MECHANISMS OF R-TYPE CALCIUM CHANNEL REGULATION IN THE ENTERIC NERVOUS SYSTEM

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ABSTRACT

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The enteric nervous system (ENS) regulates gastrointestinal function, and calcium channels are crucial to ENS function. There are distinct classes of calcium currents. L-type currents require a strong depolarization for activation, are long lasting, and are the main calcium currents recorded in muscle and endocrine cells. P/Q-type, N-type, and R-type currents, also require strong depolarizations for activation. They are expressed primarily in neurons, where they initiate neurotransmission at most fast synapses. T-type calcium currents are activated by weak depolarizations and found in neurons and cardiac myocytes. R-type calcium currents were previously recorded from guinea-pig myenteric neurons suggesting that R-type calcium channels may be important in the ENS. The aim of my dissertation was to determine their functional significance in the ENS. I investigated the role of R-type channels in the myenteric plexus at four different levels: 1) the cellular level by mapping α1E-subunit immunoreactivity in various regions of the GI tract; 2) intracellular recordings from myenteric neurons to determine whether R-type channels mediate slow synaptic excitation in intrinsic primary afferent (AH-type) neurons, and/or whether calcium entry via non-R-type channels mediate fast synaptic excitation in interneurons and motorneurons (S-type); 3) functional studies of R-

type channel-mediated neuromuscular transmission because excitatory and inhibitory motor express α1E immunoreactivity; and 4) physiologically integrative level by analyzing the effects of R-type calcium channel antagonists on peristalsis. My studies revealed novel mechanisms of R-type calcium regulation in the ENS. 1) R-type channel immunoreactivity was found in intrinsic primary afferent nerve endings, in nerve terminals of ascending interneurons, and on excitatory and inhibitory motorneurons that supply the muscle layers. 2. The R-type calcium channel antagonist $NiCl_2$ inhibited the calcium shoulder and the amplitude of the late afterhyperpolarization in AH neurons. $NiCl_2$ also acted at nerve terminals of fully cholinergic ascending interneurons to inhibit fast ganglionic neurotransmission. 3) The R-type channel couples to nitric oxide synthesis and release from nerve terminals of inhibitory motorneurons. 4) $NiCl_2$ applied to isolated ileal segments *in vitro* impaired peristalsis. Taken together, these results suggest an important role for R-type channels as mediators of neurotransmitter release or as transducers of other synaptic events in myenteric neurons. Success with a selective activator of chloride channels that relieves chronic idiopathic constipation, suggests that treatments with ion channel agonists or antagonists may help alleviate motility disorders. The R-type calcium channel may therefore be a potential drug target in combating motility disorders.

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LIST OF ABBREVIATIONS

β-nicotinomide adenine dinucleotide phosphate	β-NAD
Acetylcholine	ACh
After hyperpolarization	AHP
Area under the curve	AUC
Autonomic nervous system	ANS
Cadmium Chloride	$CdCl_2$
Calbindin	Calb
Calretinin	Calret
Central nervous system	CNS
Chloride	Cl^-
Choline acetyltransferase	ChAT
Endoplasmic reticulum	ER
Enteric nervous system	ENS
Excitatory junction potential	EJP
Fast excitatory post synaptic potential	fEPSP
Functional Gastrointestinal disorder	FGID
Gamma-aminobutyric acid	GABA
Gastrointestinal	GI
Immunoreactivity	ir
Inhibitory junction potential	IJP
Interstitial cells of Cajal	ICC

Intrinisic primary afferent neurons	IPANs
Irritable bowel syndrome	IBS
Longitudinal muscle myenteric plexus	LMMP
Migrating myoelectric complex	MMC
Neurokinin	NK
Neuropeptide Y	NPY
Neurotransmitter	NT
Nickel Chloride	$NiCl_2$
Nicotinic acetylcholine receptors	nAChRs
Nitric oxide synthase	NOS
Nitric oxide	NO
Nitro-L-arginine	NLA
Non adrenergic non cholinergic	NANC
Norepinephrine	NE
Omega-agatoxin	ω-ATX
Omega-conotoxin	ω-CTX
Phosphate buffered saline	PBS
Potassium	K^+
pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate	(PPADS)
Serotonin/5-Hydroxytryptamine	5-HT
Slow excitatory post synaptic potential	sEPSP
Sodium	Na^+
Substance P	SP

Tertiary plexus	tp
Tetrodotoxin	TTX
Tyrosine hydroxylase	TH
Vasoactive intestinal peptide	VIP
Water	H_2O

CHAPTER 1 GENERAL INTRODUCTION

THE NERVOUS SYSTEM

The nervous system is composed of the central nervous system (CNS) which includes the brain and spinal cord, and the peripheral nervous system which comprises peripheral nerves, receptors, effectors and ganglia that exist between the CNS and the periphery. In response to changes in the external environment or the internal milieu, the brain can detect, interpret and respond to these changes. This is achieved by the transduction of sensory information in the form of electrical impulses from the periphery to the CNS that are then transmitted via neurotransmitters to muscles, organs and glands. It is this capability of nerve cells to wire themselves into a network and create emergent outputs that are not simply predicted by the inputs, that is testament to the exquisite complexity of the nervous system. The British physiologist Sir Charles Sherrington referred to this elegance of neural control when he famously described the integrative function of the nervous system. That is, the ability of the nervous system to effect appropriate responses upon receiving different types of information is due to its functional interconnectivity and sensitivity. Towards this end, the manifestation of plasticity within neural circuits as a consequence of sensory inputs or motor outputs is an inherent property of our continually changing nervous system that allows us to adapt our behavior, acquire new tasks, remember past events and recognize objects. In this regard, the concept of how synaptic transmission shapes the connectivity and functions of neural circuits is crucial to understanding fundamental mechanisms of nervous system function.

STRUCTURE AND FUNCTION OF A NEURON

The great neuroanatomist Santiago Ramón y Cajal is most famous for being the primary proponent and developer of the Neuron Doctrine which states that "a neuron is an anatomically and functionally distinct cellular unit that arises through differentiation of a precursor neuroblast cell" (Guillery, 2005; Glickstein, 2006). The nerve cell, or neuron, is the structural and functional unit of the nervous system. Although several anatomical types of neurons exist, for example, pyramidal, granule, Purkinje and myenteric neurons, they all have several common features. Each neuron has a soma or cell body that contains a single nucleus which contains the genetic information for protein synthesis. The cell body is responsible for integration of incoming information. Many cellular organelles such as mitochondria, ribosomes, endoplasmic reticulum, the Golgi complex, lysosomes, and cytoskeletal proteins, reside within neurons (Shepherd, 1994). Connected to the cell body are dendrites which collect afferent information and serve to increase the surface area via which neurons can communicate. The axon begins at the soma and extends to surrounding and distant nerve cells. Their function is neurotransmission through action potentials that encode signaling information. Neurons can be differentiated according to their configuration (multipolar or unipolar) and function (sensory, interneurons and motorneurons). Sensory neurons have typically unipolar processes, while interneurons and motorneurons have multipolar processes. The classical definition of a sensory neuron is a neuron that transmits information into the CNS, and therefore has an afferent modality. Efferent (motor) neurons transmit

information to effector cells, such as muscle cells within the gastrointestinal (GI) tract. The function of interneurons is integration of information in the communication pathway between sensory and motorneurons. Neurons therefore are functionally distinct entities that have specific roles that depend on their intrinsic properties (Jessell & Kandel, 1993).

NEUROTRANSMITTERS, NEUROPEPTIDES AND SYNAPTIC TRANSMISSION

There has been an increasing and active interest in the various chemicals utilized by neurons. Advances in this area have led to an understanding of their structure, biochemical and pharmacological characteristics, functions, and how they serve as diffusible chemical messengers between neurons. Several benchmarks need to be met before a chemical is considered to be a neurotransmitter: 1) it must be synthesized and released from neurons, 2) it must act at the postsynaptic cell to produce specific changes in that cell, such as changes in input resistance, 3) its action should be blocked by known antagonists, and 4) there must be a mechanism through which its action is terminated (Deutch & Roth, 1999). Neurotransmitters are subdivided into the classical transmitters and neuropeptides.

Classical neurotransmitters

The classical neurotransmitters are small, low molecular weight chemicals that are produced in nerve terminals. Specific enzymes catalyze the biosynthesis of neurotransmitters before they are packaged into synaptic vesicles by a vesicular transporter. Acetylcholine, the biogenic amines (norepinephrine, epinephrine, dopamine, serotonin, histamine), some amino acids (glutamate and aspartate) and the ubiquitous CNS neurotransmitter gamma-aminobutyric acid (GABA) are all classical neurotransmitters (Amara & Arriza, 1993; Amara & Kuhar, 1993). Although neurotransmitter distribution is heterogeneous, specific neurotransmitters modulate activity in different regions within the CNS and peripheral nervous system. The basic stages in classical chemical neurotransmission are: a) synaptic vesicle (~ 50 nm in diameter) synthesis and docking of vesicle loaded with neurotransmitters at the active zone; b) action potential initiation at the axon hillock and invasion of the synaptic terminal by the arrival of the depolarizing action potential; c) calcium entry though voltage-gated calcium channels; d) fusion of vesicles with the plasma membrane and release of neurotransmitter into the synaptic cleft; e) binding of neurotransmitters to specific receptors; f) transduction of the signal resulting in a postsynaptic response; g) termination of neurotransmission by active reuptake of the transmitter at presynaptic nerve terminals or metabolic inactivation by degradative enzymes in the synaptic cleft (Jessell & Kandel, 1993; Kelly, 1993).

Neuropeptides

Neuropeptide synthesis is energetically more demanding than small-molecule transmitter synthesis. Whereas the small molecule neurotransmitters are synthesized in neuronal nerve terminals, peptide transmitters are synthesized in the neuron cell body. The messenger RNA (mRNA) coding for the neuropeptide is translated into a peptide precursor on ribosomes in the rough endoplasmic reticulum (ER) within the cell body. The pre-propeptide is then transported to the Golgi complex where it is packaged into large dense-core vesicles (~ 75-150 nm in diameter). These vesicles are then transported by fast axonal transport from the cell body to the nerve terminal. Importantly, neuropeptides can undergo post-translational modifications including glycosylation, methylation and sulfation. This has implications for the stability of the mature peptide. Further, large-dense core vesicles do not always undergo exocytosis at the active zones, but can release neuropeptides at extra-synaptic regions. Nonetheless, calcium influx into the nerve terminal is a critical determinant of neuropeptide release. To this end, the synaptic machinery necessary for peptide transmitter release is highly sensitive to calcium, more than that of the small molecule transmitters. Peptide transmitters therefore do not require large increases in intracellular calcium concentrations for exocytosis (Jessell & Kandel, 1993; Kelly, 1993; Goda & Davis, 2003).

THE AUTONOMIC NERVOUS SYSTEM

The autonomic nervous system (ANS) is the division of the peripheral nervous system that is responsible for involuntary sensory and motor control of visceral organs. The function of autonomic nerves is regulated by networks of nuclei that are located in the brainstem, hypothalamus and forebrain (cerebral cortex, basal ganglia, limbic system, thalamus and olfactory bulbs and tracts). These brain regions generally do not impart conscious sensation from visceral reflexes (Langley, 1922). The ANS is responsible for the co-ordination of key survival functions, such as breathing, heart-rate, digestion, and urogenital regulation. The ANS has three divisions: sympathetic, parasympathetic, and enteric (Richerson, 2003). Communication between the CNS and the sympathetic and parasympathetic pathways occurs through preganglionic neurons that are located in nuclei in the brainstem and spinal cord, and postganglionic neurons found in peripheral ganglia that receive synaptic input from preganglionic neurons. The cell bodies of sympathetic preganglionic neurons are found in the intermediolateral cell column at levels T1 through L3 in the thoracic and lumbar regions respectively of the spinal cord. Axons from these preganglionic cell bodies exit the spinal cord through the ventral root and enter the white myelinated rami. They then synapse within a sympathetic chain (paravertebral) ganglion, or may exit that paravertebral ganglion to synapse on postganglionic sympathetic neurons in prevertebral ganglia (celiac, superior mesenteric, and inferior mesenteric). Parasympathetic preganglionic neuronal cell bodies are found in the craniosacral division of the autonomic nervous

system. Preganglionic axons travel in the vagus, oculomotor, facial and glossopharyngeal cranial nerves and synapse with postganglionic parasympathetic neurons located mostly within target organs. Both sympathetic and parasympathetic preganglionic neurons use acetylcholine (ACh) as the transmitter. While postganglionic parasympathetic neurons also release ACh on to final target cells, most postganglionic sympathetic neurons release primarily norepinephrine (NE) onto the viscera. The sympathetic and parasympathetic divisions of the ANS produce opposite effects on the viscera (Richerson, 2003)

INNERVATION OF THE GASTROINTESTINAL TRACT

Extrinsic innervation of the ENS

Most enteric neurons are not directly innervated by the CNS (Galligan, 2002b). This salient feature of the GI tract implies that the gut must manifest this independence from CNS input via intact intrinsic neural networks. The small intestine and the proximal region of the large intestine are less dependent on sympathetic and parasympathetic innervation than the esophagus, stomach and distal colon (Goyal & Hirano, 1996). The ENS nonetheless does receive sympathetic, parasympathetic, extrinsic vagal and spinal neuronal input (Grundy *et al.*, 2006). Parasympathetic innervation of the gut occurs via the vagus and pelvic nerves. The myenteric plexus receives the majority of vagal efferent input, and primary afferent neurons with cell bodies in nodose ganglia branch throughout the plexus forming synapses with enteric neurons (Kirchgessner & Gershon, 1989; Holst *et al.*, 1997). Cell bodies of spinal afferent neurons reside

in dorsal root ganglia and their nerve endings mainly innervate the large intestine. Spinal afferents relay nociceptive information about the GI tract to the CNS (Cervero, 1994). These extrinsic sensory nerves, taken together, are important determinants of the physiological status of the gut, and do so on a moment to moment basis. Parasympathetic innervation of specific regions in the GI tract could mean that activation of nicotinic acetylcholine receptors (nAChRs) by nicotine needs to be strong in order for the reflex pattern of emesis and defecation to occur. Sympathetic innervation of the gut wall occurs though postganglionic neurons located with prevertebral ganglia. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for the synthesis of catecholamines including NE (Grima *et al.*, 1985). Based on the presence of TH in sympathetic nerve endings, three subtypes of branching, varicose sympathetic axons exist that supply the ENS: 1) those that target blood vessels contain TH and neuropeptide Y (NPY); 2) those that provide input to the submucosa contain TH and somatostatin; and, 3) those supplying myenteric neurons contain only TH (Macrae *et al.*, 1986). In fact, within the myenteric plexus, NE acting at α_2-adrenoreceptors blocks neurotransmitter release from ACh-containing myenteric neurons, leading to an inhibition of motility (Nishi & North, 1973b; Hirst & McKirdy, 1974; Frigo *et al.*, 1984). It has been suggested that the greater innervation of specific regions of the ENS may be because of an override system that the CNS has put into place to be able to control local gut motility.

Intrinsic innervation: the ENS

The mammalian small intestine contains approximately 100 million neurons, about as many neurons as that found in the spinal cord (Gershon, 1997). The ENS (Fig. 1.1) consists of two ganglionated plexuses: the myenteric plexus which is found in between the longitudinal and circular smooth muscle, and which innervates the GI smooth muscle, and the submucosal plexus located between the circular muscle and the mucosa, and which regulates neuroimmune interactions, local blood flow, secretion and absorption across the GI epithelium (Kunze & Furness, 1999). The myenteric and submucosal plexuses are connected by numerous fiber bundles that facilitate secretomotor and motility reflexes (Furness *et al.*, 1990). Within each plexus, the ganglia are connected via interganglionic fiber tracts, and these two nerve plexuses therefore provide the basis for all neural reflex activity in the GI tract. These reflexes are mediated by three classes of enteric neurons: intrinsic primary afferent neurons (IPANs) /sensory neurons, interneurons and motor neurons.

Between 1895 and 1899, the Russian histologist AS Dogiel conducted extensive anatomical experiments in which he traced and characterized the morphologies of human and guinea pig enteric neurons (Table 1). He found three distinct subtypes, now known as Dogiel types I, II and III. Dogiel type I neurons (Fig. 1.2) are flat, stellate cells with lamellar dendrites, are unipolar, and with a cell body diameter between 13 and 35 μm in length and 9 and 22 μm in width. Dogiel type II neurons are larger in size (from 22-47 μM) and have round/oval cell bodies (Brehmer *et al.*, 1999). A prominent feature of IPANs is that they are

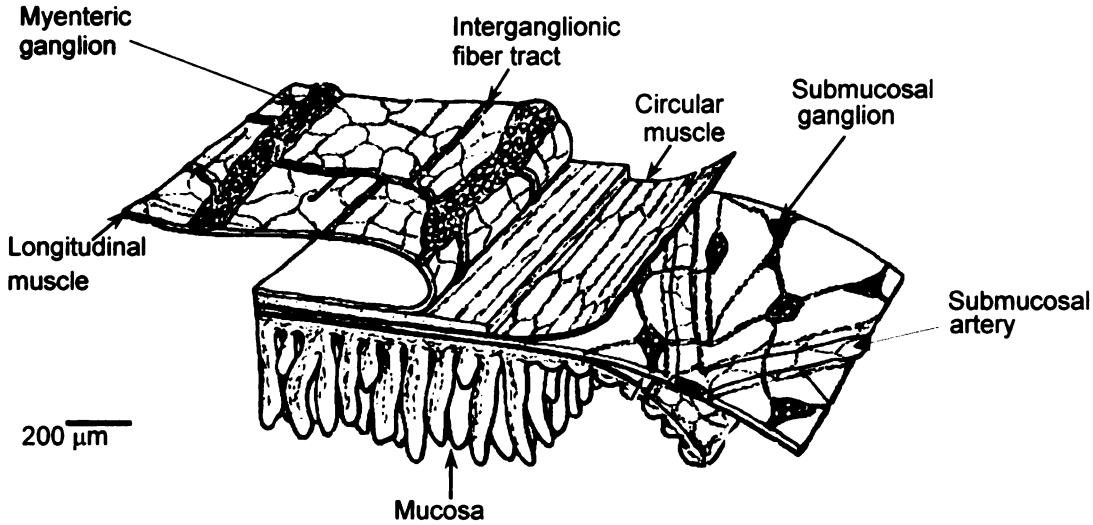


Figure. 1.1. The enteric nervous system (ENS). The mucosa with villi protrudes towards the intestinal lumen. There are two ganglionated plexuses: the myenteric plexus located between the longitudinal and circular muscle layers that controls motility, and the submucosal plexus located between the mucosa and the circular muscle. The submucosal plexus controls blood flow of arterioles across the epithelial cell layer, as well as secretion and absorption (Furness, 2006a)

Table 1.1. Descriptions of enteric neuronal morphologies as described by Dogiel (after Brehmer *et al.*, 1999)

Types and numbers of processes	Dendrites	Axonal course
I 4-20 dendrites, 1 axon	Branch and end within the ganglion of origin. Short, thick, flat, wih varicosities, lamellar.	Through neighboring ganglia, with collaterals sporadically to the muculature
II 1-16 dendrites, 1 axon	Leave ganglion of origin. Structural resemblance to axons. Ramify into long, thin, smoothly contoured branches.	To other ganglia
III 2-10 dendrites, 1 axon	Ramify and end within ganglion of origin.	Through other ganglia

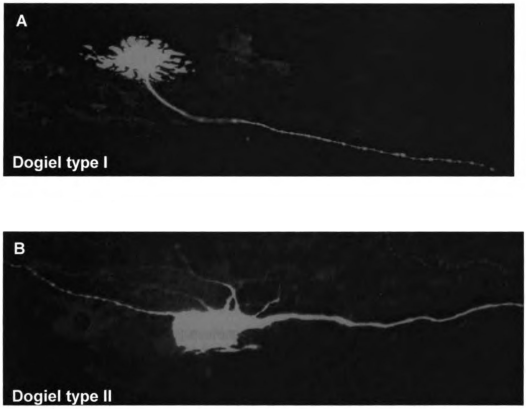


Figure. 1.2. Morphological characteristics of enteric neurons. (A) Dogiel type I neurons are flat with short lamellar dendrites and a single long axon. These neurons are the interneurons and motorneurons. (B) Dogiel type II neurons have smooth surfaces with several dendrites and multipolar axonal processes. These neurons are the IPANs (Wood, 2006a).

multipolar. In the guinea pig small intestine, IPANs constitute 26% of the total number of myenteric neurons and 11% of submucosal neurons (Furness *et al.*, 2004). Dogiel type III neurons are filamentous neurons with long branched processes (Lomax *et al.*, 1999; Furness, 2006a).

IPANs

IPANS have Dogiel II morphology (Kirchgessner & Gershon, 1988; Furness *et al.*, 1998; Pan & Gershon, 2000). This has generated some controversy since IPANs do not actually convey any conscious sensations from the intestine and are therefore not really sensory afferent neurons (Wood, 2006a). However, within the context of the intestine, the definition of IPANs are: sensory neurons in the wall of the gut that transduce mechanical or chemical stimuli into electrical activity within a local reflex arc (Furness *et al.*, 2004; Brookes & Costa, 2006). The term IPANs will be used during the course of my dissertation. Most IPANs synapse with other IPANs to form positive feedforward loops. This means that excitation within these loops or networks can build rapidly so that simultaneous activation of entire neural networks around the intestinal circumference is elicited. These bursts of excitation then drive the entire reflex circuitry towards threshold leading to the subsequent activation of interneurons and motorneurons that supply GI smooth muscle. The result is ascending contraction or descending relaxation. The axons of IPANS project from the myenteric and submucosal plexuses to the mucosal layer (Furness *et al.*, 2004). The mucosa also contains enterochromaffin (EC) cells that, when activated via

chemical or mechanical (Kunze *et al.*, 1998) stimulation of the mucosa, release 5-hydroxytryptamine (5-HT) (Kirchgessner *et al.*, 1992; Mawe *et al.*, 2006) and the other sensory mediators such as ATP (Bertrand, 2003). This indirect activation of IPANs occurs through 5-HT acting at 5-HT_3 (Bertrand *et al.*, 2000) and 5-HT_{1P} (Kirchgessner *et al.*, 1992) receptors on myenteric and submucosal AH nerve terminals respectively, and ATP acting at P2X receptors (Furness *et al.*, 2004) elicit antidromic action potentials that propagate into the soma of the IPAN. The IPAN then provides synaptic input to interneurons, which in turn activate motor neurons to produce an excitation of GI smooth muscle oral to and an inhibition anal to the stimulus site, along distinctly polarized neural pathways (Galligan, 2002b). However, it should be remembered that the mucosal processes of IPANs are also activated directly, by chemical stimuli applied to the mucosa (Kunze *et al.*, 1995; Brookes & Costa, 2006). According to Brookes and Costa (2006), a number of subsets of IPANs exist: 1) submucosal IPANs that respond to mechanical stimulation of the mucosa; 2) myenteric IPANs that respond to chemical stimulation of the mucosa; and 3) myenteric IPANs that are sensitive to tension along the gut wall as they posses mechanosensitive ion channels (Kunze *et al.*, 1998; Kunze *et al.*, 2000). Both myenteric and submucosal IPANs are neurochemically coded by the calcium-binding protein, calbindin, and this is the best marker known for IPANs (Pompolo & Furness, 1988).

Myenteric interneurons

The plasticity of the enteric neural circuitry is reflected by the presence of interneurons that are organized in a chain-like fashion along polarized pathways. All interneurons have Dogiel type I morphology (Furness *et al.*, 1993). In the guinea pig ileum, there are four classes of interneurons: a single class of ascending interneurons and three classes of descending interneurons (Brookes, 2001). Interneurons communicate via fast synaptic transmission (Galligan, 2002b). Ascending interneurons comprise 5 % of all myenteric neurons and stain positive for choline acetyltransferase (ChAT)/Calretinin/Substance P (SP)/enkephalin (Brookes *et al.*, 1997). Their primary neurotransmitter is acetylcholine (ACh). These neurons provide synaptic input to other ascending interneurons and to excitatory circular and longitudinal muscle motorneurons (Pompolo & Furness, 1993, 1995). Their functional importance is to allow rapid spread of excitation and for the effective co-ordination of motor responses of the gut smooth muscle.

In the guinea pig ileum, the first type of descending interneuron is immunoreactive for ChAT and the peptide hormone somatostatin. This class (4 % of myenteric neurons) is believed to be involved in the migrating myoelectric complex (MMC) (Furness, 2000). The second class of descending interneurons (2%) includes those that participate in the secretomotor and motility reflex. These neurons are immunoreactive for ChAT and 5-HT (at 5-HT_3 receptors) and use both ACh and 5-HT as their primary neurotransmitters. Because at least 90% of the body's 5-HT is localized within EC cells, this may represent a significant

source of the 5-HT that cause fast synaptic excitation within this interneuronal pathway. The third group of descending interneurons (5%) are those involved in the local reflex pathway. These neurons are neurochemically coded with ChAT/nitric oxide synthase (NOS)/vasoactive intestinal peptide (VIP)/gastrin-releasing peptide (GRP)/NPY, and their principal transmitters are ACh and ATP (Furness, 2000; Brookes, 2001). Evidence indicates that ACh and ATP may be the important transmitters released from nerve terminals of these neurons (LePard *et al.*, 1997). The heterogeneity of three classes of descending interneurons may be to allow diverse communication patterns to take place, from the spread of inhibition along long (~ 40 mm) distances of the gut, to co-ordinating the MMC as well as being crucial mediators of relaxation (Brookes & Costa, 2006).

Myenteric motorneurons

All enteric motorneurons have Dogiel Type I morphology. Excitatory and inhibitory circular and longitudinal muscle motorneurons produce GI smooth muscle contraction and relaxation. In the guinea pig ileum, excitatory circular and longitudinal muscle motorneurons constitute 12 % and 25 % of the total number of myenteric neurons respectively. Excitatory circular muscle motorneurons have the neurochemical phenotype ChAT/SP/enkephalin, while excitatory longitudinal muscle motorneurons immunostain positive for ChAT/calretinin/SP (Brookes & Costa, 1990). ACh and SP are the main neurotransmitters released from these neurons. Inhibitory circular and longitudinal muscle motorneurons make up 16 %

and 2 % respectively of the neurons in the myenteric plexus. These neurons have the chemical phenotype NOS/VIP/Pituitary adenylate cyclase-activating peptide (PACAP)/gamma-aminobutyric acid (GABA) (Costa *et al.*, 1986a; Furness *et al.*, 1987; Brookes & Costa, 1990). Circular muscle motorneurons are important components that are crucial to peristalsis. Longitudinal muscle motorneurons may be responsible for fine-tuning motility in a graded manner (Brookes & Costa, 2006). Substantial evidence suggests that motorneurons in each of the muscle layers are activated synchronously (Hennig *et al.*, 1999; Spencer *et al.*, 2002).

ELECTROPHYSIOLOGICAL CHARACTERISTICS OF MYENTERIC NEURONS

IPANs are electrophysiogically characterized as AH neurons (Nishi & North, 1973a; Hirst *et al.*, 1974). The defining features of AH neurons are: a resting membrane potential of approximately -70 mV, a prominent calcium shoulder on the repolarizing phase of the action potential, the long and late afterhyperpolarization (late AHP), and an absence of anodal-break excitation at the offset of a hyperpolarizing pulse (Furness *et al.*, 2004; Wood, 2006a). The major component of the upstroke during the AH somal action potential (approximately 75-110 mV in amplitude) is a tetrodotoxin (TTX)-sensitive sodium current, and a TTX-insensitive calcium current is most prominent on the falling phase of the action potential (North, 1973; Hirst *et al.*, 1985; Rugiero *et al.*, 2003). The longer-lasting calcium current is carried by N-type (α1B) calcium

channels and generates a calcium-shoulder during the falling phase of the action potential (Rugiero *et al.*, 2002). R-type calcium channels may also contribute to this calcium conductance (Rugiero *et al.*, 2002). Calcium entry induces further calcium release by activating ryanodine receptors on the endoplasmic reticulum (Hillsley *et al.*, 2000; Vogalis *et al.*, 2001). The action potential is followed by a characteristic long-lasting (2-30 s) afterhyperpolarization (AHP) mediated by the amplified level of intracellular calcium that activates an intermediate-conductance (IK) calcium-dependent potassium channel found only within the cell bodies (Nishi & North, 1973a; Morita *et al.*, 1982; Furness *et al.*, 2003). Since the AHP limits the action-potential firing rate within the cell bodies of AH neurons, these neurons cannot fire trains of action potentials and therefore control the frequency of sensory information output to interneurons and motorneurons (Hirst *et al.*, 1974). Furthermore, the hyperpolarization produced by the AHP triggers the activation of a non-selective cationic current (I_h) that increases the excitability of AH neurons (Galligan *et al.*, 1990). If IPANs/AH-type neurons are sensory neurons, then S-type neurons are the interneurons and motorneurons. In S-type neurons, the AHP following the action potential is short, lasting 20-100 ms (Hirst *et al.*, 1974). Action potentials in these neurons are generated by the voltage-dependent Na^+ and K^+ conductances in line with the classic Hodgkin and Huxley model. The resting membrane potentials of S neurons are modulated by an inwardly-rectifying potassium current (K_{ir}) (Rugiero *et al.*, 2002).

NEUROCHEMICAL CODING AND SYNAPTIC TRANSMISSION IN THE ENS

Functional classification of enteric neurons according to the neurochemical markers they express and their axonal projections is well established in the guinea pig (Brookes, 2001). As mentioned previously, ACh, SP, NO, VIP, 5-HT, the calcium-binding proteins calbindin-D28K (calbindin) and calretinin are all markers for specific subsets of enteric neurons (Costa *et al.*, 2000; Brookes, 2001; Quinson *et al.*, 2001). Moreover, there are two types of chemically-mediated synaptic mechanisms used by enteric neurons: fast and slow neurotransmission (Bornstein *et al.*, 1994; Costa *et al.*, 1996; Furness, 2000).

Slow synaptic transmission in the ENS

Long-lasting changes in neuronal excitability results from transmitter binding to a family of cell surface receptors called G-protein-coupled receptors. These are heterotrimeric seven-transmembrane domain receptors with α, β and γ subunits. G-proteins employ two main systems to mediate their effects: the cyclic AMP (cAMP), and phosphoinositol systems. A third system, the arachidonic system, is also used by G-proteins, but for the purpose of my dissertation, the arachidonic system will not be discussed here. The cAMP system is activated when a ligand (eg. norepinephrine) binds to a receptor (eg. β adrenergic receptor) that is in turn linked to a G_s transducing protein. $G_{\alpha s}$ then stimulates adenylate cyclase which then catalyzes the conversion of ATP into the second messenger cAMP. cAMP then activates protein kinase A. The transducer

of the phosphoinositol pathway is G_q. $G_{\alpha q}$ activates the primary effector phospholipase C which in turn cleaves phosphoinositol phosphate (PIP_2) to yield two second messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP_3 acts at ligand-gated IP_3 receptors on the endoplasmic reticulum (ER) to cause the release of calcium from these intracellular membrane stores. DAG remains in the membrane where it activates protein kinase C (PKC) (Siegelbaum *et al.*, 2000). Slow synaptic excitation thus serves to modulate channel and intracellular enzymes to produce a remarkable array of responses, from changing the neural circuitry to altering synaptic transmission pathways. For example, ion channels such as K^+ channels can be phosphorylated, leading to a change in their probability of opening. Once these channels are phosphorylated, they close, causing a long-lasting increase in excitability that can be experimentally detected as an increase in input resistance (Galligan, 1998; Galligan *et al.*, 2000). This particular mechanism is a hallmark of slow synaptic excitation in AH neurons. Trains of high frequency electrical stimuli (5–20 Hz) elicit sEPSPs in AH neurons (Morita & North, 1985). Slow synaptic excitation occurs via G-protein-coupled receptors due to the long latency (> 50 ms) of the sEPSP (Galligan, 2002b). sEPSPs recorded from AH neurons are mediated by ACh and SP that are co-released from the same nerve endings (Galligan, 1999). ACh acting at M_1 muscarinic receptors, however, is not the major contributor to the sEPSP in AH neurons because sEPSPs evoked in the majority of these neurons in the presence of muscarinic receptor antagonists are unaffected (North *et al.*, 1985). SP acting at neurokinin-3 (NK_3) receptors coupled to inositol phospholipid

hydrolysis and PKC is believed to be the principal mediator of slow excitatory neurotransmission in the ENS (Guard *et al.*, 1988; Bertrand & Galligan, 1995). Forskolin, a direct activator of adenylate cyclase coupled-PKA, also mimics the sEPSP implying that the PKA pathway may be also be involved in mediating the sEPSP in IPANs (Bertrand & Galligan, 1995). NK_3 receptors are located on IPANs and on interneurons that receive synaptic input from IPANs (Holzer & Holzer-Petsche, 2001). IPANs also receive slow synaptic input from other IPANs and therefore form self-reinforcing networks (Kunze & Furness, 1999). SP also acts at postsynaptic PKC-coupled NK_1 receptors on excitatory and inhibitory enteric motor neurons (Holzer & Holzer-Petsche, 2001). IPANs can therefore directly mediate slow synaptic excitation of motor neurons (Holzer & Holzer-Petsche, 2001; Galligan, 2002b). The sEPSP is due to: a) suppression of the leak potassium conductance (Galligan, 1998), b) phosphorylation of calcium-dependent potassium channels causing them to close (Bertrand & Galligan, 1995), and c) increase in chloride conductance in about 40 % of myenteric AH neurons (Bertrand & Galligan, 1994; Wood & Kirchgessner, 2004). Changes in input resistance assume a biphasic mode, where the first phase of the increase in input resistance is the closure of calcium-dependent IK channels, while increased Cl^- conductance is responsible for the second phase of decreased input resistance (Wood, 2006a). In order for the AH neuron to generate an action potential, the sEPSP has to reach threshold. Once this occurs, calcium then enters through N-type channels to trigger calcium-induced-calcium-release from the ER (Rugiero *et al.*, 2002). Calcium then activates the IK channel, increasing

its probability of opening. At the same time, protein phosphatase 2B (calcineurin) is also activated by the released calcium and dephosphorylates the IK channel, thus permitting it to re-open (Vogalis *et al.*, 2004). The IK channel is then free to mediate the late AHP that is the characteristic feature of AH neurons. What then is the functional importance of sEPSPs in the ENS? Firstly, during a sEPSP, excitatory inputs to that AH neuron will be potentiated. Second, intestinal motility requires a sustained neural response to cause prolonged excitation or inhibition in the effector systems (smooth muscle, secretory glands, blood vessels) (Bertrand & Galligan, 1995; Wood & Kirchgessner, 2004), and the electrical properties of AH neurons appear to adequately fit this description. Based on their electrophysiological properties, IPANs/sensory neurons are classified as "AH" neurons, and interneurons and motor neurons are defined as "S" neurons

Fast synaptic transmission in the ENS

A single focal stimulus applied to interganglionic connectives produces fEPSPs in S neurons (Clerc *et al.*, 1999; Alex *et al.*, 2002; Galligan, 2002b). fEPSPs recorded from S neurons are mediated by ACh acting at ligand-gated pentameric nAChRs and ATP acting at trimeric P2X receptors (Galligan *et al.*, 2000). ACh is the principal excitatory neurotransmitter in the ENS (Nishi & North, 1973a; Zhou *et al.*, 2002). nAChRs and P2X receptors are non-specific cation channels. Enteric nAChRs are also calcium-permeable (Trouslard *et al.*, 1993). In 27% of S-type myenteric neurons with oral polarity, hexamethonium, a nAChR antagonist completely inhibited the fEPSP (LePard *et al.*, 1997; LePard &

Galligan, 1999). This indicated that ACh was the only transmitter in the ascending pathway (Spencer *et al.*, 2000). ATP is a co-transmitter with ACh in approximately 67% of S neurons in selective descending pathways (LePard & Galligan, 1999; Galligan *et al.*, 2000). Further analysis revealed that 11% of S-type neurons with aboral polarity were blocked by hexamethonium and 5-HT_3, indicating that this subset of S-neuron uses both ACh and 5-HT for fast synaptic communication. In fact, in the small intestine, 5-HT_3 receptors are found in the soma of AH and S-type neurons, where they mediate fastly desensitizing inward currents (Zhou & Galligan, 1999; Galligan, 2002a). Taken together, these data indicate that most myenteric neurons mediate fast excitatory synaptic transmission predominantly by ACh and ATP. Interestingly, nAChRs have been localized in the somatodendritic region (Torocsik *et al.*, 1991) and nerve terminals (McGehee *et al.*, 1995) of myenteric interneurons and motorneurons (Galligan, 1999, 2002a). According to Galligan (2002a), the paucity of recorded 5-HT_3-mediated fEPSPs may be due to the small percentage of 5-HT-containing S-type interneurons.

NEUROMUSCULAR TRANSMISSION IN THE ENS

Autonomic nerves release neurotransmitters *en passage* from varicosities that occur at 5-15 μm intervals along axons (Blakeley & Cunnane, 1979; Blakeley *et al.*, 1982). ACh, released from excitatory longitudinal and circular muscle motor neurons, acts at presynaptic M_2 muscarinic and postjunctional M_3 cholinergic receptors (Matsui *et al.*, 2000; Eglen, 2001) and is

the major excitatory neurotransmitter responsible for contraction of GI smooth muscle (Paton, 1955; Paton & Zar, 1968; Bogeski *et al.*, 2005). Cholinergic excitatory junction potentials (EJPs) have also been recorded from guinea pig GI smooth muscle (Burnstock & Holman, 1963). SP, acting at NK_1 receptors located on interstitial cells of Cajal (ICC) and on the smooth muscle (Sternini *et al.*, 1995; Southwell & Furness, 2001), and at NK_2 receptors on the muscle (Maggi, 2000) is an excitatory co-transmitter involved in these contractions (Holzer & Holzer-Petsche, 2001; Lecci *et al.*, 2002). NK_1 receptors are localized to the soma of NOS-containing inhibitory motor neurons (Portbury *et al.*, 1996). Pharmacological evidence later showed that tachykinins excite NK_1 receptors on inhibitory motor pathways within the ENS of the guinea-pig ileum, causing those inhibitory neurons to release NO, thus depressing motor activity (Lecci *et al.*, 1999; Saban *et al.*, 1999; Bian *et al.*, 2000). nAChRs, however, are also present on the somatodendritic and nerve terminal regions (Wonnacott, 1997) of longitudinal and circular muscle motor neurons (Galligan, 1999; MacDermott *et al.*, 1999; Schneider *et al.*, 2000). Since excitatory longitudinal and circular muscle motor neurons contain both choline acetyltransferase (ChAT) and SP (Brookes, 2001), ACh and SP may be released from the same nerve endings (Galligan, 1999). Presynaptic nAChRs may therefore function as autoreceptors to facilitate SP release, causing non-cholinergic GI smooth muscle contractions (Galligan, 1999; MacDermott *et al.*, 1999).

There appears to be an oral-aboral increase in inhibitory innervation to GI smooth muscle. Burnstock and colleagues used sucrose-gap recordings with

transmural field stimulation to show that in the presence of the muscarinic receptor antagonist atropine, and guanethidine which blocks adrenergic neurotransmission, that 1 Hz stimuli pulses evoked transient hyperpolarizations or inhibitory junction potentials (IJPs) from smooth muscle from guinea pig taenia coli (Burnstock *et al.*, 1963; Burnstock & Holman, 1963; Burnstock *et al.*, 1964). TTX completely blocked the IJPs. In guinea pig, murine and human, increasing the electrical stimuli of nerve fibers supplying the muscle layer causes IJPs to become biphasic, consisting of an initial large-amplitude response, followed by a smaller-amplitude and slower second response (Wang *et al.*, 2007). Relaxation of GI smooth muscle is mediated by non-adrenergic non-cholinergic (NANC) inhibitory enteric neurons (Furness, 2000). NO is the primary mediator of this relaxation (Li & Rand, 1990; Sanders & Ward, 1992) but ATP and VIP also contribute to inhibitory NANC responses (Makhlouf & Grider, 1993; Furness, 2000). The NO-synthesizing enzyme NOS, ATP and VIP co-localize within inhibitory circular and longitudinal muscle motor neurons and may therefore act in concert, in parallel or in series to exert muscle relaxation (Costa *et al.*, 1992; Grider & Jin, 1993; Brookes, 2001). NO is synthesized on demand, diffuses into smooth muscle cells and binds to soluble guanylate cyclase. Guanylate cyclase catalyzes the conversion of guanosine-5'-triphosphate (GTP) to cyclic guanosine-3',5'-monophosphate (cGMP) (Lincoln *et al.*, 2001). If intracellular calcium levels increase via calcium entry through voltage-gated L-type calcium channels or from intracellular calcium stores via activation of inositol triphosphate (IP_3) or ryanodine receptors to cause excitation (contraction) of smooth muscle cells

(Sanders & Koh, 2006), then a decrease in intracellular calcium should produce inhibition (relaxation) of the muscle. Elevated cytosolic cGMP lowers intracellular calcium levels by activating a cGMP-dependent protein kinase (PKG) (Hofmann *et al.*, 2006). PKG phosphorylates large-conductance calcium-dependent potassium (BK) channels (Robertson *et al.*, 1993; Alioua *et al.*, 1998), thus hyperpolarizing the cell, reducing calcium influx via L-type calcium channels and relaxing GI smooth muscle (Sausbier *et al.*, 2000; Geiselhoringer *et al.*, 2004). ATP, acts at $P2Y_1$ receptors on GI smooth muscle to produce fast IJPs. In guinea pig circular muscle, the fast IJPs are completely inhibited by the selective $P2Y_1$ receptor antagonist MRS2179 (Boyer *et al.*, 1998). A recent study in mouse colonic circular muscle showed that the purinergic co-enzyme β-nicotinamide adenine dinucleotide (β-NAD) may also be a non-adrenergic non-cholinergic (NANC) inhibitory neurotransmitter (Mutafova-Yambolieva *et al.*, 2007). Hyperpolarization responses after picospritzing β-NAD onto muscle cells revealed that in the presence of β-NAD, MRS2179 drastically inhibited those responses. Those data were confirmed with calcium-imaging studies performed in human embryonic kidney (HEK)-293 cells where calcium transients to β-NAD were completely blocked by MRS2179. Therefore β-NAD mediates it effects on GI smooth muscle via $P2Y_1$ receptors (Mutafova-Yambolieva *et al.*, 2007). Moreover, electrical field stimulation of murine colonic strips maintained *in vitro* showed that ATP and β-NAD are co-released, and that their release may be mediated predominantly by N-type calcium channels (Mutafova-Yambolieva *et al.*, 2007; Smyth *et al.*, 2009).

VIP acts at G_s-coupled vasoactive-pituitary adenylate cyclase-activating peptide (VPAC) receptors on ICCs and GI smooth muscle cells to produce relaxation (Furness, 2000; Kurjak *et al.*, 2001). VIP, via cyclic AMP, may activate a calcium-dependent potassium channel to elicit this response (Kawasaki *et al.*, 1997; Bayguinov *et al.*, 1999).

SMALL INTESTINAL MOTILITY PATTERNS

Peristalsis

Peristalsis is a polarized motor reflex that is responsible for moving intestinal contents in an oral to anal direction (Trendelenburg, 2006; Gwynne & Bornstein, 2007). Phasic changes in intraluminal pressure are due to contractions and relaxations of GI smooth muscle (Huizinga *et al.*, 2006). The GI tract produces three important motor patterns. The first is non-propulsive segmental contractions that mix and churn food; this enhances absorption of essential dietary nutrients. The second type of motor patterns are those that are fully propulsive contractions that propel food anally (Trendelenburg, 2006). This latter form of peristalsis was initially described by Bayliss and Starling (1899) and is referred to as the peristaltic reflex or "the law of the intestine". Using small intestinal segments *in vitro* and *in vivo*, Bayliss and Starling proved that ascending excitation and descending inhibition occurs above and below a physiological stimulus presented within the lumen of the gut, one that mimicked a bolus of food (Bayliss & Starling, 1899). Those authors further showed that the circular and longitudinal muscle layers contract and relax synchronously to propel

food anally. The muscle layers also exhibits regular and rhythmic contractions called slow waves. Slow waves are depolarizing potentials that produce oscillations of the membrane potential of the smooth muscle, and these oscillations are close to threshold for calcium influx via L-type calcium channels (Sanders, 2008; Huizinga & Lammers, 2009). Slow waves are generated by the non-neuronal non-smooth muscle interstitial cells of Cajal, and are conducted to the adjacent smooth muscle cells via gap-junctions (Farrugia, 1999, 2008). Contraction and inhibition of the muscle occur through neurotransmitters released from enteric nerves that innervate the smooth muscle. The peristaltic reflex is mediated by both chemical stimulation of the mucosa (Bertrand *et al.*, 2000) or distention (Hennig *et al.*, 1999). Intestinal segments isolated from the guinea pig show consistent fully propagative peristaltic reflex patterns. This neurally-mediated pattern is completely abolished in the presence of the voltage-gated sodium channel blocker tetrodotoxin (TTX), but myogenic rhythmic patterns persist (Huizinga *et al.*, 1998). Experiments done with intestinal segments show that fluid-induced distention of the rat and mouse intestine *in vitro* does not evoke the classic Trendelenburg peristaltic reflex (Seerden *et al.*, 2005). Instead, oscillatory contractions of GI smooth muscle cells in those animals have been frequently demonstrated and appear to be under low neural excitation, similar to the TTX-resistant contractions and relaxations observed in the guinea pig intestine (Seerden *et al.*, 2005). Therefore, peristaltic motor patterns differ between species, and in rats and mice they are mainly due to the

high frequency of slow waves that manifest as the predominant motor patterns in those animals (Huizinga *et al.*, 1998).

The third electrical and motor pattern in the GI tract is the MMC that occurs during the interdigestive (fasting) state in mammals (Galligan *et al.*, 1989). MMCs begin in the gastroduodenal region of the GI tract and slowly propagate towards the terminal ileum as a slowly-moving wave complex (Szurszewski, 1969). This wave is created by rapid and intense contractions that sweep along the gut. Humans and guinea pigs have approximately the same number of MMC cycles that occur every 70-90 minutes (Furness, 2006a). There are four known stages of the MMC: 1) a prolonged quiescent phase; 2) an irregular phase consisting of increasing action potential frequency coinciding with an increase in motilin synthesis and release from the duodenal mucosa; 3) a peak phase where the MMC propagates; and 4) a brief period of declining and irregular activity (Carlson *et al.*, 1972). The functional significance of the MMC is the removal of undigested food, bacteria, and residual intestinal secretions (Huizinga & Lammers, 2009).

GI MOTILITY DISORDERS

The GI tract is a specialized hollow tube that consists of the oral cavity, pharynx, esophagus, stomach, small and large intestine, and anus. The function of those organs is to allow the sequential degradation of food by enzymes into smaller molecules during the transit of food. In this way, the body has access to vital minerals, nutrients, and water before they are expelled. This

goal is accomplished by the enteric nervous system (ENS), a collection of neurons embedded in the gut wall, that can function independently of input from the CNS (Gershon, 1981). Dysregulation of the ENS causes changes in motility, secretion and absorption, and leads to motility disorders (Sharkey & Kroese, 2001; Furness, 2006b). GI motility disorders are important areas for the health of the United States and the rest of the world, and comprise about 40% of GI problems for which patients seek health care (Camilleri *et al.*, 2005). They impose a heavy burden of illness, decreased quality of life, and decreased work productivity on those who are affected. Disorders of the ENS produce motor, secretory, and inflammatory and immunological disturbances of GI function. These disorders are believed to be associated with the degeneration of inflammatory cells or altered signaling of enteric neurons, and these changes are manifested as disturbances in GI transit or functional obstruction (Goyal & Hirano, 1996). Patients with FGIDs exhibit a greater altered motility response to psychological or physiological stressors when compared to control subjects (Drossman *et al.*, 2002; Parkman *et al.*, 2004). A major impact of GI disorders is its global social and economic burden. Irritable bowel syndrome (IBS), for example, has a prevalence rate of over 10% in known IBS cases and costs the United States economy at least $25 billion a year (Camilleri, 2001). IBS is probably the best known GI motility disorder. IBS is pathophysiologically complex, and a hallmark of this disorder is an alteration of the sensory innervation to the GI tract, causing visceral hypersensitivity in IBS patients. Hypersensitivity of IPANs has been implicated in patients with IBS, such that

their reduced threshold of activation would heighten motility reflexes (Galligan, 2004). There is no current cure for this condition. Patients with IBS have either increased or reduced gut motility, or alternating constipation and diarrhea in the same patient, and painful cramps (Drossman, 1999; Thompson *et al.*, 1999). Used as an index of hypersensitivity, balloon bowel-distention experiments show that IBS patients possess visceral hypersensitivity (visceral hyperalgesia), suggesting that the neural sensory system in IBS patients may be dysregulated (Ritchie, 1973; Mertz *et al.*, 1995; Bernstein *et al.*, 1996). IBS is believed to have an immune component (Furness, 2006a). In small intestinal biopsies taken from IBS patients with elevated mast cell counts, degranulating mast cells were localized close to nerve terminals that supply the mucosa (Barbara *et al.*, 2004). The resulting inflammatory insult to the GI tract increases excitability of enteric neurons (Sharkey & Kroese, 2001; Nurgali *et al.*, 2007). The manifestation of plasticity within neural circuits as a consequence of sensory inputs or motor acts is therefore an inherent property of our continually changing central and peripheral nervous systems. Moreover, most HIV/AIDS patients (50-93%) report GI problems including dysphagia, diarrhea, nausea, vomiting, weight loss, abdominal pain, anorectal disease, GI bleeding and tumors during the course of their illness (Gazzard, 1988). There is a positive correlation between a progressively compromised immune system and an increased prevalence of these GI symptoms (May *et al.*, 1993) because the small intestine is a crucial site in immune defense due to its large surface area. Understanding mechanisms of

GI disorders is therefore imperative due to their pervasiveness across many disease-types.

VOLTAGE-GATED CALCIUM CHANNELS IN THE NERVOUS SYSTEM

Overview

Voltage-gated calcium channels (Fig. 1.3) are found in virtually every excitable cell. Initial studies in the barnacle demonstrated properties of calcium channels: 1) intracellular calcium chelators favor excitability, 2) permeant divalent ions compete for entry into these channels, and 3) calcium channels are blocked by divalent metal ions including $CdCl_2$, $NiCl_2$ and Co^{2+} (Hagiwara & Byerly, 1981b, 1981a; Hille, 2001). They are physiologically crucial because they regulate a myriad of calcium-dependent intracellular events. Moreover, they possess the unique ability of translating electrical signals into chemical signals critical for neurotransmitter and hormone release, muscle contraction, gene expression, neurite outgrowth, synaptogenesis, synaptic plasticity and cell death (Berridge, 1998; Lee *et al.*, 2002; Catterall *et al.*, 2005). Structural, pharmacological and biophysical (voltage-dependence and kinetics) studies have described several distinct classes of neuronal voltage-gated calcium channels: a T-type referred to as a low-threshold current whose channel opens at more negative voltages (~ -64 mV; maximum activation ~-30 mV) with rapid inactivation (< 100 ms) (Perez-Reyes, 2003; Talavera & Nilius, 2006), and the high-threshold currents L-, N-, P/Q- and R-types whose channels open at more depolarized voltages (~ -30 mV; maximum activation ~ 0 mV) (Bean, 1989; Tsien

et al., 1991; Hoffman *et al.*, 1994) and generally inactivate slower (> 100 ms) (Yamakage & Namiki, 2002) (Table 2). Voltage-gated calcium channels are pentameric with six transmembrane α helices. These channels have five subunits: $\alpha 1$, β, $\alpha 2\delta$ and γ. The pore-forming (between S5 and S6) subunit is $\alpha 1$, and is between 190-250 kDa in size with approximately 2000 amino acid residues. The $\alpha 1$ subunit also contains the voltage sensor (S4), channel gates, and drug/toxin binding sites (Isom *et al.*, 1994; Hofmann *et al.*, 1999; Ertel *et al.*, 2000). Positive charges residing in the voltage sensor serves to activate the channel complex. The short intracellular loop between domains I and II has a $G\beta\gamma$ and PKC phosphorylation site. Interaction of $\alpha 1$ with $G\beta\gamma$ subunits reduces the rate of activation of the channel by reducing the open channel probability (Dolphin, 2003b). Phosphorylation of $\alpha 1$, however, increases the probability of calcium channel opening. A calcium-calmodulin site is positioned on the C-terminal tail. The large intracellular loop connecting domains II and III has the SNARE-binding region referred to as a synprint site. SNARES constitute the synaptic vesicle release machinery in neurons (Catterall, 2000). The synaptic membrane proteins syntaxin, synaptosome-associated protein of 25 kDa (SNAP25), and vesicle-associated membrane protein of 25 kDa (synaptobrevin) form the SNARE complex (Hofmann *et al.*, 1999). The β subunit is hydrophilic and located intracellularly. It is mainly responsible for the proper insertion of the $\alpha 1$ subunit into the membrane. β subunits dramatically enhance the cell surface

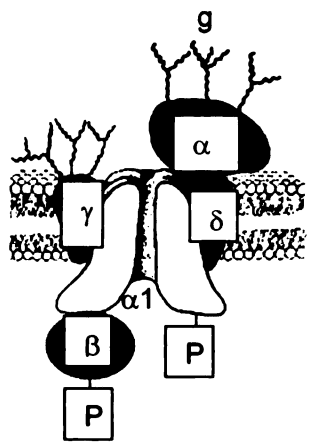


Figure. 1.3. Model depicting the membrane structure and transmembrane topology of a voltage-gated calcium channel. Arrangement of the channel in the plasma membrane. The pore forming subunit is α1. The α2δ, β, and γ subunits are the accessory subunits. Sites for channel glycosylation are indicated by "g". Voltage-gated calcium channels are tetramers, each subunit contains six transmembrane (TM) helices. Note the conduction pore-loop (green) between TM 5 and TM 6, and the voltage-sensor S4 helices (Catterall & Few, 2008).

expression of α1 subunits and affect the kinetics and voltage-gating properties of the channel (Dolphin, 2003a). The α2δ subunits are conjoined; the α2 subunit is found extracellularly and the γ subunit is a transmembrane protein. α2δ subunits also increase cell membrane expression of α1, but has less pronounced effects on channel kinetics (Davies *et al.*, 2007). The γ subunit has thus far not been intensively studied. These subunits, however, appear to have differential effects on α1 membrane expression, that is, γ subunits may either have no effect on α1 expression, or may even reduce the level of expression (Catterall, 2000). LVA channel inactivation is similar to the voltage-dependent process of Na^+ channels. However, HVA calcium channels show either slow (voltage-dependent) or fast (calcium-ion dependent) inactivation. Both serve to prevent the overloading the neuron with calcium as excessive calcium concentrations become cytotoxic. Calcium sensitive inactivation of HVA calcium channels appears to be calmodulin-mediated. HVA channels have calmodulin-binding domains on the intracellular C-terminal tail that bind calmodulin with extremely high affinity (Hofmann *et al.*, 1999). Neurons therefore use these negative feedback mechanisms for survival.

Synaptic transmission requires a dramatic elevation in intracellular calcium ions entering through voltage-gated calcium channels at concentrations of 50-100 μM (Matthews, 1996). These channels are believed to be the N- and P/Q-type calcium channels and densely associate with calcium microdomains in the nerve terminal (Hofmann *et al.*, 1999). The calcium ions then interact with the synaptic machinery. While synaptotagmin is the putative calcium sensor,

there have been reports where calcium-mediated exocytosis still occurs in synaptotagmin-knockout mice (Shao *et al.*, 1996; Sudhof & Rizo, 1996).

T-type calcium channels

T-type calcium channels produce a transient current with a tiny conductance with rapid activation and inactivation. These channels are strongly expressed in kidney and heart but have low expression in neural tissue (Perez-Reyes, 2003). T-type channels are very sensitive to high concentrations of $NiCl_2$ (Vassort & Alvarez, 1994; Vassort *et al.*, 2006). α1G, H, and I are the molecular counterparts of T-type calcium channels.

L-type calcium channels

L-type calcium channels have a large single-channel conductance and give rise to a long-lasting current. L-type channels are the principal channels in smooth, skeletal and cardiac muscle, where they mediate calcium entry into cells for excitation-contraction coupling in response to long or consistent depolarizations (Hille, 2001). L-type channels are highly sensitive to dihydropyridines (eg. nifedipine) and this is a distinctive property of these channels (Bean, 1989). α1S, C, D and F are the molecular counterparts of L-type calcium channels (Bean, 1989).

N-type calcium channels

N-type calcium channels were first localized in chick sensory neurons (Fox *et al.*, 1987). Those authors found a calcium conductance that was dihydropyridine-insensitive and intermediate to that of T- and L-type currents. N-type (N for neuronal) channels are blocked by the peptide toxin ω-conotoxin GVIA (ω-CTX). ω-CTX is a toxin isolated from the Pacific cone-shelled snail *Conus geographus* (McCleskey *et al.*, 1987; Olivera *et al.*, 1991). N-type calcium channels are important mediators of neurotransmitter release at central and peripheral synapses (Smith & Cunnane, 1996, 1997). α1B is the molecular counterpart of N-type channels (Bean, 1989).

P/Q-type calcium channels

Around the time that N-type calcium channels were being characterized with ω-CTX, Rodolfo Llinás and colleagues (1991) were working on Purkinje cell function in the cerebellum. One of the tools at their disposal was a peptide toxin, ω-agatoxin IVA (ω-ATX) from the American funnel web spider *Agelenopsis aperta*. They observed a very slowly inactivating calcium current that was especially sensitive to ω-ATX. They called this current the P (for Purkinje)-type calcium current (Hillman *et al.*, 1991). However, four years later, a second ω-ATX-sensitive calcium current was found in cerebellar granule cells that displayed rapid inactivation kinetics (Randall & Tsien, 1995). This was named the Q-type calcium current. The P/Q-currents are now combined because

they were shown to be homologous spliced variants (Bourinet *et al.*, 1999). α1A is the molecular equivalent of P/Q-type channels (Bean, 1989).

R-type calcium channels

Soong and others (1993) initially classified the R-type (R for resistant, residual or remaining) calcium current as a LVA T-type channel. However, the R-type calcium current is really a HVA current (Schneider *et al.*, 1994) that may activate at somewhat more negative voltages (~ -40 mV) and often inactivates more rapidly than the other high-threshold currents (Soong *et al.*, 1993; Yamakage & Namiki, 2002). Unlike the other calcium currents, a complete understanding of the molecular properties of the R-type calcium channel remains elusive. The principal candidate for the ion-conducting subunit of R-type calcium channels is the α1E subunit, encoded by the $Ca_V2.3$ gene (Zhang *et al.*, 1993). The α1E subunit was initially identified in the rabbit hippocampus, cerebral cortex and corpus striatum (Niidome *et al.*, 1992). Using different splice-variants of the $Ca_V2.3$ gene, α1E was expressed in *Xenopus* oocytes and localized in adult rat brain (Soong *et al.*, 1993). Recombinant α1E was subsequently expressed in HEK cells and *Xenopus* oocytes (Schneider *et al.*, 1994). Soong et al. (1993) and Schneider et al. (1994) both confirmed α1E sensitivity to blockade by $NiCl_2$. It has further been shown that the toxin SNX-482 (Newcomb *et al.*, 1998) and low concentrations of $NiCl_2$ selectively block R-type channels (Sochivko *et al.*, 2002). *In situ* hybridization and the reverse transcriptase-polymerase chain reaction both revealed extensive α1E mRNA expression in the adult mouse brainstem,

forebrain and cerebellum (Williams *et al.*, 1994). Immunohistochemical localization of α1E has been demonstrated in neurons within the rat caudate-putamen, thalamus, hypothalamus, amygdala, somata and axons of Purkinje cells (Yokoyama *et al.*, 1995; Grabsch *et al.*, 1999), cell bodies of CA1 pyramidal cells, mitral cells of the olfactory bulb (Yokoyama *et al.*, 1995), human Purkinje and cerebellar granule cells (Yokoyama *et al.*, 1995; Volsen *et al.*, 1997), rat cardiac myocytes and human distal tubules (Weiergräber *et al.*, 2000). R-type calcium channels comprise a significant portion of the calcium current recorded from murine dentate granule cells, hippocampal CA1 and cortical neurons (Sochivko *et al.*, 2002). The study by Sochivko and others (2002) was conducted in transgenic mice where the α1E gene was knocked out. Currents recorded from those CNS neurons showed that the R-type current was not eliminated but reduced. R-type calcium channel knockout-mice also show deficits in fear behavior (Lee *et al.*, 2002), spatial memory as measured by the Morris water-maze (Kubota *et al.*, 2001), and pain perception (Saegusa *et al.*, 2000). The α1E-knockout mice in the study by Saegusa and others (2000) were not susceptible to inflammatory pain. R-channels may mediate synaptic communication by those neurons in descending anti-nociceptive pathways. Moreover, another study showed that α1E mutant mice are hyperglycemic (Matsuda *et al.*, 2001). These data together highlight the physiological importance of the R-type channel in various organ systems. Thus Tottene and others (2000) used antisense oligonucleotides against the *$Ca_V2.3$* gene. Those authors observed both sensitive and insensitive R-type channel conductances in response to SNX-482 in

cerebellar granular neurons maintained in culture. In studies with P/Q-type (α1A) channel knockout mice, neurotransmitter release at the somatic neuromuscular junction occurred via R-type channels, suggesting that these channels are positioned close to presynaptic calcium sensors (Urbano *et al.*, 2003). An interaction between R-type calcium channels and the synaptic vesicle protein synaptotagmin has been demonstrated previously (Wiser *et al.*, 2002), further strengthening the importance of this channel in short-term plasticity and synaptic transmission.

Calcium channels in the ENS

Electrophysiological and immunohistochemical studies demonstrate the presence of all calcium channels, with the exception of T-type channels, in the ENS (Hirning *et al.*, 1990; Kirchgessner & Liu, 1999; Smith *et al.*, 2003). L-type calcium channels, however, are not major contributors to the synaptic excitation of enteric neurons (Reis *et al.*, 2000; Reis *et al.*, 2002). Further, the inhibition of L-type channels on GI smooth muscle reduces or abolishes spontaneous or evoked muscle movement (Horowitz *et al.*, 1996; Farrugia, 1999). P/Q type channels are expressed by myenteric neurons and contribute to neurotransmitter release from enteric nerves (Reis *et al.*, 2000; Smith *et al.*, 2003). However, weak immunoreactivity has often been associated with P/Q-type calcium channel (Kirchgessner & Liu, 1999). Nonetheless, data from guinea pig caecal submucosal (Cunningham *et al.*, 1998) and ileal myenteric neurons indicate that P/Q-type channels do contribute to neurotransmitter release (Reis *et*

al., 2000; Reis *et al.*, 2002). N-type calcium channels are ubiquitously expressed in the guinea pig plexuses (Kirchgessner & Liu, 1999). In myenteric AH neurons, N-type channels were shown to be the main contributor to the calcium shoulder on the repolarization phase of the action potential (Furness *et al.*, 1998; Vogalis *et al.*, 2002). R-type calcium currents have also been recorded in guinea pig myenteric neuron maintained in culture (Bian *et al.*, 2004).

R-type calcium channels in the ENS

R-type calcium channels are the principal subtype in the somatodendritic region of myenteric neurons maintained in primary culture (Rugiero *et al.*, 2002; Bian *et al.*, 2004), suggesting that R-type calcium channels may contribute to synaptic transmission and regulation of membrane excitability of myenteric neurons. Only two studies thus far have indicated functional roles for R-type calcium channels in the ENS (Bian *et al.*, 2004; Bian & Galligan, 2007). Bian and others (2004) used whole-cell voltage clamp recordings to show that at least 50% of the calcium current present in myenteric neurons maintained in primary culture is the R-type current. After using a strong depolarization (0 mV) pulse to activate the R-type current, they applied N-, P/Q-, and L-type calcium channel antagonists sequentially to block the calcium currents. However, 50% of the remaining calcium current remained. This residual current was only blocked after application of $NiCl_2$ (50 μM). This observation was confirmed when $NiCl_2$ and SNX-482 (0.1 μM) mutually occluded the calcium current (Bian *et al.*, 2004). That study suggested a somatodendritic role for R-type calcium channels in the ENS. A further role for R-type channels has been postulated based on a

selective coupling with α_2-adrenoreceptors (Bian & Galligan, 2007). Norepinephrine released from sympathetic postganglionic neurons inhibits GI motility (Paton & Vizi, 1969; Stebbing *et al.*, 2001). Presynaptically-located α_2-adrenoreceptors are activated by norepinephrine and inhibit neurotransmitter release by inhibiting calcium influx through voltage-gated calcium channels (Paton & Vizi, 1969; Stebbing *et al.*, 2001). R-type calcium channels couple to inhibition of α_2-adrenoreceptors via a pertussis toxin-sensitive G-protein (Bian & Galligan, 2007). R-type channels may therefore be a target for norepinephrine released from sympathetic nerve terminals (Bian & Galligan, 2007).

ION CHANNELS AS TARGETS FOR DISEASE TREATMENT IN THE ENS

The concept of how alterations in synaptic transmission shape the connectivity and functions of enteric neural circuits has a key bearing on the consideration of normal physiological versus pathophysiological changes in the ENS. Voltage-gated calcium channels are critical mediators of synaptic transmission. Excessive calcium entry, however, is also cytotoxic. Calcium influx via calcium channels must therefore be precisely regulated because alterations in calcium channel function would produce widespread cellular changes. Perturbations in ion channel function affect diverse organ systems. In the neuromuscular system, ion channel diseases results from gain-of-function mutations to cause, amongst others, epilepsy, ataxia, myotonia and cardiac arrhythmias (Cooper & Jan, 1999). In other systems, cystic fibrosis and Bartter syndrome, for example, result from defects in chloride (Cl^-) and potassium (K^+)

channels that alter the transepithelial transport gradients of salt and water (Cooper & Jan, 1999). In the ENS, the type 2 Cl^- channel (ClC-2) is found in enterocytes in the small intestine and colon (Lipecka *et al.*, 2002). ClC-2 channels regulate intracellular volume and pH levels and activation of these channels on the enterocytes increase intestinal fluid secretion (Cuppoletti *et al.*, 2004). The highly selective ClC-2 agonist lubiprostone is now being used to successfully treat patients with chronic constipation (Cuppoletti *et al.*, 2004). A recent electrophysiological study using whole-cell patch clamp recordings demonstrated that R-type calcium channels are the predominant calcium channel subtype expressed by myenteric neurons (Bian *et al.*, 2004). This raised the questions of whether a) R-type calcium channels are localized selectively within subsets of myenteric neurons, b) the mechanisms by which these calcium channels contribute to synaptic transmission at neuro-neuronal synapses and neuromuscular junctions in the myenteric plexus and GI smooth muscle, respectively, and c) the physiological significance of the large R-type currents found by Bian et al. (2003). Voltage gated calcium channels are key pharmacological targets for treating pain, stroke, epilepsy, migraine and hypertension (French & Zamponi, 2005). These observations support the concept that ion disturbances in ion channel function can contribute to the pathophysiology of some diseases, and that ion channels can also be the targets for therapeutically useful drugs. These studies will provide new insights into the contribution of R-type calcium channels to GI motility.

CHAPTER 2 HYPOTHESIS AND SPECIFIC AIMS

OVERALL HYPOTHESIS

Gastrointestinal (GI) disorders decrease the quality of life for affected patients (Drossman *et al.*, 1993; Stewart *et al.*, 1999). These disorders are classified as either structural (organic) or functional, but patients may simultaneously suffer from both types. Inflammatory bowel disease (IBD) is an organic GI disorder with clearly defined injury and inflammation that affect motility (Stewart *et al.*, 1999). When no pathological (anatomical, physiological or biochemical) etiology is apparent, the patient is diagnosed as having a functional GI disorder (FGID) (Longstreth *et al.*, 2006). There has been a surge in the diagnosis of FGIDs including IBS in recent years. The pathophysiological basis for FGIDs is unknown because FGID is due to an interaction of psychosocial factors and altered gut physiology via the brain-gut axis. This is pertinent as the GI tract has its own (enteric) nervous system (ENS) that allows the gut to function quasi-autonomously (Furness, 2000). Synaptic transmission requires voltage-gated calcium channels as mediators for a host of important biological events including neurotransmitter release and muscle contraction (Cooper & Jan, 1999; Lorenzon & Beam, 2000). R-type calcium currents can be recorded from guinea-pig myenteric neurons suggesting that pore-forming α1E calcium channel subunits are expressed in the ENS (Rugiero *et al.*, 2002; Bian *et al.*, 2004). At least 50% of the current in myenteric neurons is the R-type calcium current. This suggests an important role for R-type channels as mediators of neurotransmitter release or as transducers of other synaptic events in myenteric neurons, and implicates this channel as a potential drug target in combating motility disorders.

I therefore tested the overall hypothesis that R-type calcium channels are therefore functionally significant in controlling GI motility. This function was explored further through the following specific aims.

Specific Aim 1. Myenteric neurons express R-type calcium currents (Bian *et al.*, 2004), but the specific subset of myenteric neuron that expresses the R-type calcium channel is not known. These studies tested the hypothesis that R-type calcium channels are localized to AH neurons and/or S-type neurons.

Specific Aim 2. Fast and slow synaptic neurotransmission may be selectively modulated by calcium channel subtypes. These studies tested the hypothesis that R-type or non-R-type calcium channels are respectively responsible for substance P (SP) release as the mediator of the slow excitatory postsynaptic potential (sEPSP) in AH neurons, and acetylcholine (ACh) release as the mediator of the fast EPSP in S neurons.

Specific Aim 3. Immunohistochemical data show that α1E is present in nerve endings of excitatory motor neurons [SP-immunoreactivity (ir)], and descending interneurons and inhibitory motor neurons [vasoactive intestinal peptide (VIP)/nitric oxide synthase (NOS)-ir]. These studies tested the hypothesis that R-type calcium channels mediate nerve-to-muscle transmission in the guinea pig isolated ileum.

Specific Aim 4. My goal was to use an integrative approach to examine the functional role of R-type channels in mediating the peristaltic reflex in the guinea pig ileum. I used a modified method of Trendelenburg (Bülbring *et al.*, 1958; Huizinga *et al.*, 1998; Bian *et al.*, 2003) to test the hypothesis that R-type calcium channels are necessary or are sufficient for propulsive peristalsis *in vitro*.

CHAPTER 3 IMMUNOHISTOCHEMICAL LOCALIZATION OF R-TYPE CALCIUM CHANNELS IN THE GUINEA PIG ENTERIC NERVOUS SYSTEM

ABSTRACT

R-type calcium currents can be recorded from guinea pig myenteric neurons suggesting that α1E calcium channel subunits are expressed in the enteric nervous system. However, the neuronal subset(s) expressing α1E subunits are unknown. We used whole mounts from the stomach, intestine and colon of adult guinea pigs, and ileal myenteric neurons maintained in primary culture to map α1E-subunit expression. I also used antibodies against specific markers to determine if α1E was expressed selectively in neuronal subsets. α1E-immunoreactivity (ir) was found in colonic and gastric, but not intestinal myenteric cell bodies in the longitudinal muscle-myenteric plexus (LMMP). α1E-ir never co-localized with serotonin (a marker for descending interneurons). α1E-ir co-localized with substance P (SP) in myenteric nerve fibers and in the muscle layers throughout the gut. α1E-ir co-localized with calretinin in cell bodies and nerve fibers in primary cultured neurons, in some varicosities in the LMMP, and with calbindin in cell bodies only in dissociated neurons. α1E-ir co-localized with vasoactive intestinal peptide (VIP) and nitric oxide synthase (NOS) in myenteric nerve fibers and in muscle layers; only the colon contained α1E-VIP-ir neurons. α1E-NOS-ir neurons were found in the gastric myenteric plexus. α1E-ir co-localized with tyrosine hydroxylase in myenteric nerve fibers. α1E-ir was not detected in the submucosal plexus. I concluded that α1E-ir is expressed by cell bodies of myenteric intrinsic primary afferent neurons (calbindin-containing) and in ascending interneurons (calretinin-containing), inhibitory motorneurons (VIP/NOS-ir) and nerve endings of excitatory motorneurons (SP-ir). α1E-ir is

contained in sympathetic nerves supplying the myenteric but not the submucous plexus. R-type calcium channels could participate in synaptic transmission in the myenteric but not submucosal plexus. R-type calcium channels may also participate in neuromuscular transmission.

INTRODUCTION

The enteric nervous system (ENS), a collection of neurons embedded in the gut wall, can function independently of input from the central nervous system (Gershon, 1981). The ENS consists of two ganglionated plexuses: the myenteric plexus which provides innervation to the gastrointestinal smooth muscle, and the submucosal plexus which regulates neuroimmune interactions, local blood flow, secretion and absorption across the gastrointestinal epithelium (Kunze & Furness, 1999). Changes in enteric neuronal function can therefore cause gastrointestinal motility disorders.

Pharmacological and biophysical studies have described several distinct classes of neuronal voltage-activated calcium channels: the high-threshold currents L-, N-, P/Q- and R-types, and a T-type referred to as a low-threshold current (Hoffman *et al.*, 1994). The function of R-type calcium channels in the nervous system has not been firmly established. However, In studies using P/Q-type channel knockout mice, neurotransmitter release at the somatic neuromuscular junction occurred via R-type channels, suggesting that these channels are positioned close to sites of synaptic transmission (Urbano *et al.*, 2003). In addition, an interaction between R-type calcium channels and the synaptic vesicle protein synaptotagmin has been demonstrated previously (Wiser *et al.*, 2002), further strengthening the importance of this channel in synaptic transmission.

Neuronal calcium channels are composed of a pore-forming and voltage-sensing α1-subunit in association with accessory subunits which serve to

modulate the properties of the channel complex (Isom *et al.*, 1994; Ertel *et al.*, 2000). The pore forming subunit of R-type calcium channels is the α1E-subunit, encoded by the $Ca_V2.3$ gene. The α1E subunit was initially identified in the rabbit hippocampus, cerebral cortex and corpus striatum (Niidome *et al.*, 1992). Recombinant α1E-subunits were expressed in human embryonic kidney (HEK) cells and *Xenopus* oocytes (Schneider *et al.*, 1994) and these investigators established that R-type calcium currents could be blocked selectively by low concentrations (≤ 50 μM) of nickel chloride. It has further been shown that the spider toxin SNX-482 (0.1 μM) can also selectively block R-type calcium currents (Vajna *et al.*, 1998; Tottene *et al.*, 2000; Sochivko *et al.*, 2002).

In situ hybridization, reverse transcriptase-polymerase chain reaction and immunohistochemical studies have revealed that α1E-subunits are expressed throughout the central nervous system of mice (Williams *et al.*, 1994), rats (Yokoyama *et al.*, 1995; Grabsch *et al.*, 1999) and humans (Yokoyama *et al.*, 1995; Volsen *et al.*, 1997). α1E-subunits have also been localized to non-neuronal tissues such as cardiac myocytes from rats and human distal tubules from the kidney (Weiergräber *et al.*, 2000). α1E-subunits and R-type calcium channels are also expressed in sympathetic neurons (Murakami *et al.*, 2007), sensory neurons (Fang *et al.*, 2007) and somatic motor neurons (Urbano *et al.*, 2003; Pardo *et al.*, 2006) but localization of α1E-subunits has not been studied in other parts of the peripheral nervous system. Electrophysiological and immunohistochemical studies demonstrate the presence of all calcium channels, with the exception of T-type channels, in the ENS (Hirning *et al.*, 1990;

Kirchgessner & Liu, 1999; Smith *et al.*, 2003). L-type calcium channels, however, are not major contributors to the synaptic excitation of enteric neurons (Reis *et al.*, 2000; Reis *et al.*, 2002). Furthermore, the inhibition of L-type channels on GI smooth muscle reduces or abolishes spontaneous or evoked muscle movement (Horowitz *et al.*, 1996; Farrugia, 1999). P/Q type channels are expressed by myenteric neurons and contribute to neurotransmitter release from enteric nerves (Reis *et al.*, 2000; Smith *et al.*, 2003). R-type calcium channels may also contribute to somatodendritic action potentials or neurotransmitter release from myenteric nerve terminals (Rugiero *et al.*, 2002; Bian *et al.*, 2004). However, those studies did not identify specific functional classes of myenteric neurons: intrinsic primary afferent neurons (IPANs) or interneurons and motorneurons that express the R-type channel.

Electrophysiological studies have established that R-type calcium channels are the principal subtype in the somatodendritic region of guinea pig ileal myenteric neurons maintained in primary culture (Bian *et al.*, 2004). These data suggest that R-type calcium channels may contribute to synaptic transmission and regulation of membrane excitability of myenteric neurons. Functional classification of enteric neurons according to the neurochemical markers they express and their axonal projections is well established in the guinea pig gut (Brookes, 2001; Hansen, 2003). Substance P (SP), nitric oxide (NO), vasoactive intestinal polypeptide (VIP), 5-hydroxytryptamine (5-HT), the calcium-binding proteins calbindin and calretinin, and tyrosine hydroxylase (TH) are all markers for subsets of enteric neurons.

The predominance of R-type calcium currents in myenteric neurons suggests an important role for these calcium channels in enteric neurotransmission (Bian *et al.*, 2004). However, since the specific classes of myenteric neurons have not been identified, it remains to be determined as to whether R-type channels are localized to subsets of these neurons. Furthermore, there have been no studies of the cellular distribution of α1E calcium channel subunits in specific regions of the gut. The aim of the present investigation was to therefore localize α1E subunits with neurochemical markers for functional classes of neurons in the ENS of the guinea pig.

MATERIALS AND METHODS

Tissue collection

All animal use procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University. Adult male Hartley strain guinea pigs (n = 4; 300-400 g, Bioport, Lansing, MI) were deeply anesthetized with isoflurane (Abbott Laboratories, Chicago, IL), stunned by a blow to the back of the head, and then killed by severing the carotid arteries. Segments of the ileum, duodenum, gastric corpus, proximal and distal colon were dissected out and placed in 0.01 M phosphate-buffered saline (PBS; pH 7.2).

Protein isolation and Western blotting

The specificity and sensitivity of the α1E antibody were verified by Western blot. Longitudinal muscle-myenteric plexus (LMMP), submucosal and circular muscle preparations from the guinea pig ileum were isolated on ice and placed directly into liquid nitrogen. The rat brain and heart, and guinea pig heart served as positive controls for α1E expression. Tissues were ground to a powder in liquid nitrogen, and ice-cold homogenization buffer (125 mmol/L Tris (pH 6.8), 4% SDS, 20% glycerol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin] added. Homogenates were vortexed, sonicated briefly and transferred to a plastic centrifuge tube and spun at 4 °C to pellet debris. The supernatant was separated from the pellet and analyzed for protein concentration (BCA protein kit, Sigma-Aldrich, St. Louis, MO). Total protein (50 μg) was boiled for 5 minutes with

standard 4:1 sample buffer. Proteins were separated on 1 mm-thick, 10% SDS polyacrylamide gels using a Mini Bio-Rad® III apparatus and all samples were loaded into the gel in parallel. After transfer, the nitrocellulose membranes were blocked at 4 °C overnight in 5% milk with 0.025% NaN_3 and incubated with the α1E primary antibody (Table 1). Membranes were then rinsed three times in tris-buffered saline (TBS) with Tween (0.1%) with a final rinse in TBS and incubated with the appropriate horseradish peroxidase-linked secondary antibody (1:2000; Cell Signaling Technology, Beverly, MA, USA) for 1 hour at 4 °C with continuous shaking. ECL® reagents (Amersham Life Sciences, Arlington Heights, IL, USA) were used to visualize bands. Finally, gels were stained with Gel Code Blue (Pierce, Rockford, IL, USA) to verify protein loading.

Whole-mount preparations

Tissues were flushed of content, cut open along the mesenteric border, stretched out and pinned tautly onto a silicone elastomer-lined (Sylgard, Dow Corning, Midland, MI) petri-dish, and fixed in 10% neutral-buffered formalin (Sigma-Aldrich) overnight at 4 ^{0}C. Formalin-fixed tissues were cleared using 3 washes with dimethyl sulfoxide (J T Baker, Phillipsburg, NJ) at 10 minute intervals. Tissues were then washed in 0.01 M PBS (3 X 10 min intervals) at room temperature and stored in 0.01 M PBS at 4 ^{0}C.

Using fine scissors and forceps, the submucosal plexus, circular muscle and LMMP layers were sequentially peeled away and trimmed into ~8 mm^2

pieces. Tissues were placed into separate wells of a 24-well tissue culture plate (Corning Glass Works, Corning, NY). Each well contained 0.5 ml of 0.01 M PBS.

Primary cultures of myenteric neurons

Newborn guinea pigs (~36 h in age, ~70 g in weight) were sacrificed by severing the major neck blood vessels after deep isoflurane anesthesia. The ileum was removed and placed in cold oxygenated (95% O_2; 5% CO_2) sterile Krebs' solution of the following composition (mM): NaCl, 117; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$ 25; $NaHPO_4$, 1.2; glucose, 11. The LMMP was removed from the entire length of the ileum and cut into 5 mm segments. The dissected LMMP was divided into four aliquots and placed into 1 ml Krebs' solution containing 1600 U of trypsin (Sigma-Aldrich) for 30 min at 37 °C. The tissues were then triturated 30 times and centrifuged at 3000 g for 5 min. The supernatant was discarded and the pellet resuspended in 1 ml Krebs' solution containing 2000 U of crab hepatopancreas collagenase (Calbiochem, San Diego, CA) for 30 min at 37 °C. The suspension was again triturated and centrifuged for 5 min at 3000 g. The pellet was then resuspended in Eagle's minimum essential medium containing 10% fetal calf serum, gentamicin (10 µg/ml), penicillin (100 U/ml), and streptomycin (50 µg/ml) (Sigma-Aldrich). Cells were plated on coated poly-L-lysine glass coverslips and grown in an incubator in a 5% CO_2 atmosphere for up to 3 weeks. After 2 days in culture, cytosine arabinoside (10%) was added to the culture medium to inhibit smooth muscle and fibroblast proliferation.

Immunohistochemical procedures

All primary antibodies (see Table 1) were diluted in 0.01 M PBS/1% Triton X-100. For myenteric plexus preparations, the α1E primary antibody was incubated in combination with anti-calbindin, anti-calretinin, anti-5-HT, anti-SP, anti-VIP, anti-NOS and anti-TH primary antibodies. With the exception of anti-5-HT and anti-TH, the above antibodies were also applied to circular muscle preparations from each of the five regions. Primary antibody incubations were performed at 4 °C overnight in a humidified chamber. The tissues were then washed in 0.01 M PBS (3 X 10 min), and incubated with the appropriate secondary antibodies coupled to indocarbocyanine (CY3) or fluorescein isothiocyanate (FITC) for 90 min at room temperature. For the purposes of consistency during co-localization experiments, the α1E primary antibody was always localized with a CY3-conjugated secondary antibody while all other primary antibodies were localized with a secondary antibody conjugated to FITC. Tissue sections were subsequently washed in 0.01 M PBS (3 X 10 min) and mounted in buffered glycerol for confocal or conventional fluorescence microscopy. All photomicrographs are confocal images unless otherwise indicated.

Sequential dual-immunolocalization of α1E and nNOS. Since the α1E and NOS antibodies were raised in rabbit, an established protocol was used to study co-localization of these antigens (Franzusoff *et al.*, 1991; Negoescu *et al.*, 1994). Tissue sections were first pretreated with 10% normal goat serum (Sigma-

Aldrich) (diluted in 0.01 M PBS/ 1% Triton X-100) for 1 h at room temperature to block non-specific binding sites. The sections were then incubated with the NOS primary antibody diluted 1:200 in 0.01 M PBS/ 1% Triton X-100, in a humidified chamber at 4 °C overnight. Sections were then washed with 0.01 PBS (3 X 10 min). To prevent non-specific binding of the secondary antibody, the sections were then incubated with 0.01 M PBS/ 1% Triton X-100 containing 10% normal goat serum for 1 h at room temperature. Incubation with a goat anti-rabbit secondary antibody conjugated to FITC was performed for 2 h at room temperature. The tissues were then washed with 0.01 PBS (3 X 10 min). Non-immune rabbit serum (Pierce Biotechnology, Rockford, IL) was then applied to the tissue sections for 1 h at room temperature to saturate binding sites on the first secondary antibody (goat anti-rabbit-FITC conjugate), thus preventing the second primary antibody (α1E) binding. Monovalent goat anti-rabbit unconjugated fragment antigen binding (Fab) antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) (1:50 diluted in 1% Triton X-100) were then added to the tissue sections and incubated in a humidified chamber at 4 °C overnight. These Fab antibody fragments bind to free antigen-binding regions on the first primary antibody and to sites on the non-immune rabbit-IgG molecules, thus obstructing the binding of the second secondary antibody. The sections were then washed in 0.01 PBS (3 X 10 min) and blocked with 0.01 M PBS/ 1% Triton X-100/10% normal goat serum for 1 h at room temperature. Tissue sections were then incubated with the second primary antibody (α1E) diluted 1:400 in 0.01 M PBS/1% Triton X-100 in a humidified

chamber at 4 °C overnight, and subsequently washed in 0.01 PBS (3 X 10 min). The sections were again blocked with 0.01 M PBS/1% Triton X-100/ 10% normal goat serum for 1 h at room temperature. A goat anti-rabbit-CY3 conjugate was then applied to the tissue sections (2 h at room temperature). The tissues were rinsed in 0.01 PBS (3 X 10 min) and mounted with buffered glycerol for confocal and epifluorescence microscopy.

To eliminate potential sources of signal cross-talk (Brelje *et al.*, 1993; Schneider & Lopez, 2002) and to validate the specificity of immunostaining, three concurrent method control experiments were performed. These method controls included the omission of either one or the other primary antibody, and subsequent incubation with the corresponding secondary antibodies. The first control involved the omission of the first primary antibody, the exclusion of the second primary antibody served as the second control, and in the third control both primary antibodies were included. Antibody specificity for anti-α1E was determined using preadsorption tests on whole mount sections of the guinea pig stomach and intestine, and no α1E-ir was observed (see Fig. 3.3D inset).

Confocal and epifluorescence microscopy. Tissue sections were examined using a Leica TCS-SL laser scanning confocal system (Leica Microsystems Heidelberg, Mannheim, Germany) attached to a Leica Instruments DMLFSA microscope. Multicolor immunofluorescence analyses were then performed using a single line argon ion laser, (488 nm), which was used to excite FITC while a helium-neon laser (543 nm) excited CY3. Images were then

obtained using a 63 x 1.32 HCX PL APO oil immersion objective (Leica). The methods of Majlof and Forsgren (1993) and Schneider and Lopez (2002) were followed to facilitate accurate confocal imaging (Majlof & Forsgren, 1993; Schneider & Lopez, 2002). Sequential scan settings were used to obtain images at each wavelength individually. A xyz series range for a particular observer-determined area was defined, and a *z*-series of optical sections through the area of interest and orthogonal to the optical axis was obtained. The number of sections taken was calculated automatically by doubling the optimized step size. Paired red and green images from the different focal planes were then acquired with Leica confocal software (version 2.61) and merged to produce either an image montage or a single composite image. In the photomicrographs shown in this study, α1E-ir is always presented as red staining, with the alternate neurochemical marker presented as green staining. Yellow immunolabeling in a merged image represents antigen co-localization.

Conventional epifluorescence microscopy was used to indicate the extent of immunolabeling and to provide a histological orientation of labeled structures within each of the layers examined. Utilizing rhodamine and FITC filter sets, images corresponding to higher-resolution confocal images were viewed with a 40x numerical aperture objective coupled to a Nikon Eclipse TE2000-U inverted microscope (Nikon, Kanagawa, Japan). Images were then captured using MetaMorph 6.2r2 (Universal Imaging Corp, Downingtown, PA).

Colchicine treatment in vitro. To enhance α1E staining within small intestinal myenteric cell bodies, LMMP sections were dissected and pinned tautly onto a silicone elastomer-lined petri dish. The preparations were then incubated in sterile cell culture media comprising Eagle's minimum essential medium supplemented with fetal bovine serum (10%), gentamicin (10 μg/ml), penicillin (100 U/ml) and streptomycin (50 μg/ml) and colchicine (80 μM) (Sigma-Aldrich) for 24 h at 37 °C (Furness *et al.*, 1989). The tissues were then washed with 0.01 M PBS (3 X 10 min intervals) and then fixed in formalin overnight at 4 °C in preparation for immunohistochemistry.

Data analysis

Cell counts of co-localized labeling in neurons. Co-localized α1E/nNOS-ir cell bodies in one-hundred myenteric ganglia were counted in each of nineteen plexus preparations from four guinea pigs. These data were then exported to GraphPad Prism (GraphPad, San Diego, U.S.A) for statistical analysis. A paired t-test, or a one-way ANOVA followed by a Tukey-Kramer post-hoc test was used to determine statistical significance. $P < 0.05$ was considered significant. The data are presented as mean ± S.E.M.

Quantification of co-localization. Quantification of co-localization in merged images was assessed by a two-tiered approach: 1) line profiles, indicating pixel intensities, were drawn between structures deemed to have signal overlap (Zeiss Pascal LSM 5, Carl Zeiss Inc., Thornwood, NY); 2) object-

based analyses involving segmentation and thresholding techniques that separate and then classify varicosities as mathematically-defined individual objects (Silver & Stryker, 2000) were performed using Image Pro-Plus 4.5.1 (Media Cybernetics, Silver Spring, MD). A 7X7 pixel matrix high-gauss spatial filter (single-pass, strength 1) was applied to facilitate optimum signal-to-noise ratios. Figures were then imported into Adobe Photoshop CS 8.0 (Adobe Systems, Mountain View, CA) for further processing. Care was taken to ensure that when the overall sharpness, brightness and contrast of images were adjusted, that such manipulations did not alter the information content of the original images. Images in this thesis/dissertation are presented in color.

RESULTS

Western blot studies

Western blot analysis (Figure. 3.1) demonstrated the presence of the α1E subunit (~170 kDa) in the myenteric plexus of the guinea pig ileum. The guinea pig heart and myenteric plexus showed multiple low molecular weight bands. The strong expression of the 170 kDa α1E band in the circular muscle and its absence from the submucosa is supported by the immunohistochemical studies described below.

Summary of α1E-ir in the whole mount LMMP throughout the GI tract

α1E-ir was observed in cell bodies and axons of gastric neurons within myenteric ganglia and interconnecting nerve bundles. α1E-positive nerve fibers were abundant and diffusely distributed throughout the ganglia and interconnective fibers. This distribution pattern of α1E-ir was similar in all GI regions. In the small intestine (ileum and duodenum), no α1E-ir was detected in myenteric cell bodies. In the colon (proximal and distal), however, α1E-ir was present in cell bodies and axonal processes of some myenteric neurons. In all GI regions, strong α1E-ir was observed in nerve fibers supplying the circular smooth muscle. Longitudinal muscle nerve fibers in the tertiary plexus also exhibited α1E-ir, but this labeling was not as intense as that visualized in the circular muscle.

Summary of α1E-ir in ileal myenteric neurons maintained in primary culture

Since R-type calcium channel currents were recorded from dissociated ileal myenteric neurons in primary culture, and because no α1E-ir was observed in myenteric cell bodies from the whole-mount LMMP in the guinea pig ileum, I investigated α1E-ir in myenteric neurons in primary culture. While some neurons showed labeling throughout their cell bodies and axons, α1E-ir within the cell bodies was predominantly intracellular.

Co-labeling studies

α1E is not found in the submucosal plexus

α1E-ir was completely absent in the submucosal plexus in all regions of the gut. For example, I attempted to localize α1E-ir in submucosal ganglia of the ileum and proximal colon (Figure. 3.2) and no labeling was detected in any of these ganglia (Figure. 2A1 and C). Submucosal ganglia were revealed using an antibody raised against SP and numerous SP-ir nerve fibers were found in submucosal ganglia (Figure. 3.2A2). SP-ir but not α1E-ir was found in extrinsic sensory nerves supplying submucosal arterioles (Figure. 3.2B). TH is the rate limiting enzyme in norepinephrine synthesis and TH-ir is a marker for sympathetic nerves supplying the gastrointestinal tract. TH-ir revealed that sympathetic nerves supply submucosal ganglia and arterioles but these nerve fibers did not express α1E-ir (Figure. 3.2C, D, E).

Co-localization of α1E-ir and SP-ir in the myenteric plexus

SP-ir and α1E-ir were co-localized in varicose nerve fibers in myenteric ganglia throughout the gut, but this was not a complete overlap. This result is illustrated with data obtained in the proximal colon (Figure. 3.3A, B) and gastric corpus (Figure. 3.3C1, C2). Nerve cell bodies containing α1E-ir were also detected in myenteric ganglia of the proximal colon but SP-ir was not detected in these neurons (Figure. 3.3B). α1E-ir and SP-ir were also co-localized in nerve fibers in myenteric ganglia (Figure. 3.3C1, C2 and D) and in varicose nerve fibers supplying the longitudinal muscle layer (Figure. 3.3E) and circular muscle layers (not shown). In the duodenum, co-localization of α1E-ir and SP-ir occurred in nerve fibers in ganglia, in internodal strands and in the tertiary plexus supplying the longitudinal muscle layer. The intensity of α1E-ir labeling in the duodenal myenteric plexus was greater than that observed in the ileum, particularly in the interganglionic fiber tracts. I also identified some α1E-SP-ir in myenteric neurons in culture (Figure. 3.12C2, D2, and C3, D3).

α1E-ir with calbindin and calretinin

The calcium binding protein, calbindin, is the strongest marker for IPANs in the myenteric plexus (Song *et al.*, 1991; Costa *et al.*, 1996), while a second calcium binding protein, calretinin, is a marker for orally projecting interneurons and longitudinal muscle excitatory motorneurons in the myenteric plexus (Brookes, 2001). In whole mount LMMP sections, calbindin-ir was detected in myenteric nerve cell bodies in the stomach, ileum, duodenum and in the colon.

Representative images of calbindin-ir in nerve cell bodies are provided for the proximal colon (Figure. 3.4A, B). Although α1E-ir was readily detected in nerve fibers and nerve cell bodies in the stomach and colon, I never detected co-localization of α1E-ir and calbindin-ir in nerve cell bodies in the whole mount LMMP. These data are illustrated by results obtained in the proximal colon (Figure. 3.4A, B). α1E-calbindin co-localization was, however, present in myenteric neurons in culture (Figure. 3.12C1, D1). Calretinin-ir was detected in nerve fibers in myenteric ganglia throughout the gut and co-localized with α1E is some varicosities. These data are illustrated by results obtained in duodenal myenteric ganglia (Figure. 3.4C1, C2). Calretinin-ir was also detected in myenteric neurons of the distal colon but α1E-ir was not co-localized in calretinin-ir neurons (Figure. 3.4D1, D2). In culture, myenteric neurons showed some co-expression of α1E and calretinin in cell bodies (Figure. 3.12C4, D4) but α1E-calretinin-ir labeling was intense in axons (Figure. 3.12C5, D5).

α1E-ir and 5-HT-ir are not co-expressed in neurons

5-HT is contained in a subset of descending interneurons in the myenteric plexus and I used 5-HT as a marker for this class of neuron (Costa & Furness, 1982; Costa *et al.*, 1982). α1E and 5-HT did not co-localize in the myenteric plexus in any gut tissue examined or in dissociated neurons. α1E-ir was detected in varicose nerve fibers which formed distinct ring-shaped structures surrounding non-α1E-ir cell bodies in the ileum (Figure. 3.5A1). Varicose nerve fibers in internodal strands also showed intense and extensive α1E-ir. There

were relatively few 5-HT-ir nerve fibers but 5-HT-ir in these individual nerve fibers was intense (Figure. 3.5A2). Close analysis revealed intense α1E-ir and 5-HT-ir labeling of varicose nerve fibers within myenteric ganglia in the duodenum and 5-HT labeling was more prominent in the duodenum than in the ileum. It is possible that myenteric neurons in the duodenum contain more 5-HT than other regions (Meyer & Brinck, 1999). However, 5-HT-ir and α1E-ir were contained in separate populations of nerve fibers (Figure. 3.5B1, B2). In the corpus, strong α1E-ir was present in nerve fibers in myenteric ganglia and in interganglionic connectives. In those areas, nerve fibers displaying 5-HT-ir were more abundant than in the small intestine as reported previously (Mawe *et al.*, 1989). α1E-ir was detected in cell bodies and axonal processes in myenteric ganglia and internodal strands of the proximal and distal colon but α1E-5-HT co-localization was never observed (not shown).

Co-localization of α1E- and VIP-ir

VIP-ir is localized to cell bodies and nerve fibers in myenteric ganglia, to nerve fibers in the tertiary plexus which supplies the longitudinal muscle and to nerve fibers supplying the circular muscle layer (Costa & Furness, 1983; Brookes *et al.*, 1991b; Brookes *et al.*, 1992). I found VIP-ir nerve fibers in ileal myenteric ganglia and in the tertiary plexus and that arrangement of α1E-ir was very similar to that of VIP-ir in these regions (Figure. 3.6A1, A2). Closer examination of ileal myenteric ganglia revealed evidence of some co-localization of VIP-ir and α1E-ir in varicose nerve fibers (Figure. 3.6B1, B2). Similar overlap of VIP-ir and α1E-ir

in varicose nerve fibers was also detected in the tertiary plexus supplying ileal longitudinal muscle (Figure. 3.6C, D). Varicose nerve fibers in myenteric ganglia in the gastric corpus also displayed overlapping α1E- and VIP-ir (Figure. 3.6E, F).

Co-localization of α1E-ir with NOS-ir

I detected NOS-ir in myenteric neurons in the stomach, ileum and proximal colon (Figure. 3.7A1, B1, C and E1). However, α1E-ir labeling in neuronal cell bodies was only detected in the stomach (Figure. 3.7A2, B2) and colon (Figure. 3.7E2). In the gastric myenteric plexus I found 3.9 ± 0.60 α1E-NOS-ir neurons per ganglion, based on counts done in 100 ganglia each from gastric myenteric plexus from four guinea pigs. There was an anal-oral gradient in the numbers of α1E-NOS-ir neurons in the colon as there were 5.7 ± 0.5 neurons per ganglion in the proximal colon while there were 4.7 ± 0.6 α1-NOS-ir neurons in the distal colon ($P < 0.05$) (Figure. 3.8).

NOS-ir neurons were prominent in ileal myenteric ganglia, but α1E-ir neurons were never detected in these ganglia (Figure. 3.7C). I detected co-localization of α1E-ir and NOS-ir in nerve fibers in the ileal myenteric plexus (7D1, 7D2). However, there was not complete overlap of α1E and NOS labeling as I detected nerve fibers which were labeled by only one or the other antibody. Therefore, α1E is a marker for a subpopulation of NOS-containing nerve fibers.

I did not detect α1E-ir in myenteric nerve cell bodies in the ileum although I did detect varicose nerve fibers that contained α1E-ir and NOS-ir. It is possible

that α1E-ir does not accumulate in myenteric nerve cell bodies in this region of the gut. I used a colchicine-incubation protocol in an effort to increase α1E-ir in ileal myenteric neurons. Colchicine disrupts axonal transport and should cause an accumulation of proteins in nerve cell bodies (Costa *et al.*, 1996). However, α1E-ir was not detected in myenteric neurons even in small intestinal preparations that had been incubated with colchicine (not shown). Intense co-immunolabeling for α1E and NOS was, however, observed in the cell bodies and axons of dissociated ileal neurons (Figure. 3.13A1-A3).

Identification of excitatory and inhibitory muscle motorneurons

I next asked whether α1E-ir is co-expressed with SP-ir, VIP-ir and NOS-ir in nerve fibers supplying the muscle layers. In all regions α1E- and SP-ir co-localized in many nerve fibers supplying the longitudinal and circular muscle layers. This result is illustrated by data from nerve fibers supplying ileal circular muscle (Figure. 3.9A1, A2). SP-ir in nerve fibers in the muscle layers is a marker for excitatory motor innervation (Brookes *et al.*, 1991b; Brookes *et al.*, 1992; Brookes *et al.*, 1998). I therefore reasoned that the α1E-ir that did not contain SP-ir was present in inhibitory nerve fibers. VIP-ir and NOS-ir were used as markers for inhibitory nerve fibers (Costa and Furness, 1983; Brookes, 1993) and it was found that α1E-VIP-ir and α1E-NOS-ir were co-localized in nerve fibers supplying the muscle layers throughout the gut. This result is illustrated with data obtained in the circular muscle of the stomach (Figure. 3.9B) and proximal colon (Figure. 3.9C).

Co-localization of α1E-ir and TH-ir

As discussed above TH-ir is a marker for sympathetic nerve fibers supplying the gut. Sympathetic nerves inhibit gut motility by inhibiting myenteric neuronal activity and myenteric ganglia are innervated by sympathetic nerve fibers. I next determined if the sympathetic nerve supply of the myenteric plexus expressed α1E-ir. I found that nerve fibers in the myenteric plexus that contained TH-ir also contained α1E-ir (Figure. 3.10A, B). In the myenteric plexus brightly fluorescent rings of α1E-TH-ir varicosities circled non-α1E-ir cell bodies. Intensely labeled α1E and TH varicosities were also found in ganglionic fibers in the myenteric plexus and internodal strands of the duodenum (Figure. 3.10C). The network of ganglia and interconnecting nerve fibers in the duodenum consistently demonstrated intense α1E-ir and TH-ir. In the stomach, α1E- and TH-ir were localized to varicose nerve fibers in the myenteric ganglia and interganglionic fiber tracts. In the gastric myenteric plexus, nerve fibers co-expressing α1E-ir and TH-ir were less dense than in the corresponding areas of the other gut regions. In the colon (Figure. 3.10D), α1E-ir and TH-ir were co-localized in nerve cell bodies and nerve fibers in myenteric ganglia. The same pattern of immunohistochemical labeling applied to the distal colon, where both the α1E and TH antibodies labeled nerve cell bodies and varicose nerve fibers. There were more α1E-TH-ir neurons per myenteric ganglion in the proximal colon (2.1 ± 0.06) compared to the distal colon (1.4 ± 0.02, $P < 0.05$) based on counts in 100 ganglia in preparations from four guinea pigs (Figure. 3.11).

Furthermore, an interesting observation was that although not all α1E-cell bodies were TH-ir, all TH-ir neurons were α1E-ir.

Co-localization of α1E-synaptophysin-ir and SP-synaptophysin-ir

Synaptophysin is an integral synaptic vesicle membrane protein believed to be involved in neurotransmitter release after phosphorylation by tyrosine kinases (Evans & Cousin, 2005). Interestingly, in the guinea pig ileum, α1E and synaptophysin did not co-localize in nerve fibers in myenteric ganglia or in the tertiary plexus (Figure. 3.13A2-C2; A3-C3). Very rare co-expression of these two proteins, however, was found in some varicosities. Prominent SP-synaptophysin-ir was observed in varicosities in myenteric ganglia and longitudinal muscle in the tertiary plexus (Figure. 3.13 A4-C4).

DISCUSSION

Identification of the chemical coding of functional classes of enteric neurons has been of great help in understanding how the ENS functions. The results reported in this study identified subtypes of neurons that express R-type calcium channels. This information will assist in identifying the contribution of R-type calcium channels to the control of gastrointestinal function.

α1E is not expressed by submucosal neurons

Submucosal neurons control water and electrolyte secretion by the gastrointestinal epithelium and they also control local blood flow by providing

vasodilator input to submucosal arterioles. Submucosal arteriolar diameter is also controlled by sympathetic nerves that supply these blood vessels and by extrinsic SP-calcitonin-gene related peptide (CGRP) containing sensory nerves (Vanner & Macnaughton, 2004). SP and CGRP are both vasodilator substances (Uddman *et al.*, 1986). Neither the extrinsic nerve supply of submucosal ganglia nor arterioles (sympathetic and sensory) nor intrinsic submucosal neurons expressed α1E-ir. Although I was unable to localize α1E-ir in the submucosal plexus, I was able to confirm, using TH immunohistochemistry, the presence of periarteriolar sympathetic nerve fibers and sympathetic nerve fibers terminating in submucosal ganglia. Similarly, I was able to demonstrate the presence of SP-ir nerve fibers in submucosal ganglia and around submucosal arterioles. These data suggest that drug treatments which either block or facilitate the function of R-type calcium channels would have little effect on secretomotor activity or blood flow in the submucosa.

α1E-ir is localized to cell bodies of myenteric plexus IPANs in the small intestine

Intestinal IPANS contain calbindin and SP and they are the first neurons activated in reflex pathways responding to chemical and mechanical stimuli of the mucosa (Furness *et al.*, 1999; Furness *et al.*, 2004). In the small intestine, IPANS constitute approximately 26% of myenteric neurons and these same neurons have axons that project to other myenteric ganglia (Brookes *et al.*, 1995). In the small intestine, I could not detect α1E-ir nerve cell bodies, even in

tissues that had been incubated with colchicine in an effort to enhance cell body content of α1E-ir. Our calbindin antibody did not label nerve fibers although I was able to detect prominent α1E-ir in nerve fibers in the myenteric plexus. I could, however, assess the overlap of α1E with calbindin in small intestinal myenteric neurons maintained in culture. In these neurons, α1E-calbindin-ir was co-localized mainly within the cell soma. Very little co-staining was observed in axonal processes. This, in agreement with the study by Rugiero et al. (2002), suggests that R-type calcium channels may contribute to the tetrodotoxin-insensitive calcium current during the somal action potential in IPANs.

SP-ir is contained in IPANS which send processes to myenteric ganglia and SP-ir is also contained in ascending interneurons (Brookes, 2001). Ascending interneurons in the guinea pig myenteric plexus constitute approximately 5% of the total number of neurons present (Brookes, 2001). Motorneurons will not have processes in myenteric ganglia so they will not contribute to SP-ir in the plexus (Costa *et al.*, 2000). I found substantial co-localization of α1E- and SP-ir in myenteric ganglia. Calretinin, however, is also a marker for ascending interneurons in the myenteric plexus (Brookes *et al.*, 1991a; Brookes, 2001) and I used an antibody against this protein to localize nerve fibers in the myenteric plexus. Within the 5% of ascending interneurons, both SP and calretinin share the chemical coding in this type of neuron. In doubled-stained preparations, I detected α1E-ir and calretinin-ir nerve fibers, and found that a small population of myenteric nerve fibers expressed both α1E-ir and calretinin. Therefore, α1E-ir may be present in the nerve endings of this

subset of calretinin-containing ascending interneurons. Based on these data, I conclude that the α1E-ir nerve fibers in the small intestinal myenteric plexus arise from SP-containing and calretinin-containing excitatory interneurons.

I was able to detect α1E-ir and calbindin-ir neurons in the gastric myenteric plexus but I never detected co-localization of these two antigens in the same neuron. I did not determine their co-expression in gastric or colonic myenteric neurons in culture. Similar to my data in the small intestine, I found that, in the gastric corpus, α1E-ir and SP-ir were co-localized in nerve fibers in myenteric ganglia and in the muscle layers. SP-ir and calbindin are localized to a subset of gastric myenteric neurons but there are also SP-ir neurons which do not contain calbindin or calretinin (Furness *et al.*, 1988; Reiche *et al.*, 1999; Reiche & Schemann, 1999; Schemann *et al.*, 2001). The SP-ir but calbindin/calretinin negative neurons are either a separate population of ascending interneurons or circular muscle excitatory motorneurons (Michel *et al.*, 2000; Schemann *et al.*, 2001). My data in the ileum show that while α1E-ir co-localized with calbindin mainly in cell bodies in culture, α1E-ir was also present in calretinin-positive nerve fibers in the whole mount LMMP and in myenteric neurons in culture. It would therefore seem likely that α1E-ir nerve fibers in gastric myenteric ganglia are probably from ascending interneurons. I also show that α1E-ir and SP-ir co-localized in nerve fibers supplying the muscle layers in the stomach and these must arise from excitatory motorneurons. Therefore, R-type calcium channels may contribute to synaptic transmission in myenteric ganglia and to excitatory neuromuscular transmission in the gastric corpus.

In the colon, IPANS contain calbindin and SP-ir (Messenger & Furness, 1990; Lomax *et al.*, 1999; Lomax & Furness, 2000) and I found numerous nerve fibers containing both α1E-ir and SP-ir suggesting that nerve fibers might arise from colonic IPANS. However, unlike in the small intestine I was able to detect both α1E-ir and calbindin-ir neuronal cell bodies in the colon myenteric plexus but these proteins were expressed in separate neurons. Therefore, the nerve fibers containing α1E-ir and SP-ir are unlikely to come from calbindin-containing IPANS in the colon. There is a second set of SP-ir neurons that contain choline acetyltransferase but not calbindin in the myenteric plexus of guinea pig colon; these are ascending excitatory motorneurons supplying the circular muscle (Lomax and Furness, 2000). The SP-α1E-ir nerve fibers I detected are likely to arise from this subgroup of neurons. These data suggest that R-type calcium channels would contribute to excitatory neurotransmission to the circular muscle layer in guinea pig colon.

α1E does not co-localize with 5-HT in the myenteric plexus

5-HT is contained in some myenteric neurons and by enterochromaffin (EC) cells (Bülbring & Gershon, 1967). The importance of 5-HT in mucosal sensory transduction and as a transmitter in the regulation of secretory and motor functions in the gastrointestinal tract is well documented. In this regard, 5-HT released by EC cells may activate extrinsic and intrinsic sensory, motor and secretomotor neurons, and effector cells such as enterocytes and smooth muscle

cells (Tonini, 2005). 5-HT is also a mediator of slow synaptic excitation in some myenteric neurons (Monro *et al.*, 2005).

In the small intestine and stomach, there are ChAT-5-HT-containing descending interneurons (Clerc *et al.*, 1998; Meedeniya *et al.*, 1998; Lomax & Furness, 2000; Reiche *et al.*, 2000). In the stomach 5-HT-ir neurons also contain calbindin while 5-HT-ir descending interneurons in the distal colon are similar to the small intestine in that they contain calbindin (Lomax & Furness, 2000). I found that α1E-ir and 5-HT-ir were present in separate populations of nerve fibers in myenteric ganglia and interganglionic strands in all regions of the gut examined. Therefore, I conclude that R-type calcium channels are unlikely to contribute to 5-HT release from nerve terminals in the myenteric plexus.

α1E is expressed by enteric inhibitory neurons

NO and VIP are released from inhibitory motor neurons supplying the muscle layers in the gut (Murthy *et al.*, 1995; Xue *et al.*, 2000). I found that α1E-ir co-localized with VIP-ir and NOS-ir in cell bodies in myenteric ganglia, internodal connecting fibers and in nerve fibers supplying the longitudinal and circular muscle layers. α1E-ir and NOS-ir were co-localized in myenteric cell bodies and varicosities in the stomach and colon, and to varicose nerve fibers in myenteric ganglia, internodal strands and the muscle layers. In the ileum, VIP and NOS are present in descending interneurons, and inhibitory motor neurons. VIP but not NOS is also co-expressed with ChAT in some descending interneurons (Brookes, 2001). In the duodenum, however, NOS-ir was not

detected in VIP-containing descending interneurons, but motorneurons supplying the muscle layers in the intestine contain these neurotransmitters (Clerc *et al.*, 1998). Taken together, the localization of α1E with VIP and NOS in myenteric cell bodies and nerve fibers, and in dissociated myenteric neurons, has a number of implications: 1) α1E-VIP/NOS-ir varicose nerve fibers in the myenteric plexus of the small intestine and stomach arise from descending interneurons; 2) α1E-VIP-ir cell bodies in the colon and α1E-NOS ir neurons in the stomach and colon, are either descending interneurons or inhibitory motor neurons projecting to the circular and longitudinal muscle layers; 3) α1E-VIP-NOS labeling in nerve fibers supplying the muscle layers suggests that α1E is present in the nerve endings of inhibitory motorneurons. These results therefore suggest that R-type channels are likely involved in inhibitory neurotransmission to the muscle layers and in synaptic transmission in descending pathways in the myenteric plexus.

α1E-ir is expressed by sympathetic nerves supplying the myenteric plexus

TH is a marker for sympathetic nerve fibers and most TH-ir in the gut is extrinsic in origin. The exception is the proximal colon where amine-containing myenteric neurons have been observed (Costa *et al.*, 1971; Furness & Costa, 1971). In the present study I found that: 1) TH-ir was not present in myenteric neurons in the small intestine or stomach, but TH-ir co-localized with α1E-ir in nerve fibers in myenteric ganglia and interganglionic connectives; 2) the proximal colon exhibited a substantially greater number of TH-ir neurons than the distal colon, 3) both regions of the colon displayed a1E-ir/TH-ir nerve terminals.

Three separate subgroups of sympathetic neurons project to the gut. These are TH/NPY neurons that supply the intestinal blood vessels (Furness *et al.*, 1983), TH/somatostatin neurons that target the submucosal ganglia and mucosa (Costa & Furness, 1984) and TH-containing nerve fibers that contain another neurochemical marker and supply the myenteric plexus. My data suggest that α1E is a marker of sympathetic neurons that target the myenteric plexus and these sympathetic neurons have the neurochemical code of TH/α1E. A recent study showed that α2-adrenoreceptors couple to and inhibit the R-type calcium channel by mediating presynaptic inhibition of slow and fast synaptic transmission in the ENS (Bian & Galligan, 2007). Therefore, R-type calcium channels may be important for regulation of sympathetic neural control of gut motility.

α1E-ir does not co-localize with synaptophysin

The absence of co-localization of α1E and synaptophysin in myenteric ganglia suggests a number of possibilities: 1) since synaptophysin constitutes only 7% of the total synaptic vesicle membrane protein (Evans & Cousin, 2005), α1E may only be present on synaptophysin-negative varicosities; 2) the SNARE proteins syntaxin and SNAP-25, but not synaptobrevin, interact with N-type calcium channels by binding to the intracellular loop between domains II and III on the α1 subunit (Sheng *et al.*, 1994; Sheng *et al.*, 1996; Catterall & Few, 2008). Furthermore, N- and P/Q type calcium channels co-localize with syntaxin-1 (Catterall & Few, 2008). Putative α1E-ir varicosities may therefore contain a

neuronal vesicle population comprising of other synaptic vesicle membrane proteins such as synapsin, synaptotagmin (Wiser *et al.*, 2002), or perhaps even an as yet unidentified vesicle membrane protein; 3) synaptophysin may not be responsible for regulating neurotransmitter release from R-type calcium channel-containing nerve terminals. This is supported by evidence in CA1 hippocampal pyramidal neurons where the amplitude of excitatory postsynaptic currents, probability of vesicle release and overall synaptic transmission were unimpeded in mice that were synaptophysin-deficient (McMahon *et al.*, 1996). Those authors suggest that the function of synaptophysin may be a redundant one or that it may have a more subtle function not necessary for regulating exocytosis. This may indeed explain the surprising observation in my study that α1E and synaptophysin do not co-localize. It is unlikely that the intense and diffuse α1E-ir fibers in the myenteric plexus represent non-functional synapses or are completely non-varicose fibers, since I had observed definitive SP-synaptophysin punctate co-labeling in the LMMP (Figure. 3.13C4, D4). It would therefore be feasible in a future study to investigate the co-localization of α1E-ir with other synaptic vesicle membrane proteins, or presynaptic plasma membrane-anchored proteins such as syntaxin or neurexin.

α1E-ir does not co-localize with pan-cadherin

I did not observe any co-localization of α1E with the cell membrane marker pan-cadherin in neurons in primary culture or in the whole mount LMMP (Figure. 3.13A5-C5, A6-C6). Four auxiliary calcium channel subunits, β, $\alpha 2\delta$, and γ are

essential for the function of the α1 protein (Hoffman *et al.*, 1994) and all are expressed in neural tissue (Castellano *et al.*, 1993; Stephens *et al.*, 1997). The β subunit is located intracellularly and binds to the interaction domain on the α1 subunit, while the α2 subunit is an extracellular protein. The α2 subunit is anchored to α1 via the integral membrane protein δ. The γ subunit is also an integral membrane protein, but anchored directly to α1 (Catterall & Few, 2008). The β subunit enhances expression of the α1 protein and peak calcium current density at the cell membrane, presumably by trafficking a large number of functional calcium channels to the surface (Brice *et al.*, 1997; Hofmann *et al.*, 1999; Bichet *et al.*, 2000; Altier *et al.*, 2002). Interestingly, the expression of specific isoforms of the β subunit varies during postnatal development (Vance *et al.*, 1998). In that study, β4 protein expression increased ten-fold, concomitant with rat postnatal (P0-adult) development. α2δ subunits contribute moderately to cell membrane expression of α1 subunits, but γ subunits have no effect on α1 membrane expression (Catterall & Few, 2008). In fact, α2δ subunits strongly facilitate α1E expression and function by increasing the rate of voltage-dependent R-type calcium channel activation and inactivation (Qin *et al.*, 1998). Together, co-expression of α2δ and β calcium channel subunits with α1 increases surface expression of α1 to approximately 5 and 35% respectively (Williams *et al.*, 1992; Klugbauer *et al.*, 2003; Bernstein & Jones, 2007). Evidence suggests that the β subunit unlocks a signal motif on the I-II

intracellular loop on the α1 subunit, thus preventing retention of the α1 protein in the endoplasmic reticulum (ER) (Bichet *et al.*, 2000).

The absence of co-localization of α1E with pan-cadherin therefore suggests that: 1) since the α1E subunit has a number of glycosylation and phosphorylation sites (eg. by protein kinase C on the I-II intracellular loop), α1E may have been post-translationally modified, preventing it's folding in the rough ER and subsequent association with the accessory subunits for targeting to the cell membrane; 2) the β subunit may not have access to the putative ER-retention signal and thus cannot direct the movement of the α1E subunit from this intracellular compartment to the plasma membrane; 3) the cytoplasmic α1E staining observed could represent immature forms of the pore-forming subunit. The β subunit would then function to restrict α1E trafficking to the membrane to prevent cytotoxic insults such as uncontrolled calcium entry; 4) alternatively, α1E may be a mature protein, but the β subunit has been phosphorylated by protein kinases A and C, or undergone palmitoylation (Dolphin, 2003a). Palmitoylation regulates neurotransmission pre and postsynaptically (el-Husseini Ael & Bredt, 2002). This post-translational processing of the β (or even α2δ) protein would again mean ineffective trafficking of α1E to the plasma membrane. α1E would thus be localized mainly intracellularly; and 5) the cytoplasmic α1E staining in myenteric cell bodies in culture suggest that this may be a purely developmental occurrence. Incremental β4 expression occurs from P0 to adult in the rat brain (Vance *et al.*, 1998), and it is possible that in newborn guinea pigs that β subunits

may not be in their mature conformations, and therefore unable to facilitate efficient membrane targeting of α1E.

SUMMARY AND CONCLUSIONS

The α1E subunit of the R-type calcium channel is expressed by nerve fibers throughout the myenteric but not submucosal plexus in the guinea pig gastrointestinal tract. Based on an established neurochemical code for subclasses of myenteric neurons and myenteric nerve fibers in the guinea pig, I conclude that α1E-ir is found in the nerve terminals of sympathetic nerves supplying the myenteric plexus, ascending interneurons, inhibitory motorneurons supplying the muscle layers and the cell bodies of myenteric IPANS in the small intestine (Table 2). These observations suggest that it may be possible to selectively inhibit or facilitate neurotransmitter release from specific subsets of nerve endings using drugs which act to alter the activity of R-type calcium channels.

Table 3.1. Primary antibodies used to study the distribution of R-type calcium channels

Antibody	Dilution	Lot No	Host species and Source	Supplier's Characterization	References
α1E	1:300	AN-02	Rabbit (ACC-006, Alomone, Jerusalem, Israel).	Western blot of membrane extracts from rat brain identifies proteins at ~199 kDa and ~270 kDa. Absence of staining after preincubation with control antigen in rat cerebellar tissue.	Saegusa H, 2000 ; Fisher TE, 2000
Calbindin D-28K	1:200	113K4867	Mouse, clone CB-955 (C9848, Sigma-Aldrich, St. Louis, MO)	Immunoblotting with cultured kidney epithelial whole cell extracts labels a single protein of 28 kDa.	Katsetos CD, 1994)
Calretinin	1:200	507005828	Goat (AB1550, Millipore, Temecula, CA)	Western blot shows a single protein of 30 kDa. Preadsorption of rat cortical tissue with antibody and control antigen shows absence of immunolabeling.	Jacobowitz DM and Winsky L, 1991; Winsky et al, 1989
5-HT	1:200	522888	Mouse (AB16007, Abcam, Cambridge, MA)	Absence of staining after incubation with antibody and antigen in cryopreserved ventral mesencephalic neurons	Granberg D et al, 2000
Substance P	1:200	240907	Rat, clone NC1/34 (8450-0505, AbD Serotec, Raleigh, NC)	Complete crossreactivity for Substance P in rat brain tissues by radioimmunoassay	Cuello et al, 1979
Vasoactive intestinal peptide (VIP)	1:2000	25030911	Sheep (AB1581, Millipore)	Pre-incubation with VIP antibody and antigen abolishes immunostaining	Zhu et al, 1997

Table 3.1 (cont'd)

Neuronal nitric oxide synthase (nNOS)	1:200	LV1449629	Sheep (AB1529, Invitrogen); Rabbit (Dr. B Mayer, U of Graz, Austria)	Preadsorption of NOS antibody with antigen abolishes staining in rat frontoparietal cortex	Cauli B et al, 2004
Tyrosine hydroxylase (TH)	1:200	21071256	Mouse, clone TOHA1.1 (MAB358, Millipore)	Recognizes a~60 kDa TH protein by Western blot in rat cerebral cortex.	Rolletschek A et al, 2001; Semenenko, F. et al. 1986
Pan-cadherin	1:200	598458	Mouse, clone CH-19 (AB6528, Abcam)	Recognizes a protein ~135 kDa by Western blot in mouse hippocampal lysates.	Bae GU et al, 2008; Grimm MO et al, 2008
Synaptophysin	1:200	32K4862	Mouse, clone SVP-38 (S5768, Sigma)	Immunoblot shows a 38 kDa synaptophysin-positive band in rat brain extracts.	Eshkind and Leube, 1995

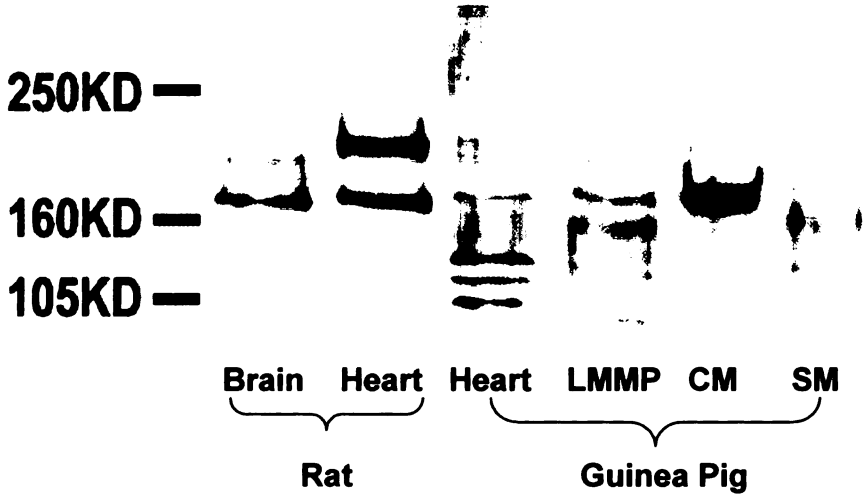


Figure 3.1. Western blot analyses of α1E subunit expression in rat brain and heart, guinea pig heart, longitudinal muscle-myenteric plexus (LMMP), small intestinal circular muscle layer (CM) and submucosa (SM). Note the multiple spliced variants detected in the myenteric plexus, and a prominent band near 170 kDa, the same size as that present in the rat brain and heart and guinea pig heart.

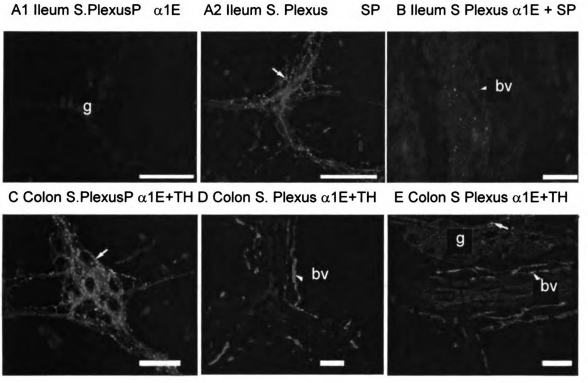


Figure. 3.2. The α1E-subunit is not expressed in the submucosal plexus in the ileum and colon. A1. SP-ir (green fluorescence) and absence of α1E-ir in a submucosal ganglion (g) in the ileum. **A2.** SP-ir (arrow) is present in submucosal ganglia. **B.** A merged image showing that arterioles (bv) in the submucosa were SP-ir (arrowhead) but not α1E-ir. **C.** A merged image showing TH-ir nerve fibers in a colonic submucosal ganglion do not contain α1E-ir. **D.** A merged image shown TH-ir (arrowhead) in periarterial nerve fibers. **E.** A merged image showing a submucosal ganglion adjacent to an arteriole; periarterial and intraganglionic nerve fibers contain TH-ir but not α1E-ir (arrow and arrowhead, respectively). Scale bars = 200 μm (A1-A2); 50 μm (B,C); 20 μm (D) and 30 μm (E).

Figure. 3.3. Co-localization of α1E- and SP-ir in nerve fibers in the LMMP in the colon, stomach and ileum. A. Merged (yellow labeling) low-magnification epifluorescence photomicrograph illustrating α1E-ir (red fluorescence) and SP-ir (green) co-localization in varicose nerve fibers (arrow) with no co-labeling in nerve cell bodies (cb) in the proximal colon. The arrowhead indicates α1E-SP co-localization in nerve fibers supplying the longitudinal muscle. Inset show a1E-ir in a myenteric ganglion and in the tertiary plexus (tp) supplying the longitudinal muscle. **B.** Confocal image showing α1E-ir and SP-ir co-localization (yellow) in varicose nerve fibers in the proximal colon (arrow). α1E-IR nerve cell bodies are also visible (arrowhead). **C1.** Co-localization of α1E and SP-ir in varicose nerve fibers in the myenteric plexus in the stomach. Arrowheads denote examples of varicosities containing both antigens. The dotted line A-B corresponds to the line profile analysis shown in **C2**. **D.** Confocal image showing α1E-SP-ir co-localization in nerve fibers in an ileal myenteric ganglion. Arrowhead indicates a varicosity containing both antigens but fibers containing either α1E-ir or SP-ir are also visible. The inset is a preadsorption control where preincubation of the α1E antibody with the antigenic peptide (1μg antibody/1μg peptide) abolished immunolabeling in the ileum LMMP. **E.** Nerve fibers of the tertiary plexus supplying the longitudinal muscle. Some nerve fibers (arrowhead in D) contained α1E- but not SP-ir. Scale bars = 200 μm (A); 100 μm (B); 18 μm (C1); 40 μm (D); 20 μm (E).

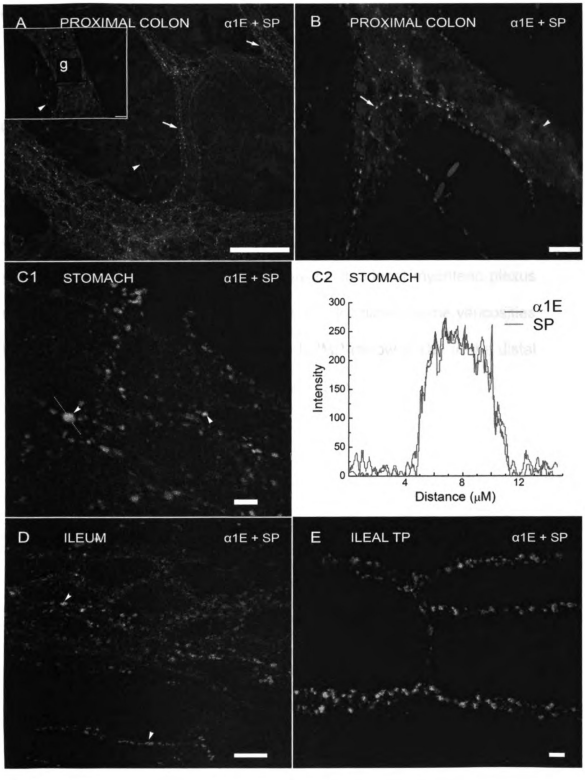

A
PROXIMAL COLON
α1E + SP
g
B
PROXIMAL COLON
α1E + SP
C1
STOMACH
α1E + SP
C2
STOMACH
α1E
SP
Intensity
300
250
200
150
100
50
0
4
8
12
Distance (μM)
D
ILEUM
α1E + SP
E
ILEAL TP
α1E + SP

Figure. 3.4. Localization of α1E-ir with calbindin-ir and calretinin-ir in the LMMP in the colon and duodenum. A. α1E-ir (red fluorescence) is contained in nerve fibers and nerve cell bodies (arrowhead) while calbindin (Calb; green fluorescence) is contained only in nerve cell bodies (arrows) in the proximal colon. **B.** Higher magnification image showing that α1E-ir (arrowhead) and calbindin-ir (arrow) are contained in different neurons in the myenteric plexus of the proximal colon. **C1, C2.** Images showing that α1E-ir (red) and calretinin (Calr; green) co-localize in some varicosities in the duodenal myenteric plexus (C2 arrowhead). **D1, D2.** α1E and calretinin also co-localize in some varicosities (arrowhead in D2) but not in cell bodies in the LMMP (arrow in D2) in the distal colon. Scale bars = 100 μm (A); 50 μm (B); 50 μm (C,D).

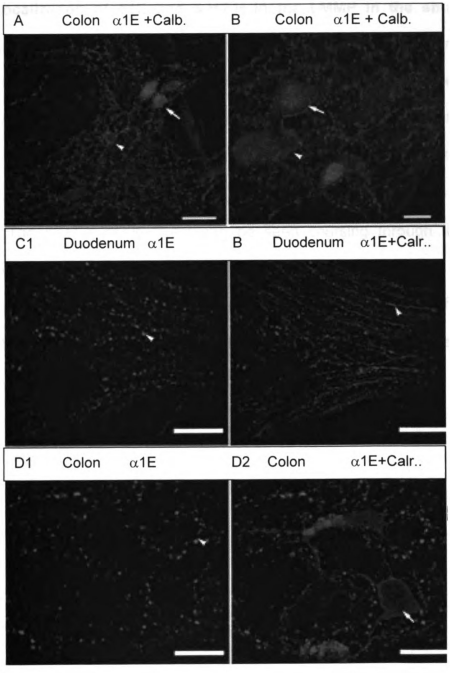

A Colon α1E +Calb.
B Colon α1E + Calb.
C1 Duodenum α1E
B Duodenum α1E+Calr..
D1 Colon α1E
D2 Colon α1E+Calr..

Figure. 3.5. Localization of α1E and 5-HT-ir in the LMMP in the small intestine. A1, A2. Low-magnification epifluorescence photomicrographs showing α1E-ir (red fluorescence) and 5-HT-ir (green) (arrowheads) are not co-localized in nerve fibers in myenteric ganglia (g) and internodal strands (is) in the ileum. Note the varicose clusters of α1E-ir that surround the neuron in (A). **B1.** Confocal image showing α1E-ir (arrowhead) and 5-HT-ir (arrow) in separate nerve fibers in the duodenum. The 5-HT-ir nerve fibers can be seen coursing through the myenteric ganglia independent of α1E-ir fibers. Similar results were obtained in the stomach ileum and colon. **B2.** The dotted line A-B indicates the pixel profile analysis from B1. This confirms that α1E-ir and 5-HT-ir are localized in different nerve fibers in the myenteric plexus. Scale bars = 100 μm (A-B); 14 μm (C).

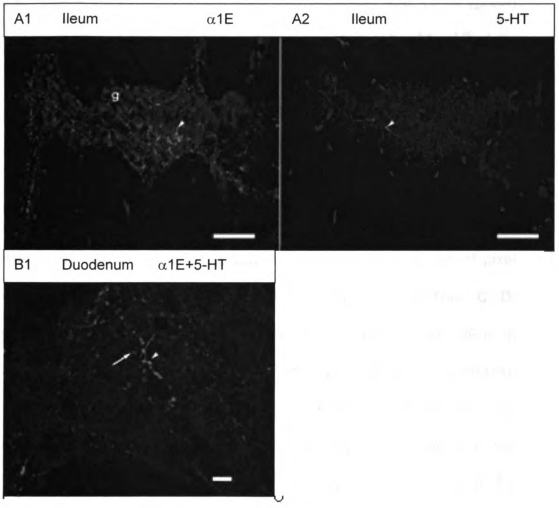

A1 Ileum α1E
A2 Ileum 5-HT
B1 Duodenum α1E+5-HT

Figure. 3.6. Co-localization of α1E-ir (red fluorescence) and VIP-ir (green fluorescence) in the LMMP in the ileum and stomach. A1, A2. Low magnification epifluorescence images demonstrating α1E and VIP-ir in varicosities (arrows) in myenteric ganglia and internodal strands in the guinea pig ileum. Note the prominent α1E-VIP positive nerve fibers in the tertiary plexus supplying the longitudinal muscle layer (arrowheads). **B1.** Confocal image of co-localized (yellow labeling) α1E-VIP-ir varicosities in the ileum. **B2.** Dotted line A-B is the line along which the pixel intensity was determined. Analysis of pixel intensities shows co-localization of α1E-ir and VIP-ir in this nerve fiber. **C, D.** Low-magnification epifluorescence photomicrograph illustrating α1E-VIP-ir in nerve fibers supplying the longitudinal muscle in the tertiary plexus (arrowheads). **E, F.** Epifluorescence (E) and confocal (F) images showing α1E-ir and VIP-ir co-localization (arrowheads) in varicose nerve fibers in a myenteric ganglion in the stomach. Scale bars = 200 μm (A1,A2); 55 μm (B); 20 μm (D-F).

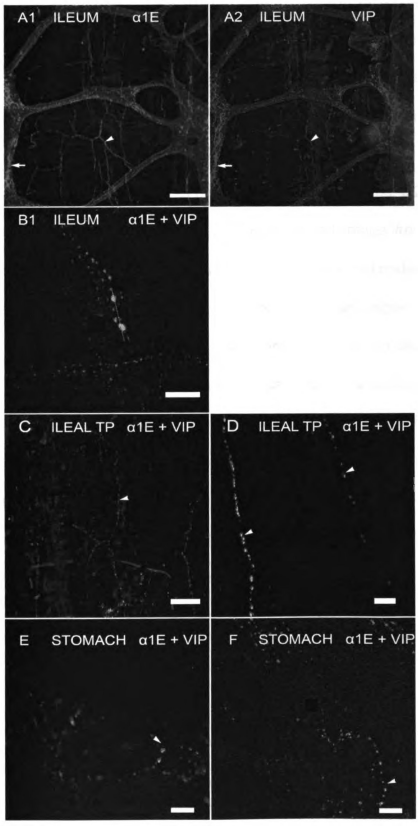
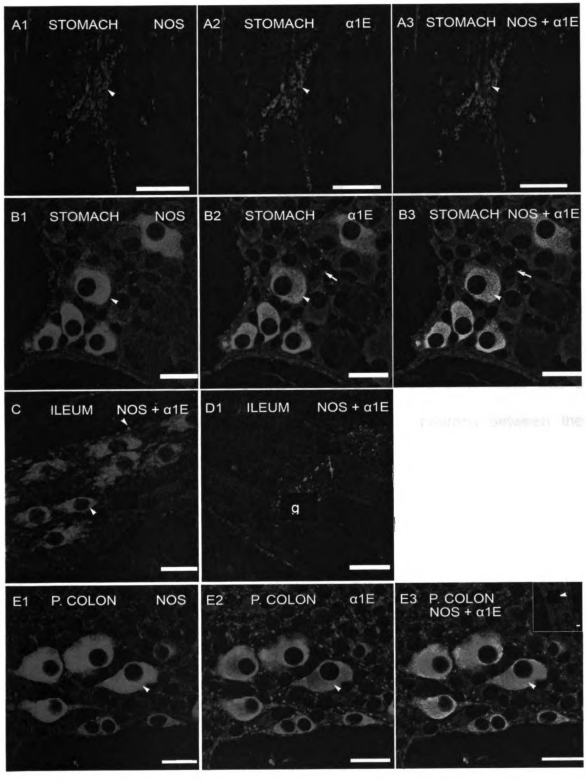

A1 ILEUM α1E
A2 ILEUM VIP
B1 ILEUM α1E + VIP
C ILEAL TP α1E + VIP
D ILEAL TP α1E + VIP
E STOMACH α1E + VIP
F STOMACH α1E + VIP

Figure. 3.7. Confocal photomicrographs showing co-localization of α1E-ir (red fluorescence) and NOS-ir (green) in the myenteric plexus. A1. NOS-ir in neurons in the gastric corpus. **A2.** the same ganglion contains α1E-ir neurons. **A3.** Merged (yellow) image of A1 and A2 showing overlap of α1E-ir and NOS-ir in gastric myenteric neurons. **B1.** High magnification images showing NOS-ir (arrowhead) localizes to myenteric neuronal cell bodies in the gastric corpus. **B2.** α1E-ir (arrowhead) is present in the same neurons. **B3.** Merged image from panels B1 and B2 shows co-localization of α1E-ir and NOS-ir in nerve cell bodies (arrowhead). Varicosities containing α1E-ir without NOS-ir are also visible in these images (arrow). **C.** Neurons (arrowheads) containing NOS-ir in an ileal myenteric ganglion. **D1.** α1E-ir and NOS-ir are co-localized in pericellular varicose nerve fibers (arrows). **D2.** Pixel intensity analysis through varicosities in D1. **E1-E3:** Co-localization of α1E-ir and NOS-ir in neurons in the myenteric plexus of the proximal colon. The inset in D3 illustrates a string of α1E-NOS-ir varicosities (arrowhead) in a myenteric ganglion from the proximal colon. Scale bars = 200 μm (A1-A3); 50 μm (B-E3); inset in D3 = 10 μm).


A1 STOMACH NOS
A2 STOMACH α1E
A3 STOMACH NOS + α1E
B1 STOMACH NOS
B2 STOMACH α1E
B3 STOMACH NOS + α1E
C ILEUM NOS + α1E
D1 ILEUM NOS + α1E
g
E1 P. COLON NOS
E2 P. COLON α1E
E3 P. COLON
NOS + α1E

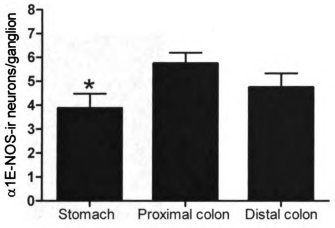


Figure. 3.8. Mean number of neurons per ganglion obtained from 100 myenteric ganglia, 19 plexus tissue sections and 4 guinea-pigs. The proximal colon displayed the highest number of α1E-nNOS-ir neurons (5.7 ± 0.5), followed by the distal colon (4.7 ± 0.6) and then the corpus (3.9 ± 0.6). There was a significant difference in the number of α1E-nNOS-ir neurons between the stomach and proximal colon.

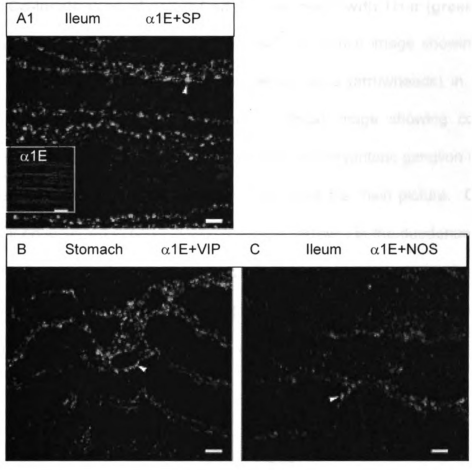
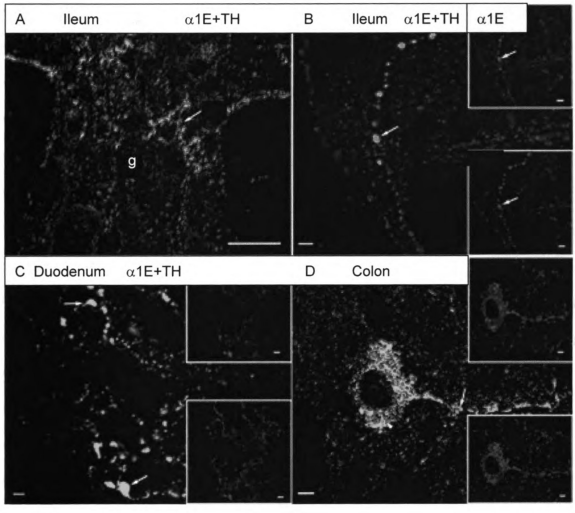


Figure. 3.9. Co-localization of α1E-ir (red fluorescence) with SP-, VIP- and NOS-ir (green fluorescence) in nerve fibers supplying the circular muscle layer. A1. α1E-ir co-localized (yellow) extensively with SP-ir in the circular muscle layer of the ileum. Inset shows a low magnification image of a1E-ir in the nerve plexus in the circular muscle layer. **A2.** Line A-B indicates a line profile analysis through the varicosities indicated by the arrowhead. **B.** α1E-ir nerve fibers in the circular muscle co-expressed VIP-ir in the stomach. **C.** α1E-ir and NOS-ir are co-localized in nerve fibers in the circular muscle layer in the proximal colon. Scale bars = 20 μm.

Figure. 3.10. Co-localization of α1E-ir (red fluorescence) with TH-ir (green) in the myenteric plexus. A. Merged (yellow) epifluorescence image showing co-localization of α1E-ir and TH-ir in varicose nerve fibers (arrowheads) in a myenteric ganglion in the ileum. **B.** Merged confocal image showing co-localization of α1E-ir and TH-ir in varicose nerve fibers in a myenteric ganglion in the ileum. Insets show individual images use to produce the main picture. **C.** α1E-ir and TH-ir co-localized in nerve fibers in ganglia (arrows) in the duodenum. Insets show individual images of α1E-ir and TH-ir. **D.** An α1E-TH-ir nerve cell body (arrowhead) and axon (arrow) in a ganglion in the proximal colon. Insets show un-merged images of α1E-ir and TH-ir. Scale bars = 200 μm (A); 20 μm (B); 16 μm (C) and 20 μm (D).


A Ileum α1E+TH
g
B Ileum α1E+TH
α1E
C Duodenum α1E+TH
D Colon

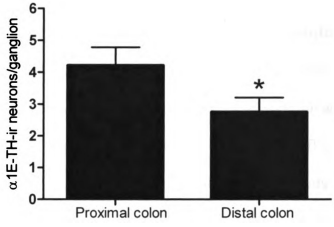


Figure. 3.11. The density of neuronal cell bodies co-localizing α1E and TH was higher in the proximal (4.2 ± 0.6) than in the distal colon (2.8 ± 0.5), and this difference was significant at P <0.05.

Figure. 3.12. Distribution of α1E (red fluorescence) with calbindin, SP, calretinin, and VIP (green fluorescence) in dissociated myenteric neurons in the guinea pig ileum. A1. α1E-ir (red) in neuronal cell bodies was cytoplasmic in all myenteric neurons observed. **B1.** Calbindin-ir was observed only in some neuronal cell bodies. **C1, D1.** Merged image (A1, B1) showing co-localization (yellow) of α1E- and calbindin in a neuronal cell body. **A2.** α1E-ir strongly localized within the cell soma and to lesser extent in axons. **B2.** Intense SP-ir was only detected around the periphery of myenteric cell bodies, and in axonal processes. **C2, D2.** This cell appears to have some co-localization (yellow, C2) at the periphery of the cell body. **A3-D3.** A cluster of three myenteric neurons, two of which are α1E-ir. The third unlabeled neuron is positive for neither α1E-ir nor SP. SP-ir was only present in neuronal processes. **A4.** Myenteric neurons immunoreactive for α1E. **B4.** Two calretinin-containing neurons show intense immunoreactivity in cell bodies. **C4, D4.** Calretinin-ir almost masks the effect of α1E-ir labeling in this image, but these two neurochemical markers do co-localize within the cell bodies. **A5-D5.** Prominent α1E-calretinin-ir in myenteric nerve fibers was observed, as evidenced by pixel intensity analysis (D5). **A6.** Cytoplasm and nerve fiber immunostained for α1E-ir. **B6.** VIP-ir was only seen in the nerve fibers of myenteric neurons in culture. No VIP immunostaining within the cell soma was visualized. **C6-D6.** Although some co-localization for α1E and VIP was present at the periphery of this α1E-ir cell, there are two distinct neurons present, one α1E-ir, the other VIP-ir. No or very little co-localization is seen along the length of the axons.

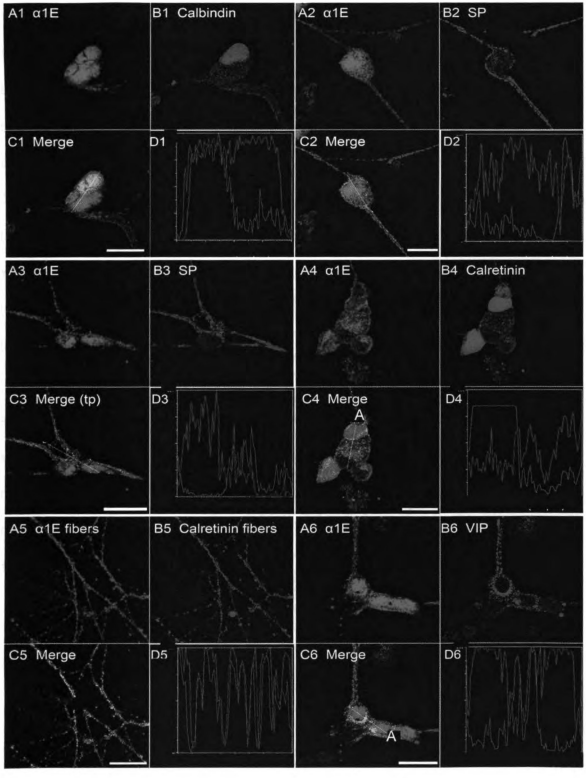
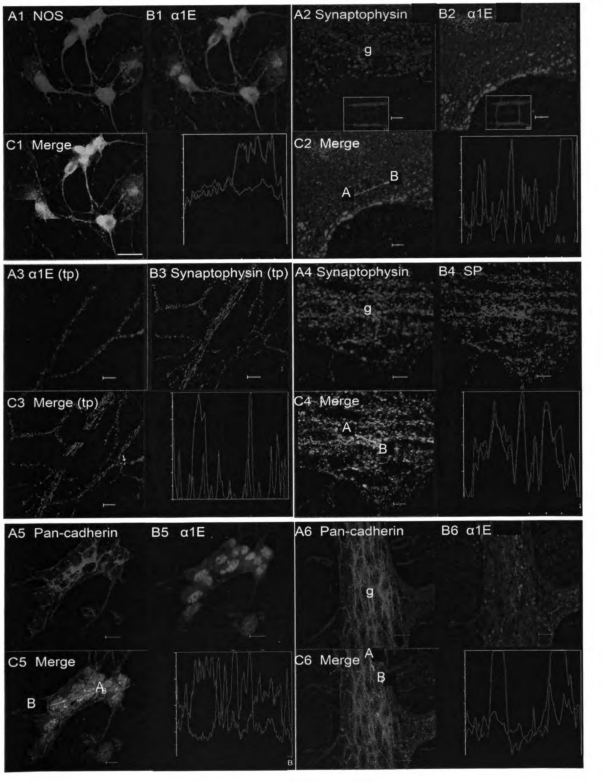

A1 α1E
B1 Calbindin
A2 α1E
B2 SP
C1 Merge
D1
C2 Merge
D2
A3 α1E
B3 SP
A4 α1E
B4 Calretinin
C3 Merge (tp)
D3
C4 Merge
A
D4
A5 α1E fibers
B5 Calretinin fibers
A6 α1E
B6 VIP
C5 Merge
D5
C6 Merge
A
D6

Figure. 3.13. Co-localization of α1E-ir with NOS, synaptophysin, SP with synaptophysin, and α1E-ir with pan-cadherin in the guinea pig ileal whole mount (WM) LMMP and myenteric neurons in culture. A1-D1. Confocal images and line pixel intensity analysis demonstrating α1E and NOS-ir co-localization (yellow) in cell bodies and axons in myenteric neurons in culture. **A2-D2.** Confocal image showing an absence of co-localization between α1E and synaptophysin-ir. Insets in A2 and B2 are low-magnification epifluorescence photomicrographs of the LMMP showing intense but restricted ganglionic (g) synaptophysin-ir; and α1E-ir throughout the LMMP respectively. **A3-D3.** α1E and synaptophysin do not co-localize in the tertiary plexus, but rare co-expression (arrow in C3) was sometimes observed. **A4-D4.** Synaptophysin and SP strongly co-localized to varicosities in the myenteric plexus. **A5-D5.** In myenteric neurons maintained in culture, α1E did not co-localize with the membrane marker pan-cadherin. **A6-D6.** In the LMMP, and with the rare exception of a few α1E-pan-cadherin-ir varicosities, co-labeling for α1E and pan-cadherin was not consistently detectable.


A1 NOS
B1 α1E
A2 Synaptophysin
B2 α1E
g
C1 Merge
C2 Merge
B
A
A3 α1E (tp)
B3 Synaptophysin (tp)
A4 Synaptophysin
B4 SP
g
C3 Merge (tp)
C4 Merge
A
B
A5 Pan-cadherin
B5 α1E
A6 Pan-cadherin
B6 α1E
g
C5 Merge
B
A
C6 Merge
A
B

CHAPTER 4 CONTRIBUTION OF R-TYPE CALCIUM CHANNELS TO THE FUNCTION OF INTRINSIC PRIMARY AFFERENT NEURONS AND ASCENDING INTERNEURONS IN THE GUINEA PIG ILEUM MYENTERIC PLEXUS

ABSTRACT

R-type calcium channels are expressed by subsets of myenteric neurons in the guinea pig ileum. In the present study, I used intracellular electrophysiological techniques preparation to determine the function of R-channels in myenteric neurons in the acutely isolated longitudinal muscle-myenteric plexus. I used the non-selective calcium channel antagonist, $CdCl_2$ (100 μM), the R-type calcium channel blockers $NiCl_2$ (50 μM) and SNX-482 (0.1 μM), and the N-type calcium channel blocker ω-conotoxin GVIA (CTX 0.1 μM). I studied the effects of these drugs on action potentials and fast and slow excitatory postsynaptic potentials (fEPSPs, sEPSPs) in S and AH neurons. CTX, but not $NiCl_2$, blocked sEPSPs in AH neurons. $NiCl_2$ (*n* = 14) and SNX-482 (*n* = 6) inhibited purely cholinergic, but not mixed cholinergic purinergic, fEPSPs in S neurons. $NiCl_2$ and SNX-482 reduced the duration and amplitude of action potentials in AH but not S neurons. $NiCl_2$ also reduced the amplitude of the late afterhyperpolarization (n = 4). These data show that R-type calcium channels do not contribute to calcium entry into nerve terminals releasing mediators of sEPSPs recorded from AH neurons but they do contribute to release of acetylcholine as the mediator of fEPSPs in a subset of S-type neurons. These data indicate that R-type calcium channels may be target for drugs that could selectively modulate activity of AH neurons or could alter fast synaptic excitation is subsets of pathways in the myenteric plexus.

INTRODUCTION

The enteric nervous system (ENS) is a division of the autonomic nervous system dedicated to local control of gastrointestinal (GI) function. The ENS exerts this control via intrinsic primary afferent neurons (IPANS), interneurons and motor neurons that reside in enteric ganglia within the gut wall. The myenteric plexus is located between the circular and longitudinal smooth muscle layers and controls GI motility. The submucosal plexus, found between the mucosa and circular muscle, facilitates absorptive and secretory responses (Costa *et al.*, 2000; Brookes, 2001). Together, these neural networks produce a myriad of coordinated GI responses (Wood, 2006a). Efficient functioning of the GI tract therefore relies on the integrative behavior of the enteric neurocircuitry.

Two types of myenteric neurons have been distinguished according to their electrophysiological properties: AH- and S-type neurons (Hirst *et al.*, 1974). AH-type neurons are the IPANs, and S-type neurons are interneurons and motorneurons (Furness *et al.*, 1998). AH neurons have a prominent shoulder on the repolarizing phase of the action potential due to inward calcium currents through one or more voltage-gated calcium channels. Calcium influx generates a long-lasting (1-20 s) late afterhyperpolarization mediated by activation of intermediate conductance calcium dependent potassium (IK) channels (Furness *et al.*, 2004). Trains of electrical stimuli applied to interganglionic nerve fibers evoke slow excitatory postsynaptic potentials (sEPSPs) that last seconds to minutes in AH neurons. A prominent mediator of the sEPSP is substance P (SP) acting at G-protein-coupled neurokinin-3 receptors (Galligan *et al.*, 2000; Furness

et al., 2004; Wood & Kirchgessner, 2004). The action potential in S-type neurons is blocked by the voltage-activated sodium channel antagonist tetrodotoxin (TTX) (Hirst *et al.*, 1974). Single stimuli applied to interganglionic nerve fibers produce a fast EPSP (fEPSP) in S-type neurons. ACh, acting at nicotinic cholinergic receptors (nAChR), is the major excitatory neurotransmitter in S neurons. However, non-cholinergic fEPSPs have also been observed and these fast synaptic responses are mediated by ATP acting at P2X purinergic receptors (LePard *et al.*, 1997; LePard & Galligan, 1999; Galligan, 2002a).

Myenteric neurons express all high-voltage-activated calcium channels (L, N, P/Q, and R-type), but not low-voltage-activated T-type calcium channels (North & Tokimasa, 1987; Kirchgessner & Liu, 1999; Reis *et al.*, 2000). Neurotransmitter release, contraction and secretory functions in the ENS are thus regulated by high-voltage-activated calcium channels (Smith *et al.*, 2003). The dihydropyridine-sensitive L-type calcium channel is found mainly on GI smooth muscle cells, and is not an active participant in synaptic excitation within the plexus (Marino *et al.*, 1993). L-, N-, P/Q- and R-type channels are present mainly on neurons and open at more depolarized voltages (~ -30 mV; maximum activation ~ 0 mV) and generally inactivate slower (> 100 ms) (Bean, 1989; Hoffman *et al.*, 1994; Yamakage & Namiki, 2002). The R (resistant) current may activate at somewhat more negative voltages (~ -40 mV) and often inactivates more rapidly than the other high-threshold currents (Fox *et al.*, 1987; Lorenzon & Foehring, 1995; Foehring *et al.*, 2000). HVA channels subtypes are blocked by different drugs. N- and P/Q-type calcium channels are blocked by ω-conotoxin

GVIA (ω-CTX) (Barajas-Lopez *et al.*, 1996; Chen & Kirchgessner, 2002) and ω-agatoxin IVA (ω-ATX) (Tsien *et al.*, 1988; Catterall, 2000; Bian *et al.*, 2004) respectively. Low concentrations of $NiCl_2$ (30-50 μM) and the spider toxin SNX-482 (0.1-1 μM) block R-type calcium channels (Randall & Tsien, 1995; Newcomb *et al.*, 1998; Wang *et al.*, 1999; Tottene *et al.*, 2000).

R-type calcium channels have been implicated in neurotransmitter release at rat hippocampal mossy and associative-commissural fiber synapses (Gasparini *et al.*, 2001), oxytocin release from rat neurohypophysial terminals (Wang *et al.*, 1999), and excitation-contraction coupling (Wu *et al.*, 1998; Albillos *et al.*, 2000). While a role for R-type calcium channels in the central nervous system (CNS) has been established, the function of the R-type calcium channel in the ENS is not known. Bian et al. (2004) showed that 50 % of the calcium current in guinea-pig ileal myenteric neurons maintained in primary culture is carried by R-type channels. The R-type channel was also shown to be coupled to the α_2-adrenoreceptor in guinea-pig myenteric neurons, implying that R-type channels may be targets for sympathetic nerve-derived norepinephrine to regulate synaptic transmission (Bian & Galligan, 2007). The co-localization of the R-type channel with SP, calretinin, vasoactive intestine peptide (VIP) and nitric oxide synthase (NOS) in varicose nerve fibers and cell bodies of calbindin-positive myenteric neurons in the guinea-pig ileum (Naidoo *et al.*, 2006) strongly suggests that R-type calcium channels may mediate calcium entry into specific subsets of myenteric neurons.

The objective of my study was to determine the contribution of R-type calcium channels to synaptic transmission in the acutely-isolated longitudinal muscle-myenteric plexus (LMMP) preparations of the adult guinea pig ileum. The native synaptic connections present in the guinea pig intestine *in vivo* are preserved in this *in vitro* preparation. I therefore investigated whether calcium entry through R-type calcium channels contributes to synaptic transmission or action potentials in guinea pig ileum myenteric neurons.

MATERIALS AND METHODS

Tissue collection and preparation

All animal use protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University. Adult male Hartley guinea pigs (250-300 g, Bioport, Lansing, MI) were anesthetized with isoflurane (Abbott Laboratories, Chicago, IL), stunned by a blow to the back of the head and exsanguinated. A segment of the ileum approximately 10 cm proximal to the ileo-cecal junction was quickly removed and placed in pre-warmed (37 °C) oxygenated (95% O_2; 5% CO_2) Krebs' solution of the following composition (in mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose. The Krebs' solution contained scopolamine (1 μM) and nifedipine (1 μM) to block muscarinic receptors and L-type calcium channels, respectively. A 5 mm^2 section of the ileum was cut open along the mesenteric border and pinned flat (Fine Science Tools, Foster City, CA) in a silicone elastomer-lined (Sylgard, Dow Corning, Midland, MI) petri dish containing Krebs' solution. The submucosa

and circular muscle were peeled away and the longitudinal muscle-myenteric plexus (LMMP) was transferred to a smaller silicone elastomer-lined perfusion chamber. The chamber was mounted on a stage of an Olympus microscope (Tokyo, Japan). The LMMP was pinned tautly and superfused continuously with warm (37 °C) Krebs' solution at a flow rate of 4 ml/min. The preparation was equilibrated for 30 min before commencing intracellular recordings.

Intracellular electrophysiological recordings

Myenteric neurons in the acutely isolated LMMP were impaled with a 2 M KCl-containing glass microelectrode (Frederick-Haer, Brunswick, ME) with a tip resistance of 80-120 MΩ. Membrane potential was recorded using an Axoclamp 2A amplifier (Axon Instruments, Foster City, USA) in bridge mode. Synaptic potentials were evoked focally with a monopolar glass electrode (tip diameter 60 μM) filled with Krebs' solution (World Precision Instruments, Sarasota, Florida) by stimulating interganglionic nerve fiber tracts. The resting membrane potential (RMP) of the impaled neuron was allowed to stabilize for 10 minutes without applying intracellular holding current. Myenteric (AH and S-type) neurons were then classified electrophysiologically according to published criteria (Hirst *et al.*, 1974; Morita & North, 1985; Wood, 1996). Short high frequency trains of stimulation (20 Hz, 500 ms), provided by a pulse generator (Master 8, A.M.P.I., Jerusalem, Israel) and a constant current stimulation unit (Grass Technologies, West Warwick, RI) were used to elicit slow excitatory postsynaptic potentials (sEPSPs). Single stimuli (0.5 ms duration, 0.1 Hz stimulus rate, 4-9 mA, 10 s

intervals) were used to evoke fast excitatory postsynaptic potentials (fEPSPs). When recording fEPSPs, the membrane potential was hyperpolarized to -90 mV to prevent fEPSPs from reaching action potential threshold. Signals were recorded using an analog to digital converter (Digidata 1322A, Molecular Devices Corp., Sunnyvale, CA) and Axoscope and Clampfit 10 software (Molecular Devices), and a desktop computer. Amplified signals were sampled at 2 kHz, filtered at 1 kHz and a digital average of 6 fEPSP sweeps was then obtained. The amplitudes of synaptic responses were measured under control conditions and after various drug treatments.

Action potentials (APs) and late afterhypolarizations (late AHPs) from AH neurons, and APs from S-type neurons, were elicited by injection of brief intracellular depolarizing current pulses (2 ms duration, 0.1 Hz, 0.02-1 nA, 6V, at 10 s intervals) through the recording electrode (De Laet *et al.*, 2002; Nurgali *et al.*, 2007). AH neurons were identified by the prominent calcium shoulder on the repolarization phase of the action potential (Hirst *et al.*, 1985). Myenteric AH and S-type APs and late AHPs were recorded at the resting membrane potentials of those neurons. I measured the amplitude, the half-maximum width, and the area under the curve (AUC) of the evoked APs and late AHPs. Half-maximum width was defined as the time at which the postsynaptic response is at half of its maximum value (Schneider, 2008). Neurons with RMPs less than -40 mV were excluded from the study.

Drug application

Local drug application was accomplished using a quartz micropipette (30 – 40 µm tip diameter) placed within 50 to 150 µm of the impaled neuron. Drugs were superfused near the impaled neuron and solution flow was gated using a VC-8 Valve Controller application system (Warner Instruments, Hamden, CT). The impaled neuron was exposed to drug treatments for at least 6 minutes before responses were recorded.

Drugs

Nifedipine, scopolamine, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS), $NiCl_2$, and mecamylamine hydrochloride were obtained from Sigma-Aldrich. ω-conotoxin GVIA (CTX), SNX-482 and tetrodotoxin (TTX) were purchased from Alomone labs (Jerusalem, Israel). All drugs were diluted in deionized water except for nifedipine which was dissolved in 95% ethanol.

Statistical analyses

Data are presented as mean values ± S.E.M for *n* neurons. Concentration-response data were fitted using non-linear regression and the Hill equation (GraphPad Prism 4, GraphPad, San Diego). Statistical differences between groups were analyzed with a t-test or one-way ANOVA with Tukey-Kramer post-hoc test comparisons. $P < 0.05$ was the criterion for determining statistical significance.

RESULTS

$CdCl_2$ inhibits action potentials in AH but not S neurons

To establish whether blockade of calcium channels would inhibit the calcium shoulder on the descending phase of the AH action potential, I applied the non-selective calcium channel antagonist $CdCl_2$ (100 μM) (Jun *et al.*, 2004) to the perfusion bath. In all 5 neurons tested, the calcium shoulder was blocked by $CdCl_2$ and this shortened the action potential half-width (Figure. 4.1A,B); $CdCl_2$ did not change action potential amplitude in AH neurons (Figure. 4.1A,C). After washout of $CdCl_2$, the voltage-gated sodium channel antagonist TTX (0.3 μM) was applied. TTX reduced action potential amplitude but not half-width (Figure. 4.1A-C). In all 5 neurons examined, $CdCl_2$ did not affect the amplitude or half-maximum width of the action potential (Figure. 4.2). However, the action potential in S neurons was blocked by TTX (Figure. 4.2).

R-type calcium channel blockers inhibit action potentials AH- but not S-neurons

I next determined the effect of the selective R-type calcium channel blocker $NiCl_2$ (50 μM) (Tottene *et al.*, 2000; Gasparini *et al.*, 2001) on the AH and S action potential. $NiCl_2$ reduced the AH neuron action potential half width by 44% and the peak amplitude by 9% ($n=12$ $P < 0.05$; Figure. 4.3). However, $NiCl_2$ (50 μM) did not change the action potential in S neurons ($n=12$, Figure. 4.4). These data provide pharmacological evidence that R-type calcium channels contribute to the action potential in AH but not S-type neurons.

Previous studies indicate that the calcium hump on the descending phase of the myenteric AH action potential is due to calcium entry through primarily N-type calcium channels (Franklin *et al.*, 1992; Furness *et al.*, 1998; Kang *et al.*, 2003). Strong immunohistochemical evidence points to the presence of N-type channels in calbindin-containing myenteric IPANS (Kirchgessner & Liu, 1999). On this basis, a significant portion of the calcium current is likely to be the N-type current. Analysis of the AH action potential in 3 neurons demonstrated that $NiCl_2$ (50 μM) and ω-CTX (0.1 μM) did not affect the amplitude of the action potential (Fig. 4.5A,B). However, $NiCl_2$ and ω-CTX produced additive reductions in the action potential half width in AH neurons.

At low concentrations, SNX-482 (0.1 μM) is a specific antagonist of R-type calcium channels (Newcomb *et al.*, 1998; Gasparini *et al.*, 2001; Bian *et al.*, 2004). I next determined whether SNX-482 inhibited action potentials in AH neurons. SNX-482 did not affect the mean amplitude of the AH action potential but it did reduce the action potential half-width (Figure. 4.5D,E). The subsequent addition of ω-CTX, produced a further inhibition of action potential half width (Figure. 4.5D) and also reduced the peak action potential amplitude. These data indicate that : 1) $NiCl_2$ and SNX-482 produce an almost identical effect on the action potential in AH neurons; and 2) N- and R-type calcium channels are the predominant contributors to the action potential in myenteric AH neurons. Since $CdCl_2$ and $NiCl_2$ did not alter the action potential of S-neurons, I therefore did not test the effects of SNX-482 or ω-CTX on the action potential of those neurons.

$NiCl_2$ inhibits the slow AHP in AH neurons

As $NiCl_2$ reduced the calcium shoulder on the action potential in AH neurons then the amplitude of the slow AHP should correspondingly decrease as the slow AHP is mediated by a calcium activated potassium channel. As anticipated, $NiCl_2$ (50 μM) reduced the peak AHP amplitude by 42 % (Figure. 4.6A,B). $NiCl_2$ also shortened the time course of the slow AHP as reflected by the reduction in the area under the curve (Figure. 4.6A,C) which was reduced by 46%.

R-type calcium channels do not contribute to slow synaptic excitation of AH neurons.

If R-type calcium channels contribute to the somal AH action potential, the next question I asked was whether R-type calcium channels contribute to release of the mediators of slow excitatory synaptic potentials (sEPSPs) in AH neurons. Enteric neurons use two means of communication, fast and slow chemical neurotransmission (Galligan, 2002b). Because neurotransmitter release from myenteric nerve terminals is regulated by voltage-gated calcium channels (Smith *et al.*, 2003), I first ensured that blockade of all calcium channels was feasible. The non-selective calcium channel blocker, $CdCl_2$ (100 μM), was used to block sEPSPs evoked by short trains (20 Hz 500 ms) of focal electrical stimulation applied to of interganglionic nerves strands, In all 5 AH neurons tested, $CdCl_2$ (100 μM) largely blocked the sEPSP (Figure. 4.7A). The mean amplitude of the control sEPSP was 21.13 ± 0.94 mV, while the mean amplitude in the presence

of $CdCl_2$ was 1.2 ± 0.3 mV. A complete concentration-response curve revealed a concentration-dependent calcium channel inhibition by $CdCl_2$ (1 – 100 μM) (*n* = 5; IC_{50} = 12 μM) (Figure. 4.7B). The decline in sEPSP amplitude caused by $CdCl_2$ was not related to a time-dependent decline in synaptic transmission because time controls revealed that sEPSP amplitude was stable over the time course of the pharmacology experiments (Figure. 4.7C).

The effect of $NiCl_2$ (50 μM) on sEPSP was studied in 16 AH neurons and it was found that $NiCl_2$ did not change sEPSP amplitude in any of these neurons (Figure. 4.8A,B). R-type calcium channels therefore do not contribute to slow synaptic excitation in AH myenteric neurons. To determine the voltage-gated channel subtype responsible for the sEPSP, I tested ω-CTX (0.1 μM). In all 4 neurons tested with $NiCl_2$ resistant sEPSPs, ω-CTX largely blocked the sEPSP (Figure. 4.9A,B). These results suggest that predominantly N-type calcium channels mediate sEPSPs in myenteric IPANS.

R-type calcium channels do not contribute to cholinergic/purinergic fEPSPs in S-type neurons

$CdCl_2$ (1–100 μM) blocked fEPSPs recorded from S neurons in a concentration-dependent manner (Figure. 4.10A,B; *n*=5; IC_{50}=10 μM). To demonstrate that this inhibition was due to calcium channel inhibition and not to a time dependent decline in synaptic transmission, time-control experiments were done where fEPSPs were evoked in drug-free conditions in 5 min intervals for 30

min. The amplitude of fEPSPs (n = 7; Figure. 4.10C) did not significantly change during this time period.

Previous studies revealed the presence of three pharmacologically distinct subsets of myenteric S-type neurons based on the mediators of fEPSPs in these cells. There are cholinergic fEPSPs (25% of neurons), mixed cholinergic/purinergic fEPSPs (67 % of neurons), and cholinergic/serotonergic fEPSPs (11 % of neurons) (Galligan & Bertrand, 1994; LePard & Galligan, 1999; Zhou & Galligan, 1999). I therefore next asked whether R-type calcium channels contribute to fast synaptic transmission in one of more of these classes of S-type neurons. To answer these questions, I used $NiCl_2$ (50 μM), the nAChR antagonist mecamylamine (10 μM), and the P2X receptor antagonist PPADS (10 μM) (Galligan, 2002a; Ren *et al.*, 2003) in an effort to block fEPSPs. Mecamylamine is a more potent nAChR antagonist than hexamethonium in myenteric neurons (Zhou *et al.*, 2002). In a subset of 15 neurons tested, $NiCl_2$ did not affect fEPSP amplitude (Figure. 4.11 A,B). Subsequent application of mecamylamine significantly reduced the fEPSP amplitude by 28 % (Figure. 4.11A,B; $P < 0.05$). Subsequent addition of PPADS further reduced the fEPSP amplitude by 77 % (Figure. 4.11A,B; $P<0.001$) compared to control. I then attempted to mimic the effect of $NiCl_2$ (50 μM) with SNX-482 (0.1 μM) on the fEPSP in 3 S-type neurons with mixed cholinergic/purinergic fEPSPs. SNX-482 did not change fEPSP amplitude in these neurons but subsequent application of ω-CTX (0.1 μM) reduced the fEPSP amplitude by 76 % (Figure. 4.12 A,B; $P<0.05$). These results indicate that calcium entry via primarily N-type, but not R-

type calcium channels mediate fEPSPs in cholinergic/purinergic S-type myenteric neurons.

R-type calcium channels contribute to cholinergic fEPSPs in S-type neurons

I next tested the hypothesis that the R-type channel is present at nerve endings using only acetylcholine as the fast synaptic transmitter. In 14 S-type neurons $NiCl_2$ (50 μM) reduced the fEPSP by 23 % (Figure. 4.13A,B; $P < 0.05$). $NiCl_2$ was washed out and in these cells mecamylamine (10 μM) inhibited the fEPSP by 95 % (Figure. 4.13A,B; $P < 0.01$). In the next experiments ($n = 6$), I first ensured that mecamylamine completely blocked the fEPSP. Then $NiCl_2$ (50 μM) reduced the fEPSP by 45 % (Figure. 4.13C, $P < 0.001$) followed by ω-CTX (0.1 μM) which inhibited the fEPSP amplitude by 42 % compared to $NiCl_2$ alone ($P < 0.05$).

I finally determined whether SNX-482 (0.1 μM) would again mimic the effect of $NiCl_2$ (50 μM). In 4 neurons examined, mecamylamine completely inhibited the fEPSP (not shown). After washout of mecamylamine SNX 482 was applied and it reduced the fEPSP by 40 % (Figure. 4.14A,B; $P<0.01$). Addition of ω-CTX (0.1 μM), then further reduced the fEPSP amplitude by 69 % (Figure. 4.14A,B; $P < 0.01$).

DISCUSSION

The ability of the ENS to effect appropriate responses upon receiving different types of information is due to its functional interconnectivity and sensitivity (Furness & Costa, 1987). The manifestation of plasticity within enteric neural circuits as a consequence of distention or chemical stimulation of the mucosa, is an inherent property of the adaptive and integrative behavior of the ENS. The neurochemistry and synaptic connections of enteric neurons shape the function of the enteric neural circuitry. The synaptic connections focus attention on voltage-gated gated calcium channels in synaptic transmission in the ENS. Voltage-gated calcium channels regulate the functions of IPANS, interneurons and motorneurons, and tailor reflex circuits to motility and secretory patterns (Holzer *et al.*, 1997). Electrophysiological studies demonstrated that ~50 % of the calcium current in ileal guinea pig myenteric neurons maintained in primary culture is the R-type calcium current (Bian *et al.*, 2004). That study, however, did not identify the specific subsets of myenteric neurons from which the R-currents were recorded. Immunohistochemical data later established the presence of R-type calcium channels in neurons and varicosities in the myenteric plexus in the adult guinea pig ileum (Naidoo *et al.*, 2006). The present study provides the first evidence that R-type calcium channels contribute to the action potential in AH-type neurons, and to synaptic transmission in a subset of S-type neurons in the guinea pig LMMP.

The AH neuron action potential is N- and R-type calcium channel-dependent

To reconcile the above descriptions of R-type calcium channels in the myenteric plexus, I first looked at the kinetics of action potentials from AH and S-type neurons. $NiCl_2$ (50 μM) and SNX-482 (0.1 μM) did not affect the action potential amplitude or half-maximum width in S-type neurons. However, in AH neurons, both $NiCl_2$ and SNX-482 significantly attenuated the mean peak amplitude and the half-maximum width in comparison with the control action potential. ω-CTX (0.1 μM) largely inhibited the remainder of the calcium shoulder on the repolarization phase of the AH action potential. The calcium shoulder is due in part to activation of a TTX-insensitive N-type channel activated calcium conductance in myenteric AH neurons (Vogalis *et al.*, 2001; Rugiero *et al.*, 2002). Moreover, strong N-type, but weak P/Q-type calcium channel immunoreactivity was found in IPANs (Kirchgessner & Liu, 1999). P/Q-type calcium channels (Rugiero *et al.*, 2002) and L-type channels do not appear to contribute to the calcium shoulder in guinea pig myenteric neurons (North & Tokimasa, 1987; Kunze *et al.*, 1994). Interestingly, Rugiero et al. (2002) found a residual calcium current after blockade of N-, L- and P/Q-type channels, and postulated a contribution by the R-type channel to the somal action potential in AH neurons. My results demonstrate that when the R-type and N-type calcium channels are blocked in tandem, the duration of the AH action potential is reduced by approximately 95 %. Furthermore, when I examined the effect of R-type channels on the late AHP, I found that $NiCl_2$ inhibited the late AHP amplitude by 42 %.

Taken together, my results confirm that primarily N-type calcium channels (~ 54 %) regulate calcium influx during action potential in AH-neurons. However, I show for the first time that R-type calcium channels (~ 40 %) also contribute significantly to calcium entry during action potential firing in the somatodendritic region of IPANs.

R-type calcium channels have been localized in neurons throughout the CNS (Gasparini *et al.*, 2001; Lee *et al.*, 2002; Dietrich *et al.*, 2003; Weiergraber *et al.*, 2008). In mouse hippocampal CA1 pyramidal neurons (Kavalali *et al.*, 1997; Isomura *et al.*, 2002; Yasuda *et al.*, 2003) and amygdala neurons (Lee *et al.*, 2002), R-type calcium channels participate in dendritic action potential generation. Inhibition of calcium channels in amygdala neurons heightened excitability and reduced the AHP amplitude in those neurons (Schiess *et al.*, 1993; Schiess *et al.*, 1999). My data indicate that in the ENS, R-type calcium channels may contribute to the AH neuron action potential by regulating somatic and dendritic excitability in IPANs. This is supported by previous evidence that R-type channels require strong depolarizations and that R-type currents have fast activation kinetics in rat cortical (Lorenzon & Foehring, 1995; Foehring *et al.*, 2000) and guinea pig myenteric neurons (Bian *et al.*, 2004). The late AHP maintains the resting conductance of AH neurons via voltage-insensitive IK channels that limit the firing rate of AH neurons to one or two action potentials in response to a depolarizing intracellular current pulse (Galligan *et al.*, 2000; Furness *et al.*, 2004). When R-type calcium channels on the somatodendritic region of IPANs were inhibited, the late AHP was correspondingly attenuated. R-

type calcium channels are therefore important contributors to the regulation of myenteric AH neuron excitability.

R-type calcium channels do not contribute to slow synaptic excitation in AH-type neurons

Slow synaptic excitation is mediated by G-protein coupled receptors that produce long-lasting slow-synaptic responses with a long latency. SP acting at neurokinin-3 (NK_3) and ACh acting at M_1 muscarinic receptors, are the principal mediators of slow excitatory neurotransmission in the ENS (Guard *et al.*, 1988; Costa *et al.*, 2000; Galligan, 2002b). A novel finding from the present study points to N-type rather than R-type calcium channels as the chief mediators of sEPSPs in AH myenteric neurons. This conclusion is based on the observation of an 89 % inhibition of the evoked sEPSP amplitude by ω-CTX. N-type calcium channels predominate on autonomic nerve terminals (Mochida *et al.*, 1996) while P/Q-channels are more prevalent on nerve terminals in the CNS (Takahashi & Momiyama, 1993). Those channel subtypes contribute to synaptic transmission in those regions (Hirning *et al.*, 1988; Miller *et al.*, 1988; Dolphin, 2003b). My data suggest that in the ENS, trains of stimulation could cause calcium entry through N-type channels to release SP. SP would then act at the postsynaptic NK_3 receptor through protein kinases A (Morita & North, 1985; Bertrand & Galligan, 1995) or C (Guard *et al.*, 1988; Bertrand & Galligan, 1995) to produce the sEPSP.

R-type calcium channels contribute to fast synaptic excitation in a subset of S-type neurons

Since R-type calcium channels are not involved in slow synaptic excitation, I next investigated their role in fast synaptic transmission. In the ENS, fast synaptic transmission in the ENS is accomplished via ligand-gated ion channels where acetylcholine (ACh) acting at nAChRs, ATP at P2X receptors and 5-HT at 5-HT_3 receptors are the predominant mechanisms of fast excitatory neurotransmission (Galligan, 2002a). My results demonstrate that nerve terminals releasing only acetylcholine as a fast synaptic transmitter in S-type neurons are sensitive to $NiCl_2$ and SNX-482. Further, R-type channels are not present on nerve terminals releasing both acetylcholine and ATP as fast synaptic transmitters as amplitude of the fEPSP does not change in the presence of either $NiCl_2$ or SNX-482. In both groups, ω-CTX inhibited the fEPSP amplitude, suggesting that N- and R-type channels are intimately involved in modulation of neurotransmitter release from nerve terminals of fully cholinergic myenteric S-neurons.

I believe that R-type calcium channels are localized to nerve terminals of fully cholinergic S-type ascending interneurons. Ascending interneurons use ACh acting at nAChRs and constitute 5 % of the total number of myenteric neurons in the guinea pig LMMP (Brookes, 2001). Descending interneurons use ACh acting at nAChRs, and ATP acting at P2X and P2Y receptors. I concluded that R-type calcium channels are present on the nerve endings of ascending interneurons because those neurons were completely sensitive to mecamylamine (Galligan &

Bertrand, 1994; LePard *et al.*, 1997; LePard & Galligan, 1999). In a second subset of S-type interneurons, PPADS (10 μM) blocked the ATP-mediated component of the mixed cholinergic/purinergic fEPSP (LePard *et al.*, 1997). In fact, the fully cholinergic $NiCl_2$-sensitive S-neurons observed in my study may be calretinin-coded (Naidoo et al. to be submitted). LePard and Galligan (1999) surgically interrupted all nerve fibers to study the ascending and descending projections of myenteric interneurons. They found that ascending interneurons use only ACh for neurotransmission, whereas descending interneurons use both ACh and ATP.

The involvement of R-type calcium channels in fast synaptic excitation in the CNS is conflicting (Gasparini *et al.*, 2001; Dietrich *et al.*, 2003). Dietrich et al. (2003) showed that presynaptic R-type calcium channels do not participate in fast neurotransmitter release at the mossy fiber-CA1 synapse in adult mice hippocampal neurons. However, separate evidence from neonatal rat hippocampal neurons showed that R-type channels contribute to fast synaptic transmission, and may be important for regulating neurotransmitter release and modulation of synaptic plasticity (Gasparini *et al.*, 2001). These contrasting observations may be due to species and/or developmental differences. Nonetheless, my data corroborate those of Gasparini and others (2001). At the mossy and associative-commissural fiber fast synapses, low concentrations of $NiCl_2$ (50 μM) and SNX-482 (0.3-1 μM) inhibited the amplitude of excitatory postsynaptic currents (EPSCs) equally (Gasparini *et al.*, 2001). The addition of SNX-482 followed by $NiCl_2$ did not further inhibit the EPSC amplitude. However,

an antagonist of N- and P/Q-type calcium channels (ω-CTX-MVIIC) significantly reduced the EPSC amplitude, indicating a role for R-type calcium channels in fast synaptic transmission. $NiCl_2$ and SNX-482 are also mutually occlusive in myenteric neurons maintained in culture (Bian *et al.*, 2004). In the present study, $NiCl_2$ (50 μM) significantly inhibited the fEPSP by 23 % (n = 14) and by 45 % (n = 6). This discrepancy could be because of low R-type channel expression in some of those (23 %-containing) S-neurons from which fEPSPs were evoked. SNX-482 attenuated the mean fEPSP amplitude by 40 %. Taken together, these results suggest that $NiCl_2$ and SNX-482 act specifically on the same pathway to inhibit fast synaptic transmission in S-type neurons. Whether $NiCl_2$ and SNX-482 are acting pre- or postsynaptically to inhibit the fEPSP remains to be determined as it is possible that R-type calcium channels also actively participate in synaptic integration.

A synaptic interaction protein site (synprint) site important for interaction with the vesicular release machinery (synaptobrevin, syntaxin and SNAP-25; the SNARE complex) was believed to be unique to N- and P/Q-type but not R-type channels (Maximov *et al.*, 1999). N- and P/Q-type channels physically and tightly couple to syntaxin 1A and SNAP-25 located on the nerve terminal membrane (Jarvis & Zamponi, 2001). R-type channels, however, may functionally interact with the SNARE complex at a putative unidentified interaction site (Bergsman & Tsien, 2000; Wiser *et al.*, 2002; Cohen *et al.*, 2003; Cohen & Atlas, 2004). This pre-association of the R-type channel with synaptic proteins suggests these calcium channels may be responsible for rapid synaptic vesicle exocytosis. My

data suggest that R- and N-type calcium channels spatially co-operate to facilitate efficient neurotransmission in fully cholinergic myenteric S neurons. In fact, this co-operativity between R- and N-type channels has been observed at the mouse endplate (Urbano *et al.*, 2003). R-type channels may be also located closer to the calcium sensor than N-type channels (Urbano *et al.*, 2003), possibly to synaptotagmin for fast synchronous neurotransmitter release (Catterall & Few, 2008). Therefore, in myenteric S-neurons, a possible mechanism for synaptic transmission via R-type channels could be: 1) a train of action potentials depolarizes the ($NiCl_2$-sensitive) S-neuron nerve terminal allowing calcium to enter though R-type channels, 2) calcium activates calcium/calmodulin-dependent (CAM) protein kinase II that then phosphorylates the cytoskeletal tethering protein synapsin on the SP-containing vesicle, 3) the vesicle is targeted to the docking site, 4) the low affinity calcium sensor synaptotagmin binds to syntaxin once calcium enters the cell, and exocytosis occurs. Taken together, R-type calcium channels may therefore contribute to fast synaptic excitation in the myenteric plexus of the guinea pig ileum.

The question then emerges, why are R-type calcium channel localized to ascending interneurons and specifically to a fully cholinergic subset of S-type neurons in the ENS? Does the ENS, by utilizing several calcium channel subtypes, employ a compensatory mechanism to allow efficient synaptic transmission? First, the topographical organization of N- and R-type calcium channels on nerve terminals may be important to short- and long-term synaptic plasticity for the fine-tuning of information encoding. Calcium channels are

modulated by Gβγ subunits released from Gi/Go proteins (Dolphin, 2003b). R-type calcium channels may be coupled to the inhibitory α_2-adrenoreceptor (Bian & Galligan, 2007). Furthermore, the SNARE complex enhances efficient exocytosis if calcium channels are close to the active zone. These properties allow synaptic transmission to be precisely regulated (Catterall & Few, 2008). The presence of both N- and R-calcium channel subtypes may serve to enhance synaptic transmission for the effective coordination of gut effector system (GI smooth muscle, secretory glands and blood vessels) responses.

Second, the guinea pig ileum contains only a single class of ascending interneurons, and these neurons provide synaptic input to other ascending interneurons and to excitatory circular and longitudinal muscle motorneurons (Brookes, 2001). It remains possible that the R-channel is present in ascending interneurons to ensure that transmission proceeds unimpeded. R-type calcium channels, located on purely cholinergic S-neurons could, by co-operating with N-type calcium channels, serve as "booster stations" for ACh release as ACh is the predominant mediator of fast synaptic excitation in the ENS. This is supported by data that R-type channels are more closely associated with the synaptic release machinery than N-type channels (Urbano *et al.*, 2003). R-type channels may not mediate fEPSPs through mixed cholinergic/purinergic S-neurons, presumably because the release of two transmitters, ACh and ATP, could increase the overall probability of successful neurotransmission. These neurons would rely on calcium entry via only N-type calcium channels for exocytosis. The functional significance of R-type channels in S-neurons may thus be one of redundancy.

SUMMARY AND CONCLUSIONS

R-type calcium channels in the guinea pig ileum make a substantial contribution to the action potential in AH neurons. The calcium inflection on the downstroke of the AH action potential is mainly mediated by both N- and R-type calcium channels. R-type channel blockers significantly inhibited the fEPSP in ascending interneurons that release only ACh. N-type channels also contribute to fast synaptic excitation in ascending pathways. I did not detect R-type channel mediation of cholinergic/purinergic S-neurons. However, N-type channels were the primary contributors to the fEPSPs in those neurons. R-type calcium channels may therefore be important to control of cholinergic synaptic excitation. This could have profound implications for the overall function of the ENS as ACh predominates fast synaptic transmission and is one of the most important neurotransmitters in ENS neurons.

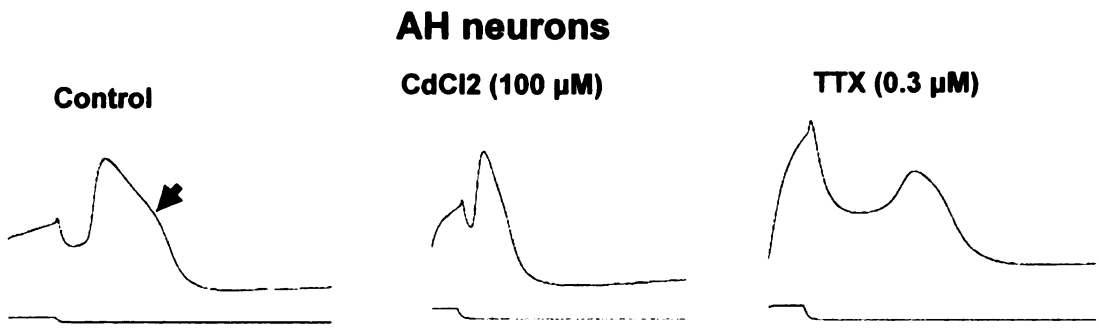


Figure. 4.1. CdCl2 inhibits action potentials in AH neurons. (A) A 2 ms intracellular depolarizing current pulse was used to evoke an action potential. The calcium shoulder (arrow in Control trace) on the repolarizing phase of the action potential was inhibited by CdCl2. Tetrodotoxin (TTX, 0.3 µM) blocked the Na^+ sensitive component during the depolarizing phase of the action potential to produce a smaller amplitude and slower calcium action potential. (B) Mean data ± S.E.M (*n* = 5) showing that CdCl2, but not TTX, reduced action potential half width (***, P< 0.01 vs Control and TTX). (C) TTX but not CdCl2 reduced action potential amplitude (*, P < 0.05 vs Control and CdCl2).

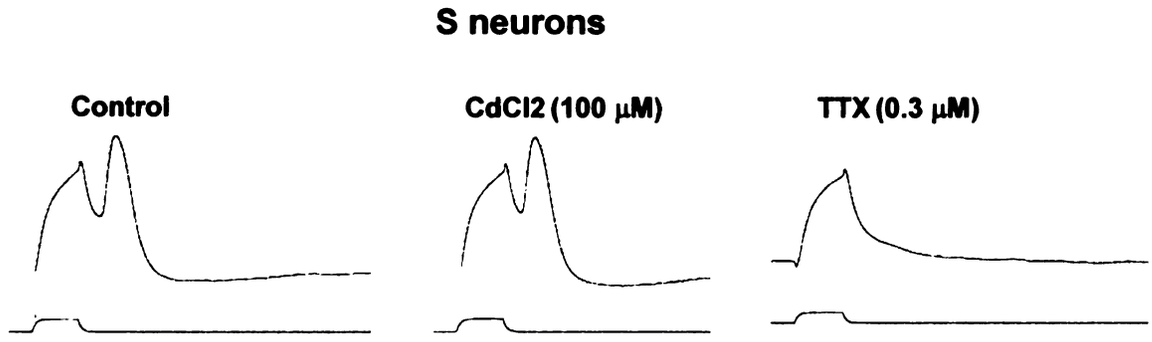


Figure. 4.2. CdCl2 does not affect action potentials in S-type neurons. (A) CdCl2 (100 μM) did not affect the action potential in S-type neurons while TTX (0.3 μM), completely blocked the action potential in the same neurons. (B) Mean data ± S.E.M (*n* = 5) from experiments similar to A.

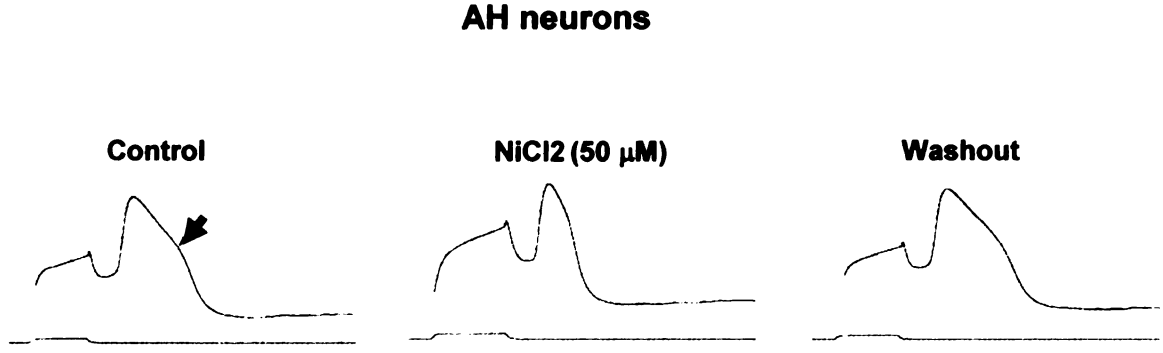


Figure. 4.3. $NiCl_2$ inhibits the action potential in AH neurons. Representative traces showing attenuation of the calcium shoulder (arrow in Control trace) on the repolarizing phase of the action potential in the presence of NiCl2. Mean data showing that NiCl2 shortened the action potential half-width (*n* = 12; *** $P < 0.001$) in a reversible manner. NiCl2 also reversibly reduced the action potential amplitude * $P < 0.05$). Data are mean ± S.E.M. *n* is the number of neurons from which AH action potentials were elicited.

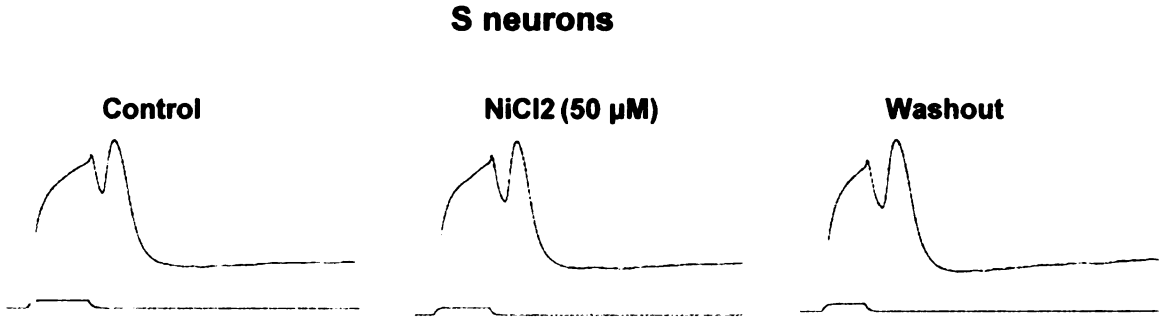


Figure. 4.4. Action potentials in S-type neurons are not sensitive to NiCl2. Representative traces showing that the amplitude and half-maximum width of the S-type action potential are unchanged by NiCl2. Mean data showing that NiCl2 (50 µM) did not change action potential half-width or peak amplitude. Data are mean ± S.E.M. *n* = 8 neurons.

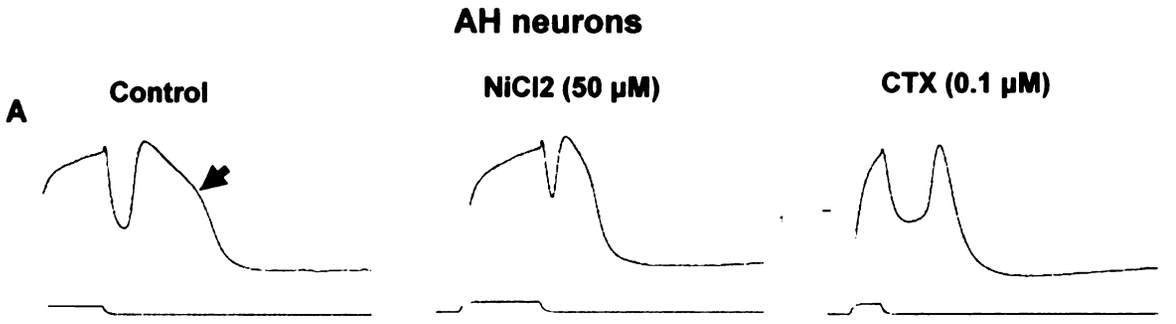


Figure. 4.5. NiCl2, SNX-482 and ω-CTX inhibit the action potential in AH neurons. Representative traces showing that NiCl2 (50 µM) reduced the calcium shoulder. The residual shoulder was then completely blocked by ω-CTX (0.1 µM). Mean data showing that the action potential half-width after $NiCl_2$-treatment was smaller compared to control (n = 3; ** = P < 0.01). ω-CTX further reduced the half-width (*** = P < 0.001 vs. control). NiCl2 and ω-CTX did not affect the peak AH action potential amplitude (n = 3; P > 0.05). SNX-482 inhibited the action potential width at half-amplitude compared to control (n = 3; * = P < 0.05). ω-CTX then further attenuated the half-maximal width (n = 3; ** = P < 0.01 vs. control). The peak amplitude was inhibited by ω-CTX but not SNX-482. Data are mean ± S.E.M. n is the number of neurons.

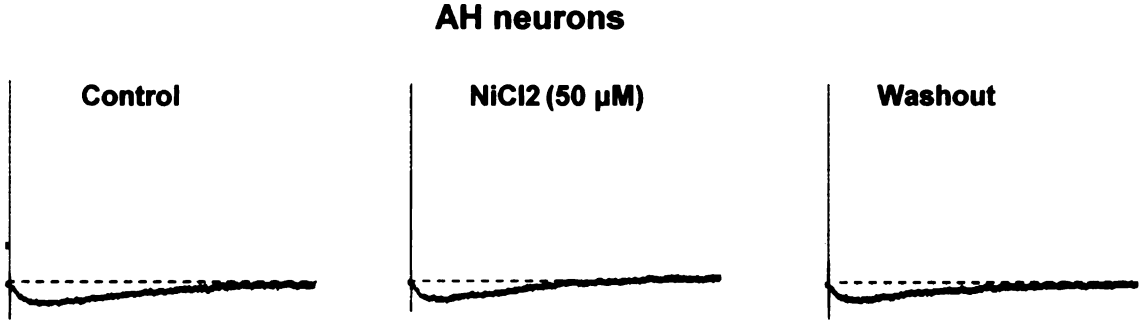


Figure. 4.6. The late afterhyperpolarization (late AHP) in AH neurons is inhibited by NiCl2. Representative traces showing that the amplitude and half-maximum width of the late AHP are inhibited after application of NiCl2 (50 µM). Mean data showing that the amplitude of the late AHP is reduced after addition of $NiCl_2$ (50 µM) treatment compared to control (n = 4; * = $P < 0.05$). The late AHP width at half-amplitude was unchanged in the presence of NiCl2 (n = 4; $P > 0.05$). NiCl2 significantly decreased the area under the curve (AUC) of the late AHP compared to the AUC of the control late AHP (n = 4; * = $P < 0.05$). Data are mean ± S.E.M. n represents the number of neurons.

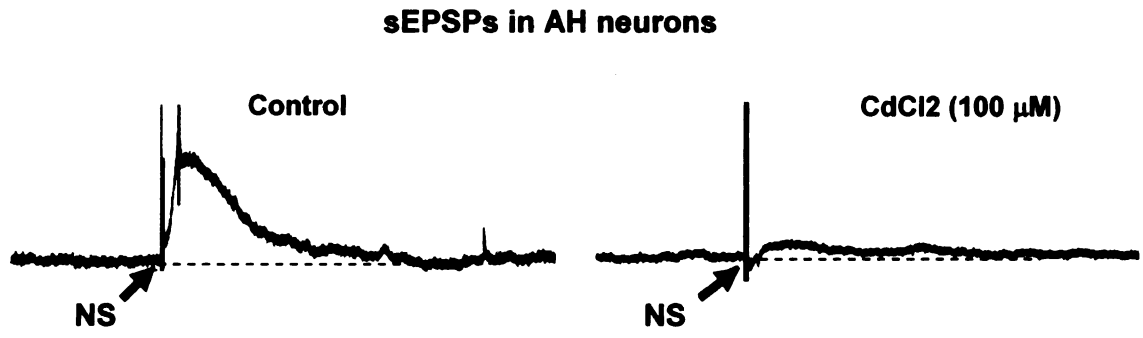


Figure. 4.7. Effect of CdCl2 on sEPSPs in AH neurons. A train of electrical pulses (20 Hz, 500 ms) applied to an interganglionic connective produces a sEPSP in an AH neuron (NS = nerve stimulation). The sEPSP is inhibited by CdCl2 (100 μM). Concentration-response curve for sEPSP inhibition by CdCl2. Curves are non-linear fits of the Hill equation. The peak amplitude of the sEPSP was stable over time ($P > 0.05$; one-way ANOVA). Data points are mean ± S.E.M. $n = 5$ neurons.

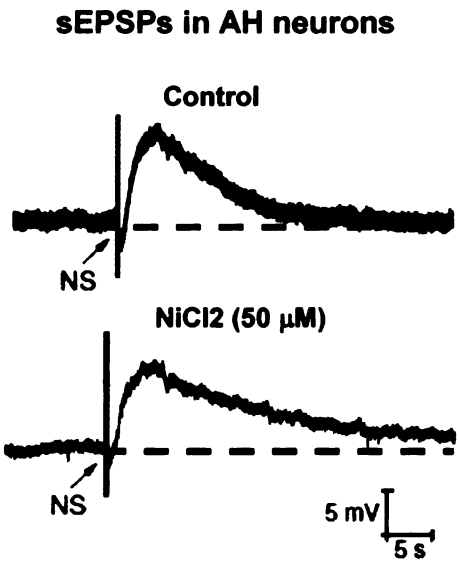


Figure. 4.8. NiCl2 (50 µM) did not inhibit sEPSPs. a sEPSP in an AH neuron is not inhibited by NiCl2.

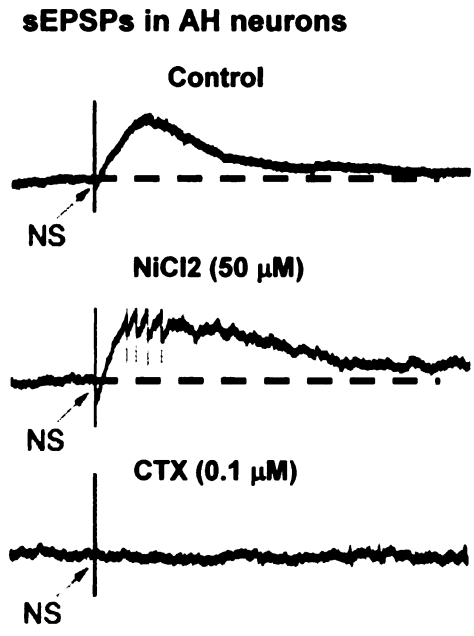


Figure. 4.9. Sensitivity of sEPSPs to ω-conotoxin (ω-CTX) (100 μM). sEPSPs in AH neurons are not inhibited by NiCl2, but are blocked by ω-CTX. Data are mean ± S.E.M similar to that shown in A. * = $P < 0.05$ (NiCl2 versus ω-CTX); ** = $P < 0.01$ (Control versus ω-CTX). *n* = 4 neurons.

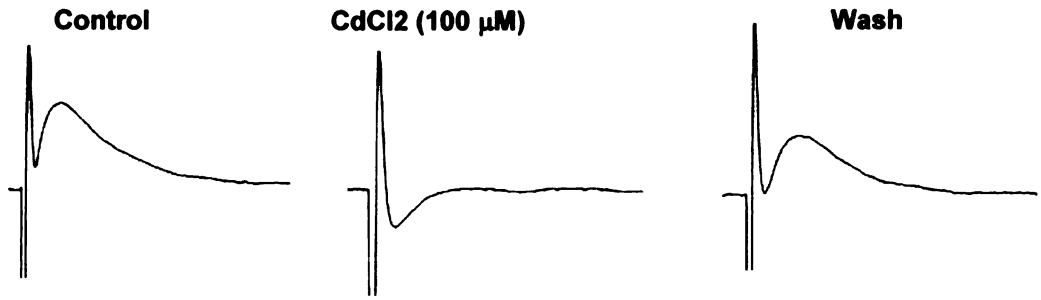


Figure. 4.10. Amplitude of fEPSPs after treatment with CdCl2 and during time controls. CdCl2 (100 μM) completely inhibited the fEPSP. Concentration-response curve for inhibition of the fEPSP by CdCl2 (n = 5). The peak amplitude of the fEPSP was stable over time (n = 7; P > 0.05). Data are mean ± S.E.M. n is the number of neurons from which fEPSPs were recorded.

Cholinergic/purinergic fEPSPs in S neurons

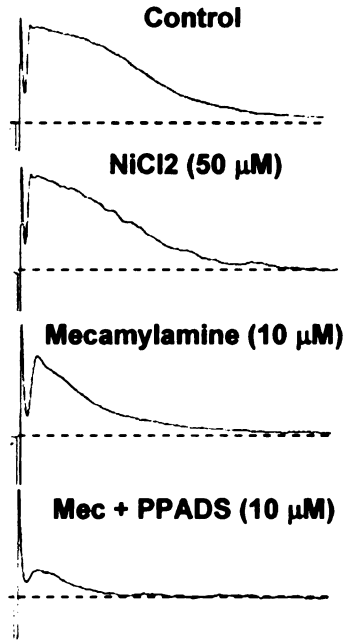


Figure. 4.11. NiCl2 does not inhibit fEPSPs in cholinergic/purinergic S-type neurons. Representative traces showing that in a subpopulation of S-type neurons, NiCl2 (50 μM) did not inhibit the fEPSP. Mecamylamine (10 μM) reduced the fEPSP. Subsequent application of mecamylamine and PPADS (10 μM) then largely inhibited the fEPSP. Mean data indicating that the amplitude of the fEPSP under control conditions is not different from the fEPSP amplitude after NiCl2 treatment (n = 15; P > 0.05). Application of mecamylamine (10 μM) reduced the fEPSP amplitude (n = 15; * = P < 0.05). Mecamylamine and PPADS (10 μM) then inhibited the fEPSP (*** = P < 0.001). Data are mean ± S.E.M. n = 15 neurons.

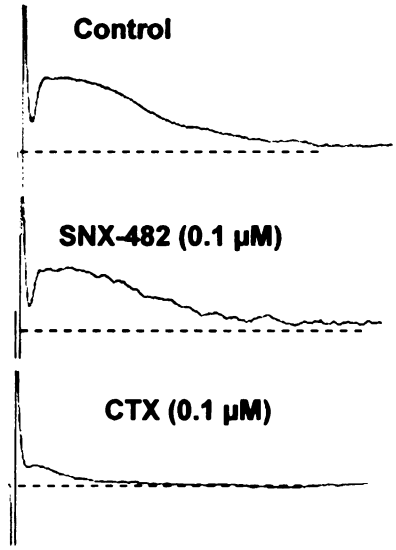


Figure. 4.12. SNX-482 does not inhibit fEPSPs in cholinergic/purinergic S-type neurons. Representative traces showing that SNX-482 (0.1 μM) has no effect on the fEPSP amplitude. Application of ω-CTX (0.1 μM) blocked the fEPSP. This population of S-type neurons is sensitive to inhibition by a combination of mecamylamine (10 μM) and PPADS (10 μM). Mecamylamine (10 μM) reduced the fEPSP amplitude (data not shown). Mecamylamine (10 μM) and PPADS (10 μM) then largely inhibited the fEPSP. Application of ω-CTX (0.1 μM) blocked the fEPSP (n = 3; * = $P < 0.05$). Data are mean ± S.E.M. n = 3 neurons.

Cholinergic fEPSPs in S neurons

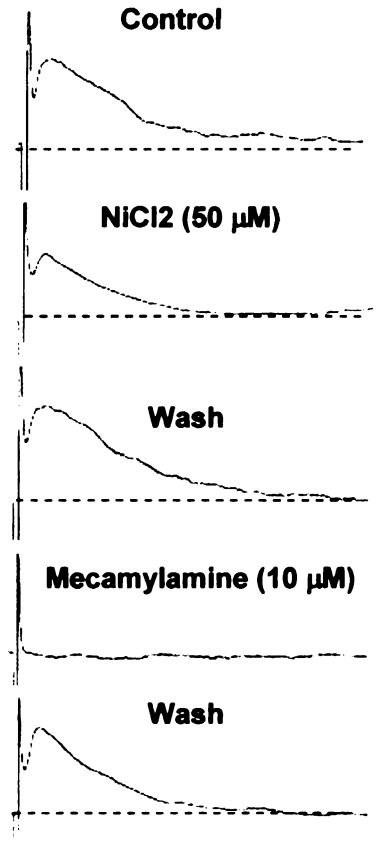


Figure. 4.13. NiCl2 and ω-CTX inhibit fEPSPs in purely cholinergic S-type neurons. Representative traces showing that in a sub-population of S-type neurons, NiCl2 (50 μM) reduced the fEPSP. Mecamylamine (10 μM) then completely blocked the fEPSP. Mean data indicating that the amplitude of the fEPSP after NiCl2 treatment is significantly smaller compared to control (* = $P < 0.05$). Mecamylamine 10 μM) reduced the fEPSP amplitude (*** = $P < 0.001$), and this was smaller compared to NiCl2 treatment (*** = $P < 0.001$). *n* = 14 neurons. (C) ω-CTX (0.1 μM) further inhibited the fEPSP (trace not shown).

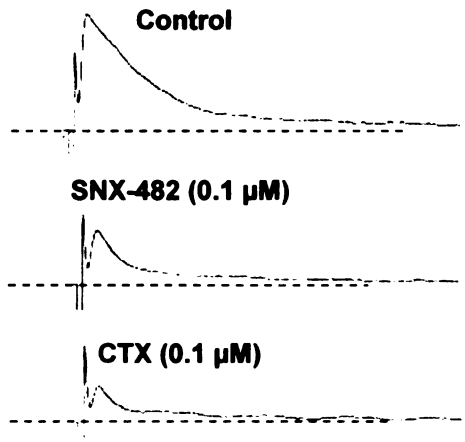


Figure. 4.14. Effect of SNX-482 and ω-CTX on purely cholinergic fEPSPs. Representative traces showing that SNX-482 (0.1 μM) reduces the fEPSP amplitude in purely cholinergic S-type neurons. ω-CTX (0.1 μM) subsequently inhibited the fEPSP. Mean data showing that SNX-482 inhibited the amplitude of the fEPSP compared to control (n = 3; *** = $P < 0.001$). Mecamylamine (10 μM) then blocked the fEPSP (data not shown). After washout of mecamylamine, ω-CTX (0.1 μM) blocked the fEPSP (n = 3; *** = $P < 0.001$). Data are mean ± S.E.M. n = 4 neurons.

CHAPTER 5 R-TYPE CALCIUM CHANNELS COUPLE TO NITRIC OXIDE-MEDIATED INHIBITORY NEUROTRANSMISSION IN THE GUINEA PIG ILEUM

ABSTRACT

Previous immunohistochemical studies showed that R-type calcium channels and nitric oxide synthase (NOS) co-localize in nerve terminals supplying the muscle layers in the guinea pig ileum. It is not known if calcium entry through R-type channels couples to NOS activation and/or ATP release. The present study determined the contribution of R-type calcium channel activation to release of NO and ATP from myenteric motorneurons supplying the muscle layers of the guinea pig ileum. I measured the effects of the NOS inhibitor, nitro L-arginine (NLA) and the R-type calcium channel blocker $NiCl_2$ on neurogenic relaxations and contractions of longitudinal muscle-myenteric plexus muscle strips maintained in vitro. Neurogenic relaxations under histamine tone and contractions in non-histamine-contracted tissues were evoked by trains (20 Hz, 0.3 ms) of transmural electrical stimulation. I also used intracellular recordings from smooth muscle cells to investigate the contribution of R-type channels to release of ATP/β-NAD as the mediators of the inhibitory junction potential (IJP). Tetrodotoxin (TTX), and the non-selective calcium channel antagonist $CdCl_2$ blocked electrically-evoked relaxations confirming that they were neurogenic. NLA inhibited neurogenic relaxations in a concentration-dependent (10-100 μM) manner; the maximum inhibition was 60%. $NiCl_2$ produced a similar inhibition of neurogenic relaxations and contractions. Co-application of $NiCl_2$ and NLA did not produce a greater inhibition of neurogenic relaxations or contractions than either blocker alone. IJPs were blocked by TTX, $CdCl_2$ and apamin. $NiCl_2$ or NLA applied alone or together did not alter IJP amplitude or time course. R-type calcium channels may couple

to NOS activation and NO-mediated muscle relaxation in the guinea pig ileum. R-channels do not couple to release of the mediator of apamin-sensitive IJPs. ATP and NO released from the same inhibitory nerves may be regulated differently, or NO and other inhibitory neurotransmitters are released from separate populations of nerve fibers.

INTRODUCTION

The enteric nervous system (ENS) controls gastrointestinal (GI) function (Furness *et al.*, 1998). The ENS is composed of two nerve plexuses: the submucosal plexus which controls the absorption of nutrients, secretion, and local blood flow across the epithelium, and the myenteric plexus which has a pivotal role in the control of GI motility (GI) (Bornstein *et al.*, 1994; Lomax & Furness, 2000). Non-adrenergic non-cholinergic (NANC) nerves mediate the majority of inhibitory responses in the ENS (Sanders & Ward, 1992). Stimulation of NANC inhibitory enteric neurons relaxes GI smooth muscle (Gao *et al.*, 2006; Burnstock, 2008) and causes inhibitory junction potentials (IJPs) (Burnstock *et al.*, 1964). Pharmacological and electrophysiological studies revealed that inhibitory neurotransmitters released from NANC nerves use varying mechanisms to cause smooth muscle relaxation (He & Goyal, 1993; Smits & Lefebvre, 1996; De Man *et al.*, 2003). The most prominent inhibitory neurotransmitters in the ENS are ATP (Burnstock *et al.*, 1997), vasoactive intestinal peptide (VIP) (Fahrenkrug *et al.*, 1979; Rekik *et al.*, 1996), carbon monoxide (Kwon *et al.*, 2001), nitric oxide (NO) (Crist *et al.*, 1992; Sanders & Ward, 1992; Mashimo *et al.*, 1996) and possibly β-nicotinamide dinucleotide (β-NAD) (Mutafova-Yambolieva et al., 2007). β-NAD/ATP, VIP and NO may all be released from the same motor nerve varicosity (Burnstock, 2008).

ATP and or β-NAD, released from enteric motorneurons, is an important inhibitory enteric neurotransmitter that acts at G_q-protein-coupled $P2Y_1$ receptors on GI circular smooth muscle (Wood, 2006b). $P2Y_1$ receptors mediate

the fast IJP (Gallego *et al.*, 2006). MRS2179 is a $P2Y_1$ receptor antagonist (Boyer *et al.*, 1998) that blocks the fast IJP in myenteric neurons (Gao *et al.*, 2006; Wang *et al.*, 2007). Apamin (0.1 μM) is a blocker of small-conductance calcium-dependent K^+ channels (SK) and selectively inhibits the fast IJP (Mackenzie & Burnstock, 1980).

NO is an important signaling molecule in the central nervous system (CNS) and the ENS. NO can function as a retrograde (Holscher, 1997) transmitter, or as a conventional anterograde transmitter where it is dependent on the calcium-dependent activation of the neuronal isoform of nitric oxide synthase (nNOS) (Ohashi *et al.*, 2007). In the ENS, nNOS is the dominant isoform of NOS (Ward *et al.*, 1992) and NO is an important inhibitory neurotransmitter (Sanders & Ward, 1992). NO relaxes GI smooth muscle (Katsoulis *et al.*, 1996; Mashimo *et al.*, 1996; Kim *et al.*, 1999). The slow IJP is NO-mediated (He & Goyal, 1993; Stark *et al.*, 1993). Regulation of nitrergic neurotransmission however, is still not well understood. The identification of the NO regulatory mechanism is complicated by the fact that NO is a highly diffusible gas that is neither pre-formed nor stored in secretory vesicles (Bredt *et al.*, 1990). Therefore its mechanism of regulation must be different from that which is used to control the release of vesicle-stored neurotransmitters. Regulation of readily releasable vesicles is consequently an important index of neurotransmitter release after each stimulation event (Rao *et al.*, 2008). NO production and release may be regulated by a catalytically active pool of nNOS via PDZ binding

domains that allow nNOS to anchor to the plasma membrane at the active zone (Rao *et al.*, 2008).

Neurotransmitter release requires calcium influx through voltage-gated calcium channels (Catterall, 2000). R-type calcium channels are high-threshold channels and require strong depolarization for activation (Foehring *et al.*, 2000) and ENS (Bian *et al.*, 2004; Bian & Galligan, 2007). R-type voltage-gated calcium channels are expressed in myenteric neurons and together with the N-, P/Q-, and L-type channels, account for the total calcium current recorded from those neurons (Bian *et al.*, 2004). The functions of R-type channels in myenteric neurons are not yet clear. In myenteric neurons maintained in primary culture, the R-type calcium channels may contribute to the somatodendritic action potential or to action potential-induced calcium entry required for neurotransmitter release from myenteric nerve terminals (Bian *et al.*, 2004). Low concentrations of $NiCl_2$ (50 μM) and SNX-482 (0.1 μM) block R-type calcium channels (Randall & Tsien, 1995; Newcomb *et al.*, 1998; Wang *et al.*, 1999; Tottene *et al.*, 2000). ω-conotoxin GVIA (ω-CTX) (0.1 μM) and ω-agatoxin IVA (0.1 μM) block N-type (Barajas-Lopez *et al.*, 1996; Chen & Kirchgessner, 2002) and P/Q-type channels (Tsien *et al.*, 1988; Catterall, 2000; Bian *et al.*, 2004) respectively.

Previous data has shown that the R-type calcium channel and nNOS co-localize in myenteric neurons, and in nerve fibers supplying the smooth muscle in the guinea pig ileum (Naidoo *et al.*, 2006). The present study investigated whether myenteric neurons mediate descending inhibition via R-type channels,

and whether R-type calcium channels and nNOS are spatially coupled on nerve endings of enteric motorneurons to cause GI smooth muscle relaxation.

MATERIALS AND METHODS

Ethical approval

All animal use protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Michigan State University.

Longitudinal muscle-myenteric plexus (LMMP) preparation

Adult male Hartley-strain guinea pigs weighing 250 g (Bioport, Lansing, Michigan; animals) were anesthetized under isoflurane inhalation (Abbott Laboratories, Chicago, IL), stunned and exsanguinated. The ileum was removed and placed in pre-warmed oxygenated (95% CO_2, 5% CO_2) Krebs' solution of the following composition (mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose. The Krebs' solution contained scopolamine (1 μM) to block muscarinic cholinergic receptors. A glass cannula was inserted into a 2 cm segment of the ileum and the longitudinal muscle attached with the myenteric plexus (LMMP) was then teased off with a cotton swab soaked in Krebs' solution. The LMMP preparation was mounted with silk ligatures between a platinum foil electrode and a stationary hook connected to an isometric force transducer (Grass Instruments, FT03C, Quincy, MA, USA) and placed in a 20 ml jacketed organ bath containing Krebs' solution at 37 °C. A resting tension of 1 g was applied to each of four 2 cm segments of LMMP preparations. Tissues were allowed to equilibrate for 60 minutes, during which the Krebs' solution was replaced in 15 minute intervals.

Transmural electrical field stimulation (EFS)

To study inhibitory mechanisms in the LMMP, each tissue was first pre-contracted with histamine (1 μM) to induce a sustained baseline resting tension (Osthaus and Galligan, 1991). Histamine acts directly at H_1 receptors on the longitudinal muscle in the guinea pig ileum to cause an increase in muscle tone (Izzo *et al.*, 1998). Non-adrenergic non-cholinergic (NANC)-mediated relaxations were induced by brief trains of transmural electrical stimuli (20 Hz, 0.3 ms pulse duration, 0.25 Hz train rate, 50 V) (Osthaus & Galligan, 1992). To determine the action of the antagonist on the inhibitory responses of the ileal longitudinal muscle, non-cumulative concentration response curves were created with a 15 minute interval between successive doses. I also investigated EFS-evoked frequency-dependent contractions in the non pre-contracted LMMP (20 Hz, 0.3 ms, 50 V). To determine the action of the antagonist on the excitatory response of the ileal longitudinal muscle, non-cumulative experiments were conducted and the effects of co-application of antagonists on the excitatory responses were studied. Transmural electrical stimuli in both set of experiments were provided by a Grass S48 stimulator and mechanical activity of the LMMP was recorded with Labscribe (iWorx, Dover, NH, USA) and a personal computer.

Tissue preparation for intracellular electrophysiological recordings

A 1.5 cm section of the ileum was cut open along the mesenteric border and pinned flat (Fine Science Tools, Foster City, CA) in a silicone elastomer-lined (Sylgard, Dow Corning, Midland, MI) petri-dish containing Krebs' solution. The

Krebs' solution contained scopolamine (1 μM) and nifedipine (1 μM) to block muscarinic receptors and L-type calcium channels on smooth muscle cells, respectively. The submucosa was removed to expose the circular muscle-longitudinal muscle-myenteric plexus (CM-LMMP). A 5 mm^2 section of the CM-LMMP was then transferred to a smaller silicone elastomer-lined recording chamber (2 ml) with the smooth muscle layer facing up. The chamber was mounted on a stage of a microscope (Olympus, Tokyo, Japan). The CM-LMMP was pinned tautly and superfused with oxygenated Krebs' solution at a flow rate of 4 ml/min at 37 °C. The preparation was equilibrated for 40 min before commencing intracellular recordings.

Intracellular electrophysiological recordings

Single smooth muscle cells in the CM-LMMP were impaled with glass microelectrodes filled with 2 M KCl (tip resistance 80-120 MΩ). Membrane potential was recorded using an Axoclamp 2A amplifier (Axon Instruments, Foster City, USA) in bridge mode. IJPs were evoked focally with a monopolar glass electrode (tip diameter 60 μM) (World Precision Instruments, Sarasota, Florida) filled with Krebs' solution by electrically stimulating myenteric nerve fibers supplying the smooth muscle. Single pulses (1 Hz, 4 ms). Short frequency trains of stimulation (5 Hz and 10 Hz, 1 s, 50 V), provided by a pulse generator (Master 8, A.M.P.I., Jerusalem, Israel) and a constant current stimulation unit (Grass Technologies, West Warwick, RI) were used to evoke IJPs. The resting membrane potential (RMP) of the smooth muscle cell was allowed to stabilize for

10 minutes without applying intracellular holding current. Cells with RMPs less than -40 mV were not included in the analysis. Signals were recorded using an A/D converter (Digidata 1322A, Molecular Devices Corp., Sunnyvale, CA), Axoscope and Clampfit 10 software (Molecular Devices), and a desktop computer. Amplified signals were sampled at 2 kHz and filtered at 1 kHz. I measured the area under the curve (AUC) as this is an integration of both IJP amplitude and duration. This allowed us to study the changes in responses induced by drugs. AUCs were measured under control conditions and after various drug treatments.

Drugs

All chemicals were obtained from Sigma-Aldrich (St Louis, MO), except for ω-CTX, ω-ATX, and tetrodotoxin (TTX) (Alomone Labs, Jerusalem, Israel). Drugs were dissolved in deionized water with the exception of nitro-L-arginine (NLA), which was dissolved in hydrochloric acid (0.1 N) and nifedipine which was dissolved in 95% ethanol.

Data Analysis

Data are presented as the mean ± S.E.M. For the EFS study, *n* refers to the number of animals from which tissues were obtained. For the intracellular IJP recordings, *n* is the number of smooth muscle cells. Concentration-response data were fitted using non-linear regression and the Hill equation (GraphPad Prism, San Diego). A one-way ANOVA, two-way ANOVA or Student's t-test for paired or

unpaired data were used when applicable. $P < 0.05$ was considered statistically significant.

RESULTS

Electrically induced relaxations of the histamine-contracted LMMP

Transmural stimulation of histamine (1 μM)-contracted LMMP preparations (n = 4) produced a triphasic response. This response was composed of a fast contraction (Figure. 5.1A*a*, B1), followed by a relaxation (Fig. 1A*b*, B1), and a rebound contraction (Figure. 5.1A*c*, B1). I first tested whether these responses were neurogenic. The voltage-dependent sodium channel blocker TTX (0.3 μM) completely inhibited the relaxation and rebound contraction of the longitudinal smooth muscle but not the initial fast contraction (Figure. 5.1B2). The $CdCl_2$ blocked EFS-induced contractions and relaxations (Figure. 5.1B3, 2A). Apamin (0.1 μM) inhibited the EFS-evoked relaxation (Figure. 5.1B4) and revealing a large-amplitude slow contraction.

R-type calcium channel blockers inhibit neurogenic relaxation of the LMMP

Previous data showed that R-type calcium channels and NOS co-localize in the LMMP (Naidoo *et al.*, 2006). The R-type calcium channel blocker, $NiCl_2$ (0.1-100 μM), caused a concentration-dependent inhibition of EFS-evoked relaxation with a maximum 41 % reduction in the control response ($P < 0.0001$) (Figure. 5.2B). NLA (0.1–100 μM) also reduced the peak relaxation amplitude in a concentration-dependent manner with a maximum inhibition of 53 % (P<

0.0001). Co-application of $NiCl_2$ with NLA (0.1–100 μM) inhibited the relaxation amplitude by 48 %. Comparison of these dose response curves revealed that $NiCl_2$ did not significantly affect the dose response curve for NLA, and the converse was also true ($P > 0.05$).

During these initial sets of experiments, I observed that $NiCl_2$ and NLA inhibited the EFS-evoked relaxation and also produced a concentration dependent increase in the amplitude of the rebound contraction. I next studied the effect of $NiCl_2$ and NLA on the amplitude of this NANC contraction in the absence of histamine induced tone. Transmural stimulation (20 Hz, 0.3 ms) of the non-pre-contracted LMMP produced a biphasic contraction response. The biphasic contraction response at the beginning of the stimulation contained an early fast contraction followed by a late slow contraction (n=10, Figure. 5.3A1, B1). There was no evidence of a time-related change in the LMMP slow contraction (Figure. 5.3A2). I then tested the effect of $NiCl_2$ (50 μM), NLA (100 μM) and apamin (0.1 μM) on this slow contraction. In these experiments I only measured the AUC (g.s) of the slow contraction because drug treatment had a pronounced effect on the amplitude and duration of the response. $NiCl_2$ increased the AUC of the contraction by 147 % (Figure. 5.3B1, 3B2, 4A; $P < 0.05$). NLA in the presence of $NiCl_2$ increased the AUC by a further 13 % and this was not statistically different from $NiCl_2$ alone (Figure. 5.3B3; Figure. 5.4A $P > 0.05$). Subsequent addition of apamin with $NiCl_2$ and NLA and increased the AUC by 53 % and this was statistically greater from the effect of $NiCl_2$ by itself (Figure. 5.3B4; $P < 0.01$), and from the effect of $NiCl_2$ in the presence of NLA ($P < 0.05$

Figure. 5.4A). TTX (0.3 μM) blocked the slow but not fast contraction while $CdCl_2$ (100 μM) blocked both responses was applied to all LMMP preparations, and subsequently blocked the slow contraction (Figure. 5.3B5 and Figure. 5.6).

In second set of experiments preparation where NLA was applied first, NLA increased the AUC of the contraction by 118 % (Figure. 5.4B, $P < 0.01$). NLA in the presence of $NiCl_2$ did not produce any additional effect on the contraction ($P > 0.05$) when compared to NLA by itself. Co-application of NLA with $NiCl_2$ and apamin increased the AUC of the contraction by 37 % which was greater than the effect of $NiCl_2$ and NLA together ($P < 0.05$). In the third set of experiments, apamin increased the AUC by 95 % (Figure. 5.4C, $P < 0.01$). NLA in the presence of apamin increased the AUC by an additional 42 % ($P < 0.01$) while subsequent addition of $NiCl_2$ did not produce any additional increase over that caused by apamin and NLA (Figure. 5.4C).

IJPs are $NiCl_2$ insensitive

I next investigated IJPs caused by focal stimulation of myenteric plexus interganglionic fiber tracts. Resting membrane potentials (RMPs) of impaled smooth muscle cells under control conditions was -51 ± 1 mV and did not change in the presence of drugs. Figures 5.5A1-A3 show control IJPs evoked in response to 1, 5 and 10 stimuli. I chose these frequencies because increasing electrical stimulus frequency causes the differential and additional release of enteric neurotransmitters from the same nerve endings (Bywater & Taylor, 1986; Crist *et al.*, 1991; Undi *et al.*, 2006; Benko *et al.*, 2007). The amplitude and

duration of the IJP increased with increasing number of stimuli and $NiCl_2$ (50 μM) did not affect IJPs (Figure. 5.5B1-B3). In the presence of $NiCl_2$, NLA (100 μM) also did not change the properties of the IJPs (Figure. 5.5C1-C3, H). In these cells, and regardless of the order of application, neither NLA nor $NiCl_2$ by themselves affected IJPs (Figure. 5.5I, n = 4; 5 Hz and 10 Hz frequencies not shown).

The selective purinergic $P2Y_1$ receptor antagonist MRS2179 (10 μM) blocked the IJPs at 1 Hz, 5 Hz and 10 Hz stimulation (Figure. 5.5D1-D3). TTX (0.3 μM) (Figure. 5.5 E) also blocked IJPs. $CdCl_2$ (1-100 μM), produced a leftward-shift in the stimulus-response curve (Figure. 5.5F, I) and caused a concentration-dependent inhibition of the IJPs (n = 4) at all stimulus frequencies. I therefore used 100 μM $CdCl_2$ in subsequent experiments. While $NiCl_2$ and NLA had no effect on the IJPs they were blocked by $CdCl_2$ (Figure. 5.5D, H). Finally, application of apamin (0.1 μM) (Figure. 5.5G) also inhibited the IJP responses. Together, these data suggest that R-type calcium channels couple to NO synthesis and release but not ATP/β-NAD release as mediator(s) of the IJP.

$NiCl_2$-insensitive IJPs are mediated by N- and P/Q-type calcium channels.

In order to provide support for selective coupling of R-type calcium channels to NO release, and because $CdCl_2$ inhibited the IJPs, I used, together with $NiCl_2$, ω-CTX and ω-ATX to inhibit N- and P/Q-type channels respectively. $NiCl_2$ did not affect the IJP (Figure. 5.6B1-B3, E) compared to control (Figure. 5.6A1-A3). Additive application of ω-CTX (0.1 μM) in the presence of $NiCl_2$

reduced IJPs evoked at all frequencies (Figure. 5.6C1-C3). Addition of ω-ATX (0.1 μM) then further reduced the AUC (Figure. 5.6D1-D3). The single stimuli IJP was almost completely blocked, while at 5 and 10 Hz a residual IJP persisted. Apamin (0.1 μM) blocked the residual IJPs. Concentration-response curves with ω-CTX (0.001-0.1 μM) and ω-ATX (0.001-0.1 μM) revealed almost complete inhibition of IJPs (Figure. 5.5I). These data indicate that N- and P/Q-type calcium channels mediate release of ATP/β-NAD as the mediators of the IJP.

DISCUSSION

This study investigated the contribution of R-type calcium channels to inhibitory neurotransmission in the ENS of the guinea pig ileum. Enteric inhibitory nerves release NO and ATP/β-NAD to relax GI smooth muscle. ATP/β-NAD act at $P2Y_1$ receptors on smooth muscle to cause apamin-sensitive inhibitory junction potentials (IJPs) and relaxation. NO increases intracellular cGMP to cause muscle relaxation. I used transmural EFS in the LMMP and conventional intracellular recordings from smooth muscle cells in the ileum to show that R-type calcium channels couple to NO to mediate relaxation of GI smooth muscle. I have further demonstrated that N- and P/Q-type calcium channels control ATP/β-NAD release.

R-type calcium channels couple to NOS release in the guinea pig LMMP

I first investigated nerve-mediated relaxations of the guinea pig LMMP using transmural EFS in the presence of scopolamine. My first experiments used histamine which caused a sustained contraction of the longitudinal muscle. EFS then evoked consistent nerve-mediated relaxations. Three responses were observed after EFS: a fast contraction, a relaxation and then a rebound contraction. The relaxation response and the rebound contraction were nerve mediated as they were blocked by TTX. The initial fast contraction, however, was TTX-resistant. There are a couple of reasons for the TTX-resistant contraction. First, calcium entry through voltage-gated calcium channels probably does not cause this contraction since blockade of those channels still produces a TTX-

resistant contraction (Kristufek *et al.*, 1999). Nicotinic ACh receptors (nAChRs) may be localized to nerve terminals of excitatory longitudinal muscle myenteric motorneurons (Galligan, 1999). nAChRs are non-specific ligand-gated cation channels that have a greater permeability to calcium than sodium and potassium ions (Vernino *et al.*, 1992). In the presence of TTX, EFS would cause a depolarization of the nerve terminal and activation of presynaptic nAChRs to allow calcium entry via the nAChR, leading to neurotransmitter release (Sacaan *et al.*, 1996; Lena & Changeux, 1997; Schneider *et al.*, 2000). Second, it is possible that high-frequency (5 Hz, and 10 Hz) nerve stimulation in myenteric neurons still causes additional calcium entry through activation of voltage-gated calcium channels, different to that observed in sympathetic neurons (Kristufek *et al.*, 1999). Calcium influx through voltage-gated calcium channels could directly cause neurotransmitter release, or indirectly by inducing calcium release from intracellular calcium stores within the smooth endoplasmic reticulum (Smith *et al.*, 2003). The TTX-resistant contraction may therefore be a calcium-mediated contraction.

Concentration-response curves with $NiCl_2$ and NLA showed that whether these drugs were co-applied, or whether they were applied alone, they produced an equivalent effect on neurogenic relaxation. Osthaus and Galligan (1992) observed that LMMP tissues exhibiting biphasic nerve-mediated response of the histamine-contracted LMMP showed: a fast relaxation followed by a rebound non-cholinergic contraction, or a fast relaxation followed by a slower and smaller amplitude relaxation. My data, however, show a fast contraction, followed by a

slower and large amplitude relaxation, and a slow rebound contraction of large amplitude. These varying responses suggest that the interplay between excitatory and inhibitory nerves is complex and that neurotransmitters released from enteric nerves can produce several motor responses. I therefore next determined the contribution of R-type calcium channels to facilitation of LMMP contractions as a measure of simultaneous release of inhibitory neurotransmitters. $NiCl_2$ and NLA, applied alone, increased the contraction and co-application of $NiCl_2$ and NLA did not produce an additive effect. The addition of apamin however, significantly increased the contraction. Apamin applied alone increased the contraction and subsequent addition of NLA or $NiCl_2$ further increased the contraction. These data suggest that R-type calcium channels couple to NO synthesis and release, but R-type channels are not coupled to ATP/β-NAD release.

R-channels do not couple to ATP release as a mediator of apamin-sensitive IJPs

I next characterized the relationship between R-type calcium channels and inhibitory neurotransmission by recording IJPs evoked from smooth muscle cells. $NiCl_2$ and NLA added independently, and together, did not affect the IJPs. However, apamin completely blocked IJPs evoked at all frequencies. Furthermore, MRS2179 also completely inhibited the IJPs. These responses were neurogenic as they were blocked by TTX. ATP, NO and VIP are released from the same enteric nerve endings on to GI smooth muscle, and IJPs with

different kinetics have been observed (Mackenzie & Burnstock, 1980; Benko *et al.*, 2007; Burnstock, 2008). ATP produces a fast IJP, NO produces a slower IJP (Hirst *et al.*, 2004), and VIP elicits very slow and tonic relaxations in the guinea pig ileal circular muscle (He & Goyal, 1993; Burnstock, 2008). In guinea pig jejunum and ileum, purinergic IJPs are either purely purinergic (26 %), partially purinergic (70 %) or non-purinergic (4 %) (Wang *et al.*, 2007). In that study, apamin (0.5 μM) and MRS2179 (2 μM) completely blocked the IJP. Wang and others (2007) used a single pulse (2 ms duration) whereas I used three different stimuli at 1 Hz, 5 Hz and at 10 Hz. In all cells tested, apamin and MRS 2179 completely blocked the IJP. This suggests that all IJPs I had evoked were ATP/βNAD responses that are not mediated by R-type calcium channels. These $NiCl_2$-insensitive IJPs were largely blocked with ω-CTX and ω-ATX indicating that the IJP is ATP-mediated. At 10 Hz, a biphasic IJP was observed. This cannot be VIP or NO-mediated since apamin completely inhibited all evoked IJPs. ATP acting at $P2Y_1$ receptors on smooth muscle causes the fast IJP (Crist *et al.*, 1992; Gallego *et al.*, 2006), thus corroborating my data. However, my results further indicate that the slower and broader duration IJPs (at 5 Hz and 10 Hz) are also be due to ATP/βNAD acting at $P2Y_1$ receptors.

Functional significance of R-type calcium channels in neuromuscular transmission

Taken together, it is possible that R-type calcium channels may couple to NO release to mediate inhibition of GI smooth muscle. R-type channels and NOS

co-localize in nerve fibers that supply the smooth muscle layer. ATP/β-NAD released from the same nerve fibers would act at $P2Y_1$ via N- and P/Q-type calcium channels. There is presently no marker for ATP localization. Therefore, whether R-type channels co-localize with ATP and NOS on the same or different myenteric motor nerve terminals remains to be determined.

R-type channels may therefore be present on separate nerve fibers to regulate NO release from motor nerve varicosities, or they may be present on the same NOS-ATP containing fibers but couple to NOS for the differential regulation of NO release on to GI smooth muscle. Accumulating evidence indicates that all three major myenteric inhibitory neurotransmitters (NO, ATP and VIP) are released from the same motor nerve terminals (Costa *et al.*, 1986b; Crist *et al.*, 1992; Furness *et al.*, 1995). It is therefore likely that R-type calcium channels are present with NO and ATP on the same nerve endings. Recent data from the mouse gut suggest a model in which enteric motor nerve terminals contain catalytically active forms of NOS (nNOSα) that are anchored via PDZ-binding domains (Lai & Lp, 2003; Kim & Sheng, 2004) to the plasma membrane (Rao *et al.*, 2008). My data therefore suggest that NOS may be docked at the nerve terminal, close to R-type calcium channel release sites. A proposed mechanism would be as follows: when the nerve terminal is depolarized by invading action potentials, calcium, entering through R-type calcium channels, binds to calmodulin to activate NOS that converts L-arginine into NO. NO then diffuses across the neuroeffector junction, binds to soluble guanylate cyclase, activates

protein kinase G (PKG) which then phosphorylates myosin light chain kinase to cause relaxation of the muscle (Lecci *et al.*, 2002)..

R-type calcium channels play important roles throughout the nervous system. For example, at hippocampal CA3-CA1 synapses, R-type channels contribute to presynaptic calcium entry (Wu & Saggau, 1995). R-type channels are also involved in excitation-contraction coupling in the brainstem (Wu *et al.*, 1998) and in chromaffin cells (Albillos *et al.*, 2000). R-type calcium channels also contribute to fast synaptic transmission at hippocampal mossy fiber and associative-commissural synapses (Gasparini *et al.*, 2001). In the ENS, R-type channels may be important to control of descending inhibition between myenteric motorneurons and effector tissues. NO and ATP are the most prominent inhibitory neurotransmitters in the ENS (Sanders & Ward, 1992; Lecci *et al.*, 2002; Furness, 2006a). It is possible that enteric motorneurons, by using multiple calcium channel subtypes, which in turn are coupled to important inhibitory neurotransmitters, ensure that transmission to the smooth muscle is achieved. The functional significance of multiple inhibitory neurotransmitters could therefore be a compensatory one, that is, deficiencies in either NOS/ATP/VIP and/or a calcium channel subtype would be compensated, at least in part, by another inhibitory neurotransmitter or calcium channel (Mashimo *et al.*, 1996; Mashimo *et al.*, 2000). The same reasoning that multiple transmitters serve as protective mechanisms to ensure continual gut function can also be ascribed to the excitatory transmitters ACh and SP. This is due to the paucity of CNS innervation

to the ENS (Galligan, 2002b), and implies that the GI tract needs to be able to function in the absence of CNS input.

SUMMARY AND CONCLUSIONS

The present study demonstrated the involvement of R-type calcium channels in neurotransmission to the GI muscle layer. My data support a model in which R-type calcium channels couple to NOS to mediate NO release from myenteric motor axon varicosities to the smooth muscle. This mediation occurs via an apamin-insensitive mechanism. Therefore R-type channels do not couple to ATP/βNAD release, but rather to NO as a mediator of relaxation responses, demonstrated by EFS of LMMP preparations, and recordings of IJPs from smooth muscle cells. All IJPs observed in this study were completely inhibited by apamin but not by $NiCl_2$ and NLA, thus providing indirect evidence of a spatial relationship and coupling between R-type calcium channels and NO release. The cross-talk between R-type channels and NOS would need to be further evaluated as to whether there is a physical coupling between the channel and the enzyme, or whether R-type channels are linked to NOS through intracellular signaling mechanisms. This study helps us understand how the inhibitory signaling pathway is involved in neuromuscular control of motility via R-type channels. This may be important as the drugs that target R-type calcium channels would help to facilitate or inhibit neurotransmitter release form nerve endings of myenteric motor neurons.

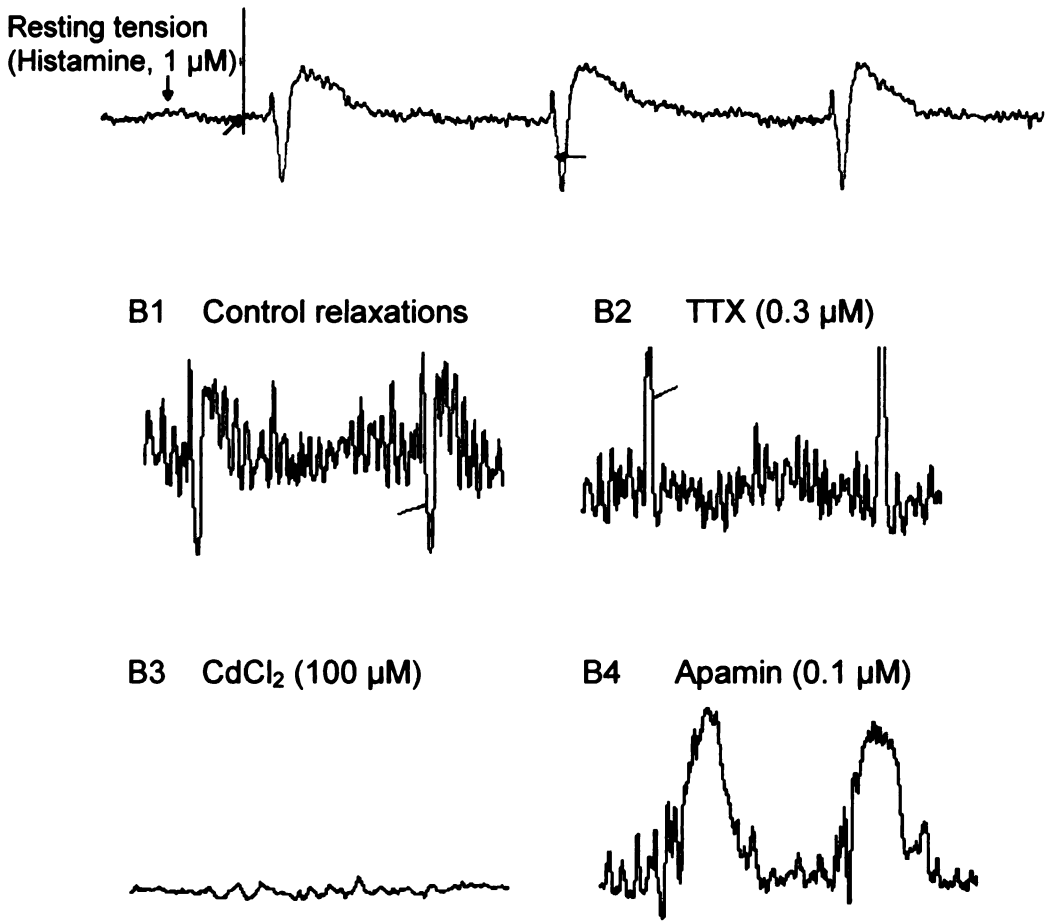


Fig. 5.1. Representative traces showing NANC responses after transmural electrical field stimulation of the guinea pig LMMP *in vitro*. Relaxations were induced in the presence of histamine (1 μM) and scopolamine (1 μM). After nerve stimulation (NS), a fast contraction (*a*) was followed by a relaxation (*b*), and then a rebound contraction (*c*) (A, B1). While LMMP relaxations were abolished by TTX (0.3 μM), a fast contraction persisted. The entire response was, however, completely sensitive to CdCl2, a non-selective antagonist of voltage-gated calcium channels. Apamin (0.1 μM) blocked the relaxation.

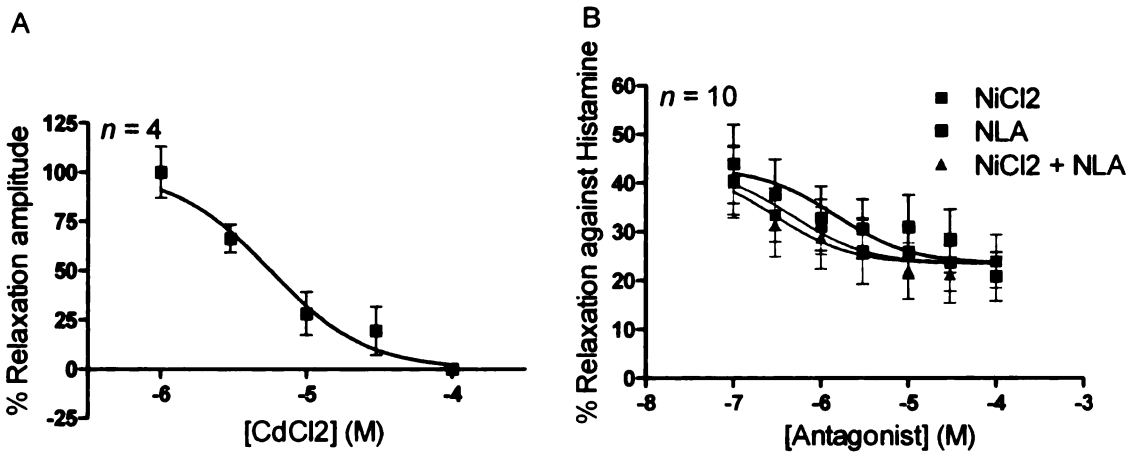
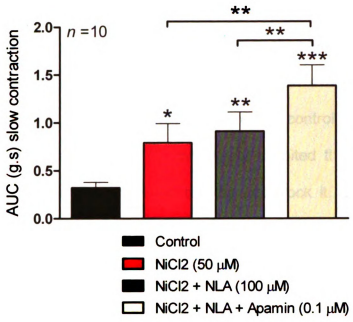


Figure. 5.2. Concentration-response curves for the area under the curve (AUC) of relaxation responses caused by EFS. CdCl2 caused a successive inhibition of the AUC (A). (B) When NLA was added first, it produced a concentration-dependent inhibition expressed as a percent of the relaxation response against the histamine contraction ($P < 0.0001$). The action of NLA was not statistically different in the presence of NiCl2 ($P > 0.05$). $NiCl_2$, added first, also caused a concentration-dependent inhibition relaxation response ($P < 0.0001$). NiCl2 + NLA together had no further effect on the relaxation ($P > 0.05$). Data are mean ± S.E.M. *n* is the number of tissues from which responses were obtained.

A.



B.

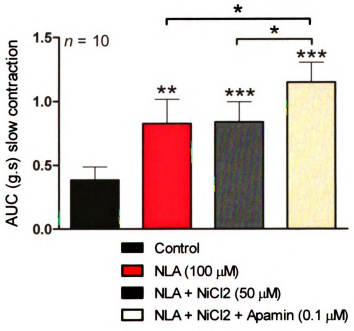


Figure. 5.3. Effects of $NiCl_2$, NLA, and apamin on LMMP contractions. Co-application of apamin + NLA + NiCl2 did not cause a significant increase compared to apamin + NLA ($P > 0.05$).

Figure. 5.4. Effect of NiCl2, and NiCl2 + NLA on inhibitory junction potentials (IJPs) evoked in guinea pig smooth muscle cells. NiCl2 (B1-B3, H), and NiCl2 + NLA (C1-C3) did not have any effect on evoked IJPs at all frequency levels of nerve stimulation (NS) compared to control (A1-A3). MRS 2179 (D1-D3), TTX (E) and apamin (G) completely inhibited the contractions. CdCl2 (F) reduced the LMMP contraction but did not block it. Concentration-response curves (I) for NiCl2 showed no effect on the IJPs, whereas CdCl2 largely inhibited contraction responses. Data are mean ± S.E.M. from *n* muscle cells.

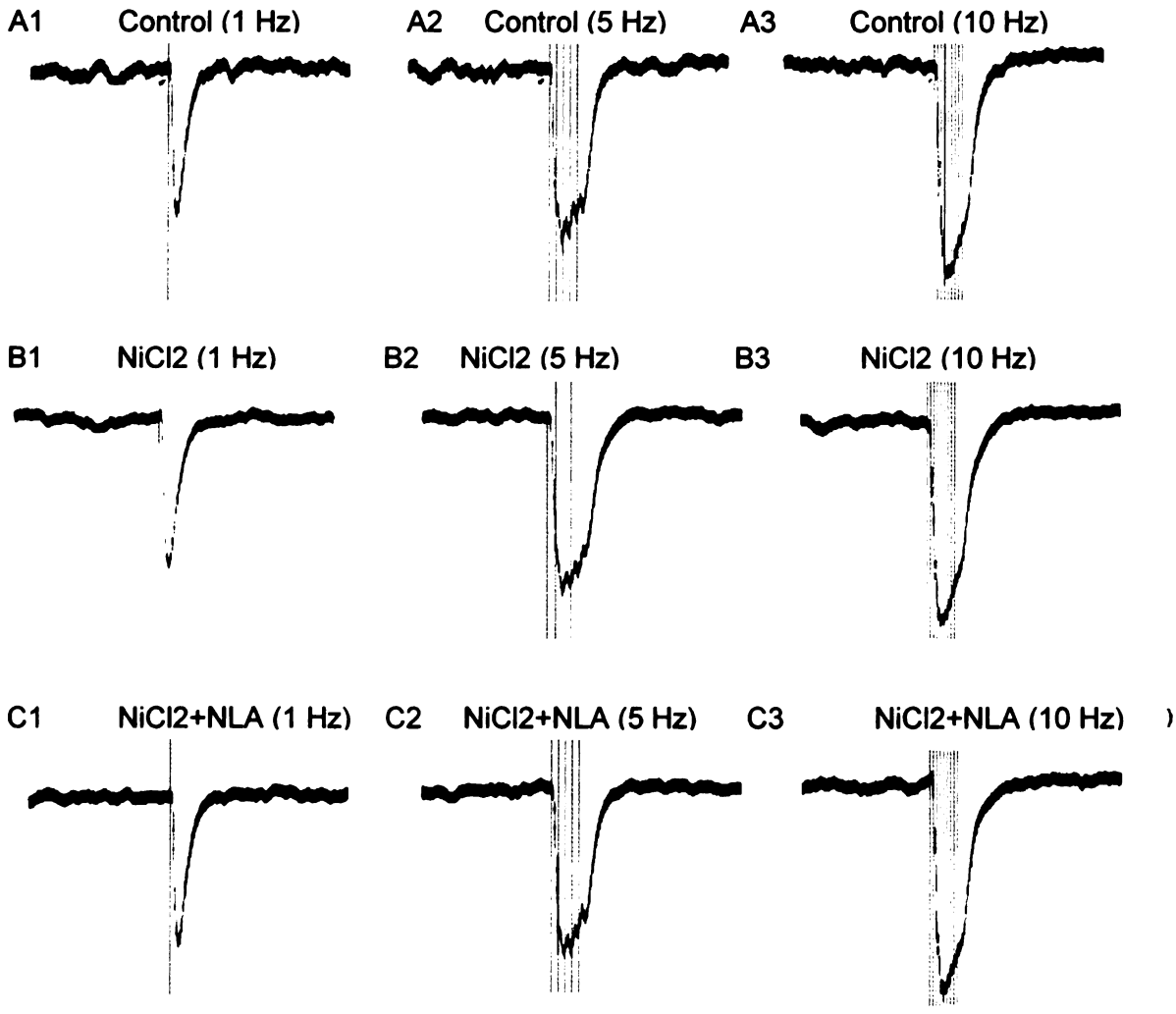
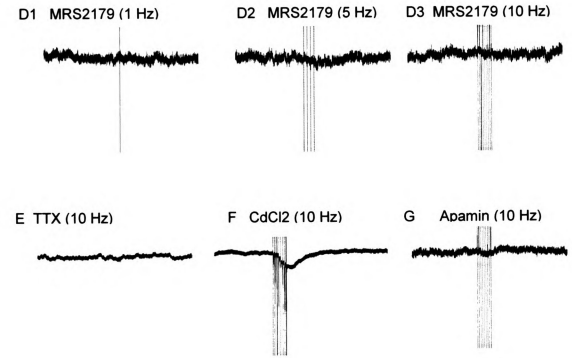
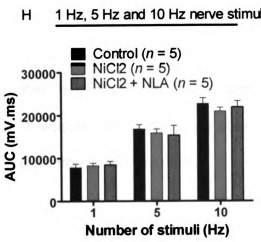
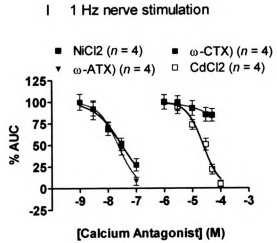

A1 Control (1 Hz)
A2 Control (5 Hz)
A3 Control (10 Hz)
B1 NiCl2 (1 Hz)
B2 NiCl2 (5 Hz)
B3 NiCl2 (10 Hz)
C1 NiCl2+NLA (1 Hz)
C2 NiCl2+NLA (5 Hz)
C3 NiCl2+NLA (10 Hz)

Figure. 5.4 (cont'd)







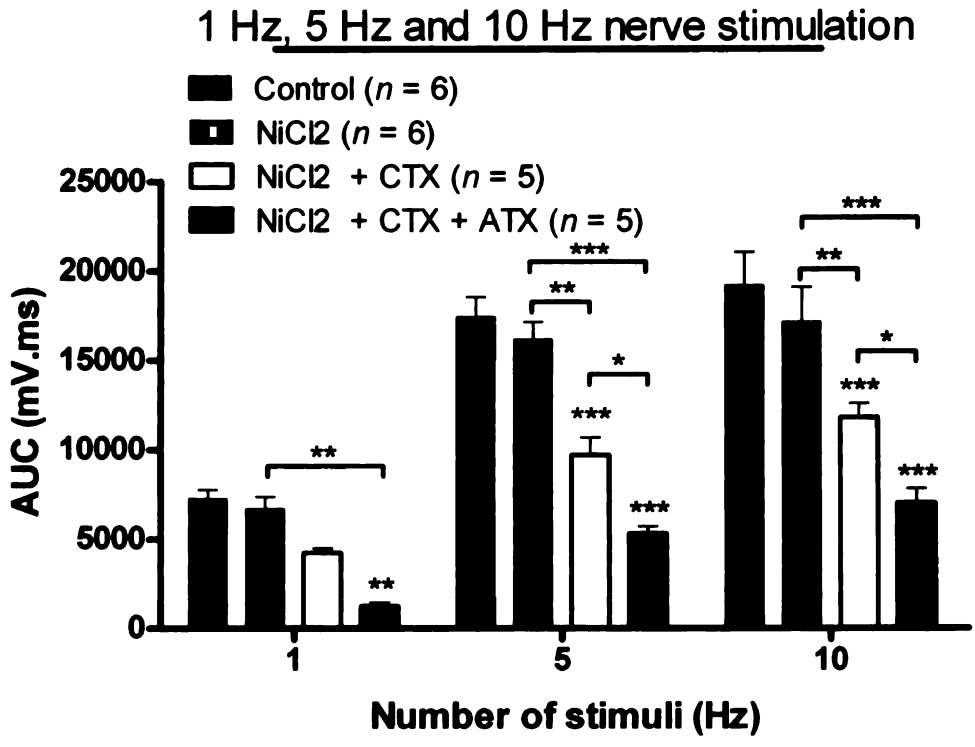


Figure. 5.5. Effect of NiCl2, and NiCl2 + ω-CTX and NiCl2 + ω-CTX + ω-ATX on IJPs. NiCl2 did not have any effect on evoked IJPs at all frequency levels of stimulation compared to control. Additive application of ω-CTX reduced the AUC. Subsequent application of ω-ATX largely blocked LMMP contractions at the 1 Hz frequency, and significantly reduced but did not block the contractions at the higher 5Hz and 10 Hz frequencies. This combined effect of NiCl2, ω-CTX + ω-ATX was similar to the result obtained after CdCl2 application. Data are mean ± S.E.M. from *n* muscle cells.

CHAPTER 6 INHIBITION OF R-TYPE CALCIUM CHANNELS IMPAIRS PERISTALSIS IN THE GUINEA PIG ILEUM

ABSTRACT

R-type calcium channels are expressed by nitric oxide synthase (NOS) containing inhibitory motorneurons and some interneurons in the guinea ileum myenteric plexus. The goals of this study were to determine if inhibition of R-type calcium channels alters fluid induced peristalsis in the guinea pig ileum *in vitro*. Tetrodotoxin (0.3 μM), and the non-specific calcium channel blocker cadmium chloride (CdCl2, 1-100 μM) blocked peristalsis confirming that the reflex was neurogenic. NiCl2 (50 μM, which blocks selectively R-type calcium channels), reduced peristaltic contractions and efficiency (measured as fluid expelled). Additional treatment with the NOS inhibitor, NLA (100 μM) further inhibited peristalsis. NLA applied alone increased contraction amplitude, frequency and efficiency and additional application of $NiCl_2$ inhibited peristalsis. ω-conotoxin GVIA (0.1 μM, a concentration which selectively inhibits N-type calcium channels), partially inhibited peristalsis, subsequent addition of $NiCl_2$ produced a further inhibitory effect. These data indicate that R-type calcium channels couple to NOS to regulate neurotransmission at neuroeffector junctions in the peristaltic reflex pathway. Selective inhibition of NO release from inhibitory motorneurons by NLA enhances peristalsis. While $NiCl_2$ also inhibits NO release from motor neurons, it has additional sites of action which cause inhibition of peristalsis. $NiCl_2$ and ω-conotoxin GVIA produce additive inhibition of peristalsis indicating that R-and N-type calcium channels are localized to different components of the reflex circuitry mediating peristalsis.

INTRODUCTION

Control of gastrointestinal (GI) motility is accomplished in part by interactions between the central nervous system (CNS), the enteric nervous system (ENS), myogenic and hormonal mechanisms (Grundy *et al.*, 2006). The myenteric plexus is the division of the ENS that is primarily responsible for controlling GI motility. The peristaltic reflex is a fundamental motility reflex that propels content along the length of the gut (Gwynne & Bornstein, 2007). Peristalsis is a reflex that is initiated by distention of the intestine and it relies on the sequential activation of intrinsic primary afferent neurons (IPANs), interneurons and motorneurons in the myenteric plexus to produce oral muscle contraction and aboral muscle relaxation relative to the stimulus (Hennig *et al.*, 1999; Huizinga *et al.*, 2006). Acetylcholine and Substance P, released from excitatory longitudinal and circular muscle motorneurons, are the major excitatory neurotransmitters responsible for contraction of GI smooth muscle and the emptying phase of peristalsis (Paton & Zar, 1968; Bogeski *et al.*, 2005). Relaxation of GI smooth muscle is mediated by non-adrenergic non-cholinergic (NANC) inhibitory enteric neurons and NO is a mediator of this relaxation (Sanders & Ward, 1992; Furness, 2000).

Contraction of GI smooth muscle is dependent in part on activation of L-type calcium channels (Horowitz *et al.*, 1996; Farrugia, 1999; Sanders, 2008). Although L-type calcium channels are also expressed in the ENS, they do not contribute to synaptic transmission (North & Tokimasa, 1987; Franklin & Willard, 1993; Reis *et al.*, 2000). N, P/Q and R-type calcium channels are also expressed

in the ENS (Hirning *et al.*, 1990; Kirchgessner & Liu, 1999; Smith *et al.*, 2003). N-type channels contribute to the calcium shoulder during the repolarizing phase on the AH action potential and to synaptic transmission by (Franklin & Willard, 1993; Rugiero *et al.*, 2002). N-type calcium channels are blocked by ω-conotoxin GVIA (McCleskey *et al.*, 1987). P/Q-type channels also contribute to neurotransmitter release in the ENS (Reis *et al.*, 2000). ω-Agatoxin IVA is a specific antagonist of P/Q channels (Mintz *et al.*, 1992).

R-type calcium currents have been recorded from guinea pig myenteric neurons maintained in primary cell culture (Bian *et al.*, 2004). R-type calcium channels also contribute to calcium currents in duodenal myenteric neurons in the intact myenteric plexus (Rugiero *et al.*, 2002; Furness, 2006a). Immunoreactivity (ir) to the pore-forming subunit (α1E) of the R-type calcium channel has been localized to subsets of myenteric neurons (Naidoo *et al.*, 2006). α1E-ir was co-localized with nitric oxide synthase (NOS) in nerve fibers supplying the muscle layers and NO contributes to non-cholinergic non-adrenergic GI smooth muscle relaxation (Lecci *et al.*, 2002). NO-mediated neurotransmission in particularly important for the peristaltic reflex (Ciccocioppo *et al.*, 1994; Waterman & Costa, 1994; Holzer *et al.*, 1997). However, the relationship between R-type calcium channel function, NO-mediated neuromuscular transmission and the peristaltic has not been studied. I used a modified Trendelenburg system (Bülbring *et al.*, 1958; Huizinga *et al.*, 1998; Bian *et al.*, 2003) to determine whether antagonism of R-type calcium channels affects fluid-induced peristalsis in the adult guinea pig ileum *in vitro*.

MATERIALS AND METHODS

Tissue collection and preparation. All animal use protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University. Adult male Hartley guinea pigs (250-300 g, Bioport, Lansing, MI) were anesthetized via isoflurane (Abbott Laboratories, Chicago, IL) inhalation, stunned by a blow to the back of the head and killed by severing the carotid arteries. A segment of the ileum approximately 10 cm proximal to the ileo-cecal junction was quickly removed and placed in pre-warmed (37 °C) oxygenated (95 % O_2; 5 % CO_2) Krebs' solution of the following composition (in mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose.

Assessment of peristalsis in vitro. Assessment of peristalsis *in vitro* was accomplished using a modified Trendelenburg preparation. A segment (6.5 cm) of the ileum was transferred to and pinned (Fine Science Tools, Foster City, CA) in a silicone elastomer-lined (Sylgard, Dow Corning, Midland, MI) petri dish containing warmed Krebs' solution. Fire-polished ends of two glass cannulas (Radnoti Glass Technology, Monrovia, CA) were inserted into the oral and aboral ends of the ileum respectively, and secured firmly with 4-0 silk suture (Harvard Apparatus, Holliston, MA). The ileal segment was mounted horizontally in a plexiglass organ bath (volume 40 ml) containing Krebs' solution and equilibrated at 37 °C for 45 min. The Krebs' solution was replaced in 15 minute intervals during the equilibration period. Fluid-induced distention was achieved by raising the level of a Krebs'-containing reservoir connected by a plastic filling tube to the

glass tube inserted into the oral end of the ileum. The filling tube was connected to a pressure transducer (TRN 050; Kent Scientific Corp., Torrington, CT) via a T-connector, allowing intraluminal pressure increases to be monitored as the reservoir height was increased. Signals from the pressure transducer were digitized and acquired using an analog to digital converter (Minidigi 1A, Molecular Devices Inc., Foster City, CA), a DC strain gage amplifier (Grass Technologies, West Warwick, RI), and Axoscope v10 software (Molecular Devices Inc). The glass tube at the aboral end of the ileum was fitted with a one-way value to prevent backflow of Krebs' solution. This tube drained into a graduate cylinder for measurement of the volume of fluid expelled during each episode (see below).

Experimental protocol. After the 45 minute equilibration period, intraluminal pressure was first increased from a basal to a threshold (T) level. T was defined as the minimum pressure (mmHg) required for the initiation of peristalsis. Peristalsis was defined as a contraction that propagated from the oral to the anal end of the segment. Typically, these contractions occurred in regular intervals throughout the distention period. The pressure was elevated for 1 minute for each episode and then returned to baseline where the tissue was left to recover for 5 minutes. A range of increasing intraluminal pressures, in 0.5 mmHg intervals, from T to T+5 mmHg was used. This allowed us to calculate the half-maximal effective intraluminal pressure (EP_{50}) for time control and antagonist concentration-response studies. Time control experiments were performed over a period of 50 min, and the Krebs' solution was replaced every 15 min. I measured the amplitude of the peak contraction (mmHg), frequency of

contractions (waves/ s), and the area under the curve (AUC; mmHg.ms) (Clampfit; Molecular Devices) during a 60 s recording period. The volume of fluid expelled (ml) at the end of each distention period was also measured.

Antagonist concentration-response curves. Concentration-response curves were obtained by using the $T+EP_{50}$ (mmHg) value at increasing antagonist concentrations for each segment of ileum. The non-selective calcium channel antagonist $CdCl_2$ (1-100 μM), and the R- and N-type calcium channel antagonists $NiCl_2$ (1–50 μM) and ω-conotoxin GVIA (ω-CTX) (0.001–1 μM), respectively, were added to the bath. The tissue was incubated in successively higher antagonist concentrations for 10 min prior to testing its effect on peristalsis.

Pressure-response curves. Pressure-response curves were obtained under control drug-free conditions and at specific antagonist concentrations using increasing intraluminal pressure values from T to T+5. The aim of the pressure-response curve experiments was to investigated the effects of $NiCl_2$ (50 μM), the nitric oxide synthase inhibitor nitro-L-arginine (NLA; 100 μM), ω-CTX, the sodium channel blocker tetrodotoxin (TTX; 0.3 μM), the small-conductance potassium channel antagonist apamin (0.1 μM) on the peristaltic reflex. All chemicals were purchased from Sigma-Aldrich (St Louis, MO), except for ω-CTX and TTX (Alomone Labs., Jerusalem, Israel).

Video imaging and spatio-temporal mapping. The construction of spatio-temporal maps was performed as previously described (de Backer *et al.*, 2008). Briefly, a camera (Logitech, Fremont, CA), placed directly above the

organ bath, was used to capture peristaltic motor movements of the ileum over a 60 s recording period (25 frames/s, 640 x 480 pixel resolution). The video file was imported into ImageJ using a plugin for QuickTime v7 (www.apple.com) (Figure 6.1A). A threshold procedure was applied to all video frames and edge-detection was used to define the gut periphery. Images were converted into binary format (Figure. 6.1B) and a region of interest was selected. Next, two further ImageJ plugins were used: Amplitude Profiler, which determines the change in intestinal diameter (mm); and Spatio-temporal Mapping software, which facilitates 3-dimensional (diameter distance and time) rendering of intestinal motility. I measured the minimum and maximum absolute diameters (mm) and the overall mean diameter (mm) of the ileum for a 60 s duration (de Backer *et al.*, 2008).

Statistics. Data are presented as mean values ± S.E.M for *n* animals from which ileal segments were obtained. Concentration-response data were fitted using non-linear regression and the Hill equation (GraphPad Prism 4, GraphPad, San Diego). Statistical analysis was performed using either Student's *t* test for paired data, or a one-way ANOVA with Tukey-Kramer multiple comparison as a post-hoc test when more than two groups were compared. The effect of drugs on measured parameter responses at various intraluminal pressures between groups was assessed using a two-way repeated-measures ANOVA; post hoc multiple comparisons were corrected using Bonferroni's procedure. $P < 0.05$ was the criterion for determining statistical significance.

RESULTS

Determining the EP_{50}. To determine the EP_{50}, I used, from a basal level, 0.5 mmHg step intervals in intraluminal pressure, ranging from T to T+5. I then calculated the EP_{50} by measuring the active area of the AUC which will incorporate frequency and amplitude of peristaltic contraction during each episode (Figure. 6.2A, B, C) during this range. The EP_{50} was 1.9 ± 0.03 mmHg (*n* = 8). Using the EP_{50}, I verified the stability of the reflex over a period of 50 minutes with peristaltic episodes at 5 minute intervals. There was no decline in the AUC or frequency of contraction during this period (Figure. 6.3A, B).

Effects of calcium channel blockers on peristalsis. I also used the EP_{50} to investigate the effect of $CdCl_2$ on peristalsis. $CdCl_2$ (1-100 μM) inhibited the peak amplitude of peristaltic contractions and their frequency. These changes are indicated by the concentration-dependent decrease in the AUC (Figure. 6.4) caused by $CdCl_2$. Previous studies suggest that the N-type calcium channel may be the predominant calcium channel in the ENS (Takahashi *et al.*, 1992; Smith *et al.*, 1999). I therefore first investigated whether ω-CTX could inhibit the peristaltic motor pattern. ω-CTX (1–100 nM) produced a concentration-dependent inhibition of the peak contraction amplitude of 39.31 % (Figure. 6.4). Low concentrations of $NiCl_2$ (50 μM) selectively blocks R-type calcium channels in the nervous system (Randall & Tsien, 1995; Tottene *et al.*, 2000). $NiCl_2$ (1-50 μM) also caused a concentration-dependent reduction in the peak amplitude, reducing it by 19.95 % (Figure. 6.4). The peristaltic reflex was impaired but not completely blocked by

either of $NiCl_2$ or ω-CTX. TTX (0.3 μM) completely inhibited the peristaltic reflex, thus confirming that this observed pattern of small intestinal motility was neurogenic through activation of myenteric neurons (data not shown).

$NiCl_2$ and NLA inhibit the peristaltic reflex

Previous immunohistochemical data show that the pore-forming subunit of the R-type calcium channel, α1E, strongly co-localizes with NOS in cell bodies and varicose nerve fibers in the myenteric plexus, and in nerve fibers innervating the circular muscle (Naidoo *et al.*, 2006). I therefore hypothesized that R-type calcium channels may couple to NOS activation and NO-mediated muscle relaxation in the guinea pig ileum. To explore the role of a possible interaction between the R-type calcium channel and NO, I performed two sets of the following experiments where either $NiCl_2$ (50 μM) or NLA (100 μM) was applied to the bath in combination with apamin (0.1 μM): 1) $NiCl_2$ alone; $NiCl_2$ + NLA; $NiCl_2$ + NLA + apamin; and 2) NLA alone; NLA + $NiCl_2$; NLA + $NiCl_2$ + apamin.

Peristaltic frequency. In experiments in which $NiCl_2$ was added first, I detected a significant decline in the peristaltic frequency compared to the control response (Figure. 6.5, 6.6A). The frequency of contractions after co-application of $NiCl_2$ and NLA compared to $NiCl_2$-treatment alone was unchanged. However, when NLA was applied first, I detected a significant increase in the number of propagating peristaltic contractions at intraluminal pressures 1.9 to 2.9 mmHg

(Figure. 6.7, 6.8A). After co-application of NLA and $NiCl_2$, the peristaltic frequency decreased below control values at 1.9 to 2.9 mmHg.

AUC. I detected a significant decrease in the AUC at 1.9 to 2.9 mmHg when $NiCl_2$ was added first (Figure. 6.5, 6.6B). Subsequent application of $NiCl_2$ and NLA did not change the AUC. In the presence of NLA first, however, the AUC increased. When NLA and $NiCl_2$ were subsequently applied together, the AUC was reduced at 1.9 to 2.9 mmHg (Figure. 6.7, 6.8B).

Volume expelled. The ileum emptied less when $NiCl_2$ was added first, deviating from the control response at 1.9 to 2.9 mmHg (Figure. 6.6C). The addition of $NiCl_2$ and NLA did not further decrease the volume of expelled fluid. When the ileum was treated with NLA first, the volume expelled increased over the control response at 2.4 and 2.9 mmHg. Subsequent application of $NiCl_2$ with NLA significantly reduced the overall volume of fluid expelled at 1.4 to 2.9 mmHg (Figure. 6.8C).

Mean Diameter. In the presence of $NiCl_2$ and then $NiCl_2$ with NLA, the mean diameter increased significantly at intraluminal pressures 1.9 to 2.9 mmHg (Figure. 6.6D).

Spatio-temporal maps. Throughout this study, peristalsis, when evoked under drug-free conditions, was consistently robust with fully propagating motor

patterns (Figures. 6.5A1, A2; 7A1, A2; 9A1, A2, 11A1, A2). My interpretation of these motor patterns relied on the concurrent analysis of simultaneously-recorded ejection pressure waves (Figure. 6.5A1). In Figure. 6.5A1, the duration of the emptying phase of peristalsis was approximately 2.5 s. Figure. 6.5A2 is a spatio-temporal map depicting the motor patterns as cycles by characterizing the propagation of peristaltic waves in time and space. Examination of a control peristaltic reflex cycle (from the midpoint of one trough to the midpoint of the next trough; Figure. 6.5A2 line *a-b*) on the map shows that the time taken for one cycle is approximately 5-6 s. This 5-6 s duration represents the emptying phase duration (see Figure 6.5A1). The spatio-temporal map therefore indicates that two fully propagating motor reflexes, each taking 2.5-3 s, traversed the ileal segment within a total time period of 5-6 s. It should therefore be noted that one cycle, as represented on my map, is a superimposition of multiple propagating motor patterns that summate to produce that cycle. When $NiCl_2$ was applied first, the time taken for one cycle to occur steadily increased (Figure. 6.5B1, B2) until the propulsive motor reflex was replaced by regular small-amplitude oscillatory contractions. $NiCl_2$ and NLA together, largely suppressed, but did not completely inhibit the reflex (Figure. 6.5C1, C2).

$NiCl_2$ and ω-CTX inhibit the peristaltic reflex

Because $NiCl_2$ did not completely inhibit the reflex (see above), I investigated the contribution of other calcium channel subtypes to peristalsis. This would help us determine the extent of R-type calcium channel involvement

in modulation of the peristaltic reflex. I chose to study the interaction between $NiCl_2$ (50 μM) and ω-CTX (100 nM). I used an identical protocol to my NLA experiments to monitor the peristaltic motor pattern, that is: 1) $NiCl_2$ first; then $NiCl_2$ + ω-CTX; and 2) ω-CTX first; then ω-CTX + $NiCl_2$.

Peristaltic frequency. When the ileum was treated initially with $NiCl_2$ alone, I observed a decrease in the frequency of peristaltic waves, and threshold pressure was unaffected. The wave frequency only decreased significantly when $NiCl_2$ and ω-CTX were co-applied (Figure. 6.9, 6.10A). However, when ω-CTX was applied first, the peristaltic reflex was impaired. In all tissues examined, peristalsis did not commence until at least 15 s after the desired level of pressure was attained; the initiation of the reflex appeared to be particularly affected. Co-application of ω-CTX and $NiCl_2$ then mostly suppressed the propulsive wave at all levels of intraluminal pressures tested (Figure. 6.11, 6.12A).

AUC. Examination of the AUC revealed a similar sensitivity of the ileum to treatment with $NiCl_2$ and ω-CTX. Initial application of $NiCl_2$ did not produce a significant decrease in the AUC, and the AUC was only reduced after incubation with both $NiCl_2$ and ω-CTX (1.4 – 2.9 mmHg) (Figure. 6.9, 6.10B). I further found that when ω-CTX was added to the bath first, and that when the ileal segment was further incubated with both calcium channel antagonists, that this had a similar diminishing effect on the AUC (Figure. 6.11, 6.12B, also compare Figure. 6.9C with Figure. 6.11C).

Volume expelled. In the presence of $NiCl_2$, the volume of fluid expelled was not different from control values (Figure. 6.10C). When the ileum was incubated with both $NiCl_2$ and ω-CTX, the volume emptied declined significantly (0.9 – 2.9 mmHg). Reversibly, the application of ω-CTX first, significantly reduced the fluid volume expelled (1.9 – 2.9 mmHg). Co-application of $NiCl_2$ and ω-CTX in both sets of experiments then caused a steep decline in fluid expulsion volume at 0.9 – 2.9 mmHg (Figure. 6.12C).

Mean diameter. The presence of both $NiCl_2$ + ω-CTX (not shown), and ω-CTX + $NiCl_2$ caused a significant increase in the mean diameter of the ileum in both experiments (Figure. 6.12D). This increase was particularly greater at higher intraluminal pressures (1.9 – 2.9 mmHg).

Spatio-temporal maps. Analysis of spatio-temporal maps showed consistent regular peristaltic reflexes under drug-free conditions (Figure. 6.9A2). $NiCl_2$ caused a progressive reduction in the strength of contraction and amplitude of each peristaltic wave. $NiCl_2$ also doubled the time taken for each motor pattern to fully propagate from 2.5 s to approximately 4.5 s (Figure. 6.9B2), and this duration increased throughout the recording session. $NiCl_2$ together with ω-CTX completely abolished the motor pattern (Figure. 6.9C2). Peristaltic activity, however, visualized as propagating oscillations uniform in amplitude, was still

observed. Reverse peristalsis was also found, indicating that in the presence of $NiCl_2$ and ω-CTX, motility is not always aborally-directed.

When I applied ω-CTX first, threshold pressure was not altered. Propagating oscillations were again present, but only for approximately 30 s from the onset of fluid-distention (Figure. 6.11B1, B2). Moreover, these oscillatory contractions were not uniform in size and/or time. The regular motor pattern then commenced and appeared to have, at least in part, recovered from the initial ω-CTX insult. Incubation of the ileum with both ω-CTX and $NiCl_2$ completely inhibited facilitation of the distension-induced motor reflex (Figure. 6.11C1, C2).

DISCUSSION

The mammalian small intestine contains approximately 100 million neurons, about as many neurons as that found in the spinal cord (Gershon, 1997). The functions of these neurons are controlled primarily by the ENS. The ENS is therefore a critical regulator of GI function by coordinating motility (peristalsis), absorption and secretory responses, thus indicating that altered enteric neuronal signaling can therefore cause GI motility disorders. In this study I therefore sought to understand the physiological role of the voltage-gated R-type calcium channels in regulating peristalsis. The major findings of this study are that the R-type calcium channels are coupled to NO release, and that this channel, together with the N-type calcium channel are the major contributors to fluid distention-induced peristalsis in vitro. Specifically, the evoked peristalsis reflex is impaired by the association of the R-channel and NO, and by the R- and N-type calcium channel subtypes. These findings have important implications for furthering our understanding of peristalsis as they provide a mechanism by which ion-channels can be targeted to control GI motility.

Voltage-gated calcium channels mediate neurotransmitter release and are therefore important regulators of synaptic transmission. I first tested for a role of calcium channels in peristalsis in the guinea pig ileum in the presence of $CdCl_2$. $CdCl_2$ inhibited the peristaltic reflex by blocking calcium channels at all synapses, consequently increasing the intraluminal threshold pressure and the amplitude of the peristaltic wave. Peristaltic motility however, was not abolished, but still present as small amplitude, shallow, and regular oscillatory contractions. These

oscillations could be due to the intrinsic myogenic mechanisms of GI smooth muscle cells via adjacent interstitial cells of Cajal (ICC) (Huizinga *et al.*, 2006; Sanders, 2008). ICCs generate the slow wave that passively transmits to the smooth muscle cell that ultimately, in conjunction with neural input, determine the frequency and strength of contractions (Farrugia, 2008). I therefore concluded that calcium channels are necessary for the release of neurotransmitters from enteric motor axons to generate peristalsis. I observed identical results when TTX was applied to the ileal segment; the reflex was inhibited, confirming neural mediation.

Inhibition of the peristaltic reflex by $NiCl_2$ and NLA.

R-type calcium channels represent a viable control point for neurotransmission if the ENS (Bian *et al.*, 2004; Bian & Galligan, 2007), and α1E subunits are localized in cell bodies of IPANS and in nerve endings of excitatory (SP-containing) and inhibitory (NOS and VIP-containing) motorneurons (Naidoo *et al.*, 2006). These channels may therefore mediate synaptic excitation in the ascending or descending reflex pathway. In my first set of experiments, I therefore investigated whether R-type calcium channels contribute to the peristaltic reflex. I used NLA to further investigate a selective coupling of the R-channel to NO inhibitory neurotransmission. $NiCl_2$ inhibited, but did not block peristalsis. Subsequent application of NLA with $NiCl_2$ did not produce any additional changes in peristalsis suggesting that NLA and $NiCl_2$ act on the same mechanism. Interestingly, when NLA was applied first the peak amplitude,

frequency, AUC and volume expelled all increased, particularly at higher threshold pressures. Subsequent application of $NiCl_2$ with NLA inhibited peristalsis. Using the peristaltic frequency as a point of reference, this then suggests at least two possible scenarios:

1) When NLA was applied first, the frequency of peristaltic contractions significantly increased. NLA inhibits NOS to increase the propagating peristaltic contraction frequency (Ciccocioppo *et al.*, 1994). However, when $NiCl_2$ was subsequently applied in the presence of NLA, the contraction frequency decreased. Since $NiCl_2$ (50 μM) inhibits calcium entry though the R-type channel, if the R-channel and NOS are selectively coupled, then calcium would not be able to activate NOS. However, it also appeared that only when the R-channel was blocked, that the ability of NLA to inhibit NOS was impaired because the resultant frequency declined. The source of this decay points to a physical interaction between the R-channel and NOS, and suggests that when the R-type channel is blocked, NOS cannot be activated and therefore NOS cannot be blocked by NLA. The fact that many peristaltic waves are still propagating suggests that $NiCl_2$ may be inducing a "braking" system on NLA-induced NOS inhibition that would otherwise produce a hyperexcitable ileum. This effect may require synaptic release of multiple inhibitory enteric neurotransmitters from motor axons on to the smooth muscle. Recent evidence indicates that enteric motor nerve terminals in the mouse may contain catalytically active forms of NOS (nNOSα) that are anchored via PDZ binding domains to the plasma membrane (Stricker *et al.*, 1997; Rao *et al.*, 2008). NOS may therefore be docked at the

nerve terminal, close to R-type calcium channel release sites. My data suggest however that NO is probably not the transmitter being released because a physical association of the R-type channel to NOS requires that calcium enters through the R-type channel to facilitate NO release. This implies that the lower peristaltic frequency may be due to mediation by another inhibitory neurotransmitter via another calcium channel subtype. After $NiCl_2$ and NLA treatment, apamin completely inhibited the peristaltic reflex. A likely candidate for this neurotransmitter is therefore ATP/βNAD as a mediator of apamin-sensitive inhibitory neurotransmission. ATP and NO are released from the same nerve terminals (Costa *et al.*, 1986b; Crist *et al.*, 1992) and constitute the major contributors to inhibitory neurotransmission in the ENS (Burnstock, 2008). Moreover, NO and ATP are reported to exert independent effects on GI motility in the rat ileum and porcine esophagus (Benko *et al.*, 2006; Farre *et al.*, 2006). When NLA and apamin were co-applied to the rabbit distal colon (Ciccocioppo *et al.*, 1994) and guinea pig small intestine (Waterman & Costa, 1994; Holzer *et al.*, 1997), the gut became hyperexcitable but with non-propulsive segmental motor patterns and very little or no emptying. However, in my study, I had added apamin after $NiCl_2$ + NLA/NLA + $NiCl_2$ and in both cases, apamin did not affect the peristaltic reflex. The resulting motor pattern was similar to the effect by TTX on the peristaltic reflex. An important test would have been to add apamin first before either $NiCl_2$ or NLA. The emptying phase of peristalsis involves the activation of excitatory and inhibitory motor neurons (Waterman & Costa, 1994). In order for efficient propagative peristalsis to occur, there must therefore be

functional synchrony between activation of these motorneuronal inputs to the smooth muscle.

2) To confirm this coupling, I did the reverse experiment. When $NiCl_2$ was added first, the frequency of contractions was reduced, suggesting the possible release of NO. Interestingly, when $NiCl_2$ with NLA were co-applied, the frequency remained the same. In the presence of $NiCl_2$, NLA was unable to inhibit NOS and could not produce an increase in the contraction frequency. This verified my conclusion from the previous experiment that entry via the R-type calcium channel may be coupled to the activation of NOS. Inhibiting the R-type channel possibly prevents inhibition of NOS. If the R-channel is inhibited, and selectively coupled to NOS, then the low peristaltic frequency observed is probably mediated by another inhibitory neurotransmitter. I observed that apamin again completely blocked peristalsis. Blocking the R-channel may alternatively also prevent the release of excitatory neurotransmitters. My data however argue that ATP release from inhibitory motor axons may be responsible for the reduced peristaltic frequency rather than low excitatory neurotransmission.

Norepinephrine, released from sympathetic nerve endings, act at α_2-adrenoreceptors to inhibit intestinal motility (Surprenant & North, 1988; Stebbing *et al.*, 2001). A selective coupling between the R-type calcium channel and α_2-adrenoreceptors on myenteric neuronal cell bodies and nerve terminals has recently been proposed (Bian & Galligan, 2007). α_2-adrenoreceptors have been detected on cell bodies of myenteric neurons but the exact phenotype of myenteric neuron expressing these receptors is not yet known (Bian & Galligan,

2007). The R-type channel may be present on the cell bodies of IPANs where it modulates the slow excitatory postsynaptic potential and somal action potential. That study showed that agonist-activated α_2-adrenoreceptors inhibit calcium entry through R-type channels, because in the presence of $NiCl_2$, inhibitory control by the α_2-adrenoreceptor over the R-type channel was removed. In my study, when NLA was applied first, it is possible that the R-type channel was unable to prevent inhibition of NOS during the peristaltic reflex due to the release of norepinephrine from sympathetic nerve endings. Norepinephrine, acting at pre-junctional α_2-adrenoreceptors on myenteric motor axons, would then inhibit calcium influx by decreasing the probability of opening of the R-type channel, or activating presynaptic K^+ channels (Hein, 2006). This principle would also hold when $NiCl_2$ was applied first. $NiCl_2$-block of the R-type channel presumably removes inhibition by the α_2-adrenoreceptor to cause either a conformational change in the calcium channel protein, or a change in the R-channel-NOS signaling cascade that renders NOS inaccessible to inhibition by NLA.

Inhibition of the peristaltic reflex by $NiCl_2$ and ω-CTX

In the present study, I demonstrate that the peristaltic reflex in the guinea pig ileum is mostly mediated by R- and N-type calcium channels. This conclusion is based on the observation that co-application of $NiCl_2$ and the N-type calcium channel antagonist ω-CTX completed inhibited the peristaltic reflex. My concentration-response curve for $NiCl_2$ and ω-CTX shows that the additive inhibitory effect of these two calcium channel antagonists is approximately 60 %.

When $NiCl_2$, was added first, the number of propagating peristaltic waves, peak peristaltic amplitude, area under the curve, and volume of fluid expelled all significantly decreased compared to control responses. These parameters appeared to be particularly affected at higher threshold pressures. The reflex was impaired but was not blocked. Application of ω-CTX then completely inhibited the peristaltic reflex. When ω-CTX was applied first, non-propulsive segmentation patterns were observed, and propagating contractions only began after at least 25 s. By itself, ω-CTX did not change the threshold pressure for initiation of peristalsis. This suggests that N-type calcium channels mediate neurotransmission between interneurons and motorneurons, and between motorneurons and the smooth muscle. However, in the presence of ω-CTX and $NiCl_2$, the reflex was abolished, signifying inhibition of neurotransmission at all synapses. I still observed intrinsic myogenic activity, indicating that peristalsis still exists, albeit with a low level of neural excitation. To date, only two studies have reported on the functional significance of R-type calcium channels in the GI tract (Bian *et al.*, 2004; Bian & Galligan, 2007). Most studies on R-type channel function have been done in the CNS and the R-channel has been localized to nerve endings at many excitatory synapses (Wu *et al.*, 1998; Tottene *et al.*, 2000; Gasparini *et al.*, 2001; Dietrich *et al.*, 2003). In wild-type mice, P/Q-type calcium channels are the major calcium channel subtype found on nerve terminals in the calyx of Held (Wu *et al.*, 1999), cerebellum (Matsushita *et al.*, 2002) and hippocampus (Qian & Noebels, 2000). Furthermore, P/Q-type channels primarily mediate acetylcholine release at mammalian endplates (Urbano *et al.*, 2003;

Pagani *et al.*, 2004). Urbano et al. (2003), using P/Q-type calcium channel knockout mice showed that: 1) R- and N-type calcium channels are upregulated, and 2) R-type channels were less numerous than N-type channels, but are located closer to presynaptic calcium sensors at the active zone. In the ENS, P/Q-type calcium channels display weak immunoreactivity weak in the soma and nerve terminals of myenteric neurons, whereas N-type calcium channel-ir was diffuse and intense (Kirchgessner & Liu, 1999). Further, P/Q-type calcium channels contribute only 20 % to the total calcium channel current (Bian *et al.*, 2004). In the present study, my data suggest that the major contributors to the peristaltic reflex are the N-and R-type calcium channels. Previous immunohistochemical and electrophysiological data indicate that α1E co-localizes with NOS, VIP and SP in enteric motor neurons, and that R-type channels are present in a specific population of purely-cholinergic calretinin-containing ascending interneurons (Naidoo and Galligan, unpublished data). The present data indicate that $NiCl_2$, in the presence of fluid-induced radial distention, clearly induces a shift in motor activity of the ileum, from neurally-mediated fully propagating reflex patterns to segmental contractions associated with non-propulsive churning movements of the fluid content. I speculate that this shift may be due to a block of neurotransmission at specific synapses along the peristaltic reflex pathway. At these synapses, the concentration of R-type channels may be high. Ascending interneurons constitute 5 % of the total number of myenteric neurons in the ileum (Furness, 2006a) and 25 % of these are fully cholinergic (Galligan *et al.*, 2000). At purely-cholinergic ascending excitatory

synapses, R-type channels would mediate all cholinergic neurotransmission from those calretinin-containing interneurons. In fact, Gasparini et al. (2001) showed that 50 μM $NiCl_2$ reduced fast glutamatergic transmission in the CNS by at least 51-59 %. Because R-channels are also present on SP- and NOS/VIP-containing enteric motorneurons, they may control excitatory and inhibitory neurotransmission to the muscle. By inhibiting calcium-entry with $NiCl_2$ at all these synapses, the reflex circuitry is dysregulated. R-type channels make an important contribution to the peristaltic reflex, but in its absence, this reflex can still continue, as I have observed under conditions of fluid-evoked distention. Inhibiting its function, however, disrupts motility. The data suggest that at least two calcium channels are necessary for the peristaltic reflex. Perhaps for the peristaltic reflex to occur, two calcium channels, from a combination of N-type with the R-type calcium channel are necessary for this facilitation. This may be part of a fail-safe mechanism that has built into the reflex circuitry to ensure the efficacy of the propagating peristaltic motor pattern in the guinea pig.

SUMMARY AND CONCLUSIONS

Inhibition of R-type calcium channels in an in vitro model of fluid-induced peristalsis suppresses but does not completely inhibit the peristaltic reflex. The R-type channel is coupled to NOS and when both are inhibited, the peristaltic reflex is abolished. The R-type and N-type calcium channels function in tandem to facilitate the peristaltic reflex. Further understanding of the interaction between the various voltage-gated channel subtypes holds promise for improving our

understanding of GI motility, and the development of therapeutic strategies against motility diseases.

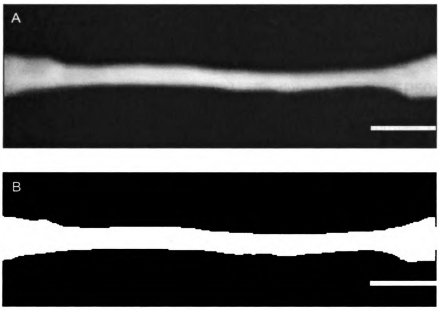
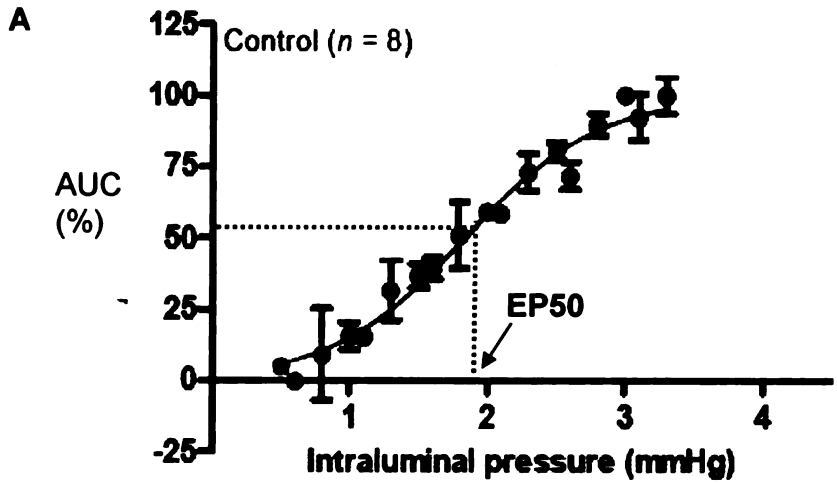
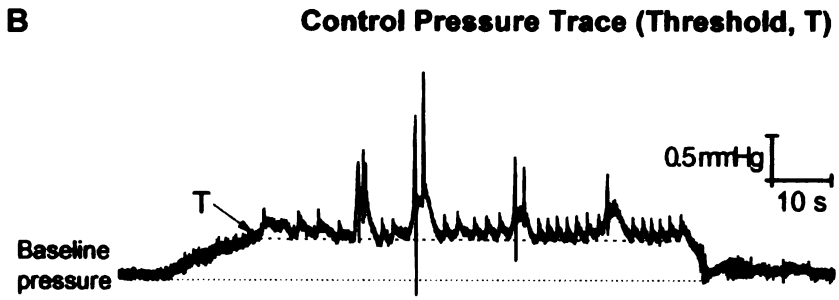


Figure 6.1. Peristalsis video imaging. A. A single frame from a peristalsis movie recording of the ileum imported into Image J. **B.** All movie frames were then converted into binary images. Image thresholding and edge detection delineated the ileum periphery. By selecting regions of the ileum and using intestinal motility software, I was able to detect changes in gut diameter. These changes were pooled and built into 3- dimensional spatio-temporal maps with an Image J-plugin linked to the graph-plotting program Gnuplot.

Figure 6.2. EP_{50} pressure-response curve and representative traces. (A) Pressure-response curve in drug-free Krebs' solution for calculating the magnitude of the area under the curve (AUC) against successive increases in intraluminal pressure. The half maximal effective pressure (EP50) was 1.9 mmHg. Data points are mean ± S.E.M. *n* represents the number of animals. Curves are non-linear fits of the Hill equation. **(B)** The reservoir was raised from a baseline pressure where no peristalsis was observed, to a threshold pressure (T, arrow) that produced a peristaltic reflex. **(C)** The area under the curve (AUC) was calculated for every pressure interval from T to T+5. These intervals were in 0.5 mmHg increments after the T value was known. Note that only the active area of the AUC was calculated. The arrow indicates the desired pressure level, and the dotted red line (at the arrow point) is the baseline for calculation of the AUC. The amplitude and frequency of peristaltic waves, in addition to the volume expelled, were also calculated.


A
Control (n = 8)
AUC
(%)
125
100
75
50
25
0
-25
EP50
1
2
3
4
Intraluminal pressure (mmHg)


B
Control Pressure Trace (Threshold, T)
0.5mmHg
10 s
T
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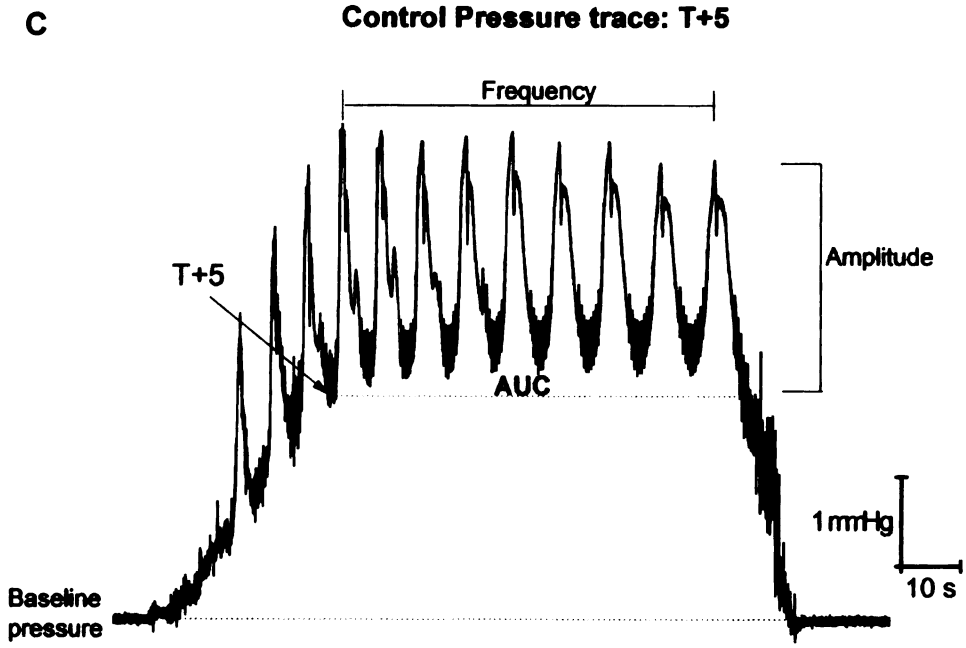
pressure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T+5
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1mmHg
10 s
Baseline
pressure

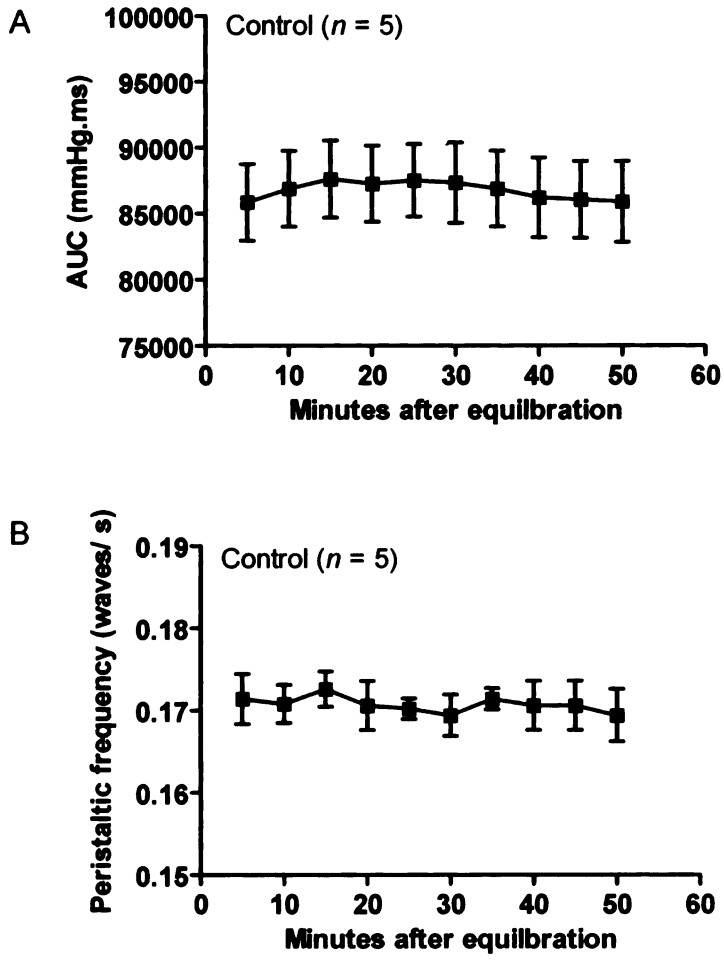


Figure. 6.3. Time controls showing stable peristaltic motor patterns after measuring (A) the AUC and (B) the peak peristaltic frequency in 5 min resting intervals for a period of 50 min after tissue equilibration. Peristalsis was evoked and recorded for 60 s. The ileal segment showed no evidence of fatigue during the 50 min experimental period.

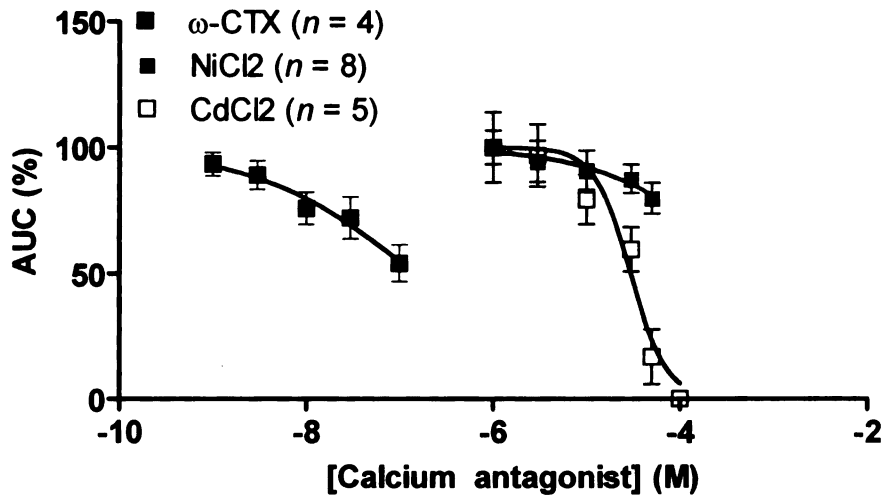
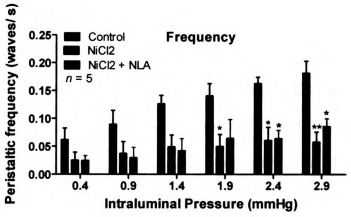


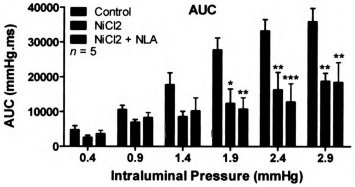
Figure. 6.4. Effect of ω-conotoxin (ω-CTX), $NiCl_2$ and $CdCl_2$ on the AUC expressed as percent change. ω-CTX (1-100 nM) and $NiCl_2$ (1-50 μM) decreased the AUC by 39% and 20% respectively. $CdCl_2$ (1-100 μM) caused a concentration-dependent inhibition of the AUC, and blocked the AUC completely at 100 μM. Data are mean ± S.E.M. *n* indicates the number of animals,

Figure. 6.5. Effect of NiCl2 and NLA on (A) frequency, (B) AUC, and (C) fluid volume expelled. These four parameters all decreased in response to NiCl2-treatment alone. Subsequent application of NLA did not produce a shift in any of the parameters. This was particularly evident at higher intraluminal pressures: frequency and AUC 1.9 – 2.9 mmHg; volume expelled 1.4 – 2.9 mmHg, and mean diameter 1.9 – 2.9 mmHg, compared to control (two-way ANOVA; $P < 0.05$). Data are mean ± S.E.M. *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$.

A


Frequency
Control
NiCl2
NiCl2 + NLA
n = 5
Peristaltic frequency (waves/ s)
0.25
0.20
0.15
0.10
0.05
0.00
0.4
0.9
1.4
1.9
2.4
2.9
Intraluminal Pressure (mmHg)

B


AUC
Control
NiCl2
NiCl2 + NLA
n = 5
AUC (mmHg.ms)
40000
30000
20000
10000
0
0.4
0.9
1.4
1.9
2.4
2.9
Intraluminal Pressure (mmHg)

C

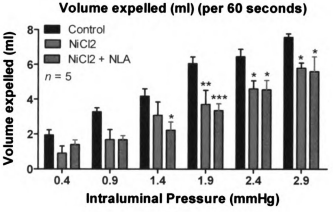
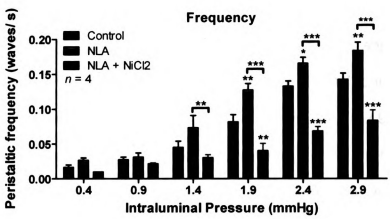
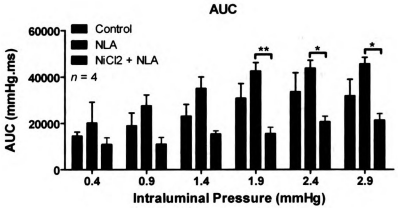

Volume expelled (ml) (per 60 seconds)
Control
NiCl2
NiCl2 + NLA
n = 5
Volume expelled (ml)
8
6
4
2
0
0.4
0.9
1.4
1.9
2.4
2.9
Intraluminal Pressure (mmHg)

Figure 6.6. Effect of NLA and NiCl2 on (A) frequency, (B) AUC and (C) fluid volume expelled (refer to Figure. 6.7). Addition of NLA first, increased all the above parameters of peristalsis. Subsequent application of NiCl2 reduced these parameters. **A.** The peak NLA contraction was significantly different from NLA + NiCl2 but not from control. **B.** The frequency of peristalsis after NLA treatment was higher compared to NLA + NiCl2 treatment, particularly at intraluminal pressures 1.4 – 2.9 mmHg. NLA, and NLA + NiCl2, were significantly different from control at pressures 1.9 – 2.9 mmHg. **C.** The AUC after NiCl2 and NLA treatment was greater than either control or NiCl2 alone, at 1.9 – 2.9 mmHg. Data are mean ± S.E.M.

A


Frequency
Control
NLA
NLA + NiCl2
n = 4
Peristaltic frequency (waves/ s)
0.20
0.15
0.10
0.05
0.00
0.4
0.9
1.4
1.9
2.4
2.9
Intraluminal Pressure (mmHg)

B


AUC
Control
NLA
NiCl2 + NLA
n = 4
AUC (mmHg.ms)
60000
40000
20000
0
0.4
0.9
1.4
1.9
2.4
2.9
Intraluminal Pressure (mmHg)

C

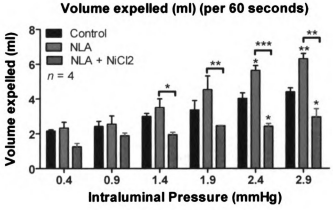
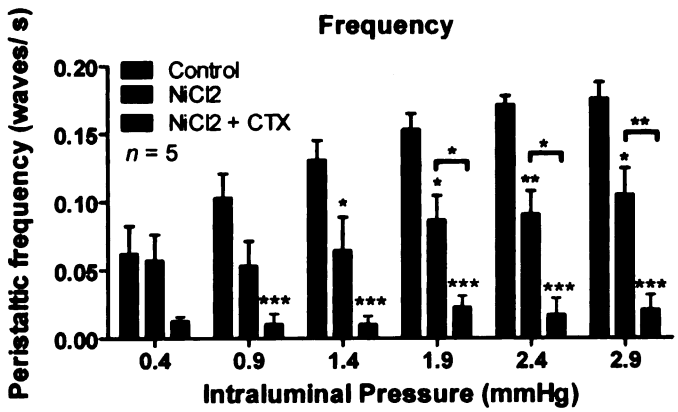
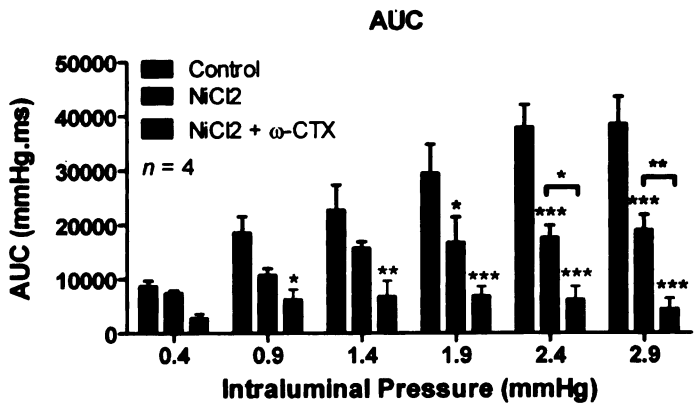

Volume expelled (ml) (per 60 seconds)
Control
NLA
NLA + NiCl2
n = 4
Volume expelled (ml)
8
6
4
2
0
0.4
0.9
1.4
1.9
2.4
2.9
Intraluminal Pressure (mmHg)

Figure 6.7. The peristaltic motor pattern changes with NiCl2, and NiCl2 + ω-CTX treatment (refer to Figure. 6.9). **A.** The frequency after NiCl2 application was reduced (1.4 – 2.9 mmHg vs. control). Addition of ω-CTX completely inhibited the reflex wave frequency (0.9 – 2.9 mmHg vs. control; (1.9 – 2.9 mmHg vs. control). **B.** The AUC was inhibited after NiCl2 treatment (1.9 – 2.9 mmHg vs. control). Subsequent application of ω-CTX then completely blocked the AUC (0.9 – 2.9 mmHg vs. control; 2.4 – 2.9 mmHg vs. NiCl2). **C.** Volume of fluid expelled decreased significantly compared to control at intraluminal pressures 2.4 and 2.9 mmHg. Following NiCl2 application, the volume ejected was significantly reduced (0.9 – 2.9 mmHg vs. control; 2.9 mmHg vs. NiCl2). **D.** Mean intestinal diameter and. Under control conditions, the ileum demonstrated a small mean diameter. After ω-CTX treatment, the diameter increased. Following ω-CTX and NiCl2 treatment, the diameter significantly increased (1.9 – 2.9 mmHg vs. control; 1.9 – 2.9 mmHg vs. ω-CTX). Data are mean ± S.E.M.

A


Frequency
Peristaltic frequency (waves/ s)
0.20
0.15
0.10
0.05
0.00
Control
NiCl2
NiCl2 + CTX
n = 5
0.4
0.9
1.4
1.9
2.4
2.9
Intraluminal Pressure (mmHg)

B


AUC
AUC (mmHg.ms)
50000
40000
30000
20000
10000
0
Control
NiCl2
NiCl2 + ω-CTX
n = 4
0.4
0.9
1.4
1.9
2.4
2.9
Intraluminal Pressure (mmHg)

C

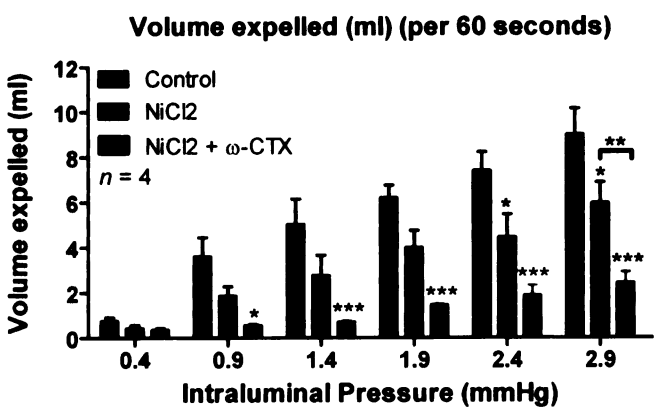
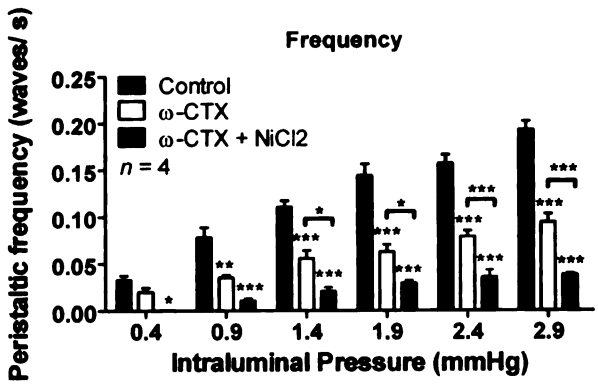
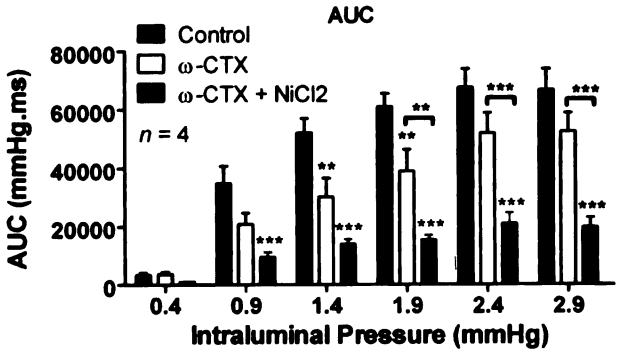

Volume expelled (ml) (per 60 seconds)
Volume expelled (ml)
12
10
8
6
4
2
0
Control
NiCl2
NiCl2 + ω-CTX
n = 4
0.4
0.9
1.4
1.9
2.4
2.9
Intraluminal Pressure (mmHg)

Figure. 6.8. Effect of ω-CTX alone, and ω-CTX with NiCl2, on peristalsis parameters. A. After ω-CTX treatment, the frequency significantly decreased compared to control (0.9 – 2.9 mmHg). Addition of NiCl2 then inhibited the frequency (0.4 - 2.9 mmHg vs. control; 1.4 - 2.9 mmHg vs. ω-CTX + NiCl2). ω-CTX together with NiCl2 blocked propagating reflex contractions, but not small oscillating contractions **B.** The AUC decreased significantly after ω-CTX treatment compared to control at intraluminal pressures 1.4 and 2.9 mmHg. The AUC was then significantly reduced after application of NiCl2 (0.9 - 2.9 mmHg vs. control; 1.9 - 2.9 mmHg vs. ω-CTX). **C.** Volume of fluid expelled declined significantly in the presence of ω-CTX (1.9 - 2.9 mmHg vs. control) and after ω-CTX + NiCl2 treatment (1.9 - 2.9 mmHg vs. ω-CTX). **D.** Under control conditions, the ileum demonstrated a small mean diameter. After ω-CTX treatment, the diameter increased. Following ω-CTX and NiCl2 treatment, the diameter significantly increased (1.9 – 2.9 mmHg vs. control; 1.9 – 2.9 mmHg vs. ω-CTX). Data are mean ± S.E.M for *n* tissues.

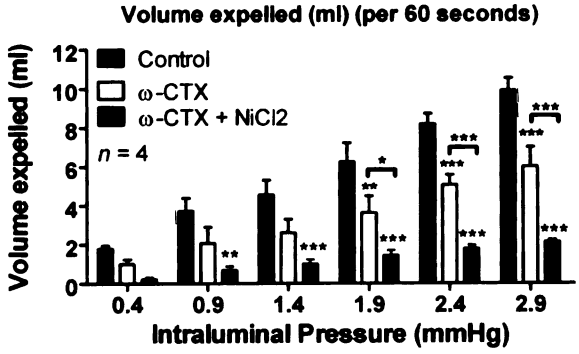
A


Frequency
Peristaltic frequency (waves/ s)
0.25
0.20
0.15
0.10
0.05
0.00
Control
ω-CTX
ω-CTX + NiCl2
n = 4
0.4
0.9
1.4
1.9
2.4
2.9
Intraluminal Pressure (mmHg)

B


AUC
AUC (mmHg.ms)
80000
60000
40000
20000
0
Control
ω-CTX
ω-CTX + NiCl2
n = 4
0.4
0.9
1.4
1.9
2.4
2.9
Intraluminal Pressure (mmHg)

C


Volume expelled (ml) (per 60 seconds)
Volume expelled (ml)
12
10
8
6
4
2
0
Control
ω-CTX
ω-CTX + NiCl2
n = 4
0.4
0.9
1.4
1.9
2.4
2.9
Intraluminal Pressure (mmHg)

CHAPTER 7 GENERAL DISCUSSION AND CONCLUSIONS

Gastrointestinal motility disorders pose a severe reduction on the quality of life for affected individuals. Despite a plethora of studies aimed at understanding the pathophysiology of GI disorders, there are still no cures. The reason probably has to do not only with the complexities of the disorders themselves, but also with the overall impact on the ENS. For example, in patients with IBS and Crohn's disease, the ENS is a specific target of inflammatory cells including lymphocytes and mast cells that alter the enteric neural circuitry. After tissue inflammation, signals generated by inflammatory cells induce changes in extrinsic sensory nerve endings, IPANs, interneurons, and motorneuronal signaling to the smooth muscle (Kirkup *et al.*, 2001). The effect of impaired or altered synaptic communication means that the ENS has to attempt to overcome inflammatory insults by deconvoluting new sensory signals and combining them within the enteric circuitry to produce an adaptive response. However, in IBD and Crohn's disease patients, drastic therapeutic strategies such as corticosteroid administration, or as a last resort, surgical intervention, are used to help patients with moderate to severe forms of those diseases. A reason for the lack of cures for GI disorders also probably has to do with the high sensitivity and semi-autonomous nature of the intrinsic reflex circuitry of the ENS. By high sensitivity I mean, for example, that even a minor alteration in the signaling pathway of the ascending excitatory pathway may have overall consequences on muscle contraction. Similarly, dysregulation of the descending inhibitory pathway would produce an altered result on muscle relaxation. IPANs are connected in feed-forward circuits, are the first neurons activated by distention or mucosal

stimulation, and act to gate excitation in the myenteric and submucosal circuitry. The integrity of the nerve endings of IPANs is therefore important for continual gut function. Furthermore, IPANs, interneurons and motorneurons are connected at multiple points along the GI tract, implying that a response to a given stimulus can occur at any given point in the gut wall. The semi-autonomous nature of the GI tract is advantageous and pragmatic in terms of having its own "little brain", in that transmission over long distances (from the CNS to the ENS) and the packaging of 100 million enteric neurons into the CNS has been avoided through evolution. However, this may also be to the detriment of the ENS. That is, because of the paucity of connections between the CNS and the ENS, the ENS is only able to respond to dysregulatory mechanisms mostly by itself. Therefore, when the GI reflex circuitry is affected by an inflammatory "soup", the outcome is severe and can be debilitating. Psycho-social factors are also believed to be involved in GI motility disorders, but conclusive evidence has yet to be presented. The search for therapeutic strategies to combat motility disorders, although challenging, is ongoing, and the studies presented in my dissertation contribute to that search. Because of the immense complexity that GI disorders present, I believe that a good strategy to target ENS dysfunction is to study the mechanisms of the ENS reflex circuitry. After all, that is the region where many GI disorders and diseases produce their overall effects.

Calcium channels are the critical conduits of synaptic transmission. They transduce electrical signals into chemical ones, and therefore regulate communication between excitable cells. They are also involved in contraction of

smooth and skeletal muscle, secretion, and gene expression. Calcium channels have also been established as key components in the ENS. Seminal experiments by Bian and Galligan (2004) demonstrated that at least 50% of the calcium current in myenteric neurons maintained in primary culture is R-type calcium channel-mediated. In light of that evidence, my dissertation addressed the functional importance of the R-type channel in the ENS. The guinea pig has been an invaluable and major contributor in furthering our understanding of ENS function. Other species, however, such as rats and mice have also been used to study the ENS. Much of our present understanding of the ENS is based on structural, morphological, neurochemical and physiological studies done in guinea pigs. The ileum is also the most studied and best characterized GI region. Study of local ENS control can also best be studied in this region because ileal myenteric neurons are sparsely innervated by vagal (parasympathetic) preganglionic cholinergic neurons and sympathetic postganglionic noradrenergic neurons located in celiac ganglia (Goyal & Hirano, 1996). CNS contribution to motility control in the ileum is therefore limited, implying that motility is predominantly locally regulated. The guinea pig was therefore my model system of choice for these studies.

A major finding in my study was that α1E-ir is not present in the submucosal plexus, and therefore does not contribute to absorptive or secretory functions in the guinea pig GI tract. However, extensive α1E-ir in varicose nerve fibers is present in the myenteric plexus in all regions of the GI tract. The

predominance of R-type calcium currents in myenteric neurons suggests an important role for this class of calcium channels in enteric neurotransmission and motility. Interestingly, there was no expression of α1E within cell bodies in the guinea pig small intestine. Cell bodies of myenteric neurons in the stomach and colon, however, do express R-type channel immunoreactivity, with the colon demonstrating a greater expression of α1E-ir. Overall, α1E staining in the colon was greater compared to the stomach and small intestine. The reasons for the intense α1E labeling in the colon LMMP could be related to the function of the colon. The colon absorbs water and electrolytes from the intestinal content and converts it into fecal matter. Furthermore, migrating myoelectric complex (MMC) patterns are absent in the colon, but slow waves are present. The MMC is an electromechanical pattern that occurs in the fasting state. It is responsible for the propulsion of residual undigested food and sloughed enterocytes from the stomach through the small intestine. It therefore serves a "housekeeping" function. The musculature in the proximal colon exhibits two basic motor patterns: segmental (haustral) contractions, and neurogenically-mediated mass peristalsis, while the distal colon only displays the former (Furness, 2006a). The function of the R-type channel, present on cell bodies and on nerve terminals, could therefore be to augment peristalsis in the colon. This could occur by mediating calcium entry during somatodendritic action potentials, or neurotransmitter release. I did not find α1E-calbindin-ir colocalization in the colon LMMP. However, I did observe strong co-localization of α1E and SP, α1E and calretinin (nerve fibers) and α1E with NOS and VIP. This suggested that α1E

was present in: 1) excitatory and inhibitory circular and longitudinal muscle motorneurons, 2) ascending interneurons, and, 3) IPANs. The prevailing dogma is that calbindin is the best known marker for myenteric IPANs. However, according to Brookes and Costa (2006), at least two types of myenteric IPANs exist: 1) an IPAN majority that respond to chemical stimulation of the mucosa; and 2) an IPAN minority that are sensitive to tension along the gut wall (Kunze *et al.*, 1998; Kunze *et al.*, 2000). In fact, a third subset of IPANs may also exist, those that are sensitive to both tension and chemical stimuli. Those data suggest that calbindin may not necessarily fit the gold-standard for confirming IPANs, and my data support this theory. SP is a marker for both IPANs and excitatory longitudinal muscle neurons. SP may therefore be present in IPAN populations that are calbindin-negative. The SP antibody I had used never labeled cell bodies, while calbindin never labeled nerve terminals. However, because SP and α1E co-localize in the myenteric LMMP, the R-type channel may therefore be present in nerve terminals of colonic IPANs that are calbindin-negative. They may also be present on nerve endings of excitatory motorneurons. There are two ways to answer these possibilities. One is to record action potentials and sEPSPs from AH neurons in LMMP preparations in the colon, in the presence of $NiCl_2$ (50 μM) or SNX-482 (0.1 μM). The second method would be to perform neuron cell culture to determine whether α1E is expressed in colonic myenteric neurons. R-type channel staining in the small intestine deviated from the stomach and the colon. The abundance of axonal varicosities in the intestinal

whole mount LMMP suggests that the function of the R-type channel may be more towards that of synaptic transmission.

Intracellular recordings of action potentials from single AH showed that $NiCl_2$ did not reduce the amplitude of the AH action potential, but did inhibit the half-width by almost 50 %. Addition of ω-CTX then completely blocked the calcium shoulder. The late AHP was also significantly reduced in the presence of $NiCl_2$. Rugiero and others (2002) showed a major contribution of N-type channels to the action potential, and also implicated the R-type channel as a contributor to the total inward calcium current. In my study, I have shown the N- and R-type calcium channels contribute equally to the somatodendritic action potential in AH neurons. $NiCl_2$ had no effect of on action potentials in S-type neurons. The effect of $NiCl_2$ on fEPSPs was interesting. I initially observed that some fEPSPs were significantly inhibited by $NiCl_2$, whereas others were completely unaffected. There is a single group of ascending interneurons but 3 groups of descending interneurons in the guinea pig ileum (Brookes, 2001). The ascending interneurons comprise just 5 % of the total number of myenteric neurons in the myenteric plexus and are immunoreactive for ChAT and calretinin. Those neurons use ACh as their neurotransmitter. It should be noted that some excitatory longitudinal muscle motorneurons (25 %) are also calretinin-ir. In the guinea pig LMMP, the axons of excitatory longitudinal muscle motorneurons are confined to the tertiary plexus (Brookes & Costa, 2006), are short, and project in this plexus close to their cell bodies (Lomax *et al.*, 1999; Furness, 2000; Brookes & Costa, 2006). In the LMMP preparation, there is therefore a greater chance of

recording from interneurons (ascending or descending) rather than longitudinal (excitatory or inhibitory) muscle motorneurons as I was stimulating interganglionic fiber tracts. Furthermore, motorneurons do not synapse with other enteric neurons (LePard & Galligan, 1999). Together, descending interneurons account for 11% of the total number of myenteric neurons in the guinea pig LMMP and use either ACh and ATP, or ACh and 5-HT for neurotransmission (LePard *et al.*, 1997; LePard & Galligan, 1999; Galligan, 2002b). A third group of descending interneurons is associated with the MMC and has Dogiel type III (filamentous) morphology. The majority of the interneurons (67%) belongs to the mixed cholinergic/purinergic group, while 25% are completely purinergic (Galligan, 2002b). Components of the fEPSP can therefore be isolated using mecamylamine (nAChR antagonist), PPADS (P2X receptor antagonist) or ondansetron (5-HT_3-receptor antagonist). My data shows that $NiCl_2$ and SNX-482 selectively act at the nerve endings of purely cholinergic nerve fibers to inhibit R-type calcium channels as the fEPSP amplitude was reduced. Neurons that use both ACh and ATP for fast transmission were unaffected by $NiCl_2$ and SNX-482 treatment. This result means that R-type calcium channels are localized to calretinin interneurons, and this is supported by my findings that α1E and calretinin co-localize in some varicosities in the LMMP. The paucity of co-localization between α1E and calretinin supports this conclusion as the R-type channel is present in just 5 % of purely cholinergic ascending interneurons. In both subtypes (cholinergic/purinergic), ω-CTX largely inhibited the fEPSP following R-channel blockade. Therefore, mainly R- and N-type channels

contribute to fast synaptic in purely cholinergic S-type interneurons, whereas only N-type calcium mostly mediate fast transmission neurons at mixed cholinergic/purinergic nerve endings. The ideal way to determine whether $NiCl_2$ is acting pre-or postsynaptically to inhibit calcium entry through R-type calcium channels would be to "puff" onto impaled neurons an agonist of the mediator of the sEPSP or fEPSP in the presence of $NiCl_2$. Previous data (DeVries M, unpublished) showed that $NiCl_2$ acts presynapticallly to inhibit synaptic trasmission. My present data further indicate that $CdCl_2$ and $NiCl_2$ do act presynaptically. This conclusion is based on: 1) $CdCl_2$ did not affect the neuronal membrane potential; 2) $NiCl_2$ did not affect the sEPSP; 3) $NiCl_2$ acts at nerve terminals in fully cholinergic S-type ascending interneurons; 4) $NiCl_2$ does not affect fEPSPs in descending interneurons. Taken together, these data rule out a general non-specific effect on the postsynaptic cell. Perhaps the reason why the R-type channel is present on nerve endings of purely cholinergic ascending interneurons is based on redundancy. That is, the ENS has included a mechanism using two calcium channels that act as fail-safes in instances of, for example, a toxin-attack that acts to block chemical neurotransmission. Having two calcium channels at a single synapse would therefore help the ENS circumvent noxious insults. Because the ENS has a single population of S-type interneurons, the R-type channel may consequently be very important in this regard. ACh is an important enteric neurotransmitter, and the importance of purely cholinergic interneurons may be to spread excitation to other cell types,

including excitatory motorneurons, to allow the coordination of motor responses over long distances in the GI tract.

R-type calcium channels also mediate inhibitory neurotransmission to the muscle layers, suggesting a functional coupling between the R-type calcium channel and NOS synthesis and NO release (Figure. 7.1). The R-channel does not appear to be linked to ATP release from nerve terminals. It is possible that the R-type channel, localized to myenteric nerve endings is coupled to NO synthesis and release, physically or via an intracellular signaling mechanism, to cause relaxation of GI smooth muscle through an ATP (and possibly β-NAD)-insensitive mechanism. This mechanism would be as follows: intracellular nNOSα would be anchored at the nerve terminal membrane, and activated by the influx of calcium. NO would be synthesized by intracellular NOS, and NO would then passively diffuse to affect the postsynaptic cell. These data therefore have important implications for the spatial organization of calcium-channel-mediated exocytosis.

The effect of $NiCl_2$ on R-type calcium channel function in the whole ileum is important for the understanding of possible therapeutic strategies against GI motility disorders, particularly in light of ion channels as potential drug targets (Lipecka *et al.*, 2002; Cuppoletti *et al.*, 2004). For example, the peptide Ziconotide is a synthetic analogue of ω-CTX used to treat chronic pain (Miljanich, 2004). Similarly, the spider toxin peptide inhibitor guangxitoxin-1 targets the delayed rectifying potassium channel Kv2.1 and enhances glucose-dependent

insulin secretion. This has important implications for the treatment of type II diabetes (Herrington *et al.*, 2006; Kaczorowski *et al.*, 2008). However, the challenges in translating ion-channel drugs from bench to bedside is difficult due to, amongst others, species differences, and just how efficacious such drugs would be in humans (Kaczorowski *et al.*, 2008).

$NiCl_2$ by itself impairs the peristalsis reflex. Peristalsis can still occur, but mixing and churning movements of the intestine were observed 30 seconds after fluid-induced peristalsis. Where exactly is $NiCl_2$ acting? $NiCl_2$: 1) reduces the calcium shoulder and the late AHP amplitude of the AH action potential; 2) acts at nerve terminals of purely cholinergic interneurons; 3) reduces inhibitory neurotransmission by coupling to NO to the muscle layers; 4) may also act at other unknown sites. By disrupting synaptic communication in a number of pathways, such as the ascending excitatory pathway, and at inhibitory neuromuscular junctions, the function of the reflex circuitry is compromised. N- and P/Q-type calcium channels appear to be localized near R-type channels, such that calcium channel co-operativity between those major calcium channel subtypes may be important for facilitation of enteric reflexes (Pietrobon, 2002, 2005). My data indicate the presence of R-type calcium channels within specific populations of myenteric neurons. R-type channels may therefore have an important function as a relay point for information signaling within those neurons. Impairing its function alters the peristaltic reflex.

A schematic of a potential coupling between voltage-gated R-type calcium channels (VGCC) and NOS on myenteric inhibitory motor nerve

terminals is shown in Figure. 7.2. In Figure. 7.2A, under drug-free conditions, fluid-distention induces a propagative peristaltic reflex. At an inhibitory neuroeffector synapse, NOS is anchored within the active zone to the plasma membrane. This synapse is also under inhibitory control by sympathetic nerves that release norepinephrine (NE). NE, acting at presynaptic G_i/G_o-coupled α_2-adrenoreceptors (α_2AR) will inhibit release of neurotransmitters from the myenteric inhibitory motor nerve fiber. If the α_2AR and R-type channel are coupled perhaps by diffusible second messengers, then the probability of the R-channel being open will be reduced, and calcium influx will be blocked. If the R-channel and NOS are coupled, then NO cannot be released if the R-channel is under the control of the α_2AR. If the R-channel and NOS are not coupled, then NO can be released via calcium entry through another calcium channel, possibly the N-type calcium channel. NO can then diffuse across the neuroeffector junction into the smooth muscle cell, bind to guanylate cyclase, to cause muscle relaxation.

When NLA is added first (Figure. 7.2B), and distention evoked, the frequency of contractions increases. NLA inhibits NOS leading to increased smooth muscle contraction. NE will again act at the α_2AR and because it is coupled to the R-channel, calcium entry through this channel will be then blocked by α_2AR activation. If the R-channel and NOS are coupled, then in the presence of NLA alone, NOS cannot be under the control of the R-channel, because the channel is still inhibited by the α_2AR. NLA is now free to exert its inhibitory actions on NOS, leading to increased contractions.

When $NiCl_2$ is added in the presence of NLA (Figure. 7.2C), and peristalsis induced, the frequency of contractions decreased. NE is still released from the sympathetic nerve terminal and activates the α_2AR. The α_2AR, however, cannot inhibit the R-type channel because $NiCl_2$ (50 μM) removes this inhibition (Bian & Galligan, 2007). Calcium entry therefore cannot occur. Because the contraction frequency is reduced (by NLA and $NiCl_2$), this suggests that NLA cannot be inhibiting NOS. Speculatively, this may be because of an intracellular coupling pathway or a physical interaction between the R-type channel and NOS. Reduced calcium entry via the R-type channel would lead to less calmodulin binding, resulting in reduced NOS activation. NO therefore cannot be synthesized via the R-channel at this synapse. The inhibitory response must be due to release of another neurotransmitter via another calcium channel. Apamin completely inhibits the peristaltic reflex, and my data also show that the R- and N-type channels act in tandem to block the reflex. Therefore, N-type (or even P/Q-) channel-mediated ATP neurotransmission must account for the low frequency of the propagating peristaltic reflex. This would also help explain the sustained inhibition in the reverse experiment when $NiCl_2$ was added first, followed by $NiCl_2$ and NLA.

Tissue damage associated with inflammation, a cellular response to injury, evokes the production of a myriad of chemical mediators from specific cell types such as mast cells and lymphocytes at the site of injury (Levine & Reichling, 1999). The initial events in tissue inflammation, provoked by chemical, thermal,

or mechanical stimuli, involve the synthesis and release of such pro-inflammatory mediators that comprise an "inflammatory soup". These compounds include, amongst others, protons, histamine, peptides (bradykinin, BK), lipids (prostaglandins), neurotransmitters (ATP and serotonin) and nerve growth factor (NGF) (Samad *et al.*, 2002). Specifically, these mediators, via the activation of intracellular signal transduction pathways alter the sensitivity of voltage-gated ion channels to produce peripheral sensitization. Sensitization may also be triggered by inflammatory mediators from nociceptive neurons themselves (neurogenic inflammation) (Julius & Basbaum, 2001). Chronic inflammatory GI diseases include ulcerative colitis and Crohn's disease and can be debilitating to affected patients. In Crohn's disease, inflammatory mediators produce long-term changes that affect the mucosal and submucosal layers, and also neuromuscular transmission. In ulcerative colitis, the mucosal and submucosal are affected. Both those conditions produce rapid intestinal transit accompanied by abdominal cramping (Koch et al., 1988). Animals models using the nematode worm *T. spiralis* or the chemical trinitro benzene sulfonate have successfully reproduced inflammation in the GI tract. Inflammation depolarizes the resting membrane potentials of GI smooth muscle cells and increases their excitability. The excitability of enteric neurons is also increased with increased input resistance (Linden et al., 2003). Under conditions of inflammation, calcium influx through L-type calcium channels is suppressed, which in turn inhibits the calcium-calmodulin-dependent phosphorylation of the myosin laight chains, thus preventing contraction. The function of L-type calcium channels is also affected

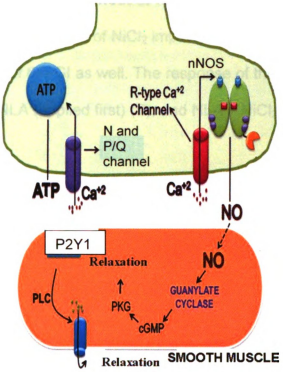
by nuclear factor κB, a ubiquitous transcription factor responsible for immune and inflammatory reactions. Interestingly, the release of calcium from intracellular calcium stores are unaffected (Shi and Sarna, 2000; Shi and Sarna, 2006). According to Shi and Sarna (2006), both neurotransmitter storage in, and release from enteric nerve terminals, are altered. Taken together, those results indicate that voltage-gated calcium channels are affected during inflammation, and that it is therefore possible to target them pharmacologically as viable control points. In my study, the R-type channel is clearly involved in intestinal motility, and it would be useful to study the function of those channels in the inflamed intestine. Inflammation increases GI transit, and I would therefore expect $NiCl_2$ to inhibit the peristaltic response. The studies reported in this dissertation show that R-type channels are integral components of the ENS. The design of therapeutic strategies using $NiCl_2$ is challenging due to its toxic nature. However, success has been achieved using toxins that block N-type calcium channels. Perhaps using SNX-482 may represent a feasible way of targeting these channels. Overall, the motility of the ileum in the presence of $NiCl_2$ is impaired. This suggests that R-type channel antagonists may be useful as anti-diarrheal drugs, so that the lives of patients who suffer with GI diseases and disorders can be improved.

Figure. 7.1. Proposed Model for Inhibitory Neuromuscular transmission.

(A1) R-type calcium channels may be present on the same nerve terminals as N- and P/Q-type calcium channels. R-channels couple to NO release, whereas N- and P/Q-type channels couple to ATP and/or β-NAD release. Both mechanisms produce relaxation of the smooth muscle.

(A2) R-type calcium channels may be present on different nerve terminals compared to N- and P/Q-type calcium channels.

A1.


nNOS
ATP
R-type Ca+2 Channel
N and P/Q channel
ATP
Ca+2
Ca+2
NO
P2Y1
Relaxation
NO
PLC
PKG
GUANYLATE CYCLASE
cGMP
Relaxation
SMOOTH MUSCLE

A2.

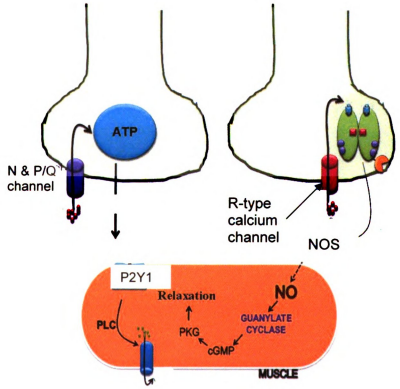
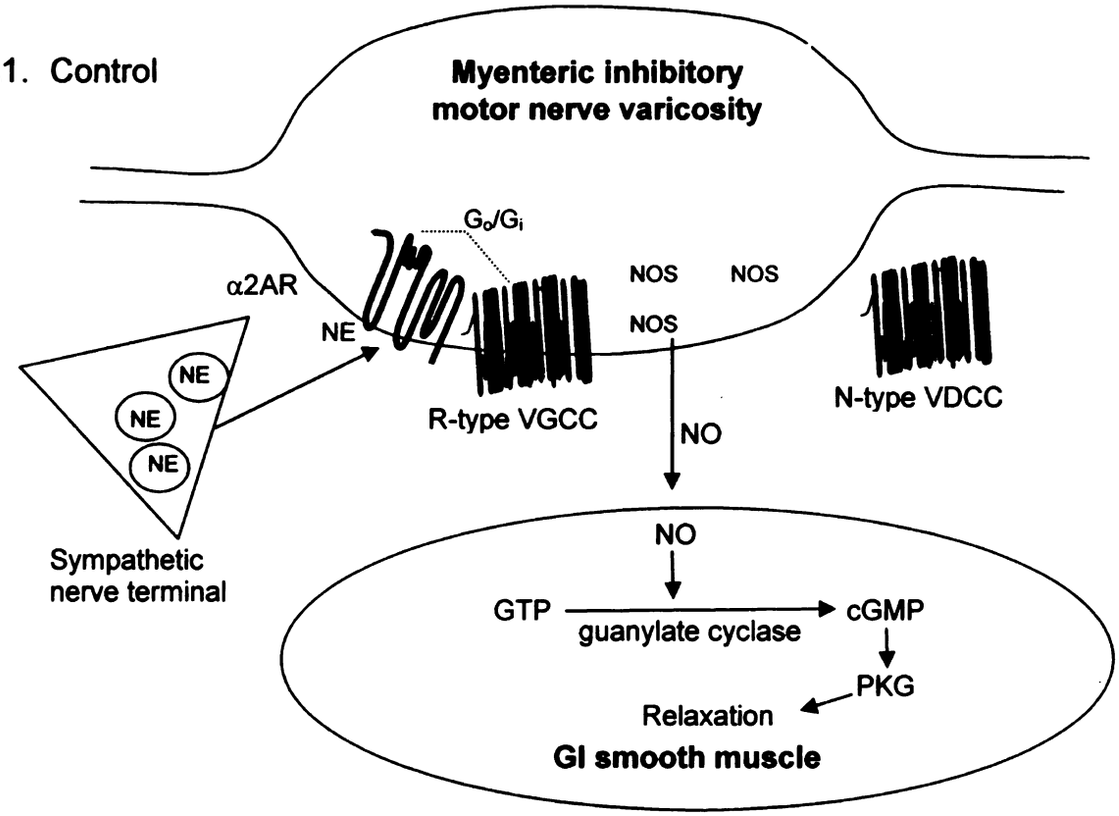

ATP
N & P/Q channel
R-type calcium channel
NOS
P2Y1
Relaxation
NO
PLC
PKG
GUANYLATE CYCLASE
cGMP
MUSCLE

Figure. 7.2. Schematic of the overall effect of $NiCl_2$ on the peristaltic reflex. Fluid induced distention in the presence of $NiCl_2$ impairs peristalsis but its effects may extend to other regions of the GI as well. The response of the ileum under control conditions (A) after NLA (applied first) (B), and NLA + $NiCl_2$ (C) treatments are shown.

A1. Control



A2. NLA first

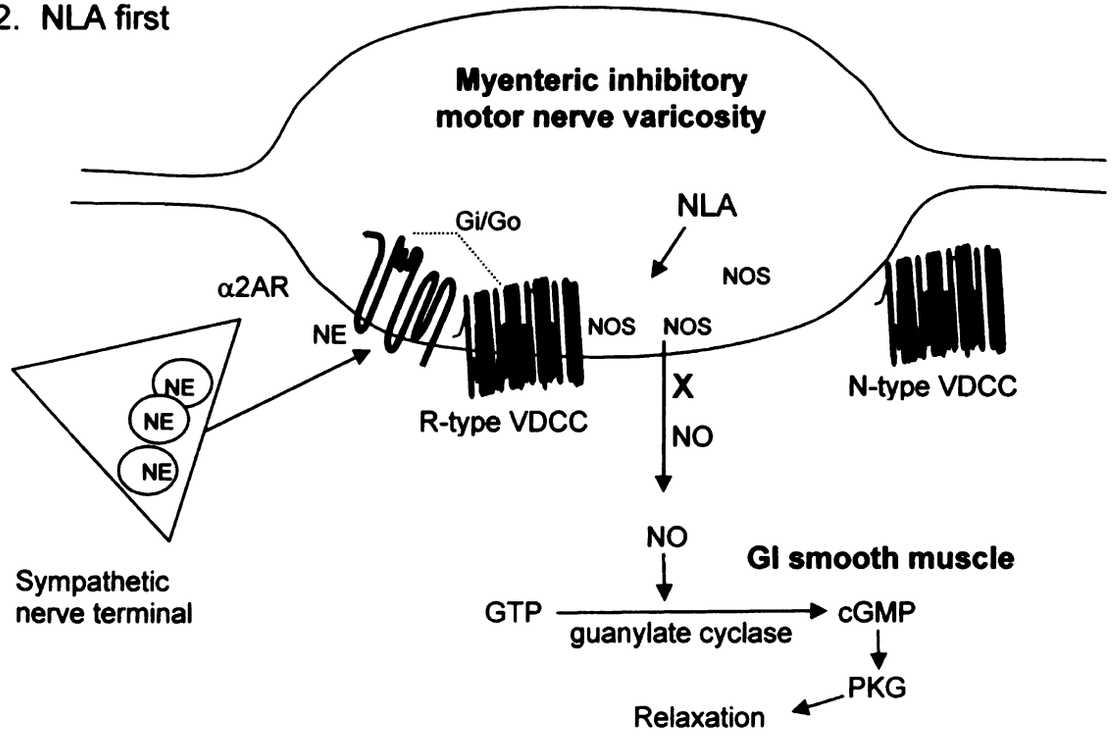
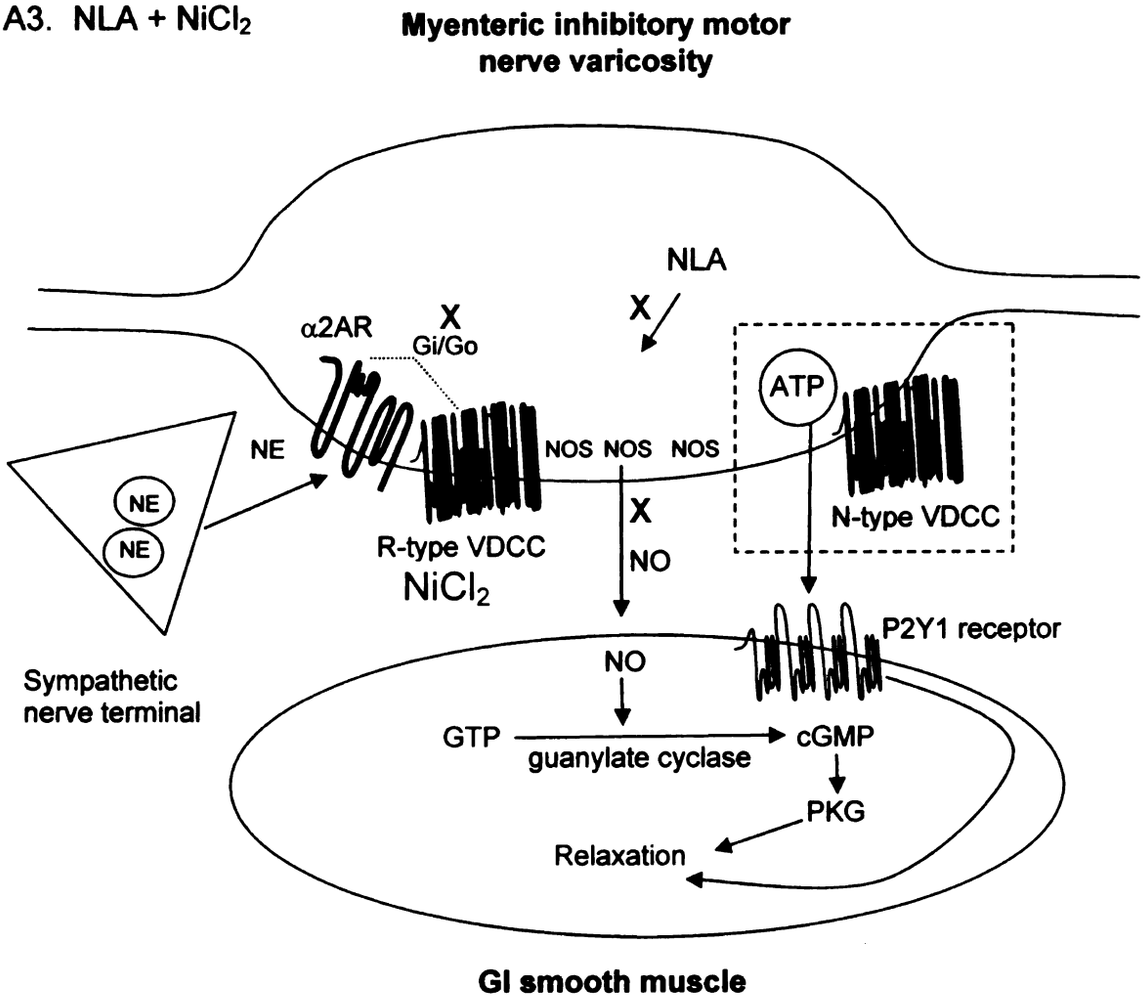


Figure. 7.2 cont'd

A3. NLA + $NiCl_2$



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