VOLTAGE-GATED Ca^{2+} CHANNEL PLASTICITY AND CONTROL OF NEUROMUSCULAR TRANSMISSION BY THE ENTERIC NERVOUS SYSTEM

By

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ABSTRACT

VOLTAGE-GATED Ca^{2+} CHANNEL PLASTICITY AND CONTROL OF NEUROMUSCULAR TRANSMISSION BY THE ENTERIC NERVOUS SYSTEM

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Functional gastrointestinal disorders (GI) disorders are characterized by alterations in the function of the GI tract that occur without clear evidence for structural or biochemical abnormalities. These disorders comprise about 41% of the total GI complications in the United States and altered motility of the GI muscles is a hallmark characteristic. The enteric nervous system (ENS) controls motility of GI muscles through an intrinsic neural circuit. Voltage-gated Ca^{2+} channels (VGCC) regulate neurotransmitter release and as such they could play a critical role in modulation of intestinal motor patterns. However, the contribution of different VGCC subtypes within the motility circuit is not very well understood. Therefore, the overall goal of these studies was to provide detail insight into the physiological role and significance of P/Q- and R-type VGCC in enteric neuromuscular transmission and investigate potential homeostatic synaptic changes in response to deficits in these VGCC subtypes. Three animal models were used to study the role of VGCC subtypes in controlling intestinal motility. Chapter 3 uses the longitudinal muscle of the guinea pig small intestine to investigate the contribution of R- and N-type VGCC in nerve-evoked contractions and relaxations. Chapter 4 uses the α1E knockout (KO) mouse, which has a genetic deletion of the gene encoding for the α1 subunit forming the pore of R-type VGCC. Here the physiological relevance of R-type channels is investigated in the colon of α1E KO mice. Chapter 5 uses the mouse called Tottering (TG), which has a spontaneous missense loss-of-function mutation in the α1A subunit of P/Q-type VGCC. Mechanical responses of the muscle and intracellular recordings of junction potentials were conducted to investigate the contribution of R- and P/Q-type VGCC to neuromuscular transmission. The studies discussed in this dissertation revealed that R- and P/Q-type

VGCC contribute to neuromuscular transmission within the ENS. (1) R-type VGCC contribute to inhibitory neuromuscular transmission of the longitudinal muscle in the guinea pig small intestine. Specifically, activation of these channels is coupled to the prominent nitrergic component of longitudinal muscle relaxation. (2) Absence of the α1E subunit of the R-type VGCC did not alter colonic motility patterns. A homeostatic plastic change was identified that explained maintenance of colonic motility in face of total absence of the α1E subunit. The nature of this change was an up-regulation in the contribution of L-type VGCC during generation of purinergic component of the inhibitory junction potentials (IJPs). The nitrergic component of the IJP was still decreased in α1E KO mice and the resting membrane potential of circular muscle cells, which is regulated by ongoing release of nitric oxide, was significantly depolarized. (3) Loss-of-function of P/Q-type VGCC only produced subtle alterations in colonic propulsive motility *in vivo* and *in vitro*. Intracellular recordings of IJPs revealed that these electrical events were either enhanced or at normal levels in TG mice. Here, an up-regulation in the contribution of L-type channels during generation of the IJPs was also identified in TG mice. Contribution of L-type channels to the IJPs could serve to produce the observed normal nerve-evoked relaxation of circular muscle rings and colonic propulsive motility. Taking together these studies provide evidence for the contribution of both R- and P/Q-type VGCCs to enteric neuromuscular transmission and for the ability of the ENS to adapt to challenges that disrupt the function of VGCC subtypes. L-type channels serve as a "back-up plan" to sustain neuromuscular transmission in-face of alterations to other VGCC subtypes. This plasticity of L-type channels maintains physiologically appropriate colonic function and overcome alterations to components of the enteric neuromuscular junction.

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KEY TO ABBREVIATIONS

ω-Nitro-L- arginine	NLA
ω-Conotoxin-GVIA	CTX-GVIA
ω-Agatoxin-IVA	ATX-IVA
Small conductance Ca^{2+}-activated K^{+} channels	SK_{Ca}
Fast inhibitory junction potential	fIJP
Slow inhibitory junction potential	sIJP
Neuronal nitric oxide synthase	nNOS
Resting membrane potential	RMP
Area under the curve	AUC
Wildtype	WT
Alpha1E knockout	α1E KO
Interstitial cells of Cajal	ICC
Interstitial cells of Cajal-Myenteric plexus	ICC-My
Interstitial cells of Cajal-submucosal plexus	ICC-SM
Voltage-gated Ca^{2+} channels	VGCC
Complementary DNA	cDNA
High voltage-activated	HVA
Low voltage-activated	LVA
Dihydropyridines	DHP
Calmodulin	CaM
Calmoduline binding domain	CBD
Calcium binding protein-1	CaBP-1
Visinin-like protein-2	VILIP-2
Tottering	TG

Lambert-Eaton Myasthenic Syndrome	LEMS
Episodic ataxia type 2	EA-2
bp	base pairs
Familial hemiplegic migraine	FHM
Spinocerebellar ataxia type 6	SCA-6
Non adrenergic non cholinergic	NANC
Longitudinal muscle myenteric plexus	LMMP
Electrical field stimulation	EFS
Gastrointestinal	GI
Enteric nervous system	ENS
Lower esophageal sphincter	LOS
Nitric oxide	NO
Vasoactive intestinal peptide	VIP
Adenosine triphosphate	ATP
Pituitary adenylate cyclase-activating polypeptide	PACAP
Nitric oxide synthase	NOS
Chronic intestinal pseudo obstruction	CIP
Ribonucleic acid	RNA
Anti-neuronal nuclear antibody	ANNA
5-Hydroxytrypyophan	5-HT
Calcium	Ca^{2+}
Tertiary plexus	tp
After-hyperpolarizing	AH
After-hyperpolarizing potential	AHP
Synaptic	S
Excitatory post-synaptic potential	EPSP
Tetrodotoxin	TTX

Alpha1	α1
Tetraethylammonium	TEA
After-depolarizing potential	ADP
Intermediate conductance Ca^{2+} activated K^{+} channels	IK
Motor neurons	MN
Interneurons	IN
Intrinsic primary afferent neurons	IPAN
Extrinsic primary afferent neurons	EPAN
Acetylcholine	ACh
Substance P	SP
Myoelectric migrating motor complex	MMC
Colonic migrating motor complex	CMMC
High amplitude propagating contractions	HAPC
Enterochromaffin cells	EC
Phospholipase C	PLC
Inositol trisphosphate	IP_3
Postsynaptic density protein-95	PSD-95
Cyclic guanosine monophosphate	cGMP
Protein kinase G	PKG
Beta-Nicotinamide adenine dinucleotide	β-NAD

CHAPTER 1: GENERAL INTRODUCTION

GASTROINTESTINAL DISORDERS

General description

Gastrointestinal (GI) disorders occur as a result of alterations in the structure or biochemical environment of the digestive system resulting in inflammation, infection, or degenerative disorders. Functional GI disorders occur when no evident structural or biochemical alteration can be associated with the presented GI symptoms. Either situation will cause neuronal dysfunction that results in altered motility, abnormal visceral perception, and secretion. Functional GI disorders comprise about 41% of the total GI problems and although not life-threatening they negatively impact patient's quality of life and impose a significant economic burden to society and the health care system (Di Giorgio et al. 2007; Gershon et al. 1994). Consequently, these disorders are acquiring great attention from both the clinical and research fields in order to understand the pathophysiology and identify viable targets for their successful treatments.

Altered motility, also known as dysmotility, is present in the majority of functional GI disorders. Motility and other functions of the digestive system are regulated by a complex neural network known as the enteric nervous system (ENS)-, which extends from the esophagus to the internal anal sphincter. The structure and function of the ENS will be described later in this chapter. Digestive neuropathies are caused by degeneration, loss, and functional impairment of enteric neural networks. These alterations are associated with uncoordinated motor activity that results in altered transit of intestinal content providing direct evidence for the critical role of this neuronal network in controlling GI motor activity (Goyal and Hirano, 1996 1996; Di Giorgio and Camilleri, 2004). The most common GI motor disorders will be discussed starting from the esophagus and ending with the large intestine.

Achalasia

Achalasia is a disorder of the esophagus characterized by a lack or absence of peristalsis, which refers to a wave-like pattern of contractions that move food along the GI tract. In addition, the lower esophageal sphincter (LOS) of patients with achalasia fails to relax preventing the passage of esophageal luminal content to the proximal stomach. Patients with achalasia complain of swallowing difficulty (dysphagia) and regurgitation. A prominent reduction in inhibitory neurons (containing nitric oxide, vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating polypeptide and adenosine triphosphate) has been identified in tissues analysis from these patients (Goyal and Hirano, 1996; Gershon et al. 1994; Di Giorgio et al. 2004). Inhibitory innervation is essential for muscle relaxation and excitatory innervation supports muscle contraction. Thus, the inhibitory denervation along with an apparently intact excitatory component is most likely a pathological mechanism underlying the motor dysfunction in achalasia (Di Giorgio et al. 1999, 2004). Achalasia can be primary, with familial and sporadic forms, and secondary to a vast number of diseases. The proposed etiological factors for the defects identified in inhibitory innervation include immune-mediated damage (including anti-neuronal antibodies), neurotropic viruses, and genetic factors leading to complete loss of myenteric ganglia (Di Nardo et al. 2008).

Current drugs used to treat the symptoms in achalasia include nitrates to induce relaxation of the LOS, phosphodiesterase-5 inhibitors to block the action of excitatory neurotransmitters, and Ca^{2+} channel blockers and botulinum toxin to relax the LOS. The efficacy of these treatments is limited and short-lived with development of tolerance and unpleasant side effects occurring relatively quickly such as headaches, low blood pressure, dizziness, flushing, dyspepsia, and nasal congestion (Di Nardo et al. 2008). Botulinum toxin has also been used to treat symptoms of achalasia, however, its used is also associated with development of adverse side effects at the site of injection such as rash and hematoma (Lacy et al., 2008).

Gastroparesis

Gastroparesis can be conceived as a paralysis of the stomach producing a significant delay in the emptying of solids and liquids. The two most common forms of gastroparesis include idiopathic (primary) and that occurring as a consequence of diabetes mellitus (Horowitz and Fraser, 1995; Patrick and Epstein, 2008). Tissue analysis from a 32-year old patient with severe idiopathic gastroparesis that underwent subtotal gastrectomy revealed neuronal dysplasia with a prominent decrease in myenteric neurons and intramuscular interstitial cells of Cajal (Zarete et al. 2003). Pharmacological treatments include serotonergic/dopaminergic prokinetics and antiemetics, antibiotics known to potentiate motility of the gut such as erythromycin, and botulinum toxin to accelerate gastric emptying in order to ameliorate some of the key symptoms (Di Nardo et al. 2008). Again some of the major comments against these pharmacological treatments are the short duration of their effectiveness together with the limited clinical evidence supporting the positive actions of these drugs. In addition, development of adverse side effects has been associated with use of these drugs. For example, cisapride (serotonin 5-HT_4 receptor agonist) was for a long time an established treatment for gastroparesis; however, this treatment is now essentially withdrawn from the market due to cardiac side effects (Abrahamsson, 2007). Metoclopramide, a dopamine receptor antagonist, also has been used to treat gastroparesis but its use is associated with risk for tardive dyskinesia, a central nervous system side effect resulting in irreversible involuntary muscle movement of the face, arms, legs, trunk or hands. Furthermore, this drug also induce neuroleptic malignant syndrome, which causes blood pressure instability, fever and a stuporous-like state (The Gastroparesis and Dysmotilities Association). As a consequence their use in the clinical setting is limited.

Congenital hypertrophic pyloric stenosis

Congenital hypertrophic pyloric stenosis typically presents clinically during infancy. Tissue analysis of the pyloric innervation showed abnormalities in the enteric neurons responsible to relax the

gastric muscle, specifically a reduction in the enzyme that produces the enteric inhibitory neurotransmitter nitric oxide (Vanderwinden et al. 1992). As a consequence of the prominent reduction in inhibitory innervation, relaxation of the muscle is virtually absent producing a sustain contraction of the pylorus and hypertrophy of muscle. Supporting the role of inhibitory innervation in the pathophysiology of this condition are animals models that lack the gene for the enzyme producing nitric oxide (NO), nitric oxide synthase (NOS). This animal model is characterized by a functional gastric obstruction similar to that observed in hypertrophic pyloric stenosis (Mashimo et al. 2000).

Chronic intestinal pseudo-obstruction

Chronic intestinal pseudo-obstruction is characterized by intestinal obstruction and impaired GI propulsion in the absence of any lesion occluding the intestinal lumen (Stanghellini et al. 2007 Coulie and Camilleri, 1999; Di Lorenzo, 1999). Chronic intestinal pseudo-obstruction (CIP) can be familial or sporadic. The sporadic form can be idiopathic or secondary to various well-known pathological conditions. Dysmotility in CIP can result from dysfunction of enteric neurons (neuropathies), interstitial cells of Cajal (mesenchymopathies), or smooth muscle cells (myopathies). Disruption of enteric neurons also known as enteric neuropathy can exist in two forms: degenerative neuropathies and inflammatory neuropathies (Schuffler et al. 1983). Altered Ca^{2+} signaling, mitochondrial dysfunction, and production of free radicals can all lead to degeneration of enteric neurons when no inflammation is detected (Hall and Wiley, 1998). Inflammatory neuropathies are characterized by a dense infiltrate of lymphocytes and plasma cells involving enteric neurons (ganglionitis). A marked decreased in the expression of nitric oxide synthase, which produces nitric oxide, within the enteric neuronal network has been identified in patients with severe gut dysmotility including idiopathic CIP (Accarino et al. 2007). This supports an impaired enteric inhibitory innervation as a pathological mechanism.

A significant humoural immune activation leading to generation of anti-neuronal antibodies has been identified in patients with severe intestinal dysmotility. Molecular targets of anti-neuronal antibodies include: RNA-binding protein, Hu proteins (type-1 anti-neuronal nuclear antibodies; ANNA-1), P/Q- and N-type voltage-gated Ca^{2+} channels, and ganglionic nicotinic cholinergic receptors (Di Nardo et al. 2008). Among these, anti-neuronal antibodies against Hu proteins are the most common (Lennon, 1996; Wood, 2007; Sutton and Winer, 2002, Darnell and Posner, 2006). Hu proteins are important players in cellular processes such as development and survival with disruption of these proteins promoting enteric neurodegenerative processes leading to intestinal dysmotility (Okano and Darnell, 1997; Wakamatsu and Weston, 1997; Lin et al. 2002).

One of the main problems on CIP and other intestinal dysmotility disorders is malnutrition caused by malabsorption and/or inadequate food intake. Pharmacological treatments to ameliorate the symptoms include the use of agents that potentiate motility of the gut in a general way (prokinetics) such as erythromycin and motilides; immunosuppressant agents have also been utilized in some instances. However, similar to the other GI motility disorders discussed above these treatments are not very effective and there is a risk of major adverse effects associated with the use of this drugs (Di Nardo et al. 2008).

Slow transit constipation

Constipation is a common gastrointestinal complaint causing physical and psychosocial problems. It has been categorized as slow transit constipation, normal transit constipation, and obstructed defecation. One of the most widely accepted definitions is two or fewer bowel movement per week or straining at stool more than 25% of the time (Drossman et al. 1982; Frattine and Nogueras, 2008). The Rome criteria describe the symptoms of constipation as straining a bowel movement, lumpy/hard stools, a sensation of incomplete evacuation, a sense of anorectal blockage, less than three

bowel movements per week, and the need for manual maneuvers to assist evacuation (Drossman et al. 2000). Morphological abnormalities of colonic innervation have been identified in slow transit constipation patients using the silver staining technique (Smith, 1967; Di Nardo et al. 2008). In addition a decrease in enteric neural elements such as cell bodies and processes, a reduction in interstitial cells of Cajal and in enteroglia cells appear to be consistent features of this condition (Porter et al. 1998; Faussone-Pellegrini, 1999; Wedel, 2002; Bassotti, 2006). It is still unclear whether alterations in enteric neurotransmitters paly a role in the pathophysiology of slow transit constipation mainly because evidence presented by multiple studies is inconsistent (Koch et al. 1988; Lincoln et al. 1990; Tzavella et al. 1996, Sjolund et al. 1997; Tomita et al. 2002). Serotonergic prokinetics, neurotrophins, and bicyclic fatty acids are some of the current pharmacological treatments for slow transit constipation. However, there is limited evidence for the clinical efficacy of these treatments and there is risk for major adverse effects especially for serotonergic prokinetics. For example, use of Tegaserod (an serotonin 5-HT_4 receptor agonist) is associated with severe diarrhea, low blood pressure, and ischemic colitis, which in severe cases require surgical removal of the bowel (The Gastroparesis and Dysmotilities Association).

In general, the focus for drug treatment of most GI motility disorders has been on compounds that have a robust increase in the kinetics of the smooth muscles. None of the current treatments attempt to modulate the enteric neuronal circuit that control intestinal motor patterns. In this line, studies aiming to comprehend the role of different components within the enteric neuronal circuit controlling motility will provide valuable insight to rationally address novel therapeutic interventions for GI motility disorders. This doctoral dissertation investigated in details the functional role and physiological relevance of one component within the enteric neuronal motility circuit: *voltage-gated calcium (Ca^{2+}) channels*.

STRUCTURE & FUNCTION OF THE ENTERIC NERVOUS SYSTEM

General Description

Digestive functions are controlled by neurons located within the central nervous system including the brain and spinal cord, prevertebral sympathetic ganglia, and within the wall of the specialized organs that make up the digestive system. The ENS is found within the walls of the entire GI tract starting from the esophagus all the way down to the anus and is also associated with the salivary glands, pancreas, and gallbladder. The enteric division together with the parasympathetic and sympathetic divisions forms what is known as the autonomic nervous system. The main function of the ENS is to integrate motility, secretions, blood flow, and immune responses in a coordinated pattern of behaviors. All this is accomplished through important neuronal networks.

Organization

The ENS is composed of nerve cells, enteric ganglia, the neural connections between these ganglia, and nerve fibers that supply effector tissues such as the smooth muscles, epithelial lining, intrinsic blood vessels, and gastroenteropancreatic endocrine cells (Furness, 2012). The ENS has approximately as many neurons as the spinal cord, which is about 400-600 million in humans (Furness, 2006). This is greater than the combined total number of neurons in parasympathetic and sympathetic ganglia.

The ENS has two ganglionated plexuses and two smooth muscle layers. The myenteric plexus is located between the longitudinal and circular muscle layers and the submucosal plexus is located between the circular muscle layer and the mucosa. Nerve fiber bundles connect the ganglia within each plexus forming the interganglionic fiber tracts. In addition, these nerve bundles form plexuses that innervate the longitudinal and circular muscle layers, muscularis mucosae, intrinsic arteries, and the mucosae (figure 1.1).

Myenteric plexus

The main function of the myenteric plexus is to control movement of the GI muscles: by generation of a coordinated pattern of contractions and relaxations. The neural network within the myenteric plexus is continuous around the circumference and along the GI tract from the esophagus to the distal part of the large intestine. The size, shape, and orientation of myenteric ganglia vary between species and between regions of the intestine. In the guinea pig small intestine, ganglia vary in size between 5 and 200 nerve somas. The ganglia are referred to as nodes of the plexuses because they lie at the junction of the interganglionic fiber tracts. The myenteric plexus has three components: primary plexus, secondary plexus, and tertiary plexus (Furness, 2006). The ganglia and the interganglionic fiber tracts comprise the primary meshwork of the myenteric plexus. The secondary meshwork is composed by fine nerve fibers within intermodal fiber tracts that do not connect adjacent ganglia but pass over a ganglion and continue in another interganglionic fiber tract. These secondary strands run parallel to the circular muscle bundles and often cross intermodal strands. The tertiary plexus is made up by fine nerve fibers that lie within the spaces formed by the primary plexus (Furness, 2000). Nerve bundles comprising the tertiary plexus identified from the ganglia, primary intermodal strands, and secondary strands and they can be found only where the longitudinal muscle is thin. Refer to figure 1.2 for a schematic representation of all three meshwork comprising the myenteric plexus.

Submucosal plexus

The main functions of the submucosal plexus are regulation of secretion, local blood flow, and absorption of nutrients. The submucosal ganglionated plexus is only found in the small and large intestines and was first described by Meissner and Billroth. As compared to the myenteric plexus the interganglionic tracts are much finer and the ganglia are much smaller (Timmermans et al. 2001). At least two layers of ganglia have been distinguished in this plexus: outer and inner layers. The inner layer

is the one closer to the intestinal lumen and the outer layer is closer to the circular muscle layer (Timmermans et al. 2001). Some of the neurons within the outer plexus supply innervation to the circular muscle and even to the longitudinal muscle (Porter et al. 1999, Timmermans et al. 1997). The majority of the nerves within the outer plexus supply innervation to the mucosa and a few neurons supply the muscles (Porter et al. 1999; Timmermans et al. 2001). In small mammals, such as the guinea pig, the submucosal plexus contains a single layer containing predominantly secretomotor neurons but not motor neurons supplying the muscle layers (Furness et al. 2003).

Innervation of the circular and longitudinal muscle layers

Innervation of the longitudinal muscle consists of the so-called longitudinal muscle plexus and the myenteric tertiary plexus (figure 1.2 A). The longitudinal muscle plexus consist of fine bundles of nerve fibers running parallel to and within the muscle and the tertiary plexus fibers lie against the inner surface of the muscle (Furness, 2006). The extent of innervation by these two plexuses to the longitudinal muscle depends on the thickness of this muscle. In large animals a longitudinal muscle plexus is clearly observed and also in small animals where this muscle is thickened such as in the teniae of the guinea pig. In animals in which the thickness of the longitudinal muscle is less than 10 muscle cells thick innervation is exclusively provided by nerve fibers coming from the tertiary component of the myenteric plexus (Llewellyn Smith et al. 1993).

The circular muscle layer contains fine nerve bundles running parallel to the length of the muscle cells and form a continuous network around the circumference and along its lengths (figure 1.2 B). In the small intestine this bundles connect with the primary and secondary components of the myenteric plexus and with the deep muscular plexus. In small mammals the nerve terminals innervating the circular muscle have their cell bodies within the myenteric plexus and some have their cell bodies within the outer layer of the submucosal plexus (Furness, 2006).

Morphology of enteric neurons

Evidence from light and electron microscopy, immunohistochemical studies, electrophysiological analysis, and retrograde tracing of neuronal projections has facilitated the identification and classification of enteric neurons. On the basis of these studies neurons within the ENS can be classified according to their morphological, neurochemical, and functional properties. Dogiel provided a comprehensive description of neuron morphologies in the myenteric and submucosal plexuses of the intestine from human, guinea pig, rabbit, rat, dog, and cat (Dogiel, 1895; 1899). He described three neuronal types, which are referred to as Dogiel type I, II, and III.

Dogiel type I

Type I neurons were described by Dogiel as flattened and slightly elongated with angular outlines (figure 1.3 A). The soma of these cells is between 13-35 μm in length and 9-22 μm in width. These cells can have 4-20 dendrites and one axon. Dendrites are flattened in the plane of the myenteric plexus extending for short distances from the cell bodies. On his descriptions, Dogiel explained that the axons of these neurons run out of the ganglia and continue through various ganglia before entering the circular muscle layer, however, not all the axons innervate this muscle layer. These suggested that Dogiel type I neurons could not be functionally grouped in a single category. In this line, Dogiel type I neurons functionally represent inhibitory motor neurons, excitatory motor neurons, and interneurons.

Dogiel type II

Dogiel type II neurons are the most prominent cell type in the myenteric and submucosal plexuses of the small and large intestine (figure 1.3 B). Specifically, within the myenteric plexus they represent 10-25% of the total neuronal population in both the small and large intestine (Furness, 2006). Dogiel described them as having 3-10 dendrites and one axon. However, later studies recognized that all the neuronal processes were axons (Hendriks et al. 1990). Some of these cells are pseudounipolar

neurons with one process extending from the soma and branching out into smaller axons. A group of these cells have somas with diameters ranging from 22-47 µm and another group with diameters ranging from 13-22 µm. By conducting dye-filling experiments Bornstein et al. (1991) identified that the long processes of these neurons within the myenteric plexus run in the circumferential direction. These long processes give rise to smaller branches that run within the same and adjacent ganglia forming synaptic connections (Pompolo and Furness 1988). Processes of Dogiel type I neurons have been identify to project to the mucosa in multiple species such as guinea pigs (Vogalis et al. 2000), pig (Hens et al. 2000), human (Hens et al. 2000), and mouse (Furness et al. 2004). Submucosal Dogiel type II neurons also have varicosities that make synaptic connections with neurons in adjacent ganglia (Furness et al. 2003). The majority of Dogiel type II neurons in the myenteric plexus of the guinea pig are immunoreactive for the Ca^{2+}-binding protein calbindin (Song et al. 1991). This has helped the identification of the chemicals expressed by these neurons as well as their synaptic connections.

Dogiel type III

Dogiel type II neurons were described to have 2-10 dendrites that branched as they extended from the cell body (figure 1.3 C). Dendritic processes are shorter than the ones described for Dogiel type II neurons ending within the ganglia of origin. Their axons emanate from a conical protrusion either from the soma or dendrites and they were identified to enter the muscle fibers.

Physiology of enteric neurons

In the 1970s intracellular recordings from enteric neurons of the guinea pig small intestine were conducted to study the passive and active electrical properties of these neurons. On the basis of these studies two main terminologies describing the electrical behavior of enteric neurons emerged: S and AH neurons. “S” stands for synaptic and these neurons exhibit large-amplitude fast excitatory post-synaptic

potentials (EPSP) when stimulated. "AH" stands for after-hyperpolarizing in recognition of the very prominent after-hyperpolarizing potential that follows action potentials in these neurons. Normally, AH neurons do not exhibit fast EPSP, however, they do show slow EPSP that can trigger action potentials (Brookes, 2001)

S neurons

S neurons are Dogiel type I in morphology. Electrically they exhibit a short action potential followed by a brief after-hyperpolarizing potential (AHP) with a duration averaging between 20-100 ms. They exhibit fast EPSP that can reach the threshold for action potential firing, however, slow EPSP have been observed in these neurons (Furness, 2006). Historically, S neurons were classified as tonically firing cells on the basis of their ability to fire action potentials in a continuous manner in response to a 50 ms intracellular depolarizing pulse (Wood et al. 1989).

AH neurons

Morphologically AH neurons are classified as Dogiel type II. The action potentials of AH neurons in the guinea pig small intestine are large, 75-110 mV in amplitude and have an inflection (hump) on the falling phase (figure 1.4). Two inward currents underlie the action potential in the soma of AH/Dogiel type II neurons: a tetrodotoxin (TTX)-sensitive Na^+ current and a TTX-insensitive Ca^{2+} current (Rugiero et al. 2003). In the presence of TTX the Ca^{2+} current is sufficient to evoke an action potential in the soma and the observed hump on the repolarizing phase of the action potential is due to this Ca^{2+} current (Kunze et al. 1994). The identity of the voltage-gated Ca^{2+} channels responsible for this hump on the AH action potentials has been investigated. L-type Ca^{2+} channels do not contribute to this Ca^{2+} inflection (Kunze et al. 1994). However, it was almost abolished by blockers of N-type Ca^{2+} channels such as conotoxin-GVIA and unaltered by blockers of P/Q-type Ca^{2+} channels such as agatoxin-IVA (Rugiero et al. 2002). This supports a critical role of N-type Ca^{2+} channels in action potential generation in AH neurons

and these neurons express immunoreactivity for the alpha 1 (α1) subunit forming N-type Ca^{2+} channels (Kirchgessner and Liur, 1999). In addition, more recent studies identified a contribution of R-type Ca^{2+} channels to generation of the Ca^{2+} inflection in these neurons and positive immunoreactivity for the α1 subunit forming R-type channels in AH neurons (Naidoo et al. 2010).

The action potentials in AH neurons are normally followed by two separate phases of hyperpolarization: an early and a late AHP (after-hyperpolarizing potential). The early AHP is continuous with the falling phase of the action potential and it lasts about 20-100 ms. Currents underlying the early AHP have been investigated. A contribution from a TEA-sensitive delayed rectifier (Zholos et al. 1999), a large conductance Ca^{2+}- and voltage-activated K^+ channels, and a 4-aminopyridine-sensitive A current have been detected (Hirst et al. 1985a). The after-depolarizing potential (ADP) follows the early AHP and it is due to a Ca^{2+}-activated depolarizing cation current (Vogalis et al. 2002a). The ADP separates the early and late AHP. The late AHP critically regulates the excitability of AH neurons. When this hyperpolarization is activated after the action potential it reduces subsequent action potentials to non-regenerative potentials. The immediate consequence of this is the inhibition of synaptic inputs to interneurons or motor neurons within a ganglion giving the late AHP a "gating role" for the control of AH synaptic outputs (Furness, 2006).

An intermediate Ca^{2+}-activated K^+ current (KCa3.1; IK; Vogalis et al. 2002b) underlies the late AHP, consequently Ca^{2+} entering the cell during the action potential (hump phase) is important for generation of the late AHP. Release of intracellular Ca^{2+} stores has been demonstrated to contribute to activation of the K^+ channels underlying the late AHP. Application of caffeine progressively increased the late AHP and both ryanodine and long-term caffeine application substantially reduced this hyperpolarizing current (Hillsley et al. 2000; Vogalis et al. 2002a). These results suggested that activation of the K^+ channels underlying the late AHP depends on Ca^{2+}-induced Ca^{2+} release from intracellular stores via a ryanodine-sensitive receptor. Vanden Bergher et al. (2002) demonstrated that

repolarization of the AHP is dependent on removal of Ca^{2+} by mitochondria because blocking the ability of the mitochondria to uptake Ca^{2+} significantly prolonged the AHP. In addition, the AHP current contributes to the resting conductance because blocking the IK with charybdotoxin depolarized the resting membrane potential of AH neurons by about 8 mV (Kunze et al. 1994). The same study observed a significant increase in the input resistance of AH neurons by blocking IK channels. This represents another piece of evidence supporting the role of the AHP currents in regulating excitability of AH neurons.

Functionally defined enteric neurons controlling muscle movement

A functional classification of enteric neurons have been achieved by combining studies investigating enteric reflexes, morphology, and targets and chemical coding of neurons regulating those reflexes.

Motor neurons

Excitatory and inhibitory motor neurons (MN) innervate the longitudinal muscle, the circular muscle, and the muscularis mucosae throughout the digestive tract. The great majority of MN have S-type electrophysiology and are classified with Dogiel type I morphology. The excitatory MN have acetylcholine and tachykinins as the main neurotransmitters and inhibitory MN have NO, ATP or a related purine, and vasoactive intestinal polypeptide (VIP) as the inhibitory neurotransmitters.

Circular muscle MN have the majority of the cell bodies in the myenteric plexus, however, a component of the circular muscle innervation has been observed to come from the submucosal plexus in rats, dog, and pig (Furness, 2006). The distance of the motor neuron projections along the intestines have been investigated using tracing and electrophysiological techniques. In general, inhibitory MN project from 8 mm to up to 30 mm anally (in relation to their cell bodies in the ganglia). Excitatory MN have shorter

projections with the great majority being less than 3 mm and a few reaching as far as 10 mm (Smith et al. 1988). The proportion of inhibitory and excitatory MN to the circular muscle in relation to the total myenteric neurons is balanced. Inhibitory MN represent about 12% and excitatory MN represent about 16%. Most MN innervate a circular muscle band that is 0.5-2 mm, however, several hundreds of MN supply a motor unit of about 2-3 mm (Furness, 2006).

Longitudinal muscle MN have their cell bodies within the myenteric plexus. However, in larger animals a small population has their cell bodies within the outer layer of the submucosal plexus. Excitatory MN to the longitudinal muscle are surprisingly abundant being about 25% of the total neurons in the myenteric plexus. The proportion of inhibitory MN is substantially smaller represented by only 2% of the myenteric neurons (Furness, 2006).

Interneurons

Interneurons (IN) form a chain of neurons within the myenteric plexus, which runs to the anal and oral directions. In the guinea pig small intestine three classes of descending IN and one type of ascending IN have been identified. The cholinergic ascending IN, which are also immunoreactive for tachykinins, are involved in local motility reflexes. Two of the three types of descending IN are also involved in regulation of the local motility reflexes; these IN are cholinergic and they are immunoreactive for NOS or 5-HT. The third type of descending IN is involved in regulation of motor patterns such as the migrating myoelectric complex in the stomach and small intestine. These IN are cholinergic and immunoreactive for somatotastin. All together, IN make about 16% of the total neuronal population within the myenteric plexus (Furness, 2006).

Intrinsic primary afferent neurons

Primary afferent neurons encode information about the state of the tissues they innervate and convey this information to integrative circuits through which the state of the organs can be modified.

The primary afferent neurons that detect the state of the intestine are the first neurons that are activated in reflex pathways in this organ. By using electrophysiological techniques and activity-dependent dyes primary afferent neurons have been shown to respond to various stimuli such as distention, luminal chemistry, and mechanical stimulation of the mucosa (Kunze et a. 1998, 1999, 2000). In this way primary afferent neurons initiate reflex pathways important for the physiological functions of the intestine including movements, blood flow, and secretions. Intrinsic primary afferent neurons (IPAN) are called *intrinsic* because their cell bodies, processes, and synaptic connections emanate and end within the enteric network. The IPAN have been identified as AH neurons with Dogiel type II morphology. The IPAN represent about 20% of neurons in the ENS and about 26% of the myenteric neurons (Furness, et al. 2004).

There are two broad classes of primary afferent neurons associated with the gut wall: the previously mentioned IPAN and extrinsic primary afferent neurons (EPAN) (figure 1.5). The EPAN have their somas in the nodose ganglion (forming the vagal afferents) and in the dorsal root ganglia (forming the spinal afferents). In addition, there are intestinofugal neurons that have their cell bodies in the gut but send projections travelling outside the ENS. With all these neurons, signals can be easily conveyed from the digestive organs to the central nervous system and trigger reflexes that act back on the gut. This communication, brain-gut axis, is important for the functional coordination between digestive organs and other body systems. Furthermore, this connection between the brain and gut relate to sensations including nausea, pain, and satiety (Furness, 2006).

Circuitry for intestinal motility

The term motility comprises various motor behaviors of the intestinal muscle including propulsive movements (peristalsis), mixing movements, and the migrating complexes of the small and large intestines. Bayliss and Starling observed the simplest reflex motor behavior related to propulsive

intestinal motility. They observed that in response to a physiological stimulus, there is a contraction of the muscle at the oral side and a relaxation of the muscle at the anal side in relation to the stimulus location. When the physiological stimulus is an artificial bolus the oral contraction and anal relaxation move the bolus along the intestine. The experiments of Langley and Maugnus demonstrated conclusive evidence for the ability of intrinsic enteric neuronal circuits to generate propulsive reflexes using a procedure known as degenerative section of nerves. These authors cut the extrinsic nerves leading to the intestine in a living animal and waited until the remaining of these nerves died; then they harvested the intestine and record propulsive reflexes *in vitro*. The results showed no difference in the propulsive reflexes between normally innervated and severed intestines (Langley and Magnus, 1905).

Figure 1.6 shows a simplified propulsive circuit within the myenteric plexus that include IPAN with varicosities in the mucosa, ascending and descending IN, and excitatory and inhibitory MN. The peristaltic reflex is initiated by either chemical or mechanical stimulation of the mucosa. This produces 5-HT release from enterochromaffin cells, which acts on 5-HT receptors on the mucosal IPAN whose cell bodies reside in the myenteric (Bertrand and Bertrand, 2010; Furness et al. 2004). In the myenteric plexus, IPAN make an excitatory synaptic connection with ascending (oral) and descending (anal) projecting IN. Ascending IN synapse with excitatory MN, which release acetylcholine (ACh) and substance P (SP) producing muscular contraction at the oral side (LePard and Galligan, 1999; Johnson et al. 1996). Descending IN synapse with inhibitory MN, which release neurotransmitters such as NO and ATP (or a related purine) producing muscular relaxation at the anal side (Grider, 2003; Gallego, 2008). The contraction on the oral side and relaxation on the anal side generates the pressure gradient required to propel content along gut.

Motor patterns in the small intestine: Myoelectric motor complex

The small intestine of mammals exhibits a motor pattern called the myoelectric migrating complex (MMC). However, the pattern of motor activity of the MMC differs between the fasted versus the fed states. Szurszewski (1969) studied the MMC and noticed that this was a recurring event from the proximal to the distal small intestine. Later studies divided the MMC motor events into four main phases: I (quiescent phase), II (irregular phase), III (rapidly occurring contraction corresponding to an MMC), IV (brief cycle of irregular activity before commencement of phase I) (Carlson et al. 1972).

Phase III of the MMC comprises rapidly occurring contractions that produce a slow movement of the complex towards the anal direction (Andrew et al. 2002). Figure 1.7 shows a typical MMC recording from different segments of the dog jejunum; from the most proximal (segment J1) to the most distal region (segment J6) (Ehrlein et al 1987). From the figure, it is clear the front part of each MMC in a given segment is more distally located in relation to the previous segment. The MMC are very powerful contractions that occlude the intestinal lumen (Ehrlein et al 1987). These occluding fast occurring contractions are responsible to propel the intestinal content in the anal direction. Due to this, the MMC has been referred as the "intestinal housekeeper".

The ENS is completely responsible for initiation and progression of the MMC. However, hexamethonium blocked the MMC showing the important role of cholinergic ganglionic enteric transmission (El Sharkawy et al. 1982). The velocity of the MMC is faster at proximal regions of the small intestine and it slows down as it approaches more distal regions. On average the propagation velocity of MMC in proximal regions of the dog small intestine is 5.3 cm/min and in distal regions is about 1.5 cm/min (Szurszewski et al 1969); a similar pattern was observed in the guinea pig small intestine (Galligan et al. 1985).

The physiological function of the MMC is to remove epithelial cells, secretions, and undigested food from the lumen of the small intestine. If impaired, accumulation of unwanted bacteria will occur leading to malabsorption and ileal infections (Summer, 2003).

Motor patterns in the colon: Colonic migrating motor complex

The colonic migrating motor complex (CMMC) is a major motor pattern in the large intestine. The CMMC are neurally mediated, rhythmic, and long lasting pattern of contractile activity that occur without the application of nerve-stimulation. On average they occur at regular intervals of approximately 3 minutes in the murine colon (Smith et al. 2014; Dickson et al. 2010; Dickson et al. 2009; France et al. 2012). Electrically, the CMMC consists of a brief hyperpolarization followed by fast oscillations with action potentials superimposed on a slow depolarization of the muscle cells (Dickson et al. 2010; Smith et al. 2014). The human counterpart of CMMC is known as the high amplitude propagating contractions (HAPC). When HAPC occur in the human colon *in vivo* they resemble the CMMC pattern in the murine colon both in frequency and duration *in vivo* and *in vitro* conditions (Smith et al. 2014; Zarate and Spencer, 2011; Spencer et al. 2012). Similar to the previously described MMC, the generation and propagation of the CMMC is totally dependent on the ENS because this motor pattern occurs in isolated colons devoid of extrinsic inputs (Bayguinov et al. 2010; Lyster et al. 1995). The main function of the CMMC pattern is to regulate fecal pellet propulsion. This has been demonstrated using an animal model for slow transit constipation, which shows disrupted pattern of CMMC both at the mechanical and electrical level (Heredia et al. 2012).

The CMMC pattern originates in the proximal colon and propagates towards the more distal direction. A regular CMMC pattern can be observed when a stationary artificial pellet is placed in the lumen of the colon. In this condition a CMMC pattern consists of an oral contraction followed, with some delay, by an anal contraction (Heredia et al. 2009, 2013). This pattern of contractile activity is called propagating CMMC.

Figure 1.8 is presenting a proposed model for the enteric neural control of CMMC generation. (1) Tonic inhibition to the circular muscle is provided by spontaneously active descending IN containing 5-HT. This provides a continuous activation of inhibitory motor neurons. (2) Mechanical or chemical

stimulation of the mucosa induces release of 5-HT from enterochromaffin cells (EC) activating 5-HT_3 receptors on the mucosal endings of IPAN. This will induce action potential firing in IPAN. (3) Activation of IPAN leads to synaptic stimulation of ascending IN. IPAN release ACh and SP as excitatory neurotransmitters (Johnson et al., 1996). (4) Release of ACh from IPAN leads to activation of excitatory MN producing contraction of the circular muscle at the oral side. (5) Release of both ACh and SP from IPAN leads to synaptic stimulation of descending IN. (6) Consequently, inhibitory MN will be further activated and relaxation of the circular muscle will be produced at the anal side. This relaxation determines the direction of propagation. (7) The contraction at the oral side and relaxation at the anal side (in relation to fecal pellet location) will move the pellet anally similar to the peristaltic reflex observed in the small intestine. At the new location, the pellet will start the cycle (Dickson et al. 2010; Dickson et al. 2009, 2009; Bayguinov 2010; Smith et al. 2014).

Regulation of smooth muscle motility by motor neurons

As we have seen in the previous sections, motility patterns are under the control of enteric neurons. GI muscles receive innervation from both excitatory and inhibitory MN (Bennett et al. 1966; Bennett, 1966).

Excitatory neurotransmitters

The electrical and mechanical response of the smooth muscle depends upon the action of excitatory and inhibitory neurotransmitters. ACh is the most prominent excitatory neurotransmitter and the post-junctional responses are mediated by the muscarinic receptors (M2 and M3) (Sanders et al. 2012). Acetylcholine-mediated contraction activates the phospholipase C beta 1/inositol trisphosphate (PLC-β_1/IP_3) pathway. This pathway leads to release of intracellular Ca^{2+}, which binds to calmodulin forming the Ca^{2+}-calmodulin complex. This complex then activates the myosin light chain kinase to

initiate contraction (Murthy, 2006). Cholinergic transmission refers to synaptic communication using ACh as the neurotransmitter. However, a component of excitatory transmission is resistant to block of cholinergic transmission suggesting the role of another excitatory neurotransmitter. In this line, neurokinins (SP and neurokinin A) bind to neurokinin 1 and neurokinin 2 receptors and activate pathways leading to contraction of the muscle (Sanders et al. 2012). This communication is known as non-cholinergic excitatory transmission.

Inhibitory neurotransmitters

NO is considered one of the predominant inhibitory neurotransmitter in the GI tract. This is a non-traditional neurotransmitter because the dynamic of the mechanisms regulating its release from the pre-synaptic nerve and its post-synaptic targets do not resemble the ones described for typical neurotransmitter molecules such as ACh (Rao et al. 2008; Chaudhury et al. 2009, 2011). During inhibitory neuromuscular transmission NO is produced by the enzyme NOS in the nerve terminals (Chaudhury et al. 2009). The type of NOS able to synthetize NO is a dimer of the neuronal-NOS-α (nNOSα) that is associated with the membrane of the nerve terminal (Rao et al. 2008). Upon arrival of the action potential or nerve stimulation, the active nNOSα binds calmodulin in a Ca^{2+}-dependent manner to produce NO (Rao et al. 2008; Thatte et al. 2009; Chaudhury et al. 2009). Chaudhury et al. (2009) investigated the nature of the interaction allowing association of nNOSα with the membrane. The active nNOSα is associated with the scaffolding protein known as PSD95 (post-synaptic density protein-95). This scaffolding protein has three PDZ (acronym for PSD95, *Drosophila* septate junction protein Discs-large, and the epithelial tight junction protein ZO-1) binding domains, a SH3, and a guanylate kinase domain. PSD95 is attached to the varicosity membrane by palmitoylation at the PDZ1 domain. Specifically, nNOSα is attached to PDZ2 domain of PSD95 and the latter is anchored to voltage-gated Ca^{2+} channels (Chaudhury et al. 2009). All these interactions between nNOSα, PDS95, and Ca^{2+} channels resemble those described for the "priming" of the traditional neurotransmitters and the SNARE

complex. The clustering of nNOSα to the membrane allow fast response to Ca^{2+} influx and thus timely NO production during inhibitory neuromuscular transmission. NO, upon production, diffuses into the muscle and activate guanylyl cyclase leading to production of cyclic guanosine monophosphate (cGMP), activation of protein kinase G (PKG), activation of K^+ channels, and decreased Ca^{2+} sensitivity (Sanders et al. 2012). All these mechanisms lead to hyperpolarization and mechanical relaxation of the muscle cells. The identity of the purinergic neurotransmitter is a current controversy in the field of inhibitory neuromuscular transmission. Some groups have provided evidence supporting a role for ATP (Burnstock, 2009; Belai and Burnstock, 2000, 1994) and yet other provided evidence favoring β-nicotinic adenine dinucleotide (β-NAD) as the true purinergic neurotransmitter (Mutafova-Yambolieva et al. 2007; Hwang et al. 2011). The purinergic neurotransmitter is stored in synaptic vesicles and the vesicular nucleotide transporter might provide a mechanism for packing this molecule inside vesicles and importantly it represents a potential molecular maker for purinergic nerves (Sawada et al. 2008). Purines bind to P2Y1 purinergic receptors in muscle cells activating the PLC/IP_3/diacylglycerol pathway leading to activation of small conductance Ca^{2+}-activating K^+ channels and hyperpolarization (Kurahashi et al. 2011; Gallego et al. 2012; Hwang et al. 2012).

Peptides also participate in inhibitory neuromuscular transmission. VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) contribute to inhibitory transmission however, frequencies higher that 10 Hz are needed in order to unmask this component (Goyal et al. 1980; McConalogue et al. 1995).

Interstitial cells of Cajal: "Gut pacemaker cells"

GI muscles display autonomous and rhythmic activity that is intrinsic to these muscles and as such do not depend on neural input. However, neural inputs can modulate the intensity of the autonomous activity in order to regulate the strength of muscle motor behaviors. The identity of the

cell responsible for generation of the pacemaker activity is now known to be interstitial cells of Cajal (ICC) (Sanders, 1996). Isolated ICC exhibits spontaneous electrical rhythmicity similar to the electrical activity of intact muscles (Langton et al. 1989; Zhu et al. 2009). c-Kit is a proto-oncogene encoding a receptor protein kinase and it was accidentally found to be expressed in ICC (Maeda et al. 1994). Animals with loss-of-function in c-Kit signaling lack pacemaker ICC in the small intestine and do not express pacemaker activity (Ward and Burns, 1994; Torihashi et al. 1995; Huizinga, et al. 1995) supporting a role for these cells as the gut pacemakers.

There are pacemaker regions throughout the GI tract including the stomach, small intestine, and colon. In the stomach the pacemaker region is located between the circular and longitudinal muscle layers (myenteric plexus) of the corpus, antrum, and pylorus. Pacemaker slow waves can be recorded in these areas from gastric ICC located in the myenteric plexus (IC-MY) (Sanders, 1996). In small intestine pacemaker activity comes primarily from the border between the circular and longitudinal muscle layers and thus ICC located between these two muscle layers (IC-MY) are responsible for this activity. In the colon two pacemaker regions have been reported: submucosal (IC-SM) and myenteric plexus. However, the participation of the IC-SM in pacemaker activity of the colon might not be true in all species (Sanders, 1996).

The critical inward currents necessary for pacemaker activity in ICC have been investigated. The resting membrane potential of cells in the slow wave pacemaker region in situ is about -80 mV (Smith et al. 1987). Conducting current recordings from IC-SM provided evidence for the existence of T-type voltage-gated Ca^{2+} channels in these cells in addition to the known L-type currents (Lee and Sanders 1993; Sanders, 1996). T-type Ca^{2+} currents are low-threshold currents meaning that they activate at very negative potentials and they do not fully inactivate. As such these channels are suitable for initiation of pacemaker activity, as it was established for cardiac pacemaker cells (Bean, 1985). Near the resting membrane potential of IC-SM the T-type Ca^{2+} channels will open providing a small but sustained

Ca^{2+} influx that will slowly depolarize these cells toward the threshold for activation of L-type Ca^{2+} channels. ICC has a high input impedance meaning that a small influx of current will produce a significant depolarization (Sanders, 1996). Eventually, L-type Ca^{2+} channels in ICC will be activated amplifying the inward currents. Due to the coupling between ICC and smooth muscle cells, this amplified pacemaker current will be spread to muscle cells bringing them to threshold regulating initiation and regeneration of rhythmic activity.

ICC might mediate neurotransmission

Many researchers including Cajal had observed that ICC are functionally interposed between the terminals of motor neurons and the muscle (Ward and Sanders, 2001). This is now referred as the intercalation hypothesis. Torihashi et al. (1995) disrupted the ICC from the myenteric plexus and deep muscular plexus by treating rats with antibodies to c-Kit. The authors found that excitatory and inhibitory transmission to the ileum was decreased and transmission to the colon was abolished supporting an important role for ICC in mediating neuromuscular transmission. ICC express receptors or post-synaptic targets for neurotransmitters such as NO (Shuttlerworth et al. 1993), VIP (Epperson et al. 2000), and ATP (Burnstock and Lavin, 2002), ACh (Epperson et al. 2000), and tachykinin (Portbury et al. 1996) supporting a role for these cells in neuromuscular transmission.

VOLTAGE-GATED Ca^{2+} CHANNELS IN THE NERVOUS SYSTEM

Calcium transients mediate a host of neuronal functions including intracellular signaling development, gene expression, cell death, cell metabolism, excitation, and finally regulation of neurotransmitter release (Berridge, 1998; Choi, 1992; Clapham 1995; Ginty 1997). The latter is of especial interest for the studies described in this dissertation. Voltage-gated Ca^{2+} channels (VGCC) represent one important mechanism for the control of Ca^{2+} levels in the cell. These channels are

heteromeric protein structures and are sensitive to changes in the membrane potential of the cell where they are expressed (Catterall, 2011). Molecular, pharmacological, and electrophysiological studies had identified different types of VGCCs: L-, P-, Q-, R-, and T-type VGCC (Randal and Tsien, 1995; Caterall, 2011; Snutch et al. 1990; Zhang et al. 1993).

Molecular basis of VGCC: structure and diversity

VGCC contain multiple subunits: a principal pore-forming α1 subunit of about 190 kDa in association with an intracellular β subunit of about 55 kDa, and a disulfide-linked α2δ subunit of 170 kDa. In the skeletal neuromuscular junction VGCC also have a γ subunit of about 33 kDa. The α1 subunit is a protein of 2000 amino acids residues and the organization is similar to that described for the voltage-gated Na^+ channels (Caterall, 2011). Figure 1.9 is showing the structural organization of the α1 subunit. The amino acid residues are organized into four domains (I-IV). Each domain contains six transmembrane segments (S1-S6) and a membrane associated loop between transmembrane segments S5 and S6. This region is depicted in green color in Figure 10 and is especially important for forming the pore of the channel. The narrow external end of the pore is lined by the pore loop, which contains a pair of glutamate residues in each domain. This structural feature confers Ca^{2+} selectivity and is unique for Ca^{2+} channels (Heinemann et al. 1992). The segment S4 within each of the four domains is the voltage sensor and under the influence of an electrical field it moves outward and rotates initiating a conformational change that open the pore of the channel (Catterall, 2011).

Biochemical analysis revealed structure of intracellular β subunits, which consisted of α helices but no transmembrane segments (Takahashi et al. 1987). The α2 subunit was identified to be an extracellular glycoprotein attached to the membrane through a disulfide linkage to the δ subunit (Gurnett et al 1996). The δ subunit is encoded by the same gene as the α2 subunit and the mature forms of these two subunits results from post-translational proteolytic processing and disulfide linkage (De Jongh et al.

1990). The δ subunit was originally predicted to be anchored to the membrane by a single transmembrane segment. However, Davis et al. (2010) reported solid evidence showing that post-translational processing cleaves the predicted transmembrane segment and the δ subunit is anchored to the membrane through a glycophosphatidylinositol membrane anchor. The γ subunit of the skeletal VGCCs is glycoprotein with four transmembrane segments (Jay et al. 1990; Milstein and Nicoll, 2008).

Molecular diversity of VGCC is due to the expression of different α1 subunits. Ten different forms have been characterized by cDNA cloning and functional expression in mammalian cells or Xenopus oocytes (Catterall, 2011). These subunits can be divided in three subfamilies: Cav1, Cav2, and Cav3 (Snutch and Reiner, 1992; Erthel et al. 2000). Cav1 subfamily (Cav1.1-1.4) conduct L-type Ca^{2+} current and the pore of the channel is form by either α1D, α1C, α1F or α1S subunits (Snutch et al 1991; Williams et al. 1992a; Hell et al. 1993). Cav2 subfamily is divided into three groups. Cav2.1 conduct both P- and Q-type Ca^{2+} currents and they are form by the α1A subunits. Although P- and Q-type channels have different biophysical properties they are believe to be splice variants of the same gene. This is supported by Jun et al. (1999) findings in which mice deficient in the α1A gene do not expressed neither P- nor Q-type currents. Cav 2.2 subfamily conducts N-type currents and these channels are formed by the α1B subunit (Snutch et al. 1990). Cav 2.3 subfamily conducts R-type currents and at least a component of the total R-type channels are form by the α1E subunit (Wilson et al. 2000). Finally, Cav3 subfamily conduct T-type currents and these channels are form by α1G, α1H, α1I subunits (Perez-Reyes, 1998).

Functional diversity of Ca^{2+} channels

One of the classifications of VGCC takes into account the magnitude of the depolarization required for channel activation. According to this, two groups of VGCCs can be separated: high voltage-

activated (HVA) channels and low voltage-activated (LVA) channels. HVA Ca^{2+} channels are L-, N-, P-, Q-, and R-type channels while LVA Ca^{2+} channels are designated as T-type channels.

T-type Ca^{2+} Channels

Channels classified as LVA were designated T-type because the current flowing through these channels was transient in duration and the conductance was tiny. In addition, these channels open at more negative voltages (approximately -64 mV) and express fast inactivation (<100 ms) (Perez-Reyes, 2003; Talavera and Nilius, 2006). T-type channels are sensitive to high concentrations of $NiCl_2$ (Vassort and Alvarez, 1994; Vassort et al. 2006).

L-type Ca^{2+} channels

HVA L-type channels have a large conductance and a very slow inactivation, which together give rise to a long-lasting Ca^{2+} current. L-type channels are predominantly express in smooth, cardiac, and skeletal muscles where they mediate excitation contraction coupling (Hille, 2001). L-type channels are highly sensitive to block by dihydropyridines (DHP) and this is a unique feature that distinguish L-type channels from the other subtypes. This DHP-sensitivity is clinically used for treatment of cardiovascular diseases (Catterall, 2011).

N-type Ca^{2+} channels

When first discovered in the chick sensory neurons, the current through N-type Ca^{2+} channels was neither tiny/transient nor large/long-lasting (Fox et al. 1987) and thus the properties of these channels were intermediate to those observed in T-type and L-type Ca^{2+} channels. They were denominated N for neuronal. N-type channels are sensitive to block by the peptide neurotoxin called ω-conotoxin GVIA isolated from the Pacific cone snail Conus geographus (Olivera et al. 1991).

P/Q-type Ca^{2+} channels

Close to the time when N-type channels were identified by using the peptide ω-conotoxin-GVIA, additional pharmacological approaches aided the identification of more VGCC subtypes. P- and Q-type VGCCs were identified in the cerebellum specifically in Purkinje cells and cerebellar granule cells, respectively. They express differential sensitivities for ω-agatoxin IVA, which is from the American web spider Agelenopsis aperta, in addition to different inactivation kinetics. Specifically, P-type channels express a slower inactivation and a higher sensitivity to block by ω-agatoxin IVA than Q-type channels (Mintz et al. 1992; Sather et al. 1993; Zhang et al. 1993).

R-type Ca^{2+} channels

Tsien and co-workers identified a residual current in rat cerebellar granule cells after pharmacological block of L-, N-, P, and Q-type VGCCs (Zhang et al. 1993; Randall and Tsien, 1995). In cerebellar granule cells, a component of the R-type current is reduced by SNX-482, a toxin isolated from the tarantula *Hysterocarates gigas* (Tottene et al. 2000; Wilson et al. 2000). A heterogeneous population of calcium channels generates the total R-type current and they express different pharmacological and permeation properties (Tottene et al. 1996; Wilson et al. 2000; Tottene et al. 2000). At least in the cerebellum the two populations are denominated as G2 and G3. The G2 channels share features of the recombinant α1E channels: (1) high sensitivity to Ni^{2+} and SNX-482 block, (2) larger macroscopic current with Ca^{2+} than Ba^{2+}, (3) Single channel conductance of 12-15 ps, (4) activate at approximately 15 mV more negative potentials than G3 channels. The G3 channels are more similar to the other HVA channels (α1A, α1B, α1C): (1) resistant to SNX-482 and Ni^{2+}, (2) single channel conductance of 20 ps, (3) larger macroscopic current with Ba^{2+} than Ca^{2+}, (4) activate a more depolarized potentials (Tottene et al. 1996). The aforementioned properties of R-type Ca^{2+} currents have been studied in the cerebellum. The contribution of these two channel populations to the total R-type current might vary in different neuronal populations (Bian et al. 2004; Naidoo et al. 2010)

Functional role of Ca^{2+} channel subunits

In general, the α1 subunit by itself is capable of producing a functional Ca^{2+} channel albeit with low expression and abnormal kinetics and voltage-dependence of the Ca^{2+} current. Combination of the α1 subunit with the auxiliary subunits has an important influence on Ca^{2+} channel function (Catterall, 2000; Hofmann et al. 1999). Structurally the β subunit has a SH3 domain and a guanylate cyclase domain and they are very important for protein interactions. For example, the guanylate cyclase domain interacts with the intracellular loop connecting domains I and II (fig 10). This association occurs in such a way that the SH3 domain is completely exposed to the intracellular environment and available for other protein interactions. The β subunit shifts the voltage-dependence of activation and inactivation to more negative potentials, increases the rate of inactivation, and also enhances the level of expression of Ca^{2+} currents (Catterall, 2000; Hofmann et al. 19994; Catterall and Leal, 2013). The α2δ subunit enhance cell-surface expression of the α1 subunits (Davies et al. 2007) and is important for efficiently couple Ca^{2+} influx to exocytosis at active zone in the nerve terminals (Hoppa et al. 2012).

Regulation of neurotransmitter release: VGCCs and pre-synaptic proteins

Neurotransmitter release is predominantly regulated by the Cav2 subfamily of VGCCs. The Cav2.1 (P/Q-type channel) has a major role in subserving neurotransmitter release in the skeletal neuromuscular junction and in central synapses (Dunlap et al. 1995). The Cav2.2 (N-type channel) is critical at synapse within the autonomic nervous system but is also involved in central synaptic transmission (Olivera et al. 1994). The Cav2.3 (R-type channel) also contribute to neurotransmitter release at central synapses (Gasparini et al. 2001) and is also involved in neuromuscular transmission to smooth muscle of the gut (Naidoo et al. 2010).

There are two forms of Ca^{2+}-dependent neurotransmitter release: fast synchronous (phasic) and slow asynchronous (tonic) (Catterall and Few, 2008). Synchronous release results in a large, fast post-

synaptic response and is driven by the precisely timed pre-synaptic Ca^{2+} influx (Sabatini and Regher, 1996). After the action potential there is a residual Ca^{2+} remaining in the terminal that evokes a basal or tonic level of neurotransmitter release and this is what constitute the asynchronous form of neurotransmission (Lu and Trussell, 2000). It is established that neurotransmitter release is proportional to the third or fourth power of Ca^{2+} influx (Catterall and Leal, 2008). Consequently, pre-synaptic Ca^{2+}channels tightly regulate neurotransmitter release and small changes in these channels are expected to cause notable alteration in transmission. However, due to the plasticity of the synapse some of these changes in Ca^{2+} channels occur without major alterations in transmission due to the ability of the synapse to cope with perturbations.

The pre-synaptic terminals contain an area called *active zone*, where neurotransmitter release is highly successful because it clusters multiple proteins that work to increase the effectiveness of synaptic vesicle exocytosis and thus neurotransmitter release. Calcium channels represent one of the proteins clustered at active zones. In this line, it has been shown that the release probability of a single synaptic vesicle increases with the number of Ca^{2+} channels at the active zone (Holderith et al. 2012; Sheng et al. 2012; Südhof, 2012). Ca^{2+}-triggered synaptic vesicle exocytosis also depends on the assembly of the SNARE complex. This complex is composed by the vesicle-associated v-SNARE protein synaptobrevin (VAMP) that interacts with two plasma membrane associated t-SNARE proteins, SNAP-25 and syntaxin-1 (Bajjalieh and Sheller, 1995; Sudhof, 2004, 1995). In order for the SNARE complex to be matured it needs synaptotagmin, which is an integral protein of the synaptic vesicle membrane. Synaptotagmin is a Ca^{2+}-binding protein and a sensor providing the Ca^{2+}-dependent regulation of the proteins that are part of the fusion machinery. Upon activation of VGCCs, Ca^{2+} influx into the pre-synaptic terminal binds synaptotagmin inducing a conformational change in the SNARE complex from a *trans* to a *cis* state. This change results in the fusion of the nerve terminal membrane with the synaptic vesicle membrane releasing the neurotransmitters into the synaptic cleft (Catterall and Few, 2008).

Cav2.1 and Cav2.2 channels interact with the SNARE complex. Syntaxin-1 and SNAP-25 but not the synaptic vesicle SNARE synaptobrevin specifically interact with the Cav2.2 channels by binding to the intracellular loop between domains II and III at the synaptic protein interaction (*synprint*) site (Sheng et al. 1994) (figure 1.10). Interaction of the SNARE proteins with the *synprint* site regulates expression, localization, and function of Cav2 channels (Catterall and Leal, 2013). This interaction is Ca^{2+}-dependent with maximal binding at 20 μM Ca^{2+} and reduced binding at either lower or higher concentrations (Sheng et al. 1996). Cav2.1 channels have an analogous *synprint* site and different channel subtypes have distinct associations with syntaxin-1 and SNAP-25 (Kim and Catterall, 1997; Rettig et al. 1996). Synaptotagmin-1 has two binding domains for Ca^{2+}, which initiate synchronous neurotransmitter release (Sudhof, 2004). In addition, synaptotagmin binds to the *synprint* site of both Cav2.1 and Cav2.2 (Sheng et al. 1997). Interesting, syntaxin-1 has a Ca^{2+} -dependent competitive interaction with the *synprint* site of Ca^{2+} channels and synaptotagmin. For example, at low a Ca^{2+} concentration syntaxin-1 interacts with the *synprint* site and at higher Ca^{2+} concentration (>30 μM) its association with synaptotagmin increases (Sheng et al. 1996). These Ca^{2+}-depended interactions are thought to support the fusion of the terminal membrane with the vesicle membrane during exocytosis.

Other pre-synaptic proteins (RIM, Munc13, RIM-binding protein, liprin-α, and ELKS) interact with the SNARE complex in order to place synaptic vesicles immediately next to the membrane of the nerve terminal ("dock" and "prime" vesicles) and in close apposition to Ca^{2+} channels (Südhof, 2012). Other interaction that is required for recruitment of Cav2 channels to active zones is the binding of the SNARE-interacting protein RIM to the distal C terminus of Cav2.1 channels and with the β-subunits (Kiyonaka et al. 2007; Han et al. 2011; Kaeser et al. 2011).

Short-term plasticity of Ca^{2+} channels by Ca^{2+} and calmodulin

Facilitation in response to short trains of stimulation and inactivation in response to longer trains are examples of short-term Ca^{2+} channel plasticity. These changes have been studied in Cav2.1 (P/Q-type) channels in the large calyx of Held synapse and in transfected neuronal and non-neuronal cells (Lee et al. 1999, 2000; Mochida et al. 2008; Borst et al. 1998; Cuttle et al. 1998). Facilitation and inactivation are both dependent upon Ca^{2+} and calmodulin (CaM), however, facilitation has a higher affinity for Ca^{2+} than inactivation (Lee et al 2000). CaM interacts with two sites of the Cav2.1 channel: IQ-like domain (IM motif) and a CaM-binding domain (CBD) (Lee et al. 1999; De Maria et al. 2001; Lee et al. 2003). Mutations in the C-terminal of CaM and in IM motif of Cav2.1 channels reduce facilitation. On the other hand, mutations in the N-terminal and deletion of the CBD of Cav2.1 channels reduce inactivation Lee et al. 1999; De Maria et al. 2001; Lee et al. 2003). Therefore, local Ca^{2+} influx can induce facilitation through interactions between Ca^{2+}/CaM and IM motif whereas a more general increase in Ca^{2+} level will induce inactivation via a Ca^{2+}/CaM and CBD interaction.

Synaptic depression is another example of short-term plasticity regulating Ca^{2+} channel function and it reduces the strength of synaptic transmission. Some studies using prolonged stimulation had suggested that a depletion of the vesicles present at the readily releasable pool is what causes synaptic depression (Schneggenburger et al. 2012). However, this idea has been challenged by studies showing that even after complete depletion of the readily releasable pool of synaptic vesicles by repetitive stimulation significant neurotransmitter release can be elicited (Wadel et al. 2007; Catterall and Leal, 2013). Interesting, studies in the calyx of Held showed that Ca^{2+}-dependent inactivation of Cav2.1 channels correlates with synaptic depression and inhibition of CaM (which is known to mediate inactivation) reduces the observed depression events (Forsythe et al. 1998; Xu and Wu, 2005; Wu and Borst, 1999). As it was previously mentioned, deletion of CBD of transfected Cav2.1 channels blocks inactivation and synaptic depression (Lee et al. 1999,2000; Mochida et al. 2008). These studies provided persuasive evidence supporting the role of Ca^{2+}/CaM binding to CBD in the process of inactivation and

directly relate this as a cause of synaptic depression. Overall, Ca^{2+} channel inactivation works to prevents excessive Ca^{2+} entry and it contributes to the phenomenon of depression during synaptic transmission.

Other neuronal Ca^{2+} sensor proteins regulate the activity of Cav2.1 channels. Ca^{2+}-binding protein-1 (CaBP1) and visinin-like protein-2 (VILIP-2) regulate Cav2.1 channel activity in opposite ways. CaBP1 accelerates inactivation of Cav2.1 in a Ca^{2+}-independent manner channels (Lee et al. 2002). VILIP-2 increases Cav2.1 channel facilitation and also inhibits inactivation (Lautermilch et al. 2005). Studies showed that interaction between VILIP-2 and IM domain of Cav2.1 channels mediates the enhancing effects in facilitation and interactions between CaBP1 and CBD caused the acceleration of inactivation (Leal et al. 2012). Thus CaBP1 and VILIP-2 fine-tune synaptic facilitation and depression by a "pull and push" regulation of Cav2.1 function.

LONG-TERM PLASTICITY INVOLVING Ca^{2+} CHANNELS

Neuroplasticity drives changes in synaptic output and these changes are fundamentally important for neurological phenomena such as learning and memory. Homeostatic synaptic plasticity, a more stabilizing form of neuroplasticity, work to ensure that synaptic outputs are maintained at physiologically normal level when exposed to challenges. Now, these challenges are exemplified by perturbations against synaptic function. Under this condition homeostatic plasticity include changes in: neurotransmitter release, expression of neurotransmitter receptors, ion channel density or conductance properties (Perez-Otaño and Ehlers, 2005). Some of the most exciting questions in this field relates to the ability of synapses to use particular homeostatic mechanisms to overcome perturbations against synaptic components as well as the specific nature of these mechanisms and how they change across different neuronal populations. A critical role for VGCC has been proposed for homeostatic control of synapse function in several experimental conditions using either invertebrates such as fruit flies and

mammals. In this line, genetic mutations or pharmacological block of VGCC impairs both neurotransmission and homeostatic forms of neuroplasticity (Catterall and Few 2008; Norton and McDonough, 2008).

Synaptic plasticity at the invertebrate neuromuscular junction

A battery of studies showing the role of VGCC in homeostatic synaptic plasticity has been conducted in the neuromuscular junction of *Drosophila melanogaster*. The larval neuromuscular junction is a glutamatergic synapse expressing a prominent capacity for homeostatic regulation (Frank, 2014). After chronic or acute perturbations to excitability the neuromuscular junction retains normal levels of post-synaptic excitation (Petersen et al. 1997). Cav2 channels and several active zone proteins have been shown to be important players during development of homeostatic changes. Pharmacological inhibition of post-junctional glutamate receptors resulted in an increased expression of pre-synaptic active zones proteins such as Bruchpilot (homolog for ELKS/CAST) (Weyhersmuller et al. 2011). Chronic and acute glutamate receptor impairments induced significant increases in pre-synaptic Ca^{2+} influx through a Cav2 channels because a partial loss-of-function of these channels occludes the increase (Müller and Davis, 2012).

Pre-synaptic signaling has been shown to be involved in enhancing Cav2 function after glutamate receptor impairments. The Khc-73 is a member of the kinesin super family and loss-of-function mutations in this kinesin completely impairs the homeostatic changes related to Cav2 function and active zones (Tsurudome et al. 2010). Expression of Khc-73 is negatively regulated and loss of this regulation produces an overexpression of this kinesin. Consequently, an enhanced pre-synaptic Cav2 current is observed most likely due to enhanced Cav2 function by kinesin (Tsurudome et al. 2010). The pre-synaptic protein, RIM, is also involved in the homeostatic changes by producing increases in the size of the readily releasable pool of pre-synaptic vesicles (Müller et al. 2012). Pre-synaptic epithelial

sodium channels (ENaC) are also required for the synaptic homeostasis (Younger et al. 2013). The ENaC functions as a voltage-insensitive cation channel (Schild, 2010). Younger et al (2013) demonstrated that ENaC work to depolarize the membrane potential providing a depolarizing resting pre-synaptic current that can enhance Cav2 activity contributing to maintenance of synaptic function.

Mammalian homeostatic synaptic plasticity

Challenges to synaptic activity can also result in profound homeostatic changes to components of the mammalian pre-synaptic machinery and this has been supported using hippocampal cultures. AMPA receptor blockade with NBQX or CNQX induces two main homeostatic responses: incorporation of new GluA2-lacking AMPA receptors post-synaptically and a retrograde signaling process resulting in increased pre-synaptic release properties (Thiagarajan et al. 2005; Gong et al. 2007). Co-application of N- and P/Q-type channel blockers completely abolishes the enhanced pre-synaptic activity supporting a role for Cav2 function in mediating the observed homeostatic changes (Jakawich et al. 2010).

Cav1 channels are involved in homeostatic post-synaptic changes

Cav1 (L-type) channels are expressed in pre- and post-synaptic neuronal components and also in non-neuronal excitable cells such as muscles. When Thiagarajan et al. (2005) identified the homeostatic changes induced by blockade of AMPA receptors-that is, the increased expression of post-synaptic AMPA receptors, it was also reported that blockade of Cav1 function induced the same changes. In fact, the authors demonstrated that AMPA receptor blockade leads to a loss of Cav1 channel activity resulting in the observed compensatory increase in AMPA receptors. It seems that loss of Ca^{2+} influx through post-synaptic Cav1 channels is involved in homeostatic potentiation. Is the opposite true? Goold and Nicoll (2010) investigated this utilizing optogenetics to provide constant stimulation of CA1 pyramidal

neurons for a period of 24 hours. They observed a down-regulation of AMPA receptor mediated post-synaptic responses and blocking Cav1 channels with nifedipine occluded the compensatory events. Together, this studies shows that Cav1 channels contribute to homeostatic changes.

Homeostatic plasticity in response to Ca^{2+} channel perturbations

Ensuring physiologically normal Ca^{2+} signaling could be conceived as a priority for the nervous system and as a result many, but not all, neuronal populations are equipped with a molecular machinery to undergo compensatory changes. The mammalian neuromuscular junction of the Tottering (Tg) mouse exemplifies this phenomenon. The Tg mice have a spontaneous occurring mutation leading to a loss-of-function in the Cav2.1 (P/Q-type) channels (Wakamori et al. 1998). The Cav2.1 channels are the predominant regulators of acetylcholine release at the skeletal neuromuscular junction. The Tg mutation, despite altering function of the primary subtype of Ca^{2+} channel normally involved in acetylcholine release, does not cause neuromuscular dysfunction. This suggests the action of compensatory mechanism allowing other Ca^{2+} channel subtypes subserve acetylcholine release in face of the Tg-mediated perturbation of the skeletal neuromuscular junction. Using immunohistochemical and electrophysiological methods Pardo et al. (2006) showed that in Tg mice Cav2.2 (N-type) and Cav2.3 (R-type) channels assume control of acetylcholine release at motor nerve terminals and these homeostatic changes are sufficient to maintain synaptic function.

Lambert-Eaton Myasthenic Syndrome (LEMS) is a paraneoplastic neuromuscular disorder in which autoantibodies target Cav2.1 channels at the motor nerve terminals. Passive transfer of LEMS to mice by repeated administration of plasma from LEMS patients reduced the amplitude of pre-synaptic Cav2.1 currents and unmasked a component of the current mediated by Cav1 channels (Xu et al. 1998). Later Flink and Atchison (2002) demonstrated that the unmasked Cav1 currents in the motor nerve

terminal was involved in acetylcholine release and thus these channels become actively involved in synaptic transmission at the neuromuscular junction of a LEMS animal model.

Cav2.1 channels are one of the most important Ca^{2+} channels mediating neurotransmitter release at central synapses (Dunlap et al. 1995). Basal forebrain neurons of Tg mice express increased whole cell current densities, despite a loss-of-function in Cav2.1 channels. This homeostatic change is accomplished by a functional increase in the component of the current mediated by Cav1 channels (Etheredge et al. 2007). Synaptic transmission in the cerebellum of Tg mice becomes more dependent on Cav2.2 (N-type) channels (Leenders et al. 2002). In a similar way, synaptic transmission in hippocampal slices is maintained in Tg mice due to an increase in the reliance of Cav2.2 (N-type) channels (Quian and Noebels, 2000). A deletion of the Cav2.1 gene does not impaired synaptic transmission in hippocampal slices due to compensatory increase in the involvement of Cav2.2 (N-type) and Cav2.3 (R-type) channels (Jun et al. 1999). The same authors showed an increase in Cav1 function in Purkinje neurons of Cav2.1 knockout mice.

Altogether these studies demonstrate the ability of neurons to respond to perturbations against Ca^{2+} channels. Specifically, these studies show that recruitment of VGCC in order to offset deficit of a particular type of Ca^{2+} channel is a common mechanism of neuronal plasticity. The "mystery" here is which conditions underlie recruitment of Ca^{2+} channels and what determines the channel subtype that is recruited.

DYSFUNCTION OF CAV2 CHANNELS AND NEUROLOGICAL DISEASES

On the basis of the essential functions of Ca^{2+} channels in neuronal and synaptic function it is not surprising the fact that disruptions in the function of these channels are associated with the pathophysiology of neurological diseases. These neurological diseases are referred as Ca^{2+} channelopathies because they are related to defects in VGCCs.

CAv2.1 (α1A)

Mutations associated with Cav2.1 cause a rare disorder termed episodic ataxia type 2 (EA-2) characterized by paroxysmal attacks of cerebellar ataxia that can last for several days. Ophoff et al. (1996) identified two mutations in unrelated patients with EA-2 that mapped to the Cav2.1 gene. One mutation involves a base deletion and the other occurs at a splice junction site. However, both are predicted to lead to a frameshit such that the Cav2.1 protein is truncated prematurely after the S1 region of domain III. The incomplete channel is thought to be non-functional or incorrectly folded and trafficked. Generally, the clinical signs of EA-2 are evident prior to adulthood. A case of a 9 base pair (bp) insertion in the cytoplasmic domain II-III linker was associated with a late onset EA-2. This alteration was located just downstream the *synprint* site of Cav2.1 channels, however the exact effect of the mutation in channel function is unclear. Studies indicate disruption in the gating of the channels due to a substantial reduction in current and a 20 mV depolarization shift in activation threshold together with some change in activation and inactivation kinetics (Mckeown et al. 2006).

Another channelopathy is a rare autosomal dominant disorder known as familial hemiplegic migraine (FHM). It is characterized by intense attacks of migraine with aura, which last for several days. Several missense mutations have been found within the Cav2.1 gene that lead to increase Ca^{2+} influx through the expressed channels (Trettel et al. 2000) but the biophysical characteristics conferred to the Cav2.1 channels are not identical. Alterations that have been reported are changes in gating properties, such as faster or slower inactivation (Kraus et al. 1998), activation at more negative potentials (Kraus et al. 2000). Electrophysiological studies from a transgenic FHM mouse model bearing the human R192Q mutation reported increased current densities from cerebellar granule cells and channel activation at more negative voltages (van den Maagdenberg et al. 2004). In addition, this FHM mouse model expressed enhanced neurotransmission and susceptibility to cortical spreading depression supporting the fact that this channelopathy is due to a gain-of-function mutation.

Spinocerebellar ataxia type-6 (SCA-6) is also associated with mutations in Cav2.1 channels. The onset of SCA-6 is early middle age and it is characterized by a mild progressive cerebellar atrophy causing dysarthia, nystagmus, ataxia, loss of gait, and even death (Mantu, 2005). This disorder is also characterized by peripheral neuropathy that includes symptoms of autonomic dysfunction related to intestinal dysmotility. SCA-6 arises through a shift in the reading frame and triplet (CAG) repeats expansion at the distal carboxyl terminus (Zhuchenko et al. 1997). It appears that the critical size of the CAG encoded polyglutamine stretch is 19 repeats; longer repeats are associated with a greater severity of the symptoms (Ishikawa et al. 1997). The consequences of the polyglutamine expansions in the function of Cav2.1 channels are still unclear, however, a hyperpolarizing shift in channel inactivation was reported in an animal model of SCA-6 (Mckeown et al. 2006). In addition, other studies had suggested that the polyglutamine expansion is cleaved *in vivo* and transported to the nucleus where it can be highly toxic to neurons especially Purkinje cells (Kordasiewicz et al. 2006).

Lambert-Eaton Myasthenic Syndrome (LEMS) is an autoimmune neurological disorder characterized by 3 cardinal symptoms: skeletal muscle weakness, areflexia, and autonomic dysfunction**.** Approximately 60% of LEMS patients have an underlying tumor, most commonly small cell lung carcinoma (O'Neill et al. 1998). The pathology underlying LEMS is believed to be due to circulating autoantibodies generated as an immune response to the presence of the tumor. One fact supporting this is the ability to transfer the electrophysiological and morphological features of LEMS to mice via injections of IgG from LEMS patients (Newsom-Davis and Murray, 1984). This creates an animal model of the disease that can be utilized to reveal the pathological mechanisms. Using the aforementioned animal model of LEMS, it is now very well established that LEMS patient IgGs reduces ACh release at skeletal neuromuscular junction by attacking VGCCs causing a decrease in their number and organization (Flink and Atchison, 2003). At the adult skeletal neuromuscular junction P/Q-type VGCC is the predominant subtype mediating ACh release and there are studies demonstrating disruption in this

VGCC subtype by LEMS IgG (Katz et al. 1995; Xu et al. 1998; Flink and Atchison, 2002; Pardo et al. 2006). Recent evidence suggests that LEMS autoantibodies recognize multiple subtypes of VGCCs in addition to P/Q-type channels, including: N-, and R-type channels bringing insight into potential new mechanisms underlying LEMS pathology (Hajela et al. 2015).

Autonomic dysfunction in LEMS is widespread involving dysfunction in the enteric, sympathetic, and parasympathetic divisions (Waterman, 2001). The mechanisms underlying autonomic dysfunction in LEMS are not well understood. Approximately 30% of LEMS patients suffer from GI motility dysfunction with symptoms such as constipation and poor control of bowels (Waterman, 2001). One study reported that acute exposure of LEMS IgGs to the guinea pig taenia caeci caused a significant decrease in the nerve-evoked contraction of this muscle (Houzen et al 1998).

Cav2.2 (α1B)

Mutations in the Cav2.2 have not been identified in the human population and this is a remarkable observation due to the importance of this channel in neuronal transmission. Studies using mice in which Cav2.2 gene has been deleted provide important information about potential problems. Using such animal models deficit in N-type Ca^{2+} channel function is associated with altered nociception (Saegusa et al. 2002) and dysfunction of the sympathetic nervous system (Ino et al. 2001).

Cav2.3 (α1E)

These channels are found in dendrites, soma, and nerve terminals suggesting that these channels contribute to neuronal excitability, integration of synaptic inputs, and neurotransmitter release. No human disorders have been identified related to mutation in Cav2.3 gene. Studies using

mice with deficit in these channels suggest problems with glucose-stimulating insulin release from pancreatic beta cells (Jing et al. 2005).

VOLTAGE-GATED Ca^{2+} CHANNELS IN ENTERIC NEUROMUSCULAR TRANSMISSION.

Immunohistochemical studies have shown that the ENS expresses the full complement of VGCC: P/Q-, N-, R-, and L-type channels (Naidoo et al. 2010; Waterman, 2000). Excitatory neuromuscular transmission in the GI tract is mediated in part by N-type VGCC. Supporting the role of N-type VGCC subserving cholinergic and non-adrenergic non-cholinergic (NANC) excitatory neurotransmitter release are experiments conducted in small intestinal longitudinal muscle (Hong et al. 1996; Boot, 1994), colonic circular (Maggi et al. 1994) and longitudinal muscle (De Lucca et al. 1990), taenia caeca (Houzed et al. 1998), and trachea (Chung-Hung et al. 2010) of rodents. Most of these studies show a component of the response that is resistant to N-type VGCC block. In this line, studies of various motor responses of peripheral organs revealed that responses resistant to N-type VGCC block are more pronounced when higher frequencies of nerve stimulation are used (Waterman, 2000). This suggests that multiple subtypes of VGCC cooperate at the peripheral nerve terminals to subserve neurotransmitter release as observed in the central nervous system.

Various studies have investigated the identity of the non-N-type VGCC mediating neurotransmitter release. A role for P/Q-type in mediating the responses resistant to N-type VGCC block has been suggested by studies conducted in the guinea pig small intestine (Boot et al. 1996). However, studies in the guinea pig trachea have argued against a role for P/Q-type VGCCs in mediating release of enteric neurotransmitters, at least in this preparation (Chung-Hung et al. 2010). More studies are required to clearly define the role of P/Q-type VGCCs in enteric excitatory neuromuscular transmission.

The role of N-type VGCC on inhibitory neuromuscular transmission as compare with excitatory transmission is much more variable. N-type VGCCs mediate release of inhibitory neurotransmitters in the gastric fundus and colon of rats (De Lucca et al. 1990; Borderies and Jimenez, 1996), however, Maggi et al (1988) showed no involvement of this channel in the rat proximal duodenum. In guinea pigs, N-type VGCC was reported to participate in inhibitory transmission in the trachea (Chung-Hung et al. 2010), however, inhibitory responses of the colonic longitudinal and circular muscle in the same species were resistant to N-type block (Zagorodnyuk and Maggi, 1994; Humphreys and Costa, 1994). A number of studies have supported the idea that a non-N-type channel is involved in release of inhibitory neurotransmitters (Waterman, 2000). The R-type VGCC has been suggested as one channel subtype cooperating with the N-type during inhibitory transmission in guinea pig trachea (Chung-Hung et al. 2010) and studies that attempted to identify a role for P/Q-type VGCC have provide evidence that argue against an involvement of this channel subtype (Borderies and Jimenez, 1996). However, recent studies from our lab had shown that inhibitory junction potentials in the guinea pig small intestine are reduced by blockade of both N- and P/Q-type VGCC. Further studies are needed in order to understand the specific roles of different VGCC subtypes during inhibitory neuromuscular transmission.

APPENDIX

	HVA	HVA	LVA
Nomenclature			
Ca^{2+} current type	L	P/Q, N, R	T
Structural nomenclature	Ca_v 1.1, 1.2, 1.3, 1.4	Ca_v 2.1, 2.2, 2.3	Ca_v 3.1, 3.2, 3.3
Snutch gene class (previous α_1 nomenclature)	S, C, D, F	A, B, E	G, H, I
Gating properties			
Activation range	Positive to -10 mV	Positive to -20 mV	Positive to -70 mV
Inactivation range	-60 to -10 mV	-120 to -30 mV	-100 to -60 mV
Inactivation	Very slow (τ>500 ms)	Partial (τ~50-80 ms)	Complete (τ~2-=50 ms)
Deactivation rate	Rapid	Slow	Rapid
Pore properties			
Single-channel conductance	25 ps	13 ps	8 ps
Single-channel opening	Continual reopening	Long burst	Brief burst, inactivation
Relative conductance	Ba^{2+}>Ca^{2+}	Ba^{2+}>Ca^{2+}	Ba^{2+} = Ca^{2+}
Pharmacology			
ω-conotoxin GVIA	Resistant	Cav2.2 sensitive	Resistant
Dihydropyridines	Sensitive	Resistant	Resistant
ω-agatoxin IVA	Resistant	Cav2.1 sensitive	Resistant
SNX-482	Resistant	Cav2.3 sensitive	Resistant
Divalent block	Cd^{2+}>Ni^{2+}	Cd^{2+}>Ni^{2+}	Ni^{2+} < Cd^{2+}

Table 1.1 Summary of the properties of voltage-gated Ca^{2+} channel subtypes. Adapted from Hille et al. 2001.

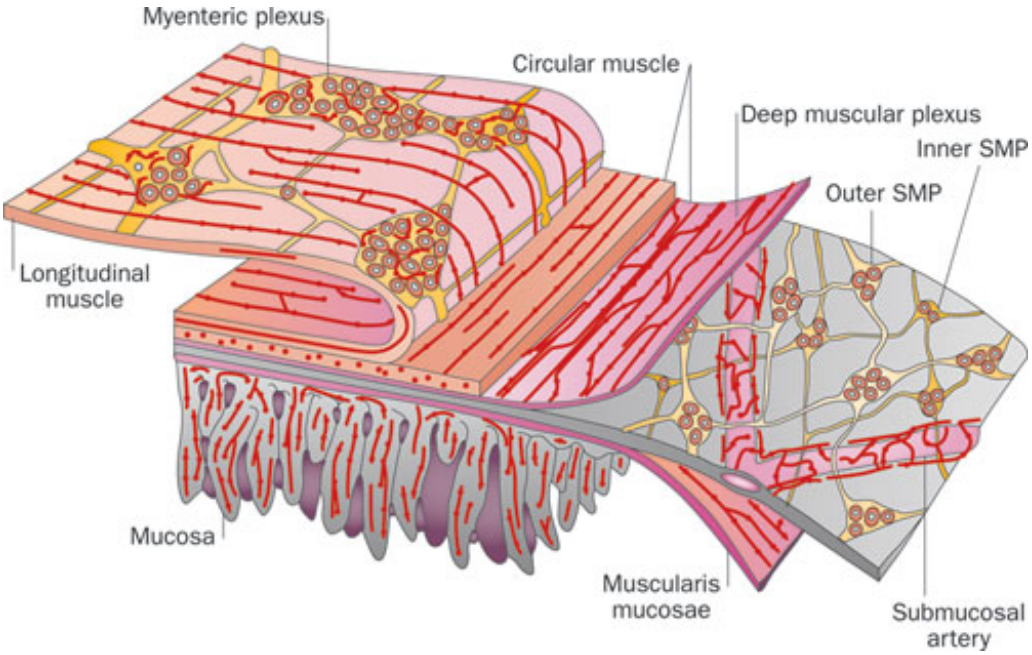

Figure 1.1: Organization of the enteric nervous system (ENS). The ENS has two ganglionated plexus, the myenteric plexus between the longitudinal and circular muscle layers and the submucosal plexus between the circular muscle and the mucosa layers. Nerve fiber bundles connect the ganglia and form plexuses that innervate the longitudinal muscle, circular muscle, muscularis mucosa, intrinsic artieris, and mucosa. Modified from Furness, JB. The Enteric Nervous System (Balckwell, Oxford, 2006).

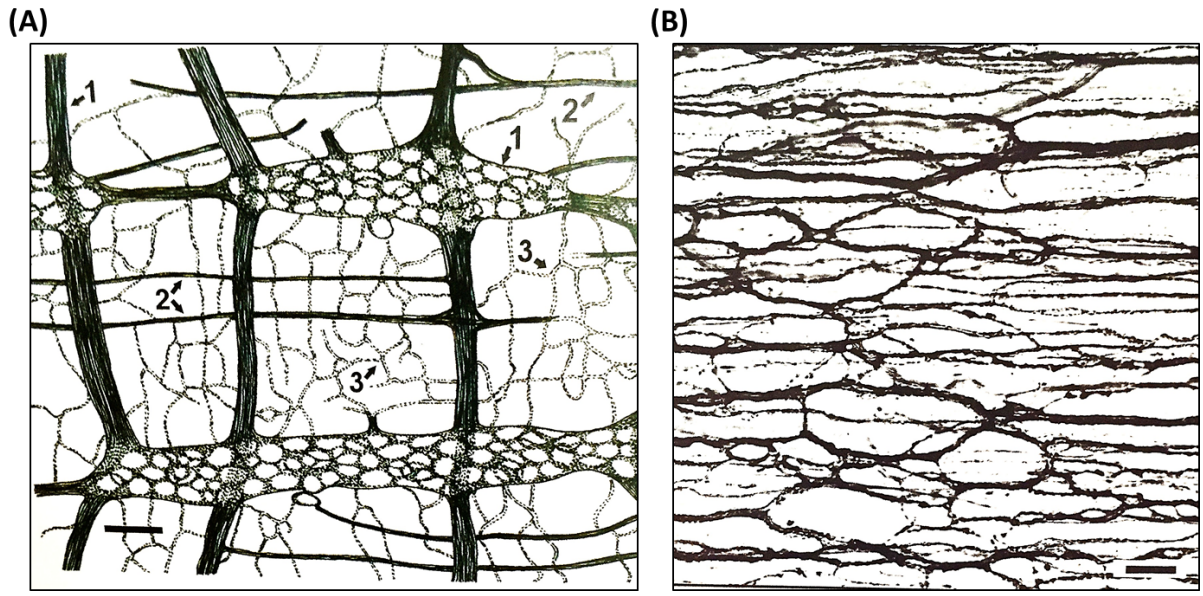

Figure 1.2: Nerve plexuses innervating the longitudinal and circular muscle layers. (A) The different components of the myenteric plexus are shown in this whole mount representation of the guinea pig small intestine. (1) Primary component: consistent of the ganglia and internodal fiber tracts. (2) Secondary component: consist of nerve strands lying parallel to the circular muscle. (3) Tertiary component: found only where the longitudinal muscle is thin and provide innervation to the longitudinal muscle. Neuron somas are represented as white ovals in the ganglia. (B) Nerve fibers providing innervation to the circular muscle layer. This drawing is a representation of a whole mount preparation of the circular muscle from he guinea pig small intestine, stained with the Champy-Maillet iodine and osmium technique. Major nerve fiber bundles run parallel to the long axes of the muscle cells and many connections occur between these bundles. Modified from Furness, JB. The Enteric Nervous System (Balckwell, Oxford, 2006).

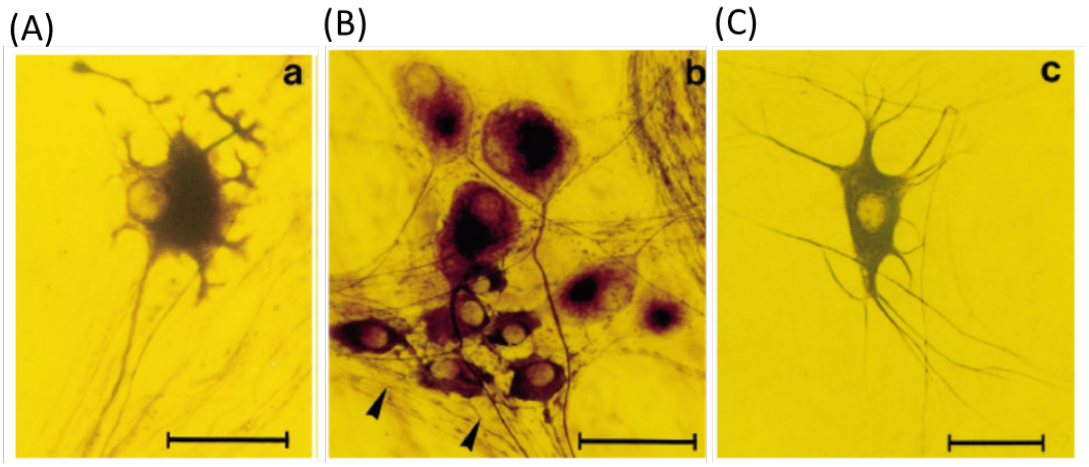

Figure 1.3: Representations of the distinct morphologies of enteric neurons. (A) Examples of Dogiel type I neurons (B) Examples from Dogiel type II neurons and (C) Examples of Dogiel type III. Modified from Furness, JB. The Enteric Nervous System (Balckwell, Oxford, 2006).

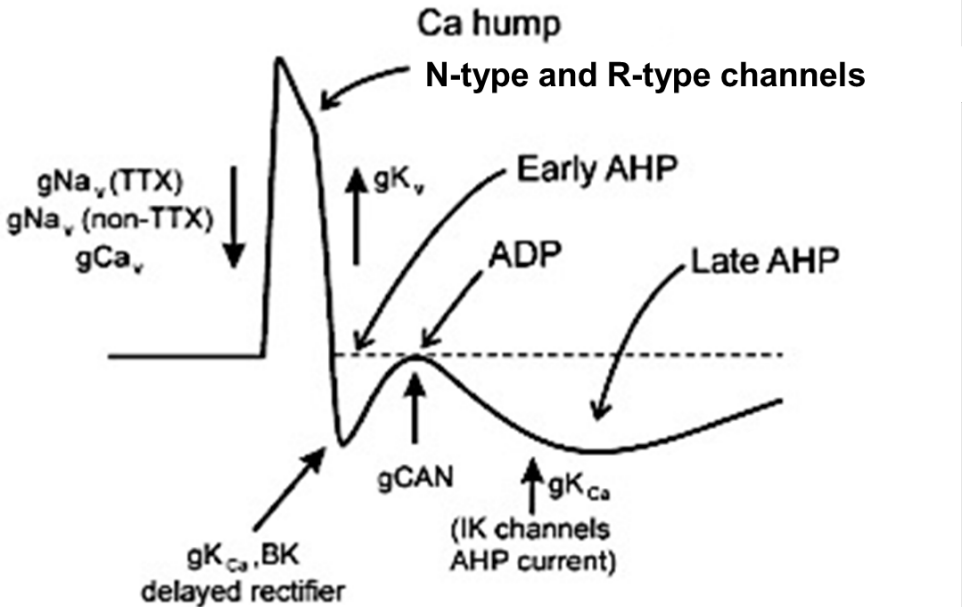

Figure 1.4: Action potential in AH neurons showing the early and late AHP. AH neurons are functionally referred as intrinsic primary afferent neurons. Action potentials generated at the some of these cells are initiated by opening of voltage-gated Na channels generating a Na^{+} conductance (gNav) and voltage-gated Ca^{+2} channels generating a Ca^{+2} conductance (gCav). The Ca^{+2} conductance is carried by both N- and R-type voltage-gated Ca^{+2} channels. There is also a tetrodotoxin (TTX) resistance sodium conductance (gNav). The Ca^{+2} current outlasts the Na^{+} current and is responsible for the inflection ("hump") during the repolarization phase. The action potential is terminated by the decline in Na^{+} and Ca^{+2} conductance and the activation of at least three K^{+} conductances that contribute to an early after hyperpolarizing potential (AHP). The latter is followed by a late AHP, which is carried by a Ca^{+2}-activated K^{+} conductance (gKca: intermediate conductance K^{+} channels). An afterdepolarizing potential (ADP) due to Ca^{+2} activation of a mixed cation conductance (gCAN) occurs between the early and late AHPs. Modified from Furness, JB. The Enteric Nervous System (Balckwell, Oxford, 2006).

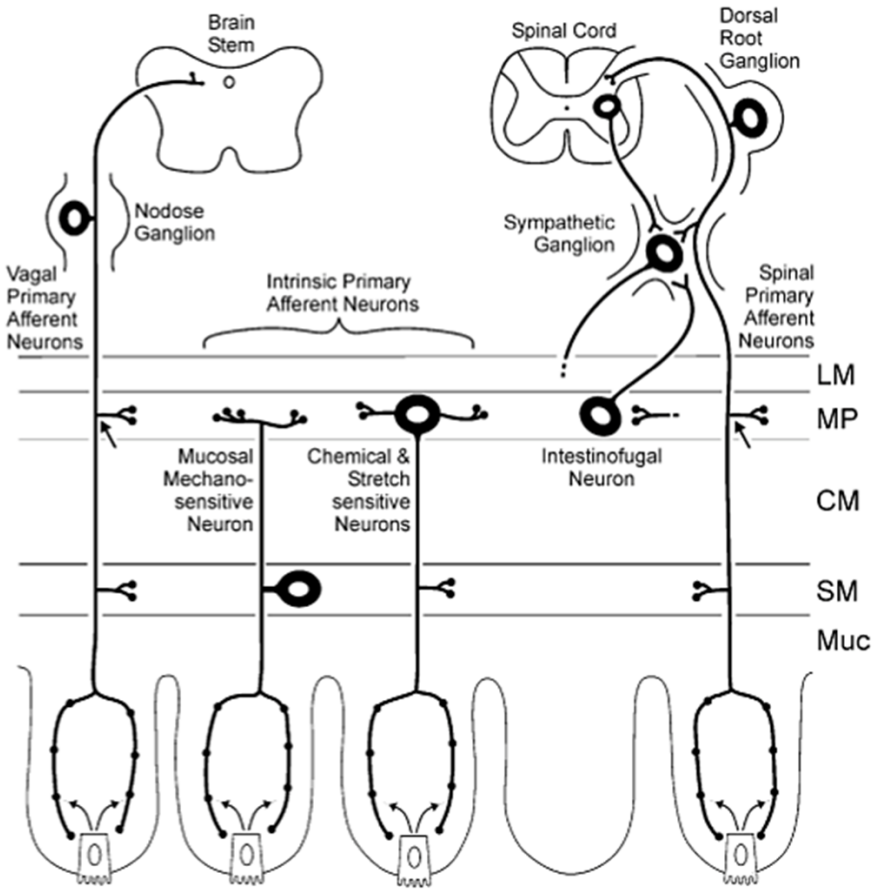

Figure 1.5: The afferent neurons of the digestive tract. There are two classes of intrinsic primary afferent neurons (IPNAs): myenteric IPANs responding to mechanical or chemical stimulation of the fibers innervating the mucosa and submucosa IPANs that detect mechanical distortion of the mucosa and luminal chemistry. Extrinsic primary afferent neurons have cell bodies in dorsal root ganglia (spinal primary afferent neurons) and vagal (nodose and jugular) ganglia. Spinal afferent neurons supply collateral branches in sympathetic ganglia and in the gut wall. Intestinofugal neurons are part of the afferent limbs of entero-enteric reflex pathways. LM: longitudinal muscle, CM: myenteric plexus, CM: circular muscle, SM: submucosa, Muc: mucosa. Modified from Furness, JB. The Enteric Nervous System (Balckwell, Oxford, 2006).

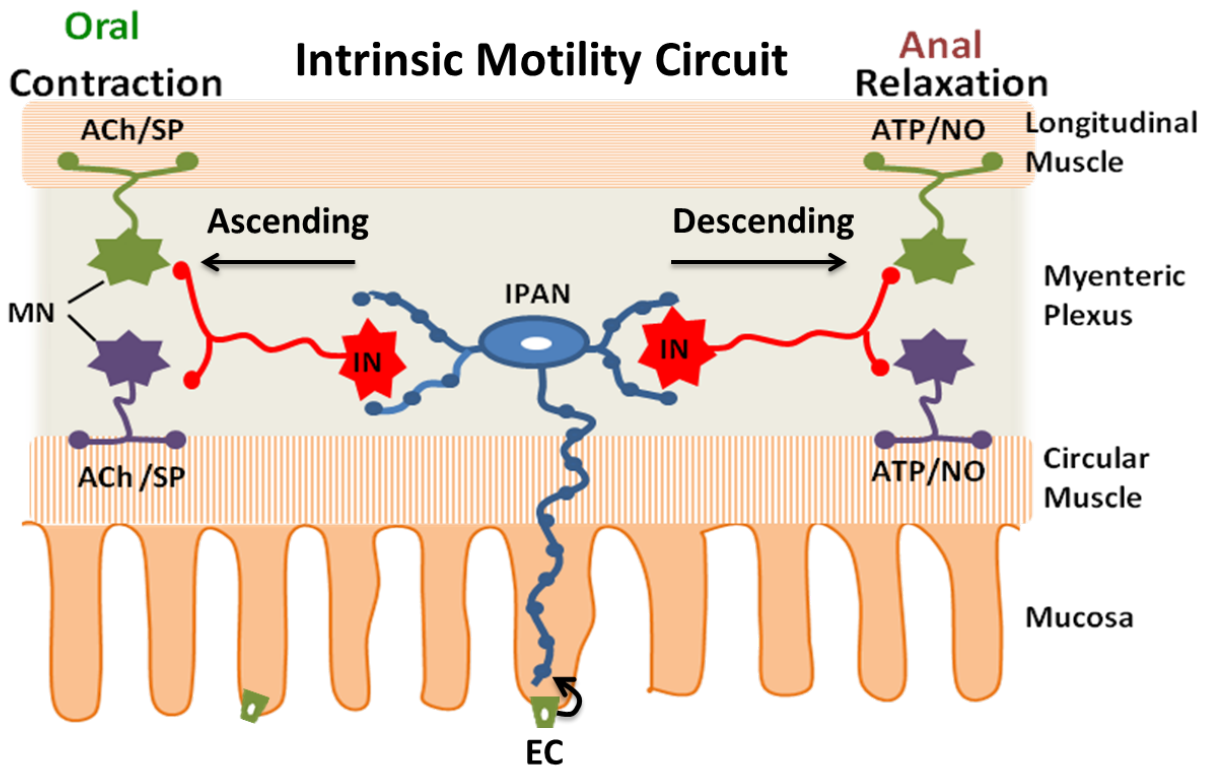

Figure 1.6: Propulsive reflex neuronal circuit in the intestine. Motility is initiated through IPANs, which connect interneurons that run orally (ascending) and anally (descending) within the myenteric plexus and also connect directly with motor neurons (not shown). The interneurons connect with motor neurons that innervate the muscle layers. The peristaltic reflex is initiated by either chemical or mechanical stimulation of the mucosa. This produces 5-HT release from entero-chromaffin cells, which acts on 5-HT receptors on the mucosal IPANs whose cell bodies reside in the myenteric. In the myenteric plexus, IPANs make an excitatory synaptic connection with ascending and descending projecting interneurons using Ach and SP as the neurotransmitters (Johnson et al., 1996). IPANs release ACh to activate ascending interneurons. Ascending interneurons release ACh to stimulate excitatory motor neurons (LePard and Galligan, 1999), which release both acetylcholine and substance P producing muscular contraction at the oral side (Jonhson et al., 1996). IPANs release both ACh and ATP to activate descending interneurons (Johnson et al., 1996). Descending interneurons release both ACh and ATP to

Figure 1.6 (cont'd)

activate inhibitory motor neurons (LePard and Galligan, 1999), which in turn release neurotransmitters such as nitric oxide and ATP (or a related purine) producing muscular relaxation at the anal side (Grider, 2003; De Gallego et al., 2008; Mutafova-Yambolieva et al., 2007). The contraction on the oral side and relaxation on the anal side generates the pressure gradient required to propel content along gut. IPAN: intrinsic primary afferent neurons. IN: interneuron, MN: motor neurons (excitatory or inhibitory), EC: entero-chromaffin cells, ACh: acetylcholine, SP: substance P, NO: nitric oxide, ATP: adenosine triphosphate

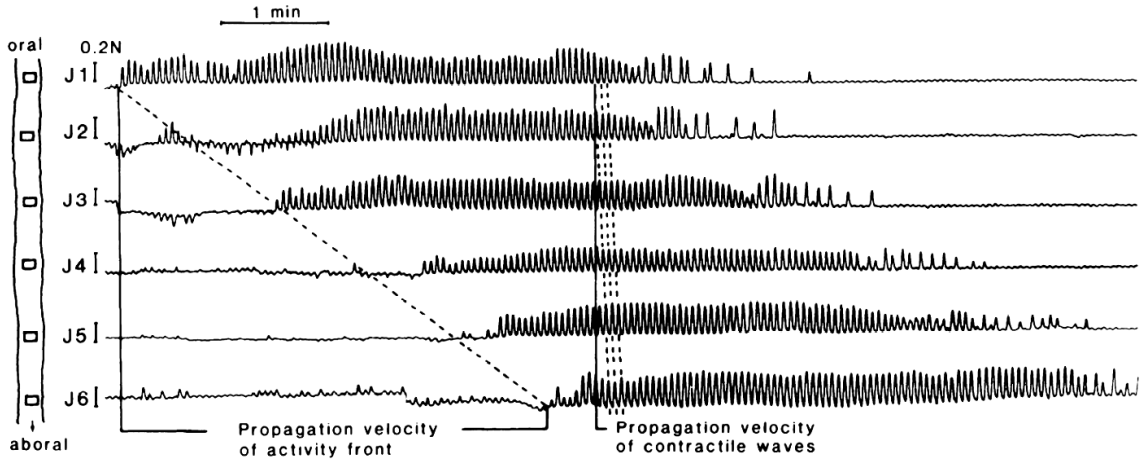

Figure 1.7: Representation of the migrating myoelectric complex (MMC). This motor pattern was recorded from closely spaced electrodes in the dog jejunum. The MMC progresses slowly along the intestine. This pattern consists of super imposed fast-moving contractions. The contractile waves are initiated at progressively more distal (anal) regions as the complex advances anally. Reproduced from Ehrlein et al. 1987.

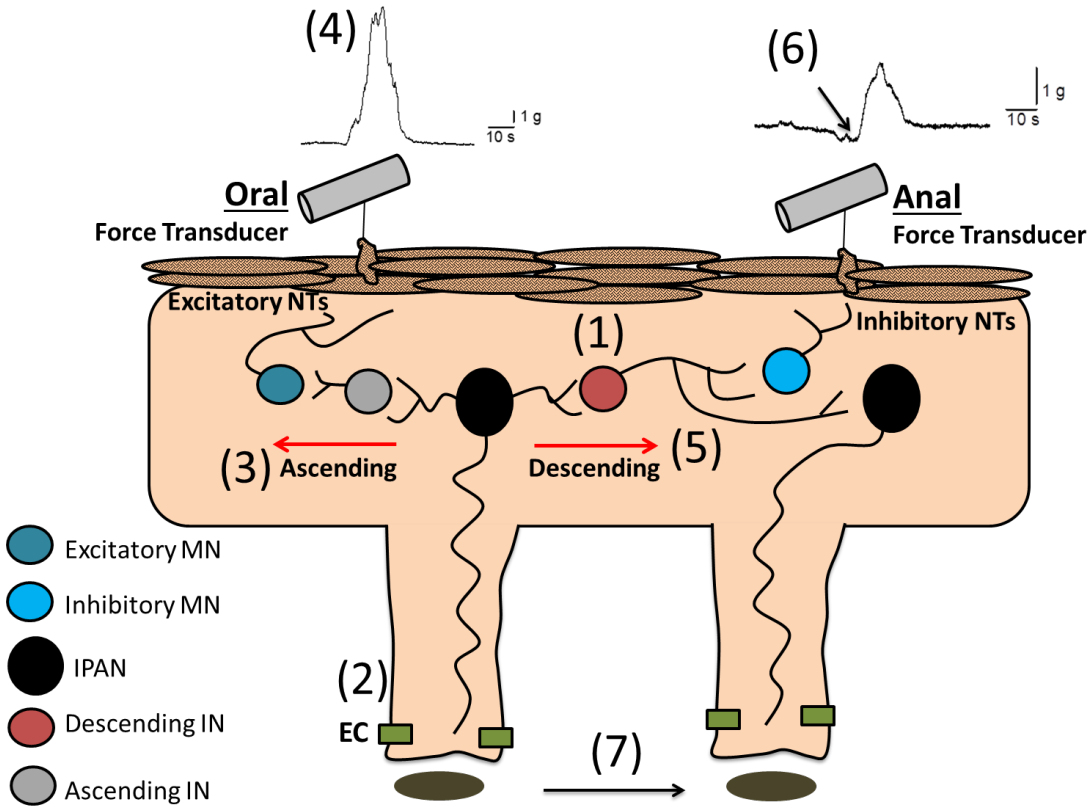

Figure 1.8: Proposed model for the enteric neural circuitry underlying generation of the CMMC. (1) Tonic inhibition to the circular muscle is provided by spontaneously active descending interneurons containing 5-HT. This provides a continuous activation of inhibitory motor neurons. (2) Mechanical or chemical stimulation of the mucosa induces release of 5-HT from EC cells activating 5-HT_3 receptors on the mucosal endings of IPANs. This will induce action potential firing in IPANs. (3) Activation of IPANs leads to synaptic stimulation of ascending interneurons. (4) This, in turn activates excitatory motor neurons producing contraction of the circular muscle at the oral side. (5) Activation of IPANs will also lead to synaptic stimulation of descending interneurons. (6) Consequently, inhibitory motor neurons will be further activated and a relaxation of the circular muscle will be produced at the anal side. This relaxation determines the direction of propagation. (7) The contraction at the oral side and relaxation at

Figure 1.8 (cont'd)

the anal side (in relation to fecal pellet location) will move the pellet anally similar to the peristaltic reflex observed in the small intestine. At the new location, the pellet will start the cycle (Dickson et al 2010; Dickson et al. 2009, 2009; Bayguinov 2010; Smith et al. 2014).

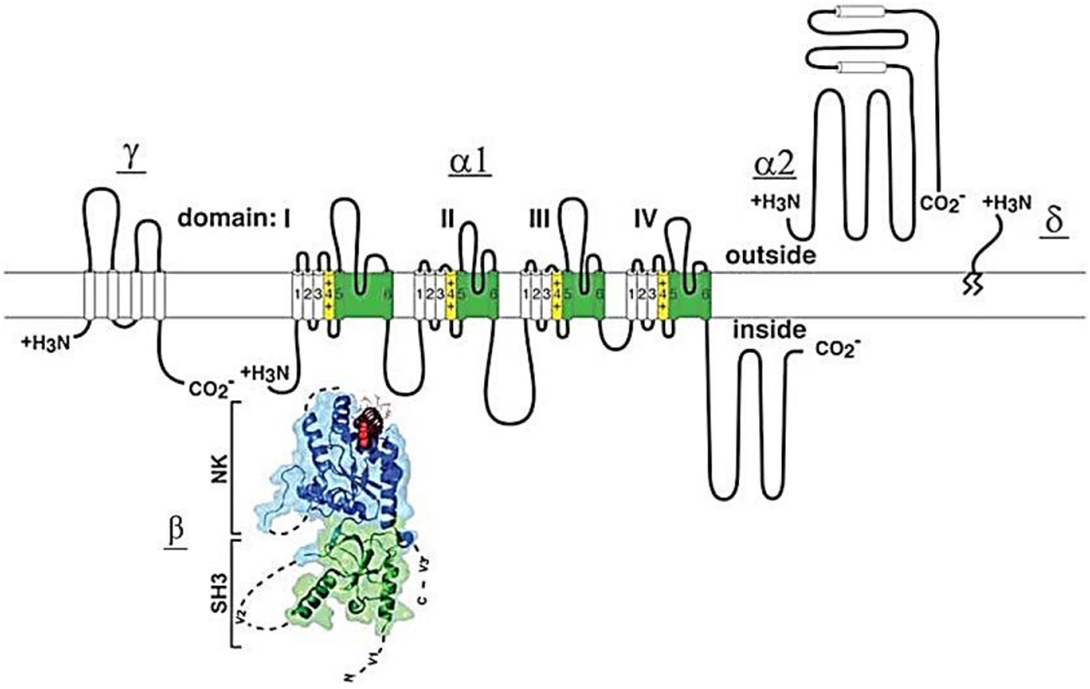

Figure 1.9: Molecular structure of the alpha1 subunit of voltage-gated Ca^{2+} channels. The amino acid residues are organized into four domains (I-IV). Each domain contains six transmembrane segments (S1-S6) and a membrane associated loop between transmembrane segments S5 and S6. This region is depicted in green color and is especially important for forming the pore of the channel. The segment S4 within each of the four domains is the voltage sensor and under the influence of an electrical field it moves outward and rotates initiating a conformational change that open the pore of the channel. The intracellular β subunits consists of α helices but no transmembrane segments. The α2 subunit was identified to be an extracellular glycoprotein attached to the membrane through a disulfide linkage to the δ subunit. The latter is anchored to the membrane through a glycophosphatidylinositol membrane anchor. The γ subunit of the skeletal VGCCs is glycoprotein with four transmembrane segments. However this subunit might not be a component of VGCC in other parts of the nervous system such as the brain where related γ-like proteins are modulators of AMPA receptors. Modified from Catterall and Leal, 2013.

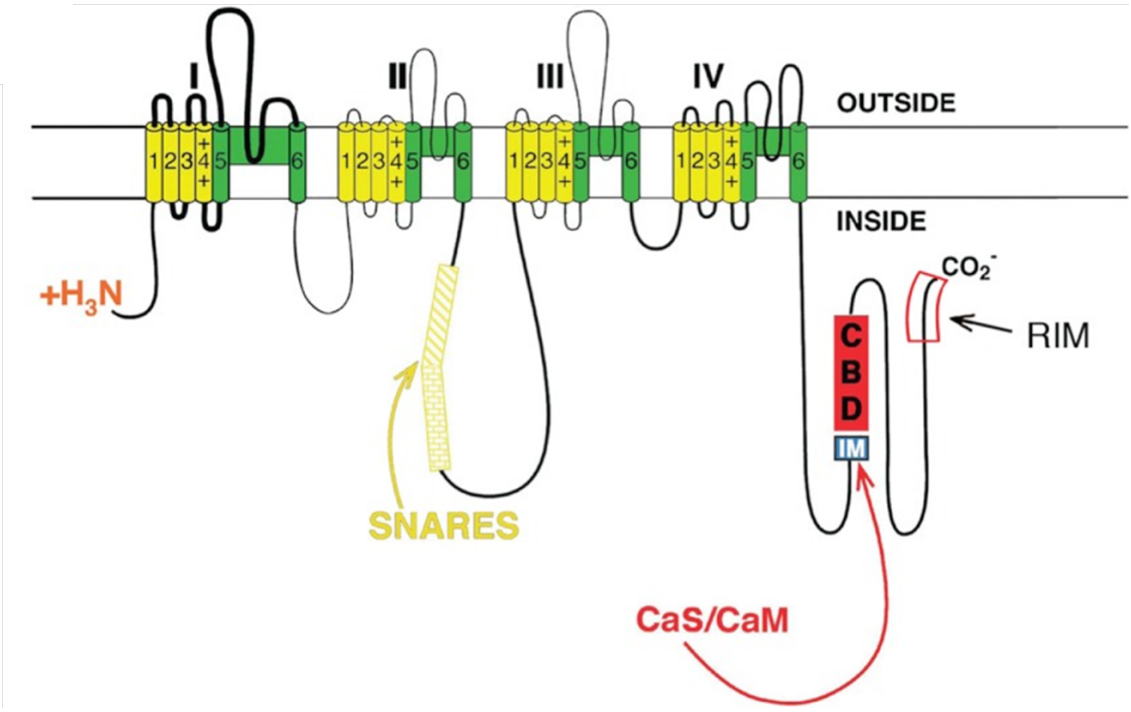

Figure 1.10: Alpha 1 subunit structure including the *synprint* site and CBD interacting domains. The *synprint* site refers to a **syn**aptic **pr**otein **int**eraction site located in the intracellular loop between domains II and III. SNARE proteins (Syntaxin-1 and SNAP-25) and this interaction regulate expression, localization, and function of Cav2. This interaction is Ca^{2+}-dependent with maximal binding at 20 µM Ca^{2+} and reduced binding at either lower or higher concentrations. Cav2.1 channels have an analogous *synprint* site and different channel subtypes have distinct associations with syntaxin-1 and SNAP-25. The SNARE-interacting protein RIM binds to the distal C terminus of Cav2.1 channels in order to recruitment of Cav2 channels to active zones. The Ca^{2+} sensor proteins and calmodulin (CaM) interact with the CBD and IM domains to regulate facilitation and inactivation of Ca channels (see text). Modified from Catterall and Leal, 2013.

CHAPTER 2: HYPOTHESIS & SPECIFIC AIMS

OVERALL GOALS

Gastrointestinal (GI) disorders are characterized by structural or biochemical alterations, which are characteristic of inflammatory and infectious diseases. However, GI disorders can also be caused by functional alterations without underlying structural abnormalities and these are known as functional GI disorders. The latter comprises about 41% of the total GI complications seen by physicians in the United States and altered motility of the GI muscles is a hallmark characteristic of these disorders. The enteric nervous system controls motility of GI muscles through an intrinsic neural circuit consisting of afferent neurons, interneurons, and excitatory and inhibitory motor neurons. Voltage-gated Ca channels (VGCCs) tightly regulate neurotransmitter release and as such these channels could play a critical role in modulation of intestinal motor patterns. Thus, within the enteric motility circuit there are multiple sites where VGCCs could be targeted in order to modulate intestinal motility patterns. However, the contribution of different VGCC subtypes within the motility circuit is not very well understood. It is established that N-type VGCCs contribute to neurotransmitter release within the enteric ganglia and at the enteric neuromuscular junction. Therefore, N-type channels are physiologically essential in order to maintain normal intestinal function. Conversely, the physiological role and significance of other VGCC subtypes, such as P/Q-type and R-type is not clearly settled. Interesting, alterations in the function of P/Q-type VGCCs are associated with neurological disorders characterized not only by voluntary motor dysfunction but also autonomic alterations that include GI motor dysfunctions. This line of evidence provides a basis for studying the role and relevance of non-N-type VGCCs in enteric transmission. Thus, the overall goals of these studies are to provide detailed insight into the physiological role and significance of P/Q- and R-type VGCCs in enteric neuromuscular transmission and investigate potential homeostatic synaptic changes in response to deficits in VGCC subtypes.

OVERALL HYPOTHESIS & AIMS

Overall Hypothesis

These studies tested the hypothesis that deficit in the function of either P/Q-type or R-type VGCCs will alter intestinal propulsive motility by disrupting enteric neuromuscular transmission. This overall hypothesis was tested through completion of the following specific aims.

Specific Aim 1

Myenteric neurons controlling express R-type Ca^{2+} currents and they are involved in ascending ganglionic transmission. However, the contribution of R-type channels during neuromuscular transmission is not established. Thus, these studies tested the hypothesis that R-type Ca^{2+} channels contribute to neuromuscular transmission to the small intestine.

Specific Aim 2

Preliminary studies showed that R-type Ca^{2+} channels contribute to inhibitory neuromuscular transmission to the intestines. However, the physiological relevance of R-type Ca^{2+} channels in controlling intestinal propulsive motility is not yet established. These studies tested the hypothesis that genetic deletion of R-type Ca^{2+} channels will reduce inhibitory neuromuscular transmission to the colonic muscle disrupting colonic propulsive motility.

Specific Aim 3

P/Q-type Ca^{2+} channels participate in excitatory neuromuscular transmission; however, their role in inhibitory transmission is not completely settled. These studies tested the hypothesis that a loss-of-function mutation in P/Q-type Ca^{2+} channels will disrupt neuromuscular transmission and colonic propulsive motility.

CHAPTER 3: R-TYPE VOLTAGE-GATED CALCIUM CHANNELS CONTRIBUTE TO RELAXATION OF THE LONGITUDINAL MUSCLE IN THE GUINEA PIG ILEUM

ABSTRACT

Myenteric neurons, which control motility of the intestines, express R-type Ca^{2+} currents and they are involved in fast cholinergic ganglionic transmission within the ENS. However, the contribution of R-type Ca^{2+} currents to neuromuscular transmission is not yet established. Thus, these studies tested the specific hypothesis that R-type Ca^{2+} channels contribute to neuromuscular transmission to the longitudinal muscle of the guinea pig ileum. Isometric mechanical responses of the longitudinal muscle myenteric plexus (LMMP) preparations were studied. Neurogenic relaxations and contractions were evoked by trains (20 Hz, 1 s) of transmural electrical field stimulation (EFS). The effect of NLA (NOS inhibitor), $NiCl_2$ (R-type Ca^{2+} channel blocker), CTX-GVIA (N-type Ca^{2+} channel blocker), apamin (SK_{Ca} channel blocker), and MRS 2179 (P2Y1 receptor antagonist) on nerve-evoked mechanical responses of the LMMP preparation was investigated. Intracellular recordings were employed to record nerve-evoked junction potentials from LMMP preparations. The effect of $NiCl_2$ (R-type Ca^{2+} channel blocker), CTX-GVIA (N-type Ca^{2+} channel blocker), and MRS 2179 (P2Y1 receptor antagonist) on the electrical responses of the LMMP preparation was investigated. $NiCl_2$ and NLA produced a concentration dependent inhibition of the longitudinal muscle relaxation and co-application did not produce a greater inhibition than that observed by either drug alone. A similar pattern was observed when non-cholinergic contractions were studied. Both $NiCl_2$ and NLA potentiated non-cholinergic contractions and co-application did not produce a further effect than that caused by either drug alone. Apamin and CTX-GIVA but not MRS 2179 inhibited the relaxation of the muscle. Nerve-evoked junction potentials consisted of a small hyperpolarization followed by a long lasting depolarization. $NiCl_2$ treatment decreased the hyperpolarization leaving unaltered the depolarization. On the other side, MRS 2179 treatment decreased the depolarization without affecting the hyperpolarization. Immunoreactivity for the α1 subunit of the R-type channel (α1E) was localized in the nerve varicosities tertiary plexus and some of these co-localized with immunoreactivity for NOS. Taking together these studies provide

evidence for contribution of R-type Ca^{2+} channels in neuromuscular transmission to the longitudinal muscle. Specifically, the R-type Ca^{2+} currents may couple to NOS activation to nitrergic relaxation of the muscle. Furthermore, activation of purinergic P2Y1 receptors might be coupled to excitatory and not inhibitory pathways to the longitudinal muscle of the guinea pig ileum.

INTRODUCTION

Non-adrenergic non-cholinergic (NANC) nerves reside within the myenteric plexus and activation of these neurons is responsible for inhibitory neuromuscular transmission to the longitudinal and circular smooth muscle layers. This type of transmission produces hyperpolarization of the smooth muscle membrane potential and contributes to relaxation of these muscle layers. Pharmacological and electrophysiological studies revealed that there are several inhibitory neurotransmitters released from NANC nerves and several post-junctional signaling mechanisms linked to smooth muscle relaxation.

In general, inhibitory neuromuscular transmitters in the enteric nervous system (ENS) include nitric oxide (NO) (Sanders et al. 1992; He et al. 1993; Osthaus et al. 1992; Smits et al. 1996), purinergic neurotransmitters which identity is still under current investigation but has been suggested to be either ATP (Smits et al. 1996; Crist et al. 1992; De Man et al. 2003, Burnstock et al. 2008) or β-nicotinamide dinucleotide (β-NAD) (Mutafova-Yambolieva et al. 2007). In addition, peptides such as vasoactive intestinal peptide (VIP) have been shown to be involved in inhibitory transmission to the muscle layers (Crist et al. 1992; Grider et al. 1990). The purinergic neurotransmitter acts at $P2Y_1$ receptors on GI smooth muscle (Benko et al. 2007; Wang et al. 2007; Gallego et al. 2006) and interstitial cells of Cajal (Ward et al. 2001). Activation of $P2Y_1$ receptor is linked to Ca^{2+} release from intracellular stores producing opening of apamin-sensitive small conductance Ca^{2+}-activated K^+ channels (SK_{ca}) (Pluja et al. 1999). Activation of SK_{ca} leads to a high-amplitude transient hyperpolarization known as the fast inhibitory junction potential (fIJP) (Benko et al. 2007; Wang et al. 2007; Gallego et al. 2006; Pacaud et al.

1996; Zhang et al. 2010). NO production requires Ca^{2+}-dependent activation of the neuronal isoform of nitric oxide synthase (nNOS) (Mashimo et al. 1996; Rao et al. 2008; Chaudhury et al. 2009). NO diffuses from inhibitory nerve terminals to target smooth muscle cells or ICC where it activates soluble guanylate cyclase causing cyclic guanosine 5- triphosphate (cGMP) formation. cGMP activates protein kinase G which can target K^+ and Ca^{2+} channels to favor hyperpolarization or it can modulate the dynamic of the actin filaments and thus directly regulates mechanical relaxation (Lino et al. 2009; De Man et al. 2007). VIP is also released from inhibitory motor neurons in a Ca^{2+}-dependent manner and VIP receptors activate the adenylate cyclase-protein kinase A signaling pathway to cause smooth muscle relaxation (Rekik et al. 1996; Makhlouf and Murthy, 1997).

In the longitudinal muscle of the guinea pig ileum NO has a prominent role in mediating the relaxation while ATP contributes via smooth muscle P_2 purinoceptors to contractions (Patel et al. 2010; Zizzo et al. 2007; Ivancheva et al. 2001; Matsu et al. 1997; Osthaus et al. 1992). Some of these studies had directly suggested a post-junctional antagonism between NO and ATP in the longitudinal muscle of the distal regions of the small intestine (Ivancheva et al. 2001). In the circular muscle of the guinea pig ileum both NO and ATP contribute to inhibitory neuromuscular transmission (He and Goyal, 1993; Crist et al. 1992). A similar interaction between ATP and NO in the colonic muscle has been observed. In proximal regions of the colon ATP and NO contribute to inhibitory pathways to both longitudinal and circular muscle layers (Rozsai et al. 2006). A change in the dynamics between NO and ATP is observed when more distal regions of the colon are investigated. In the longitudinal muscle of mouse distal colon ATP is involved in excitatory neuromuscular transmission (Zizzo et al. 2007, 2008) while in the circular muscle both ATP and NO contribute to relaxation mechanisms of this muscle layer (Zizzo et al 2006; Zagorodnyuk and Maggi, 1994). These studies clearly showed the complexity of the interactions between enteric neurotransmitters controlling motor activity of the intestinal muscle layers.

Release of all neurotransmitters depends on activation of VGCC expressed by nerve terminals. R-type ($Ca_V2.3$) (Bian et al. 2004, 2007), N-type ($Ca_V2.2$), P/Q type ($Ca_V2.1$), but not L-type ($Ca_V1.2$) VGCC contribute to neurotransmitter release from enteric neurons (Takahashi et al. 1992; Lippi et al. 1998; Cunningham et al. 1998; Kirchgessner et al. 1999; Reis et al. 2002, 2000). From all the known VGCC subtypes the role and physiological significance of R-type channels are the least understood. However, studies have shown that R-type Ca^{2+} channels are involved in enteric ganglionic transmission within the myenteric plexus. For example, R-type Ca^{2+} currents couple to fast synaptic excitation in ascending pathways in the myenteric plexus where acetylcholine is the excitatory neurotransmitter (Naidoo et al. 2010). Fast synaptic excitation in descending pathways in the myenteric plexus use acetylcholine and ATP as the excitatory neurotransmitters with both N- and P/Q-type Ca^{2+} channels subserving their release (Naidoo et al. 2010). These studies demonstrate that N-, P/Q-, and R-type VGCC regulate neurotransmitter release within the ganglionic neuronal circuit controlling motility of the intestines.

At the neuromuscular junction the role of different VGCC during inhibitory neuromuscular transmission is not well understood. Studies had used ω-conotoxin GVIA (CTX-GVIA) to investigate the role of N-type VGCC in inhibitory neuromuscular transmission. Overall, these studies have shown that there are two main components in this type of transmission: one sensitive and another resistant to CTX-GVIA (see review from Waterman, 2000). These studies revealed the importance of N-type VGCC in inhibitory pathways to the muscle. However, they also clearly showed that N-type channels are not the only VGCC subtype involved in inhibitory neuromuscular transmission. In this line, the specific contribution of other VGCC subtypes such as R-type and P/Q-type to inhibitory neuromuscular transmission has not yet been established in the literature.

The main objective of this study is to investigate the contribution of R-type VGCC during inhibitory neuromuscular transmission to the longitudinal muscle layer of the guinea pig ileum. This will provide important insight into the functional role of R-type VGCC in enteric neuromuscular transmission.

In addition, this study aimed to re-examine the contribution of SK_{ca} channels and purinergic transmission to the longitudinal muscle.

MATERIALS AND METHODS

Longitudinal muscle-myenteric plexus (LMMP) preparation

All animal use protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Michigan State University. Adult male Hartley-strain guinea pigs weighing 250-350 g (Emergent BioSolutions, Lansing, Michigan) were anesthetized using isoflurane inhalation (Abbott Laboratories, Chicago, IL), stunned and exsanguinated. The ileum was removed and placed in oxygenated (95% CO_2, 5% CO_2) Krebs solution of the following composition (millimolar): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose. A glass rod 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. One end of the LMMP preparation was mounted with silk ligatures to a stationary hook at the bottom of a tissue holder and the other end was connected to an isometric force transducer (Grass Instruments, FT03C, Quincy, MA, USA). The tissue holder also contained two platinum foil electrodes and the LMMP preparations were positioned between the electrodes. The tissue holder and attached LMMP were placed in a 20 ml jacketed organ bath containing oxygenated Krebs solution at 37 °C. A resting tension of 1 g was applied to each of the 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 sustained baseline resting tension (Bian et al. 2004). Neurogenic relaxations

(the muscarinic receptor antagonist scopolamine, 1 μM) was added to the Krebs solution) were induced by brief trains of transmural electrical stimuli (20 Hz, 0.5 ms pulse duration, 1 s train duration, 0.25 Hz train rate, 40 mV) (Osthaus and Galligan, 1992). To determine the action of antagonists on relaxations, non-cumulative concentration response curves were created with a 15-minute interval between successive doses.

Single electrical stimuli (0.1 Hz, 0.5 ms, 40 mV) were used to evoke neurogenic cholinergic contractions (scopolamine free Krebs solution) (Galligan, 1993). Trains of stimuli (20 Hz, 1 s, 0.5 ms pulse duration, 40 mv, 0.25 Hz train rate) were used to evoke non-cholinergic contractions (scopolamine, 1 mM present). To determine the action of antagonists on both types of contraction, non-cumulative concentration response curves were created with a 15-minute interval between additions of successive antagonist concentrations. Transmural electrical stimuli were provided by a Grass S48 stimulator and mechanical activity of the LMMP was recorded using Labscribe software (iWorx, Dover, NH, USA) and a personal computer.

Intracellular electrophysiological recordings

A 1.5 cm section of the ileum was cut open along the mesenteric border and pinned flat 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 Ca^{2+} channels on smooth muscle cells, respectively. The submucosa was removed to expose the LMMP. A 5 mm^2 section of the LMMP was then transferred to a smaller silicone elastomer-lined recording chamber (2 ml) with the circular muscle layer facing up. The chamber was mounted on a stage of a microscope (Olympus, Tokyo, Japan). The LMMP was pinned tautly and superfused with oxygenated (95% O_2, 5% O_2) Krebs' solution at a flow rate of 4 ml/min at 37 °C. The preparation was equilibrated for 40 min before commencing intracellular recordings.

Single smooth muscle cells 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. The stimulating electrode was positioned near the recording site and single stimuli (0.5 ms duration) and short trains of stimulation (20 Hz, 1 s, 40 mV), 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 smooth muscle cells was allowed to stabilize for 10 minutes. Signals were recorded using an A/D converter (Digidata 1322A, Molecular Devices Corp., Sunnyvale, CA), Axoscope 10 software (Molecular Devices), and a desktop computer. Amplified signals were sampled at 2 kHz and filtered at 1 kHz. We 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 during drug treatment.

Immunohistochemical studies

Ileal segments were cut open along the mesenteric border, stretched and pinned flat in a silcone elastomer-lined Petri dish, which was then filled with paraformaldehyde fixative. Tissues were fixed overnight (4 ^{o}C) and then washed three times with 0.01 M PBS at 10-min intervals. Whole-mount LMMP preparations were dissected and then incubated in 4% normal donkey blocking serum diluted in 0.01 M PBS containing 0.3% Titon X-100. All primary antiserum were diluted to 1:200 in PBS containing 0.3% Triton X-100. Tissues were incubated with primary antibodies overnight at room temperature. NOS immunoreactivity was localized using a sheep polyclonal antibody (Millipore, USA, Cat. No: AB1529). A rabbit polyclonal α1E antiserum was used to localize $Ca_V2.3$ (Alomone laboratories,

Jerusalem, Cat. No. ACC-006). The antibody was raised against the intracellular loop connecting domains II and III of the rat $Ca_V2.3$ protein. After overnight incubation, unbound antibody was rinsed away with 3 washes of PBS at 10 min intervals. The preparations were then incubated in fluorescein isothiocyanate (FITC) (1:40 dilution in PBS containing 0.3% Triton X-100) conjugated secondary antibodies (Jackson Immunochemicals, West Grove, PA, USA) for 2 h at room temperature. The tissue was washed in PBS 3 times at 10 min intervals and then mounted in buffered glycerol (pH = 8.6) for fluorescence microscopy. Staining was viewed using a Nikon fluorescence microscope (model TE 2000-U; Nikon Corporation, Tokyo, Japan) and MetaMorph Software (Molecular Devices, Sunnyvale, CA, USA) or a Leica TCS SL laser scanning confocal microscope using a Leica DMLFSA microscope body (Leica Microsystems Inc., Buffalo Grove, IL USA).

Drugs

All drugs were obtained from Sigma-Aldrich (St Louis, MO), except ω-conotoxin GVIA (ω-CTX GVIA), ω-agatoxin ATX IVA (ω-ATX IVA) and SNX-482 (Alomone Labs, Jerusalem, Israel). Drugs were dissolved in deionized water with the exception of ω-nitro-L-arginine (NLA), which was dissolved in HCl (0.1 N) and nifedipine, which was dissolved in 95% ethanol.

Data Analysis

Data are presented as the mean ± S.E.M. "*n*" refers to the number of animals from which tissues were obtained. Data were analyzed using a one-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Transmural nerve stimulation in histamine (1 μM) pre-contracted LMMP preparations (scopolamine 1 μM present) produced a relaxation followed by a slowly developing rebound contraction in most preparations (figure 3.1 A). The relaxation and rebound contraction were both blocked by tetrodotoxin (TTX, 0.3 μM) (not shown). The non-selective Ca^{2+} channel blocker $CdCl_2$ produced a concentration-dependent and complete inhibition of the relaxation and rebound contraction (figure 3.1 A, B) with an IC_{50} value of 5.6 ± 1.2 μM (n = 7).

$NiCl_2$ and ω-nitro-L-arginine (NLA) inhibited neurogenic relaxations

At concentrations ≤ 50 μM, $NiCl_2$ can selectively block R-type Ca^{2+} channels (Wu et. 1998; Wang et al. 1999; Tottene et al. 2000; Gasparini et al. 2001). We found that $NiCl_2$ (0.1-100 μM) caused a concentration-dependent inhibition of neurogenic LMMP relaxations (figure 3.2 A). The NOS inhibitor, NLA (0.1–100 μM), also reduced the peak relaxation with a maximum inhibition of 20 ± 5%. Co-application of $NiCl_2$ with NLA inhibited the relaxation amplitude by 21 ± 5%. There were no differences in the concentration response curves for $NiCl_2$, NLA or $NiCl_2$ + NLA for inhibition of the NANC relaxations ($P > 0.05$) (Figure 3.2 A).

While $NiCl_2$ blocks R-type Ca^{2+} channels, it could also block other Ca^{2+}-dependent mechanisms to alter smooth muscle tone. Therefore, we tested the actions of $NiCl_2$ on cholinergic contractions of the LMMP caused by single electrical stimuli applied at 0.1 Hz (Galligan, 1993). These responses were blocked by scopolamine (1 μM) indicating that they are mediated by acetylcholine acting at muscarinic receptors on the longitudinal muscle (not shown) (Galligan, 1993). $NiCl_2$ (1-100 μM, n=6) did not alter cholinergic contractions while $CdCl_2$ (1-100 μM) produced a concentration dependent inhibition of the same responses (figure 3.2 B); the IC_{50} was 8.2 ± 1.4 mM (n=6). We further verified the specificity of $NiCl_2$ for NANC relaxations by testing its effects on non-cholinergic contractions evoked by short trains of stimulation (10 Hz, 1 s) in the presence of scopolamine (1 μM) to block muscarinic receptors. These

non-cholinergic contractions are mediated predominately by substance P acting at neurokinin type 1 receptors (Galligan, 1993). $NiCl_2$ produced a concentration-dependent (EC_{50} = 24 ± 1.1 mM, n=3) *increase* in the amplitude of the non-cholinergic contractions (figure 3.2 C). $CdCl_2$ (IC_{50} = 35 ± 9.7 µM, n=6) and the N-type VGCC blocker ω-CTX (IC_{50} = 6.9 ± 2.4 nM, n=3) both inhibited the non-cholinergic contractions (figure 3.2 C). This data suggests that $NiCl_2$-sensitive R-type VGCC contribute to NANC transmission specifically in relaxation pathways to the longitudinal muscle since blocking these channels produced potentiation of non-cholinergic contractions and inhibition of relaxations.

$NiCl_2$ potentiates non-cholinergic contractions to a similar extent as NLA and apamin

Treatments targeted to inhibit or block NOS and SK_{ca} channels have been shown to potentiate contractile activity in the muscle due to the fact that they both contribute to inhibitory neuromuscular transmission and thus relaxation mechanisms. Therefore, to further investigate the role of $NiCl_2$-sensitive R-type VGCCs in relaxation mechanisms to the longitudinal muscle the effect of $NiCl_2$ (50 µm), NLA (100 µm), and apamin (0.1 µm) was tested on non-cholinergic nerve-evoked contractions. In these experiments, the AUC of the contractions was measured because drug treatments increased both the amplitude and duration of these responses. $NiCl_2$ increased the AUC by 147% (figure 3.3 A, B; $P < 0.05$) and cumulative addition of NLA did not produce a further increase of the contraction. Subsequent addition of apamin produced a 53% increase in the AUC of the contractions. The magnitude of this effect was statistically greater than the effect of $NiCl_2$ and $NiCl_2$ + NLA (figure 3.3 B; $P<0.05$ for n = 10). In the following experiments the sequence of drug application was changed. NLA applied first increased contraction AUC by 118% (figure 3.3 C, $P<0.05$) while addition of $NiCl_2$ did not produce a further increase in the contraction. Cumulative addition of apamin caused an additional 37% increase in contraction AUC, which was greater than the effect of NLA and NLA + $NiCl_2$ (figure 3.3 C, $P<0.05$). Apamin applied first increased the contraction AUC by 95% (figure 3.3 D, $P<0.05$). Cumulative addition of NLA increased

the AUC by an additional 42% ($P < 0.05$) while subsequent addition of $NiCl_2$ did not produce a further increase over that caused by apamin and NLA (fgure 3.3 D). Overall, $NiCl_2$ and NLA produced a potentiation of the non-cholinergic contractile activity through a common pathway because their effects were occluded by each other. Apamin also potentiates the contractions; however, the data suggest it does it through an additional mechanism not including the targets of $NiCl_2$ or NLA.

SK_{ca} channels and N-type VGCC contribute to relaxation of the longitudinal muscle

The contraction experiments described above suggested that apamin-sensitive SK_{ca} channels contribute to relaxation through a mechanism not including NOS or $NiCl_2$-sensitive R-type VGCC. To further investigate this observation in a more direct manner, the effect of apamin was studied on nerve-evoked relaxations of the longitudinal muscle. The relaxations were evoked as described for figure 3.1 and 3.2 A. Co-application of NLA and $NiCl_2$ significantly inhibited the relaxation by 62%. Cumulative application of apamin abolished the remaining relaxation and this effect was statistically significant when compared to NLA/$NiCl_2$ effect ($P<0.05$) (figure 3.4B). Similar results were obtained when apamin was applied first. Addition of apamin produced a significant inhibition of the relaxation by about 36%. Cumulative application of NLA/$NiCl_2$ abolished the apamin-resistant relaxation and this effect was statistically significant ($P<0.05$) (figure 3.4 C). Taking together this result demonstrate that activation of SK_{ca} channels contribute to relaxation of the longitudinal muscle through a pathway different from the one used by $NiCl_2$-sensitive R-type VGCC and NOS.

A similar experiment was conducted to investigate whether or not N-type VGCCs contribute to relaxation through the pathway targeted by NLA/$NiCl_2$. If that is the case, we should expect no inhibitory effect of ω-CTX-GVIA when this toxin is applied after NLA/$NiCl_2$ treatment. NLA/$NiCl_2$ once again produced a significant inhibition of the relaxation of about 74% inhibition ($P<0.05$). Cumulative

application of ω-CTX-GVIA abolished the resistant relaxation and this effect was statistically significant ($P<0.05$) (figure 3.5 A and B). ω-CTX-GVIA by itself produced a significant 46% inhibition of the relaxation and sequential application of NLA/$NiCl_2$ abolished the ω-CTX-GVIA-resistant relaxation (figure 3.5 C). This piece of data suggests that N-type VGCC contribute to relaxation of the longitudinal muscle in a general fashion. The results suggest that N-type VGCC do contribute to nitrergic relaxation because the inhibitory effect of ω-CTX-GVIA and NLA/$NiCl_2$ when combined surpasses 100% (add 74% plus 46% for NLA/NiCl2 and CTX-GVIA, respectively). However, activation of N-type VGCC is not compromised to NOS activation. It is possible that activation of N-type VGCC regulate the release of other inhibitory neurotransmitters such as the purinergic molecule or peptides such as VIP in addition to contribute to NOS activation.

Purinergic neuromuscular transmission is not critical for relaxation of the longitudinal muscle

Contribution of purinergic neuromuscular transmission to relaxation of the longitudinal muscle was investigated using the P2Y1 receptor antagonist, MRS 2179 (10 μM). Blocking of P2Y1 receptors with MRS 2179 produced about 9% inhibition of the relaxation and this effect was not statistically significant ($P>0.05$) (figure 3.6 A and B). As described by the previous results of the present study, sequential addition of NLA and apamin completely abolished the relaxation (figure 3.6 B). NLA by itself produced about 61% inhibition of the relaxation; effect considered statistically significant ($P<0.05$) (figure 3.6 C). Sequential application of MRS 2179 did produce a small inhibition that represented about 38% of the NLA-resistant relaxation. However, when the effect of MRS 2179 was compared to control relaxation the percent inhibition was about 15%; none of these effects were statistically significant (figure 3.6 C). This result suggested that activation of P2Y1 receptor, a critical component of purinergic neuromuscular transmission (Gallego et al. 2012), has a minor role in relaxation of the longitudinal muscle. The data also demonstrate the critical role of NOS activation for relaxation of the longitudinal

muscle and thus it shows that the predominant component of relaxation mechanisms in this muscle layer is nitrergic transmission.

N-type and R-type VGCC contribute to relaxation of the longitudinal muscle

In order to clearly investigate the contribution of N- and R-type channels to nitrergic relaxation of the longitudinal muscle the effect of ω-CTX-GVIA and $NiCl_2$ was evaluated. To obtain a predominantly nitrergic relaxation MRS 2179 was added to eliminate the small component mediated by activation of P2Y1 receptor. Under this condition, ω-CTX-GVIA produced a 43% inhibition of the relaxation and this effect was statistically significant ($P<0.05$) (figure 3.7 A and B). Sequential addition of $NiCl_2$ produced an additional significant inhibition of the relaxation of about 41%. Interesting, when $NiCl_2$ (50 μM) was added first it did not produce an inhibitory effect but sequential addition of ω-CTX-GVIA did inhibit the relaxation producing about 50% inhibition ($P<0.05$) (figure 3.7 C). This data suggested that both N- and R-type VGCC contribute to nitrergic relaxation of the longitudinal muscle. However, in this case the role of R-type is better appreciated when N-type VGCC contribution is pharmacologically eliminated.

Sensitivity of longitudinal muscle junction potentials to $NiCl_2$ and MRS 2179

Junction potentials from the longitudinal muscle were recorded to evaluate if $NiCl_2$-sensitive R-type VGCC play a role as it was observed in the mechanical relaxations. In addition, since P2Y1 receptors played such a minor role in the mechanical relaxation of the muscle, the effect of MRS 2179 on junction potentials was evaluated to identify any potential explanation. Longitudinal muscle junction potentials were evoked (20 Hz, 1 s train) in the presence of scopolamine (1 μm) to block muscarinic receptors. These junction potentials were composed of a small hyperpolarization (inhibitory junction potential or IJP) followed by a high-amplitude long lasting depolarization (excitatory junction potential or EJP) (figure 3.8 A). $NiCl_2$ (50 μM) produced a significant decrease in the amplitude of the IJP by about ~50% ($p<0.05$)

(figure 3.8 B). However, this treatment did not affect the EJPs (P>0.05) (figure 3.8 C). MRS 2179 (10 μM) produced a small inhibitory effect of the IJP but this was not considered statistically significant (P>0.05; figure 3.8 D). Surprisingly, MRS 2179 did produce a significant ~50% inhibition of the amplitude of the EJPs (figure 3.8 E). The mean resting membrane potentials of longitudinal muscle cell was not altered by either treatment (figure 3.9). Inhibition of EJP by blocking P2Y1 receptors explains the observed minor role of purinergic inhibitory transmission to the longitudinal muscle relaxation because it appears that activation of this receptor is coupled to depolarization of the muscle.

Localization of the α1E subunit of $Ca_V2.3$ in guinea pig ileum

Immunohistochemical studies revealed α1E immunoreactivity in varicose nerve fibers in myenteric ganglia and in the tertiary plexus that supplies the longitudinal muscle layer (Figure 3.10 A). Preparations were co-stained with the α1E antibody and a nNOS antibody. α1E- and nNOS-immunoreactivity were co-localized in nerve fibers in the tertiary plexus (Figure 3.10 B-G).

DISCUSSION

R-type and N-type VGCCs couple to nitrergic relaxation of the longitudinal muscle

This study investigated the contribution of R-type Ca^{2+} channels to excitatory and inhibitory neuromuscular transmission in guinea pig ileum. We used $NiCl_2$ as a tool to test the function of R-type Ca^{2+} channels because it blocks these channels somewhat selectively at concentrations ≤ 50 μM (Wu et al. 1998; Wang et al. 1999; Tottene et al. 2000; Gasparini et al. 2001). About 45% of the whole cell Ca^{2+} current recorded from myenteric neurons is R-type Ca^{2+} current and this was identified after addition of drugs blocking currents through L-, N-, and P/Q-type VGCCs (Bian et al., 2004). This myenteric R-type Ca^{2+} current is totally abolished by either $NiCl_2$ (50 μM) or the toxin SXN-482 (Bian et al. 2004). This study clearly shows that $NiCl_2$ at 50 μm can be used to study R-type VGCCs in the ENS.

In histamine pre-contracted tissues, electrical field stimulation caused transient relaxations followed by a rebound contraction. This pattern of responses confirms that electrical stimulation induce release of various neurotransmitters producing inhibitory and excitatory responses on the smooth muscle (Hoyle and Burnstock, 1989, Lundberg, 1996). TTX and $CdCl_2$, a non-selective Ca^{2+} channel blocker, abolished both responses indicating their neurogenic nature and dependence of Ca^{2+} influx. As shown previously in the longitudinal muscle of the guinea pig ileum the NOS inhibitor, NLA, produced a concentration dependent but incomplete inhibition of the neurogenic relaxation (Osthaus and Galligan, 1992). $NiCl_2$ produced a similar inhibitory effect on the neurogenic relaxation while combined application of $NiCl_2$ and NLA did not produce an effect that differed from either blocker alone. These data suggest that $NiCl_2$ and NLA act on a common mechanism to inhibit neurogenic relaxations of the longitudinal muscle. Since NOS is a Ca^{2+}-dependent enzyme, R-type VGCCs can provide the Ca^{2+} required for activation and production of NO to initiate the nitrergic relaxation of the longitudinal muscle.

Although $NiCl_2$ is a potent inhibitor of R-type Ca^{2+} channels, it is possible that it could directly alter muscle contractility or block other Ca^{2+} permeable channels. This issue was addressed in part by studying the actions of $NiCl_2$ on neurogenic contractions caused by single electrical stimuli of the LMMP. These contractions are mediated by acetylcholine acting at muscarinic receptors on the longitudinal muscle (Galligan, 1993). $NiCl_2$ did not alter the cholinergic contraction while both $CdCl_2$ and the N-type VGCC blocker, ω-CTX-GVIA, abolished this response. These data indicate that $NiCl_2$ does not have a generalized inhibitory effect on neurotransmitter release or muscle contractility. It is possible that $NiCl_2$ could interact directly with NOS to inhibit its function. Studies of nNOS activity in brain homogenates revealed that $NiCl_2$ could directly inhibit nNOS activity (Mittal et al. 1995). However, in these studies, nNOS inhibition only occurred at a very high $NiCl_2$ concentration, as the K_i value for $NiCl_2$ acting directly on nNOS was 360 μM. This is > 7-fold higher than the concentrations of $NiCl_2$ used in our studies to

inhibit neurogenic relaxations and therefore direct inhibition of nNOS is unlikely to account for the inhibitory effect of $NiCl_2$ observed in the present study.

ω-CTX-GVIA is a potent blocker of N-type VGCCs (D'Ascenzo et al. 2004) and we used this toxin to investigate the role of these Ca^{2+} channel subtype in the nitrergic relaxations of the longitudinal muscle. ω-CTX-GVIA produced a substantial inhibition of the relaxation with or without pre-inhibition of NOS with NLA. This suggested that N-type VGCC contribute to both the predominant nitrergic relaxation and also to the small residual relaxation resistant to NLA block. N-type Ca^{2+} channels contribution to neuromuscular transmission in gastrointestinal muscles has been extensively studied. In this line, the major contributions of N-type Ca^{2+} channels to release of both excitatory and inhibitory neurotransmitters from the stomach to the colonic smooth muscle have been reported (Waterman, 2000). An important observation reported by multiple studies is that inhibitory neuromuscular transmission has a component resistant to block of N-type VGCC suggesting involvement of others yet unidentified VGCC subtypes (De Lucca et al. 1990; Maggi et al. 1988; Cayabyab et al 1997; Borderies et al. 1997; Lundy and Frew, 1994; Boeckxstaens et al. 1993; Zagorodnyuk and Maggi, 1994; Humphreys and Costa, 1992; Watson et al. 1991). The present study provides insight about the identity of the unidentified VGCC contributing to inhibitory neuromuscular transmission. Evidence for the involvement of R-type VGCC in generating relaxation and hyperpolarization of the longitudinal muscle from guinea pig ileum is clearly demonstrated by the results presented in the current study. Accordingly to the data presented in this study both N-type and R-type VGCCs contribute to inhibitory neuromuscular transmission to the longitudinal muscle by providing the Ca^{2+} required for NOS activation, which leads to the nitrergic relaxation of this muscle.

Inhibition of R-type VGCCs potentiates non-cholinergic excitatory neuromuscular transmission

In our studies of neurogenic relaxation, it was found that the rebound contraction increased in amplitude and duration in the presence of $NiCl_2$ and NLA. As $NiCl_2$ and NLA reduced inhibitory neuromuscular transmission, simultaneous excitatory responses would be enhanced. We used the enhanced rebound contraction as an additional test for coincident sites of action of $NiCl_2$ and NLA on neuromuscular transmission. We also varied the sequence of drug application. $NiCl_2$ and NLA alone both increased the non-cholinergic contraction but the effects of these blockers did not work in a synergistic fashion. However, addition of apamin after NLA and $NiCl_2$ treatment caused a further increase in the non-cholinergic contraction. Similarly, apamin alone increased the non-cholinergic contraction with either $NiCl_2$ or NLA producing a further increase. These data indicate that $NiCl_2$ and NLA acted at a shared target (NLA directly inhibiting nNOS activation and $NiCl_2$ eliminating the R-type Ca^{2+} current needed for nNOS activation) while apamin further increased the NANC contraction by blocking SK_{Ca} channels on the muscle. These are two non-overlapping pathways causing smooth muscle relaxation and when they are pharmacologically inhibited excitatory mechanisms are potentiated. This represents an indirect way of supporting the role of R-type VGCCs in inhibitory pathways to the longitudinal muscle.

Blocking of N-type VGCC with ω-CTX-GVIA virtually abolished both the cholinergic and non-cholinergic contractions when applied at the highest concentration used in this study (100 nM). Involvement of N-type channels in enteric neurotransmitter release occurs during both excitatory and inhibitory neuromuscular transmission (Waterman, 2000) and the present study supports this notion. On the other side, R-type VGCCs appear to contribute to neuromuscular transmission specifically regulating release of inhibitory neurotransmitters within the so-called NANC myenteric nerves.

R-type VGCC contribute to IJPs recorded from longitudinal muscle cells

The observation that R-type Ca^{2+} channels participate in the nitrergic relaxations of the longitudinal muscle suggested a contribution of these channels during generation of junction potentials in these muscle cells. Recordings of this electrical events revealed a biphasic junction potential consisting of hyperpolarization (IJP) followed by a depolarization (EJP). Blocking of R-type Ca^{2+} channels with $NiCl_2$ reduced the hyperpolarization phase strongly supporting the role of these channels during inhibitory pathways. It is known that R-type Ca^{2+} currents are functionally expressed by myenteric neurons (Bian et al. 2004) and that these channels participate in ganglionic transmission within the ENS (Naidoo et al. 2010). The present study furnished the currently known role of R-type VGCC by demonstrating an involvement of these channels in nerve-evoked relaxations and in the electrical events underlying these mechanical responses.

Activation of P2Y1 receptors contribute to excitatory neuromuscular transmission

Release of the purinergic neurotransmitter from enteric nerves mediates both excitatory and inhibitory events in different regions of the gastrointestinal tract via activation of P2 purinergic receptors. In the longitudinal muscle of guinea pig ileum ATP contributes to contraction by activating P2 purinoceptors (Ivancheva et al. 2000). This is also true for the mouse distal colon where ATP or a related purine induced excitatory actions via activation of ADPβS-sensitive P2Y purinoceptors (Zizzo et al. 2007). In addition, Zizzo et al. in the same study showed that nerve-evoked responses consisted of a nitrergic relaxation followed by a contraction that was cholinergic and purinergic in nature. The present study showed a minor role of P2Y1 receptors in mechanical relaxations of the longitudinal muscle because blocking this receptor with MRS 2179 did not significantly alter the relaxation. Junction potential recordings from the longitudinal muscle demonstrated an inhibitory effect of MRS2179 during the depolarization phase and leaving intact the hyperpolarization phase of this electrical event. This result supports a role for activation of P2Y1 receptors during excitatory mechanisms in the longitudinal muscle

and explains the lack of a significant effect of MRS 2179 on the nerve-evoked relaxation. Various studies have used pharmacological approaches in order to unmask the identity of the purinoceptors coupled to excitatory pathways without any success. The present study suggests that P2Y1 receptors are coupled to the excitatory machinery in the longitudinal muscle, at least in the guinea pig ileum. However other studies have shown pharmacological evidence arguing against a role of P2Y1 receptors in excitatory mechanisms within the longitudinal muscle of mouse distal colon (Zizzo et al. 2008). Taking together this suggests that this phenomenon dependent not only on the type of smooth muscle but also the specie under study.

Functional implications for enteric neuromuscular transmission to the longitudinal muscle

R-type Ca^{2+} channels couple to NOS activation and NO release to cause smooth muscle relaxation. N-type Ca^{2+} channels are one of the main VGCC regulating neurotransmitter release and as such, together with R-type channels, contribute to NO release and longitudinal muscle relaxation. Current descriptions of the nerve supply of the longitudinal muscle layer suggest that there is only one population of inhibitory neurons that is identified by NOS immunostaining (Brookes, 2001). Therefore, the assumption is that these neurons release both NO and ATP/β-NAD to cause muscle electrical and mechanical responses. Figure 3.11 provides a hypothetical model that consolidates data presented by the current and previously conducted studies. The catalytically active form of NOS (nNOSα) is anchored via PDZ-binding domains to the plasma membrane and to VGCCs (Rao et al. 2008; Chaudhury et al. 2009). Thus, the active pool of nNOS resembles synaptic vesicles located at the active zones in that this pool is docked at the nerve terminal in close apposition to VGCCs. Therefore, upon arrival of the action potential VGCCs will open allowing influx of Ca^{2+} through N- and R-type VGCCs (the present study). This Ca^{2+} influx activates the enzyme NOS to produce NO, which then diffuses into the longitudinal muscle cells producing hyperpolarization and relaxation. The purinergic neurotransmitter is stored in synaptic

vesicles but Identification of the VGCC subtype responsible for its release was not possible in the present study, although the most likely candidate will be N-type VGCC. Upon release the purinergic neurotransmitter will activate P2Y receptors causing contraction of the longitudinal muscle (present study; Ivancheva et al. 2007; Zizzo et al. 2007, 2008). This excitatory response as a result of P2Y receptor activation is dependent upon release of intracellular Ca^{2+}, which has been showed to occur through activation of PLC/IP3 pathway and a ryanodine-sensitive mechanism (Zizzo et al. 2008). The present study was able to observe an inhibitory effect of MRS 2179 on the EJPs suggesting a role for P2Y1 purinoceptors in mediating the excitatory responses.

It is possible that there are two separate populations of inhibitory motor neurons supplying the longitudinal muscle; one population using NO as the transmitter and a second population using ATP/β-NAD. The present study suggests that in this situation R- and N-type Ca^{2+} channels will be present on the nitrergic nerve terminals. A molecular marker for purinergic nerves will be of great value to finally determine which hypothetical model describe the enteric population of inhibitory neurons. Unfortunately, there are no reliable markers available at this time for purinergic nerve fibers in the ENS, although the vesicular nucleotide transporter (VNUT, SLC17A9) may prove to be the needed marker (Sawada et al. 2008). We localized α1E immunoreactivity in myenteric ganglia and in the tertiary plexus that supplies the longitudinal muscle. It was also possible to co-localize α1E- and NOS-immunoreactivity in individual varicosities in the tertiary plexus. These data support our conclusion that R-type Ca^{2+} channels couple to NOS activation in nerve fibers supplying the longitudinal muscle.

APPENDIX

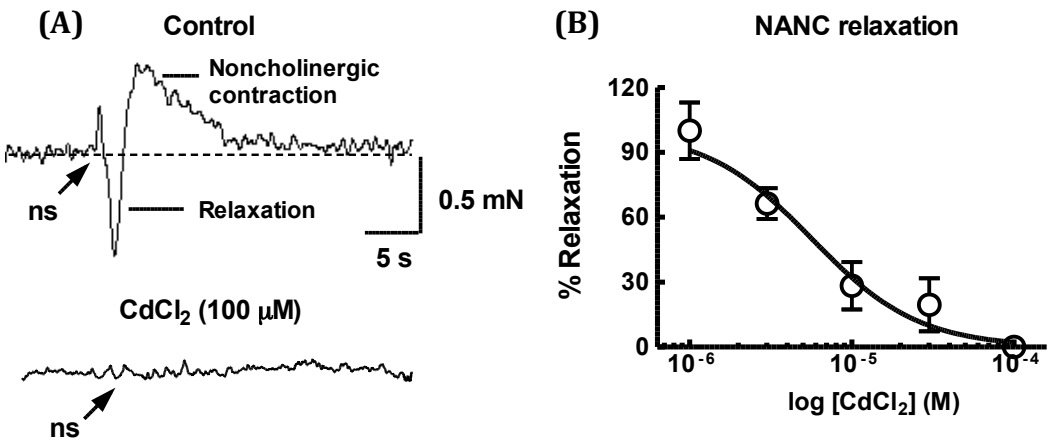

Figure 3.1: Representative traces showing neurogenic responses after transmural electrical field stilation of the guinea pig LMMP *in vitro*. (A) top, Relaxations were induced in the presence of histamine (1 mM) to increase tone and scopolamine (1 mM) to block muscarinic receptors. Nerve stimulation (ns, 20 Hz, 1 s) caused a relaxation followed by a non-cholinergic contraction. (A) bottom, the relaxation and rebound contraction were blocked by $CdCl_2$ (100 mM). (B) Inhibition of the NANC relaxation by $CdCl_2$ was concentration dependent (n=6).

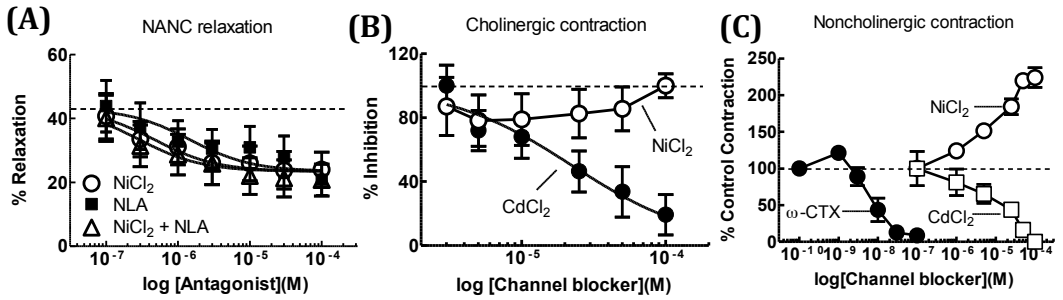

Figure 3.2: $NiCl_2$ inhibits neurogenic relaxations but not neurogenic cholinergic contractions of the LMMP. (A) $NiCl_2$ (50 µM) and NLA (100 µM) produced concentration dependent inhibition of the relaxation evoked by nerve stimulation (20 Hz, 1 s). The control relaxation was approximately 40% of histamine-induced tone. This relaxation was reduced by ~50% by NLA or $NiCl_2$. Combined application of $NiCl_2$ and NLA did not produce a greater inhibition than either drug alone (n=7). (B) $NiCl_2$ did not inhibit LMMP contractions caused by single shocks (0.1 Hz). These contractions are blocked completely by the muscarinic antagonist scopolamine (not shown). $CdCl_2$ produced a concentration- dependent inhibition of the neurogenic cholinergic contraction (n=6). (C) The N-type Ca^{2+} channel blocker w-conotoxin GVIA (CTX-GVIA; 100 nM) and $CdCl_2$ blocked non-cholinergic contractions of the LMMP (20 Hz 1 s, scopolamine 1 mM present). $NiCl_2$ produced a concentration dependent increase in the amplitude of the non-cholinergic contraction (n=6)

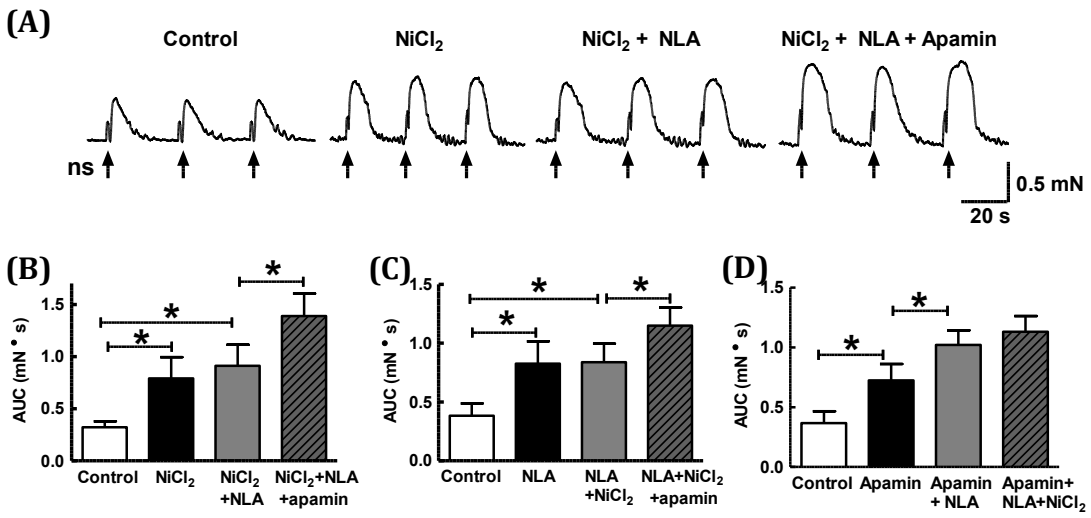

Figure 3.3: Potentiation of longitudinal muscle non-cholinergic contractions. (A) Non-cholinergic contractions (scopolamine 1 μM present) were evoked by a train of nerve stimulation (20 Hz, 1 s). The AUC of the contractions was used to analyze the effect of different treatments because both the amplitude and duration of these responses were affected. $NiCl_2$ (50 mM) significantly increased the AUC of the contractions and subsequent addition of NLA (100 mM) did not produce any further increase. However, addition of the SK_{ca} channel blocker apamin (0.1 mM) further increased contraction AUC ($P<0.05$). (B) Quantitative data for experiment illustrated in "A". (C) Data from experiment similar to that shown in "A" except the sequence of drug application was altered with NLA application preceding $NiCl_2$ followed by apamin. NLA increased the contraction while addition of $NiCl_2$ did not produce a further increase. However, sequential addition of apamin did produce an additional increased in contraction AUC. (D) Sequence of drug application was changed now with application of apamin first and then NLA followed by addition of $NiCl_2$. Apamin and then NLA produced sequential significant increase in the contraction while $NiCl_2$ did not produce a further increase. For all figures * indicates $P<0.05$, n=10 for each experiment.

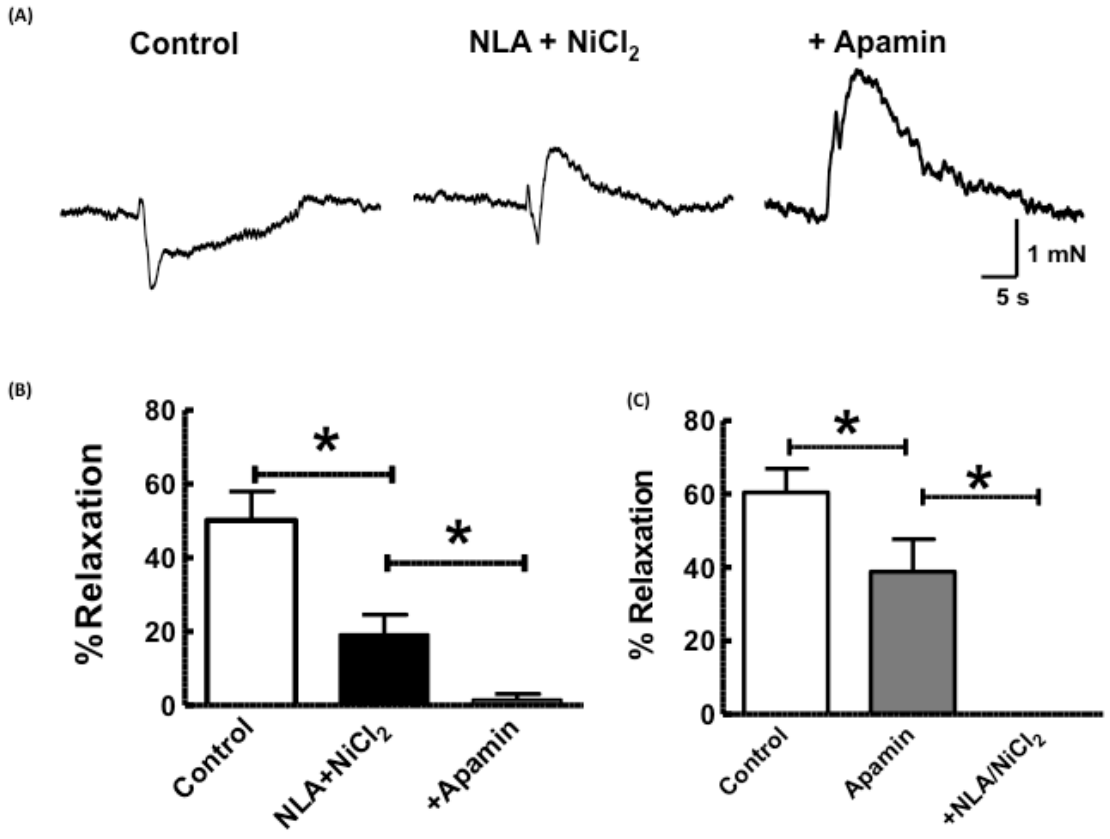

Figure 3.4: Additive effects of $NiCl_2$/NLA and apamin on neurogenic relaxation of longitudinal smooth muscle. (A) Representative recordings of neurogenic relaxations in control (no drug), NLA (100 mM) + $NiCl_2$ (50 mM), and NLA + $NiCl_2$ + apamin (0.1 mM) treated tissues. (B) NLA + $NiCl_2$ produced ~62% inhibition of the relaxation and subsequent addition of apamin completely blocked this response. (C) Similar results were obtained when the drug application sequence was altered with apamin reducing the relaxation by ~36% and subsequent addition of NLA + $NiCl_2$ blocked the remaining response. *P<0.05 compared to control or other drug treated groups; n = 7.

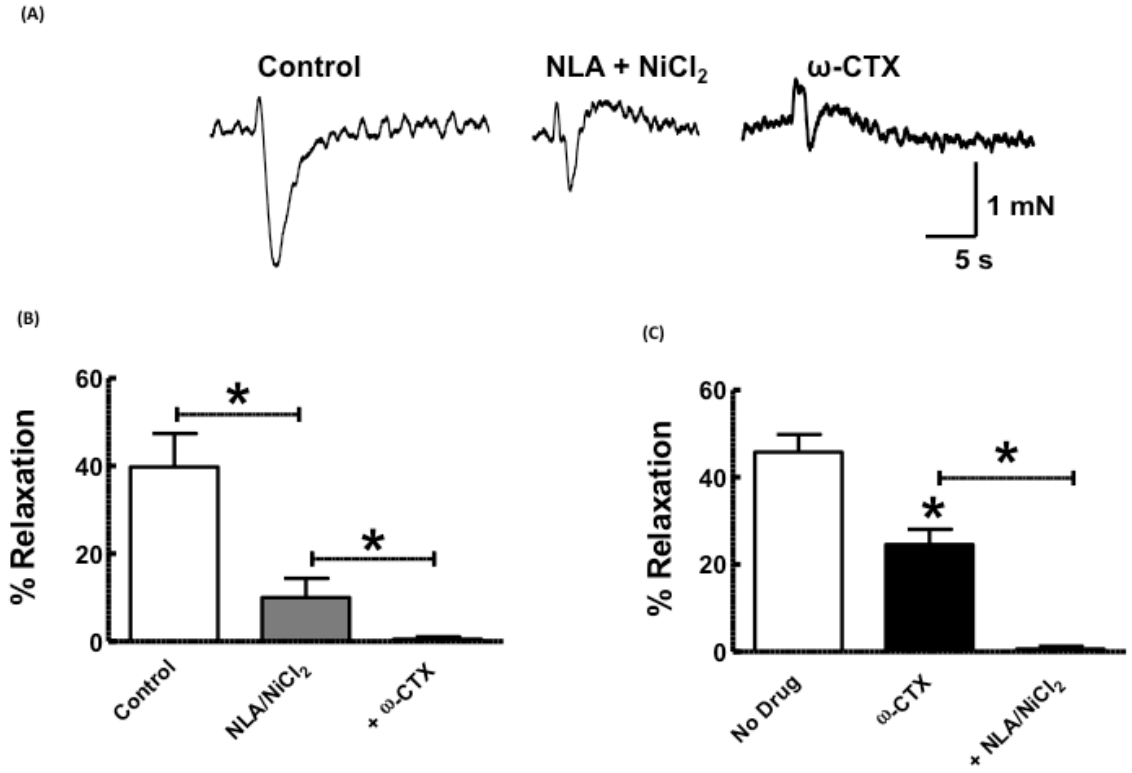

Figure 3.5: Additive effects of $NiCl_2$/NLA and ω-CTX on neurogenic relaxation of longitudinal smooth muscle. (A) Representative recordings of neurogenic relaxations in control (no drug), NLA (100 mM) + $NiCl_2$ (50 mM), and NLA + $NiCl_2$ + w-CTX (100 nM) treated tissues. (B) NLA + $NiCl_2$ produced ~74% inhibition of the relaxation and subsequent addition of w-CTX completely blocked the remaining response. (C) Similar results were obtained when the drug application sequence was altered with w-CTX reducing the relaxation by ~46% and subsequent addition of NLA + $NiCl_2$ blocked the remaining response. *P<0.05 compared to control or other drug treated groups; n = 4.

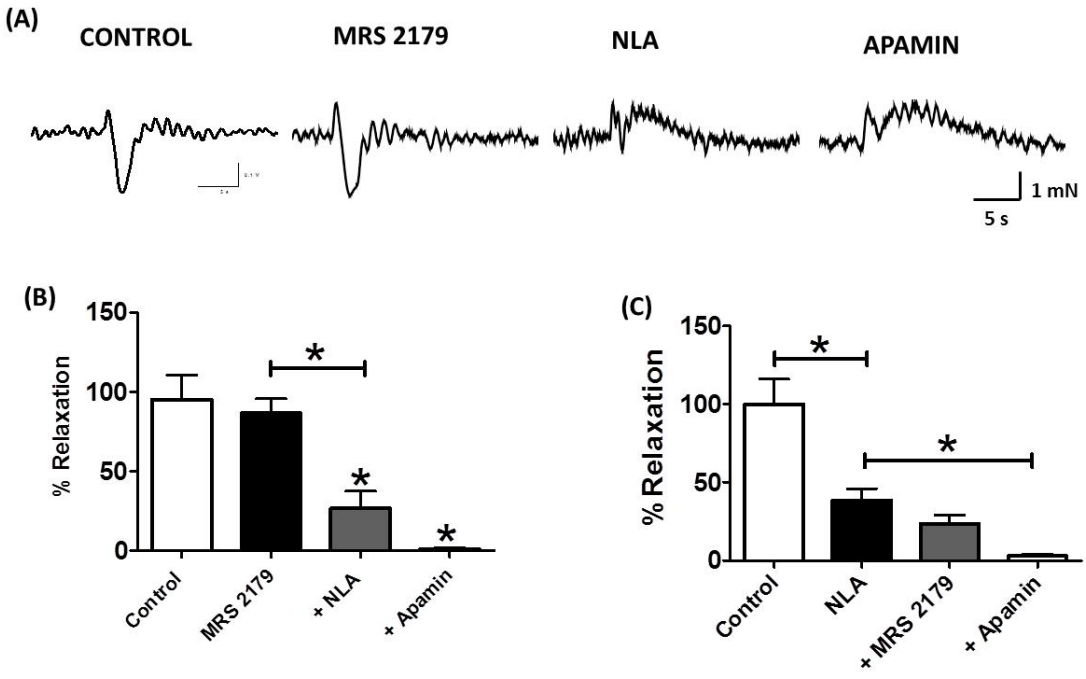

Figure 3.6: Nitric oxide and SK_{ca} channel activation but not P2Y1 receptors are the major mediators of longitudinal muscle relaxation. (A) Representative tracings of longitudinal muscle relaxation to various treatments; quantification of this experiment is presented in panel B. (B) Application of MRS 2179 did not produce a significant inhibition of the relaxation (~9% inhibition, $P>0.05$), however, cumulative addition of NLA and apamin significantly inhibit the relaxation compared to relaxations in control and MRS 2179 conditions ($P<0.05$). (C) NLA inhibited relaxations by ~61% inhibition and this effect was significant when compared to control relaxation ($P<0.05$). Cumulative addition of MRS 2179 = did not produce any further inhibition. Addition of apamin abolished the NLA-resistant relaxation and this effect was statistically significant (for both experiments n = 7).

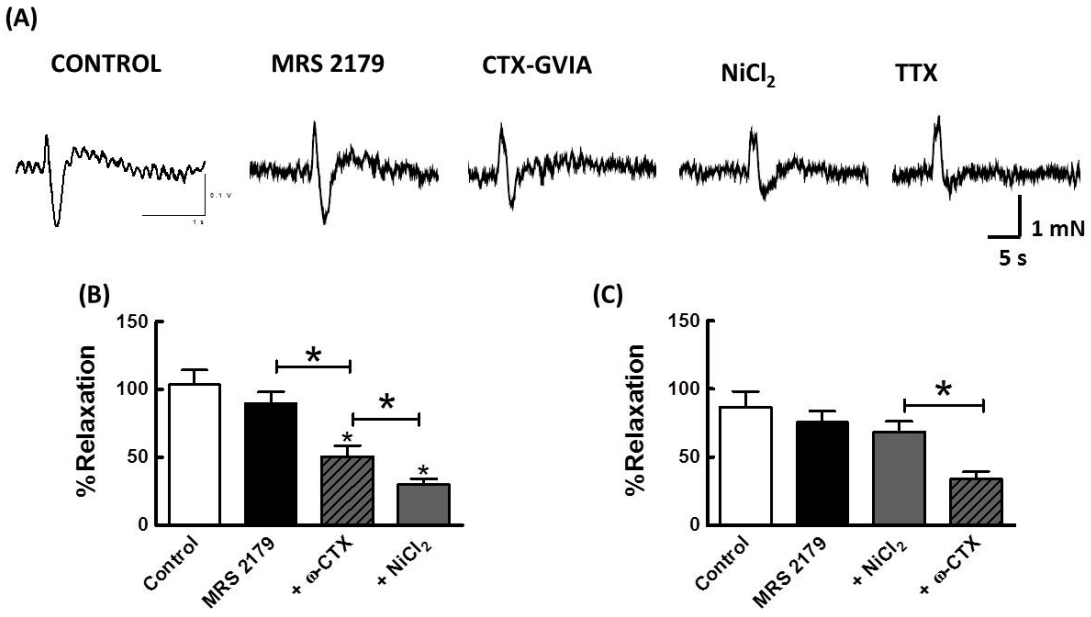

Figure 3.7. Role of N-type and R-type Ca^{2+} channels on predominantly nitrergic relaxations of longitudinal smooth muscle. (A) Representative tracings of longitudinal muscle relaxation to various treatments; quantification of this experiment is presented in panel B. (B) MRS 2179 was added in order to eliminate the small purinergic component of the relaxation. As a result, the relaxation presented is mostly nitrergic. CTX-GVIA (100 nM) inhibited the relaxation and application of $NiCl_2$ (50 µM) produced a further inhibition. The remaining relaxation was TTX resistant and thus non-neurogenic. (B) Quantification of the experiment presented in "A" (*P<0.05 compared to control and between indicated groups; n=6). (C) $NiCl_2$ did not inhibit the neurogenic relaxation while subsequent addition of CTX-GVIA inhibited the relaxation by ~60% (*P < 0.05 vs $NiCl_2$; N=7).

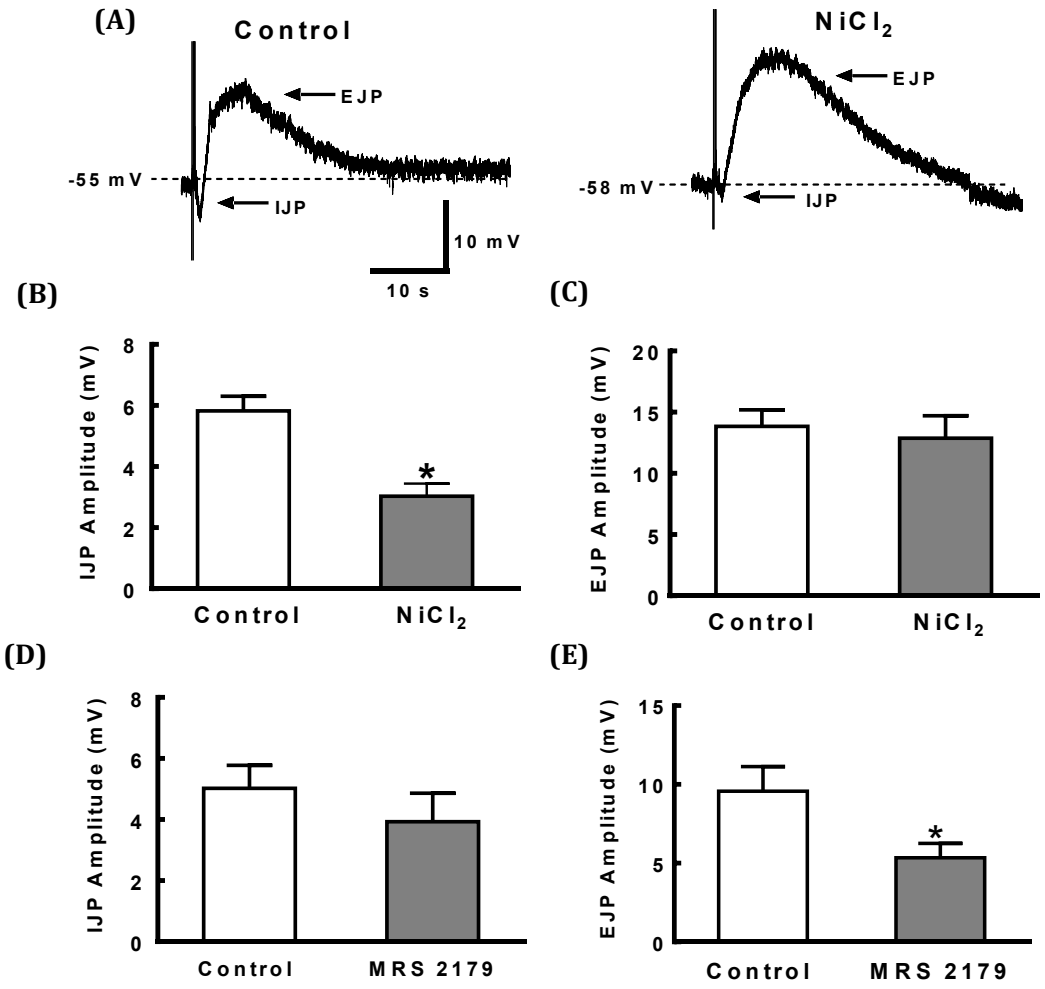

Figure 3.8. Inhibitory (IJP) and excitatory (EJP) junction potentials recorded from longitudinal smooth muscle cells. (A) A Representation of the junction potential recorded from the longitudinal muscle. The small hyperpolarization (IJP) is followed by a large depolarization (EFJ). (B) and (C) $NiCl_2$ (50 mM) inhibits the IJP but not the EJP (B, *P < 0.05 vs. control). (D) The $P2Y_1$ receptor antagonist, MRS 2179, did not alter the IJP but reduced the EJP by ~50% (*P < 0.05 vs. control).

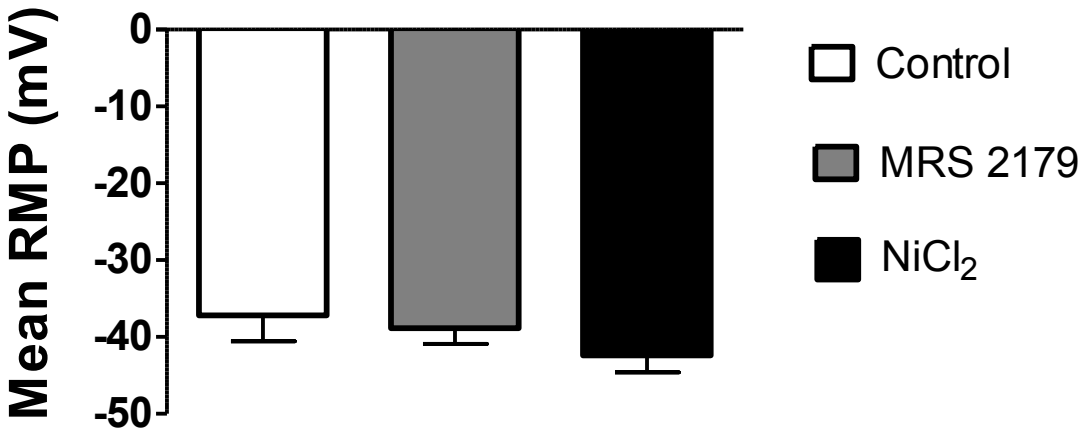

Figure 3.9: Resting membrane potential is not altered by $NiCl_2$ or MRS 2179. Neither NiCl2 nor MRS 2179 altered resting membrane potential of cells from which junction potentials were recorded. Thus, any changes produced by these treatments are more likely to occur at the level of the nerve terminals without any post-junctional actions.

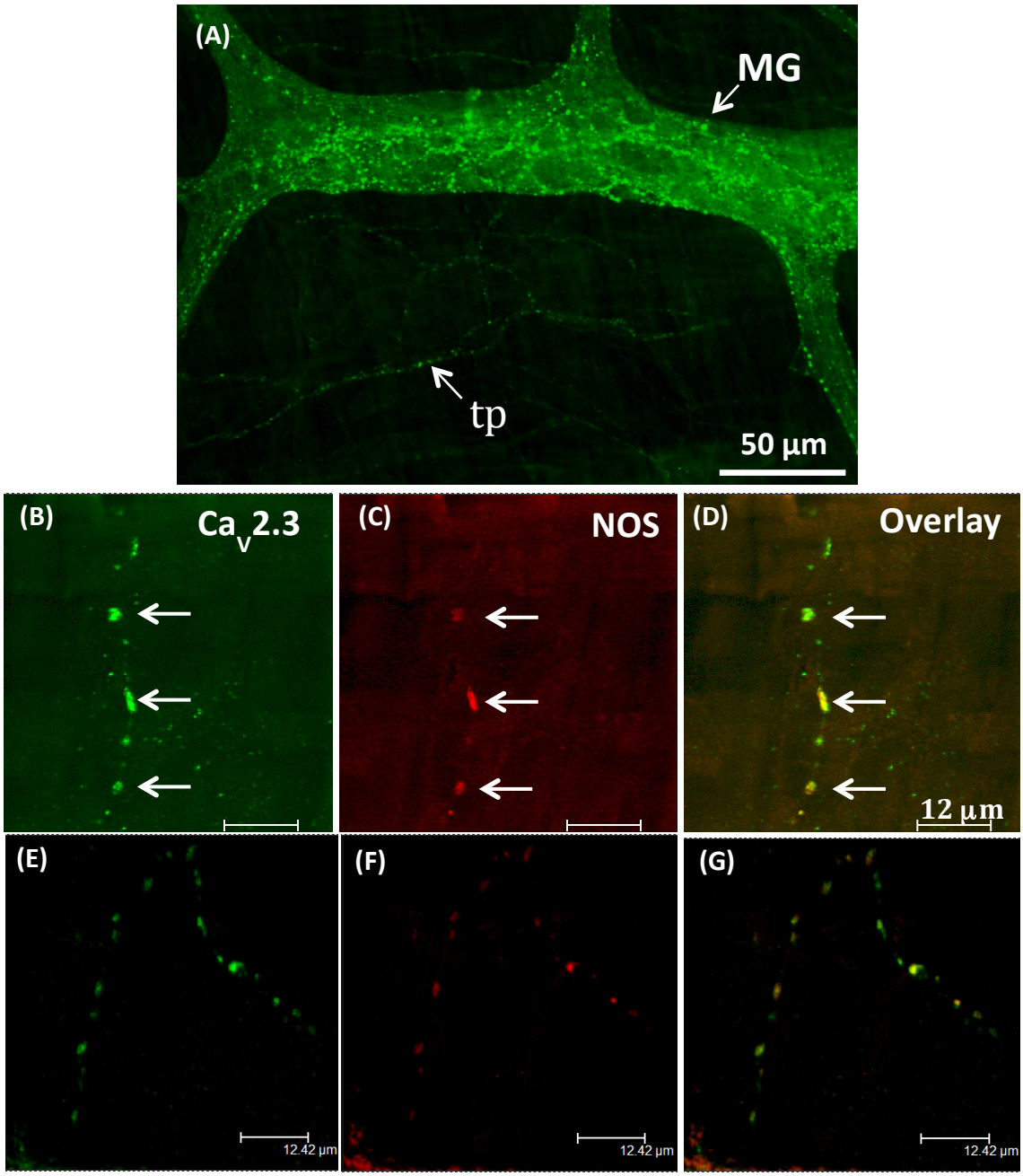

Figure 3.10: Immunohistochemical localization of $Ca_V2.3$ and neuronal NOS. (A) Image shows distribution of $Ca_V2.3$-immunoreactive varicose nerve fibers in myenteric ganglia and in the tertiary plexus that supplies the longitudinal muscle (arrowhead). (B-D) High magnification confocal images of a single nerve fiber in the tertiary plexus that co-expresses $Ca_V2.3$ and nNOS-immunoreactivity

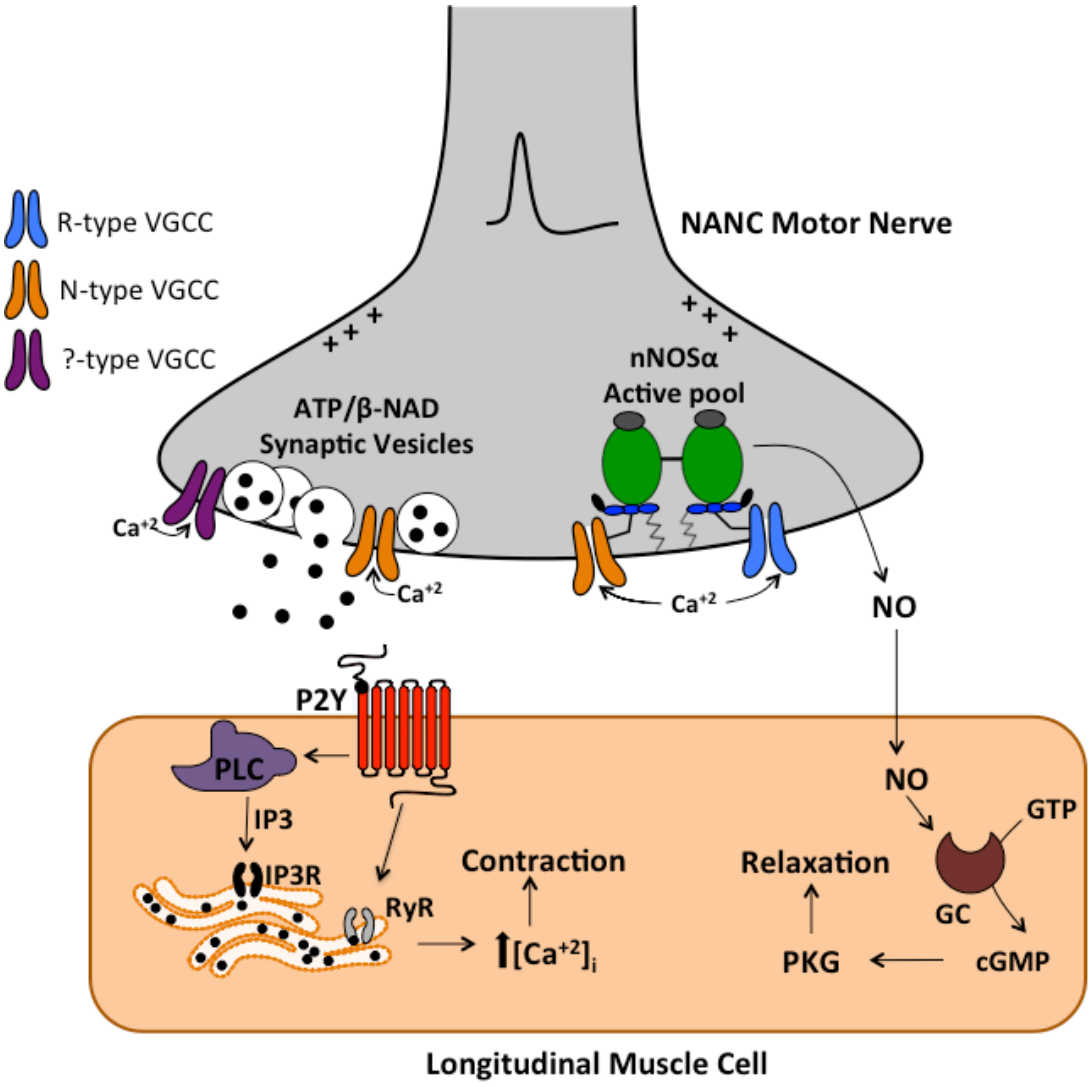

Figure 3.11: Proposed working model for inhibitory neuromuscular transmission to longitudinal muscle. The catalytically active form of NOS (nNOSα) is anchored via PDZ-binding domains to the plasma membrane and to VGCCs (Rao et al. 2008; Chaudhury et al. 2009). Thus, the active pool of nNOS resembles synaptic vesicles located at the active zones in that this pool is docked at the nerve terminal in close apposition to VGCCs. Therefore, upon arrival of the action potential VGCCs will open allowing influx of Ca^{2+} through N- and R-type VGCCs (the present study). This Ca^{2+} influx activates the enzyme NOS to produce NO, which then diffuses into the longitudinal muscle cells producing hyperpolarization

Figure 3.11 (cont'd)

and relaxation. The purinergic neurotransmitter is stored in synaptic vesicles but Identification of the VGCC subtype responsible for its release was not possible in the present study, although the most likely candidate will be N-type VGCCs. Upon release the purinergic neurotransmitter will activate P2Y receptors causing contraction of the longitudinal muscle (present study; Ivancheva et al. 2007; Zizzo et al. 2007, 2008). This excitatory response as a result of P2Y receptor activation is dependent upon release of intracellular Ca^{2+}, which has been showed to occur through activation of PLC/IP3 pathway and a ryanodine-sensitive mechanism (Zizzo et al. 2008). The present study was able to observe an inhibitory effect of MRS 2179 on the EJPs suggesting a role for P2Y1 purinoceptors in mediating the excitatory responses.

CHAPTER 4: FUNCTIONAL UP-REGULATION OF L-TYPE CHANNELS IN THE COLON OF α1E KNOCKOUT MICE

ABSTRACT

Preliminary studies showed that R-type Ca^{2+} channels contribute to inhibitory neuromuscular transmission to the intestines. However, the physiological relevance of R-type Ca^{2+} channels in controlling intestinal propulsive motility is not yet established. These studies tested the hypothesis that genetic deletion of R-type Ca^{2+} channels (α1E KO) will reduce inhibitory neuromuscular transmission to the colonic muscle disrupting colonic propulsive motility. Fecal output and the colonic motor pattern known as CMMC were studied to investigate colonic propulsive motility *in vivo* and *in vitro*, respectively. Intracellular recordings of inhibitory junction potentials (IJPs) and electrical spontaneous firing pattern from circular muscle cells were conducted. All experiments were conducted in α1E KO and wildtype (WT) mice. Both the *in vivo* and *in vitro* studies of colonic propulsive motility showed no alteration in colonic function in α1E KO mice. Pharmacological examination of the contribution of N- and R-type channels to the CMMC pattern revealed that N-type Ca^{2+} channels are critical regulators of this motor pattern. However, blocking R-type channels with $NiCl_2$ did not affect the CMMC pattern in WT mice but it did alter this pattern in α1E KO suggesting that R-type currents could be up regulated in α1E KO mice. IJP recordings from α1E KO mice revealed a compensatory contribution of L-type channels to generation of this electrical event. The L-type channel blocker, nifedipine, produced a 34% inhibition of the IJP, which was similar to the inhibition produced by the R-type channel selective toxin, SNX-482. Nifedipine only produced about 15% inhibition of the IJP in WT mice. The fast component of the IJP (purinergic) was at the level of WT, however, the slow (nitrergic) component was decreased. The resting membrane potential of circular muscle cells in α1E KO mice were about 5 mV more depolarized than WT cells. The firing pattern of circular muscle cells did not differ between α1E KO and WT mice. The presented results demonstrated that absence of R-type channels induced homeostatic changes at the neuromuscular junction in order to maintain colonic motility. The identified adaptive change is a functional up-regulation in the contribution of L-type channels to generation of IJPs. This L-type channel up-regulation

seems to “boost” purinergic transmission but not nitrergic transmission since this component was still decrease in α1E KO mice. Resting membrane potential and colonic muscle tone is regulated by spontaneous release of NO and this could explain the depolarization of the resting membrane potential in α1E KO cells. However, it seems that the maintenance of purinergic inhibitory transmission is physiologically sufficient to retain colonic propulsive motility intact in α1E KO mice.

INTRODUCTION

Neuromuscular transmission finely regulates colonic motility (Spencer and Smith, 2001; Sanders et al. 2012). The immediate consequence of communication between enteric motor neurons and smooth muscle cells is an electrophysiological event known as junction potential. Activation of excitatory motor neurons generates excitatory junction potentials represented by depolarization and contraction of the muscle. Activation of inhibitory motor neurons generates inhibitory junction potentials (IJPs) represented by hyperpolarization and relaxation of the muscle. Junction potentials can be evoked by electrical field stimulation (EFS) and these responses have been used to study the neurotransmitters and receptors involved in neuromuscular transmission in various animal species (Watson et al. 1991; Cayabyab et al. 1997; Borderies et al. 1997; Zagorodnyuk and Maggi, 1994).

It is well established in the literature that EFS-evoked IJPs are generated by two main inhibitory neurotransmitters. The action of these two neurotransmitters produce a bi-phasic IJP composed of a fast and a slow component (Gallego et al. 2012). The fast component, which is a transient high amplitude hyperpolarization of the membrane potential, is mediated by purinergic neurotransmitter acting at P2Y1 receptors (Gallego et al. 2012; Fernandez et al. 2008). The identity of the purinergic molecule is still under active investigation but has been suggested to be either ATP or β-NAD (Hwang et al. 2011; Xue et al. 1999). The slow component, which is a long-lasting small amplitude hyperpolarization, is mediated by activation of nitric oxide synthase (NOS) and production of nitric oxide

(NO) (Keff et al. 1993; Shuttleworth et al. 1997; Dalziel et al. 1991). Release of these neurotransmitters from the motor nerves is calcium dependent and thus regulated by voltage-gated calcium channels (VGCC) (Rao et al. 2007; Chaudhury et al. 2009; Crist et al. 1992; Gallego et al. 2011; Mutafova-Yambolieva et al. 2007). Consequently, through regulation of transmitter release VGCC could play an important role during inhibitory neuromuscular transmission and thus in the control of colonic motility.

The pore of VGCC is formed by the α1 subunit and diversity of these channels is due to expression of genes encoding different isoforms of the α1 subunit (Catterall, 2011). There are three main subfamilies of the α1 subunit: Cav1 (α1C and α1D), Cav2 (α1A, α1B, and α1E), and Cav3 (α1G, α1H, α1I). Cav1 corresponds to the L-type VGCC and they play a major role in excitation-contraction coupling in striated and smooth muscles (Sanders et al. 2012; Hille et al. 2001). Cav2 corresponds to P/Q- (Cav2.1), N- (Cav2.2), and R-type (Cav2.3) VGCCs and these channels regulate neurotransmitter release from nerve terminals and modulate neuronal excitability (Smith and Cunnane 1997; Wheeler et al. 1995; Naidoo et al. 2010). Together these four types of VGCC belong to the HVA calcium channels. Cav3 forms the T-type VGCC, also known as LVA calcium channels, and they are key for generation of pacemaker activity and repetitive firing (Sanders, 1996). N- and P/Q-type VGCC are well known for their role in regulation of neurotransmitter release within the nervous system including the enteric nervous system (Waterman, 2000). R-type VGCC have been shown to regulate neurotransmitter release from enteric neurons (Naidoo et al. 2010), however, the role and physiological significance of this channel subtype is not clearly understood.

R-type Ca^{2+} currents were first described in cerebellar granule cells because after pharmacological block of L-type, P/Q-type, and N-type VGCCs there was still a current resistant to all these treatments (Zhang et al. 1993; Randall and Tsien, 1995). Due to this observation this current was denominated as R (resistant)-type Ca^{2+} current. Studies in cerebellar granule neurons showed that R-

type Ca^{2+} currents are form by a heterogeneous population of calcium channels known as G2 and G3 (Tottene et al. 1996; Forti et al. 1994). Table 4.1 describes the characteristics of each type of Ca^{2+} channel. In summary G2 channels have a single channel conductance of 12-15 pS, larger macroscopic current with Ca^{2+} than with Ba^{2+}, high sensitivity to Ni^{2+} and SNX-482 block, and activate at approximately 15 mV more negative potentials than their counterpart. G3 Ca^{2+} channels have a single channel conductance of 20 pS, larger macroscopic current with Ba^{2+} than with Ca^{2+}, resistant to Ni^{2+} and SNX-482 block, and activate at more depolarized potentials than G2 channels. The pore of the channels conducting R-type Ca^{2+} currents has been demonstrated to be the α1E subunit (Cav2.3) (Tottene et al. 1996; Tottene et al. 2000). However, a study using a α1E knock-out mice showed that α1E subunit forms the pore of a component of the total R-type Ca^{2+} current with the rest of the current resulting from the expression a still unidentified calcium channel subunit (Wilson et al. 2000).

Myenteric neurons within the ENS control movement of the smooth muscle (Sanders et al. 2012). Whole cell current recordings from myenteric neurons in culture from guinea pig small intestine showed that 44.4% of the total current in these neurons is R-type Ca^{2+} current (Bian et al. 2004). Either Ni^{2+} or SNX-482 treatment abolished the R-type currents recorded from these neurons. The sensitivity to Ni^{2+} and SNX-482 block suggests that R-type current in myenteric neurons result from opening of the previously described G2 Ca^{2+} channels. The study of Bian et al. 2004 clearly established the presence of R-type currents in enteric neurons controlling motility of the muscle layers. The question that arises is what is the role and physiological relevance of the R-type Ca^{2+} currents in the ENS?

These studies were conducted to provide detailed insight for the role and relevance of R-type VGCC in enteric inhibitory neuromuscular transmission to the colonic muscle using a mouse in which the gene that encodes for α1E subunit has been deleted (α1E knock-out; KO). In addition, the ability of the ENS to compensate for the loss of α1E channels was investigated.

MATERIALS AND METHODS

Mice

All animal use protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University. α1E KO mouse strain is 129SV x C57Bl/6J. α1E KO frozen embryos were implanted in wildtype C57BL/6J females to generate a heterozygous offspring. Heterozygous breeding pairs were maintained to obtain a balance number of wildtype and α1E KO mice. Colonies were maintained at Michigan State University Laboratory Animal Resources (East Lansing, MI). Litters were genotyped at weaning, which occurred 3 weeks after birth in order to identify wildtype (+/+) and homozygote (-/-) mice. All animals were fed normal diet and were studied between 4 and 5 months of age (male). Mice were euthanized using isoflurane anesthesia followed by cervical dislocation.

In vivo study of colonic propulsive motility

Colonic propulsive motility was studied by evaluating fecal output in WT and α1E KO mice (n = 6). Mice were individually housed with a pre-measured amount of water and food. For 3 consecutive days each mouse were removed from the original cage and placed in an individual cage with no water and food for 1 hour. This was always conducted at the same time, between 10 am and 11 am. Fecal pellets produced by each mouse during this period were counted and weighted. After this, all fecal pellets were placed in a 60°C oven overnight and dry weight was determined. Fecal number and wet and dry weight from each animal was averaged over the 3-day period. This averaged value from each mouse was used for statistical comparisons.

Propulsive colonic motility studies in vitro: Colonic Migrating Motor Complex

Preparation of colonic segments for recordings of the Colonic Migrating Motor Complex (CMMC) was performed as described previously (Devries et al. 2010). Briefly, after the mice were sacrificed the entire colon was removed and placed into oxygenated and warm Krebs solution. Immediately after this, the luminal content was gently flushed out with Krebs solution. A stainless-steel rod was inserted into the lumen and the tissue was secured at each end with a surgical silk. Metal clips (Fine Science Tools, Foster City, CA, USA) were attached to the oral and anal end of the tissue 1.5 cm apart. Both metal clips were then connected to separate force transducers using surgical silk. The rod holding the tissue was secure in a 60 ml bath containing oxygenated Krebs solution at 37 °C. The oral and anal ends of the colon were stretched to an initial tension of 1 g. The tissue was allowed to equilibrate for 30 minutes during which a regular pattern of propagating contractions was established and that is known as the CMMC. The CMMC pattern was then recorded for an additional hour and a 30-minute segment was selected for analysis. Frequency, amplitude, and AUC of the contractions were measured. Propagated CMMC were determined as a complex in which a contraction occurred first at the oral recording site followed by a contraction recorded at the anal site. The effect of $NiCl_2$ (50 μM) and ω-CTX-GVIA (30 nM and 100 nM) was investigated to study the role of R-type and N-type VGCC, respectively. A baseline of 30-minutes was established to record control CMMCs and then either treatment was applied for a period of 20 minutes. The effect of these treatments on the amplitude, AUC, and frequency of CMMC was investigated. Recordings were obtained using two Grass Instruments CP122A strain gauge amplifiers. The output of these amplifiers was fed to an analog/digital converter (Minidigi 1A, Molecular Devices; http://www.moleculardevices.com/) and Labchart software 7 (Molecular Devices).

Intracellular recordings of inhibitory junction potentials and smooth muscle cell electrical activity

Conventional intracellular electrophysiological techniques were used to obtain recordings of IJP and smooth muscle electrical activity in the distal colon of WT and α1E KO mice. A 2-cm segment of the

distal colon was harvested from the mice immediately after euthanasia and placed in a petri dish containing pre-warmed (37°C) and oxygenated (95% O_2; 5% CO_2) Krebs solution. The Krebs solution contained scopolamine (1 μM) to block muscarinic receptors and in some experimental conditions nifedipine (1 μM) was added to block L-type Ca^{2+} channels. The colonic segment was cut open along the mesenteric border and pinned flat on the petri dish with the mucosal layer facing upwards. The mucosa and submucosa layers were gently removed using fine forceps. A 5-mm^2 section was cut and transferred to a 5 ml silicone elastomer-lined recording chamber. The section was stretched lightly and pinned to the chamber floor using small stainless steel pins. The chamber was mounted on a stage of an inverted microscope and it was then perfused with oxygenated Krebs solution at a flow rate of 5 ml/min. The preparations were equilibrated for 30 minutes before commencing of intracellular recordings.

Smooth muscle cells were impaled with a 2 M KCl-containing glass microelectrode (Frederick-Haer, Brunswick, ME) with a tip resistance of 60-100 MΩ. To avoid muscle movement in experimental conditions were nifedipine was not present a smaller window was created in the middle of the preparation using micro-pins (0.125 mm from World Precision Instruments, Inc). This technique successfully restrained muscle movement making possible to maintain impalement for periods of 2-30 minutes. Upon successful impalement the resting membrane potential of circular muscle cells was allowed to stabilize for a period of 2 minutes. Membrane potential was recorded using an Axoclamp 2A amplifier, a Digidata 1322A analog-digital converter, and Axoscope 9.2 software (all from Molecular Devices, Sunnyvale, CA, USA). Amplified signals were sampled at 2kHz and filtered at 1kHz. Data were stored on a computer hard drive and Clampfit 10.3 software was used for the analysis of membrane potentials.

IJPs were evoked using a pair of silver wired stimulating electrodes (A-M Systems, Inc.) inserted into the recording chamber parallel to the circular muscle fibers to electrically stimulate the nerve fibers

supplying the muscle cells. Electrical stimuli consisted of 1 s train duration, 0.8 ms pulse duration, 10 Hz, and 10-90 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). Two parameters of the IJPs were characterized in WT and α1E KO mice. The peak amplitude of the fast IJP was measured to obtain information about the purinergic component of the inhibitory neuromuscular transmission. The AUC of the IJP was measured to obtain information about both the purinergic and nitrergic components of this type of transmission. These two parameters were always compared between WT and α1E KO mice and were used to examine the action of different treatments in generation of IJPs.

Drug application system

Two types of drug application systems were used to study the effect of different treatments on IJPs. In one application system, drugs were added in known concentrations to the flowing Krebs solution using a system of three-way stopcocks. Drug concentrations reached steady state in the recording chamber within 4 minutes. The other was a local drug application system, which was accomplished using a quartz micropipette (30-40 μm tip diameter) placed within 50 to 150 μm of the impaled muscle cell. Before impalement, Krebs solution was superfused through the quartz micropipette near the recording microelectrode. This prevented disruption of membrane potential recordings by the turning on of the local application system. After recordings of control responses the Krebs solution flow was replaced with flow of different drug treatments using a VC-8 Valve Controller application system (Warner Instruments, Hamden CT). The impaled muscle cells were exposed to drug treatments for at least 5 minutes before responses were recorded.

Drugs

Nifedipine, scopolamine, ω-CTX-GVIA, ω-ATX-IVA, SNX-482, NLA, TTX, and MRS 2179 were obtained from Sigma-Aldrich. All drugs were diluted in deionize water except nifedipine which was dissolved in DMSO.

Statistical analysis

Data are presented as mean values ± S.E.M. for n representing the number of mice for *in vivo* and *in vitro* CMMC and circular muscle ring responses studies. For intracellular recording studies the n value represents the number of cells. Statistical differences between groups were analyzed with a two-way ANOVA followed by a Bonferroni post-hoc test or when applicable a 2-tailed student t-Test was used. In either case, $P<0.05$ was the criterion for determining statistical significance.

RESULTS

Colonic propulsive motility in vivo is not altered in α1E KO mice

To investigate if the absence of the α1E subunit of R-type VGCCs disrupts colonic propulsive motility *in vivo*, fecal pellet output was monitored for 1 hour during 3 consecutive days in WT and α1E KO mice. Body weight, food intake, and water intake was measured for the duration of the study in order to rule out the possibility that these parameters are contributing to any change observed. Body weight was similar between WT and α1E KO mice (32 ± 1.8 g for WT and 34 ± 2.1 g for α1E KO; $P > 0.05$). Food and water intake were both higher in α1E KO as compared to WT mice, however, statistical significance was only detected for food intake (figure 4.1).

To compare fecal output between WT and α1E KO mice the number of pellets produced in 1-hour and the weight of the collected fecal material were measured for the 3 consecutive days and the averaged value for each mouse was used for comparison purposes. The results showed the mean pellet

number was higher in α1E KO mice, however that was not statistically significance when compared to WT mice (6.6 ± 1.6 g in WT and 9.8 ± 0.9 g in α1E KO; P = 0.1170) (figure 4.2 A). Both the pellet wet and dry weights were larger in α1E KO mice as compared to WT but statistical significance was only detected for pellet dry weight (for wet weight: 0.18 ± 0.04 g in WT and 0.23 ± 0.02 g in α1E KO, P = 0.2695; for dry weight: 0.07 ± 0.01 g in WT and 0.11 ± 0.006 in α1E KO, P = 0.0416) (figure 4.2 B-C). Percentage of water content in fecal material was also investigated by dividing the difference between wet and dry pellet weight into wet pellet weight for each mouse. Overall, percentage of water content was similar between WT and α1E KO mice (49.2 ± 7.5% in WT and 51.1 ± 2.1% in α1E KO; P = 0.8096) (figure 4.2D). The percentage of fecal output was calculated by diving the total dry weight into either the food intake (figure 4.3 A) or the body weight (figure 4.3 B). In either case the fecal output percentage was not statistically different between WT and α1E KO mice.

Colonic propulsive motility in vitro is not altered in α1E KO mice

Colonic propulsive motility was investigated *in vitro* by studying the CMMC. The CMMC is a critical neutrally mediated (figure 4.4) rhythmic contractions observed in the large intestine of many mammals, including humans (Smith et al. 2014). The main function of the CMMC pattern is to regulate transit of fecal material along the length of the colon (Bassotti et al. 1988; Bharucha et al. 2012). A propagating CMMC pattern is composed by a contraction that starts in the oral side and then migrates towards the anal side. This pattern on contractions is called an oral-anal propagating contraction. A representation of the CMMC pattern from WT and α1E KO mice is provided in figure 4.5. The percentage of propagating contractions was determined in WT and α1E KO mice by studying the CMMC pattern in the colon of both types of mice. The results showed that about 93% and 85% of the total observed contractions were propagating contractions in WT and α1E KO mice, respectively (figure 4.6 A). The small difference between these two values was not statistically significant (93 ± 3.3% in WT and

85 ± 6% in α1E KO; P > 0.05) demonstrating that the proportion of propagating contractions is not altered in α1E KO mice. The time for an oral contraction to migrate to the anal side was measured and this is called propagation time. Propagating contractions in WT and α1E KO mice had similar propagation times averaging 14.8 ± 1.3 s and 15.6 ± 2.7 s, respectively (P = 0.7964) (figure 4.6 B).

Since the pattern of CMMC was not altered in α1E KO mice, the nature of the contractions (force and duration) was studied by measuring the peak amplitude and AUC of oral and anal contractions. The amplitude and AUC of oral contractions was similar between WT and α1E KO mice (figure 4.7 A-B). The same pattern was observed for anal contractions; no significant differences were detected for the amplitude and AUC of anal contractions between WT and α1E KO mice (figure 4.7 C-D). The number of oral and anal contractions generated in 30 minutes was counted in order to determine frequency of oral and anal contractions. Data showed that frequency of oral and anal contractions was not different between WT and α1E KO mice (figure 4.8 A-B). The anal contractions were preceded by a small relaxation and this is thought to be important for guiding the direction of propagation (facilitating propagation towards the anal side) (Smith et al. 2014). The amplitude and AUC of those relaxations were measured and compared between WT and α1E KO mice. There was a tendency for the amplitude and AUC of the relaxations to be larger in α1E KO mice. However, none of these parameters were significantly different between the two types of mice (figure 4.9 A-B). The frequency of relaxations, in a period of 30 minutes, was also measured and this value was similar between WT and KO mice (figure 4.9 C).

These CMMC studies demonstrate that there are not alterations in α1E KO mice that point towards disruptions in colonic propulsive motility. Therefore, the *in vitro* studies support the *in vivo* studies showing no alterations in fecal output in α1E KO mice.

N-type Ca^{2+} currents are essential for generation of the CMMC

The role of N-type VGCCs in generation of the CMMC pattern was investigated in WT and α1E KO mice. The data showed that ω-CTX-GVIA at 100 nM and at 30 nM was able to completely abolish the CMMC pattern (figure 4.10). The effect of ω-CTX-GVIA was very similar to the effect of TTX (figure 4.4) suggesting that N-type VGCCs contribute to generation of the CMMC at multiple synaptic sites within the myenteric circuit mediating this motor pattern.

Pharmacological block of R-type calcium current altered CMMC in alpha1E KO mice

We have seen until now that N-type VGCC are critical for the neurally mediated CMMC most likely because this VGCC subtype is contributing to generation of CMMC at multiple sites within the neural circuit controlling this motor patter. Investigating this motor pattern in the α1E KO mouse suggested that R-type Ca^{2+} channels are not critical for generation of CMMC because these events were generally normal in these mice. Another possibility is the occurrence of an adaptation in response to loss of R-type VGCC that is masking the role of this channel subtype. In order to investigate this, I preceded to pharmacologically block the R-type VGCC with $NiCl_2$ (50 μM) in WT mice. This represents an acute pharmacological block/loss of R-type VGCC function. Thus it will not allow enough time for the system to undergo adaptive changes in order to offset the pharmacologically compromised function of these channels.

Overall, $NiCl_2$ treatment did not produce any significant effect neither on the oral or anal contractions nor in the propagation time of oral-anal propagated contractions (figure 4.11). The frequency of oral and anal contractions was not affected either by $NiCl_2$ treatment (figure 4.12). In addition, $NiCl_2$ did not alter the nature or frequency of the relaxations preceding the CMMCs (figure 4.13). The effects of $NiCl_2$ were also investigated in α1E KO mice and the results were surprisingly different as those observed in WT mice. The AUC of the oral contractions was significantly decreased by

$NiCl_2$ treatment (figure 4.14 A). Interesting, the AUC of the anal contractions was significantly increased by $NiCl_2$ treatment (figure 4.14 B). However, propagation time was not significantly altered by $NiCl_2$ treatment (figure 4.14 C). In this same line, neither the frequency of oral nor the frequency of anal contractions was significantly affected by $NiCl_2$ treatment (figure 4.15). In terms of the relaxations preceding the CMMCs, it appears that $NiCl_2$ decrease these responses but this effect was not statistically significant (figure 4.16 A). The frequency of the relaxations was not significantly affected by treatment with $NiCl_2$ (figure 4.16 B). Taking together the WT data showed that $NiCl_2$-sensitive R-type currents are not critical for generation of CMMCs. However, the fact that $NiCl_2$ had an effect on the CMMC in alpha1E KO suggest that total R-type current is regulated and thus there is still a component sensitive to block by $NiCl_2$ treatment.

At the end of these *in vivo* and *in vitro* studies I have data that demonstrate normal colonic propulsive motility in α1E KO mice. One explanation is that channels formed by the α1E subunit, absent in the KO mice, are not critical for maintaining normal motility patterns in the colon or the ENS is able to adapt and compensate the loss of this population of VGCC. To further investigate this I preceded to study inhibitory neuromuscular transmission to the colon, which produces hyperpolarization and mechanical relaxation of the smooth muscle. This type of neuromuscular transmission is critical for normal regulation of colonic motility and function. Thus, by investigating the electrical response to inhibitory neuromuscular transmission I will be able to identify any changes occurring at the level of the neuro-muscle communication that could be correlated with normal colonic motility in α1E KO mice.

Characterization of the colonic IJPs in α1E KO mice

Two pieces of data demonstrated a role of R-type VGCC during generation of IJPs in the colonic circular muscle cells of WT mice. First, IJPs were evoked by applying different frequencies of nerve

stimulation (1-60 Hz) and this produced a frequency-dependent increase in the AUC of the IJPs (figure 4.17). Application of 50 μM $NiCl_2$ produced a statistically significant decrease in the AUC of these IJP responses. A representative trace is provided (figure 4.17 B) showing that $NiCl_2$ preferentially decrease the slow nitrergic component of the IJP leaving intact the purinergic component. This piece of data suggested that when R-type VGCC are pharmacologically blocked a decrease in IJP can be observed especially during the slow phases of the IJP. Therefore, $NiCl_2$-sensitive R-type currents appear to participate in generation of IJPs particularly in the nitrergic slow phase of this electrical event. The second piece of evidence supporting a role for R-type channels in IJP generation is the fact that SNX-482 (neurotoxin highly sensitive for R-type currents) produced a 35% inhibition of the IJP in WT mice (figure 4.18).

Now, the question is: would a knockout of the α1E subunit, which forms the pore of R-type Ca^{2+} channels, produce a similar effect as the pharmacological block of this channel subtype? Or are any adaptive changes occurring in order to compensate the loss of the α1E subunit in the ENS? I recorded IJPs from WT and α1E KO mice to clearly answer these questions and provide insight into the role and significance of R-type currents in enteric neuromuscular transmission.

IJPs were recorded form circular muscle cells and both the amplitude and AUC were measured to obtain information about the two main components of the IJP. The amplitude of the IJPs provides information about the purinergic fast component and the AUC provides information about both, purinergic and the nitrergic slow component of the IJP. These IJPs were recorded under nifedipine conditions (1 μM) to prevent movement of the muscle and achieve a more stable impalement. Under this condition, the amplitude and AUC of the IJP were significantly reduced in α1E KO mice (figure 4.19). This observation favors a role of R-type VGCCs during generation of the IJP. The fact that both

parameters of the IJP were reduced by knocking-out α1E subunit suggested a contribution of this channel subtype to both purinergic and nitrergic components of the IJP.

L-type VGCCs are responsive to perturbations around them, especially those altering the function of other VGCC subtypes (Etheredge et al. 2007; Flink and Atchison, 2002). To unmask any potential compensatory contribution of L-type channels, I conducted IJP recordings in nifedipine-free conditions. As a result, calcium-dependent action potentials were present producing movement of the colonic muscle. In order to successfully impale a muscle cell, movement was mechanically prevented by creating a small window in the center of the preparation using small pins. Under nifedipine-free conditions the amplitude of the IJPs in α1E KO and in WT mice were at the same level (figure 4.20 A). The AUC of the IJP was still significantly lower in α1E KO mice compared to WT (figure 4.20 B). This experiment suggested that when L-type channels are available for activation the previously observed disruptions in the IJP events (figure 4.19) are either eliminated as occur with the amplitude or less evident as in the case of the AUC. It could be possible that nifedipine is changing IJPs in WT and not in the α1E KO mice. In order to determine if nifedipine is changing IJPs in WT mice, IJPs recorded in the presence and absence of nifedipine were grouped and compared. The results showed that IJPs recorded in nifedipine-free conditions were significantly smaller than IJP recorded in presence of nifedipine; this applies for both amplitude (figure 4.21 A) and AUC (figure 4.21 B). The same analysis was conducted in α1E KO mice and whether or not nifedipine was present in the recording conditions the amplitude and AUC of the IJPs were not altered at all (figure 4.21 C-D). Is not that IJPs in α1E KO mice are restored in nifedipine-free conditions, as suggested figure 20, but IJPs in WT mice are decreased in this condition. This gives the impression that there is a “restoration” of IJPs in α1E KO mice when in fact these IJPs remain the same irrespective of nifedipine being in the recording conditions or not.

L-type channels do not participate in neurotransmitter release and they do not play a major role in generation of the IJPs (Reis et al. 2002, 2000; Cunningham et al. 1999). Therefore, it was puzzlingly to observe a decrease in WT IJPs in response to removing nifedipine from the recording conditions. The resting membrane potential (RMP) of all those cells from which IJPs were compared in Figure 4.19 and 4.20 was measured in order to identify a change that could explain the results in WT IJPs. In WT, cells showed RMP averaging -44 ± 1.5 mV in nifedipine-free conditions and -39.5 ± 2.4 mV in presence of nifedipine (figure 4.22 A). This represents a 4.5 mV depolarization in RMP of cells impaled in presence of nifedipine, but this effect was not statistically significant (P = 0.1303). However, it is possible for this 4.5 mV depolarization to be physiologically significant by increasing the driving force for K^+ to move outside the cell and thus generating a larger hyperpolarization in the presence of nifedipine. In α1E KO, the presence of nifedipine did not change RMP of cells at all (-38.7 ± 1.3 mv in nifedipine-free conditions and -38.8 ± 1.5 mV in presence of nifedipine; P>0.05) (figure 4.22 B). That could explain the lack of effect on the IJPs in response to changes in the recording conditions related to nifedipine.

To clearly investigate the potential role of L-type channels in inhibitory neuromuscular transmission, IJPs were recorded from cells in control conditions and after 10 minutes application of 1 μM nifedipine. The control and treated IJPs were recorded from the same cell and therefore this represents a pair comparison. Nifedipine treatment did not produce a significant change in the amplitude or AUC of the IJPs from WT mice (figure 4.23 A-B). The RMP of these cells were not significantly affected by application of nifedipine (-42 ± 4.7 control vs. -40.2 ± 3.6 in nifedipine conditions; P = 0.6644) (figure 4.23 C). Therefore, L-type channels do not play a role in generation of IJP in WT mice. In α1E KO mice nifedipine treatment produced a significant inhibition of the amplitude (figure 4.24 A) but not the AUC (figure 4.24 B) of the IJPs. Even though nifedipine decreased IJP amplitude, this treatment did not alter RMP of the cells from where those IJP were recorded (-40.5 ± 2.8 in control and -38.1 ± 2.7 in nifedipine treatment; P = 0.5613) (figure 4.24C). The percent inhibition

produced by nifedipine in alpha1E KO IJPs almost double the one produced in IJPs from WT mice (15.6 ± 4.7% inhibition in WT and 34 ± 8.2%; P = 0.0645) (figure 4.25).

Taking together these results showed α1E KO mice have a normal purinergic IJP, however the slow nitrergic component appears to be reduced. There is a nifedipine-sensitive component in IJPs from α1E KO mice that is not present in WT mice. The component of the IJP that is sensitive to nifedipine in α1E KO mice is the fast purinergic phase of the IJP because the slow phase was not significantly inhibited by this treatment. Since the specific R-type VGCC blocker, SNX-482, produced a 34% inhibition of the IJPs in WT mice it is possible that development of nifedipine-sensitivity of IJPs occur as a compensatory adaptation in response to the α1E subunit knockout.

Ongoing inhibitory innervation and circular muscle cell excitability in α1E KO mice

The colonic muscle receives ongoing or spontaneous innervation from inhibitory motor neurons and this regulates colonic motility and function (Gil et al. 2010). Both the purinergic and nitrergic neurotransmitters contribute to this spontaneous innervation, however they have distinct roles. Purinergic neurotransmitter regulates generation of spontaneous IJPs and the nitrergic neurotransmitter regulates RMP and muscle tone (Gil et al., 2010). The RMP of circular muscle cells was measured and compared between WT and α1E KO mice to investigate changes in ongoing colonic inhibitory innervation. Cells from WT mice had a RMP averaging - 43.7 ± 1.7 mV and α1E KO cells had a RMP averaging -38 ± 1.3 mV and the difference between these values was statistically significant (P = 0.0169) (figure 4.26 A). Consequently, there is a 5.7 mV depolarization of the RMP in cells from α1E KO mice as compared to WT cells. Under nifedipine conditions the RMP of WT and α1E KO mice was very similar (-39.5 ± 2.4 mV in WT and -39.7 ± 1.4 mV in α1E KO; P = 0.9649) (figure 4.26 B), mainly because this treatment produced a small depolarization in RMP of WT cells. Because the nitrergic component of the

ongoing inhibitory innervation to the colon regulates RMP, this data suggested that some alterations might occur in α1E KO mice in the nitrergic component of inhibitory transmission.

Circular muscle cells have an intrinsic firing activity. In this line, two types of firing patterns were identified by recording spontaneous electrical activity in muscle cells. The first one is called a bursting pattern (figure 4.27 A) consisting of a slow wave depolarization with action potential firing overlapping at the plateau phase of the depolarization. The second one is called continuous firing pattern and consist of cells able to fire in a non-bursting fashion (figure 4.27 B). In an attempt to investigate if the 5.7 mV depolarization in RMP of cells from α1E KO mice was enough to produce changes in excitability of circular muscle cells I characterize the firing pattern profile of all cells impaled in my studies. The AUC of the slow wave depolarization and the number of action potentials fired during the depolarization were measured and no significant difference was observed between WT and α1E KO mice (figure 4.28). The RMP of these cells was actually not different between WT and α1E KO mice (figure 4.28 D). For continuous firing cells the number of action potentials fired in one minute was counted. This value is not significantly different between WT and α1E KO mice (figure 4.29 B). However, the RMP of continuous firing cell in α1E KO was about 6.7 mV more depolarized than WT cells (-44.5 ± 1.8 mV in WT and -37.7 1.3 mV in α1E KO; P = 0.0062) and that was statistically significant (figure 4.29 C). These results suggest that although there is a statistical significant depolarization of the RMP in cells from α1E KO mice it is not enough to change the firing pattern or excitability of circular muscle cells.

DISCUSSION

Calcium transients generated by opening of VGCCs play a host of neuronal functions including tight regulation of neurotransmitter release. This role places VGCC in a critical position within the

neuronal circuits controlling motility of the intestinal muscle. Enteric neurons express the full complement of HVA Ca^{2+} channels: P/Q-type, N-type, R-type, and L-type VGCC (Kirchgessner et al. 1999). Of all these channel subtypes the physiological role and significance of R-type VGCC are the least understood particularly within the ENS.

R-type Ca^{2+} currents are formed by a heterogeneous population of VGCC

R-type Ca^{2+} currents have been extensively studied in cerebellar granule neurons and various studies showed that the α1E subunit forms the pore of the R-type VGCC (Wilson et al. 2000; Tottene et al. 2000). However, studies using α1E KO mice showed the existence of a substantial R-type current suggesting that other α1 subunits must conduct this type of currents. Wilson and colleagues (2000) demonstrated that about 31% and 34.7% of the total current recorded in cerebellar granule neurons were R-type current in α1E KO and WT, respectively. Despite the fact that α1E KO mice have a deletion of the gene encoding for the α1E subunit they expressed R-type currents that were comparable to the one recorded in WT mice. However, the component of the R-type current sensitive to SNX-482 block was nearly loss in α1E KO mice (Wilson et al. 2000). The authors concluded that the α1E subunit is responsible for just a component of the total R-type current, specifically the component sensitive to SNX-482 block. A detail that was not mentioned by the authors of this study is the fact that their animal model is a whole body constitutive knock-out and thus the possibility of the occurrence of compensatory changes exists. These changes could confer other α1 subunits resistance to their respective blockers and as a result they can now contribute to the total R-type current. Jun et al (2000) showed an 80% reduction of the R-type current in cerebellar granule neurons of α1A (P/Q-type VGCC) KO mice suggesting the capacity of this subunit to contribute to R-type currents. Together these studies suggested that total R-type currents are conducted by a heterogeneous population of Ca^{2+} channels formed at least by two α1 subunits: α1E and α1A.

α1E KO mice have normal colonic propulsive motility

To investigate the physiological role and significance of R-type VGCC in enteric neuromuscular transmission I used the whole body constitutive α1E knock-out mouse. This model has a deletion of the α1E (Cav2.3) gene and thus neither the transcript nor the protein could be present in this animal (Wilson et al. 2000). This provided a valuable opportunity to identify the role of these channels in regulation of colonic propulsive motility.

Colonic propulsive motility was evaluated through *in vivo* and *in vitro* studies. Fecal output was monitored for three consecutive days and α1E KO mice did not show any major alterations that could indicate disruptions in colonic propulsive motility. A similar outcome was observed by studying the CMMC motor pattern. I showed that VGCC, specifically N-type channels, are essential for regulation of motor patterns in the colon. However, the fact that no alterations were observed in α1E KO mice suggested that either R-type channels are not critical for colonic propulsive motility or the ENS is able to offset the loss through compensatory changes in order to maintain colonic motility. Evidence for the occurrence of compensatory mechanisms in α1E KO mice has been reported. Whole cell patch-clamp recordings in Purkinje neurons from heterozygous mice showed deficits in the generation of long-term depression (LTD), which is a critical event underlying motor learning. Interestingly, the generation of LTD in α1E KO mice was not different from WT mice suggesting that compensatory mechanisms must come into play. However, the authors of that study were unable to identify the nature of the compensatory mechanisms (Osanai et al. 2006). The question that arises is the following; do compensatory changes occur in the ENS in order to maintain normal colonic motility? In the present study, the results showing an effect of $NiCl_2$ in the CMMC of α1E KO but not WT mice point toward the possibility of an increase in the $NiCl_2$-sensitive currents of α1E KO mice. R-type channels that are not form by the α1E subunit most likely generate this potentially new $NiCl_2$-sensitive component present in α1E KO mice.

R-type VGCCs participate in colonic inhibitory neuromuscular transmission

Although the specific function of R-type currents in the ENS is unclear, these channels are the predominant conductors of Ca^{2+} currents in myenteric neurons activated by corticotrophin release factor (Bisschops et al. 2006). In addition, another study demonstrated that about 44.4% of the Ca^{2+} current conducted by myenteric neurons maintained in primary culture is R-type current (Bian et al. 2004). Functionally these R-type VGCC contribute to action potentials but not slow synaptic transmission in intrinsic primary afferent neurons, also known as AH neurons (Naidoo et al. 2010). The role of R-type VGCC in neurotransmitter release within the ENS is less clearly understood but there are studies that support a role of these channels during this process. Specifically, R-type VGCC contribute to release of ACh in order to mediate fast excitatory post-synaptic potentials in a subset of myenteric neurons, known as S neurons (Naidoo et al. 2010).

The present studies showed that pharmacological block of R-type VGCCs using $NiCl_2$ and the neurotoxin SNX-482 produced a significant inhibition of the IJPs in WT mice, demonstrating a contribution of these channels to inhibitory neuromuscular transmission. The physiological role and significance of R-type channels was further investigated using α1E KO mice. IJP recordings from α1E KO mice showed a significant reduction of both components of this electrical event. These results suggested participation of R-type VGCCs in both the purinergic and nitrergic components of the IJPs. My studies contribute to the current body of evidence supporting a role for R-type VGCC within the ENS especially during regulation of neurotransmitter release to the colonic smooth muscle.

In this part of my studies IJP recordings were conducted under conditions where L-type channels were blocked in order to achieve successful cell impalements. The rationale for this is that block of these channels prevent muscle movement without affecting IJPs because these channels do not contribute to active or passive properties of enteric neurons or neurotransmitter release (Kunze et al. 1994; Reis et al. 2000; Reis et al. 2002). However, L-type channels are responsive to perturbations

around them especially those targeting the function of other VGCC subtypes (Etheredge et al. 2007; Flink et al. 2002; Thibault et al. 2001). Therefore, in order to unmask any potential compensatory contribution IJP recordings were conducted in conditions where L-type channels were not block (nifedipine-free conditions). Under this conditions IJPs from WT and α1E KO mice appeared to be at the same level, specifically the fast phase of these events corresponding to the purinergic component. The reduction in the nitrergic component remained irrespective of the status of L-type VGCC. Are these observations suggesting a compensatory contribution of L-type channels to inhibitory neuromuscular transmission to the colonic muscle of α1E KO mice?

IJPs from Alpha1E KO mice exhibit a nifedipine-sensitive component

The present study provided evidence demonstrating that the purinergic fast phase of the IJP in α1E KO mice is sensitive to L-type VGCC block by nifedipine. This phenomenon is not observed in IJPs recorded from WT mice suggesting this could be a compensatory adaptation to overcome the deletion of the α1E (Cav2.3) gene. Specifically, nifedipine caused about 34% inhibition of the IJP in α1E KO mice compared to 16% observed in WT mice. Interesting, the 34% inhibition caused by nifedipine in α1E KO mice is similar to the percent inhibition produced by SNX-482 in IJPs recorded from WT mice. This evidence further supports the idea that L-type channel contribution is occurring as a compensatory response to the deletion of the α1E gene. This compensatory contribution of L-type channels to inhibitory neuromuscular transmission appears to only "boost" purinergic transmission. Evidence supporting this statement mainly arises from the observation that nifedipine did not significantly reduce the AUC of the IJP and this parameter was still significantly smaller in recording conditions where L-type channels were available for activation.

There still important questions that remain to be answered. For example, what is the mechanism allowing participation of L-type channels in IJP generation? Why the nifedipine sensitivity

occurs only in the purinergic component of the IJP? Could this be related to the possibility of the existence of two different populations of inhibitory motor neurons: one containing the purinergic neurotransmitters stored in synaptic vesicles and another population containing the Ca^{2+}-dependent enzyme NOS? Or there is co-transmission of these neurotransmitters and L-type contribution occurs to favor the purinergic component due to the traditional dynamics of the purinergic neurotransmitter. Availability of immunohistochemical tools to detect purinergic nerves in combination with the currently available tools to detect nitrergic nerves and L-type channels will be valuable to answer these questions.

Adaptive changes related to L-type channels have been observed in the ENS. Chronic stress induced an enhancement of the mRNA and protein expression of L-type channels (CAV1.2 channels) in the colonic muscle after 9 days (Choudhury et al. 2009). These changes are associated with faster colonic transit and increased defecation rate demonstrating that the observed alterations in L-type expression are functional. The enhanced L-type channel expression was observed within 1 hour after exposure to stress and the authors identified transcriptional and post-transcriptional modifications as a mean to increase expression of L-type channels (Li and Sarna, 2011). These studies support the notion that L-type Ca^{2+} channels are responsive to perturbations occurring within the ENS.

Resting membrane potential of cell in Alpha1E KO mice are depolarized

Ongoing release of NO regulates RMP of colonic muscle cells (Gil et al. 2010). The present study found a 5.7 mV depolarization of the resting membrane potential in colonic cells from α1E KO mice. This depolarization could be a consequence of the impairment in the AUC of the IJP, which suggests disruptions in the nitrergic component of inhibitory neuromuscular transmission. In addition, the depolarizing change RMP could facilitate activation of L-type channels since the membrane potential is now closer to the threshold for activation these channels (Chen et al. 2006). A depolarizing change in RMP of circular muscle cells can increase the excitability and firing pattern o these cells (France et al.

2012). However, it was interesting to observe that in α1E KO the depolarizing change in membrane potential did not altered excitability of circular muscle cells mice. As it was explained earlier the fast phase of the IJP is maintained in α1E KO mice and this could provide enough inhibitory innervation to keep the excitability of circular muscle cells unaltered. In this line, ongoing release of the purinergic neurotransmitter is responsible for generation of spontaneous IJPs contributing to the inhibitory tone present in the colonic muscle (Gil et al. 2010).

Active pool of nNOS and VGCC complex

In mouse gastrointestinal tissues nNOS exists in two pools: catalytically active and inactive (Rao et al. 2007; Chaudhury et al. 2009). The nature of the catalytically active pool of nNOS has been described and consists of a dimer of nNOSα associated with the membrane of the nerve terminal through interaction with the scaffolding protein PSD95. The latter is attached to the nerve terminal membrane by palmitoylation and importantly it is also anchored to VGCCs through an interaction that is yet unknown. All these protein interaction cluster nNOS with Ca^{2+} channels giving rise to the active pool of nNOS and the VGCC complex. Arrival of action potentials to the nerve terminal will open VGCC allowing a Ca^{2+} influx to the nerve terminal. The Ca^{2+} influx induces binding of calmodulin to the close by nNOS and the production of NO (Rao et al. 2007; Chaudhury et al. 2009).

It is possible that R-type VGCC cluster with the nNOS active pool through interaction with the PSD95 protein. Thus, a deletion of the α1E gene could produce a reduction in the active pool of this enzyme if this pool is unable to interact with other VGCC subtypes. Identification of the exact mechanism allowing interaction between PSD95 proteins and VGCC will provide information about the ability of nNOS to interact with different VGCC subtypes.

APPENDIX

R-type Current	Characteristics
G2	**Share features of recombinant α1E channels** (1) High sensitivity to Ni and SNX-482 block (2) Larger macroscopic current with Ca than Ba (3) Single channel conductance of 12-15 pS. (4) Activate ~ 15 mV more negative potentials than G3
G3	**Share features of HVA α1A (P/Q), α1B (N), and α1C (L)** (1) Resistant to SNX-482 and Ni block (2) Single channel conductance of 20 pS (3) Larger macroscopic current with Ba than Ca (4) Activate a more depolarized potentials

Table 4.1 Properties of the two main populations of R-type Ca^{2+} currents as identified in cerebellar granule cells. (Tottene et al. 1996; Forti et al. 1994)

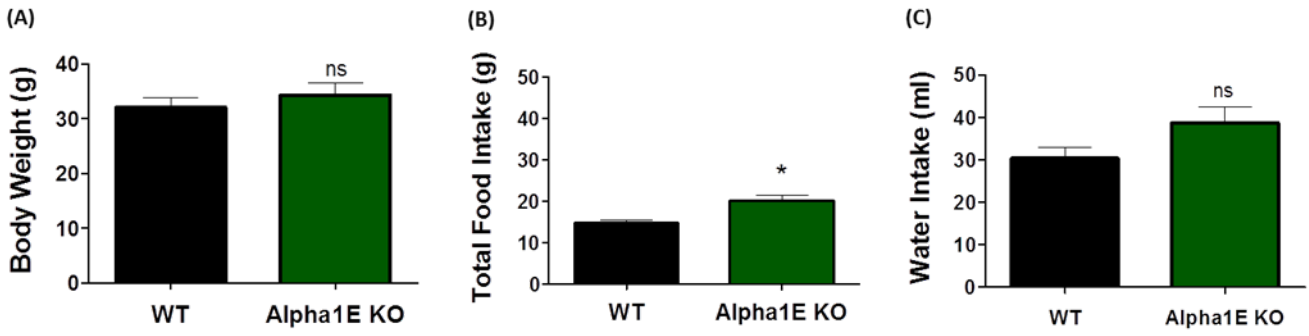

Figure 4.1: Body weight and total food and water intake during the *in vivo* fecal output study. (A) Body weight was similar between WT and KO mice (32 ± 1.8 g for WT and 34 ± 2.1 g for KO; $P > 0.05$). (B) Food intake was higher in KO mice and this increase was statistically significant (14.7 ± 0.7 g in WT and 20 ± 1.4 g in KO; $P = 0.0072$). (C) Water intake was also higher in KO mice, however, this increase was not statistically significant when compared to WT mice (30.3 ± 2.4 ml in WT and 38.7 ± 3.7 ml in KO; $P = 0.0907$). Data represent mean ± S.E.M. for n = 6 mice. Student's t-Test was used to compare averaged values and to detect statistical significance.

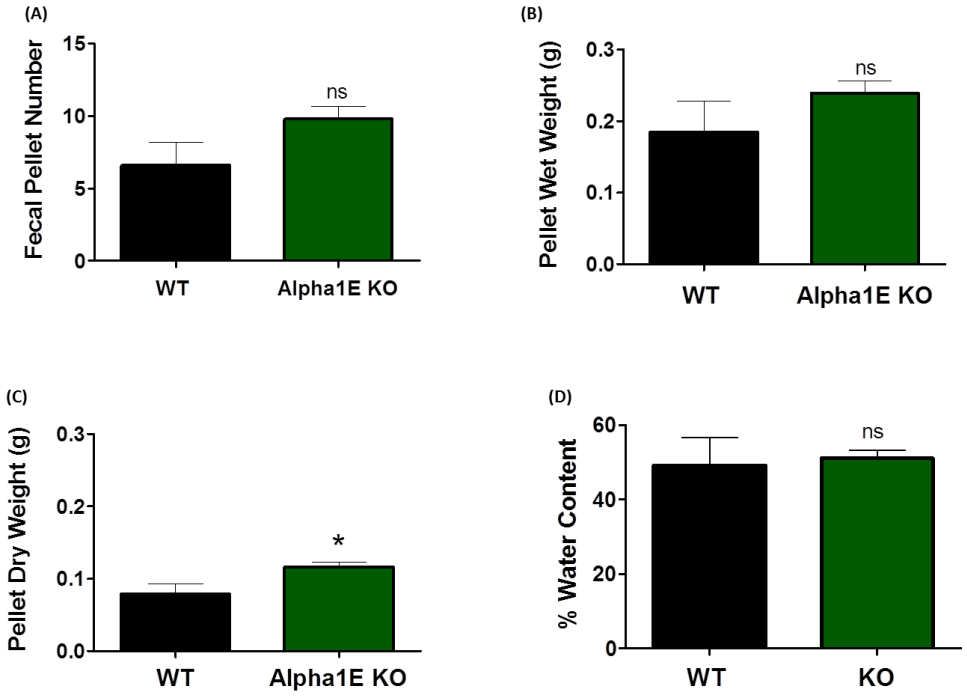

Figure 4.2: Mean fecal pellet output was higher in α1E KO mice. Fecal pellet output was monitored over a period of 1 hour for 3 consecutive days in each mouse. The 3 day average for each mouse was used to compare fecal output between WT and KO mice. (A) The mean pellet number was higher in KO mice, however, that increase did not reach statistical significance when compared to WT mice (6.6 ± 1.6 g in WT and 9.8 ± 0.9 g in KO; P = 0.1170). (B) Wet weight of the collected fecal material was measured each day and the averaged value showed there is not statistical difference between WT and KO, although the mean weight tend to be larger in KO mice (0.18 ± 0.04 g in WT and 0.23 ± 0.02 g in KO; P = 0.2695). (C) Pellets were placed at 60C overnight and dry weight was measured. Data showed there is a small but statistical significant increase in the dry weight of the fecal material in KO mice (0.07 ± 0.01 g in WT and 0.11 ± 0.006 in KO; P = 0.0416).(D) The percentage of water content was calculated by dividing the difference between pellet wet and dry weight into pellet wet weight. That difference was then multiplied by 100 to get the percentage of water content. These values were very similar between WT and KO mice (49.2 ± 7.5% in WT and 51.1 ± 2.1% in KO; P = 0.8096). Data represent mean ± S.E.M.

Figure 4.2 (cont'd)

for n = 6 mice. Student's t-Test was used to compare averaged values and to detect statistical significance.

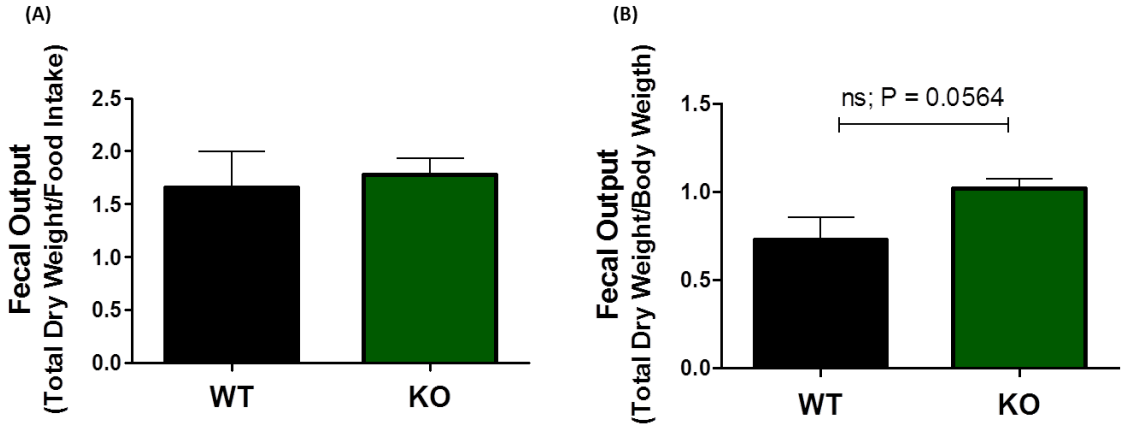

Figure 4.3: *In vivo* propulsive motility is not altered in α1E KO mice. (A) and (B) are showing the calculated percentage fecal output in WT and KO mice. (A) Fecal output was calculated by dividing total pellet dry weight for each mouse into its total food intake and multiplied that by 100. This value was similar between the two types of animals (1.6 ± 0.33% in WT and 1.8 ± 0.15% in KO; P = 0.7612). (B) Fecal output was also calculated by dividing total fecal dry weight of each mouse into its body weight and multiplied that by 100. This value was larger in KO mice, however, the increase did not reach statistical significance although the P value was very close to be considered significant (0.72 ± 0.12% in WT and 1.02 ± 0.05% in KO; P = 0.0564). Data represent mean ± S.E.M. for n = 6 mice. Student's t-Test was used to compare averaged values and to detect statistical significance.

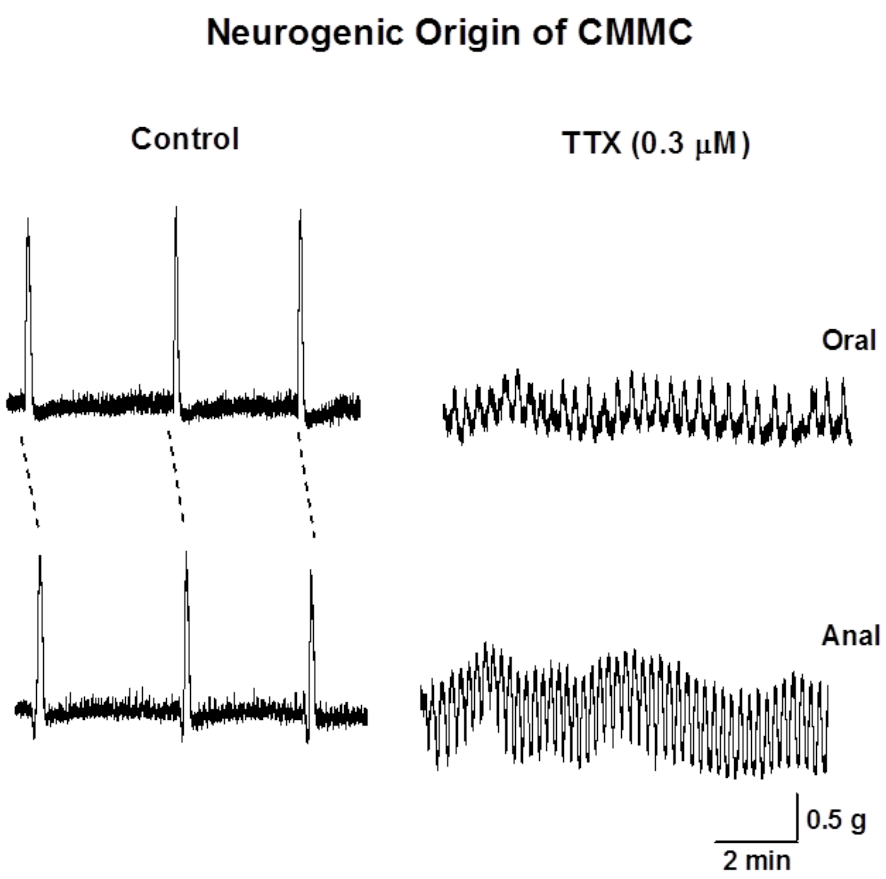

Figure 4.4: The neurogenic nature of the CMMC motor pattern. Addition of the Na^+ channel blocker tetrodotoxin (TTX) completely disrupted the organized CMMC pattern. This demonstrates that this motor patter is dependent upon neural communication. Note the increase in myogenic contractions after addition of TTX.

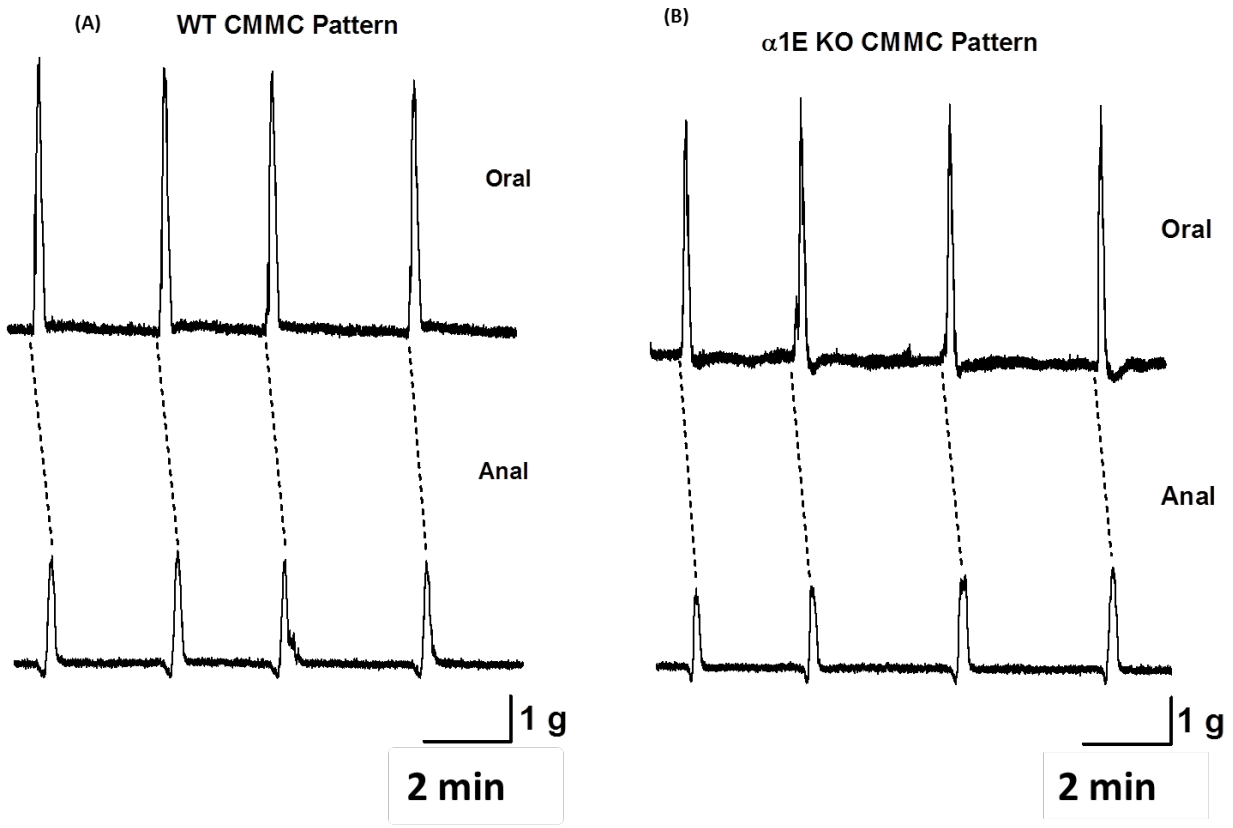

Figure 4.5: Representation of the CMMC pattern in WT and α1E KO mice. Oral-anal propagating contractions are presented for WT (A) and KO (B) mice. In general, the CMMC pattern was very similar between WT and KO mice.

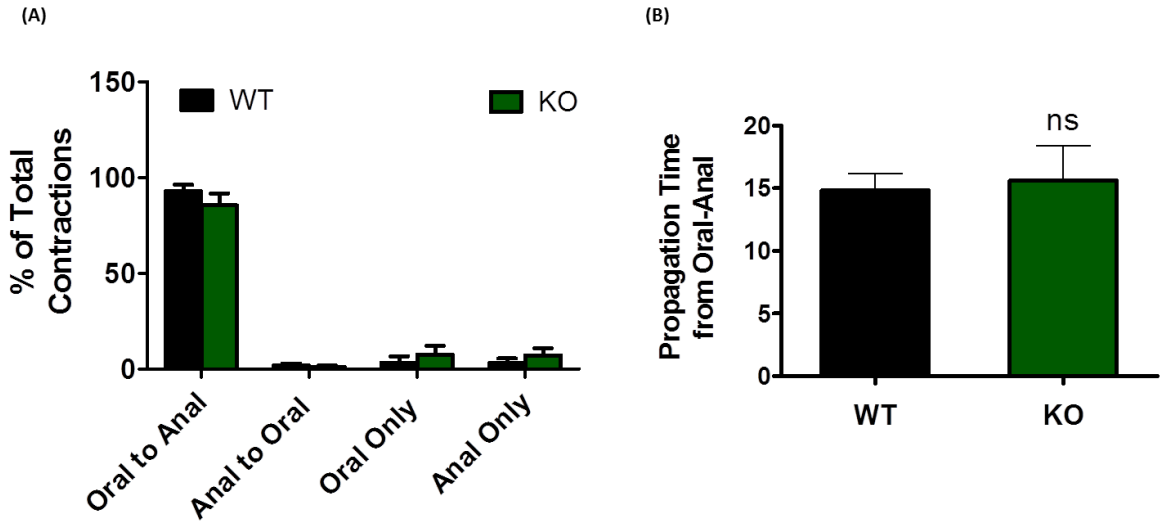

Figure 4.6: Propagation pattern of contractions is not altered in α1E KO mice. CMMC pattern of contraction was divided into oral-anal propagated contractions, anal-oral retrogradely propagated contractions, contractions only at the oral side and only at the anal side. (A) The proportion of contractions propagating from oral-anal was not different between WT and KO mice (93 ± 3.3% in WT and 85 ± 6% in KO). The remaining contractions were propagating in a retrograde direction or occurring only at the oral or anal side. The distribution did not differ between WT and KO mice. (B) Propagation time of oral-anal propagating contractions was similar between WT and KO mice (14.8 ± 1.3 s in WT and 15.6 ± 2.8 s in KO; P = 0.7964). Data represent mean ± S.E.M. for n = 10 mice. Student's t-Test was used to compare averaged values and to detect statistical significance.

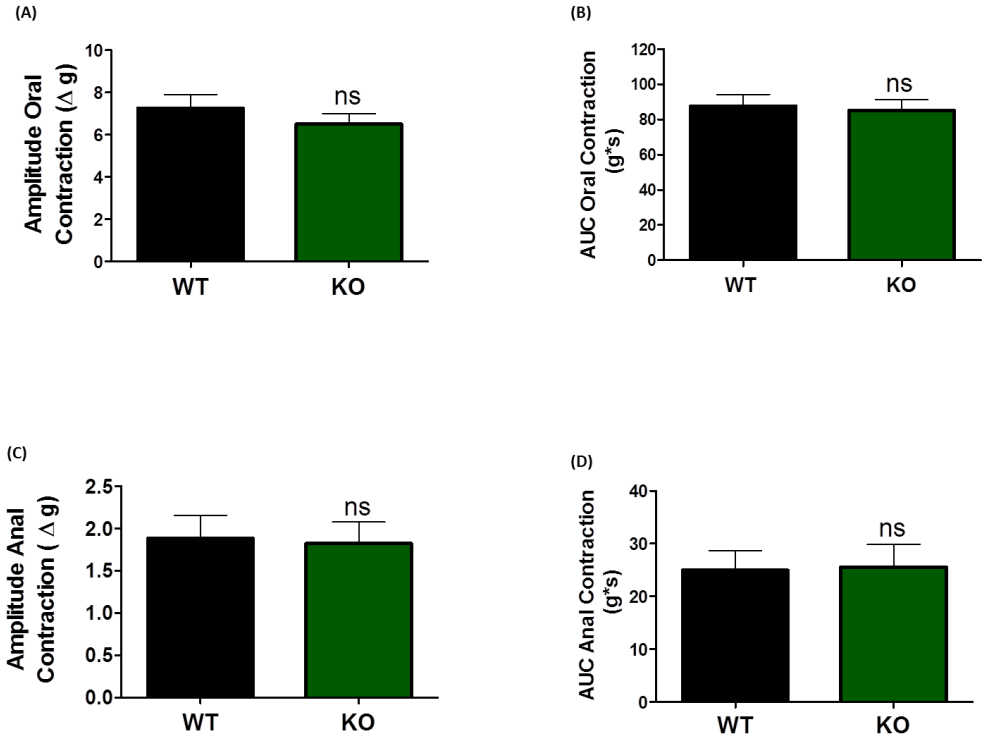

Figure 4.7: The nature of the contractions generating the CMMC is not altered in α1E KO mice. The amplitude (A) and AUC (B) of the oral contractions were not different between WT and KO mice. Similarly, the amplitude (C) and AUC (C) of the anal contractions were not different either between WT and KO mice. Data represent mean ± S.E.M. for n = 10 mice. Student's t-Test was used to compare averaged values and to detect statistical significance.

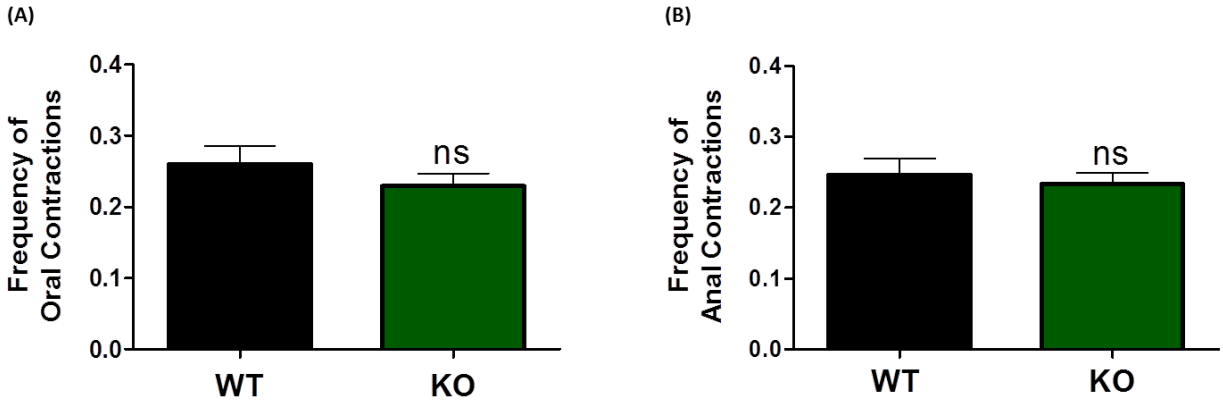

Figure 4.8: Frequency of oral and anal contractions during the CMMC is not altered in α1E KO mice. The number of oral and anal contractions generated in 30 minutes was counted in order to determine frequency of these contractions. Oral contractions occurred at a frequency of 0.26 ± 0.02 Hz in WT and 0.23 ± 0.01 Hz in KO and the small difference was not statistically significant (P = 0.3504). Anal contractions occurred at a similar frequency in WT and KO mice (0.24 ± 0.02 Hz in WT and 0.23 ± 0.01 Hz in KO mice; P = 0.6445). Data represent mean ± S.E.M. for n = 10 mice. Student's t-Test was used to compare averaged values and to detect statistical significance.

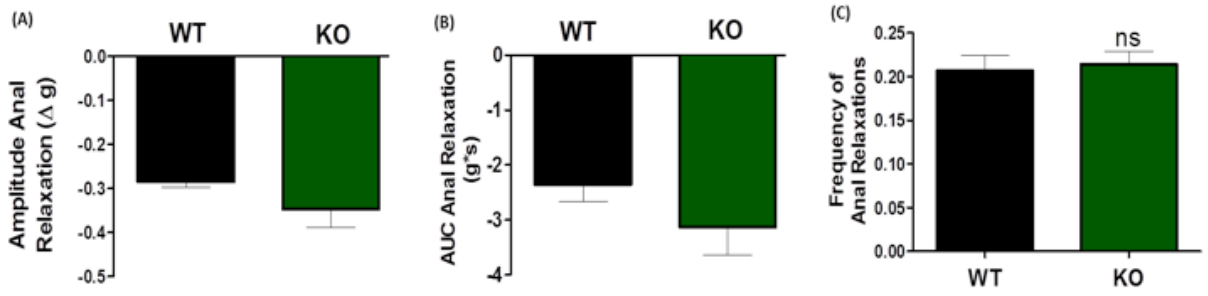

Figure 4.9: Relaxations preceding each colonic migrating contraction were not altered in α1E KO mice. There was a tendency for the amplitude (A) and AUC (B) of the relaxations to be larger in KO mice. However, none of these parameters were significantly different between WT and KO mice. (C) The frequency of anal relaxations, in a period of 30 minutes, and this value was similar between WT and KO mice. Data represent mean ± S.E.M. for n = 10 mice. Student's t-Test was used to compare averaged values and to detect statistical significance.

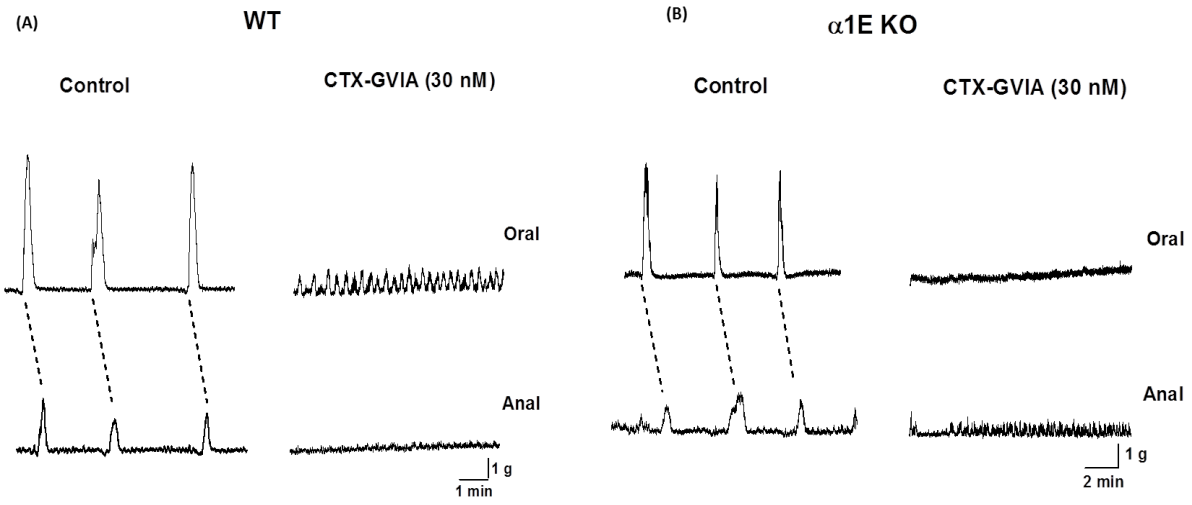

Figure 4.10: Blocking of N-type VGCCs abolished the CMMC pattern. Block of N-type channels with 30 nM CTX-GVIA abolished the CMMC pattern in WT (A) and KO mice (B). CTX-GVIA at 100 nM acted had a similar action (not shown). Interesting, after block of N-type channels the myogenic contractions in KO mice were smaller than WT.

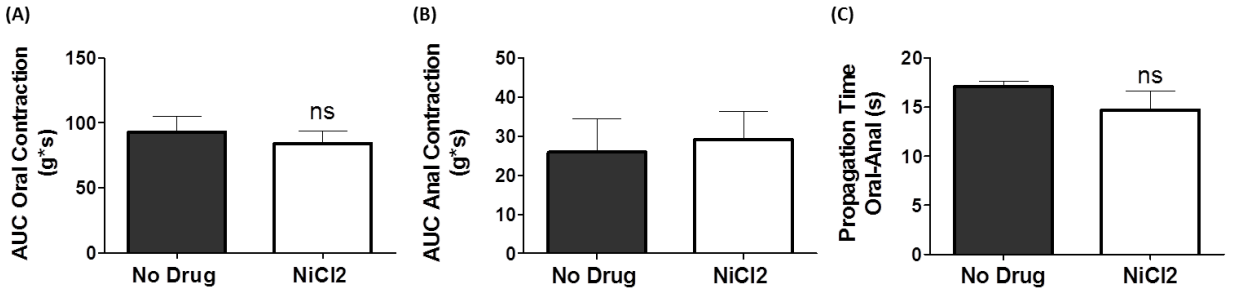

Figure 4.11: Pharmacological block of R-type VGCCs with $NiCl_2$ did not alter CMMC in WT mice. Acute block of R-type calcium currents with NiCl2 (50 um) did not significantly altered the AUC of oral (A) and anal (B) contractions. This treatment did not alter either the amplitudes of oral and anal contractions (data not shown) of WT mice. (C) Propagation time of oral-anal propagated contractions was shorter after NiCl2 treatment, but this difference was not statistically significant. Data represent mean ± S.E.M. for n = 7 mice. Student's t-Test was used to compared averaged values and detect statistical significance.

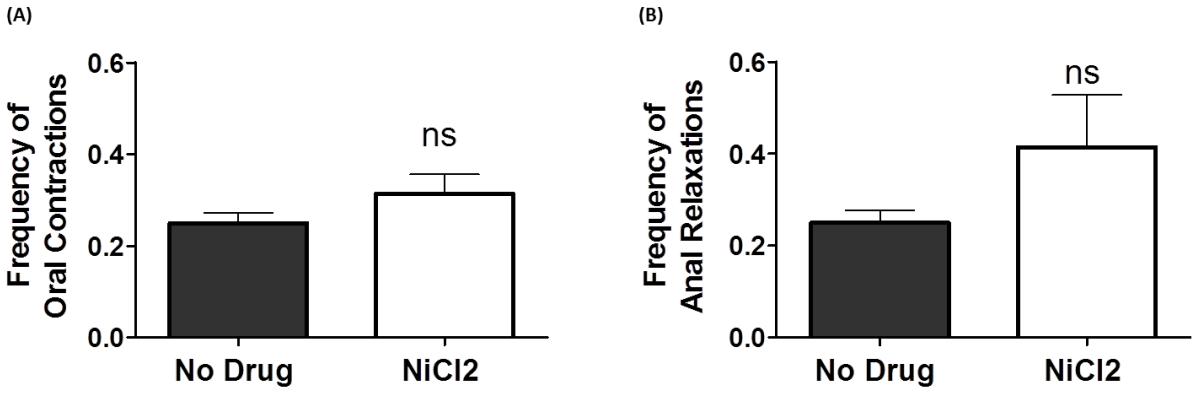

Figure 4.12: Frequency of CMMC is not altered by $NiCl_2$ in WT mice. $NiCl_2$ treatment produced a small increase in the frequency of oral (A) and anal (B) contractions. However, this change was not statistically significant for neither the oral nor the anal contractions.

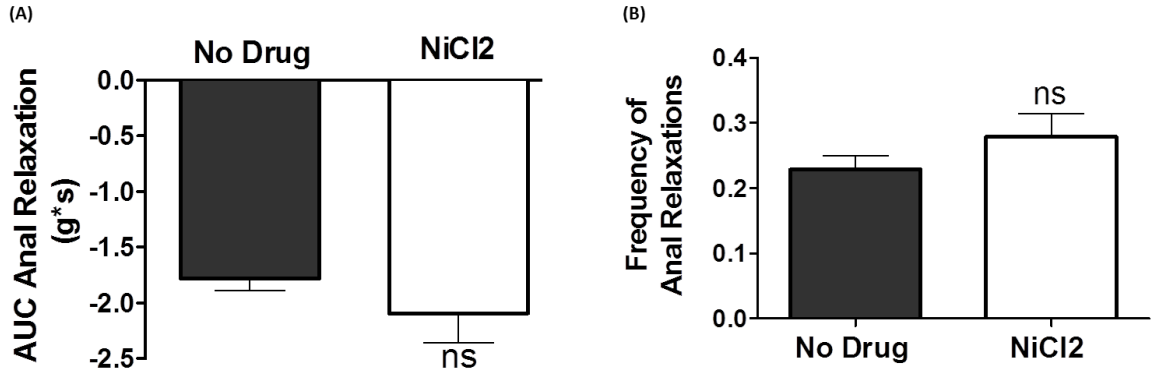

Figure 4.13: Relaxations preceding CMMC were not altered by $NiCl_2$ in WT mice. (A) The AUC of the relaxations occurring before the contractions were slightly increased by $NiCl_2$, however this effect was not statistically significant. (B) The frequency of these relaxations was not significantly altered either by NiCl2 treatment.

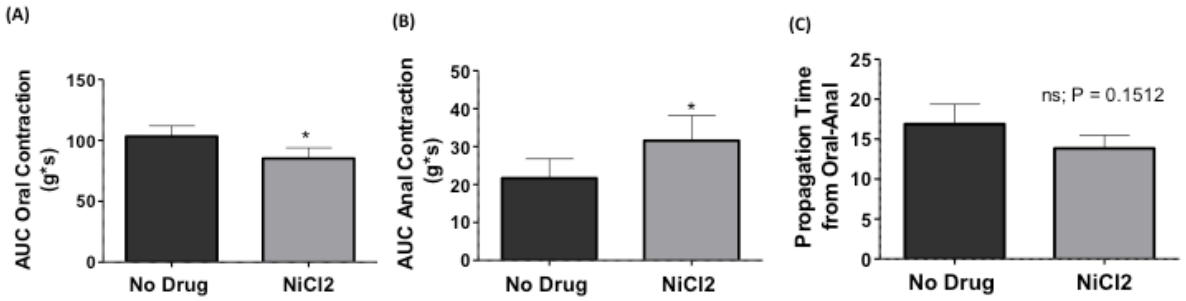

Figure 4.14: Pharmacological block of R-type VGCCs with $NiCl_2$ altered CMMC in α1E KO mice. (A) $NiCl_2$ produced a significant decrease in the AUC of the oral contractions. (B) However, $NiCl_2$ treatment produced a significant increase in the AUC of the anal contractions. (C) Propagation time of oral-anal propagated contractions was shorter after $NiCl_2$ treatment; however, this decrease was not statistically significant.

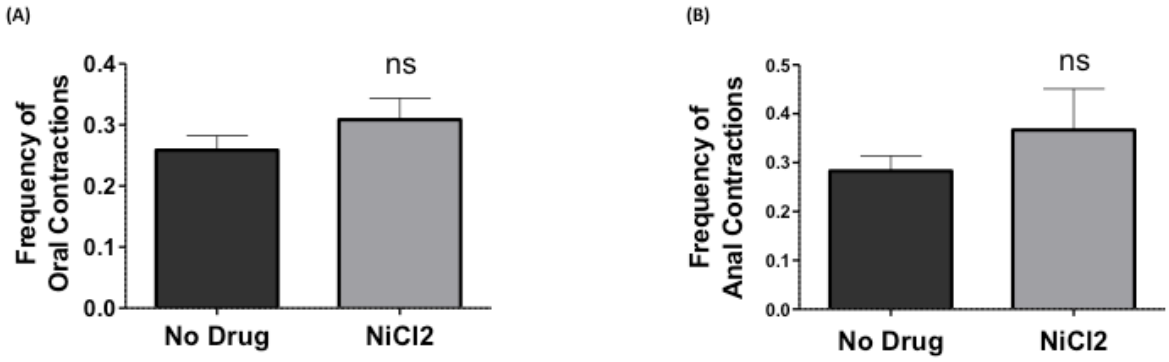

Figure 4.15: Frequency of CMMC was not altered by $NiCl_2$ in α1E KO mice. $NiCl_2$ treatment produced a small increase in the frequency of oral (A) and anal (B) contractions. However, this change was not statistically significant for neither the oral nor the anal contractions.

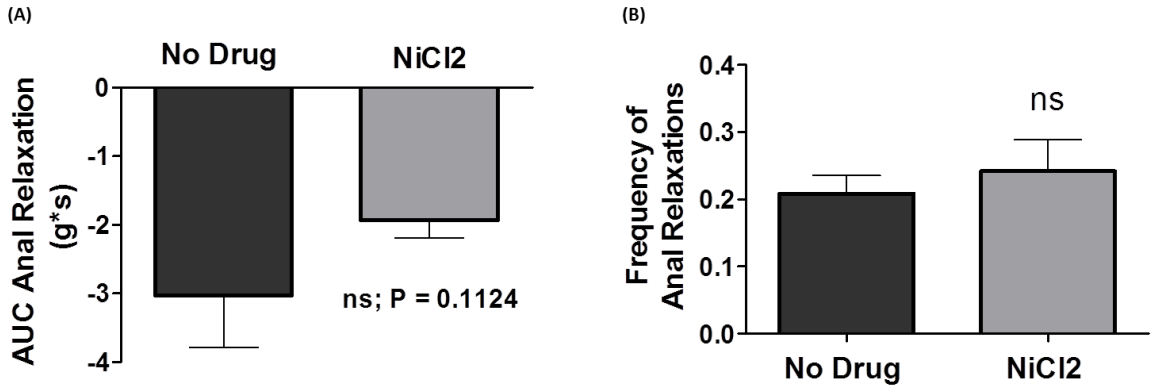

Figure 4.16: Relaxations preceding CMMC were slightly altered by $NiCl_2$ in α1E KO mice. (A) $NiCl_2$ treatment produced a decrease in the relaxations preceding the CMMCs, however this change was not statistically significant. (B) Frequency of relaxations was not significantly affected by $NiCl_2$ treatment.

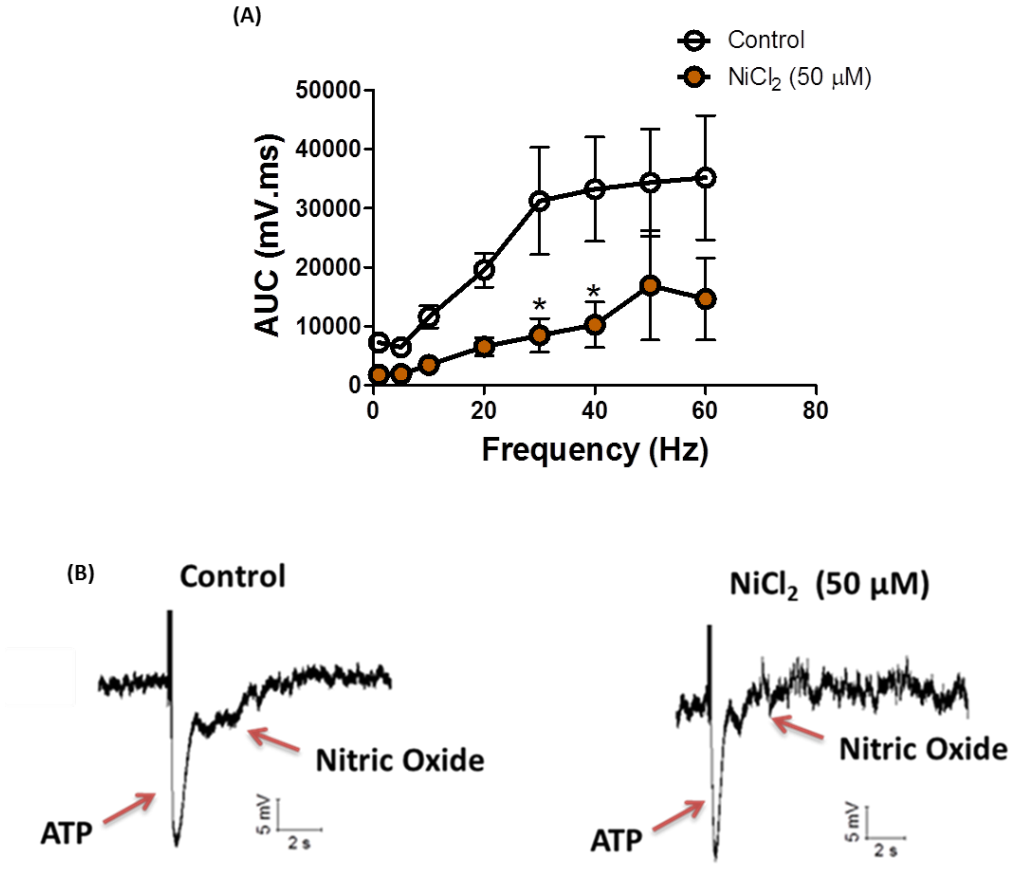

Figure 4.17: $NiCl_2$ significantly reduced IJPs recorded from the colon of WT mice. (A) There is a frequency-dependent increase in the AUC of IJPs recorded from WT mice. Application of 50 um $NiCl_2$ significantly reduced the AUC of the IJPs ($P<0.05$). (B) Representative control IJP and after 10 min application of 50 um $NiCl_2$. Note $NiCl_2$ decreased the slow nitrergic component of the IJP and this effect will be reflected as a reduced AUC as shown in panel A. Two-Way ANOVA was used to detect statistical differences. Data represent mean ± SEM for n = 11 cells.

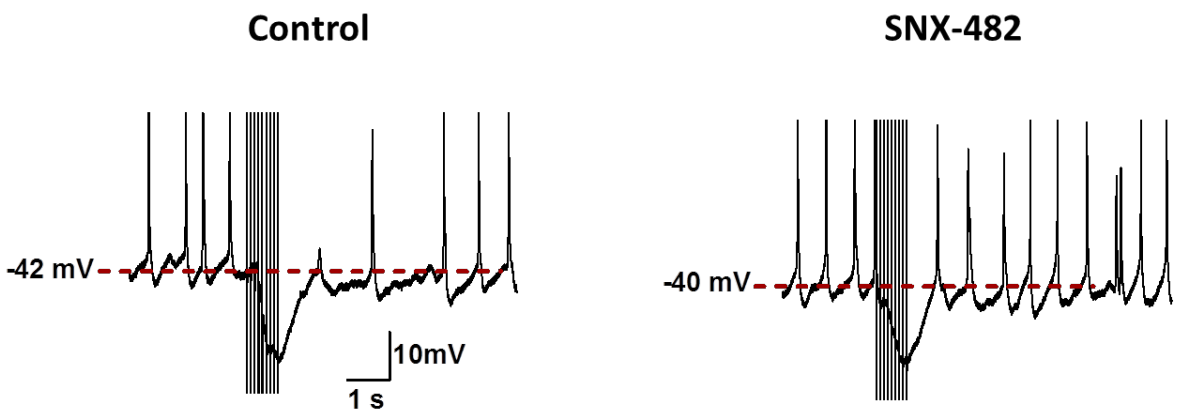

Figure 4.18: SNX-482 produces about 36% inhibition of the IJP in WT mice. Representative trace showing the effect of the R-type channel specific neurotoxin SNX-482. This toxin produced a percent inhibition averaging 35.6 ± 6.4% in WT mice. This result suggested a contribution of SNX-482-sensitive R-type calcium channels in generation of colonic IJPs.

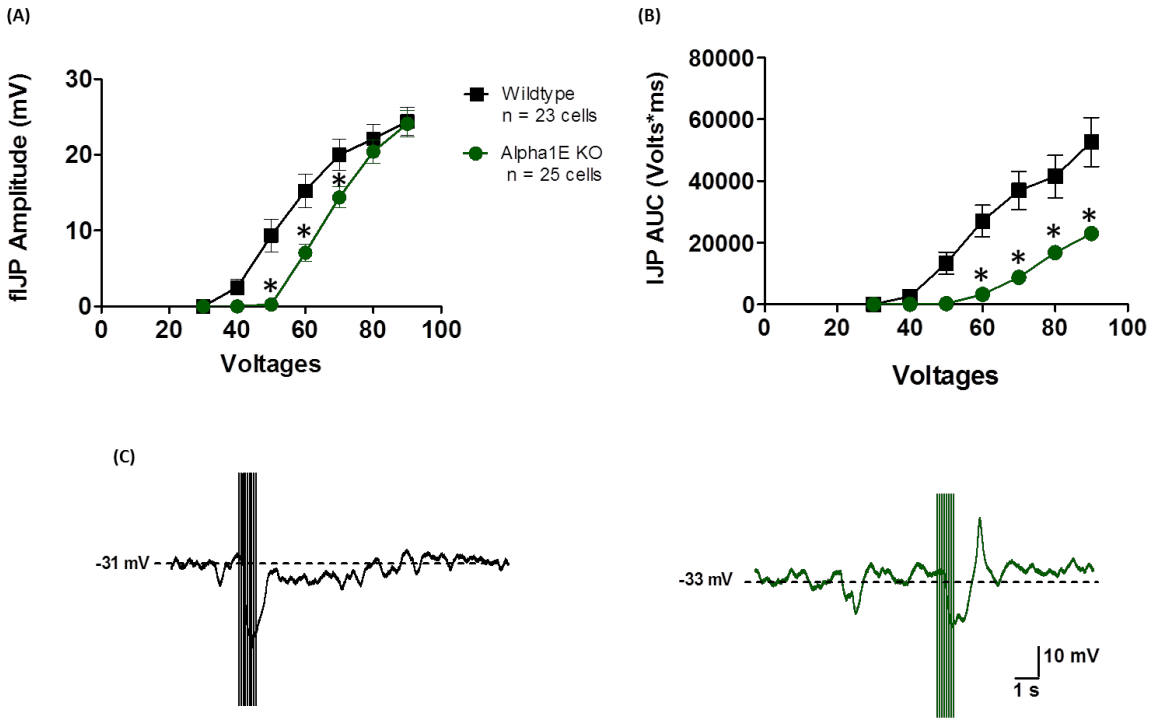

Figure 4.19: Amplitude and AUC of the IJPs are decreased α1E KO mice. IJPs were recorded under nifedipine conditions (1 um) to prevent movement of smooth muscle and achieve stable and longer impalements. There is a voltage-dependent increase in the amplitude and AUC of the IJPs (voltage-effect: $P<0.05$) in both WT and KO mice. (A) However, KO mice presented a significant decrease in the amplitude of the IJPs (genotype-effect: $P<0.05$). (B) In a similar way, KO mice presented a significant decrease in the AUC of the IJPs (genotype effect: $P<0.05$). A significant interaction was detected ($P<0.05$), meaning that genotype does not have the same effect on amplitude and AUC at all values of voltages used to evoke IJPs. (C) Representative IJPs recorded from WT and KO circular muscle cells. Note the decrease in amplitude and duration of the IJP recorded from a KO circular muscle cell. . Two-Way ANOVA was used to detect statistical differences. Data represent mean ± SEM for n = 23 cells for WT and n = 25 cells for KO.

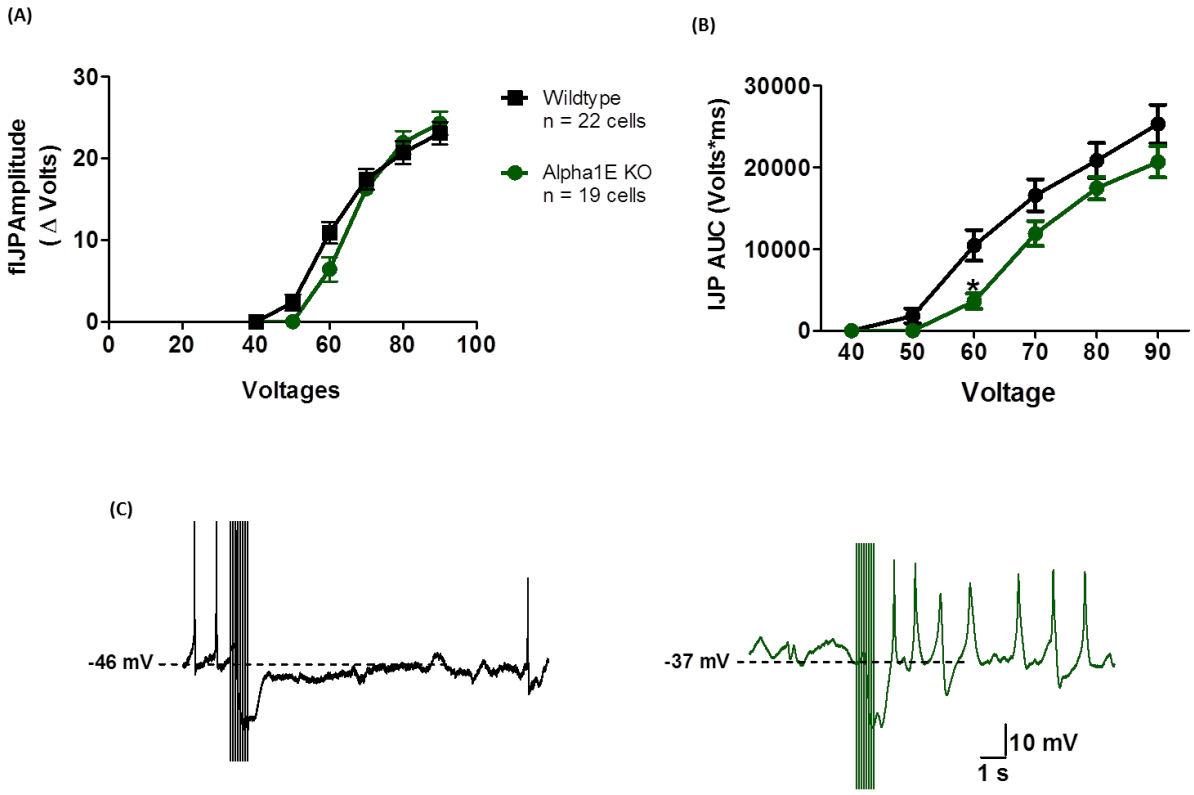

Figure 4.20: In nifedipine-free conditions the fast component of the IJP in α1E KO mice is at WT levels. IJPs were recorded in nifedipine-free conditions in order to determine if L-type channels are contributing to IJP generation. There is a voltage-dependent increase in the amplitude and AUC of the IJP (voltage-effect: $P<0.05$). (A) The amplitude of the IJPs in KO mice are now at the level of WT (genotype-effect: $P<0.05$). (B) The AUC is still significantly lower in KO mice as compared to WT (Genotype-effect: $P>0.05$). No significant interaction was detected ($P>0.05$). (C) Representative IJP recording from WT and KO circular muscle cells under nifedipine-free conditions. Note the calcium-dependent action potential firing in both cells. . Two-Way ANOVA was used to detect statistical differences. Data represent mean ± SEM for n= 22 cells for WT and 19 cells for KO.

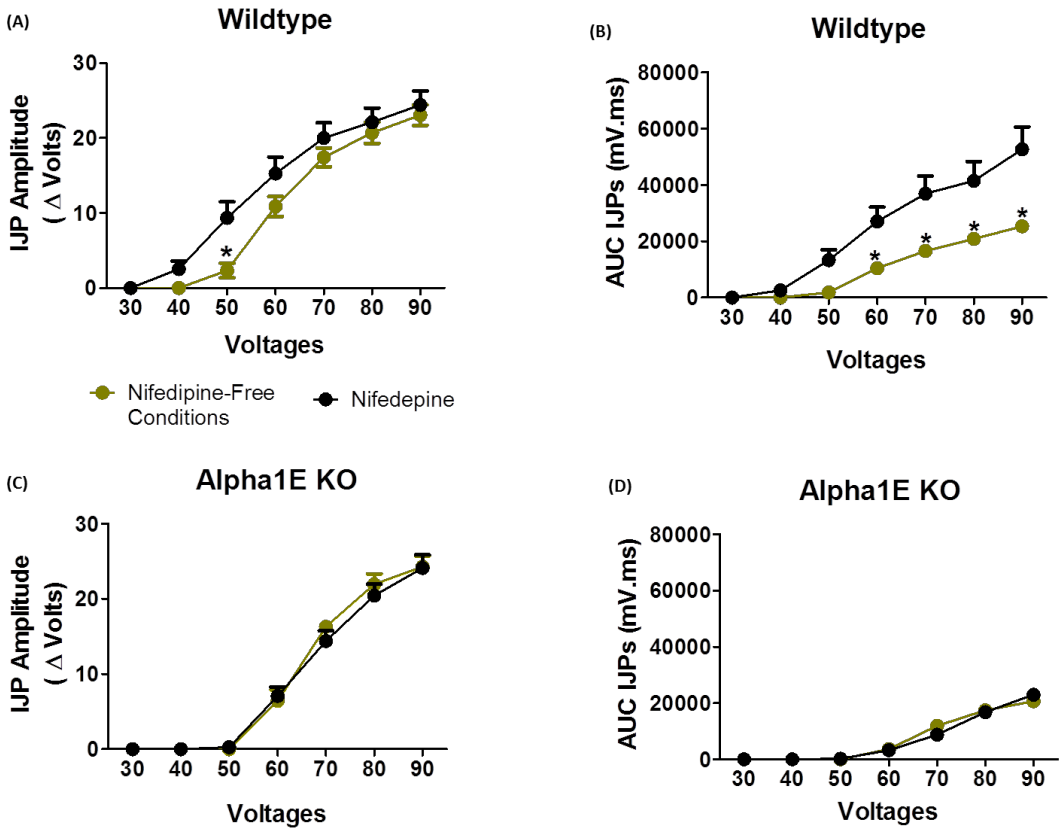

Figure 4.21: The presence of nifedipine altered IJP only in WT mice. IJPs recorded in the absence and presence of nifedipine (1 μM) were pooled and compared in WT and KO mice. This represents a non-paired comparison of the IJPs. This analysis revealed that presence of nifedipine in recording conditions significantly increases the amplitude (A) and AUC (B) of the IJPs (Treatment effect: $P<0.05$) in WT mice. (B) However, a similar comparison in KO mice revealed that presence of nifedipine in recording conditions did not change neither the amplitude (C) nor the AUC (D) of IJPs in these mice (Treatment effect: $P>0.05$).

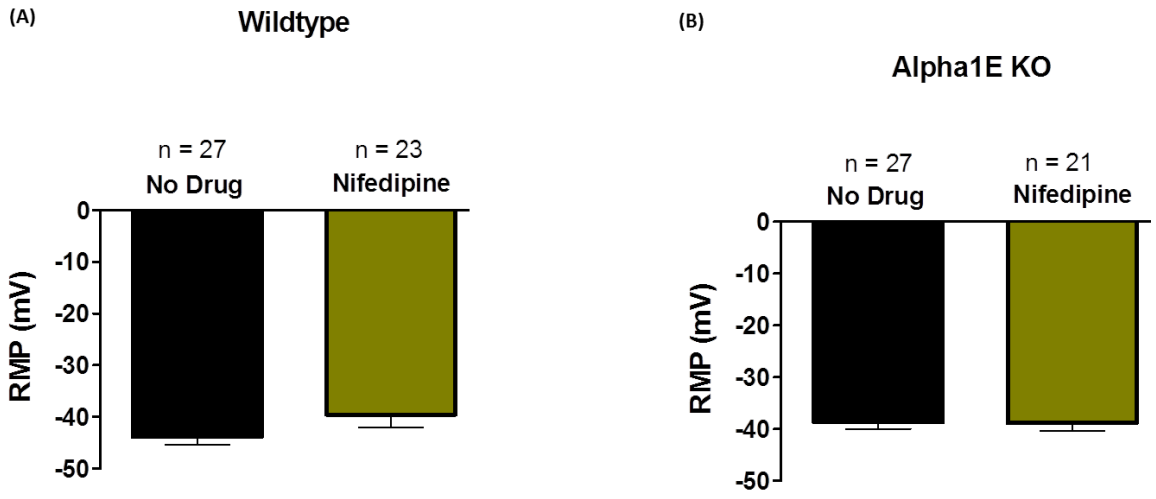

Figure 4.22: Resting membrane potential is not altered by nifedipine. The resting membrane potential (RMO) of circular muscle cells impaled in nifedipine-free conditions was compared with those impaled in the presence of nifedipine. These RMP values correspond to the cells from which IJPs are shown in figure 19. This is again an unpaired comparison of the effect of nifedipine in RMP. (A) In WT, cells show a RMP averaging -44 ± 1.5 Mv in nifedipine-free conditions and -39.5 ± 2.4mV in presence of nifedipine. There is a 4.5 Mv depolarization in RMP of cells impaled in presence of nifedipine, however, this effect was not statistically significant (P = 0.1303). (B) In KO, the presence of nifedipine did not change RMP of cells at all (-38.7 ± 1.3 mv in nifedipine-free conditions and -38.8 ± 1.5mv in presence of nifedipine; P = 0.9469). Student t-Test was used to detect statistical differences. Data represent mean ± SEM.

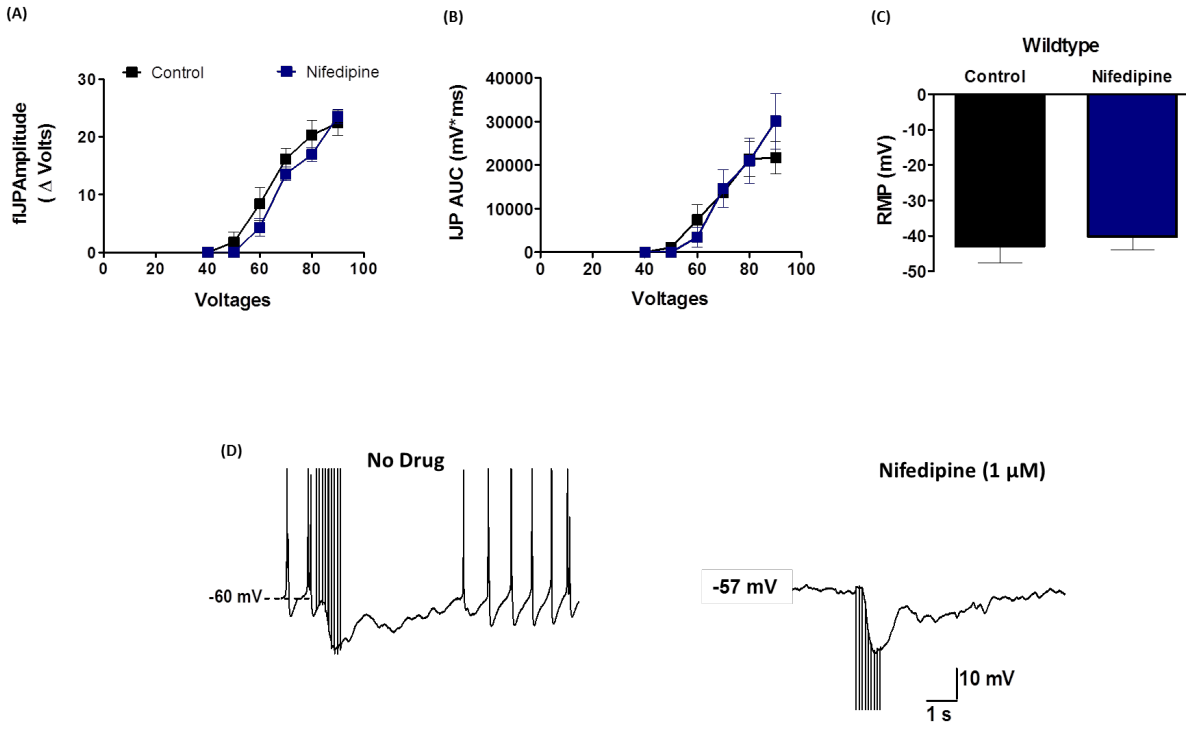

Figure 4.23: IJPs in WT mice are not sensitive to block of L-type channels by nifedipine. To better evaluate sensitivity of IJPs to the L-type channel blocker nifedipine, IJPs were recorded in control conditions and after 10 min application of 1 um nifedipine. Nifedipine did not produce any significant change in the amplitude (A) or AUC (B) of the IJPs. (C) Nifedipine did not change the RMP of cell from WT mice (-42 ± 4.7 control 40.2 ± 3.6 in nifedipine conditions; P = 0.6644). (D) Representative IJPs recorded in control and after application of 1 um nifedipine in WT mice. . Two-Way ANOVA was used to detect statistical differences in panels A and B. Student t-Test was used for panel C. Data represent mean ± SEM for n= 6 cells.

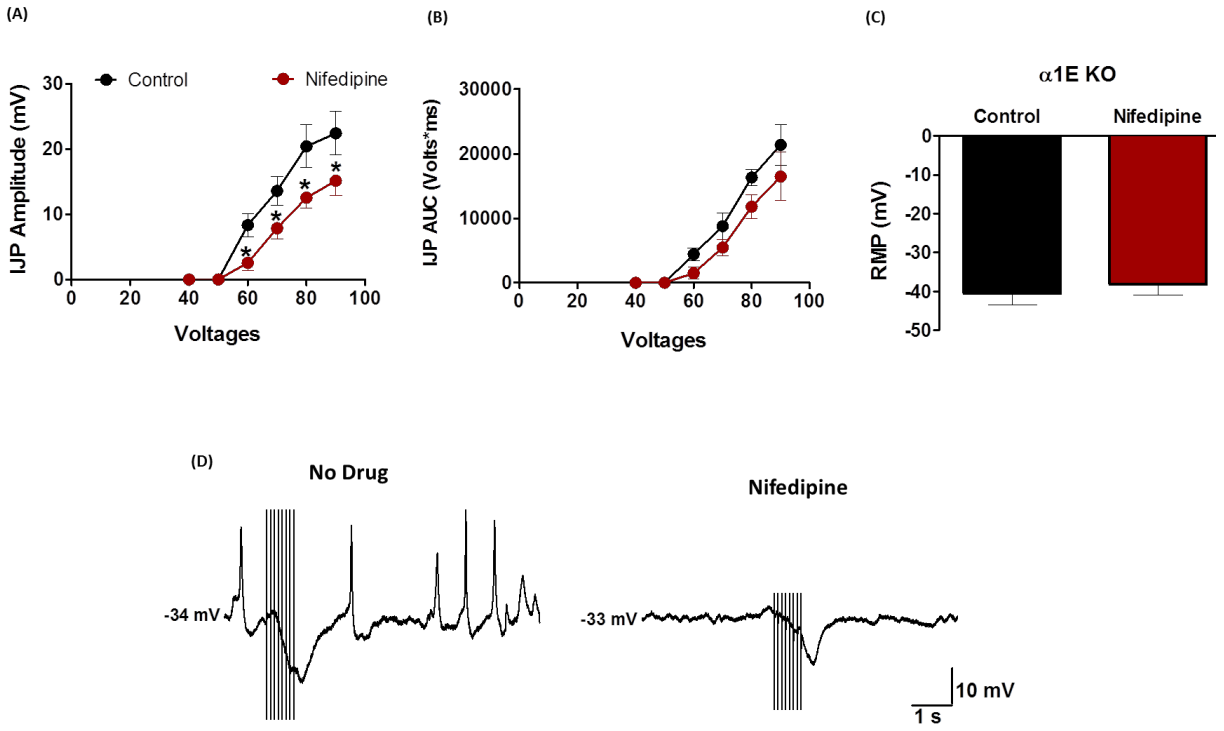

Figure 4.24: Fast IJPs α1E KO mice are sensitive to block of L-type channels by nifedipine. The sensitivity of IJPs to block of L-type channels by nifedipine was also evaluated in KO mice by recording IJPs in control and after 10 min application of 1 um nifedipine. (A) The amplitude of the IJPs was significantly decreased after perfusion of nifedipine (treatment effect: $P<0.05$). (B) The AUC of the IJPs was slightly decreased by nifedipine but this effect was not statistically significant when the effect at each voltage used was compared between control and nifedipine treatment. (C) Even though nifedipine did decrease the IJP amplitude, this treatment did not alter RMP of the cells from where those IJP were recorded (-40.5 ± 2.8 in control and -38.1 ± 2.7 in nifedipine conditions; $P = 0.5613$). (D) Representation of this inhibitory effect in alpha1E KO IJPs. Two-Way ANOVA was used to detect statistical differences in panels A and B. Student t-Test was used for panel C. Data represent mean ± SEM for n= 7 cells.

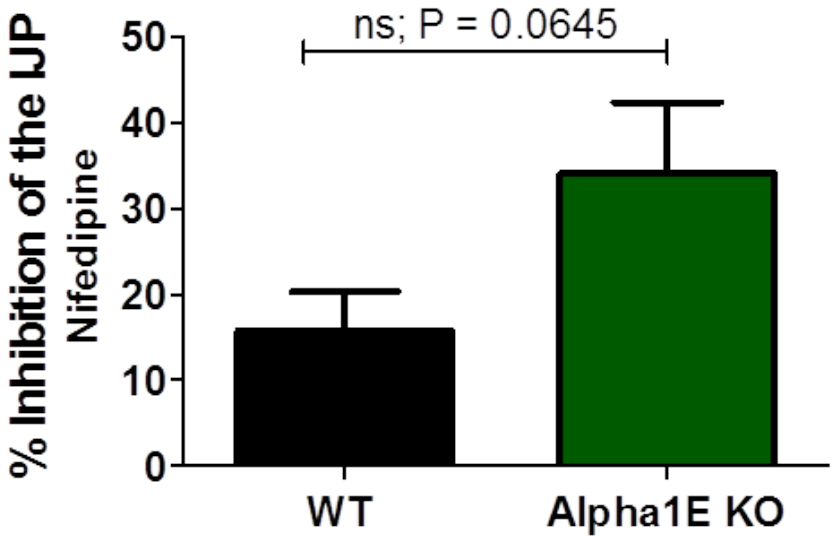

Figure 4.25: IJPs from α1E KO mice have a nifedipine-sensitive component. Nifedipine treatment produced a 15.6 ± 4.7% inhibition in WT and 34 ± 8.2% inhibition in KO mice. The difference in percent inhibition between WT and KO mice was substantial however it did not reach statistical significance although the P value was very close to be considered significant, P = 0.0645. Student t-Test was used to detect statistical significance. Data represent mean ± SEM for n= 8 cells for WT and n = 7 cells in KO.

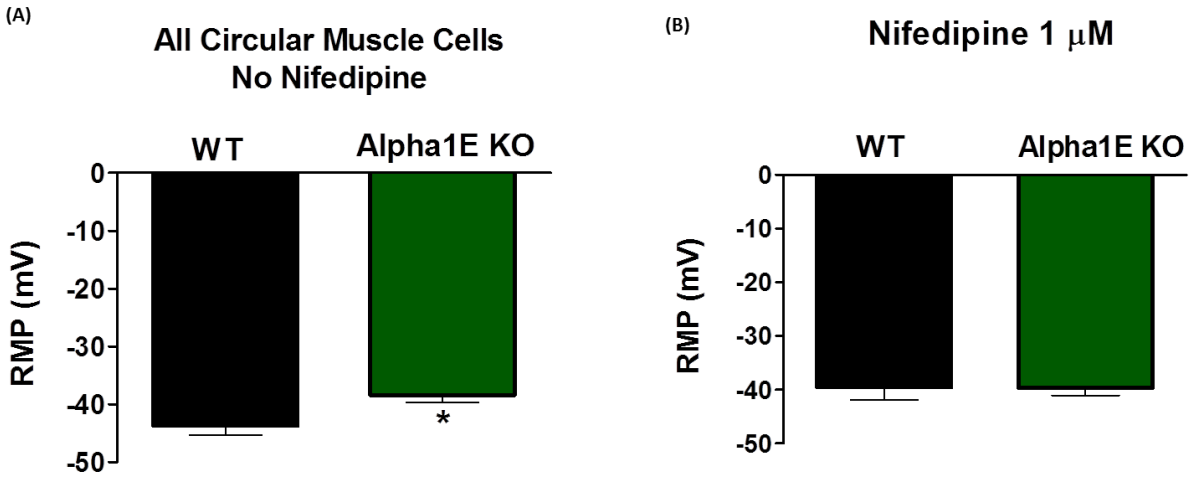

Figure 4.26: Cells from α1E KO mice have a depolarized resting membrane potential. The RMP of all the cells recorded from WT and KO mice was measured and compared. (A) Overall cells from WT mice had a RMP averaging -43.7 ± 1.7mv and KO cells had a RMP averaging -38 ± 1.3mv. When compared these values were statistically different, P = 0.0169. Thus, there is a 5.7 mV depolarization of the RMP in cells from KO mice. (B) However, when nifedipine is present in recording conditions this difference disappears. WT and KO cells have a RMP averaging -39.5 ± 2.4mv and -39.7 ± 1.4mv, respectively (P = 0.9649). Student t-Test was used to detect statistical significance. Data represent mean ± SEM for n= 27 cells for WT and KO in panel A and n = 23 cells in WT and n = 25 cells in KO in panel B. .

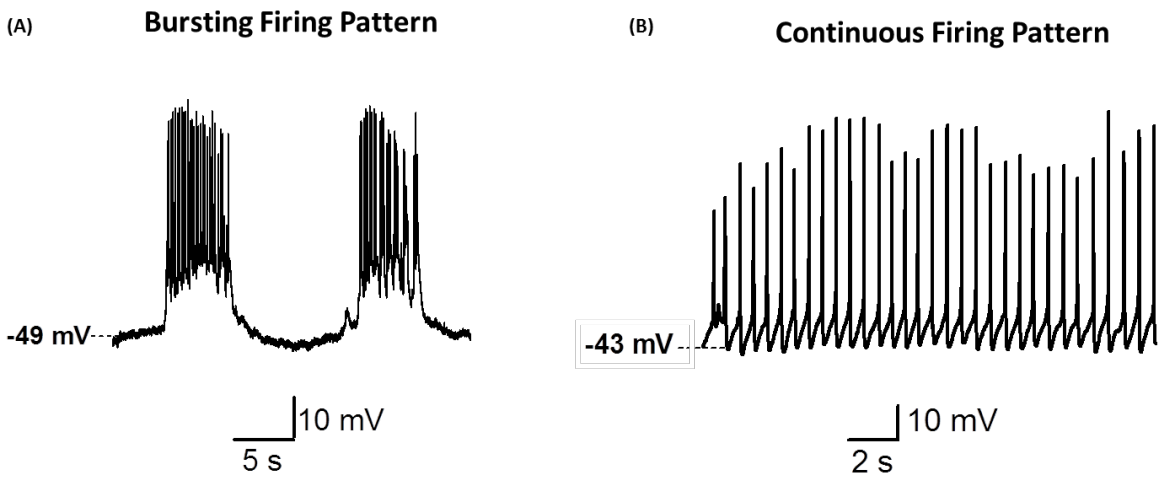

Figure 4.27: Types of firing patterns observed from circular muscle cells. Circular muscle cells presented two types of firing patterns. (A) Bursting Firing pattern: consisted of a slow wave of depolarization with firing of action potentials overlapping the slow wave depolarization. (B) Continuous firing pattern: consisted of cells able to fire actions potentials in a non-bursting fashion. Both types of firing patterns were observed in cells from WT and KO mice.

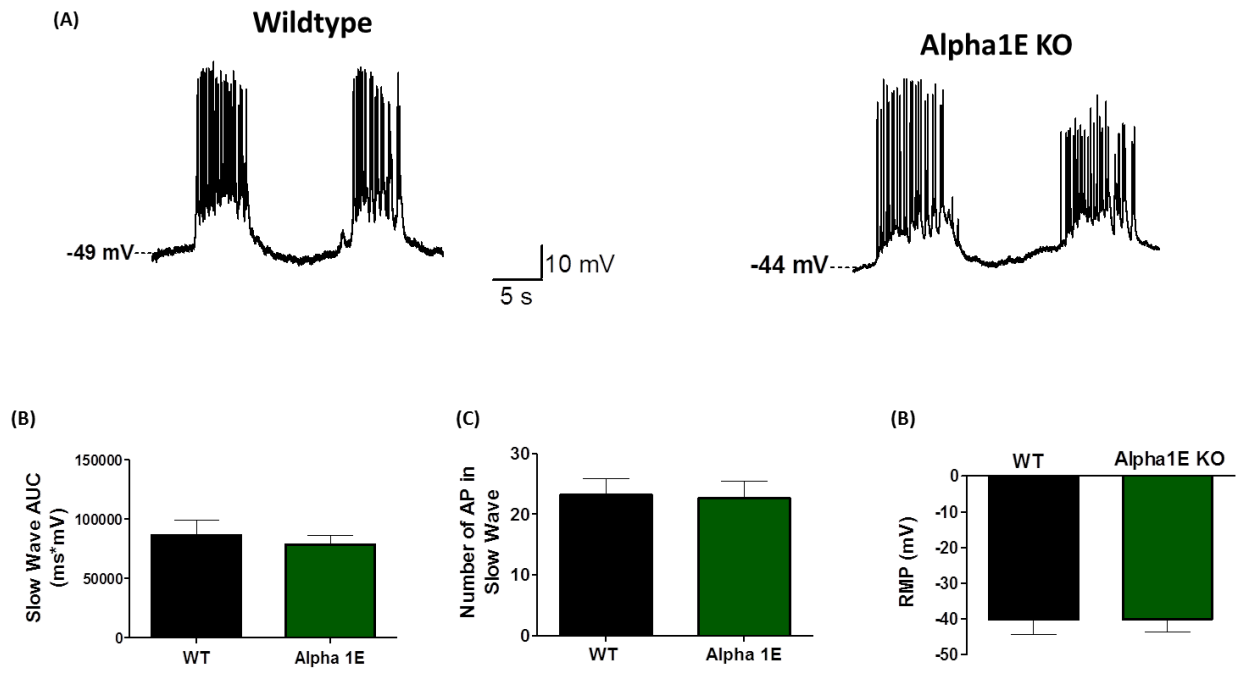

Figure 4.28: Bursting pattern was similar between WT and α1E KO cells. The AUC of the slow wave depolarization and the number of action potentials fired within each wave was measured to compare bursting activity in cells from WT and KO mice. (A) Representation of the bursting pattern in cells from WT and KO mice. Neither the AUC of the slow wave depolarization (A) nor the number of action potentials fired (B) were different between WT and KO mice. The amplitude and duration of the waves were measured and no differences were detected either (data not shown). (C) RMP of bursting cells was about the same in WT and KO mice ($P>0.05$). Student t-Test was used to detect statistical significance. Data represent mean ± SEM for n = 8 cells in WT and n = 16 cells in KO.

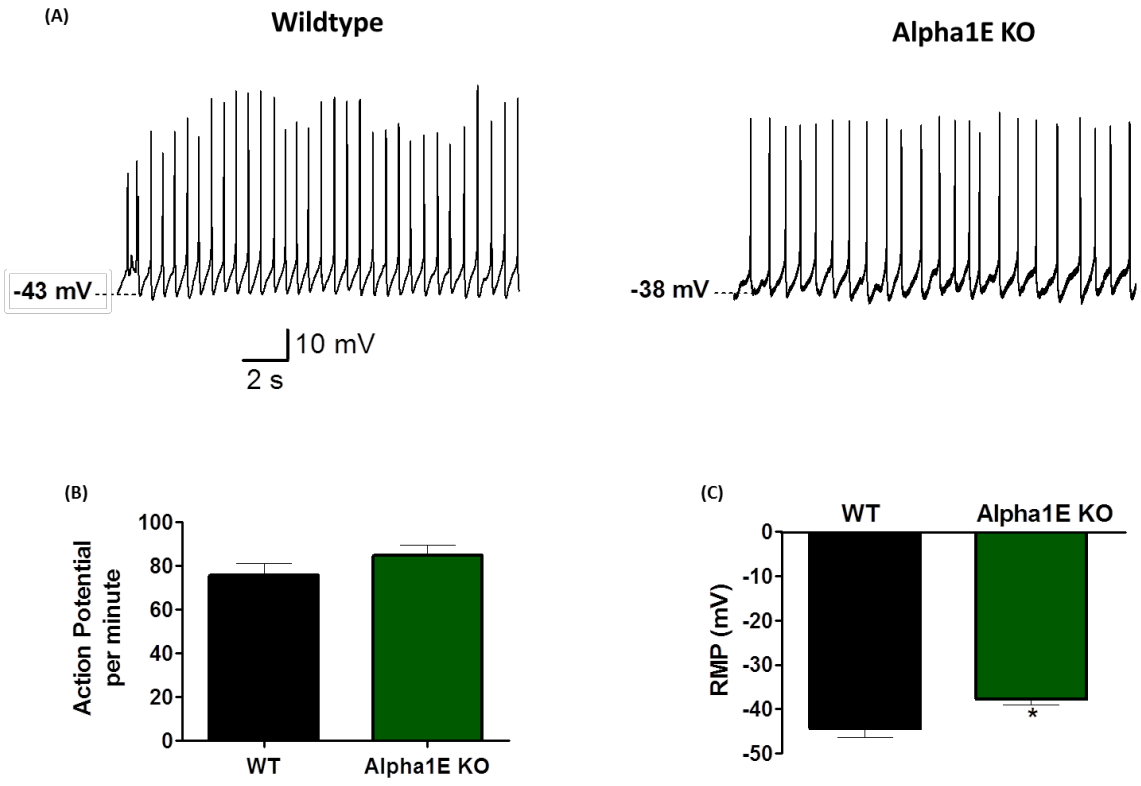

Figure 4.29: Continuous firing pattern was similar between WT and α1E KO cells. The number of action potentials fired in one minute was counted in cells from WT and KO mice to evaluate if differences existed between WT and KO mice regarding this firing pattern. Representation of a continuous firing cells in WT and KO mice. (A) Quantification of the data showed no statistically significant differences between the numbers of action potential fired in one minute. (B) RMP of continuous firing cells was more depolarized in KO as compared to cells from WT mice (-44.5 ±1.8 mv in WT and -37.7 1.3mv in KO; P = 0.0062). Student t-Test was used to detect statistical significance. Data represent mean ± SEM for n = 24 cells in WT and n = 21 cells in KO.

CHAPTER 5: FUNCTIONAL UP-REGULATION OF L-TYPE CHANNEL IN THE COLON OF P/Q-TYPE DEFICIENT (TOTTERING) MICE

ABSTRACT

Intestinal motility is finely regulated by Inhibitory neuromuscular transmission, which mainly uses NO and a purinergic signaling molecule as the inhibitory neurotransmitters. By regulating neurotransmitter release VGCC could play a key role during inhibitory neuromuscular transmission and thus in the control of intestinal motility. The P/Q-type VGCC contribute to excitatory neuromuscular transmission; however, their role in inhibitory transmission is not completely understood. The tottering (TG) mouse has a spontaneous missense mutation in the α1A subunit forming the pore of P/Q-type channels. Thus, these studies used the TG mouse to test the hypothesis that a loss-of-function mutation in P/Q-type Ca^{2+} channels will disrupt neuromuscular transmission and colonic propulsive motility. Colonic propulsive motility was studied *in vivo* by monitoring fecal output and *in vitro* by studying the CMMC motor pattern. Nerve-evoked mechanical relaxations and contractions were investigated as well as intracellular recordings of inhibitory junction potentials and electrical spontaneous activity of circular muscle cells. Distal colonic propulsive motility was only modestly altered in TG mice. The number of fecal pellets was slightly decreased in TG mice; however, the percentage of fecal output was not statistically different between WT and TG mice. Regarding the CMMC pattern, the contractions at the anal side of the colon were increased in TG mice. In general, IJP recorded from circular muscle cells were either larger in TG mice or at normal level as compared to WT mice. A portion of the IJP in TG mice is sensitive to the L-type channel blocker, nifedipine. The latter blocks about 35% and 14% of the IJP in TG and WT mice, respectively. The contribution of N- and R-type channels was larger in TG mice as compared to WT. The resting membrane potential of the circular muscle cells from TG mice was not altered; however, the bursting firing activity of these cells was modestly longer in TG mice. Nerve-evoked relaxations of circular muscle rings in TG mice were normal but contractions were significantly enhanced. In conclusion, these results demonstrate that a functional deficit in P/Q-type channels does not significantly altered colonic propulsive motility. This is due to the ability of the ENS to compensate

for this functional loss through an up-regulation in the contribution of other VGCC subtypes specifically L-type channels. This compensation serves to maintain inhibitory neuromuscular transmission and physiologically normal colonic motility.

INTRODUCTION

Motility in the GI tract is finely regulated by enteric excitatory and inhibitory motor neurons (Burnstock et al. 1963; Bennett, 1966; Spencer and Smith, 2001). Inhibitory neuromuscular transmission is responsible for generation of hyperpolarization and mechanical relaxation of the smooth muscle. This transmission is accomplished by release of various inhibitory neurotransmitters such as NO (Sanders and Ward, 1992) and a purinergic signaling molecule which identity is still under investigation but has been suggested to be ATP or β-NAD and/or its metabolites (Xue et al. 1999; Mutafova-Yambolieva et al. 2007; Hwang et al. 2011). In addition, peptides such as VIP and PACAP contribute to inhibitory neuromuscular transmission (Christ et al. 1992; Shuttleworth and Keef, 1995). Several studies had demonstrated the importance of inhibitory neuromuscular transmission for the regulation of functions such as the control of colonic propulsive motility and transit (Hwang et al. 2007; Strong et al. 2010).

Activation of inhibitory neuromuscular transmission to colonic muscle produces post-junctional responses characterized by two main components (Keef et al. 1993' Hwanf et al. 2012; Gallego et al. 2012). The first component is a large-amplitude transient hyperpolarization of the membrane potential known as the fast inhibitory junction potential (fIJP). This is mediated by release of the purinergic neurotransmitter and post-synaptic activation of the G-protein coupled P2Y1 receptors (Gallego et al. 2012). Activation of this receptor in the muscle leads to increases in intracellular Ca^{2+} concentrations and opening of Ca^{2+}-activated K^{+} channels generating the transient hyperpolarization that distinguishes the fIJP (Koh et al. 1997). The second component consists of a small-amplitude long-lasting hyperpolarization of the membrane potential that follows the fIJP and is known as the slow IJP (sIJP).

Activation of NOS, production of NO, and diffusion of this gas into the muscle initiate generation of the sIJP (Shuttleworth et al. 1997). In the muscle NO leads to increases in the production of proteinase kinase G which can modulate the activity of myosin light chain kinase and and thus regulate the dynamics of actin filaments to favor mechanical relaxation. In addition, it can phosphorylate and open K^+ channels to produce hyperpolarization of the membrane potential in the muscle (Sanders, 2008; Murthy, 2005). Release of these inhibitory neurotransmitters is Ca^{2+} dependent and consequently regulated by activation of VGCC (Burnstock, 2009; Gallego et al. 2008).

Influx of Ca^{2+} through VGCC triggers neurotransmitter release at central and peripheral synapses (Augustine and Charlton, 1986). By regulating neurotransmitter release VGCC could play a key role during enteric inhibitory neuromuscular transmission and thus in the control of colonic motility. The pore of the VGCC is formed by the α1 subunit and the diversity of these channels is due to expression of multiple genes encoding different subtypes of the α1 subunit. Pharmacological and electrophysiological studies showed there are at least five main α1 subunits for HVA calcium channels. The pore of P/Q-type (Cav2.1), N-type (Cav2.2), and R-type (Cav2.3) channels are formed by the α1A, α1B, and α1E subunits, respectively. L-type (Cav1.2-1.3) channels are form by the α1C, α1D, α1S, and α1F subunits (Zhang et al. 1993; Tsien et al. 1991).

The role of N-type and P/Q-type during excitatory neuromuscular transmission in the peripheral nervous system is very well established (Waterman, 2000). However, the contribution of different VGCC subtypes during inhibitory neuromuscular transmission is not clearly understood yet. In-depth comprehension of the VGCC subtypes involved in enteric neuromuscular transmission will provide insight into the neuronal circuits mediating intestinal motility unveiling potential targets for the treatment of GI motility disorders.

In humans, perturbations in the Cav2.1 (P/Q-type VGCC) gene are associated with neurological disorders also known as channelopathies (Burgess and Noebels, 1999; Pietrobon, 2002). Specifically, mutations in this gene are linked to the pathophysiology of episodic ataxia type-2 and autosomal dominant spinocerebellar ataxia type-6 (Ophoff et al. 1996; Zhuchenko et al. 1997). In general, these disorders predominantly present dysfunction of voluntary motor coordination and balance. Lambert-Eaton syndrome (LEMS) is an autoimmune condition in which the autoantibodies inhibit the function of P/Q-type VGCCs causing skeletal muscle weakness (Lambert-Elmqvist, 1971). Besides presenting motor dysfunction some of these disorders also include alterations in the autonomic nervous system. One of such alterations includes GI motility dysfunction (Waterman, 2001; Houzen et al. 1998). This provided the basis for studying the role and physiological significance of P/Q-type VGCC in enteric neuromuscular transmission.

Several spontaneous mutations of the P/Q-type channels have been identified in mice and one of them is the Tottering (TG) mutant mouse. The TG mutation encodes for a proline-leucine amino acid substitution in the S4-S5 linker region of domain II of the α1A subunit (Fletcher et al. 1996). The functional consequences were identified to produce a decrease in whole cell current density and voltage-dependent inactivation during prolonged hyperpolarization in Purkinje cells (Wakamori et al. 1998). These changes occur without altering the single channel properties and conductance of P/Q-type VGCC. TG mice express a phenotype characterized by ataxia, episodes of behavioral arrest, and motor seizures (Green and Sidman, 1962; Noebels and Sidman, 1979). Synaptic plasticity involving VGCC has been demonstrated at multiple TG synapses. Synaptic transmission in the cerebellum and hippocampus is maintained due to an increase in the reliance of N-type VGCC (Leenders et al. 2002; Quian and Noebels, 2000). Skeletal neuromuscular transmission in TG mice is sustained by an up-regulation in the contribution of both N- and R-type VGCC (Pardo et al. 2006). Whole cell current in basal forebrain neurons from TG mice are unaltered due to a functional up-regulation in the role of L-type VGCC

(Etheredge et al. 2005). These studies demonstrate the capacity of the nervous system to respond to alterations in VGCC and the functional redundancy of these channels in certain populations of the nervous system.

By using the TG mouse as an animal model I sought to determine the functional consequences of a loss-of-function of P/Q-type VGCCs in enteric neuromuscular transmission and thus in the control of colonic propulsive motility. In addition, I aimed to investigate whether a functional deficit in P/Q-type channels unmask compensatory mechanisms involving other VGCC subtype in the ENS.

MATERIALS AND METHODS

Mice

All animal use protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University. Breeding pairs of heterozygote C57BL/6J-tg mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Colonies were maintained at Michigan State University Laboratory Animal Resources (East Lansing, MI). Litters were genotyped at weaning, which occurred 3 weeks after birth in order to identify wildtype (WT) and homozygote (tg/tg) mice. In addition to that, homozygote (tg/tg) mice were also identified by their characteristic phenotype consisting of ataxia, motor seizures, and episodes of behavioral arrest also known as absence epilepsy. All animals were fed normal diet and were studied between 4 and 5 months of age (male). Mice were euthanized using isoflurane anesthesia followed by cervical dislocation.

Propulsive colonic motility studies in vivo

Fecal pellet output was evaluated in WT (n= 10) and TG mice (n = 9). Mice were individually housed with a pre-measured amount of food and water. The cages were equipped with the normal bedding material to avoid any stress especially in TG mice. Fecal output was monitored for a period of

48-hours and at the end of this period fecal pellets were collected and counted. Fecal pellets were placed into a 60 °C oven overnight and dry weight was then determined. This protocol is different from the one previously reported by our laboratory (France et al. 2012) in which each mice is placed in a clean cage and fecal pellets are collected only for a 1-hour period during 3 consecutive days. That protocol was too stressful for TG mice preventing a reliable assessment of *in vivo* colonic propulsive motility in these mice.

Propulsive colonic motility studies in vitro: Colonic Migrating Motor Complex

Preparation of colonic segments for recordings of the Colonic Migrating Motor Complex (CMMC) was performed as described previously (Devries et al. 2010). Briefly, after the mice were sacrificed the entire colon was removed and placed into oxygenated and warm Krebs solution. Immediately after this, the luminal content was gently flushed out with Krebs solution. A stainless-steel rod was inserted into the lumen and the tissue was secured at each end with a surgical silk. Metal clips (Fine Science Tools, Foster City, CA, USA) were attached to the oral and anal end of the tissue 1.5 cm apart. Both metal clips were then connected to separate force transducers using surgical silk. The rod holding the tissue was secured in a 60-ml bath containing oxygenated and pre-warmed Krebs solution at 37 °C. The oral and anal ends of the colon were stretched to an initial tension of 1 g. The tissue was allowed to equilibrate for 30 minutes during which a regular pattern of propagating contractions was established and that is known as the CMMC. The CMMC pattern was then recorded for an additional hour and a 30-minute segment was selected for analysis. Frequency, amplitude, and AUC were measured in the oral and anal side. Propagated CMMC were determined as a complex in which a contraction occurred first at the oral recording site followed by a contraction recorded at the anal site. Recordings were obtained using two Grass Instruments CP122A strain gauge amplifiers. The output of these amplifiers was fed to an

analog/digital converter (Minidigi 1A, Molecular Devices; http://www.moleculardevices.com/) and Labchart software 7 (Molecular Devices).

Intracellular recordings of inhibitory junction potentials and smooth muscle cell electrical activity

Conventional intracellular electrophysiological techniques were used to obtain recordings of inhibitory junction potentials and smooth muscle electrical activity in the distal colon of WT and TG mice. A 2-cm segment of the distal colon was harvested from the mice immediately after euthanasia and placed in a petri dish containing pre-warmed (37 °C) and oxygenated (95% O_2; 5% CO_2) Krebs solution. The Krebs solution contained scopolamine (1 μM) to block muscarinic receptors and in some experimental conditions nifedipine (1 μM) was added to block L-type Ca^{2+} channels. The colonic segment was cut open along the mesenteric border and pinned flat on the petri dish with the mucosal layer facing upwards. The mucosa and submucosa layers were gently removed using fine forceps. A 5 mm^2 section was cut and transferred to a 5-ml silicone elastomer-lined recording chamber. The section was stretched lightly and pinned to the chamber floor using small stainless steel pins. The chamber was mounted on a stage of an inverted microscope and it was then perfused with oxygenated Krebs solution at a flow rate of 5 ml/min. The preparations were equilibrated for 30 minutes before commencing of intracellular recordings.

Smooth muscle cells were impaled with a 2 M KCl-containing glass microelectrode (Frederick-Haer, Brunswick, ME) with a tip resistance of 60-100 MΩ. To avoid muscle movement in experimental conditions were nifedipine was absent a smaller window was created in the middle of the preparation using micro-pins (0.125 mm from World Precision Instruments, Inc). This technique successfully restrained muscle movement making possible to maintain impalement for periods of 2-60 minutes. Upon successful impalement the resting membrane potential of circular muscle cells was allowed to stabilize for a period of 2 minutes. Membrane potential was recorded using an Axoclamp 2A amplifier, a

Digidata 1322A analog-digital converter, and Axoscope 9.2 software (all from Molecular Devices, Sunnyvale, CA, USA). Amplified signals were sampled at 2kHz and filtered at 1kHz. Data were stored on a computer hard drive and Clampfit 10.3 software was used for the analysis of membrane potentials.

IJPs were evoked using a pair of silver wired stimulating electrodes (A-M Systems, Inc.) inserted into the recording chamber parallel to the circular muscle fibers to electrically stimulate the nerve fibers supplying the muscle cells. Electrical stimuli consisted of 1 s train duration, 0.8 ms pulse duration, 10 Hz, and 10-90 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). Two parameters of the IJPs were characterized in WT and TG mice. The peak amplitude of the fast IJP was measured to obtain information about the purinergic component of the inhibitory neuromuscular transmission. The area under the curve (AUC) of the IJP was measured to obtain information about both the purinergic and nitrergic components of this type of transmission. These two parameters were always compared between WT and TG mice and were used to examine the action of different treatments in generation of IJPs.

Drug application system

Two types of drug application systems were used to study the effect of different treatments on IJPs. In one application system, drugs were added in known concentrations to the flowing Krebs solution using a system of three-way stopcocks. Drug concentrations reached steady state in the recording chamber within 4 minutes. The other was a local drug application system, which was accomplished using a quartz micropipette (30-40 μm tip diameter) placed within 50 to 150 μm of the impaled muscle cell. Before impalement, Krebs solution was superfused through the quartz micropipette near the recording microelectrode to prevent disruption of membrane potential recordings by turning on of the local application system. After recordings of control responses the Krebs

solution flow was replaced with flow of different drug treatments using a VC-8 Valve Controller application system (Warner Instruments, Hamden CT). The impaled muscle cells were exposed to drug treatments for at least 5 minutes before responses were recorded.

Nerve-evoked mechanical responses in colonic circular muscle rings

The distal colon of WT and TG mice was dissected into 1-cm long circular muscle rings. Each segment 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 oxygenated Krebs solution at 37 °C. A resting tension of 1 g was applied to each preparation and then tissues were allowed to equilibrate for 30 minutes. During this time the tissues were washed with fresh Krebs solution in 10 minutes intervals and tension was re-established to 1 g if it was necessary. After equilibration, bethanechol (10 μM) and sodium nitroprusside (SNP; 5 μM) were applied to each bath in order to produce a maximal contraction and relaxation, respectively. The peak amplitude of these responses was used to normalize the nerve-evoked contractions and relaxations. From this point on, scopolamine (1 μM) was present in the experimental conditions in order to isolate non-cholinergic inhibitory neuromuscular transmission.

Contractions and relaxations were induced by transmural electrical stimuli (20 V, 0.1 ms pulse duration, and a frequency response curve of 1-25 Hz). The different frequencies of nerve stimulation were applied to each tissue in 2-minute intervals. Each preparation was subjected to the frequency response curve twice. The responses induced by the second frequency response curve were used for analysis in all tissue preparations. Tetrodotoxin (TTX; 0.3 μM) was used to demonstrate the neurogenic nature of the contractions and relaxations. MRS 2179 (10 μM) and ω-Nitro L-arginine (NLA; 100 μM) were used to study the purinergic and nitrergic components of the relaxation, respectively. Each of these drugs was applied for a period of 10 minutes after which the frequency response curve was

conducted on each tissue preparation in order to examine the action of these drugs on circular muscle rings mechanical responses. Transmural electrical stimuli were provided by a Grass S48 stimulator and mechanical activity of the muscle rings was recorded with Labscribe (iWorx, Dover, NH, USA) and a computer.

For analysis purposes the peak amplitude of contractions and relaxations was measured. These values were normalized using the peak amplitude of the bethanechol-induced contraction or the peak amplitude of the SNP-induced relaxation. With that information the percent contraction or the percent relaxation was determined and compared between WT and TG mice. In addition, that was the value analyzed in order to examine the effect of different treatments in mechanical responses of circular muscle rings.

Drugs

Nifedipine, scopolamine, ω-conotoxin-GVIA (ω-CTX-GVIA), ω-agatoxin-IVA (ω-ATX-IVA), SNX-482, TTX, ω-Nitro L-arginine (NLA), sodium nitroprusside (SNP), bethanechol, and MRS 2179 were obtained from Sigma-Aldrich. All drugs were diluted in deionize water except for NLA which was dissolved in 0.1N HCl and nifedipine which was dissolved in DMSO.

Statistical analysis

Data are presented as mean values ± S.E.M. for n representing the number of mice for *in vivo* studies, *in vitro* CMMC studies, and circular muscle ring responses studies. For intracellular recording studies the n value represents the number of cells. Statistical differences between groups were analyzed with a two-way ANOVA followed by a Bonferroni post-hoc test or when applicable a 2-tailed student's t-Test was used. In either case, $P < 0.05$ was the criterion for determining statistical significance.

RESULTS

Distal colonic propulsive motility is modestly altered in TG mice in vivo and in vitro

To investigate colonic function *in vivo*, fecal pellet output was measured continuously for a period of 48 hours. The mean weight of WT and TG mice was 35.9 ± 2.5 g and 21.5 ± 0.9 g, respectively. On average TG mice were about 40% smaller than WT mice and this difference in body weight was statistically significant (figure 5.1 A). Water intake was significantly smaller in TG mice, however food intake was the same (figure 5.1 B-C). TG mice showed a small but statistically significant decrease in the number of fecal pellets produced in a 48-hour period (figure 5.2 A). There was a subtle decrease in the mean dry weight of the collected fecal material in TG mice, however this was not statistically significant when compared with WT mice (figure 5.2 B). The percentage of fecal output was calculated by dividing the total dry weight of the collected fecal material into the total food intake for the 48-hour period. Although the results showed a modest decrease in TG mice, the percentage of fecal output was not significantly different between WT and TG mice (figure 5.2 C).

The CMMC is a motor pattern of propagating contractions that occur in the mouse colon *in vitro* (Smith et al. 2014). In WT mice, about 88 ± 4% of the contractions that began in the oral side propagated to the anal side and these are known as propagating contractions. In TG, a similar percentage was observed with 94 ± 2.5% being propagating contractions. Oral-anal simultaneous occurring contractions were only identified in 6 ± 3.8% of the total contractions observed in WT mice. Contractions occurring only at oral side were identified in about 5.5 ± 3.6% and 5.3 ± 2.4% of the total contractions observed in WT and TG, respectively. Retrograde propagating contractions or contractions occurring only at the anal side were never observed in WT or TG mice (figure 5.3 A). The propagation time for oral-anal propagating contractions tended to be longer in TG mice (slower propagation) but when compared to WT it was not statistically significant (figure 5.3 B). There was a tendency for the AUC of the oral and

anal contractions to be larger in TG mice. However, this increase was only significant for the AUC of anal contractions in TG mice (figure 5.3 C-D). The frequency of oral and anal contractions was similar between WT and TG mice (figure 5.4). Pharmacological or genetic inhibition of NOS increases the frequency of the CMMC pattern in mice by relieving the colonic muscle of ongoing inhibitory innervation (Dickson et al. 2010). Thus, the fact the TG mutation did not alter frequency of CMMC suggests no alterations in the ongoing nitrergic inhibitory innervation to the colonic muscle. A representation of CMMC pattern in WT and TG mice is showed in figure 5.5.

These results revealed that TG mutation produces only subtle alterations in colonic propulsive motility. There are two possible explanations for these results: P/Q-type VGCCs are not critical for colonic propulsive motility or the ENS is able to compensate for the functional deficit and maintain physiologically appropiate colonic function.

IJPs recorded from circular muscle cells are larger in TG mice

Inhibitory neuromuscular transmission finely regulates propulsive colonic motility. In order to investigate if adaptive changes were taking place in the colon of TG mice the electrical events (IJPs) produced by inhibitory neuromuscular transmission were studied. IJPs were recorded from circular muscle cells in the absence of the L-type VGCC blocker, nifedipine, to unmask any potential role of these channels in IJP generation. On average there was a statistically significant increase in the amplitude and AUC of the IJPs recorded from TG as compared to WT mice (figure 5.6 A-B). This change could not be attributed to a difference in the driving force to K^+ ions efflux because there was no difference in resting membrane potential of circular muscle cells from TG and WT mice (figure 5.6 C). This data suggested a statistically significant enhancement of the IJP in face of a loss-of-function of P/Q-type VGCCs in TG mice.

No alterations in the purinergic and nitrergic components of the IJP in TG mice

Not all the IJPs recorded from TG mice were larger than IJPs recorded from WT. In this regard, a group of TG mice presented IJPs that were at the level of WT and these responses were used to investigate the purinergic and nitrergic components of this electrical event.

The purinergic P2Y1 receptor antagonist, MRS 2179 (10 μM), abolished the fast phase of the IJP in both TG and WT mice demonstrating once again the purinergic nature of this phase. In WT, MRS 2179 produced a substantial decrease in the AUC of the IJP. However, the effect of MRS 2179 did not reach statistical significance when compare no control IJPs (figure 5.7 A). MRS 2179 produced a statistically significant decrease in the AUC of the IJP in TG mice (figure 5.7 B). Under MRS 2179 conditions the slow nitrergic component of the IJP is isolated and that was not different between TG and WT mice (figure 5.7 C). A representation of these experiments is provided in figure 5.7 D. The NOS inhibitor, NLA, produced a statistically significant decrease in the AUC of the IJP in both WT and TG mice (figure 5.8 A-B). Under NLA conditions, the fast purinergic component of the IJP is isolated and the AUC of this component was not different between TG and WT mice (figure 5.8 C). NLA treatment did not change the amplitude of the fast phase of the IJP in WT or TG mice (figure 5.9). This demonstrates the fact that nitrergic transmission generates the slow phase of the IJP and blocking this component has little effect on the peak amplitude of the fast IJP. This explains why the AUC is very sensitive to changes in the nitrergic component of the IJPs.

A portion of the IJP in TG mice is sensitive to the L-type VGCC blocker, nifedipine

The results showing enhancement of the IJP in TG mice suggested that a compensatory adaptation is taking place in order to maintain function in the colon of these mice. To identify potential adaptive mechanisms IJPs were now recorded in the presence of 1 μM nifedipine. This protocol helped

us to investigate if L-type VGCCs were playing a role in generation of IJPs in TG mice. When nifedipine was present, both the amplitude and AUC of the IJPs were significantly smaller in TG mice (figure 5.10 A-B). To further investigate the effect of nifedipine, the IJPs recorded in the absence and in the presence of nifedipine were grouped and compared in WT mice. The same analysis was conducted in TG mice. The results showed that in WT, IJPs recorded in the absence of nifedipine were slightly smaller than IJP recorded in the presence of nifedipine (figure 5.11 A). The opposite was observed in TG mice. IJPs recorded in the absence of nifedipine were significantly larger than IJPs recorded in the presence of nifedipine (figure 5.11 B). These set of experiments suggested that L-type VGCCs are contributing to generation of IJPs in TG mice.

To investigate the sensitivity of IJPs to block of L-type VGCC the inhibitory effect of nifedipine in these responses was studied in TG and WT mice. IJPs were recorded in control conditions and after 10-minute application of 1 μM nifedipine in the same circular muscle cell. The results showed that nifedipine produced a reduction in the amplitude of the IJP of about 5 ± 3.7% and 32 ± 4.9% inhibition in WT and TG mice, respectively (figure 5.12 A). In terms of the AUC of the IJP, nifedipine produced a 14.8 ± 6% and 35 ± 7% inhibition in WT and TG mice, respectively (figure 5.12 B). When compared, the percent inhibition of nifedipine on IJPs was always significantly larger in TG mice. This data suggests a role of L-type channels in generation of colonic IJPs in TG mice that is not evident in WT mice.

The profile of VGCC subtypes generating IJP is different in TG mice

Since a significant portion of the IJP is now generated by activation of L-type VGCCs in TG mice, I sought to investigate if there was a further change in the profile of VGCCs contributing to the IJP. This was investigated pharmacologically by using neurotoxins specific for each VGCC subtype. Blocking P/Q-type VGCCs with ω-ATX-IVA had a 40.8 ± 5% inhibition in WT and 3 ± 2.1% inhibition in TG mice; this difference was statistically significant (figure 5.13 A). The low percent inhibition by agatoxin-IVA in IJP

from TG mice was expected since this mutation causes a loss-of-function in the α1A subunit forming the pore of the channel. Contribution of R-type VGCCs was investigated using SNX-482. This toxin produced a 35.6 ± 6.5% inhibition in WT and a 52.4 ± 6.2% inhibition in TG (figure 5.13 B). The percent inhibition by SNX-482 was larger in TG mice, however it did not reach statistical significance (P = 0.08) although the P value was very close to be consider significant. ω-CTX-GVIA was used to block current through N-type VGCCs and the results showed a 47 ± 4% inhibition in WT and a 61 ± 7% inhibition in TG mice (figure 5.13 C). The percent inhibition produced by blocking the N-type VGCCs was again lager in TG mice, however it did not reach statistical significance (P = 0.1240). In general there was a tendency for both ω -CTX-GVIA and SNX-482 to produce a larger percent inhibition in IJPs from TG mice. However, when comparing the inhibitory effects of these toxins between WT and TG they were not statistically significant although these values might still be physiologically significant. The percent inhibition by nifedipine is included (figure 5.13 D) to better appreciate the change in the profile of VGCC subtypes regulating generation of IJP in TG mice. N-type, P/Q-type, and R-type VGCCs dominate generation of IJPs in WT conditions. However, due to the functional deficit present in TG mutants this VGCC profile is shifted. In TG mice L-type, N-type, and R-type VGCCs govern generation of the IJPs. All these channels have a larger contribution to the IJP in TG mice as compared to their contribution to the IJP in WT mice.

Relaxations of circular muscle were not altered and contractions were enhanced in TG mice

To investigate if the observed changes in IJPs were translated into normal relaxation of the colonic muscle, nerve-evoked mechanical responses of circular muscle rings were studied. SNP (5 μM) produced a relaxation that was similar between TG and WT circular muscle rings. Therefore, this response was used to normalize the peak amplitude of the nerve-evoked relaxations. There was a frequency-dependent increase in the percent relaxation of the muscle that occurred to the same degree

in TG and WT mice. Therefore, the genotype did not significantly affect the percent relaxation induced at each frequency of nerve stimulation (figure 5.14).

Inhibiting NOS with NLA significantly reduced the relaxations in both WT and TG circular muscle rings (figure 5.15 A-B). These results showed the important contribution of nitrergic inhibitory transmission for the mechanical relaxation of the muscle. Blocking the purinergic receptor, P2Y1, with MRS 2179 (10 um) did not inhibit relaxations in WT mice (figure 5.16 A). This result demonstrates the redundancy of inhibitory neurotransmitters in the ENS. In this case, it appears that nitrergic neurotransmission can sustain relaxation in the absence of the purinergic component of inhibitory neuromuscular transmission. This phenomenon has been called the "safety factor" of inhibitory neuromuscular transmission (Waterman and Costa, 1994; Waterman et al. 1994). In TG, MRS 2179 produced a small but significant inhibitory effect in the percent relaxation (figure 5.16 B). This result suggested two possibilities. First, there could be a reduction of the inhibitory "safety factor" and thus MRS 2179 is able to produce a small inhibition of the relaxation as was observed in TG mice. The second refers to the possibility that there is an increase in the contribution of the purinergic neurotransmitter to the relaxation in TG muscles, in which case MRS 2179 could produce a small inhibition. In order to further investigate this, the isolated purinergic and nitrergic components of the relaxation were studied. The isolated purinergic component of the relaxation (in the presence of NLA) was not different between WT and TG mice (figure 5.17 A). In addition, the ATP-induced relaxation of the muscle was not different between TG and WT mice (figure 5.17 B). This ruled out any change in the contribution of the purinergic neurotransmitter to mechanical relaxation of the muscle in TG mice. The isolated nitrergic component (in the presence of MRS 2179) of the relaxation was slightly smaller in TG mice (figure 5.17 C). The relaxation response of the muscle to a NO donor (SNP) was similar between TG and WT mice, suggesting that there is no difference in the ability of the muscle to respond to NO (figure 5.17 D). These results favor the possibility that there is a reduction of the inhibitory "safety factor", which in this particular

case implies a small reduction in the nitrergic component of the relaxation. Because there are no differences in the ability of the muscle to respond to NO between TG and WT mice, the decrease in the nitrergic component must occur at the level of the nerve.

The ATP induced hyperpolarization was also investigated in impaled cell from TG and WT mice. ATP (5 mM) was applied for different durations (10-35 ms) at a pressure of 5 psi. The amplitude and AUC of the hyperpolarization were measured. The results showed that both amplitude and AUC of the ATP-induced hyperpolarization were smaller in TG mice as compared to WT (figure 5.18). Even though the ATP-induce relaxation was not altered in TG mice, the electrical response to ATP was significantly reduced.

Nerve-evoked contractions of the muscle were also investigated. The bethanechol-induced contractions were not different in WT and TG mice. Therefore, these responses were used to normalize the nerve-evoked muscle contractions. There was a frequency-dependent increase in the percent contractions of the muscle in WT and TG mice (figure 5.19). However, the contractions were significantly larger in TG as compared to WT contractions. It is possible that the observed small decrease in the nitrergic component is able to potentiate excitatory neuromuscular transmission.

Characterization of resting membrane potential and firing patterns of circular muscle cells in TG mice

The colonic muscle is under ongoing inhibitory innervation known as inhibitory tone. The purinergic and nitrergic neurotransmitters contribute to this ongoing inhibitory tone through different mechanisms: purinergic neurotransmitter regulates spontaneous generation of IJPs and the nitrergic neurotransmitter regulates resting membrane potential (RMP) and colonic muscle tone (Gil et al. 2010). It is thought that this inhibitory tone also modulates spontaneous electrical activity of circular muscle cells. In order to investigate the status of the colonic inhibitory tone the RMP and firing activity of

circular muscle cells were measured. Distal circular muscle cells of WT and TG mice had RMP averaging -43.7 ± 0.9 mV and -43.62 ± 0.7 mV, respectively (figure 5.20). The fact that the RMP was about the same in WT and TG mice suggested no alterations in the component of the ongoing inhibitory innervation that regulates RMP in TG mice.

Circular muscle cells present a spontaneous and rhythmic pattern of electrical activity. In these studies two different patterns of electrical activity were observed in the muscle cells. One pattern was characterized by the rhythmic occurrence of slow wave depolarization with overlapping generation of action potentials, known as bursting firing pattern (figure 5.21 A). The second observed pattern was characterized by a continuous firing of action potentials without generation of the slow wave depolarization (figure 5.21 B). This was called a continuous firing pattern. The RMP of cells firing in a continuous pattern was always more depolarized than cells firing in a bursting pattern. Specifically, bursting cells in WT mice had RMP averaging -47 ± 1.3 mV and continuous cells had RMP averaging -42 ± 1 mV and this depolarization was statistically significant (figure 5.22 A). Bursting cells in TG mice had RMP averaging -48.5 ± 1.4 mV and continuous firing cells had RMP averaging -42.4 ± 0.86 mV; that depolarization was also statistically significant (figure 5.22 B). However, when comparing the RMP of either bursting or continuous firing cells between WT and TG the mean values were about the same (figure 5.2 2C-D).

In bursting cells the amplitude, duration, and AUC of the slow wave were characterized. In addition the action potential frequency within the slow waves was measured and all of these parameters were compared between WT and TG (figure 5.23). The slow wave duration was the only parameter that was significantly different between the two types of mice. The duration of the slow wave in WT mice was about 10 ± 0.5 s and 12 ± 0.7 s in TG mice. This represents a small but statistically significant difference in the slow wave duration between WT and TG mice (figure 5.23 B). The number of action potentials fired in 1 minute was quantify in continuous firing muscle cells and compared

between the different mice. The results showed no significant difference in the number of action potentials fired between WT and TG mice (figure 5.24).

DISCUSSION

These studies investigated the functional consequences of the TG mutation (loss-of-function in P/Q-type VGCCs) in enteric inhibitory neuromuscular transmission. Specifically, I tested the hypothesis that loss-of-function in P/Q-type VGCCs will be enough to disrupt enteric inhibitory neuromuscular transmission and colonic propulsive motility. In addition, I investigated if the TG mutation was able to unmask compensatory changes involving other VGCC subtypes. In summary these studies showed that despite a substantial functional deficit in P/Q-type VGCCs colonic propulsive motility *in vivo* and *in vitro* was for the most part normal. TG mutant mice showed IJP events that were slightly enhanced and a normal mechanical relaxation of the circular muscle. A modest decrease in the nitrergic component of the relaxation was observed, but this was not enough to alter resting membrane potential or excitability of muscle cells. This apparent maintenance of colonic function is accomplished at least in part by a significant compensatory contribution of L-type VGCCs during inhibitory transmission to the colonic muscle.

P/Q-type VGCCs contribute to inhibitory neuromuscular transmission to the colon

P/Q-type VGCCs are considered among the most abundant calcium channels controlling both neuronal excitability and neurotransmitter release at multiple synapses with the nervous system (Catterall, 1998; Dunlap et al. 1995; Wheeler et al. 1995). Therefore, it is not surprising the fact that perturbations in this channel are associated with neuronal dysfunction and development of channelopathies including familial hemiplegic migraine, episodic ataxia type-2 and autosomal dominant spinocerebellar ataxia type-6 (Ophoff et al. 1996; Zhuchenko et al. 1997). Besides presenting voluntary

motor dysfunction, some of these disorders present symptoms suggesting dysfunction of the component of the enteric nervous system mediating motility of the smooth muscle (Waterman, 2001); suggesting a physiologically significant role of P/Q-type VGCC in enteric neuromuscular transmission.

The present study provided evidence demonstrating a role for P/Q-type VGCCs in inhibitory transmission to the mouse colonic muscle. This was demonstrated by the fact that ω-agatoxin-IVA, which specifically block current flowing through the Cav2.1 channel, produced about 40% inhibition of the IJP in WT mice. P/Q-type VGCCs has been shown to be involved in mediating the component of excitatory neuromuscular transmission resistant to block of N-type VGCCs (Waterman, 2000; Hong et al. 1996; Boot et al. 1994). The non-N-type VGCC component was unmasked when higher frequencies (> 5 Hz) of nerve stimulation were used. This suggests involvement of P/Q-type channels in producing a more general increase in intracellular calcium concentrations leading to release of both acetylcholine and the peptide substance P. The role of P/Q-type channels during inhibitory neuromuscular transmission is less clear with studies presenting conflicting evidence for the role of this channel during inhibitory transmission (Borderies et al. 1996).

The present study provided important insight into the functional role of P/Q-type VGCCs in enteric inhibitory transmission to the smooth muscle. Normally, IJPs are recorded in condition where L-type VGCCs are blocked in order to abolish muscle action potential generation, and as a consequence muscle movement. Block of L-type channels does not alter active or passive properties of enteric neurons (Kunze et al. 1994; Kunze and Furness, 1999). Furthermore, contribution of L-type channels to neurotransmitter release from enteric nerves is not significant because L-type channel blockers do not alter neurotransmitter release in the intestines (Cunningham et al. 1998; Lippi et al. 1998; Reis et al. 2000; Reis et al. 2002). Thus, adding nifedipine to the recording conditions does not alter IJP generation in normal mice. My studies showed that under these conditions the purinergic and nitrergic components of the IJP were significantly smaller in TG mice. This data suggested that P/Q-type VGCCs

regulate release of the purinergic neurotransmitter and activation of NOS contributing to purinergic and nitrergic component of the IJPs. Under nifedipine conditions the resting membrane potential of circular muscle cells was not altered in TG mice. The nitrergic component of spontaneous or ongoing inhibitory innervation to the colonic muscle regulates resting membrane potential (Gil et al. 2010). In this line, the resting membrane potential of circular muscle cells was unchanged in TG mice suggesting no alterations in the nitrergic component of spontaneous inhibitory innervation to the colon.

Functional compensation of L-type VGCCs works to maintain inhibitory neuromuscular transmission in the colon of TG mice

L-type VGCCs respond to functional perturbations against other calcium channel subtypes (Flink and Atchison, 2002; Xu et al. 1998). Thus, I sought to investigate if the TG mutation induces compensatory adaptations involving other subtypes of VGCCs. The first experiments that suggested a functional compensation in the contribution of L-type VGCCs to inhibitory neuromuscular transmission were IJP recordings in the absence and then in the presence of the L-type channel blocker, nifedipine. Within TG mice, IJPs recorded in nifedipine-free conditions were larger than IJPs recorded in the presence of nifedipine. In WT mice the opposite was observed-that is, IJPs recorded in nifedipine-free conditions were modestly decreased compared with IJPs recorded in the presence of this drug. Overall the results showed that only under nifedipine-free conditions the majority of the IJPs recorded from TG mice were either enhanced or at the same level of WT mice. Specifically, block of L-type VGCCs caused about 35% inhibition of the IJP in TG mice while in WT it only caused about 14% inhibition. This clearly shows that a portion of the IJP in TG mice is now sensitive to block of dihydropiridine-sensitive L-type VGCCs. The present study also showed a small up-regulation in the role of N-type and R-type VGCCs in generation of IJPs. However, the L-type VGCC compensation was the most predominant adaptive change observed in TG mutant mice.

It appears the functional compensation in the role of L-type VGCCs together with the overall enhancement in the contribution of N-type and R-type VGCCs works to produce normal IJP in face of a functional deficit in P/Q-type VGCCs. A normal or even enhanced IJP in TG mice could be enough to produce the observed normal mechanical relaxation of circular muscle rings. As I mentioned previously, inhibitory neuromuscular transmission finely regulates colonic propulsive motility and transit. Therefore, the identified adaptive changes in response to functional deficits in P/Q-type VGCCs provide an explanation for the results of *in vivo* and *in vitro* studies of colonic propulsive motility, which suggested an overall maintenance of normal colonic function in TG mice.

Compensatory adaptations in response to defective P/Q-type channels in other neuronal populations

Plasticity involving adaptive changes in the role of different VGCCs in response to P/Q-type channels disruptions has been demonstrated in different parts of the nervous system. P/Q-type channels conduct 90% of the whole cell current in Purkinje neurons (Wakamori et al. 1998). In TG mice there is a substantial decrease in the whole cell current recorded from these neurons without clear evidence of any functional compensation in the contribution of other VGCC subtypes to whole cell currents (Wakamori et a. 1998; Campbell and Hess, 1999). Other studies have been conducted in other neuronal populations in TG mutant mice to identify if adaptive mechanisms do occur. Synaptic transmission in the cerebellum and hippocampus of TG mice is maintained due to an increase in the contribution of N-type VGCCs to neurotransmitter release (Leenders et al. 2002; Quian and Noebels, 2000). Although P/Q-type VGCC is the predominant calcium channel subserving acetylcholine release at the skeletal neuromuscular junction, there are no major alterations during this communication in TG mice (Pardo et al. 2006; Plomp et al. 2000). This is due to the fact that there is a compensatory contribution in the role of N-type and R-type VGCC in regulating acetylcholine release to the skeletal muscle (Pardo et al. 2006). Basal forebrain neurons express the full complement of VGCCs (Griffith et al.

1994). Whole cell currents in basal forebrain neurons from TG mice are maintained at normal levels and this is accomplished by an adaptive increase in the component of the current conducted by L-type VGCCs (Etheredge et al. 2005). Perturbations to P/Q-type VGCCs also result under autoimmune conditions. In Lambert-Eaton Myasthenic Syndrome autoantibodies bind to P/Q-type VGCCs decreasing the function and expression of this channel subtype especially at the skeletal neuromuscular junction (Lambert and Elmqvist, 1971; Lambert and Lennon, 1988). However, neuromuscular transmission is maintained due to a development of the L-type channels contribution to acetylcholine release (Flink and Atchison, 2003). My studies add to this body of evidence demonstrating the ability of enteric neurons to offset the functional deficit in P/Q-type VGCCs presented by TG mice. I presented electrophysiological evidence showing the enhancement of inhibitory neuromuscular transmission in TG mutant mice. In addition, this evidence was combined with pharmacological evidence clearly unmasking the compensatory contribution of L-type VGCCs to this type of enteric synaptic communication.

All together this body of information points towards the fact that different neuronal populations have different mechanisms to offset perturbations in VGCCs. Yet, there are neuronal populations such as Purkinje neurons that simply are unable to undergo plastic changes involving VGCC subtypes at the cell soma and dendrites (where they can regulate excitability) at least in regards to the TG mutation. The nature of the compensatory mechanisms depends on how diverse is the molecular machinery that can be put into action to successfully compensate disturbances in the function of VGCCs. The results presented in the current study show that enteric neurons have compensatory molecular machinery that at least entails giving the “green light” to L-type channels to contribute to neurotransmitter release.

Potential mechanisms underlying increase in L-type VGCC function

Most of the studies providing evidence for a functional up-regulation of L-type VGCCs have not been successful at identifying the mechanism of action underlying this compensatory event. Etheredge et al., 2005 identified a compensatory contribution of L-type VGCCs to the whole cell current in basal forebrain neurons and investigated if up-regulated transcription was part of the mechanism. However, the reported basal forebrain mRNA eliminates up-regulated transcription as a mechanism for the compensation in L-type channel function (Etheredge et al. 2005). A compensatory involvement of L-type calcium channels in transmitter release from adult mammalian motor nerve terminals was identified after passive transfer of Lambert-Eaton plasma to mice. Lambert-Eaton human plasma contains antibodies directed to P/Q-type VGCCs causing disruptions in the function and expression of these channels. However, the authors were unable to identify the mechanisms underlying this compensatory change in L-type function (Flink and Atchsion, 2002).

Chronic stress induced an enhancement of the mRNA and protein expression of the α1C subunit of L-type channels (CAV1.2 channels) in the colonic muscle after 9 days (Choudhury et al. 2009). These changes depend of release of norepinephrine from the adrenal medulla and are associated with faster colonic transit and increased defecation rate. The enhanced α1C expression is observed within 1 hour after exposure to stress and thus transcriptional up-regulation alone is not able to explain such a rapid increase in protein synthesis (Li and Sarna, 2011). In this line, the authors were able to show that norepinephrine can rapidly increase α1C expression by diminishing degradation and increasing translation of existing α1C transcripts. These studies support the notion that L-type calcium channels are responsive to perturbations occurring within the enteric nervous system. In addition, these authors presented transcriptional and post-transcriptional modifications as a mean to enhance expression of L-type channels without the necessity of *de novo* synthesis. However, according to the studies described above (Li and Sarna, 2011; Choudhury et al. 2009) the plasticity of L-type calcium channels takes place at the post-junctional level and not at the motor nerve.

My studies suggested the observed plasticity of L-type channels occur at the level of the nerve because the muscle response to exogenous application of agonist for post-junctional transmitter targets is not altered in TG mutant mice. In fact, the α1C subunit of L-type VGCCs is expressed in the motor nerve fibers innervating the circular muscle cells in the intestine (Kirchgessner and Liu, 1999). In addition, α1C-immunireactivity is observed both in the nerve fibers within the myenteric plexus and within the tertiary plexus (Kirchgessner and Liu, 1999). Whole cell recording of myenteric neurons from the guinea pig small intestine showed a 20% sensitivity of this current to block of L-type channels with nifedipine (Bian et al. 2004). Suggesting a role for this channel subtype in regulation of neuronal excitability and synaptic input integration. However, despite the fact that α1C channels are expressed in nerve varicosities no studies have found a role for these channels in transmitter release under normal conditions.

The observed compensatory L-type channel contribution to inhibitory neuromuscular transmission to the colon of TG mice could reflect recruitment of existing L-type channels in the motor nerve fibers. The question is: how does the functional unmasking of L-type channels occur in TG mice? One possible mechanism is a reduction in the calcium-activated potassium channel (K_{ca}) currents at the motor nerve terminals. This mechanism has been proposed to explain the compensatory involvement of L-type channels in synaptic transmission from motor nerve terminals after LEMS treatment (Flink and Atchison, 2002, 2003) In this line, blocking of K_{ca} channel with iberiotoxin unmasked the development of a contribution of L-type channels to acetylcholine release from normal mammalian motor nerve terminals (Flink and Atchison, 2003b). Several studies have shown K_{ca} channels to be in close proximity to the VGCCs localized at active zones regulating transmitter release (Robitaille and Charlton, 1993; Xu and Atchison, 1996). Thus, calcium influx through VGCCs can activate K_{ca} generating potassium outflow and hyperpolarization. Consequently, this hyperpolarization will limit the extent and duration of action potential-induced depolarization at the nerve terminal. The TG mutation compromises the function of

P/Q-type VGCCs reducing calcium current and thus activation of K_{ca}. Therefore, depolarization at the nerve terminal could be now enhanced allowing activation of the normally silent L-type channels. L-type channels are most likely distantly located from the active zone. However, because calcium current flowing through L-type channels is large and long lasting it can reach the site where exocytosis is taking place and thus contribute to neurotransmitter release.

In conclusion, the TG mutation represents a valuable model to study plasticity of VGCC and its role in regulation of colonic propulsive motility. These studies presented clear evidence showing the ability of the enteric nervous system to compensate functional deficit of P/Q-type VGCC subtype, which serve to support the physiological role of this channel in colonic motility. Importantly, these studies unmask the potential of L-type VGCC to detect perturbations and contribute to homeostatic synaptic plasticity to sustain inhibitory neuromuscular transmission.

APPENDIX

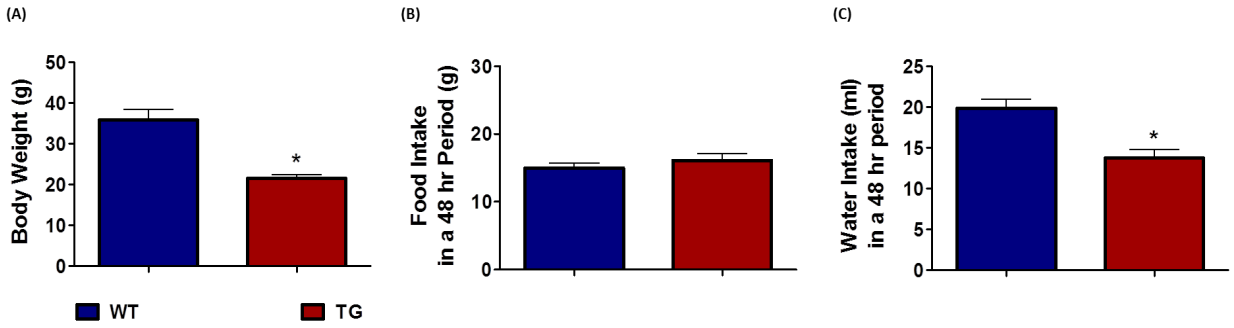

Figure 5.1: Measurement of body weight and food and water intake water during the *in vivo* study.

(A) The mean weight of TG and WT mice were 21.5 ± 0.9 g and 35.9 ± 2.5 g, respectively. On average TG mice were about 40% smaller than WT mice. The difference in body weight was statistically significant ($P<0.05$). During the in vivo study food (B) and water (C) intake was measured in WT and TG mice. Food intake was not different in these mice, however water intake was significantly smaller in TG mice (20 ± 1 ml for WT and 14 ± 0.9 ml for TG, $P<0.05$). Data represent mean ± S.E.M. for n = 9 TG mice and 10 WT mice.

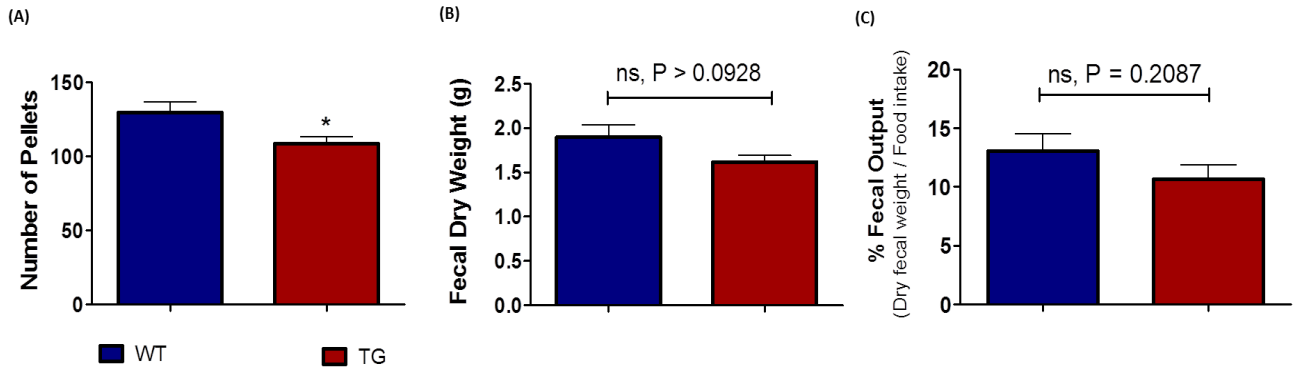

Figure 5.2: *In vivo* fecal output is only modestly altered in TG mutant mice. (A) The mean total number of pellets collected after the 48-hour is shown for WT and TG mice. TG mice showed a small but statistically significant decrease in the number of fecal pellets produced in a 48-hour period (130 ± 7 for WT and 109 ± 4 for TG, P = 0.01). (B) The same pattern was observed for fecal dry weight, however the small decrease in fecal dry weight presented by TG was not statistically significant (1.9 ± 0.1 g for WT and 1.6 ± 0.07 for TG, P = 0.09). (C) Percentage Fecal output was calculated by diving total dry fecal weight into total food intake. There is a small decrease in the mean percentage fecal output in TG mice compared with WT, however, this was not statistically significant (13 ± 1.4% for WT and 10 ± 1.2% for TG, P = 0.2087). Data represent mean ± S.E.M. for n = 9 TG mice and 10 WT mice.

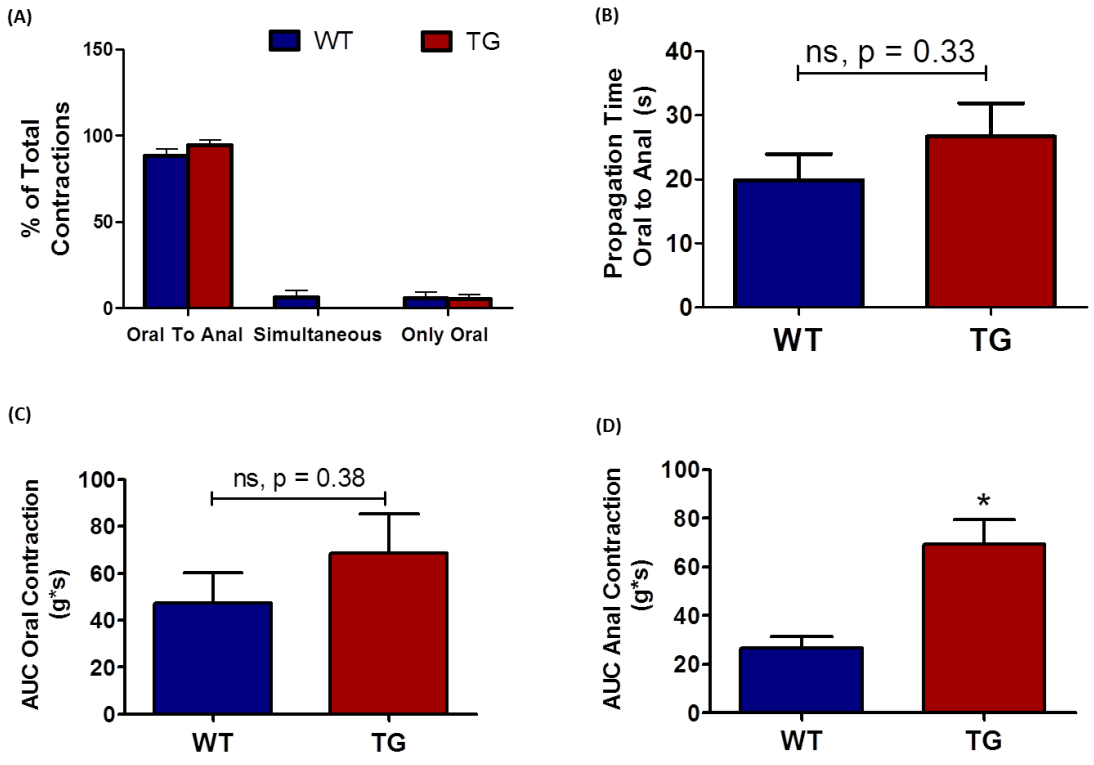

Figure 5.3: Characterization of the colonic migrating motor complex in TG mutant mice. (A) The mean percentage of oral-anal propagating contractions was similar between WT and TG mice (88 ± 4% for WT and 94 ±2.5% for TG, P>0.05). There were no oral-anal simultaneous contractions in TG mice and only a small percentage of the total contractions occurred in a simultaneous fashion in WT mice (6 ± 4%). Only a small percentage of contractions occurred only at the oral side in WT and TG (5 ± 3.6% for WT and 5 ± 2.4%for TG, P>0.05) (B) The propagation time from oral-anal propagating contractions tend to be longer in TG mice (slower propagation) but this was not statistically significant. No anal only contractions were detected in WT and TG mice. (C) The AUC of the oral contractions tend to be larger in TG mice but it was not statistically different from WT (47 ± 13 g.s for WT and 68 ± 17 g.s for TG, P = 3387). (D) The AUC of the anal contractions was also larger in TG mice and this increase was statistically significant (26.5 ± 5 g.s for WT and 69 ± 10 g.s for TG, P = 0.0035). Data represent mean + S.E.M. for n = 6 TG mice and 6 WT mice.

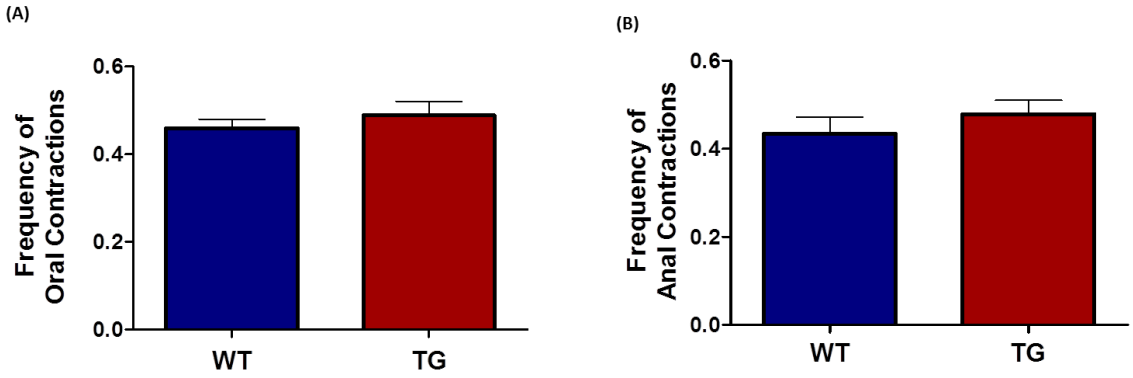

Figure 5.4: TG mutation did not alter frequency of oral and anal contractions. A 30-minute window of regular CMMC activity was selected and the numbers of oral and anal contractions were counted to calculate frequency. (A) The frequency of oral contractions in a period of 30 minutes was not altered in TG mice as compared to WT. (B) Similarly, the TG mutations do not altered frequency of anal contractions recorded in 30 minutes. Data represent mean + S.E.M. for n = 6 TG mice and 6 WT mice.

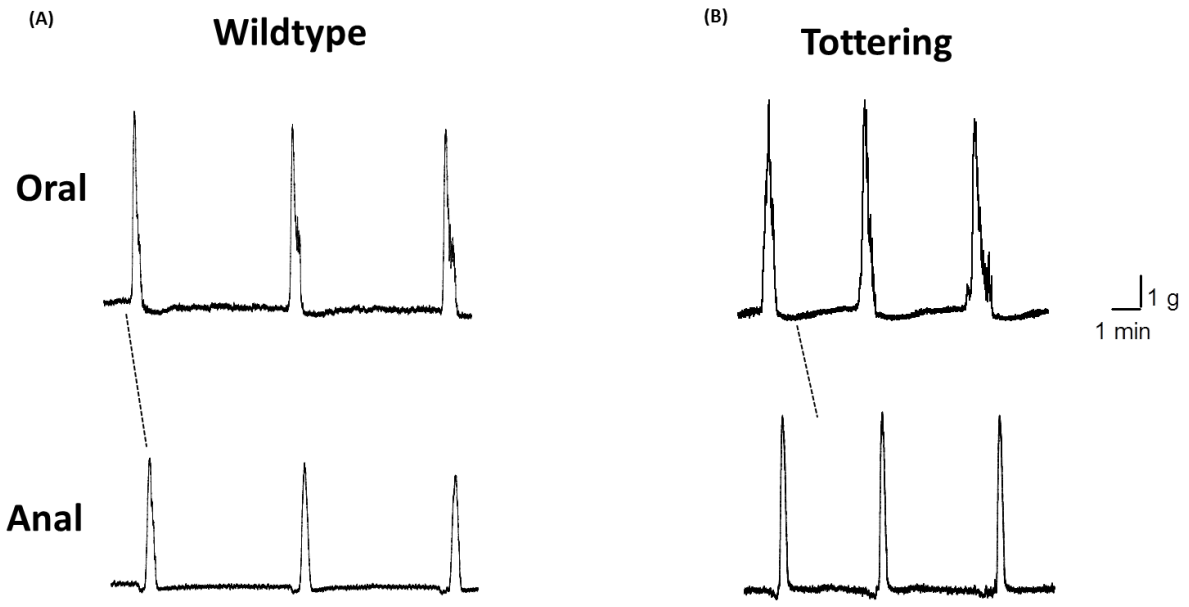

Figure 5.5: Representation of the CMMC pattern. (A) CMMC pattern observed in TG in a segment of the colonic muscle. (B) CMMC pattern observed in WT in a segment of the colonic muscle. The three presented contractions are called oral-anal propagating contractions because a contraction first starts at the oral side and after some delay a contraction is generated at the anal side. That migration induced propulsion of the colonic content along the length of the intestine.

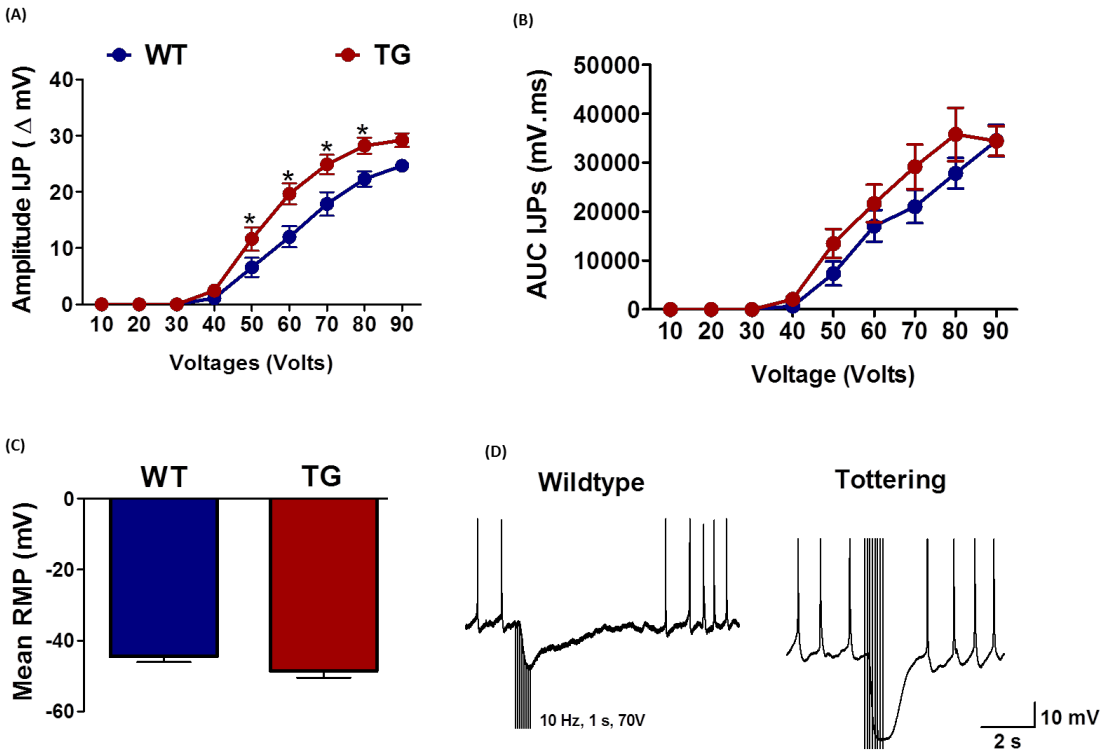

Figure 5.6: IJPs recorded from circular muscle cells are larger in TG mice. IJPs were characterized by measuring the amplitude of the fast purinergic phase and AUC of the whole response. (A) There is a voltage-dependent increase in the amplitude of the IJP in both WT and TG (voltage effect: $P<0.05$). The amplitude of the IJP is significantly larger in TG mice as compared with WT mice (genotype effect: $P<0.05$). A significant interaction was detected meaning the genotype affects the amplitude of the IJPs differently in the range of voltages used for nerve stimulation. (B) There is a voltage-dependent increase in the AUC of the IJP in both WT and TG (voltage effect: $P<0.05$). The AUC of the IJP is slightly larger in TG mice as compared with WT mice (genotype effect: $P<0.05$). No significant interaction was detected ($P>0.05$). (C) The mean resting membrane potential of circular muscle cells was not different between WT and TG mice ruling out a change in the driving force for K^+ out-flux as a mean for the enhanced hyperpolarization. (D) Representative IJPs recorded from WT and TG circular muscle cell to clearly show the enhanced IJP observed in TG mice. Data represented as mean ± SEM, n = 21 cells in

Figure 5.6 (cont'd)

WT and 19 cellS in TG. Student's t-Test and Two-Way ANOVA were used to determine statistical significance.

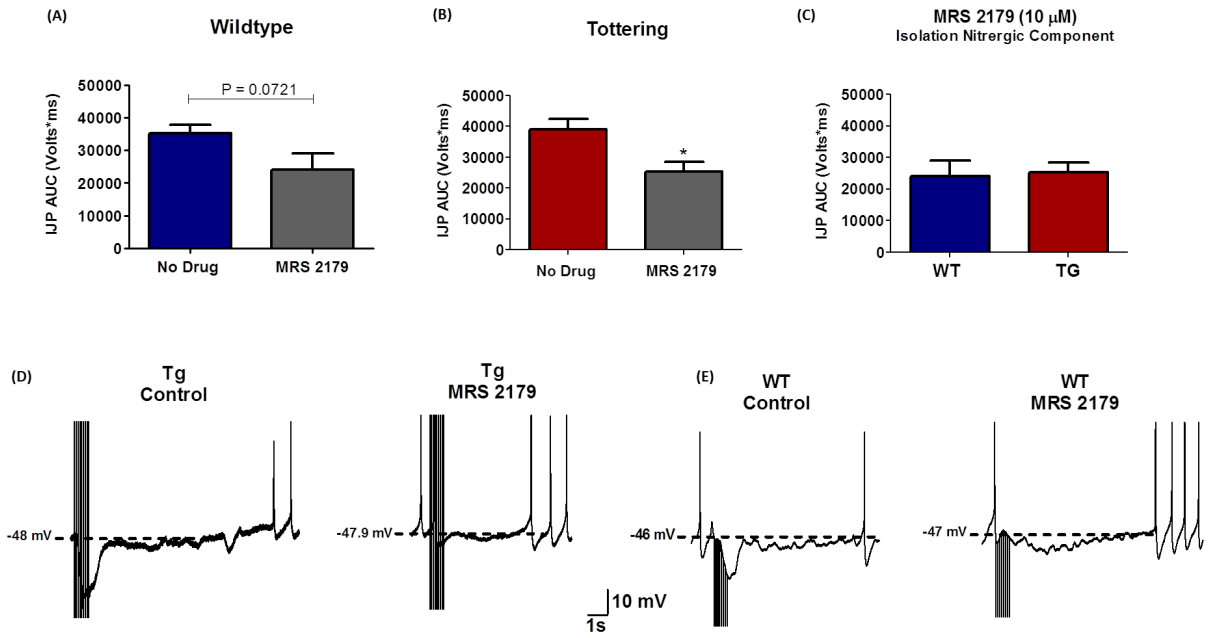

Figure 5.7: The purinergic receptor antagonist, MRS 2179, inhibit IJPs in WT and TG mice. The effect of the P2Y1 purinergic receptor antagonist, MRS 2179 (10 μM) was investigated in TG and WT mice. (A) In WT mice, MRS 2179 abolished the fast phase of the IJP. This produced a substantial decrease in the AUC of the IJP, however it did not reach statistical significance (P = 0.0721). (B) In TG mice, MRS 2179 abolished the fast phase of the IJP as well. In this case MRS 2179 did produce a statistical significant decrease in the AUC of the IJP (P = 0.0137). (C) Addition of MRS 2179 isolates the slow nitrergic component of the IJP. A comparison of this component revealed no differences between WT and TG mice (P = 0.8490) . Data represented as mean ± S.E.M., n =7 cells in WT and 6 cells in TG. Student's t-Test was used to determine statistical significance

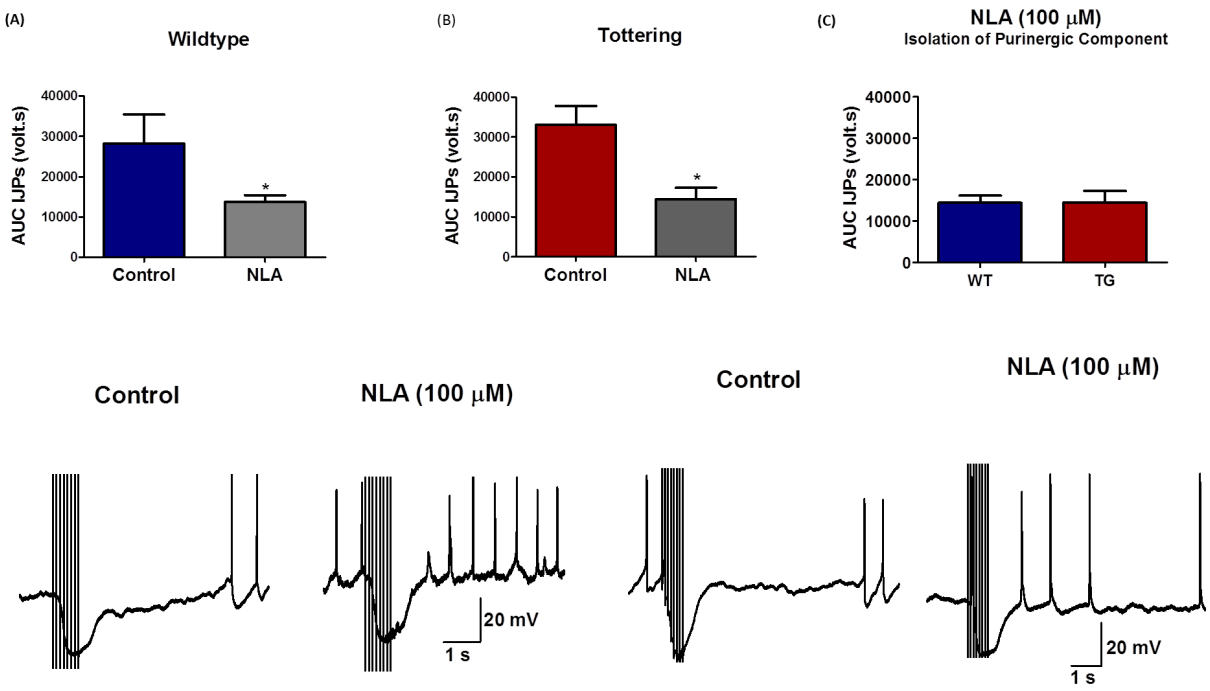

Figure 5.8: The nitric oxide synthase inhibitor, NLA, decreased IJPs in WT and TG mice. The effect NLA (100 μM) on the amplitude and AUC of the IJP was investigated. NLA produced a significant inhibition of the IJP AUC in both WT (A) and TG (B) mice (P = 0.0452 for WT and P = 0.0078 for TG). (C) NLA treatment isolates the fast purinergic component of the IJPs. Under this condition, the purinergic component of the IJPs in TG and WT mice are in the same (P = 0.9931). Data represented as mean ± S.E.M., n =9 cells in WT and 7 cells in TG. Student's t-Test was used to determine statistical significance

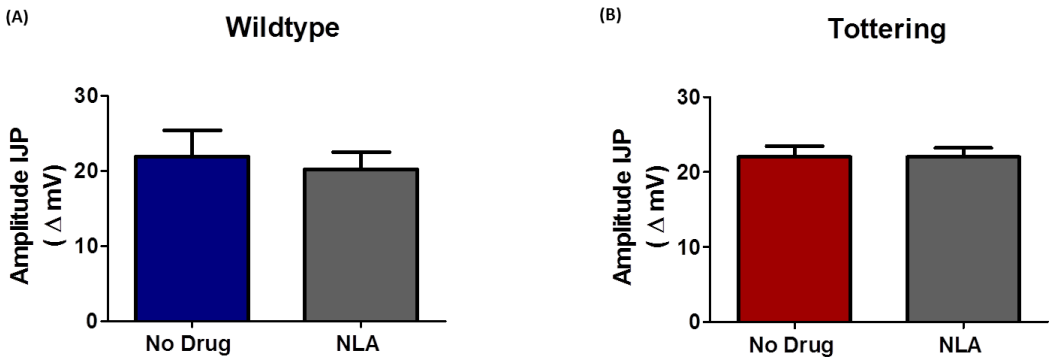

Figure 5.9: Amplitude of the fast IJP is not affected by inhibition of the nitrergic component. NLA treatment did not change the amplitude of the fast purinergic component neither in WT (A) or TG (B) mice. These results confirm the fact that fast IJPs are mainly purinergic and nitrergic transmission does not alter significantly the fast phase of the IJP. Data represented as mean ± S.E.M., n =9 cells in WT and 7 cells in TG. Student t-T was used to determine statistical significance. Data represented as mean ± S.E.M., n =9 cells in WT and 7 cells in TG. Student's t-Test was used to determine statistical significance.

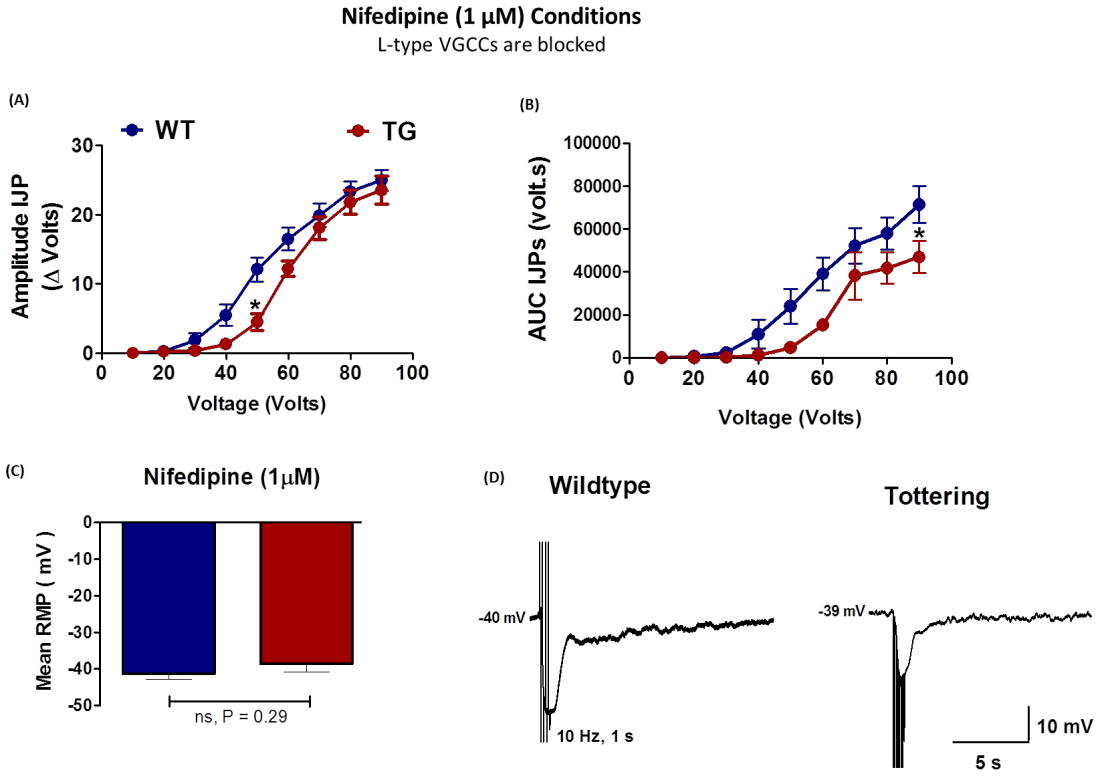

Figure 5.10: Under nifedipine conditions the IJPs in TG mice are smaller. (A) There is a voltage-dependent increase in the amplitude of the IJP in both WT and TG (voltage effect: P <0.05). The amplitude of the fast IJP is significantly smaller in TG mice (genotype effect: P<0.05). This occurs in conditions where L-type channels are blocked with 1 μM nifedipine. (B) There is a voltage-dependent increase in the amplitude of the IJP in both WT and TG (voltage effect: P <0.05). The AUC of the IJP in TG mice is also significantly smaller as compared with WT mice (genotype effect: P<0.05). No significant interaction was detected in either case. Data represented as mean ± S.E.M., n = 31 cell for WT and 20 cell for TG. Two-Way ANOVA was used to detect statistical significance.

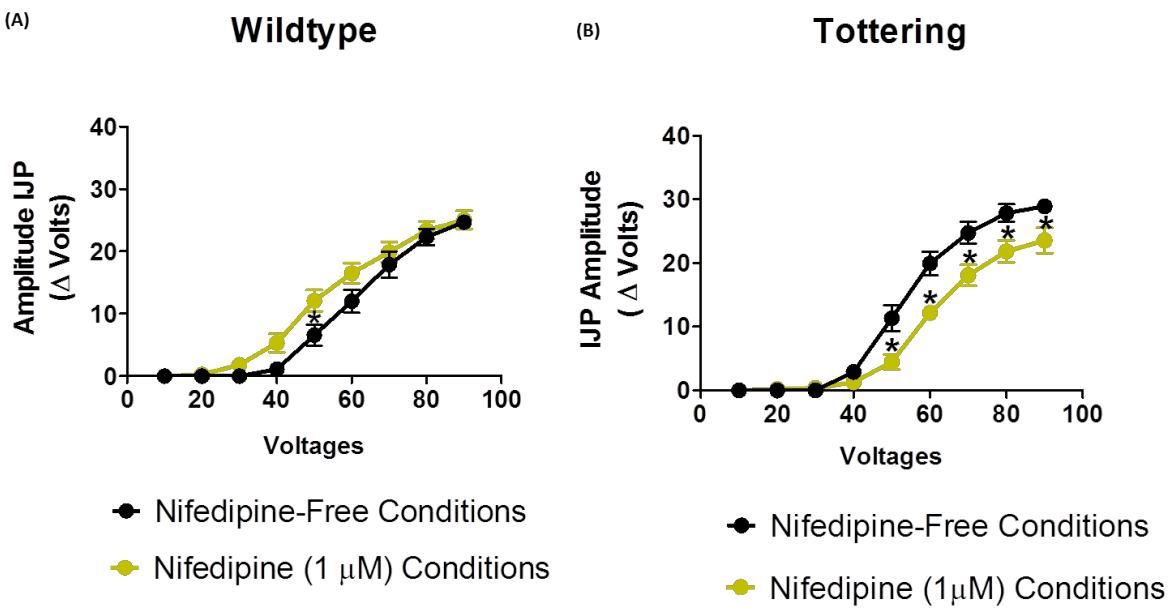

Figure 5.11: The presence of nifedipine enhances IJPs in TG but not in WT mice. IJPs recorded in the absence and presence of nifedipine (1 μM) were pooled and compared. (A) In WT, IJPs recorded in nifedipine-free conditions (n = 21 cells) were always slightly smaller than IJPs recorded in the presence of nifedipine (n= 32 cells). This difference was significant (nifedipine effect: $P<0.05$). No significant interaction was detected. (B) In TG, IJPs recorded in the nifedipine-free conditions (n = 19 cells) were larger than IJPs recorded in the presence of nifedipine (n = 20 cells); this is also significant (nifedipine effect: $P<0.05$). A significant interaction was detected meaning that nifedipine does not have the same effect on all the voltage used for nerve stimulation. Data represented as mean ± S.E.M. Two-Way ANOVA was used to detect statistical significance.

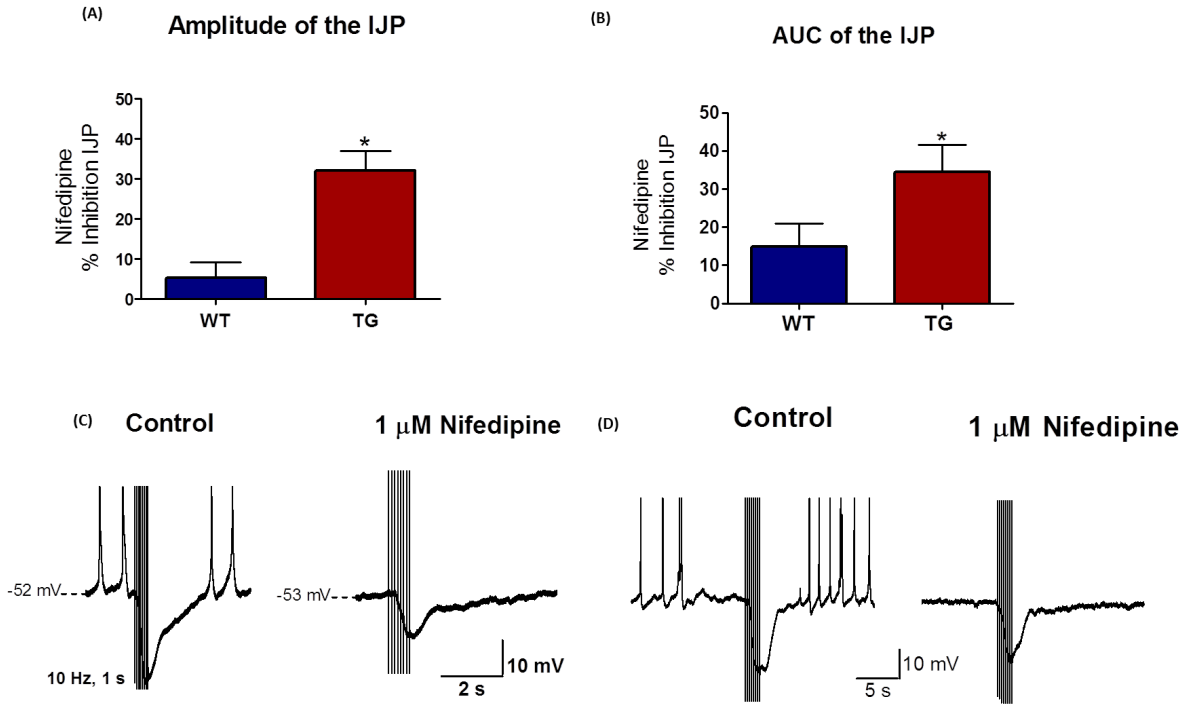

Figure 5.12: Nifedipine caused up to 35% inhibition of the IJP in TG mice. (A) Nifedipine produced 5 ± 3.7% and 32 ± 4.9% inhibition in the amplitude of the IJP in WT and TG mice, respectively. This difference was statistically significant (P = 0.0015). (B) Nifedipine produced a 14.8 ± 6% and 35 ± 7% inhibition in the AUC of the IJP in WT and TG mice, respectively. This difference was also statistically significant (P = 0.0480). The percent inhibition of nifedipine on IJPs was always significantly larger in TG mice. Data represented as mean ± S.E.M., n =12 cells. Student's t-Test was used to determine statistical significance.

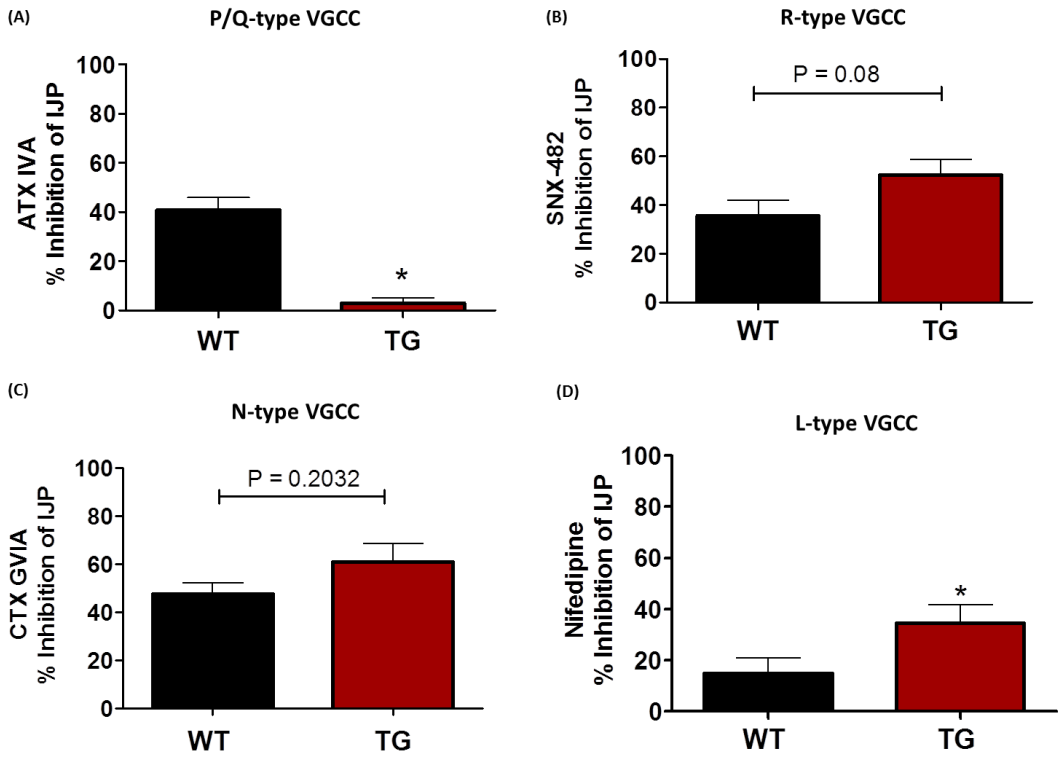

Figure 5.13: A different profile of VGCC subtypes mediate IJPs in TG mice. (A) Blocking P/Q-type VGCCs with agatoxin-IVA had a 40.8 ± 5% inhibition in WT and 3 ± 2.1% inhibition in TG mice, this difference was statistically significant ($P < 0.0001$; n = 10 cells in WT and 9 cells in TG) . (B) Contribution of R-type VGCCs was investigated using SNX-482. This toxin produced a 35.6 ± 6.5% inhibition in WT and a 52.4 ± 6.2% inhibition in TG, this difference did not reach statistical significance ($P = 0.0803$; n = 9 cells) . (C) Conotoxin-GVIA was used to block current through N-type VGCCs and the results showed a 47 ± 4% inhibition in WT and a 61 ± 7% inhibition in TG mice. Although the percent inhibition was larger in TG mice, the difference did reach statistical significance ($P = 0.0232$; n = 9 cells in WT and 7 cells in TG). (D) For comparison purposes the 35 ± 7% inhibition of nifedipine in TG is presented. It is clear that the percent inhibition by nifedipine tries to replace the percent inhibition produced by agatoxin-IVA in WT mice. Data represented as mean ± S.E.M. Student's t-Test was used to determine statistical significance.

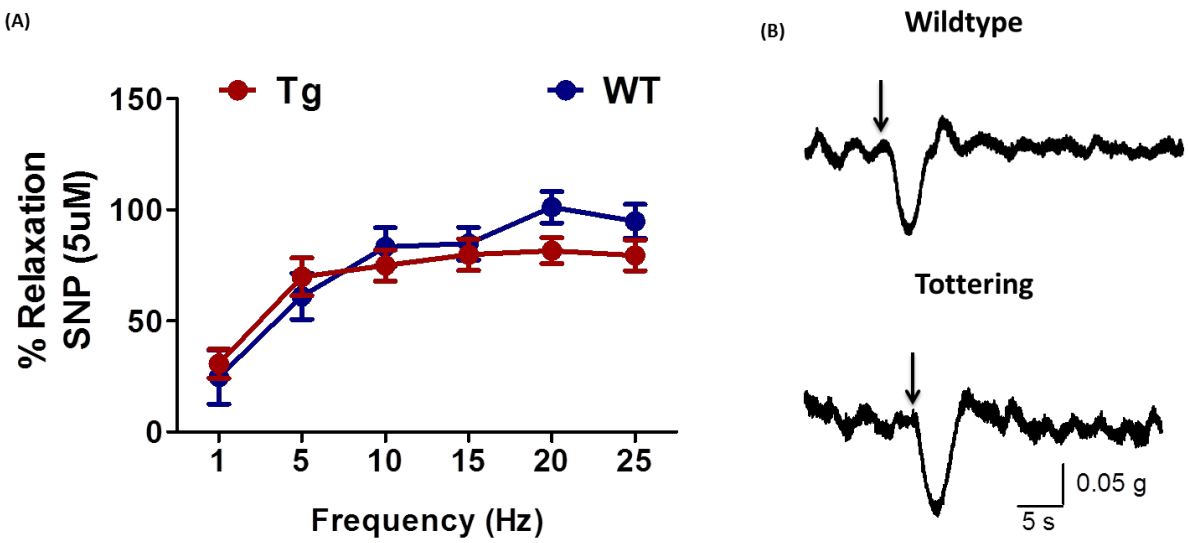

Figure 5.14: Mechanical relaxations of the circular muscle are not altered in TG mice. (A) There is a frequency-dependent increase in the percent relaxation of the muscle in both WT and TG mice (frequency effect: $P<0.05$). However, this frequency-dependent increase occurred to the same extent in WT and TG mice (genotype effect: $P>0.05$). No significant interaction was detected meaning that genotype has the same effect at all frequencies used to evoke relaxations. (B) Representative relaxation of circular muscle rings in WT and TG mice. Data represent mean ± S.E.M., n = 5 mice in each group. Two-Way ANOVA was used to determine statistical significance.

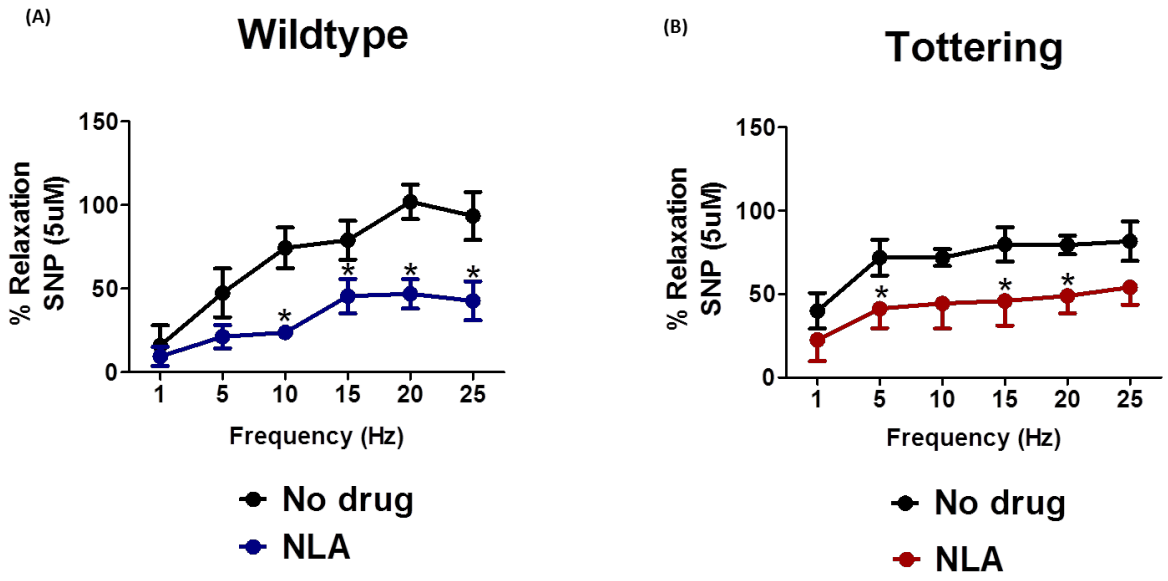

Figure 5.15: Inhibition of nitric oxide synthase decreases muscle relaxation in both WT and TG mice. There is a frequency-dependent increase in the relaxations in both panels (frequency effect: $P<0.05$). In WT mice (A) and TG mice (B) inhibition of NOS by NLA significantly decreased the relaxations in the circular muscle (NLA effect: $P<0.05$). For panel (A) the interaction was significant meaning that NLA does not has the same effect at all the frequencies use to evoke relaxations. For (B) no significant interaction was detected. Data represent mean ± S.E.M., n = 5 mice in each group. Two-Way ANOVA was used to determine statistical significance.

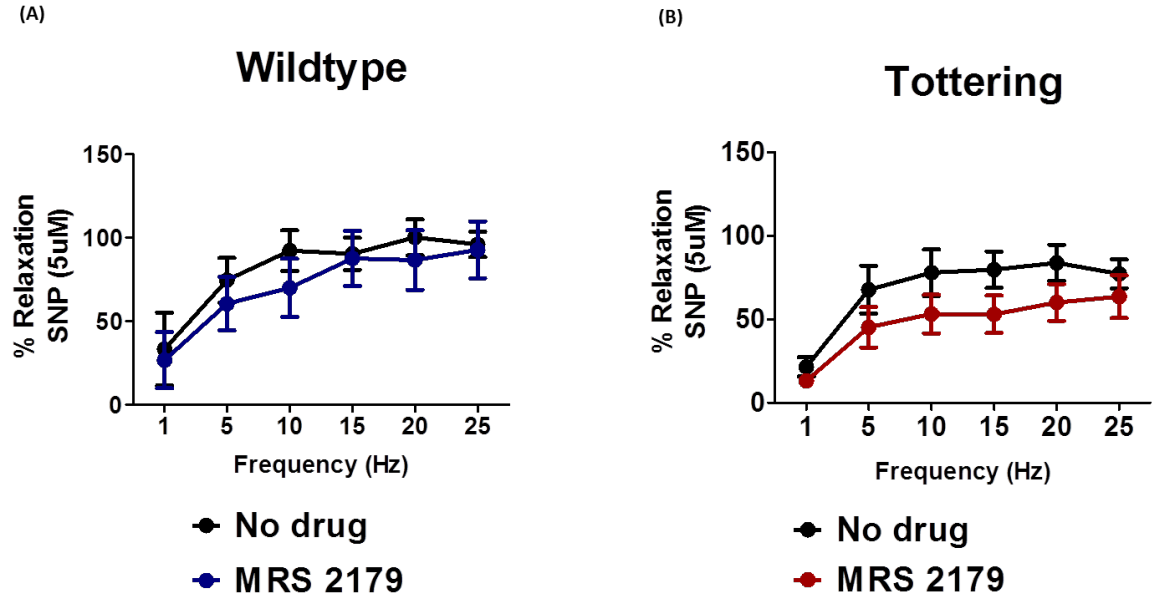

Figure 5.16: Blocking the purinergic receptor, P2Y1, modestly inhibited relaxations in TG mice. (A) MRS 2179 (10 µM) had no inhibitory effect on relaxations of the circular muscle in WT mice (MRS 2179 effect: $P>0.05$ and Frequency effect: $P<0.05$). (B) The same treatment had a slight inhibitory effect on relaxation of the muscle in TG mice (MRS 2179 effect: $P<0.05$ and Frequency effect: $P<0.05$). There is no significant interaction in either WT or TG mice. Data represent mean ± SEM, n = 5 mice in each group. Two-Way ANOVA was used to determine statistical significance.

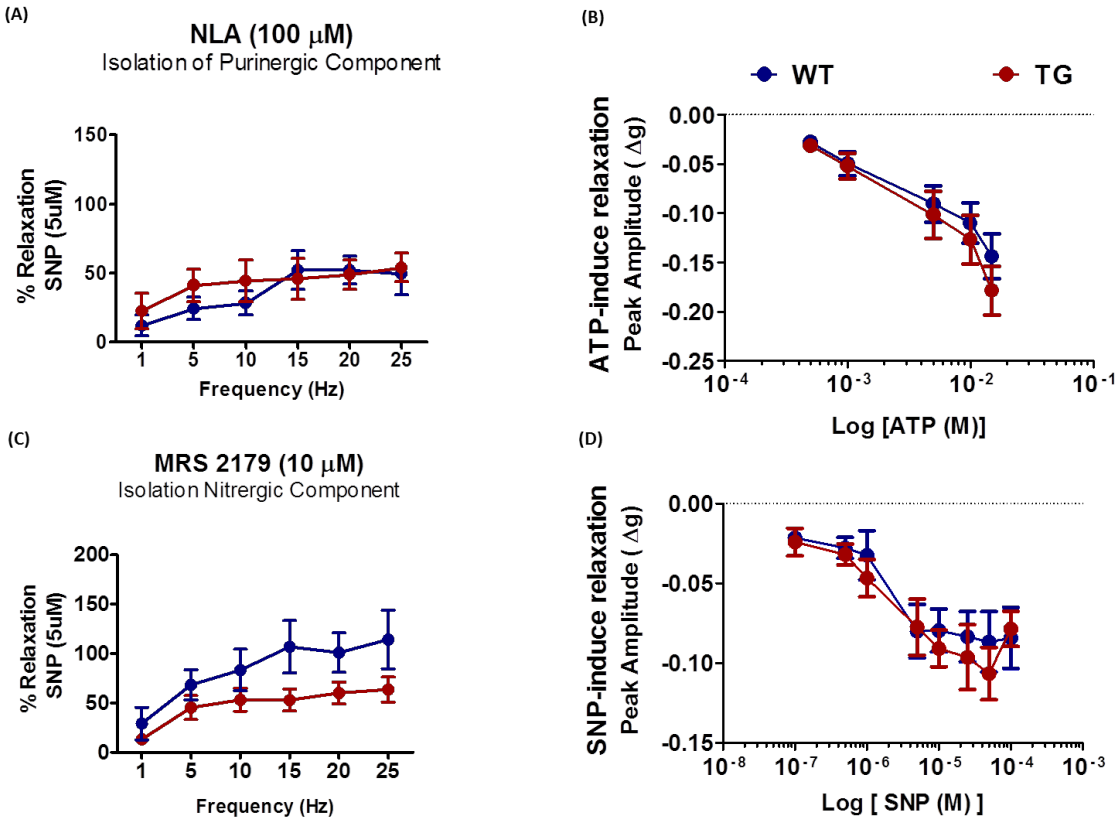

Figure 5.17: Characterization of the purinergic and nitrergic components of the relaxation. (A) Purinergic component: There is a frequency-dependent increase in the relaxations (frequency effect: $P<0.05$). The purinergic component of the relaxation was not different between WT and TG mice (genotype effect: $P>0.05$). No significant interaction was detected. (B) There is a concentration-dependent increase in ATP-induced relaxation of the muscle (ATP effect: $P<0.05$). However, the genotype does not affect the ATP -induced relaxations (genotype effect: $P>0.05$). No significant interaction was detected. (C) Nitrergic Component: There is a frequency-dependent increase in the relaxations (frequency effect: $P<0.05$). The nitrergic component of the relaxation was slightly smaller in TG mice and it was significant (genotype effect: $P<0.05$). No significant interaction was detected. (D) There is a concentration-dependent increase in SNP-induced relaxation of the muscle (SNP effect: $P<0.05$). The genotype does not affect the SNP-induced relaxation (genotype effect: $P>0.05$). No

Figure 5.17 (cont'd)

significant interaction was detected. Data represent mean ± SEM, n = 5 mice in each group. Two-Way ANOVA was used to determine statistical significance.

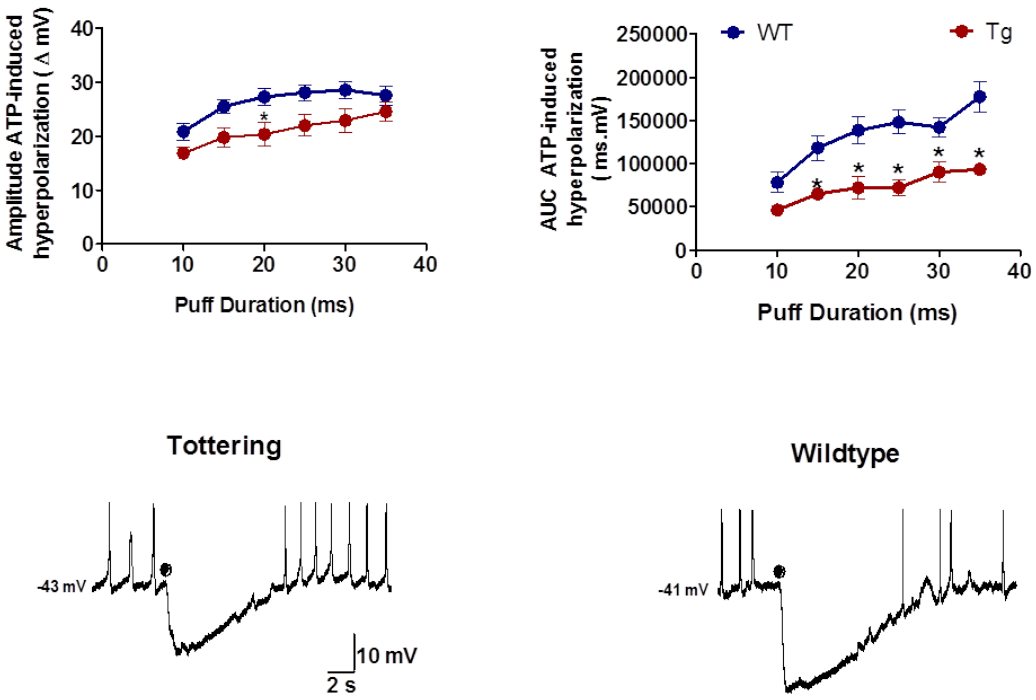

Figure 5.18: ATP-induced hyperpolarization is smaller in TG mice. ATP (5 µM) was pressure-applied close to the recording site to investigate the ATP-induced hyperpolarization in WT and TG mice. The amplitude (A) and AUC (B) of the hyperpolarization was significantly smaller in TG as compared to WT. (C) Representative response to ATP in TG and WT mice. Two-Way ANOVA was used to determine statistical significance. Data represents mean ± SEM, n = 10 cells.

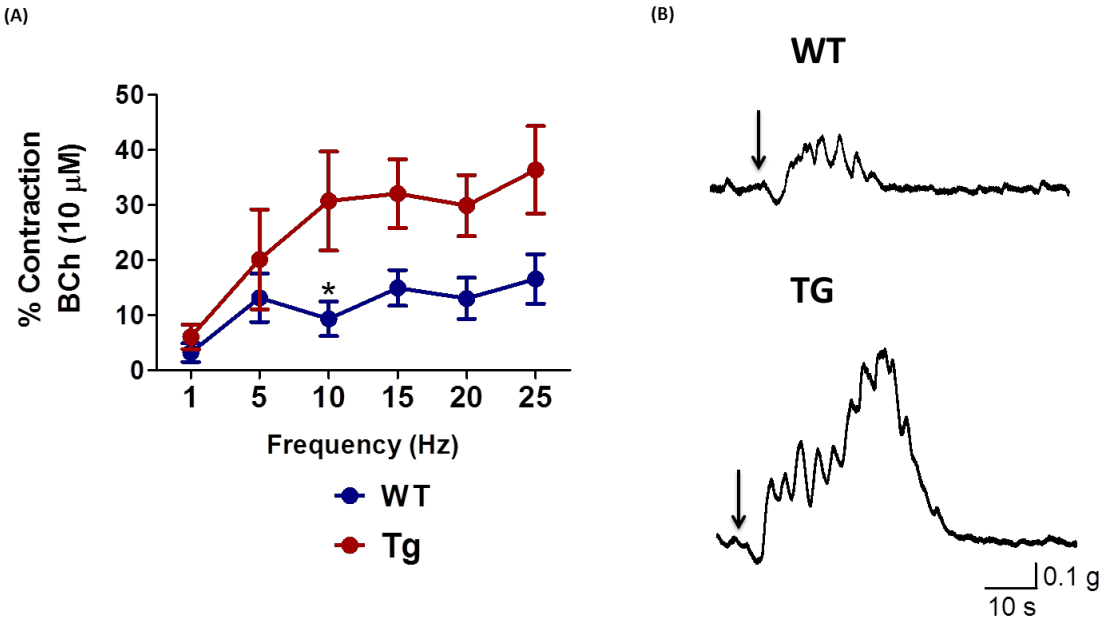

Figure 5.19: Nerve-evoked contractions of circular muscle are larger in TG mice. (A) There is a frequency-dependent increase in the muscle contractions in both WT and TG mice (frequency effect: $P<0.05$). The contractions are significantly larger in TG as compared to WT mice (genotype effect: $P<0.05$). (B) Representative traces showing a contraction in WT and TG mice. No significant interaction was detected. Note the contractions are preceded by a small relaxation. Data represent mean ± S.E.M., n = 5 mice in each group. Two-Way ANOVA was used to determine statistical significance.

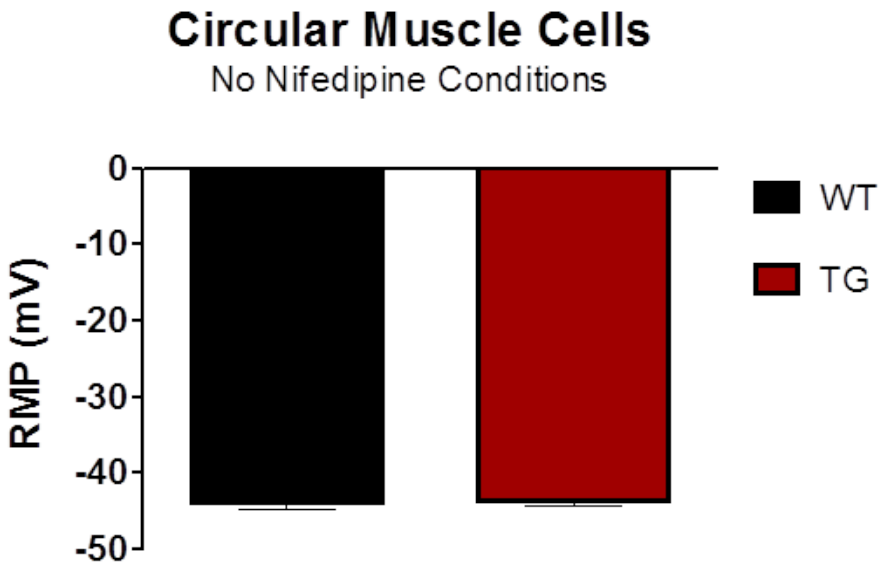

Figure 5.20: Characterization of the resting membrane potential in circular muscle cells. Distal circular muscle cells of WT and TG mice had RMP averaging -43.7 ± 0.9 mV and -43.62 ± 0.7 mV, respectively. No statistical difference was detected. Data represented as mean ± S.E.M; n = 106 cells in WT and 109 cells in TG. Student's t-Test was used to determine statistical significance.

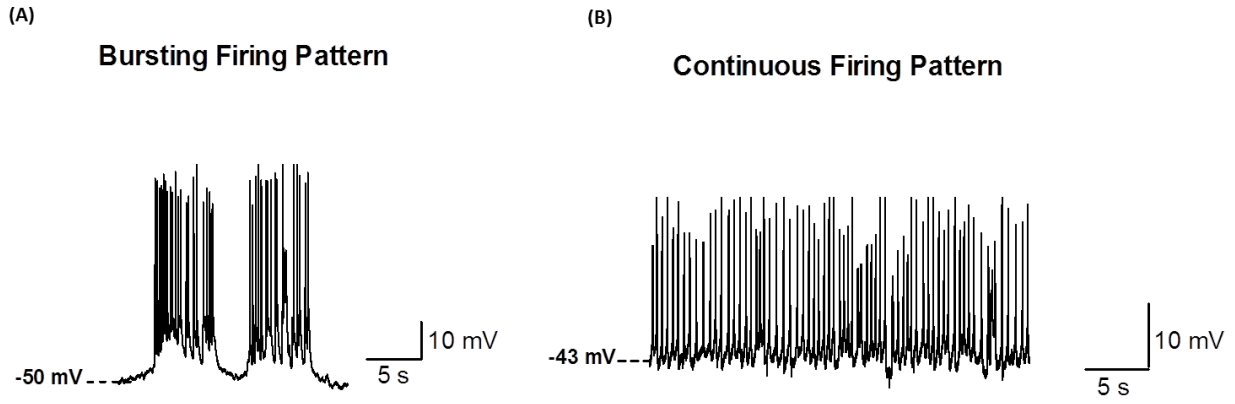

Figure 5.21: Types of firing patterns observed from circular muscle cells. Circular muscle cells presented two types of firing patterns. (A) Bursting Firing pattern: consisted of a slow wave of depolarization with firing of action potentials overlapping the slow wave depolarization. (B) Continuous firing pattern: consisted of cells able to fire actions potentials in a non-bursting fashion. Both types of firing patterns were observed in cells from WT and TG mice.

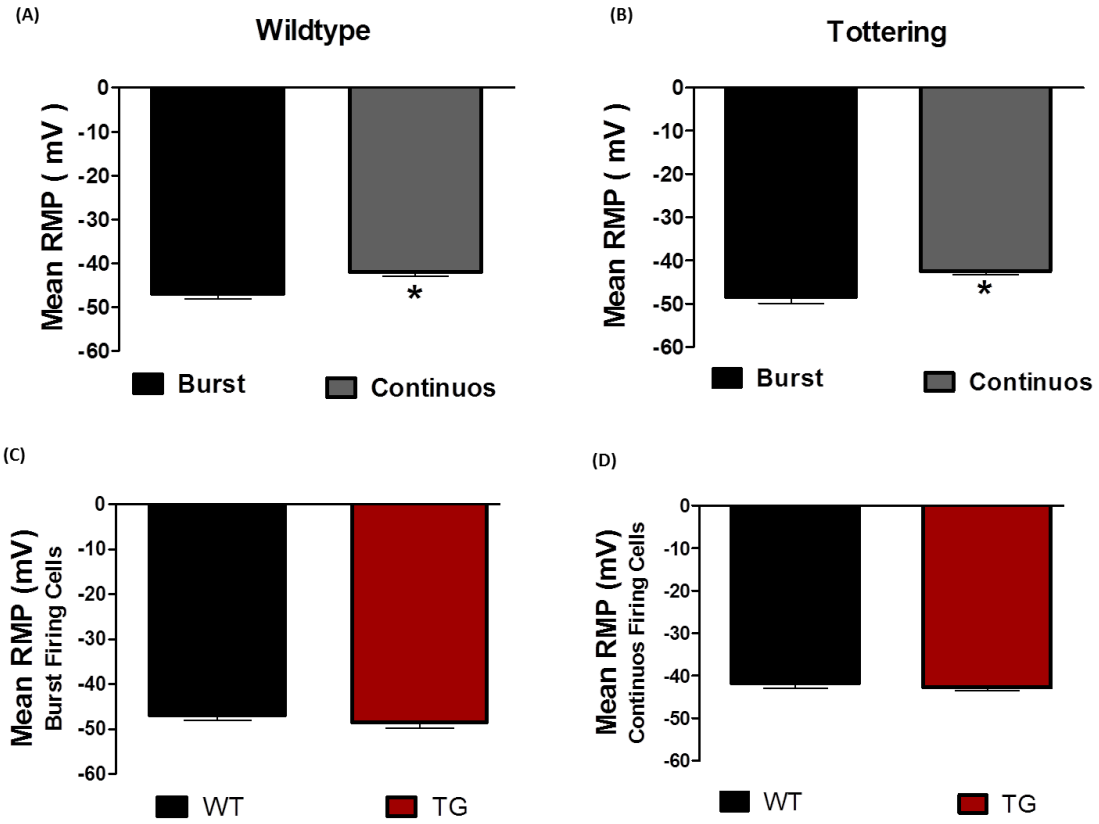

Figure 5.22: Bursting cells have a more depolarized resting membrane potential than continuous firing cells. (A) Bursting cells in WT mice had RMP averaging -47 ± 1.3 mV and continuous cells had RMP averaging -42 ± 1 mV and this depolarization was statistically significant (P =0.005; n = 40 bursting cells and 64 continuous cells) . (B) Bursting cells in TG mice had RMP averaging -48.5 ± 1.4 mV and continuous firing cells had RMP averaging -42.4 ± 0.86 mV; that depolarization was also statistically significant (P = 0.0043); n = 19 bursting cells and 101 continuous cells). When comparing the RMP of either bursting (C) or continuous (D) firing cells between WT and TG the mean values were about the same. Data represent mean ± S.E.M. Student's t-Test was used to determine statistical significance.

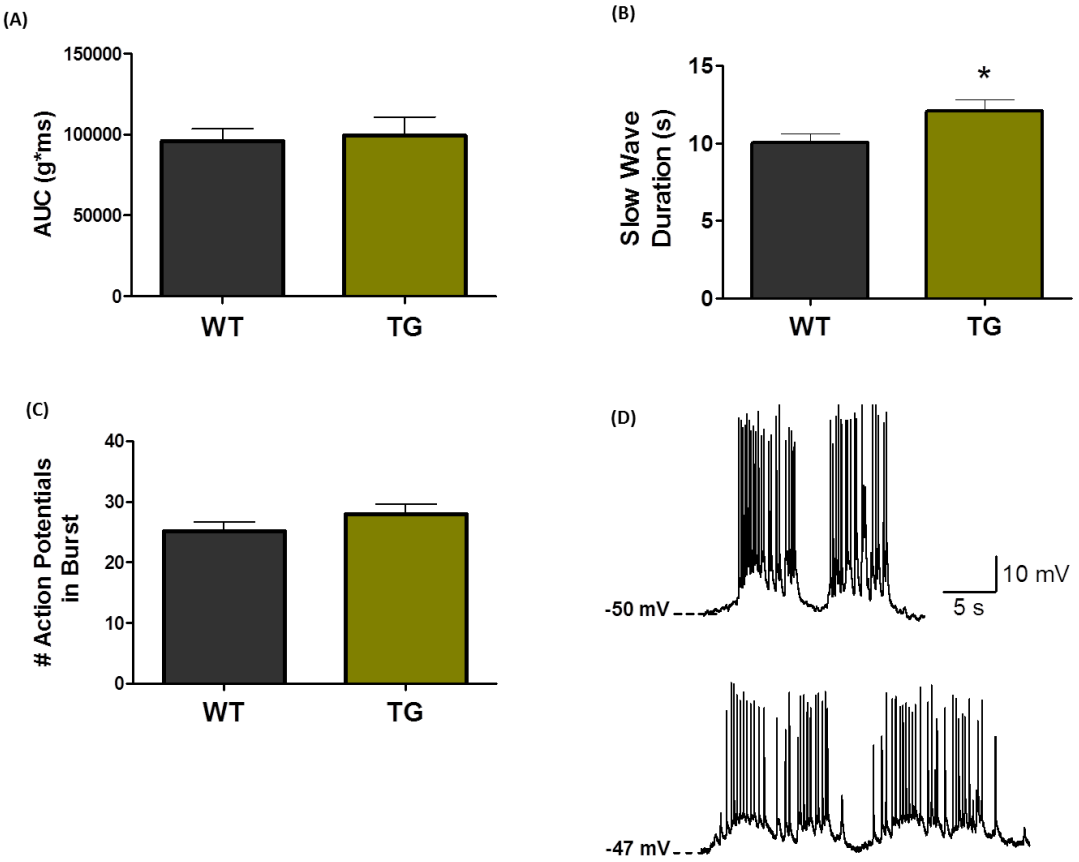

Figure 5.23: Slow wave duration of bursting cells was modestly longer in TG mice. (A) The AUC of the slow wave depolarization was similar between WT and TG mice. (B) Duration of the slow wave depolarization was slightly increased in TG mice and this change was statistically significant (10 ± 0.5 s in WT and 12 ± 0.7 s in TG mice, P = 0.0340). (C) The number of action potential fired within the slow depolarization was quantified and there is no significant difference in this parameter between WT and TG mice. (D) Representation of the bursting pattern in WT and TG mice emphasizing the longer duration of the slow depolarization in TG mice. Data represent mean + S.E.M. for n. Data represent mean ± S.E.M., n = 35 cell for WT and 21 cell for TG. Student's t-Test was used to determine statistical significance.

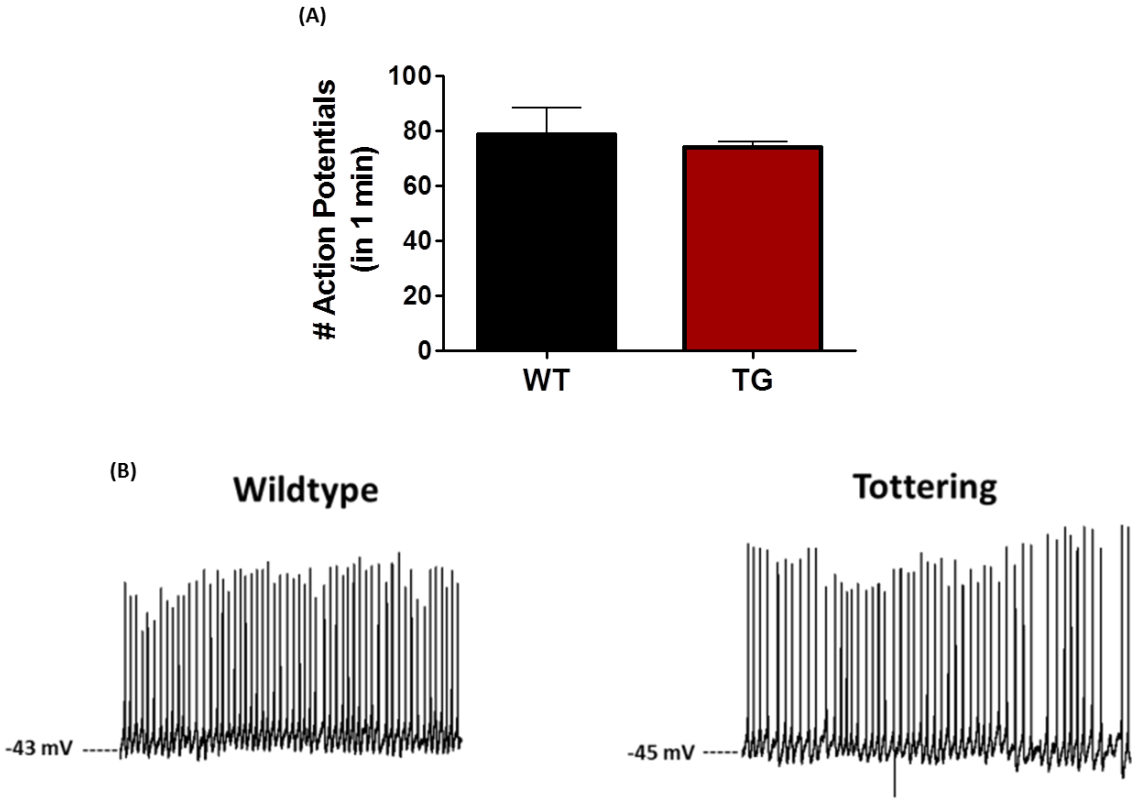

Figure 5.24: The pattern of continuous firing is similar between TG and WT mice. (A) The number of action potentials fire in one minute was quantified in WT and TG continuous firing cells. The result showed no difference in the number of action potentials firing between WT and TG muscle cells (P = 0.4790). (B) Representation of continuous firing cells in WT and TG mice. Data represent mean ± S.E.M., n = 16 cell for WT and 55 cell for TG. Student's t-Test was used to determine statistical significance.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

SUMMARY AND GENERAL CONCLUSIONS

Gastrointestinal (GI) functional and motility disorders are characterized by altered sensory, motor, and even secretory functions of the GI tract. They are referred as "functional" because the symptomatology cannot be clearly linked to structural or biochemical abnormalities of the digestive system. Therefore, the symptomatology presented by these disorders is associated with dysfunction of the neuronal circuit controlling sensory, motor, and secretory functions of the GI. Some of the most common functional GI motility disorders in practice are achalasia, gastroparesis, intestinal pseudo-obstruction, and slow-transit constipation (Di Nardo et al. 2008). Functional GI disorders are gaining serious attention from the healthcare systems. For example, irritable bowel syndrome, which is characterized by abdominal pain and intestinal dysmotility, has an estimated prevalence of 15-22% in western society and by itself account for up to 60% of the referrals to secondary care (Ford et al. 2008).

The current pharmacological approaches for GI motility disorders act generally to either increase (prokinetics) or decrease (antidiarrheal) propulsive motility rather than modulating motility patterns of the smooth muscles. In addition, the clinical efficacy of the current pharmacological treatments is poorly documented, short in duration, and accompanied with major adverse side-effects (reviewed by Nardo et al. 2008). Thus, there is a serious and explicit need for novel therapeutic targets for the successful management of GI motility disorders.

Motility of the GI muscles is under the control of the enteric nervous system (ENS), which contains an intrinsic polarized neural circuit that generates coordinated motility patterns from the esophagus to the intestines. The electrical and coupling activity of GI effector cells, interstitial cells of Cajal and smooth muscle cells, are crucial factors regulating motility patterns. Enteric neurons are important regulators of the intensity and precision of the rhythmic electrical activity of these effector cells. Understanding in exquisite detail motility neuronal circuits and electrical rhythmic activity of effector cells is crucial in order to rationally address targets for pharmacological intervention.

An important step toward this goal is to comprehend which ion channels are indispensable for regulation of motility and electrical activity of the GI smooth muscles. Voltage-gated ion channels (VGICs) are essential components within the neuronal circuit controlling motility of the gut. This has been demonstrated by studying colonic propulsive motility in vitro. Block of voltage-gated Na^+ channels and thus neuronal action potentials completely stops transit of an artificial pellet along the colonic segment. However, there are multiple types of VGICs within the ENS such as K^+ and Ca^{2+} channels. The studies completed in this dissertation provide insight into the physiological role and relevance of voltage-gated Ca^{2+} channels (VGCCs) within the enteric neuronal circuit controlling motility patterns of the large intestine.

The rationale for considering VGCCs as important regulators of intestinal motility emanates from their crucial role in regulating neurotransmitter release. In addition, neurological disorders associated with perturbations in VGCCs present autonomic alterations that include GI motility dysfunction. Animal models with perturbations in VGCC subtypes provide a unique opportunity to investigate the physiological significance of VGCCs in enteric transmission. In this dissertation two animals models were used to investigate the physiological role of P/Q- and R-type VGCC subtypes and these were the tottering (TG) and α1E KO, respectively. The overall hypothesis of this dissertation is that functional deficit in P/Q- and R-type VGCCs disrupts enteric neuro-muscle communication and propulsive colonic motility. Since both animal models induced homeostatic plasticity in the central and peripheral nervous system, the ability of the ENS to compensate the loss-of-function of these VGCCs was investigated.

R-type VGCCs contribute to inhibitory neuromuscular transmission to the longitudinal muscle of the guinea pig ileum

The third chapter of this dissertation aimed to investigate the role of R-type VGCCs in enteric neuromuscular transmission to the longitudinal muscle of the small intestine. To do that

electrophysiological and pharmacological approaches were employed using the guinea pig ileum as a model. Studies from cerebellar granule cells indicate that a heterogeneous population of Ca^{2+} channels generates total R-type currents and the two main types of currents were called G2 and G3 (Tottene et al. 2000, 1996). The G2 currents have the characteristics of recombinant alpha1E subunit: abolished by $NiCl_2$ and SNX-482 treatment, larger macroscopic current with Ca^{2+} than Ba^{2+}, single channel conductance of 12-15 ps, activated at ~15 mV more negative potentials than G3. The G3 currents share properties of P/Q-, N-, and L-type VGCCs: resistant to NiCl2 and SNX-482 block, larger macroscopic current with Ba^{2+} than Ca^{2+}, single channel conductance of 20 ps, activate at more negative potentials. Whole cell recordings from enteric myenteric neurons, which control muscle movement, showed that 40% of the total current is R-type (Bian et al. 2004). The same authors were able to completely abolish the R-type current with either $NiCl_2$ or SNX-482 treatment. This demonstrates that R-type currents in the ENS are likely to be conducted by a single population of Ca^{2+} channels and due to the pharmacological sensitivity this current is likely to be conducted by G2 type Ca^{2+} channels formed by the α1E subunit. Later, it was demonstrated that R-type channels contribute to ascending synaptic transmission within the enteric circuit controlling motility of the muscle and to the action potential in intrinsic primary afferent neurons (Naidoo et al 2010). However, a function for R-type VGCCs during neuromuscular transmission has not been clearly settled in the literature.

The studies completed in this third chapter demonstrate that R-type VGCCs contribute to inhibitory neuromuscular transmission to the longitudinal muscle of the guinea pig ileum. The relaxation of the longitudinal muscle is prominently reduced by inhibition of NOS. Therefore, this relaxation is mostly mediated by activation of NOS and production of NO. The studies showed that inhibition of NOS and block of R-type VGCCs reduced the relaxation to the same extend. This suggested that R-type VGCCs provide the Ca^{2+} needed for NOS activation but it is unlikely that this Ca^{2+} contribute to generation of the non-nitrergic component of the relaxation. The dynamics of nitrergic transmission is

atypical, but it resembles the dynamics of traditional neurotransmission regarding regulation of neurotransmitter release. Pre-synaptically, there is an active pool of NOS that resembles the profile of synaptic vesicles at the active zones. The active pool of NOS consist of a dimer of nNOSα attached to the scaffolding protein PSD95 which in turn is anchored to the membrane of the nerve terminal by palmitoylation and to VGCCs (Rao et al. 2007; Chaudhury et al. 2009). These protein interactions place NOS in close apposition to VGCCs allowing a timely response to Ca^{2+} influx induced by arrival of action potentials to the nerve terminal.

Contribution of R-type VGCCs to enteric neuromuscular transmission is specific for the inhibitory component because blocking R-type channels did not reduce contraction of the muscle. In fact, block of these channels potentiated non-cholinergic contractions and left unaltered contractions mediated by acetylcholine. Pharmacological treatments that are well known to reduce relaxation or inhibitory transmission to the muscle such as nitric L-arginine (Keef et al. 1993) and apamin (Koh et al. 1997) also potentiated contractions supporting the involvement of R-type VGCCs in inhibitory neuromuscular transmission.

The role of N-type VGCCs in regulating neurotransmitter release in the peripheral nervous system is very well established especially during autonomic neuroeffector transmission (Waterman, 2000). Application of ω-conotoxin-GVIA to block N-type VGCCs once again demonstrated contribution of N-type currents to excitatory and inhibitory neuromuscular transmission to the longitudinal muscle of the guinea pig ileum and this result is supported by previous studies (reviewed by Waterman, 2000). The results from the present studies showed that N-type VGCCs contribute to nitrergic but also to the non-nitrergic component of the relaxation. The working model (refer to figure 3.11) has R- and N-type VGCCs closely apposed to the active pool of NOS contributing to nitrergic relaxation; however, N-type VGCCs are also contributing to release of the other mediator of the longitudinal muscle relaxation. This

can occur in different domain within the same nerve terminal or in two different populations of inhibitory nerves.

The identity of the non-nitrergic component of the relaxation was not identified in these studies. This component is smaller than the nitrergic component and sensitive to block of small conductance Ca^{2+}-activated K^{+} channels. The other main inhibitory neurotransmitter is a purinergic molecule which identity is still on debate but has been suggested to be either ATP or β-NAD (Hwang et al. 2011; Belain and Burnstock, 1994). The present studies showed that the purinergic neurotransmitter does not mediate the non-nitrergic component because block of the purinergic receptor with MRS 2179 had a minor inhibitory effect on the relaxation. Bi-phasic junction potentials were recorded from the longitudinal muscle consisting of a small hyperpolarization followed by a large and long-lasting depolarization. MRS 2179 did not alter the hyperpolarization but reduced the depolarization phase of the junction potentials. This suggested that the purinergic neurotransmitter is couple to excitatory pathways to the longitudinal muscle. Excitatory actions of purinergic neuromuscular transmission had been shown before in the longitudinal muscle of guinea pig ileum (Ivancheva et al. 2000) and mouse distal colon (Zizzo et al. 2007). Blocking of R-type VGCCs inhibited the hyperpolarization without affecting the depolarization phase supporting involvement of these channels in inhibitory neuromuscular transmission.

Purinergic neuromuscular transmission to the circular muscle is coupled to hyperpolarization and relaxation of the muscle in both small (Crist et al. 1992) and large intestine (Gallego et al. 2008). It is interesting the fact that purinergic actions in the longitudinal muscle have been reported to be excitatory in particular areas along the intestines, especially distally located regions (Zizzo et al. 2008, 2007; Ivancheva et al. 2000). The two muscle layers have been reported to express different behaviors during propulsive activity: relaxation in the circular muscle coupled to contraction of the longitudinal muscle (Wood, 1998). Thus the different outcomes of purinergic neuromuscular transmission to the

longitudinal and circular muscle might be critical for physiologically appropriate propulsive activity. R-type VGCCs is not involved in release of the purinergic transmitter or mediation of the excitatory transmission because $NiCl_2$ did not inhibit the mechanical contractions or the depolarization phase of the junction potentials from the longitudinal muscle.

It is still unknown whether NO and the purinergic neurotransmitter are expressed by the same population of enteric motor neurons or if they are expressed by at least two different populations. One of the limiting factors in addressing this question is the fact that there are no reliable markers for purinergic nerves although the vesicular nucleotide transporter (VNUT, SLC17A9) may prove to be the needed molecular marker for these nerves (Sawada et al. 2008). The current assumption is that NO and the purinergic neurotransmitter are expressed by the same population of motor neurons but in different domains. The data presented in this chapter suggest that one domain has the active pool of NOS closely opposed to both R- and N-type VGCCs. A different domain could express the purinergic neurotransmitter (presumably ATP) stored in synaptic vesicles and the present study showed that N-type VGCCs regulate release of this transmitter. Blocking these channels abolished the non-cholinergic contractions of the longitudinal muscle, which according to the effect of MRS 2179 on the depolarization phase of junction potentials, should in part be mediated by ATP or other purinergic molecule. This working model is depicted in figure 3.11. It is well established that these two neurotransmitters have inhibitory actions to the proximal intestine regardless of the muscle layers (Rozsai et al 2001; Wang et al. 2007) and in the circular muscle of distally located intestinal regions (Crist et al. 1992; Gallego et al. 2008; He and Goyal, 1993). A similar working model can apply in this instance. The neurotransmitters will produce their characteristic electrical responses in the muscle (the transient and large purinergic hyperpolarization followed by the small and long lasting nitrergic hyperpolarization). There is evidence supporting a role for N-type VGCCs in regulating release of both neurotransmitters (Waterman, 2000). However, the contribution of other channel subtypes such as R- or P/Q-type is not clearly addressed.

Therefore, more studies aiming to elucidate the role of these non-N-type VGCCs in enteric transmission will provide important insight into the general role of VGCCs during regulation of intestinal motility.

P/Q- and R-type VGCCs are physiologically relevant because the ENS has a "back-up plan" to overcome perturbations against these channels

In order to investigate the physiological role and relevance of P/Q- and R-type VGCC in the regulation of colonic motility two animal models were used: TG mouse and α1E KO mouse. The TG mouse has a spontaneous missense mutation in the α1A (P/Q-type VGCC) subunit close to the pore forming region of the channel. The functional consequences of this mutation were studied in Purkinje cell of the cerebellum and the study reported a reduction in the whole-cell current density and voltage-dependent inactivation during a prolonged depolarization (Wakamori et al 1998). As a result, the function of P/Q-type VGCCs is compromised. The α1E KO has a genetic constitutive whole body deletion of the gene encoding for the α1E subunit. The latter forms the pore of the channel conducting R-type Ca^{2+} current. As a consequence, no α1E protein is expressed and thus no R-type Ca^{2+} current is expressed at least the one originated from the α1E subunit.

The consequences of these mutations in colonic propulsive motility were investigated. *In vivo* colonic propulsive motility was studied by monitoring fecal output and in general no major alterations were identified in animals with a loss-of-function in either P/Q- or R-type VGCCs. *In vitro* colonic motility was assessed by studying the colonic migrating motor complex (CMMC). The CMMC is a motor pattern consisting of a series of contractions occurring at the oral and anal segments of the colon in relation to the fecal content. Normally an oral contraction is followed by an anal contraction and this pattern of contractile activity is critical for normal transit of fecal material along the length of the colon (see chapter 1). No major alterations were found in the pattern of the CMMC in neither of the two animal models. Therefore, the outcomes of the *in vivo* and *in vitro* studies of colonic propulsive motility are

consistent: no major alteration in fecal output together with no alterations in the CMMC motor pattern. Thus, there are two main possibilities: P/Q- and R-type VGCCs are not critical for regulating colonic motility or in fact they are so important that the ENS has a "back-up plan" to offset the functional loss of these channels in order to maintain function.

To further investigate these possibilities neuromuscular transmission to the colon was studied by recording inhibitory junction potentials (IJP). The rationale supporting this approach is that inhibitory neuromuscular transmission finely regulates intestinal motility and disruptions in the components of this transmission have been reported for several GI motility disorders (Di Nardo et al. 2007; Burnstock, 2013). The contribution of several VGCC subtypes to generation of the IJP was investigated using peptide toxins specific for each channel subtype. The results showed that blocking P/Q-, R-, and N-type VGCCs produced a 41%, 36%, and 47% inhibition of the IJP in wildtype mice, respectively. Combination of the percentages overpass 100% inhibition, thus the Ca^{2+} currents provided by these channels have common targets meaning these currents regulate release of both neurotransmitters to different extents. The fact that pharmacological inhibition of either P/Q- or R-type VGCCs had a substantial reduction of the IJP suggested they play an important role during inhibitory transmission to the muscle. Yet, genetic alterations leading either to a loss-of-function or a total absence of these channels do not cause major disruptions in colonic propulsive motility. Caution has to be taken when compare the effects of pharmacological interventions with the effects of genetic mutations in certain proteins (Spencer, 2013).

Recordings of junction potentials normally are conducted under conditions in which L-type VGCCs are block in order to prevent movement of the smooth muscle. Dihydropyridines such as nifedipine are used to selectively block current through L-type channels. This approach does not affect neurotransmission because L-type channels do not contribute to the passive or active properties of enteric neurons or to neurotransmitter release (Kunze et al. 1994; Reis et al. 2002, 2000). However, L-type channels contribute to homeostatic synaptic plasticity (Frank, 2014) and compensatory changes

involving these channels have been reported for Tg mice (Etheredge et al 2007). Due to this, IJP recordings in both animal models were conducted in the absence and presence of nifedipine. In general the result showed that there is a component of the IJP that is sensitive to block of L-type VGCCs in both TG and α1E KO mice. In nifedipine-free conditions IJPs in TG and α1E KO mice were at the level or even higher than wildtype. In both animal models nifedipine block about 35% of the IJP while in wildtype mice the same treatment block less than 15% of the IJP. Taking together these results showed that genetic alterations leading to either loss-of-function in P/Q- or absence of R-type channels unmask an L-type contribution to the IJP that is not present in wildtype mice. The identified contribution of L-type channel to IJPs in TG and α1E KO mice (35%) is similar to the contribution of P/Q- (41%) and R-type VGCCs (34%) to IJPs in wildtype mice supporting that the involvement of L-type channels is compensatory and effective. It is clear now why TG and α1E KO mice showed no major alteration in colonic propulsive motility in face of having functional deficit in either P/Q- or R-type VGCCs. The L-type channels compensatory contribution works as a homeostatic synaptic mechanism to maintain physiologically appropriate colonic function.

The studies completed in this dissertation suggested that the L-type channel compensation "boost" more effectively the purinergic than the nitrergic component. This observation was more evident in α1E KO mice because the AUC of the IJPs, which is regulated by the nitrergic component, was always smaller as compared to wildtype mice. In addition, nifedipine treatment had very little effect of the AUC but significantly inhibited the amplitude of the IJP, which is mostly regulated by the purinergic component. The slight decrease in nitrergic component was further supported by the observation that circular muscle cells had a depolarization in the resting membrane potential of about 5 mV. Resting membrane potential in colonic cells is regulated by ongoing release of NO (Gil et al. 2010). In addition R-type VGCCs activate at more negative potentials than the rest of the HVA Ca^{2+} channels, at around -40 mV (Tottene et al. 2000). Taking together these results suggest that R-type VGCCs contribute to ongoing

release of NO to regulate colonic resting membrane potential. A loss of these channels, as it occurs in α1E KO mice, will be accompanied by an impairment in nitrergic transmission reflected as a reduction in the nitrergic slow IJP and a depolarized resting membrane potential of colonic circular muscle cells.

The rhythmic electrical activity of circular muscle cells was investigated in both animal models to determine any changes in the neuronal modulation of the electrical activity. Two main firing patterns were identified in all mice studied: bursting pattern consisting of slow depolarization with overlapping oscillatory activity that include action potentials and a continuous pattern consisting of action potentials fired in a non-burst fashion. In general, the characteristics of both firing patterns did not differ between the mutated mice and wildtype controls. The only difference was identified in TG mice, which consisted of a bursting pattern of slightly longer duration as compared to wildtype mice. A burst of longer duration can be associated with more excitability of the cells and a depolarized resting membrane potential (France et al. 2012). In TG mice the resting membrane potential was not altered, however, the muscle contractions either spontaneous or nerve-evoked were substantially larger than wildtype contractions. This could either be a consequence of the slight increase in muscle excitability or could be due to a potential contribution of L-type channels to excitatory neurotransmission. The latter possibility was not addressed by the present study.

As it was mentioned previously compensatory mechanisms has been reported for TG and α1E KO mice. In TG, synaptic transmission in the cerebellum becomes more dependent on N-type VGCCs to offset the loss of P/Q-type channels (Leenders et al. 2002; Matsushita et al. 2002). A similar finding was reported for hippocampal slices where pre-synaptic currents and thus neurotransmitter release becomes more dependent on N-type VGCCs in Tg mice (Quian and Noebels, 2000). In an animal model of the autoimmune condition Lambert-Eaton Myasthenic Syndrome (LEMS) there is an autoantibody-mediated decrease in Cav2.1 (P/Q-type) channels and a compensatory L-type contribution to acetylcholine was reported (Flink and Atchison, 2002). Pre-synaptic release of acetylcholine at the

skeletal neuromuscular junction in TG mice is more dependent on N- and R-type VGCCs (Pardo et al. 2006). The whole cell Ca^{2+} currents of basal forebrain neurons in TG mice were sustained despite a loss-of-function in P/Q-type channels. This was achieved by a compensatory increase in the component of the current mediated by L-type channels (Etheredge et al. 2007).

Evidence for the occurrence of compensatory mechanisms in α1E KO mice has been reported. Whole cell patch-clamp recordings in Purkinje neurons from α1E -/+ showed deficits in the generation of long-term depression (LTD), which is a critical event underlying motor learning. Interestingly, the generation of LTD in α1E KO mice was not different from wildtype mice suggesting that compensatory mechanisms must come into play. However, the authors of that study were unable to identify the nature of the compensatory mechanisms (Osanai et al. 2006).

Pharmacological challenges to synaptic activity can also result in profound homeostatic changes to components of the mammalian pre-synaptic machinery and this has been supported using hippocampal cultures from rodents. Pharmacological block of AMPA receptor induced two main homeostatic responses: incorporation of new GluA2-lacking AMPA receptors post-synaptically and a retrograde signaling process resulting in increased pre-synaptic release properties (Thiagarajan et al. 2005; Gong et al. 2007). Co-application of N- and P/Q-type channel blockers completely abolishes the enhanced pre-synaptic activity supporting a role for Cav2 function in mediating the observed homeostatic changes (Jakawich et al. 2010).

This body of literature demonstrates that homeostatic plasticity occurs in the nervous system in response to pharmacological or genetic challenges. In fact, the central nervous system possesses an immense capacity to be plastic enabling compensation for either genetics or environmental disturbances. With this in mind it is not surprisingly the observation that mice with a functional deficit in Ca^{2+} channels, known to be generally important for neurotransmitter release, are capable to adapt and successfully compensate the functional loss.

The "back-up plan" identified by the present studies is the contribution of L-type VGCCs to inhibitory neuromuscular transmission to the colonic muscle (figure 6.1). The question that arises now is: What are the mechanisms underlying participation of L-type channels during inhibitory neuromuscular transmission? The first step is to determine where is the L-type channel compensation occurring: pre-synaptically or post-junctionally. The studies conducted in this dissertation suggested that it is unlikely for the L-type channels to be up-regulated at the level of the muscle. This is due to the fact that the post-junctional response to exogenous application of excitatory and inhibitory agents did not differ between the mice, at least in Tg mice. Thus, any changes in the contribution of L-type channels must occur at the level of the nerve terminal. The present study was not able to address the specific mechanisms that could underlie contribution of L-type channels to neurotransmitter release. However, potential mechanisms that could allow active participation of L-type channels during inhibitory neuromuscular transmission are discussed below.

Potential mechanisms underlying involvement of L-type channels in homeostatic synaptic plasticity

Most of the studies providing evidence for a functional up-regulation of L-type VGCCs have not been successful at identifying the mechanism of action underlying this compensatory event. The studies that identified a compensatory contribution of L-type VGCCs to the whole cell current in basal forebrain neurons were unable to determine a mechanism of action, however, the authors were able to eliminate up-regulated transcription as a mean for the compensatory events (Etheredge et al. 2005). In a similar way, the mechanism underlying compensatory involvement of L-type calcium channels to transmitter release from adult mammalian motor nerve terminals in an animal model of LEMS is unknown (Flink and Atchison, 2002).

The observed compensatory L-type channel contribution to inhibitory neuromuscular transmission could reflect recruitment of existing L-type channels in the motor nerve fibers. The

question is, how does the functional unmasking of L-type channels occur? The α1C subunit of L-type VGCCs is expressed in the motor nerve fibers innervating the circular muscle cells in the intestine (Kirchgessner and Liu, 1999). In addition, α1C-immunireactivity is observed both in the nerve fibers within the myenteric plexus and within the tertiary plexus (Kirchgessner and Liu, 1999). Using electrophysiological and pharmacological tools it was demonstrated that about 20% of the whole cell current recorded from myenteric neurons is conducted by L-type channels (Bian et al. 2004). These studies support the molecular and functional presence of L-type channels in the myenteric plexus of the ENS.

Blocking of calcium-activated K^+ channels (K_{ca}) channels with iberiotoxin unmasked the development of L-type channels contribution to acetylcholine release from normal mammalian motor nerve terminals (Flink and Atchison, 2003b). This was an acute pharmacological block of K_{ca} and thus synthesis of new L-type channel protein is unlikely. Te authors suggested recruitment of existing L-type channels in the nerve terminal is a more feasible mechanism. K_{ca} channels are in close proximity to VGCCs localized at active zones for transmitter release (Robitaille and Charlton, 1993; Xu and Atchison, 1996). Thus, calcium influx through VGCCs can activate K_{ca} generating potassium outflow and hyperpolarization. This hyperpolarization will limit the extent and duration of action potential-induced depolarization at the nerve terminal. Loss of a source of extracellular Ca^{2+}, such a VGCCs, will reduce activation of K_{ca} and this could work to recruit L-type Ca^{2+} currents to the active zones. How is this recruitment exactly happening? Is there a physical trafficking of L-type channels to the active zones? That would be unlikely because the α1 subunit of L-type channels does not have the *synprint* site required to interact with proteins of the SNARE complex or other active zone proteins (Catterall and Leal, 2013). However, the mechanism underlying unmasking of L-type channels can use the kinetics of L-type currents. These channels have a large conductance that inactivates very slowly generating a large and long-lasting Ca^{2+} current (Catterall, 2011; Hille, 2001). Thus, it is possible that the nature of the L-

type current once activated is enough to reach the place where neurotransmitters are being release. A decrease in the capacity of the nerve terminal to buffer Ca^{2+} can also contribute to the ability of the L-type currents to reach the active zones or in general to maintain the amplitude of Ca^{2+} currents. This has been reported to occur in Purkinje cells of the cerebellum from TG mice and is thought to work as a compensatory mechanism to deal with the functional deficit in Cav2.1 (P/Q-type) since this channel subtype carry about 90% of the current in these cells (Dove et al. 2000; Murchison et al. 2002).

Part of the identified "back-up plan" in enteric neurons is also to increase the overall contribution of Cav2 channels such as R-type and N-type VGCCs, which normally contribute to neurotransmitter release. What could be the mechanism increasing contribution of these channels? A recent concept that is now used to describe the local regulation of VGCCs is known as the effector checkpoint model of Ca^{2+} channel regulation. This provides a functional checkpoint of the readiness of Ca^{2+} channels to carry out its physiological role and enhances its activity if it passes this checkpoint criterion (Caterall, 2012). One example of this regulatory process is the fact that interaction with an incomplete SNARE complex inhibits activity of Cav2 channels, however, successful formation of the complete SNARE complex disinhibit Cav2 channels and enhances their function (Bezprozvanny et al. 1995; Wiser et al. 1996; Zhong et al. 1999). Binding of Ca/CaM protein kinase II to Cav2.1 (P/Q-type) channels enhances their activity (Jiang et al. 2008). Another piece of evidence is the observation that binding of RIM, an active zone protein that regulates the SNARE complex, to β subunits increases Cav2 channel activity (Kiyonaka et al. 2007). These represent mechanisms that can be put into action to enhance the activity of existing and "ready to work" VGCCs at the nerve terminal.

In summary completion of the studies described in this dissertation provided important insight for the physiological role and significance of P/Q- and R-type VGCCs in enteric neuromuscular transmission. Both of these channels are involved in inhibitory transmission to the colon and thus they can regulate colonic motility. However, the ENS is prepared to deal with functional deficits to either of

these channels and I called this phenomenon: the enteric “back-up plan”. This homeostatic mechanism consisted in allowing L-type channels to participate in inhibitory transmission and induce an overall increase in the contribution of the unaffected Cav2 channels. These studies demonstrate the capacity of the ENS to be plastic and overcome challenges around it.

Are either P/Q- or R-type VGCCs novel targets for the treatment of GI motility disorders? Taking together these studies point that acute pharmacological block of these channels will modulate intestinal motility patterns. The system is redundant regarding the function of VGCCs; there is always a VGCC subtype that can take the lead and maintain function and this can be done by an unaltered Cav2 channel or by Cav1 channels. This is important because the current goal for treatment of GI motility disorders is to modulate instead of generally increasing or decreasing motility of the intestines. We still have a long way to go in order to rationally address targets for pharmacological intervention but we are in the right path.

APPENDIX

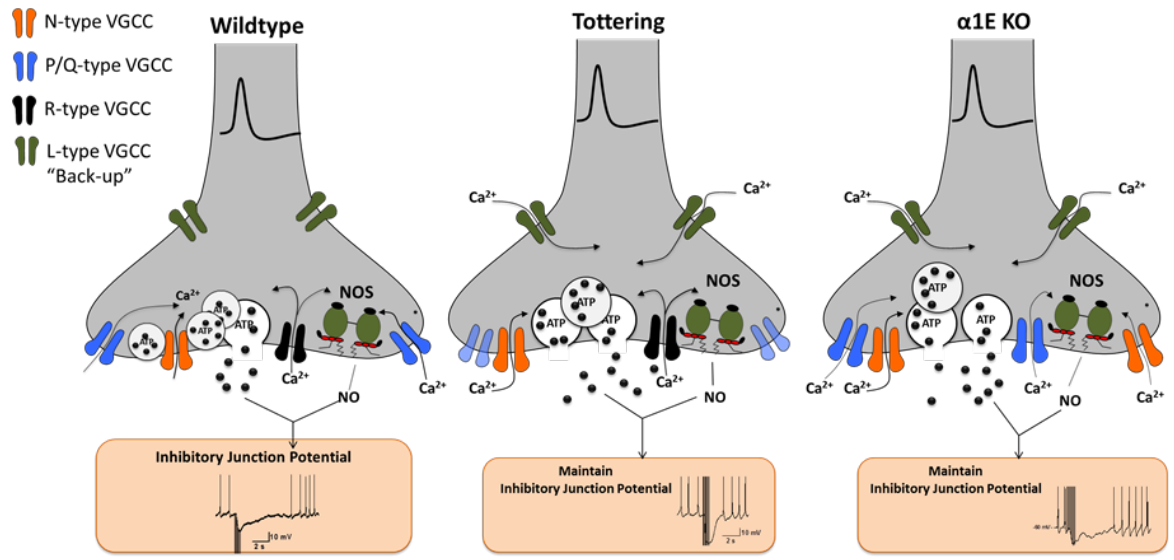

Figure 6.1: L-type Ca^{2+} channels as a "back-up plan" to sustain enteric inhibitory neuromuscular transmission. In WT mice release of neurotransmitters is mediated by P/Q-, R-, and N-type VGCCs. L-type channels are expressed by enteric neurons but they do not contribute to neurotransmitter release. The Tottering (TG) and α1E KO mice present either a functional deficit in P/Q-type channels (TG) or a knockout of the R-type channel (α1E KO mice). Thus, these two animal models represent a synaptic challenge because they do not have at least one VGCC subtype contributing to neurotransmitter release. Completion of the studies discussed in this dissertation demonstrated that the ENS is able to overcome these alterations in VGCCs and sustain physiologically appropriate colonic function. One of the mechanisms allowing the ENS to be successful at this is a functional up-regulation in the contribution of L-type channels to neurotransmitter release. Both animal models showed a component of the inhibitory neuromuscular transmission sensitive to block of L-type channels and this supports an active role of these channels. L-type channels have a large and long-lasting Ca2+ current that can potentially reach the site where neurotransmitters are being release without the need to be trafficked to active zones in the nerve. (Modified and adapted from Flink and Atchison, 2003).

REFERENCES

REFERENCES

Abrahamsson H. Treatment options for patients with severe gastroparesis. Gut 2007; 56: 877-883.

Accarino A, Colucci R, Barbara G, et al. Mast cell neuromuscular involvement in patientswith severe gastrointestinal motility disorders. Gut 2007; 39(Suppl. 1): A18.

Andrew JM, O'Donovan DG, Hebbard GS, Malbert CH, Doran SM, Dent J. Human duodenal phase IIImigrating motor complex activity is predominantly antegrade as revealed by high-resolution manometry and colour pressure plots. Neurogastroenterol Motil 2002; 14: 331-338.

Augustine GJ and Charlton MP. Calcium dependence of presynaptic calcium current and post-synaptic response at the squid giant synapse. J Physiol 1986; 381: 619-640.

Bajjalieh, S.M., and Scheller, R.H. The biochemistry of neurotransmitter secretion. J. Biol. Chem. 1995; 270: 1971–1974.

Bassotti G, Gaburri M. Manometric investigation of high-amplitude propagated contractile activity of the human colon. Am J Physiol 1988; 255: G660-G664.

Bassotti G, Villanacci V, Maurer CA, et al. The role of glial cells and apopto- sis of enteric neurones in the neuropa- thology of intractable slow transit constipation. Gut 2006; 55: 41–6.

Bayguinov PO, Hennig GW, Smith TK. Calcium activity in different classes of myenteric neurons underlying the migrating motor complex in the murine colon. J Physiol. 2010; 588: 399–421.

Bean BP. Two kinds of calcium channels in canine atrial cells: Difference in kinetics, selectivity, and pharmacology. J Gen Physiol 1985; 86:1-30.

Belai A, Burnstock G. Evidence for coexistence of ATP and nitric oxide in non-adrenergic, non-cholinergic (NANC) inhibitory neurones in rat ileum, colon and anococcygeus muscle. Cell Tissue Res 1994; 278: 197-200.

Belai A, Burnstock G. Pattern of distribution and co-localization of NOS and ATP in the myenteric plexus of human fetal stomach and intestine. Neurorepot 2000; 11:5-8.

Benko R, Undi S, Wolf M, Vereczkei A, Illenyi L, Kassai M, Cseke L, Kelemen D, Horvath OP, Antal A, Magyar K, Bartho L. P2 purinoceptor antagonists inhibit the non-adrenergic, non-cholinergic relaxation of the human colon in vitro. Neuroscience 2007; 147: 146-152.

Bennett MR. Transmission from intramural excitatory nerves to the smooth muscle cells of the guinea-pig taenia coli. J Physiol 1966; 185: 132-147.

Bennett, M. R., Burnstock, G. & Holman, M. Transmission from intramural inhibitory nerves to the smooth muscle of the guinea-pig taenia coli. J Physiol 1966; 182: 541–558.

Berridge, MJ. Neuronal calcium signaling. Neuron 1998; 21: 13–26.

Bertrand PP., Bertrand RL. Serotonin release and uptake in the gastrointestinal tract. Auton Neurosci 2010; 153: 47-57.

Bezprozvanny I, Scheller RH, Tsien RW. Functional impact of syntaxin on gating of N-type and Q-type calcium channels. Nature 1995; 378: 623–626.

Bharucha AE. High amplitude propagated contractions. Neurogastroenterol Motil 2012; 24: 977-982.

Bian X, Galligan JJ. Alpha2-adrenoceptors couple to inhibition of R-type calcium currents in myenteric neurons. Neurogastroenterol Motil 2007; 19: 845-855.

Bian X, Zhou X, Galligan JJ. R-type calcium channels in myenteric neurons of guinea pig small intestine. Am J Physiol Gastrointest Liver Physiol 2004; 287: G134-142.

Bisschops R, Vanden Berghe P, Sarnelli G, Janssens J, and Tack J. CRF-induced calcium signaling in guinea pig small intestine myenteric neurons involves CRF-1 receptors and activation of voltage-sensitive calcium channels. Am J Physiol 2006; 290: G1252-G1260.

Boeckxstaens GE, De Man JG, Pelckmans PA, Cromheeke KM, Herman AG, Van Maercke YM. Ca^{2+} dependency of the release of nitric oxide from non-adrenergic non-cholinergic nerves. Br J Pharmac 1993; 110: 1329-1334.

Boot JR. Differential effects of omega-conotoxin GVIA and MVIIC on nerve stimulation induced contractions of guinea-pig ileum and rat vas deferens. Eur J Pharmac 1994; 258: 155-158.

Borderies JR, Gonalons E, Angel F, Vergara P, Jimenez M. Effect of different calcium channel blockers on inhibitory junction potentials and slow waves in porcine ileum. Life Sci 1997; 60: 883-892.

Borderies JR, Jimenez m, Angel F. Non-adrenergic, non-cholinergic inhibitory junction potential in rat colonic circular muscle is partly sensitive to ω-conotoxins GVIA and resistant to L-, P-, or Q-type calcium channel blockers. Neurosci Letters 1996; 210: 91-94.

Bornstein JC, Hendriks R, Furness JB, Trussell DC. Ramifications of the axons of AH-neurons injected with the intracellular marker biocytin in the myenteric plexus of the guinea pig small intestine. J Comp Neurol 1991; 314: 437-451.

Borst, J. G., and Sakmann, B. Facilitation of presynaptic calcium currents in the rat brainstem. J Physiol 1998; 513, 149 –155.

Brookes SJ. Classes of enteric nerve cells in the guinea-pig small intestine. Anat Rec 2001; 262: 58-70.

Burgess DL and Noebels JL. Voltage-dependent calcium channel mutations in neurological diseases. Ann NY Acad Sci 1999; 868: 199-212.

Burnstock G. Purinergic cotransmission. Exp Physiol 2009; 94: 20-24. Burnstock G, Bennett M and Holman M. Inhibition of the smooth muscle of the taenia coli. Nature 1963; 200: 581-582.

Burnstock G, Lavin S. Interstitial cells of Cajal and purinergic signaling. Autonom Neurosci 2002; 97:68-72.

Burnstock G. The journey to establish purinergic signalling in the gut. Neurogastroenterol Motil 2008; 20 Suppl 1: 8-19.

Campbell DB and Hess EJ. L-type calcium channels contribute to the tottering mouse dystonic episodes. Mol Pharmacol 1999; 55, 23-31.

Carlson GM, Bedi BS, Code CF. Mechanism of propagation of intestinal interdigestive myoelectric complex. Am J Physiol 1972; 222: 1027-1030.

Caterall WA. Voltage-gated calcium channels. Cold Spring Harb Perspect Biol 2011; 3:a003947.

Catteral WA. Structure and function of neuronal Ca^{2+} channels and their role in neurotransmitter release. Cell Calcium 1998; 24: 307-323.

Catteral WA. Structure and function of voltge-gated ion channels. Annu Rev Cell Dev Biol 2000; 16: 521-555.

Catterall WA, Few AP. Calcium channels regulation and pre-synaptic plasticity. Neuron 2008; 59: 882-901.

Catterall WA, Leal KL. Calcium channels and short-term synaptic plasticity. J Biol Chem 2013; 288: 10742-10749.

Catterall WA. Voltage-gated calcium channels 2011. Cold Spring Harbor Perspect Biol 2011; 3: a003947.

Cayabyab FS, Jimenez M, Vergara P, de Bruni H, Daniel EE. Influence of nitric oxide and vasoactive intestinal peptide on the spontaneous and triggered electrical and mechanical activities of the canine ileum. Can J Physiol Pharmac 1997; 75: 383-397.

Chaudhury A, He XD, Goyal RK. Myosin VA plays a key role in nitrergic neurotransmission by transporting nnosalpha to enteric varicosity membrane. Am J Physiol GastroenterolLiver Physiol 2011; G498-G507.

Chaudhury A, He XD, Goyal RK. Role of PSD95 in membrane association and catalytic activity of nnosalpha in nitrergic varisosities in mice gut. Am J Physiol Gastrointest Liver Physiol 2009; 297: G806-813.

Chen RS. Calcium channel gamma-6 subunit: in search of its functions and mechanisms of actios. In Neuroscience , pp 7 2006. University of Illinois at Urbana-Champaing, Illinois.

Choi, D.W. Excitotoxic cell death. J Neurobiol 1992; 23: 1261–1276.

Choudhury BK, Shi XZ, Sarna SK. Norepinephrine mediates the transcriptional effects of heterotypic chronic stress on colonic motor function. Am J Physiol Gastrointest Liver Physiol 2009; 296: G1238-1247.

Chung-Hung S., Hsuin-Te H., Kuo-Hsien W., et al. Calcium channel subtypes for cholinergic and nonadrenergic noncholinergic neurotransmission in isolated guinea pig trachea. Naunyn-Schmied Arch Pharmacol 2010; 382: 419-432.

Clapham, DE. Calcium signaling. Cell 1995; 80: 259–268.

Coulie B, Camilleri M. Intestinal pseudo-obstruction. Annu Rev Med 1999; 50: 37–55.

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

Cunningham SM, Mihara S, and Higashi H. Presynaptic calcium channels mediating synaptic transmission in submucosal neurones of the guinea-pig caecum. J Physiol 1998; 509: 425–435.

Cuttle, MF Tsujimoto, T, Forsythe, ID, and Takahashi, T. Facilitation of the presynaptic calcium current at an auditory synapse in rat brainstem. J Physiol 1998; 512: 723–729.

D'Ascenzo M, Vairano M, Andreassi C, Navarra P, Azzena GB, Grassi C. Electrophysiological and molecular evidence of L-(Cav1), N-(Cav2.2), and R-(Cav2.3) type Ca^{2+} channels in rat cortical astrocytes. Glia 2004; 45: 354-363.

Darnell RB, Posner JB. Paraneoplastic syndromes affecting the nervous sys- tem. Semin Oncol 2006; 33: 270–98.

Davies A, Kadurin I, Alvarez-Laviada A, Douglas L, Nieto-Rostro M, Bauer CS, Pratt WS, Dolphin AC. The a2d subunits of voltage-gated calcium chan- nels form GPI-anchored proteins, a posttranslational modification essential for function. Proc Natl Acad Sci 2010; 107: 1654–1659.

Davies, A, Hendrich, J, Van Minh, AT, Wratten, J, Douglas, L, Dolphin, AC. Functional biology of the □2□ subunits of voltage- gated calcium channels. Trends Pharmacol Sci 2007; 28: 220 –228.

Daziel HH, Thornbury KD, Ward SM, and Sanders KM. Involvement of nitric oxide synthetic pathway in inhibitory junction potentials in canine proximal colon. Am J Physiol Gastrointest Liver Physiol 1991; 260: G789-G792.

De Giorgio R, Camilleri M. Human enteric neuropathies: morphology and molecular pathology. Neurogastroenterol Motil 2004; 16: 515–31.

De Giorgio R, Di Simone M, Stanghel- lini V, et al. Esophageal and gastric nitric oxide synthesizing innervation in primary achalasia. Am J Gastroenterol 1999.

De Giorgio R, Guerrini S, Barbara C, Cremon C, Stanghellini V, Corinaldesi R. New insights into human enteric neuropathies. Neurogastroenterol Motil 2004; 16: 143-147.

De Jongh KS, Warner C, Catterall WA. Subunits of purified calcium channels. a2 and d are encoded by the same gene. J Biol Chem 1990; 265: 14738–14741.

De Luca A, Li CG, Rand MJ, Reid JJ, Thaina P, Wong-Dusting HK. Effects of omega-conotixin GVIA on autonomic neuroeffector transmission in various tissues. Br J Pharmac 1990; 101: 437-447.

De Man JG, De Winter BY, Herman AG, Pelckmans PA. Study on the cyclic GMP-dependency of relaxations to endogenous and exogenous nitric oxide in the mouse gastrointestinal tract. Br J Pharmacol 2007; 150: 88-96.

De Man JG, De Winter BY, Seerden TC, De Schepper HU, Herman AG, Pelckmans PA. Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. Br J Pharmacol 2003; 140: 1108-1116.

De Maria, CD, Soong, TW, Alseikhan, BA, Alvania, RS, and Yue, DT. Calmodulin bifurcates the local calcium signal that modulates P/Q-type calcium channels. Nature 2001; 411: 484 – 489.

Devries MP, Vessalo M, Galligan JJ. Deletion of P2X2 and P2X3 receptor subunits does not alter motility of the mouse colon. Front Neurosci 2010; 4, 1–7.

Di Lorenzo C. Pseudo-obstruction: cur- rent approaches. Gastroenterology 1999; 116: 980–7.

Di Nardo G, Blandizzi C, Volta U, Colucci R, Stanghellini V, Barabaras G, Del Tacca M, Tonini M, Corinaldesi R, De Giorgios R. Review article: molecular, pathological and therapeutic features of human enteric neuropathies. Aliment Pharmacol Ther 2008; 28:25-42.

Dickson EJ, Heredia DJ, McCann CJ, Henning GW, and Smith TK. The mechanisms underlying the generation of the colonic migrating motor complex in both wild-type and nNOS knockout mice. Am J Physiol Gastrointest Liver Physiol 2009; 298: G222-G232.

Dickson EJ, Heredia DJ, Smith TK. Critical role of 5-TH_{1A}, 5-HT_3, 5-HT_7 receptor subtypes in the initiation, generation, and propagation of the murine colonic migrating motor complex. Am J Physiol Gastrointest Liver Physiol 2010; 299: G144-G157.

Dogiel AS. Uber den Bau der Ganglien in den Geflechten des Darmes und der Gallenblase des Menschen und der Saugetiere. Arch Anat Physiol Leipzig Anat Abt Jg 1899; 130-158.

Dogiel AS. Zur Frage uber die Ganglion der Darmgeflechte bei den Saugetieren. Anat Anat Anz 1895; 10: 517-528.

Drossman DA, Corazziari E, Talley NJ, Thompson WG, Whitehead WE. Rome II: The Functional Gastrointestinal Disorders. McLean, VA: Degnon Associates; 2000.

Drossman DA, Sandler RS, McKee DC, Lovitz AJ. Bowel patterns among patients not seeking health care. Gastro- enterology 1982; 82: 529–534.

Dunlap K, Luebke JI, Turner TJ. Exocytotic Ca^{2+} channels in mammalian central neurons. Trends Neurosci 1995; 18: 89-98.

Ehrlein HJ, Schemann M, Siegle ML. Motor patterns of small intestine determined by closely spaced extraluminal transducers and videofluoroscopy. Am J Physiol 1987; 253: G259-G267.

El Sharkawy TY, Markus H, Diamant NE. Neural control of the intestinal migrating myoelectric complex. A pharmacological analysis. Can J Physiol Pharmacol 1982; 60: 794-804.

Epperson A, Hatton WJ, Callaghan B et al. Molecular markers in cultured and freshly isolated interstitial cells of Cajal. Am J Physiol 2000; 279: C529-C539.

Ertel EA, Campbell KP, Harpold MM, Hofmann F, Mori Y, Perez-Reyes E Schwartz A, Snutch TP, Tanabe T, Birn- baumer L, et al. Nomenclature of voltage-gated calcium channels. Neuron 2000; 25: 533–535.

Etheredge JA, Murchison D, Abott LC, and Griffth WH. Functional compensation by other voltage-gated Ca^{2+} channels in mouse basal forebrain neurons with $Cav_{2.1}$ mutations. Brain Res 2005; 1140: 105-119.

Faussone-Pellegrini MS, Infantino A, Matini P, Masin A, Mayer B, Lise M. Neuronal anomalies and normal muscle morphology at the hypomotile ileoce- cocolonic region of patients affected by idiopathic chronic constipation. Histol Histopathol 1999; 14: 1119–34.

Fernandez E, Guo X, Vergara P, and Jimenez M. Evidence supporting a role for ATP as a non-adrenergic non-cholinergic inhibitory transmitter in the porcine ileum. Life Sci 1998; 62: 1303-1315.

Fletcher CF, Lutz CM, O'Sullivan TN, Shaughnessy Jr JD, Hawkes R, Frankel WN, Copeland NG, Jenkins NA. Absence epilepsy in tottering mutant mice is associated with calcium channel defects. Cell 1996; 87: 607-617.

Flink MT and Atchison WD. Passive transfer of Lambert-Eaton syndrome to mice induces dihydropyridine sensitivity of neuromuscular transmission. J Physiol 2002; 543: 567-576.

Flink MT, Atchison WD. Ca^{2+} channels as targets of neurological disease: Lambert-Eaton Syndrome and other Ca^{2+} channelopathies. J Bioenerg Biomembr 2003a; 35: 697-718.

Flink MT and Atchison WD. Iberiotoxin-induced block of Ca2+-activated K+ channels induces dihydropyridine sensitivity of Ach release from mammalian motor nerve terminals. J Pharmacol Exp Ther 2003b; 305: 646-656.

Ford AC, Talley NJ, Veldhuyzen van Zanten SJ, Vakil NB, Simel DL, Moayyedi P. Will the history and physical examination help establish that irritable bowel syndrome is causing this patient's lower gastrointestinal tract sympotoms? FAMA 300: 1793-1805.

Forsythe, ID, Tsujimoto, T, Barnes-Davies, M, Cuttle, MF, and Taka- hashi, T. Inactivation of presynaptic calcium current contributes to synaptic depression at a fast central synapse. Neuron 1998; 20: 797– 807.

Forti L, Tottene A, Moretti A, and Pietrobon. Three novel types of voltage-dependent calcium channels in rat cerebellar neuons. J Neurosci 1994; 14: 5243-5256.

Fox AP, Nowycky MC, Tsien RW. Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurons. J Physiol 1987; 394: 149-172.

France M, Bhattarai Y, Galligan JJ, Hui X. Impaired propulsive motility in the distal but not proximal colon of BK channel β1-subunit knockout mice. Neurogastroenterol Motil 2012; 24: e450-e459.

Frank AC. How voltage-gated calcium channels gate forms of homeostatic synaptic plasticity. Frontiers in Cellular Neuroscience 2014; 8: 1-14.

Frattine JC and Nogueras MD. Slow transit constipation: a review of a colonic functional disorder. Clin colon Rectal Surg 2008; 21: 146-152.

Furness JB, Alex G, Clark MJ, Lal VV. Morphologies and projections of defined classes of neurons in the submucosal of the guinea-pig small intestine. Anat Rec 2003; 272A: 475-483.

Furness JB, Jones C, Nurgali K, Clerc N. Intrinsic primary afferent neurons and nerve circuits within the intestine. Prog Neurobiol 2004; 72: 143-164.

Furness JB, Robbins HL, Xiao J, Stebbing MJ, Nurgali K. Projections and chemistry of Dogiel type II neurons in the mouse colon. Cell Tissue Res 2004; 317: 1-12.

Furness JB. The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol 2012; 9: 286-294.

Furness JB, Jone C, Nurgali et al. Intrinsic primary afferent neurons and nerve circuits within the intestine. Prog Neurobiol 2004; 72: 143-164.

Furness, JB. The Enteric Nervous System (Blackwell, Oxford, 2006).

Furness, JB. Types of of neurons in the enteric nervous. J Auton Nerv Syst 2000; 81: 87-96.

Gallego D, Gil V, Aleu J, Auli M, Clave P, and Jimenez M. Purinergic and nitrergic junction potential in the human colon. Am J Physiol Gastrointest Liver Physiol 2008; 295: G522-G533.

Gallego D, Gil V, Aleu J, Martinez-Cutillas M, Clave P, and Jimenez M. Pharmacological characterization of purinergic inhibitory neuromuscular transmission in the human colon. Neurogastroenterol Motil 2011; 23: 792-802.

Gallego D, Gil V, Martinez-Cutillas M, Mane M, Martin MT, Jimenez M. Purinergic neuromuscular transmission is absent in the colon of P2Y1 knocked out mice. J Physiol 2012: 590, 1943-1956.

Gallego D, Hernandez P, Clave P, Jimenez M. $P2Y_1$ receptors mediate inhibitory purinergic neuromuscular transmission in the human colon. Am J Physiol 2006; 291: G584-594.

Galligan JJ, Costa M, Furness JB. Gastrointestinal myoelectric activity in conscious guinea pigs. Am J Physiol 1985; 249: G92-G99.

Galligan JJ. Differential inhibition of cholinergic and noncholinergic neurogenic contractions by mu opioid and alpha-2 adrenergic agonists in guinea pig ileum. J Pharmacol Exp Ther 1993; 264: 375-383.

Gasparini S, Kasyanov AM, Pietrobon D, Voronin LL, Cherubini E. Presynaptic R-type calcium channels contribute to fast excitatory synaptic transmission in the rat hippocampus. J Neurosci 2001; 21: 8715-8721.

Gershon MD, Kirchgessner AL, Wade PR. Functional anatomy of the enteric nervous system. In: Johnson LR, eds. Physiology of the Gastrointetsinal tract: Raven Press: 1994; 381-422.

Gil V, Gallego D, Grasa L, Martin MT, Jimenez M. Purinergic and nitrergic neuromuscular transmission mediates spontaneous neuronal activity in the rat colon. Am J Physiol Gastroenterol Liver Physiol 2010; 299: G158-G169.

Ginty, D.D. Calcium regulation of gene expression: isn't that spatial. Neuron 1997; 18: 183–186.

Gong, B, Wang, H, Gu, S, Heximer, SP, Zhuo, M. Genetic evidence for the requirement of adenylyl cyclase 1 in synaptic scaling of forebrain cortical neurons. Eur. J. Neurosci. 2007; 26: 275–288.

Goold, CP, and Nicoll, RA. Single-cell optogenetic excitation drives homeostatic synaptic depression. Neuron 2010; 68: 512–528.

Goyal RK and Hirano I. The enteric nervous system. New Engl J Med 1996; 25: 1106–15.

Goyal, R. K., Rattan, S. & Said, S. I. VIP as a possible neurotransmitter of non-cholinergic non-adrenergic inhibitory neurones. Nature 1980; 288: 378–380.

Green MC and Sidman RL. Tottering - a neuromuscular mutation in the mouse. J Hered 1962; 53: 79-94.

Grider JR, Rivier JR. Vasoactive intestinal peptide (VIP) as transmitter of inhibitory motor neurons of the gut: evidence from the use of selective VIP antagonists and VIP antiserum. J Pharmacol Exp Ther 1990; 253: 738-42.

Grider JR. Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. J Pharmacol Exp Ther 2003; 307: 460-467.

Griffith WH, Taylor L, Davis MJ. Whole-cell and single-cell calcium currents in guinea pig basal forebrain neurons. J Neurophysiol 1994; 71: 2359-2376.

Gurnett CA, De Waard M, Campbell KP. Dual func- tion of the voltage-dependent Ca2þ channel a2d subunit in current stimulation and subunit interaction. Neuron 1996; 16: 431–440.

Hajela RK, Huntoon KM, Atchison WD. Lambert-Eaton syndrome antibodies target multiple subunits of voltage-gated Ca2+ channels. Muscle Nerve 2015; 51:176-84.

Hall KE and Wiley JW. Neural injury, repair and adaptation in the GI tract. I. New insights into neuronal injury: a cautionary tale. Am J Physiol 1998; 274: G978–83.

Han, Y, Kaeser, PS, Südhof, TC, and Schneggenburger, R. RIM determines Ca^{2+} channel density and vesicle docking at the presynaptic active zone. Neuron 2011; 69: 304 –316.

He XD, Goyal RK. Nitric oxide involvement in the peptide VIP-associated inhibitory junction potential in the guinea pig ileum. J Physiol 1993; 461: 485-499.

Heinemann SH, Terlau H, Stu¨hmer W, Imoto K, Numa S. Calcium channel characteristics conferred on the sodium channel by single mutations. Nature 1992; 356: 441 – 443.

Hell JW, Westenbroek RE, Warner C, Ahlijanian MK, Prystay W, Gilbert MM, Snutch TP, Caterall WA. Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel alpha 1 subunits. J Cell Biol 1993; 123: 949-962.

Hendriks R, Bornstein JC, Furness JB. An electrophysiological study of the projections of putative sensory neurons within the myenteric plexus of the guinea-pig ileum. Neurosci Lett 1990; 110: 286-290.

Hens J, Schrodl F, Brehmer A, et al. Mucosal projections of enteric neurons in the porcine small intestine. J Comp Neurol 2000; 421: 426-436.

Heredia DJ, Dickson EJ, Bayguinov PO, Hennig GW, Smith TK. Localized release of serotonin (5-hydroxytryptamine) by a fecal pellet regulates migrating motor complexes in murine colon. Gastroenterology. 2009; 136: 1328–1338.

Heredia DJ, Gershon MD, Koh SD, Corrigan RD, Okamoto T, Smith TK. Important role of mucosal serotonin in colonic propulsion and peristaltic reflexes: in vitro analyses in mice lacking tryptophan hydroxylase 1. J Physiol 2013; 591: 5939–5957.

Heredia DJ, Grainger N, McCann CJ, Smith TK. Insights from a novel model of slow-transit constipation generated by partial outlet obstruction in the murine large intestine. Am J Physiol Gastrointest Liver Physiol. 2012; 303: G1004–G1016.

Hille B. Voltage-gated calcium channels Sinauer Associates, Sunderland, MA. 2001.

Hillsley K, Kenyon JL, Smith TK. Ryanodine-sensitive stores regulate the excitability of AH neurons in the myenteric plexus of guinea-pig ileum. J Neurophysiol 2000; 84: 2777-2785.

Hirst GDS, Johnson SM, van Helden DF. The calcium currents in a myenteric neurone of the guinea-pig ileum. J Physiol (Lond) 1985; 361: 297-314.

Hofmann, F, Lacinová, L, and Klugbauer, N. Voltage-dependentcalcium channels: from structure to function. Rev. Physiol. Biochem Pharmacol 1999; 139: 33– 87.

Holderith, N., Lorincz, A., Katona, G., Rózsa, B., Kulik, A., Watanabe, M., and Nusser, Z. Release probability of hippocampal glutamatergic terminals scales with the size of the active zone. Nat. Neurosci. 2012; 15: 988 –997.

Hong SJ, Roam YF, Chang CC. Inhibition of neuromuscular transmission in the myenteric plexux of guinea pig ileum by ω-conotoxins GVIA, MVIIA, MVIIC, and SVIB. Br J Pharmac 1996; 118: 797-803.

Hoppa, MB, Lana, B, Margas, W. Dolphin, AC, and Ryan, TA. α2δ expression sets presynaptic calcium channel abundance and release probability. Nature 2012; 486: 122–125.

Horowitz M, Fraser RJ. Gastroparesis: diagnosis and management. Scand J Gastroenterol 1995; 213(Suppl.): 7–16.

Houzed H, Hattori Y, Kanno M, et al. Functional evaluation of inhibition of autonomic transmitter release by auto-antibody from Lambert-Eaton myasthenic syndrome. Ann Neurol 1998; 43: 677-680.

Hoyle CHV, Burnstock G. Neuromuscular transmission in the gastrointestinal tract. In: The Handbook of Physiology, Section 6: The Gastrointestinal System, Vol. 1, pp. 435-464. Ed. GM Macklouf. Washington DC: American Physiology Society.

Huizinga, J. D. et al. W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. Nature 1995; 373: 347–349.

Humphreys CMS, Costa M. The effect of omega-conotoxin on the transmission from enteric inhibitory motor neurons in the guinea pig distal colon. Proc Aust Neurosci Soc 1992; 3: 204.

Hwang SJ, Durnin L, Dwyer L, Rhee PL, Ward SM, Koh SD, Sanders KM and Mutafova-Yambolieva VN. β-Nicotinamide adenine dinucleotide is an enteric inhibitory neurotransmitter in human and nonhuman primate colons. Gastroenterology 2011; 140: 608-617.

Hwang, S. J. et al. P2Y1 Purinoreceptors are fundamental to inhibitory motor control of murine colonic excitability and transit. J Physiol 2012; 590: 1957–1972.

Iino S, Horiguchi K, Nojyo Y, Ward SM, Sanders KM. Interstitial cells of Cajal contain signalling molecules for transduction of nitrergic stimulation in guinea pig caecum. Neurogastroenterol Motil 2009; 21: 542-550, e12-13.

Ino M, Yoshinaga T, Wakamori M, Miyamoto N, Takahashi E, Sonoda J, et al. Functional disorders of the sympathetic nervous system in mice lacking the alpha 1B subunit (Cav 2.2) of N-type calcium channels. Proc Natl Acad Sci USA 2001; 98: 5323–8.

Ishikawa K, Tanaka H, Saito M, Ohkoshi N, Fujita T, Yoshizawa K, et al. Japanese families with autosomal dominant pure cerebellar ataxia map to chromosome 19p13.1-p13.2 and are strongly associated with mild CAG expansions in the spinocerebellar ataxia type 6 gene in chromosome 19p13.1. Am J Hum Genet 1997; 61.

Ivancheva C, Itzev D, Radomirov. Functional antagonism between nitric oxide and ATP in the motor responses of guinea-pig ileum. J Autonom Pharmacol 2000; 20: 147-156.

Jakawich, SK, Nasser, HB, Strong, MJ, McCartney, AJ, Perez, AS, Rakesh, N, et al. Local presynaptic activity gates homeostatic changes in presynaptic function driven by dendritic BDNF synthesis. Neuron 2010; 68: 1143– 1158.

Jay SD, Ellis SB, McCue AF, Williams ME, Vedvick TS, Harpold MM, Campbell KP. Primary structure of the g subunit of the DHP-sensitive calcium channel from skeletal muscle. Science 1990; 248: 490–492.

Jiang X, Lautermilch NJ, Watari H, Westenbroek RE, Scheuer T, Catterall WA. Modulation of Cav2.1 channels by Ca2þ/calmodulin-dependent protein kinase II bound to the C-terminal domain. Proc Natl Acad Sci 2008; 105: 341 – 346.

Jing X, Li DQ, Olofsson CS. Salehi A, Surve VV, Caballero J, et al. CaV2.3 calcium channels control second-phase insulin release. J Clin Invest 2005; 115: 146–54.

Johnson PJ, Bornstein JC, Yuan SY et al. Analysis of contributions of acetylcholine and tachykinins to neuro-neuronal transmission in motility reflexes in the guinea pig ileum. Br J Pharmacol 1996; 118: 973-983.

Jun K, Piedras-Rentera E, Smith SM, Wheeler DB, Lee SB, Lee TG, Chin H, Adams ME, Scheller RH, Tsien RW, and Shin HS. Ablation of the P/Q-type Ca^{2+} channel currents, altered synaptic transmission, and progressive ataxia in mice lacking the α1A subunit. Proc Natl Acad Sci USA 2000; 96: 15245-15250.

Dunlap K, Luebke JL, Turner, TJ. Exocytotic Ca^{2+} channels in mammalian central neurons. Trends Neurosci 1995; 18: 89–98.

Kaeser PS, Deng L, Wang Y, Dulubova I, Liu X, Rizo J, and Südhof TC. RIM proteins tether Ca2 channels to presynaptic active zones via a direct PDZ-domain interaction. Cell 2011; 144: 282–295.

Katz E, Ferro PA, Cherksey BD, et al. Effects of Ca^{2+} channel blockers on transmitter release and pre-synaptic currents at the frog neuromuscular junction. J Physiol 1995; 486: 695-706.

Keff KD, Du C, Ward SM, McGregor B and Sanders KM. Enteric inhibitory neural regulation of human colonic circular muscle: role of nitric oxide. Gastroenterology 1993; 105: 1009-1016.

Kim D.K, Catteral WA. Ca2+-dependent and -independent interactions of the isoforms of the α_{1A} subunit of brain Ca2+ channels with presynaptic SNARE proteins. Proc. Natl. Acad. Sci. USA 1997; 94: 14782–14786.

Kirchgessner AL, Liu MT. Differential localization of Ca^{2+} channel alpha1 subunits in the enteric nervous system: presence of alpha1B channel-like immunoreactivity in intrinsic primary afferent neurons. J Comp Neurol 1999; 409: 85-104.

Kiyonaka S, Wakamori M, Miki T, Uriu Y, Nonaka M, Bito H, Beedle AM, Mori E, Hara Y, De Waard M, et al. RIM1 confers sustained activity and neurotrans- mitter vesicle anchoring to presynaptic calcium channels. Nat Neurosci 2007; 10: 691–701.

Koch TR, Carney JA, Go L, Go VL. Idio-pathic chronic constipation is associated with decreased colonic vasoactive intestinal peptide. Gastroenterology 1988; 94: 300–10.

Koh SD, Dick GM and Sanders KM. Small-conductance Ca^{2+}-dependent K^+ channels activated by ATP in murine colonic smooth muscle. Am J Physiol Cell Physiol 1997; 273: C2010-C2021.

Kordasiewicz HB, Thompson RM, Clark HB, Gomez CM. C-termini of P/Q-type Ca^{2+}channel {alpha} 1A subunits translocate to nuclei and promote polyglutamine-mediated toxicity. Hum Mol Genet 2006; 15: 1587–99.

Kraus RL, Sinnegger MJ, Glossmann H, Hering S, Striessnig J. Familial hemiplegic migraine mutations change alpha(1A)Ca (2+) channel kinetics. J Biol Chem 1998; 273: 5586–90.

Kraus RL, Sinnegger MJ, Koschak A, Glossmann H, Stenirri S, Carrera P, et al. Three new familial hemiplegic migraine mutants affect P/Q-type Ca(2+) channel kinetics. J Biol Chem 2000; 275: 9239–43.

Kunze WA and Furness JB. The enteric nervous system and regulation of intestinal motility. Annu Rev Physiol 1999; 61: 117–142.

Kunze WA, Bornstein JC, Furness JB, Hendriks R, and Stephenson DS. Charybdotoxin and iberiotoxin but not apamin abolish the slow after-hyperpolarization in myenteric plexus neurons. Eur J Physiol 1994; 428: 300–306.

Kunze WAA, Clerc N, Bertrand PP., Furness JB. Contractile activity in intestinal muscle evokes action potential discharge in guinea-pig myenteric neurons. J Physiol (Lond) 1999; 517: 547–561.

Kunze WAA, Clerc, N, Furness JB., Gola M. The soma and neurites of primary afferent neurons in the guinea-pig intestine respond differentially to deformation. J Physiol (Lond) 2000; 526: 375–385.

Kunze WAA, Furness JB, Bertrand PP, Bornstein JC. Intracellular recording from myenteric neurons of the guinea-pig ileum that respond to stretch. J Physiol (Lond) 1998; 506: 827–842.

Kurahashi M. et al. A functional role for the 'fibroblast-like cells' in gastrointestinal smooth muscles. J. Physiol 2011; 589: 697–710.

Lacy BE, Weiser K, Kennedy A. Botulinum toxin and gastrointestinal tract disorders. Gastroenterol Hepatol 2008; 4: 283-295.

Lambert EH and Elmqvist D. Quantal components of endplate potentials in the myasthenic syndrome. Annals of the New York Academy of Sciences 1971; 183: 183-199.

Lambert EH and Lennon VA. Selected IgG rapidly induces Lambert-Eaton myasthenic syndrome in mice: complement independence and EMG abnormalities. Muscle and Nerve 1988; 11: 1133–1145.

Langley JN and Magnus R. Some observations of the movement of the intestines before and after degenerative section of the mesenteric nerves. J Physiol (Lond) 1905; 33: 34-51.

Langton P, Ward SM, Carl A, Norell MA and Sanders KM. Spontaneous electrical activity of interstitial cells of Cajal isolated from canine proximal colon. Proc. Natl Acad. Sci USA 1989; 86: 7280–7284.

Lautermilch NJ, Few AP, Scheuer T, and Catterall WA. Modulation of CaV2.1 channels by the neuronal calcium-binding protein visinin-like protein-2. J Neurosci 2005; 25: 7062–7070.

Leal K, Mochida S, Scheuer T, and Catterall WA. Fine-tuning synaptic plasticity by modulation of CaV2.1 channels with calcium sensor proteins. Proc Natl Acad Sci USA 2012; 109: 17069 –17074.

Lee HK, Sanders KM. Comparison of ionic currents from interstitial cells and smooth muscle cells of canine colon. J Physiol (Lond) 1993; 460: 135-152.

Lee A, Scheuer T, and Catterall WA. Ca^{2+}/calmodulin-dependent facilitation and inactivation of P/Q-type Ca^{2+} channels. J Neurosci 2000; 20: 6830-6838.

Lee A, Westenbroek RE, Haeseleer F, Palczewski K, Scheuer T, and Catterall WA. Differential modulation of CaV2.1 channels by calmodulin and calcium-binding protein 1. Nat. Neurosci.2002; 5: 210 –217.

Lee A, Wong ST, Gallagher D, Li B, Storm DR, Scheuer T, and Catterall WA. Calcium/calmodulin binds to and modulates P/Q-type calcium channels. Nature 1999; 399, 155–159.

Lee A, Zhou H, Scheuer T, and Catterall WA. Molecular determinants of calcium/calmodulin-dependent regulation of CaV 2.1 channels. Proc Natl Acad Sci USA 2003; 100: 16059 –16064.

Leenders AG, van den Maagdenberg AM, Lopes da Silva FH, Sheng ZH, Molenaar PC, Ghijsen WE. Neurotransmitter release from tottering mice nerve terminals with reduced expression of mutated P- and Q-type Ca^{2+}-channels. Eur J Neurosci 2002; 15: 13-18.

Lennon VA. Calcium channel and related paraneoplastic disease autoanti- bodies. In: Peter JB, Shoenfeld Y, eds. Textbook of Autoantibodies. Amster- dam: Elsevier Science 1996: 139–47.

LePard KJ and Galligan JJ. Analysis of fast synaptic pathways in myenteric plexus of guinea pig ileum. Am J Physiol 1999; 276: G529-538.

Li Q and Sarna SK. Chronic stress targets posttranslational mechanisms to paridly upregulate α1C-subunit of Cav1.2b calcium channels in colonic smooth cells. Am J Physiol Gastrointest Liver Physiol 2011; 300: G154-163.

Lin Z, Gao N, Hu HZ, et al. Immunore- activity of Hu proteins facilitates iden- tification of myenteric neurones in guinea-pig small intestine. Neurogastroenterol Motil 2002; 14: 197–204.

Lincoln J, Crowe R, Kamm MA, Burn- stock G, Lennard-Jones JE. Serotonin and 5-hydroxyindolacetic acid are increased in the sigmoid colon in severe idiopathic constipation. Gastro- enterology 1990; 98: 1219–25.

Lippi A, Santicioli P, Criscuoli M and Maggi CA. Depolarization evoked co-release of tachykinins from enteric nerves in the guinea-pig proximal colon. Naunyn-Schmiedeberg's Arch Pharmacol 1998; 357: 245-251.

Llewellyn Smith IJ, Costa M, Furness JB, Bornstein JC. Structure of the tertiary component of the myenteric plexus in the guinea-pig small intestine. Cell Tissue Res 1993; 272: 509-516.

Lu, T, and Trussell, LO. Inhibitory transmission mediated by asynchro- nous transmitter release. Neuron 2000; 26: 683–694.

Lunderberg JM. Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. Pharmacol Rev 1996; 48: 113-178.

Lundy PM, Frew R. Effect of ω-agatoxin IVA on autonomic neurotransmission. Eur J Pharmac 1994; 261: 79-84.

Lyster DJ, Bywater RA, Taylor GS. Neurogenic control of myoelectric complexes in mouse isolated colon. Gastroenterology. 1995; 108: 1371–1378.

Maeda H, Yamagata A, Nishikawa S, Yoshinaga K, Kobayashi S, Nishi K, Nishikawa S. Requirement of c-kit for development of intestinal pacemaker system. Development 1992; 116: 369-375.

Maggi CA, Patacchini R, Santicioli P, Lippe IT, Giuliani S, Geppetti P, Del Bianco E, Selleri S, Meli A. The effect of omega conotoxin GVIA, a peptide modulator of the N-type voltage sensitive calcium channels, on motor responses produced by activation of efferents and sensory nerves in mammalian smooth muscle. Naunyn Schmiedeberg's Arch Pharmac 1988; 338: 107-113.

Maggi CA, Holzer P, Giuliani S. Effect of ω-conotoxin on cholinergic and tachykinergic excitatory neurotransmission to the circular muscle of the guinea-pig colon. Naunyn Schmiedeberg's Arch Pharmac 1994; 350: 529-536.

Makhlouf GM, Murthy KS. Signal transduction in gastrointestinal smooth muscle. Cell Signal 1997; 9: 269-276.

Manto MU. The wide spectrum of spinocerebellar ataxias (SCAs). Cerebellum 2005; 4: 2–6.

Mashimo H, He XD, Huang PL, Fishman MC, Goyal RK. Neuronal constitutive nitric oxide synthase is involved in murine enteric inhibitory neurotransmission. J Clin Invest 1996; 98: 8-13.

Mashimo H, Kjellin A, Goyal RK. Gas- tric stasis in neuronal nitric oxide syn- thase-deficient knockout mice. Gastroenterology 2000; 119: 766–73.

Matsu K, Katsuragi T, Fujiki S, Sato C, Furukawa T. ATP release and contraction mediated by different P2-receptor subtypes in guinea-pig ileal smooth muscle. Br J Pharmacol 1997; 121: 1744-1748.

McConalogue K. et al. Histochemical, pharmacological, biochemical and chromatographic evidence that pituitary adenylyl cyclase activating peptide is involved in inhibitory neurotransmission in the taenia of the guinea-pig caecum. J Auton Nerv Syst 1995; 50: 311–322.

Mckeown L, Robinson P, Jones OT. Molecular basis of inherited calcium channelopathies: role of mutations in pore-forming subunits. Acta Pharmacologica Sinica 2006; 27: 799–812.

Milstein AD, and Nicoll RA. Regulation of AMPA receptor gating and pharmacology by TARP auxiliary subunits. Trends Pharmacol. Sci. 2008; 29: 333–339.

Mintz IM, Venema VJ, Swiderek KM, Lee TD, Bean BP, Adams ME. P-type calcium channels block by the spider toxin omega Aga-IVA. Nature 1992; 355: 827-829.

Mittal CK, Harrell WB, Mehta CS. Interaction of heavy metal toxicants with brain constitutive nitric oxide synthase. Mol Cell Biochem 1995; 149: 263-265.

Mochida S, Few AP, Scheuer T, and Catterall WA. Regulation of presynaptic CaV2.1 channels by Ca2 sensor proteins mediates short-term synaptic plasticity. Neuron 2008; 57: 210 –216.

Müller M, and Davis GW. Trans-synaptic control of presynaptic Ca(2)(+) influx achieves homeostatic potentiation of neurotransmitter release. Curr Biol 2012; 22: 1102–1108.

Müller M, Liu KS, Sigrist SJ, and Davis GW. RIM controls homeostatic plasticity through modulation of the readily-releasable vesicle pool. J Neurosci 2012; 32: 16574–16585.

Murthy KS. Signaling for contraction and relaxation in smooth muscle of the gut. Annu Rev Physiol 2006; 68: 345-374.

Mutafova-Yambolieva VN, Hwang SJ, Hao X, Chen H, Zhu MX, Wood JD, Ward SM and Sanders KM. β-Nicotinamide adenine dinucleotide is an inhibitory neurotransmitter in visceral smooth muscle. Proc Natl Acad Sci USA 2007; 104: 16359-16364.

Naidoo V, Dai X, Galligan J. R-type Ca^{2+} channels contribute to fast synaptic excitation and action potentials in subsets of myenteric neurons in the guinea pig intestine. Neurogastroenterol Motil 2010; 22: e353-e363.

Newsom-Davis J and Murray NM. Plasma exchange and immunosuppressive drug treatment in the Lambert-Eaton myasthenic syndrome. Neurology 1984; 34: 480-485.

Noebels JL and Sidman RL. Inherited epilepsy: spike-wave and focal motor seizures in the mutant mouse tottering. Science 1979; 204: 1334-1336.

Norton RS, and McDonough SI. Peptides targeting voltage-gated calcium channels. Curr Pharm Des 2008; 14: 2480–2491.

O'Neill JH, Murray NM, Newsom-Davis J. The Lambert-Eaton myasthenic syndrome: a review of 50 years. Brain 1998; 111: 577-586.

Okano HJ, Darnell RB. A hierarchy of Hu RNA binding proteins in developing and adult neurons. J Neurosci 1997; 17: 3024–37.

Olivera BM. Imperial JS, Cruz LJ, Bindokas VP, Venema VJ, Adams ME. Calcium channel-targeted polypeptide toxins. Ann NY Acad Sci 1991; 635: 114-122.

Olivera BM, Miljanich GP, Ramachandran J, and Adams ME. Calcium channel diversity and neurotransmitter release: the ω-conotoxins and ω-agatoxins. Annu Rev Biochem 1994; 63, 823– 867.

Ophoff RA, Terwindt GS, Vergouwe MN, van Eijk R, Oefner PJ, Hoffman SMG, Lamerdin JE, Mohrenweiser HW, Bulamn DE, Ferrari M, Haan J, Lindhout D, van Ommen G-JB, Hofker MH, Ferrari MD, Frants RR. Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations of the Ca^{2+} channel gene CACNL1A4. Cell 1996; 87: 543-552.

Osanai MO, Saegusa H, Kazuno A, et al. Altered Cerebellar function in mice lacking Cav2.3 Ca2+ channel. Biochem Biophys Res Com 2006; 344: 920-925.

Osthaus LE, Galligan JJ. Antagonists of nitric oxide synthesis inhibit nerve-mediated relaxations of longitudinal muscle in guinea pig ileum. J Pharmacol Exp Ther 1992; 260: 140-145.

Pacaud P, Feolde E, Frelin C, Loirand G. Characterization of the P2Y-purinoceptor involved in the ATP-induced rise in cytosolic Ca^{2+} concentration in rat ileal myocytes. Br J Pharmacol 1996; 118: 2213-2219.

Pardo NE, Hajela RK, Atchison WD. Acetylcholine release at neuromuscular junctions of adult tottering mice is controlled by N-(Cav2.2) and R-type (CAV2.3) but not L-type (Cav1.2) Ca^{2+} channels. J of Pharmacol and Exp Therap 2006; 319: 1009-1020.

Patel BA, Dai X, Burda JE, Zhao H, Swain GM, Galligan JJ, and Bian X. Inhibitory neuromuscular transmission to ileal longitudinal muscle predominates in neonatal guinea pigs. Neurogastroeneteol Motil 2010; 22: 909-918.

Patrick A, Epstein O. Review article: gastroparesis. Aliment Pharmacol Ther 2008; 27: 724–40.

Pérez-Otaño, I, and Ehlers, MD. Homeostatic plasticity and NMDA receptor trafficking. Trends Neurosci 2005; 28: 229–238.

Perez-Reyes E. Molecular characterization of a novel family of low voltage activated, T-type, calcium channels. J Bioenerg Biomembr 1998; 30:313-318.

Perez-Reyez E. Molecular physiology of low voltage activated T-type calcium channels. Physiol Rev 2003; 83: 117-161.

Petersen SA, Fetter RD, Noordermeer JN, Goodman CS, and DiAntonio A. Genetic analysis of glutamate receptors in Drosophila reveals a retrograde signal regulating presynaptic transmitter release. Neuron 1997; 19, 1237– 1248.

Pietrobon D. Calcium channels and channelopathies of the central nervous system. Mol Neurobiol 2002; 25, 31-50.

Plomp JJ, Vergouwne MN, Van Den MaagdenbergAM, Ferrari MD, Frants RR, and Molenaar PC. Abnormal transmitter release at neuromuscular junctions of mice carrying the tottering α_{1A} Ca^{2+} channel mutation. Brain 2000; 123: 463-471.

Pluja L, Fernandez E, Jimenez M. Neuroral modulation of the cyclic electrical mechanical activity in the rat colonic circular muscle: putative role of ATP and NO. Br J Pharmacol 1999; 126: 883-892.

Pompolo S, Furness JB. Ultrastructure and synaptic relationship of calbindin-reactive, Dogiel type II neurons, in myenteric ganglia of guinea pig small intestine. J Neurocytol 1988; 17: 771-782.

Portbury AL, Furness JB, Young HM, Southwell BR, Vigna SR. Localisation of NK1 receptor immunoreactivity and interstitial cells of the guinea-pig gastrointestinal tract. J Comp Neurol 1996; 367: 342-351.

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

Porter AJ, Wattchow DA, Hunter A, Costa M. Abnormalities of nerve fibers in the circular muscle of patients with slow transit constipation. Int J Colorec- tal Dis 1998; 13: 208–216.

Quian J and Noebels JL. Pre-synaptic Ca^{2+} influx at a mpuse central synapse with Ca^{2+} channel subunit mutations. J Neurosci 2000; 20: 163-170.

Randall A, and Tsien RW. Pharmacological dissection of multiple types of ca^{2+} channel currents in rat cerebellar granule neurons. J Neurosci 1995; 15: 2995-3012.

Rao MY, Chaudhury A, and Goyal RK. Active and inactive pools of nNOS in the nerve terminals in mouse gut: implications for nitrergic neurontransmission. Am J Physiol Gastrointest Liver Physiol 2008; 294, G627-G634.

Reis HJ, Biscaro FV, Gomez MV, and Romano-Silva MA. Depolarization-evoked GABA release from myenteric plexus is partially coupled to L-, N-, and P/Q-type calcium channels. Cell Mol Neurobiol 2002; 22: 805–811.

Reis HJ, Massensini AR, Prado MA, Gomez RS, Gomez MV, and Romano-Silva MA. Calcium channels coupled to depolarization-evoked glutamate release in the myenteric plexus of guinea-pig ileum. Neuroscience 2000; 101, 237–242.

Rettig J, Sheng ZH, Kim DK, Hodson CD, Snutch TP, Catterall WA. Isoform-specific interaction of the α_{1A} subunits of brain Ca2+ channels with the presynaptic proteins syntaxin and SNAP-25. Proc Natl Acad Sci USA 1996; 93: 7363–7368.

Rekik M, Delvaux M, Tack I, Frexinos J, Bueno L. VIP-induced relaxation of guinea-pig intestinal smooth muscle cells: sequential involvement of cyclic AMP and nitric oxide. Br J Pharmacol 1996; 118: 477-484.

Robitaille R, Garcia ML, Kaczorowski GJ, Charlton MP. Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. Neuron 1993; 11: 645–655.

Rozsai B, Lazar Z, benko R, Batho L. Inhibition of the NANC relaxation of the guinea-pig proximal colon longitudinal muscle by the purinoceptors antagonist PPADS, inhibition of nitric oxide synthase, but not by a PACAP/VIP antagonist. Pharmacol Res 2001; 43: 83-87.

Rugiero F, Gola M, Kunze WAA, Reynaud JC, Furness JB, Clerc N. Analysis of whole cell current by pathc clamp of guinea-pig myenteric neurones in intact ganglia. J Phyiol (Lond) 2002; 538: 447-463.

Rugiero F, Mistry M, Sage D, et al. Selective expression of a persistent TTX-resistant Na^{+} current and Nav 1.9 subunit in myenteric sensory neurons. J Neurosci 2003; 23: 2715-2725.

Sabatini, BL, and Regehr, WG. Timing of neurotransmission at fast synapses in the mammalian brain. Nature 1996; 384: 170–172.

Saegusa H, Matsuda Y, Tanabe T. Effects of ablation of N- and R-type Ca(2+) channels on pain transmission. Neurosci Res 2002; 43: 1–7.

Sander KM, Koh SD, Ro S, and Ward SM. Regulation of gastrointestinal motility-insights from smooth muscle biology. Nat Rev Gastroenterol Hepathol 2012; 9: 633-645.

Sanders KM. A case for interstitial cells of cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. Gastroenterol 1996; 111: 492-515.

Sanders KM. Regulation of smooth muscle excitation and contraction. Neurogastroenterol Motil 2008; 20 Suppl 1:39-53.

Sanders KM and Wards SM. Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. Am J Physiol Gastrointest Liver Physiol 1992; 262, G379-G392.

Sanders KM. Beta-nicotinamide adenine dinucleotide is an inhibitory neurotransmitter in visceral smooth muscle. Proc Natl Acad Sci USA 2007; 104: 16359-16364.

Sather WA, Tanabe T, Zhang JF, Mori Y, Adams ME, Tsien RW. Distinct biophysical and pharmacological properties of class A (BI) calcium channel alpha1 subunits. Neurons 1993; 11: 291-303.

Sawada K, Echigo N, Juge N, Miyaji T, Otsuka M, Omote H, Yamamoto A, Moriyama Y. Identification of a vesicular nucleotide transporter. Proc Natl Acad Sci USA 2008; 105: 5683-5686.

Schild L. The epithelial sodium channel and the control of sodium balance. Biochim Biophys Acta 2010; 1802: 1159–1165. doi: 10.1016/j.bbadis.2010.06.014

Schneggenburger R, Sakaba T, and Neher E. Vesicle pools and short-term synaptic depression: lessons from a large synapse. Trends Neurosci. 2002; 25: 206 –212.

Schuffler MD, Baird HW, Fleming CR, et al. Intestinal pseudo-obstruction as the presenting manifestation of small-cell carcinoma of the lung. A paraneo- plastic neuropathy of the gastrointesti- nal tract. Ann Intern Med 1983; 98: 129–34.

Sheng J, He L, Zheng H, Xue L, Luo F, Shin W, Sun T, Kuner T, Yue DT, and Wu LG. Calcium-channel number critically influ- ences synaptic strength and plasticity at the active zone. Nat. Neurosci. 2012; 15: 998 –1006.

Sheng ZH, Rettig J, Cook T, and Catterall WA. Calcium-dependent interaction of N-type calcium channels with the synaptic core-complex. Nature 1996; 379: 451–454.

Sheng ZH, Rettig J, Takahashi M, and Catterall WA. Identification of a syntaxin-binding site on N-type calcium channels. Neuron 1994; 13: 1303–1313.

Sheng ZH, Yokoyama C, Catterall WA. Interaction of the synprint site of N-type Ca2+ channels with the C2B domain of synaptotagmin I. Proc Natl Acad Sci USA, 1997; 94: 5405–5410.

Shuttleworth CWR and Keef KD. Roles of peptides in enteric neuromuscular transmission. Regul Pept 1995; 56: 101-120.

Shuttleworth CWR, Xue C, Ward SM, De Vente J, Sanders KM. Immunohistochemical localization of of 3'-5' cyclic guanosine monophosphatase in the canine proximal colon: responses to nitric oxide and electrical stimulation of enteric inhibitory neurons. Neuroscience 1993; 56: 513-522.

Shuttleworth CWR, Conlon SB and Sanders KM. Regulation of citrulline recycling in nitric oxide-dependent neurotransmissionin the murine proximal colon. Br J Pharmacol 1997; 20: 707-713.

Sjolund K, Fasth S, Ekman R, et al. Neuropeptides in idiopathic chronic constipation (slow transit constipation). Neurogastroenterol Motil 1997; 9: 143– 50.

Smith AB and Cunnane TC. Omega-conotoxin GVIA-resistant neurotransmitter release in postganglionic sympathetic nerve terminalks. Neuroscience 1996; 70, 817-824.

Smith AB and Cunnane TC. Multiple calcium channels control neurotransmitter release from rat postganglionic nerve terminals. J Physiol 1997; 499: 341-349.

Smith B. Myenteric plexus in Hirschprung's disease. Gut 1967; 8: 308–12.

Smith TK, Furness JB. Reflex changes in circular muscle activity elicited by stroking the mucosa: an electrophysiological analysis in the isolated guinea-pig ileum. J Auton Nerv Syst 1988; 25: 205-218.

Smith TK, Park KJ, Hennig GW. Colonic migrating motor complexes, high amplitude propagating contractions, neuronal and mucosal serotonin. J Neurogastroenterol Motil 2014; 20, 423-446.

Smith TK, Reed JB, Sanders KM. Origin and propagation of electrical slow waves in circular muscle of the canine proximal colon. Am J Physiol 1987; 252: C215-C224.

Smits GJ, Lefebvre RA. ATP and nitric oxide: inhibitory NANC neurotransmitters in the longitudinal muscle-myenteric plexus preparation of the rat ileum. Br J Pharmacol 1996; 118: 695-703.

Snutch TP, Leonard JP, Gilbert MM, Lester HA, Davidson N. Rat brain expresses a heterogeneous family of calcium channels. Proc Natl Acad Sci USA 1990; 87: 3391-3395.

Snutch TP, Reiner PB. Calcium channels: Diversity of form and function. Curr Opin Neurobiol 1992; 2: 247–253.

Snutch TP, Tomlinson WJ, Leonard JP, Gilbert MM. Distinct calcium channels are generated by alternative splicing and are differentially expressed in the mammalian CNS. Neuron 1991; 7: 45-57.

Song ZM, Brookes SJH, Costa M. Identification of myenteric neurons which project to the mucosa of the guine pig small intestine. Neurosci Lett 1991; 129: 294-298.

Spencer NJ and Smith TK. Simultaneous intracellular recordings from longitudinal and circular muscle during the peristaltic reflex in guinea-pig distal colon. J Physiol 2001; 533: 787-799.

Spencer NJ, Kyloh M, Wattchow DA, et al. Characterization of motor patterns in isolated human colon: are there differences in patients with slow-transit constipation? Am J Physiol Gastrointest Liver Physiol 2012; 302: G34–G43.

Stanghellini V, Cogliandro RF, De Giorgio R, Barbara G, Salvioli B, Corinaldesi R. Chronic intestinal pseudo- obstruction: manifestations, natural history and management. Neurogastro-enterol Motil 2007; 19: 440–52.

Südhof, TC. The presynaptic active zone. Neuron 2012; 75: 11–25.

Sudhof, TC. The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature 1995; 375: 645–653.

Sudhof, TC. The synaptic vesicle cycle. Annu Rev Neurosci 2004; 27, 509–547.

Summers RW. Approach to the point patient with ileus and obstruction. In: Yamada T (ed.) Gastroenterology, vol 1 pp 829-843. Lippincott Williams & Wilkins, Philadelphia, PA.

Sutton I, Winer JB. The immunopathogenesis of paraneoplastic neurological syndromes. Clin Sci (Lond) 2002; 102: 475–86.

Szurszewski JH. A migrating electric complex of the canine small intestine. Am J Physiol 1969; 217: 1757-1763.

Takahashi M, Seagar MJ, Jones JF, Reber BF, Catterall WA. Subunit structure of dihydropyridine-sensitive calcium channels from skeletal muscle. Proc Natl Acad Sci 1987; 84: 5478–5482.

Takahashi T, Tsunoda Y, Lu Y, Wiley J, Owyang C. Nicotinic receptor-evoked release of acetylcholine and somatostatin in the myenteric plexus is coupled to calcium influx via N-type calcium channels. J Pharmacol Exp Ther 1992; 263: 1-5.

Talavera K and Nilius B. Biophysics and structure-function relationship of T-type Ca^{2+} channels. Cell Calcium 2006; 40: 97-114.

Thatte HS, He XD, Goyal RK. Imaging of nitric oxide in nitrergic neuromuscular neurotransmission in the gut. PLoS One 2009; 4: e4990.

Thiagarajan TC, Lindskog M, and Tsien RW. Adaptation to synaptic inactivity in hippocampal neurons. Neuron 2005; 47: 725–737.

Thibault O, Hadley R, and Landfield PW. Elevated postsynaptic $[Ca^{2+}]_I$ and L-type calcium channel activity in aged hippocampal neurons: relationship to impared synaptic plasticity. J Neurosci 2001; 21: 9744-9756.

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

Timmermans JP, Hens J, Adriaensen D. Other submucocous plexus: an intrinsinc nerve network involved in both secretory and motility processes in the intestine of large mammasl and humans. Anat Rec 2001; 262: 71-78.

Tomita R, Fujisaki S, Ikeda T, Fukuzawa M. Role of nitric oxide in the colon of patients with slow-transit constipation. Dis Colon Rectum 2002; 45: 593–600.

Torihashi S, Ward SM, Nishikawa SI, Nishi K, Kobayashi S, Sanders KM. c-kit-Dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. Cell Tissue Res 1995; 280: 97-111.

Torihashi S. et al. c-kit-dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. Cell Tissue Res 1995; 280, 97–111.

Tottene A, Moretti A, Pietrobon D. Functional diversity of P-type and R-type calcium channels in rat cerebellar neurons. J Neurosci 1996; 16: 6353-6363.

Tottene A, Volse S, and Pietrobon D. α1E subunits form the pore of three cerebellar R-type calcium channels with different pharmacological and permeation properties. J Neurosci 2000; 20: 171-178.

Trettel F, Mantuano E, Calabresi V, Veneziano L, Olsen AS, Georgescu A, et al. A fine physical map of the CACNA1A gene region on 19p13.1-p13.2 chromosome. Gene 2000; 241: 45–50.

Tsien RW, Ellinor PT, and Horne WA. Molecular diversity of voltage-dependent Ca^{2+} channels. Trends Pharmacol Sci 1991; 12: 349-354.

Tsurudome K, Tsang K, Liao EH, Ball R, Penney J, Yang JS, et al. The Drosophila miR-310 cluster negatively regulates synaptic strength at the neuromuscular junction. Neuron 2010; 68: 879–893.

Tzavella K, Riepl RL, Klauser AG, Voderholzer WA, Schindlbeck NE, Muller-Lissner SA. Decreased substance P levels in rectal biopsies from patients with slow transit constipation. Eur J Gastroenterol Hepatol 1996; 8: 1207– 11.

Van den Maagdenberg AMJM, Pietrobon D, Pizzorusso T, Kaja S, Broos LAM, Cesetti T, et al. A Cacnala knockin migraine mouse model with increased susceptibility to cortical spreading depression. Neuron 2004; 41: 701–10.

Vanden Berghe P, Kenyon JL, Smith TK. Mitochondrial Ca^{2+} uptake regulates the excitability of myenteric neurons. J Neurosci 2002; 22: 6962-6971.

Vanderwinden JM, Mailleux P, Schiff- mann SN, Vanderhaegen J-J, De Laet M-H. Nitric oxide synthase activity in infantile hypertrophic pyloric stenosis. New Engl J Med 1992; 327: 511–5.

Vassort G, Alvarez J. Cardiac T-type calcium current: pharmacology and roles in cardiac tissues. J Cardiovasc Electrophysiol 1994; 5: 376-393.

Vassort G, Talavera K, Alvarez JL. Role of T-type Ca^{2+} channels in the heart. Cell Calcium 2006; 40: 205-220.

Vogalis F, Harvey JR, Lohman RJ, Furness JB. Action potential afterdepolarization mediated by a Ca^{2+}-activated cation conductance in myenteric AH-neurons. Neurosci 2002a; 115: 375-393.

Vogalis F, Harvey JR, Lohman RJ, Furness JB. TEA- and apamin-resistant KCa channels in guinea-pig myenteric neurons: slow AHP channels. J Physiol (Lond) 2002b; 538: 421-433.

Vogalis F, Hillsley K, Smith T. Recording ionic events from cultured, DiI-labelled myenteric neurons in the guinea pig proximal colon. J Neurosci Met 2000; 96: 25-34.

Wadel K, Neher E, and Sakaba, T. The coupling between synaptic vesicles and Ca2 channels determines fast neurotransmitter release. Neuron 2007; 53, 563–575.

Wakamatsu Y, Weston JA. Sequential expression and role of Hu RNA-binding proteins during neurogenesis. Development 1997; 124: 3449–60.

Wakamori M, Yamazaki K, Matsunodaira h, Teramoto T, Tanaka I, Niidome T, Sawada K, Nishizawa Y, Sekiguchi N, Mori E, Mori Y, Imoto K. Single tottering mutations responsible for the neuropathic phenotype of the P-type calcium channel. J Biol Chem 1998; 273: 34857-34867.

Wang G, Dayanithi G, Newcomb R, Lemos JR. An R-type Ca^{2+} current in neurohypophysial terminals preferentially regulates oxytocin secretion. J Neurosci 1999; 19: 9235-9241.

Wang GD, Wang XY, Hu HZ, Liu S, Gao N, Fang X, Xia Y, Wood JD. Inhibitory neuromuscular transmission mediated by the $P2Y_1$ purinergic receptor in guinea pig small intestine. Am J Physiol 2007; 292: G1483-1489.

Ward SM, Sanders KM. Interstitial cells of Cajal: primary targets of enteric motor innervation. Anat Rec 2001; 262: 125-135.

Ward SM, Burns AJ, Torihashi S and Sanders KM. Mutation of the proto-oncogene c-kit blocks development of interstitial cells and electrical rhythmicity in murine intestine. J Physiol 1994; 480: 91–97.

Waterman SA. Voltage-gated calcium channels in autonomic neuroeffector transmission. Pro Neurobiol 2000; 60: 181-210.

Waterman SA. Autonomic dysfunction in Lambert-Eaton Myasthenic Syndrome. Clinical Autonomic Research 2001; 11: 145-154.

Waterman SA and Costa M. The role of enteric inhibitory motorneurons in peristalsis in the isolated guinea-pig small intestine. J Physiol 1994; 477: 459-468.

Waterman SA, Costa M, and Tonini M. Enteric inhibitory reflexes mediate accommodation in the isolated guinea-pig small intestine. J Physiol 1994; 474: 539-546.

Watson MJ, Mosse P, Taylor GS, Bywater RAR. Effect of ω-conotoxin GVIA (CTX) on excitatory and inhibitory junction potentials recorded from the circular muscle cells of the mouse distal colon. Proc Aust Physiol Pharmac Soc 1991; 22: 172.

Wedel T, Roblick UJ, Ott V, et al. Oli- goneuronal hypoganglionosis in patients with idiopathic slow-transit constipation. Dis Colon Rectum 2002; 45: 54–62.

Weyhersmüller, A, Hallermann, S, Wagner, N, and Eilers, J. Rapid active zone remodeling during synaptic plasticity. J. Neurosci 2011; 31: 6041–6052.

Wheeler DB, Randa A, Sather WA, Tsien RW. Neuronal calcium channels encoded by by the alpha1A subunit and their contribution to excitatory synaptic transmission in the CNS. Prog Brain Res 1995; 105: 65-78.

Williams ME, Feldman DH, McCue AF, Brenner R, Velicelebi G, Ellis SB, Harpold MM. Structure and functional expression of alpha 1, alpha 2, and beta subunits of a novel human neuronal calcium channel subtype. Neuron 1992;8: 71-84.

Wilson SM1, Toth PT, Oh SB, Gillard SE, Volsen S, Ren D, Philipson LH, Lee EC, Fletcher CF, Tessarollo L, Copeland NG, Jenkins NA, Miller RJ. The status of voltage-dependent calcium channels in alpha 1E knock-out mice. J Neurosci 2001; 20: 8566-8571.

Wiser O, Bennett MK, Atlas D. Functional interaction of syntaxin and SNAP-25 with voltage-sensitive L- and N-type Ca2þ channels. EMBO J 1996; 15: 4100–4110.

Wood JD. Enteric neuropathobiology. In: Phillips SF, Wingate DL (eds), Functional disorders of the gut. Churchill Livingstone: London 1998: pp. 19–42.

Wood JD. Electrical and synaptic behavior of enteric neurons. In: Schultz SG, Wood JD, Ranner BB (eds) Handbook of Physiology: The Nervous System pp465-516. American Physiological Society, Maryland.

Wood JD. Neuropathophysiology of functional gastrointestinal disorders. World J Gastroenterol 2007; 13: 1313– 32.

Wu LG, Borst JG, Sakmann B. R-type Ca^{2+} currents evoke transmitter release at a rat central synapse. Proc Natl Acad Sci USA 1998; 95: 4720-4725.

Wu, LG, and Borst, J G. The reduced release probability of re-leasable vesicles during recovery from short-term synaptic depression. Neuron 1999; 23: 821– 832.

Xu YF, Atchison WD. Effect of ω-agatoxin IVA and ω-conotoxin MVIIC on perineurial Ca++ and Ca++-activated K+ currents of mouse motor nerve terminals Journal of Pharmacology and Experimental Therapeutics 1996; 279: 1229–123.

Xu YF, Hewett S, Atchison WD. Passive transfer of Lambert-Eaton myasthenic syndrome induces dihydropyridine sensitivity of I_{Ca} in mouse motor nerve terminals. J Neurophysiol 1998; 80: 1056-1069.

Xu, J, and Wu, LG. The decrease in the presynaptic calcium current is a major cause of short-term depression at a calyx-type synapse. Neuron 2005; 46: 633– 645.

Xue L, Farrugia G, Sarr MG, and Szurszewski JH. ATP is a mediator of the fast inhibitory junction potential in human jejunal circular smooth muscle. Am J Physiol Gastrointest Liver Physiol 1999; 276, G1373-G1378.

Younger, MA, Müller, M, Tong, A, Pym, EC, and Davis, GWA. Presynaptic ENaC channel drives homeostatic plasticity. Neuron 2013; 79: 1183–1196.

Zagorodnyuk V and Maggi CA. Electrophysiological evidence for different release mechanism of ATP and NO as inhibitory NANC transmitters in guinea pig colon. Br J Pharmac 1994; 112: 1077-1082.

Zarate N, Mearin F, Wang XY, Hewlett B, Huizinga JD, Malagelada JR. Severe idiopathic gastroparesis due to neuro- nal and interstitial cells of Cajal degen- eration: pathological findings and management. Gut 2003; 52: 966–70.

Zarate N, Spencer NJ. Chronic constipation: lessons from animal studies. Best Pract Res Clin Gastroenterol 2011; 25: 59–71.

Zhang JF, Randall AD, Ellinor PT, Horne WA, Sather WA, Tanabe T, Schwarz TL and Tsien RW. Distinctive pharmacology and kinetics of cloned neuronal Ca^{2+} channels and their possible counterparts in mammalian CNS neurons. Neuropharmacology 1993; 32: 1075-1088.

Zhang Y, Lomax AE, Paterson WG. $P2Y_1$ receptors mediate apamin-sensitive and -insensitive inhibitory junction potentials in murine colonic circular smooth muscle. J Pharmacol Exp Ther 2010; 333: 602-611.

Zholos AV, Baidan LV, Starodub AM, Wood JD. Potassium channels of myenteric neurons in guinea pig small intestine. Neurosci 1999; 89: 603-618.

Zhong H, Yokoyama C, Scheuer T, Catterall WA. Reciprocal regulation of P/Q-type calcium channels by SNAP-25, syntaxin and synaptotagmin. Nat Neurosci 1999; 2: 939 – 941.

Zhu, M. H. et al. A Ca^{2+}-activated Cl^- conductance in interstitial cells of Cajal linked to slow wave currents and pacemaker activity. J. Physiol. 2009; 587: 4905–4918.

Zhuchenko O, Bailey J, Bonnen P, Ashizawa T, Stockton DW, Amos C, Dobyns WB, Subramony SH, Zoghbi HY, Lee CC. Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the α1A-voltage-dependent calcium channel. Nat Genet 1997; 15: 62-69.

Zizzo MG, Mule F, Serio R. Activation of P2Y receptor by ATP and by its analogue, ADPBS, triggers two calcium signal pathways in the longitudinal muscle of mouse distal colon. Eur J Pharmacol 2008; 595: 84-89.

Zizzo MG, Mule F, Serio R. Evidence that ATP or a related purine is an excitatory neurotransmitter in the longitudinal muscle of mouse distal colon. Br J Pharmacol 2007; 151: 152-160.

Zizzo MG, Mule F, Serio R. Mechanisms underlying hyperpolarization evoked by P2Y recptor activation in mouse distal colon. Eur J Pharmacol 2006; 21: 1