Neural control mechanisms underlying motility in guinea pig and human intestine

A thesis submitted in total fulfillment of the requirements of the degree of Doctor of Philosophy

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Summary

The patterns of motor activity in the lower gastrointestinal tract of mammals and the mechanisms underlying their generation are incompletely understood. In this thesis, experiments were performed to provide greater insight into the role of the enteric nervous system in the generation of different propulsive motor patterns in the isolated guinea pig and human lower gastrointestinal tract.

In Chapter 2, we revealed the presence of a novel form of colonic peristalsis that was surprisingly preserved despite complete blockade of major excitatory neurotransmitters (acetylcholine, tachykinins) at both enteric neuro-neuronal and neuro-muscular junctions. It was also shown that following blockade of major excitatory neuro-neuronal and neuro-muscular transmitters an intrinsic oral-aboral polarity underlying neurogenic propulsive motor patterns was always preserved.

In Chapters 3-4, the role of endogenous serotonin in the generation and propagation of colonic peristalsis was investigated. It was found that in preparations acutely depleted of all endogenous serotonin, peristalsis was still preserved with remarkably few deficits. We also demonstrated that selective antagonists of 5-HT3 and 5-HT4 receptors could still exert a temporary blockade of peristalsis despite the absence of any detectable endogenous serotonin. These raise support for the notion that 5-HT3 and 5-HT4 receptors can display constitutive activity and the antagonists can behave as inverse agonists.

Experiments in Chapters 5 were conducted on isolated segments of human bowel and the patterns of motor activity characterised in terminal ileum and colon *ex vivo*. Long segments of bowel were preserved *ex vivo* which allowed us to preserve enteric neural activity and record propulsive neurogenic motor patterns. From our small bowel studies, we report in this thesis that propagating motor patterns are only preserved in longer segments of bowel tissue, suggesting that an intact neural circuitry is vital for their generation.

Experiments on the human colon *ex vivo* allowed for characterisation of the motor activity in what we considered "experimental control tissue" and compared these activities with those obtained from colonic specimens from patients with slow transit constipation (STC) (Chapter 6). We have recorded similar motor activities and contractile patterns, even in specimens from patients with STC. The presence of an underlying contractile activity that appeared similar to that seen in healthy controls raises the possibility that the aetiology underlying slow transit constipation may be induced by alterations in extrinsic neural inputs, rather than any overt dysfunction of the ENS. These experiments pave the way for an exciting future of experimentation.

Declaration

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

Tiong Cheng Sia, August 2014

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Publications

Sia, TC; Brookes, S; Dinning, P; Wattchow, D; Spencer, NJ. 'Peristalsis and propulsion of colonic content can occur after blockade of major neuro-neuronal and neuro-muscular transmitters in isolated guinea pig colon' **American Journal of Physiology** (2013) 305: G933-G939

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Sia, TC; Flack, N; Robinson, L; Kyloh, M; Nicholas, S; Brookes, SJ; Wattchow, DA; Dinning, P; Oliver, J; Spencer, NJ. 'Is Serotonin in enteric nerves required for distension-evoked peristalsis and propulsion of content in guinea pig distal colon?' **Neuroscience** (2013) 240: 325-335

Sia, TC; Kuizenga, M; Dodds, K; Wiklendt, L; Arkwright, J; Thomas, T; Brookes, S; Spencer, NJ; Wattchow, DA; Dinning, P; Costa, M. 'Neurally mediated discrete clustered contractions propagating in *ex vivo* segments of human ileum'. **American Journal of Physiology** (2014) (electronically published ahead of print)

Raghupathi, R; Duffield, M; Zelkas, L; Meedeniya, A; Brookes, S; Sia, TC; Wattchow, D; Spencer, NJ; Keating, D. 'Identification of unique release kinetics of serotonin from guinea pig and human enterochromaffin cells' **Journal of Physiology** (2013) 591: 5959-5975

Spencer, NJ; Nicholas, SJ; Sia, TC; Staikopoulos, V; Kyloh, M; Beckett, EA. 'By what mechanism does ondansetron inhibit colonic migrating motor complexes: Does it require endogenous serotonin in the gut wall?' **Neurogastroenterology & Motility** (2013) 25:677-685

Spencer, NJ; Kyloh, M; Wattchow, DA; Sia, TC; Nicholas, S; Brookes, SJ. Characterization of motor patterns in isolated human colon: are there differences in patients with slow-transit constipation? **American Journal of Physiology** (2011) 302: G34-43

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Conference proceedings

2014 Royal Australasian College of Surgeons Paper Day

- "Motility recordings *in vivo* and *ex vivo* shed insight into the etiology of slow transit constipation" (Oral)

Special Seminar, Centre for Neuroscience, Flinders University, SA

- "Investigations in the neural mechanisms that govern peristalsis in the guinea pig and human lower gastrointestinal tract" (Oral)

Australian Neuroscience Society Annual Conference, Adelaide, SA

- "Mechanisms underlying hexamethonium and atropine resistant peristalsis in the guinea pig distal colon" (poster)

2013 Royal Australasian College of Surgeons (SA) Paper Day

- Is there a functional role for serotonin neurotransmission in peristalsis (Oral)

- Evidence of peristalsis in the *ex vivo* human small bowel (Oral)

Royal Australasian College of Surgeons Annual Scientific Meeting Tripartite- WA, SA, NT

- "Investigations into the mechanisms underlying peristalsis in the human small bowel" (Oral)

Flinders Medical Centre Grand Round

- *Ex vivo* Peristalsis in the Human Lower Gastrointestinal Tract: Where are we now? (Oral)

Australian Neuroscience Society Annual Scientific Conference (ANS ASC), Melbourne, VIC

- A human model for understanding small bowel motility (Oral)

Royal Australasian College of Surgeons Annual Scientific Congress, Auckland, New Zealand

- Comparison of human *ex vivo* and *in vivo* ileal motor patterns; is myogenic activity preserved in an organ bath? (Oral)

2012 Surgical Research Society of Australasia Annual Scientific Meeting, Adelaide, SA

- An *ex vivo* model for studying human small bowel and colonic motor activity (Oral)

Australian Neuroscience Society Annual Scientific Conference, Gold Coast, QLD

- Endogenous Serotonin is Not required in the Generation of Colonic Peristalsis (Oral)

- *Ex vivo* characterization of the human colon: a pioneering effort

Royal Australasian College of Surgeons (SA) Paper Day

- Insights into serotonergic pathways in colonic motility (Oral)

- Behaviour of the human small bowel and colon in vitro: some early findings (Oral)

Neurogastroenterology and Motility Annual Convention, Bologna, Italy

- Identification of different patterns of propagating motor activity in the isolated human colon (Poster)

Digestive Diseases Week, San Diego, CA, USA

- *Ex vivo* Characterisation of the Human Colon (Poster)

Royal Australasian College of Surgeons Annual Scientific Congress, Kuala Lumpur, Malaysia

- Is there a need to target serotonergic pathways in colonic dysmotility? (Poster)

- *Ex vivo* analysis of colonic motility from extended lengths of resected human colon (Poster)

2011 Oral- Royal College of Surgeons (SA) Paper Day

- Role of serotonin in Colonic Motility (Oral)

Chapter 1: Regulation of motility in the lower gastrointestinal tract

1.1. ANATOMY OF THE LOWER GASTROINTESTINAL TRACT

1.1.1. Components of the gastrointestinal tract

The gastrointestinal (GI) tract is an elongated tube commencing at the oral cavity, ending at the anus. The entire GI-tract consists of the oral cavity, oesophagus, stomach, small intestine (including the duodenum, jejunum and ileum), caecum, colon (ascending, transverse, descending, sigmoid), rectum and anus. Each of these distinct anatomical structures plays an essential, but distinct role in digestion, absorption and propulsion of luminal contents. This thesis is focused on understanding the motor activity of the isolated small bowel and colon and the role of the endogenous neurotransmitters, particularly serotonin, in the generation of these motor patterns.

The human small bowel measures approximately 7 metres in length and the colon approximately 1 metre (1). This is based on cadaveric measurements, but in humans *in vivo*, the total length of small intestine may be considerably shorter, estimated at 3 to 4 metres (2, 3). This discrepancy is significant when physiological parameters such as velocity and distance of contractile propagations are measured. In these circumstances careful interpretation between *in vitro*/*ex vivo* and *in vivo* studies are required.

The small intestine occupies the central region of the abdominal cavity, suspended on a mesenteric root that runs obliquely from the left side, superiorly, and extending to the right side, inferiorly. The primary vascular supply to the small bowel is via

jejunal branches that arise from the superior mesenteric artery. The terminal ileum receives ileal branches from the ileocolic pedicle, also a tributary of the superior mesenteric artery. A segment of terminal ileum is usually excised in colonic resection surgeries for cancer within the right hemicolon as this arterial supply is ligated as part of the procedure.

1.1.2. The bowel wall

The wall of the GI tract is composed of five primary layers: the serosa, two distinct muscle layers: an outer longitudinal and inner circular, the submucosa and the mucosa (consisting of the muscularis mucosae, lamina propria, and the epithelial layer luminally). The serosa is the outermost layer of the GI-tract, comprised of a thin film that envelopes the outer longitudinal muscle layers and inner circular muscle. There is no serosa overlying the oesophagus, nor in the rectum distally, for which the anterior 1/3 and posterior 2/3 are extraperitoneal. The myenteric plexus consists of a network of enteric ganglia, which is arranged between the two muscle layers. The mucosal plexus lies within the submucosa. The mucosa is the innermost layer of the bowel wall that facilitates biochemical and mechanical contact with ingested luminal contents. It is in this layer that specialised cells involved in secretion and absorption are present, and specifically where enterochromaffin cells that secrete serotonin and other neurotransmitters are localised. Interstitial Cells of Cajal (ICC) are found within the muscular layers and of the inner circular and outer longitudinal muscles (4). Of note is the human colon, where the outer longitudinal muscle layers are organised into three discreet longitudinal fibres equidistant from one another, named taeniae, which coalesce as the colon becomes the rectum.

Interestingly, sacculations form as a result of the taenia being slightly shorter than the rest of the colonic tissue, creating the appearance of haustrations (5). There is overwhelming evidence that the complex activity of neuronal plexuses in the submucosa and between the two muscular layers (myenteric plexus) initiates and propagates complex motor activities, such as peristalsis.

1.1.3. Inter-species correlation

It is now known that the neurochemistry of the enteric nervous systems is remarkably well preserved across species. There is an extraordinary similarity in the electrophysiological characteristics between enteric neurons in human (6) and other animal species (7, 8) including even invertebrates (9). For example, the major neurotransmitters that have been identified in laboratory animals have also been identified in human GI-tract (10-16). This is perhaps best exemplified by the guinea pig enteric nervous system, where similar neurochemical coding of enteric neurons can be correlated directly with human intestine (17). It is for these reasons that animal models have proved important in understanding functional deficits to intestinal motility in humans. Age and weight variation can be minimised in animals, and a greater range of interventions performed.

However, while it is noteworthy that the major motor patterns underlying propulsion of contents identified in humans *in vivo* have also been recognised in laboratory animals, subtle differences are known to occur between species (3, 18). This necessitates experimentation with human tissue to further understand basic physiology of the GI tract. This will be discussed in more detail below.

1.2. CLASSIFICATION OF NEURONS IN THE GI TRACT

There is now sound evidence that neurons of the ENS can be classified into functional and morphological groups (8, 19, 20). The earliest morphological classification of enteric neurons were by Dogiel (21). Since the initial descriptions of 3 main classes, (Dogiel type I-III), this is now expanded to include a list of other cells (22).

The density of myenteric neurons in the ENS of mammals is high. For example, in the guinea pig intestine, a 10mm section of myenteric plexus includes some 6,500 intrinsic primary afferent neurons, 1,200 ascending and 3,000 descending interneurons, 3,000 excitatory and 4,000 inhibitory interneurons. These neurons fire in a coordinated network to ensure orderly progression of propagating motor activity along the bowel (23).

Electrophysiological findings revealed the presence of 2 types of neurons in the myenteric plexus. These were first classified by Hirst *et al*. in 1974 as AH and S-type neurons (24) and coincidentally characterised by Nishi & North as Type 1 and Type 2 neurons (25). The terminology AH and S type neurons is nowadays more often used. “AH”-type neuron is an acronym for “after-hyperpolarising”, since they were found to exhibit long lasting after hyperpolarisations (up to 30 seconds duration) following single somal action potentials (24). S-neurons (S- synaptic) on the other hand discharges a tonic burst of action potentials in response to current injection. Furthermore, AH neurons were seen morphologically to be large bodied cells with multiple axons, (Dogiel Type II) while S neurons are smaller and uniaxonal (Dogiel type I) (8, 19). A variety of functional neuronal groups exist in

the bowel wall including intrinsic primary afferents, motor neurons to differing layers of the gut wall, interneurons and intestinofugal neurons. Studying C-Fos reactivity as a marker of neuronal activity (C-Fos messenger-RNA is upregulated in neuronal activity) initially revealed intrinsic sensory nerves to be AH neurons, leading Kirchgessner and Gershon in 1988 to coin the terms “Intrinsic primary afferents” (26), as they “sense” changes in its environment (whether these changes refer to distension, chemical or mechanical stimulation), and then convey these “senses” through to effector neurons within the ENS via synaptic connections with chains of interneurons. In 1995, Kunze and colleagues confirmed that AH neurons were intrinsic sensory neurons by demonstrating that they respond to acid puffed onto the mucosa in a low calcium solution that blocked all synaptic transmission (27). It is now clear that these sensory neurons synapse with interneurons and motor neurons, which also have S-type electrophysiology (28, 29). More recently, it has been shown that a population of myenteric S neurons can also behave functionally as intrinsic sensory neurons, one that responds to circumferential stretch (30). By blocking excitatory postsynaptic potentials (EPSP) in a low calcium, high magnesium solution, stretch-induced firing in this population of S neurons persisted, while AH neurons were electrically quiescent. It was found that these stretch-sensitive myenteric S-neurons were morphologically identified as interneurons in the myenteric plexus. Other classes of interneurons simply convey synaptic signals in both an oral and anal direction (31, 32). Although evidence exists that intrinsic sensory nerve cell bodies lie within the myenteric plexus, another hypothesis has been argued that extrinsic primary afferent with cell bodies in dorsal root ganglia are responsible for sensory reflexes in the GI tract. In other words, no intrinsic sensory neurons lie within the bowel (33).

Immunohistochemical techniques over the past 20 years have revealed major insights into morphology and neurochemistry of the enteric nervous system of laboratory animals (28, 29) and human intestine (11-14, 16, 34). This has facilitated clear identification of the roles of differing types of neurons in healthy and diseased bowel.

Unlike skeletal muscle, both excitatory and inhibitory motor neurons exist in the GI tract, supplying both the longitudinal and circular muscles (35, 36). These motor neurons are S-type uni-axonal neurons. While all motor neurons supplying the circular and longitudinal muscles of small laboratory animals arise from the myenteric plexus, a component of the circular muscle may be innervated from the submucosal plexus in other species (8).

1.3. NEURAL CONTROL OF PERISTALSIS- INTRINSIC INNERVATION

At the turn of the last century, Bayliss and Starling (1899) first described peristalsis in the conscious dog intestine. They demonstrated that when devoid of extrinsic innervation, the intestine was able to contract in coordination and propel digesta anally leading to the proposition of the "Law of the intestine" (37). They also clearly documented that local stimulation of the bowel evoked a peristaltic contraction consisting an ascending excitatory and descending inhibitory neural reflex response. At a similar time to the pioneering studies of Bayliss and Starling, Langley also suggested that enteric neurons were able to function independently of the CNS (38). This was following his description of the large numbers of neurons in the ENS, its

degree of independence from the central nervous system, the presence of entire reflex pathways within the ENS, and relatively few efferent axons entering the gut compared to the number of enteric neurons within it (17, 38).

1.3.1. Myenteric plexus

The myenteric (Auerbach's) plexus is sandwiched between the inner circular and outer longitudinal muscles of the bowel wall.

Acetylcholine and tachykinins have traditionally been implicated as the main initiators of peristalsis. However, multiple other neurotransmitters have now been implicated in mediating fast synaptic transmission at different functional classes of myenteric neurons. These include adenosine triphosphate (ATP) acting on P2 receptors and serotonin acting on 5-HT3 receptors (39).

While cholinergic neurotransmission is clearly the major excitatory neurotransmitter in the gut wall (8, 19, 40), it is also known that hexamethonium (nicotinic receptor antagonist) resistant peristalsis can occur in the ENS. The nature of the neurotransmitters that underlie hexamethonium resistant peristalsis remains elusive. Bartho and colleagues showed that atropine and spantide (NK-2 antagonist) blocked peristalsis in specimens that were resistant to hexamethonium (41). More recently, Spencer and others revealed the presence of hexamethonium resistant peristalsis in the guinea pig colon. They showed that in the colon, hexamethonium resistant peristalsis was also resistant to NK-3 receptor, 5-HT3 receptor and P2 purinoceptor antagonism (39). In Chapter 2, I will be describing experiments that ascertain

whether blockade of major excitatory receptors at the neuro-muscular junctions inhibit hexamethonium resistant peristalsis in the colon.

1.4. MEDIATORS OF EXCITATORY POST-SYNAPTIC POTENTIALS

1.4.1. Acetylcholine (ACh)

Overwhelming evidence has demonstrated that ACh is the primary excitatory neurotransmitter in the gastrointestinal tract. In 1934, Dale and Feldberg detected the release of ACh upon vagal stimulation of the stomach and concluded that ACh was released from nerve endings (42). Subsequently, many others have also demonstrated an excitatory effect of ACh on neural pathways in the gut wall. The use of multi-chambered organ baths has proved very useful in unraveling the role of various neurotransmitters in different parts of the enteric neural circuitry. For example, Johnson and colleagues revealed that ascending excitatory pathways were significantly reduced in the presence of hexamethonium and a desensitising concentration of the NK-3 receptor agonist senktide. This was further abolished in the presence of the cholinergic neuro-muscular receptor blocker hyoscine (43). Further evidence for cholinergic-dependent transmission is obtained from experiments where it has been shown that muscarinic receptor antagonists, such as atropine or hyoscine consistently blocked excitatory neurotransmission. In conditions of ACh enzymatic breakdown (by acetylcholinesterases) inhibition, excitatory motor activity is facilitated (8). With the emergence and subsequent development of immunohistochemical and electrophysiological methods, the role of

ACh as the major excitatory transmitter underlying GI motility in enteric neurotransmission is now overwhelmingly clear.

In some instances however, it is known that atropine resistance occurs. This is discussed below in relation to tachykinins and their roles in enteric neurotransmission, and forms the basis for our experiments in Chapter 2.

1.4.2. Tachykinins

Tachykinin receptors are seven transmembrane class-A G-protein coupled receptors (44). Clinically associated with altered GI motility, secretion and visceral sensitivity (45), tachykinin receptor families have been studied in detail over the past 20 years. Immunohistochemistry has revealed that ACh and tachykinins are most commonly co-localised (8, 46). Tachykinins are likely to play a lesser, albeit still significant role in generating excitatory post-synaptic potentials compared to ACh (47). Where this comes into significance is in regards to atropine-resistant peristalsis, where tachykinin receptors play a major role.

There are three well characterised tachykinin receptors- NK1, NK2 and NK3 (44, 48). Strong evidence suggests that NK-1 and NK-2 receptors are involved in regulating GI motility, forming the major component of non-adrenergic non-cholinergic excitatory transmission (47, 49, 50). NK-1 and NK-2 receptor antagonists were shown to block peristalsis in the presence of atropine (51). This suggested that atropine-resistant peristalsis was largely mediated by tachykinins. Tachykininergic transmission (via NK-2 receptors) together with cholinergic neurotransmission (through muscarinic receptors) plays the major excitatory

pathways to the circular muscles (52). It has been shown that when cholinergic neurotransmission was blocked at the neuro-neuronal junction, only NK-2 receptor antagonists were able to further attenuate this hexamethonium-resistant peristaltic activity (51). Also, hexamethonium-sensitive but atropine-resistant peristalsis is suppressed by the NK-2 receptor blocker MEN 10376 (53).

Interestingly, ICCs are also consistently found to exhibit NK-1 receptors and it is thought that substance P release regulates pacemaker currents via these channels (54-56). NK-3 receptors have been shown to be less likely to contribute to any excitatory input on motility. Of the available NK-2 antagonists, ibodutant has been shown to have amongst the highest binding affinity with a pKi of 9.9 (saredutant= 9.2, nepadutant=8.4) (57). This is one of the reasons we chose this antagonist for our following experimentations looking into the nature of atropine resistant peristalsis.

1.4.3. Serotonin

Serotonin, or 5-Hydroxytryptamine (5-HT) is one of many monoamine neurotransmitters. First isolated by Vittorio Erspamer in 1935, it was initially named enteramine after its anatomical source. Following findings of its role in blood vessel contraction the term “serotonin” came to be used (58).

5-HT has been involved in various physiological processes including regulation of muscular contraction, platelet aggregation, appetite, mood, and wakefulness (59). It has also been implicated in various endocrine roles regulating coagulation, liver regeneration and bone formation (60).

Most importantly, and central to the discussions in this thesis, is the perception that endogenous 5-HT is a requirement for the initiation and/or generation of peristalsis in the small intestine (61, 62) and colon (63-66) and colonic migrating motor complexes (CMMCs) in the colon (67). Our motivations are following recent evidence that have cast serious doubt on the original hypothesis that endogenous serotonin is essential for peristalsis (39, 68-71). This will be discussed in detail in the following chapters.

1.4.3.1. Why endogenous 5-HT was thought to be important in the generation of peristalsis

The majority of endogenous serotonin is synthesised within the gut wall (72). In fact, more than 95% of endogenous serotonin is derived from the enterochromaffin cells (EC) within the mucosa, with minute quantities being released from enteric neurons. Serotonin is also found in platelets, but only in a storage capacity.

Edith Büllbring's laboratory was the first to propose that endogenous 5-HT release from the mucosa was essential for peristalsis to occur (61, 62, 73). In her early experiments, Büllbring showed that when mechanical stimuli were applied to the mucosal epithelium, serotonin was released luminally and this was noted to coincide with the occurrence of peristaltic contractions. The fact that her laboratory noted a one to one correlation between release of endogenous 5-HT and the onset of peristalsis was perceived to be sound evidence that release of 5-HT was the cause, not the effect of peristalsis.

In further support for the early hypothesis that endogenous 5-HT was essential for peristalsis to occur, it was found that exogenous application of 5-HT to the intestine potently initiated peristalsis. Evidence that endogenous 5-HT may be a major player in the generation of peristalsis comes from numerous studies that have revealed that specific agonists of 5-HT receptors can initiate or enhance peristalsis. For example, 5-HT4 receptor agonists have been shown to accelerate transit of contents in the lower GI tract, both *in vivo* (74, 75) and *ex vivo* (64, 76). Conversely, a number of studies have shown that various antagonists of specific 5-HT receptors, can reduce the velocity of propulsion in a dose-dependent manner (64), possibly via mechanisms that involve inhibition of calcitonin gene-related peptide (CGRP) (63).

It is interesting that Kadowaki found that individual blockade of 5-HT3 and 5-HT4 receptors had no effect on motility, and only when both receptors were blocked, or in the added presence of hexamethonium was motility blocked. It was suggested then by the authors that these receptors work in parallel, but at least some cholinergic activity is essential (65). This was significant, as for the first time it was noted that many prior experiments concluding an essential role for serotonin in lower GI tract motility have been derived from non-specific antagonists of serotonin receptors, such as tropisetron and benzamides.

While studying cohorts of mice with tryptophan hydroxylase-1 and 2 knockouts and comparing them with their wild type littermates, Li and colleagues reported decreased motility under conditions where neuronal 5-HT was depleted, but not when only mucosally-synthesised 5-HT was depleted (77). Following depletion of mucosal 5-HT, total GI transit time was found not to be reduced in TPH1 (mucosal)

knockouts (77). This suggests that neuronally derived serotonin may be important in motility of the GI tract, but not mucosal derived serotonin. It is important to note, however, that in response to genetic ablation of neuronally synthesised 5-HT, major neuro-architectural changes also occurred, which could have accounted for the reduction in transit. This is in contrast to previous assumptions that the comparative abundance of EC cell derived serotonin implicates it as an important driver of peristalsis (78). However, it is difficult to reconcile these significant effects given that serotonergic neurons only account for less than 1% of all within the myenteric plexus (79).

We speculated that if endogenous serotonin was indeed essential for peristalsis to occur in the lower GI tract; and, in light of the fact that >95% of endogenous serotonin is stored within EC cells of the mucosa, then one would presume that removal of the mucosa would abolish peristalsis.

Recent work by Bertrand (80) has suggested that 5-HT release from EC cells is caused by the muscle contraction that underlies peristalsis (as EC cells are potently mechanosensitive). Indeed, it was recently shown in the colon that when real time amperometric recordings were made, serotonin was released at the same time as many, but not all colonic migrating motor complexes (68). This result is consistent with the early experiments by Büllbring *et al*. in the small intestine where it was shown that 5-HT release into the lumen correlated with the onset of peristalsis. Many have argued that this correlation implies that endogenous 5-HT release in the colon is also responsible for the initiation of peristalsis (64). However, this notion was revised recently when Spencer and colleagues removed the mucosa from the colon.

These experiments showed that all release of 5-HT ceased, but distension-evoked peristalsis persisted (81). This was a defining experiment which proved that 5-HT release was a consequence of peristalsis and not the underlying cause of it. In this thesis, I will present new data describing the effects of removing the mucosa and submucosal plexus on distension-evoked peristalsis in the colon in conjunction with experiments that also depleted neuronally synthesised 5-HT.

Ultimately, pharmacological efficacy of serotonin based drugs in human disease (82-84), and correlation of serotonin bioavailability with various conditions of GI dysmotility have been considered grounds to propose that serotonin plays a significant role in GI motility (78, 85, 86).

1.3.3.2. Serotonin biochemistry and enterochromaffin cell release kinetics

Serotonin is derived from tryptophan. Hydroxylated to 5-hydroxy-L-tryptophan from the amino acid L-tryptophan, it is then catalysed by the rate limiting enzyme tryptophan hydroxylase (TPH) to serotonin, and stored within enterochromaffin (EC) cells. Calcium influx into the EC cells stimulates release of serotonin via activation of L-type Ca^{2+} channels. Interestingly, recent evidence points to only miniscule amounts of serotonin released, perhaps just enough for activation of the mucosal 5-HT receptors in each fusion event, at about 70 times less than that seen for catecholamine release from similar sized large dense core vesicles (87).

1.4.3.3. Enterochromaffin cells (EC) and their paracrine effects

While evidence suggests 5-HT may be a neurotransmitter in the enteric nervous system the largest amount of serotonin in the body is not formed within the neural circuitry, but in specialized enterochromaffin cells (EC) found in enteric mucosal epithelium. To this effect up to 95% of bodily serotonin is found within the mucosa, from a process that involves the rate limiting enzyme tryptophan hydroxylase 1 (TPH1), found primarily in EC cells (88). The remaining production is found within neurons- and these involve a separate group of enzymes, P2 (89). On the other hand, platelets (carrying serotonin intravascularly) have none of these enzymes, and are therefore unable to produce serotonin.

Serotonin is stored within large electron dense secretary granules and is released following various stimuli including that of taste and mechanical deformation of the EC cells. This leads to an increase in calcium availability from both voltage gated calcium channel activation or intracellular release of calcium stores (90). Despite the extensive debates on the roles and significance of serotonin release from EC cells, cellular level understanding in this field is still lacking. The fact that only now is basic information in relation to its release kinetics been clarified (87) is testimony to this.

Unlike other neuro-neuronal junctions, where there is close apposition between nerve ending and receptors on the receiving neuron, serotonin release from EC cells has no direct physical connection to any neuronal post-synaptic receptors. It is

therefore often described that 5-HT exerts a paracrine effect *in vivo*. It is thought that the hypersecretion seen from EC cells evolved as a compensatory mechanism as the EC cell and receptor do not physically come into contact. In addition, it has been postulated that the high concentrations of serotonin from EC cells could act on the mucosal projections of AH neurons or 5-HT1a receptors on mucosal mast cells, to stimulate enteric neural circuitry underlying GI motility (91).

Similar to other neurotransmitters, serotonin is constantly catabolised by monoamine oxidases (MAO) into its corresponding aldehyde. Specialised serotonin reuptake transporters (SERT) mediate this reuptake into epithelial cells, platelets and other cells. This is significant as MAOs are not freely present in the synaptic space and therefore blocking the actions of reuptake with MAO inhibitors (MAOIs) presents itself as a means of potentiating the presence of available serotonin in the synaptic junction. Aldehyde is then metabolised by aldehyde dehydrogenase to 5-hydroxyindolacetic acid (5-HIAA) in the liver and excreted via the kidneys. 5-HIAA allows for measurements indicating increased levels of circulating serotonin and assists in the diagnosis and follow up of clinical conditions involving serotonin dysregulation.

1.4.3.4. The serotonin receptor family and its downstream effects of activation

Complex arrays of different 5-HT receptors are expressed in the bowel walls. Understanding the functional role of these receptors is vital if we are to fully understand the potential of serotonin receptor agonists and antagonists in the treatment of clinical states of dysmotility.

Seven classes of serotonin receptors have been identified, of which some have been further subclassified. Of these, 5-HT3 and 5-HT4 receptors have long been accepted as primary receptors that are thought to underlie changes in GI motility patterns. 5-HT3 receptors are ligand gated ion channels which are found on enteric neurons, mediating fast post-synaptic excitatory transmission. On the other hand, 5-HT4 receptors are G-protein coupled receptors and are found predominantly in the mucosa mediating slow excitatory neurotransmission. These receptors primarily co-localise on cholinergic nerve terminals, and when stimulated released ACh at nicotinic/neuro-neuronal synapses. This suggests that 5-HT4 mediated response is more modulatory than excitatory, and at least some cholinergic activity is essential (65).

Upon activation by high concentrations of paracrine-secreted serotonin from EC cells, 5-HT3 receptors on intrinsic primary afferent neurons (IPANs) and extrinsic nerve terminals are activated, leading to the initiation of peristalsis and secretory reflexes. This initiation has also been proposed to involve co-release of ACh and CGRP from these neurons to generate both fast and slow excitatory neurotransmission (63). In experiments performed on guinea pig colon, circular muscle contractions were thought to be mediated by serotonin acting on 5-HT3 receptors. In the longitudinal muscle concentration dependent atropine inhibition of serotonin induced contraction would suggests that the mechanism of contraction here depends on the subsequent release of ACh and substance P (92). 5-HT4 receptors located on cholinergic neurons are found pre-synaptically and amplify the strength of contractions by enhancing release of ACh (93).

To further add to the complexity of these receptor families, 5-HT4 receptors are also found on the ICCs, and contradictingly mediate smooth muscle relaxation (94).

5-HT3 and 5-HT4 receptors will form a major focus in one of the chapters in this thesis.

1.4.3.5. Clinical relevance and pharmacology of serotonergic neurotransmission

EC cells and the corresponding bioavailability of gut-derived serotonin have been implicated in many functional gastrointestinal dysmotility states. Clinical conditions of irritable bowel syndrome, slow transit constipation and various inflammatory conditions of the bowel including Crohn's disease (95, 96) have each been shown to correlate with changes in EC cell populations and distribution, with resultant altered serotonin levels. Whether these changes represent a "cause or effect" of the diseases is not clear. Based on these findings, some investigators have determined the functional benefits of potentiating synaptic serotonin availability and mixed results have been obtained with the use of selective serotonin receptor reuptake inhibitors (SERT) (97).

Many therapeutic agents currently available are known to modulate serotonergic receptors and/or transmission (98). Some, for the lack of complete pharmacological understanding, were approved for release but subsequently withdrawn. This was secondary to concerns of specificity and resultant adverse effects. One example is tegaserod (Zelnorm, Zelmac), a 5-HT4 agonist that has been shown to be efficacious

in accelerating gastric emptying, shortening small bowel transit and accelerating colonic transit (99, 100), in addition to neuroprotective roles (101). As a result it underwent clinical trials and was approved for the treatment of constipation predominant irritable bowel syndrome (C-IBS) and chronic constipation. Unfortunately, it was later withdrawn following increased risks of ischaemic cardiovascular events (102). Similarly, another 5-HT4 receptor agonist cisapride (Propulsid) was also withdrawn, due to side effects of prolonged QT intervals leading to cardiac arrhythmias with devastating effects including that of polymorphic ventricular tachycardia (103, 104). Alosetron (Lotronex), a 5-HT3 antagonist, was initially approved for use in diarrhoea predominant irritable bowel syndrome (D-IBS) (98, 105), but its use is now very limited following concerns of ischaemic colitis (78, 106). The withdrawal of these drugs for non-GI related side effects highlights our inadequate understanding of the mechanisms underlying serotonin receptor activation and downstream effects.

More recently, prucalopride (Resolor, Resotrans), a selective 5-HT4 receptor agonist with higher levels of specificity compared to cisapride. has been shown in phase III trials to be beneficial for patients suffering from slow transit constipation (82, 107, 108). While our understanding of drug pharmacodynamics is lacking, the incidence of side effects will inevitably arise over time.

1.4.3.6. Depletion of serotonin as a means of studying its role in motility

Attempts to deplete serotonin from the gut wall have produced mixed results. Earlier experiments using reserpine to deplete serotonin appeared not to affect motility in

the guinea pig colon (109). Both tryptophan free diets and experiments using parachlorophenylalanine (pCPA) to deplete serotonin were effective in doing so but did not affect gastrointestinal transit (110, 111). In experiments where 5,6-dihydroxytryptamine (5,6-DHT) and 5,7-dihydroxytryptamine (5,7-DHT) were used to cause extensive damage to serotonergic neurons, impairment of motility was evident. However, poor specificity of the 5-DHT drugs preclude any significant conclusions to be drawn (112).

A more recent example is the use of tryptophan hydroxylase-1 (TPH1) blockers to deplete serotonin. In these experiments, originally designed to assess effects of gut derived serotonin on bone formation, gastrointestinal motility was found to not be affected in TPH1 inhibitor treated mice. This was especially interesting, as the concentrations used were not negligible amounts, up to 250mg/kg body weight of the mice (70). Under these circumstances, the possibility of plastic and development changes, especially given the administration of drug in days leading up to the experiments led to motility adaptations and should of course be considered. Simply depleting serotonin, without consideration of the site of production or the accommodation changes has rendered these experiments difficult to interpret.

What remains clear, is the fact that mucosal serotonin was not required for the generation of peristalsis. With this in mind, the natural suggestion would be that the enteric neurons that synthesise 5-HT in the ENS play a major role in control of peristalsis. While this remains a plausible, it is difficult to fathom that the minute population of serotonergic neurons (comprising only of <1% of all enteric neurons) could be responsible for such a major function in the bowel. Recent studies

investigated the effects of selective genetic deletion of serotonin synthesis from EC cells, by deleting the TPH1. This led to an inhibition of 99% of endogenous 5-HT levels, but no decrease in motility *in vivo* (113). , Following earlier observation of motility preservation in the setting of TPH1 knockout, Li and colleagues investigated the role of TPH2 and concluded that total GI transit, small bowel transit and colonic motility were abnormally slow in the TPH2 knockout specimens (77). One wonders if the disproportionate role of neuronal serotonin in mediating peristalsis was related to its established role in enteric neuronal development and survival, thus leading to its significant decrease in motility parameters.

In this thesis, I have used methods to deplete endogenous stores of serotonin from enteric neurons in our specimens. By immediate pretreatment of reserpine 24 hours before the experiments and upon resection of the specimen for *ex vivo* analysis we are confident we have performed the most comprehensive of methods to deplete serotonin from our specimens without chance of accommodation or plasticity.

1.4.3.7. Concluding points on serotonin in GI motility

In our view, it is vital to understand the role of endogenous 5-HT in the generation of complex motility patterns. Early studies in the 1950's provided compelling but indirect evidence that endogenous serotonin played an important role in the initiation of peristalsis (61). However, if we are going to continue to develop drugs in the future that modulate peristalsis, it is vital we understand the role of endogenous 5-HT in the generation of this complex motility pattern. I have in this thesis explored the role of endogenous serotonin in the generation and propagation of peristalsis.

Firstly, we aimed to completely deplete serotonin from the guinea pig distal colon, using reserpine treatment the day before study of the bowel segments, then meticulously dissected the mucosa away from the colon to remove all available stores of serotonin Depletion of all serotonin stores were confirmed using immunohistochemistry and mass spectrometry. By doing so acutely (just prior to *ex vivo* experimentation) we remove any uncertainties associated with accommodation post depletion and found that peristalsis indeed is robust and present in the absence of GI tract derived serotonin (69).

Under these circumstances, we were intrigued by the efficacy of serotonin receptor agonists and antagonists in facilitating or retarding intestinal peristalsis. Interestingly the antagonists used consistently retarded and abolished peristalsis, albeit transiently (71). It is likely that serotonin receptors do play a role in peristalsis; but their role is at most modulatory, not essential.

Modulating the serotonin pathways are no doubt a very significant and potentially promising way of treating patients with GI dysmotility conditions. But until today available pharmacological agents acting onto the various serotonergic receptors seem to be more an exercise in the balance of risk versus benefit, with multiple drugs so far being removed from the market.

Some evidence suggest an autonomous role for certain 5-HT receptors, in particular those incorporating the 5-HT3b subunit (114). These findings then beg the question: Could evidence of serotonergic neurotransmission over the last 60 years be a larger

result of underlying constitutive activity of the receptors themselves, rather than involving the activation of these receptors by release of endogenous 5-HT from enterochromaffin cells?

1.4.4. Adenosine triphosphate (ATP)

ATP is a major excitatory and inhibitory neurotransmitter at many autonomic junctions. ATP acting on P2 receptors has complex manifestations, with some subunits being excitatory and at some inhibitory depending on the neuro-neuronal or neuromuscular junction, respectively. In the enteric nervous system there is extensive data to suggest a role for P2X mediated transmission. P2X receptor activation in the myenteric plexus has been shown to contribute to fast excitatory synaptic transmission (115-117). In the guinea pig distal colon, it was found that fast excitatory postsynaptic potentials in the myenteric plexus that were resistant to nicotinic blockade were significantly reduced by the P2 receptor antagonist pyridoxal phosphate-6-azo tetrasodium salt hydrate (PPADS) (118).

Extensive evidence has been presented to support the notion that ATP is involved in inhibitory neurotransmission in the gut (119). Evidence of such activity is conveniently established in the utility of apamin to block apamin-sensitive potassium channels, the specific mechanism by which ATP exerts its inhibitory effects (120-122). Other known and postulated inhibitory neurotransmitters- vasoactive intestinal peptide (VIP) and nitric oxide (NO) (123) are not generally affected by apamin (122). Over the past decade substantial evidence has been presented to strengthen the purinergic theory of inhibitory neurotransmission- including the release and detection of presence of ATP during stimulation of NANC neurons, and the fact that

NANC neuronal related response by ATP and desensitisation of ATP receptors blocked responses to NANC neuronal stimulation (124).

1.5. MEDIATORS OF INHIBITORY POST-SYNAPTIC POTENTIALS

Smooth muscle in the gastrointestinal tract receives a prominent inhibitory innervation. The presence of an inhibitory component in the smooth muscles of the gastrointestinal tract were reported when Langley noted that in the presence of atropine, stimulation of the vagus nerve produced relaxation of the stomach. Further stimulation of the autonomic centres in the brain led to relaxation in the stomach confirming that this was mediated by the vagus itself (38).

1.5.1. Non adrenergic, Non cholinergic inhibitory neurotransmission (NANC)

A period of time passed before Geoffrey Burnstock noted in 1963 that inhibitory neurons in the gut were neither adrenergic nor cholinergic (125, 126). Initial work with cholinergic and adrenergic blocking agents then revealed surprising results that active inhibitory neurons were not dependent on these 2 major pathways, thought at the time to be the basis of all neurotransmission in the GI tract (124). This was in line with findings confirming the presence of inhibitory junction potentials in the presence of atropine (muscarinic blockade) and guanethidine (sympathetic-inhibitory blockade) (127). Subsequently, inhibitory junctional potentials, characterised by transient periods of hyperpolarisation in the smooth muscle membrane potentials were found to be present in various regions of the gut and across various mammalian species (128-131).

1.5.2. Nitric oxide (NO)

Nitric oxide (NO) is well characterised as an inhibitory neurotransmitter in the gastrointestinal tract. NO is synthesised from a guanidine group of L-arginine and can be produced by all mammalian cells that express nitric oxide synthase (NOS). At present, 3 distinct NO producing enzyme systems are identified- 1) A constitutive enzyme present primarily in the vascular endothelium, 2) An inducible enzyme (iNOS), induced by activation of cytokines and lipopolysaccharides during inflammation, and 3) A neurally associated, constitutive enzyme found in the brain and in the enteric nervous system (132). The discovery of NO as a mediator of inhibitory effects on the GI tract was most fascinating and pertinent to our discussion here. NO is a compound that not only differs from other neurochemical transmitters in that it is gaseous in its steady state, it is also produced on demand by its synthesizing enzyme nitric oxide synthase (NOS) (133). Blockade of NOS results in reduction in inhibitory neurotransmission in different regions of the GI tract in many species and has been identified in inhibitory neurons supplying the GI tract musculature (133, 134).

A series of experiments in the early 80's conducted by Bywater *et al* showed the presence of 2 different pathways of inhibition in the guinea pig ileum, one of which is characterised by an initial "fast" NANC hyperpolarization, and one that is slow, and apamin resistant (135-137). In the presence of apamin-resistant NANC inhibition in the gut, this slower hyperpolarization (1-2 seconds, compared to the fast hyperpolarization seen consistent with ATP) was then blocked by inhibition of NOS. In the guinea pig at least, this has been shown in to be a generalised response in various regions of the GI tract- e.g. ileum (138, 139), and colon (140). Further

experiments investigating the roles of NO, VIP and ATP (the postulated NANC inhibitory transmitters) now show that NO is the primary candidate (141).

1.5.3. Vasoactive intestinal peptide (VIP)

VIP has been detected immunohistochemically (46) and physiologically found to potently relax GI musculature (142-144). VIP was identified as a constituent of inhibitory neurons and plays a role in inhibitory neurotransmission (145). The antiserum against VIP potently inhibits descending relaxation in a concentration dependent manner (143). VIP containing neurons also project from the intestines to the prevertebral sympathetic ganglia, and are involved in intestino-intestinal reflexes- these are likely to involve intestinofugal neurons (146).

1.5.4. Opioid peptides

Despite common misconception, opioid peptides do not directly contribute to inhibitory neurotransmission in the GI tract. In clinical conditions of GI disturbance, morphine, or opioid like substances leading to decreased GI motility and increased transit times. Abdominal symptoms of bloating, pain and constipation can be misinterpreted as a result of μ-receptor activation causing GI motility impairment. μ-receptor activation in fact has no effect on myogenic activity, and has been shown to lead to reduced output of ACh from excitatory neurons (147), and like GABA are found on the excitatory neurons modulating the output of ACh (148).

1.6. MYOGENIC FACTORS IN GASTROINTESTINAL MOTILITY

1.6.1. Smooth muscles in the gastrointestinal tract

Smooth muscles are the single most basic contractile unit within the GI tract. With exclusion of the upper 2/3 of the human oesophagus, smooth muscle cells are the mechanical effectors of contraction for the rest of the gastrointestinal tract. But unlike in skeletal muscle, smooth muscle cells are connected to its adjacent cells by gap junctions, and contractile activity is coordinated by an intrinsically driven electrical activity now clearly derived from the ICC (see below), acting in a syncytial fashion when activated. The individual cells exhibit a phasic depolarisation and repolarisation of their membrane potentials, with each depolarisation leading to a contraction upon reaching threshold. These contractions exhibit variable frequencies, amplitude and shape depending on the site of gastrointestinal tract studied. Commonly, each contraction commences with an upstroke phase followed by a plateau phase, which lasts a few seconds. An example of this would be the human colon, where slow waves have been recorded about 2-4 cycles per minute (149). Recorded in the early 1950s by the likes of Bortoff, Prosser and Tomita with their respective colleagues, this repetitive contractile activity, for the reasons of its relatively long contractile phase is now commonly described as slow waves (150-152), and is likely modulated by the interstitial cells of Cajal (ICC). In addition to phasic contractions of the intestinal musculature generated by ICCs it is clear that smooth muscle, particularly in the terminal gastrointestinal tract has been shown to be maintained under tonic neurogenic inhibition via enteric inhibitory neurons (153).

1.6.2. Interstitial cells of Cajal (ICC)

Smooth muscle cells are the effector cells underlying contraction in the gut wall. But contraction is not possible without input from some form of a pacemaker, akin to the sino-atrial node in the heart. This pacemaker system has now been clearly identified in the gut as comprising of individual interstitial cells of Cajal (ICC) (4).

The earliest morphological discovery of ICCs seems to be due to the work of Santiago Ramon y Cajal, although this has been sometimes disputed (154). It was in 1889 that he first published on the discovery of a fibroblast-like cell mediating smooth muscle contraction. It was however thought at that time that ICCs were likely of neuronal origin. This was postulated following Cajal's use of the Golgi stain and Ehrlich's methylene blue staining (superior forms of staining nerves at that time) that these networks of interstitial cells received neural input and regulated contraction of smooth muscle around them.

Interestingly, it was during this time that Sir Arthur Keith suggested that these cells constituted a pacemaker system within the walls of the intestine (154). It was not until much later that more conclusive evidence of this was presented. Early data to suggest this shown that when smooth muscle cells were isolated and studied with patch clamping, slow waves were never observed (155). Alternatively, when ICCs were isolated, smooth muscle exhibited spontaneous depolarisation with the same characteristics as slow waves (156).

ICCs are interspersed between layers of the gut wall, and as such are classified according to their position: the ICCs found in the myenteric plexus as ICC-MY, and

ICC-SM when found in the submucous plexus. ICCs are also found within the circular and longitudinal muscles and are then named ICC-IM (intramuscular). These cells form connections via gap junctions with other ICCs and also with smooth muscle cells. Interestingly, in certain species there exists different patterns of motility that is not neurally mediated, suggesting that different pacemaker systems can exist in a single segment of bowel, either by location, or by type. An example of this would be the rabbit caecum (157), whereby in the presence of hexamethonium two distinct phasic motility patterns are seen. Rat (158), dog (159) and human colon (149) have also been shown to have more than one pacemakers systems in place within the bowel walls.

One significant advance in the study of ICCs was the discovery that ICCs themselves express the tyrosine kinase receptor, Kit (160). Following this discovery, an antibody to the Kit receptor was developed, c-Kit, which proved essential in morphologically characterising all the different classes of ICC in the gut wall.

While working on neutralising antibodies to block Kit signalling to determine the effects of reduced Kit function, Maeda and colleagues observed pathological gastric and intestinal distension and abnormal contractile behaviour in the newborn mice (161). In addition, Huizinga and colleagues showed that in animals with the W/W^{v} mutation, these mice failed to generate any slow waves (160). In humans, treatment of gastrointestinal stromal tumours (GISTs) and or haematological malignancies e.g. chronic myeloid leukemia with imatinib (a specific inhibitor of tyrosine kinase) commonly results in diarrhoeas (162, 163). This has been postulated to be secondary to inhibition of Kit on ICCs, but details on this effect are still lacking.

In addition to its spontaneous pacemaker-like activity, ICCs also respond to neural input. Excitatory and inhibitory effects on the smooth muscle of the GI tract are at least in part relayed by the ICCs. Receptors for NO (164) and tachykinins (165, 166) have been identified on ICCs. Excitatory neural input has been shown to increase the amplitude of slow waves resulting in an increase in smooth muscle Ca entry and stronger contractions. The reverse has also been demonstrated. These in particular, involve specific ICC's located in the smooth muscular layers, the ICC-IM (intramuscular) (167).

It is also likely that ICC networks display some level of plasticity. Certain neural factors can affect ICC redistribution in pathological states. ICC numbers were decreased proximal to an induced obstruction, with this network disruption seen with a loss of response to enteric neural stimulation, which was only partially restored after 30 days (168).

In clinical medicine there is now an abundance of data suggesting that aberrations in ICC populations can lead to conditions of gastrointestinal dysmotility (169). These include common conditions, such as both type 1 and type 2 diabetes, chronic constipation (including both chronic idiopathic constipation and slow transit constipation) to rare conditions such as the Chagas's disease involving the colon resulting in dysmotility (170-173), substantiating an involvement of ICCs in motility in health.

1.7. EXTRINSIC INNERVATION OF THE INTESTINE

The extrinsic innervation of the gastrointestinal tract consists of 3 major divisions: vagal, sympathetic and sensory. The vagal pathways are anatomically craniosacral (8). The craniosacral pathways are predominantly of parasympathetic origin, while the thoracolumbar pathways contribute sympathetic input into the gut. The primary parasympathetic innervation of the small intestine and colon is via the vagus nerve (174), cell bodies of which are located within the brainstem in the dorsomotor nucleus and nucleus ambiguous (175). The more distal regions of the colon and rectum receive parasympathetic input from the sacral nerve roots (S2-4) arising from the lumbosacral spinal cord. Emerging evidence suggest that sensory afferents are specialised, such that their nerve endings originate within the gut wall, but projecting centrally to the dorsal horn of the spinal cord. There are, at present, 5 different functional classes of spinal afferent that respond to different sensory modalities (176). The location of the nerve endings of spinal afferents includes intraganglionic laminar endings in the myenteric ganglia, mucosal endings, intramuscular endings, muscular-mucosal endings close to the muscularis mucosae, and vascular endings on the blood vessels feeding into the gut (177).

The sympathetic innervation of the GI tract, derived from the thoracolumbar region of the spinal cord (T5-L2), forms neuronal centres in larger mammals such as the human or pig. There are three major prevertebral ganglia which are known as the coeliac ganglia, superior mesenteric and inferior mesenteric ganglia (178). Of significance to the lower gastrointestinal tract are the latter 2 ganglia, with the superior mesenteric ganglia supplying the stomach, small intestines and the proximal colon, and the inferior mesenteric ganglia supplying the distal colon. These cell

bodies contain noradrenaline. In addition, a small population of neurons are derived from the paravertebral ganglia (179). A unique feature of prevertebral ganglia is that they receive fast excitatory synaptic input from a specialised subset of enteric neurons- the viscerofugal neurons. Because most of the known viscerofugal neuron populations are centred in the distal colon, prevertebral ganglia serve the important role of connecting the more distal regions of colon with more proximal regions (178).

Sympathetic nerves release noradrenaline in the GI tract which is generally inhibitory, leading to decreased motility, decreased secretion, decreased blood flow and depressed cell renewal of the mucosa (37, 109, 180, 181). Intracellular recordings from myenteric neurons showed that when stimulated, they inhibit spontaneous and evoked fast synaptic potentials within the myenteric plexus (182). It is important to note that while the enteric nervous system is autonomous, extrinsic sympathetic innervation still plays an important role in the regulation of intestinal activity. The primary role of the sympathetic nervous system is to decrease GI-motility and reduce blood flow to the gut wall. This contrasts with release of noradrenaline from sympathetic nerves that innervate the heart, which have the opposite effect of causing an increase in heart rate.

1.8. MOTILITY PATTERNS IN THE SMALL INTESTINE

1.8.1. Peristalsis and the "Law of the intestine"

Bayliss and Starling first proposed the "Law of the Intestine" based on their observations in the denervated dog small intestine. They demonstrated the presence

of an intrinsic reflex that underlies the propulsion of luminal contents (37). Upon stimulation with e.g. a pinch, or a bolus distension of Vaseline coated cotton wool, onto the canine small intestine, a coordinated reflex contraction and relaxation of the smooth muscle was evoked, oral and anal to the point of stimulation. It was on these observations that the term "Law of the intestines" was coined. In verbatim:

"*Local stimulation of the gut produces excitation above and inhibition below the stimulated region. These effects are dependent on the activity of the local nervous mechanism*".

Further experiments conducted by other investigators using electrophysiological methods also demonstrated a polarised reflex that generates depolarisation and corresponding tissue contractile activity proximal to the stimulus, and hyperpolarization of the nerve cells and corresponding inhibition distally appeared to have confirmed this phenomenon (130, 183, 184).

The "Law of the intestine" was however not always reproducible and was found to not be the case in the guinea pig ileum. In experiments performed by Spencer *et al*., the guinea pig ileum was found to contact both orally and anally across both muscle layers simultaneously (185). This was significant as the guinea pig ileum was, and still remains one of the most reliable and widely used models for studying peristalsis in the GI tract.

Cannon originally described the presence of 4 mechanical descriptions of motility in the GI tract- that of rhythmic segmentation, katastalsis (antegrade segmenting

patterns) and anastalsis (retrograde segmenting patterns) and diastalsis (186). Clinically in most animal species antegrade and retrograde events are clearly seen in conditions of normal physiology (e.g. antegrade transit of gastric content, chyme and stool, as well as defecation) and disease states (e.g. emesis, diarrhoeal illness). Today, these are generally simplified into 2 main groups of contractile activity-segmenting contractions (incorporating the concepts of antegrade and retrograde segmenting patterns) and the already mentioned peristaltic contractions (Cannon's diastalsis).

1.8.2. Segmenting contractions

Segmenting contractions are rhythmic contractions of circular and longitudinal muscle, so named as they result in an appearance of the small bowel as a series of segments (186), and is crucial in mixing luminal contents and aiding in absorption. As such, they play an important role in GI transit, as transit itself depends on the relative proportion of mixing and propulsive motor patterns (187). Lasting for a few seconds, these almost simultaneous contractions and relaxations force digesta in both directions, leading to the occurrence of multiple local contractions that result in a mixing pattern of the digesta.

Evidence showing pharmacological agents affecting and blocking segmentation, as well as experiments demonstrating concurrence of contractions with excitatory junction potentials distinct from slow wave type frequencies would suggests that is an ENS driven phenomenon (188).

1.8.3. Migrating Motor Complexes (MMC)

MMCs are cyclic motor patterns that occur in the GI tract from the stomach and propagate to the distal small bowel (189, 190). Despite detailed characterisation of these complexes, the functional role of the MMC remains unclear, though many now accept its role as a intestinal "housekeeper" propelling digesta and other material such as mucosal epithelial debris in an aboral direction.

Boldyreff described the occurrence of this phenomenon in 1902, recording then the presence of 4 distinct motor activity in the canine stomach which were interspersed with periods of quiescence and interrupted by feeding. This was at a time when radiological studies of GI transit was commonly performed and in many cases periodicity of contractions were noted. In fact at there was little realisation at the time that periodicity or contractile activity had a myoelectrical basis. Decades later, in 1969 Joseph Szurszewski made the observation from implanted electrodes along the length of the canine colon that these activity propagate in an aboral fashion, culminating in what we now classify as its migratory component (190). It was only in 1972 that Grivel and Ruckebusch first established the correlation of myoelectrical activity from implanted electrodes in the dog small intestine to its mechanical changes in the bowel (191).

In 1975, work by Code and Marlett on the canine stomach and later small bowel described MMCs into 4 phases that span the length of the stomach and small bowel over a period of 1.5-2 hours: 1) a period of relative inactivity, 2) irregular transitional phases, 3) a period of intense contractile activity, and 4) a period of quiescence (192).

It is now known that in non ruminants e.g. human and dog, the MMC occurs only in the fasted state. They consist of cyclical propagating contractions that sweep through the bowel, starting in the stomach and propagating into the ileum. This is in contrast to ruminants that display MMCs in both the fed and fasted states (193). This is probably an adaptive mechanism, as, interestingly it was also noted that when ruminants were fed only twice a day, they were then observed to exhibit interruptions in MMC. (73, 194).

MMCs are controlled by a complex interaction of hormonal and neuronal inputs. Various peptides are implicated in the control of MMCs. These include motilin, somatostatin, serotonin, insulin and ghrelin amongst others (195). In fact, erythromycin acting on motilin receptors (196, 197) is well accepted clinically to decrease GI transit time by inducing phase III contractions (198, 199).

When perfusing the vasa recta of healthy dogs with atropine, hexamethonium or TTX, Sarna *et al* noted blockade of already propagating phase III contractions but at the same time inducing new phase III contractions (200). Extrinsically, the vagus had been implicated in regulating MMCs, especially in the stomach. Blockade of vagal activity by either cooling or vagotomy have led to disruption of periodic activity in the stomach (195). But it was also noted that neither vagotomy, nor sympathectomy, nor complete extrinsic denervation interrupts the initiation or progression of the MMC (201-203). While neither sympathetic nor parasympathetic activity were required for the present of MMCs *in vivo* (204), enteric nervous system is involved, evident from the abolishing effects of tetrodotoxin (TTX) on

these specimens (200). Auto-transplantation work performed by Sarr and colleagues in the 80's also provided interesting insight into MMC in the canine small bowel. Unlike the stomach, in their experiments, while certain features of the inter-digestive motor complexes were also affected, MMCs still persisted in the small bowel when devoid of vagal innervation (205). Interestingly, over a period of time most of these features recover, with the exception of motor responses of the small bowel to food (206).

1.9. MOTILITY PATTERNS IN THE LARGE INTESTINE

1.9.1. The peristaltic reflex and the "Law of the intestine" in the colon

The "Law of the intestine" was first postulated by Bayliss and Starling based on experiments performed on the canine small bowel (207). Bayliss and Starling followed up with investigations into the colon and confirmed their findings in the small bowel (208), with others following and demonstrating the same polarized phenomenon in the colon (109, 209). Although the Law of the intestine has been demonstrated in many species, the presence of a polarised intrinsic neural reflex response to distension has been controversial (see above), especially in the small bowel. In the colon however, this "Law" seems to be more consistently recorded. Part of the reason may be that the small intestine typically propels a liquid composition, and the need for a muscular relaxation is more important in the colon where a solid composition is present.

Crema demonstrated the presence of an intact peristaltic reflex in 1970, consistent with the "Law of the intestine" in the guinea pig and cat colon (109). Costa and Furness showed in 1976 that when placed in isolation, under physiological conditions, a length of guinea pig colon is able to propel an acutely inserted guinea pig faecal pellet in an aboral direction (210). Similarly, in the mouse isolated whole colon faecal pellets inserted into the oral end were shown to be propelled approximately 1-2mm/s and expelled (211). The fact that these preparations are sensitive to tetrodotoxin strongly suggests not only preservation of an intact enteric nervous system, but also that propagation is likely to be neural in origin (210). To date, this remains a reliable organ bath model for studying colonic physiology.

Velocities of propagation seen then, measuring 1-2mm/sec still hold true, consistent with results described in this thesis (69, 212).

Electrophysiological evidence confirming the peristaltic reflex is well established. It is well known that excitatory junction potentials (EJPs) can be evoked orally, and inhibitory junction potentials evoked anally to a stimulus (184, 213). Hirst and McKirdy showed that when distended, most long synaptic pathways in the myenteric plexus lead anally, exhibiting inhibitory junction potentials (183, 184). More recently, when studying simultaneous intracellular recordings of the circular muscles in the guinea pig circular muscle, Spencer *et al* showed not only was an ongoing discharge of excitatory junction potentials (EJPs) evoked oral to the stretch, and inhibitory junction potentials (IJPs) anal to it, it was clear that both these potentials were time locked (214). This suggested that stretch stimulated synchronous activation of ascending and descending interneurons. In addition, these were preserved only in longer specimens (>7mm), suggesting further that preservation of interneuronal connections were essential.

1.9.2. Colonic migrating complexes (CMMCs)

Colonic migrating complexes have been recorded from many different mammals, including dog colon (215), mouse colon (153), pig and human colon (216). They consist of cyclical propagating contractions that sweep over long lengths of large bowel.

Whilst there are many similarities between CMMCs in the colon and MMCs in the small intestine, there are some distinct characteristics that clearly differentiate both

motor patterns from each other. For example, in the colon CMMCs occur in both fed and fasted state, compared to MMCs in the small bowel which only occur in the fasted state (189, 190).

Sarna and colleagues reported 2 distinct types of contractions observed in the colon- a long duration contraction occurring at about 0.5-2 cycles/min and a shorter duration contraction occurring at 4-6 cycles/min in the dog colon (215). These contractile types are likely related to the 4 types of electrical activities reported in the small bowel. In addition, colonic MMCs are seen to propagate in both directions, contrary to the small bowel where MMCs only propagate in an aboral direction (217).

In the human colon, studies *ex vivo* have largely been restricted to short segments of tissue, until recently (218). Koch *et al* in 1988 studied the individual characteristics of muscle contraction looking at differences between normal and diseased, in this case- ulcerative colitis specimens, and were unable to find significant differences in parameters such as resting membrane potential, mean slow wave frequency and amplitudes, or inhibitory junction potentials (219). Martinez-abad *et al* reported in short segments of human colon strips very limited findings, with most specimens not showing any spontaneous activity and only 20% of all specimens presenting evaluable motility (220). The longest segments appear to be from those of the Korean group, Choe *et al*. They had studied segments measuring up to 4cm in length, and in these specimens noting the presence of propagating contractions, culminating in the findings that the right hemicolon exhibits more retrograde propagating contractions (consistent with its role in mixing digesta and allowing for

more fluid reabsorption time) (221). Despite this, on average it still appears that short segments of tissue offer limited insight into the behaviour of the human colon, and longer segments, with preservation of interneuronal connections may be required for proper analysis of the colon's intrinsic activity. It is clear from clinical examples of Hirschsprung's disease (whereby short segments of the colon, due to incomplete migration of the neural networks are devoid of networks of the ENS), colonic motility is disrupted leading to a clinical presentation of bowel obstruction. Interestingly, while this clinical condition was described in the late 19th century (222) prior to the descriptions of the ENS, correlation between the clinical phenomenon and its actual pathophysiology was not made until 1940 (223).

Working on the principle that longer lengths of colon are required for proper study of motility, our laboratories at Flinders University have now early data on extended lengths of human colon procured from hemicolectomy and total colectomy specimens. Early works on these experiments have been interesting (218). By studying whole lengths of human colon resected for slow transit constipation we have recently reported that 1) rhythmic colonic motor complexes were seen in both patients with and without slow transit constipation, and 2) that the frequencies were found to be higher in the *ex vivo* experiments compared to *in vivo* recordings (218). There are many possibilities to explain these results, which may involve enteric or extrinsic nervous systems. One example is that the loss of enteric ganglia associated with autoimmune enteric neuropathies may contribute to slow transit constipation, in line with what has been described in irritable bowel syndrome (224).

MMCs in the small intestine and CMMCs in the colon are clearly neurogenic in origin, since they are both blocked by nerve conduction blockers. (225, 226). Interestingly the neurogenic activity underlying CMMC in the colon is still preserved following paralysis of the smooth muscle with L-type Ca^{2+} channel blockers (136).

1.10. QUANTIFICATION OF *IN VIVO* MOTOR PATTERNS USING DIFFERENT TECHNOLOGIES

Ultimately the benefits of understanding the control mechanisms underlying GI motility have their greatest relevance in the treatment of human dysmotility. For various logistical reasons experimentation on human intestine has been slow, compared to advancements in animal models. The basic mechanisms underlying contractility in the small intestine and colon have been ascertained (149, 219, 227). These include information gathered from immunohistochemical, electrophysiological and short segment mechanical muscle strip experimentation. What is lacking in the human model, unlike in the animal model, is an integrative way of studying whole segments of human gut tissue in isolation from the patient.

In the earlier parts of this century transit measurements utilised radiological techniques. In the last 30 years, luminal recordings of pressure in the distal GI tract, especially in the colon have now provided insightful data on the characteristics of contraction in the human colon *in vivo*.

1.10.1. Transit studies

The earliest visualisations of transit in the human gastrointestinal tract were performed in the early days of radiography, prior to when the adverse effects of radiation were seen. Haustral contractions and mass movements within the colon were recorded by the likes of Holzneckt (228), Cannon (229), Schwarz and Alvarez (230). These early experiments gave insight into the contractile activity and transit along the bowel, but were largely descriptive.

1.10.2. Manometry

While radiological transit studies provide a visual image of contractile events, manometric and luminal recordings of the lower GI tract provide more quantitative analysis of the characteristics of contractile activity. Length of contractile propagations, frequencies of events and amplitudes of contractions are possible with luminal pressure recordings.

Manometric studies of the colon in the past have focused on the use of water perfused catheters. Earlier experiments with balloon measuring pressure were more commonly described as balloon kymography. Hardcastle and Mann in 1968 described early experiments of pressure changes using a balloon catheter looking at the underlying characteristics of contraction in the colon. The findings from their simplistic experiments looking at pressure changes within the colonic lumen yielded important data including that the presence of infrequent spontaneous activity increased with the use of laxatives (bisacodyl and oxyphenisatin), measurements of speed of propagation (then already consistently measured at 25 cm/min), that these

activity were likely neurally mediated (application of lidocaine abolished activity) and that there were no peristaltic contractions in the rectum (231).

Since then experiments utilising water based colonic manometry has been able to shed further insight into the actual mechanical characteristics of colonic motility. Based on data obtained from these studies, various motor patterns have been described- including: 1) Antegrade high amplitude propagating sequences (HAPCs), 2) Low amplitude propagating sequences, 3) Non-propagating contractions, and 4) Retrograde propagating pressure waves (232). There have been descriptions of diurnal and regional variations in propagating pressure waves in the prepared colon utilising a perfusion pressure manometer (233). There also appears to be promising signs of a correlation between clinical symptoms and distinct motility patterns. An example of this correlation is the fact that Dinning *et al* showed in 2004
increased retrograde activity of decreased amplitude but no increase in frequency of propagating sequences in in the distal colon of patients with obstructed defecation (234). Despite the information obtained from these studies, many of these studies have been largely descriptive, and significant end points were difficult to validate as human tissue is inherently variable, difficult to standardise and low in experimental numbers. As a consequence very few studies have translated into true clinical biomarkers.

Since the early manometric studies, it has been acknowledged that these perfusion studies are less than ideal because of the large geographical spacings between pressure sensors. Newer high sensitivity catheter recordings have since shown that the an older perfusion catheter may erroneously determine directionality of a

propagating contractile event (235). High sensitivity recordings have been made in oesophageal manometry more extensively compared to its recent use in the colon- the relative ease of recording in oesophagus has been attributed to easy placement of the catheter and lumen occlusion. This resulted in vast amounts of literature on manometry in the upper gastrointestinal tract. Conditions including gastro-oesophageal reflux and achalasia rely on manometry as part of its diagnostic and therapeutic work up. Given the complexity of MMC recordings- the variation of contraction activity spanning the length of the bowel and the minute mechanical changes within the bowel wall,, fibre-optic catheter based manometry is now the only method that facilitates reliable measurement of the MMC and its migratory movements (195).

To date only a small number of papers have described findings obtained utilising high-resolution catheters. These include studies utilising solid-state sensors and water perfused sensors. While water perfused catheters are cheaper, robust, relatively flexible and autoclave friendly, they are limited by the bulky external components including transducers and pumps, and suffer from slow pressure rise rates. Solid-state catheters have the advantage of a more simplistic set up and high frequency response rates, but were until recently hampered by limitations on the length from limited sensor sites. More recently at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) (the Australian national science agency), new technology has now enabled shorter sensor spacing lengths down to 10mm (236). Using this technology, a new window of opportunity has arisen to visualise precisely the directionality of motor patterns in the GI-tract. An early example has been seen in the paediatric population. Using high-resolution manometry, Giorgio and

colleagues were likely the first to establish a biomarker for neuropathy in slow transit constipation. By using bisacodyl as a stimulant for bowel activity, they found that the area under the curve in high amplitude propagating events were increased in patients with STC, consistent with a lack of motor quiescence indicative of a neuromuscular pathology (237). In adults, Patton and colleagues, used high resolution recordings to show that in patients with incontinence, sacral nerve stimulation increased the rate of retrograde activity in the sigmoid colon retarding entry of content to the rectum, leading to an improvement in continence (238).

Much is unknown about human colonic contractility patterns in health and disease and high-resolution manometry is an exciting tool in this fertile field.

1.10.3. *Ex vivo* Investigations

Despite the advancements of studying motility in the human colon *in vivo*, inherent limitations still apply. Diet, medical comorbidities, medications amongst others contributes to vast variations in individuals GI tract function. The extrinsic innervation adds an additional layer of complexity in the understanding of an already complex system. In addition, movement artifacts hinder high resolution *in vivo* recordings of human GI tract motility.

Because of our weak understanding of human colonic motility, pioneering work is underway at the Department of Physiology at Flinders University to study the intact human lower GI tract in an isolated, organ bath set up. In collaboration with specialist colorectal surgeons at Flinders Medical Centre and working closely with

the Department of Surgical Pathology, we have obtained specimens of extended length with the aims of utilising our combined experiences in the laboratories in organ bath physiology, to better understand the characteristics of small bowel and colonic motility in the human *ex vivo*.

1.11. SPECIFIC AIMS OF THIS THESIS

Firstly, using the guinea pig distal colon as an animal model, to:

a. Understand the basis of neurogenic peristaltic activity in the large intestine, and the phenomenon of hexamethonium resistant peristalsis
b. Determine the role of endogenous serotonin in the generation of distension-evoked peristalsis
c. Determine whether the inhibitory effect of 5-HT3 and 5-HT4 receptor antagonists requires the presence of endogenous serotonin in the large intestine

Secondly, using an *ex vivo* human model to:

d. Establish the feasibility of using human terminal ileum as a model to study motility in an organ bath
e. Characterise the major patterns of motor activity in the human small bowel ex vivo
f. Characterise the major patterns of motor activity in the human colon *ex vivo* and compare this motility pattern with the motility recorded from the same colon *in vivo*, in patients with slow transit constipation

Chapter 2: Novel insights into neuro-neuronal and neuro-muscular transmission underlying distension-evoked colonic peristalsis in the guinea pig colon

2.1. ABSTRACT

Background: We have recently identified hexamethonium-resistant peristalsis in the guinea pig colon. We showed that following acute blockade of nicotinic receptors, peristalsis recovers leading to normal propagation velocities of faecal pellets along the colon. This raises the fundamental question as to what mechanisms underlie hexamethonium-resistant peristalsis. **Aims**: In this study we investigated whether blockade of the major receptors that underlie excitatory neuro-muscular transmission are required for hexamethonium-resistant peristalsis to occur. **Methods**: Video imaging of colonic wall movements was used to make spatio-temporal maps and determine the velocity of peristalsis. Propagation of artificial faecal pellets in the guinea pig distal colon was studied in hexamethonium, atropine, ω-conotoxin (GVIA), ibodutant (MEN15596) and tetrodotoxin (TTX). **Results**: Hexamethonium and ibodutant alone did not retard peristalsis. In contrast, ω-conotoxin abolished peristalsis in a proportion of preparations, and reduced the velocity of propagation in all remaining specimens. In fact, peristalsis could still occur, in a proportion of animals, in the combined presence of hexamethonium, atropine, ibodutant and ω-conotoxin. Peristalsis never occurred in the presence of tetrodotoxin. **Discussion**: The major finding of the current study is the unexpected observation that peristalsis can still occur after blockade of the major excitatory neuro-neuronal and neuro-muscular transmitters. Also, the colon retained an intrinsic polarity in the presence of these antagonists and was only able to expel pellets in an aboral direction. The nature of the mechanism(s)/ neurotransmitter(s) that generate peristalsis and facilitates natural faecal pellet propulsion, after

blockade of major excitatory neurotransmitters, at the neuro-neuronal and neuro-muscular junction remains to be identified.

2.2. INTRODUCTION

The mechanisms underlying distension-evoked colonic peristalsis have been the subject of much investigation over the past century, but remain incompletely understood. What is clear is that the enteric nervous system (ENS) plays an essential role (8, 39, 210). Major advances have recently been made with regards to the cell types that are required for distension-evoked peristalsis to occur (81). For example, it was once thought that the mucosa, and release of serotonin from the mucosa, was necessary to initiate distension-evoked peristalsis (62-65). However, recent studies performed in-vitro have shown that distension-evoked peristalsis (68, 69) and colonic motor complexes (239) were not abolished when endogenous 5-HT is chemically depleted from enteric nerves and the mucosa is physically removed, which contains the largest store of serotonin in the body. Similar findings have now been reported in mice with genetic mutations to block synthesis of mucosally derived 5-HT (70). Based on recent findings it seems more likely that endogenous 5-HT plays, if anything, a modulatory, not essential role in intestinal motility.

It has been suggested from earlier work that nicotinic receptor activation is essential for peristalsis to occur (210, 240). This is highly consistent with the major role of fast nicotinic synaptic potentials recorded from most myenteric neurons (40, 241). Despite extensive evidence that nicotinic receptor activation plays a major role in enteric neuro-neuronal transmission (7, 8), recent work has shown that after

an acute blockade of peristalsis with hexamethonium, intestinal (41) and colonic (39) peristalsis can recover, whilst in the continued presence of nicotinic antagonists. In the colon, it was further shown that hexamethonium-resistant peristalsis persisted following further exposure to antagonists of neurokinin-3 (NK-3) receptors, 5-HT_3 receptors, and P2 purinoreceptors (39). This raises the fundamental question as to what are the intrinsic mechanisms/neurotransmitters that underlie hexamethonium-resistant peristalsis in the guinea pig distal colon.

Since peristalsis can occur following blockade of major excitatory neuro-neuronal transmitters, we were particularly interested in whether hexamethonium-resistant colonic peristalsis would still occur when major receptors for excitatory neuro-muscular transmitters on the smooth muscle (acetylcholine and tachykinins) were also blocked. Based on findings from earlier studies in the guinea pig small intestine, it has been shown that atropine abolishes hexamethonium-resistant peristalsis (41), however it is unclear whether this phenomenon exists in the colon.

2.3.1. Aims

A major aim of the current study was to determine whether hexamethonium-resistant peristalsis in guinea pig distal colon requires activation of muscarinic receptors, and if peristalsis persists in the presence of hexamethonium and atropine, would it still occur after subsequent blockade of NK-2 tachykininergic receptors, which mediate the major component of non-cholinergic neuro-muscular transmission (242). We have also investigated the mechanisms underlying the intrinsic polarity of hexamethonium-resistant aboral propulsion of colonic content.

2.4. METHODS

Adult guinea pigs, weighing between 250-400grams, were killed by a blow to the occipital region and exsanguinated, in a manner approved by the Animal Welfare Committee of Flinders University. The terminal 12-15cm of distal colon, taken approximately 4cm from the anus, was removed and placed in either warmed Krebs solution which was constantly bubbled with carbogen gas (95% O_2/ 5% CO_2). Endogenous pellets were naturally expelled. The isolated distal colon from each animal was then further segmented to yield two preparations, each approximately 6cm in length. Video imaging of colonic wall movements and mechanical recordings were made from the circular muscle (see below) using the protocol described below.

2.4.1. Experimental protocol

Spatio-temporal maps were generated from peristaltic contractions propagating an acutely inserted natural faecal pellet coated in epoxy resin. Expulsion and reinsertion of the pellets was maintained throughout the experiment in 5 min intervals. A period of 20 minutes was allowed before recording the effects of the administered antagonist drugs, allowing for >3 bath volume changes. During this period pellets were continually inserted during at the same intervals.

2.4.2. Mechanical recordings from the circular muscle during peristalsis and faecal pellet propulsion

We recorded the force generated during each peristaltic contraction by inserting an artificial faecal pellet (with ligature attached), into the oral end of colon and allowed to naturally propagate midway along the length of colon. The pellet was then fixed at this site so that we could record peristaltic waves with an isometric recording transducer (Grass (FT-03C; Grass, Quincy, M.A., USA) connected to the ligature. Data was recorded from the force transducers onto a computer running LabChart 6 (AD Instruments, Australia) via two custom made preamplifiers (Biomedical Engineering, Flinders University) and a PowerLab (model: 4/30; AD Instruments, Bella Vista, NSW, Australia). These experiments are described in the results as the maintained distension experiments induced by a fixed pellet, since the pellet was not free to move along the entire length of the preparation.

2.4.3. Video imaging of peristalsis and generation of spatio-temporal maps

Circular muscle contractions of the gut wall were recorded using the Gastrointestinal Motility Monitoring system (GIMM; Med-Associates Inc., Saint Albans, VT, USA). In brief, the colonic preparations were illuminated from beneath and a digital video camera was used to record the propagation of a single faecal pell*et al*ong the length of the colon. Spatio-temporal maps were constructed from the digital videos that were acquired from individual pellet runs.

2.4.4. Measurements and Statistics

The propagation velocity of inserted pellets was determined from spatio-temporal maps. Propulsion of faecal pellets along the colon was characterised as, i) continuous (i.e. did not pause along the length of the preparation at any point); ii) staggered propagation – where pellets remained stationary for one or more periods ≥5s before resuming propagation and being expelled. This usually occurred as multiple interruptions in the continuous propulsion of the faecal pellet down the length of the colon. This typically led to a prolonged delay of expulsion from the colon; iii) propagation with incomplete expulsion of the pellet; or, iv) the absence of propagation, where inserted pellets did not move at all from the site of insertion. Only preparations that showed complete expulsion of pellets were included in analysis of propagation velocity. All data are presented as means ± S.E.M. The "n" refers to the number of preparations on which observations were made. A maximum of two preparations of distal colon was obtained from any one animal. Data sets were considered statistically significant where $P<0.05$. Statistical analysis of velocities was conducted for all experiments using within-fields/repeated measures analysis on Statistical Package for Social Sciences (SPSS, IBM) given the nature of experiments using repeated addition of receptor antagonists.

2.4.5. Drugs and Solutions

The Krebs solution used contained (in mMol): NaCl, 118; KCl, 4.7; $NaHPO_4.2H_20$, 1.0; $NaHCO_3$, 25; $MgCl.6H_20$, 1.2; D- Glucose, 11; $CaCl_2.2H_20$, 2.5. Hexamethonium (500μM), atropine (1μM, 3μM) and TTX (TTX 1μM) were

purchased from Sigma, MO, USA. ω-Conotoxin (GIVA; 0.1 μM) was purchased from Alomone Labs, Jerusalem, Israel, and Ibodutant (MEN15596; 1μM, 3μM) was gifted from Dr Vladimir Zagorodnyuk, sourced from the Menarini Group, Florence, Italy.

2.5. RESULTS

In total, 32 preparations of distal colon were removed from 16 guinea pigs. In 32/34 (94%) of these preparations, insertion of a single faecal pellet into the oral end of isolated distal colon elicited a peristaltic wave, which had a continuous propagation along the colon that led to expulsion of the pellet from the anal end. In the remaining two specimens, a staggered propulsion of the faecal pellet was observed that led to complete expulsion (Fig. 3A). The average velocity of faecal pellets during these continuous runs was recorded at 1.98 +/- 0.05 $mm.sec^{-1}$ (205 runs, n=32).

2.5.1. Hexamethonium resistant peristalsis

In previous studies on isolated guinea pig distal colon intracellular recordings from myenteric neurons showed that hexamethonium blocked all fast synaptic transmission (30). Hexamethonium (500 μM) was applied to 16 preparations (n=16). Of these, 10/16 (63%) displayed a continuous propagation along the colon (Fig. 1 Aii; Fig. 3A). Of the remaining 3/16 preparations (19%) the presence of hexamethonium induced a staggered propulsion that led to complete expulsion of pellets from the colon (Fig. 3A). In addition, in these 13/16 preparations the mean velocity in hexamethonium was 2.59 +/-0.1 $mm.sec^{-1}$, and in control was 1.92 +/-

0.08 mm.sec^{-1} (70 runs, n=13, P=0.09, Fig. 4A). In the remaining 3/16 preparations (19%), initial staggered propulsion was evident where pellets were propelled a short distance along the colon but failed to be expelled from the gut (Fig. 3A).

2.5.2. The effect of atropine on hexamethonium-resistant peristalsis

We were particularly interested in whether atropine would inhibit or abolish hexamethonium-resistant peristalsis, as has been demonstrated in the guinea pig ileum (41). Surprisingly, we found that peristalsis and the propulsion of faecal pellets persisted in the presence of atropine (1 μM) and hexamethonium (500 μM) (n=13). Of a total of 13 preparations, none showed continuous propulsion of faecal pellets along the colon. In 7 of these a staggered propulsion was observed leading to expulsion (Fig. 2), 4 did not run at all, and 2 ran in a staggered fashion but were not expelled.

Overall, in the presence of atropine and hexamethonium, the mean propagation velocities were significantly diminished to 0.42mm.sec^{-1} (27 runs; n=13; P=0.02; Fig. 3A, 4A). In 2/13 preparations (15%) pellets were propelled a short distance along the colon, but were never expelled and remained within the lumen for the entire experiment (Fig. 3A). In the remaining 4/13 (31%) preparations tested, pellets did not move from the oral site of insertion. Whilst in the presence of hexamethonium and atropine, we further tested whether the N-type Ca^{2+} channel blocker, ω-conotoxin (0.1 μM) would abolish peristalsis. It was found that in the presence of hexamethonium, atropine and ω-conotoxin peristalsis could still occur. In total, in all 5 preparations tested, staggered propulsion was preserved, and

remarkably, pellets could still propagate along the full length of colon, albeit at very slow velocities (Fig. 3A). No significant differences in velocity were observed when compared to hexamethonium and atropine alone (7 runs, n=5, P=0.25; Fig. 4A).

2.5.3. The effects of NK-2 receptor blockade on hexamethonium- and atropine-resistant peristalsis

When the NK-2 antagonist, ibodutant (1μM) was applied to the colon alone, peristalsis was unaffected (Fig. 1Bii). The mean propagation velocity was recorded at 2.36 +/- 0.1mm.sec^{-1}, compared to controls at 1.92 +/- 0.11 mm.sec^{-1}. This was not statistically significant (28 runs, n=8, P=0.62; Fig. 4B). Subsequently, hexamethonium and atropine were added to the colon segments, which abolished peristalsis in 5/8 (63%) of preparations. In the remaining 3 preparations, staggered propulsion occurred that led to expulsion of pellets (Fig. 3B), with velocities of 0.05 +/- 0.01mm.sec^{-1} (6 runs, n=3, P=0.015; Fig. 4B). Further addition of ω-conotoxin had no effect on the remaining active specimens (0.1 +/- 0.02mm.sec^{-1}, 6 runs, n=3, P=0.11, Fig. 4B).

We tested whether a higher concentration of ibodutant (3 μM) would lead to any differences in peristalsis compared to 1 μM ibodutant. In 4 preparations, ibodutant at 3 μM resulted in no difference in propagation velocity of pellets (control: 1.62 +/- 0.22 mm.sec^{-1}, ibodutant: 1.19 +/- 0.34 mm.sec^{-1}, n=4, P=0.33). This higher dose resulted in staggered propulsion in 1 of 4 preparations, with other preparations showing continuous propulsion with no changes in the characteristics of propulsion. Furthermore, these 4 preparations were subjected to a higher concentration of atropine (3 μM). In these preparations, 2 of 4 preparations still propelled, albeit in

staggered manner with velocities averaging 0.045 mm.sec^{-1}(n=2, P=0.18). In the other 2 preparations, peristalsis was abolished.

2.5.4. Is there recovery of peristalsis after blockade with TTX?

It is well established that TTX blocks colonic peristalsis (210). In our preparations, pellets could not be inserted into the colon in the presence of TTX (1μM) due to increased constriction of the oral region and the conspicuous change in wall compliance. This precluded insertion of the pellet into the oral end. To circumvent this problem we applied TTX to the colon when the pellet was already inserted and fixed at a point mid way along the colon (see methods). Under these conditions, TTX consistently abolished any peristaltic activity induced by a fixed pellet. We were particularly interested to determine, if peristalsis would recover following prolonged exposure to TTX; similar to what we found with hexamethonium. We found that peristalsis never recovered following maintained exposure to TTX (1μM; >1hr; n=6).

2.5.5. Effects of ω-conotoxin on distension-induced peristalsis

N-type Ca^{2+} channels are known to play a major role in neurotransmitter release from enteric neurons (243, 244). We were interested in whether ω-conotoxin added alone (in the absence of hexamethonium) would affect peristalsis. We found that ω-conotoxin abolished peristalsis in 3/8 (38%) of preparations (n=8). Of the remaining 5/8 preparations, 2 showed continuous propagation of the faecal pellet that led to expulsion from the colon (25%), while the other 3 (38%) showed a staggered propagation that still led to expulsion (Fig. 3C). The mean propagation

velocity in these 5 preparations, (that still showed expulsion of faecal pellet), were reduced from 2.07 +/- 0.10 mm.sec^{-1} (control) to 0.67 +/- 0.17 mm.sec^{-1} (16 runs, n=5, P=0.004, Fig. 4C). Further addition of hexamethonium and atropine abolished propagation in 2/5 preparations (40%) (Fig. 3C), and in the remaining 3 preparations where peristalsis persisted, propagation velocity was further reduced to 0.04 +/- 0.01 mm.sec^{-1} (P=0.07; Fig. 4C).

2.5.6. An intrinsic polarity underlying peristalsis in the presence of neuro-neuronal and neuro-muscular antagonists

It is known that in the isolated guinea pig colon, propulsion of faecal pellets can only occur in an oral to anal direction (39, 210, 240). We investigated whether peristalsis that persists in the presence of these neuro-neuronal and neuro-muscular antagonists would still maintain an oral to anal directionality. In the presence of hexamethonium, atropine, ibodutant, and ω-conotoxin, faecal pellets inserted into the anal end of the colon never propagated orally (Fig. 5). In the same preparations, pellets inserted into the oral end were propelled in the anal direction in all preparations. This non-nicotinic, non-muscarinic, non-NK-2 receptor mediated propagation did not occur in the presence of TTX (n=6). Attempts at inserting a pellet from an anal direction encountered resistance in all conditions of control and drugs in all instances.

2.6. DISCUSSION

2.6.1. Presence of a novel form of peristalsis

The major finding of this study is that peristalsis and the propulsion of faecal content could still occur not only in the presence of hexamethonium, but also following blockade of muscarinic receptors, NK-2 receptors and ω-conotoxin sensitive Ca^{2+} channels. Whilst this study confirms that nicotinic and muscarinic receptors play a major, but not critical role in the generation and propagation of peristalsis, we revealed that in the presence of major antagonists of neuro-neuronal and neuro-muscular antagonists, a novel form of peristalsis persisted that is capable of propelling faecal contents in only an oral to anal direction.

We expected that faecal pellet propulsion would not occur in the combined presence of both hexamethonium and atropine. What we found was that in a proportion of animals where pellets were propelled in the presence of both these antagonists, the velocity of propulsion was significantly slower due to the highly staggered nature of propagating contractions. We further evaluated the mechanisms underlying this propulsion by observing propulsion in the presence of the NK-2 receptor antagonist ibodutant, hexamethonium and atropine. It is known that NK-2 receptors are localized predominantly on the neuro-muscular junction, mediating the excitatory reflex responses of the guinea pig colon (53, 245, 246). We found that ibodutant on its own had no effect in reducing the velocity of peristalsis. Interestingly, Lecci *et al*. found that in guinea pig colon *in vivo,* NK-2 receptor antagonists also did not block peristalsis, and in fact found significantly increased

speed of propulsion of an artificially inserted rectal balloon (50). Whilst peristalsis has never been shown to occur in the presence of hexamethonium and atropine, *in vivo* or in-vitro, a further novelty of our experiments is that peristalsis can still occur in the presence of hexamethonium, atropine and ibodutant (Fig. 3B).

In light of the fact that peristalsis could still occur in a proportion of preparations in hexamethonium and atropine, we were interested in whether this non-nicotinic, non-muscarinic pathway would still require N-type Ca^{2+} channels, which are known to play a major role in enteric neurotransmission (243). ω-Conotoxin inhibits depolarization-evoked acetylcholine release (247), and together with tachykinins, is inhibited at a pre-junctional level (248). In the presence of hexamethonium and atropine, further addition of ω-conotoxin did not affect the propagation velocity. This suggests that the mechanisms that underlie hexamethonium, atropine and NK-2 resistant peristalsis do not require N-type Ca^{2+} channels. When applied in the absence of any other drug, ω-conotoxin blocked peristalsis in 3/8 preparations. The fact that ω-conotoxin had a significant effect suggests that N-type Ca^{2+} channel antagonists play a major role in the generation of peristalsis, but their activation is not a prerequisite for peristalsis to occur.

2.6.2. Is hexamethonium, atropine and NK-2 receptor resistant-peristalsis mediated by a neural phenomenon?

Whilst peristalsis could still occur in the presence of hexamethonium, atropine, an NK-2 receptor antagonist and ω-conotoxin, it never occurred in TTX. If peristalsis that occurs in the presence of these antagonists was truly neural in origin, then it is particularly interesting by what mechanism the enteric motor neurons can still be

excited to cause muscle contraction. It seems difficult to believe that the enteric motor neurons can still excite smooth muscle after the blockade of the major neuro-neuronal and neuro-muscular transmitters. If the enteric nervous system is responsible for the generation of peristalsis in the presence of these antagonists, the neurotransmitters responsible must activate enteric excitatory motor neurons via synaptic potentials that do not require nicotinic, muscarinic nor NK-2 receptors. It is possible that peristalsis, which persists in the presence of these antagonists, is myogenic in origin, and that TTX abolishes this motor pattern by acting on TTX-sensitive Na^+ channels in the smooth muscle (to change compliance and prevent propulsion), in addition to enteric neurons. In support of this, there is abundant of evidence of the presence of TTX-sensitive Na^+ channels in the mouse vas deferens (249), sheep lymphatics (250), rabbit pulmonary artery (251), rat uterus (252)and the human colon (253, 254). Interestingly, there has also been recent evidence suggesting TTX- resistant peristalsis (240).

2.6.3. Why is there an intrinsic polarity underlying peristalsis in the presence of hexamethonium, atropine, NK-2 receptor antagonists and ω-conotoxin?

A major finding of our current study was that in the presence of hexamethonium, atropine, ibodutant and ω-conotoxin, faecal pellets were always expelled in an oral to anal direction. We also noted that pellets inserted into the anal end never propagated orally. The reason why pellets never propagated orally is not clear. What is clear is that the interstitial cells of Cajal are localized within various anatomical layers of the gastrointestinal wall (255-257), and act as myogenic pacemaker cells that generate phasic contractility in the gut wall (154, 160, 258, 259). Also, there is abundant evidence that a gradient in slow wave frequency due

to interstitial cells of Cajal exists along the length of the gastrointestinal tract (260-262). While the mechanism underlying the preservation of an intrinsic polarity in the colon in the presence of all antagonists is unclear, one could postulate that a gradient in myogenic activity generated by interstitial cells of Cajal is involved.

2.7. CONCLUSION

The major finding of the current study is that peristalsis and the propulsion of faecal pellets can occur following blockade of the major neuro-neuronal and neuro-muscular transmitters in the distal colon of guinea pigs. The mechanisms underlying this hexamethonium resistant peristalsis were abolished by TTX, which suggests a neurally mediated phenomenon is essential. Alternatively, a myogenic process may underlie peristalsis under these conditions, but require TTX sensitive Na^+ channels in smooth muscle or other cell types. Of particular interest is why an intrinsic polarity still prevails after blockade of nicotinic, muscarinic and NK-2 receptors. This will form the basis of future investigations.

Figures: Chapter 2

FIGURE 1. PRESERVATION OF PERISTALSIS IN ANTAGONISTS OF MAJOR NEURO-NEURONAL AND NEURO-MUSCULAR JUNCTIONS

Spatio-temporal maps of faecal pellet runs in control conditions compared to in the presence of antagonists of neuro-neuronal and neuro-muscular transmission. In each panel, controls recordings are shown in panel Ai to Di. Recordings showing peristalsis in the presence of various antagonists, see panel Aii to Dii; A-D refers to results obtained from 4 separate preparations from 4 separate animals. It can be seen that peristalsis and propulsion of faecal pellet still occurs in the presence of hexamethonium (Aii), ibodutant (Bii), ω-conotoxin (Cii), and hexamethonium and atropine (Dii), although velocity is much slower in the latter two drug combinations, due to the staggered rather than continuous propagation.

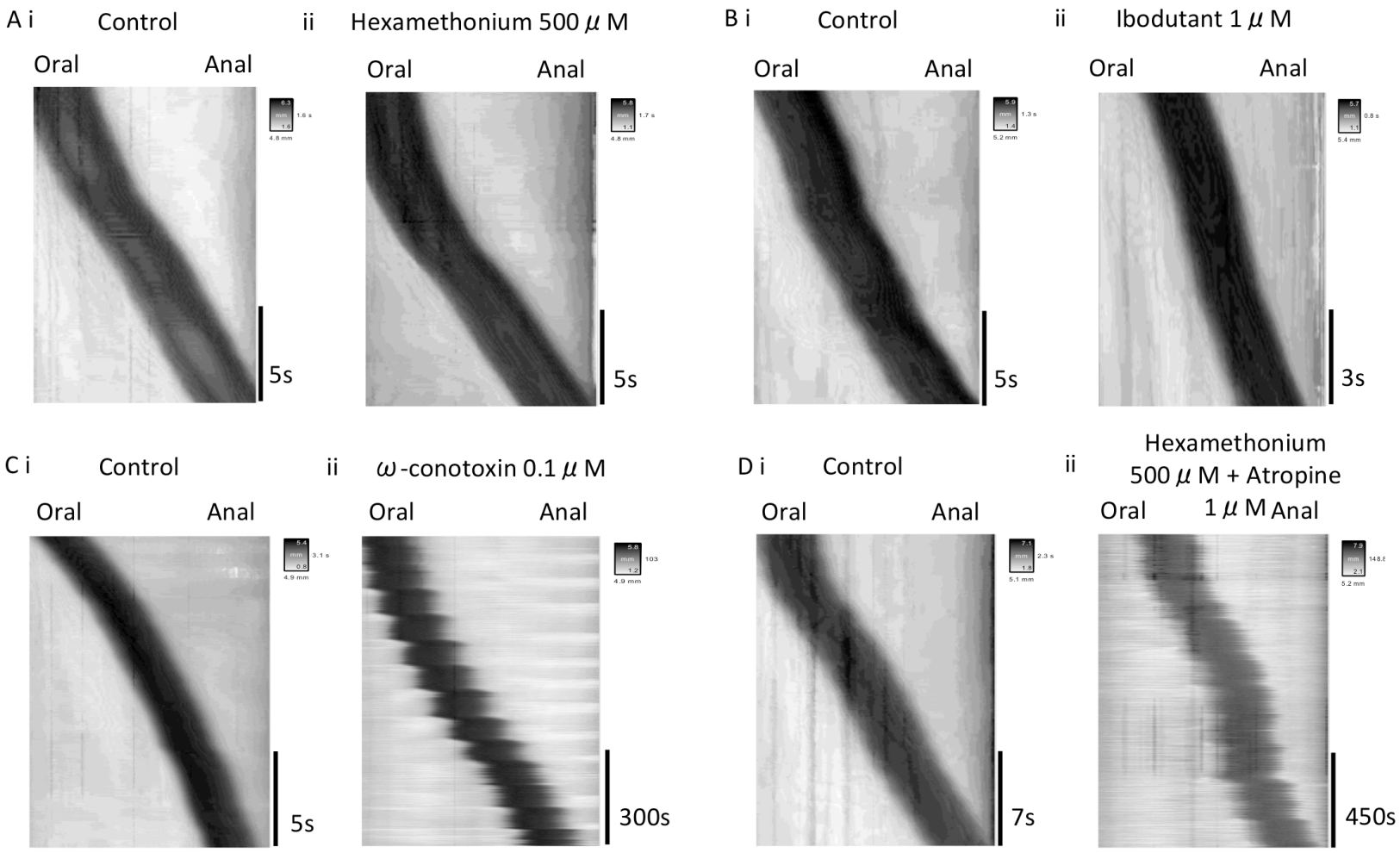

A i
Control
ii
Hexamethonium 500 μ M
Oral
Anal
Oral
Anal
5s
5s
B i
Control
ii
Ibodutant 1 μ M
Oral
Anal
Oral
Anal
5s
3s
C i
Control
ii
ω-conotoxin 0.1 μ M
Oral
Anal
Oral
Anal
5s
300s
D i
Control
ii
Hexamethonium
500 μ M + Atropine
1 μ M
Oral
Anal
Oral
Anal
7s
450s

FIGURE 2. PRESERVATION OF PERISTALSIS IN THE PRESENCE OF COMBINATIONS OF ANTAGONISTS

Spatio-temporal maps showing the presence of peristalsis and propulsion of faecal pellets whilst in the presence of various neuro-neuronal and neuro-muscular antagonists. A-C, each show two examples of spatio-temporal maps from 2 different animals. A. Preservation of peristalsis in hexamethonium and atropine, B. Presence of peristalsis in hexamethonium, atropine and ω-conotoxin. C. Peristalsis occurs in hexamethonium, atropine, ω-conotoxin and ibodutant. Note the marked increase in transit times required, and the staggered character of propagation.

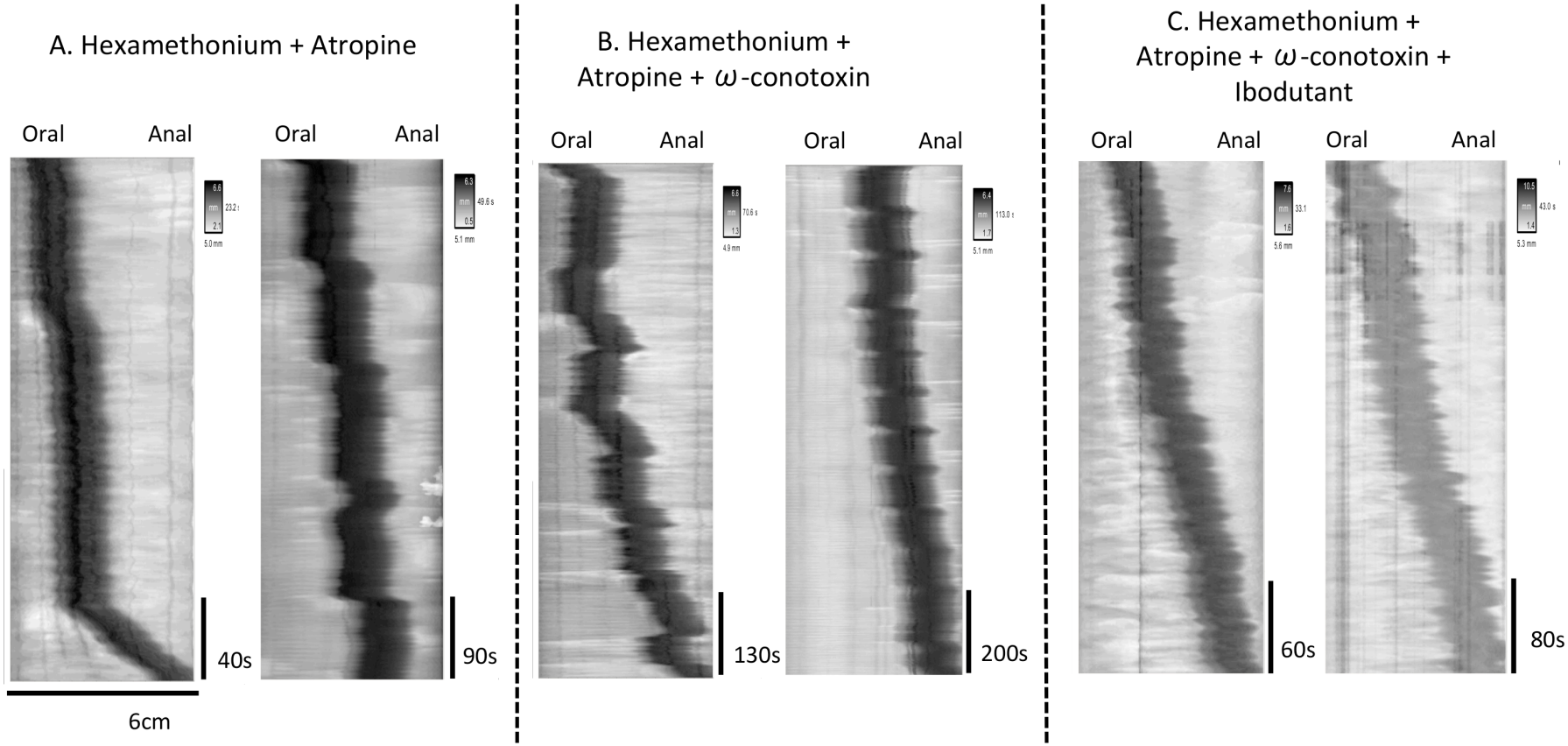

A. Hexamethonium + Atropine
Oral
Anal
Oral
Anal
40s
90s
6cm
B. Hexamethonium +
Atropine + ω-conotoxin
Oral
Anal
Oral
Anal
130s
200s
C. Hexamethonium +
Atropine + ω-conotoxin +
Ibodutant
Oral
Anal
Oral
Anal
60s
80s

FIGURE 3. PROPORTIONAL BREAKDOWN OF THE EFFECTS OF VARIOUS ANTAGONISTS USED INDEPENDENTLY AND IN COMBINATION

Graphs showing relative proportion of preparations where peristalsis and propulsion of faecal pellets is preserved following sequential addition of neural antagonists. The data is presented with respect to the proportion of preparations where runs are observed as either a smooth continuous run, staggered run, an initial propagation with no expulsion of pellet, and pellets that did not run. The individual graphs A, B and C represent data obtained from separate groups of animals. A. Experimental protocol showing a recording period of control, followed by sequential addition of hexamethonium (500uM), atropine (1uM) and ω-conotoxin (0.1uM). B. Addition of ibodutant after control recordings followed by combined application of hexamethonium and atropine, then further addition of ω-conotoxin. C. ω-conotoxin applied directly after a control recording then subsequent addition of hexamethonium and atropine whilst in the presence of ω-conotoxin. While propagation could still occur in the presence of multiple antagonists, no continuous runs were observed.

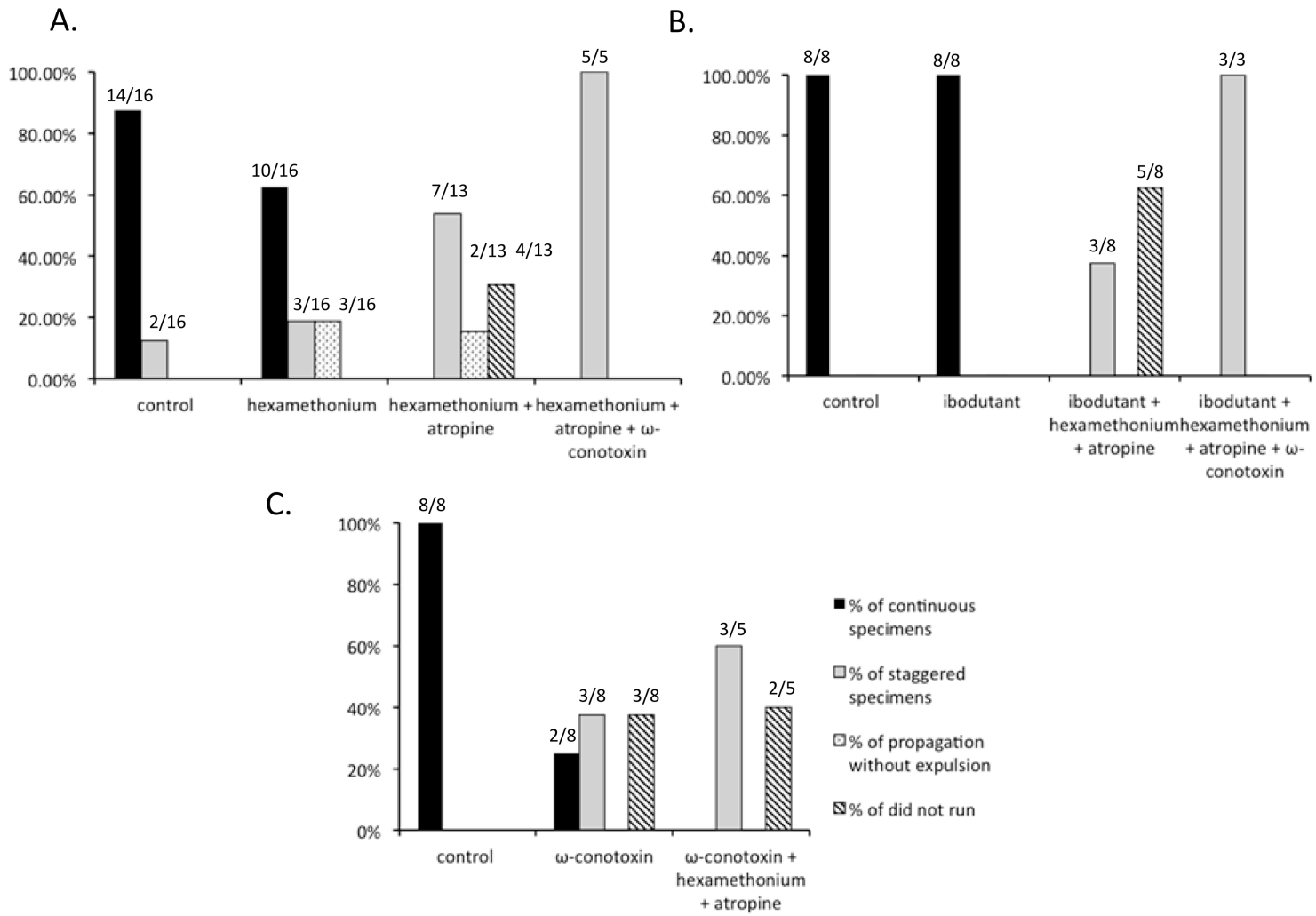

A.
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
14/16
2/16
10/16
3/16
3/16
7/13
2/13
4/13
5/5
control
hexamethonium
hexamethonium + atropine
hexamethonium + atropine + ω-conotoxin
B.
8/8
8/8
3/8
5/8
3/3
control
ibodutant
ibodutant + hexamethonium + atropine
ibodutant + hexamethonium + atropine + ω-conotoxin
C.
100%
80%
60%
40%
20%
0%
8/8
2/8
3/8
3/8
3/5
2/5
control
ω-conotoxin
ω-conotoxin + hexamethonium + atropine
% of continuous specimens
% of staggered specimens
% of propagation without expulsion
% of did not run

FIGURE 4. VELOCITIES OF PROPAGATION IN PRESENCE OF MAJOR NEURO-NEURONAL AND NEURO-MUSCULAR JUNCTION ANTAGONISTS

Velocities of pellet propulsion after exposure to various antagonists. Note that diagrams A, B, C show sequential additions of drugs following control runs. A. Propagation velocities are significantly reduced in the presence of hexamethonium and atropine and in ω-conotoxin. B. Peristalsis was unaffected by ibodutant but significantly reduced by further addition of hexamethonium and atropine, with or without ω-conotoxin. C. ω-conotoxin alone significantly reduced the velocity of peristalsis. There was no further significant reduction of velocity when hexamethonium and atropine were applied. Statistical measurements performed using within fields/repeated measures analysis and significance expressed in P-values. The control data sets in A, B, and C are from different animals and represent a completely different cohort of experiments.

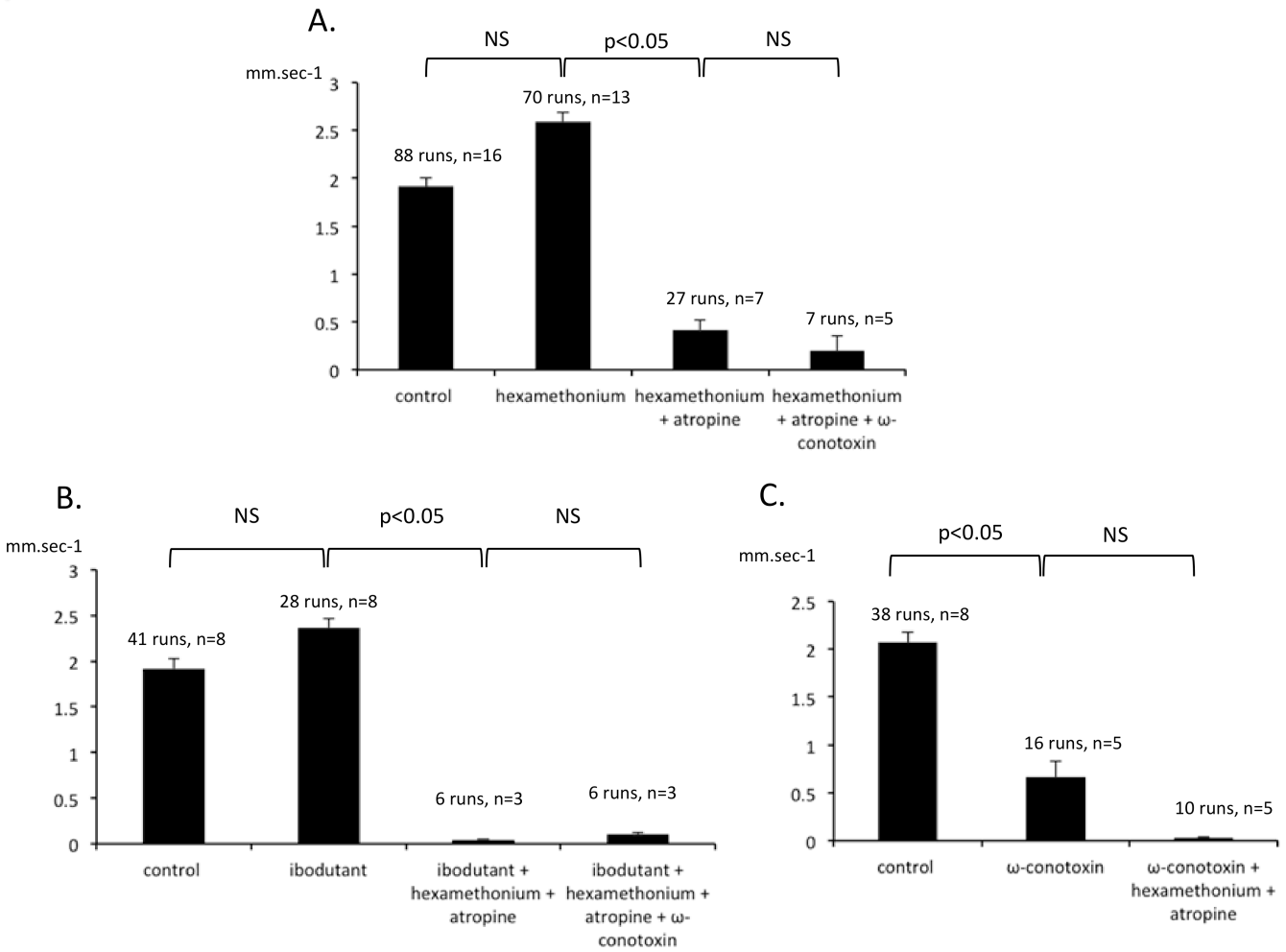
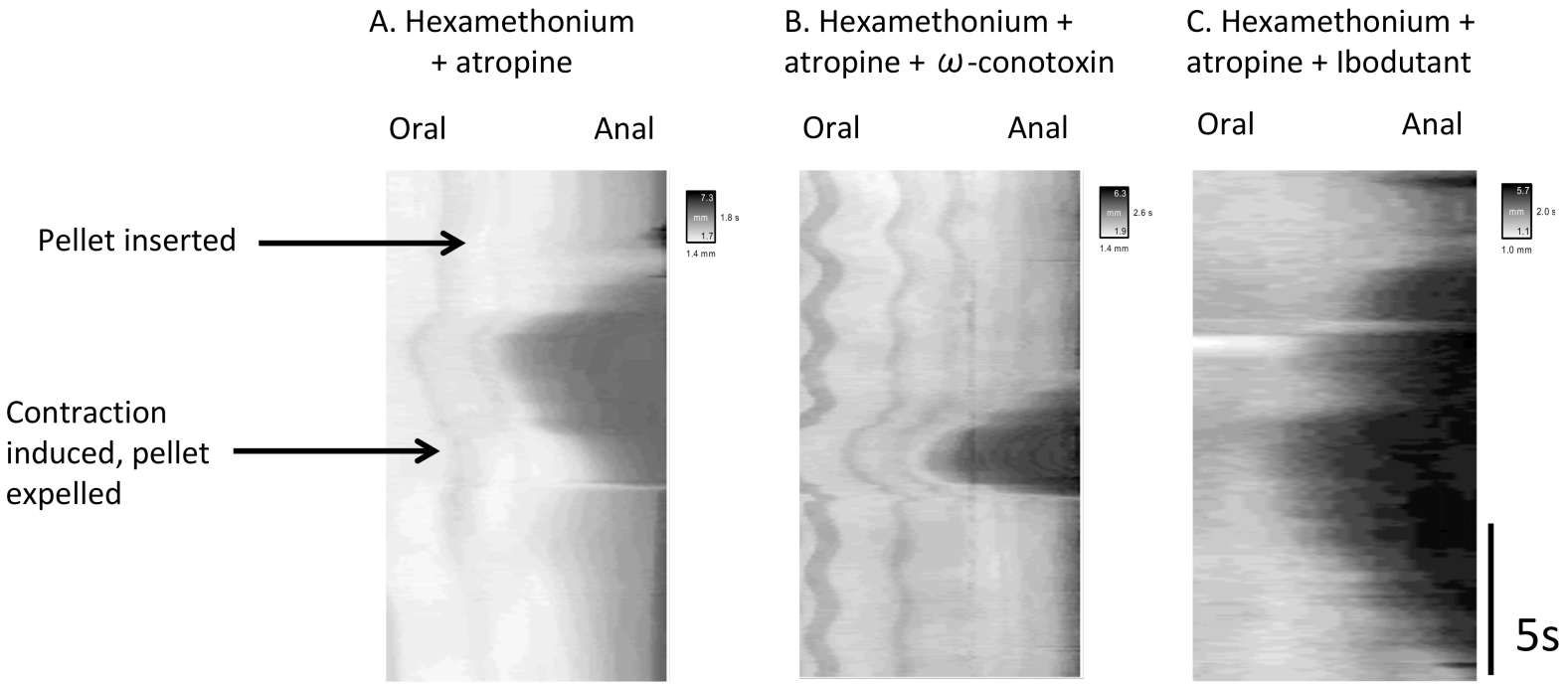

A.
NS
p<0.05
NS
mm.sec-1
3
2.5
2
1.5
1
0.5
0
88 runs, n=16
70 runs, n=13
27 runs, n=7
7 runs, n=5
control
hexamethonium
hexamethonium + atropine
hexamethonium + atropine + ω-conotoxin
B.
NS
p<0.05
NS
mm.sec-1
41 runs, n=8
28 runs, n=8
6 runs, n=3
6 runs, n=3
control
ibodutant
ibodutant + hexamethonium + atropine
ibodutant + hexamethonium + atropine + ω-conotoxin
C.
p<0.05
NS
mm.sec-1
38 runs, n=8
16 runs, n=5
10 runs, n=5
control
ω-conotoxin
ω-conotoxin + hexamethonium + atropine

FIGURE 5. PRESERVATION OF AN INTRINSIC POLARITY IN THE PRESENCE OF MAJOR NEURO-NEURONAL AND NEURO-MUSCULAR JUNCTION BLOCKADE

Insertion of faecal pellets into the anal end of isolated colon failed to induce orally migrating peristalsis. A. In the presence of hexamethonium and atropine, a pellet was inserted into the anal end and a contraction was elicited (see arrow) at which point the pellet was immediately expelled. B. In a separate preparation from a different animal, the same result was obtained in hexamethonium and ω-conotoxin. C. In another different preparation, in the presence of hexamethonium, atropine and ibodutant, again no orally migrating peristalsis was triggered.


A. Hexamethonium + atropine
B. Hexamethonium + atropine + ω-conotoxin
C. Hexamethonium + atropine + Ibodutant
Oral
Anal
Oral
Anal
Oral
Anal
Pellet inserted
Contraction induced, pellet expelled
7.3
mm
1.7
1.8 s
1.4 mm
6.3
mm
1.9
2.6 s
1.4 mm
5.7
mm
1.1
2.0 s
1.0 mm
5s

Chapter 3: Is serotonin in enteric nerves required for distension-evoked peristalsis and propulsion of content in guinea pig distal colon?

3.1. ABSTRACT:

Background: Recent studies have shown genetic deletion of the gene that synthesises 5-Hydroxytryptamine (5-HT) in enteric neurons (tryptophan hydroxylase-2, TPH2) leads to a reduction in intestinal transit. However, deletion of the TPH2 gene also leads to major developmental changes in enteric ganglia, which could also explain changes in intestinal transit. **Aims**: To investigate this further by acutely depleting serotonin from enteric neurons over a 24 hour period, without the confounding influences induced by genetic manipulation. **Methods**: Guinea pigs were injected with reserpine 24-hours prior to euthanasia. Video-imaging and spatio-temporal mapping was used to record peristalsis evoked by natural faecal pellets, or slow infusion of intraluminal fluid. Immunohistochemical staining for 5-HT was used to detect the presence of serotonin in the myenteric plexus. **Results**: It was found that endogenous 5-HT was always detected in myenteric ganglia of control animals, but never in guinea pigs treated with reserpine. Interestingly, peristalsis was still reliably evoked by either intraluminal fluid, or faecal pellets in reserpine-treated animals that also had their entire mucosa and submucosal plexus removed. In these 5-HT depleted animals, there was no change in the frequency of peristalsis or force generated during peristalsis. In control animals, or reserpine treated animals, high concentrations (up to 10μM) of ondansetron and SDZ-205-557, or granisetron and SDZ-205-557 had no effect on peristalsis. **Discussion**: Acute depletion of serotonin from enteric nerves does not prevent distension-evoked peristalsis, nor propulsion of luminal content. Also, we found no evidence that 5-HT3 and 5-HT4 receptor activation is required for peristalsis, or propulsion of contents to occur. Taken together, we suggest that the intrinsic mechanisms that generate peristalsis and

entrain propagation along the isolated guinea pig distal colon are independent of 5-HT in enteric neurons or the mucosa, and do not require activation of 5-HT3 or 5-HT4 receptors.

3.2. INTRODUCTION

Antagonists of 5-HT3 receptors, such as ondansetron (Zofran) and alosetron (Lotronex), have been commonly prescribed in clinics for relief of symptoms in patients with diarrhea-predominant irritable bowel syndrome (D-IBS). Whilst it is clear that these antagonists can prolong gastrointestinal (GI) transit in humans and laboratory animals, the mechanisms by which these drugs inhibit motility and reduce transit is still poorly understood. The long-standing presumption is that 5-HT antagonists inhibit GI-motility and prolong GI-transit by preventing endogenous 5-HT from activating 5-HT receptors on enteric nerves or enterochromaffin (EC) cells in the mucosa (115, 263, 264). Indeed, there is substantial circumstantial evidence to support a role for endogenous 5-HT and/or 5-HT receptors in the control of GI-motility, in healthy and diseased bowel (263). This is based largely on the fact that the largest quantity of serotonin in the body is synthesised in the intestinal mucosa (265) and high concentrations of 5-HT can be dynamically released from the mucosal layer of the colonic wall (68, 81). In addition, exogenous 5-HT- can evoke slow synaptic potentials in a subset of enteric neurons (266). Also, in a small number of studies endogenous 5-HT mediated fast synaptic potentials have been reported in approximately 10% of all myenteric neurons (115) and a wide variety of antagonists of 5-HT receptors can potently inhibit, or block peristalsis and reduce transit (63, 65).

Despite abundant circumstantial evidence that endogenous 5-HT is involved in the control of GI-motility, the idea that endogenous 5-HT plays a major role in peristalsis and the control of GI-transit was substantially revised in the last couple of years, in light of recent findings from two different laboratories. The first of these studies employed the first real time recordings of 5-HT release from the colonic mucosa during peristalsis (81) and showed that when the mucosa was completely removed from the colon, it abolished all release of 5-HT, but did not prevent the initiation of peristalsis (267), nor the generation of colonic migrating motor complexes (CMMCs) (68). This showed that the release of 5-HT from the mucosa was a consequence of peristalsis and CMMCs; and was clearly not the underlying cause of their generation (68, 81). In support of this, another independent study demonstrated that selective inhibition of 5-HT synthesis from enterochromaffin (EC) cells within the mucosa abolished 5-HT levels *in vivo*, but did not have any effect on transit time, gastric emptying or colonic motility (70, 77). These recent findings are significant because the vast majority of 5-HT in the body (>95%) is synthesised within the intestinal mucosa, with only minor quantities synthesised in the enteric nervous system. In fact, fewer than 1% of enteric neurons synthesise 5-HT (28) and studies have shown that 5-HT-mediated fast synaptic potentials are rare in guinea pig enteric neurons (8, 19) and have never been detected in the mouse colon (268, 269), rat small intestine (270) or human colon enteric neurons (6), where all fast EPSPs have been shown to be abolished by nicotinic antagonists. In light of the difficulty in identifying fast synaptic transmission in enteric neurons that is mediated by endogenous 5-HT, it is puzzling why antagonists of 5-HT3 receptors can potently inhibit or abolish GI motility patterns in these species (271, 272).

Since recent studies have shown that removal of the mucosa does not prevent peristalsis (81), or CMMCs *ex vivo* (68) and has no inhibitory effect on GI-transit *in vivo* (70), this raises the fundamental question as to whether neuronal 5-HT plays an important role in the control of GI-motility of mammals ? Could it be that mucosal 5-HT in the body has little effect (68), or no effect on colonic motility (70), but that neuronal 5-HT plays a major role? A recent study showed that genetic deletion of the enzyme that synthesises 5-HT in enteric neurons (Tryptophan Hydroxylase 2; TPH2) leads to an increase in intestinal and colonic transit, suggesting that neuronally synthesised 5-HT may be important for regulating GI-propulsion (77). However, in these knockout mice, there were major developmental and neurochemical changes that occurred in their enteric nervous system (77), which could also have led to the changes in transit. Therefore, at present, it is still unclear whether the changes in GI-transit reported are due to the depletion of enteric neuronal 5-HT, or the development deficits that occurred in enteric ganglia, as a consequence of deletion of the TPH2 gene. This prompted us to determine whether the acute depletion of enteric neuronal 5-HT would lead to changes in colonic motility, but without the confounding influences caused by genetic deletions of specific genes.

3.3. METHODS

3.3.1. Preparation of tissues

Adult male guinea pigs (350–450g) were killed by a blow to the occipital region and exsanguinated, in a manner approved by the Animal Welfare Committee of Flinders University. The distal colon (5-10cm from the anus) was removed and placed in

warm Krebs solution which was constantly bubbled with carbogen gas (95% O_2/ 5% CO_2). After a period of time (usually <20min), faecal pellets naturally present were expelled from the colon. A segment of distal colon (6cm in length) was mounted in an organ bath and left to equilibrate for 30 minutes. After this time, video imaging or mechanical recordings were made from the circular muscle using the protocol described below.

3.3.2. Technique to deplete endogenous 5-HT from the enteric nervous system

The enteric nervous system was depleted of endogenous 5-HT using the technique first demonstrated by Costa & Furness (273). This involves a single intraperitoneal injection of reserpine (at a concentration of 0.5mg/Kg) between 18-24 hours prior to euthanasia. 5-HT immunoreactivity is not detected in the enteric nervous system after this procedure (273). However, because reserpine does not deplete 5-HT from the mucosa, we further employed our recently published method (81) to remove the mucosa, submucosa and submucosal plexus by sharp dissection from the colon. This allows us to test whether acute depletion of 5-HT from enteric nerves and the absence of the mucosa and submucosal plexus impair colonic motility, without potential complications such as compensation induced in genetically modified animals.

3.3.3. Terminology used to define different types of preparations

Throughout the results we refer to control preparations as those which were not treated with reserpine and had their mucosa and submucosal plexus present. We refer

to “reserpine treated” preparations as those that have been treated with reserpine and which also have their mucosa and submucosal plexus removed. Therefore, reserpine-treated preparations are devoid of all known sources of endogenously synthesised 5-HT.

3.3.4. Protocol for measuring faecal pellet propagation

To induce peristalsis in vitro, using faecal pellets we followed our recent protocol (81). In brief, natural faecal pellets that had been expelled from guinea pigs weighing between 350-450 g were obtained and coated in epoxy resin to form a hard coat. A single pellet was gently inserted into the oral end of colon every 5 minutes and the characteristics of peristalsis noted. The propagation velocity of inserted pellets was determined from spatio-temporal maps. In these cases, the propagation of pellets along the colon was characterised as either continuous (i.e. did not pause propagation at any point), or showed a staggered propagation – where pellets remained stationary for periods ≥10s, >60s, >5min, or where inserted pellets did not propagate at all after insertion. Only preparations that showed continuous propagation of pellets were included in analysis of propagation velocity.

3.3.5. Mechanical recordings of circular muscle contractility during peristalsis evoked by a fixed faecal pellet

We recorded the force generated during each peristaltic contraction by inserting an artificial faecal pellet with ligature attached, into the oral end of colon and allowing the pellet to naturally propagate midway along the length of colon (81). The pellet was then fixed at this site so that we could record cyclical peristaltic waves with an

isometric recording transducer Grass (FT-03C; Grass, Quincy, M.A., U.S.A) connected to the ligature. These experiments were described in the results section as the maintained distension experiments induced by a fixed pellet, since the pellet was not free to move along the colon. The isometric force transducers were connected to two custom made preamplifiers (Biomedical engineering, Flinders University) and then to a PowerLab (model: 4/30; AD Instruments, Bella Vista, N.S.W, Australia). LabChart version 6.0 (AD Instruments, Australia) was used for analysis of data.

3.3.6. Fluid-infused peristalsis

Preparations approximately 6cm in length were studied either intact, or after the mucosa and submucosal plexus had been removed and were mounted in an organ bath constantly perfused with Krebs solution at 36^0C. The lumen of the colon was also perfused with Krebs solutions at 36^oC with a flow rate of 2.4mL/min. The propagation velocity and frequency of peristaltic waves were determined by video imaging of colonic wall movements using spatio-temporal maps.

3.3.7. Video imaging of peristalsis and generation of spatio-temporal maps

The contractions of the circular muscle of the gut wall were recorded using the Gastrointestinal Motility Monitoring system (GIMM; Med-Associates Inc., Saint Albans, VT, USA). The colon was illuminated from beneath and a digital video camera was used to record the propagation of the faecal pell*et al*ong the colon. Spatio-temporal maps of the changes in circumferential diameter (D-maps) were constructed from the digital videos that were acquired during individual peristaltic waves of single faecal pellets propelled along the colon.

3.3.8. Measurements and Statistics

The half duration and peak amplitude of each peristaltic wave were measured from tension recordings, as was the interval between each cyclical peristaltic contraction. Data in the results section are presented as means ± S.E.M. The use of "N" in the results section refers to the number of animals on which observations were made. Data sets were considered statistically significant if $P < 0.05$. Students unpaired t-test was used to compare data.

3.3.9. Immunohistochemistry

Isolated segments of guinea pig distal colon were fixed by pinning sheet preparations of colon under constant tension in a Sylgard lined Petri dish (Dow Corning Corp., Midland, MI, USA) and immersing overnight in Zamboni's fixative (5% Formaldehyde and 15% saturated picric acid in 0.1M phosphate buffer; pH 7.2) at 4°C. Preparations were then cleared in dimethyl sulfoxide (10 min immersion, repeated three times), tissue was washed in phosphate buffered saline (PBS); (0.2M sodium phosphate buffer, pH 7.2) and a whole mount of the myenteric plexus and longitudinal muscle was prepared by removing the mucosa, submucosa and circular muscle with the aid of a dissecting microscope. Goat 5-HT antisera (Immunostar, Cat: 20079) was applied at 1:1500 overnight at room temperature then washed 3 x 10 mins in PBS. Tissue was then incubated for a further 2 hours in secondary antisera (Donkey anti Goat CY3; Jackson Immunoresearch Laboratories Inc.) at 1:400 then washed 3 x 10 mins in PBS and mounted in bicarbonate- buffered glycerol (pH 8.6).

3.3.10. Drugs and Solutions

The Krebs solution used contained (in mMol): NaCl, 118; KCl, 4.7; $NaHPO_4.2H_20$, 1.0; $NaHCO_3$, 25; $MgCl.6H_20$, 1.2; D- Glucose, 11; $CaCl_2.2H_20$, 2.5. Ondansetron hydrochloride, granisetron and SDZ-205-557 were obtained from Sigma Chemical Co. St. Louis. Mo. U.S.A.

3.4. RESULTS

3.4.1. Effects of reserpine pretreatment on endogenous 5-HT in myenteric ganglia

We found that in all animals injected with reserpine 24 hours prior to euthanasia (N=5), no 5-HT immunoreactivity was detected in the myenteric plexus of the distal colon, consistent with previous studies (273), (Fig. 1). Once we removed the distal colon from reserpine treated animals, we then removed the mucosa and submucosal plexus from the colon, using our recently described technique (81).

3.4.2. Effects of depletion of endogenous 5-HT on faecal pellet distension induced peristalsis

We were interested in whether peristalsis would still occur in reserpine-treated animals. To test this, we inserted a natural faecal pellet covered in epoxy resin into the oral end of colon removed from reserpine-treated guinea pigs, and closely monitored its progression along the colon (81, 210). In these preparations, the propulsion of faecal pellets were still consistently evoked. During each peristaltic wave, we determined whether the propulsion of each faecal pellet showed continuous propagation, staggered propagation along the colon or whether pellets did not propel

at all along the colon. There was no discernable difference in the characteristics of peristaltic waves evoked between control preparations (Fig. 2C) and those that were reserpine-treated (Fig. 2F). However, the propagation velocity of faecal pellets was slower in reserpine-treated preparations which had their mucosa and submucosal plexus removed (1.5 ± 0.07mm.sec-1; P=0.006; 37 runs; N=4) compared to control preparations (2.0 ± 0.25mm.sec-1; 123 peristaltic runs; N=10). This reduction in propagation velocity was also found in previous study (81), when guinea pigs also had their mucosa and submucosal plexus removed, but were not injected with reserpine. This showed that the reserpine treatment in itself was not responsible for the decreased propagation velocity, but rather occurred when the mucosa and submucosal plexus was removed. This is further supported by the observation that in reserpine-treated animals that had their mucosa preserved there was no difference in their propagation velocities (1.8 ± 0.25 mm.sec-1; N=4) compared to controls (2.0 ± 0.25mm.sec-1; 123 peristaltic runs; P>0.05; N=10). We then investigated whether the propulsive force of peristalsis against a stationary pellet was affected by depletion of neuronal 5-HT. The peak force generated during each peristaltic wave was not different between reserpine-treated preparations (9.7 ± 1.5gm; N=4) when compared with controls (10.6 ± 0.9gm; N=4) (Fig. 3). Also, the mean interval between peristaltic waves was not significantly different between controls (278 ± 62sec; N=4) and those that were reserpine-treated (398 ± 124 sec; N=4)(Fig. 3). These results suggest that endogenous serotonin in enteric nerves was not required for the mechanisms that regulate the force underlying peristaltic contractions, nor the frequency of the underlying pattern generator that regulates their timing.

3.4.3. Effects of 5-HT3 and 5-HT4 receptor antagonists on peristalsis evoked by natural faecal pellets

Previous studies have shown that the combined application of the 5-HT3 antagonist, ondansetron at 1μM and the 5-HT4 antagonist, SDZ-205-557 at 1μM substantially reduces the velocity of peristalsis evoked by natural faecal pellets in guinea pig colon (65). In light of this, we tested the effects of these antagonists in control preparations and compared responses to reserpine-treated preparations. To do this, we inserted a single faecal pellet into the oral end of colon, and then measured the velocity of continuously propagating pellets that were expelled from the anal end. This was performed for 15 consecutive peristaltic waves, performed every 5 minutes (see Fig. 4A). After the 15th control peristaltic wave, we infused either a combination of ondansetron (1μM) and SDZ 205-557 (1μM), or granisetron (1μM) and SDZ-205-557 (1μM) and then waited 45 minutes. After perfusion of these antagonists, the 16th peristaltic wave was evoked by pellet insertion. On the 16th peristaltic wave we noted a decreased trend (not significant; $P>0.05$) in the velocity of continuously propelled pellets (Fig.4A), and an increase in the proportion of pellet runs that showed a staggered type of propagation (Fig. 4B). However, from the 17th run onwards, the velocity of pellets and the proportion of continuous runs recovered. To test whether these slight decrease detected on the 16th was an effect of the antagonists, or might be due to the period of inactivity during the 45 minute period of perfusion of the antagonists, we repeated the experimental protocol, but omitted adding the antagonists to the perfusing solution. We found that without the antagonists present, a similar result was obtained (see Fig. 4A- Krebs, Fig. 4C). This meant that the 45-minute period of inactivity, and not the antagonists per se, was responsible for the insignificant decrease in propagation velocity (Fig. 4A) and the increased proportion

of staggered runs. Overall, none of this data suggested that the antagonists had any effect on velocity of pellet propulsion.

3.4.4. Effects of depletion of endogenous neuronal 5-HT on fluid-distension induced peristalsis

Peristalsis can also be evoked in guinea pig distal colon by intraluminal fluid infusion (81). Therefore, we were also particularly interested in whether acute depletion of endogenous neuronal 5-HT would disrupt, or prevent, the generation of peristalsis and disrupt the propulsion of intraluminal fluid content. In response to slow constant infusion of warm (36^{o}C) Krebs solution into the oral end of colon, peristalsis was consistently elicited at the oral end of reserpine-treated preparations which propelled fluid out of the anal end cyclical peristaltic waves (as in Fig. 5B). In response to constant intraluminal fluid infusion of control preparations, there was on average 2.2 ± 0.4 (N=6) peristaltic waves per minute, which was not different from reserpine-treated preparations: 1.9 ± 0.5 peristaltic waves per minute (Fig. 5; P=0.10; N=6). Interestingly, in reserpine-treated preparations, the mean propagation velocity of individual peristaltic waves evoked by fluid infusion was (3.3 ± 1.4 mm.sec^{-1}), which was significantly slower than control preparations (6.5 ± 0.2mm.sec^{-1}; P=0.04; N=4). Similar to the results with faecal pellets, the slower propagation velocities of peristalsis with fluid infusion are unlikely due to the reserpine treatment, because it was previously shown that removal of the mucosa and submucosal plexus, without reserpine treatment, also significantly reduced propagation velocities of peristalsis to the same degree (81).

3.4.5. Effects of 5-HT3 and 5-HT4 receptor antagonists on peristalsis evoked by slow constant fluid infusion

Since faecal pellet propulsion experiments above were unaffected by 5-HT3 and 5-HT4 antagonists (Fig. 4), we sought to investigate whether peristalsis evoked by slow intraluminal fluid infusion may be inhibited by the combined application of these antagonists (Fig. 6). We then investigated the effects of the combined application of the antagonists. We compared the immediate effects of the antagonist infusion (represented as "equilibration of antagonists" on the X-axis of Fig. 6). This represented the mean effect of the antagonists on the frequency of peristaltic waves after the first 30 minutes of perfusion of the antagonists into the organ bath. The second period, represented by "antagonists present" on Fig. 6 refers the mean effect of the antagonists on the frequency of peristaltic waves after the perfusion from 30-60minutes after entry into the organ bath. Overall, in control preparations (Fig. 6A & 6B) and in reserpine-treated (Fig. 6C & 6D), there was no effect on the frequency of peristaltic waves of either a combination of high concentrations of ondansetron (10μM) and SDZ-205-557 (10μM) or granisetron (10μM) and SDZ-205-557 (10μM)(Fig.6; N=5; P>0.05). In the first cohort of experiments on control animals, the overall mean frequency of peristaltic waves was 2.3 ± 0.4 peristaltic waves/min, which was unchanged in the presence of ondansetron and SDZ 205-557 (10μM) at 1.8 ± 0.3 peristaltic waves/minute (Fig. 6A; P=0.29; N=5). Similarly, the frequency of peristaltic waves in control preparations (1.8 ± 0.3 peristaltic waves/min) was unchanged in the presence of granisetron and SDZ 205-557 both at 10μM (1.2 ± 0.5 peristaltic waves/min) (see Fig. 6B; P=0.07; N=5). In reserpine-treated preparations, the mean frequency of peristaltic waves before antagonists was 2.1 ± 0.8 peristaltic waves/min, which was unchanged in ondansetron and SDZ 205-557 (10μM) at 1.9 ±

0.9 (Fig.6C; P=0.9; N=5). Again, in these 5-HT depleted preparations, there was no overall difference in the frequency of peristaltic waves in granisetron and SDZ 205-557 (10μM), which occurred at 1.9 ± 0.3 peristaltic waves/min (Fig. 6D; P=0.28; N=5). Interestingly, in some preparations, we found that the antagonists actually transiently increased the frequency of peristaltic waves in both reserpinised preparations and in control preparations (Fig.7).

Finally, we investigated whether the propagation velocity of peristaltic waves was affected by the combined presence of both granisetron (10μM) and SDZ 205-556 (10μM). In control animals, the mean propagation velocity of peristaltic waves in control solution (6.5 ± 0.2mm/s; N=4) was unchanged after both antagonists (7.6 ± 0.8mm/s; N=4; P=0.21). In reserpine-treated animals, the mean propagation velocity (3.3 ± 1.4mm/s; N=4) was also unchanged after both antagonists (5.1 ± 0.6mm/s; N=4; P=0.22).

3.5. DISCUSSION

3.5.1. Peristalsis is reliably reproduced following complete depletion of endogenous serotonin

The major aim of the current study was to determine whether acute depletion of 5-HT from enteric neurons would prevent, or disrupt peristalsis evoked by faecal pellets, or intraluminal fluid distension. To address this question, we used a technique that acutely depletes 5-HT from enteric neurons in guinea pig (273). We found that after 5-HT has been acutely depleted from enteric neurons, and when the mucosa and submucosal plexus had been removed from the colon, peristalsis was

still consistently evoked by either faecal pellets or fluid distension. A further observation of the current study was that high concentrations of specific 5-HT3 and 5-HT4 antagonists did not inhibit the frequency, or the propagation velocity of peristalsis. In this regard, we are unable to verify previous work that suggests endogenous 5-HT from either the mucosa, or enteric nervous system plays a major role in the control of colonic peristalsis.

In a previous study, using the same preparation (65), it was reported that the velocity of faecal pellet propulsion was significantly reduced by the combined application of ondansetron and granisetron, when both antagonists were used at a concentration of 1μM. In contrast, when used at the same concentration or higher, we found these antagonists had no effect on the propagation velocity of faecal pellets along the colon, nor the velocity of peristaltic waves evoked by intraluminal fluid. Ondansetron and granisetron are highly potent and highly selective antagonists of 5-HT3 receptors, with an EC50 of 100nM and 0.3nM (274), respectively. The concentrations of ondansetron and granisetron we used in this study (at 1μM or 10μM) are in the order of 100-1000 times greater than the EC50 for these antagonists. Yet, despite these excessively high concentrations, we still found no significant effect on peristalsis evoked using the identical methods as those reported by previous investigators (65). The concentration of ondansetron we used in this study is 10 fold greater than the concentration used previously, see (39), where we identified the concentration of antagonist required to block 5-HT3 receptors in this preparation.

3.5.2. The role of endogenous 5-HT in synaptic transmission and its receptors in peristalsis

Whilst it is now clear that mucosal release of 5-HT is not required for the generation of colonic peristalsis in guinea pig distal colon *ex vivo* (81), nor the generation of migrating complexes in mouse colon *ex vivo* (68), what has remained unclear is the role of neuronally synthesised 5-HT in the control of GI-motility. A recent study showed that genetic deletion of the gene TPH2, which synthesises 5-HT in enteric nerves, leads to a significant decrease in intestinal and colonic transit (77). However, in that study, genetic deletion of the TPH2 gene also led to major developmental changes in the enteric nervous system, which could also be responsible for the changes in GI-transit seen. If endogenous 5-HT in enteric nerves played a major role in the generation of peristalsis, then we presumed that acute depletion of neuronal 5-HT would cause major disruptions to peristalsis. None of our data obtained from this study supported this notion. What we did find was that the propagation velocities of peristaltic waves were consistently and significantly slower after removal of the mucosa and submucosal plexus. However, this decrease in propagation velocity was first detected in a previous study when we removed the mucosa from the colon (81) was independent of any treatment with reserpine. This is supported by the fact that reserpine-treated preparations that had their mucosa and submucosal plexus present had no different propagation velocities to control animals.

Identifying a functional role for endogenous serotonin in the generation or propagation of propulsive motor patterns, such as peristalsis, or migrating complexes, has proved challenging. A number of studies have reported that different 5-HT antagonists can inhibit migrating complexes and peristalsis during *in vivo* or *ex*

vivo recordings made from the small and large intestine. However, these conclusions, including those from our own previous work (272), have been largely speculative because the site of action of the antagonists is completely unknown. What is clear is that 5-HT mediated synaptic potentials are rarely, or never recorded in mammalian enteric neurons (8); and when they have been reported, they are of small amplitude (8). It is clear that nicotinic transmission is the major type of fast synaptic transmission recorded in the enteric nervous system (7, 8, 30). Indeed, during repetitive activation of peristaltic reflex pathways in the guinea pig distal colon, direct recordings from myenteric S- or AH-type neurons never revealed any evidence of synaptic potentials due to 5-HT (30). In fact, in independent intracellular electrophysiological studies from human colon (6), rat colon (270), guinea pig colon (30) and mouse colon (268) all showed that fast synaptic transmission in myenteric S neurons was blocked by nicotinic antagonists, and no 5-HT-mediated synaptic potentials have been reported. In those studies which have recorded 5-HT mediated fast synaptic potentials, they have been reported to occur in a very small proportion of neurons and very small amplitude (269, 275). This is inconsistent with a view that endogenous 5-HT plays a major role in synaptic transmission in the enteric nervous system. There is no doubt that 5-HT receptors are abundant in the gut wall, and that the largest quantity of 5-HT in the body is synthesised in the gut itself. But, these characteristics do not necessitate a major role for endogenous 5-HT in enteric synaptic transmission.

A number of studies in the past have implicated 5-HT as a major player in both the migrating motor complex (67, 276, 277), or in the generation of colonic peristalsis (65). From our studies (68, 81), and the findings of the current study, we have not

found any data that suggests that endogenous 5-HT in enteric nerves, or the mucosa plays a role in peristalsis in guinea pig, or the colonic migrating complex in mouse, at least in vitro. Perhaps more importantly, we have obtained no data to suggest that 5-HT3 and 5-HT4 antagonists inhibit peristalsis via mechanisms that involve endogenous 5-HT. It is possible that *in vivo*, endogenous 5-HT plays a much more significant role in gastrointestinal motility, when blood flow and extrinsic nerves are preserved. Although, based on the recent findings from the Gershon laboratory, this appears also not to be the case, since genetic deletion of the gene that synthesises 5-HT in EC cells has no effect on GI-transit *in vivo* in the upper and lower regions of the GI-tract (70). When the gene that synthesises 5-HT in enteric neurons in genetically deleted it causes a reduction in transit, but this mutation also significantly modifies the development which could also be responsible for the reduction in transit (77).

3.6. CONCLUSION

The major finding of the current study shows that acute depletion of 5-HT from enteric nerves does not prevent colonic peristalsis, nor inhibit propulsion of liquid or solid colonic content. We also obtained no data to suggest blockade of 5-HT3 and 5-HT4 receptors causes any significant effect on peristalsis induced by acute faecal pellet distension, or by fluid infusion. Taken together, our observations lead us to propose that activation of 5-HT3 and/or 5-HT4 receptors is not a prerequisite for peristalsis to occur following physiological distension stimuli, at least in vitro. These findings have important ramifications for the future development of therapeutic agents that are intended to target the actions of endogenous 5-HT.

Figures: Chapter 3

FIGURE 1. IMMUNOHISTOCHEMICAL EVIDENCE OF 5-HT DEPLETION IN THE MYENTERIC PLEXUS OF GUINEA PIG DISTAL COLON

Reserpine depletes endogenous 5-HT from the myenteric plexus. A & B shows two tissues from control animals where endogenous 5-HT is present fine varicose nerve fibres. C & D shows preparations from two different animals after reserpine treatment. No 5-HT is detected.

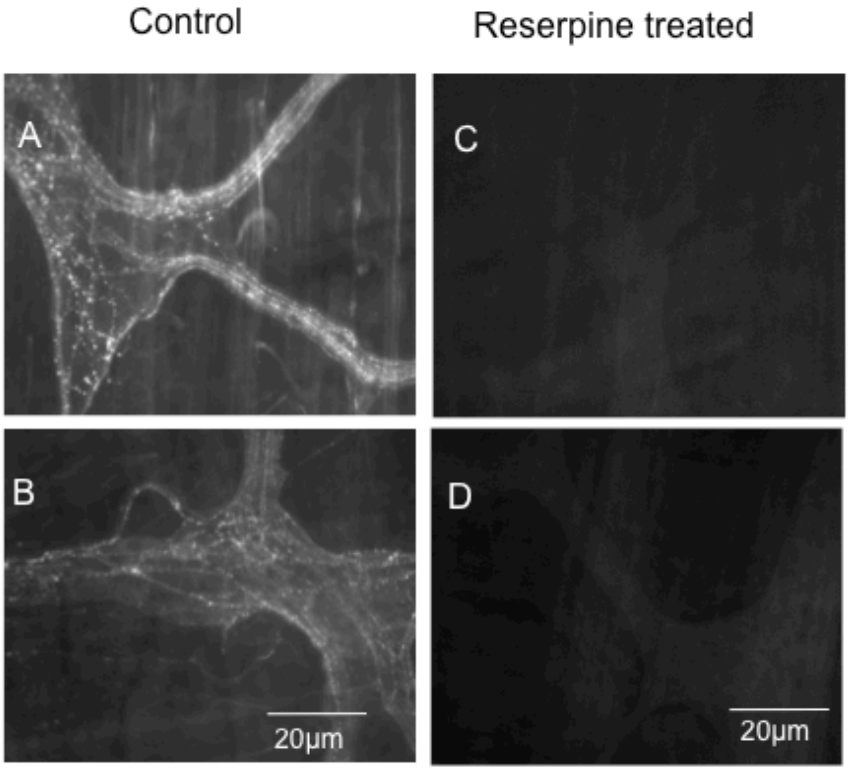

Control
Reserpine treated
A
C
B
D
20μm
20μm

FIGURE 2. EVIDENCE OF PERISTALSIS IN SEROTONIN DEPLETED GUINEA PIG DISTAL COLON *EX VIVO*

Peristalsis persists in the distal colon after depletion of endogenous 5-HT in enteric nerves and removal of the mucosa and submucosal plexus. A. Photomicrographs showing the propagation of a single faecal pellet in a control segment of colon (that contains 5-HT in enteric nerves, with mucosa and submucosal plexus present). B. Spatio-temporal map shows propagation of a single pellet during peristalsis evoked in the preparation from panel A. C. Graph shows the relative proportion of peristaltic waves that showed continuous or staggered propagation along the colon, or simply did not run at all in control animals. D. Peristalsis in reserpine-treated colon that also had mucosa and submucosal plexus removed. E. Spatio-temporal map of a single peristaltic wave from the preparation shown in D. F. Relative proportion of peristaltic waves that showed continuous propagation, staggered propagation, or did not run in reserpine-treated preparations with mucosa and submucosa removed.

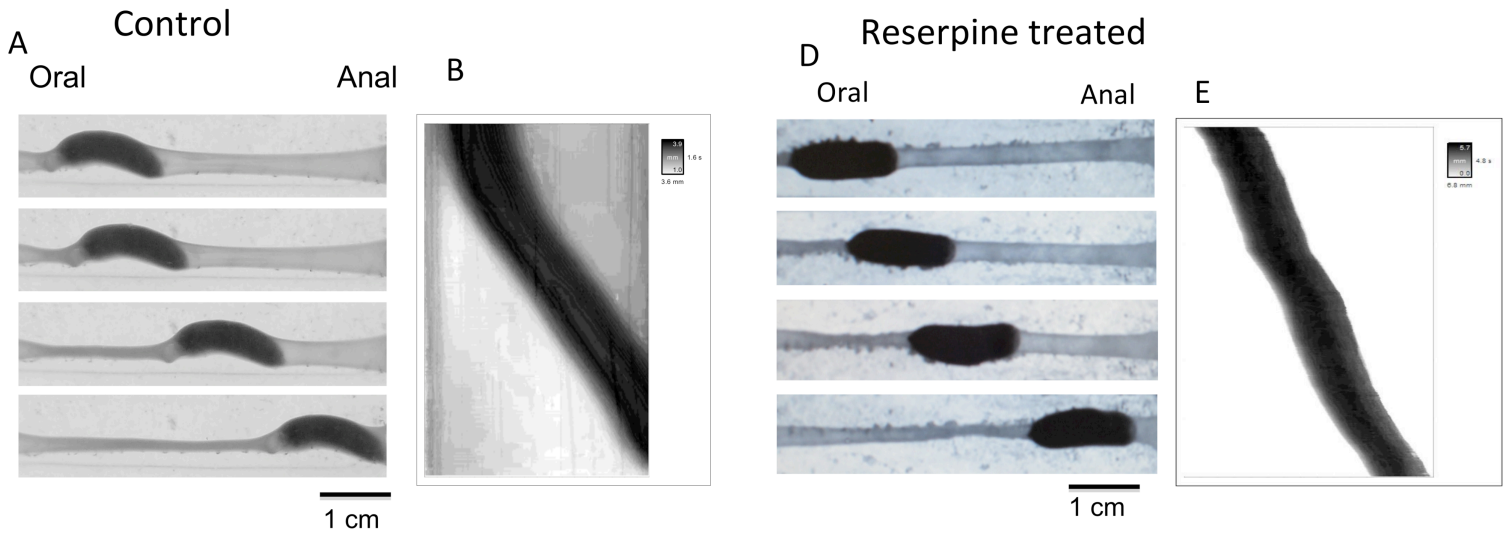

A
Control
Oral
Anal
B
1 cm
D
Reserpine treated
Oral
Anal
E
1 cm
C
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CONTINUOUS
STAGGERED>10SEC
STAGGERED>60SEC
STAGGERED>5MINS
DID NOT RUN
F
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CONTINUOUS
STAGGERED>10SEC
STAGGERED>60SEC
STAGGERED>5MINS
DID NOT RUN

FIGURE 3. CHARACTERISTICS OF PERISTALTIC EVENTS UNCHANGED IN 5-HT DEPLETED GUINEA PIG DISTAL COLON *EX VIVO*

Depletion of neuronal 5-HT and removal of mucosa and submucosal plexus does not reduce the force, or frequency of cyclical peristaltic waves evoked by maintained distension induced by a fixed faecal pellet. A. Photomicrograph of the preparation used to record the force generated during peristalsis. The black arrow indicates an intense contraction of the circular muscle that was occurring during a peristaltic contraction against the fixed pellet. B & C shows mechanical recordings of peak propulsive force generated during peristalsis in control preparations (that contain mucosa and submucosal plexus and not treated with reserpine) (B); and preparations of colon treated with reserpine and devoid of mucosa and submucosal plexus (C). No detectable differences in interval between peristaltic waves (D) or force generated during peristalsis evoked by a fixed pellet.

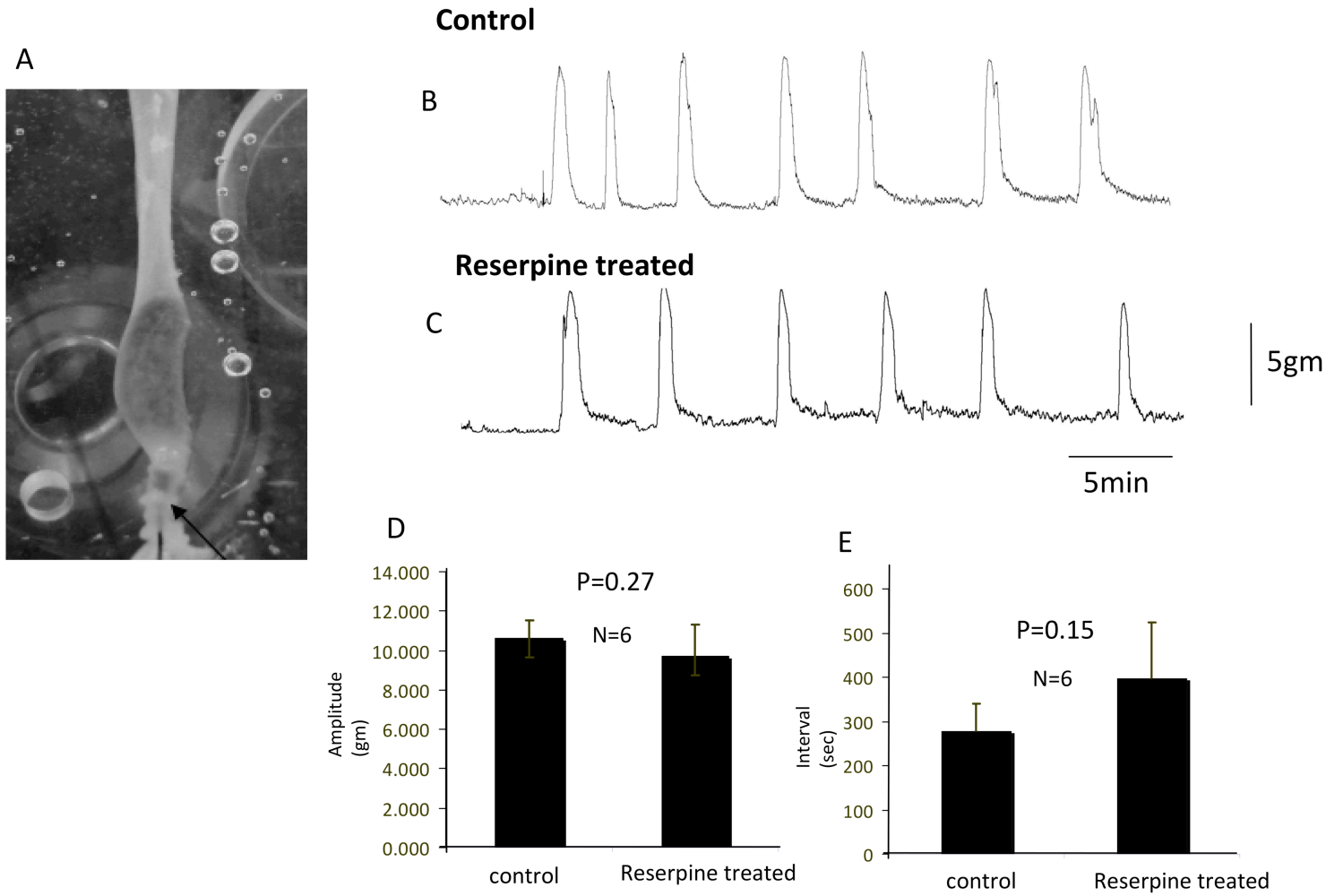

A
B
Control
C
Reserpine treated
5gm
5min
D
P=0.27
N=6
Amplitude (gm)
14.000
12.000
10.000
8.000
6.000
4.000
2.000
0.000
control
Reserpine treated
E
P=0.15
N=6
Interval (sec)
600
500
400
300
200
100
0
control
Reserpine treated

FIGURE 4. CHARACTERISTICS OF VELOCITY OF PERISTALSIS AND PROPORTION OF CONTINUOUS OR STAGGERED PERISTALTIC RUNS BEFORE AND AFTER EXPOSURE TO ANTAGONISTS OF 5-HT3 AND 5-HT4 RECEPTORS.

A, shows the propagation velocity of single faecal pellets over 15 consecutive runs, separated by 5 minutes intervals. The graph shows that the velocity of propulsion of faecal pellets in the first 15 runs is relatively constant. After the 15th run, a 45-minute period of infusion of either ondansetron and SDZ-205-557 or granisetron and SDZ-205-557 was imposed on the preparations, where no pellets were inserted. The 16th run was the first peristaltic run made after the 45-minute infusion of antagonists. At this point, there was a slight (insignificant) decrease in velocity of peristalsis with either combination of antagonists. However, when the antagonists were omitted from the solution and Krebs only solution was perfused for a 45 minute period, a similar decrease in velocity of peristalsis was detected in the 16th peristaltic run (not significant). B. Graph shows the proportion of peristaltic runs that had continuous or staggered runs before and after addition of the ondansetron and SDZ 205-557. Graph shows that after the 15th run, where the antagonists are perfused for 45 minutes, there was an increase in proportion of staggered runs (16th run onwards). C. This graph shows the pooled data when the antagonists were replaced with Krebs solution, so that no antagonists were applied to the colon. The same response shown in panel B was also found in panel C. This means that the 45-minute period of not stimulating the colon (no pellets inserted) when Krebs only was infused (but not the antagonists) was responsible for the increase in staggered runs. By the 23rd and 24th peristaltic runs, in preparations exposed to either antagonists (C) all peristaltic runs had recovered to 100% continuous propagation. This shows that the antagonists caused no long-term change in peristalsis.

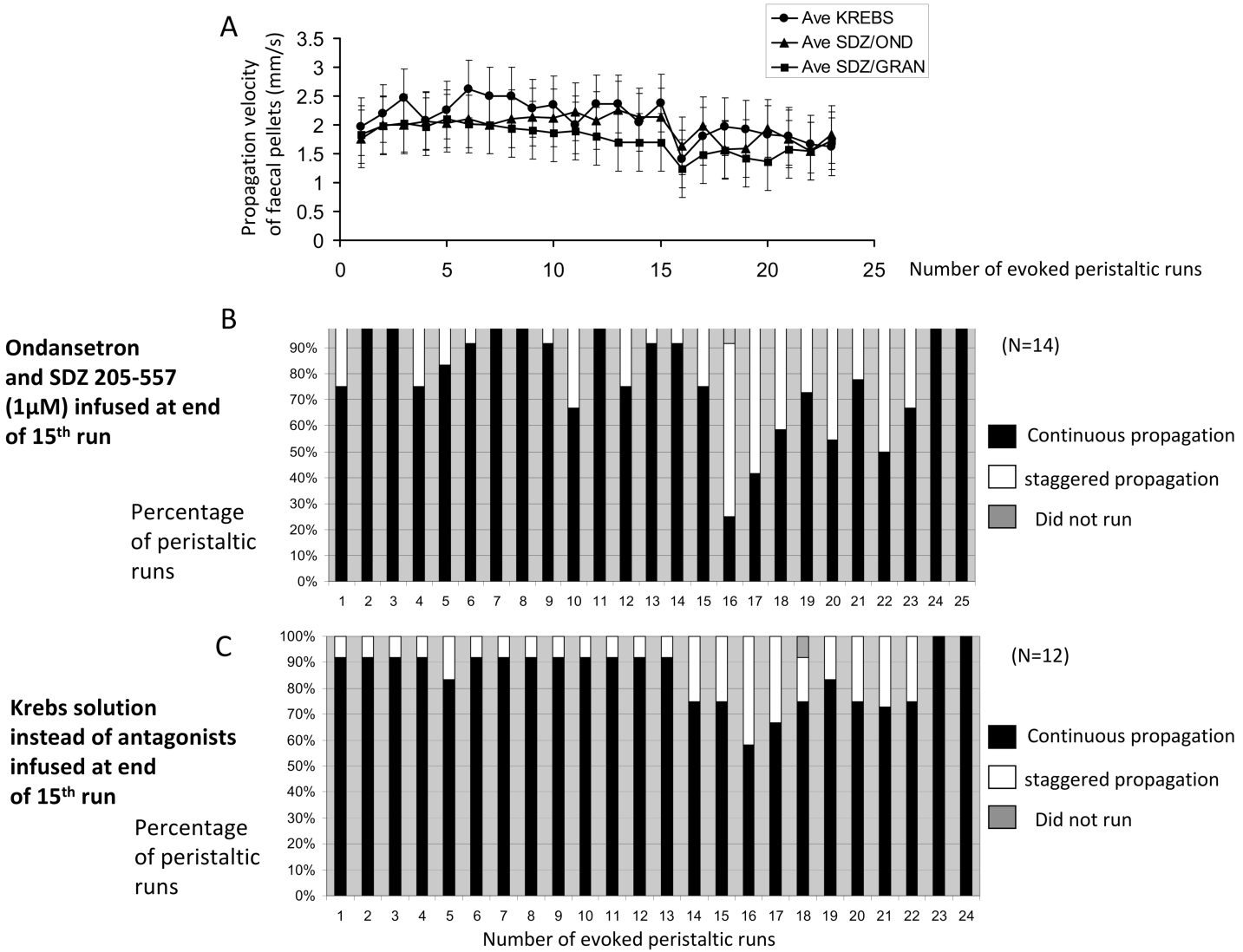

A
Ave KREBS
Ave SDZ/OND
Ave SDZ/GRAN
Propagation velocity of faecal pellets (mm/s)
3.5
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
Number of evoked peristaltic runs
B
Ondansetron and SDZ 205-557 (1μM) infused at end of 15th run
Percentage of peristaltic runs
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
(N=14)
Continuous propagation
staggered propagation
Did not run
C
Krebs solution instead of antagonists infused at end of 15th run
Percentage of peristaltic runs
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Number of evoked peristaltic runs
(N=12)
Continuous propagation
staggered propagation
Did not run

FIGURE 5. FLUID DISTENSION EVOKED PERISTALTIC EVENTS UNAFFECTED BY 5-HT DEPLETION

Fluid distension evoked peristalsis is unaffected by depletion of 5-HT in enteric nerves and removal of the mucosa and submucosal plexus. A, preparation used to infuse constant flow of warm (36^{o}C) Krebs solution into the oral end of colon to induce cyclical peristaltic waves. B. Spatio-temporal map showing repetitive peristaltic waves evoked in control preparations of colon. C. Peristalsis was unaffected in reserpinised preparations that also had mucosa and submucosal plexus removed. D. Mean data showing no significant difference in the intervals between peristaltic waves in either control or 5-HT depleted preparations. E. Photomicrographs showing propulsion of fluid in a reserpinised preparation with mucosa and submucosal plexus removed.

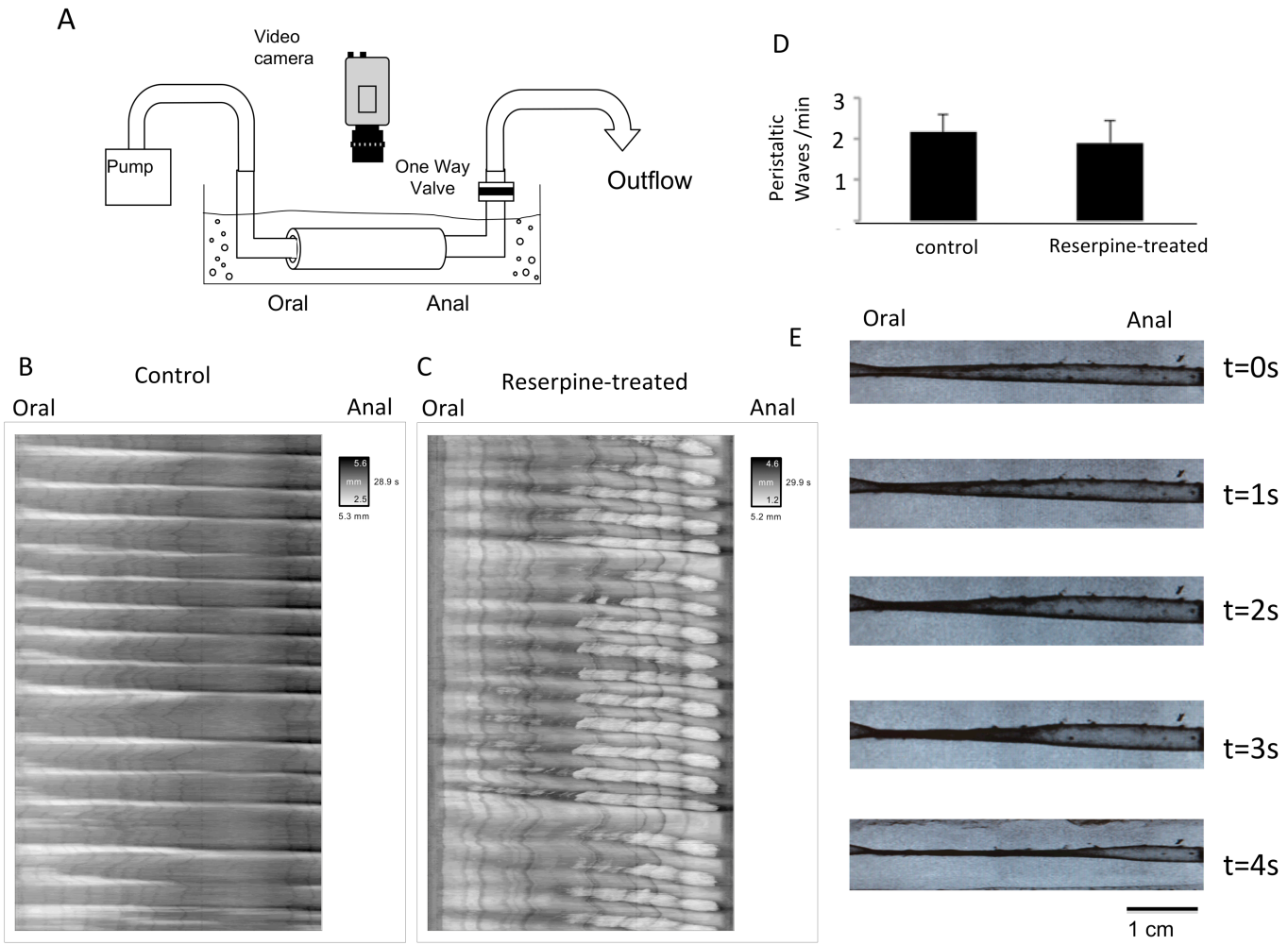

A
Video camera
Pump
One Way Valve
Outflow
Oral
Anal
D
Peristaltic Waves /min
3
2
1
control
Reserpine-treated
B
Control
Oral
Anal
5.6
mm
2.5
28.9 s
5.3 mm
C
Reserpine-treated
Oral
Anal
4.6
mm
1.2
29.9 s
5.2 mm
E
Oral
Anal
t=0s
t=1s
t=2s
t=3s
t=4s
1 cm

FIGURE 6. EFFECT OF 5-HT3 AND 5-HT4 ANTAGONISTS ON FREQUENCY OF PERISTALTIC EVENTS

Combined application of 5-HT3 and 5-HT4 receptor antagonists had no effect on the frequency of peristalsis evoked by slow fluid infusion in control preparations, or, in reserpine-treated preparations. A & B shows peristalsis evoked in control preparations was unaffected by ondansetron (10μM) and SDZ-205-557 (10μM) (see A), or granisetron (10μM) and SDZ-205-557 (10μM) (see B). C & D. In reserpine-treated preparations devoid of mucosa and submucosal plexus, there was also no significant effect on the frequency of peristalsis when both ondansetron (10μM) and SDZ-205-557 (10μM) were applied (see C), or granisetron (10μM) and SDZ-205-557 (10μM) (see D). The "control period" represents the period where only Krebs solution was applied to the colon, for 30 minutes prior to any drug addition. The "equilibration of antagonists" period represents the initial 30 min period from when the antagonists first entered the organ bath. The period represented by "antagonists present" on each graph refers to measurements of the number of peristaltic waves that occurred between 30min to 60min after antagonists first entered the organ bath.

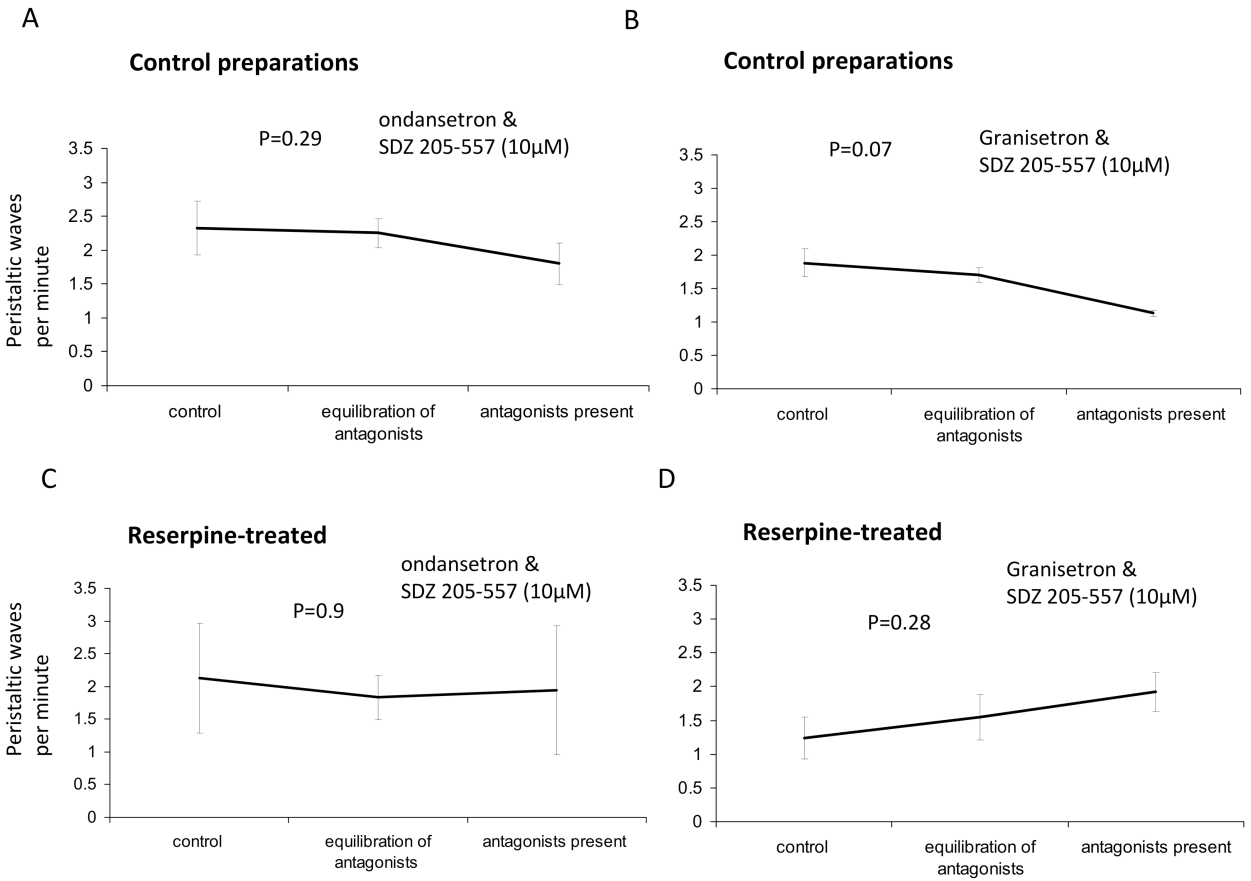
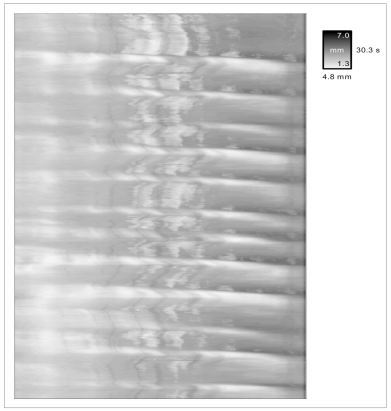

A
Control preparations
P=0.29
ondansetron &
SDZ 205-557 (10µM)
Peristaltic waves per minute
3.5
3
2.5
2
1.5
1
0.5
0
control
equilibration of antagonists
antagonists present
B
Control preparations
P=0.07
Granisetron &
SDZ 205-557 (10µM)
C
Reserpine-treated
P=0.9
ondansetron &
SDZ 205-557 (10µM)
D
Reserpine-treated
P=0.28
Granisetron &
SDZ 205-557 (10µM)

FIGURE 7. SPATIO-TEMPORAL MAPPING SHOWING PERISTALSIS UNAFFECTED IN THE PRESENCE OF 5-HT3 AND 5-HT4 ANTAGONISTS

High concentrations of ondansetron and SDZ-205-557, or granisetron and SDZ-205-557 did not block peristalsis evoked by slow fluid infusion in control preparations, or reserpine-treated preparations that had mucosa and submucosa removed. Ai shows a spatio-temporal map generated in Krebs solution, from a reserpine-treated preparation that also had mucosa and submucosal plexus removed. Aii shows the same preparation after ondansetron (10μM) and SDZ-205-557 (10μM) was infused to the colon. Peristalsis was not reduced in frequency by the antagonists. Bi. Spatio-temporal map of peristalsis also evoked by fluid infusion in a different animal that was also treated with reserpine and had mucosa and submucosal plexus removed. Bii shows that the combined effects of ondansetron (10μM) and granisetron (10μM) also had no inhibitory effect on peristalsis.

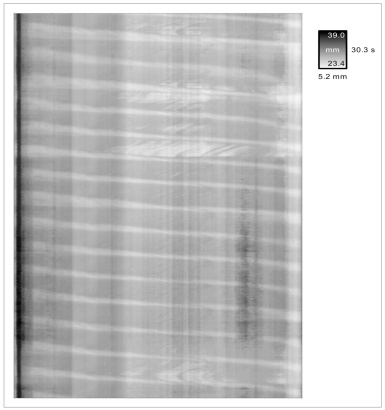
Ai

Reserpine-treated



Aii

+ Ondansetron (10µM) & SDZ 205-55 (10µM)

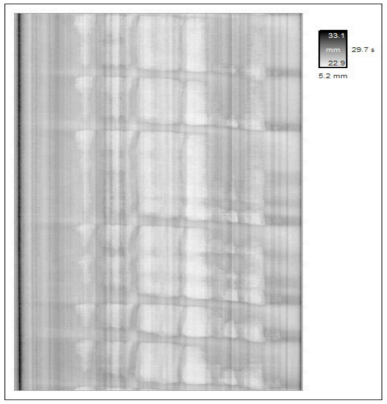


Bi

Reserpine-treated

Bii

+ Granisetron 10µM & SDZ 205-55 (10µM)



Chapter 4. 5-HT3 and 5-HT4 antagonists inhibit peristaltic contractions in guinea pig distal colon by mechanisms independent of endogenous 5-HT

4.1. ABSTRACT

Background: Recent studies have shown that endogenous serotonin is not required for colonic peristalsis in vitro, nor gastrointestinal (GI) transit *in vivo*. However, a variety of different antagonists of 5-Hydroxytryptamine (5-HT) receptors are known to retard peristalsis and GI-transit in mammals, including humans. This raises the question of how these antagonists inhibit GI-motility and transit, if depletion of endogenous 5-HT does not cause any significant inhibitory changes to GI-motility or transit? **Aims**: We investigated the mechanism by which 5-HT3 and 5-HT4 antagonists inhibit peristaltic contractions in isolated guinea pig distal colon, and whether the effects of these antagonists differ in preparations of colon that have endogenous serotonin depleted. **Methods**: In control animals, repetitive peristaltic contractions were evoked in response to fixed faecal pellet distension. Serotonin depletion as established using tandem mass spectrometry facilities here at Flinders University. **Results**: Interestingly, the amplitude and frequency of distension-evoked peristaltic contractions were unaffected in reserpine treated animals (to deplete neuronal 5-HT) with mucosa and submucosal plexus removed. In these reserpine-treated animals, tandem mass-spectrometry and immunohistochemistry did not detect the presence of any endogenous serotonin, whereas 5-HT was always detected in controls. In control animals, peristaltic contractions were blocked temporarily by ondansetron (1-10μM) and SDZ-205-557 (1-10μM), giving the impression that these antagonists blocked peristaltic contractions permanently. Surprisingly, ondansetron (1-10μM) and SDZ-205-557 (1-10μM) also had potent inhibitory effects on peristaltic contractions evoked in preparations with no detectable 5-HT. **Discussion**: HT3 and 5-HT4 antagonists can inhibit distension-evoked peristaltic contractions

independently of endogenous 5-HT. We suggest that 5-HT3 and 5-HT4 antagonists act on constitutively active 5-HT3 and 5-HT4 receptors to reduce enteric neuronal excitability, regardless of the presence or absence of endogenous 5-HT.

4.2. INTRODUCTION

Since the early 1950's, endogenous serotonin release from the gastrointestinal tract has been suggested to play an important role in the control of different gastrointestinal motility patterns, such as peristalsis, in the small and large intestine. Evidence to support this hypothesis comes from the fact that the largest quantity of serotonin in the body is synthesised in enterochromaffin cells (265) and high concentrations of 5-HT can be dynamically released from the mucosa (68, 80, 81). In addition it is well know that exogenous 5-HT potently stimulates GI-motility (61, 62, 68, 81) and a variety of antagonists of 5-HT receptors can potently inhibit, or block peristalsis and reduce transit (63, 65, 67).

Despite significant circumstantial data supporting a role for endogenous 5-HT in the control of GI-motility, the notion that endogenous 5-HT played a major role in control of GI-motility was substantially revised in the past couple of years, based on findings from independent laboratories (69, 70, 77, 81). These recent studies showed that when 5-HT was depleted from enteric neurons; followed by removal of the mucosa and submucosal plexus, distension-evoked peristalsis still occurred without any significant deficits (69). Similar findings have now been reported for colonic migrating motor complexes (CMMCs) in the mouse colon (239). Interestingly, in the mouse colon, it was found that antagonists of 5-HT3 receptors, such as ondansetron,

still inhibited CMMCs equally, or significantly more effectively in 5-HT depleted preparations (239). These recent findings are difficult to reconcile with earlier suggestions that endogenous 5-HT played a major role in the control of these motor patterns. Coincidently, recent findings from the laboratory of Gershon and colleagues (70, 77), demonstrated that deletion of the gene responsible for 5-HT synthesis in enterochromaffin (EC) cells led to no inhibitory effects on GI-transit in live mice (70, 77). These recent findings are important because the vast majority of 5-HT in the body (>95%) is synthesised within the intestinal mucosa, with only minor quantities synthesised in the enteric nervous system. In fact, less than 1% of enteric neurons have been shown to synthesise 5-HT (28). Also, 5-HT-mediated synaptic potentials are rarely recorded from myenteric neurons. When they have been reported, they occur in a very small proportion of enteric neurons and are of small amplitude (8, 115, 278). Studies have shown that 5-HT-mediated fast synaptic potentials are extremely rare in guinea pigs (8, 19); and have never been detected in the mouse colon (268, 269), rat small intestine (270) or human colon (6). In these species, all fast synaptic potentials in enteric neurons are abolished by nicotinic antagonists, and no serotonergic fast synaptic potentials have been reported. Of particular interest is why do 5-HT receptor antagonists potently inhibit GI motility and reduce transit in these same species where no serotonergic fast EPSPs have been detected (271, 272)?

Whilst there is no doubt that antagonists of 5-HT3 and 5-HT4 receptors can delay gastrointestinal (GI) transit in both humans and laboratory animals, the mechanisms by which these drugs induce their inhibitory effects on GI-motility is poorly understood. Recently, we reported that 5-HT3 and 5-HT4 receptor antagonists had no

significant effect on colonic peristalsis in guinea pig distal colon, when peristalsis was evoked by acute luminal distension using faecal pellets, or slow intraluminal fluid distension (69). These results were inconsistent with data obtained from other laboratories where it was shown that the same 5-HT3 and 5-HT4 antagonists potently inhibited peristalsis, evoked by the same stimuli and in the same preparation (64, 65). In the current study, we used a different stimulus to evoke peristaltic contractions. We applied maintained distension of the colonic wall using an artificial faecal pellet that was fixed and not able to be freely propelled along the colon. This is a different type of distension stimulus to that applied in our recent study using the same preparation (69). The aim of this preparation was to determine whether antagonists of 5-HT3 and 5-HT4 receptors may inhibit repetitive peristaltic contractions, evoked by maintained colonic distension, and if so, would the effects of these antagonists be altered in preparations which lacked the mucosa and were depleted of endogenous 5-HT.

4.3. METHODS

4.3.1. Preparation of tissues

Adult male guinea pigs, weighing between 350–450g, were euthanised by an acute blow to the occipital region and exsanguinated, in a manner approved by the Animal Welfare Committee of Flinders University. The distal colon (5-10cm from the anus) was removed and placed in warm Krebs solution which was constantly bubbled with carbogen gas (95% O_2/ 5% CO_2). After a period of time (usually <20min), faecal pellets naturally present were expelled from the colon. A segment of distal colon (6cm in length) was mounted in an organ bath and left to equilibrate for 30 minutes.

After this time, mechanical recordings were made from the circular muscle using the protocol described below.

4.3.2. Technique to deplete endogenous 5-HT from the enteric nervous system

The enteric nervous system was depleted of endogenous 5-HT using the technique first demonstrated by Costa & Furness (69, 273). This involves a single subcutaneous injection of reserpine (at a concentration of 0.5mg/Kg) between 18-24 hours prior to euthanasia. 5-HT immunoreactivity is not detected in the enteric nervous system after this procedure (273). However, because reserpine does not deplete 5-HT from the mucosa, we further employed our recently published method (81) to remove the mucosa, submucosa and submucosal plexus by inverting the colon and using sharp dissection. This allows us to test whether acute depletion of 5-HT from enteric nerves and the absence of the mucosa and submucosal plexus impair colonic motility, without potential complications such as compensation induced in genetically modified animals.

4.3.3. Terminology used to define different preparations

Throughout the results we refer to 'control' preparations as those which were not treated with reserpine and had their mucosa and submucosal plexus intact. We refer to 'reserpine treated – mucosa present' preparations as those which have been treated with reserpine and which also have their mucosa and submucosal plexus present. Preparations that had been treated with reserpine but also had their mucosa and submucosal plexus removed were referred to as 'reserpine-treated, mucosa removed'.

4.3.4. Mechanical recordings of circular muscle contractility during peristalsis evoked by a fixed faecal pellet

We recorded the isometric force generated by the circular muscle during each peristaltic contraction by inserting an artificial faecal pellet into the oral end of colon and allowing the pellet to naturally propagate midway along the length of colon (81). The pellet was attached to fine surgical cotton thread that was then fixed in the mid region of the preparation so that it could not be expelled. Cyclical peristaltic contractions were recorded with an isometric recording transducer Grass (FT-03C; Grass, Quincy, M.A., U.S.A) connected to the ligature. These experiments were described in the results section as the maintained distension experiments induced by a fixed pellet, since the pellet was not free to move along the colon. The isometric force transducers were connected to two custom made preamplifiers (Biomedical engineering, Flinders University) and then to a PowerLab (model: 4/30; AD Instruments, Bella Vista, N.S.W, Australia). LabChart version 6.0 (AD Instruments, Australia) was used for analysis of data.

4.3.5. Measurements and Statistics

Measurements of the half duration and peak amplitude of each peristaltic contraction were measured from tension recordings, as was the interval between each cyclical peristaltic contraction. Data in the results section are presented as means ± S.E.M. The use of ‘n’ in the results section refers to the number of animals on which observations were made. Data sets were considered statistically significant if $P < 0.05$. Students unpaired t-test was used to compare data.

4.3.6. Immunohistochemistry

Isolated segments of guinea pig distal colon were fixed by pinning sheet preparations of colon under constant tension in a Sylgard lined Petri dish (Dow Corning Corp., Midland, MI, USA) and immersing overnight in Zamboni's fixative (5% Formaldehyde and 15% saturated picric acid in 0.1M phosphate buffer; pH 7.2) at 4°C. Preparations were then cleared in dimethyl sulfoxide (10 min immersion, repeated three times), tissue was washed in phosphate buffered saline (PBS); (0.2M sodium phosphate buffer, pH 7.2) and a whole mount of the myenteric plexus and longitudinal muscle was prepared by removing the mucosa, submucosa and circular muscle with the aid of a dissecting microscope. Goat 5-HT antisera (Immunostar, Cat: 20079) was applied at 1:1500 overnight at room temperature then washed 3 x 10 mins in PBS. Tissue was then incubated for a further 2 hours in secondary antisera (Donkey anti Goat CY3; Jackson Immunoresearch Laboratories Inc.) at 1:400 then washed 3 x 10 mins in PBS and mounted in bicarbonate- buffered glycerol (pH 8.6).

4.3.7. Drugs and Solutions

The Krebs solution used contained (in mMol): NaCl, 118; KCl, 4.7; $NaHPO_4.2H_20$, 1.0; $NaHCO_3$, 25; $MgCl.6H_20$, 1.2; D- Glucose, 11; $CaCl_2.2H_20$, 2.5. Ondansetron hydrochloride and SDZ-205-557 were obtained from Sigma Chemical Co. St. Louis. Mo. U.S.A, and were made up at a stock concentration of 10^{-2}M.

4.3.8. Chemical Analysis of Tissue Extracts for 5-HT by Tandem Mass Spectrometry

The amount of 5-HT present in tissues was measured using solid-phase extraction, reversed-phase liquid chromatography and electrospray tandem mass spectrometry (LC-MSMS) with isotope dilution, essentially as described by (279). Liquid chromatography was performed using an Atlantis T3 column (3 µm particle size, 150 x 2.1 mm, Waters), while mass spectrometry used a 3200 Q-Trap instrument (ABSCIEX Toronto, Canada) tuned to measure ion transitions with m/z 177>160 for 5-HT, and 181>164 for tetra-deuterated 5-HT as internal standard. Quantitation was carried out using peak area ratios and Analyst v.1.5 software with linear calibration plots covering the sample concentration range 0 to 1000 nmol/L. The limit of quantitation (S/N 5) was 0.5-1 nmol/L.

4.4. RESULTS

4.4.1. Peristaltic contractions in control environments

We investigated the effects of maintained colonic wall distension by inserting an artificial faecal pellet into the oral end of colon. The pellet was free to propel anally to a point midway along the colon, at which point the pellet was fixed in place via fine suture thread, so that it could not be further expelled, as previously described (81), (see Fig. 1). In response to maintained distension of control animals with mucosa present, cyclical peristaltic contractions were evoked at a mean interval of 278.10 ± 62.3s (N=6), or reserpine-treated animals with mucosa and submucosal plexus removed (398.8 ± 124s; N=4; P=0.15). This revealed that control mechanisms that regulated the frequency of peristaltic contractions were independent of the

mucosa or submucosal plexus, or, endogenous 5-HT in enteric neurons. Similarly, the mean peak amplitude of circular muscle contractions induced during each peristaltic contraction was 10.64 ± 0.8g in control animals, which, was no different from reserpine-treated animals with mucosa present (11.4 ± 0.8g; P=0.61; N=16), or reserpine-treated animals with mucosa and submucosal plexus removed (9.7 ± 1.5g; P=0.07; N=4).

4.4.2. Effects of 5-HT3 or 5-HT4 receptor antagonist on peristaltic contractions

We were particularly interested in the effects of the selective 5-HT3 or 5-HT4 antagonists, ondansetron and SDZ 205-557, on repetitive peristaltic contractions evoked in control preparations and in preparations that were treated with reserpine but retained mucosa. Initially, we applied ondansetron or SDZ 205-557 to control segments of colon, when reliable peristaltic contractions were evoked by maintained fixed pellet distension (e.g. Fig. 1A). Overall, it was found in majority of preparations (see Fig. 2), the initial application of either antagonist caused a blockade of peristaltic contractions which recovered after continued application (Fig. 2 & Fig. 3). Interestingly, when these contractions did recover in the presence of either antagonist, there was no difference in peak amplitude (Fig.4). It was found that in each preparation, application of ondansetron or SDZ-205-557 caused a temporary blockade of peristaltic contractions at each increasingly higher concentration of antagonist (Fig. 2 & Fig. 3). When these contractions recovered in the continued presence of either antagonist, it was found that the intervals between repetitive peristaltic contractions was not significantly different from controls, suggesting that either receptor were not actually required for their generation (Fig. 5). Only at

excessively high concentrations between 5-10μM was peristaltic contractions permanently abolished (Fig. 3).

It has been suggested that the combined application of a 5-HT3 and 5-HT4 receptor antagonist is required to block peristalsis (63, 65, 67). Therefore, we tested the effects of both antagonists applied together. Similar results were obtained in preparations of colon from control mice; and reserpine-treated mice with intact mucosa and submucosal plexus (Fig.6). When both ondansetron or SDZ 205-557 were applied together, again the antagonists led to a sudden blockade of peristaltic contractions, which recovered typically within 10-30min after application (Fig. 6A). After application of both these antagonists, contraction amplitudes were not different from control (Fig.4 & 6B). The concentration of either ondansetron or SDZ-205-557 required to block peristaltic contractions in these reserpine-treated animals was similar to controls (Fig.3). And, only at excessively high concentrations (5-10μM) of either antagonist was it possible to permanently cause blockade (Fig.3B & 3D). These concentrations required to block peristaltic contractions are not consistent with a view that either receptor subtype is necessary for the generation of this motor pattern. We confirmed in reserpine-treated animals that myenteric ganglia were depleted of neuronal 5-HT, using immunohistochemical staining for 5-HT (Fig.6C).

Of particular interest to us was the combined effect of ondansetron and SDZ on preparations of colon that had been reserpine-treated and had their mucosa and submucosal plexus removed (Fig.7). In these preparations, there was no detectable 5-HT using mass spectrometry (Table 1), yet interestingly, application of both antagonists together still had a potent inhibitory effect on peristaltic contractions

(Fig.7). One striking observation was that in reserpine-treated animals that also had their mucosa and submucosal plexus removed, combined application of ondansetron and SDZ 205-557 (1μM) was actually more effective in abolishing these contractions than in control preparations (Fig. 8B). Fewer preparations showed a temporary blockade of contractions, followed by recovery. All contractions were abolished at 3μM (Fig. 8B). These major inhibitory effects on peristaltic contractions occurred, despite the fact that there was no detectable 5-HT in these preparations (Table 1).

4.4.3. Confirmation of depletion of 5-HT from enteric nerves using Immunohistochemistry and mass spectrometry

Tandem-mass spectrometry was used to quantify the level of endogenous 5-HT in control colonic specimens and colonic specimens treated with reserpine. In control specimens, mass spectrometry was consistently able to detect 5-HT in concentrations between 1-10nM (mean 1.9 ± 0.31nM; N=4; Table 1). However, in reserpine-treated segments of colon, that also had their mucosa and submucosal plexus removed, mass spectrometry never detected the presence of serotonin to a sensitivity level of 0.5-1nM (N=4; Table 2). This data was further independently verified using immunohistochemical labeling for 5-HT (273), in which control samples of colon always revealed the presence of 5-HT containing varicose fibres. However, in reserpine-treated animals, immunohistochemical labeling consistently revealed the absence of any endogenous 5-HT in internodal strands of myenteric ganglia (Fig. 5D).

4.5. DISCUSSION

In this study, we evoked repetitive peristaltic contractions in the distal colon by maintained intraluminal distension, using artificial faecal pellets that were fixed within the lumen. This is a different method of distension compared with the approach we used in a recent study from our laboratory (69), where peristalsis was evoked by insertion of faecal pellets that were free to move along the colon or by slow intraluminal fluid distension. We found no overall significant effect of selective 5-HT3 and 5-HT4 antagonists on peristaltic contractions evoked by either of these stimuli, although transient inhibitory or excitatory effects were observed. Those published findings are in contrast to the findings of the current study where we found that the same antagonists could have significant inhibitory effects on repetitive peristaltic contractions evoked by maintained distension. What was particularly noteworthy was that the inhibitory effects of both ondansetron and SDZ-205-557 were equally, or more effective in preparations of colon that had mucosa and submucosal plexus removed; and had no detectable 5-HT, using mass spectrometry. These findings are not consistent with a view that endogenous 5-HT is required for peristaltic contractions to occur, nor that 5-HT3 or 5-HT4 antagonists act by blocking the action of endogenous 5-HT on 5-HT3 or 5-HT4 receptors.

It has been known for some time that a variety of 5-HT antagonists can have robust inhibitory effects on GI-transit and on different motility patterns in the GI-tract. Despite these well-known inhibitory effects, there has been considerable speculation regarding the site of action of these antagonists. It has been presumed that these antagonists act within the gut wall since these antagonists cause inhibition of GI-

motility and propulsion in isolated segments of intestine removed from the animal. Equally unclear is the mechanism by which they inhibit GI-motility patterns, such as peristalsis or cyclical migrating complexes in the small or large intestine. One popular hypothesis has been that 5-HT antagonists could act to inhibit GI-motility by inhibiting the release of endogenous 5-HT from enterochromaffin (EC) cells in the mucosa, or by blocking serotonergic synaptic transmission in the enteric nervous system. The findings of the current study suggest this is unlikely to be the case, at least in the distal colon, since we found that 5-HT3 and 5-HT4 antagonists still inhibited peristaltic contractions in preparations of colon in which no endogenous 5-HT. Furthermore, our data shows that the primary mechanisms by which 5-HT3 and 5-HT4 antagonists inhibit cyclical peristaltic contractions induced by maintained distension, must occur independently of the mucosa, or submucosal plexus.

4.5.1. By what mechanism could 5-HT antagonists cause temporary or sustained inhibition of peristaltic contractions in preparations depleted of endogenous 5-HT?

A major finding of the current study was that in the majority of preparations, the presence of ondansetron and/or SDZ 205-557 caused a rapid blockade of peristaltic contractions. However, in the continued presence of the drugs peristaltic contractions gradually recovered had characteristics not significantly different from controls. The fact that peristaltic contractions still occurred robustly in reserpine-treated animals with mucosa and submucosal plexus removed confirms our previous work that endogenous 5-HT was not required for peristalsis to occur. Also, the fact that the antagonists potently inhibited peristaltic contractions in 5-HT depleted preparations

showed that the antagonists did not require release of endogenous 5-HT for their inhibitory effects to occur (Fig.7A).

One possible explanation for these findings is that 5-HT3 and 5-HT4 receptors may be constitutively active in the absence of any endogenous ligand (i.e. 5-HT). This would explain why the antagonists could still potently inhibit contractions, without any endogenous 5-HT being present in the colon. Indeed, the ligand-gated 5-HT3 receptor (114) and the G-protein coupled 5-HT4 (280) receptor have both been reported to display constitutive activity. If the antagonists reduce this constitutive activity (acting as inverse agonists), this could reduce the background excitability of the enteric neurons that express these receptors and inhibit the neuronal circuitry required for this motor pattern to occur. The reason for the recovery of peristaltic contractions in the continued presence of the antagonists is remarkably similar to the results we reported with hexamethonium, which also temporarily blocked peristalsis, then recovered (39). We speculate that a sudden reduction of electrical excitability in enteric circuits, evoked by antagonists, may be followed by a gradual increase in neuronal excitability that partially compensates, explaining why peristaltic contractions recover. This mechanism would not require endogenous 5-HT.

4.5.2. Why do the same 5-HT3 and 5-HT4 antagonists have different effects on peristaltic contractions evoked in the same preparation of distal colon?

We evoked peristalsis via a different method to the method used in this current study. In our recent study (69), we had evoked peristalsis by slow constant fluid infusion, or acutely inserted faecal pellets. In these cases, intraluminal contents were free to be propelled along the colon and expelled by each peristaltic wave. In contrast, in the

current study, we recorded peristaltic contractions that were evoked by maintained colonic wall distension, by a fixed faecal pellet that was not free to be propelled along the colon. Interestingly, despite using the same region of colon from the same species, we found that the same antagonists had very different effects on the two different patterns of motor activity evoked. Why the same antagonists, used at the same concentrations, had different effects is unclear. What is clear is that the inhibitory effects we recorded in the current study are unlikely to be due to the blockade of endogenous 5-HT acting on either 5-HT3 or 5-HT4 receptors. This is because the inhibitory effects of these antagonists occurred in colonic preparations that had no detectable 5-HT. This is highly consistent with our recent conclusions and data which showed that endogenous 5-HT was not required for peristalsis (69). If the peristaltic contractions were blocked by 5-HT3 and 5-HT4 antagonists because endogenous 5-HT was important for their activation, then depletion of all endogenous 5-HT would be expected to cause the same inhibitory effects. This did not happen. We found no significant deficits in distension-evoked peristaltic contractions in 5-HT depleted preparations. This leads us to believe that that antagonists were acting on 5-HT3 and 5-HT4 receptors to change the excitability of the enteric nervous system independent of the presence of endogenous 5-HT. Indeed, the same effects were also reported with ondansetron on colonic migrating motor complexes in the isolated mouse colon, where it was found that in mucosa-free colonic preparations treated with reserpine, ondansetron was actually significantly more effective at inhibiting CMMCs (239).

4.6. CONCLUSION

The findings of the current study show the repetitive peristaltic contractions induced by maintained colonic wall distension do not require the mucosa, submucosal plexus, nor the presence of 5-HT in myenteric neurons. Consistent with this conclusion is that antagonists of 5-HT3 and 5-HT4 receptors were able to inhibit peristaltic contractions in preparations of colon that did not contain any detectable levels of 5-HT. We suggest that 5-HT3 and 5-HT4 antagonists inhibit peristaltic contractions by blocking constitutively active 5-HT3 and 5-HT4 receptors, which can modulate the excitability of enteric nervous system, independent of the presence of endogenous 5-HT. An alternate explanation to account for these results is that 5-HT antagonists act to cause axonal conduction failure similar to the actions of nicotine causing depolarising block on unmyelinated c-fibres.

Figures: Chapter 4

FIGURE 1. EXPERIMENTAL SET UP

Schematic representation of the method used to evoke repetitive peristaltic contractions. A. An artificial faecal pellet is inserted into the oral end of the isolated colon attached to fine cotton thread. The pellet was allowed to naturally propel along the colon, until it reached a point midway along the colon where it was fixed and isometric force measurements made of the degree of tension generated on the pellet, during each cyclical peristaltic contraction. B shows a photomicrograph of the pellet fixed in the colonic lumen. The arrow indicates a robust circular muscle contraction active on the oral side of the pellet.

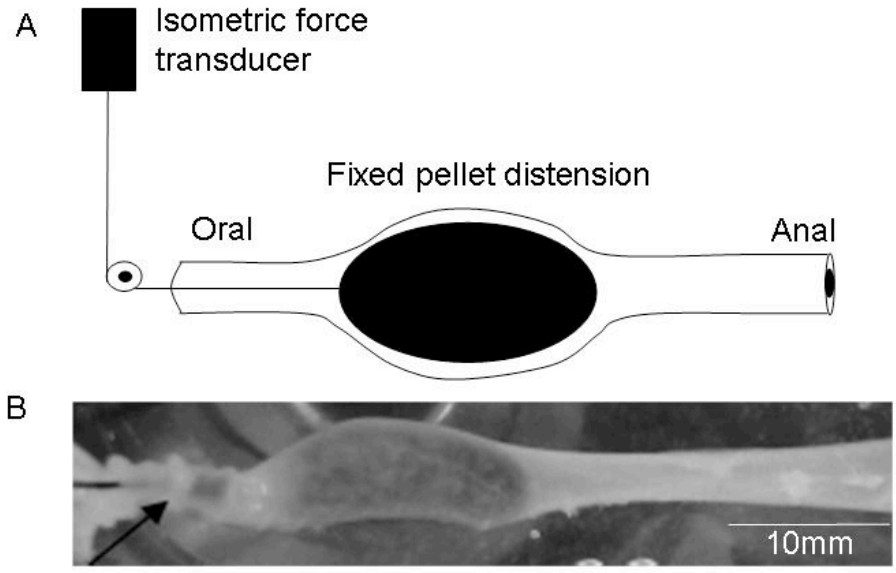

A
Isometric force transducer
Fixed pellet distension
Oral
Anal
B
10mm

FIGURE 2. TRANSIENT EFFECT OF COMBINED ANTAGONISM OF 5-HT3 AND 5-HT4 RECEPTORS IN GUINEA PIG DISTAL COLON *EX VIVO*

Effects of ondansetron and SDZ 205-557 on a control colonic preparation. On each occasion when increasing concentrations of both antagonists were applied a temporary blockade of peristaltic contractions occurred. However, in the continued presence of both drugs, contractions recovered with characteristics not detectably different from controls. B shows that when peristaltic contractions recovered at 1 and 3μM, the amplitude of these contractions was not different from control prior to drug addition. C. Immunohistochemical staining for 5-HT revealed the presence of immunoreactive varicose axons in internodal strands and ganglia.

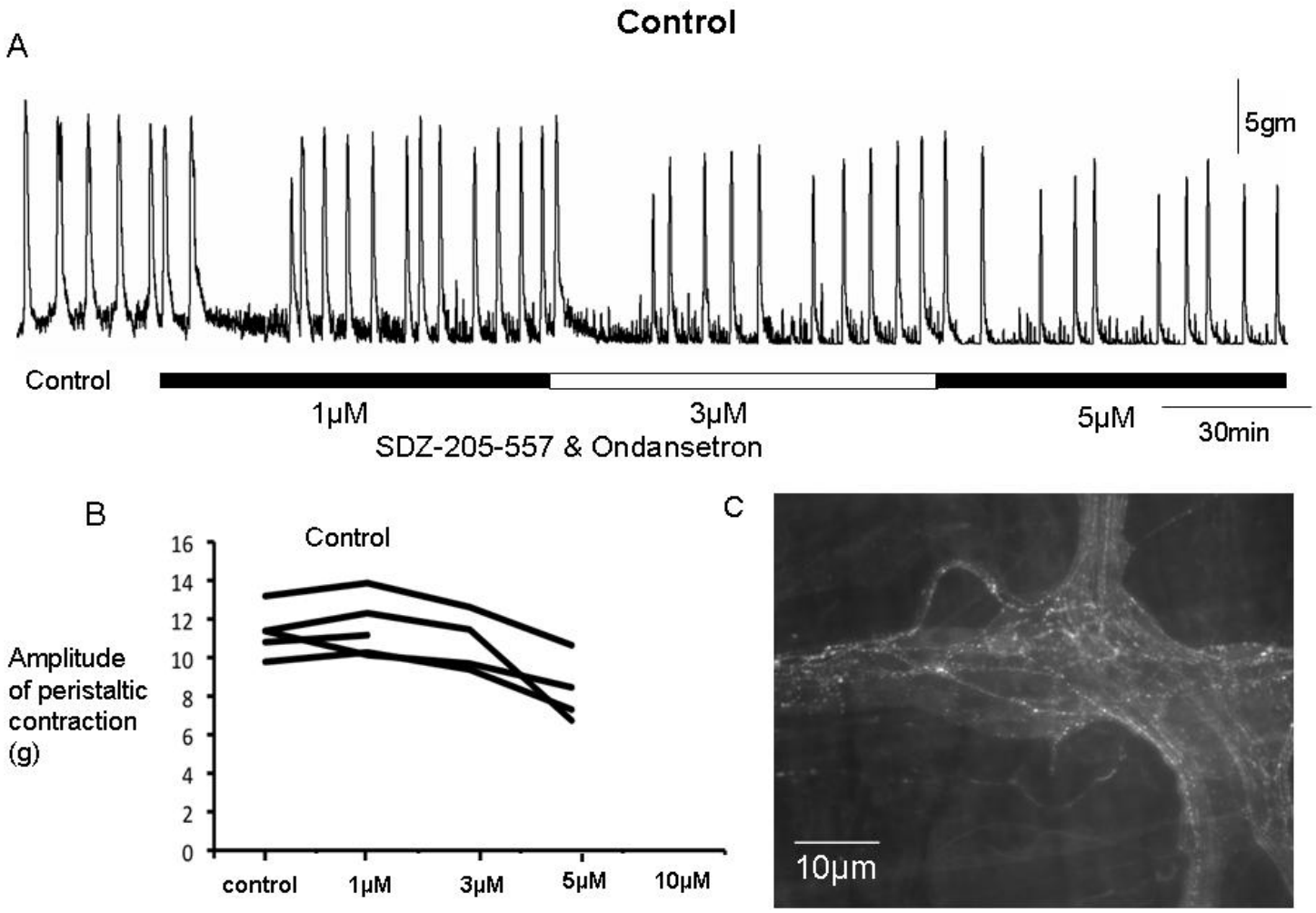
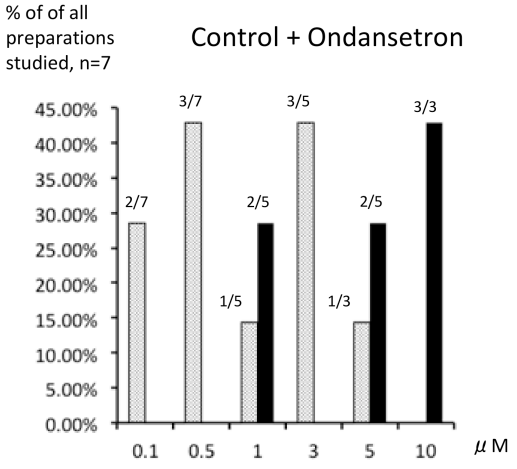

A
Control
5gm
Control
1μM
3μM
5μM
30min
SDZ-205-557 & Ondansetron
B
Control
Amplitude of peristaltic contraction (g)
16
14
12
10
8
6
4
2
0
control
1μM
3μM
5μM
10μM
C
10μm

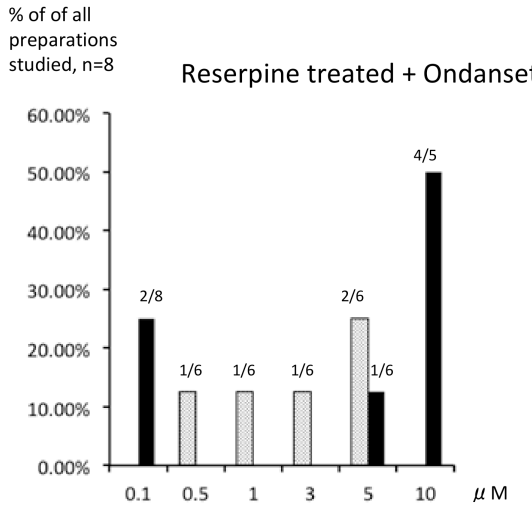
FIGURE 3. PROPORTIONAL BREAKDOWN OF THE EFFECT OF 5-HT3 AND 5-HT4 ANTAGONISTS ON GUINEA PIG DISTAL COLON *EX VIVO*

Graph shows the number of preparations of colon from separate animals, that showed a temporary or permanent blockade of peristaltic contractions in control preparations and preparations obtained from animals treated with reserpine prior to being euthanized. A. In control animal specimens, some proportion of preparations showed a temporary blockade of peristaltic contractions upon application of ondansetron at increasing concentrations of antagonists. Similar results were obtained in reserpine-treated preparations with mucosa present (B). C and D. Increasing concentrations of SDZ-205-557 control preparations and reserpine-treated preparations showed a temporary blockade of peristaltic contractions. At higher concentrations peristaltic contractions were blocked permanently.

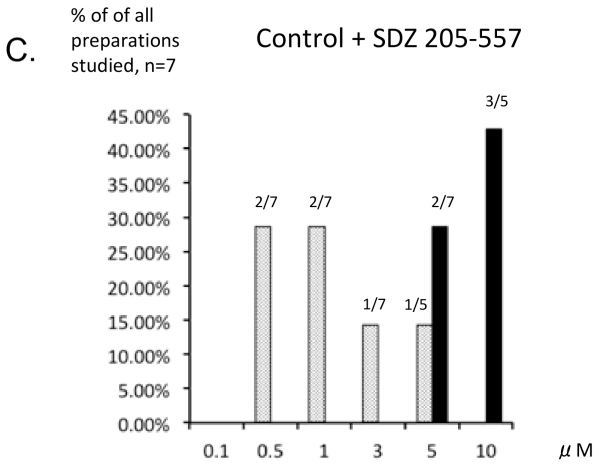
A.


% of of all preparations studied, n=7
Control + Ondansetron
45.00%
40.00%
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
2/7
3/7
1/5
2/5
3/5
1/3
2/5
3/3
0.1
0.5
1
3
5
10
μ M

B.


% of of all preparations studied, n=8
Reserpine treated + Ondansetron
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
2/8
1/6
1/6
1/6
2/6
1/6
4/5
0.1
0.5
1
3
5
10
μ M

C.


% of of all preparations studied, n=7
Control + SDZ 205-557
45.00%
40.00%
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
2/7
2/7
1/7
1/5
2/7
3/5
0.1
0.5
1
3
5
10
μ M

D.

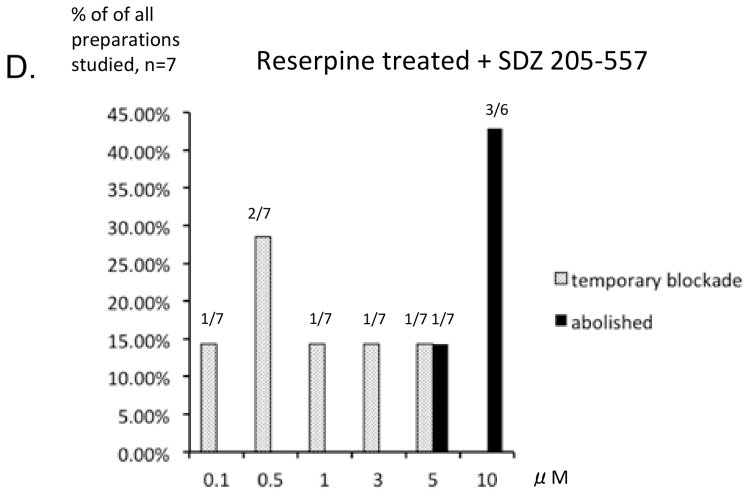

% of of all preparations studied, n=7
Reserpine treated + SDZ 205-557
45.00%
40.00%
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
1/7
2/7
1/7
1/7
1/7
1/7
3/6
0.1
0.5
1
3
5
10
μ M
temporary blockade
abolished

FIGURE 4. EFFECT OF 5-HT3 AND 5-HT4 ANTAGONISTS ON FORCE OF PERISTALTIC CONTRACTIONS

Graphs show mean changes in amplitude of peristaltic contractions, in response to the combined application of ondansetron and SDZ-205-557. A shows that in control animals, the amplitudes of peristaltic contractions showed little change after recovery and whilst in the continued presence of both antagonists. B. Also in reserpine-treated animals with mucosa present, the amplitudes of peristaltic contractions were no different following recovery, after an initial temporary blockade.

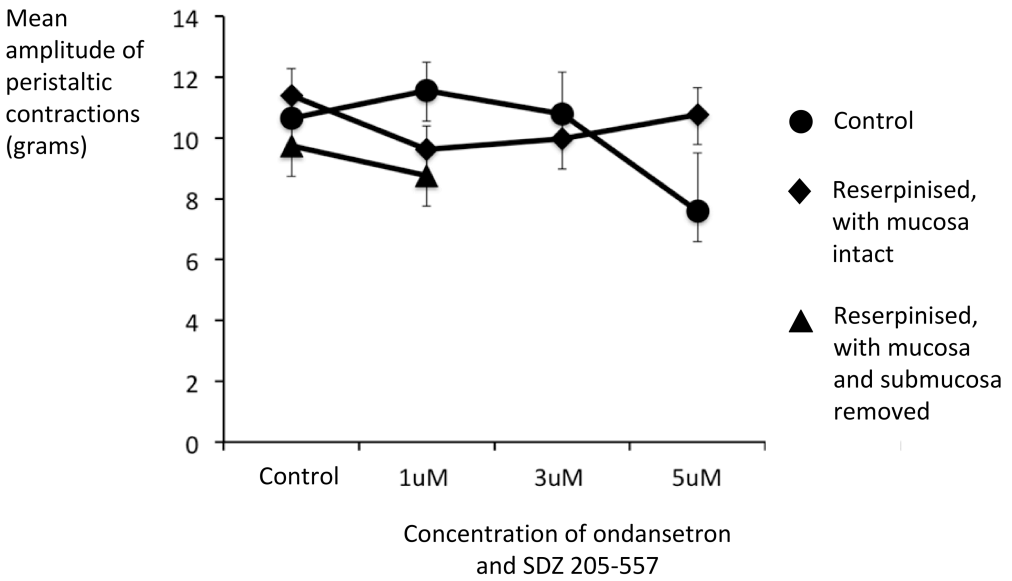

Mean amplitude of peristaltic contractions (grams)
14
12
10
8
6
4
2
0
Control
1uM
3uM
5uM
Concentration of ondansetron and SDZ 205-557
Control
Reserpinised, with mucosa intact
Reserpinised, with mucosa and submucosa removed

FIGURE 5. EFFECT ON FREQUENCY OF CONTRACTIONS IN THE PRESENCE OF 5-HT3 AND 5-HT4 RECEPTOR ANTAGONISTS

Effects of application of SDZ-205-557 or granisetron on the intervals between repetitive peristaltic contractions. A, show a graphical representation of the responses of individual preparations of colon to SDZ-205-557. No clear changes are seen. B Similar lack of effect of SDZ-205-557 on reserpine treated animals, in which the mucosa was present. C shows the mean changes in interval between peristaltic contractions in control (A) and reserpine-treated (B) preparations overlaid on the same graph. D, shows the same experiment as in panel A, but granisetron is used instead of SDZ-205-557. Again, no major changes in intervals were detected. E shows the lack of effect of granisetron on intervals between peristaltic contractions in reserpine-treated animals, with mucosa present. F, shows mean data of panels D & E superimposed. No overall significant effects were detected.

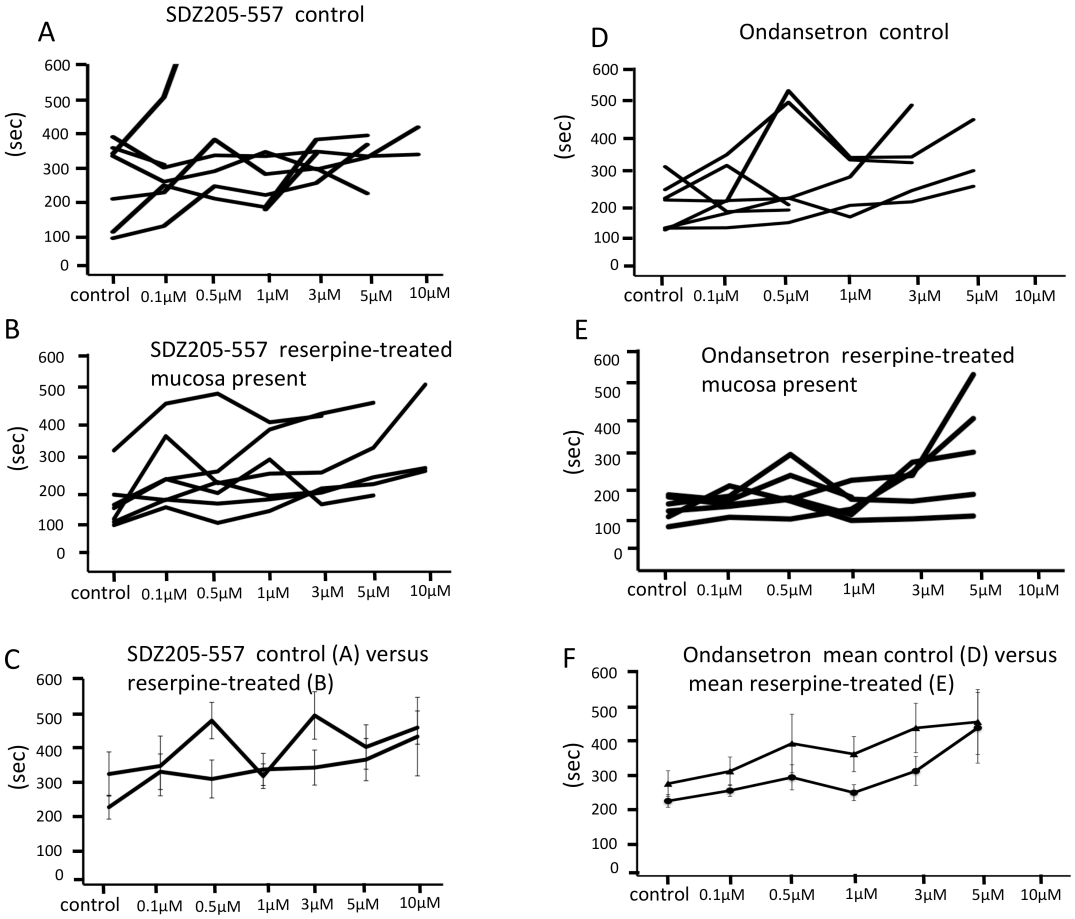
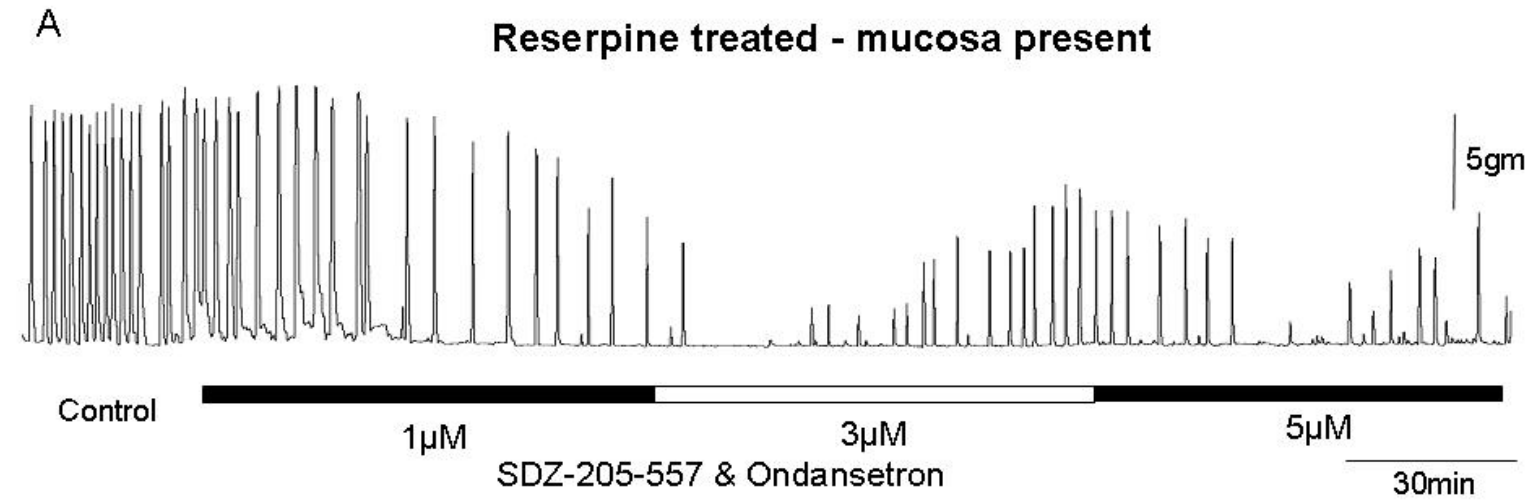
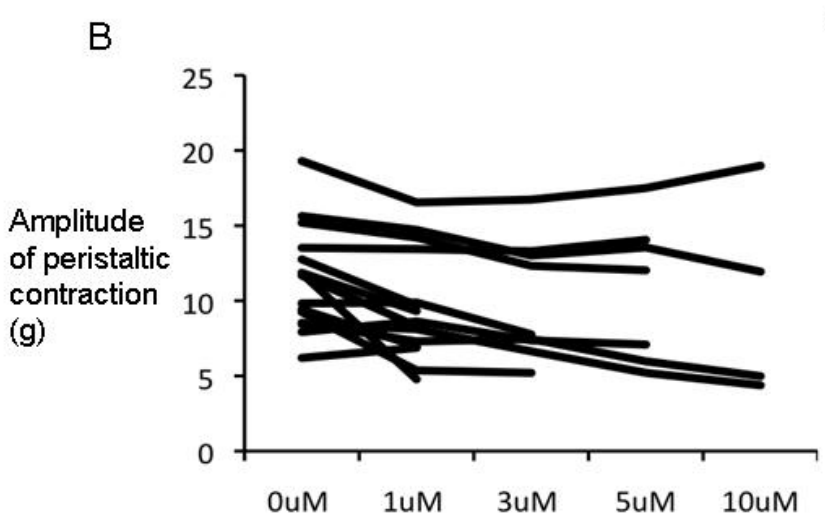

A
SDZ205-557 control
(sec)
600
500
400
300
200
100
0
control 0.1μM 0.5μM 1μM 3μM 5μM 10μM
D
Ondansetron control
(sec)
600
500
400
300
200
100
0
control 0.1μM 0.5μM 1μM 3μM 5μM 10μM
B
SDZ205-557 reserpine-treated
mucosa present
(sec)
600
500
400
300
200
100
0
control 0.1μM 0.5μM 1μM 3μM 5μM 10μM
E
Ondansetron reserpine-treated
mucosa present
(sec)
600
500
400
300
200
100
0
control 0.1μM 0.5μM 1μM 3μM 5μM 10μM
C
SDZ205-557 control (A) versus
reserpine-treated (B)
(sec)
600
500
400
300
200
100
0
control 0.1μM 0.5μM 1μM 3μM 5μM 10μM
F
Ondansetron mean control (D) versus
mean reserpine-treated (E)
(sec)
600
500
400
300
200
100
0
control 0.1μM 0.5μM 1μM 3μM 5μM 10μM

FIGURE 6. RECOVERY OF PERISTALSIS IN THE PRESENCE OF 5-HT3 AND 5HT4 RECEPTOR ANTAGONISTS

In a reserpine-treated animal with mucosa present, combined application of SDZ-205-557 and granisetron caused temporary inhibition of peristaltic contractions which recovered even with increasing concentrations up to 5μM. B shows changes in amplitude of peristaltic contractions from individual animals. It is apparent that when these contractions recovers the amplitudes are unaffected in the majority of preparations, even up to 10μM in concentration. C shows that 5-HT has been depleted from myenteric ganglia in response to reserpine treatment.


A
Reserpine treated - mucosa present
5gm
Control
1μM
3μM
5μM
SDZ-205-557 & Ondansetron
30min


B
Amplitude of peristaltic contraction (g)
25
20
15
10
5
0
0uM
1uM
3uM
5uM
10uM

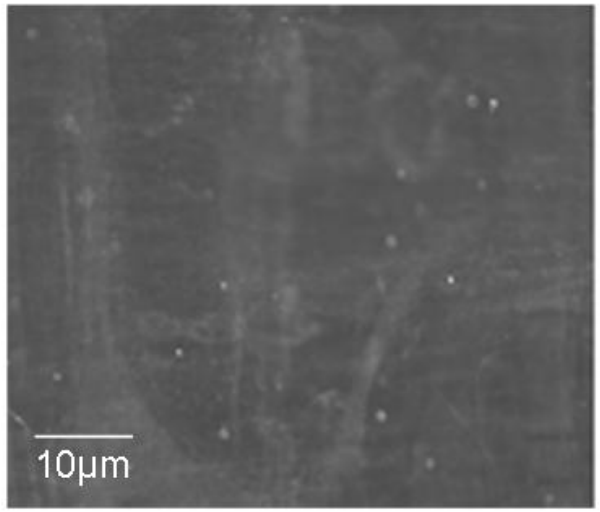

C
10μm

FIGURE 7. EFFECT OF 5-HT3 AND 5-HT4 RECEPTOR ANTAGONISTS IN GUINEA PIG DISTAL COLON WITH MUCOSA RESECTED

In a reserpine-treated animal, with mucosa and submucosal plexus removed, the combined application of SDZ-205-557 and granisetron had potent inhibitory effects on peristaltic contractions, even despite the lack of any 5-HT detected in these preparations with mass spectrometry or immunohistochemistry. A. The recording showing peristalsis slowed at 1μM and is abolished at 3μM. B. Graph showing that 5-HT depleted preparations showed greater sensitivities to both antagonists. All preparations were permanently abolished by both antagonists at 3μM. C. Immunohistochemistry confirmed the absence of 5-HT in myenteric ganglia and internodal strands.

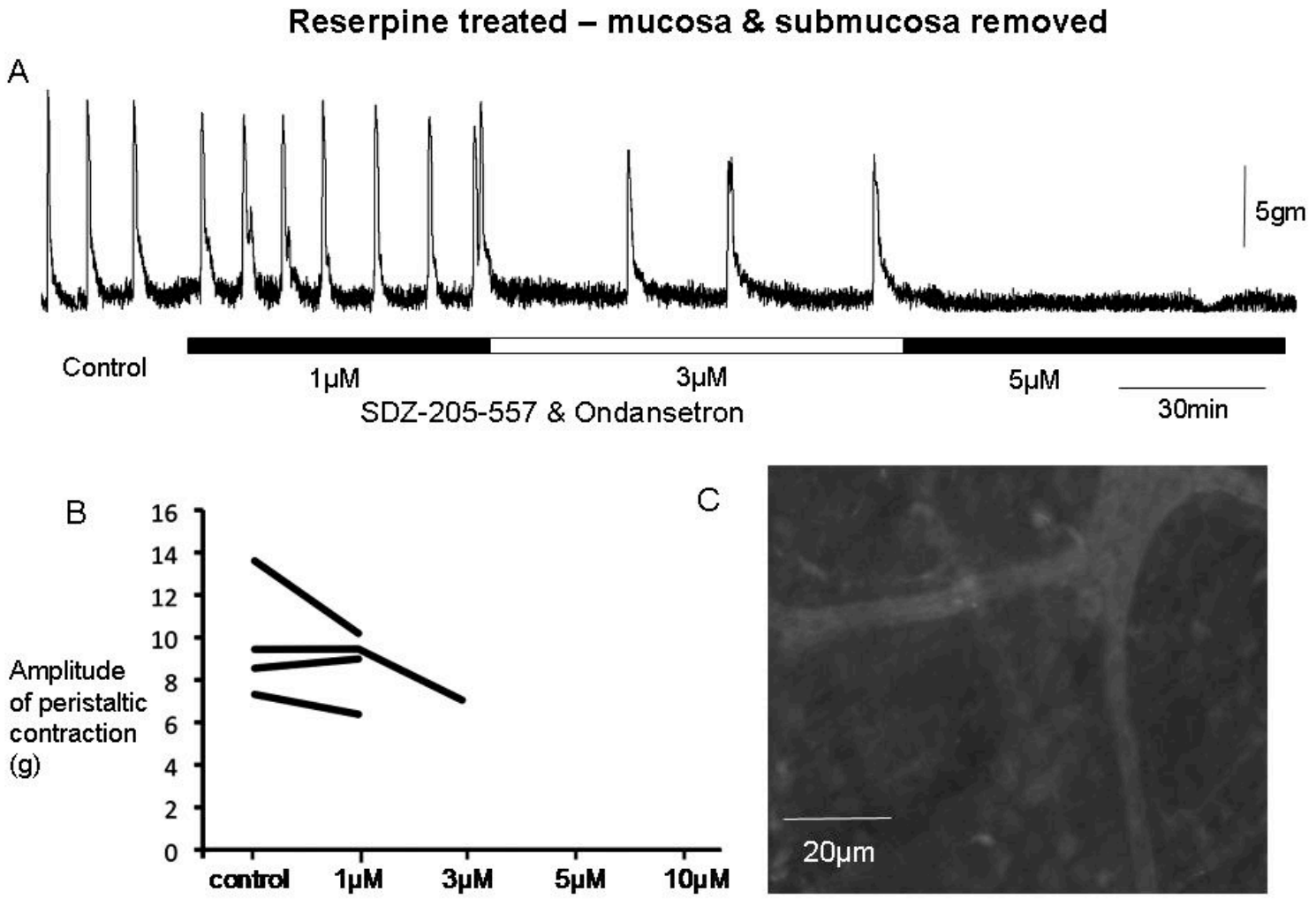
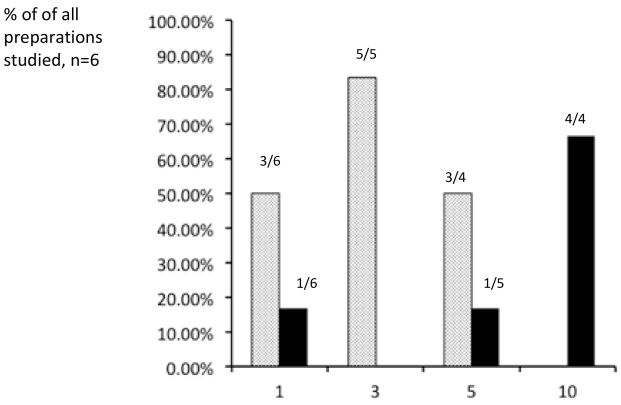

Reserpine treated – mucosa & submucosa removed
A
5gm
Control
1μM
3μM
5μM
30min
SDZ-205-557 & Ondansetron
B
Amplitude of peristaltic contraction (g)
16
14
12
10
8
6
4
2
0
control
1μM
3μM
5μM
10μM
C
20μm

FIGURE 8. PROPORTIONAL BREAKDOWN OF THE EFFECT OF 5-HT3 AND 5-HT4 RECEPTOR ANTAGONISTS

Sensitivity of individual experiments to the combined effects of receptor antagonists to 5-HT3 and 5-HT4 receptors. Control data shows the proportion of individual colonic preparations that showed repeated temporary blockade of repetitive peristaltic contractions with increasing concentrations of SDZ-205-557 and granisetron. The table shows responses of 6 control animals to consecutive application of increasing concentrations of granisetron and ondansetron (1-10μM) and responses to 5 preparations treated with reserpine and also with mucosa and submucosal plexus removed. Interestingly, in reserpinised preparations, with mucosa and submucosal plexus removed, peristaltic contractions rarely recovered in the presence of both antagonists and most 3 of 4 animals were blocked at 1μM, whereas in control animals, 4 out of 6 animals required 10μM to permanently block these contractions.

Control Ondansetron + SDZ 205-557



Serotonin depleted Ondansetron + SDZ 205-557

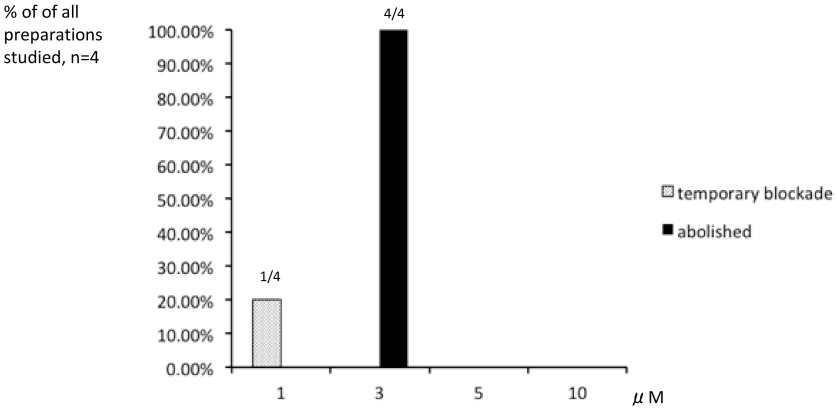


TABLE 1. EFFICACY OF RESERPINE INDUCED NEURONAL 5-HT DEPLETION AS MEASURED BY MASS SPECTROMETRY

Levels of 5-HT detected from control guinea pig colon and from animals treated with reserpine and devoid of mucosa and submucosal plexus. In all control animals, 5-HT was detected from control preparations in the range of 1-3nM. However, in preparations treated with reserpine that also had mucosa and submucosal plexus removed, mass spectrometry did not detect the presence of 5-HT.

controls	concentration (nM)
Animal 1	2.7
Animal 2	1.5
Animal 3	1.6
Animal 4	1.9

Reserpine treated / mucosa & submucosal plexus removed	concentration (nM)
Animal 1	n.d
Animal 2	n.d
Animal 3	n.d
Animal 4	n.d

Chapter 5: Identification of neurogenic peristalsis in the *ex vivo* human small intestine

5.1. ABSTRACT

Background: The motor activity of the human digestive tract arises from complex interactions between spontaneous myogenic activity driven by pacemaker cells and neurogenic mechanisms. However, these relationships remain poorly characterised in the human intestine. **Aims**: To characterise the motility patterns of the human terminal ileum (TI) *ex vivo* and compare these data to *in vivo* recordings from the terminal ileum. **Methods**: Segments of isolated human TI (12.1 ± 1.4 cm, 7-26cm, n=20) were obtained from right hemicolectomy specimens and studied *ex vivo* in an organ bath. Using a combination of simultaneous mechanical recording techniques with external force transducers, luminal fibre-optic manometry and video recordings of gut movement we recorded resting contractility and gut contraction in response antagonists of nerve conduction (lidocaine; 100uM). The *in vivo* recordings of resting terminal ileum motility were obtained from healthy controls using intraluminal water perfused manometry (n=8). **Results**: Propagating pressure waves were seen in all the *ex vivo* preparations with a length >13cm (n=4) and in these preparations lidocaine blocked the propagating activity. Lidocaine had no effect upon the frequency of phasic activity. In shorter preparation of terminal ileum no propagating activity was observed. The frequencies of propagating events were measured at 0.5 +/- 0.03 events per minute. The velocity of propagation was 1.7 +/- 0.8 $mm.sec^{-1}$. In the *ex vivo* recordings phasic activity was seen in all specimens, with frequencies of 5.0 ± 0.6 (force transducer) and 6.1 ± 0.3 (fibre-optic) counts per minute (cpm) *ex vivo*. This frequency was significantly lower than that recorded *in vivo* (10.0 ± 0.3 cpm; $p<0.05$). **Discussion**: Using a combination of different *ex vivo* techniques, we have been able to record both myogenic and neurogenic motor

patterns in the human terminal ileum. The presence of propagating activity in longer segments of human terminal ileum would suggest that a certain length of intact intrinsic neural circuitry is required for generation of propagating activity. In future studies this preparation will allow us to unravel the different enteric neural and myogenic motor patterns in the human gastrointestinal tract. These data will provide key information on the mechanisms that underpin small bowel motility.

5.2. INTRODUCTION:

Despite significant advances in our understanding of the mechanisms of gastrointestinal motor patterns in humans, significant gaps in our understanding remain (281).

The human terminal ileum is one of the most inaccessible regions of the human digestive tract and therefore one of the least studied. Manometric and transit studies suggest specialised motor patterns exist within the region. For example, in 1913 through the use x-rays, Hertz observed that a bismuth labelled meal could sit at the terminal ileum for one hour or more before any content would pass into the caecum and that the ileum remain full for up to 6 hours after content has left the stomach. Later scintigraphic studies confirmed these early observations, and revealed postprandial states of relaxation in the distal ileum (282, 283). This proposed ileal brake (284) allows for the absorption of several bile salts and vitamin B_{12}, processes that only occur in the terminal ileum (285).

When the terminal ileum does empty it does so in a pulsatile fashion (286, 287). A number of intraluminal manometric studies have demonstrated a series of specialised motor patterns that are likely to be related to this pulsatile flow. These consist of a mixture of prolonged propagating contractions and discrete clustered contractions (multiphasic pressure wave recording in one channel at a frequency between 4-12 cycles/min) (288-290). Utilizing combined manometry and scintigraphy, Dinning *et al* showed that propagating pressures in the terminal ileum were temporally associated with both caecal filling and caecal propagating sequences (288). Furthermore the same study demonstrated that these ileal propagating pressure sequences were associated with inhibition of the phasic activity recorded in the ileo-colonic junction, presumably allowing ileo-caecal flow to occur (288).

As with other regions of digestive tract the motility of the terminal ileum is composed of a complex interaction between extrinsic neural inputs, enteric nerves and intrinsic spontaneous movements generated by pacemaker cells (myogenic mechanisms) (8, 291). While such mechanisms can be shown to exist in animal preparations (292), elucidating the role neural/myogenic mechanisms play in human *in vivo* motility is difficult.

However, *ex vivo* experimentation allows for a controlled environment in which motor patterns can be studied. Such studies remove the influence of the extrinsic nerves thus allowing investigation into the underlining contractility of the small bowel. In addition, *ex vivo* experiments also allows for the effects of more invasive intervention otherwise not possible *in vivo*, such neural blockade, electrical stimulation and mechanical distension to be studied.

Recently we published results from mechanical recordings of the human colon in a large organ bath. In those experiments using a row of force transduces we were able to record motility from entire sections of the human colon (218). Utilising a similar setup, we have now performed *ex vivo* recordings of human terminal ileum removed from right hemicolon resections performed for colonic malignancy. In addition to the existing set-up, a fibre-optic catheter was incorporated for measuring luminal contractile responses (see methods). These data have then been compared to *in vivo* terminal ileal manometry recordings made previously by Dinning and colleagues while studying the human colon (293).

5.3. METHODS:

5.3.1. Ethics approval

Prior to the surgical procedures all patients gave written and informed consent and the study was approved by the Flinders Clinical Research Ethics Committee; approval number 50/07.

5.3.2. *Ex vivo* recordings

5.3.2.1. Tissue preparation

Segments of terminal ileum were removed from right hemicolectomy resection specimens for colonic malignancy. Variable lengths of small bowel between 7 to 26cm were obtained. This length was dependent upon the surgeons' confidence of

adequate anastomotic end vascularisation following ileo-colic artery high ligation for lymphovascular assessment. In our earlier experiments, whole right hemicolectomy resections- including the segment of terminal ileum, caecum and right hemicolon were placed in the organ bath to study motility in the terminal ileum. In our latter experiments, in consultation with the Department of Surgical Pathology, the terminal ileum was excised from the specimen for study. The mesentery was dissected off the small bowel and left to remain with the colonic portion of specimen. The oral end of the specimen was labeled to ensure correct orientation in the organ bath, as well as no disruption to subsequent pathological assessment. Upon resection, specimens were placed in warm oxygenated Krebs solution and transported to the laboratories within the same building as the operating theatres. The time from vascular occlusion ranged from 10 -20 minutes, and transport time from operating theatres to placement within the organ bath was <30 minutes. The specifically constructed organ bath consisted of a 7 litre fluid containment filled with Krebs (NaCl 118mMol, KCl 4.7mMol, $NaHPO_4$ $2H_20$, $NaHCO_3$ 25mMol, $MgCl6H_2O$, D- glucose 11mMol, $CaCl_22H_2O$ 2.5mMol). This was perfused with carbogen and maintained at 36.5+/- 0.5 ^{0}C surrounded by temperature regulating water jacket.

5.3.2.2. Recording techniques

3 separate techniques outlined below were utilised for these experiments, either in combination or individually (Fig 1, 2, 3). Patients from whom these specimens were obtained were fasted for >6 hours prior to their surgical procedure. Prior to placement in the organ bath intraluminal content was expelled by flushing Krebs through the segment.

5.3.2.2.1. Force transducers

Alligator clips attached to force transducers via 3-0 silk suture threads were clipped onto discrete sites along the serosal surface of the small bowel. These were spaced at 1-2cm intervals (Fig 2). Tension generated by circular muscle contraction was relayed onto independent isometric recording transducers (Grass FT-03C; Grass, Quincy, MA) and connected to individual custom made preamplifiers (Biomedical Engineering, Flinders University) and onto a Powerlab (model 4/30; AD Instruments, Bella Vista, NSW, Australia) and a notebook Personal Computer (PC) running LabChart 7.0 (AD Instruments, Australia).

5.3.2.2.2. Fibre-optic manometry recording

The fibre-optic catheter incorporated 60 sensors spaced at 1cm intervals and had an outside diameter of 3mm (294). The catheter was attached to a spectral interrogator unit (FOS&S FBG-scan 804. FOS&S, Geel, Belgium) and pressures were recorded in real time using a custom written LabVIEW program (National Instruments, TX, USA). The recorded manometric traces were viewed and analysed using software (PlotHRM) developed by one of the authors. The software was written in Matlab (The MathWorks, MA, USA) and Java (Sun Microsystems, CA, USA). The fibre-optic catheter was attached to a metal rod and the rod was passed through the lumen of the specimen. Cable ties secured the oral and anal ends of the specimen rod (Fig 3).

5.3.2.2.3. Video recording of diameter changes

A digital video camera (Canon Legria HF S20. Ota, Tokyo, Japan) was positioned on profile to record diameter changes alongside the superior aspect of the bowel placed in the organ bath. This was used to record movies of terminal ileal wall motion in clips of 10 minutes duration (Fig. 1, 3). These were then re-sampled down to 4 frames per second in QuickTime (Apple Inc. Cupertino, CA, USA). The videos generated were then converted to spatio-temporal maps of changes in diameter ("Dmaps") with software written in Matlab, adapted from the methods developed in our laboratory (Hennig *et al*., 1999). Briefly, the diameter at each point along the preparation was calculated for each frame and converted into grey scale, to create a spatio-temporal map of diameter changes.

Our initial experiments (n=13) were conducted using either one of force transducers or fibre-optics manometry recordings. Following experiments were conducted using various combinations of both force transducers, and fibre-optic manometry as well as video recording of diameter changes to generate spatio-temporal maps.

5.3.2.3. Experimental setup

In every experiment (bar 3 additional experiments, explained below), to hold the specimens in place within the bath, alligator clips were attached to the oral and anal ends of each segment, and wire attached to the alligator clips were wrapped around screws placed at the ends of the organ bath. The specimen was anchored with a thin luminal rod for which cable ties were used to secure both ends.

In an attempt to optimize the *ex vivo* recordings a range of recording techniques were tested. Single technique experiments included those utilising force transducers (n=7) or fibre-optic catheter (n=6) only, as described in sections 5.3.2.2.1 - 5.3.2.2.3 of this thesis. These experiments allowed for assessment of frequencies of phasic contractile events and propagating activity in each set-up and allowed for analytic comparisons between the groups.

In a separate series of experiments (n=5), combined recordings of both the fibre-optic and force transducer were conducted. These were performed with the aim of observing if similar contractile activity were recorded by both techniques. In addition, in these experiments we conducted video recordings on 4 experiments to generate spatio-temporal maps. Video recordings were only feasible in a clean preparation not tarnished by surrounding fatty tissue from the mesentery.

In an additional series of experiments, 3 specimens were recorded with the catheter placed luminally, without the inner securing aluminium rod. In these cases the bowel was secured in place by the clips on either end similar to that described above.

In all specimens a 30-minute basal recording was performed before any intervention. This formed the control period of every experiment. Initial experiments were conducted to study contractile activity in control environments. Subsequently, in 8 specimens lidocaine was added (100uM; Sigma Chemicals Co., St Louis, Mo., U.S.A) (please refer to Table. 1) to abolish enteric neural activity. This was not

carried out in the other 12 specimens. At the end of the period of recording the specimens were taken to pathology.

In a single experiment a size 8 Fr. rubber tubing was secured luminally on the oral end of the specimen with cable ties. Krebs solution was infused through the tubing in a 1-2ml increments. This allowed us to simulate a gradual distension of the lumen induced by content filling in normal physiological states.

5.3.3. *In vivo* recordings

In vivo recordings of human terminal ileum motility were provided by Dinning *et al*. (unpublished observations) (293). In these experiments, a water-perfused catheter (5 metres, 16 side holes at 7.5cm intervals, Dentsleeve, Wayville, SA) was placed per-nasally. Ileal and colonic recordings were performed over a 24hour period in an unprepared bowel. To minimize the impact of meals and morning waking on gut motility, an hour-long period of terminal ileal motor activity was selected for each volunteer during a fasted period, one hour after the subject woke. *In vivo* data were analysed only for phasic contractile activity.

5.3.4. Measurements and statistics:

5.3.4.1. Analysis of phasic contractile activity

In both force transducer and fibre-optic recordings, the frequency of phasic contractile activity was calculated. This frequency was obtained across multiple sites within a single specimen in both control and intervention periods. Phasic contractile

activity present during the recordings, were characterised as a repetitive ongoing activity incorporating a discernable onset, peak and offset, and did not have the features of pressure increases generated by artifact. In the *in vivo* recordings, straining or respiratory oscillations were removed from the analysis (293).

5.3.4.2. Analysis of propagating activity

Propagating activity in the *ex vivo* setup was defined as 4 or more pressure waves in adjacent channels (i.e. ≥ 4 cm). Pressure peaks in adjacent channels had to fall within a time window of ± 5 seconds of each other and not be deemed synchronous. These propagating sequences were further classified as anterograde (anally propagating) or retrograde (orally propagating) (235). The propagation speed (velocity) was measured in mm/s.

Propagating activity was not analysed in the *in vivo* data. It was in our opinion that these spacings (7.5cm) were not adequately sensitive to resolve directionality of propagation. In addition, the reliability of determining what constitutes a propagating activity under these resolutions is poor (235).

5.3.4.3. Statistical analysis

Students Paired T-tests were used for comparison analysis between groups unless specified. To determine and change in the propagating activity over time, the hour long recording were divided into 4 epochs (15 minutes each) with propagating sequences in each epoch compared to others using a one-way ANOVA.

In experiments comparing frequencies of phasic activity over time, 3 distinct 10-minute epochs were chosen- 1. Early (5^{th} to 15^{th} minute), 2. Mid experiment (25^{th} to 35^{th} minute, and 3. Late (45^{th} to 55^{th} minute). The frequencies from these groups were also compared using one-way ANOVA.

5.3.4.4. Fast Fourier Transformation (FFT)

FFT algorithm was employed to establish the presence of a dominant frequency. In essence the FFT divides the contractile activity temporally into partially overlapping smaller segments and computes the dominant frequency within the smaller temporal region. The single dominant frequency is plotted on a power spectrum at any point in time. In the presence of a clear dominant frequency a red line appears (Fig 4C). FFT was used on the only 3 experiments performed utilising luminal fibre-optic catheter recordings. Lidocaine was used as the pharmacological agent of choice in abolishing neural activity.

5.4. RESULTS:

A total of 20 human terminal ileal specimens were studied. These ranged from 7cm to 26cm (mean=12.0 cm, median 9.5 cm, n=20) (Table 1).

5.4.1. Presence of an underlying phasic contractile activity in the human terminal ileum

In *ex vivo* recordings distinct periods of phasic activity was seen, and interspersed with periods of quiescence.

Recordings by external force transducer and luminal fibre-optic revealed phasic cyclical contractile activity that was similar in waveform. This was consistent across all specimens (Fig. 4. Ai, Bi). Using force transducer experiments, frequencies were recorded at 5.01 +/- 0.61 cpm (n=7). Similar frequencies were seen in the fibre-optic recordings at 5.74 +/- 0.43 cpm (n=7, P=0.16) (Fig. 4. C).

When 3 distinct epochs (10 minutes duration- i. early: 15^{th} to 25^{th} minute, ii. mid: 25^{th} to 35^{th} minute, late: 45^{th} to 55^{th} minute) within an hour-long recording were compared, there was no difference in frequencies across each group (one-way ANOVA; force transducer recordings, P=0.54; luminal fibre-optic catheter recordings, P=0.68; Fig. 4. Aii, Bii).

5.4.2. Propagating events

Propagating contractile activity was recorded from 4 of 20 specimens, each obtained from a different patient. These specimens measured 13 cm, 18 cm, 23 cm and 26 cm in length (Table 1). The propagating activity occurred at 0.45 +/- 0.03 events per minute and at a speed of 1.72 +/- 0.79 $mm.sec^{-1}$ (Fig. 6). None of the specimens shorter than 13 cm showed signs of propagating activity regardless of the technique used.

In 3 of 4 specimens propagating contractions were recorded simultaneously with force transducers, luminal fibre-optic manometry and high-resolution video imaging to generate spatio-temporal maps. Propagating contractions, when present were seen across all 3 recording modalities. Contractions that propagated were recorded in both

anterograde and retrograde directions were most prominent in the spatio-temporal maps (Fig. 5). In the single other experiment the propagating activity was recorded on a luminal fibre-optic catheter recording only. To test the role of the enteric nervous system in the generation of these propagating contractions we applied lidocaine to the organ bath. Lidocaine consistently abolished the prolonged propagating contractions (Fig. 6, 7).

5.4.3. Presence of enteric nervous system modulating the underlying phasic contractile activity

Propagating contractions, when present, were consistently abolished following lidocaine infusion into the organ bath. Phasic contractile activity, appearing most clearly in the presence of lidocaine was consistently recorded across all 3 recording modalities (Fig. 8).

The presence of lidocaine did not significantly change the frequency of underlying phasic contractile activity (Lidocaine 6.03 +/- 0.37 cpm, vs. baseline 5.97 +/- 0.35 cpm, n=8, P=0.93) (Fig. 9).

Fast Fourier Transformation (FFT) was employed to determine if there was indeed a dominant frequency. FFT was performed on the 3 luminally obtained fibre-optic recordings that were not connected to external force transducers. This was performed to isolate specimens whereby no other confounding influences were present that could account for change in underlying myogenic activity (e.g. pinching of the bowel). Despite similarities in frequency before and after lidocaine, in experiments conducted before lidocaine no clear dominant frequency of contractile activity was

observed. In lidocaine treated preparations, a dominant frequency of 6 cpm was observed in 2/3 of specimens examined (Fig. 9).

5.4.4. *In vivo* water perfused recordings

Distinct periods of distinct high amplitude activity were seen interspersed between periods of lower amplitude activity. The amplitudes of the lower frequency events measure 11.9 +/- 1.2 mmHg and the higher amplitude contractions measure 68.1 +/- 4.5 mmHg. Frequencies of phasic contractile activity measured in higher amplitude activity regions were 10 +/- 0.3 events per minute. In the lower amplitude periods, frequencies were higher, measuring 14.53 +/- 1 events per minute ($n=8$, $P<0.05$). When compared to *ex vivo* recordings, *in vivo* high amplitude phasic periods exhibited higher frequencies (5.0 +/- 0.6 cpm *ex vivo* c.f. 10 +/- 0.25 cpm *in vivo*, $P<0.05$, Fig. 11).

5.4.5. Example of luminal distension that modifies the directionality of motor activity in isolated human small bowel

Slow luminal distension with fluid was performed in one experiment ($n=1$, 26cm length). In this preparation propagating activity was seen occurring in both anterograde and retrograde directions prior to commencement of infusion. Step-wise increments of 1ml Krebs solution from the oral end resulted in an apparent re-organisation of these propagating sequences into an anterograde only direction. This was observed in all 3 recording techniques (Fig. 10).

5.4.6. Video imaging of contractile activity

High-resolution video recordings used to generate spatio-temporal maps resulted in recordings of contractile activity and its propagation (when present), in a non-interrupted manner. Directionality of propagation, and underlying phasic contractile activity were clearly seen on spatio-temporal maps generated.

In these experiments, propagating contractile activity detected on spatio-temporal maps could be temporally correlated with both force transducer recordings and luminal fibre-optic recordings (Fig, 5, 8, 10) in a clear 1:1 correlation.

5.5. DISCUSSION

Using variable lengths of excised human terminal ileum and several different recording techniques in our *ex vivo* preparation we have been able to record both propagating and non-propagating terminal ileal motor patterns. Following the application of lidocaine we were able to determine the existence of both myogenic and neurogenic motor patterns. To the best of our knowledge these are the first studies to record motility of large segments of isolated human terminal ileum *ex vivo*.

5.5.1. Confirmation of the role of the enteric nervous system in the generation of motor activity in the isolated human small intestine

Lidocaine was used in these experiments to block neural activity via blockade of fast voltage gated Na^+ channels. Phasic contractile activity was present throughout the recordings, even prior to lidocaine infusion into the bath. This suggested firstly, that

these phasic activities were likely to represent slow wave mediated contractions that do not require the neuronal input. There was, however, in the presence of lidocaine, a more regular phasic activity, shown as an emergence of a dominant frequency band on FFT in following addition of lidocaine suggest the presence of an intact neural circuitry modulating the underlying myogenic activity in an *ex vivo* setting (Fig. 9). This neural activity was present until the infusion of lidocaine, at times up to more than an hour after the specimen had been removed from the patient. The fact that neural activity was present in specimens studied at this time from removal from the patient indicated that hypoxia had not abolished neural activity. We are conscious however of the possibility that the myogenic phasic motor rhythm had been induced by nerve conduction blockade with TTX and this could have affected our interpretation of our FFT analysis.

When propagating activity was seen (n=4), especially in longer specimens (>13cm), lidocaine effectively abolished these propagating contractile events. From this it is clear that neural activation was required for the generation of propagating contractile events.

5.5.2. Role of combining multiple simultaneous recording techniques

In these experiments, we have utilised simultaneous recording methods that involved force transducers, fibre-optic manometry and high-resolution video imaging to record motility patterns seen in the *ex vivo* terminal ileum. Previously many experiments have attempted to record motility patterns by using recording techniques with wide spacing between recording sites. For example in human *in vivo* colonic and small bowel manometry studies motor patterns have been described on the basis

of recordings made from sensors spaced at > 7cm (295). Similarly *ex vivo* recordings of colonic motility utilized force transducers spaced at 10cm intervals. (218). By utilising a fibre-optic catheter, Dinning *et al* were able to study variable intervals in resolution width, and concluded that lower resolution recordings poorly reflect underlying contractile activity. It was found that doubling cm wide resolutions resulted in loss of half of all propagating sequences (seen in 1cm wide resolution), with tripling of the resolution resulting in a 30% chance of incorrectly labelling the directionality of propagation (235). From the data it appears that the force transducers may record similar motility patterns seen on the fibre-optic catheter, when placed at close intervals (Fig. 5, 8, 10). However, this set-up of clips on the serosa of the bowel, coupled to individual force transducers, proved to be cumbersome, especially when clips were placed in close proximity, even at resolution intervals of 2-3cm, in contrast to the luminal fibre-optic catheter with consistent 1 cm-wide resolution intervals.

The force transducers and its clip placements, resulted in the bowel specimen being 'lifted up' and approximating the inferior edge of the colon onto the luminal fibre-optic catheter, placed on the underside of the pull through securing rod (see Fig.1, 2, 3).

From the small number of specimens available, propagating activity was seen predominantly in the longer specimens (>13 cm, n=4), with no specimen less than 13 cm showing propagating activity. This may suggest that a preservation of length of interneurons is required in the maintenance of propagating activity. The 'Laws of the intestines', discussed in earlier parts of this theses stipulate that a proximal

contraction occurs as a response to a stimulus- this in essence required the presence of interneuronal connections to the oral end from the actual stimulus. It was previously shown that sectioning of the cat colon into shorter specimens (2cm) abolished the migrating spike bursts, leading Christensen and colleagues to postulate that the genesis of this migrating motor activity requires a significant length of intact enteric nervous system (217). In humans, retrograde labelling showed that interneurons with Dogiel type 1 morphology projected up to 68mm aborally (34); one could thus suggest that in the shorter preparations (i.e. 7cm and under) many of the pathways underlying peristaltic activity would have been severed. Further studies will be required to investigate the optimal length required for the preservation of this motor activity.

5.5.3. Increased frequency of phasic contractile activity *ex vivo* compared to *in vivo*

Frequencies generated in an *ex vivo* environment were significantly slower than that seen *in vivo*. Multiple postulates are possible in this setting, including the lack of extrinsic innervation, a weaker neural presence *ex vivo* modulating underlying myogenic activity as well as differing characteristics of ICCs rhythmicity in an *ex vivo* environment. Published historical data of *in vivo* frequencies using intraluminal pressure sensors in the human terminal ileum were recorded at 8.5 +/- 0.2 cpm 10cm proximal to the ileo-caecal junction (296). Our results showing frequencies *in vivo* of 10 +/- 0.3 cpm *in vivo* were consistent with this.

The differences in frequency of phasic contractions encountered *in vivo* and *ex vivo* may be related to the possibilities of denervation of the extrinsic nervous system

and/or transient potential loss of vascular blood supply and physiological conditions while in transit. While valid concerns, we know from animal studies that the intrinsic pacemaker generation in the mouse small bowel is robust and shows remarkable recovery in response to insults e.g. obstruction (168) and colonic motility is preserved in resected specimens in animals, and that the intestinal tract is viable still up to 12 hours from onset of ischemia (297).

5.5.4. Are *ex vivo* experiments representative of *in vivo* physiological conditions?

In our experiments, cyclical phasic contractile activity is likely to represent the mechanical recordings of electrical slow waves generated by these pacemaker cells. The fact that these events seen in both *ex vivo* and *in vivo* recordings are morphologically similar reflects the intrinsic myogenic nature of these pacemaker systems.

In addition, when the contractile activity was seen *ex vivo*, it was likened by the operating surgeons involved to the segmentation behaviour seen *in vivo* on anaesthetised patients, observed during laparotomy (Fig. 12). As luminal distension provides good stimulus for the generation of the propagating contractions (8, 109, 298), in this case we postulated the augmentation into an anterograde direction of propagation as an effect likely to occur when a fluid bolus is introduced from an oral direction in the *in vivo* small bowel. It appears likely, that in the gradual infusion of fluid from the oral end, a clear anterograde propagating contraction can be evoked that traverses the length of the small bowel specimen. This one observation warrants

further exploration with a defined protocol of fluid infusion into an intact specimen, and utilizing the methods of recording as developed for this thesis.

5.6. CONCLUSION

These are the first *ex vivo* studies of extended intact segments of the human terminal ileum. We present evidence that blocking enteric neural activity can modulate the underlying frequency of myogenically driven phasic contractile elements. This series of experiments shows the ability to record propagating contractile sequences *ex vivo* and allows for further interventions in the future to be performed in human terminal ileal tissue.

Figures: Chapter 5

FIGURE 1. CROSS SECTIONAL CARTOON OF EXPERIMENTAL SET UP

Cross sectional view of the experimental set up. The bowel (orange ring) is seen placed within the water jacket, with constant oxygenation. A. The force transducer arms are attached to clips that clip onto the bowel walls. Each circular contraction is recorded onto the LabChart software. B. The catheter is placed on the underside of the bowel, with its fibre-optic sensors abutting the bowel wall. This also is the mesenteric edge. C. A high-resolution video camera is placed externally, oriented to record the lateral aspect of the bowel wall and focused on the upper edge/ anti-mesenteric border of the terminal ileum for generation of spatio-temporal maps.

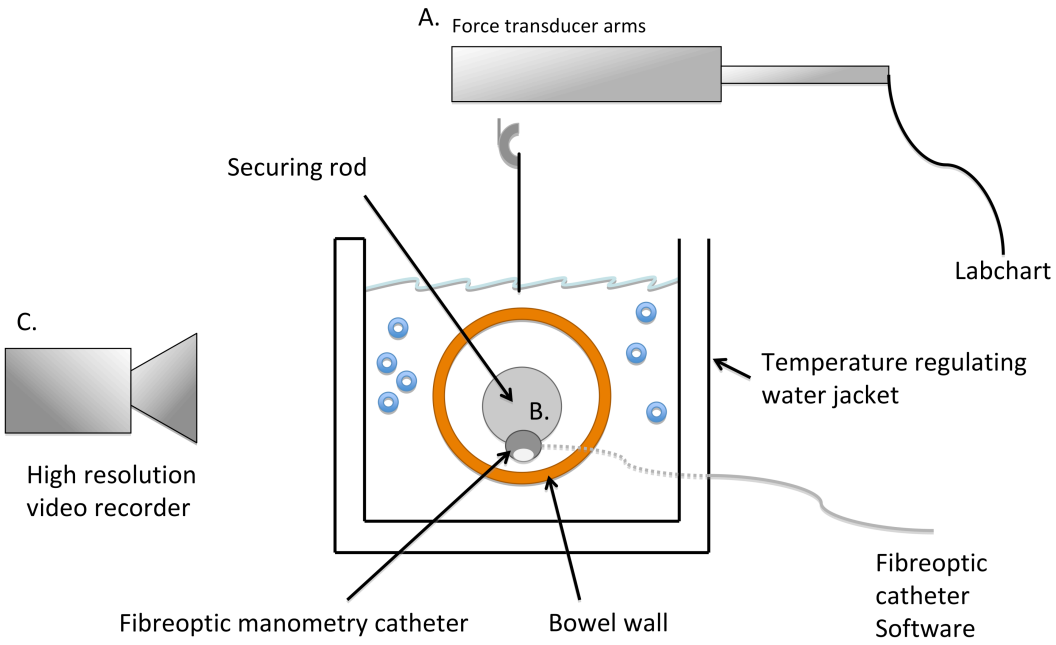

A.
Force transducer arms
Securing rod
Labchart
C.
B.
Temperature regulating
water jacket
High resolution
video recorder
Fibreoptic
catheter
Software
Fibreoptic manometry catheter
Bowel wall

FIGURE 2. PHOTOGRAPH OF EXPERIMENTAL TECHNIQUE

In this image the clips, seen in the prior image are connected to suture threads and onto force transducers. The number of force transducers utilised in each set up are dependent on the length of the tissue (5 used here, from channels 2-6).

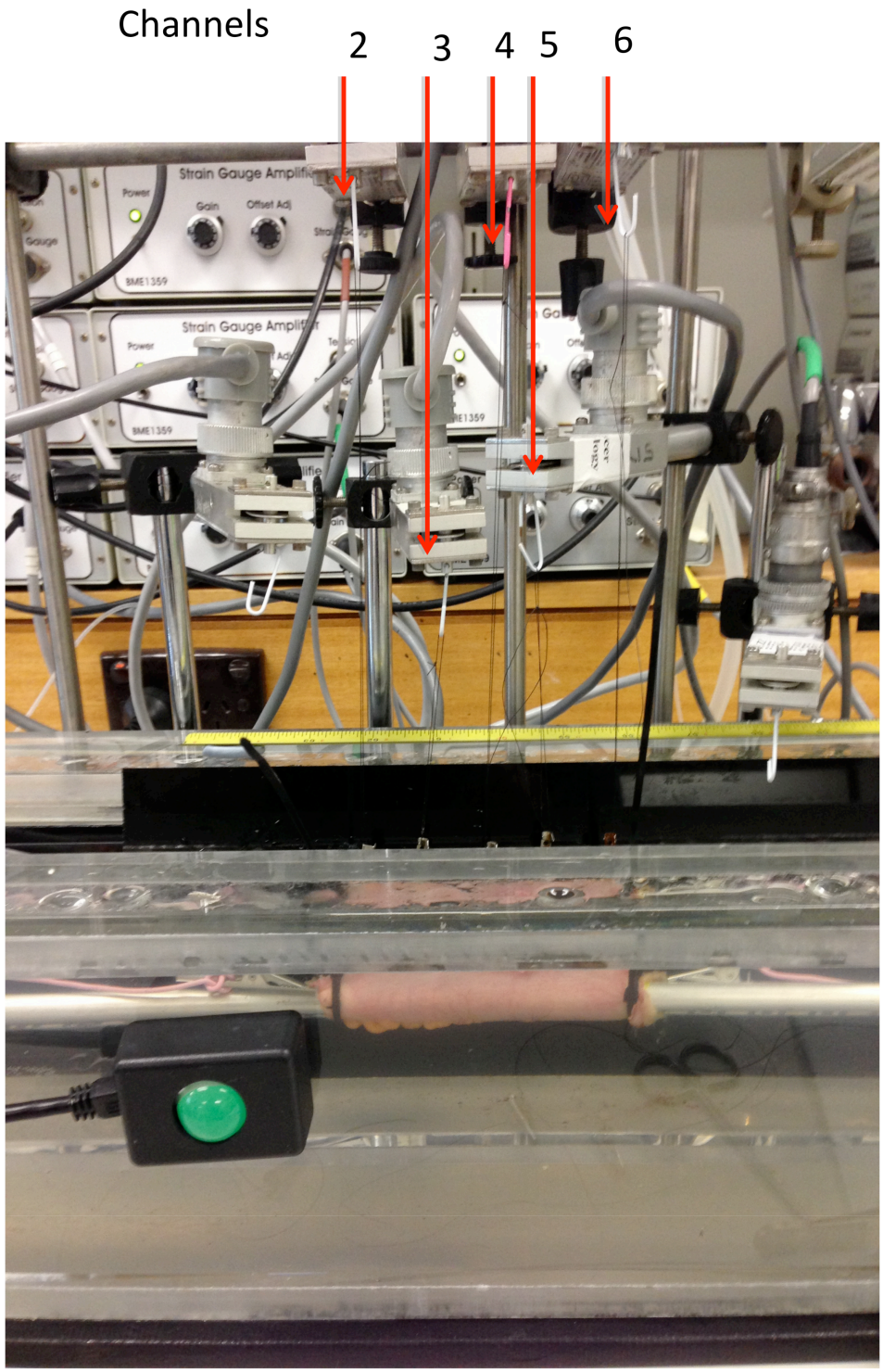

Channels
2
3
4
5
6
Strain Gauge Amplifier
Power
Gain
Offset Adj
9ME1359

FIGURE 3. VIEW FROM HIGH-RESOLUTION VIDEO IMAGING FOR GENERATION OF SPATIO-TEMPORAL MAPPING

This is an image taken from the screen of high-resolution video camera showing a specimen of human terminal ileum placed within the organ bath, filled with Krebs and oxygenated. This 9cm segment is anchored on both ends by cable ties, oriented with the oral end to the left, and anal end to the right, with the fibre-optic catheter placed on its mesenteric edge, inferiorly in the picture. Clips on both ends secure the placement of the tissue. 5 clips are used in this recording configuration; each spaced about 1-2cm apart and connected to force transducers (please refer to following image). A green signalling light is used for temporal alignment of the recording.

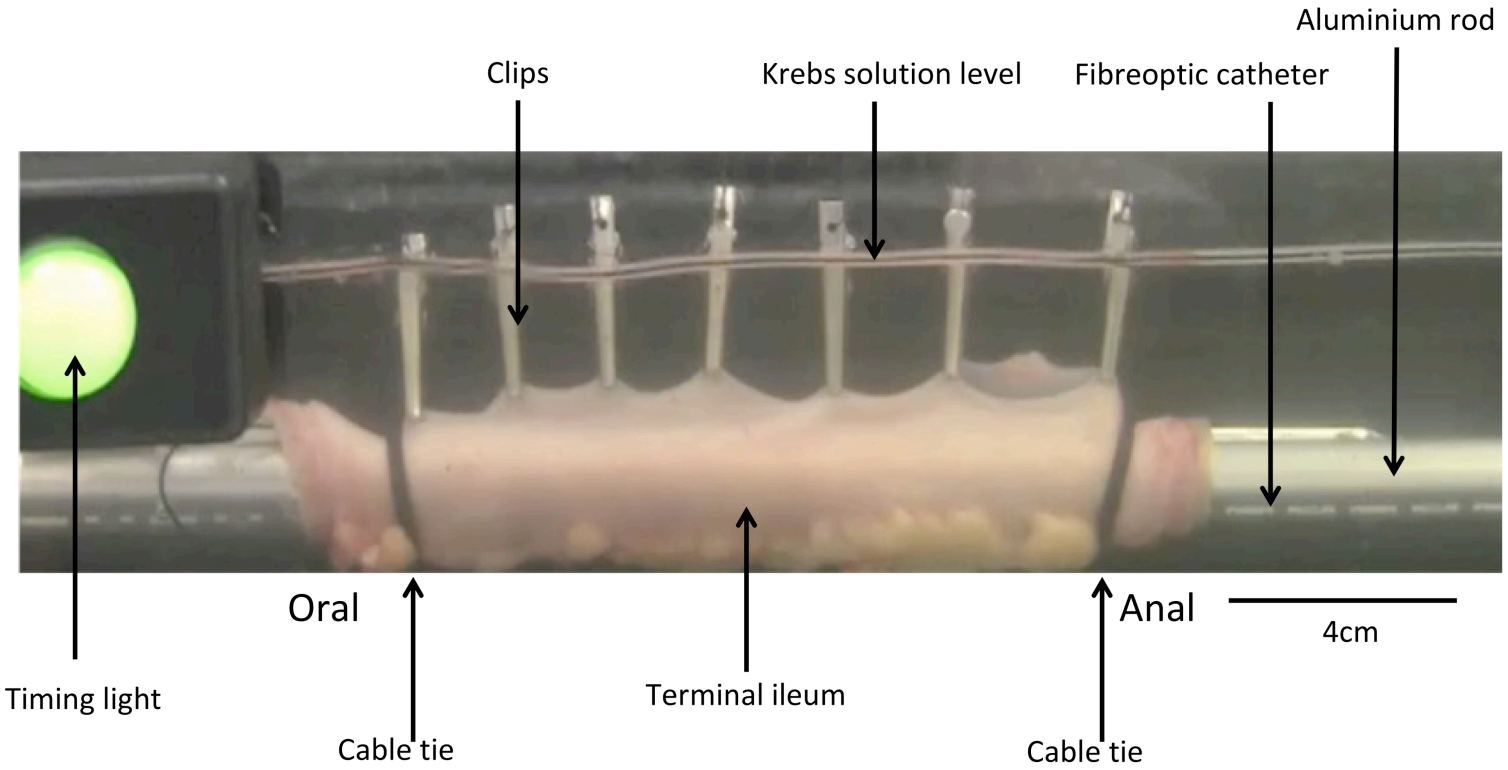

Aluminium rod
Clips
Krebs solution level
Fibreoptic catheter
Oral
Anal
4cm
Timing light
Cable tie
Terminal ileum
Cable tie

FIGURE 4. CHARACTERISTICS OF CONTRACTIONS GENERATED BY BOTH FORCE TRANSDUCER AND FIBRE-OPTIC CATHETER RECORDINGS

This figure shows the tracings obtained from both force transducer recordings (A), and luminal fibre-optic manometer recordings (B). Both these traces A.i and B.1. show morphologically similar and regular phasic contractile activity. When compared over 3 distinct 10 minute segments A.ii and B.ii, at 5-15mins, 25-35mins and 45-55mins, no significant difference is seen in both experimental recording techniques C. Also, no significant difference is seen in both force transducer and luminal fibre-optic recordings of frequencies as well.

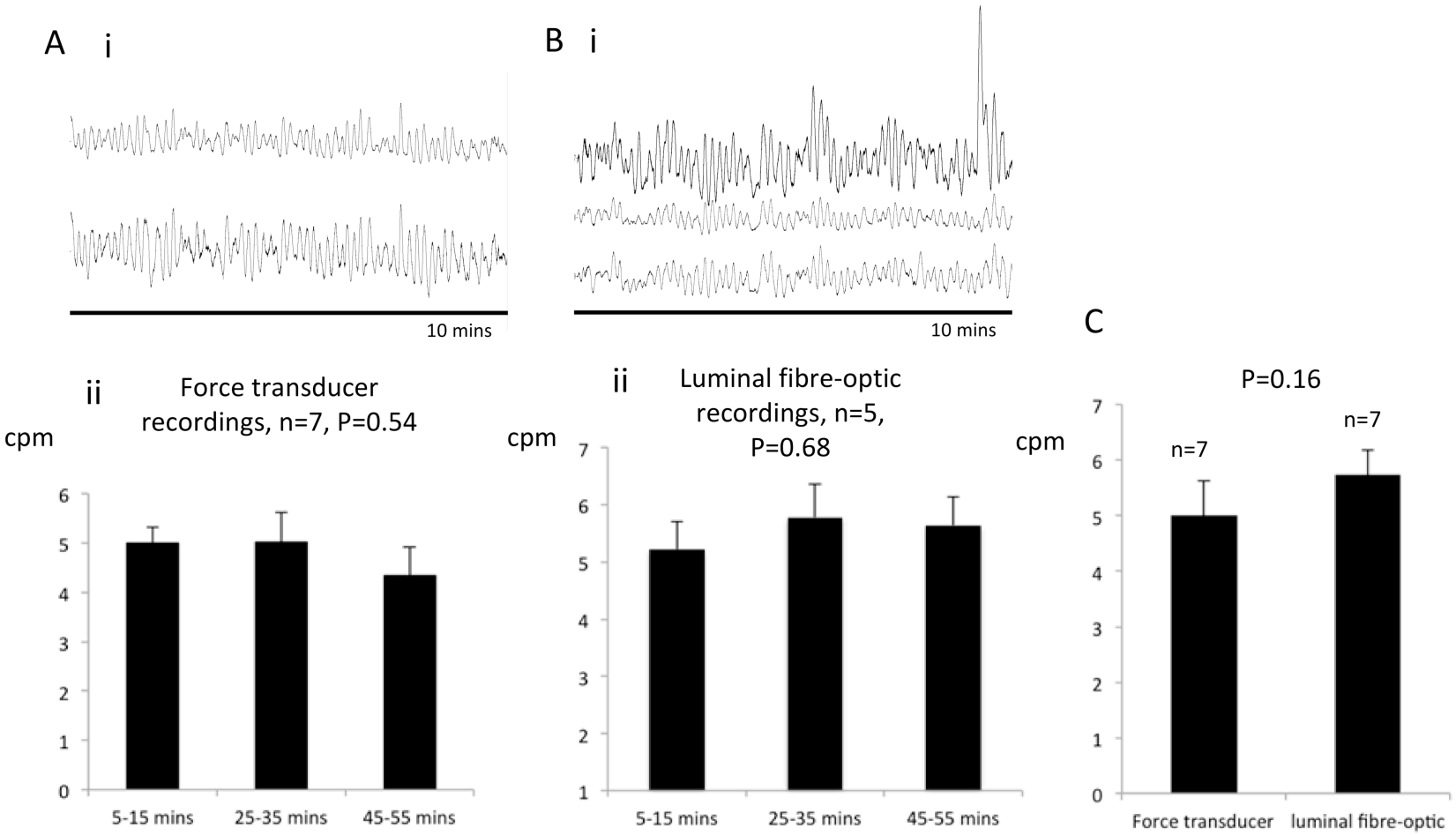

A i
10 mins
B i
10 mins
ii
Force transducer recordings, n=7, P=0.54
cpm
5-15 mins
25-35 mins
45-55 mins
ii
Luminal fibre-optic recordings, n=5, P=0.68
cpm
5-15 mins
25-35 mins
45-55 mins
C
P=0.16
cpm
n=7
n=7
Force transducer
luminal fibre-optic

FIGURE 5. PRESENCE OF PROPAGATING ACTIVITY IN THE *EX VIVO* HUMAN TERMINAL ILEUM RECORDED USING 3 SEPARATE RECORDING TECHNIQUES

Presence of propagating activity in a 26cm long segment of human terminal ileum recorded over 30 minutes. A. 2 hour-long recording generated from a fibre-optic manometer catheter. B, C and D are 20-minute traces seen early in the experiment (B. Force transducer recording, C. Fibre-optic catheter recording, and D, spatio-temporal maps generated from changes in diameter noted from high-resolution video recordings). All 3 recordings show similar activity, but this was clearly more pronounced in the spatio-temporal maps. Also note the bi-directional propagating activity present in the control period, before any intervention was instituted.

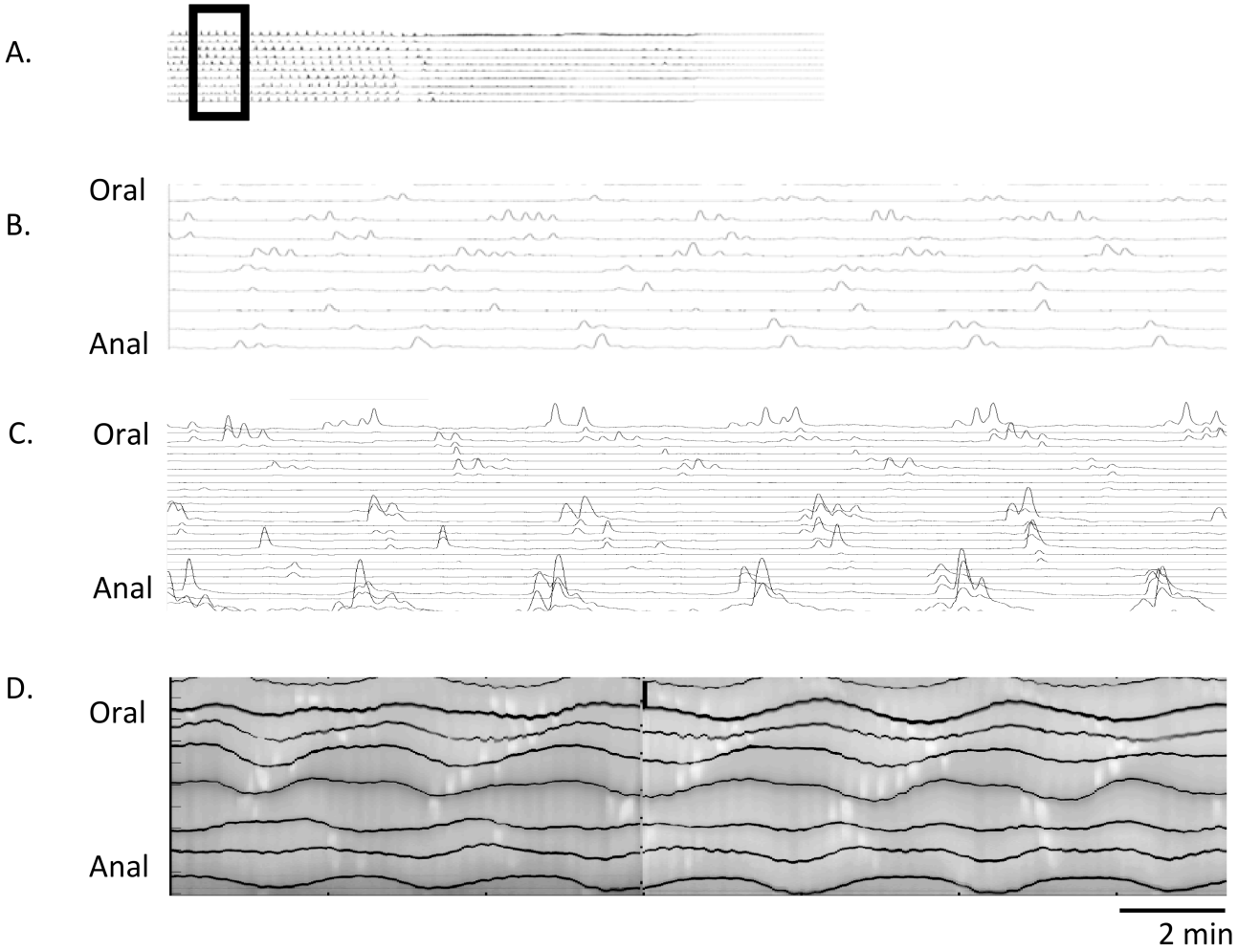

A.
B.
Oral
Anal
C.
Oral
Anal
D.
Oral
Anal
2 min

FIGURE 6. CHARACTERISTICS OF PROPAGATING CONTRACTIONS RECORDED FROM ISOLATED HUMAN TERMINAL ILEUM

In longer specimens propagating contractions was seen *ex vivo*. A. This shows a segment of bowel measuring 20cm exhibiting propagating activity. B. Close inspection of the contractions revealed its frequencies at 0.45+/-0.03 events per minute, and at velocities of 1.72+/-0.79 mm/sec. C. Lidocaine blocks this activity.

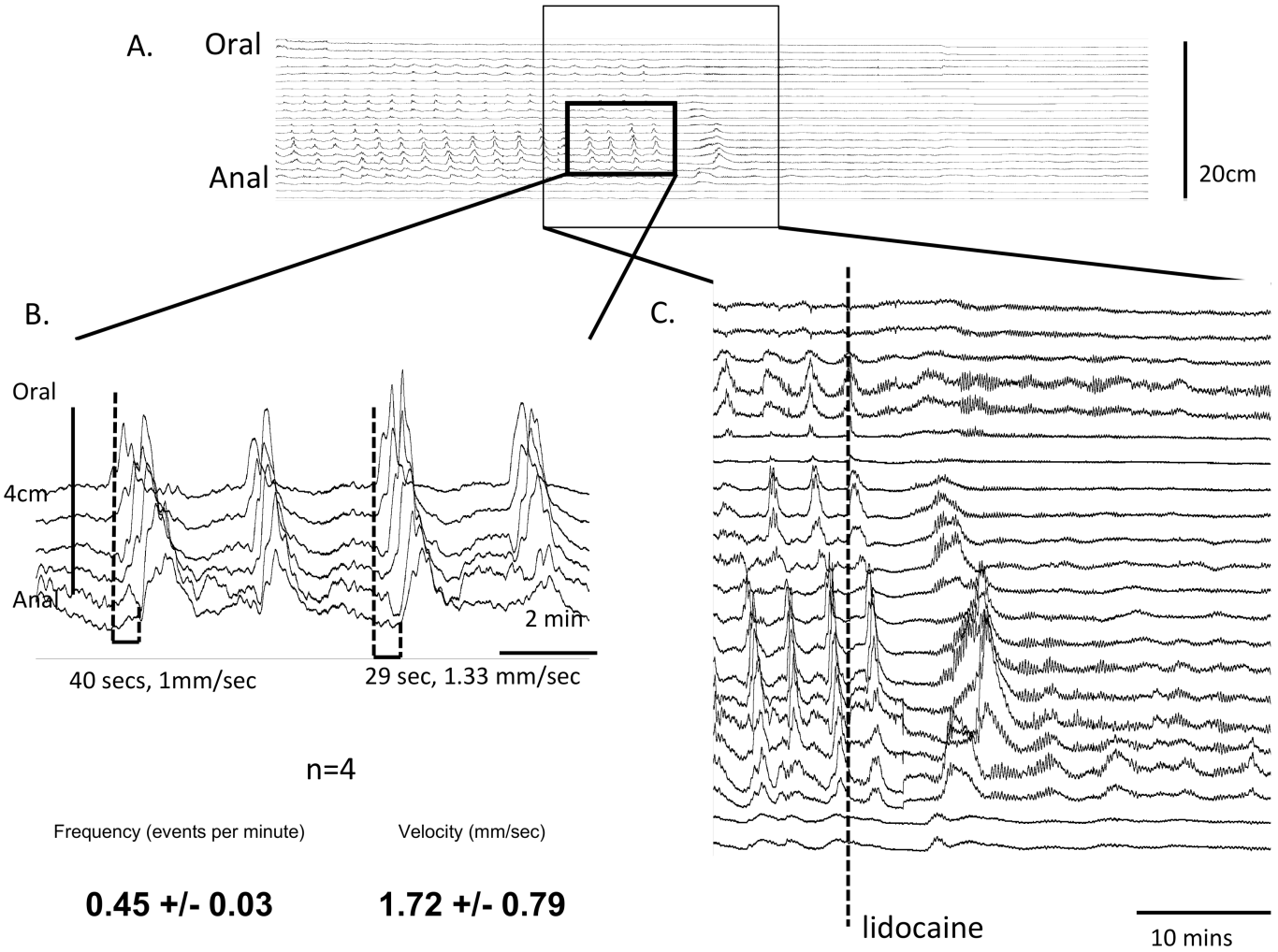

A.
Oral
Anal
20cm
B.
C.
Oral
4cm
Anal
2 min
40 secs, 1mm/sec
29 sec, 1.33 mm/sec
n=4
Frequency (events per minute)
Velocity (mm/sec)
0.45 +/- 0.03
1.72 +/- 0.79
lidocaine
10 mins

FIGURE 7. EFFECT OF LIDOCAINE ON PROPAGATING ACTIVITY

A. 2 hour-long recording of the human terminal ileum. Simultaneous recordings made using: B, mechanical force transducers, C, fibre-optic manometry catheter, D, video imaging for spatio-temporal mapping. After Lidocaine, a marked abolition of propagating sequences was seen.

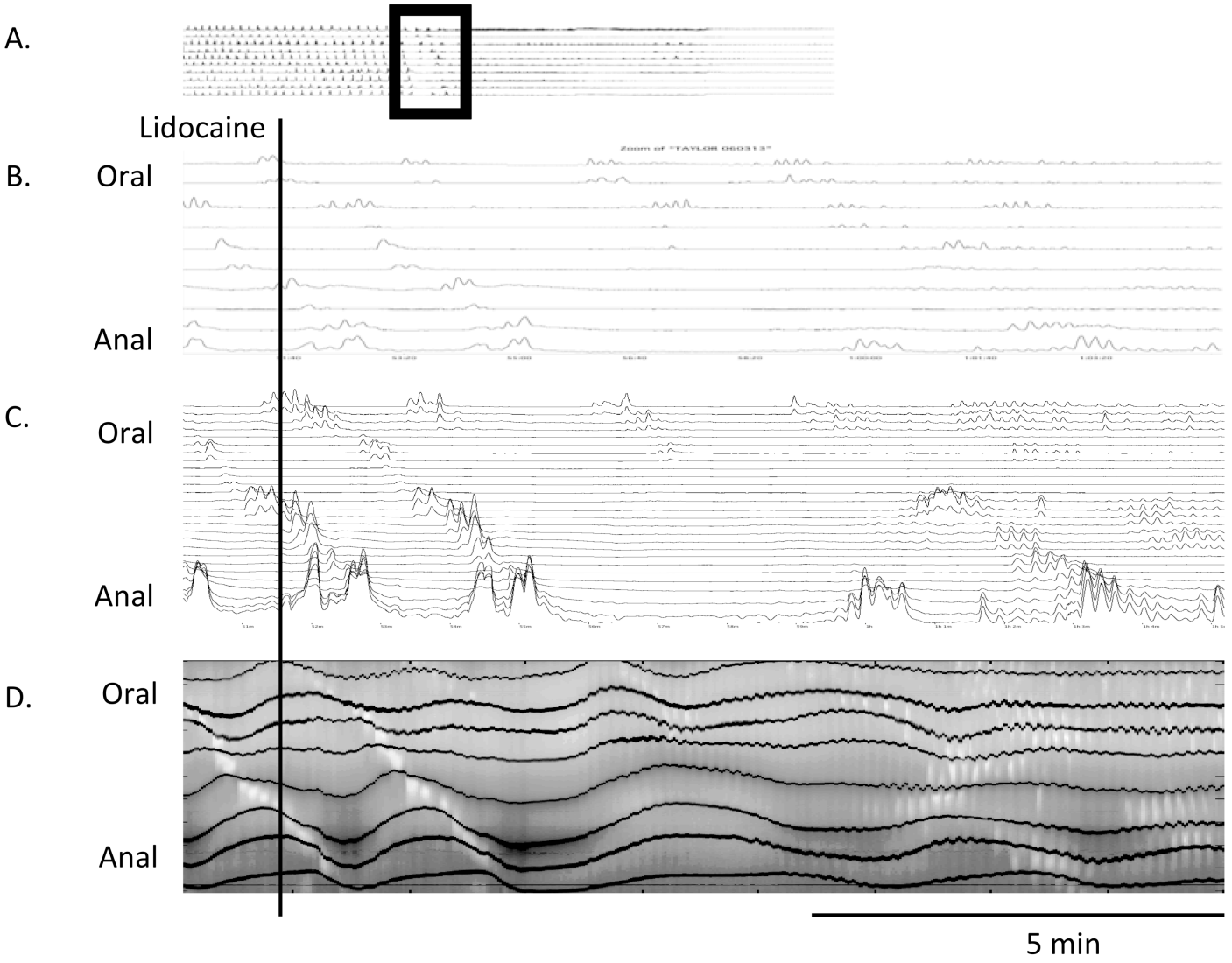

A.
Lidocaine
B.
Oral
Anal
C.
Oral
Anal
D.
Oral
Anal
5 min

FIGURE 8. PRESENCE OF AN UNDERLYING PHASIC CONTRACTILE ACTIVITY UNMASKED IN THE PRESENCE OF LIDOCAINE

In the presence of lidocaine, propagating sequences were abolished. The presence of an underlying re-occurring phasic activity was clearly seen in all modalities. A. Complete 2 hour recording recorded on the fibre-optic catheter, B, C and D are zoomed in 15-minute views obtained in the presence of Lidocaine. (B. Force transducer recording, C. Fibre-optic recording, D. Spatio-temporal map generated from high-resolution video recording). In this example, frequencies of these activities averaged 6.3 +/- 0.5 cpm.

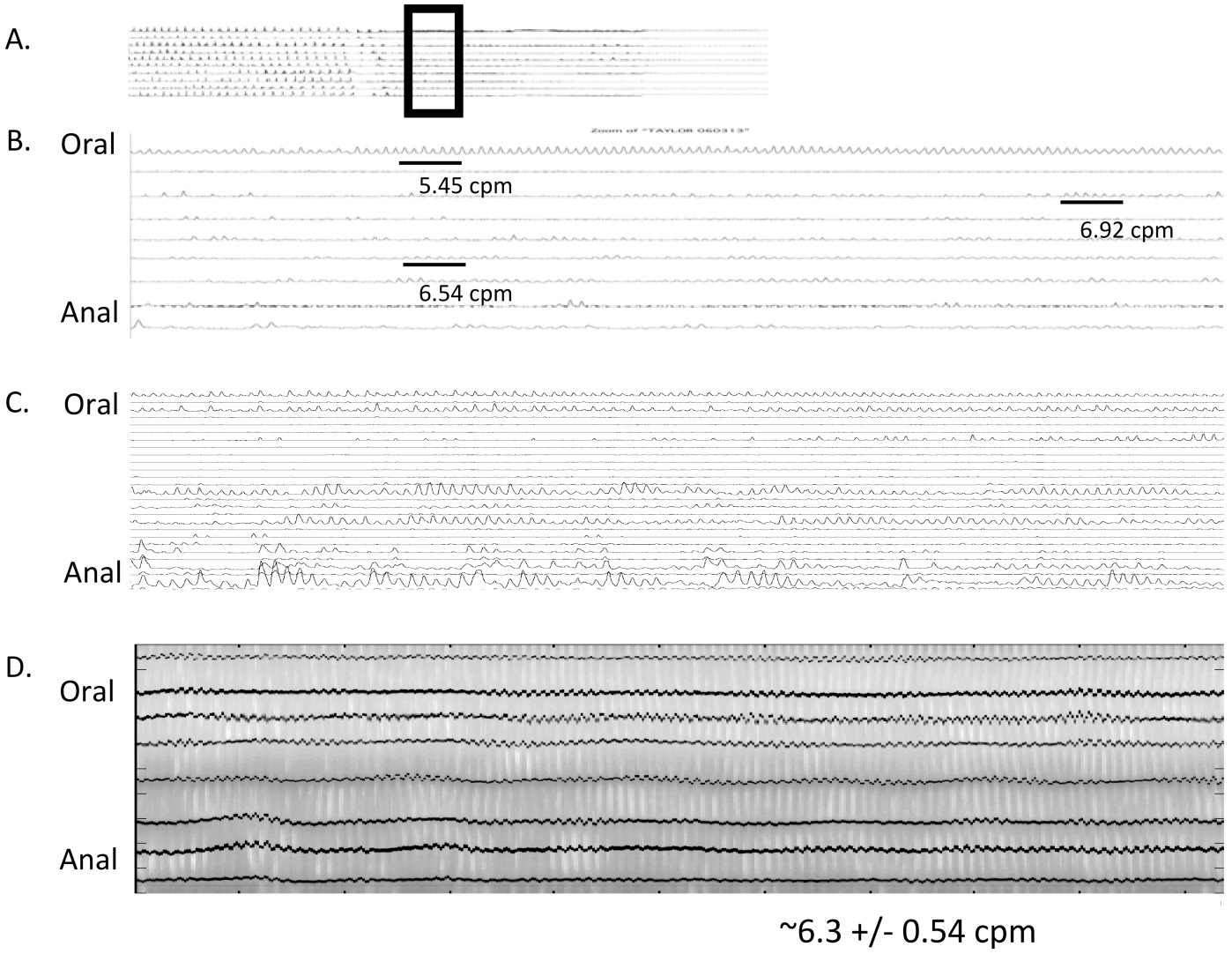

A.
B.
Oral
5.45 cpm
6.92 cpm
6.54 cpm
Anal
C.
Oral
Anal
D.
Oral
Anal
~6.3 +/- 0.54 cpm

FIGURE 9. PRESENCE OF A DOMINANT UNDERLYING PHASIC CONTRACTILE FREQUENCY AS SEEN USING FAST FOURIER TRANSFORMATION

A. A single trace from a recording is exemplified. Lidocaine was added at the arrow point to block neural activity. B. Frequencies measured before, and after lidocaine showed no significant difference. C. Fast Fourier Transformation analysis showed a dominant band appeared in lidocaine at about 6 cpm (not seen before addition of lidocaine when a broad spectrum of frequencies were seen).

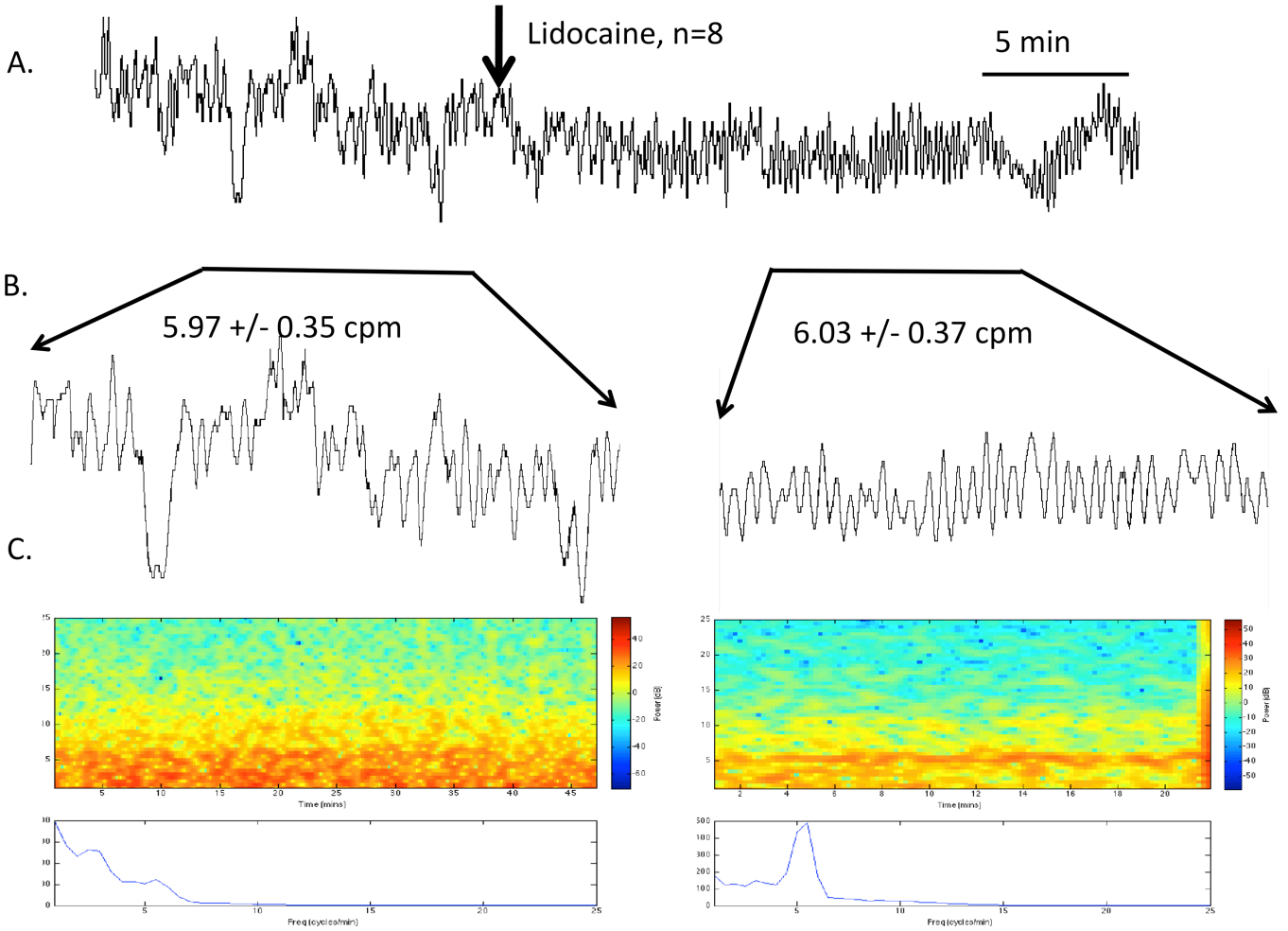

A.
Lidocaine, n=8
5 min
B.
5.97 +/- 0.35 cpm
6.03 +/- 0.37 cpm
C.
Time (mins)
Power (dB)
Freq (cycles/min)

FIGURE 10. EFFECT OF LUMINAL DISTENSION ON HUMAN TERMINAL ILEUM *EX VIVO*

A. 2 hour recording in a segment of human terminal ileum. B. Force transducer recording of the highlighted time period. Note the vertical lines superimposed onto this trace, indicating the timepoints of which 1cm boluses of Krebs solution were introduced into the lumen. Prior to infusion contractile events were seen to propagate in both anterograde and retrograde directions. The 1ml boluses created a gradual distension that led to the observation of a clear anterograde propagating event. This same timeframe was represented by recordings made with the fibre-optic manometry catheter (C) and spatio-temporal map (D).

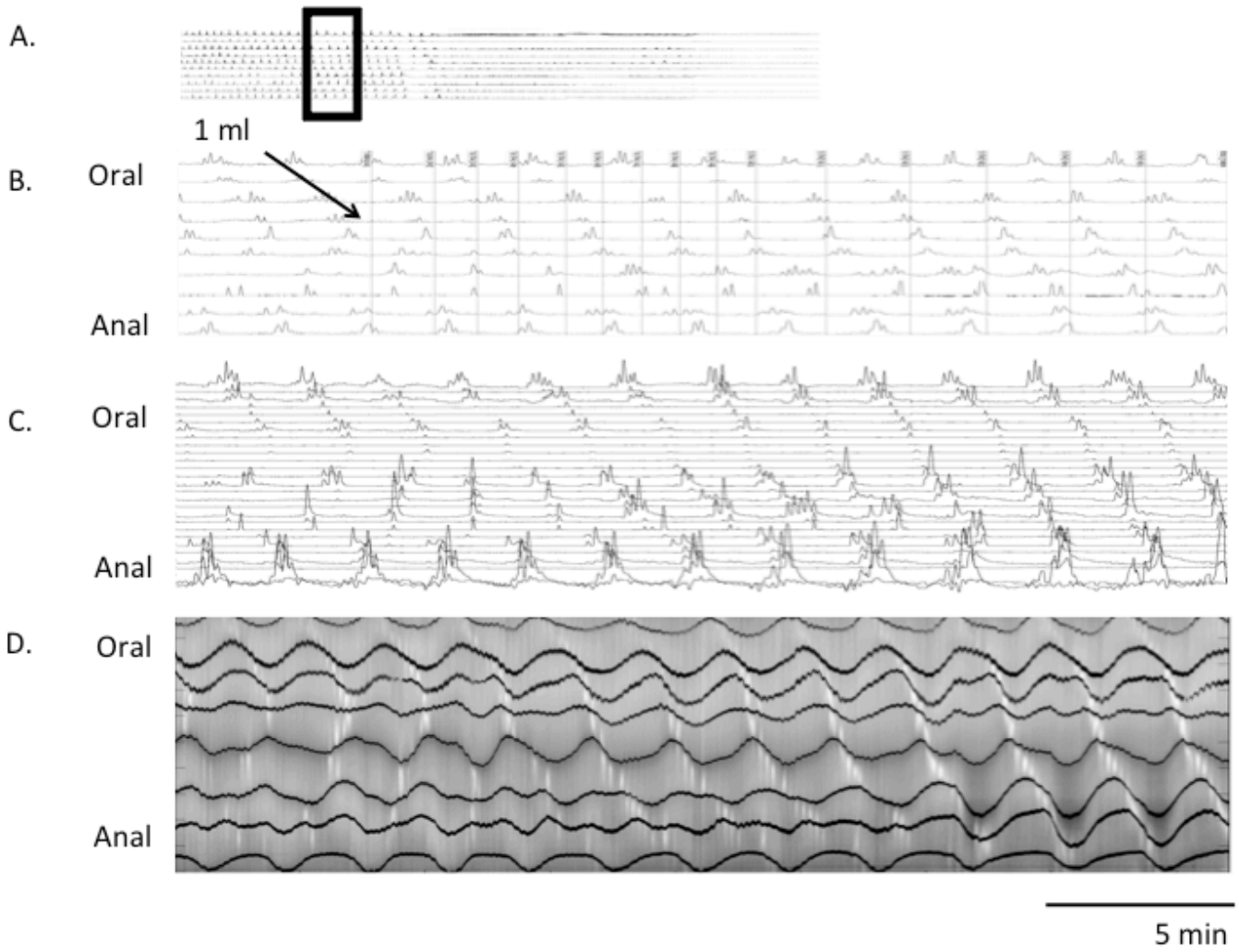

A.
1 ml
B.
Oral
Anal
C.
Oral
Anal
D.
Oral
Anal
5 min

FIGURE 11. *IN VIVO* RECORDING ALLOWS FOR COMPARISON OF PARAMETERS SEEN *EX VIVO*

This group of diagrams shows results obtained from *in vivo* experimentation. A. Presence of the catheter *in vivo* (presence of the catheter within the lumen of the colon was highlighted by a thickened line). We examined the segment of terminal ileum just proximal to the ileocaecal junction. B. Example of an hour-long trace from the *in vivo* recordings, showing periods of high amplitude activity, mixed with periods of relative quiescence. C. Frequency of the high amplitude activity was measured at 9.98+/-0.25 cpm. D. This is higher than that seen *ex vivo*.

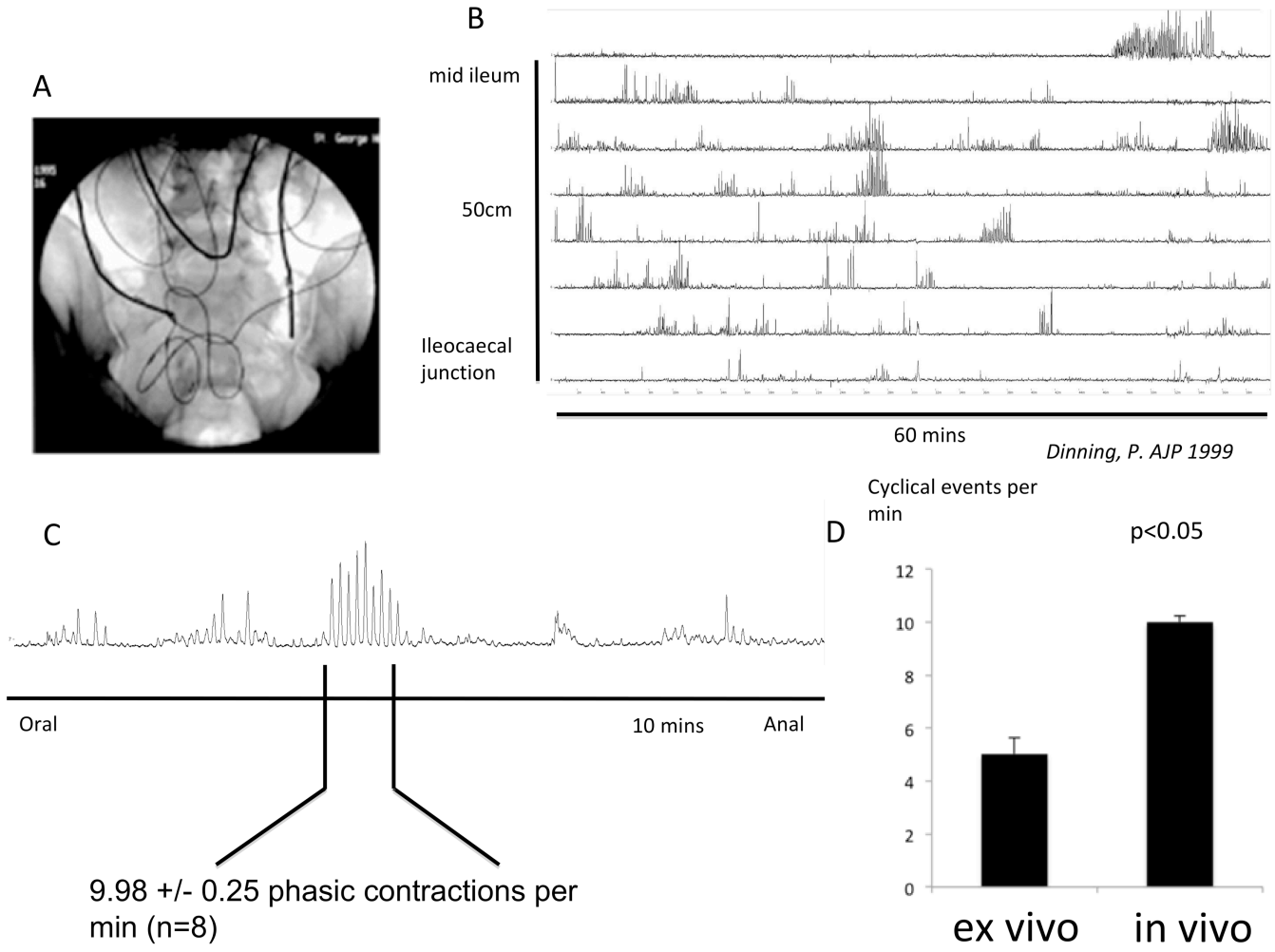
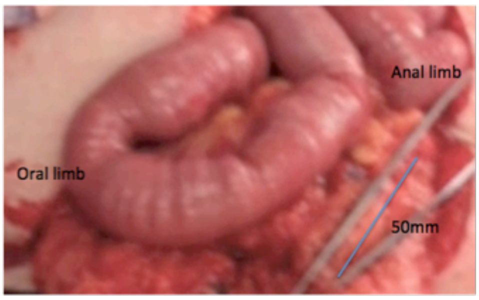
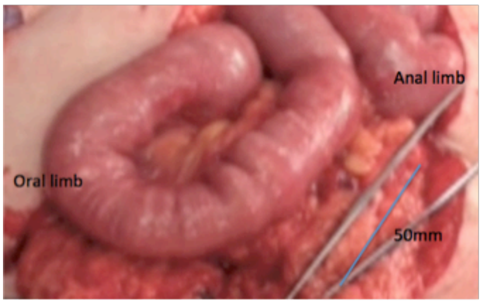
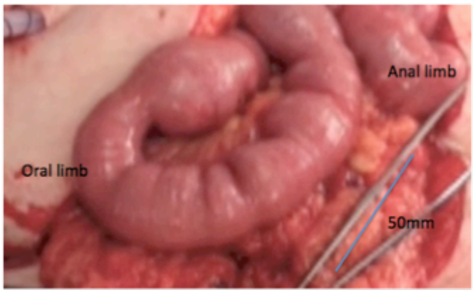

A
B
mid ileum
50cm
Ileocaecal junction
60 mins
Dinning, P. AJP 1999
C
Oral
10 mins
Anal
9.98 +/- 0.25 phasic contractions per min (n=8)
D
Cyclical events per min
p<0.05
12
10
8
6
4
2
0
ex vivo
in vivo

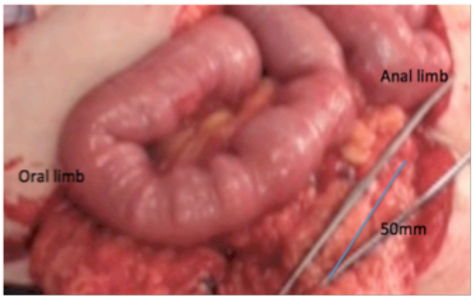
FIGURE 12. PRESENCE OF SEGMENTAL CONTRACTILE ACTIVITY SEEN *IN VIVO* IN THE SMALL BOWEL OF ANAESTHETISED HUMAN PATIENTS

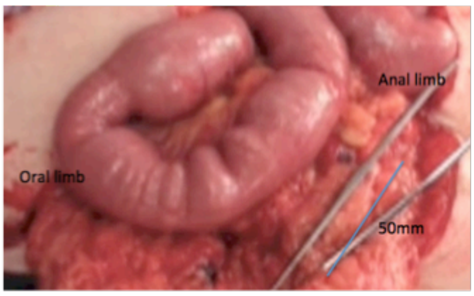
Six images taken sequentially, 3 seconds apart during a laparotomy for colonic resection. From top down, the images depict chronologically, the emergence of circumferential contractions that move in both directions of the human small bowel. The oral and anal ends of the small bowel are marked, as well as a marker of distance.

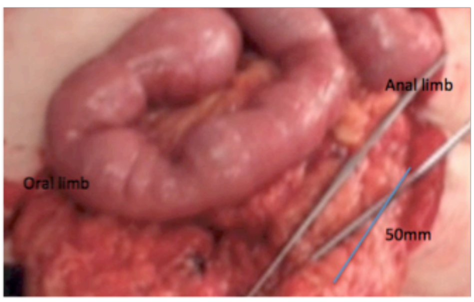

Anal limb
Oral limb
50mm


Anal limb
Oral limb
50mm


Anal limb
Oral limb
50mm


Anal limb
Oral limb
50mm


Anal limb
Oral limb
50mm


Anal limb
Oral limb
50mm

Chapter 6: Comparison of *in vivo* with *ex vivo* recordings of the human colon in slow transit states reveals insight into mechanisms of peristalsis and disease pathophysiology

6.1. ABSTRACT

Background: The pathophysiology underlying slow transit constipation (STC) is still unclear. Intractable constipation, refractory to medical and biophysical interventions may lead to colectomy. We present the findings from a unique opportunity to study both the *in vivo* and *ex vivo* motor patterns of two patients presenting with STC for colectomy. **Methods**: Both patients with scintigraphically confirmed STC, and no evidence of biochemical aberrations or rectal obstructive disorders underwent fibre-optic colonic manometry recordings two weeks prior to colectomy. Following resection the entire colonic specimen was transferred to an organ bath and the motility from the descending and sigmoid colon was recorded with the fibre-optic manometry catheter. These *ex vivo* recordings were compared to the respective *in vivo* recordings and to two *ex vivo* control specimens obtained from anterior resections from cancer operations. Propagating sequences (anterograde or retrograde) were identified in all preparations, and measurements of total number, velocities, amplitudes and distances propagated by each propagating contractile event recorded. **Results**: Propagating sequences were observed in all preparations. The characteristics between groups, including number of anterograde and retrograde propagating events, extent/ distance travelled, amplitudes and velocities of each propagating event were similar. An underlying contractile activity occurring at 2-4 cycles per minute was also observed across all three groups. **Discussion**: Similar propagating contractile activity can be recorded from the *ex vivo* and *in vivo* colon from patients with slow transit constipation. The prevalence of propagating activity in the *ex vivo* setting suggests that these motor patterns are generated from the enteric nervous system. The similarity in frequency and extent of propagation between the

motor patterns in the *ex vivo* control and constipated patients, suggests that the colonic contractile abnormalities described from *in vivo* slow transit recordings may be due to aberrant input from extrinsic nerves.

6.2. INTRODUCTION

Slow transit constipation (STC) is common, and carries significant societal and financial burden (299). For some patients quality of life measures are comparable to that of serious chronic medical conditions such as diabetes and osteoarthritis (300). Up to a third of patients do not gain adequate relief from standard pharmacological therapy or biofeedback (301, 302). While surgery, in the form of subtotal colectomy or segmental colectomy provides symptom relief in some patients, it is associated with various morbidities, as well as the real risk of recurring symptoms post operatively (303). The known risk of incomplete symptom resolution after surgical resection highlights the complex nature underlying the pathogenesis of slow transit constipation.

In patients with STC, abnormal colonic contractility is clearly implicated. In comparison to healthy controls, colonic manometric recordings have shown an absent or diminished frequency of the propulsive high amplitude propagating sequences, compared to healthy controls (234, 304, 305), and a diminished colonic response to a high calorie meal (306, 307) and/ or morning waking (307, 308). As the generation of high amplitude propagating sequences and the colonic responses to physiological stimuli are both thought to be neurally mediated (309, 310), one possibility is that the underlying cause of STC may involve attenuated extrinsic neural activity.

There are a number of studies that have shown various alterations within the colonic enteric nervous system in patients with STC including redistribution of cholinergic and nitrenergic neurons, (15, 172, 311, 312), a reduction in total number of neurons within the myenteric plexus in general (313), and changes in populations of the pacemaker cells within the bowel wall, the interstitial cells of Cajal (ICC) (172, 314). In addition introduction of recombinant human brain derived neurotrophic factors into the gut reduced transit times and improve symptoms in both the healthy and constipated patient (315), further suggesting that the deterioration in populations of enteric neurons play a reversible role in the manifestation of slow transit symptoms. Collectively these observations have led to a number of investigations that suggest the aetiology of slow transit constipation may result from a reduction in the enteric neurons and pacemaker systems essential for the initiation of colonic propagating activity in the colon. However, in contrast the only known experiment on extended lengths of human colon *ex vivo* demonstrated a presence of robust contractile activity in patients with slow transit constipation (STC) (218), thus suggesting intrinsic neural pathways are at least partially preserved.

More recently our group has begun to conduct colonic manometry studies utilising high-resolution fibre-optic manometry. In healthy controls these studies have shown that a high-calorie meal induces an increase in distal colonic cyclic (2-6 per minute) propagating motor patterns (316). In patients with STC, while pressure waves with a frequency of 2-6 per minute are present throughout the colon, the increase in postprandial cyclic propagating activity is absent (Dinning, *et al.* 2014 unpublished). The slow wave frequency of these motor patterns in health suggests involvement of the intrinsic pacemaker cells of the Interstitial Cells of Cajal (ICC), while the rapid increase after a meal suggests increased activity of extrinsic neural pathways (316).

Thus while the fibre-optic recordings provide a greater insight into motor patterns that exist in both health and disease, we remain unsure of the cause of STC.

In 2013, two patients with severe STC that had undergone the fibre-optic colonic manometry recordings mentioned above, went on to have a total colectomy and ileo-rectal anastomoses. Utilising our *ex vivo* technique for recording motility patterns (see chapter 5) we were in a unique position to record and compare the motility of these patients both *in vivo* and *ex vivo*. We postulated that should the etiology of STC be found within the bowel walls itself, high-resolution recordings obtained from *ex vivo* colonic specimens of patients with STC would yield lower frequencies, slower speeds of propagation and weaker contractile amplitudes compared to controls.

6.3. METHODS

6.3.1. Overview

The data presented here was obtained from 2 STC patients that underwent both *in vivo* and *ex vivo* colonic manometry. The overall comparison of *in vivo* manometry findings between healthy controls and STC forms part of an ongoing study. Here the motor patterns between the *in vivo* and *ex vivo* recordings of these 2 patients have been compared. In addition *ex vivo* colonic motor patterns from two other patients, with normal bowel function, that underwent colorectal resections for rectal malignancy were recorded. These recordings provide "control" *ex vivo* colonic motility and the motor patterns from these specimens have been compared to the *ex vivo* STC colons (Fig. 1).

6.3.2. Patient selection

Patients studied for control parameters of colonic motility had underlying rectal malignancy with non-obstructing symptoms leading to time of resection (n=2). Rectal malignancy resulting in anterior resections provided useful specimens as our experimental "controls". This is because the resection specimen itself includes a segment of healthy colon proximal to the malignancy. This is performed as the distal descending and sigmoid colons drain into the same lymphatic basin as the upper rectum. Lymph node assessment of the specimens aid in pathological assessment of the malignancy. In addition, the inferior mesenteric artery for which the lymphatic drainage followed was sacrificed with intention to achieve a tension free anastomosis. Following retrieval of the specimen from the operating theatres, the descending and sigmoid colons were excised away from the cancerous region with a minimal margin of 5cm. This again ensured adequate margins for pathological assessment. The excised healthy tissue was then useable for *ex vivo* recoding.

The two patients with STC underwent colonic placement of the fibre-optic catheter for *in vivo* recordings of motility performed at Flinders Medical Centre. These 2 patients exhibited the following characteristics of clinically diagnosed constipation: i) aged 18 – 75; ii) Rome III criteria for constipation; iii) slow transit constipation, confirmed by isotope colonic transit study; iv) failed symptomatic response to standard therapies including laxatives, dietary modification and exercise; v) failed colonic response to a high calorie meal (Dinning et al. unpublished); and vi) normal colonoscopy within 5 years of enrolment (with the exception of colonic melanosis coli and non-malignant colonic polyps).

These patients were also confirmed to be absent of the following: i) metabolic, neurogenic or endocrine disorder(s) known to cause constipation (e.g. hypercalcaemia, hypothyroidism, diabetes, multiple sclerosis, Parkinson's, scleroderma); ii) consumed drugs which list constipation as a potential side effect deemed to be clinically relevant by the referring physician (e.g. calcium channel blockers); iii) prior abdominal radiotherapy; iv) prior abdominal surgery (except cholecystectomy, appendectomy, inguinal hernia repair); v) current or planned pregnancy; vi) current or prior history of malignancy.

All 4 participants in the study had given written, informed consent and the studies were approved of by The Southern Adelaide Health Service / Flinders University Human Research Ethics Committee (*in vivo* recording: 419.10; *ex vivo* recording: 50.07).

6.3.3. Profiles for control patients

Control patient 1:

A 40-year old male underwent laparotomy for an ultra-low anterior resection for metastatic rectal adenocarcinoma to the liver. This was in the background of a diagnosis of hereditary non-polyposis colorectal cancer. He had presented with rectal bleeding necessitating colonoscopy and subsequent operative input. His operative procedure was without complication.

Control patient 2:

A 55-year old man underwent laparotomy for an abdomino-perineal resection (APR) for metastatic rectal adenocarcinoma to the liver. His initial presentation to medical attention was following painful defecation, with rectal cancer diagnosed on colonoscopy. His operative procedure was without complication.

6.3.4. Profiles for STC patients

STC Patient 1 (Table 1, Fig. 2)*:*

A 45-year-old female with a 25-year history of constipation, non-responsive to conservative and medical treatment measures and scintigraphically confirmed STC. At the time of the procedure she reported having up to 45mg of bisacodyl (9 x 5mg tablets) a day, with small loose bowel actions as a result. Her symptoms satisfied the list of criteria on the ROME III classification for constipation (Table 1). Biochemical abnormalities for slow transit were excluded, and she underwent further investigations including endo-anal ultrasound (EAUS) and colonoscopic evaluation for a non-functional cause for her constipatory state.

Patient 1 had a total colectomy and stapled ileo-rectal anastomosis. Postoperatively she had a protracted recovery period complicated by ileus with resultant ongoing abdominal pain, distension and vomiting. She was discharged on the 8th postoperative day after her symptoms improved and she was opening her bowels.

STC Patient 2 (Table 1; Fig. 3)*:*

A 25-year-old female presented with a 14 year history of constipation with intervals of bowel opening up to 14 days on average with the aid of laxatives (polyethylene glycol- Movicol). Again in this patient, ROME III criteria were met for a clinical diagnosis of functional constipation, and subsequent transit studies confirmed STC. She underwent total colectomy with ileo-rectal hand sewn end-to-end anastomosis. Her postoperative care was uneventful and only reported some nausea secondary to morphine administration. This was resolved with a change to fentanyl for analgesia. She was discharged on day 4 postoperatively.

6.3.5. Colonic manometry

Colonic manometry, both *in vivo* and *ex vivo*, was recorded with a high-resolution fiber optic manometry catheter (236, 317). The catheter contained 72 sensors spaced at one-centimeter intervals. The fiber-optic catheter was attached to a spectral interrogator unit (FBG-scan 804; FOS&S, Geel, Belgium) and pressures were recorded in real time on a custom-written LabVIEW© program (National Instruments, Austin, Texas, USA).

Analysis of the fiber-optic data was performed manually using software (PlotHRM) developed in house. PlotHRM was written in Matlab© (The MathWorks, Natick, Massachusetts, USA) and JavaTM (Sun Microsystems, Santa Clara, California, USA).

6.3.6. Colonoscopic placement of the fiber-optic catheter

The placement technique of the colonic catheters has been described in detail previously (318). On the day prior to the procedure the bowel was cleared using sodium picosulphate and polyethylene glycol (Pharmatel Fresenius Kabi Pty Ltd, Hornsby Australia). Patients drank clear fluids overnight. Lying in the left lateral position, with conscious sedation using midazolam and fentanyl, the manometry catheter was introduced with a colonoscope and clipped to the mucosa of the hepatic flexure in patients 1 and 2 using Endoclips (Resolution Clip® Boston Scientific, Massachusetts, USA.).

6.3.7. *Ex vivo* recording of colonic motility

Following resection, the colectomy specimen was collected in theatre and immediately placed in warm (35^0C) oxygenated Krebs solution en route to placement within the organ bath, located in the laboratories within the same operating theatre complex. Time from intracorporeal removal to placement within the organ bath were both under 15 minutes. The organ bath comprised of an external wall functioning as a water jacket, regulating temperature and heavy oxygenation. Upon arrival to the laboratories, and throughout the experiments the specimens were healthy in appearance with no macroscopic evidence of ischaemic change. The specimen was secured by luminal placement of an aluminium rod. This rod anchored the fibre-optic catheter on its inferior aspect. Both ends of the specimen were secured with cable ties. Force transducers are placed onto the superior aspect of the bowel wall.

Alligator clips attached to force transducers via 3-0 silk suture threads and were clipped onto the serosal aspect of the colon, spaced at 2-4cm intervals (Fig. 4). Tension generated by circular muscle contraction was relayed onto independent isometric recording transducers (Grass FT-03C; Grass, Quincy, MA) and connected to individual custom made preamplifiers (Biomedical Engineering, Flinders University) and onto a PowerLab (model 4/30; AD Instruments, Bella Vista, NSW, Australia) and a notebook Personal Computer (PC) running LabChart 7.0 (AD Instruments, Australia).

6.3.7.1. Ex vivo recordings of control specimens (Fig. 4)

Left sided colonic resection specimens, performed for cancer resections were used for controls (n=2). The mesentery was excised off the colon and kept adherent to the cancer segment of tissue. This was immediately delivered to the Department of Surgical Pathology, for assessment of the malignancy.

6.3.7.2. Ex vivo recordings of STC specimens

Colonic resections from the 2 patients who underwent total colectomy and ileorectal anastomosis were obtained and studied in the organ bath in its entirety. While the length of the colon approximated 1-1.5 metres, only the distal 60cms of colon were studied *ex vivo* given logistical ability to only record this length of colon (Fig. 5).

6.3.8. Data Analysis

Each recording was analysed for the existence of anterograde or retrograde propagating sequences (PSs). A PS were defined as an array of three or more pressure waves recorded in adjacent recording sites. Pressure waves were deemed to be linked to one another if there was no period of quiescence between a pressure wave in one channel ending and the upstroke of the pressure wave in the adjacent channel. The direction of propagation was determined as anterograde (anal) or retrograde (oral) using these criteria. If a pressure wave returned to baseline before a pressure wave in a proximal or distal adjacent channel began to form, then the pressures waves were not considered part of the same event.

Number of propagating contractile events, velocities of individual contractions, distances of propagation and amplitudes were studied for all three groups of *ex vivo* controls, *ex vivo* STC colons and *in vivo* STC colons. In the *in vivo* STC colons, propagating sequence characteristics were obtained from the fasted period, prior to a standardized test meal.

6.4. RESULTS

The characteristics of the anterograde and retrograde propagating sequences have been summarised in table 2 & 3. Anterograde and retrograde propagating sequences were recorded in all preparations, both *in vivo* and *ex vivo*. As can be seen in the tables, the counts, velocity, extent of propagation and amplitude of the propagating event were remarkably similar between the *ex vivo* control and STC colons.

6.4.1. Table 1: Anterograde propagating sequences

	STC		Control *Ex vivo*
	In vivo	*Ex vivo*	
Number of events (/half hour)	6.25 +/- 13.44	10.57 +/- 1.51	7.29 +/- 1.24
Velocity (mm/s)	7.01 +/- 6.51	8.33 +/- 1.50	4.61 +/- 0.81
Amplitude (mmHg)	26.92 +/- 1.38	83.87 +/- 23.75	41.02 +/- 8.08
Extent (mm)	40.68 +/- 13.18	113.87 +/- 10.74	119.43 +/- 16.32

6.4.2. Table 2: Retrograde propagating sequences

	STC		Control *Ex vivo*
	In vivo	*Ex vivo*	
Number of events (/half hour)	11.75 +/- 8.44	6.29 +/- 0.90	7.29 +/- 1.71
Velocity (mm/s)	3.90 +/- 1.37	8.51 +/- 0.83	4.03 +/- 0.71
Amplitude (mmHg)	33.58 +/- 0.99	73.55 +/- 20.76	55.01 +/- 13.67
Extent (mm)	44.22 +/- 2.37	122.55 +/- 15.39	107.91 +/- 11.66

6.4.3. Non-propagating activity

Isolated examples of pressure wave rhythmic patterns occurring at 2-4 cpm could be identified in both STC patients *in vivo*. This was readily apparent in *ex vivo* and *in vivo* STC colon (Fig. 6), but less clearly seen in *ex vivo* controls

6.5. DISCUSSION:

Slow transit constipation (STC) is a troubling condition with a third of young females in Australia seeking medical advice for their constipation, unable to obtain unsatisfactory results from current therapies (301, 302). While surgical colonic resection remains an option it is only reserved only for the most recalcitrant of these patients. Therefore the opportunity to study extended segments of human colon with clinical diagnosis of slow transit constipation, in an *in vivo*, and *ex vivo* setting combined with the utility of a high-resolution pressure catheter is unique. This represents the most severe end of the spectrum of disorders.

While the cause of slow transit constipation is incompletely understood, abnormal colonic contractility is implicated, which has been suggested to be neurogenic or myogenic in origin (309). The colon of these patients often fail to respond to physiological stimuli such as meals and/or morning waking, and several studies have show abnormalities within the ENS (15, 172, 313, 319). Given the potential abnormalities within the wall of the colon it could be expected the *ex vivo* STC colon would show less activity when compared to control tissue. However, as with the

previous study by Spencer *et al* (218), our results show that the motility patterns in the *ex vivo* STC colon are similar to that recorded from *ex vivo* control tissue.

In interpreting these results it is important to acknowledge that the motility patterns in this study were recorded in very different settings and therefore the motor patterns recorded *in vivo* may reflect activity from a different mechanism than those recorded *ex vivo*. To address this we can look at the data generated from a study of colonic manometry recently completed by our group. Using fibre-optic manometry in the healthy adult colon, five distinctive propagating motor patterns were identified (316). These motor patterns included high amplitude propagating sequences, cyclic propagating motor patterns (that made up 70% of all propagating motor patterns) and three types of isolated propagating sequences, which were distinguished from one another by their speed and extent of propagation. On the basis of discriminant and multivariate analysis of the duration, gradient, and amplitude of the pressure events that made up each of the propagating motor patterns, we were able to distinguished two distinct types of pressure events: those belonging to the high amplitude propagating sequences and those belonging to all other propagating motor patterns (cyclic and the three isolated propagating motor patterns) (316). Therefore, these data suggest that the human colonic propagating motor patterns might have two independent origins. As several previous studies have suggested that the high amplitude events are likely require extrinsic neural input (320), these motor patterns were labeled as neurogenic.

Of the other propagating motor patterns recorded from healthy colon *in vivo*, the most common was the cyclic motor pattern, which closely resembled the *ex vivo e* myogenic, slow-wave-dependent activity recorded previously from human colon

(149, 227, 321) and was therefore considered to be myogenic in origin. As the isolated propagating motor patterns, recorded in the healthy colon, could not be statistically distinguished from the cyclic activity, they were considered to also share the same underlying myogenic mechanism (316).

On the basis of our finding in healthy controls, the *in vivo* motor patterns recorded in the slow transit colon *in vivo* were all likely to be myogenic in origin (no high amplitude propagating sequences were identified). Furthermore as the motor patterns record *ex vivo* cannot be influenced by extrinsic neural input, they are also likely to be myogenic in origin. Therefore although the motor patterns in the *ex vivo* and *in vivo* setting were recorded under different conditions, we are confident that they originate from the same fundamental mechanism.

On this basis the findings from our current study may help to shed some light upon the pathophysiology that underpins STC. For example if the activity recorded from the *ex vivo* colon is of myogenic origin and it occurred at the same frequency as that recorded from the control *ex vivo* preparation, then the slow transit abnormality may not lie with the intrinsic pathways of the of colon. Furthermore as this myogenic activity is rapidly influenced by a meal in healthy controls (316) and not in these slow transit constipation patients (Dinning, *et al.* 2014 unpublished) it suggests that the abnormality might lie within the extrinsic innervation of the colon. Similarly in paraplegic patients, while distal colonic phasic activity is readily apparent prior to a meal, the meal itself does not change the activity (322, 323)

In conclusion, whilst drawing definitive conclusions from just two patients is clearly difficult, our results do suggest that in slow transit constipation, the primary pathology may lie extrinsic to the gut wall itself. This may also suggest that the

known changes seen in both neuronal and pacemaker cell populations may well be a result of, rather than the actual etiology underlying STC.

Figures: Chapter 6

FIGURE 1. EXPERIMENTAL PROTOCOL

Study protocol demonstrating the 6 separate experiments analysed for experiments from this chapter.

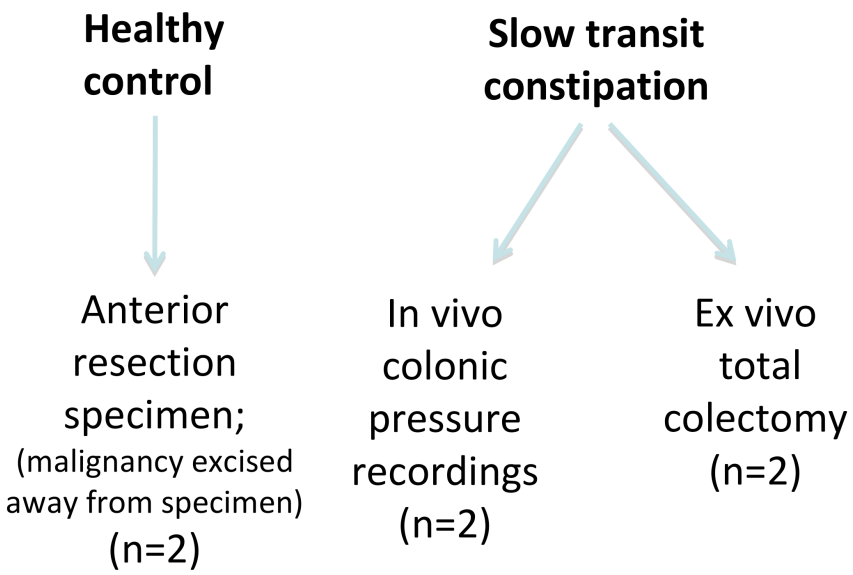

Healthy control
Slow transit constipation
Anterior resection specimen; (malignancy excised away from specimen) (n=2)
In vivo colonic pressure recordings (n=2)
Ex vivo total colectomy (n=2)

TABLE 1. STC PATIENT PROFILES

Characteristics of presentation, and procedures performed on the 2 patients with a history of slow transit constipation included in this study. Data obtained from the colonic recordings of these 2 patients allowed for comparisons *in vivo* and *ex vivo*; looking into the parameters of contractile activity.

	patient 1 (Pt1)	patient 2 (Pt2)
sex	Female	Female
age	44	25
medical history	back pain, depression, endometriosis, hysterectomy (laparoscopic)	vestibular neuronitis, social anxiety disorder
BMI	21 (60kg, 170cm)	33 (77kg, 152cm)
medications	gabapentin, mersyndol, moduretic, slow k, stemetil, valium, zolpidem	OCP, dulcolax, movicol

Rome III criteria for clinical STC 2006 (symptoms in at least 25% of defecation episodes)

straining	yes	yes
lumpy or hard stools	yes	yes
sensation of incomplete emptying in	yes	yes
sensation of ano-rectal obstruction/blockage	yes	yes
need for manual manouevres to facilitate	yes	no
fewer than 3 defecation episodes per week	yes	yes

Diagnosis

colonic scintigraphy	no expulsion of 100% of contents after 4 days	no expulsion of 100% of contents after 3 days
gastric scintigraphy	normal, no delay	normal, no delay
endo-anal US	intact internal, external sphincters, puborectalis identified	intact internal, external sphincters, puborectalis identified
colonoscopy	normal (more than 3- 2002, 2005, 2008, 2013 last)	normal (2013)
manometry	yes	yes
biochemical analysis	No significant derangements	No significant derangements
thyroid functions tests (TFT)	normal, TSH 2.56mIU/L, FT4 15 pmol/L	normal, TSH 4.5uIU/L, FT4 18pmol/L

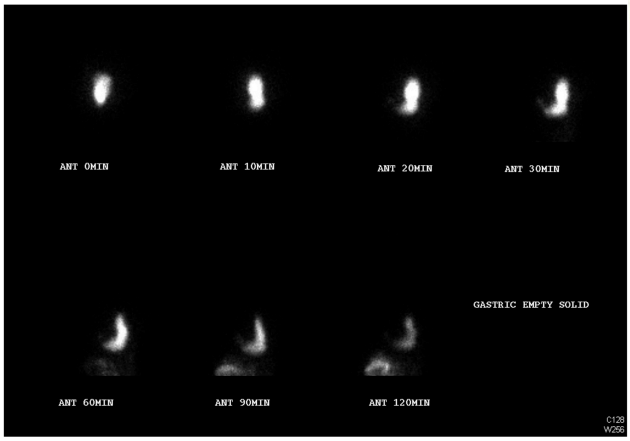
Perioperative information

Surgical procedure	total colectomy and ileorectal anastomosis(stapled)	total colectomy and ileorectal anastomosis (hand sewn)
Perioperative status	ileus and bowels not open for 8 days	uneventful recovery, discharged day 4 post procedure
Pathology assessment	no definitive anatomical pathology observed	no definitive anatomical pathology observed

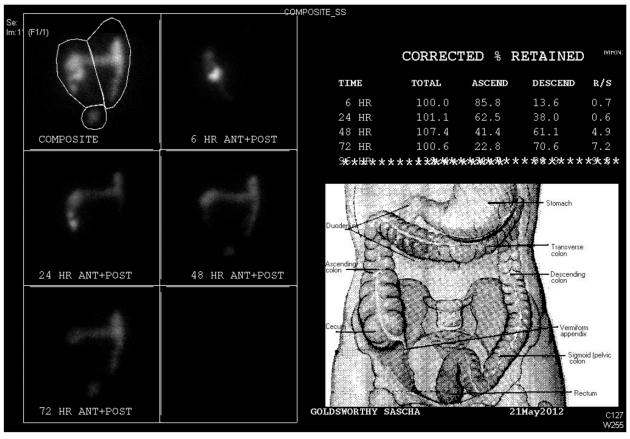
FIGURE 2. EVIDENCE FOR STC AND EXCLUSION OF OTHER CONDITIONS IN PATIENT 1

A. Gastric emptying study: Upon ingestion of the radioactive meal there is prompt filling of the stomach. By 20 minutes, there is passing of the tracer into the first part of the duodenum. By 60 minutes, tracer activity is seen in the proximal small bowel. Gastric half emptying time was within the normal limits at 59 minutes. Gastric emptying was therefore assessed as normal. B. These images demonstrate scintigraphy tracer activity in the right hemicolon at 6 hours, with subsequent images demonstrating some passage of activity to the left hemicolon. However, at 72 hours, approximately 100% of tracer activity still remained within the large bowel (normal range <10%). C. The top image refers to the most distal region of the anal canal, heading superiorly on the following images below. The puborectalis sling was thinned out, but the internal and external sphincters were intact. The results from A, B and C conclude that primary dysmotility in this patient is likely confined to the colon.

A.


ANT 0MIN
ANT 10MIN
ANT 20MIN
ANT 30MIN
GASTRIC EMPTY SOLID
ANT 60MIN
ANT 90MIN
ANT 120MIN
C128
W256

B.


COMPOSITE_SS
COMPOSITE
6 HR ANT+POST
24 HR ANT+POST
48 HR ANT+POST
72 HR ANT+POST
CORRECTED % RETAINED
TIME TOTAL ASCEND DESCEND R/S
6 HR 100.0 85.8 13.6 0.7
24 HR 101.1 62.5 38.0 0.6
48 HR 107.4 41.4 61.1 4.9
72 HR 100.6 22.8 70.6 7.2
Stomach
Duodenum
Transverse colon
Ascending colon
Descending colon
Caecum
Vermiform appendix
Sigmoid (pelvic) colon
Rectum
GOLDSWORTHY SASCHA
21May2012
C127
W255

C.

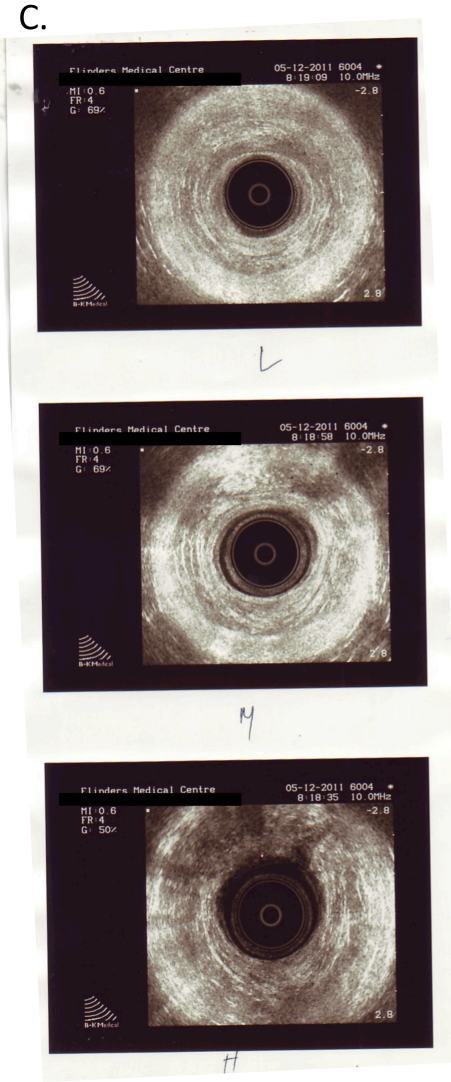
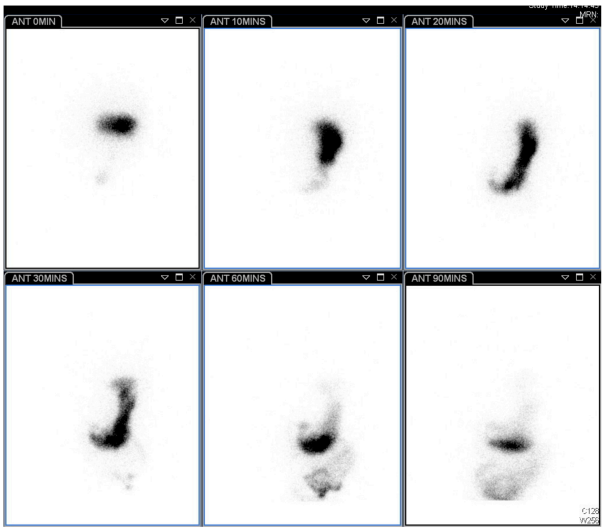

Flinders Medical Centre
05-12-2011 6004
8:19:09 10.0MHz
MI 0.6
FR 4
G 69%
-2.8
2.8
L
Flinders Medical Centre
05-12-2011 6004
8:18:58 10.0MHz
MI 0.6
FR 4
G 69%
-2.8
2.8
M
Flinders Medical Centre
05-12-2011 6004
8:18:35 10.0MHz
MI 0.6
FR 4
G 50%
-2.8
2.8
H

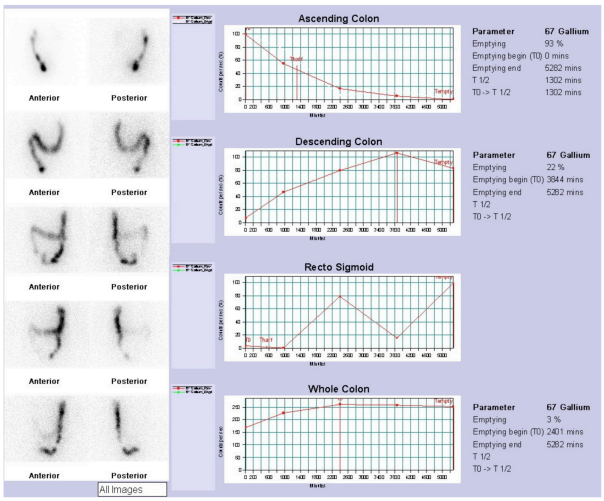
FIGURE 3. EVIDENCE FOR STC AND EXCLUSION OF OTHER CONDITIONS IN PATIENT 2

Again these results here show, A. Normal gastric emptying, B. Delayed colonic transit with persistent tracer accumulation to 96 hours in the descending colon, and C. Intact internal and external sphincters.

A.


ANT 0MIN
ANT 10MINS
ANT 20MINS
ANT 30MINS
ANT 60MINS
ANT 90MINS

C.


Anterior
Posterior
All Images
Ascending Colon
Descending Colon
Recto Sigmoid
Whole Colon
Parameter
67 Gallium
Emptying
93 %
Emptying begin (T0)
0 mins
Emptying end
5282 mins
T 1/2
1302 mins
T0 -> T 1/2
1302 mins
Emptying
22 %
Emptying begin (T0)
3944 mins
Emptying end
5282 mins
T 1/2
T0 -> T 1/2
Emptying
3 %
Emptying begin (T0)
2401 mins
Emptying end
5282 mins
T 1/2
T0 -> T 1/2

C.

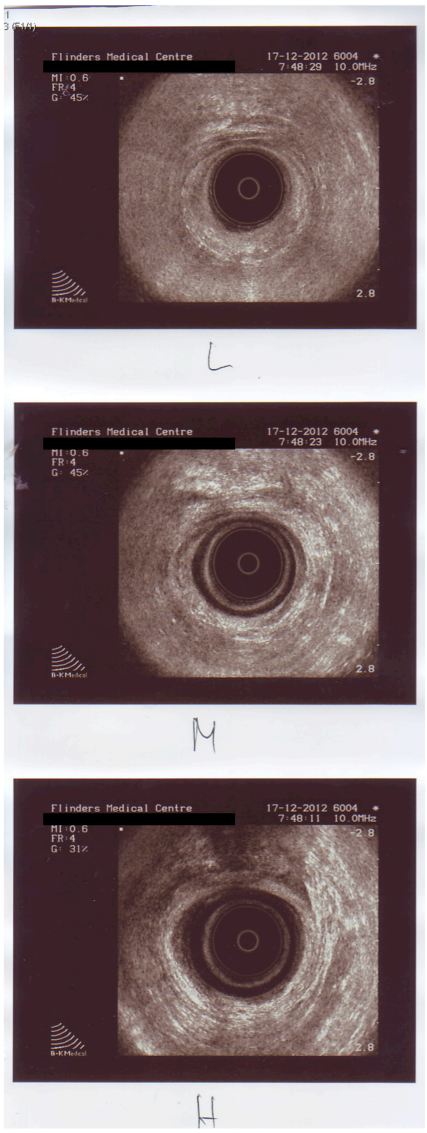

Flinders Medical Centre
17-12-2012 6004
7:48:29 10.0MHz
MI:0.6
FR:4
G: 45%
-2.8
2.8
L
Flinders Medical Centre
17-12-2012 6004
7:48:23 10.0MHz
MI:0.6
FR:4
G: 45%
-2.8
2.8
M
Flinders Medical Centre
17-12-2012 6004
7:48:11 10.0MHz
MI:0.6
FR:4
G: 31%
-2.8
2.8
H

FIGURE 4. PRESENCE OF CONTRACTILE ACTIVITY IN THE CONTROL SPECIMENS OF HUMAN COLON *EX VIVO*

Control experiments showing the presence of contractile activity in the *ex vivo* colon using a high-resolution recording catheter. A. Placement of the control colon specimen within an organ bath, B. An hour-long recording shown, C. A magnified view of the 20 minutes of recording in amongst the hour-long recording, highlighting the presence of robust contractile activity within the bowel walls of the control colon.

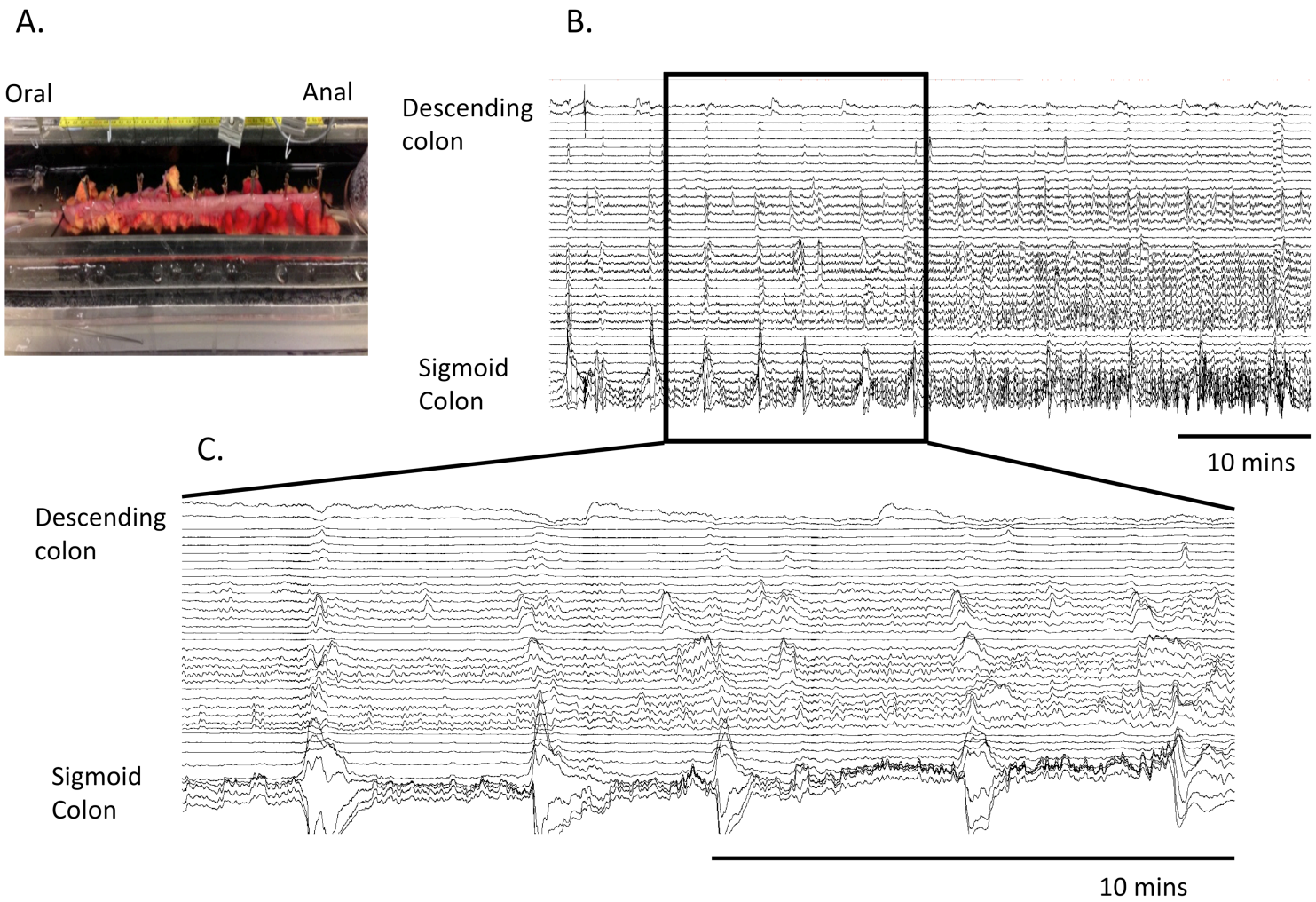

A.
Oral
Anal
B.
Descending colon
Sigmoid Colon
10 mins
C.
Descending colon
Sigmoid Colon
10 mins

FIGURE 5. PRESENCE OF CONTRACTILE ACTIVITY IN THE HUMAN STC COLON *EX VIVO*

Example from patient 2 with STC showing the presence of contractile propagating sequences in the descending and sigmoid colon. A. Colectomy specimen prior to placement within organ bath, in anatomical orientation (upper) and orientation within organ bath (lower), B. 2 hour recording of the STC colon *ex vivo* as recorded by a fibre-optic manometry catheter, C. 10 minute segment expanded from a 2 hour recording, again, highlighting the presence of robust contractile activity within the STC colon.

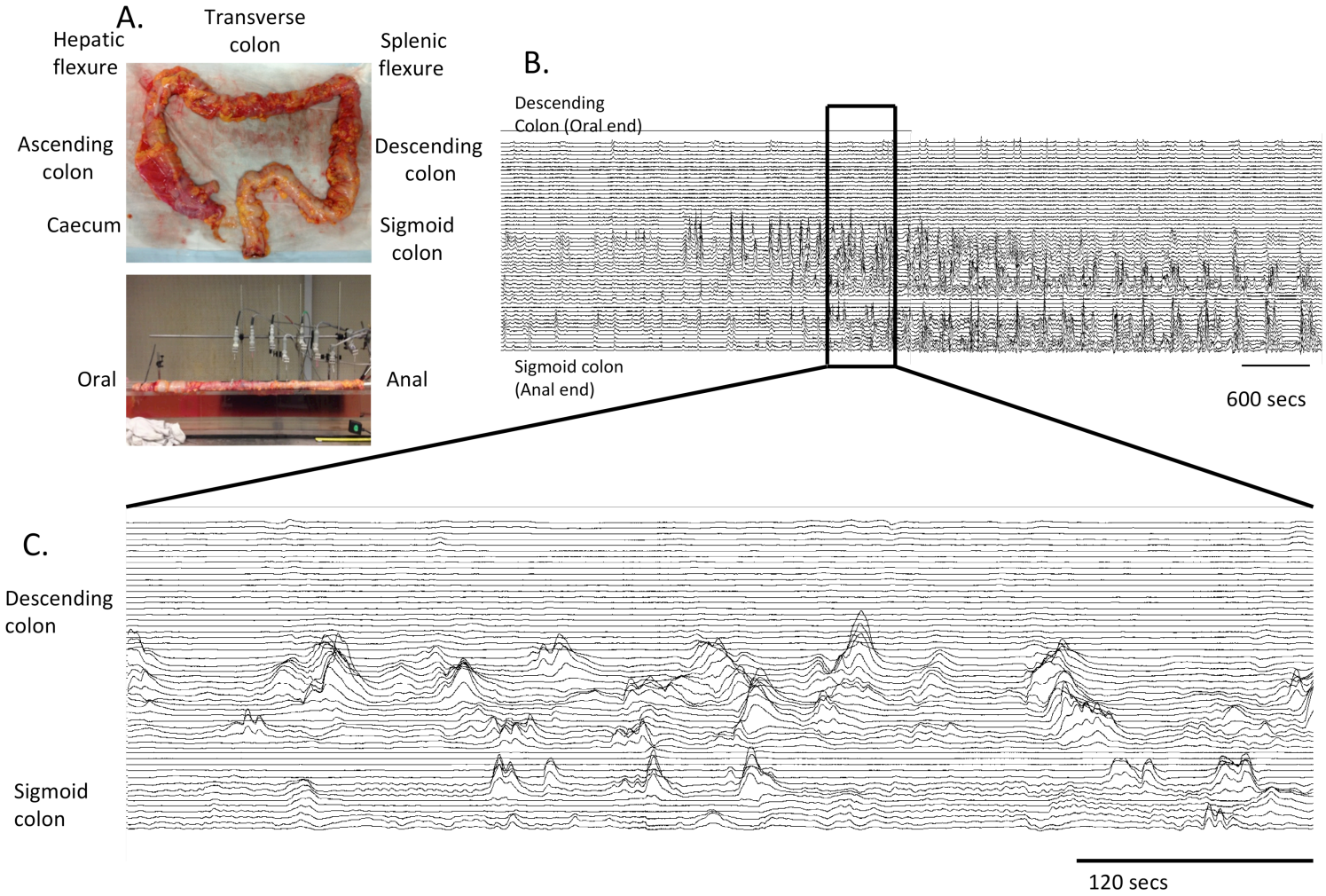
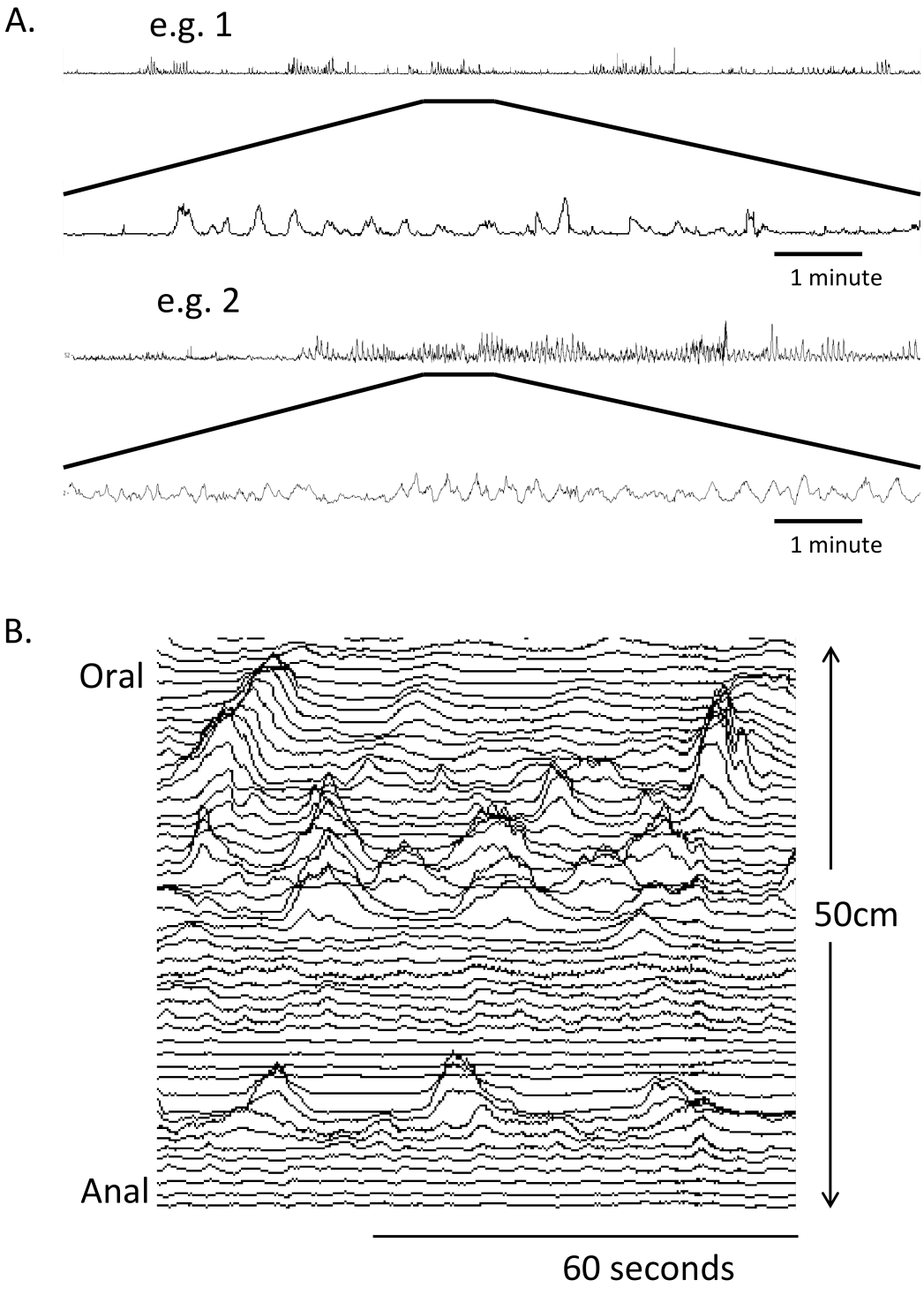

A.
Transverse colon
Hepatic flexure
Splenic flexure
Ascending colon
Descending colon
Caecum
Sigmoid colon
Oral
Anal
B.
Descending Colon (Oral end)
Sigmoid colon (Anal end)
600 secs
C.
Descending colon
Sigmoid colon
120 secs

FIGURE 6. PRESENCE OF AN UNDERLYING PHASIC CONTRACTILE ACTIVITY IN THE *EX VIVO* COLON

A. Presence of an underlying cyclical, low amplitude contractile activity, measuring 2-6 cycles per minute; B. emergence of propagating sequences noted from these cyclical activity. These events were clearly seen in *in vivo* and *ex vivo* STC experimentation.


A.
e.g. 1
1 minute
e.g. 2
1 minute
B.
Oral
Anal
50cm
60 seconds

Chapter 7: Discussion

Studies in this thesis investigate a number of aspects of the intrinsic mechanisms that underlie propulsive motor patterns in the lower gastrointestinal tract of guinea pigs and humans. A primary aim was to compare data obtained in animal models with motility in human intestine, and to examine if similar patterns of motility exist in large *ex vivo* segments of human bowel as are observed in smaller laboratory mammals.

In Chapter 2 the phenomenon of hexamethonium-resistant peristalsis was investigated. Whilst hexamethonium-resistant peristalsis had been described some time ago in the small intestine (41, 324), it has only recently been shown in the large intestine (39). We confirmed that hexamethonium-resistant peristalsis does exist in the distal colon of guinea pigs, and we have extended our understanding of this phenomenon. The major finding of Chapter 2 was the demonstration that peristalsis can still occur in the presence of antagonists of major neuro-neuronal and neuro-muscular transmitters (212). One might expect that blockade of excitatory neurotransmission at these cholinergic junctions would lead to a total abolition of peristalsis. Surprisingly, peristalsis was resistant to blockade of cholinergic receptors at both neuro-neuronal and neuro-muscular junctions in many, but not all preparations of colon. In experiments detailed in this chapter, it was also revealed that in the presence of hexamethonium and atropine, further addition of the NK-2 receptor antagonist ibodutant and the calcium channel antagonist ω-conotoxin GVI-A led to the blockade of peristalsis in most specimens. However, a small proportion of specimens still demonstrated propagation of faecal pellets in the combined presence of all of these drugs. Pellets moved slowly, in a staggered fashion along the colon, but ultimately traversed the full colonic length. In most experiments blockade

of major transmitters at these neuro-neuronal and neuro-muscular junctions slowed peristalsis in these preparations (from controls that typically took less than 30 seconds) to transit times of up to 1 hour in a short (~8cm) segment of guinea pig colon.

It might be argued that myogenic mechanisms underlie the propulsion that was resistant to hexamethonium, atropine, ibodutant and ω-conotoxin GVI-A. The observation that tetrodotoxin (TTX) always abolished peristalsis suggests that the underlying mechanism was dependent upon voltage-dependent Na^+ channels within enteric nerves. We presumed that, in the presence of TTX, any propagating activity is likely to be myogenic in nature. If peristalsis could be generated by myogenic activity, it would be reasonable to test whether the normal oral-to-anal polarity was preserved when enteric synaptic transmission was effectively blocked. We showed that whenever peristalsis persisted in the presence of all antagonists tested (except TTX), it always propagated aborally. This suggests that the pathways regulating peristalsis are hard-wired into the ENS and do not require major excitatory neurotransmitters to entrain an aboral directionality of propulsion. This body of work for the first time characterises peristalsis following blockade of a combination of the major neuro-neuronal and neuro-muscular transmitters, and suggests a novel mechanism of neural coordination in the ENS that is yet to be clarified.

The question of whether or not hexamethonium-resistant peristalsis involves neurotransmitters in the ENS is valid. There is indeed strong evidence for the role of tachykinins in the generation of peristalsis (242), but there are many other postulated

excitatory neurotransmitters in the ENS. Of these, serotonin/ 5-hydroxytryptamine (5-HT) has been implicated in many gastrointestinal functions.

We therefore sought to explore the role of endogenous 5-HT in the generation of colonic peristalsis in Chapters 3 and 4. There is substantial background literature regarding the role of endogenous 5-HT in peristalsis. Since the 1950s serotonin has been thought to play a major role in the generation of peristalsis. In fact to date, many have suggested that the initiation of peristalsis is triggered by serotonin release from the enterochromaffin cells (EC) located within the mucosa (61-63, 325, 326). The mechanism proposed has involved mechanical distortion or chemical stimulation of EC cells, which lead to release of serotonin. Serotonin in turn is postulated to activate mucosal afferent endings in intrinsic sensory neurons. While sounding plausible, these hypotheses have been somewhat speculative, because the site of action of endogenous serotonin release from EC cells has been elusive (detailed in Chapter 3). Many of these experiments relied on the observation that selective antagonists of 5-HT receptors blocked peristalsis (64, 65), however, their cellular site of action was not always clear.

Until recently (68, 327), no direct real time recordings of 5-HT release had been made from the mucosa in any region of the GI-tract. The long standing notion that mucosally released 5-HT was critical for peristalsis was challenged by Spencer and colleagues, who showed in a series of experiments that distension-evoked peristalsis was still preserved in preparations where the entire mucosa was removed (EC cell-depleted) from the guinea pig distal colon (81). However in their preparations neuronal 5-HT was still present in the nervous system. It could be argued that release

of 5-HT was still necessary, perhaps from the ENS rather than EC cells, was essential for peristalsis to occur; even though the levels of 5-HT synthesised in enteric neurons (<1% of neurons) are tiny compared with high concentrations synthesised in EC cells. Interestingly, observations by Gershon and colleagues showed that genetic depletion of serotonin by means of TPH1 blockade resulted in loss of mucosal/EC cell production of serotonin, but no change in *in vivo* motility in the small intestine (70). We considered it worthwhile to test whether enteric neuronal 5-HT was sufficient for peristalsis, bearing in mind that fewer than 1% of enteric neurons contain this neurotransmitter. Furthermore, endogenous serotonergic synaptic potentials have rarely been recorded in the ENS of guinea pigs (115) and never from human (6), rat (270) or mouse (268, 269).

In our experiments in Chapter 3 we sought to investigate whether neuronal 5-HT was essential for peristalsis to occur. When no serotonin was detectable in the colon (following excision of the mucosa, and pretreatment with reserpine to deplete 5-HT from myenteric neurons), peristalsis was still preserved with no change in contractile force (69).

Our new data (69) provides evidence that endogenous serotonin in enteric nerves and the mucosa is not essential for peristalsis in lower gastrointestinal tract. This is significant because serotonergic receptor antagonists are widely prescribed for the treatment of conditions of gastrointestinal dysmotility, but their site of action and mechanism of action is unclear (84, 100, 328-330). While these drugs had been used with purported good efficacy, they can lead to significant side effects including cardiac arrhythmias and ischaemic colitis. As a result, there has been restricted

prescription, and in some cases these drugs have been taken off the market altogether, e.g. Lotronex (alosetron), Propulsid (cisapride) (106, 331). A clear understanding of the mechanisms of action of these pharmacological agents might help alleviate the known potential side effects, and guide us towards more targeted and effective treatments (332).

In Chapter 4, the effects of selective 5-HT3 and 5-HT4 antagonists were investigated on the cyclical generation of peristalsis evoked by a fixed pellet. In these experiments, we found that selective 5-HT3 and 5-HT4 antagonists reliably induced blockade of peristaltic contractions, as reported previously. However, the blockade was transient. When applied for longer periods (up to an hour), peristalsis often recovered, in the continued presence of the antagonists (71). This may provide an important hint about the time course of therapeutics actions of serotonergic agents.

In summary, as a result of the findings in chapters 2-4, we deduce that release of endogenous 5-HT in enteric neurons or EC cells is not essential for the generation of colonic peristalsis (69, 71). Also, these experiments revealed that the presence of the endogenous natural ligand (5-HT) is not required *per se* for the activation of serotonergic receptors that have been postulated to play an essential role in peristalsis. Peristalsis and propagating activity were present in the apparent complete absence of endogenous 5-HT. In addition, antagonists of 5-HT3 and 5-HT4 receptors could still inhibit or block peristalsis even when there is no 5-HT available to bind to the receptors. This suggests that at least some of the inhibitory effects of these antagonists are likely due to blockade of constitutively active 5-HT3 and/or 5-HT4 receptors i.e. the antagonists may actually function as inverse agonists.

Constitutively active receptors can maintain some activity without the binding of endogenous ligands. For example, the 5-HT3B receptor subunit can display constitutive channel opening in the absence of any 5-HT (114). Many G-protein coupled receptors (GPCR) can exhibit some form of constitutive activity or can be modified to display constitutive activity (333, 334). If clinical symptoms of motility in the lower GI tract are indeed influenced by constitutive activity 5-HT receptors, this might be important in the targeting of new therapeutics to modulate bowel motility (335). Such inverse agonists may have a future in GI motility, but it will be important to further define their roles. Our findings point the way towards additional research in this area.

While data presented in this thesis, together with data from Dr Gershon's laboratories (70, 77) have shown that endogenous 5-HT is not required for peristalsis or *in vivo* transit, 5-HT may be involved in other biological processes in the gut. From the present study, it should not be concluded that endogenous serotonin plays absolutely no role in gut motility patterns. It is entirely feasible that in disease states changes in populations of EC cells could trigger changed transit times in the colon, leading to symptoms of diarrhoea. Indeed, rare secretory tumours, e.g. carcinoid tumours are clear examples of this. While the functional role of EC-derived 5-HT remains unclear, studies have suggested that neuronal 5-HT may play a role in the development of enteric neurons (77) and in bone metabolism (60, 70, 336). Our data simply shows that endogenous 5-HT in enteric neurons and EC cells is required for the single motor pattern of peristalsis. Indeed myenteric neurons potently respond to exogenous 5-HT causing depolarisation via 5-HT3 and 5-HT4 receptors (266, 337,

338), suggesting that it may play an important, yet-to-be-discovered role in neural control or gut function.

Our unique collaboration with the Department of Surgery at Flinders Medical Centre allowed us to characterise motility in isolated specimens of the human intestine. Studies of this size of preparation of human gastrointestinal tract have never been performed *ex vivo* in a controlled environment, devoid of circulating hormones, extrinsic innervation, and ongoing anaesthetic related interference. The insights from early and rudimentary experimentation on the human colon were encouraging and provided exciting prospects of experimentation onto the human ENS (218).

In Chapter 5, experiments were performed on long, intact segments of human terminal ileum. These experiments revealed the presence of motility patterns including propagating activity and phasic contractile activity. We were able to capture these motility patterns simultaneously using three recording techniques. Spatio-temporal mapping of diameter changes was used to measure frequencies and velocities of motor patterns, and by comparing with *in vivo* recordings provided by Dr Phil Dinning we were also able to examine the likely effects of the central nervous system upon gut motility.

A distinct advantage of our study over previous ones is the ability to use long lengths of intestine. While studies on shorter segments (~4cm) of human small intestine provide local information on the contractile pattern frequency (221), the limited length may prevent the study of propagating contractile activity. Propagating neurogenic motor activity has been consistently shown in the isolated whole colon of

many species (211, 339, 340), including humans (218). In the isolated cat colon, it has been shown that sectioning of the preparation into 10mm segments inhibits propagating cyclical neurally-mediated colonic spike bursts (217). We have now demonstrated similar findings in the human small intestine, as our experiments in longer specimens (up to 26cm) clearly demonstrated propagating activity. In shorter segments (<13cm), propagating activity was never seen. This provides evidence that the length of the specimen (perhaps because of the preservation of a sufficient population of long enteric interneurons) were required for the generation and propagation of these motor patterns in the human terminal ileum. Indeed it is known from retrograde tracing of human enteric neurons, that axonal projections to the muscle layers are less than 1cm long, whereas interneuronal projections can be up to at least 8cm long (34).

In addition, we established in the isolated human small intestine, that the presence of cyclical propagating contractile activity requires enteric neuronal activity. Lidocaine consistently abolished propagating events, and revealed an underlying phasic contractile activity that is cyclical in nature. These lidocaine-resistant cyclical contractions are likely orchestrated by endogenous pacemakers - the Interstitial Cells of Cajal (ICC). The ENS may modulate pacemaker driven activity (as shown in our Fast Fourier analysis in Figure 9 of Chapter 5 as an appearance of a dominant frequency in the presence of neuronal blockade). This is consistent with recent descriptions of neural activation modifying the frequency of myogenic contractions in *ex vivo* strips of human colonic tissue (227).

In this thesis, we have developed a number of *ex vivo* studies using animal models, and from these studies gained significant new insights into GI motility patterns. This allowed us to address a number of questions that had proved intractable in the past. When compared to animal models, motility in the human intestine is poorly understood. Armed with our understanding of organ bath physiology, pharmacology of the ENS in animals, knowledge on the anatomical neural circuitry of the human GI tract and now an understanding of the basic contractile activity in the human terminal ileum, we are in an exciting position to develop further our understanding the ENS of the human bowel in health and disease. It is conceivable that experiments performed in animal models can be replicated more readily in human tissue, *ex vivo*, now that the underlying characteristics of its contractile behaviour has been outlined. Eliciting reflex behaviour in isolated segments of human intestine will allow for further analysis using pharmacological dissection.

It is with this in mind, that the experiments in Chapter 6 were devised. The opportunity to study the total colectomy specimens of patients with slow transit constipation (STC) *ex vivo* was utilised to investigate the role of the ENS in the control of complex propagating motor patterns. By comparing these findings with colectomies performed for non-obstructing cancers, we were able to identify some distinct *ex vivo* motor activities and correlate these patterns with those recorded from the colons taken from patients with STC.

These recordings allowed us to further clarify the basic pathophysiology of a clinical condition that is highly prevalent, yet poorly understood. Studies of human colonic tissue have revealed some evidence of disruption to the ENS in the slow transit

colon, including altered enteric neurochemistry, enteric structural changes and ICC redistribution (15, 172, 219, 311, 313, 314, 341). One would deduce, given the clinical presence of constipation that these enteric nervous changes contribute to impaired contractile activity.

The main findings in our *ex vivo* experiments was that robust contractile propagating activity could be recorded in both the control and slow transit human colon. The measures we made of contractile activity including the number of propagating events, amplitudes of individual events and velocities were largely similar in the STC colon and control colons. The fact that the STC colon *ex vivo* behaved in a similar manner to the control colon *ex vivo* led us to speculate that the mechanisms underlying the onset of STC may affect the extrinsic innervation of the colon rather than the enteric neural circuits themselves. It is therefore also possible that any dysfunction of enteric neural processing in STC patients may be a result of delayed transit, rather than reflecting underlying cause. While the complex effects of the extrinsic nervous system were not examined in this study, what remains evident from our series of experiments is the presence of similar motor activity in the slow transit colon *ex vivo* to that in control colon *ex vivo*.

Slow transit constipation cases rarely result in colectomy. As more patients are studied, we will be able to analyse the contractile activity within these specimens in the same manner with aims for objective quantification of contractile activity within the human STC patient. This is now an established method, and future similar experiments are much anticipated.

The translation of basic understanding gained from animal models to human bowel leads to a greater understanding of human GI motility. This is only possible by close collaboration between basic scientists and clinicians. The immediate future will yield many new findings with significant relevance to medical care. We certainly look forward to it.

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