies.

Circumferential distribution

Viscerofugal neuron cell bodies are located predominantly in ganglia toward the mesenteric border of the small and large intestines of guinea pig (Kuramoto and Furness, 1989, Furness et al., 1990c, Messenger and Furness, 1992, Sharkey et al., 1998, Tassicker et al., 1999a, Ermilov et al., 2003), and rat (Luckensmeyer and Keast, 1995a, 1996, Furness et al., 2000b); and in the large intestine of mouse (Miller and Szurszewski, 2002) and pig (Barbiers et al., 1993, Barbiers et al., 1994). In the rectum, viscerofugal nerve cell bodies are more evenly distributed circumferentially, around the gut wall (Luckensmeyer and Keast, 1995a, 1996).

The layer distribution of viscerofugal nerve cell bodies

Layer of gut	Species	Gut region	Reference
Viscerofugal nerve cell bodies identified in the myenteric plexus **but not** the submucous plexus	Guinea Pig	Stomach, small intestine, large intestine	Kuramoto and Furness, 1989, Furness et al., 1990c, Messenger and Furness, 1992, 1993, Anderson et al., 1995, Zagorodnyuk et al., 2001, Ermilov et al., 2003
	Rat	Stomach, small intestine, large intestine	Lee et al., 1986, Hamaji et al., 1987, Domoto et al., 1995, Luckensmeyer and Keast, 1995a, Furness et al., 2000b
	Mouse	Large intestine	Miller and Szurszewski, 2002
	Dog	Large intestine	Li and Masuko, 1997
Viscerofugal nerve cell bodies identified in **both** the submucous and myenteric plexus	Guinea Pig	Large intestine	Dalsgaard and Elfvin, 1982
	Cat	Small intestine	Feher, 1982, Feher and Vajda, 1982
	Pig	Large intestine	Barbiers et al., 1993, Barbiers et al., 1994

Table 1.01

The locations of viscerofugal nerve cell bodies revealed by neuroanatomical tracing from prevertebral ganglia or mesenteric nerve trunks. This table lists studies that identified viscerofugal neurons in both the myenteric plexus and submucous plexus or only in the myenteric plexus where both of the plexuses were been inspected for retrogradely-labelled nerve cell bodies. In the guinea pig, all but a single study has reported viscerofugal nerve cell bodies were only located in the myenteric plexus.

The size of viscerofugal nerve cell bodies

Measure	Gut region	Nerve cell body size	Reference
Major & minor axis length	Small intestine	42±7 x 19±4μm	Kuramoto and Furness, 1989
		26±4 x 16±3μm	Tassicker et al., 1999
	Colon	42±16 x17±4μm	Sharkey et al., 1998
		33.4±1.3 x 18.9±0.7μm_{α}	Ermilov et al., 2003
		39.4±4.0 x 21.3±1.5μm_{β}	Ermilov et al., 2003
		48.6±1.1 x 16.1±0.6μm	Miller and Szurszewski, 2002
Cross-sectional area	Oesophagus	651 ± 35 μm^2	Zagorodnyuk and Brookes, 2000
	Stomach	631 ± 47 μm^2	Zagorodnyuk et al., 2001
	Rectum	338 ± 11 μm2	Olsson et al., 2004
Volume	Colon	2770 ± 250μm^3_{α}	Ermilov et al., 2003
		4490 ± 730μm^3_{β}	Ermilov et al., 2003
α – Dogiel type I morphology, β – Dogiel type II morphology shaded cells – mouse data, unshaded cells – guinea pig data			

Table 1.02

Reported sizes of viscerofugal nerve cell bodies. This table shows all reports of viscerofugal nerve cell body size as the length of their two major axes, area, or cell volume. Their sizes are comparable to those of lamellar and simple Dogiel type I neurons (Furness et al., 1988). Note that these studies use different neuronal tracers, which can vary in the type and fullness of labelling. Also note that in one study, Dogiel type I and II viscerofugal neurons were reported (these are indicated; Ermilov et al., 2003).

Longitudinal distribution

Several retrograde tracing studies report that the number of viscerofugal neuron cell bodies increase distally along the gut; the greatest numbers are reported in the rectum (Messenger and Furness, 1993, Barbiers et al., 1994, Luckensmeyer and Keast, 1995a, 1996, Li and Masuko, 1997). To date, the most comprehensive quantitative assessments of the gastrointestinal viscerofugal neuron population has been studies by Messenger and Furness (Messenger and Furness, 1992, 1993) who retrogradely labelled neurons projecting to all prevertebral ganglia in guinea pig. They reported population densities of below 1 viscerofugal cell body per cm^2 in stomach, about 20 cells/cm^2 in distal ileum, 25-100 cells/cm^2 in distal colon, and 200 cells/cm^2 in the rectum (this data is summarized in **figure 1.04**). These studies account for the majority viscerofugal neurons in the gut, although populations known to exist that project to pelvic ganglia (Luckensmeyer and Keast, 1995a, 1996) and through vagal nerve pathways (Zagorodnyuk and Brookes, 2000, Zagorodnyuk et al., 2001) were not included. It has been noted that the gradient in the density of viscerofugal nerve cell bodies along the gut contrasts with the more even distribution of sympathetic innervation (Costa and Gabella, 1971, Messenger and Furness, 1993). This may indicate a tendency for intestino-intestinal reflexes to operate in a distal to proximal direction.

Distribution of viscerofugal nerve cell bodies along the gut, traced from the abdominal prevertebral ganglia

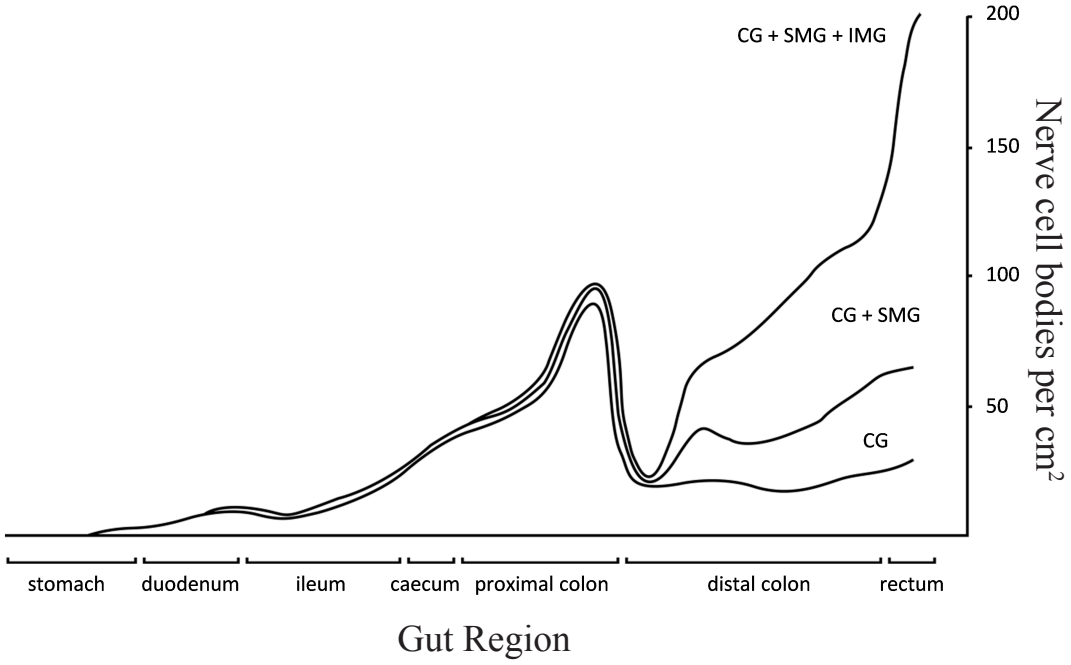

Figure 1.04

The distribution of viscerofugal neurons along the gut. This graph shows the numbers of viscerofugal nerve cell bodies retrogradely traced from the prevertebral ganglia in guinea pig (Messenger and Furness, 1992, 1993). Almost all viscerofugal neurons from the stomach to the proximal colon make projections to the coeliac ganglia (CG). In the distal colon and rectum increasing numbers of viscerofugal neurons make projections to the superior mesenteric ganglia (SMG) and inferior mesenteric ganglia (SMG). This graph shows that besides a small peak in the proximal colon, the total number of viscerofugal neurons in the gut gradually increase from proximal to distal regions. Viscerofugal neurons that project to the spinal cord and major pelvic ganglia from the distal gut, and those which make projections through the vagus nerve are not shown here. Figure adapted from Messenger and Furness (1993).

Morphological characteristics of viscerofugal neurons

Dimensions of viscerofugal nerve cell bodies

The dimensions of viscerofugal neuron cell bodies in several reports are summarized in **table 1.02**. The type of tracers and methodologies used varied between studies.

Soma-dendritic morphology

The morphological detail of viscerofugal neurons has been best revealed by intracellular dye injections following retrograde tracing (Sharkey et al., 1998, Ermilov et al., 2003), DiI tracing in fixed tissue (Ermilov et al., 1998, Ermilov et al., 2003) and biotinamide tracing in live tissue (Tassicker et al., 1999a). Almost all retrograde tracing studies using fluorescent dyes, particularly Fast Blue, report that viscerofugal neurons are exclusively uniaxonal with short flattened dendrites, or without dendrites, described as Dogiel type I (Kuramoto and Furness, 1989, Furness et al., 1990c, Messenger and Furness, 1992, 1993, Furness et al., 2000b, Lomax et al., 2000). Although Fast Blue did not always fill neuronal processes (Kuramoto and Furness, 1989), these findings have been corroborated by the high quality cytoplasmic labelling of biotinamide tracing (Tassicker et al., 1999a, Olsson et al., 2004, Hibberd et al., 2012c, b), as well as intracellular dye injections of biocytin (Sharkey et al., 1998). Interestingly, the use of DiI tracing in fixed and live tissue, as well as intracellular lucifer yellow injections have yielded both Dogiel type I and type II morphologies among populations of viscerofugal neurons (Ermilov et al., 1998, Ermilov et al., 2003). The latter type had larger smooth cell bodies, multiple axons, tapering dendrites and constituted about 30% of viscerofugal neurons; they also have less nicotinic receptor immunoreactivity than type I neurons and some

surround type I neurons with their neurites (Ermilov et al., 1998, Ermilov et al., 2003). Whether the morphological differences identified in the latter studies reflect different functional populations in viscerofugal neurons is not known.

Projections of viscerofugal neuron axons

By definition, viscerofugal neuron axons leave the gut. The largest and best characterised population of viscerofugal neurons are those which terminate within the sympathetic prevertebral and pelvic ganglia which form 'intestino-intestinal' reflex circuitry with gut-projecting sympathetic neurons (Messenger and Furness, 1992, 1993). The aforementioned retrograde tracing studies by Messenger & Furness identified the locations of viscerofugal neurons along the gut that project to sympathetic prevertebral ganglia, summarized in **figure 1.04**. In general, the more caudal prevertebral ganglia are targeted by viscerofugal neurons located in more caudal/distal the regions of gut but there was tendency of viscerofugal neurons from distal regions of gut to also project to rostral prevertebral ganglia. Contrasted with the more evenly distributed projections of sympathetic neurons, this led the authors to suggest viscerofugal neuron activation of sympathetic neurons generally affects more proximal gut regions. In addition to these well characterized populations, there are several more obscure populations of 'viscerofugal' neurons. In the oesophagus some neurons project from the myenteric plexus into the trachea (Fischer et al., 1998). Also, viscerofugal neurons in the oesophagus and stomach project via the vagus nerve, but their terminations are not known (Holst et al., 1997). A small population of myenteric neurons in the small intestine project into the pancreas (Kirchgessner and Gershon, 1990, Kirchgessner et al., 1994, Kirchgessner et al., 1996). Finally, some neurons in the rectum project via pelvic nerves, through the

dorsal roots, into spinal cord segments L_6 to S_1 (Doerffler-Melly and Neuhuber, 1988, Neuhuber et al., 1993).

Neurochemical coding in the guinea pig

Some chemical coding of viscerofugal neurons has been directly identified by combining retrograde tracing with immunohistochemistry as well as indirectly by observing losses of axonal immunoreactivity in prevertebral ganglia after sectioning mesenteric nerves. The synthetic enzyme choline acetyltransferase (ChAT) has been identified within viscerofugal neuron cell bodies, consistent with their putative cholinergic phenotype (Mann et al., 1995, Sharkey et al., 1998, Tassicker et al., 1999a, Furness et al., 2000b, Zagorodnyuk and Brookes, 2000). Other putative transmitters of viscerofugal neurons have been identified, including the related peptides VIP (Messenger and Furness, 1991, 1992, 1993), peptide histidine isoleucine, and PACAP (Lindh et al., 1988, Ermilov et al., 2004b). In guinea pig, NOS and calbindin occurs in viscerofugal neurons of the large intestine but not small intestine (Mann et al., 1995). A summary of neurochemical content reported in retrogradely traced viscerofugal nerve cell bodies in the guinea pig is presented in **table 1.03**.

Neurochemical content of viscerofugal nerve cell bodies in the guinea pig

Substance	Oesophagus	Stomach	Small intestine	Large intestine	Rectum	Reference
Serotonin			-			Tassicker et al., 1999
Bombesin		+	+	+	+	Dalsgaard et al., 1983, Hamaji et al., 1989b, Mann et al., 1995
Calbindin			-	+	+	Kuramoto and Furness, 1989, Furness et al., 1990c, Messenger and Furness, 1991, 1992, 1993, Mann et al., 1995, Sharkey et al., 1998, Tassicker et al., 1999
Calretinin			-	-		Sharkey et al., 1998, Tassicker et al., 1999
Cholecystokinin			+	+		Dalsgaard et al., 1983, Hamaji et al., 1989b
Calcitonin gene-related peptide	-		-		-	Tassicker et al., 1999, Zagorodnyuk and Brookes, 2000, Olsson et al., 2004
Choline acetyltransferase	+		+	+	+	Mann et al., 1995, Sharkey et al., 1998, Tassicker et al., 1999, Zagorodnyuk and Brookes, 2000, Olsson et al., 2004
Nitric oxide synthase	+		-	+	+	Furness and Anderson, 1993, Mann et al., 1995, Sharkey et al., 1998, Tassicker et al., 1999, Zagorodnyuk and Brookes, 2000, Olsson et al., 2004
Pituitary adenylate cyclase activating peptide				+		Ermilov et al., 2004
Somatostatin			-			Tassicker et al., 1999
Tyrosine hydroxylase	-		-		-	Tassicker et al., 1999 Zagorodnyuk and Brookes, 2000, Olsson et al., 2004
Vesicular acetylcholine transporter					-	Olsson et al., 2004
Vesicular glutamate transporter 1					-	Olsson et al., 2004
Vesicular glutamate transporter 2					-	Olsson et al., 2004
Vasoactive intestinal peptide		+	+	+	+	Costa and Furness, 1983, Dalsgaard et al., 1983, Hamaji et al., 1989b, Kuramoto and Furness, 1989, Furness et al., 1990c, Messenger and Furness, 1991, 1992, 1993, Tassicker et al., 1999, Ermilov et al., 2004
Vesicular monoamine transporter					-	Olsson et al., 2004

Table 1.03

Neurochemical content of viscerofugal neurons in guinea pig. This table summarizes immunohistochemical studies that report immunoreactive content in retrogradely traced viscerofugal nerve cell bodies. Plus (+) signs indicate that a proportion of viscerofugal neurons contained immunoreactivity, minus (-) signs indicate viscerofugal neurons were not immunoreactive. Unlabelled boxes indicate regions that have not been tested. ChAT, BOM and VIP have been observed in viscerofugal neurons throughout the gut, while NOS and calbindin appear to be located in the large intestine only. Note that NOS has been reported in viscerofugal neurons in the oesophagus, but their projections were not identified (Zagorodnyuk and Brookes, 2000).

Synaptic terminals of viscerofugal neurons in prevertebral ganglia

The synaptic terminations of viscerofugal neurons occur in the abdominal sympathetic prevertebral ganglia which lie at the junctions of the coeliac and mesenteric arteries with the abdominal aorta. As described previously, prevertebral postganglionic sympathetic neurons may be classified by their targets and expression of peptide transmitters: NA/NPY-expressing vasomotor neurons innervate the vasculature; NA/SOM-expressing neurons selectively inhibit enteric secretomotor neurons; and NA-only neurons inhibit enteric motor neurons (Macrae et al., 1986). VIP-expressing viscerofugal neuron terminals selectively appose somata and dendrites of motor and secretomotor postganglionic sympathetic neurons at a similar spatial density to sympathetic preganglionic synapses – effectively doubling the density of synapses received by these classes compared to vasoconstrictor neurons. Additionally, the dendritic fields of neurons which receive viscerofugal neuron synaptic terminations are about three times larger than those which do not (Gibbins and Morris, 2006).

Fast Ca^{2+} dependent exocytotic release of transmitter in cholinergic neurotransmission requires synaptosomal-associated protein 25, syntaxin, synaptophysin, synaptobrevin and synaptotagmin for vesicle fusion to the membrane while the transport protein VAChT is required for loading acetylcholine into vesicles (Sudhof, 2004). Quantitative immunohistochemical studies of these presynaptic proteins revealed that terminals of VIP-IR viscerofugal neuron boutons contain significantly less SNARE proteins, as well as VAChT, than those of sympathetic preganglionic neurons (Gibbins et al., 2003a). It is hypothesized that the decreased expression of synaptic proteins explain the lower synaptic efficacy (‘weak”

synapses) of viscerofugal neurons inputs relative to spinal preganglionic inputs, which are more likely to evoke postganglionic action potentials when stimulated (Gibbins et al., 2003a).

Neurophysiology of viscerofugal neurons

Electrophysiology

Intracellular recordings of synaptic input from populations of viscerofugal neurons

The firing behaviour of viscerofugal neurons has been characterised most extensively from intracellular recordings of the noradrenergic postganglionic sympathetic neurons upon which they terminate (Crowcroft et al., 1971b). This technique yields information from multiple converging viscerofugal neurons. In combination with simultaneous recordings of intraluminal pressure and volume of an intestinal segment attached by mesenteric/colonic nerves (see **figure 1.05**), the mechanical and chemical sensitivities of viscerofugal neurons have been deduced.

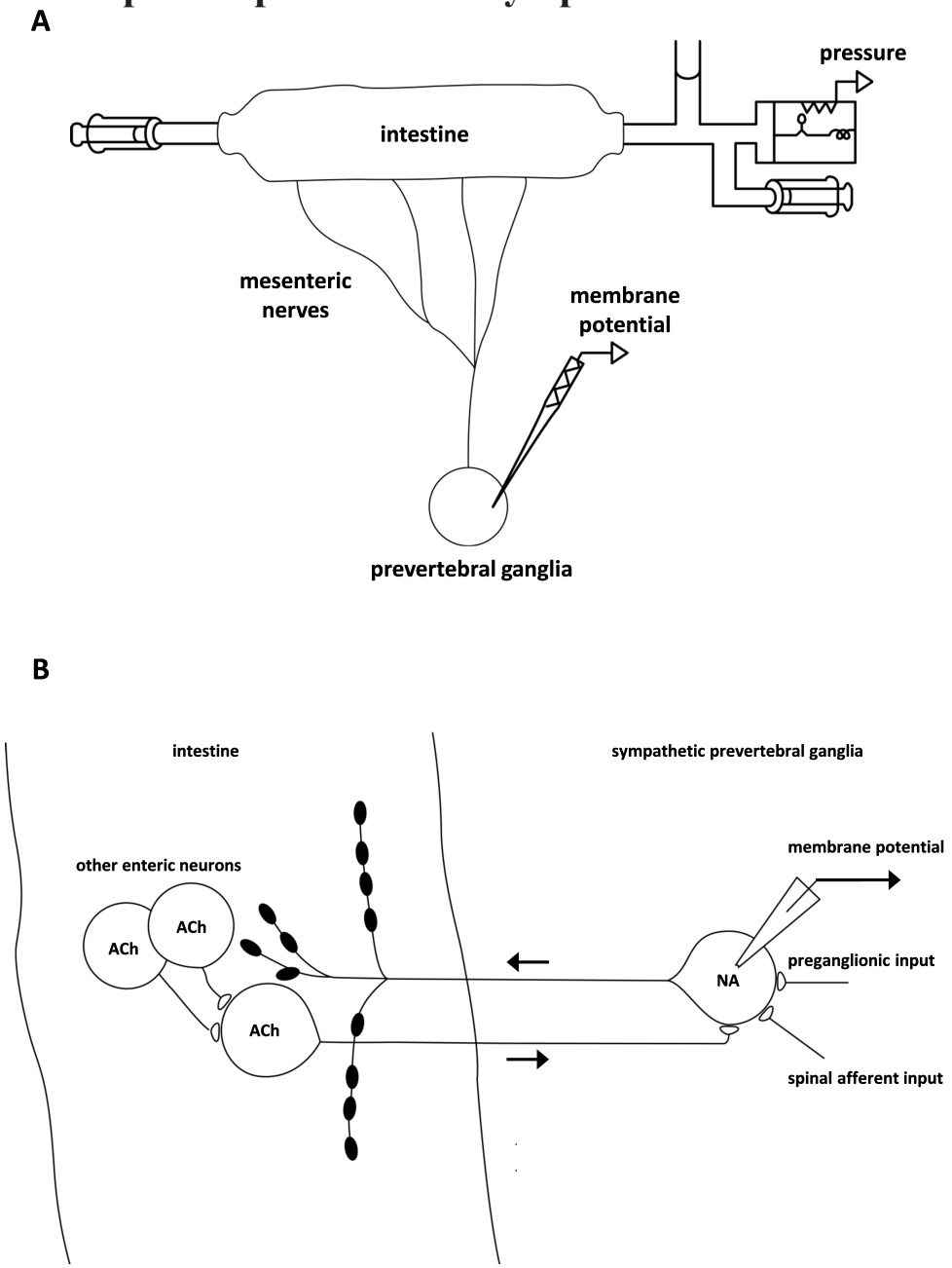

Figure 1.05

Tubular preparations of gut attached to sympathetic prevertebral ganglia via mesenteric nerves. Shown in **A** is a schematic diagram of an experimental setup used to record intracellularly from sympathetic neurons that receive synaptic inputs from viscerofugal neurons while monitoring pressure and volume in the gut lumen. Shown in **B** is a diagram showing the sources of synaptic inputs to sympathetic neurons. Viscerofugal neurons projecting axons out of the gut wall supply nicotinic inputs to sympathetic neurons which project axons back into the gut. In addition to inputs from viscerofugal neurons, sympathetic neurons also receive nicotinic inputs from preganglionic neurons projecting out of the spinal cord and peptidergic inputs from spinal sensory neurons that project axon collaterals into prevertebral ganglia. Abbreviations: ACh – acetylcholine; NA – noradrenaline. **A** adapted from (Kreulen and Szurszewski, 1979); **B** adapted from (Crowcroft et al., 1971).

Distension of the attached intestine with air or saline increases the rate of fast EPSPs in the IMG (Crowcroft et al., 1971a, Szurszewski and Weems, 1976, Weems and Szurszewski, 1977, 1978, Kreulen and Szurszewski, 1979b, Peters and Kreulen, 1986, Shu et al., 1987, Anthony and Kreulen, 1990, Bywater, 1993, Parkman et al., 1993, Stapelfeldt et al., 1993, Ermilov et al., 2004b), SMG (Kreulen and Szurszewski, 1979b, a, Miller and Szurszewski, 1997, Skok et al., 1998, Miller and Szurszewski, 2002, 2003), and CG (Kreulen and Szurszewski, 1979b, a, Stebbing and Bornstein, 1993) of mouse and guinea pig. The firing response to a prolonged distension is described as an initial rapid burst of activity that diminishes to a sustained rate of discharge that is significantly greater than basal levels. If synaptic or nicotinic transmission is blocked in the intestine, both ongoing and distension evoked fast EPSPs in prevertebral ganglia are depressed. Thus, it is likely that other enteric neurons are involved in driving viscerofugal neuron firing.

However, these reports also show that fast EPSPs are not completely abolished by these treatments. Bywater (1993) found that synaptic blockade in the gut caused more profound depression of the sustained discharge rather than the initial rapid burst of firing evoked by distension. The author speculated there were first- and second-order subtypes of viscerofugal neurons that directly and indirectly transduce responses to distension. However, this could also reflect the presence of spinal afferent neurons that release acetylcholine in the prevertebral ganglia, or that viscerofugal neurons may be both driven by other enteric neurons, and be capable of directly transducing mechanical stimuli. The issue has not been resolved.

Fast EPSPs and action potentials in decentralized sympathetic ganglia vary in frequency with propulsive contractile activity in the large intestine. This relationship is most apparent at higher intraluminal pressures in the guinea-pig (Weems and Szurszewski, 1977, Anthony and Kreulen, 1990) than in the mouse (Miller and Szurszewski, 1997, 2002, 2003). In mouse, increased intestinal volume coincides with large bursts of firing, prior to emptying contractions. Holding intraluminal volume constant, before or after fluid-emptying contractions, maintained increased and decreased viscerofugal neuron firing rates, respectively. These observations prompted the hypothesis that viscerofugal neurons are volume (circumferential length) sensors (Miller and Szurszewski, 2002, Szurszewski et al., 2002, Miller and Szurszewski, 2003). In support of this hypothesis, isometric relaxation of the gut with nifedipine did not affect frequency of fast EPSPs in sympathetic nerve cell bodies (Miller and Szurszewski, 2003).

Direct intracellular recordings of viscerofugal nerve cell bodies

Direct intracellular electrophysiological recordings have been made from viscerofugal neurons in the guinea pig colon (Sharkey et al., 1998). They had membrane potentials, time constants and input resistance which were consistent with enteric S, or type 3 neurons (Browning and Lees, 1996). All viscerofugal neurons received high amplitude fast EPSPs. Twelve of 19 impaled viscerofugal neurons fired 1-3 action potentials during direct depolarizing current pulses. The remaining one third of cells fired no action potentials. Unexpectedly, viscerofugal neurons did not receive slow excitatory postsynaptic potentials, unlike 67% of randomly impaled myenteric S neurons. In a single neuron tested, all electrically evoked fast EPSPs were nicotinic. The inhibitory effects of nicotinic receptor blockade in the gut on fast

EPSP frequency in sympathetic neurons suggested that viscerofugal neurons receive nicotinic synaptic input from other enteric neurons and therefore could function as interneurons (Crowcroft et al., 1971b, Szurszewski and Weems, 1976, Bywater, 1993, Parkman et al., 1993, Stebbing and Bornstein, 1993, Miller and Szurszewski, 1997, 2002). The study by Sharkey and colleagues (1998) provided the first direct evidence of this. A drawback of direct intracellular recordings is that the effects of physiological mechanical stimuli could not easily be tested because tissue movements may dislodge electrodes from impaled cells.

Direct extracellular recordings of viscerofugal neurons

Extracellular electrophysiological recordings from extrinsic nerve trunks to the gut could potentially provide a means to record directly viscerofugal neuron activity and the effects of physiological stimuli. However, in addition to viscerofugal neuron axons and those of autonomic efferent nerves, centrally projecting spinal and vagal sensory neurons also run through these nerve trunks, which obscure the source of the recorded action potentials. Interestingly, a single study by Pilipenko (1956) probably achieved the first ever electrophysiological recordings made from viscerofugal neurons. He recorded action potentials from the peripheral stumps of sectioned mesenteric nerves in the cat in vivo, 14-27 days after surgery. This is ample time to allow the degeneration of both spinal sensory neurons and autonomic efferent neurons in these nerve trunks, leaving only the intact axons of viscerofugal neurons. The author may not have been aware of this achievement at the time, since the recorded nerve activity was attributed to Dogiel type II neurons, without reference to the earlier work of Kuntz (1940) and Kuntz and Saccomanno (1944) who had unequivocally established the existence of peripheral sensory neurons that projected

to the sympathetic ganglia. Pilipenko (1956) showed that distending the small intestine increased the rate of action potential discharge recorded from mesenteric nerves. This is consistent with a mechanosensory function of viscerofugal neurons. Later, Cervero and Sharkey (1988) used neonatal capsaicin treatment in rats to ablate SP-containing spinal afferent neurons before making recordings from mesenteric nerves. These recordings were not significantly different to control recordings. In the absence of a method to positively identify viscerofugal neurons, solid conclusions could not be reached. The recorded neurons in capsaicin treated rats (6 units) were mechanosensitive and activated by acetylcholine (Cervero and Sharkey, 1988). Outside these studies, viscerofugal neurons have not been unequivocally identified in extracellular recordings of mesenteric nerve trunks. This is despite many studies having recorded from nerve trunks that should, in theory, contain axons of viscerofugal neurons.

Neurochemistry

Acetylcholine

The primary transmitter released from viscerofugal neurons onto sympathetic neurons in the prevertebral ganglia is acetylcholine. Nicotinic receptor antagonists added to the ganglion (but not intestine) block distension-evoked fast EPSPs in guinea and mouse sympathetic neurons (Crowcroft et al., 1971a, Kreulen and Szurszewski, 1979a, Peters and Kreulen, 1986, Love and Szurszewski, 1987, Anthony and Kreulen, 1990, Stapelfeldt et al., 1993, Miller and Szurszewski, 1997, Skok et al., 1998, Ermilov et al., 2004b). In the rabbit and guinea pig IMG, muscarinic receptors may also contribute to a slow EPSP (Mo and Dun, 1984,

Simmons and Dun, 1985). Tritiated acetylcholine release into decentralized IMG is significantly enhanced by distension of the intestine (Parkman et al., 1993). Nicotinic blockade in the gut diminishes the amount of acetylcholine released into the ganglia but does not affect SP release (contained within spinal afferent neurons), strongly implying acetylcholine is released from viscerofugal neurons. The cholinergic phenotype of viscerofugal neurons is supported by the detection of ChAT in retrogradely labelled viscerofugal neuron cell bodies throughout the gut in guinea pig (Mann et al., 1995, Sharkey et al., 1998, Tassicker et al., 1999a, Zagorodnyuk and Brookes, 2000) and rat (Furness et al., 2000b). Furthermore, the terminals of viscerofugal neurons are immunoreactive for ChAT and VAChT (Gibbins et al., 2003a). Viscerofugal neuron terminals contain less immunoreactivity for ChAT and VAChT than preganglionic neuron terminals within the same ganglion. This probably reflects their weaker, subthreshold synaptic inputs compared to sympathetic preganglionic inputs (Gibbins et al., 2003a).

Peptide transmitters

Neuropeptides VIP, PACAP and probably peptide histidine isoleucine are released by viscerofugal neuron terminals onto sympathetic neurons. VIP has been detected in retrogradely labelled viscerofugal neuron cell bodies throughout the gut in guinea pig (Hamaji et al., 1989a, Kuramoto and Furness, 1989, Furness et al., 1990c, Messenger and Furness, 1991, 1992, 1993, Tassicker et al., 1999a, Ermilov et al., 2004b), pig (Barbiers et al., 1993, Timmermans et al., 1993, Barbiers et al., 1994), rat stomach (Lee et al., 1986), and dog colon and rectum (Li and Masuko, 1997). PACAP has been detected in viscerofugal neuron cell bodies in the guinea pig colon and in cholinergic terminals in IMG (Ermilov et al., 2004b). VIP and peptide histidine

isoleucine contained within putative viscerofugal neuron terminals has been identified in guinea pig (Costa and Furness, 1983, Dalsgaard et al., 1983, Lindh et al., 1988, Masuko and Chiba, 1988, Webber and Heym, 1988) and rat (Hamaji et al., 1989b). Detection of VIP-IR varicosities has been used for identification of viscerofugal neuron terminals in CG (Anderson et al., 2002). Radioimmunoassay of VIP and PACAP show their release into prevertebral ganglia is significantly increased by intestinal distension (Parkman et al., 1993, Ma and Szurszewski, 1996a, Ermilov et al., 2004b). Distension-evoked VIP release is abolished by nicotinic receptor antagonists added to the gut and unaffected by *in vivo* capsaicin pretreatment, implying VIP is released from viscerofugal neurons but not spinal afferent neurons (Parkman et al., 1993, Ma and Szurszewski, 1996a). The effects of VIP and PACAP are mediated by GPCRs: VPAC and the PAC-1 receptors, respectively, occurring pre- and postsynaptically (Ermilov et al., 2004b). The excitability of sympathetic neurons is enhanced by VIP and PACAP, which evoke slow membrane depolarizations and increased input resistance, significantly increasing the efficacy of nicotinic neurotransmission and the likelihood of postsynaptic action potentials (Love and Szurszewski, 1987, Ermilov et al., 2004b). Both peptides also depolarize presynaptic terminals, evoking exocytosis of acetylcholine, increasing the rate of nicotinic fast EPSPs received by sympathetic neurons (Ermilov et al., 2004b). Exogenous VIP and PACAP in the IMG (but not the gut) can inhibit ongoing colonic contractions, and relax the gut wall (Ermilov et al., 2004b).

Aims of this project and summary of findings

Early morphological and functional studies demonstrated that axons of viscerofugal neurons are present in mesenteric nerve trunks, and are activated by physiological stimuli, including distension (Kuntz, 1938, Kuntz, 1940, Kuntz and Saccomanno, 1944). Electrophysiological recordings from sensory C-fibres in visceral nerves were made in the 1930's (Adrian et al., 1932). Early recordings demonstrated that both visceral and cutaneous sensory neurons were sensitive to a variety of stimuli including chemicals, mechanical forces, and temperature. In theory, action potentials conducted along the axons of viscerofugal neurons ought to be detected in electrophysiological recordings from mesenteric nerve trunks. This has not yet been shown, probably because of the confounding presence of spinal sensory neurons in the same nerve trunks. These have made it difficult to identify the origin of recorded nerve activity with certainty (Cervero and Sharkey, 1988). We asked whether the action potentials of viscerofugal neurons can be detected in mesenteric nerves to the gut.

In the present studies we aimed to identify the action potentials of viscerofugal neurons in electrophysiological recordings from mesenteric nerve trunks to the gut. In **chapter 2**, we show that organotypic culture (Brookes and Costa, 1990, Song et al., 1995) may be used to selectively ablate extrinsic nerves in isolated preparations of guinea pig distal colon. After organ culture, extracellular electrophysiological recordings of mesenteric nerve trunks revealed action potential firing consistent with single viscerofugal neurons. We show for the first time that action potentials of viscerofugal neurons may be identified in recordings from mesenteric nerve trunks.

Additionally we describe their firing behaviour and relationship with mechanical stimuli and contractility in the gut. Some of these studies have been published recently (Hibberd et al., 2012b).

Characterization of cutaneous sensory neurons is more comprehensive than visceral sensory neurons, in part because many cutaneous endings are morphologically specialized structures that are discriminable using basic histological techniques. This enables localized stimuli applied during physiological and electrophysiological investigations to be correlated with the sensory endings, thus linking structure to function (Gray and Matthews, 1951, Iggo and Andres, 1982). Sensory endings in the gut on the other hand are simpler in structure. They usually have fine unmyelinated fibres that ramify extensively among the dense nerve networks of intrinsic enteric nerves and extrinsic sympathetic and parasympathetic efferent nerves. Thus, sensory nerves are largely indistinguishable without using neuroanatomical labelling approaches. Specific neuroanatomical tracing methods have been developed that are suitable for use in isolated preparations of gut (Tassicker et al., 1999a). As described previously, paired physiological mapping of mechanosensory transduction sites with rapid neuroanatomical tracing has enabled selective structure and function correlational studies of extrinsic sensory neurons in the gut (Zagorodnyuk and Brookes, 2000). We asked whether similar methods could be used to identify action potentials of viscerofugal neurons in recordings from mesenteric nerve trunks in fresh preparations, where extrinsic sensory neurons were present.

In **chapter 3** we aimed to make identified recordings from viscerofugal neurons in mesenteric nerve trunks to “fresh” preparations of distal colon. These preparations

also contain spinal sensory neurons (Song et al., 2009). Thus, viscerofugal neurons were required to be discriminated. We demonstrated that focal ejection of a nicotinic receptor agonist stimulated firing activity detected in mesenteric nerves that correlated with local viscerofugal nerve cell bodies. These studies revealed viscerofugal neurons were identifiable in such preparations by their combination of sensitivity to a nicotinic receptor agonist and insensitivity to capsaicin (which activates the majority of spinal sensory neurons in this region; Song et al., 2009). These data are exploited in **chapter 4** to characterize the effects of mechanical stimuli on single viscerofugal neurons in detail. As described previously, indirect studies of populations of viscerofugal neurons suggested that a proportion may be directly mechanosensitive. We found that all identified viscerofugal neurons are directly mechanosensitive. In addition we show that viscerofugal neurons are sensitive to changes in gut wall length, rather than tension and that they may be directly activated by distension in both the circumferential and longitudinal axes. Results presented in **chapter 3** and **chapter 4** have been published (Hibberd et al., 2012c).

Observations made in **chapters 2** and **chapter 3** that suggested there were two types of colonic viscerofugal neurons based on their morphology. In **chapter 5** we aimed to characterize these populations of viscerofugal neurons further. We show that viscerofugal nerve cell bodies labelled in fine detail by biotinamide had either a simple or lamellar morphology, using previously described classifications (Furness et al., 1988). Immunohistochemical analysis of ChAT and NOS content revealed that viscerofugal neurons with lamellar morphology predominantly contained both ChAT and NOS, while those with simple morphology contained mainly ChAT alone. In

addition we present evidence that these two groups of viscerofugal neurons are differentially distributed along the gut (longitudinally) and across the gut wall (circumferentially).

As described previously, viscerofugal neurons also function as interneurons. They receive fast synaptic inputs from other enteric neurons (Sharkey et al., 1998). Prominent nicotinic inputs were observed in extracellular recordings described in **chapter 2**. The sources of these inputs are unknown. In **chapter 6**, we aimed to map the location of other enteric neurons that supply synaptic inputs to colonic viscerofugal neurons. We made targeted intracellular recordings from viscerofugal neurons retrogradely labelled by DiI applied to mesenteric nerves. By focally activating myenteric neurons, we demonstrated that viscerofugal neurons receive fast synaptic inputs from both ascending and descending enteric neural pathways (in press; Hibberd et al., 2014).

The major themes arising from the results of all studies presented here are presented and discussed in **chapter 7**.