MECHANISMS UNDERLYING RHYTHMIC ACTIVITIES OF THE GASTROINTESTINAL TRACT

MECHANISMS UNDERLYING RHYTHMIC ACTIVITIES OF THE GASTROINTESTINAL TRACT

BY,

MARC J PISTILLI HBSc

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2012) McMaster University
(Medical Sciences) Hamilton, Ontario

TITLE: Mechanisms Underlying Rhythmic Activities in the Gastrointestinal Tract

AUTHOR: Marc J Pistilli, HBSc (McMaster University)

SUPERVISOR: Professor Jan D Huizinga PhD

NUMBER OF PAGES: xii, 98

"On no subject in physiology do we meet with so many discrepancies of fact and opinion as in that of the physiology of the intestinal movements."

- Bayliss and Starling (1899)

ABSTRACT

The organs of the gastrointestinal (GI) tract display a variety of motor patterns, involved in grinding, mixing, enhancing absorption and propulsion of nutrients and waste products. Specialized motor patterns are generated by unique mechanisms inherent to the GI segment in which they are found. Rhythmic contractions are a feature of most motor patterns. Slow wave driven peristalsis is an acknowledged motor pattern associated with interstitial cells of Cajal (ICC) pacemakers, but propulsive motor patterns which are blocked by tetrodotoxin are seen to be exclusively generated by the enteric nervous system (ENS). This has not been proven, however, and the origin of rhythmicity of propulsive motor patterns needs further study, particularly related to a potential role of the pacemaker ICC found throughout the GI tract. The aim of this study was exploring the mechanisms which underlie various GI motor patterns, with particular focus on the origin of rhythmicity of these patterns.

I have demonstrated with manometry and spatiotemporal maps that murine rhythmic propulsion requires a myogenic pacemaker which is evoked by acetylcholine and substance P; nitric oxide is not involved. Calcium imaging evidence suggests that the pacemaker is the ICC of the deep muscular plexus, as these cells rhythmically activate to substance P. I observed rhythmic contractility patterns in human antrum, pylorus and duodenum when stimulated with carbachol. The hypothesis emerged that the ENS modifies the pyloric pacemaker into unique rhythmic patterns. Colonic muscle strip contractility from the rat has a low frequency rhythmic pattern which is myogenic. This

pattern is augmented by the conditioned media from the probiotic *E. coli Nissle 1917* through a non-neural mechanism.

The current explanation of entirely ENS generated motor patterns is not accurate. The ENS plays an important role in stimulating and regulating GI motor patterns in conjunction with myogenic pacemakers. It is only through acknowledgment of all GI cell types that we can understand the mechanisms governing motility.

AKNOWLEDGEMENTS

Funding for this project was provided by an operating grant for Dr. Jan Huizinga from CIHR. I would also like to acknowledge the Ontario Graduate Scholarship and the Queen Elizabeth II Graduate Scholarship in Science and Technology I received over my two years.

I have made significant progress both professionally as a student of science but also personally as I move forward in life. Dr. Jan Huizinga has been of utmost importance on this journey and for that I am grateful. His dedication to science has left an everlasting impression on me and helped reinforce my interests in *normal* physiology. I also want to thank Jan for the countless opportunities to participate in research, discussions, publications and presentations. These opportunities are invaluable to my future employment and success, and have also made graduate school meaningful and enjoyable.

The guidance that my supervisory committee provided was excellent. Both Dr. Luke Janssen and Dr. Denis Crankshaw helped make this project a realistic and worthwhile endeavor. I specifically want to thank Dr. Janssen for challenging me over these two years, as I have learned confidence in myself and the scientific ideas I may hold. I also want to specifically thank Dr. Crankshaw for fostering the early ideas of research within me and teaching me techniques that I still use in my research. Without his guidance and supervision I might never have entered the field of scientific research nor pursue graduate school.

I want to thank all the members of the Huizinga lab for making it an enjoyable place to work. I really enjoyed coming in everyday to see you all. I would specifically

like to thank Bobbi-Jo Lowie, Sarah-Lynn Martz and Dr. Sean Parsons for teaching me the techniques that I used throughout my research and in the preparation of this thesis. I would also like to thank Amir Khoshdel and George Wright for technical assistance in performing the motility study and the editing of this document. I want to thank all the remaining members for the countless discussions we have had and helping me develop a better understanding of gastrointestinal physiology.

Finally, I would like to thank members of my family, particularly my wife Joanna and parents for supporting me in countless ways throughout these last two years. Your patience and understanding have allowed me to focus on my education, and succeed during this time.

LIST OF FIGURES

LIST OF ABBREVIATIONS

7TM – seven-transmembrane receptors; G-protein coupled receptors

ACE – angiotensin-converting enzyme
AM – (acetoxymethyl ester)
ANOVA – analysis of variance
AUC – area under the curve

cpm - contractions per minute

ICC – interstitial cells of Cajal
ICC-DMP – deep muscular plexus interstitial cells of Cajal
ICC-MP – myenteric plexus interstitial cells of Cajal
IP3 - inositol 1,4,5-trisphosphate

L-NNA - N_{ω}- Nitro-L-Arginine

mAChR – muscarinic acetylcholine receptors
M_n – muscarinic acetylcholine receptor "n" subtype

nAChR - nicotinic acetylcholine receptors
NEP - neutral endopeptidase
Nissle - *Escherichia coli* Nissle 1917
NKA – neurokinin A
NKB – neurokinin B
NK_n – tachykinin receptor "n" subtype
NO – nitric acid

PAO – phenylarsine oxide
PMC – propulsive motor complex
PMP – propulsive motor pattern

ROI – region of interest

SEM – standard error of the mean
SWAC - Slow wave associated contractions

TTX – tetrodotoxin

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Anatomy of the small intestine

The small intestine consists of various layers including mucosa, submucosa, and circular and longitudinal smooth muscle. These layers work in conjunction with one another to facilitate digestion, and act as a barrier to the outside (lumen) of the body (Stockinger et al., 2011). Located between the longitudinal and circular muscle layers is the myenteric plexus (Fig 1.1) (Komuro, 2006). The plexus consists primarily of enteric nervous system cell bodies, including sensory neurons (intrinsic primary afferent neurons) excitatory and inhibitory motor neurons (S-neurons) as well as interneurons (Costa et al., 2000). Located alongside the neurons is a network of myenteric plexus interstitial cells of Cajal (ICC-MP) (Komuro, 2006). A second network of interstitial cells of Cajal (ICC) is found between the submucosa and the circular muscle layer (Fig 1.1); these cells are termed the deep muscular plexus interstitial cells of Cajal (ICC-DMP) (Komuro, 2006). Most motor patterns of the small intestine do not rely on central innervation (Schemann, 2005), thus it is a combination of smooth muscle layers, enteric neurons and ICC which contribute to this aspect of intestinal function.

1.2 Motor patterns of the small intestine

The musculature of the small intestine facilitates mixing and propulsion of luminal contents and also plays a role in absorption of nutrients (Gwynne and Bornstein, 2007). These physiological necessities are either fully or partially governed by the various

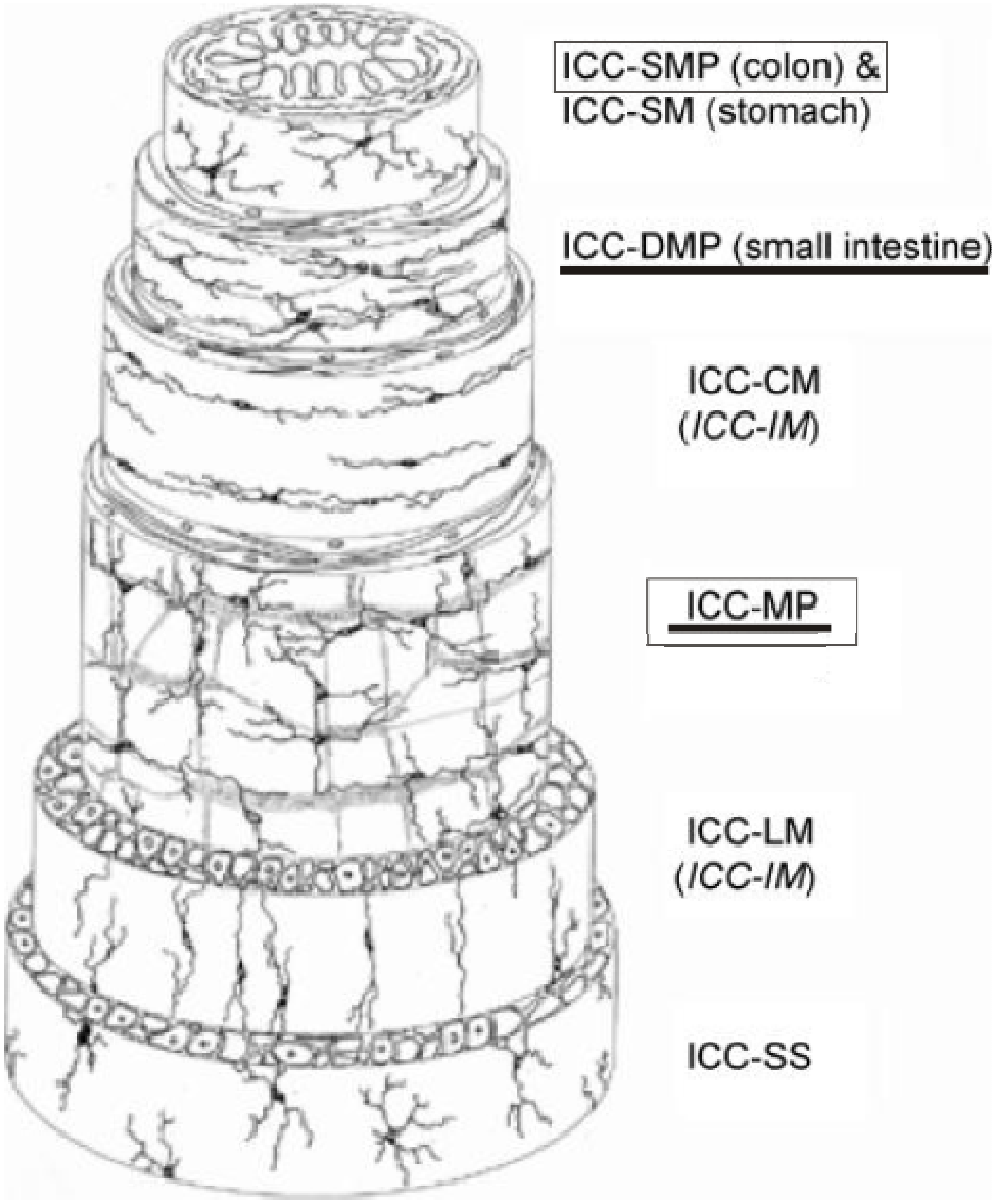

Figure 1.1. Location of gastrointestinal interstitial cells of Cajal. The lumen of the gut (top of figure) is directly contacted by the mucosa sitting atop the submucosa. Below are the deep muscular plexus and then the circular smooth muscle layers. The myenteric plexus lies between the circular muscle and the longitudinal smooth muscle layers, which is all capped off by the serosa. ICC which are underlined are found in the mouse small intestine; those boxed are found in the rat colon. Figure adapted from Komuro (2006).

patterns of contraction seen in the small intestine, whereby different patterns yield the required physiological response. Motility patterns fall into two main categories, peristalsis and segmentation, both of which are patterns of circular muscle contractions but have overlapping physiological functions (Huizinga and Lammers, 2009; Gwynne and Bornstein, 2007).

1.2.1 Peristalsis

Peristalsis is a general term used to define propagating waves. In the gastrointestinal tract peristalsis is defined as a propagating wave of contraction, where the wave exhibits either complete or fractional constriction of the lumen (Huizinga and Lammers, 2009). Peristaltic waves are able to move luminal content along short or long distances depending on the underlying pattern of contraction. Since peristalsis has a general definition, any motility pattern consisting of waves of contraction is classified as such, and thus peristalsis can be broken down into a variety of different patterns. This generality has led to much confusion, as the term is used as a catch-all for different "peristaltic" motor patterns with different functions and underlying mechanisms (Spencer et al., 2001; Huizinga and Lammers, 2009).

A common peristaltic pattern in the small intestine is that of a series both caudad peristaltic and retroperistaltic contractions. These waves of contractions can travel along the length of the small intestine but are insufficient in propelling content over long distances on their own (Wang et al., 2005). These contractile waves are associated with the slow waves (Hennig et al., 2010; Huizinga et al., 2009; Sanders and Ward, 2006) generated from the interstitial cells of Cajal of the myenteric plexus (MP) (Der-Silaphet et

al., 1998). This underlying mechanism has been confirmed using a variety of techniques, not least of which was the utilization of an ICC-MP knockout model where the slow waves were not present (Huizinga et al., 1995). Since the aforementioned peristaltic pattern results in very little net content movement caudad, it is important to make a distinction between it and peristaltic motility patterns which are primarily propulsive.

Bayliss and Starling (1899) first described the propulsive motor complex as a bolus preceded by (caudal to) an area of relaxation and followed by (oral to) an area of contraction. This observation was termed the "law of the intestine" (Spencer et al., 1999). Research in the field of the enteric nervous system has led to the development of an underlying mechanism to explain this observed propulsive contraction. Immunohistochemical evidence shows orally projecting excitatory motor neurons and caudal projecting inhibitory motor neurons (Fig. 1.2) (Costa et al., 2000). Initiation of propulsion is by a bolus of content which activates the nearby sensory neurons which in turn activate the above mentioned neural networks, leading to relaxation caudal and contraction oral to the bolus (Costa et al., 2000), as the law of the intestine suggests (Spencer et al., 1999). This mechanism is known as the peristaltic reflex. Assessment of the peristaltic reflex has shown that although there is neural involvement in a "reflex" to a stretch of the small intestinal wall, the peristaltic reflex is not supported by *in vitro* evidence (Spencer et al., 1999). Specifically, a caudad relaxation was not seen in response to luminal stretch as the peristaltic reflex suggests (Spencer et al., 1999; White et al., 1934). Also, apamin, which blocks small conductance calcium-activated potassium

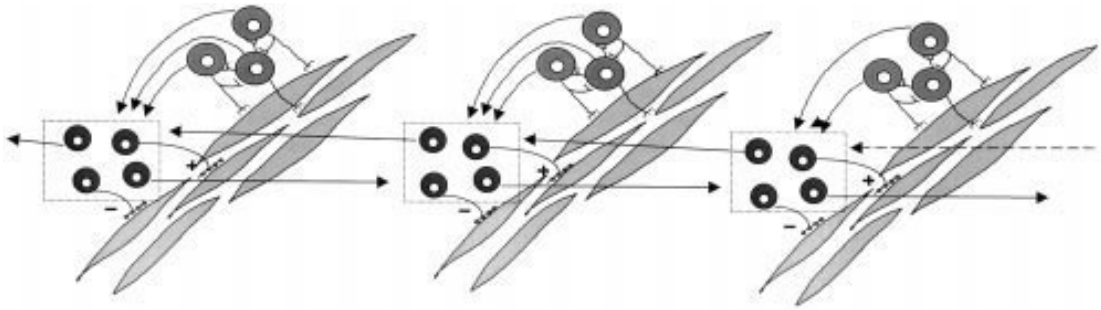

Figure 1.2. Model of the peristaltic reflex. Grey circles represent sensory neurons relaying the stretch stimulus from the smooth muscle (elongated structures). Black circles represent inhibitory and excitatory motor neurons as well as interneurons. Inhibitory neurons project caudad and excitatory neurons project oral. These pathways are repeated sequentially along the length of the small intestine. Figure adapted from Costa et al. (2000).

channels responsible for inhibitory junction potentials in neurons and smooth muscle relaxation, had little effect on the propulsive contractions of the small intestine (Spencer et al., 1999). Thirdly, earlier observations of small intestinal motor patterns observed contractions which propagated away from the stimulus in both directions in response to a stretch stimulus (Alvarez and Starkweather 1919), and not the caudal directionality as suggested by the law of the intestine. Although it is clear there is a role for neurons in propulsion, the current mechanisms have the glaring omission of being unable to account for the inherent rhythmicity of the propulsive motor complexes seen in the small intestine. A new mechanism has been proposed to explain propulsion in the colon whereby a mixture of both neural inputs and ICC rhythmicity are responsible for propulsion (Huizinga et al., 2011). This model suggests neurons account for the inducibility of propulsive patterns and the ICC govern the rhythmicity of the motor pattern (Huizinga et al., 2011). It is possible that a similar mechanism may be responsible for propulsion in the small intestine; however the roles of ICC and content propulsion in this segment of the gastrointestinal system have yet to be properly assessed.

The effects of muscle tone on patterns of propulsion have been partially studied. Propulsion in the small intestine was originally thought to be an “all-or-none” process (Kosterlitz and Lees, 1963), whereby a bolus of content eventually stretched the lumen to the point where a propulsive contraction occurred (Spencer et al., 2001). Spencer et al., (2001) showed that propulsive contractions were graded with respect to small intestinal tone, whereby increases in stretch led to increased force of contraction. The same study also demonstrated that propulsive contractions propagated caudad even when segments of

the small intestine were paralyzed pharmacologically with the calcium channel blocker nifedipine. Conversely, initiation of a propulsive motor complex was prevented by paralyzing the initial stretch stimulated area. Thus, the current underlying mechanism of propulsion requires physical distention for initiation but contraction propagation is an inherent property of the small intestine and does not require further luminal stimulus (Spencer et al., 2001). However, that study only assessed instantaneous stretch events and ignored a more physiologically relevant model where luminal distention occurs over large periods of time, not just acutely. In experiments where a constant distention is applied to the small intestine over longer periods of time, a rhythmic pattern of propulsion results with consistent frequency of contractions (Trendelenberg, 1917). The current neural understanding of propulsion fails to account for the rhythmic nature that propulsive peristalsis exhibits when the small intestine is distended, leaving much still not understood about the underlying mechanism of propulsion.

1.2.2 Segmentation

Segmentation is a motility pattern limited to the small intestine, whereas peristalsis is found throughout the gastrointestinal tract (Wood, 1999). Segmentation is characterized by circular muscle contractions that are not continuous but regularly spaced by areas of relaxation. The resulting contractions push the content in the lumen in both directions resulting in a series of uniform segments (Fig. 1.3) (Cannon, 1902). This pattern of contractions releases and then is reformed with different circular muscle sections contracting (Fig. 1.3 (**3**)). Reformation of regular spaced groups of contracted small intestine (Fig. 1.3 (**4**)), hence the original name “rhythmic segmentation”.

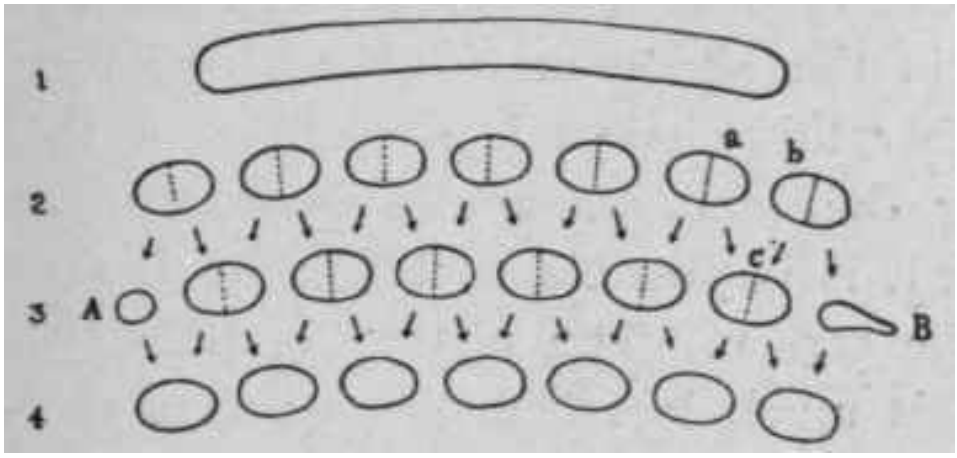

Figure 1.3. Segmentation of the small intestine. (**1**) A segment of small intestine containing chyme. Regularly spaced contractions separate the chyme into smaller segments that are separated by uniform distances (**2**). The small intestine relaxes and then segments the chyme again, but areas of contraction are shifted (**3**). This pattern continues for many cycles with little net movement of content (**4**). Figure adapted from Cannon (1902).

Segmentation functions to mix intraluminal content and enhance epithelial contact with the content to promote absorption (Gwynne and Bornstein, 2007). Segmentation has been seen most consistently using *in vivo* techniques such as radiological imaging (Cannon, 1902; Wang et al., 2005).

Initiation of the segmentation pattern is not completely understood but has been described to be highly dependent on intraluminal content (Cannon, 1902). Content may be involved in the initiation of segmentation either by the intraluminal distention caused by the content or by direct sensation of particular components of the content, the former having not been assessed *in vitro*. It has been shown that the presence of fatty acids (Gwynne et al., 2004A) or amino acids (Gwynne et al., 2004B) in the lumen of the small intestine may be responsible (or partially responsible) for the segmentation pattern. Gwynne and Bornstein (2007) have attempted to develop an *in vitro* model of segmentation, employing decanoic acid (a saturated fatty acid) as the initiator of the pattern. Mechanistically, it was found that inhibitory junction potentials were very important in the segmentation pattern, since their blockade by apamin resulted in the segmentation pattern returning to a peristaltic pattern (Gwynne and Bornstein, 2007). Although there is a neural component to segmentation, a role for slow waves or ICC has not been ruled out. In fact, the proposed enteric nervous system mechanism of segmentation fails to account for the rhythmicity that is strongly associated with the pattern. Furthermore, the *in vitro* model does not fully resemble the *in vivo* observations, as segmentation was only shown to occur in very short segments with only two or three segmental contractions occurring at any given moment.

The inherent rhythmicity is an important part of segmentation since the contractions follow very stable patterns (Cannon, 1902; Henderson 1909). For example it has been shown that in rabbit small intestine, the number of rhythmic contractions per minute slows in a linear fashion the further from the pylorus (Alvarez, 1914). The frequency decay was confirmed in feline (Hightower Jr, 1959), murine (Hightower Jr, 1959) and canine (Puestow, 1931; Castleton, 1934) studies where it was shown that the decay in frequency ranged between 40-60% in the distal small intestine compared to the respective duodenum (Hightower Jr, 1959). This pattern is also seen in the small intestine with the decay in slow wave frequency the more distal from the pylorus (Lammers and Stephen, 2008). A second similarity between the rhythm of segmentation and slow waves is the different frequencies seen across species. Comparing slow wave frequencies and segmentation patterns (Table 1.1), it can be seen that not only the trend in frequencies across species is the same but that absolute values within species are quite close (Puestow, 1940). Although only circumstantial, it may be that the slow wave and segmentation frequencies are linked physiologically by a currently unknown mechanism as originally suggested by Puestow in 1940.

1.3 Interstitial cell of Cajal

Interstitial cells of Cajal have been described to form fully functioning and independent networks within the small intestine, and indeed examples such as slow wave associated contractions is proof of this (Huizinga and Lammers, 2009). However, ICC have also been though to play the role of a neural-smooth muscle interface, facilitating motor patterns in conjunction with the enteric nervous system (Ward and Sanders, 2001).

Table 1.1 Slow wave and segmentation frequencies

Species	Segmentation Frequency (cycles / min)	Slow Wave Frequency (slow waves / min)	Reference
Human	11	12	Hightower Jr, 1959; Code et al., 1952; Lin et al., 2006
Dog	18	17-22	Hightower Jr, 1959; Lin et al., 2000
Rabbit	15-20	14.24 ± 0.44	Alvarez, 1914; Hightower Jr, 1959; Shea-Donohue et al., 1997
Cat	24	10-15	Hightower Jr, 1959; Dahms et al., 1987; Lammers and Stephen, 2008

1.3.1 Interstitial cell of Cajal pacemaking

Smooth muscle contractions of the small intestine are controlled by rhythmic electrical signals termed slow waves. The slow wave shifts the membrane potential of myocytes to a potential which increases the open probability of voltage sensitive calcium channels. When open, these channels allow calcium into the cell and facilitate contraction (Lee et al., 1999). The slow wave is a rhythmic electrical signal that is generated by the ICC-MP found in the small intestine (Sanders et al., 2006; Der-Silaphet et al., 1998). Similarly, the ICC-DMP produce a rhythmic electrical signal not unlike the slow wave (for simplicity the term "slow wave" will only be used for the electrical signal originating from the ICC-MP) (Jiménez et al., 1996). The electrical signal generated by the ICC-DMP has been suggested to be a redundant "back-up" mechanism for controlling small intestine motility; its true physiological role is unknown. Others have shown that the network is coupled to both neurons and smooth muscle, and thus may mediate neural input of small intestinal motor patterns (Iino and Horiguchi, 2006).

Being the most active pacemaker system, the ICC-MP generate a slow wave that has been shown to correlate with rhythmic contractions of the small intestine. Low amplitude contractions observed in the gut occur at the same frequency that the ICC-MP pace (Hennig et al., 2010; Huizinga et al., 2009; Sanders and Ward, 2006). The slow wave associated contractions (SWAC) are the peristaltic contractions described previously that are able to traverse the length of the small intestine but with limited ability for content propulsion.

Although the function of ICC-DMP has yet to be elucidated, there is some evidence for its involvement in gut motility. Wang and coworkers (2005) have shown that destruction of the neural and ICC networks of the deep muscular plexus via *Trichinella spiralis* infection leads to a loss of the propulsive motor pattern (not SWAC) described above. Allowing time for the infection to resolve and the deep muscular plexus (and ICC) to repair, the propulsive pattern returned. This suggests that the destruction of ICC-DMP and their associated nerves leads to abnormal motility, since it is this propulsive pattern which is associated with content propulsion.

A known marker of the slow wave activity is a corresponding calcium oscillation seen in the ICC associated with the generation of slow waves (Torihashi et al., 2002). The calcium oscillations of the ICC-MP are synchronized between cells and correlate strongly with contractions seen in the small intestine (Lowie et al., 2011).

1.3.2 Innervation of interstitial cells of Cajal

Both ICC-MP and ICC-DMP networks are closely associated with nerve endings and smooth muscle cells, suggesting a possible mechanism for neural regulation of gut pacemaking and motility (Iino et al., 2004). Specifically, there are more nerve contacts with the ICC-DMP network than the ICC-MP network, perhaps suggesting it is the network most responsible for neural input from the enteric nervous system (Inio et al., 2004). Excitatory nerves associated with the ICC-DMP contain the neuropeptide substance P (Faussone-Pellegrini, 2006). Additionally, the ICC-DMP in this interaction express the NK_1 - tachykinin receptor (Inio et al., 2004); the subtype at which substance P is most selective. These nerves also contain acetylcholine with membrane expression of

muscarinic acetylcholine receptors on ICC-DMP (Ward et al., 2006). Similarly, inhibitory motor neurons which release nitric oxide are also found in close proximity to the ICC-DMP (Wang et al., 1999; Ward et al., 2006). There is thus a logical pathway that could potentially explain neural regulation of small intestinal motility through ICC-DMP pacemaking via substance P, acetylcholine and nitric oxide messengers; however, the true biological roles of substance P, acetylcholine and nitric oxide on ICC-DMP have yet to be elucidated.

1.4 Acetylcholine and acetylcholine receptors

The neurotransmitter acetylcholine plays a prominent role in the excitatory axis of the enteric nervous system of the small intestine. Acetylcholine is a product of the enzymatic esterification by choline acetyltransferase of the compounds choline and acetic acid (Oda, 1999). There are two classes of acetylcholine receptor, those sensitive to nicotine termed nicotinic acetylcholine receptors (nAChR) and those sensitive to muscarine termed muscarinic acetylcholine receptors (mAChR) (Lukas et al., 1999; Caulfield and Birdsall, 1998). There are currently ten nAChR subtypes (α1- α10), all of which are ligand-gated ion channels (Lukas et al., 1999). Activation of nAChRs upon binding of acetylcholine opens the channel allowing the passive flow of sodium ions (Na^+) in to the cell and potassium ions (K^+) out of the cell with a net depolarizing effect. Nicotinic acetylcholine receptors are expressed throughout the enteric nervous system, both on post-synaptic neurons (mediating acetylcholine signaling) but also on presynaptic excitatory neurons, facilitating a positive feedback loop whereby acetylcholine activates nAChRs on presynaptic neurons leading to a further release of acetylcholine (Mandl and

Kiss, 2007). There are five mAChR subtypes (M1, M2, M3, M4, M5), all of which are seven-transmembrane (G-protein coupled receptors) receptors (7TM) (Caulfield and Birdsall, 1998). The odd numbered mAChR subtypes primarily couple to $G_{q/11}$ where-as the even numbered subtypes couple with $G_{i/o}$ (Alexander et al., 2008). Muscarinic acetylcholine receptors are found throughout the small intestine, particularly on smooth muscle cells and ICC. The M2 and M3 receptor subtypes are found on both smooth muscle cells and ICC, specifically the ICC-DMP (Chen et al., 2007). The cellular mechanism of action of the M2 and M3 receptor are distinct upon activation, but they tend to both be excitatory (especially with regards to smooth muscle contraction) in the gastrointestinal tract (Ehlert et al., 2012). M2 receptors couple to $G_{i/o}$, which inhibits adenylyl cyclase leading to a reduction in the relaxat agent cyclic AMP and therefore cellular excitation (Ehlert et al., 2012). M3 receptors couple to $G_{q/11}$ leading to the liberation of inositol 1,4,5-trisphosphate (IP3) and the subsequent release of calcium from intracellular stores (Ehlert et al., 2012), which is part of active smooth muscle and ICC function (Ehlert et al., 2012; Lowie et al., 2011).

Acetylcholine signal termination is achieved primarily through two mechanisms: enzymatic breakdown and receptor internalization/desensitization. Once in the synaptic space, acetylcholine is rapidly broken down by the enzyme acetylcholinesterase. Acetylcholinesterase breaks down acetylcholine into synaptic inactive acetate and choline (van Koppen and Kaiser, 2003). The enzymatic breakdown is quite rapid and it has been suggested to effectively limit the dispersion of acetylcholine past the synapse in which it was released, preventing inappropriate receptor activation. Pharmacologically,

acetylcholinesterase inhibitors have been developed, such as neostigmine and physostigmine, which compete for the active site of the enzyme and prevent the break down of acetylcholine at the synapse. More convenient for experimentation is the pharmacological agent carbachol which is an acetylcholine receptor agonist that is not broken down by acetylcholinesterase (Nishioka et al., 2007), thus avoiding the effects of this enzyme. However, use of carbachol experimentally limits signal termination. Concurrently, activation of mAChR (like other seven transmembrane receptors) leads to the phosphorylation of the active receptor by cytoplasmic 7TM kinases. These phosphorylation events have the dual capacity to desensitize and internalize mAChRs (van Koppen and Kaiser, 2003). Desensitization occurs via a conformational change towards a state which does not interact with G-proteins or ligands as well, thus desensitizing the agonist-receptor activation response. The same phosphorylation also leads to the recruitment of β-arrestin, and interaction with clathrin and dynamin leading to the budding and formation of a clathrin-coated vesicle inside the cell, removing the receptor from the cell surface and thus terminating the signal (van Koppen and Kaiser, 2003). As with other membrane bound proteins in vesicles they can be targeted for degradation or recycled back to the cell membrane (Bomberger et al., 2012).

1.5 Substance P and tachykinin receptors

Tachykinins are neuropeptide ligands associated mostly with nerves, but also have non-neural origin in some instances (Pennefather et al., 2004). The tachykinins are often associated with excitatory pathways of the nervous system both centrally and peripherally (Pennefather et al., 2004). There are three tachykinin receptor subtypes and three

endogenous tachykinins. The subtype nomenclature is simply NK_1, NK_2 and NK_3 (Faussone-Pellegrini, 2006). The three tachykinins are substance P, neurokinin A (NKA) and neurokinin B (NKB) (Faussone-Pellegrini, 2006). The NK_1 receptor is mostly activated by substance P (Pennefather et al., 2004) but can also be non-selectively activated by NKA and NKB. The NK_2 and NK_3 receptors are preferentially activated by NKA and NKB respectively (Faussone-Pellegrini, 2006). As mentioned previously, there are substance P producing neurons of the enteric nervous system in the small intestine. Further evidence of the role of substance P in small intestine motility is that ICC-DMP express the NK_1 receptor which is preferentially activated by substance P, but does not express the NK_2 or NK_3 receptors (Shimizu et al., 2008), selective for the other tachykinins.

Signaling via substance P and NK_1 receptors is a highly regulated process (Kirkwood et al., 2001). Termination of a substance P signal is achieved via two main mechanisms, substance P metabolism and tachykinin receptor internalization (Kirkwood et al., 2001). Since substance P is a short polypeptide, it is susceptible to metabolism by a variety of enzymes and proteases. One of the main substance P metabolizers is the enzyme enkephalinase (Djokic et al., 1989). Specifically, neutral endopeptidase (NEP), also known as neprilysin, has been implicated in substance P metabolism at other smooth muscle junctions, namely in the trachea, but also in the gut (Djokic et al., 1989). Other enzymes capable of metabolizing substance P include angiotensin-converting enzyme (ACE) involved in the renin-angiotensin system, serine proteases and acetylcholinesterase (Djokic et al., 1989). The second method of substance P signal termination is the

internalization of NK_1 receptors on the postsynaptic cell, for example the ICC-DMP of the small intestine. Upon activation of peptide binding 7TMs, as in the case of tachykinin receptors, activation of the secondary messenger system feeds back on the 7TMs, leading to the formation of clathrin-coated pits in proximity to the receptors. Eventually the pit closes through a process of pinching and budding and the receptor is fully internalized via endocytosis (Kirkwood et al., 2001; Sagan and Lavielle 2001). Since the receptor is fully internalized within the cell it is no longer available for activation from extracellular signaling molecules. In the case of the small intestine, NK_1 receptor internalization at ICC-DMP may facilitate neural signal cessation. Once in the cell, the receptor either proceeds to the lysosome for degradation or is recycled back to the cell surface to receive the next signal (Grady et al., 1995). The processes of substance P metabolism and NK_1 internalization prevent over stimulation by a signal and contribute to fine tune neural and effector responses.

1.6 Objectives

To gain a better understanding of myogenic and neural control mechanisms of rhythmic motor patterns in preparations that have different physiological functions. The main focus was on the mouse small intestine where rhythmic motor patterns propel luminal content. I used functional motility studies and calcium imaging specifically on the mouse small intestine to:

i) Describe the physiological characteristics of rhythmic propulsion.

ii) Explore the role of neurotransmission as a mechanism for modulating the pacemaker activity of ICC-DMP and rhythmic propulsion.

iii) Explore the patterns of ICC-DMP calcium oscillations, paying particular attention to rhythmic patterns which may be associated with slow waves, and correlate these oscillations with rhythmic motor patterns.

1.7 Hypothesis

The general hypothesis is that both myogenic and neurogenic control systems are involved in rhythmic motor patterns in the gastrointestinal tract. With respect to the murine small intestine the hypothesis is further articulated as follows:

Based on the significant lack of explanation for the rhythmicity involved in propulsion by current enteric nervous system models, it is likely that ICC play a role in this pattern. Interstitial cells of Cajal are already known to supply a rhythmic stimulus to the smooth muscle in general peristaltic terms and thus could facilitate the rhythmicity found in propulsion. It may be the ICC-DMP of the small intestine that facilitate this rhythmicity as the ICC-MP already pace a known pattern of peristalsis (and possibly even segmentation) in the small intestine. We expect it to be shown using functional organ bath studies and employing neural blockade just as was previously done in the rat colon (Huizinga et al., 2011). Perhaps content propulsion in the small intestine originates as is suggested in the colon, where propulsion is an inducible pattern that is highly regulated by stretch and the enteric nervous system.

2. METHODOLOGY

2.1 Animals

All research involving animals was approved by the Animal Research Ethics Board (AREB) at McMaster University. Animal handling and use was done in accordance with the standards set out by the Canadian Council on Animal Care (CCAC). Adult CD-1 mice (Charles River International Inc., Wilmington MA), aged approximately 16 weeks were housed in conventional cages in the central animal facility of McMaster University with *ad libitum* access to food and water under a 12 hour light : 12 hour dark cycle. On the days of organ bath experimentation, animals were fasted for four hours prior to experimentation, to void the jejunum of content, but were given *ad libitum* access to water, animals were not fasted for calcium imaging experiments. For calcium imaging experiments, neonatal CD-1 mice (Charles River International Inc., Wilmington MA) aged 5-15 days were also used. Neonatal mice were housed with their mother in the same condition as the adult mice. Finally, adult Sprague-Dawley rats, (Charles River International Inc., Wilmington MA) weighing 200-250 grams, were used for the *in vitro* colonic muscle strip experiments. All animals were euthanized by cervical dislocation. Rats were first anesthetized via chloroform inhalation. Once dead, the abdominal cavity was opened and the small and large intestines were carefully dissected out. The intestines were then rinsed with Krebs' solution in order to remove any excess blood, and placed into fresh Krebs' solution to mimic physiological ionic and nutrient concentrations as well as maintain a constant pH.

2.2 Organ bath technique

Dissected and cleaned small intestinal segments were required for organ bath experiments. Intestines were pinned out in a dissecting dish containing fresh Krebs' (gassed with 95% O_2, 5% CO_2) under finite longitudinal tension. The mesenteric fat was cut away with fine point scissors, and then the tissue was cut into a 5 to 8 centimeter segment, depending on the quality of fat removal and appearance of luminal content (empty segments were preferred). Each small intestine yielded one high quality, cleaned segment for organ bath experimentation.

The organ bath apparatus consisted of various parts necessary for keeping the tissue alive, experimentation and data recording. The main organ bath was filled with 200 mL of fresh, gassed Krebs' solution. The bath was connected to a water heater/pump which circulated warm water to the bath in order to heat the Krebs' solution to 37 ^{o}C while not directly contacting the Krebs' solution. Adjacent to the organ bath was reservoir containing room temperature Krebs' solution. The reservoir was mounted on a height adjustable track; thus, by raising and lowering the reservoir, increased and decreased pressures could be simulated. The bottom of the reservoir was attached to a valve and small "inflow" tube. The valve allowed for the controlled timing of content flow. The inflow tube was used to cannulate that oral end of the intestinal segment. The inflow tube was pierced on the side and a second smaller tube was inserted. This smaller tube was inserted further into the small intestine than the inflow tube. The other end of the small tube was attached to a fluid pressure recording device (Argon Medical Devices Inc., Athens TX). The pressure signal was amplified using a Grass LP122 AC/DC

amplifier (Astro-Med Inc., West Warwick RI). The amplified signal was digitized using an Axon Instruments MiniDigi 1A digitizer (Molecular Devices, Sunnyvale CA) attached to a personal computer. Data were recorded using AxoScope 10.3 software (Molecular Devices, Sunnyvale CA) which maintained a digital copy and was also capable of performing raw data extraction. Concurrently, an "outflow tube" measuring the same diameter as the inflow tube was used to cannulate the caudal end of the intestinal segment. The outflow tube was attached to an empty reservoir containing a second small pressure recording tube. This pressure recording tube recorded the pressure changes in the reservoir whenever luminal content was propelled out of the small intestine. A digital reading of the outflow was achieved in the same manner as the intraluminal pressure recording. Since both recordings were performed via the same program, a perfect time match record was created for both. Finally, located above the organ bath, was an HDR-SR11 digital HD video camera recorder (Sony Corporation, Tokyo Japan), which recorded the visual contractions of the tissue. Video recordings were started simultaneously with pressure and outflow recordings to have time matched data throughout.

2.3 Calcium imaging technique

Both adult and neonatal intestinal preparations devoid of mucosa and submucosa were required for calcium imaging. An approximately 1 cm segment of small intestine gathered in the manner stated in section 2.1 was cut open along the mesenteric border. In a Sylgard 184 silicone elastomer (Dow Corning Corporation, Midland MI) bottom dish containing continually gassed Krebs' solution, the resulting approximate 1 cm by 1 cm

square segment was then pinned under light but finite tension with the mucosal layer (luminal surface) facing away from the bottom of the dish. In order to expose the ICC-DMP for imaging, the mucosal and submucosal layers were dissected away using blunt dissection with forceps. The resulting preparation consisted of an exposed circular muscle layer with the deep muscular plexus lying above it. The preparation was then returned to a large reserve of Krebs' solution and equilibrated for a period of one hour.

The remainder of the experiment was conducted under minimal light conditions as the imaging agent was light sensitive. Intestinal preparations were removed from the large Krebs' reservoir and incubated for 15 minutes with 1 mL Krebs' solution containing 5 μM Molecular Probes fluo-4 AM (acetoxymethyl ester) (Life Technologies Corporation, Carlsbad CA), 10 μM probenecid (Sigma-Aldrich, St. Louis MO) and 0.02% pluronic F-127 (Sigma-Aldrich, St. Louis MO). Pluronic F-127 is a reagent used to aid in the solubilization of acetoxymethyl esters. Acetoxymethyl esters have difficulty crossing the cell membrane as well as remaining in aqueous solutions which are required in keeping cells alive using Krebs' solution. Fluo-4 AM is a calcium chelating dye which only emits fluorescent light upon excitation and binding of calcium. Without calcium, the dye does not emit light even when excited. Upon entry into the cell the acetoxymethyl ester group of fluo-4 AM is cleaved by non-specific cytosolic esterases, yielding a free fluo-4 which can not easily transfer across the cell membrane. Finally, probenecid is a nonselective membrane pump blocker which prevents the active pumping of the fluo-4 dye out of the cell helping to limit the dye to the cytosol.

Once the dye was loaded into the cell, tissue preparations were constantly perfused with fresh Krebs' solution heated to 37 ^{o}C. Intestinal preparations were viewed using a Nikon eclipse FN1 microscope (Nikon Corporation, Tokyo, Japan) employing either 20X or 40X objectives with a GFP filter allowing for the excitation of fluo-4 with light at a wavelength of 488 nm. Digital videos were created using a QuantEM 512SC camera (Photometrics, Tucson AZ) capturing digital images and recorded with Nikon-NIS Elements software (Nikon Corporation, Tokyo, Japan) installed on a personal computer.

2.4 Muscle strip organ bath technique

2.4.1 Human pyloric region muscle strips

Human tissue was acquired from leftover segments from an immunohistochemical study of ICC at the level of the pyloric interface. Informed consent was obtained from all patients before samples were taken. Tissue was obtained from patients undergoing gastrointestinal resections associated primarily with pancreatic cancer. Tissues were transported to the laboratory in fresh Krebs' solution where they were transferred to a dissecting tray. A trained expert in gastrointestinal physiology dissected out two strips, measuring approximately 10mm x 3mm x 5mm, of antrum, pylorus and duodenum from each patient sample, oriented along the circular muscle axis.

Dissected muscle strips were tied with thread with a loop at one end and a long piece at the other. The loop was slipped onto a hook located at the bottom of a 20 mL organ bath containing fresh Krebs' solution continually gassed. The long thread was mounted and tied to a FT-03 force displacement transducer (Astro-Med, Inc., West

Warwick RI). Signal amplification was achieved using a Grass 7D polygraph (Astro-Med, Inc., West Warwick RI), captured by acquisition hardware DI 205 and DI 720 (DataQ Instruments, Akron OH) displayed on a computer using Windaq Data acquisition software (DataQ Instruments, Akron OH). The resulting data were output as force of contraction over time for the duration of the experiment.

2.4.2 Rat colon muscle strips

Once dissected out of the rat, a 5 cm section of colon was cut away and placed in a dissecting dish containing fresh gassed Krebs' solution. The segment was cut along the mesenteric border and pinned open revealing the mucosal layer. Using forceps, the mucosal and submucosal layers were removed via blunt dissection, with the muscle layers remaining. Six full diameter circular muscle strips (approximately 5 mm wide) were cut from the segment and tied with a loop on one end and a free thread on the other. The loop was slipped onto a hook located at the bottom of a 20 mL organ bath containing fresh Krebs' solution continually gassed. The long thread was strung-up and tied to a FT-03 force displacement transducer (Astro-Med, Inc., West Warwick RI). Signal amplification was achieved using a Grass 7D polygraph (Astro-Med, Inc., West Warwick RI), captured by acquisition hardware DI 205 and DI 720 (DataQ Instruments, Akron OH) displayed on a computer using Windaq Data acquisition software (DataQ Instruments, Akron OH). The resulting data were output as force of contraction over time for the duration of the experiment.

2.5 Solutions, drugs and reagents

All experiments were performed using Krebs' solution comprised of (in mM): NaCl 118.1; NaH_2PO_4 1.0; $MgSO_4$ 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25, and glucose 11.1; continually gassed with 95% O_2, 5% CO_2. All salts were of highest purity from local sources. All drugs were dissolved in deionized water except for N_ω-Nitro-L-Arginine (L-NNA) in 50 mM HCl_{aq}, pluronic F-127 and probenecid in DMSO and fluo-4 in pluronic F-127/DMSO solution. Drugs were dissolved daily as required except for pluronic F-127 (stored at room temperature), fluo-4 (stored at -20 ^oC), tetrodotoxin (TTX) (stored at -20 ^oC) and methoxyverapimil (stored at 4^oC).

2.6 Data Analysis and Statistics

Raw data from intraluminal pressure recording and luminal outflow recording were extracted from AxoScope using a personal computer. The parameters of contraction frequency, amplitude and duration (see section 3.2 for details) were calculated from pressure recordings. Video recordings were converted to .mov format and transformed into spatiotemporal maps using a specifically designed ImageJ plugin. The plugin tracked the diameter changes along the small intestinal segment over time. Spatiotemporal maps were created along a grey scale where the darker the pixel the smaller the diameter of the segment at that point in time. Further analysis of these maps can convert the grey scale directly into numerical diameter changes in millimeters for analysis of baseline diameter, change in diameter during contraction and degree of tonic contraction.

Calcium imaging videos were analyzed using the region of interest (ROI) function of the Nikon-NIS Elements software. This function allowed for the encirclement of a cell

of interest and data output of the intensity of light (as proportional to the amount of fluo-4 dye bound to calcium) versus time of the recording.

Muscle strip contraction data (both human pyloric interface and rat colon) was analyzed using the WindDaq software used for recording the data. The software allowed for transformation of contractions on a relative time scale for calculation of frequency. Likewise, force of contraction data could be exported versus time for graphing purposes or used directly from the software for individual maximum amplitude calculations.

All data are presented as mean ± standard error of mean (SEM) of n experiments, where n is a value from a different animal, except in the case of human muscle strips where n is the number of strips due to the lack in viable tissue (no individual supplied more than 2 strips). Data means were compared using paired and unpaired Student's t-test or one way analysis of variance (ANOVA) employing a Bonferroni correction, where appropriate.

3. MECHANISM OF PROPULSION IN THE MOUSE SMALL INTESTINE

3.1 Introduction

Content propulsion in the small intestine has largely been attributed to a phenomenon known as the peristaltic reflex (Bayliss and Starling, 1899). The peristaltic reflex is a reflex of the small intestine entirely driven by the enteric nervous system. The reflex is characterized by caudal projecting inhibitory motor neurons and orally projecting excitatory motor neurons which when triggered by distention to a bolus of chyme lead to a relaxation caudal to the bolus and a contraction oral to the bolus (Costa et al., 2000). This mechanism is repeated along the length of the small intestine creating a wave of peristalsis, propelling content. Although anatomically possible, there are some questions as to whether the peristaltic reflex is relevant under physiological conditions (Spencer et al., 1999; White et al., 1934; Alvarez and Starkweather 1919). Specifically, the reflex is rarely studied under physiological conditions of large amounts of content but rather individual distention events which are immediately ceased (Spencer et al., 2001). Since a constant distention stimulus is rarely studied, the intrinsic rhythm of propulsion is not usually noticed. However, even when rhythmic propulsion is studied, the aspects of rhythmicity are not assessed nor commented upon (Holzer et al., 1998), and the role of other anatomical features of the small intestine (i.e. interstitial cells of Cajal) are ignored or poorly assessed for their role in propulsive peristalsis (Spencer et al., 2001).

A defining feature of the propulsive motor complex (PMC) is the inherent rhythmicity with which it is repeated. The propulsive motor pattern (PMP) is a poorly characterized pattern of the small intestine, which has been seen in guinea-pig (Holzer et

al., 1998; Spencer et al., 2001) and mouse (Wang et al., 2005). The slow frequency with which it occurs has been defined as a secondary pattern when compared to the slow wave associated contractions. It has been shown that a destruction of the ICC and neurons of the deep muscular plexus is associated with the loss of this lower frequency pattern (Wang et al., 2005). It is also known that the pattern is sensitive to tachykinin receptor antagonism and thus, employs substance P in the underlying mechanism (Holzer et al., 1998). A complete mechanism of rhythmic propulsion remains elusive, as those studying it to date have either assumed it followed the mechanism of the peristaltic reflex and thus ignored a more basic assessment of the pattern or only provided preliminary mechanistic evidence for the pattern. Recently, a mechanism explaining the rhythmicity of content propulsion of the rat colon has been published where it is suggested that an ICC pacemaker system independent of the slow wave generating ICC of the colon are responsible for the low frequency pattern of propulsion (Huizinga et al., 2011). Likewise, the small intestine contains a fully developed ICC network at the level of the deep muscular plexus which is independent of the slow wave generating ICC-MP (Komuro, 2006). Interestingly, the ICC-DMP are the cells which were associated with the loss of rhythmic propulsion as described above (Wang et al., 2005) and, they are the pacemakers that express high levels of tachykinin receptor subtype NK1 (Faussone-Pellegrini, 2006) and have a large degree of innervation (Ward et al., 2006).

The objective of this study was to describe the physiological characteristics of rhythmic propulsion and further assess the mechanism by which rhythmic propulsion originates in the mouse small intestine.

3.2 Methods

Adult CD-1 intestinal segments were used for all organ bath experiments. Information on animal handling and intestinal extraction data can be found in sections 2.1 and 2.2 respectively.

3.2.1 Organ bath experiments proper

Once cannulated and warmed in the organ bath for 30 minutes, simultaneous intraluminal pressure, luminal outflow and video monitoring of contractions were initiated. Baseline recordings were done with the reservoir valve closed for 5 minutes. The reservoir valve was then opened while the reservoir remained in the zero pressure position for another 5 minutes. The Krebs' solution reservoir was raised in increments of 1 cm spaced apart by 10 minute recordings up to a total of 3 cm or until rhythmic propulsion was seen on the intraluminal pressure recording or via visual inspection. Reservoir heights above 3 cm were not used as the resulting pressure induced a large degree of intestinal distention which normal contractions could not overcome.

After the entire 10 minute control recording of rhythmic propulsion, the pharmacological agents used to assess the pattern were, 0.5 μM tetrodotoxin, 0.2 mM L-NNA, or 10 nM substance P. Tissues that displayed rhythmic propulsion which did not last the 10 minute recording were treated with carbachol (5 μM). Carbachol (5 μM) was subsequently added in to tissues that had rhythmic propulsion blocked by TTX in an attempt to regain the pattern. All drugs were added directly into the organ bath and left in contact with the intestinal segments for the duration of the experiment.

3.2.2 Data analysis

All raw data extraction from the intraluminal pressure records was done using the AxoScope 10.3 software, which was the same software used to record the data during experimentation. Subsequent transformations of data were done using Microsoft Excel (Redmond, WA). The following intraluminal pressure parameters were calculated as follows:

Frequency of PMP = Number of PMC / t (Fig 3.1A)

where PMC is an individual contraction; t is time in minutes.

Maximum Pressure of PMP (Amplitude) = $\frac{(\max A_{PMC} - \text{baseline})}{(\max A_{SWAC} - \text{baseline})}$ (Fig 3.1A)

where $\max A_{PMC}$ is the maximum amplitude of PMCs; baseline is the lowest amplitude reading prior to a PMC; $\max A_{SWAC}$ is the average maximum amplitude of slow wave associated contractions.

Duration of PMC = $t_{1PMC} - t_{0PMC}$ (Fig 3.1 A)

where t_{0PMC} is the time at the beginning of a PMC; t_{1PMC} is the time at the end of a PMC

Data extracted from spatiotemporal maps were from direct measurement of the maps or image J transformations. The following spatiotemporal map parameters were calculated as follows:

Frequency of PMP = Number of PMC / t (Fig 3.1 B)

where PMC is an individual contraction; t is time in minutes.

Duration of PMC = $t_{1PMC} - t_{0PMC}$ (Fig 3.1 B)

where t_{0PMC} is the time at the beginning of a PMC; t_{1PMC} is the time at the end of a PMC.

$$Velocity = L_{gut} / (\Delta t_{Mid}) \quad \text{(Fig 3.1B)}$$

where L_{gut} equals the length of gut that the PMC traveled from start to finish in cm; Δt_{Mid} is the caudal midpoint PMC duration time minus the oral midpoint duration time in seconds.

$$Percent\ Luminal\ Occlusion\ (Amplitude) = \frac{(MAX_{contraction} - Baseline)}{Baseline} \quad \text{(Fig 3.1B)}$$

where $MAX_{contraction}$ is the smallest luminal diameter achieved during contraction; Baseline is the largest luminal diameter achieved during relaxation.

3.3 Results

3.3.1 Characteristics of the propulsive motor pattern

Slow wave associated contractions were readily observed in most organ segments via changes in intraluminal pressure. More elusive were the low frequency, large amplitude contractions (Fig 3.2A) which required a distention stimulus for initiation. The low frequency, large amplitude contractions were associated with content expulsion (Fig. 3.2B) and thus the pattern was termed the propulsive motor pattern; one contraction in this pattern being referred to as the propulsive motor complex. The slow wave associated contractions showed small movements of intraluminal content upon visual inspection, but insufficient force was generated to obtain outflow in response to these contractions. Once activated, the propulsive motor pattern often persisted at a frequency of 1.0 ± 0.1 contractions per minute (cpm) throughout the duration of the experiment allowing for pharmacological manipulation.

Spatiotemporal mapping of the entire intestinal segment revealed a distinct "banding" pattern associated with the propulsive motor complexes. Rhythmic propulsion

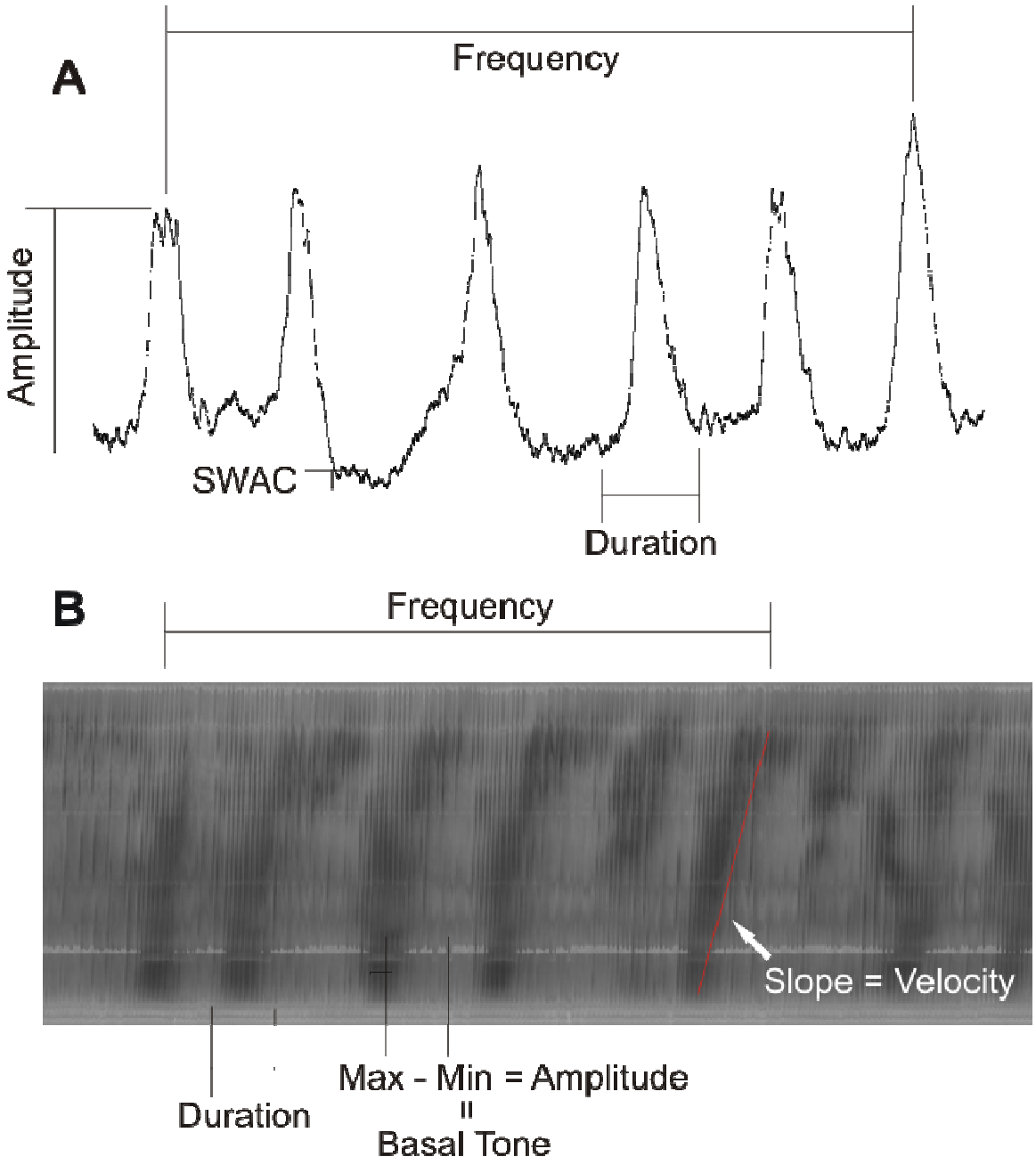

Figure 3.1. Analysis of organ bath experiments. Raw trace output from intraluminal pressure recording (**A**) measured as absolute pressure over time. For the effect of a drug, the same parameters are recorded before and after addition of the compound. Spatiotemporal map (**B**) created from video recording of intestinal contractions. The darker the area on the grey scale, the more contracted the tissue. Time proceeds from left to right, while gut length proceeds from bottom to top.

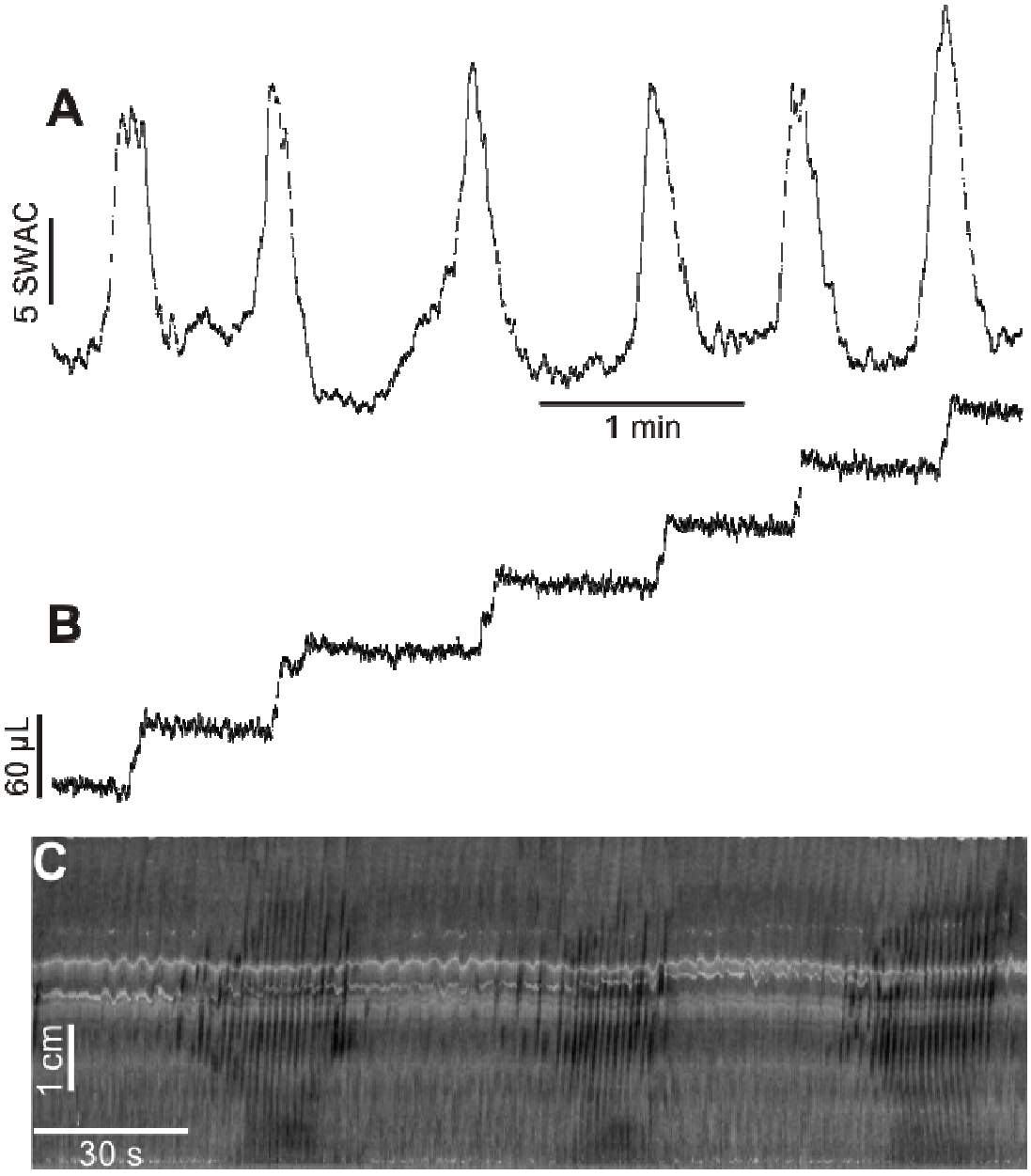

Figure 3.2. Content propulsion in the small intestine. Intraluminal pressure recording of the propulsive motor pattern (**A**) and the time-matched outflow of content (**B**) associated with the PMCs. Pressure is normalized to the pressure generated by slow-wave associated contractions (SWAC). Spatiotemporal map (**C**) reveals the alternating contractions of the propulsive motor pattern with superimposed slow wave associated contractions during times of activity. The darker the area on the map along the grey scale corresponds with smaller luminal diameter or increased level of contraction.

was seen as a pattern of periods of distinct slow wave associated peristaltic contractions followed by a period of little to no high frequency contractions (Fig. 3.2C). These bands occurred at a regular interval of 0.9 ± 0.1 contractions per minute (cpm) under the conditions of 1-3 cm of intraluminal pressure. The banding pattern was also characterized as an increase in force of contraction and increase in luminal occlusion compared to regular slow wave associated contractions. It was the increased force and luminal occlusion which was able to propel content though the lumen of the small intestine and achieve outflow.

The propulsive motor pattern is highly dependent on the amount of stretch that the muscle wall experiences. Although examples were seen where the pattern emerged under limited luminal distention, the majority of the time, an initial or sustained stretch of the tissue was required to stimulate the pattern (Fig. 3.3A). The reciprocal was not necessarily true, whereby increases in intraluminal distention did not always initiate the propulsive motor pattern.

Not only was stretch a large determinant in initiating the propulsive motor pattern, but it was also highly correlated with the frequency of the individual contractions. In tissue segments that displayed a consistent propulsive motor pattern, a decrease in intraluminal pressure of 1 cm changed the frequency from 1.4 to 0.8 cpm; subsequently the resulting pattern reduced to a point where the rhythm ceased and contractile amplitudes were low. Returning the intraluminal pressure back to the previous level restored the propulsive motor pattern (Fig. 3.3B).

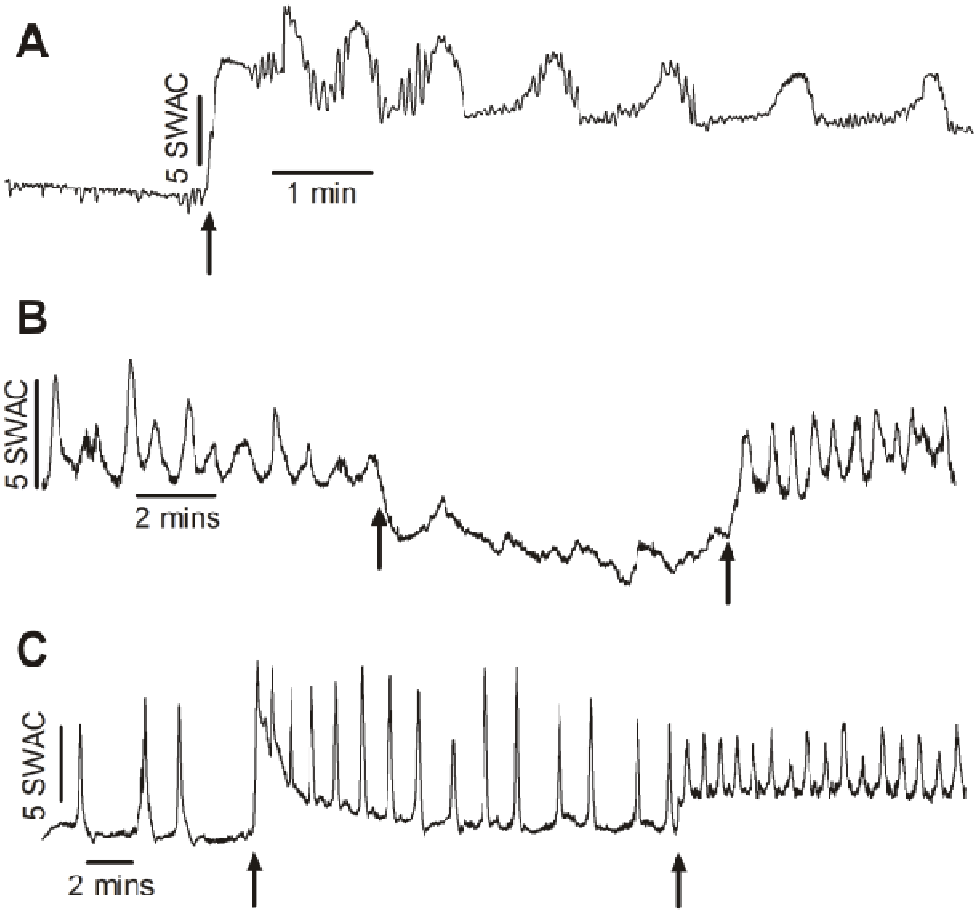

Figure 3.3. Effect of distention on the propulsive motor pattern. A large increase in intraluminal distention initiated the PMP (**A**). Reducing intraluminal pressure (**B**, left arrow) abolished PMP; restoring luminal distention (**B**, right arrow) restored the PMP. Increasing levels of distention (**C**, both arrows) and corresponding increase in PMP frequency. Pressure is normalized to the pressure generated by slow wave associated contractions (SWAC).

Similarly, a step up by 1 cm of water in intraluminal pressure increased the frequency from 0.47 cpm to 0.69 cpm (Fig. 3.3C). A further increase in pressure of 1 cm was able to raise the frequency again to 1.3 cpm (Fig. 3.3C), demonstrating the graded influence of intraluminal stretch on propulsive motor complex frequency.

3.3.2 Stimulation of the propulsive motor pattern with carbachol

Addition of 5 µM carbachol stimulated the PMP into a consistent high amplitude pattern (Fig 3.4A) in segments that had previously displayed rhythmic propulsion. Conversely, the PMP was not initiated via carbachol in tissues that had not exhibited rhythmic propulsion during the organ bath experimentation. The frequency of carbachol induced PMCs was almost twice that compared to stretch induced control propulsion (Table 3.1). Intraluminal pressure recordings indicated no significant difference in duration or maximum amplitude of control compared to carbachol induced PMCs (Table 3.1). Spatiotemporal mapping (Fig 3.4B) confirmed both the change in frequency and unaltered duration and amplitude (percent occlusion) to carbachol treatment (Fig 3.4C; Table 3.1). Mapping also revealed that the velocity of contractions was unaltered between control and carbachol driven rhythmic propulsion (Table 3.1).

3.3.3 Abolishing the propulsive motor pattern with tetrodotoxin

Blocking of all enteric neural transmission with tetrodotoxin resulted in two main effects on the PMP. In some cases (2 out of 6) the tissue responded by increasing the basal tone to the same level as the max amplitude of the PMC (Fig 3.5A). This result agreed with the analysis of the spatiotemporal maps which showed that the level of

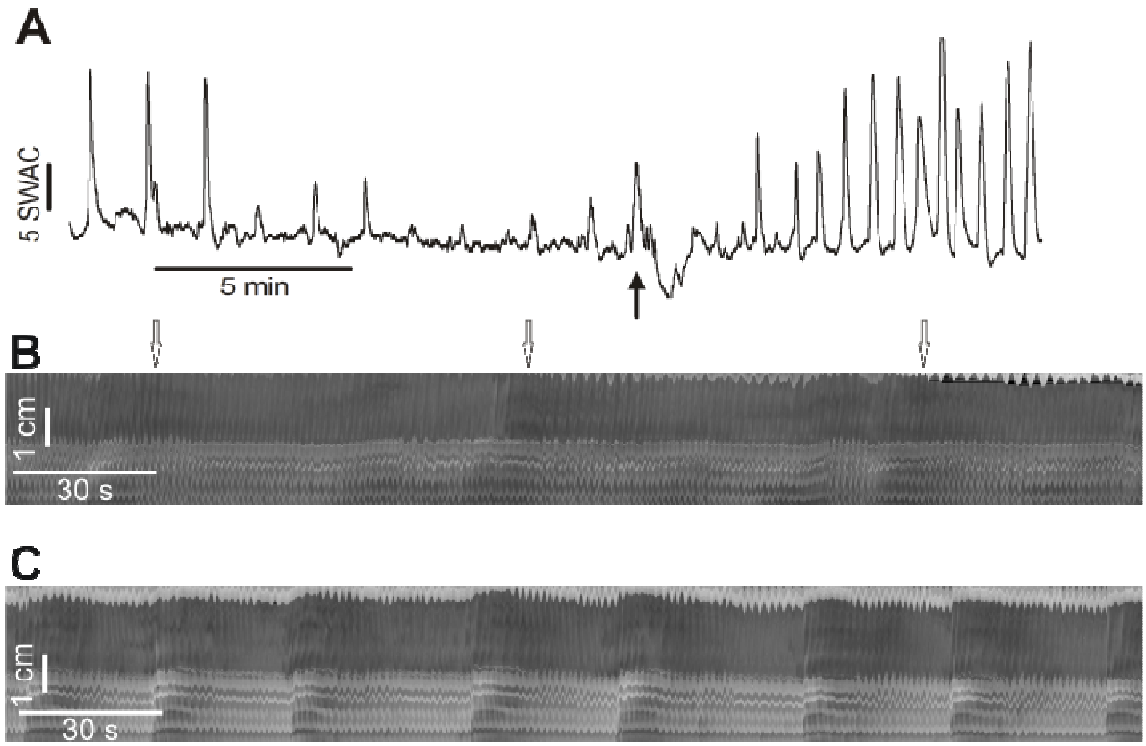

Figure 3.4. Restoration of rhythmic propulsion with carbachol. Intraluminal pressure recording (**A**) showing the recovery of rhythmic propulsion after addition of 5 µM carbachol (black arrow). Pressure is normalized to the pressure generated by slow wave associated contractions (SWAC). Control spatiotemporal map (**B**) with indicated PMCs (white arrows). PMCs after carbachol stimulation (**C**; entire duration of the map). The darker the area on the map along the grey scale corresponds with smaller luminal diameter or increased level of contraction.

Table 3.1 The Effect of Carbachol on the Parameters of Rhythmic Propulsion

	Intraluminal Pressure Recording		**Spatiotemporal Mapping**	
	Control	**Carbachol (5 µM)**	**Control**	**Carbachol (5 µM)**
Frequency (cpm)	0.9 ± 0.2 n = 4	1.9 ± 0.1** n = 4	0.9 ± 0.2 n = 3	2.0 ± 0.1** n = 3
Duration (s)	25.1 ± 5.6 n=4	30.4 ± 1.7 n = 4	55.6 ± 40.3 n = 3	12.3 ± 1.9 n = 3
Amplitude (%SWAC; mm)	11.1 ± 3.1 n = 4	17.8 ± 4.3 n = 4	1.3 ± 0.2 n = 3	1.5 ± 0.3 n = 4
Velocity (cm / s)	N/A	N/A	0.9 ± 0.2 n = 3	0.7 ± 0.1 n = 4

** $p < 0.01$; * $p < 0.05$

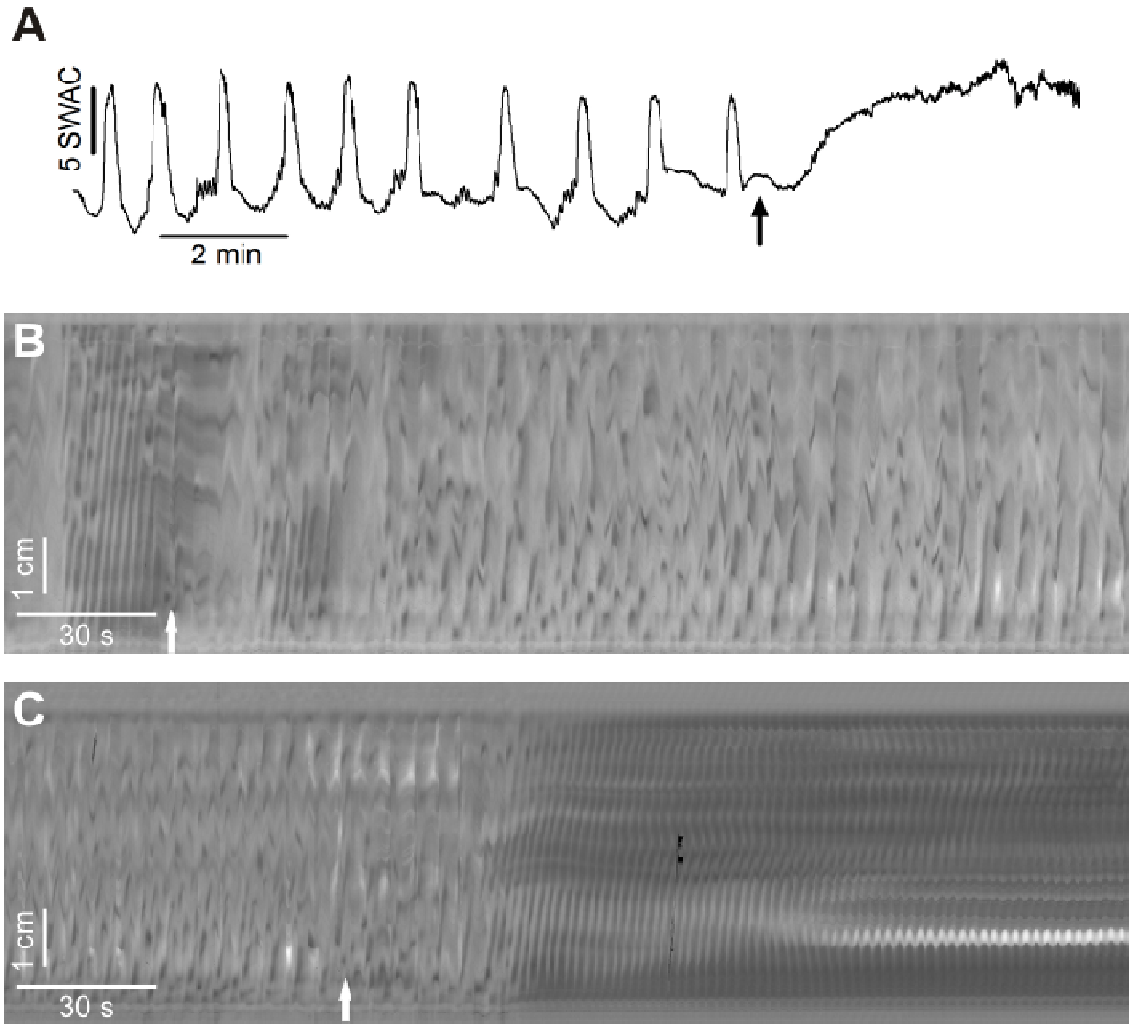

Figure 3.5. Effect of neural blockade on intestinal motor patterns. Intraluminal pressure recoding (**A**) shows a tonic increase in pressure in response to the addition of 0.5 mM tetrodotoxin (black arrow). Pressure is normalized to the pressure generated by slow wave associated contractions (SWAC). Spatiotemporal mapping reveals the abolishment of PMCs and a shift towards segmentation (**B**) upon addition of 0.5 µM tetrodotoxin (white arrow). Addition of 2 µM carbachol (**C**; white arrow) shifted segmentation to slow wave associated contractions. The darker the area on the map along the grey scale corresponds with smaller luminal diameter or increased level of contraction.

luminal occlusion (amplitude) to PMCs was the same as the contraction induced by TTX (Table 3.2). In this case, rhythmic propulsion was no longer seen, however, it was not discernible whether the pattern was abolished or merely overcome by the tonic increase in basal tone and resultant increase in intraluminal pressure. Conversely, in some intestinal segments, the PMP was replaced with segmentation like patterns without remnants of the PMP (Fig 3.5B). Segmentation patterns were identified with two main parameters. Firstly, the spatiotemporal maps showed neither rhythmic propulsion nor peristaltic slow wave associated contractions. Secondly, the remaining contractile pattern consisted of areas of interrupted slow wave associated contractions that were not uniform throughout the time period (Fig 3.6). Addition of carbachol (2 μM) did not restore the PMP in segments treated with TTX; however, it did result in a dominant pattern of slow wave associated contractions (Fig 3.5C). Interestingly, the spatiotemporal map showed a significant reduction in the frequency of slow wave associated contractions which was not seen via the intraluminal pressure recording (Table 3.2).

3.3.4 Modifying the propulsive motor pattern with L-NNA

Like acetylcholine, nitric oxide (NO) is a major mediator of intestinal motility, except on the inhibitory (smooth muscle relaxation) side of the enteric nervous system. Prevention of NO production by inhibiting nitric oxide synthase with L-NNA increased the basal pressure of the small intestine and acutely abolished the propulsive motor pattern (Fig. 3.7A). After a short period, the pattern reemerged superimposed on top of the increased basal pressure (Fig. 3.7A). The restored PMP had the same frequency, duration and amplitude of contractions as the control situation (Table 3.3).

Table 3.2 The Effect of TTX on the Parameters of Rhythmic Propulsion

	Intraluminal Pressure Recording		**Spatiotemporal Mapping**	
	Control	**TTX (0.5 µM)**	**Control**	**TTX (0.5 µM)**
Frequency (cpm)	1.2 ± 0.2 n = 3	N/A	0.8 ± 0.1 n = 6	N/A
Duration (s)	28.3 ± 4.7 n = 3	N/A	23.0 ± 3.2 n = 6	N/A
Amplitude (%SWAC; %Baseline)	13.4 ± 3.1 n = 3	9.6 ± 2.8 n = 3	37.2 ± 3.7 n = 6	39.8 ± 5.4 n = 6
Baseline (mm)	N/A	N/A	4.9 ± 0.3 n = 5	4.9 ± 0.5 n = 5
Velocity (cm / s)	N/A	N/A	1.48 ± 0.5 n = 5	1.0 ± 0.2 n = 3
Frequency of SWAC (cpm)	29.7 ± 2.6 n = 3	23.5 ± 3.3 n = 3	33.9 ± 2.9 n = 6	16.4 ± 5.9* n = 4

* $p < 0.05$

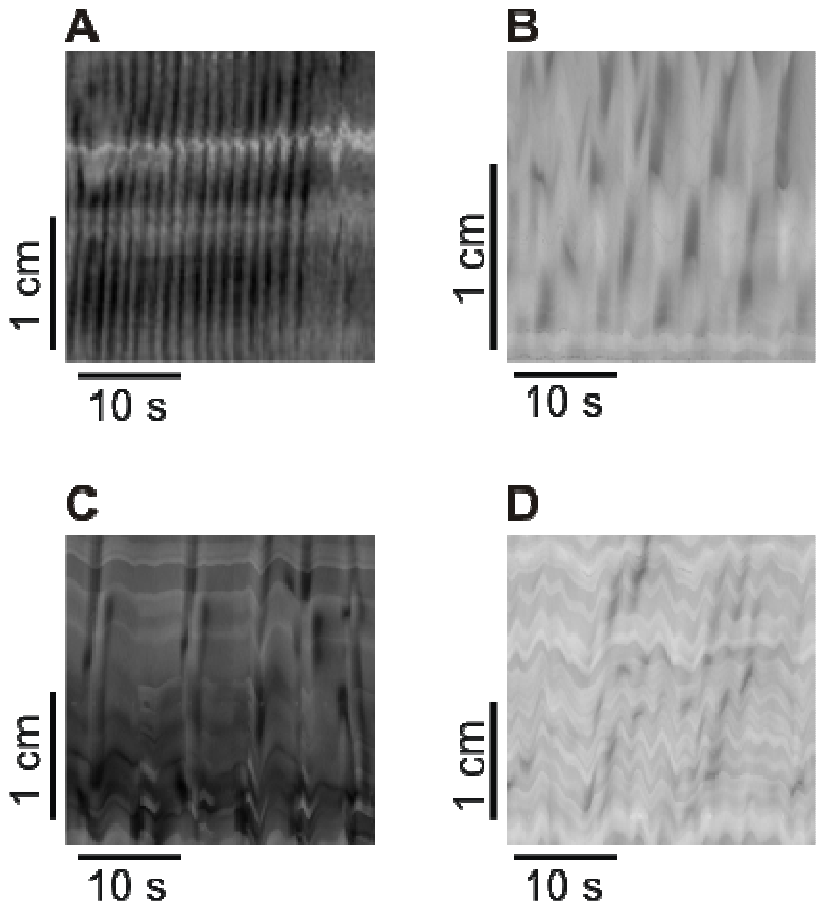

Figure 3.6. Segmentation motor pattern. Appearance of slow wave associated contractions (**A**) as measured by spatiotemporal maps. Examples of short duration, segmental contractions (**B**, **C** and **D**) with the impression of interrupted slow wave associated contractions, in response to 0.5 μM tetrodotoxin.

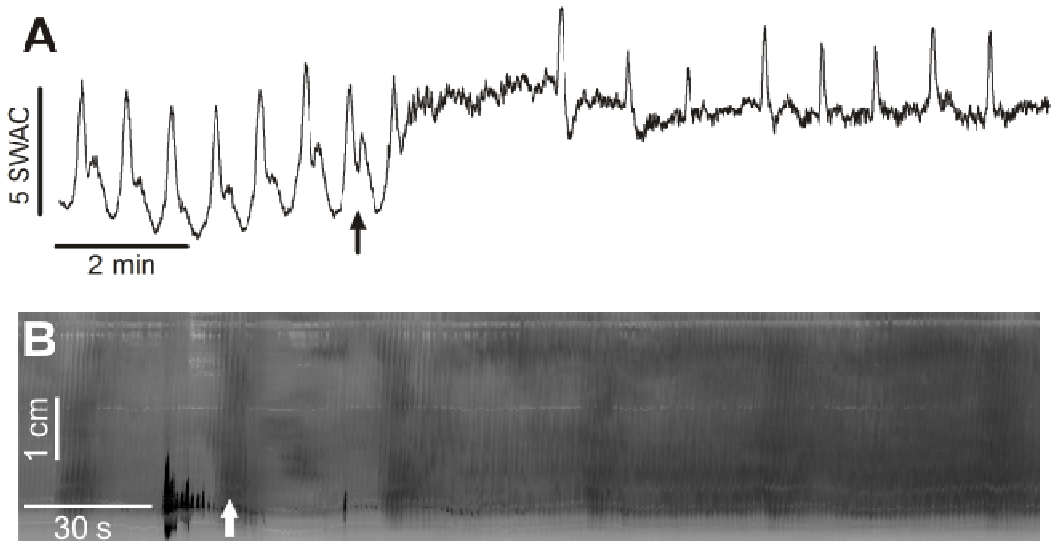

Figure 3.7. Effect of nitric oxide blockade on rhythmic propulsion. Intraluminal pressure recording (**A**) revealed both a tonic increase in baseline pressure and continuation of rhythmic propulsion in the presence of 0.2 mM L-NNA (black arrow). Pressure is normalized to the pressure generated by slow wave associated contractions (SWAC). Spatiotemporal mapping (**B**) consistently revealed the tonic contraction of the intestine after addition of L-NNA (white arrow) but also the continuation of the PMCs. The darker the area on the map, along the grey scale, corresponds with reduced luminal diameter or increased level of contraction.

Table 3.3 The Effect of L-NNA on the Parameters of Rhythmic Propulsion

	Intraluminal Pressure Recording		**Spatiotemporal Mapping**	
	Control	**L-NNA (0.2 mM)**	**Control**	**L-NNA (0.2 mM)**
Frequency (cpm)	1.1 ± 0.2 n = 5	1.3 ± 0.03 n = 3	1.1 ± 0.2 n = 5	1.3 ± 0.1 n = 3
Duration (s)	25.8 ± 2.3 n = 5	22.4 ± 6.8 n = 3	12.3 ± 2.3 n = 5	9.6 ± 1.2 n = 3
Amplitude (%SWAC; %Baseline)	4.5 ± 2.3 n = 5	7.7 ± 2.1 n = 3	31.0 ± 5.3 n = 5	22.0 ± 4.6 n = 5
Proximal Baseline (mm)	N/A	N/A	4.6 ± 0.6 n = 5	4.6 ± 0.4 n = 5
Distal Baseline (mm)	N/A	N/A	4.3 ± 0.6 n = 5	3.5 ± 0.9 n = 4
Velocity (cm / s)	N/A	N/A	0.7 ± 0.3 n = 3	N/A
Frequency of SWAC (cpm)	32.4 ± 5.3 n = 5	34.2 ± 6.9* n = 4	38.6 ± 6.2 n = 5	42.1 ± 9.2 n = 3

* $p < 0.05$

Spatiotemporal mapping of the effect of L-NNA on the propulsive motor pattern confirmed the results obtained via intraluminal pressure recordings. It is clear that L-NNA does not abolish the propulsive motor pattern (Fig. 3.7B) but induces an increased basal contractile state of the circular muscle (Fig. 3.7B). Once again, the resulting PMP pattern had unaltered parameters compared to control (Table 3.3). Upon visual inspection, there seemed to be a location bias where the proximal half contracted more than the distal half of the segment, however empirical data did not support this observation (Table 3.3). There was a statistically significant increase in slow wave associated contractions as measured by the intraluminal pressure recording; however, the increase is not likely to be physiologically relevant due to the absolute magnitude in change. Also, spatiotemporal mapping did not confirm the statistical significance of this observation.

3.3.5 Modifying the propulsive motor pattern with substance P

In cases where rhythmic propulsion was not as stable (2 out of 6), addition of substance P normalized the pattern to resemble a more typical propulsive motor pattern (Fig 3.8A). In the remaining experiments where rhythmic propulsion appeared stable and typical, substance P had little effect on either the intraluminal pressure recordings (Fig 3.8B) or the spatiotemporal maps (Fig 3.8C and D). The elevated control frequency of the PMP was significantly reduced by the addition of substance P to a frequency more in line with typical control parameters. This reduction in frequency was only seen via intraluminal pressure recordings and not spatiotemporal mapping. All remaining parameters, measured from both manometry (Table 3.4) and spatiotemporal mapping,

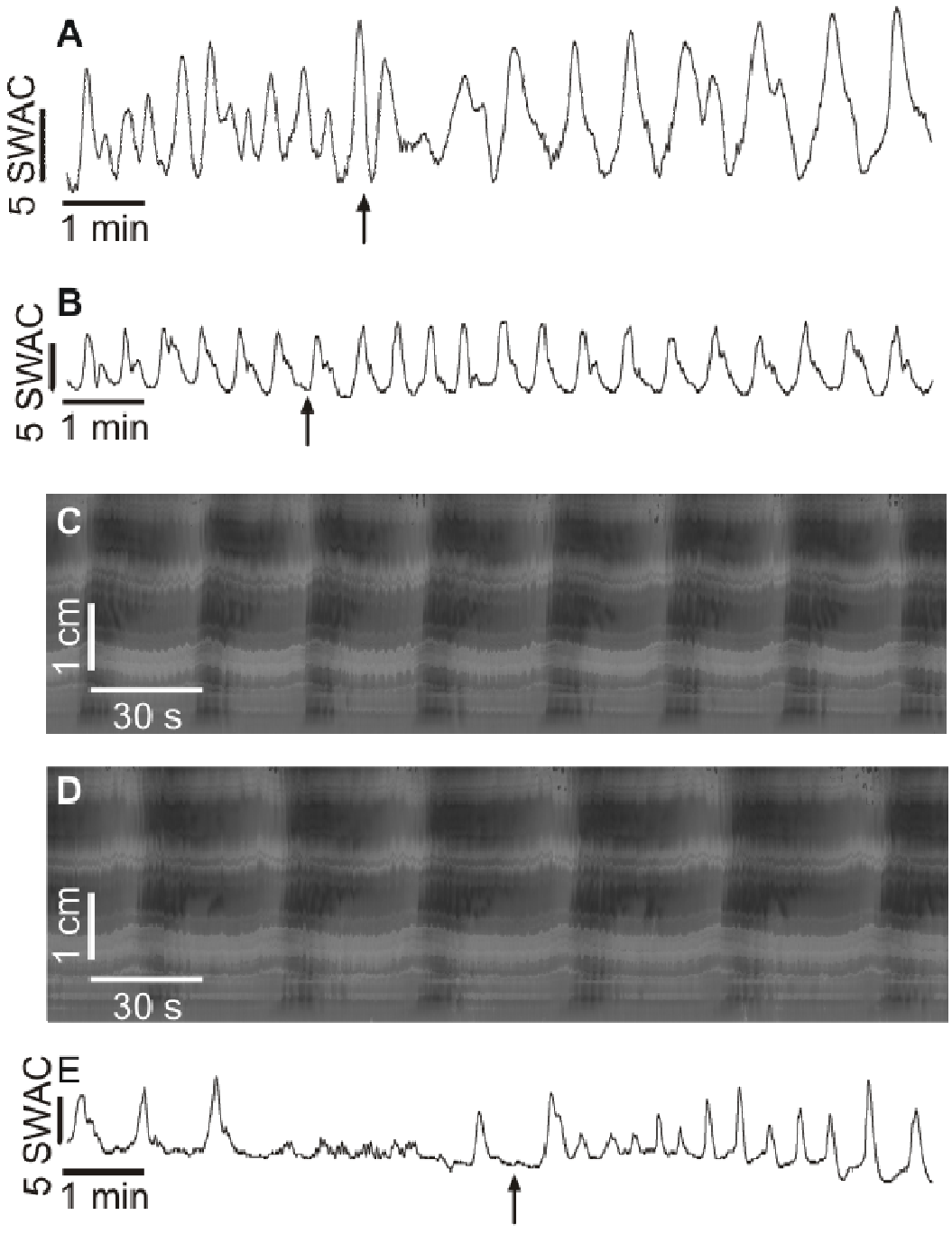

Figure 3.8. Effect of substance P on rhythmic propulsion. Intraluminal pressure recording showed a normalization (**A**) as well as little effect (**B**) in response to 10 nM substance P (black arrows), depending on control activity. Spatiotemporal mapping of control PMP (**C**) and the marginal effect of 10 nM substance P (**D**). Addition of 5 µM carbachol restored (**E**; black arrow) the PMP in jejunal segments where rhythmic propulsion was inhibited by 50 nM substance P.

Table 3.4 The Effect of Substance P on Intraluminal Pressure Parameters of Rhythmic Propulsion

	Control	Substance P (10 nM)	Sustance P (50 nM)
Frequency (cpm)	2.0 ± 0.3 n = 5	1.3 ± 0.1* n = 5	1.1 ± 0.1* n = 5
Duration (s)	23.8 ± 2.8 n = 5	31.4 ± 3.2 n = 5	32.5 ± 3.8 n = 5
Amplitude (%SWAC)	10.1 ± 2.5 n = 5	15.7 ± 3.9 n = 5	12.4 ± 3.1 n = 4
Frequency of SWAC (cpm)	17.6 ± 2.0 n = 6	24.6 ± 6.3 n = 5	25.7 ± 7.1 n = 5

* $p < 0.05$

Table 3.5 The Effect of Substance P on Spatiotemporal Mapping of Rhythmic Propulsion Parameters

	Control	Substance P (10 nM)	Sustance P (50 nM)
Frequency (cpm)	1.7 ± 0.3 n = 5	1.6 ± 0.2 n = 6	1.4 ± 0.2 n = 5
Duration (s)	11.8 ± 1.7 n = 5	15.5 ± 2.9 n = 6	18.5 ± 4.2 n = 5
Amplitude (%Baseline)	42.2 ± 4.9 n = 5	39.9 ± 4.1 n = 6	40.8 ± 8.4 n = 5
Baseline (mm)	6.0 ± 0.5 n = 5	5.5 ± 0.5 n = 6	4.8 ± 0.8 n = 5
Velocity (cm / s)	1.4 ± 0.3 n = 5	1.8 ± 0.3 n = 6	1.6 ± 0.4 n = 5
Frequency of SWAC (cpm)	52.5 ± 1.5 n = 5	50.2 ± 1.7 n = 5	56.6 ± 2.6 n = 5

* $p < 0.05$

(Table 3.5) were unchanged in response to both 10 nM and 50 nM substance P. Finally, in two cases, substance P caused a loss of the PMP which was reversed by the addition of 5 μM carbachol, restoring rhythmic propulsion (Fig 3.8E).

3.4 Discussion

3.4.1 Rhythmic propulsion

The strong, rhythmic nature of small intestinal propulsion is not a novel observation. This pattern of propulsion has been seen by various researchers often studying the role of the enteric nervous system on intestinal motility (Costa et al., 2000; Holzer et al., 1998; Spencer et al., 2001; Spencer et al., 1999). What is novel about exploring propulsion is characterizing the pattern under more physiological parameters and acknowledging the inherent rhythmicity.

Rhythmic propulsion is an inducible pattern that requires a stimulus. This can be thought of as two components, firstly, the physiological stimulus which triggers and modifies propulsion, and secondly, the underlying mechanism which drives the motor pattern. I have demonstrated the inducibility of the pattern: providing intraluminal distention with fluid content can initiate the pattern. Distention is a powerful trigger of peristaltic contractions which has been documented and assessed rigorously (Tonini et al., 1989; Grider, 2003). Distention induced propulsion has been mainly attributed to the peristaltic reflex (Spencer et al., 1999). Under laboratory conditions with short bursts of distention the peristaltic reflex performs well in explaining the resulting contractile activity. However, constant distention, which is more physiologically relevant, has only recently been studied with a growing realization of the rhythmicity with which propulsion

occurs (Holzer et al., 1998; Huizinga et al., 2011). Distention not only stimulates rhythmic propulsion, but the degree of distention also modifies the frequency at which it occurs.

I have demonstrated the graded effect of luminal distention on rhythmic propulsion. In my experiments an increase in muscular distention resulted in an increased frequency of propulsive contractions. Similarly, Spencer et al., (2001) using different experimental conditions, found that there was a graded effect of contractile amplitude and luminal distention. These two results demonstrate the ability of the small intestine to modify content propulsion under differing physiological conditions. These findings are in contrast to the slow wave associated contractions, which are peristaltic but not very propulsive. The slow wave associated contractions are also inducible but the underlying pacemaker remains active even in the absence of contractile activity (Huizinga and Lammers, 2009). Finally, the slow wave associated contraction frequency does not easily change in response to differing physiological states, but rather exists as an ingrained feature of the ICC-MP pacemaker system, which can be modified by targeting direct aspects of the cellular mechanism within ICC (Malysz et al., 2001; Lowie et al., 2011).

Pharmacological experimentation using the small intestine allows for a direct assessment of the role of the enteric nervous system on motility. Carbachol was used to mimic the usually stimulatory effects of acetylcholine. Acetylcholine is released from excitatory motor neurons, which according to the peristaltic reflex model, interacts directly with the smooth muscle to bring about contraction (Costa et al., 2000). Although smooth muscle cells express muscarinic acetylcholine receptors (Chen et al., 2007),

motor neurons additionally project to ICC-DMP, which also express these receptors (Wang et al., 2003; Ward and Sanders, 2001). Acetylcholine is able to raise the resting membrane potential of smooth muscles, thereby increasing the likelihood that they reach threshold and contract (Inoue et al., 1998). The effect of acetylcholine directly on ICC-DMP remains elusive (Wang et al., 2003). The peristaltic reflex model does not account for the origin of rhythmicity inherent to the propulsive motor pattern. In my experiments, carbachol was in contact with the tissue at a constant concentration and yet a rhythmic pattern persisted. A role of acetylcholine in stimulating the pattern is likely; however, the persistence of rhythmicity after carbachol addition suggests that it is the target of carbachol which is the pacemaker, in contrast to the rhythmic release of acetylcholine from excitatory motor neurons. Carbachol alone was only able to induce the PMP in segments that were previously propelling content *in vitro*, which suggests that acetylcholine is required, but not sufficient in initiating propulsion. This is in contrast to the rat colon which exhibits a recovery in rhythmic propulsion with the addition of carbachol, even in the presence of complete neural blockade with lidocaine (Huizinga et al., 2011).

Nitric oxide in the small intestine is primarily responsible for setting the basal tone of the muscle layer (Dijkstra et al., 2004). A significant source of nitric oxide is the inhibitory motor neurons of the enteric nervous system. Nitric oxide is thus an integral part of the peristaltic reflex facilitating the region of relaxation caudal to the triggering bolus of content (Grider, 2003). Since the rhythmicity of the propulsive motor pattern persisted superimposed on top of a tonic increase in baseline pressure in the absence of

nitric oxide (nitric oxide synthase inhibited by L-NNA), it cannot be caused by rhythmic nitric oxide release. Since the contractions of the PMP have an amplitude the same as the tonic increase in pressure, it could be thought that the PMC was achieved just by removing nitric oxide from a short period of time, which is not the case. Thus, neither rhythmic acetylcholine nor nitric oxide release can explain the physiologically relevant rhythmicity of propulsion. Similar to the experiments with carbachol, L-NNA did not alter any of the PMC parameters, affirming their dependence on the pacemaker and not the stimulating signal from the enteric nervous system.

Often publications have focused on mechanisms of motility which ignore input from myogenic sources, and suggest mechanisms which are limited, not entirely based on all scientific findings or not physiologically relevant (Huizinga and Lammers, 2009). This oversimplification has led to assumption of the control capacity of enteric neurons for which there is no evidence, namely the origin of rhythmicity of intestinal propulsion. The origin of rhythmicity of the propulsive motor pattern is likely myogenic, but this does not mean that there is no input from the enteric nervous system. Acetylcholine h plays a prominent excitatory role on intestinal motility and content transit (Kilbinger and Weihrauch, 1982), which is consistent with the stimulatory role of carbachol on rhythmic propulsion that I have demonstrated. In my experiments, the enteric nervous system was required for the PMP because tetrodotoxin abolished the pattern completely. Since carbachol could not restore rhythmic propulsion after addition of TTX, the underlying mechanism must be different from that of the rat colon, where carbachol was sufficient in driving rhythmic propulsion in the presence of neural blockade (Huizinga et al., 2011). It

is likely then that acetylcholine is required for rhythmic propulsion alongside another factor such as substance P.

It is unclear whether substance P plays a stimulatory or regulatory role in rhythmic propulsion. Substance P did not modify any parameters except for frequency. The basal frequency of the PMP in these experiments was elevated compared to typical control values. Substance P normalized the frequency and the consistency of the pattern towards control values. Substance P working through NK_1 receptors (primarily found on the ICC-DMP) is inhibitory with regards to propulsive peristalsis (Holzer et al., 1995). My results agree with their findings but not entirely with the conclusion. In my case, substance P reduced frequency, but the higher concentration did not further lower the frequency past what is typical for rhythmic propulsion in the mouse small intestine. Thus, substance P can only modify rhythmic propulsion when it is not at a physiologically appropriate frequency. However, substance P is excitatory at intestinal smooth muscle cells because it interacts with NK_2 receptors expressed on these cells (Holzer et al., 1995). Therefore, substance P can promote propulsive contractions and simultaneously help set the pacemaker frequency of these contractions.

The results I have presented above provide strong evidence against the functions of the peristaltic reflex in rhythmic propulsion. The enteric nervous system does play a role in rhythmic propulsion from a stimulatory and maintenance capacity, but the rhythmicity associated with the pattern is myogenic. It is possible that increased distention is signaled by neurons through acetylcholine release at ICC-DMP leading to increased frequency of contractions, since the PMP is distention sensitive. Conversely,

substance P release may counter the excitation to help maintain the pattern and may be released in response to reductions in distention. The combination of substance P and acetylcholine to drive propulsive contractions has been seen previously in mouse small intestine; where mucosal stimulation induced propagation of propulsive peristalsis which was attenuated, but not abolished, by antagonizing muscarinic acetylcholine receptors or tachykinin receptors. Using both receptor antagonists concurrently completely abolished all propagation of the contraction (Grider, 2003). Although nitric oxide plays an important role in the peristaltic reflex model, it does not play a role in rhythmic propulsion. Since neither substance P nor acetylcholine can stimulate or recover rhythmic propulsion alone, it is likely that they are both required for the PMP although whether they target the ICC-DMP, smooth muscle or both remains unclear.

3.4.2 Segmentation

The primary role of the segmentation motor pattern is to mix intraluminal contents for greater intestinal absorption (Gwynne et al., 2004B). The underlying pattern shows a high degree of rhythmicity *in vivo* (Cannon, 1902; Wang et al., 2005). Using an *in vitro* model, I have demonstrated that the segmentation pattern follows the rhythmicity of the slow wave frequency and is for that, dependent on ICC-MP. Work in our lab (unpublished) suggests that the rhythmic interference pattern characteristic of segmentation occurs because of interfering electrical slow waves, but the origin and mechanisms are undetermined. Gwynne and Bornstein (2007) showed that nutrients, such as short chain fatty acids, can induce the segmentation motor pattern. This is seen *in vivo* where lipid meals require increased processing time and thus a longer measured intestinal

transit (Higham and Read, 1990). Nutrient induced segmentation was explained as neural in origin; however, neither ICC nor the origin of rhythmicity in the pattern were properly assessed (Gwynne and Bornstein, 2007) which is inappropriate, considering it is already known that the rhythmicity of segmentation follows closely the slow wave frequencies across species (Table 1). Conversely, I have shown that segmentation can be induced in the presence of neural blockade with tetrodotoxin, and thus the pattern has a myogenic origin, whereas, the opposite conclusion was made by Gwynne and Bornstein (2007) who claimed that segmentation was entirely neurogenic without myogenic input. Gwynne and Bornstein (2007) employed a guinea-pig model which has low level slow wave activity compared to other animals, and they admit that it may not represent a true model of segmentation in all other species. The consistency of cholinergic SWACs in the absence of neural activity, and the fact that the slow waves clearly underlie segmentation suggests that these patterns occur on a spectrum which is predominated by SWACSs when acetylcholine acts at ICC-MP or smooth muscle directly. *In vitro*, segmentation occurs in the absence of enteric nervous system activity, however, this does not rule out a potential physiological role for neurons in segmentation. As mentioned above, the nerves were shown important for *in vitro nutrient induced* segmentation. It is thus possible that the pattern is governed by a myogenic mechanism, but is activated physiologically though enteric nervous system sensation of specific luminal contents, such as lipids and proteins (Gwynne et al., 2004B).

4. CACLIUM OSCILATIONS IN ICC OF THE DEEP MUSCULAR PLEXUS

4.1 Introduction

Excitability of intestinal cells, including neurons, smooth muscle and ICC is often experimentally signaled by increases in intracellular calcium which outlast the excitatory event (Tack and Smith, 2004). This calcium signal has given researchers the ability to study the actions of cells participating in intestinal motility on a cellular level. Specifically, a rhythmical change in intracellular calcium in ICC-MP is equivalent in frequency with the frequency and generation of slow waves (Yamazawa and Iino, 2002). Intracellular calcium measurements have revealed a great degree of synchronicity and cooperation of ICC-MP in generating and propagating of slow waves (Lowie et al., 2011). Poorly understood is the role of intracellular calcium oscillations and relations to ICC-DMP electrical pacemaking. Our lab has preliminary data showing that calcium activity in ICC-DMP differs from that of the ICC-MP by a great deal, specifically, ICC-DMP do not flash calcium but rather oscillate it in a polar manner within the cell. Also, ICC-DMP calcium activity is not synchronized between neighboring cells, whereas ICC-MP oscillate intracellular calcium as a network at or near an instantaneous manner as their neighboring ICC (Lowie, 2011).

The ICC-DMP are highly innervated by the enteric nervous system. This innervation has led many to postulate a transduction role of the ICC-DMP, facilitating a communication link between nerves and smooth muscle (Iino et al., 2004). Although anatomically plausible, there are currently no physiological data to provide an explanation for the high degree of innervation of ICC-DMP (Ward et al., 2006).

Similarly, it is known that ICC-DMP are innervated by cholinergic (Wang et al., 2003), tachykinergic (Faussone-Pellegrini, 2006) and nitrergic (Wang et al., 2003) neurons, however, there are little data reporting how acetylcholine, substance P or nitric oxide effect ICC-DMP activity. A recent hypothesis by Huizinga and colleagues (2011) has suggested a dual pacemaker network responsible for rhythmic propulsion in the rat colon. This hypothesis consists of a summative effect between the slow wave generating ICC and a second innervated ICC network in the colon. Likewise, the hypothesis may be extended to the small intestine, which also contains a slow wave generating network of ICC (ICC-MP) and the highly innervated ICC-DMP network.

4.2 Methods

Adult and neonatal CD-1 intestinal preparations were used for all calcium imaging experiments. Information on animal handling can be found in sections 2.1. Information on intestinal preparations and calcium imaging can be found in section 2.3.

4.2.1 Calcium imaging experiments proper

The remainder of the experiment was conducted under minimal light conditions as the imaging agent was light sensitive. Intestinal preparations were removed from the large Krebs' solution reservoir and incubated for 15 minutes with 1 mL Krebs' solution containing 5 µM fluo-4 AM (acetoxymethyl ester), 10 µM probenecid and 0.02% pluronic F-127. Once the dye was loaded, preparations were perfused constantly with fresh Krebs' solution gassed with 95% O_2; 5% CO_2 heated to 37 ^{o}C. Intestinal preparations were viewed using a Nikon eclipse FN1 microscope employing either 20X

or 40X objectives with a GFP filter allowing for the excitation of fluo-4 with light at a wavelength of 488 nm. Digital videos were created using a QuantEM 512SC camera capturing visual images and recorded with Nikon-NIS Elements software loaded on a personal computer. Upon visual identification of ICC-DMP (actively oscillating calcium or quiescent), control videos were recorded followed by the addition of agonists substance P (10 nM), carbachol (5 μM) or phenylarsine oxide (PAO; 0.1 μM), in order to mimic the activity of the tachykinergic and cholinergic enteric nervous system or reduce receptor internalization, respectively. A reduced light exposure protocol was also used where a 0.2 second image was recorded followed by a 0.8 second dark period and repeated for the duration of the experiment to facilitate longer recording periods.

4.2.2 Data analysis

Data were extracted as the fluorescence intensity versus time of specified cells. Cells confirmed as ICC-DMP based on their morphology as bipolar, ovoid cells lying in the same orientation as the circular muscle were circled using the ROI function of the Nikon-NIS Elements software. The software allowed for the data extraction of multiple cells from the same field of view, simultaneously. Due to the preliminary nature of the experiments, no further transformation was done to the data, which is not inconsistent with previous publications (Lowie et al., 2011). Likewise, the exploratory nature of the experiments did not present enough data to perform adequate statistical analysis.

4.3 Results

4.3.1 Neonatal ICC-DMP

The ICC-DMP, of neonatal small intestine, cycled calcium from side to side within a cell at ever-changing rates. This contrasts with the calcium cycling in ICC-MP which exhibited a flash of calcium throughout the entire cell body at the same time. The calcium oscillations did not correspond with those of neighboring ICC-DMP and thus synchronization of oscillations was not observed. In an interesting experiment, after addition of 10 nM substance P, both calcium oscillation frequency, as well as baseline calcium increased (Fig. 4.1A). With regards to baseline $[Ca^{2+}]_i$, the observed increase returned back to normal shortly after elevation, but was followed by subsequent increases in a rhythmic fashion (Fig. 4.1A). In some instances, addition of substance P increased basal calcium without a return to normal or the presence of rhythmic changes (Fig 4.1B). By using the reduced exposure protocol, experiments in excess of 10 minutes revealed a second phasic elevation in calcium longer in duration than those previously seen, however at a much lower frequency (Fig. 4.2). Coadministration of PAO to reduce NK_1 receptor internalization did not reveal any obvious change in duration of low frequency rhythmic activity initiated by 10 nM substance P.

4.3.2 Adult ICC-DMP

Tissue preparations exposing the deep muscular plexus in adult mice yielded few active ICC-DMP. Imaging revealed either no obvious ICC-DMP, or showed ICC-DMP which were not oscillating calcium. The latter group exhibit characteristics of properly loaded cells, but neither substance P nor acetylcholine initiated calcium activity in these

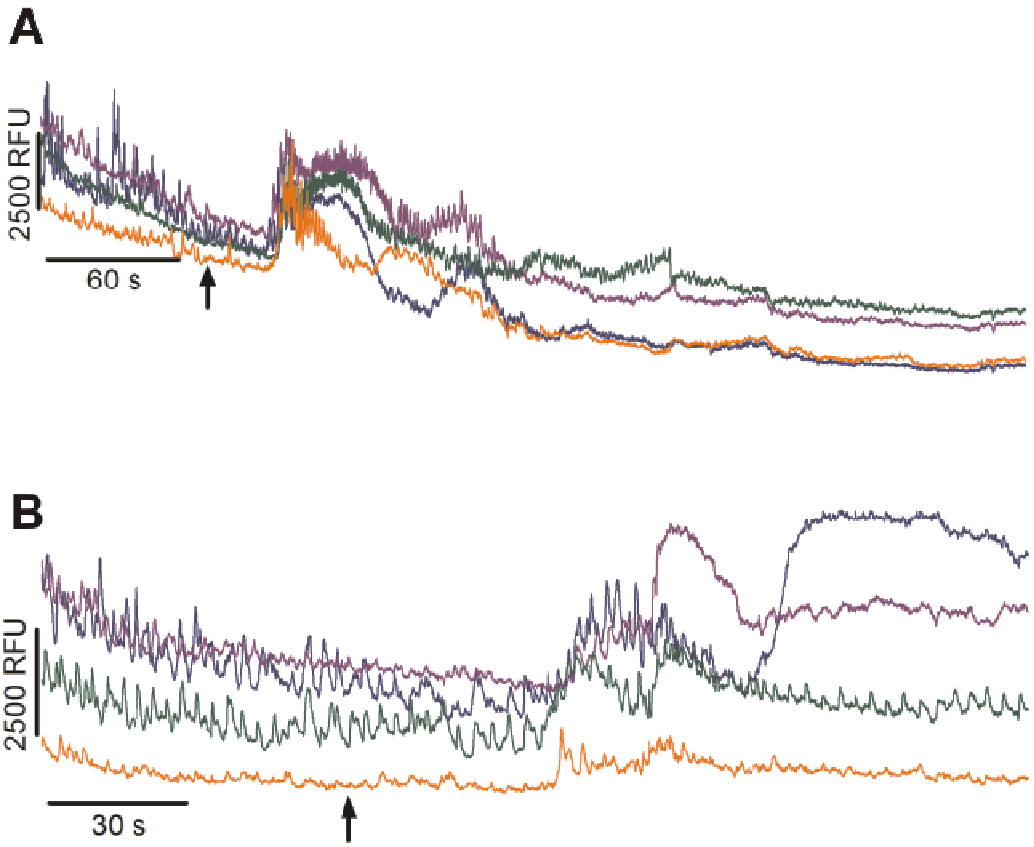

Figure 4.1. Effect of substance P on intracellular calcium concentration of neonatal ICC-DMP. Each line indicates the intensity of fluorescence (relative fluorescence unit - RFU) of fluo-4 bound to calcium, over time in a separate ICC-DMP. Addition of 10 nM substance P (black arrow) induced a short-lived rhythmic increase in basal florescent intensity (**A** and **B**). In some instances, the increase in basal florescence was tonic (**B**).

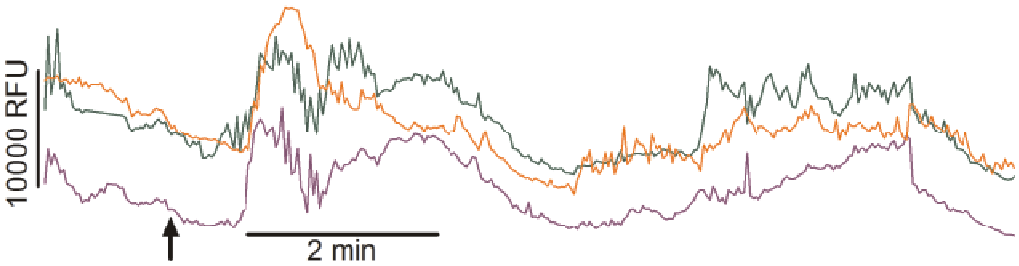

Figure 4.2. Effect of long-term substance P exposure on intracellular calcium concentration of neonatal ICC-DMP. Each line indicates the intensity of fluorescence (relative fluorescence unit - RFU) of fluo-4 bound to calcium, over time in a separate ICC-DMP. Addition of 10 nM substance P (black arrow) induced a short-lived low frequency pattern. Also seen was a second lower frequency pattern of oscillating baseline calcium levels.

cells. In ICC-DMP that had active calcium oscillations, substance P increased the frequency of oscillations and the baseline calcium in segments of the cell (Fig. 4.3B), but the net effect on the whole cell seems to be unchanged (Fig. 4.3A). The independence of these cells has yet to be elucidated as preparations have only yielded one or two active cells at a time and not entire networks as seen with the neonatal preparations.

4.4 Discussion

Although a relatively new technique, intracellular calcium imaging has allowed for a precise measurement of the activity found in ICC-MP. A similar assessment of ICC-DMP has not been performed. As mentioned above, an increase in intracellular calcium in ICC-MP is a marker of the pacemaker potential (Torihashi et al., 2002). ICC-DMP also generate an electrical signal in some instances (Jiménez et al., 1996), but it is unclear if this activity is associated with a rise in intracellular calcium. ICC-DMP shifted calcium concentration from side to side within the cell body rather than flashing a simultaneous increase in calcium concentration though out the entirety of the cell body like in the ICC-MP. The origin of calcium in ICC-DMP is currently unknown. There may be an unequal opening of extracellular calcium channels or intracellular calcium stores, the latter being the primary source of calcium in ICC-MP activity (Lowie et al., 2011). Since there is rarely equal calcium activity across an individual ICC-DMP at one time, it is not likely that this cycling signals electrical activity. The large rhythmic increase in baseline calcium induced by substance P may signal calcium activity because the increase in calcium was uniform throughout the cell. Another major difference from ICC-MP is that

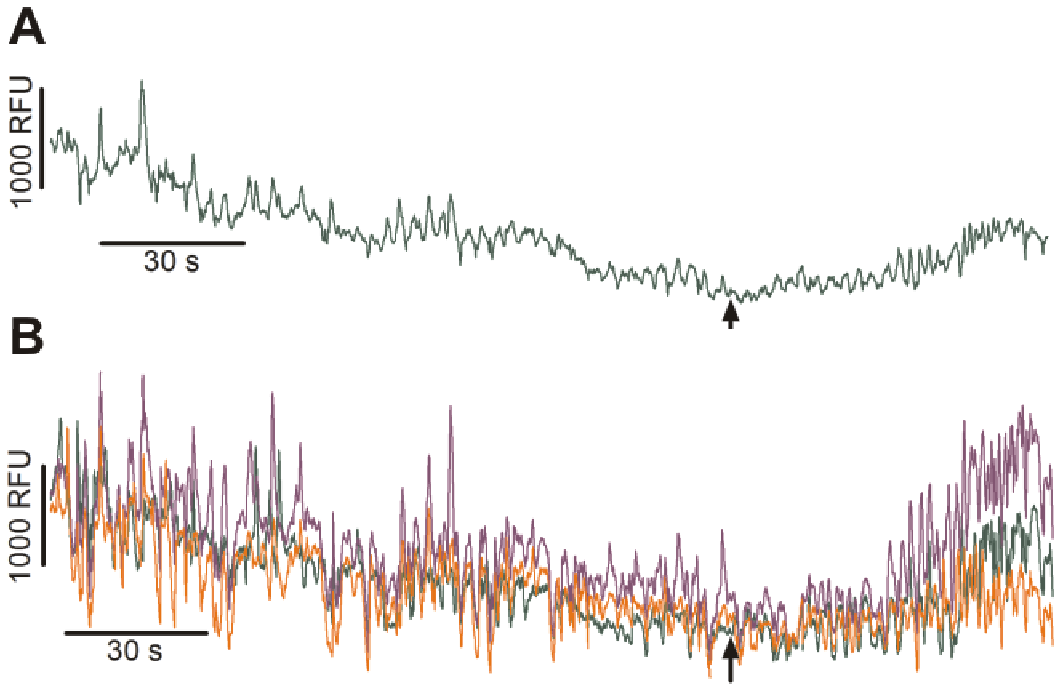

Figure 4.3. Effect of substance P exposure on intracellular calcium cycling within adult ICC-DMP. The overall fluorescence activity representing intracellular calcium concentration bound to fluo-4 of a single ICC-DMP (A) and its response to the addition of 10 nM substance P (black arrow). Fluorescence within the same cell split into thirds (one line for each; **B**) enhancing the visibility of calcium activity upon addition of 10 nM substance P (black arrow).

the ICC-DMP do not synchronize calcium cycling between neighboring cells. This is interesting because the cells have a high degree of coupling as judged by the presence of gap junctions between one another in the network, and also with smooth muscle cells (Kobilo et al., 2003). The only synchronization seen between neighboring ICC-DMP was the rhythmic rise in baseline intracellular calcium in response to substance P. This suggests that ICC-DMP activity may be signaled by changes in baseline calcium and not the individual internal calcium oscillations. Calcium oscillations did not persist for a very long time. The termination of the pattern was not a result of tachykinin receptor internalization, but may be attributed to receptor desensitization (Defea et al., 2000). The termination could also be due to a complex inhibitory feedback system, from the enteric nervous system preventing inappropriate cellular actions to absent content or distention stimuli, as substance P was artificially introduced to the system.

Neonatal ICC-DMP calcium activity was quite abundant, however, the adult ICC-DMP were rarely spontaneously active. This may be a result of the further development of both the ICC and neural networks between neonates and adults. The adult ICC-DMP are highly innervated by both excitatory and inhibitor motor neurons (Wang et al., 2003). The inhibitory motor neurons may be influencing the lack of activity seen in these cells in adulthood. Enteric nerves are fully formed by post natal day 10 in mice, however, their function is not developed to the point of an adult neural system (Foong et al., 2012). Secondly, it is likely that the ICC-DMP pacemaker is inducible and not always present like the ICC-MP produced slow wave (Wang, 2005), as seen by the increase in calcium activity in response to substance P and no spontaneous rhythmicity. This suggests that the

neural connections form the basis for triggering this inducible pacemaker network. Excitatory motor neurons respond to input from sensory neurons, in response to stimulus such as distention or nutrients (Schemann and Mazzuoli, 2010; Mourad and Saadé, 2011). If the correct stimulus for ICC-DMP network stimulation is not present in the calcium imaging experiments performed, it is reasonable to expect the cells to be quiescent. Finding the correct conditions/stimulants of ICC-DMP calcium and pacemaker activity will allow for a better understanding of how this ICC network performs under physiological conditions and which motor patterns it may be controlling or modifying.

5. CONTRACTILE PATTERNS OF THE HUMAN PYLORIC REGION

5.1 Introduction

Understanding the anatomy of the gastrointestinal system is paramount to understanding how motor functions originate and persist. One of the key cell types involved in rhythmic motor patterns in both the stomach and the small intestine are the ICC. The ICC set the frequency of rhythmic motor patterns as well as aid in the propagation of motor patterns (Huizinga and Lammers, 2009). Animal studies have shown that the ICC networks found in the antrum of the stomach are completely independent of the intestinal ICC networks (Wang et al., 2005). Electrical activity is unable to cross the pylorus either from the stomach to the intestine or vice versa (Wang et al., 2005). These two findings fit well with our understanding of intestinal and stomach motor patterns, specifically the high frequency with which contractions occur in the duodenum compared to those of the antrum (Wang et al., 2005). What remains unclear is if the motor patterns from the stomach and intestine can interact in other ways other than through ICC. Also, most of our understanding of anatomical features of the ICC networks has come from animal models, so studying these cells in the human pyloric region is important to understanding normal human physiology. The following is a miniature project designed to assess the rhythmic contractile patterns of smooth muscle strips from the antrum, pylorus and duodenum. The project was undertaken to correlate contractility data with the anatomical localization of ICC in the region of the pylorus. I hypothesized that both the antrum and duodenum would respond to stimulation with a strong rhythmic

pattern with the antrum displaying a frequency much slower than that found in the duodenum.

5.2 Methods

Adult circular muscle strips were used for all muscle strip experiments. Methods on tissue acquisition and muscle strip preparation can be found in section 2.4.1.

5.2.1 Muscle strip experiments proper

Once the tissues were strung up in individual organ baths, they were warmed to 37^{o}C and a resting tension of 10 mN was applied to each. Tissues were allowed a 1 hour equilibration time and then successive 2 mN stretches were applied to the tissue in 12 minute intervals. After four successive stretches, regardless if phasic contractions were stimulated, 1 μM carbachol was added to all tissues and remained in contact with the tissue for 25 minutes.

5.2.2 Data analysis

Due to the dearth in stretch-induced activity in muscle strip experiments, analysis was only done on carbachol-stimulated contractions. Frequency of contractions was calculated over a 10 minute interval after a 2 minute equilibration period post carbachol addition. Where appropriate, a second frequency calculation was done to assess the reduction/stabilization in tissue contractions. Finally, in pyloric strips exhibiting tonic contraction caused by 1 μM carbachol, the strength (amplitude) of these contractions was also calculated, subtracting the preset baseline tension prior to carbachol addition.

5.3 Results

5.3.1 Effect of stretch on circular muscle strip contractility

Although circular muscle strips responded to stretch, this did not happen in a consistent manner. Stretching of tissues from 10 – 18 mN did not evoke contractions in most strips. Figure 5.1 shows an example of increasing stretch and response in the pylorus where tissues responded to stretch in 2 out of 10 cases. The antrum and duodenal muscle strips only responded with contractions to stretch in 2 out of 9 and 1 out of 10 strips, respectively.

5.3.2 Effect of carbachol on circular muscle strip contractility

Antrum (5 of 10 strips), pylorus (4 of 10 strips) and duodenum (7 of 10 strips) muscle strips responded more consistently to carbachol, even in cases where there was no response to stretch. The distal antrum showed a strong rhythmic pattern at a frequency of 4.3 ± 0.9 cpm (n =4). Following the initial response, the frequency was either maintained (Fig 5.3A) over a period of 25 minutes or slowed down (Fig. 5.2A) to 2.4 ± 0.7 cpm (n = 5). The pyloric circular muscle strips reacted to carbachol consistently with a marked increase in tone, not seen in distal antrum or proximal duodenum (Fig 5.2B and Fig 5.3B). The maximal amplitude of the tone was 39.7 ± 6.8 mN, compared to 24.2 ± 4.7 mN initial tone. The tonic contraction progressed into a pattern of stable phasic contractions with a frequency of 12 ± 3.2 cpm (n=3) (Fig 5.2B). This frequency was either maintained (Fig 5.3B) or was seen to halve (Fig 5.2B). The duodenal circular muscle strips reacted to carbachol consistently with a pattern of strong rhythmic contractile activity with two frequency groups; 12.1 ± 0.5 cpm (n = 5) (Fig 5.2C) or 4.0 ±

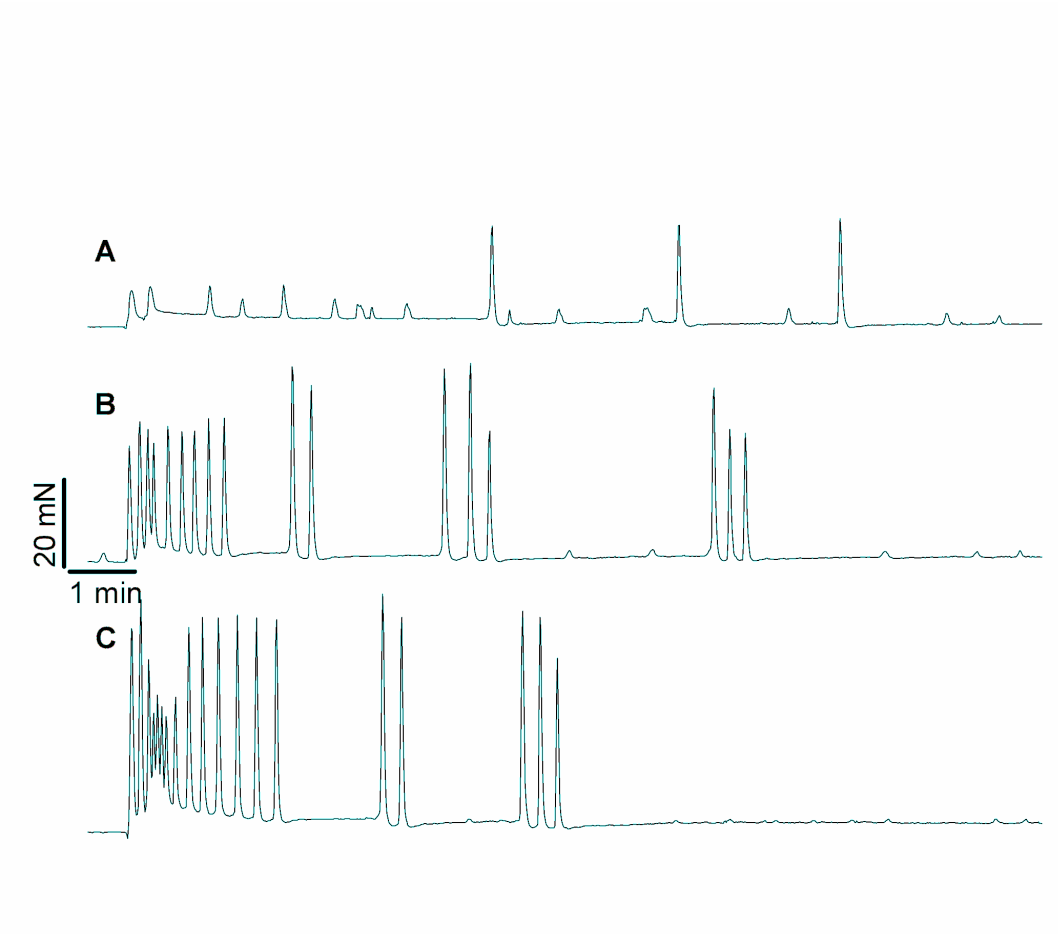

Figure 5.1. Effect of stretch on a human pyloric muscle strip. Data are from a single pyloric muscle strip presented as force over time. The first stretch (**A**) induced some contractions which persisted over time. Additional stretches (**B** and **C**) increase the response time and strength of contractions of the human pylorus.

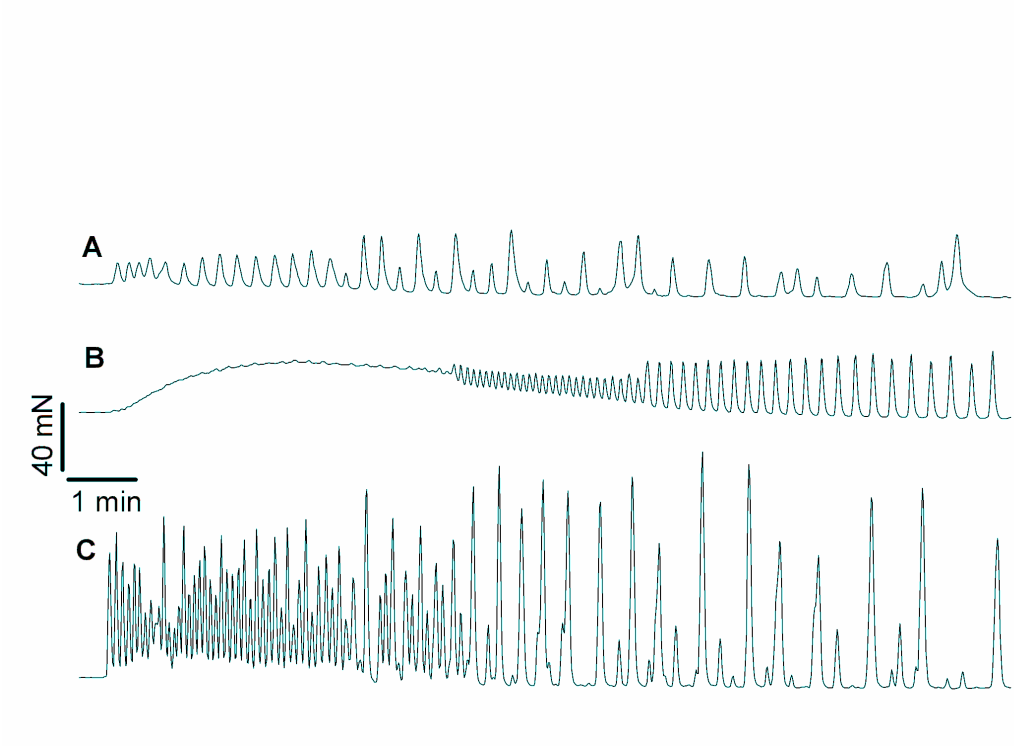

Figure 5.2. Evolving effect of carbachol on the human circular muscle strips. Addition of 1 µM carbachol at the beginning of the traces induced phasic contractions in both the distal antrum (**A**) and proximal duodenum (**C**), but a tonic contraction in the pyloric strip (**B**). All initial patterns devolved over time with a reduction in force and frequency of contractions. Data obtained from the same individual for all three traces.

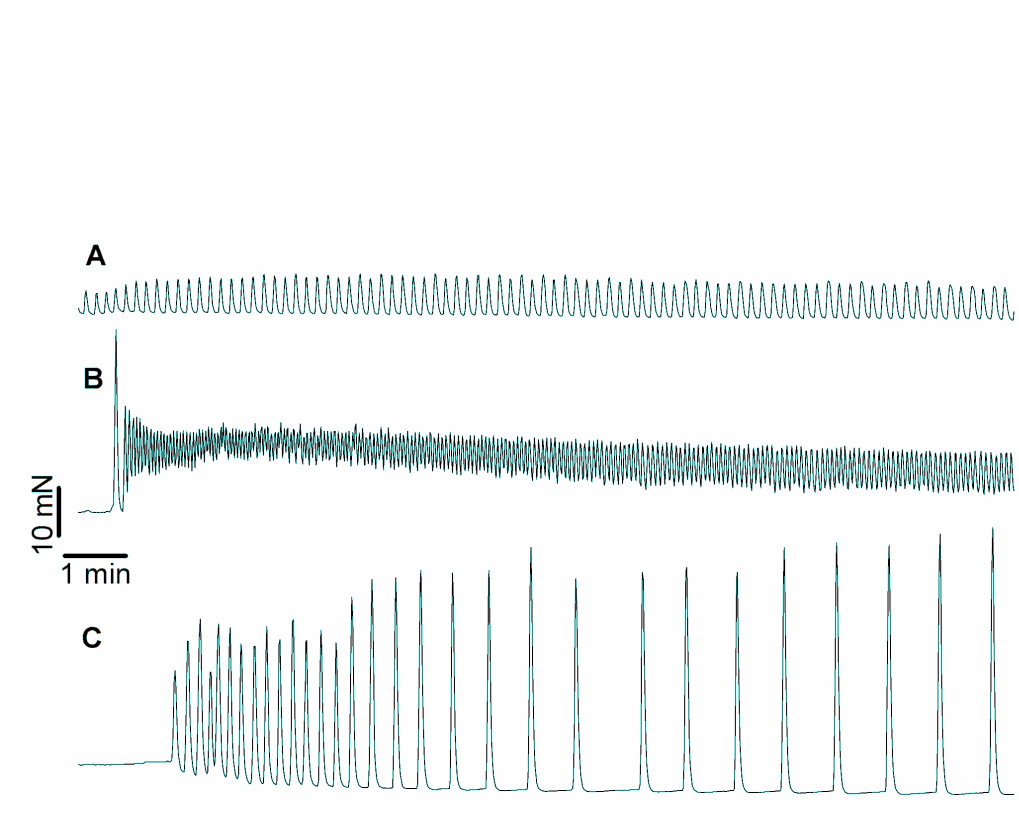

Figure 5.3. Stable effect of carbachol on human circular muscle strips. Addition of 1 µM carbachol at the beginning of the traces induced phasic contractions in the antrum (**A**), pylorus (**B**) and proximal duodenum (**C**) that were consistent over time. The phasic contractions of the pylorus were superimposed on top of a tonic contraction in this strip. Data obtained from the same individual for all three traces.

0.7 cpm (n=3) (Fig 5.3C). These two groups developed over a 25 minute period into a single rhythmic motor pattern of 1.6 ± 0.2 cpm (n=8) (Fig 5.2C and Fig 5.3C).

5.4 Discussion

The pylorus is a circular muscle sphincter designed to hold content in the stomach facilitating the physical break-down of content. The pylorus also controls the release of content into the duodenum (Ramkumar and Schultze 2005). These distinct physiological processes require differing motor patterns, thus it is not a surprise to find differing cellular anatomies of the three regions governing these patterns. Rodents have a distinct separation of pacemaker ICC-MP in the stomach and small intestine separated by the pylorus which did not contain these cells (Wang et al., 2005). The findings of a concurrent anatomical study from which muscle strips for my data were provided, did not have the same distinction in ICC networks in humans. The stomach contained low levels of ICC-MP, which was also seen in the pylorus itself, unlike the rodent (Wang et al., 2005). Interestingly, the proximal duodenum did not display ICC-MP or ICC-DMP, as is typically seen in rodents (Wang et al., 2005). Thus, the similarities between antrum and pylorus, and the lack of ICC in the duodenum, did not fully explain the distinct motor patterns that are required by each muscle region.

Although the underlying pacemaker physiology of the human pyloric region was not as distinct as predicted, the functional contractility data yielded typical distinct patterns in each of the three regions studied. The stomach requires a great deal of force to break down large pieces of food into more manageable sizes, which is achieved via rhythmic low frequency high powered contractions (Cullen and Kelly, 1993). This

contrasts with the proximal duodenum where motility patterns only need to mix content to maximize intestinal contact with content for absorption. This is achieved with weak rhythmic contractions that are at a higher frequency than the stomach (O'Grady et al; Erickson et al., 2009; Schulze, 2006). The muscle strip data I have presented confirms the frequency difference between stomach and duodenum, although some duodenal strips had frequencies closer to that of the stomach. The absolute frequency values compare favourably with *in vivo* physiological studies where the stomach and duodenal contractions occurred at a rate of 2.8 (O'Grady et al., 2010) and 15.8 (Erickson et al., 2009) per minute, respectively. The fact that there are few ICC in the duodenum of samples studied suggests that there should not be rhythmic contractions at slow wave frequencies in these strips, which was not the case. It was difficult to determine a distinct border between the pylorus and duodenum due to a large population of ICC-MP in this region (data not published). It is likely that some of these ICC were included in the muscle strip preparations and thus provided the pacemaker for phasic contractions. Although anatomically difficult to distinguish, the duodenum strips never exhibited a tonic contraction, and thus can be claimed confidently to originate from the small intestine and not the pylorus.

Under normal circumstances, the pylorus remains closed (contracted) far more than it is open (relaxed) (Desipio et al., 2007). The function of the pylorus is thought not to be primarily governed by gastrointestinal pacemakers, but rather by direct innervation (Allescher and Daniel, 1994). This is likely true for rodents where no ICC-MP pacemaker activities were found. However, my data suggest that in humans, a myogenic pacemaker

might be an important part of sphincter physiology. The fact that rhythmic activities of the antrum and pylorus were clearly distinct might be due to a differing neuronal regulation of the pacemaker activities. Acetylcholine stimulates tonic contraction to keep the pylorus closed, achieving the compartmentalization required for stomach digestion (Allescher and Daniel, 1994). Addition of carbachol in my experiments mimicked the tonic contraction as would be expected from this muscle layer. Interestingly, there were phasic contractions superimposed on top of the tonic contraction of the pyloric muscle strips, which has previously been seen in humans via manometry recordings (Friedenberg et al., 2008). These phasic contractions are likely attributed to the low levels of ICC-MP expressed (data not published) in the human pylorus. The role of the low level ICC-MP and resulting phasic contractions in the pylorus is not clear, but it may facilitate low level release of stomach content into the duodenum between typical pyloric relaxation and stomach emptying events. Although the rodent model clearly links anatomical features with motor function, the human pyloric region is not as easily defined, but I have shown that it still maintains its proper underlying motor patterns in isolated circular muscle strips.

6. EFFECT OF E. COLI NISSLE 1917 ON RAT COLON CONTRACTILIY

6.1 Introduction

Symptomology of gastrointestinal disorders almost always involves negative effects in motility. The most common motility issues are constipation and diarrhea which effect individuals of all ages (Liu, 2011). Often these symptoms present with no etiology or as part of a complex disease, such as inflammatory bowel disease, for which the underlying cause is still unknown (Obermeir et al., 2010). There has been a recent drive towards understanding the role of intestinal bacteria and normal gastrointestinal health. It has led to the postulation that probiotics may be used to help promote a healthy intestine-bacteria symbiosis, and potentially alleviate symptoms of constipation (Valerio et al., 2010). One such probiotic is *Escherichia coli* Nissle 1917 (Nissle), which is currently used in some European countries to help alleviate constipation (Sonnenborn and Schultze, 2009). Nissle has been tested in clinical studies where it increases stool frequency and thus alleviates constipation compared to control (Sonnenborn and Schultze, 2009). However, the studies have yet to elucidate how the motility modulating effects occurred. Nissle has also been studied on *in vitro* human colonic muscle strips, where it acts to increase contractile activity (Bär et al., 2009). The same study also showed that the effect was not neurally mediated and was likely a direct effect on smooth muscle (Bär et al., 2009). The true biological mechanism of action for Nissle on smooth muscle contractility is yet to be elucidated. How the increase in contractility might affect the motor patterns of the colon is currently being studied. The following miniature study was conducted as a supplement to a motility study being performed using rat colon. The purpose of the mini

study was to confirm the data of the human study as well as further elucidate the basic mechanism of action of Nissle on colonic smooth muscle. I hypothesized that the effects of Nissle would be excitatory on smooth muscle contractions, and that the effect would be independent of the enteric nervous system, as well as the colonic mucosa and submucosa.

6.2 Methods

Adult Sprague-Dawley rat colonic circular muscle strips were used for all the following muscle strip experiments. Information on animal handling can be found in sections 2.1. Information on colonic strip preparation can be found in section 2.4.2.

6.2.1 Muscle strip experiments proper

Once the tissues were strung up in individual organ baths, containing gassed Krebs' solution, tissues were warmed to 37^{o}C and a resting tension of 10 mN was applied to each. Tissues were allowed a 1 hour equilibration time and then stretched back to a 10 mN baseline. Since the contractile pattern of interest was not neurally mediated, 1 µM lidocaine was added to negate the effects of the enteric nervous system. A 15 minute control period after lidocaine addition was recorded followed by three successive additions (1:300 dilution, 1:200 dilution and 1:150 dilution) of either conditioned (containing dissolved bacterial products) or unconditioned (bacterial growth medium only) media. Between each addition a 12 minute equilibration and record period occurred. At the end of the final 12 minute period, 10 µM D-600 was added to halt all contractions.

6.2.2 Data analysis

Spontaneous contractions were usually present after the 1 hour equilibration period, thus control data, including frequency, force of contraction (amplitude) and area under the curve (AUC) were calculated for the final 10 minutes of the 15 minutes control period. Frequency was calculated as the number of contractions during the 10 minute recording period divided by 10 minutes. Amplitude was calculated as the average max force of contraction for those contractions falling in the 10 minute recording period minus the baseline tension directly before each contraction. Finally, the area under the curve was calculated as the integral of the force-time recording over the same 10 minute period. The same procedure was applied for calculating frequency, amplitude and AUC for the 10 minute recording period of each medium addition. A final area under the curve was calculated after the addition of methoxyverapimil, which was subtracted from each AUC value calculated to remove the artificially created baseline tension. Data are presented as mean ± SEM from three animals; the addition of treatment with conditioned and unconditioned media were performed in triplicate.

6.3 Results

Most muscle strips displayed strong rhythmic contractions after the one hour incubation period. These high-amplitude low-frequency contractions were unaffected by neural blockade using lidocaine (Fig. 6.1). The frequency of contractions, after the addition of lidocaine, was 0.6 ± 0.02 cpm (Fig 6.2). Addition of the two lowest concentrations of media had no effect on the contractions. The 1:150 dilution of conditioned media showed

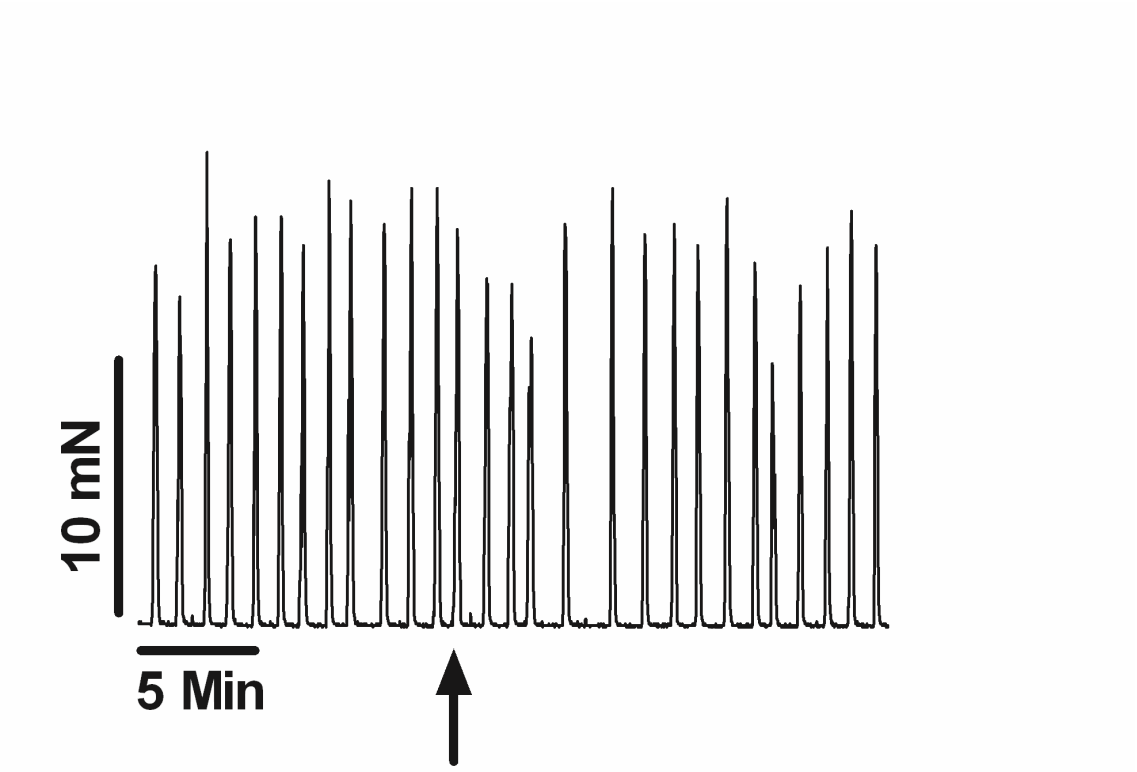

Figure 6.1. Effect of neural blockade on high-amplitude low-frequency contractions of the rat colon muscle strips. Representative force versus time graph of a rat colon circular muscle strip. Addition of 1 μM lidocaine (black arrow) showed no effect on either the frequency or the force of contractions of the high-amplitude low-frequency contractions.

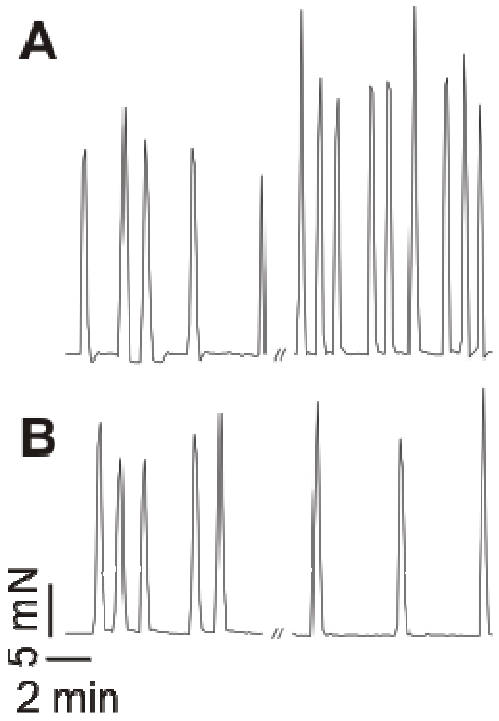

Figure 6.2. The effects of conditioned (A) and unconditioned media (B) on muscle strips from the colon devoid of mucosa. Tissues were treated with lidocaine (1 μM) (shown as the first 10 minutes of the figure) and then cumulative additions of treated or untreated media up to a dilution factor of 1:150 (second 10 minutes of the figure). Addition of lower concentrations of treated media lasted 30 minutes (not shown; period represented with the double slash).

excitatory effects for all parameters calculated (Fig 6.2). Frequency of contractions increased by 101% ± 39 compared to control, whereas the unconditioned media caused a decrease of 18% ± 14 of control (Fig 6.2). Conditioned media increased the amplitude of contractions by 67% ± 11 and the unconditioned media only had a 6% ± 6 effect on amplitude. Finally, the overall force of contraction increased 172% ± 7 of control in response to the conditioned media. The unconditioned media increase the overall force by only 52% ± 14 of control.

6.4 Discussion

The high-amplitude low-frequency contractions responsible for content propulsion are thought to be governed by a secondary, non-neural pacemaker (Huizinga et al., 2011). I have confirmed via muscle strip contractility that this is the case, as lidocaine did not have an effect on these contractions. Physiologically, however, a neural component may be required for propagation of the contractions, as they are abolished via neural blockade in a rat colon motility study (Huizinga et al., 2011). However, the same authors showed that there is still some rhythmic activity in the proximal colon at the same frequency as the propulsive contractions. It is likely that the lidocaine insensitivity, in my experiments, is due to the proximal location from where the strips were taken. Interestingly, others have also observed the neural-independent contractions in human colonic muscle strips (Gonzalez and Sarna, 2001). Several authors have shown the importance of excitatory neurotransmitters on propulsion in the colon, and this may attest to the inducible nature of the pattern. As Huizinga et al., (2011) have demonstrated, colonic propulsion is

dependent on a myogenic pacemaker, but still requires a cholinergic stimulus to persist, even when all other neural activity is blocked.

The motility experiments performed in conjunction with the muscle strip experiments provided support for the use of Nissle to relieve idiopathic constipation. Martz (2011) showed that there was an increase in frequency and intraluminal pressure generated by propulsive contractions in the colon, in response to Nissle. The increases in the parameters of propulsion were superimposed on the decrease in motility seen in response to the unconditioned media, further emphasizing the excitatory motor effects of Nissle. My muscle strip data confirmed the results seen in the motility study (Martz, 2011) where frequency, amplitude and overall contractile force were augmented by Nissle. The motility study did not provide definitive evidence for the mechanism by which Nissle acts. My study, performed in the presence of lidocaine, and using tissues devoid of mucosa and submucosa suggests that the conditioned media is interacting directly with the smooth muscle or the pacemaker cells. Considering the barrier function of the mucosal layers, it remains uncertain if the results seen via contractility and motility studies are clinically relevant, since the contractility data were obtained in tissues which lacked this barrier. Likewise, the bacterial products were exposed to the smooth muscle layers and not the lumen during the motility study. Conversely, a recent study (Wikoff et al., 2009) showed that many bacterial products are absorbed and are detectable in the circulation of the host. These molecules may reach pharmacologically relevant concentrations, allowing them to interact with the smooth muscle, ICC or enteric nervous system via the blood steam. This pathway eliminates the condition of direct luminal

contact with each cell type and renders the mucosal barrier less of an obstacle for these molecules.

Bacterial products range from simple molecules, like sugars and short chain fatty acids, to large complex proteins (Macfarlane and Macfarlane, 2012). The molecules produced specifically by Nissle have not been isolated or characterized. A better understanding of the Nissle compounds with motility effects could lead to better drugs and a better understanding of their biological target. For example, a recent study showed that bacterial products from *Bifidobacterium longum* NCC 3001 is able to directly interact with the enteric nervous system (Bercik et al., 2011) and potentially bring about motor changes through this mechanism. Others have shown that molecules, such as short chain fatty acids, promote segmentation (Gwynne and Bornstein, 2007; Gwynne et al., 2004B), reducing propulsion and thus a potential starting point to finding better treatments for diarrhea. My results have provided the basis for understanding the mechanism of Nissle products on colonic motility and may spur further research into the pharmacological targets for treating constipations and diarrhea.

7. GENERAL DISCUSSION

7.1 Summary of findings

The focus of my research has been on the rhythmicity of motor and contractile patterns in the mouse small intestine, human stomach, pylorus and small intestine, as well as the rat large intestine. All segments studied, as well as calcium imaging preparations, displayed rhythmicity inherent to the organ of study. The origin of rhythmicity was not always the same across species and locations, but the presence of rhythmic patterns in the gastrointestinal tract is undeniable.

The major focus was to better understand rhythmic propulsion in the mouse small intestine. I have shown that the propulsive motor pattern is an inducible pattern that often responds to intestinal wall distention. The underlying mechanism of rhythmic propulsion, based on the evidence from my research, requires input from the enteric nervous system, as well as ICC. Unlike rhythmic propulsion in the colon (Huizinga et al., 2011), acetylcholine, together with myogenic control systems, are insufficient for the PMP in the mouse small intestine. It seems that the enteric nervous system must provide both substance P and acetylcholine for the PMP. Acetylcholine may act directly on ICC-DMP, as revealed from the calcium imaging data, to regulate the frequency of the pacemaker. Acetylcholine may also act on ICC-DMP, and perhaps smooth muscle cells, to help bring the propulsive electrical signal to threshold for smooth muscle contraction. The appearance of rhythmicity, even in the presence of constant concentrations of carbachol or substance P, points to a myogenic origin of rhythmicity. Inhibitory nitric oxide does not play a role in the propulsive motor pattern, as the pattern persisted in the absence of

nitric oxide production. Excitatory motor neurons are required because blocking neural transmission abolishes the propulsive motor pattern.

Rhythmic patterns were also prominent in the human antrum, pylorus, duodenum and rat colonic muscle strips. The human patterns were unique from one another, confirming the underlying roles of each tissue type. The pattern of ICC expression did not fully predict the observed contractile patterns, and thus the true origin of rhythmicity of these patterns remains unclear. The rhythmicity of the low-frequency pattern measured in the rat colon muscle strips was unaffected by neural transmission blockade, which was the opposite result from the related motility study. The discrepancy may be a result of the proximal nature of the strips used, as this is the region under the least amount of inhibitory neural influence. Finally, the procontractile effects of Nissle on circular muscle strips were in agreement with the prokinetic effects observed in the colonic motility study. The mechanism of action remains elusive, however, the procontractile effects are not likely to be neurogenic as neural transmission was blocked during the experiments.

Final conclusions:

The studies conducted during my thesis work have focused on the interactions between myogenic and neurogenic control systems. My research and a careful assessment of the literature have convinced me that only by full recognition of the two control systems will gastrointestinal motor patterns be fully explained. This is critical for drug development, since it is important to find targets for modifying motor activities.

Rhythmic propulsion in the mouse small intestine requires both substance P and acetylcholine stimulation from enteric motor neurons, but the rhythmicity of the pattern is myogenic with ICC-DMP likely being the pacemaker. The mouse small intestine experiments showed that myogenic and neurogenic control systems cannot be seen as separate systems which are independent and summative. The neural control system can induce pacemaker activity and so a complicated, but intriguing interdependent control system governs propulsion.

Unique rhythmic motor patters of the distal antrum, pylorus and proximal duodenum are not easily explained by simply assuming that separate ICC pacemaker systems give the three regions their independent activities. The hypothesis from our work is that ICC networks can be present and similar in regions such as distal antrum and pylorus, but that their distinct patters are due to differences in innervation of these pacemaker systems. It has been amply demonstrated (unpublished data) that the pylorus is uniquely innervated, which is consistent with my findings.

Nissle products have procontracile effects on colonic muscle strips, acting through a myogenic mechanism. This upholds its use in the treatment of constipation. My study provided important information on the design of experiments to assess neural control of motility. I found a contractile pattern that is entirely myogenic. However, the probable equivalent of this pattern in intestinal motility studies is in part neurogenic. The important lesson from these experiments is that muscle strips may not have the neuronal circuitry to modify or elucidate neural control mechanisms. This is not always appreciated as muscle strips are often used to study neural control of contractile patterns, through electrical

stimulation of motor neurons. It may be that more physiological and complex neural responses are not present in isolated muscle strips.

7.2 Experimental limitations

Although *in vitro* experimentation on the gastrointestinal system has been ongoing for over 100 years, there are still some limitations to the techniques that are currently being employed in intestinal motor function studies. My main project required both intraluminal pressure recordings, as well as spatiotemporal maps of the intestinal diameter changes. The limitation of using manometry is that in the current setup, I could only gather pressure data from a single proximal point within the small intestine. Thus, it is difficult to fully quantify the effect of some of the pressure changes as I do not know if they were always propulsive. The luminal outflow measurements aided in determining outflow/propulsion, but due to the small diameter of the mouse small intestine, it was quite easy for the outflow tube to become obstructed, thus making it seem like there is no outflow when the pattern of contractions was clearly propulsive. The spatiotemporal maps were a valuable tool in better understanding the makeup of the propulsive motor pattern and the peristaltic nature of the associated contraction(s). Unfortunately, spatiotemporal maps are rendered difficult to assess circular muscle contractions if the experiment had too many longitudinal muscle contractions, which in the mouse small intestine occur occasionally under the current experimental design.

Since calcium imaging is a relatively new technique in the field of ICC research, there are still some aspects limiting the types of experiments which can be performed. The most significant limitation of calcium imaging is that the calcium chelating dye fluo-

4 is light sensitive and rapidly loses intensity when being imaged. This limits the duration that a region with active ICC can be studied. Reducing the light exposure via shutter manipulation has enabled me to prolong the experiments, however, it is still not ideal for observing low-frequency rhythmic patterns of calcium enabled fluorescence over time. A second limitation to specifically studying the ICC-DMP is the lack of baseline activity in these cells. As mentioned above, the ICC-DMP form an inducible pacemaker and thus are only activated under specific conditions. Because the exact conditions for active ICC-DMP are not fully understood, it is difficult to stimulate them and then assess the activity of these cells via calcium imaging.

Finally, the muscle strip experiments of the human pylorus and the rat colon have their own limitations. The technique is very useful at studying simple effects of smooth muscle contractility; however, it does not give a full glimpse of the *in vivo* activity. Since circular muscle strips were employed, most longitudinal projecting neurons are severed, with unknown resulting effects. A major feature of motor patterns of the gastrointestinal tract is the peristaltic nature of the contractions. By using muscle strips, one can only understand how the individual region behaves and not how it responds to a propagating contraction. Finally, a specific limitation to the human pylorus study is the large time lapse that occurred between the removal of the tissue sample from the patient, and when the experiments were performed. This was a result of poor coordination between clinical and basic science teams, as well as the movement of surgeries to an offsite location mid-way through the study. It is difficult to predict the level of deterioration in the tissue from the start of surgery to the beginning of the experiments, often eight hours later.

7.3 Future experiments

The work I have done has provided a better understanding of rhythmic motor patterns throughout the gastrointestinal tract, in multiple species. There are many avenues of exploration which can build on the research presented here, both from basic science and clinical perspectives.

Although inducing rhythmic propulsion in the small intestine does not always occur in response to distention, *in vivo* intestinal motility experiments remain a relatively easy way to further study the propulsive motor patter. From a basic science perspective, further research must be done to understand the targets of both substance P and acetylcholine, and how they work cooperatively to induce rhythmic propulsion. From a human health perspective, probiotics and pharmaceuticals can be tested for their propulsion-modifying effects, and may help lead to safer and more effective treatments for dysmotility.

ICC-DMP activity, observed using calcium imaging, holds the potential for a better understanding of the pacemaker activity of this cell type. Firstly, with regards to propulsion, a better understanding of how acetylcholine and substance P act on these cells will assist in replicating the rhythmicity with which the propulsive motor pattern presents in motility studies. Secondly, a detailed assessment of the cellular mechanism (channels, receptors etc.) of ICC-DMP could be conducted, just like the studies performed on the ICC-MP pacemaker network. A better understanding of the cellular mechanism of ICC-DMP pacemaking could potentially lead to novel targets for the treatment of dysmotility,

as these are the cells that are likely responsible for content propulsion in the small intestine.

The pattern of ICC populated within and around the human pylorus has not led to a simple understanding of the rhythmicity inherent to the motor patterns of each region. Thus, the opportunity remains to further assess which cell types are responsible for the inherent rhythmicity seen in these tissues. Ideally, larger sections of human pylorus, antrum and duodenum would allow for an assessment of the changes in motor pattern rhythmicity, in relation to the distance from the pylorus. The same could be done using immunohistochemistry, where one observes the changes in ICC expression at varying distances from the pylorus. This will allow for a more accurate assessment of which cell types align with the specific motor patterns of the antrum, pylorus and duodenum, respectively.

Finally, muscle strip experiments with Nissle on rat colon strips in conjunction with motility studies have provided the basis for further characterization of the excitatory effects of the bacterial products. Specifically, it is important to determine which Nissle products have the observed prokinetic ability and upon which intestinal target they act. Once again, this will allow for the development of better and more targeted treatments for dysmotility.

REFERENCES

Alexander SPH, Mathie A and Peterson JA (2008). Guide to receptors and channels (GRAC), 3rd edn. *Br J Pharmacol* **(Suppl. 2)**: S1-S209.

Allescher HD and Daniel EE (1994) Role of NO in pyloric, antral, and duodenal motility and its interaction with other inhibitory mediators. *Dig Dis Sci* **39**: 73S-75S.

Alvarez WC (1914) Functional variations in contractions of different parts of the small intestine. *Am J Physiol* **35**: 177-193.

Alvarez WC and Starkweather E (1919) Conduction in the small intestine. *J Physiol* **50**: 252-265.

Bär F, von Koschitzky H, Roblick U, Bruch HP, Schultze L, Sonnenborn U, Böttner M and Wenedl T (2009) Cell-free supernatants of Escherichia coli Nissle 1917 modulate human colonic motility: evidence from an in vitro organ bath study. *Neurogastroenterol Motil* **21**: e16-e17.

Bayliss WM and Starling EH (1899) The movements and innervation of the small intestine. *J Physiol* **24**: 99-143.

Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, Deng Y, Blennerhassett PA, Fahnestock M, Moine D, Berger B, Huizinga JD, Kunze W, McLean PG, Bergonzelli GE, Collins SM and Verdu EF (2011) The anxiolytic effect of *Bifidobacterim longum* NCC3001 involves vagal pathways for gut-brain communication. *Neurogastroenterol Motil* **23**: 1132-1139.

Bomberger JM, Marameswaran N and Spielman WS (2012) Regulation of GPCR trafficking by RAMPs. *Adv Exp Med Biol* **744**: 25-37.

Cannon WB (1902) The movements of the intestines studied by means of the Röntgen rays. *Am J Physiol* **6**: 251-277.

Castleton KB (1934) An experimental study of the movements of the small intestine. *Am J Physiol* **107**: 641-664.

Caulfield MP and Birdsall NJM (1998) International union of pharmacology. XVII classification of muscarinic acetylcholine receptors. *Pharmacol Rev* **50**: 279-290.

Chen H, Redelman D, Ro S, Ward SM, Ordög T and Sanders KM (2007) Selective labeling and isolation of functional classes of interstitial cells of Cajal of human and murine small intestine. *Am J Physiol cell Physiol* **292**: C497-C507.

Code CF, Hightower Jr NC and Morlock CD (1952) Motility of the alimentary canal in man. Review of recent studies. *Am J Med* **13**: 328-351.

Costa M, Brookes SJH and Hennig GW (2000) Anatomy and physiology of the enteric nervous system. *Gut* **47**: iv15-iv19.

Cullen JJ and Kelly KA (1993) Gastric motor physiology and pathophysiology. *Surg Clin North Am* **73**: 1145-1160.

Dahms V, Prosser CL and Suzuki N (1987) Two types of 'slow waves' in intestinal smooth muscle of cat. *J Physiol* **392**: 51-69.

Defea K, Schmidlin F, Déry O, Grady EF and Bunnett NW (2000) Mechanisms of initiation and termination of signaling by neuropeptide receptors: a comparison with the proteinase-activated receptors. *Biochem Soc Trans* **28**: 419-426.

Der-Silaphet T, Malysz J, Hagel S, Arsenault LA and Huizinga JD (1998) Interstitial cells of Cajal direct normal propulsive contractile activity in the mouse small intestine. *Gastroenterology* **114**: 724-736.

Desipio J, Friedenberg FK, Korimilli A, Richter JE, Parkman HP and Fisher RS (2007) High-resolution solid-state manometry of the antropyloroduodenal region. *Neurogastroenternol Motil* **19**: 188-195.

Dijkstra G, van Goor H, Jansen PL and Moshage H (2004) Targeting nitric oxide in the gastrointestinal tract. *Curr Opin Investig Drugs* **5**: 529-536.

Djokic TD, Sekizawa K, Borson DB and Nadel JA (1989) Neutral endopeptidase inhibitors potentiate substance P-induced contraction in gut smooth muscle. *Am J Physiol* **256**: G39-G43.

Ehlert FJ, Park KJ and Griffin MT (2012) Muscarinic agonists and antagonists: effects on gastrointestinal function. *Handb Exp Pharmacol* **208**: 343-374.

Erickson JC, Obioha C, Goodale A, Bradshaw LA and Richards WO (2009) Detection of small bowel slow wave frequencies from noninvasive biomagnetic measurements. *IEEE Trans Biomed Eng* **56**: 2181-2189.

Faussone-Pellegrini MS (2006) Relationships between neurokinin receptor-expressing interstitial cells of Cajal and tachykininergic nerves in the gut. *J Cell Mol Med* **10**: 20-32.

Foong JP, Nguyen TV, Furness JB, Bornstein JC and Young HM (2012) Myenteric neurons of the mouse small intestine undergo significant electrophysiological and morphological changes during postnatal development. *J Physiol* **590**: 2375-2390.

Friedenberg FK, Desipio J, Korimilli A, Bohning M, Sum E, Parkman HP, Richter JE and Fisher RS (2008) Tonic and phasic pyloric activity in response to CCK-octapeptide. *Dig Dis Sci* **53**: 905-911.

Gonzalez A and Sarna SK (2001) Neural regulation of *in vitro* giant contractions in the rat colon. *Am J Physiol Gastrointest Liver Physiol* **281**: G275-G282.

Grady EF, Garland AM, Gamp PD, Lovett M, Payan DG and Bunnett NW (1995) Delineation of the endocytic pathway of substance P and its seven-transmembrane domain NK1 receptor. *Mol Biol Cell* **6**: 509-524.

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

Gwynne RM and Bornstein JC (2007) Mechanisms underlying nutrient-induced segmentation in isolated guinea pig small intestine. *Am J Physiol Gastrointest Liver Physiol* **292**: 1162-1172.

Gwynne RM, Dombek A, Chang E, Tan LL and Bornstein JC (2004A) Video mapping and extracellular recordings studies on nutrient-induced segmentation in isolated guinea-pig small intestine. *Gastroenterology* **126**: A-223.

Gwynne RM, Thomas EA, Goh SM, Sjovall H and Bornstein JC (2004B) Segmentation induced by intraluminal fatty acid in isolated guinea-pig duodenum and jejunum. *J Physiol* **556**: 557-569.

Higham SE and Read NW (1990) Effect of ingestion of fat on ileostomy effluent. *Gut* **31**: 435-438.

Holzer P, Lippe IT, Heinemann A and Barthó L (1998) Tachykinin NK1 and NK2 receptor-mediated control of peristaltic propulsion in the guinea-pig small intestine *in vitro*. *Neuropharmacology* **37**: 131-138.

Henderson Y (1909) Acapnia and shock. III. Shock after laparotomy: its prevention, production and relief. *Am J Physiol* **24**: 66-85.

Hennig GW, Spencer NJ, Jokela-Willis S, Bayguinov PO, Lee HT, Ritchie LA, Ward SM, Smith TK and Sanders KM (2010) ICC-MY coordinate smooth muscle electrical and mechanical activity in the murine small intestine. *Neurogastroenterol Motil* **22**: e138-e151.

Hightower JR NC (1959) Chapter 98: Motor action of the small bowel. Handbook of Physiology: a critical comprehensive presentation of physiological knowledge and concepts, sec. 6 iv The Alimentary Canal, pp 2001-2024. American Physiology Society: Washington DC.

Holzer P, Schluet W and Maggi CA (1995) Substance P stimulates and inhibits intestinal peristalsis via distinct receptors. *J Pharmacol Exp Ther* **274**: 322-328.

Huizinga JD and Lammers WJEP (2009) Gut peristalsis is governed by a multitude of cooperating mechanisms. *Am J Physiol Gastrointest Liver Physiol* **296**: G1-G8.

Huizinga JD, Martz S, Gil V, Wang XY, Jiménez M and Parsons S (2011) Two independent networks of interstitial cells of Cajal work cooperatively with the enteric nervous system to create colonic motor patterns. *Front Neurosci* **5**: 93.

Huizinga JD, Thuneberg L, Klüppel M, Malysz J, Mikkelsen HB and Bernstein A (1995) W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature* **373**: 347-349.

Huizinga JD, Zarate N and Farrugia G (2009) Physiology, injury and recovery of interstitial cells of Cajal: basic and clinical science. *Gastroenterology* **137**: 1548-1556.

Iino S and Horiguchi K (2006) Interstitial cells of Cajal are involved in neurotransmission in the gastrointestinal tract. *Acta Histochem Cytochem* **39**: 145-153.

Inoue R, Morita H, Yanagida H and Ito Y (1998) Potentiating actions of lanthanum on Ach-induced cation current in guinea-pig ileal smooth muscle cells. *J Smooth Muscle Res* **34**: 69-81.

Iino S, Ward SM and Sanders KM (2004) Interstitial cells of Cajal are functionally innervated by excitatory motor neurons in the murine intestine. *J Physiol* **556**: 521-530.

Jiménez M, Cayabyab FS, Vergara P and Daniel EE (1996) Heterogeneity in electrical activity of the canine ileal circular muscle: interaction of two pacemakers. *Neurogastroenterol Motil* **8**: 339-349.

Kilbinger H and Weihrauch TR (1982) Drugs increasing gastrointestinal motility. *Pharmacology* **25**: 61-72.

Kirkwood KS, Bunnett NW, Maa J, Castagliolo I, Liu B, Gerard N, Zacks J, Pothoulakis C and Grady EF (2001) Deletion of neutral endopeptidase exacerbates intestinal inflammation induced by *Clostridium difficile* toxin A. *Am J Physiol Gastrointest Liver Physiol* **281**: G544-G551.

Kobilo T, Szurszewski JH, Farrugia G and Hanani M (2003) Coupling and innervation patterns of interstitial cells of Cajal in the deep muscular plexus of the guinea-pig. *Neurogastroenterol Motil* **15**: 635-641.

Komuro T (2006) Structure and organization of interstitial cells of Cajal in the gastrointestinal tract. *J Physiol* **576**: 653-658.

Kosterlitz JH and Lees GM (1963) Pharmacological analysis of intrinsic intestinal reflexes. *Pharmacol Rev* **16**: 301-339.

Lammers WJ and Stephen B (2008) Origin and propagation of individual slow waves along the intact feline small intestine. *Exp Physiol* **93**: 334-346.

Lee JC, Thuneberg L, Berezin I and Huizinga JD (1999) Generation of slow waves in membrane potentials is an intrinsic property of interstitial cells of Cajal. *Am J Physiol* **277**: G409-G423.

Lin AS, Buist ML, Smith NP and Pullan AJ (2006) Modeling slow wave activity in the small intestine. *J Theor Biol* **242**: 356-362.

Lin X, Hayes J, Peters LJ and Chen JD (2000) Entrainment of intestinal slow waves with electrical stimulation using intraluminal electrodes. *Ann Biomed Eng* **18**: 582-587.

Liu LW (2011) Chronic constipation: current treatment options. *Can J Gastroenterol* **25**: 22B-28B.

Lowie BJ (2011) Spontaneous calcium oscillations in two networks of interstitial cells of Cajal in the mouse small intestine. *Unpublished Master of Science thesis*. McMaster University, Hamilton Canada.

Lowie BJ, Wang XY, White EJ and Huizinga JD (2011) On the origin of rhythmic calcium transients in the ICC-MP of the mouse small intestine. *Am J Physiol Gastrointest Liver Physiol* **301**: G835-G845.

Lukas RJ, Changeux J-P, Le Novere N, Albuqurque EX, Balfour DJ, Berg DK, Bertrand d, Chiappinelli VA, Clarke PB, Collins AC, Dani JA, Grady SR, Kellar KJ, Lindstrom JM, Marks MJ, Quik M, Taylor PW and Wonnacott S (1999) International union of pharmacology XX. Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits. *Pharmacol Rev* **51**: 397-401.

Macfarlane GT and Macfarlane S (2012) Bacteria, colonic fermentation, and gastrointestinal health. *J AOAC Int* **95**: 50-60.

Malysz J, Donnelly G and Huizinga JD (2001) Regulation of slow wave frequency by IP(3)-sensitive calcium release in the murine small intestine. *Am J Physiol Gastrointest Liver Physiol* **280**: G439-G448.

Mandl P and Kiss JP (2007) Role of presynaptic nicotinc acetylcholine receptors in the regulation of gastrointestinal motility. *Brain Res Bull* **72**: 194-200.

Martz SL (2011) *Escherichia coli* Nissle 1917 conditioned media promotes peristalsis by increasing colonic motor patterns governed by the ICC-MP and enteric nervous system. *Unpublished Bachelor of Science thesis*. McMaster University, Hamilton Canada.

Mourad FH and Saadé NE (2011) Neural regulation of intestinal nutrient absorption. *Prog Neurobiol* **95**: 149-162.

Nishioka K, Shibata O, Yamaguchi M, Yoshimura M, Makita T and Sumikawa K (2007) Succinylcholine potentiates acetylcholine-induced contractile and phosphatidylionositol responses of rat trachea. *J Anesth* **21**: 171-175.

Obermeier F, Hofmann C and Falk W (2010) Inflammatory bowel diseases: when natural friends turn into enemies – the importance of CpG motifs of bacterial DNA in intestinal homeostasis and chronic intestinal inflammation. *Int J Inflam* **2010**: 641910.

Oda Y (1999) Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system.

O'Grady G, Du P, Cheng LK, Egbuji JU, Lammers WJEP, Windsor JA and Pullan AJ (2010) Origin and propagation of human gastric slow-wave activity define by high resolution mapping. *Am J Physiol Gastrointest Liver Physiol* **299**: G585-G592.

Pennefather JN, Lecci A, Candenas ML, Patak E, Pinto FM and Maggi CA (2004) Tachykinins and tachykinin receptors: a growing family. *Life Sci* **74**: 1445-1463.

Puestow CB (1940) Intestinal motility of the dog and man. University of Illinois Press: Urbana Il.

Puestow CB (1931) Studies on the activity of isolated intestinal segments. *Proc Staff Meetings Mayo Clinic* **6**: 131-132.

Ramkumar D and Schultze KS (2005) The pylorus. *Neurogastroenterol Motil* **17**: 22-30.

Sagan S and Lavielle S (2001) Internalization of [3H]substance P analogues in NK-1 receptor transfected CHO cells. *Biochem Biophys Res Commun* **282**: 958-964.

Sanders KM and Ward SM (2006) Interstitial cells of Cajal: a new perspective on smooth muscle function. *J Physiol* **576**: 721-726.

Schemann M (2005) Control of gastrointestinal motility by the "gut brain" – the enteric nervous system. *J Pediatr Gastroenterol Nutr* **41**: S4-S6.

Schemann M and Mazzuoli G (2010) Multifunctional mechanosensitive neurons in the enteric nervous system. *Auton Neurosci* **153**: 21-25.

Schultze K (2006) Imaging and modeling of digestion in the stomach and the duodenum. *Neurogastroenterol Motil* **18**: 172-183.

Shea-Donohue T, Goldhill JM, Montcalm-Mazzilli E, Colleton C, Piñeiro-Carrero VM and Sjogren RW (1997) Role of sensory afferents in the myoelectric response to acute enteric inflammation in the rabbit. *Am J Physiol* **273**: G447-G455.

Shimizu Y, Matsuyama H, Shiina T, Takewaki T and Furness JB (2008) Tachykinins and their functions in the gastrointestinal tract. *Cell Mol Life Sci* **65**: 295-311.

Sonnenborn U and Schultz J (2009) The non pathogenic Escherichia coli strain Nissle 1917 – features of a versatile probiotic. *Microb Ecol Health Dis* **21**: 122-158.

Spencer NJ, Smith CB and Smith TK (2001) Role of muscle tone in peristalsis in guinea-pig small intestine. *J Physiol* **530**: 295-306.

Spencer NJ, Walsh M and Smith TK (1999) Does the guinea-pig ileum obey the 'law of the intestine'? *J Physiol* **517**: 889-898.

Stockinger S, Hornef MW and Chassin C (2011) Establishment of intestinal homeostasis during the neonatal period. *Cell Mol Life Sci* **68**: 3699-3712.

Tack J and Smith TK (2004) Calcium imaging of gut activity. *Neurogastroenterol Motil* **16**: 86-95.

Tonini M, Galligan JJ and North RA (1989) Effects of cisapride on cholinergic neurotransmission and propulsive motility in the guinea pig ileum. *Gastroenterology* **96**: 1257-1264.

Torihashi S, Fujimoto T, Trost C and Nakayama S (2002) Calcium oscillation linked to pacemaking of interstitial cells of Cajal. *J Biol Chem* **277**: 19191-19197.

Trendelenburg P (1917) Physiologische und pharmakologische versuche über die dünndarmperistaltik. *Naunyn Schmiedebergs Arch Pharmacol* **81**:55-129.

Valerio F, Russo F, de Candia S, Riezzo G, Orlando A, Lonigro SL and Lavermicocca P (2010) Effects of probiotic Lactobacillus paracasei-enriched artichokes on constipated patients: a pilot study. *J Clin Gastroenterol* **44**: S49-S53.

van Koppen CJ and Kaiser B (2003) Regulation of muscarinic acetylcholine receptor signaling. *Pharmacol Ther* **98**: 197-220.

Wang XY, Lammers WJ, Bercik P and Huizinga JD (2005) Lack of pyloric interstitial cells of Cajal explains distinct peristaltic motor patterns in stomach and small intestine. *Am J Physiol Gastrointest Liver Physiol* **289**: G539-G549.

Wang XY, Paterson C and Huizinga JD (2003) Cholinergic and nitrergic innervation of ICC-DMP and ICC-IM in the human small intestine. *Neurogastroenterol Motil* **15**: 531-543.

Wang XY, Sanders KM and Ward SM (1999) Intimate relationship between interstitial cells of Cajal and enteric nerves in the guinea-pig small intestine. *Cell Tissue Res* **295**: 247-256.

Wang XY, Vannucchi MG, Nieuwmeyer F, Ye J, Faussone-Pellegrini MS and Huizinga JD (2005) Changes in interstitial cells of Cajal at the deep muscular plexus are associated with loss of distention-induced burst-type muscle activity in mice infected by *Trichinella spiralis*. *Am J Pathol* **167**: 437-453.

Ward and Sanders (2001) Interstitial cells of Cajal: primary targets of enteric motor innervation. *Anat Rec* **262**: 125-135.

Ward SM, McLaren GL and Sanders KM (2006) Interstitial cells of Cajal in the deep muscular plexus mediate enteric motor neurotransmission in the mouse small intestine. *J Physiol* **573**: 147-159.

White HL, Rainey WR, Monaghan B and Harris AS (1934) Observations on the nervous control of the ileocaecal sphincter and on intestinal movements in an unanesthetized human subject. *Am J Physiol* **108**: 449-457.

Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC and Siuzdak G (2009) Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proc Natl Acad Sci U S A* **106**: 3698-3703.

Wood JD (1999) Enteric nervous control of motility in the upper gastrointestinal tract in defensive states. *Dig Dis Sci* **44**: 44S-52S.

Yamazawa T and Iino M (2002) Simultaneous imaging of Ca^{2+} signals in interstitial cells of Cajal and longitudinal smooth muscle cells during rhythmic activity in mouse ileum. *J Physiol* **538**: 823-835.