

# **A FUNCTIONAL ANALYSIS OF GASTROINTESTINAL MOTILITY IN THE GUINEA PIG AND HUMAN**

**A thesis submitted in total fulfilment of the requirements of  
the degree of doctor of philosophy**

**Simona Elisa Carbone**

**Bachelor of Medical Science, Bachelor of Science (Honours)**

Discipline of Human Physiology

Flinders Medical Science and Technology

Centre for Neuroscience

School of Medicine, Flinders University

Adelaide, South Australia

**August 2012**

# TABLE OF CONTENTS

<b>THESIS SUMMARY .....</b>	<b>VI</b>
<b>DECLARATION .....</b>	<b>VIII</b>
<b>ACKNOWLEDGEMENTS .....</b>	<b>IX</b>
<b>PUBLICATIONS AND CONFERENCE PRESENTATIONS.....</b>	<b>XI</b>
Refereed journal articles:.....	xi
Conference proceedings: .....	xi

## **1. CELLULAR MECHANISMS UNDERLYING SMOOTH MUSCLE EXCITABILITY IN HUMAN AND ANIMAL GASTROINTESTINAL TRACT. .1**

<b>1.1 The anatomy of the gastrointestinal tract .....</b>	<b>1</b>
1.1.1 The layers of the gastrointestinal tract.....	1
1.1.2 The enteric nervous system .....	1
<b>1.2 Smooth muscle cells .....</b>	<b>2</b>
1.2.1 Morphology .....	3
1.2.2 Contractile apparatus .....	3
1.2.3 Electrophysiology .....	4
1.2.4 Ionic basis for the modulation of smooth muscle cell membrane potential.....	6
1.2.5 Calcium release from intracellular stores .....	9
<b>1.3 Slow waves.....</b>	<b>10</b>
1.3.1 Electrophysiology .....	11
1.3.2 Ionic basis for slow waves.....	11
<b>1.4 Interstitial cells of Cajal .....</b>	<b>12</b>
1.4.1 ICC and the pacemaker region .....	12
1.4.2 ICC and neurotransmission .....	16
1.4.3 Generating spontaneous activity in ICC.....	19
1.4.4 Coordinating and propagating spontaneous activity of ICC .....	21
<b>1.5 Cellular Junctions .....</b>	<b>22</b>
<b>1.6 Gap junctions .....</b>	<b>23</b>
1.6.1 Morphology of gap junctions .....	23
1.6.2 Channel formation.....	24

1.6.3 Degradation .....	25
1.6.4 Gap junctions and metabolic coupling.....	26
1.6.5 Gap junctions and electrical coupling.....	26
1.6.6 Factors influencing gap junction conductance .....	27
1.6.7 Clinical significance of gap junctions.....	31
1.6.8 An electrical syncytium in the gastrointestinal tract? .....	31
<b>1.7 Motility patterns of the gastrointestinal tract.....</b>	<b>35</b>
1.7.1 Muscle contractions .....	35
1.7.2 Peristalsis .....	35
<b>1.8 Peristaltic reflex and ‘the law of the intestine’ .....</b>	<b>36</b>
1.8.1 Ascending excitatory pathway .....	37
1.8.2 Descending pathways.....	39
1.8.3 Propagating response .....	42
1.8.4 Occult reflex .....	42
<b>1.9 Other forms of peristalsis.....</b>	<b>43</b>
1.9.1 Migrating motor complex.....	43
1.9.2 Colonic migrating motor complex .....	43
1.9.3 Giant migrating motor complex .....	44
1.9.4 Propagating between muscle layers .....	45
<b>1.10. Human studies .....</b>	<b>45</b>
1.10.1 Immunohistochemistry: highlighting the neural pathways .....	45
1.10.1 Spontaneous oscillatory activity in human colon .....	47
1.10.3 Neuromuscular transmission in the human colon .....	49
<b>1.11 Summary .....</b>	<b>50</b>
<b>2. CHANGES IN THE PHYSIOLOGY OF SMOOTH MUSCLE CELLS OF THE GUINEA PIG ILEUM AND COLON FOLLOWING DISSECTION .....</b>	<b>52</b>
<b>2.1 Introduction .....</b>	<b>52</b>
<b>2.2 Methods.....</b>	<b>53</b>
2.2.1 Dissection .....	53
2.2.2 Intracellular recording.....	55
2.2.3 Measuring the force of contractions.....	56
2.2.4 Data Analysis .....	56
<b>2.3 Results .....</b>	<b>57</b>
2.3.1 Electrophysiological properties of circular smooth muscle cells following dissection.....	57

2.3.2 The electrophysiological properties of longitudinal muscle cells.....	63
2.3.3 Effects on colonic circular smooth muscle cells .....	63
2.3.4 Effects of dissection on nitregeric responses. ....	65
2.3.5 Excitatory junction potentials in the ileum and colon .....	65
2.3.6 Neurotransmission between enteric neurons was not blocked in the first 30 minutes .....	68
2.3.7 The recovery of spontaneous contractions following dissection .....	68
<b>2.4 Discussion .....</b>	<b>75</b>
2.4.1 Post dissection ‘unresponsiveness’ .....	75
2.4.2 Loss of gap junction coupling .....	76
2.4.3 Reduction of neuromuscular transmission .....	77
2.4.4 Resting membrane potential .....	78
2.4.5 Spontaneous contractions .....	78
<b>2.5 Conclusions.....</b>	<b>79</b>
<b>3. EVIDENCE FOR THE INVOLVEMENT OF GAP JUNCTION COUPLING IN NEUROMUSCULAR TRANSMISSION .....</b>	<b>80</b>
<b>3.1 Introduction .....</b>	<b>80</b>
<b>3.2 Materials and Methods.....</b>	<b>81</b>
3.2.1 Electrophysiological responses to exogenous ATP .....	81
3.2.2 Visualising carboxyfluorescein labelling in sections.....	82
3.2.3 Immunohistochemistry .....	82
3.2.4 Measuring the force of contractions.....	83
3.2.5 Data Analysis .....	83
<b>3.3 Results .....</b>	<b>83</b>
3.3.1 Effects of gap junction blockers on the excitability of circular muscle cells.....	83
3.3.2 The effects of carbenoxlone on slow inhibitory junction potentials (sIJP) and excitatory junction potentials (EJP) .....	88
3.3.3 Effects of exogenous ATP.....	88
3.3.4 Responses to sodium nitroprusside and bethanacol.....	91
3.3.5 Visualising dye fills in sectioned preparations.....	96
3.3.6 Recording from a cell other than circular muscle cell .....	96
3.3.7 Spontaneous contractions following inhibition of gap junctions.....	99
<b>3.4 Discussion .....</b>	<b>99</b>
3.4.1 Gap junction uncoupling and the loss of junction potentials .....	102
3.4.2 Interstitial Cells of Cajal and neuromuscular transmission.....	103
3.4.3 Fibroblast-like cells and neuromuscular transmission.....	103

3.4.4 Gap junction coupling and spontaneous contractions.....	105
3.4.5 The specificity of gap junction blockers.....	105
3.4.6 Exogenous ATP activates inhibitory motoneurons in the ileum but not the colon .....	107
3.5 Conclusion .....	108

#### **4. UNDERSTANDING THE MECHANISMS THAT LEAD TO GAP JUNCTION UNCOUPLING AND ASSOCIATED UNRESPONSIVENESS FOLLOWING DISSECTION OF THE GUINEA PIG ILEUM..... 109**

<b>4.1 Introduction .....</b>	<b>109</b>
<b>4.2 Methods.....</b>	<b>111</b>
4.2.1 Experimental design.....	111
4.2.2 Drugs.....	112
4.2.3 Data analysis.....	112
<b>4.3 Results .....</b>	<b>113</b>
4.3.1 Temperature as a cause for initial suppression of responses.....	113
4.3.2 Cutting a responsive preparation.....	113
4.3.4 Stretching a responsive preparation .....	117
4.3.5 Limiting influx of Ca <sup>2+</sup> from Krebs solution during dissection.....	120
4.3.6 Prostaglandins as a mediator of uncoupling.....	120
4.3.7 Stabilising mast cells to limit the loss of responses from dissection .....	121
4.3.8 Cyclic Adenosine monophosphate (cAMP) as modulator of gap junction coupling.....	121
4.3.9 Cyclic guanosine 3'-5'-monophosphate (cGMP) as mediator of gap junction uncoupling...	123
4.3.10 The recovery of responses in partially dissected preparations with the mucosa intact. ....	123
<b>4.4 Discussion .....</b>	<b>128</b>
4.4.1 Involvement of other factors .....	129
4.4.2 Limiting damage .....	129
4.4.3 Potential mediators of gap junction uncoupling.....	130
4.4.4 Phosphorylation and gap junction intercellular coupling .....	131
4.4.5 Clinical applications.....	132
4.5 Conclusion .....	133

#### **5. SPONTANEOUS AND ELECTRICALLY EVOKED CONTRACTIONS IN SMALL SEGMENTS OF HUMAN COLON..... 134**

<b>5.1 Introduction .....</b>	<b>134</b>
<b>5.2 Materials and Methods.....</b>	<b>135</b>
5.2.1 Tissue preparation .....	135
5.2.2 Variations in contractility for preparations differing in cross sectional area. ....	136

5.2.3	Frequencies of contractions .....	136
5.2.4	Ascending excitatory neurons and colonic contractility.....	137
5.2.5	Drugs used .....	137
5.2.6	Data analysis.....	139
<b>5.3</b>	<b>Results .....</b>	<b>139</b>
5.3.1	The frequencies of contractions in muscle strips of varying size. ....	139
5.3.2	Slow phasic contractions .....	140
5.3.3	Intermediate contractions.....	147
5.3.4	High frequency contractions.....	147
5.3.5	Spontaneous activity in ‘T’ segments of colon .....	147
5.3.6	Stimulation of ascending interneuronal pathways and its effects on slow phasic contractions .....	149
5.3.7	Inhibiting nicotinic pathways: effects on premature contractions. ....	149
<b>5.4</b>	<b>Discussion .....</b>	<b>153</b>
5.4.1	Spontaneous slow phasic contractions.....	156
5.4.2	Ascending excitatory pathways in the human colon.....	157
5.4.3	The importance in studying different sized preparations. ....	158
<b>5.5</b>	<b>Conclusion .....</b>	<b>159</b>
<b>6.</b>	<b>GENERAL DISCUSSION AND CONCLUSIONS.....</b>	<b>161</b>
6.1	Summary of findings.....	161
6.2	Other considerations.....	164
6.2.1	Post dissection loss of responses- not just gap junction coupling.....	164
6.2.2	Gap junction coupling and neuromuscular responses: the role of coupling between smooth muscle cells, ICC and fibroblast like cells.....	165
6.2.3	Release of neurotransmitter is not affected by preparation setup .....	166
6.2.4	2-APB blocks gap junctions and IP3 receptors .....	167
6.2.5	Is the lack of responses following preparation setup specific to the guinea pig? .....	167
6.2.6	The loss of responses is “just damage” .....	168
6.2.7	Ascending pathways in the human colon: cholinergic and tachykinergic.....	168
6.2.8	An alternative pattern generator in the colon .....	169
6.3	Conclusion .....	170
<b>BIBLIOGRAPHY .....</b>	<b>171</b>	

## THESIS SUMMARY

Gastrointestinal motility results from coordinated contractions and relaxation of smooth muscle layers of the gut wall. Motor patterns mix or propel content along the gastrointestinal tract. The circular and longitudinal muscle layers within the gut wall are comprised of smooth muscle cells and interstitial cells of Cajal (ICC), which work together to make up the myogenic component of gastrointestinal motility. Smooth muscle cells and ICC are electrically and metabolically coupled to themselves and each other by a series of gap junctions. Gap junction coupling has been shown in several cellular systems to be modulated by: pH,  $\text{Ca}^{2+}$ , second messengers, voltage differences and by various neurotransmitters and pharmacological agents. Gastrointestinal motility is also controlled by intrinsic and extrinsic neural inputs. Mechanical and/or electrical recordings of smooth muscle cells from segments of the gut wall have been extensively studied *in vitro* to understand mechanisms underlying gastrointestinal motility. Immediately following setup, many of these preparations display a curious physiological feature: they appear to be completely inactive. These preparations lack tone, they fail to develop spontaneous contractions, they lack excitatory or inhibitory junction potentials, and these preparations fail to respond to electrical or pharmacological stimuli. Responses develop and then stabilise over the next 60-120 minutes. This phenomena is often implicitly reported in the methods section of papers, however the mechanistic causes for the apparent loss of responsiveness is unknown.

One of the aims of this project was to understand the mechanisms that account for the loss of responses after dissection. In chapter 2, the electrical properties of circular smooth muscle cells from specimens of guinea pig ileum and colon were monitored as preparations recovered from dissection. Junction potentials (primarily fast inhibitory junction potentials), resting membrane potential, input resistance and dye coupling were monitored over this period. The role of gap junction coupling in neuromuscular transmission was investigated in chapter 3. In chapter 4, the role of various elements of the setup procedure were studied, in an attempt to highlight which factors activated the uncoupling process. This problem was approached from 2

directions: by testing whether manipulations could evoke a second loss of response, and by testing whether omitting the use of other manipulations reduced the initial loss of responses. In chapter 5 the role of cholinergic interneuronal pathways in generating an ascending contraction in segments of human colon was studied. Few functional *in vitro* studies have considered the role of interneuronal pathways in human tissue due to limited supply of specimens of an appropriate size required for this analysis. The frequency of spontaneous contractions in narrow segments versus larger segments was also considered. These studies identified two mechanisms that may contribute to the control of gastrointestinal motility *in vitro*. Their physiological significance *in vitro* remains to be determined.



## **DECLARATION**

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

Simona E. Carbone, August 2012

## ACKNOWLEDGEMENTS

I am very grateful to have been primarily supervised by Professor Simon Brookes. I have appreciated his patience and knowledge while teaching me the basics of electrophysiology, the art of scrutinising a scientific paper and the process of explaining complex scientific ideas to an audience. Simon's ability to simplify what came across to me as being the most complex of ideas or analysis always amazed (and frustrated) me. Simon has been my supervisor from my first undergraduate project through to now. While his approach to science and the quality of the work produced by his laboratory was the main reason why I stayed, he has also been a wonderful mentor and I have appreciated the many laughs I've had with him too.

I have been co-supervised by Professor David Wattoo and Associate Professor Nick Spencer. David conducted many of the surgeries within the Flinders Medical Centre and Flinders Private Hospital. His enthusiasm and dedication to basic science research on human gastrointestinal samples is one of the main reasons we are able to work with these precious specimens in our laboratories. I admire his passion and commitment to the cause; I will endeavour to continue this work. I have appreciated his warm nature and our many chats that often drift towards conversations of Italy. Nick has overseen many aspects of this thesis, and there have been countless times I have run into his office very confused about one thing or another. He has always patiently and calmly attempted to solve my queries. I have learnt a lot about being a confident researcher from his approach to science and if I can be half as confident as he is than I'd be doing pretty well.

I have been fortunate enough to have had additional mentorship from Professor Marcello Costa and Associate Professor Philip Dinning. Marcello's neuroscience lectures first sparked my interest in the field. I have no idea how one person can carry so much knowledge, but I am grateful that he is willing to part with some of it. I have never come away from a conversation with him without having learnt something new. Phil has provided much career mentorship in the time he has been at Flinders. He has always offered encouragement and direction when I have felt lost and his "get on with it" attitude has often pulled me into line. I'd also like to thank Doctor Vladimir Zagorodnyuk whose appraisals and reviews of my work have

been hugely beneficial. His input was particularly valuable in the first 2 results chapters.

My research project was conducted in the Neurogastroenterology Laboratory at Flinders University, with additional support from by the Visceral Neurophysiology Laboratory. The lab members have changed many times over the course of my project and these individuals have been instrumental during the time they were here: Sarah Nicholas, Alison Wadey and Kirsty Hendi. Petra Unterweger and Kelsi Dodds have provided a fresh, bright energy to the laboratory, and have been wonderful in providing assistance over the final stretch of this thesis. To Doctor Dayan de Fontgalland, for encouraging me to take that step and commence a PhD. To Tim Hibberd for providing mutual PhD student support. I'd like to thank Ms Bao Nan Chen (Nanny) for her experimental expertise, her laboratory knowledge, but most importantly her generous spirit. Our many chats have been very helpful and spurred me to persevere in this project, and I am grateful for her words of Chinese wisdom. A special thank you to Melinda Kyloh, for everything that she does. Despite her heavy experiment load, Mel will always have time to help anyone, no matter what the problem. I cannot express how many times she has done this for me and I am eternally grateful for her support and friendship.

I'd like to thank the Clinician's Special Purpose Fund for funding my PhD scholarship. Thank you to the Centre for Neuroscience for providing a platform for students to practice the art of oral presentation and for providing assistance so that I could attend the Australian Course of Advanced Neuroscience.

Thank you to my Mum and Dad, for providing me with the opportunity so that I could achieve my utmost and for encouraging me to reach for my goals. You haven't always understood my scientific problems, but you have listened anyway. To Nonna Liliana, thank you for the many pasta dinners - my brain food.

Finally, to Andrew Maitland, my rock. Thank you for ensuring that my life is balanced, for picking up the pieces when I drop them and for always making me laugh. Your unwavering support and belief in me has made this thesis happen.

## **PUBLICATIONS AND CONFERENCE PRESENTATIONS**

### **Refereed journal articles:**

**Carbone SE, Dinning PG, Costa M, Spencer NJ, Brookes SJH and Wattchow DA (2013)** ‘Ascending excitatory neural pathways modulate slow phasic myogenic contractions in the isolated human colon’ *Neurogastroenterol and Motil* In press

**Carbone SE, Wattchow DA, Spencer NJ and Brookes SJH (2012)** ‘A loss of responsiveness of circular smooth muscle cells from the guinea pig ileum is associated with changes in gap junction coupling.’ *Am J Physiol: Gastrointest Liver Physiol*. Vol. 302, pp: G1434-G1444.

Wattchow D, Brookes S, Murphy E, **Carbone S**, de Fontgalland D, Costa M. (2008) ‘Regional variation in the neurochemical coding of the myenteric plexus of the human colon and changes in patients with slow transit constipation.’ *Neurogastroenterol Motil*. vol. 20 no. 12, pp:1298-305.

### **Conference proceedings:**

#### Presenting author

**Neurogastroenterology and Motility conference (2012)** SE Carbone, Dinning PG, NJ Spencer, SJH Brookes & DA Wattchow ‘Functional evidence of ascending excitatory pathways in the human colon.’ (oral presentation)

**VIIth International symposia on ICC (2012)** Carbone SE, Wattchow DA, Spencer NJ, Brookes SJH ‘The responsiveness of smooth muscle cells in the guinea pig ileum is affected by elements of dissection.’ (poster presentation)

**Australian Neuroscience Society (ANS) conference (2012)** Carbone SE, Wattchow DA, Spencer NJ, Brookes SJH ‘Understanding the mechanisms which uncouple smooth muscle cells of the guinea pig ileum following dissection.’ (poster presentation)

**Collaborators Day (2011)** Carbone SE, Wattchow DA, Spencer NJ, Brookes SJH ‘A lack of response of smooth muscle cells to electrical and chemical stimuli is associated with changes in gap junction coupling.’ (poster presentation)

**Digestive Disease Week (DDW) abstract (2011)** Carbone SE, Wattchow DA, Spencer NJ, Brookes SJH ‘Why smooth muscle preparations are initially unresponsive to chemical and electrical stimuli in vitro: association with changes in gap junction coupling.’ (poster presentation)

**ANS abstract (2011)** Carbone SE, Wattchow DA, Spencer NJ, Brookes SJH ‘Purinergic junction potentials and responses to exogenous ATP by gut smooth muscle cells both require an ‘equilibration’ period.’ (oral presentation)

**Australian Physiological Society (AuPS) conference abstract (2010)** Carbone SE, Wattchow DA, Spencer NJ, Brookes SJH ‘Gap junction coupling between smooth muscle cells modulates responses to inhibitory motorneurons and exogenous ATP.’ (oral presentation)

**ANS abstract (2010)** Carbone SE, Wattchow DA, Spencer NJ, Brookes SJH ‘Why gut smooth muscle needs to “equilibrate” before enteric motor innervation becomes fully functional.’ (oral presentation)

**ANS abstract (2009)** Carbone SE, Brookes SJH, Wattchow DA and Spencer, NJ  
'Mechanisms underlying ascending excitation in the human colon.' (oral  
presentation)