THE ROLE OF LUMINAL 5-HT_4 RECEPTORS IN COLONIC MOTILITY

REGULATION OF COLONIC MOTOR FUNCTION VIA ACTIVATION OF LUMINAL 5-HT_4 RECEPTORS

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LAY ABSTRACT

Functional gastrointestinal (GI) disorders including constipation and Irritable Bowel Syndrome (IBS) constitute the most widespread digestive disorders that could involve GI dysmotility and altered serotonin (5-HT) signaling. Current treatments include oral intake of prokinetic drugs such as serotonin sub-type 4 receptor (5-HT_4) agonists that activate 5-HT_4 receptors located on nerves in the gut wall. However, these receptors are also found on the luminal side of enterochromaffin cells in the colonic epithelium where more than 90% of the body's 5-HT is synthesized. Therefore, activation of luminal 5-HT_4 receptors by using a delivery system that releases the drug inside the colonic lumen without it being first absorbed in the upper GI tract, can result in the release of 5-HT and increase in colonic motility. This could significantly minimize the adverse side effects associated with systemic absorption of such drugs. In this study, first the rabbit animal model was used to test the effects of prucalopride after administration inside the colon (ex vivo). Results showed significant increase in propulsive motor patterns and their properties such as pressure and force. Such potent prokinetic effects occurred even in the presence of simulated fecal impaction, an acute complication of chronic constipation. Using high-resolution colonic manometry (HRCM), all aspects of propulsive motility including the colo-anal reflex and simultaneous pressure waves (SPW) were studied in vivo in healthy volunteers; then, the effects of intraluminal prucalopride was evaluated with HRCM in a human case study. Similar to the animal model, marked increase in propulsive motor activity was observed. This project shows the SPW and the colo-anal reflex have potential diagnostic values in patients with colonic dysmotility or abdominal bloating and prucalopride incorporated in colon-specific drug delivery systems has the potential to become the preferred prokinetic for the treatment of constipation. It also encourages further research into the role of luminal 5-HT in generating normal colonic motor function.

ABSTRACT

Activating luminal 5-HT_4 receptors in the colonic mucosa could result in the release of serotonin (5-HT) from enterochromaffin cells into the lamina propria to stimulate the myenteric neurons. The main objective of this thesis was to investigate the possibility of increasing colonic peristalsis by stimulating luminal 5-HT_4 receptors via administration of prucalopride, a highly selective 5-HT_4 receptor agonist intraluminally. To this end, both animal and human studies were performed. In the rabbit animal model, motor patterns of the isolated proximal colon were studied ex vivo using simultaneous spatiotemporal diameter mapping and pressure sensing in response to intraluminal prucalopride and exogenous 5-HT. Intraluminal prucalopride and 5-HT stimulated propulsive motor activity in a dose dependent and antagonist sensitive manner by increasing the contraction amplitude, pressure, frequency, velocity and degree of propagation of the colonic motor complex in a graded manner; that is, depending on the level of excitation, properties of motor patterns were enhanced or a more forceful colonic motor complex was initiated. To further investigate the effects of the drug in the presence of fecal impaction as an acute complication of severe chronic constipation, a rapid barostat bag was used to simulate bulk of fecal matter. Long-distance contractions (LDCs) with significantly higher contraction amplitudes and duration compared to baseline were evoked by the drug which propagated fully along the colon even in the presence of simulated fecal impaction.

High resolution colonic manometry (HRCM) with an 84-sensor water-perfused catheter (sensor spacing 1cm) was used in human in vivo studies. All aspects of propulsive motility including the colo-anal reflex and the simultaneous pressure wave (SPW) were investigated using luminal interventions in healthy subjects. Afterward, a HRCM case study was performed in a healthy volunteer to assess the effects of intraluminal prucalopride. Two dominant types of SPWs were observed that were associated with anal sphincter relaxation; pan-colonic SPWs and SPWs that emerged at the termination of a proximal high amplitude propagating pressure wave (HAPW). Meal and bisacodyl induced higher numbers of SPW associated anal sphincter relaxation compared to baseline. The average amplitude of SPWs

associated with anal sphincter relaxation was higher than the ones without quantifiable relaxation. Gas expulsion was always associated with a SPW and SPWs of significantly higher amplitudes were associated with gas expulsion and/or urge. Bisacodyl induced a significantly higher degree and duration of HAPW associated sphincter relaxation. Proximal balloon distension markedly increased the number of SPWs associated with gas expulsion. SPWs could consist of clusters of high frequency SPWs and could occur in a rhythmic fashion. Intraluminal prucalopride increased the frequency of HAPWs and SPWs compared to both baseline and after meal intake. It also significantly increased the amplitude of pan-colonic SPWs. Motor patterns evoked by intraluminal prucalopride could be associated with significant internal anal sphincter relaxation and urge to defecate.

This project shows that intraluminal prucalopride results in strong prokinetic effects in both the rabbit and the human colon suggesting the role of 5-HT_4 receptor-mediated endogenous 5-HT in regulating colonic motor function. SPW was found to be a dominant propulsive motor pattern and likely part of the gastrocolic reflex. Association of SPWs with anal sphincter relaxation suggests a neurogenic program underlying the SPW and the influence of prucalopride on its amplitude indicates the role of 5-HT in its generation. Rhythmic occurrence of SPWs suggests that networks of interstitial cells of Cajal (ICC) might govern part of their pattern. Association with gas expulsion suggests the SPW to be a biomarker for gas transit while association with urge to defecate and expulsion of the balloon suggests involvement in normal defecation. Hence, the SPW may become a biomarker for evaluating colonic motor activity involved in gas transit, the gastrocolic reflex and extrinsic neural responses to rectal stimulation with diagnostic values in patients with colonic dysmotility and bloating. This project suggests documentation of the colo-anal reflex as part of the manometric assessment. It also supports incorporating 5-HT_4 receptor agonists in colon-specific drug delivery systems to treat dysmotility and promotes further research into the role of luminal 5-HT in normal colonic motor function. It may also be possible to adjust luminal 5-HT through diet or modifying 5-HT producing microbiota, in particular when patients with constipation show reduced luminal 5-HT.

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LIST OF ABBREVIATIONS

5-HT	5-Hydroxytryptamine (serotonin)
5-HTR	5-Hydroxytryptamine receptor
FPC	Fast propagating contraction
LDC	Long distance contraction
HBC	Haustral boundary contraction
EC	Enterochromaffin
ICC	Interstitial cells of Cajal
IPAN	Intrinsic primary afferent neuron
CDD	Colon targeted drug delivery
SPW	Simultaneous pressure wave
HAPW	High amplitude propagating pressure wave
GI	Gastrointestinal
ENS	Enteric nervous system
MAOA	Monoamine oxidase A
TPH	Tryptophan hydroxylase
IBS	Irritable bowel syndrome
IBD	Irritable bowel disease
FGID	Functional gastrointestinal disorder
CNS	Central nervous system
NO	Nitric oxide
ATP	Adenosine triphosphate
VIP	Vasoactive intestinal peptide
SERT	Serotonin reuptake transporter
CGRP	calcitonin gene-related peptide
EPSP	Excitatory post-synaptic potential
TTX	Tetrodotoxin
RAIR	Recto-anal inhibitory reflex
AH	After hyper-polarization
hERG	Human ether-a-go-go-related gene
HRCM	High resolution colonic manometry

IAS	Internal anal sphincter
SSRI	Selective serotonin reuptake inhibitor
SCFA	Short chain fatty acid
FAERS	FDA adverse event reporting system
CCK	Cholecystokinin
IGLE	Intraganglionic laminar endings
IMA	Intramuscular array
NTS	Nucleus tractus solitarius
L-NMMA	N(G)-monomethyl-L-arginine
cAMP	Cyclic adenosine monophosphate
DC	Dendritic cell
CREB	cAMP response element binding
5-HIAA	5-hydroxyindoleacetic acid
PBS	Phosphate-buffered saline
RAIR	recto-anal inhibitory reflex

DECLARATION OF ACADEMIC ACHIEVEMENT

All animal experiments (chapters 2,3) were designed by Mitra Shokrollahi and Dr. Jan D Huizinga and were performed by Mitra Shokrollahi. Modifications of the experimental set up was identified and performed by Mitra Shokrollahi. All analysis design, quantitative image data analysis, statistical analysis and graphical representation of data for animal experiments were executed by Mitra Shokrollahi. Interpretation of all results related to all animal experiments was done by Mitra Shokrollahi under the guidance of Dr. Jan D Huizinga. Insights into further animal experiments were offered by Dr. Jihong Chen. A modified short version of chapter 2 was published (https://doi.org/10.1111/nmo.13598) with Mitra Shokrollahi as the first author. Contribution of authors to this paper was as follows: Mitra Shokrollahi and Dr. Jan D Huizinga designed the research study; Mitra Shokrollahi performed all the experiments, designed the analysis and analyzed all data; Mitra Shokrollahi wrote the manuscript, designed and created all figures and tables and executed all statistical analysis. Mitra Shokrollahi, Dr. Jan D Huizinga and Dr. Jihong Chen revised the manuscript.

All High-Resolution Colonic Manometry experiments in chapter 4 were performed by Dr. Jihong Chen and designed by Dr. Jan D Huizinga and Dr. Jihong Chen. Mitra Shokrollahi designed the analysis of the anal sphincter relaxation project and performed anal sphincter relaxation analysis associated with all motor patterns (the colo-anal reflex project), performed all analysis related to the association of motor patterns with gas/liquid expulsion and gender-based differences, interpreted all data related to these projects under the guidance of Dr. Jan D Huizinga, had major contribution in quantitative image analysis of the rest of the data as presented in Table 12, in interpretation of these data and in statistical analysis. A different short version of chapter 4 was published with Mitra Shokrollahi as the third author (doi: 10.3389/fphys.2018.01248). Mitra Shokrollahi's contribution to this paper included all the above mentioned plus critical revision of the manuscript for important intellectual content and revision of the final draft. The colo-anal reflex project

was presented as a poster at Canadian digestive disease week conference (CDDW) 2018, with Mitra Shokrollahi as the second author.

The High-Resolution colonic Manometry experiment in chapter 5 was performed by Dr. Jihong Chen. All analysis design, quantitative image data analysis, statistical analysis and graphical representation of data related to chapter 5 was executed by Mitra Shokrollahi. Interpretation of results related to this chapter was done by Mitra Shokrollahi under the guidance of Dr. Jan D Huizinga. All BON cell experiments were designed by Mitra Shokrollahi and Dr. Jan D Huizinga, performed by Eric Kwon and insights were offered by Dr. Waliul Khan. Interpretation of results were done by Mitra Shokrollahi under the guidance of Dr. Jan D Huizinga and Dr. Waliul Khan. Insights into further interpretation and experiments in general were offered by Dr. Jihong Chen and Dr. Elyanne Ratcliffe. Results from animal studies in chapter 2 and HRCM case study in chapter 5 were presented as a poster at McMaster Institute for Research on Aging (MIRA) 2019 conference with Mitra Shokrollahi as the first author.

This manuscript was written by Mitra Shokrollahi and edited by Dr. Jan D Huizinga.

CHAPTER 1- INTRODUCTION

PHYSIOLOGICAL CONTROL OF COLONIC MOTILITY

Motility encompasses many complex processes that are dependent on contraction of longitudinal and circular muscle layers resulting in moving luminal contents through the gastrointestinal (GI) tract (Miller et al., 2018; Furness, 2008). Motility is influenced by the enteric nervous system (ENS), the central nervous system (CNS), sympathetic and parasympathetic nervous system, the hormonal milieu, the receptors dispersed throughout the GI tract, the gut microbiota and the luminal contents (Miller et al., 2018; Furness, 2008). Our knowledge of the mechanisms of the interaction between these various control elements of the GI tract are still at its infancy (Costa, 2016).

Anatomically, the colon is comprised of multiple layers (from inside-out): the epithelium, the lamina propria, the muscularis mucosa, the sub-muscular plexus, the circular muscle layer, the myenteric plexus and the longitudinal muscle layer (Costa, 2016). The activity of the colon can be separated into “propagating” and “non-propagating” motor activity with the latter making up a larger portion of contractions in the distal colon. The propagating motor patterns usually produce a significant “propulsion” of stool forward at least to the mid-colon (Bharucha, 2012; Miller et al., 2018). The major function of colonic motility is the mixing of content which is important for creating optimal exposure of content to the absorptive surface of the gut and for bacterial homeostasis (Huizinga et al., 2011); even propulsive motor activity that generates propulsion of stool, predominantly exposes the contents to the mucosal surface because propulsion only rarely ends in evacuation of content from the body (Huizinga, 2016).

Propulsive motor activity or “peristalsis” is defined as the activity of the gut musculature that can propel content into the anal (antegrade peristalsis) or oral (retrograde peristalsis)

directions (Huizinga, 2018). According to the classical "law of the intestine" the local distention of the intestinal wall by a bolus stimulates inhibitory neurons to relax the musculature in front of the bolus while triggering excitatory neurons at the oral end of the bolus which results in the movement of the bolus in anal direction. This is known as the "Bayliss and Starling peristaltic reflex" originally postulated by Bayliss and Starling in 1899 (Huizinga, 2018; Spencer et al., 2004). However, this reflex is not the only mechanism that can propel intestinal content (Huizinga, 2018) and numerous investigators have failed to observe it in many species (Spencer et al., 2004; Huizinga, 2016). Moreover, under many conditions that require propulsion, there is no bolus but only slushy content or harder content of various lengths. Therefore, the term "peristaltic reflex" is best reserved for bolus-induced peristalsis while the term "peristalsis" is used for all the other mechanisms that propel content (Huizinga, 2018).

Propulsive motor activity mainly results from an interplay between interstitial cells of Cajal (ICC) and the enteric nervous system (Huizinga et al., 2014) though extrinsic nerves also play a role in orchestrating peristalsis (Huizinga, 2018). In humans, lesions to extrinsic nerve pathways or spinal cord damage leads to abnormal colonic motility and often constipation (Anderson, 2004), which can be improved by stimulation of defecation centres in the spinal cord (Ferens et al. 2011; Ellis et al. 2015) indicating the important role of extrinsic innervation in the control of colonic motility. The rhythmicity and propagating characteristics of the peristaltic movements are governed by ICC networks and the occurrence and force of contraction of peristaltic movements are dependent on enteric neural activity. Peristaltic activity can be evoked by enteric nerves that trigger stimulus-dependent pace-making in a network of ICC, illustrating the interaction of the ENS and ICC (Huizinga, 2018).

ICC are mesenchymal cells originating from the same precursors as circular smooth muscle cells (Young, 1999) and they are regarded as part of the myogenic control system of gut motility (Huizinga et al., 2014). ICC are unique cells that have either intrinsic pacemaker

activity or perform stimulus-induced pace-making (Huizinga, 2018). ICC act as networks connected to inhibitory and excitatory intrinsic and extrinsic nerves that influence the network activity. Therefore, ICC networks play an important role in both excitatory and inhibitory enteric motor neurotransmission and their absence or scarcity has been associated with various GI disorders (Huizinga, 2018). Specifically, abnormalities in ICC networks are increasingly recognized as being associated with dysfunction of peristalsis (Huizinga et al., 2014).

The segmentation motor activity of the gut is different from peristalsis in that it contains only stationary or very short distance propagating contractions without major forward propulsion of the luminal contents; and it is specialized for only mixing and absorption of nutrients (Huizinga, 2016; Huizinga & Chen, 2014; Mawe & Hoffman 2013). In the mouse small intestine, segmentation motor pattern is easily expressed after total blockade of the enteric nervous system, which implies that a myogenic control system underlies this activity (Huizinga et al., 2014). Huizinga and colleagues demonstrated that segmentation emerges when the amplitude of the slow wave activity of the dominant pacemaker ICC associated with the myenteric plexus (ICC-MP), is modulated by the phase of induced lower frequency rhythmic transient depolarizations generated by ICC associated with the deep muscular plexus (ICC-DMP; Huizinga et al., 2014). Nevertheless, this does not exclude the role of the ENS in the development of segmentation in vivo. The ENS provides an essential stimulus for the motor activity to develop and several components of the ENS have been shown to be involved in the segmentation motor activity (Huizinga, 2016; Gwynne & Bornstein 2007). The ENS neural activity works in concert with ICC to generate the segmentation motor patterns (Huizinga & Chen 2014).

Ultimately, the contractile activity of the gut is generated by the smooth muscle cells which respond to various stimuli to contract. Gut smooth muscles contract when calcium enters the cell cytoplasm via voltage-activated calcium channels which require significant depolarization to open (Huizinga, 2018). The smooth muscle cells are constantly subjected

to rhythmic depolarizations from the ICC pacemaker cells and other stimuli that trigger contraction such as distention and neural activity to further depolarize the cell. This brings the slow-wave plateau above the threshold to generate smooth muscle action potentials which lets calcium into the cells and subsequently results in smooth muscle contraction (Huizinga, 2018).

The GI tract is the only internal organ with its own independent nervous system, known as the ENS, which is concealed within the gut wall and can function independently of neural inputs from the central nervous system (Hu & Spencer, 2018). The main function of the myenteric plexus is to generate neurogenic contractions and relaxation of the smooth muscle layers; while the submucosal plexus is largely associated with secretomotor reflexes and absorption (Hu & Spencer, 2018). The ENS is comprised of various neurons including motor neurons, interneurons and afferent neurons.

Many hormones and neurotransmitters can affect motor and secretory pathways in the colon resulting in excitation and increasing secretion (e.g. acetylcholine, serotonin) or inhibition and increasing absorption (e.g. somatostatin, norepinephrine; Miller et al, 2018; Furness et al, 1998; Kunze et al., 2000). The cell bodies of the motor neurons are located in the myenteric plexus with endings on either circular or longitudinal muscles. They can be inhibitory or excitatory. Inhibitory motor neurons release either nitric oxide (NO), vasoactive intestinal peptide (VIP) or adenosine triphosphate (ATP). On the other hand, excitatory motor neurons release acetylcholine or tachykinins (Furness, 2008). Interneurons are located in the myenteric plexus and transfer information from a primary neuron to the effector neuron (Furness, 2008).

The colon is equipped with two groups of afferent neurons: those with cell bodies in cranial and dorsal root ganglia (extrinsic), and those with cell bodies in the gut wall (intrinsic). Most of the intrinsic and extrinsic primary afferent neurons have endings which project into the lamina propria of the mucosal layer where they can be exposed to serotonin,

5-hydroxytryptamine (5-HT), released from enterochromaffin (EC)-cells (Blacksaw et al., 2007). These nerve endings include vagal afferent fibers arising from the nodose ganglion (extrinsic), spinal afferent fibers arising from dorsal root ganglia (extrinsic), and intrinsic afferent AH neurons with cell bodies located in submucosal and myenteric ganglia (Mawe & Hoffman, 2013).

Extrinsic primary afferent neurons mediate communication between the gut and the CNS. The gut is innervated by several classes of extrinsic neurons that have distinct combinations of properties making them sensitive to particular mechanical and chemical stimuli (Brooks et al., 2013). Vagal afferent fibres arising in the upper GI tract send messages that result in a variety of responses upon activation by 5-HT. In vivo and ex vivo studies of extrinsic colonic afferent nerves have shown that spinal afferents express 5-HT_3Rs on their peripheral endings (Mawe & Hoffman, 2013). Intrinsic primary afferent neurons (IPAN) synapse with ICC (Zhu et al., 2014) and activate myenteric motor neurons in response to stimuli. Both stretch and chemical stimuli can trigger IPANs. Stretch-sensitive IPANs have been shown to be physically connected to the gut musculature and their activation is proportional to the degree of distension (Kunze et al., 2000).

Activation of receptors on the luminal side of the epithelial cells can secrete chemicals that stimulate the endings of IPANs. For instance, 5-HT can be released from the EC cells via both mechanical distention and chemical stimulation which subsequently stimulates IPANs (Mawe & Hoffman, 2013). IPANs then activate the myenteric neurons. This neuronal circuitry also involves glial cells and ICC (Smith et al., 2014). Therefore, motility can be evoked and regulated via a myriad of stimuli including activation of luminal receptors which is the main topic of this thesis.

DIVERSE ROLES OF 5-HT IN THE GI TRACT

Serotonin (5-HT) is an important gastrointestinal signaling molecule predominantly synthesized and released from EC cells within the colonic mucosa and to a lesser extent by serotonergic neurons located in the myenteric plexus (Mawe & Hoffman, 2013). Evolutionarily speaking, 5-HT existed in plants before animals and it may be tied to the evolution of life itself particularly through the role of tryptophan as its precursor molecule which is a unique essential amino acid with light-absorbing properties. Serotonin-like molecules in plants direct the growth of light-absorbing structures. This property also occurs in animal cells in which 5-HT influences the cytoskeleton alternation and regulates cell proliferation, migration and maturation in a variety of cell types including lung, kidney, endothelial cells, mast cells, neurons and astrocytes (Azmitia, 2001).

5-HT is a highly conserved ancestral monoamine distinctly regulated by two different rate-limiting enzymes, tryptophan hydroxylase-1 (TPH1, mucosal 5-HT) and tryptophan hydroxylase-2 (TPH2, neuronal 5-HT). 5-HT specifically has an important role in secretomotor and sensory functions of the gut through activation of a myriad of GI receptors. These receptors are dispersed throughout the mucosa, lamina propria, and the ENS (Hoffman et al., 2012; Yaakob et al., 2015). The 5-HT receptor (5-HTR) subtypes 5-HT_2R, 5-HT_3R, 5-HT_4R and 5-HT_7R in the human colon, were found to be the most common subtypes detected in all layers of the tissue (Chetty et al., 2009).

5-HT has been shown to affect a variety of physiological and pathophysiological states in the gut, such as peristalsis, epithelial secretion, vasodilation, nausea, and visceral hypersensitivity (Mawe & Hoffman 2013; Gershon 2013; Gwynne et al., 2014). The actions of 5-HT are ended via being transported back by the serotonin reuptake transporter (SERT) into epithelial cells (Martel et al., 2003) and then being degraded by monoamine oxidase A (MAOA) to 5-hydroxyindoleacetic acid (5-HIAA) (Banskota et al., 2018; Mawe & Hoffman 2013; Gershon 2013; Gwynne et al., 2014; Bellono et al., 2017; Kim & Khan, 2014; Li et al., 2011; Gershon 2013). Despite efforts over the past six decades to understand 5-HT signaling, many unanswered questions still remain (Yu et al., 2015).

EC cells produce more than 90% of the body's serotonin and represent the largest endocrine cell population in the gut although they constitute only 1% of total intestinal epithelia (Kim & Khan 2014; Gunawardene et al., 2011). These triangular-shaped cells originate from intestinal stem cells near the base of crypts and usually migrate towards the villous tips. EC cells are distributed throughout the human GI tract with maximum numbers in the duodenum and rectum (Spiller, 2008). Intrinsic and extrinsic afferent nerves of the gut do not reach into the lumen where they could sample for nutrients, toxins or other cues to initiate reflex responses. Therefore, EC cells function as sensory transducers to respond to various stimuli (Bellono et al., 2017). The apex of EC cells extends out into the lumen with their base in contact with the base membrane. Their microvilli facilitate their role as polymodal stimulus detectors that form a direct line of communication between the gut epithelium and primary afferent nerve fibers (Spiller, 2008; Bellono et al., 2017). EC cells can be activated via luminal stimuli causing them to release serotonin from the dense granules found both apically and basally. The 5-HT released into the lumen (apical release) or lamina propria (basal release) can mediate gastrointestinal functions (Spiller, 2008; Bellono et al., 2017). 5-HT acts either on enterocytes or on mucosal afferents in the lamina propria, initiating secretion and propulsive motor patterns (Spiller, 2008).

EC cells have recently been shown to express taste receptors and fluorescent calcium imaging studies show stimulation of cells by a range of tastants including thymol (bitter taste of thyme), results in 5-HT release (Braun et al., 2007). Short chain fatty acids, bacterial products normally found only in the colonic lumen, have also been shown to stimulate 5-HT release (Fukumoto et al., 2003) and luminal administration of butyrate can elicit peristalsis (Vincent et al., 2018).

In addition to being chemo-sensitive, EC cells are mechanosensitive and release 5-HT when the mucosa is distorted by circular muscle contraction or by stroking (Borstein & Foong, 2018). The trauma induced by stroking may release a range of mediators including ATP which can activate EC cells directly (Spiller, 2008). Mechanical stimulation also evoked 5-HT outflow from BON cells, an EC cell line derived from pancreatic cancer line (Kim et al., 2001).

Although the mucosa of the GI tract is the major depository of the body's 5-HT, enteric neurons also express 5-HT. It is estimated that only around 2% of the ENS neurons are serotonergic (Costa et al., 1996). Similar to the neurons in the brain, synthesis of 5-HT in the enteric neurons depends on TPH2. Numerous studies have demonstrated that neuronal 5-HT can contribute to both fast and slow excitatory postsynaptic potentials (EPSPs; Mawe & Hoffman, 2013). Activation of neuronal 5-HT_4 and 5-HT_3 receptors of the gut have been extensively studied. Since this thesis project is focused on 5-HT_4Rs, the role of both epithelial and neuronal 5-HT_4Rs in GI motility will be reviewed in a separate section.

5-HT_3Rs are expressed on the intrinsic and extrinsic afferent nerves that extend into the mucosa, interneurons, inhibitory and excitatory motor neurons, ICCs, smooth muscle and enterocytes. 5-HT_3Rs are involved in the activation of intrinsic and extrinsic afferent nerves by 5-HT released from EC cells. Bertrand and colleagues demonstrated that AH neurons in the myenteric plexus, which serve as primary afferent neurons and project to the mucosa, are directly activated by 5-HT applied to the mucosa, and that these responses are exclusively mediated by 5-HT_3Rs in the guinea pig (Mawe & Hoffman 2013; Bertrand et al., 2000). Many studies have demonstrated that 5-HT_3Rs can participate in the activation of propulsive motility and secretory responses in the gut (Bertrand et al., 2000; Mawe & Hoffman 2013; Yu et al., 2015) and 5-HT_3 receptor agonists have been developed for the treatment of different GI disorders including constipation and irritable bowel syndrome (IBS).

5-HT also exerts other actions in the gut such as promoting inflammation (Mawe & Hoffman 2013; Banskota et al., 2018; Manocha & Khan 2012; Kim & Khan, 2014). Alternations in 5-HT signaling, and the number of 5-HT positive EC cells have been observed in various models of intestinal inflammation with 5-HT manifesting a pro-inflammatory function (Kim & Khan, 2014). Blocking 5-HT synthesis using an oral TPH inhibitor has been shown to effectively reduce intestinal inflammation (Kim et al., 2013). Many serotonergenic receptors have been found on various immune cells such as B and T

lymphocytes, monocytes, macrophage, and dendritic cells (DC). Moreover, mast cells, macrophage, and T cells also have the ability to synthesize 5-HT from tryptophan (Manocha & Khan 2012). The precise mechanisms of 5-HT pro-inflammatory roles remains to be elucidated. However, various studies on immune cells have revealed its regulatory effects on immune response modulation (Kim & Khan, 2014; Wang et al., 2007; Manocha & Khan 2012).

Moreover, 5-HT regulates pancreatic enzyme secretion (Banskota et al., 2018), has epithelial protective effects (Spohn et al., 2016) and serves as a trophic factor to promote the development and maintenance of neurons and interstitial cells of Cajal (Mawe & Hoffman, 2013; Liu et al., 2009). 5-HT also influences bone function. EC cell-derived 5-HT in the gut acts as a hormone to inhibit bone formation by decreasing osteoblast proliferation via activation of 5-HTR_{1B} receptors located on pre-osteoblasts while neuronal 5-HT acts as a neurotransmitter to promote bone growth (Banskota et al., 2018).

5-HT SIGNALING & CLINICAL GASTROINTESTINAL DISORDERS

Functional gastrointestinal disorders (FGIDs) including constipation, IBS, functional dyspepsia and gastroparesis together constitute the most common diseases of the digestive tract and significantly affect patients' quality of life (Jeong et al., 2018). The pathophysiological mechanisms responsible for these disorders have not been fully elucidated, but patients with FGIDs frequently have GI dysmotility (Manabe et al., 2010). A number of studies have reported altered 5-HT signaling activity in intestinal disorders with the emphasis on the key role of 5-HT in regulating GI motor functions (Kim & Khan, 2014; Banskota et al., 2018; Spiller, 2008).

Although knowledge of abnormalities of 5-HT metabolism in human diseases is still lagging (Spiller et al., 2008), alterations in EC cells and 5-HT signaling have been

associated to a number of GI disorders including chronic constipation, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and enteric infections; highlighting the important role of 5-HT signaling in gut homeostasis (Kim & Khan, 2014; Beattie & Smith, 2008; Spiller et al., 2008; El-Salhy et al., 2012). Numerous investigators are beginning to link disturbed 5-HT physiology with the pathophysiology of GI disorders. Nevertheless, the role of 5-HT in the pathophysiology of GI diseases can vary. Among the most important roles of 5-HT is its influence on GI motility and its ability to modulate the immune system (Manocha & Khan, 2012).

Inflammatory bowel disease (IBD) includes two chronic GI diseases: ulcerative colitis and Crohn's disease. Inflammation of intestinal mucosa has been found to affect 5-HT signalling in both humans and animal models. In addition, changes in EC cell numbers and 5-HT content have been reported in both Crohn's disease and ulcerative colitis (Manocha & Khan, 2012). Increase in 5-HT content have been observed in experimental models of colitis and consumption of selective serotonin reuptake inhibitors (SSRIs) is associated with microscopic colitis (Manocha & Khan, 2012; Banskota et al., 2018).

Irritable bowel syndrome (IBS) is associated with abdominal pain, cramping, and changes in the bowel movement. Patients can experience predominantly constipation (IBS-C), diarrhea (IBS-D) or mixed of both (IBS-M). IBS-D is associated with elevated 5-HT while IBS-C is associated with decreased levels of 5-HT in the colonic mucosa (Manocha & Khan, 2012; Camilleri, 2009; Spiller, 2008). Several studies on human and experimental animal models suggest the association of symptoms of IBS with the level of 5-HT in the gut, which depends on the number of EC cells, altered SERT expression in mucosal biopsies and mRNA level of TPH (Banskota et al., 2018). Studies of the 5-HT signaling in IBS-C have reported contradictory results ranging from decreased 5-HT content in mucosal samples (El-Salhy et al., 2012), to increased, or unchanged (Costedio et al., 2010) and 5-HT_4 receptor agonists have been shown to be effective in treating IBS-C (Spiller et al., 2008; Camilleri et al., 2008; El Salhy et al., 2012).

Causes of constipation may be primary (also called functional or idiopathic) or secondary (also called organic). Secondary constipation is related to disease or medications. Primary constipation is usually sub-divided into normal-transit, slow-transit and evacuation constipation (Andrews & Storr, 2011, Koch et al., 1997; Yang & Ma, 2017; Costilla & Foxx-Ornestein, 2014). The pathophysiology of normal-transit constipation is similar to IBS-C (Rey et al., 2014) which is traditionally believed to involve altered bowel motility and hypersensitivity although the precise mechanisms of the symptoms remain unclear (Yang & Ma, 2017). Evacuation constipation is commonly due to dysfunction of the pelvic floor and anal sphincters (Rao et al., 1998) while slow-transit constipation is characterized by significant increased total bowel transit time (Yang & Ma, 2017). There is evidence for absence or reduction in the number of ICCs in slow-transit constipation (Tong et al., 2004; Lyford et al., 2005; He et al., 2000; Wang et al., 2008). However, the pathophysiology underlying constipation is multifactorial and remains poorly understood (Andrews & Storr, 2011; Yang & Ma, 2017; Said, 2018). It is often not easy to distinguish chronic constipation from IBS-C, and it has been proposed that they actually represent a single condition with patients situated at various sites along a pain and discomfort spectrum (Costedio et al., 2010).

Altered 5-HT signaling in patients with chronic constipation is well documented although studies have yielded somewhat contradictory results. Chronic constipation has been associated with increase in TPH1 transcript, 5-HT content, and 5-HT release under basal and stimulated conditions (Costedio et al., 2010). 5-HT levels were significantly elevated in the sigmoid colon in patients with severe idiopathic constipation as well (Lincoln et al., 1990). The number of EC cells and SERT transcript levels were not altered in a study on chronic constipation (Costedio et al., 2010); however, El-Salhy and colleagues reported that the number of 5-HT-immunoreactive cells per unit area of epithelial cells is lower in colons from patients with slow transit constipation (El-Salhy, 1999). Therefore, findings do not provide direct evidence for a causal relationship between 5-HT decrease and chronic constipation; nevertheless, in studies that provide evidence for increased level of 5-HT in

chronic constipation, it is possible that elevated synthesis and release of 5-HT in the colonic mucosa could contribute to constipation due to receptor desensitization. In vitro studies have shown that exposure of guinea pig distal colon to desensitizing concentrations of 5-HT or to fluoxetine (SERT inhibitor), decreases the rate of propulsive motility (Costedio et al., 2010) indicating that this could be a possible mechanism involved in chronic constipation.

On balance, there is enough evidence to conclude that altered 5-HT signaling is an important factor in diseases affecting the GI tract. The therapeutic drugs that target selective modulation of 5-HT activity including SSRIs, 5-HT_3 and 5-HT_4 antagonists and agonists, have been used in the treatment of functional GI disorders, however, they have been associated with adverse side effects (Spiller et al., 2008; Cole et al., 2004) emphasizing the need for more studies on the impact of 5-HT in GI pathophysiology (Spiller et al., 2008).

5-HT SIGNALING & GASTROINTESTINAL MOTILITY

The role of 5-HT in GI motility has been riddled with controversy although it has been studied for decades. In the early 1950s Bulbring & colleagues showed that 5-HT is released from the mucosa at a similar time as peristalsis occurred in the guinea pig small intestine. This was inferred as EC-cell-derived 5-HT having an important role in initiating peristalsis (Keating & Spencer, 2018; Banskota et al., 2018). Later this idea was challenged through studies that failed to correlate temporal release of 5-HT with propagating neurogenic motor patterns in the guinea pig intestine and distal colon using real time amperometry (Spencer et al., 2011; Keating & Spencer, 2010).

Additionally, other investigators revealed that the absence of the mucosa by dissecting it away did not prevent peristalsis (Keating & Spencer, 2018; Gershon, 2013; Banskota et al., 2018). For instance, Keating & Spencer (2010), showed that removal of mucosa, submucosa and submucosal plexus did not prevent spontaneous generation of peristalsis in

mouse isolated colon. In vivo studies on gut motility in the absence of the enzyme TPH1 also demonstrated that deletion of TPH1 did not inhibit GI transit in conscious mice (Yadav et al., 2010; Li et al., 2011; Keating & Spencer, 2018). Therefore, it was postulated that the EC-cell-derived 5-HT is not a prerequisite for generation of peristalsis, and it is not necessary to initiate neurogenic motor patterns.

Nevertheless, in vitro analysis of propulsive motility in the colon from the same TPH1 depleted mice indicated that 5-HT released by EC cells facilitates normal propulsion. In the knock out animals, reflex responses to distension were significantly reduced and only larger fecal pellets were propelled (Heredia et al., 2013). Other studies demonstrated that genetic removal of EC cell 5-HT synthesis, deletion of SERT or the removal of the mucosa by dissection results in "abnormalities" in the neurogenic motor patterns (Keating & Spencer, 2018; Smith et al., 2015). Keating & Spencer (2010) reported that the properties of motor patterns were different after the removal of mucosa. Li et al., (2011), found out that total GI transit time was significantly longer in both TPH2 and TPH1/2 knock out animals compared to the wild type littermate controls of each. In addition, recent studies on short chain fatty acid (SCFA)-induced 5-HT release demonstrated that TPH1 knock out mice had "abnormal" propulsive motor patterns and were unable to sense and propel the smaller fecal pellets. Administration of 10mM butyrate failed to increase the frequency of neurogenic propulsive motor patterns in these mice, suggesting that this response requires mucosal 5-HT (Vincent et al., 2018).

Electrophysiological studies of guinea pig ileum also indicate that mucosal terminals of AH neurons can be stimulated by mucosal 5-HT. These neurons have cell bodies in the myenteric plexus and can be stimulated via 5-HT receptors (Bertrand et al., 1997, 2000; Bertrand & Bornstein, 2002; Gwynne et al., 2014). According to Bertrand et al., (2000), mucosal 5-HT also evokes slow excitatory postsynaptic potentials (EPSPs) in myenteric neurons. These slow EPSPs increase the firing of distension sensitive neurons; thus, enhancing reflexes evoked by distension. Most slow EPSPs in myenteric neurons are

blocked by a specific tachykinin receptor antagonist (Alex et al., 2001; Johnson & Bornstein, 2004; Gwynne et al., 2014) which suggests that luminally applied 5-HT facilitates propulsive reflexes via the release of a tachykinin.

Although the role of endogenous 5-HT in *initiating* peristalsis has been controversial, there is no doubt that 5-HT is a potent *modulator* of neurogenic motor patterns and gastrointestinal transit in the gut wall (Martin et al., 2017; Keating & spencer, 2010, 2018; Spencer et al., 2011; Gwynne et al., 2014). Therefore, the fact that distention-induced neurogenic motor patterns can be evoked without the involvement of EC-cell derived 5-HT does not negate its important role in regulation of colonic motor function (Heredita et al., 2013; Bornstein, 2012; Vincent et al., 2018).

PROKINETIC EFFECTS OF 5-HT_4 RECEPTOR AGONISTS

5-HT_4 receptor (5-HT_4R) agonists have been known to enhance gastrointestinal motility via acting on neuronal 5-HT_4Rs resulting in the increase of acetylcholine release from presynaptic myenteric neurons and subsequently stimulating smooth muscle contraction. Several 5-HT_4R agonists have been shown in animals to induce intestinal secretion and propulsion and in humans to facilitate peristalsis (Mawe & Hoffman, 2013; Spiller, 2008; Hoffman et al., 2012; Gershon, 2013).

The neuronal 5-HT_4R pathway model is supported by a number of investigations that show 5-HT_4Rs are located on enteric nerve terminals (Hoffman et al., 2012). Additionally, bath application of 5-HT_4R agonists increases peristalsis in organ bath studies of animal models such as guinea pig, mice and rat colon (Hoffman et al., 2012; Tonini et al., 1989; Galligan et al., 2005; Mawe & Hoffman, 2013). However, it is important to mention that there are other studies that have failed to detect any alternation in propulsive motility after application of 5-HT_4R agonists serosally (Hoffman et al., 2012). Moreover, transgenic mice

that lack 5-HT_4Rs show slowed colonic motility indicating that these receptors are physiologically activated to regulate propulsive motility (Mawe & Hoffman, 2013).

In addition to animal studies that support the neuronal mechanism of action of 5-HT_4R agonists, many randomized clinical trials have demonstrated that "oral" 5-HT_4Rs, which act via the neuronal 5-HT_4R pathway, are effective therapeutic agents for promoting gut motility in humans. For instance, studies report that oral prucalopride can stimulate contractions in colonic longitudinal smooth muscles through acetylcholine release via activation of 5-HT_4Rs on presynaptic neurons as well as causing relaxation of human colonic rectal circular smooth muscles (Wong et al., 2010). Several placebo-controlled clinical trials report that a significant percentage of people who took oral prucalopride showed improvement in at least one clinical efficacy endpoint such as the number of spontaneous bowel movements per week (Tack et al, 2013).

Nevertheless, data suggests that besides stimulating neuronal 5-HT_4Rs, 5-HT_4R agonists can also act through epithelial 5-HT_4Rs in the colonic mucosa (Hoffman et al., 2012). This is possible because 5-HT_4Rs are also expressed in all epithelial cells of the mucosal layer including enterochromaffin cells of the mouse and human colon (Galligan et al., 2005; Grider et al., 1996; Hoffman et al., 2012; Mawe & Hoffman, 2013). 5-HT_4R transcripts were detected in all regions of the human intestinal mucosa, with the highest level in the terminal ileum (Hoffman et al., 2012). Hence, another mechanism of action of 5-HT_4R agonists could be through activation of this class of 5-HT_4Rs. Molecular, histological and amperometry data from mouse and human mucosal biopsies as well as motility studies of rat and guinea pig colon have shed light on the mucosal mechanism of action of 5-HT_4R agonists (Hoffman et al, 2012). Hoffman et al., (2012) reported that 5-HT_4Rs are expressed on two subtypes of epithelial cells; EC cells and goblet cells. Other studies demonstrated that they are also expressed on enterocytes (Mawe & Hoffman, 2013). Examining 5-HT as an oxidation current in the presence of cisapride (5-HT_4R agonist), demonstrated that its mucosal application induced 5-HT release from EC cells which was inhibited by a 5-HT_4R

antagonist in the guinea pig distal colon and in mice. This response persisted after application of tetrodotoxin (TTX) which indicates that cisapride stimulated 5-HT release from the mucosa by directly activating EC cells rather than via a neural mechanism (Hoffman et al., 2012).

On the other hand, activation of epithelial 5-HT_4Rs resulted in mucus secretion from goblet cells which could be visualized as large vacuoles in the epithelial layer, referred to as cavitation (Hoffman et al., 2012; Mawe & Hoffman, 2013). Mucus release was TTX insensitive and 5-HT_4R antagonist sensitive. In addition, mucosal application of tegaserod (partial 5-HT_4R agonist, previously known as HTF 919) stimulated goblet cell cavitation in guinea pig distal colon in a TTX insensitive and antagonist sensitive manner which provided further evidence that 5-HT_4R agonists have a mucosal site of action. Pellet propulsion velocity in response to luminal vs. serosal application of naronapride (5-HT_4R agonist) in guinea pig distal colon also revealed that intraluminal naronapride significantly increased the rate of propulsive motility while addition of naronapride to the bathing solution did not have the same effect (Hoffman et al., 2012).

Other studies have demonstrated that luminal application of 5-HT_4R agonists promotes propulsive motility. For instance, intraluminal perfusion with 5-HT_4R agonists, tegaserod and prucalopride, increased the velocity of pellet propulsion in guinea pig distal colon (Jin et al.,1999). The effect of 5-HT_4R agonists were dose-dependent with pellet propulsion velocity increasing significantly in higher concentrations. Application of 5-HT_4R antagonists reflected a similar concentration dependent manner of decrease in pellet propulsion velocity (Jin et al., 1999). Mucosal application of 5-HT_4R partial agonist tegaserod also promoted peristalsis in human jejunal and rat and guinea pig colonic segments (Grider et al.,1996).

Despite the clinical effectiveness of “oral” 5-HT_4R agonists, classical 5-HT_4R agonists such as tegaserod, cisapride and mosapride have been associated to serious adverse side

effects including but not limited to drug induced arrhythmia and sudden cardiac arrest due to their high selectivity for human cardiac hERG potassium channels (Manabe et al, 2010; Guidicessi et al., 2018). Therefore, cisapride and tegaserod, have already been removed from the market due to concerns associated with cardiovascular adverse events (Tack et al., 2012; Guidicessi et al., 2018). However, even among the currently available prokinetics, the safety of some drugs is still not fully confirmed. For instance, Metoclopramide a dopamine receptor antagonist which is still extensively used as an intravenous prokinetic drug to treat delayed gastric emptying and to facilitate early enteral feeding (Van der Meer et al., 2014), has been reported in FDA adverse event reporting system (FAERS) as of 2018 to have caused 17356 cardiovascular adverse events out of which 1129 resulted in death. Considering that post-marketing adverse event reporting is subject to bias and underreporting, the real number could be even higher (Guidicessi et al., 2018).

Prucalopride (previously known as R093877 and R108512) is the first representative of the benzofuran class to have been reported to have low potency for hERG potassium channel blockade and high 5-HT_4R agonism while being devoid of non-specific inhibitory activity at other 5-HT receptors (Wong et al., 2010; Briejer et al., 2001; Chey WD et al., 2011). Binding studies have demonstrated that it has a high affinity for the human 5-HT_4a and 5-HT_4b receptors, with mean pK(i) of respectively 8.60 and 8.10. Therefore, in contrast to other 5-HT_4R agonists, affinities of prucalopride for all other receptors are low, yielding an almost 300-fold selectivity for the 5-HT_4Rs (Tack & Corsetti 2012). Agonist binding to the G protein-coupled 5-HT_4 receptor activates adenylate cyclase and increases intracellular cyclic adenosine monophosphate (cAMP) (Wong et al., 2010).

Nevertheless, oral prucalopride had been limited to the treatment of chronic constipation in only adult female patients in whom laxatives had failed to provide adequate relief (Blandizzi et al., 2012). Recently, in December 2018, oral prucalopride with the generic name of “Motegrity” was approved by the FDA to be used for the treatment of chronic idiopathic constipation. However, in general, most clinical trials of this compound were

focused on primary constipation excluding other types of constipation (Sajid et al., 2016). Additionally, trials in the elderly, the high-risk population that manifest the highest incidences of chronic constipation while usually having associated renal and cardiovascular co-morbidities, are still deficient (Sajid et al., 2016). In clinical trials that did include older subjects (> 65 years old), plasma concentrations of prucalopride were ~30% higher than in younger adults (Wong et al., 2010). Although oral prucalopride has the lowest potency for cardiac hERG blockade compared to classical 5-HT_4R agonists and been reported safe by various extensive cardiac monitoring studies, most of these trials were only 4 weeks in duration and FAERS database shows that it has caused at least 27 serious post-marketing adverse events as of 2018 (Guidicessi et al., 2018).

LUMINAL SENSING & COLON TARGETED DRUG DELIVERY

Recently, various luminally acting agents have been developed that exert their primary impact on bowel-related disorders through local or luminal effects. Luminally acting agents can modify colonic motility and mucosal absorption and secretion (Menees et al., 2012; Yang & Ma, 2017). They lead to physiological effects through modulation of the luminal microenvironment and/or a variety of receptor-mediated pathways, usually with multiple modes of action (Menees et al., 2012). In general, luminally acting agents for constipation focus on either enhancing motility of the colon (prokinetic) or increasing secretion (pro-secretory) in the colon (Yang & Ma, 2017). However, they can improve constipation symptoms by a number of various mechanisms that include increasing stool bulk, reducing stool viscosity, providing an osmotic load, stimulating smooth muscle contractions, increasing intestinal secretion, promoting chloride secretion, increasing colonic bile acid concentrations and/or altering the gut microbiota (Menees et al., 2012).

As mentioned in previous sections, IPANs and some vagal and pelvic afferent endings come into close proximity to the mucosal epithelium although they never penetrate into the lumen (Vanner et al., 2016; Mawe & Hoffman, 2013). This exposes them to chemicals absorbed across the mucosal epithelium or released from enteroendocrine cells whose

apical membrane is exposed to luminal content. This is similar to the relationship between taste buds and gustatory mucosal afferents in the mouth (Vanner et al., 2016). Remarkably, the same G-protein-coupled receptors and ion channels found in the mouth are also expressed within the GI tract. This relationship between the afferents and the mucosal epithelium is important for controlling reflex responses on motility and secretion in the digestive tract (Vanner et al., 2016). EC cells are one of a diverse family of enteroendocrine cells that are scattered in the GI mucosa and their mediators can act in a paracrine manner on afferent nerve fibers (Vanner et al., 2016; Kendig & Grider, 2015; Mawe & Hoffman, 2013). The evidence for the action of 5-HT_4R agonists through luminal 5-HT_4Rs and EC-cell-derived release of 5-HT discussed in previous sections, indicates that colonic mucosa could be a site for drug delivery of 5-HT_4 receptor agonists. Prucalopride administrated directly to the mucosa has the potential to become the preferred prokinetic for the treatment of constipation and abdominal pain in visceral hypersensitivity as it restricts adverse side effects while promoting colonic motility (Hoffman et al., 2012; Mawe & Hoffman, 2013).

Besides minimizing systemic side effects, another advantage of the delivery of drugs directly to the colon without them being first absorbed in the upper GI tract, is that it allows for a higher concentration of the drug to reach the colon via mucosa (Amidon et al., 2015; Kumar et al., 2010; Philip et al., 2010; Hoffman et al., 2012). Oral colon-targeted drug delivery (CDD) systems are the more popular form of colon-specific drug delivery compared with the rectal CDDs due to their convenience, flexibility in manufacturing and design and ease of administration (Philip et al., 2010). Such oral formulations are designed to avoid gastric release. Currently, there are no standardized colon release media (Wahlgren et al., 2019). Traditionally, colon targeted delivery can be achieved via pH-controlled release systems. These formulations are based on the difference in pH along the GI-tract and film coatings of different polymers that dissolve at different pH levels (Wahlgren et al., 2019). The major drawback of these systems is the large intra-individual variation in pH along the GI-tract, especially for patients with GI-tract diseases. Due to the large variation of pH in the GI-tract, CDDs that are only dependent on pH difference have a

major risk of both premature drug release and no release at all (Maroni et al., 2017; Wahlgren et al., 2019).

CDDs can also be based on time-controlled release which relies on slow dissolving polymer films (Hu & Peppercorn, 2008). In such systems, the polymer which is responsible for the release should not be sensitive to pH, enzymes or other components of the lumen such as bile salts. These types of formulations are partly affected by transport of water and components in the GI tract fluid into the formulation. Moreover, the rate of gastric emptying also affects the release of the time delayed formulations in the colon. It is well known that the gastric emptying time varies significantly for larger objects such as a tablet (Wahlgren et al., 2019). Time-dependent and pH-sensitive systems can be integrated for instance in a pulsincap that uses both strategies (Amidon et al., 2015).

The large intestine is colonized by a vast number of bacteria creating a complex microbial community that affect the release from oral formulations targeting the colon (Wahlgren et al., 2019). Therefore, a more recent strategy is to use excipients that are degraded by the microorganisms in the colon. The bacteria of the colon produce a large repertoire of enzymes including amylases, pectinases and β-d-galactosidases to mention a few. Such enzymes have the ability to hydrolyze polysaccharide-specific bonds. Therefore, polysaccharides that can only be degraded by the microorganisms in the colon such as dextran, inulin and chitosan are used as coatings for this purpose (Wahlgren et al., 2019).

With the advancement of nanotechnology, various types of nanoparticle formulations for colon delivery have also been investigated by numerous researchers. These can be based on lipids, chitosan or silica, and the nanoparticles can target inflamed mucosa (Zhang et al., 2017). In spite of the excitement about these novel formulations, there is a need for more attention to the practical design of the final dosage forms to successfully deliver the nanoparticles to the colon (Wahlgren et al., 2019).

COLO-ANAL MOTOR COORDINATION IN THE DEFECATION REFLEX

For normal defecation to occur, the aboral mass movement of the colonic contents must occur in coordination with anal sphincter relaxation. This coordination mechanism is referred to as “the colo-anal reflex” (Sintusek et al., 2018). The colo-anal reflex is defined as a decrease in pressure of the internal anal sphincter occurring simultaneously with aboral propagation of high amplitude propagating pressure waves (HAPWs, De Schryver et al., 2003; Chen et al., 2017), also called high amplitude contractions (HAPCs, Bassotti & Gaburri, 1988) or high amplitude propagating sequences (Dinning et al., 2004), in the human colon (Sintusek et al., 2018). The mechanisms by which anal sphincter relaxation occurs in coordination with aboral colonic mass movements are still poorly understood (Rodriguez et al., 2012). However, recent studies have demonstrated that this coordination mechanism is integral to our understanding of the physiology of defecation (Bajwa et al., 2009; Sintusek et al., 2018).

Animal studies showed that the anal sphincter is under the influence of both the autonomic nervous system and the enteric nervous system despite exhibiting spontaneous myogenic tone (Sintusek et al., 2018; Bassoti et al., 1996). The ano-rectum is composed of four muscles including the internal and external anal sphincters, the conjoined longitudinal muscles, and levator ani muscle. The internal anal sphincter is a circular smooth muscle and it is innervated extrinsically via the sacral nerves while the external anal sphincter is a skeletal muscle and it is innervated by the pudendal nerve and the perineal branch of the forth sacral nerve (Sintusek et al., 2018; Bajwa et al., 2009). The anal sphincters together contribute to the sphincter tone which is on average between 25 and 120 mmHg (Sintusek et al., 2018).

The colo-anal reflex could be evoked with the onset of HAPW in the left or right colon (Rodriguez et al., 2012; Sintusek et al., 2018); but in most cases, internal anal sphincter (IAS) relaxation started when peristalsis was migrating through the left colon (Rodriguez

et al., 2012). Based on its timing and duration, this reflex should not be confused with the transient relaxation of the internal anal sphincter in response to rectal distention called the "recto-anal inhibitory reflex" (RAIR; Sintusek et al., 2018; Rodriguez et al., 2012).The recto-anal inhibitory reflex (RAIR) which is triggered by rectal distention is a motor pattern mediated by inhibitory neurons of the intrinsic enteric nervous system releasing nitric oxide whose action is dependent on the presence of a network of ICCs (De Lorjin et al., 2005; Sintusek et al., 2018). The RAIR is typically absent in Hirschsprung's disease, where the myenteric plexus ganglia are absent (Sintusek et al., 2018; Sangwan et al., 1998).

Malcolm et al., (2000) noted that IAS relaxation induced by yohimbine, clonidine or meal in healthy adults occurred just before the recorded onset of HAPW in the descending colon (14 ± 4s) and significantly before (88 ± 7s) the arrival of the HAPW in the rectum. Hence, they demonstrated that sphincter relaxation was mainly associated with the onset of the HAPWs suggesting a coloanal reflex that may facilitate defecation during mass movements independently of the recto-anal inhibitory reflex. However, in their study HAPWs were recorded only in the descending colon. Hence, it is highly probable that the onset of HAPWs in the proximal colon could not be detected.

Rodriguez et al., (2012) noted that in 64% of HAPW events in pediatric patients evaluated for intractable constipation, baseline, bisacodyl or meal induced IAS relaxation started when the HAPWs propagated into the left colon. However, a third of sphincter relaxation events occurred while the HAPWs were still in the right colon. They noticed that the colo-anal reflex may start when HAPWs originate proximal to the splenic flexure or when their amplitude is lower than 75 mmHg. Comparison between the HAPW associated anal sphincter relaxation and RAIR associated relaxations revealed that the percentage and duration of relaxation were significantly larger in the former compared to the latter, suggesting that the colo-anal reflex may have physiological importance. Consistent with Malcolm et al., (2000), due to the strong temporal association of HAPWs with the IAS

relaxation, they hypothesized that the colo-anal reflex is neurally mediated. However, the neural pathways involved in this process were not identified.

To further investigate the physiology of the colo-anal reflex, Sintusek et al., (2018) studied the colo-anal reflexes of a group of children with chronic constipation and Hirschsprung's disease, who had either disrupted continuity of the colon due to colostomy formation or absence of RAIR as sign of an abnormal anal myenteric pathway. HAPWs were recorded postprandially and after bisacodyl administration. The colo-anal reflex persisted even when the enteric nervous system innervation was disrupted with a sigmoidoctomy and formation of colostomy. The reflex was also present in two patients with Hirschsprung's disease. This suggests that the colo-anal reflex is mediated via the extrinsic nervous system (Sintusek et al., 2018). Furthermore, they demonstrated that in patients with disrupted bowel continuity, the onset of the colo-anal reflex was associated with arrival of the HAPWs into the proximal colon (right, mainly mid transverse colon), whereas in the control group, the colo-anal reflex was initiated with the progression of the HAPWs to descending/sigmoid colon (left colon).

Overall, data strongly suggests that the colo-anal reflex is an important aspect of defecation. Therefore, with the increasing use of the High-Resolution Colonic Manometry (HRCM) as a diagnostic tool for colonic motility disorders, it is expected that documentation of the presence or absence of the colo-anal reflex and/or abnormalities associated with coordination between anal sphincter relaxation and propagating motor patterns, will be an important part of the manometric assessment (Sintusek et al., 2018).

HYPOTHESES & AIMS

The overall aim of this thesis was to investigate the regulation of colonic motor activity and pharmacological involvement in colonic motor function via "luminal" stimulation. The focus of these studies was on the hypothesis that activation of luminal 5-HT_4Rs could

modulate peristaltic motor activity via the release of 5-HT from EC cells into the lamina propria and the lumen. Hence, it would be possible to increase propulsive activity of the colon via intraluminal administration of prucalopride, a highly selective 5-HT_4R agonist. To improve understanding of the intricacies of the physiology of defecation and in light of previous findings discussed above, all aspects of propulsive motility including the colo-anal reflex and the simultaneous pressure wave (SPW) as a common yet largely ignored motor pattern in the human colon (Rao et al., 2001; Chen et al., 2017), were also characterized and studied.

Animal Studies

Two series of animal studies were performed. The purpose of animal studies was to increase our understanding of the prokinetic effects of intraluminal prucalopride and to deepen our ability to interpret results from future human High-Resolution Colonic Manometry (HRCM) experiments.

The first project (chapter 2) aimed at evaluating changes in colonic motor patterns of the whole isolated proximal and mid rabbit colon ex vivo in response to activation of luminal 5-HT_4Rs in the colonic mucosa via intraluminal administration of prucalopride and exogenous 5-HT. The hypothesis was that activating luminal 5-HT_4Rs via prucalopride and exogenous 5-HT increases various characteristics of colonic motor patterns possibly through 5-HT outflow from EC cells into the lamina propria and subsequent stimulation of the myenteric neurons.

The second project (chapter 3) investigated the effects of intraluminal prucalopride in the presence of simulated fecal impaction as a common acute complication in constipation. The large bulk of content in the colon was mimicked by a fixed inflated rapid barostat bag which was modified to fit the rabbit colon and distend it to almost double its baseline diameter.

Human Studies

The first human study aimed at characterizing colo-anal motor coordination as an integral part of defecation and SPWs as common propulsive motor patterns in healthy volunteers using HRCM with a custom made 84-sensor water perfused catheter (1cm spacing) which allowed for increased precision. It also explored generation and/or changes in characteristics of various motor patterns in vivo in response to different luminal interventions including intraluminal bisacodyl, meal and balloon distention. The second HRCM research is a case study aiming at exploring the effects of intraluminal prucalopride in a healthy volunteer in vivo. The hypothesis was that intraluminal prucalopride could increase propulsive motor activity via the release of EC-cell-derived 5-HT as described in the rabbit animal model.

SECTION I

RABBIT ANIMAL MODEL STUDIES

CHAPTER 2- INTRALUMINAL PRUCALOPRIDE INCREASES PROPULSIVE MOTOR ACTIVITY IN THE ISOLATED PROXIMAL AND MID RABBIT COLON

INTRODUCTION

As mentioned in chapter 1, serotonin plays a role as a neurotransmitter within the myenteric plexus, and as a paracrine substance synthesized in enterochromaffin cells within the epithelial cell layer of the intestinal mucosa (Mawe & Hoffman, 2013; Kendig & Grider, 2015). EC cells can be stimulated from the luminal side through their many receptors and transporters. EC cells in the mouse colon express sugar transporters (SGLT1, GLUT1, GLUT2, GLUT5), sugar sensors (T1R3, SGLT3), G protein-coupled receptors for fatty acids (FFAR1-4, GPR84), amino acids (GPR92) and lipid amides (GPR119; Martin et al., 2017). Luminal butyrate elicits peristaltic activity (Girder et al., 2007; Vincent et al., 2018), and activation of muscarinic receptors evokes 5-HT release and motor responses in a TTX insensitive manner (Bertrand, 2006).

It was previously discussed that morphological evidence exists for expression of 5-HT_4R at the apical surface of EC cells in mouse, rat, guinea pig, and human mucosal epithelium (Mawe & Hoffman, 2013; Hoffman et al., 2012). Studies also demonstrated that enteric neurons project into the mucosa with nerve endings that form synaptic like contacts with EC cells, and 5-HT strongly activates these nerve endings (Bellono et al., 2017). Therefore, the EC-cell-derived 5-HT is regarded as a potential target for the treatment of dysmotility disorders (Costedio et al., 2007).

The 5-HT containing granules of EC cells are predominantly located at the basal surface which release into the interstitial space of the lamina propria (Mawe & Hoffman, 2013); however, 5-HT is also released in the lumen in response to mechanical stimuli, vagal nerve

stimulation, food intake, luminal acidification (Tsukamoto et al., 2007) and signals from the gut microbiota which can enhance peristalsis (Hata et al., 2017). One of the functions of luminal 5-HT may be to lower the threshold of pressure required to elicit peristaltic activity (Gwynne et al., 2014). 5-HT is also released in the lumen in response to an increase in intraluminal pressure (Tsukamoto et al., 2007).

With the emergence of HRCM, efforts are underway to evaluate human motor patterns as clinical biomarkers of healthy colonic motor activity (Chen et al., 2018; Chen et al., 2017; Dinning et al., 2015; Corsetti et al., 2017). In high resolution manometry, colonic motility is evaluated as pressure patterns. The rabbit colon has been used previously to investigate the relationship between circular muscle contraction patterns and colonic pressure waves by Huizinga and colleagues (Quan et al., 2017). In the present study, the same rabbit model is used to evaluate colonic motor patterns and associated pressure development involved in the prokinetic effects of intraluminal prucalopride. Whole organ motility studies are essential to unravel the local effects of 5-HT_4R agonists since motor patterns are clinically evaluated as biomarkers of healthy colonic motor function (Chen et al., 2018; Chen et al., 2017; Dinning et al., 2016; Liam et al., 2014). The aim of this study was to evaluate the pharmacological effects of intraluminal prucalopride as a highly selective 5-HT_4R agonist as well as potential physiological effects of intraluminal 5-HT on motility patterns of the rabbit colon. In addition, it is expected that this study helps with understanding the effects of luminal prokinetics on human colon manometric pressure patterns.

MATERIALS AND METHODS

Animals: The rabbit proximal and mid colon are taeniated similar to the human colon which makes it an ideal model for gastrointestinal studies (Quan et al., 2017; Lentle et al., 20008). All experiments were approved by the Animal Research Ethics Board at McMaster University (Animal Utilization Protocol 14-12-49). All methods were performed in accordance with the guidelines and regulations of the Animal Research Ethics Board of McMaster University and the Canadian Institutes of Health Research. The whole proximal

and mid colon (~ 26 cm) was removed from adult New Zealand white rabbits (N= 32, female=2, male=30) of 1.6–2.5 kg body weight, euthanized with 2ml sodium pentobarbital per 4.5kg body weight through ear vein injection.

Tissue preparation: After a midline incision, the whole proximal colon from the 3-taeniated end to the 1-taeniated was removed and placed in room temperature continuously oxygenated (5%CO_2 and 95%O_2) Krebs solution (pH 7.3~7.4) of (mM) 120 NaCl, 5.9 KCl, 15.5 $NaHCO_3$, 1.2 NaH_2PO_4, 0.1 citric acid, 0.1 aspartic acid, 2.5 $CaCl_2$, 1.2 $MgCl_2$, and 6 glucose. The mesentery and cecum were removed, and colonic content was gently flushed out using a gavage needle.

Experimental setup: The prepared organ was cannulated using a steel tube (made from an 18-gauge needle) with a plastic collar and mounted in an organ bath containing 2 liters of continuously oxygenated Krebs solution. A heating tube connected to a hot water bath, was placed in the organ bath and used to adjust the temperature to 35°C. A row of 10 cameras were mounted 65 cm above the organ bath by means of a horizontal L-bar supported by two clamp stands. Camera signals were recorded by a 16-channel digital video recorder at 30 frames per second (Parsons & Huizinga, 2015).

Drugs were perfused in the lumen at a rate of ~1 ml/min via a proximal polyethylene (PE) inflow tube (ID 1.19mm, OD 1.7mm) connected to a digital peristaltic pump (Peri-Star ™ Pro). The anal cannula was attached to a PE outflow tube fixed at the edge of a beaker which was placed on top of an adjustable crank to ensure the height of the outflow tube remained at the level of the colon so that it would not change the intraluminal pressure. The intraluminal pressure and the motor activity were determined by the rate of inflow. Slow perfusion rate was used to avoid change in luminal distention while optimizing the survival of the epithelial cells. The perfusion rate was kept constant during the experiment to ensure that the propulsive motility was exclusively induced by the prokinetic test compounds.

After a 30-minute acclimatization, motility recordings were initiated. Each session was recorded for ~ 30 minutes.

Intraluminal pressure recordings took place simultaneous with video recordings of contractile activity in 19 experiments. Pressure was measured using a PE catheter (ID 1.57 mm, OD 2.08mm) connected to a pressure transducer (Argon Medical Devices, Inc.) attached to a 10-ml syringe filled with water which was fixed ~ 35 cm above the level of the colon. The transducer was connected to a Grass amplifier (Astro-Med, Inc., RI, USA) which amplified the initial signal and was then digitized using a MiniDigi 1B (Axon CNS molecular devices). After calibration, the catheter was inserted into the proximal colon through another PE tube fixed alongside the oral cannula to minimize possible movement of the catheter during the experiment. The catheter's tip was positioned at the section of the colon that was being recorded by the third digital camera ~ 4cm from the oral end to measure intraluminal pressure of motor patterns at the time of initiation.

Mapping and analysis: Changes in propulsive activity were studied through both the colonic diameter and intraluminal pressure changes. For spatiotemporal mapping of contractile activity, data acquisition occurred through Microsoft LifeCam software. Spatiotemporal maps were then created from video recordings. A "spatiotemporal map" is an image which represents the motor activity based on the change in colon diameter over time (i.e. a D-map). Colon width is calculated at each pixel along the colon's length (image Y-axis), for each video frame (image X-axis) with black signifying contraction or decrease in colon diameter and white relaxation or increase in colon diameter. Changes in colon diameter associated with motor patterns were quantified using ImageJ with plugins developed by Sean Parsons (Parsons & Huizinga, 2015).

The reduction in the diameter of the colon is due to circular muscle contraction. The degree of colon diameter reduction corresponding to the force of contraction is calculated based on the difference between the maximum and the minimum colon diameter associated with

the motility pattern. The stronger the contraction, the higher the degree of colon diameter reduction. This is referred to as "contraction amplitude". The motor pattern was selected freehand on ImageJ and different parameters of the motor patterns were measured including contraction amplitude, length of propagation, frequency, velocity and duration. Pressure recordings were analyzed using Axoscope 10 (Molecular devices, CA, USA) software. Spatiotemporal and pressure maps were matched, and pressure amplitude associated with motility patterns was measured. "Pressure amplitude" shows the degree of change in intraluminal pressure associated with a motility pattern. Since the propulsive motor patterns are pan-colonic and the pressure measurements are performed with a single sensor, the association between contraction and pressure amplitude is correlative. 3-D figures were made using MATLAB (Mathworks, Natick, MA, United States).

Statistics: Data is presented as mean ± SEM. Significant difference between motility results and intraluminal pressure was determined by t-test or one-way ANOVA as stated in table footnotes using Prism 7 software (GraphPad, United States), $P < 0.05$ was considered significant.

Test compounds: Prucalopride (4-Amino-5-chloro-2,3-dihydro-N-[1-(3-methoxypropyl)-4-piperidinyl]-7-benzofurancarboxamide) and serotonin hydrochloride **(**3-(2-Aminoethyl)-5-hydroxyindole hydrochloride) were purchased from Sigma-Aldrich (St Louis, MO, USA). GR113808 (1-methyl-1*H*-indole-3-carboxylic acid, [1-[2-[(methylsulfonyl)amino] ethyl]-4-piperidinyl] methyl ester) was purchased from Tocris (Bio-Techne Canada). Prucalopride and GR113808 were dissolved in DMSO and serotonin hydrochloride was dissolved in distilled water. To avoid oxidation, new serotonin hydrochloride solutions were made up for each experiment.

Nomenclature: The nomenclature used for propulsive motor patterns in animal models of colonic motility is not consistent in the literature. The major propulsive motor pattern in all animal models is a pan-colonic rhythmic activity that has been called "colonic migrating

motor complex" (Dinning et al., 2012), "mass peristaltic event" (Lentle et al., 2008) or "giant contraction" occurring at a frequency around 0.5cpm in the mouse (Powel e al., 2003) and the rabbit colon (Quan et al., 2017) and up to 2cpm in the guinea pig (Costa et al., 2015). At a consensus meeting it was agreed to use the term "Colonic Motor Complex" (Corsetti et al., 2019). In the present study, we show that the characteristics of this motor complex are dramatically different at different levels of excitation. Here it is demonstrated that Long Distance Contractions (LDCs) and clusters of Fast Propagating Contractions (FPCs) are both expressions of the Colonic Motor Complex at different levels of excitation.

The rabbit colon exhibits motor patterns that are neurogenic and have been associated with propulsive activity (Chen et al., 2016). Neurogenic activity refers to the activity of the enteric nervous system resulting in specific motor patterns. In the rabbit, mouse and rat, we and others have identified the Long Distance Contractions (LDC) as the most forceful circumferential propulsive contraction evoked by distention that propels content down the colon (Chen et al., 2013; Chen et al., 2016; Lentle et al., 2008; Quan et al., 2017; Vincent et al., 2018; Yu et al., 2015; Kending et al., 2015). The propulsive nature of LDCs can be measured by their association with 3-5ml outflow of PBS (Chen et al., 2016). LDCs have a characteristic triangular shape in spatiotemporal maps due to the fact that the most proximal part of the colon remains contracted while the front of the contraction propagates anally, preceded by relaxation (Chen et al., 2016). LDCs are neurogenic in that they are abolished by TTX or hexamethonium (Lentle et al., 2008; Yu et al., 2015; Chen et al., 2016).

Fast propagating contractions (FPCs) are also called fast phasic activity (Lentle et al., 2008). FPCs are circular muscle contractions that occur at a higher frequency and propagate at a higher velocity compared to LDCs usually in antegrade direction. They are less forceful than LDCs and they may occur in clusters of several FPCs (Quan et al., 2017). Clusters of FPCs have been associated with 1-3 ml PBS outflow (Chen et al., 2016). Although single

FPCs are myogenic (Lentle et al., 2008), the formation of FPC clusters is neurogenic (Chen et al., 2016).

The myogenic activity in the rabbit is manifested through "ripples" which are slow-wave-driven ring contractions of the circular muscle, independent of the enteric nervous system (Chen et al., 2016). Since the proximal rabbit colon is haustrated, it exhibits haustral boundary contractions. Haustral boundary contractions (HBC) are slowly propagating circular muscle contractions dividing the colon into pockets of haustra. They usually propagate in the anal direction and interact with ripples (Chen et al., 2016). Several studies have concluded that the rhythmic propagation of the haustral boundaries is facilitated by ICC generated slow waves, an example of a neurally stimulus-dependent slow wave (Hanman et al., 2019). HBCs are segmental in nature due to their continuous interaction with ripples (Chen et al., 2016; Lentle et al., 2008; Huizinga et al., 2014). Haustral boundary contractions are neurogenic in that they are abolished by TTX or hexamethonium (Dinning et al., 2012; Chen et al., 2016; Lentle et al., 2008).

RESULTS

Effects of Intraluminal Prucalopride on Long Distance Contractions (LDCs) and Fast Propagating Contractions (FPCs)

Taking into account the most forceful expression of the colonic motor complex, the LDC, intraluminal prucalopride significantly enhanced LDC activity including its frequency, contraction amplitude, velocity and the degree of propagation along the whole proximal and mid colon (Table1, Fig 1 & 2C,D). At baseline, LDCs occurred at a low frequency of 0.09 ± 0.03 cpm with a contraction amplitude of 0.76 ± 0.05 cm. Intraluminal prucalopride, resulted in a dose dependant significant increase in contraction amplitude and frequency of LDCs; whereas the average LDC duration did not differ significantly (Table 1). The 5-HT_4 receptor antagonist GR113808 (10μM) inhibited the effects of intraluminal prucalopride (N=6) (Fig 1).

Table 1. Characteristics of LDCs in Response to Intraluminal Prucalopride

Long distance Contractions (LDCs)				
	Baseline (N=19, n=49)	**Intraluminal prucalopride 2μM (N=9, n=84)**	**Intraluminal prucalopride 5μM (N= 13, n=105)**	**Intraluminal prucalopride 10μM (N= 15, n=133)**
Frequency (cpm)	0.09 ± 0.03	0.34 ± 0.08 ***	0.35 ± 0.08 **	0.37 ± 0.05 ****
Contraction amplitude (cm)	0.76 ± 0.05	1.00 ± 0.01 ****/###	1.17 ± 0.02****/†††	1.20 ± 0.02 ****/$$$
Velocity (cm s $^{-1}$)	2.30 ± 0.13	2.82 ± 0.09 *	3.00 ± 0.08 ***	2.50 ± 0.08
Duration (s)	6.84 ± 0.37	6.90 ± 0.33	6.30 ± 0.3	7.67 ± 0.25 ††
Propagation length (cm)	16.50 ± 0.9	19.60 ± 0.6 *	19.80 ± 0.4 *	20.80 ± 0.3 *
% of propagation along the colon	64.30 ± 6.5	79.90 ± 4.2 *	78.06 ± 4.6 *	78.17 ± 2.3 *

Values are mean ± SEM. Significant difference determined by One-way ANOVA. * compared to baseline conditions (*P< 0.05, **P < 0.01, ***P< 0.001, ****P < 0.0001), † compared to the immediate lower concentration (††P<0.01, †††P<0.001), $ compared to the lowest drug concentration ($$$P< 0.001), # compared to both higher concentrations (###P < 0.001)

In 14/19 experiments, FPCs were present at baseline. In 9/14 experiments, single FPCs appeared at random (no regular rhythmicity was noted) with mean contraction amplitude of 0.42 ± 0.01 cm and propagating in antegrade direction (n=120). In 5/14 experiments, the rabbit colon exhibited clusters of several FPCs with mean contraction amplitude of 0.61 ± 0.04 cm (n=59) which was significantly higher than that of single FPCs indicating their stronger force of contraction (Fig 3D). These clusters could contain LDCs or transient LDC-like motor patterns (N=4). Administration of luminal prucalopride resulted in a significant increase in contraction amplitude of FPCs (Table 2). The frequency of single erratic FPCs significantly *decreased* after the administration of the lowest concentration of prucalopride (2μM) compared to baseline conditions whereas the frequency of FPC clusters significantly *increased* (Table 2).

Prucalopride at 5μM, resulted in a marked *decrease* of the frequency of FPC clusters compared with 2μM. This is because administration of higher doses of the drug prompted

the generation of more forceful motor patterns. Firstly, forceful FPCs were formed in cluster format in response to a lower dose (2μM) while further increase in drug concentration resulted in development of LDCs that gradually replaced the FPC clusters. This indicates a shift in colonic contractile activity towards developing the most forceful propulsive motor pattern (LDC) with increase in drug concentration (Table1,2; Fig1). Particularly, at higher concentrations of the drug (5μM and 10μM), single FPCs were not observed and only clusters of FPCs, at times with LDCs developing among them, emerged. This could happen regardless of the baseline already manifesting spontaneous FPCs (Table 2, Fig3). Similar to LDCs, FPCs did not develop after the administration of prucalopride in the presence of the antagonist GR113808 (10μM) (N=6) (Fig1).

Table 2. Characteristics of FPCs in Response to Intraluminal Prucalopride

	Single FPCs		**Cluster of FPCs**			
	Baseline (N=9, n=120)	**Intraluminal prucalopride 2μM (N=5, n=20)**	**Baseline (N=5, n=59)**	**Intraluminal prucalopride 2μM (N=3, n=95)**	**Intraluminal prucalopride 5μM (N=13, n=178)**	**Intraluminal prucalopride 10μM (N=15, n=157)**
Contraction amplitude (cm)	0.42 ± 0.01	0.68 ± 0.03 ****	0.61 ± 0.04 ****	0.80 ± 0.01 ††††	0.94 ± 0.02 ††††	0.90 ± 0.02 ††††
Frequency (cpm)	0.97 ± 0.40	0.14 ± 0.02 *	0.40 ± 0.13	1.08 ± 0.62 †	0.58 ± 0.2 #	0.41 ± 0.1
Velocity (cm s^{-1})	6.60 ± 0.20	8.4 ± 1.2 *	6.40 ± 0.30	9 ± 0.5 †	8.2 ± 0.31 †	8.4 ± 0.67 †
Duration (s)	3.40 ± 0.10	3.4 ± 0.3	3.60 ± 0.12	3.1 ± 0.16	3 ± 0.06	3.7 ± 0.2
Propagation length (cm)	20.8 ± 0.22	22.7 ± 0.73 **	22.27 ± 0.34	22 ± 0.40	21.2 ± 0.31	20 ± 0.33
% of propagation along the colon	81.5 ± 1.3	80.1 ± 3.6	80.7 ± 4.80	88.5 ± 4.00	80.7 ± 2.8	80 ± 2.3

Values are mean ± SEM. Significant difference determined by Student's t-test for single FPC data, and by one-way AOVA with multiple comparisons for cluster of FPCs. * compared to single FPCs at baseline conditions (*P< 0.05, **P < 0.01, ****P < 0.0001), † compared to baseline in cluster of FPCs (†P< 0.05, ††††P < 0.0001), # compared to the immediate lower concentration (#P <0.05).

The 5-HT_4 receptor antagonist GR113808 (10μM), was administrated in the lumen 30 minutes before prucalopride and intraluminal exogenous 5-HT. In 6/6 experiments, no LDCs were observed while FPC clusters were present in 2/6 experiments at baseline with the frequency of 0.63 ± 0.03 cpm, contraction amplitude of 0.57 ± 0.02 cm and velocity of 5.40 ± 0.46 cm s^{-1} propagating along the 56 ± 2.6 % of the length of the colon (n=38, Fig1I). Baseline activity remained unaffected by the antagonist alone. No LDCs were observed and characteristics of FPC clusters did not change significantly. They continued manifesting at the frequency of 0.60 ± 0.13 cpm with contraction amplitude of 0.60 ± 0.03 cm, velocity of 5.33 ± 0.34 cm s^{-1} propagating along the 58 ± 2.5 % of the colon (n=36, Fig1J).

Effects of Intraluminal exogenous 5-HT on Propulsive Contractions

In 7/7 experiments no LDCs were present at baseline while after administration of exogenous 5-HT inside the lumen, LDCs gradually developed and their frequency increased significantly in response to higher 5-HT concentrations. Additionally, the LDC contraction amplitude was significantly enhanced in higher 5-HT concentrations indicating their more forceful nature (Table 3, Fig 4). The velocity, duration and degree of propagation of LDCs along the colon remained unaffected (Table 3).

In 3/7 experiments FPCs were present at baseline with 2/3 experiments manifesting single FPCs. In 4/7 experiments high frequency single FPCs developed after the administration of intraluminal 5-HT (2μM) which were significantly faster and more forceful than baseline single FPCs (Table 3). They also propagated along a significantly higher percentage of the colon. In the first ~10 minutes after increasing the concentration of 5-HT to 5μM and 10μM, single FPCs remained present but they were significantly more forceful than baseline showing marked increase in contraction amplitude in a dose dependant manner. Furthermore, single FPCs were significantly faster at 5-HT 5μM and 10μM compared with baseline (Table 3).

In time, single FPCs disappeared and clusters of FPCs developed with significant increase in contraction amplitude in comparison with control (Table 3, Fig 5). However, the frequency of FPC clusters did not show any significant change with increase in 5-HT concentration. The 5-HT_4 receptor antagonist (GR113808 10μM) inhibited the effects of 5-HT on propulsive motor patterns. In 6/6 experiments no propulsive motor patterns were generated in the presence of the antagonist in response to intraluminal exogenous 5-HT (Fig 4).

Table 3. Characteristics of LDCs and FPCs as a Result of Intraluminal 5-HT

		Contraction amplitude (cm)	Frequency (cpm)	Velocity (cm s^{-1})	Duration (s)	Propagation length (cm)	% of propagation along the colon
LDC	**Baseline (N=7,n=0)**	**0**	**0**	**0**	**0**	**0**	**0**
	5-HT 2μM (N=7,n=16)	$1 \pm 0.03^{****}$	0.1 ± 0.03	2 ± 0.1	10.4 ± 0.6	20 ± 0.7	80 ± 4.6
	5-HT 5μM (N=7,n=34)	$1.3 \pm 0.0^{****/††}$	$0.3 \pm 0.1^{***/†}$	2.2 ± 0.1	9.7 ± 0.5	19.7 ± 0.4	79.5 ± 5.7
	5-HT 10μM (N=6,n=37)	$1.5 \pm 0.05^{****/††/####}$	$0.3 \pm 0.07^{***/\#}$	2 ± 0.1	10 ± 0.5	18.7 ± 0.8	81 ± 8.2
Single FPC	**Baseline (N=2,n=90)**	0.48 ± 0.01	1.9 ± 1.7	11.4 ± 0.4	1.9 ± 0.1	18.3 ± 0.2	85.8 ± 3.7
	5-HT 2μM (N=4,n=137)	$0.85 \pm 0.01^{****}$	1.1 ± 0.9	14 ± 0.5	1.5 ± 0.07	18 ± 0.2	89 ± 3.2
	5-HT 5μM (N=2,n=119)	$1 \pm 0.01^{****/††††}$	2 ± 1.4	$15.5 \pm 1^{*}$	2.5 ± 0.1	22 ± 0.1	87 ± 3
	5-HT 10μM (N=3,n=117)	$1 \pm 0.01^{****/††/####}$	0.9 ± 0.6	$17.2 \pm 1.2^{**}$	1.8 ± 0.1	21.3 ± 0.1	93 ± 3.3
FPC cluster	**Baseline (N=1,n=10)**	0.67 ± 0.01	0.43	8.4 ± 0.7	2 ± 0.16	18.2 ± 0.8	81.3
	5-HT 2μM (N=2,n=29)	$0.9 \pm 0.05^{*}$	0.45 ± 0.2	6.4 ± 0.3	3 ± 0.09	18.4 ± 0.3	85 ± 5.6
	5-HT 5μM (N=3,n=76)	$0.93 \pm 0.02^{**}$	0.96 ± 0.3	6.5 ± 1	5 ± 0.3	18.2 ± 0.3	83 ± 6
	5-HT 10μM (N=3,n=48)	$0.94 \pm 0.03^{**}$	0.42 ± 0.05	9.1 ± 1.6	4.5 ± 0.4	18.2 ± 0.5	80 ± 7.1

Values are mean ± SEM. Significant difference determined by One-way ANOVA with multiple comparisons. * compared to baseline ($^{*}P< 0.05$, $^{**}P < 0.01$, $^{***}P< 0.001$, $^{****}P < 0.0001$), † compared to the immediate lower concentrations ($^{††}P <0.01$, $^{††††}P <0.0001$), $^{\#}$ compared to the lowest concentration ($^{\#}P <0.05$,$^{\#\#\#\#}P<0.0001$)

Intraluminal Pressure Patterns as a Consequence of LDCs and FPCs

At baseline, LDCs with ~ 0.76 cm contraction amplitude, were accompanied by intraluminal pressure increases of 3 ± 0.2 cmH_2O in prucalopride experiments while no LDCs were present at baseline in 5-HT experiments (Table 4). Prucalopride at all concentrations significantly increased the transient pressure amplitude as a consequence of LDCs compared to baseline which mimics the dose dependant increase in contraction amplitude (Fig 5E,F). However, while the LDC contraction amplitude as a result of prucalopride 10μM was not significantly different compared with 5μM (Table 1), the corresponding LDC pressure amplitude significantly increased at 10μM compared with both lower concentrations (Table 4). Prucalopride at 5μM and 10μM significantly raised the amplitude of intraluminal pressure patterns associated with FPCs. Even relatively small diameter changes of single erratic FPCs resulted in detectable pressure increases at the same frequency as the motor patterns (Fig 5D). Application of 5-HT (2μM) resulted in development of LDCs with mean pressure amplitude of 5.3 ± 0.9 cmH_2O. Increasing the concentration of intraluminal exogenous 5-HT, significantly raised the pressure amplitude of LDCs and FPCs in a dose dependant manner (Table 4).

Table 4. Intraluminal Pressure Increase as a Consequence of LDCs and FPCs

Condition	Mean pressure amplitude of LDC (cmH_2O)	Mean pressure amplitude of FPC (cmH_2O)
Baseline (Prucalopride experiments)	3 ± 0.2 (N= 11, n=12)	0.6 ± 0.1 (N=11, n=57)
Intraluminal prucalopride 2μM	3.7 ± 0.3 ** (N=6, n=21)	0.9 ± 0.05 (N=6, n=22)
Intraluminal prucalopride 5μM	5 ± 0.3 $^{***/\dagger\dagger}$ (N=11, n=34)	1.1 ± 0.08 *** (N=11, n=47)
Intraluminal prucalopride 10μM	6.8 ± 1 $^{***/\dagger/\#\#\#}$ (N=9, n=29)	1.13 ± 0.2 * (N=9, n=19)
Baseline (5-HT experiments)	NA (N=7, n=0)	0.9 ± 0.04 (N=7, n=97)
5-HT 2μM	5.3 ± 0.9	1.5 ± 0.06 ****

	(N=5, n=14)	(N=7, n=162)
5-HT 5μM	11.4 ± 4.4 † (N=5, n=23)	1.7 ± 0.05 $^{****/\dagger}$ (N=5, n=147)
5-HT 10μM	17.7 ± 4 $^{\dagger/\#\#\#}$ (N=4, n=35)	2.1 ± 0.1 $^{****/\dagger\dagger\dagger\dagger/\#\#\#\#}$ (N=4, n=140)

Values are mean ± SEM. Significant difference determined by One-way ANOVA with multiple comparisons. * compared to baseline (*P <0.05, **P < 0.01, ***P< 0.001, ****P < 0.0001), † compared to the immediate lower concentrations (†P< 0.05, ††P < 0.01, ††††P<0.0001), $^{\#}$ compared to the lowest concentration ($^{\#\#\#}$P<0.001, $^{\#\#\#\#}$P<0.0001)

Gradual Transformation of the Colonic Motor Complex

The effects of intraluminal prucalopride and intraluminal exogenous 5-HT were dependent on the state of excitation of the colon. When no propulsive activity was present at baseline, FPCs would develop; whereas if FPCs were already present, LDC activity would emerge. Figure 6 shows the different levels of excitation expressed as different motor pattern configurations. At low levels of excitation, first single erratic FPCs would develop, which could increase in various characteristics including contraction and intraluminal amplitude, frequency, velocity and/or degree of propagation upon further excitation. Subsequently, these single FPCs would become organized in clusters, followed by further increase in amplitude and contraction force. FPCs when manifested in cluster configuration resulted in a more forceful contraction compared to single FPCs at all conditions. Development of LDCs took place in a gradual progressive manner usually beginning inside the FPC clusters. In time, FPCs gradually disappeared while LDCs slowly developed into full propagating forceful motor patterns. Due to this gradual nature of development, a spectrum of "transient" motor patterns could be identified which were neither FPCs nor fully developed LDCs but reminiscent of the previous motor patterns (Fig 2E,F).

Increasing the concentration of prucalopride or 5-HT always resulted in either enhancing different characteristics of the same motor patterns or evoking a more forceful expression of the colonic motor complex. This included prompting the development of FPC clusters

when preceded by single FPCs, and development of LDCs when the colon was already exhibiting FPC clusters (Fig 6). However, when LDCs fully developed or in rare cases were already present at baseline, contractile activity was intensified only through increasing the frequency and/or amplitude of LDCs. Therefore, each level of excitation was expressed via a specific motor pattern configuration and several propulsive motor patterns preceded the LDCs as the most forceful propulsive motor pattern in the rabbit colon (Fig 6).

Effects of Intraluminal Prucalopride and 5-HT on Ripple Frequency

The baseline frequency of ripples in prucalopride experiments was 9.2 ± 0.43 cpm (N=9) which significantly *decreased* after drug administration and ripples gradually disappeared as LDCs and FPCs developed (Table 5, Fig 7). However, this response was not dose dependant. The ripple frequency decreased to 4 ± 0.73 cpm at 2μM and to 3.5 ± 0.84 and 3.9 ± 0.74 cpm at 5μM and 10μM respectively.

In intraluminal exogenous 5-HT experiments, ripple frequency at baseline was at 9.75 ± 0.84 cpm (N=6) which significantly diminished compared to the baseline conditions after 5-HT administration. Ripples gradually disappeared as more forceful propulsive motor patterns developed. However, similar to the prucalopride experiments, the decrease in ripple frequency and their subsequent disappearance did not occur in a dose dependant manner. Reduction in ripple frequency in response to intraluminal prucalopride and exogenous 5-HT was not observed in the presence of the 5-HT_4R antagonist GR113808 10μM (N=5, Table 6).

Table 5. Ripple Frequency in Various Conditions

Intervention	Frequency (cpm)
Baseline (Prucalopride experiments), (N=9)	9.2 ± 0.43
Intraluminal prucalopride 2μM, (N=4)	4 ± 0.73 ***
Intraluminal prucalopride 5μM, (N=6)	3.5 ± 0.84 ****
Intraluminal prucalopride 10μM, (N=10)	3.9 ± 0.74 ****

Baseline (5-HT experiments), (N=6)	9.75 ± 0.84
5-HT 2μM, (N=5)	4.6 ± 0.50 **
5-HT 5μM, (N=5)	5.8 ± 1.00 *
5-HT 10μM, (N=5)	5.7 ± 0.9 *

Values are mean ± SEM. Significant difference determined by One-way ANOVA. * compared to baseline conditions (*P< 0.05, **P < 0.01, ***P< 0.001, ****P < 0.0001).

Table 6. Effects of the Antagonist on Ripple Frequency

Intervention	Frequency (cpm)
Baseline before antagonist (N=5)	8 ± 0.75
Baseline after antagonist (N=5)	7.8 ± 0.64
Intraluminal prucalopride 2μM, (N=5)	7.7 ± 0.80
Intraluminal prucalopride 5μM, (N=5)	8.2 ± 0.90
5-HT 2μM, (N=5)	7.4 ± 0.80
5-HT 5μM, (N=5)	7.4 ± 0.76

Values are mean ± SEM.

Effects of Intraluminal Prucalopride and 5-HT on Haustral Boundary Contractions

Haustral boundary contractions (HBC) were a prominent motor pattern of the rabbit colon. The frequency of HBCs at baseline in prucalopride experiments was 0.63 ± 0.08 cpm (N=19) and they propagated very slowly at the velocity of 0.014 ± 0.004 cm s^{-1} (N=19) along the colon. The velocity of HBCs was remarkably similar comparing colons from different rabbits and compared to a previous study (Chen et al., 2015). The frequency of HBCs *decreased* significantly after administration of intraluminal prucalopride 5 μM and 10μM compared to the baseline while HBC velocity remained unaffected. This response was not dose dependent (Table 7).

Table 7. Comparison of HBC characteristics in different conditions

	Frequency (cpm)	Velocity (cm s^{-1})
Intraluminal Prucalopride		
Baseline (N=19, n=25)	0.63 ± 0.08	0.014 ± 0.004
2µM (N=9, n=17)	0.50 ± 0.1	0.015 ± 0.01
5µM (N=13, n=12)	0.40 ± 0.05 *	0.013 ± 0.002
10µM (N=15, n=30)	0.35 ± 0.05 *	0.014 ± 0.001
Intraluminal Exogenous 5-HT		
Baseline (N=7, n=18)	0.70 ± 0.06	0.013 ± 0.003
2µM (N=7, n=19)	0.41± 0.06 *	0.015 ± 0.001
5µM (N=7, n=18)	0.38 ± 0.09 *	0.015 ± 0.002
10µM (N=7, n=17)	0.33 ± 0.15 *	0.016 ± 0.002
Antagonist (GR113808)		
Baseline before antagonist (N=6, n=16)	0.57 ± 0.05	0.014 ± 0.001
Baseline after antagonist (N=6, n=15)	0.55 ± 0.05	0.013 ± 0.001
Prucalopride 2µM (N=6, n=16)	0.55 ± 0.06	0.015 ± 0.001
Prucalopride 5µM (N=6, n=16)	0.56 ± 0.05	0.015 ± 0.002
5-HT 2µM (N=4, n=10)	0.55 ± 0.02	0.013 ± 0.002
5-HT 5µM (N=4, n=9)	0.52 ± 0.18	0.014 ± 0.003

Values are mean ± SEM. Significance was determined using one-way ANOVA with multiple comparisons. *compared to baseline (* $P<0.05$).

The frequency of HBCs at baseline conditions in 5-HT experiments was 0.7± 0.06 cpm (N=7) and they propagated at the velocity of 0.013 ± 0.003 cm s^{-1} (N=7). Administration of 5-HT in all concentrations inside the lumen resulted in the marked *decrease* of HBC frequency compared to baseline but this did not happen in a dose dependent manner either. 5-HT did not affect the HBC velocity (Table 7). In 6/6 experiments, decrease in HBC frequency in response to prucalopride and 5-HT was inhibited by the antagonist. Antagonist alone did not affect neither the frequency nor the velocity of HBCs at baseline conditions (N=6).

FIGURES

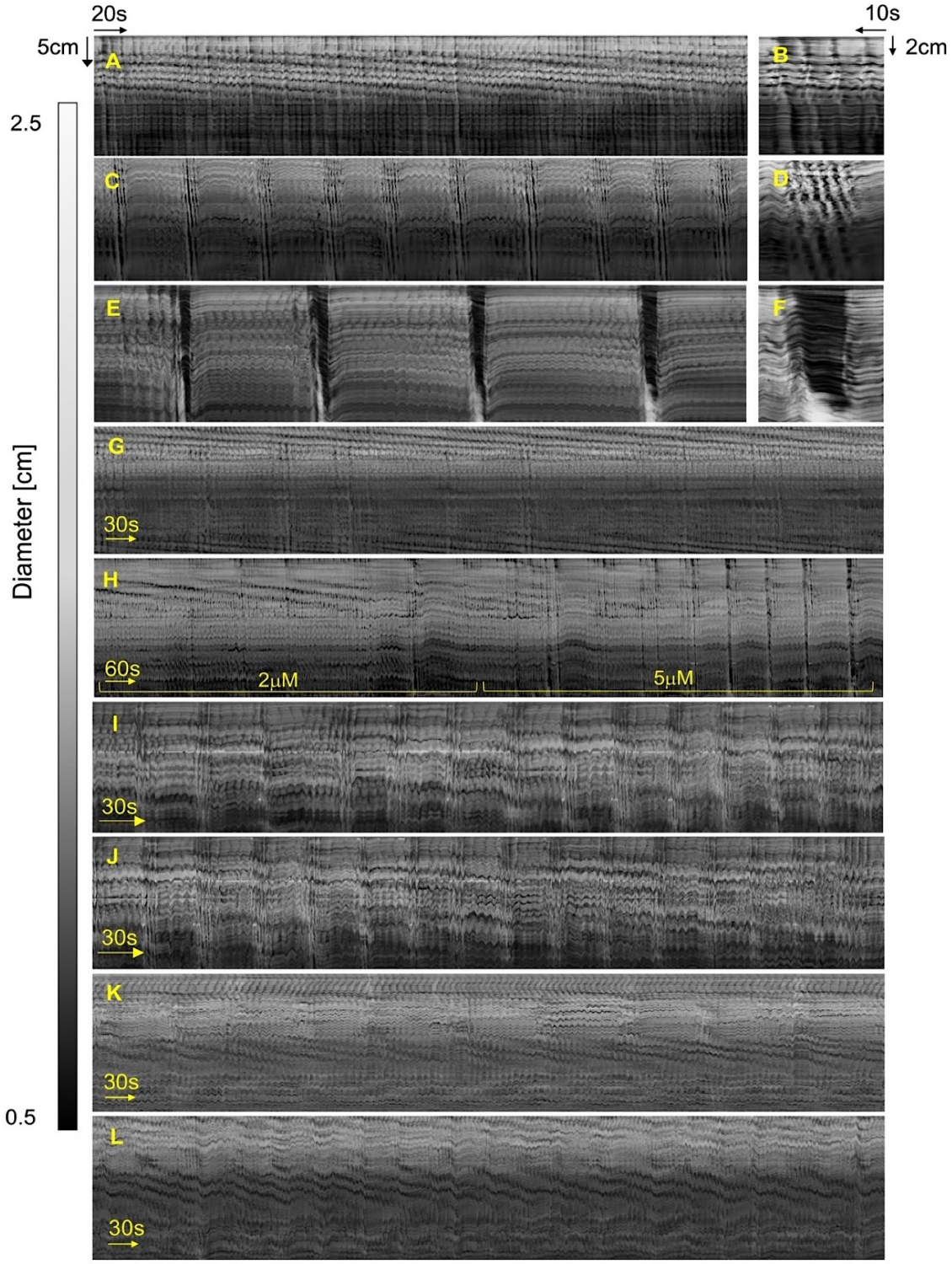
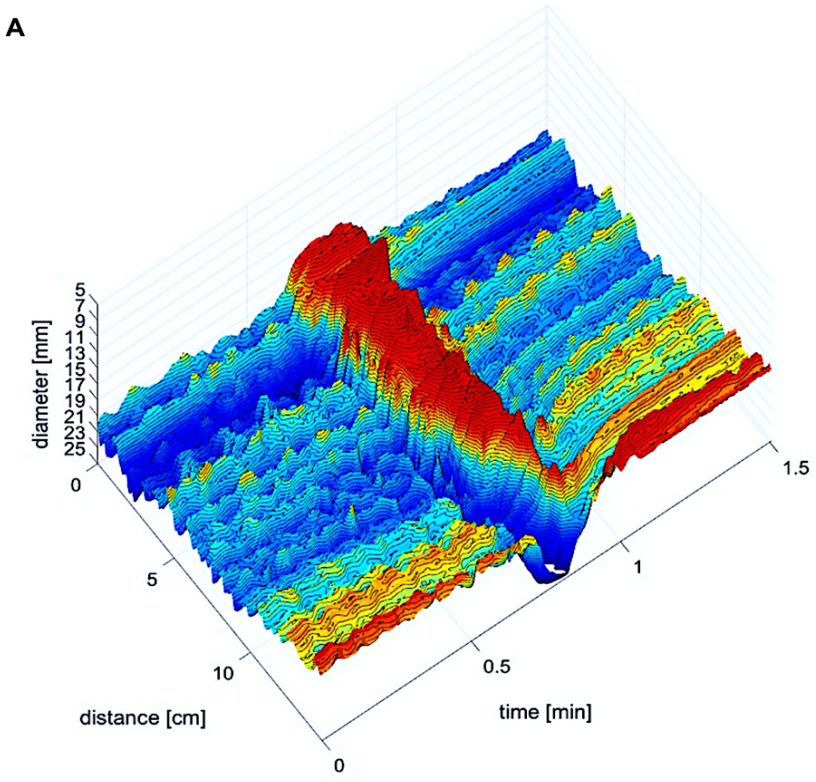
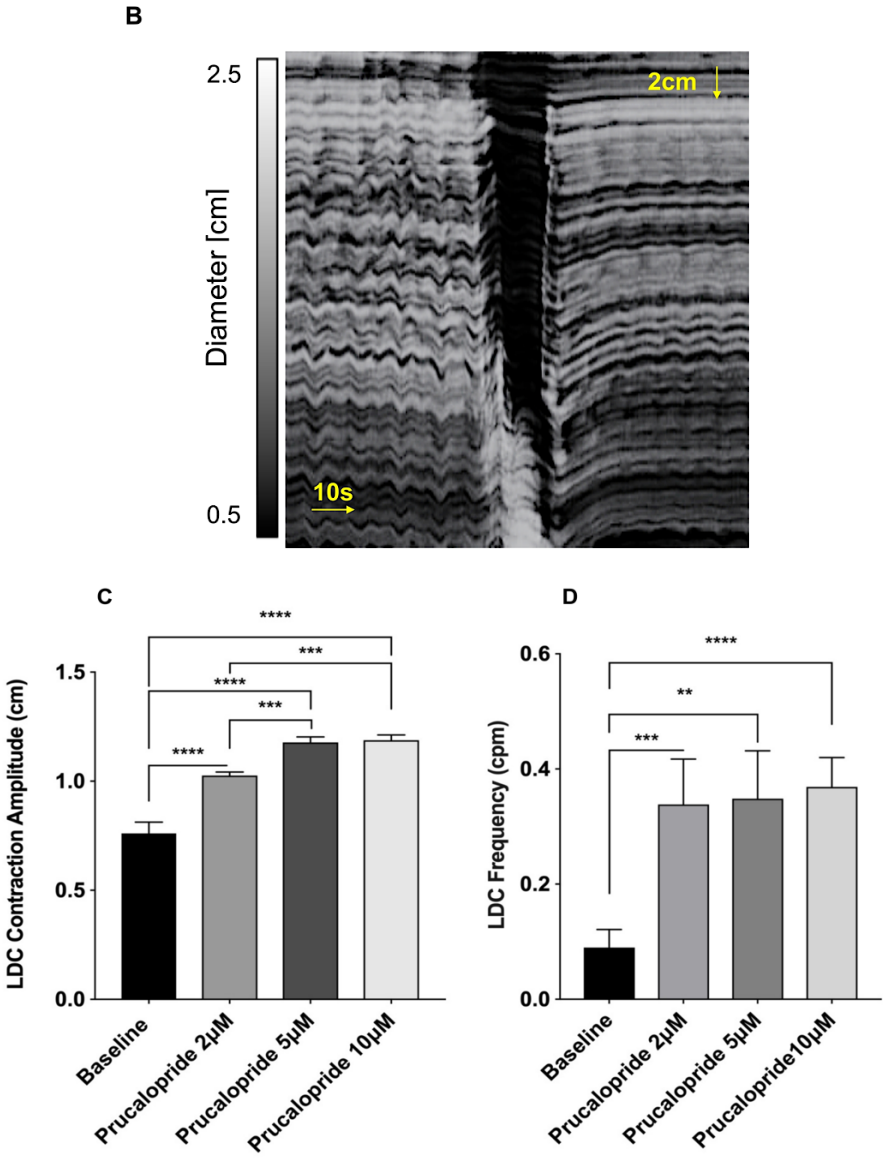


Figure 1.Intraluminal Prucalopride Increases Propulsive Motor Activity

Experiment 1 (A-F), (A,B) Baseline manifests individual FPCs. (C,D) Prucalopride, 5μM results in clusters of forceful FPCs. (E,F) Prucalopride, 10μM results in propagating forceful individual LDCs across the entire colon. Experiment 2 (G,H), (G) The last 15 minutes of baseline recording showing no major motor patterns (some incomplete FPC-like patterns are present). (H) Development of motor patterns in response to intraluminal prucalopride (2 and 5μM) showing 15 minutes of each intervention in the same colon. Note the gradual development of FPC clusters and LDCs. Experiment 3 (I,J), 5-HT_4 receptor antagonist GR113808 (10μM) had no significant effect on baseline activity. (I), 10 minutes recording of baseline activity showing FPC clusters. (J) 10 minutes recording of baseline + GR113808 with no significant difference in FPC clsuters. Experiment 4, (K,L), Neither LDCs nor FPCs are generated in response to intraluminal prucalopride at the presence of the 5-HT_4 receptor antagonist GR113808 (10μM), (K) 10 minutes recording after prucalopride 2μM + GR113808, (L) 10 minutes recording after prucalopirde 5μM + GR113808.




B
2.5
Diameter [cm]
0.5
2cm
10s
C
LDC Contraction Amplitude (cm)
1.5
1.0
0.5
0.0
****
***
****
***
****
Baseline
Prucalopride 2μM
Prucalopride 5μM
Prucalopride 10μM
D
LDC Frequency (cpm)
0.6
0.4
0.2
0.0
****
**
***
Baseline
Prucalopride 2μM
Prucalopride 5μM
Prucalopride10μM

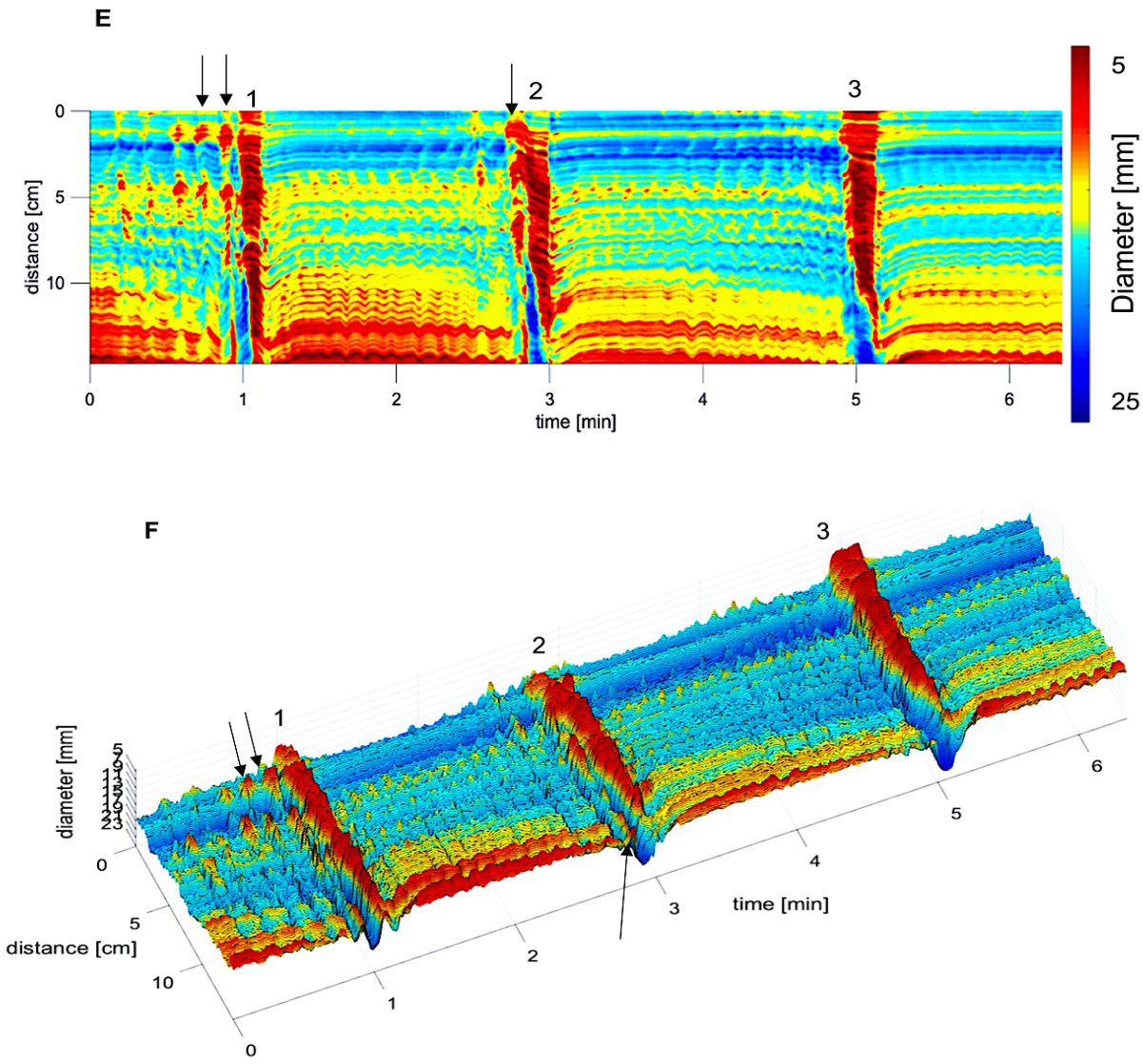
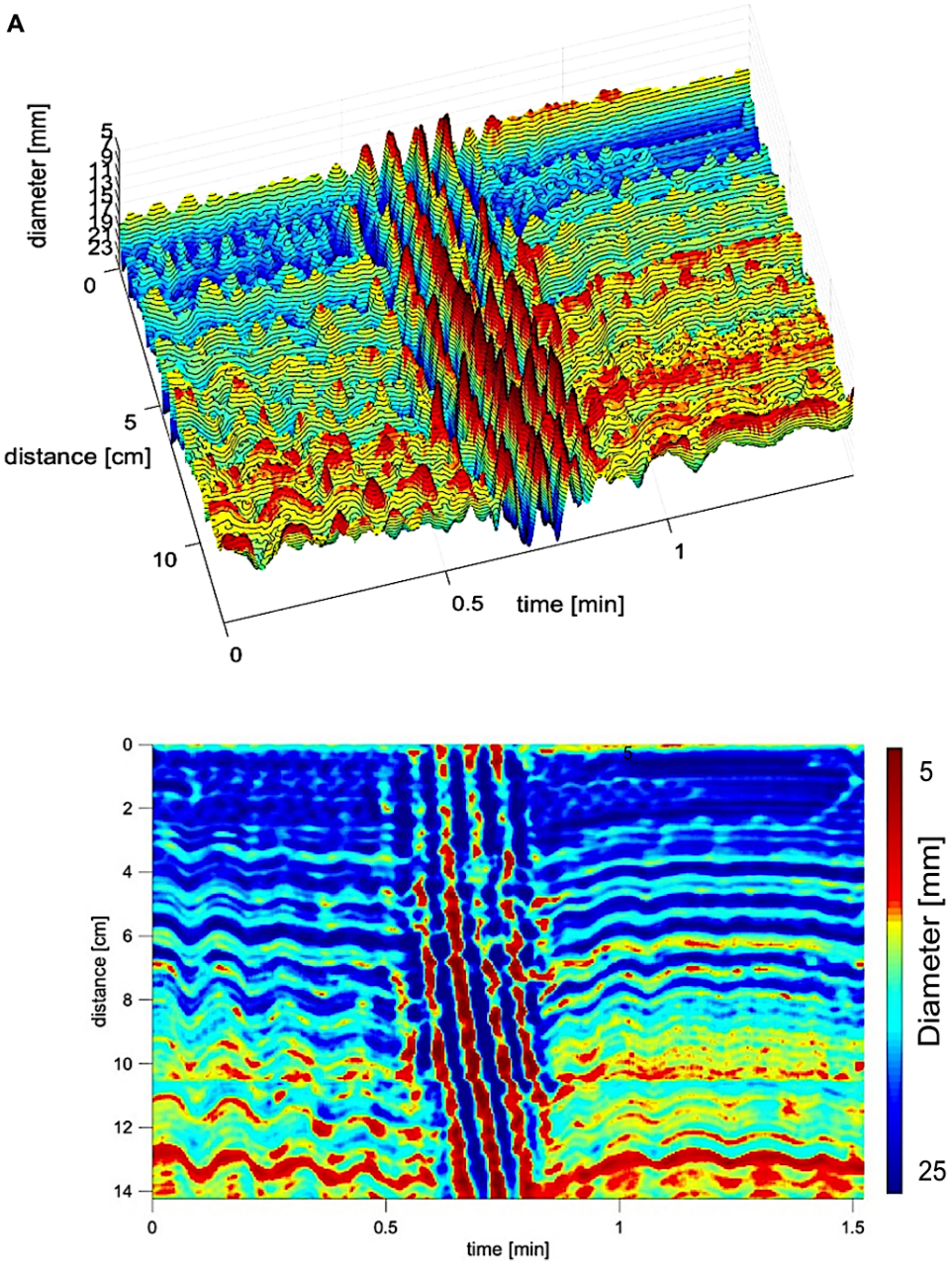


Figure 2. Propulsive Characteristics of LDCs Enhanced Significantly in Response to Intraluminal Prucalopride

(A) An LDC (contraction amplitude of 1.01 cm) evoked by intraluminal prucalopride 10μM shown as a 3-D image and (B) 2-D spatiotemporal diameter map. Red in 3-D plot and black in spatiotemporal D-map show contraction or decrease in colon diameter while blue and white show relaxation respectively. (C) Effects of intraluminal prucalopride on LDC contraction amplitude (D) Effects of intraluminal prucalopride on LDC frequency (** P< 0.01, *** P< 0.001, **** P < 0.0001). (E,F) Gradual development of more forceful LDCs (Labelled as 1, 2, 3 with contraction amplitudes of 0.8, 1.0 and 1.2 cm respectively) is observed as well as gradual increase in amplitude of preceding relaxation in response to intraluminal prucalopride 10μM. Shown as 2-D and 3-D plots. Arrows show remnants of a cluster of FPCs.


A
diameter [mm]
5
23
0
5
distance [cm]
10
0
0.5
time [min]
1
0
2
4
6
8
10
12
14
distance [cm]
0
0.5
1
1.5
time [min]
5
25
Diameter [mm]

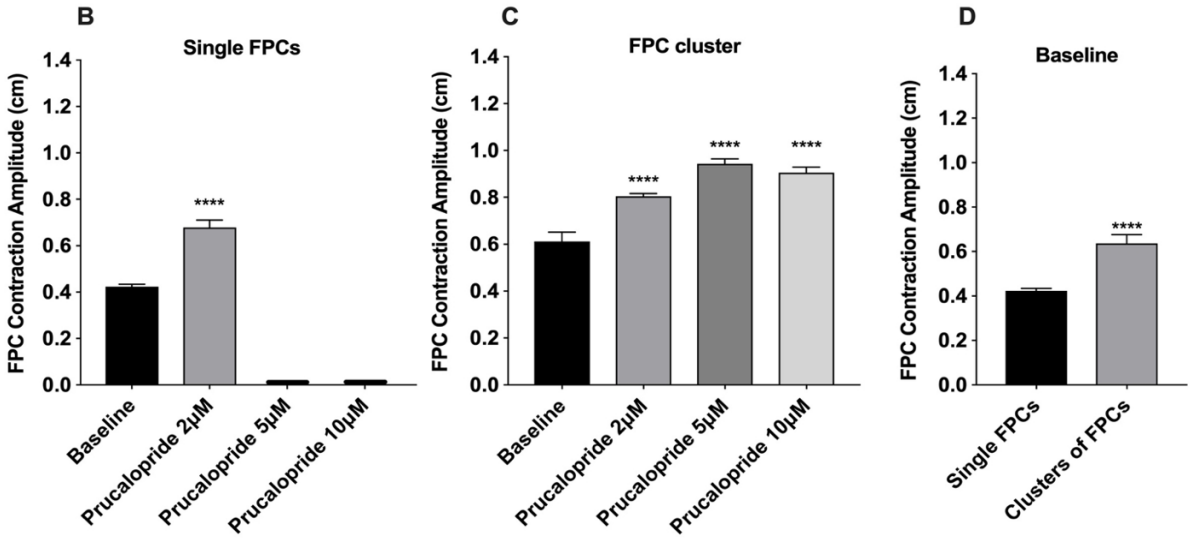
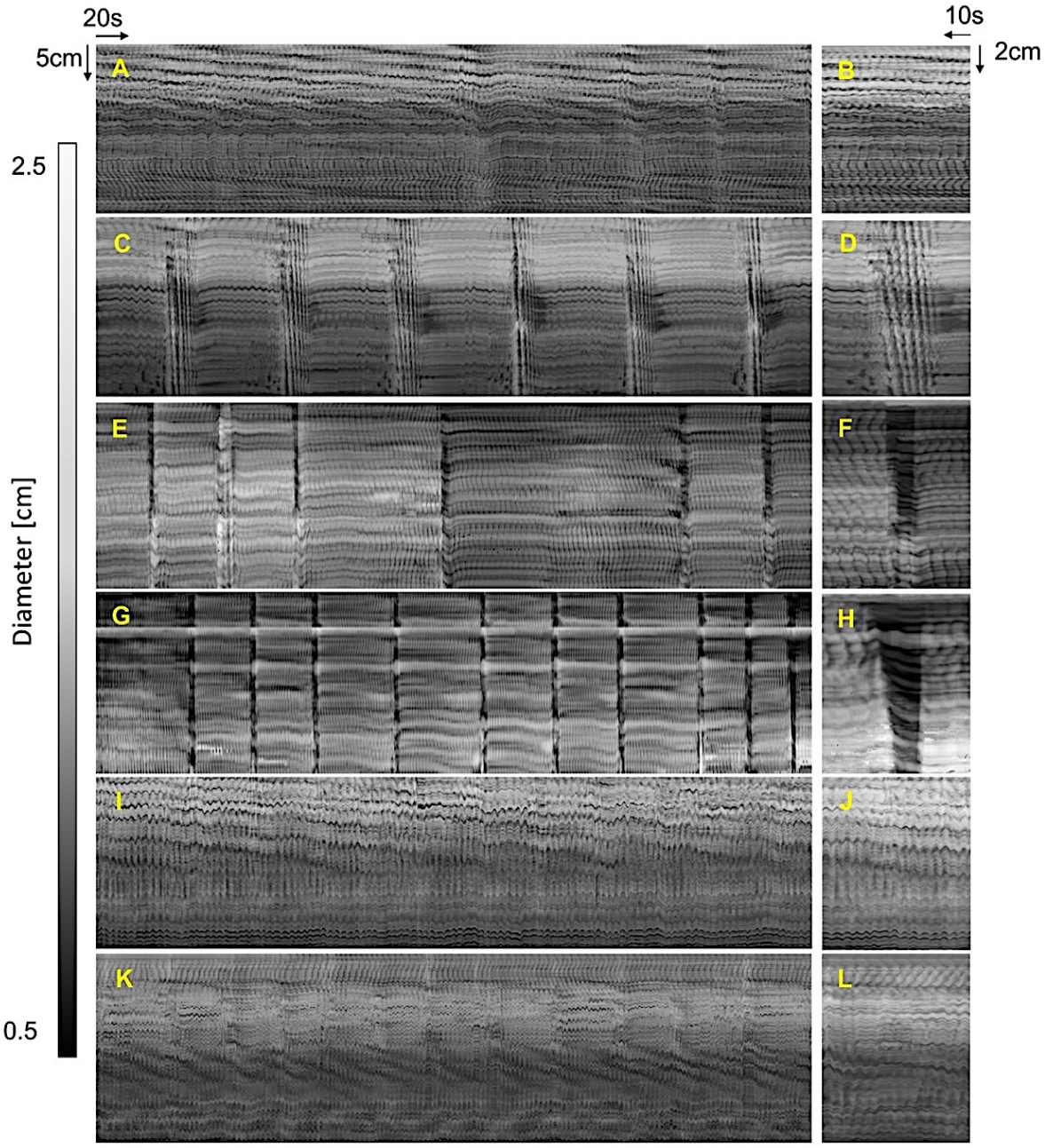
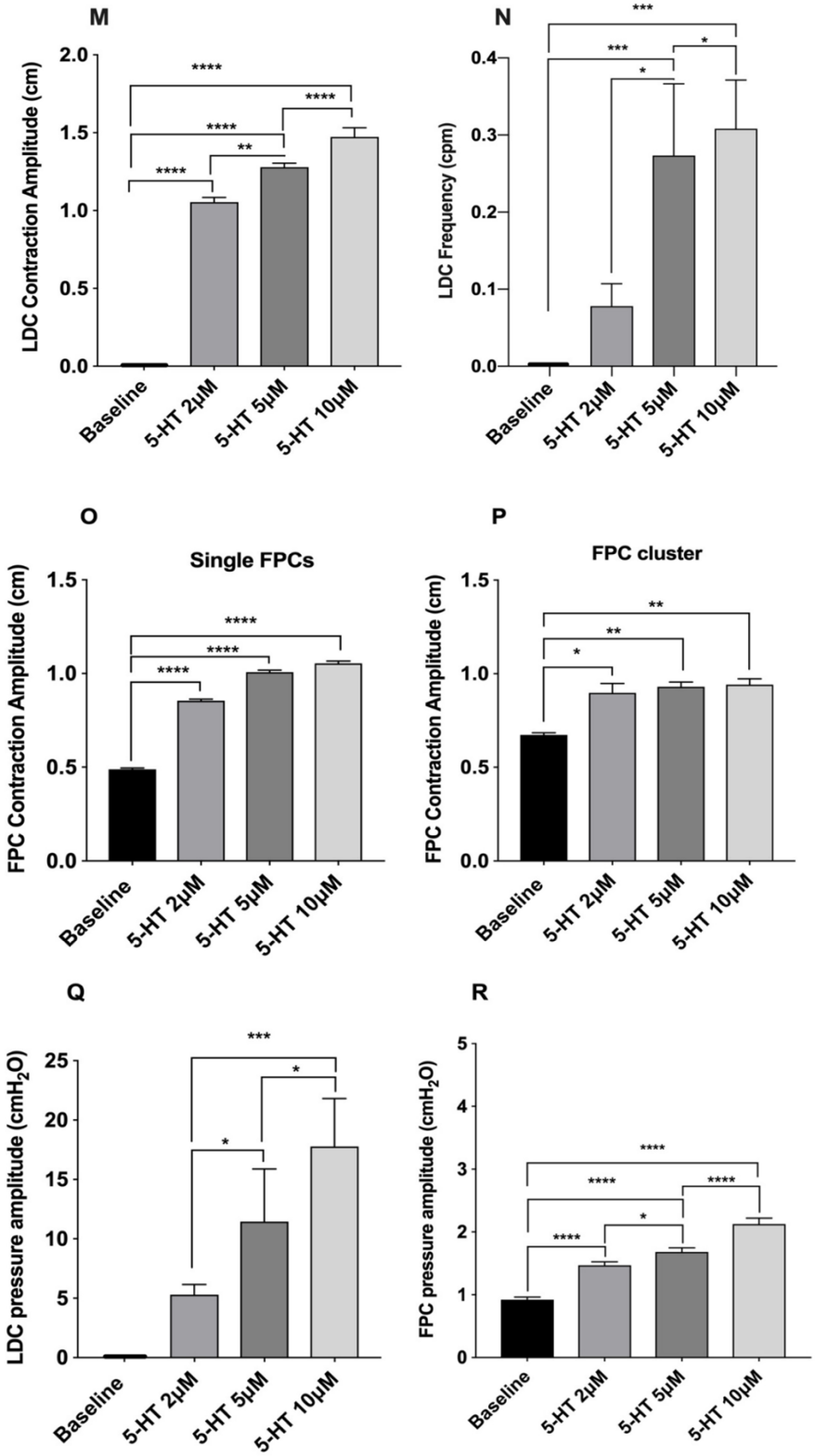


Figure 3. Propulsive Characteristics of FPCs Enhance Significantly in Response to Intraluminal Prucalopride

(A) A typical cluster of forceful FPCs developed in response to prucalopride 5μM shown as 2-D and 3-D plots. (B) Effects of intraluminal prucalopride on FPC contraction amplitude. Note that no individual FPCs were developed at higher drug concentrations. (C) Effects of intraluminal prucalopride on contraction amplitude of FPC clusters. (D) Comparison of contraction amplitude between single FPCs and FPCs arranged in a cluster at baseline conditions without any intervention (**** $P < 0.0001$).


20s
10s
5cm
2cm
A
B
C
D
E
F
G
H
I
J
K
L
2.5
Diameter [cm]
0.5


M
LDC Contraction Amplitude (cm)
2.0
1.5
1.0
0.5
0.0
****
****
****
**
****
Baseline
5-HT 2μM
5-HT 5μM
5-HT 10μM
N
LDC Frequency (cpm)
0.4
0.3
0.2
0.1
0.0
***
***
*
*
Baseline
5-HT 2μM
5-HT 5μM
5-HT 10μM
O
Single FPCs
FPC Contraction Amplitude (cm)
1.5
1.0
0.5
0.0
****
****
****
Baseline
5-HT 2μM
5-HT 5μM
5-HT 10μM
P
FPC cluster
FPC Contraction Amplitude (cm)
1.5
1.0
0.5
0.0
**
**
*
Baseline
5-HT 2μM
5-HT 5μM
5-HT 10μM
Q
LDC pressure amplitude (cmH2O)
25
20
15
10
5
0
***
*
*
Baseline
5-HT 2μM
5-HT 5μM
5-HT 10μM
R
FPC pressure amplitude (cmH2O)
5
4
3
2
1
0
****
****
****
*
****
Baseline
5-HT 2μM
5-HT 5μM
5-HT 10μM

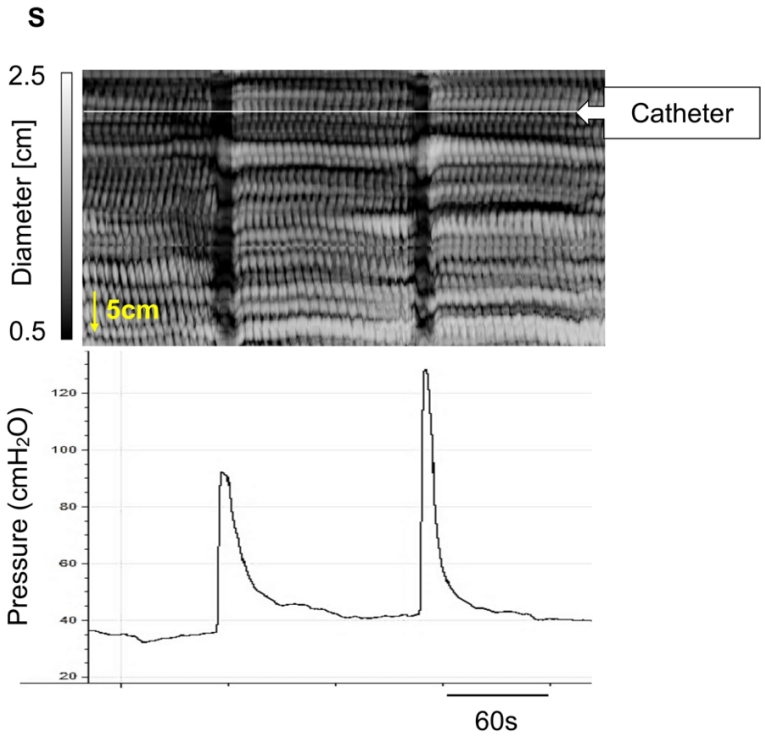
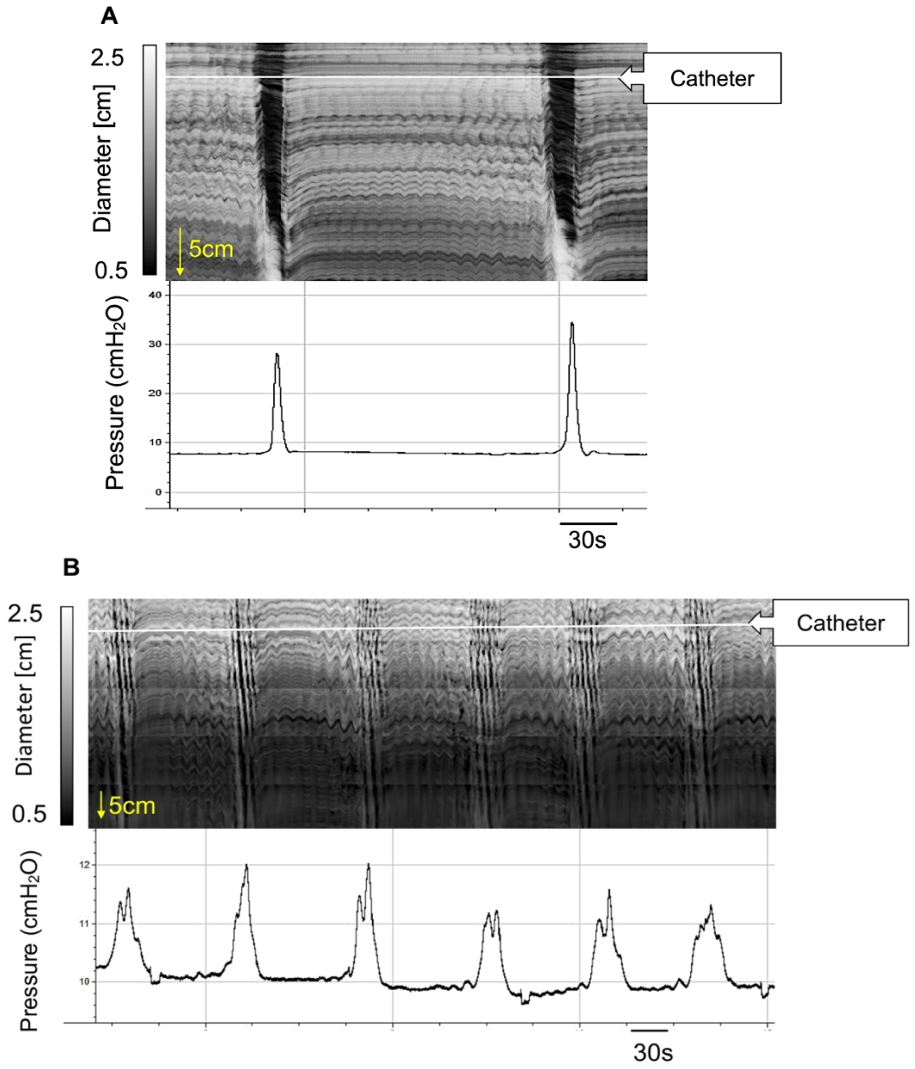
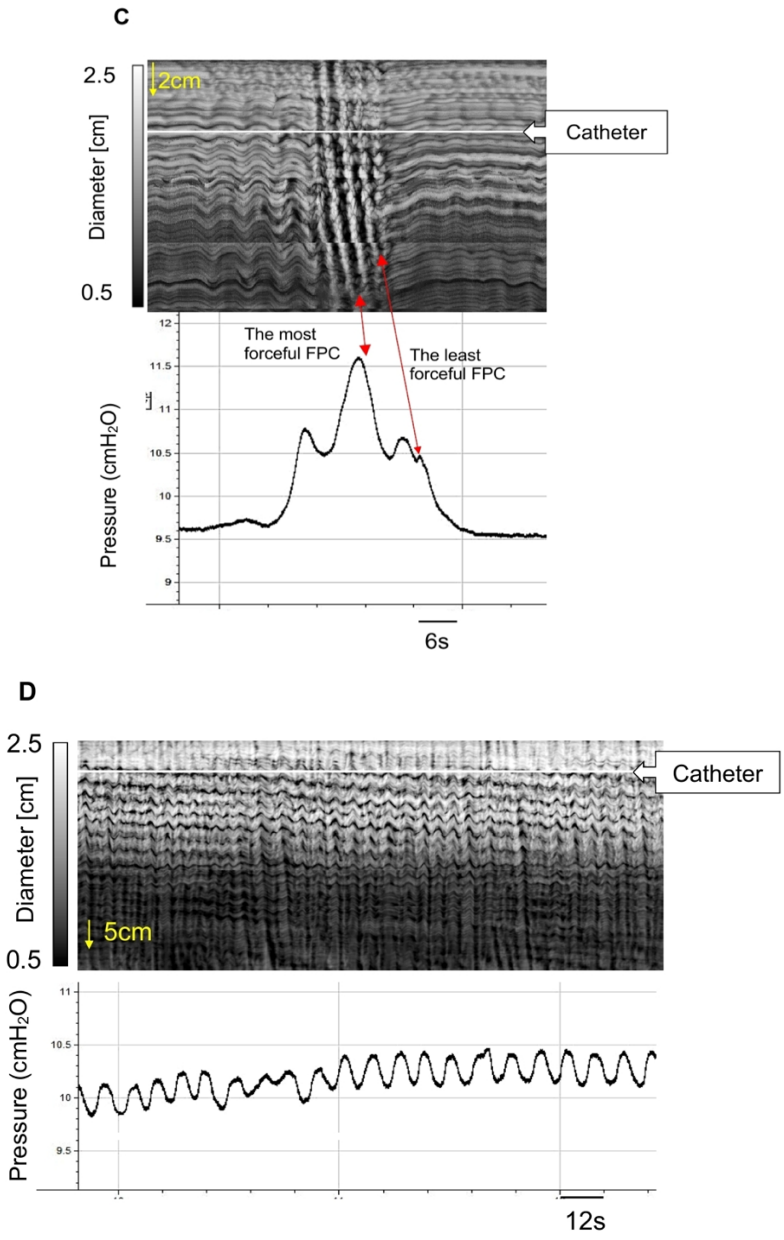


Figure 4. Intraluminal Exogenous 5-HT Enhances Propulsive Motor Activity

Experiment 1 (A,B) D-map of baseline, haustral boundary contractions and ripples are present while no FPCs or LDCs are observed. (C,D) Cluster of FPCs formed after intraluminal 5-HT 2μM, (E,F) LDCs formed after intraluminal 5-HT 5μM, (G,H) Significantly more forceful LDCs are formed with a significantly higher frequency after intraluminal 5-HT 10μM.
Experiment 2 (I,J) Neither LDCs nor FPCs develop after 5-HT 2μM in the presence of the antagonist GR113808 (10μM), (K,L) Neither LDCs nor FPCs develop after 5-HT 5μM in the presence of the antagonist GR113808 (10μM), (M) The effect of intraluminal 5-HT on LDC contraction amplitude, (N) The effect of intraluminal 5-HT on LDC frequency, (O) The effect of intraluminal 5-HT on single FPC contraction amplitude, (P) The effect of intraluminal 5-HT on contraction amplitude of FPC clusters, (Q) The effect of intraluminal 5-HT on LDC pressure amplitude as a consequence of intraluminal 5-HT, (R) The effect of intraluminal 5-HT on FPC pressure amplitude as a consequence of intraluminal 5-HT ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).
Experiment 3 (S) D-map and intraluminal pressure profile of two forceful LDCs (57 and 86 cmH_2O) formed after intraluminal 5-HT 10μM. The white line shows the position of the catheter.


A
2.5
Diameter [cm]
0.5
5cm
Catheter
Pressure (cmH2O)
40
30
20
10
0
30s
B
2.5
Diameter [cm]
0.5
5cm
Catheter
Pressure (cmH2O)
12
11
10
30s


C
2.5
2cm
Diameter [cm]
0.5
Catheter
The most forceful FPC
The least forceful FPC
Pressure (cmH2O)
6s
D
2.5
Diameter [cm]
0.5
Catheter
5cm
Pressure (cmH2O)
12s

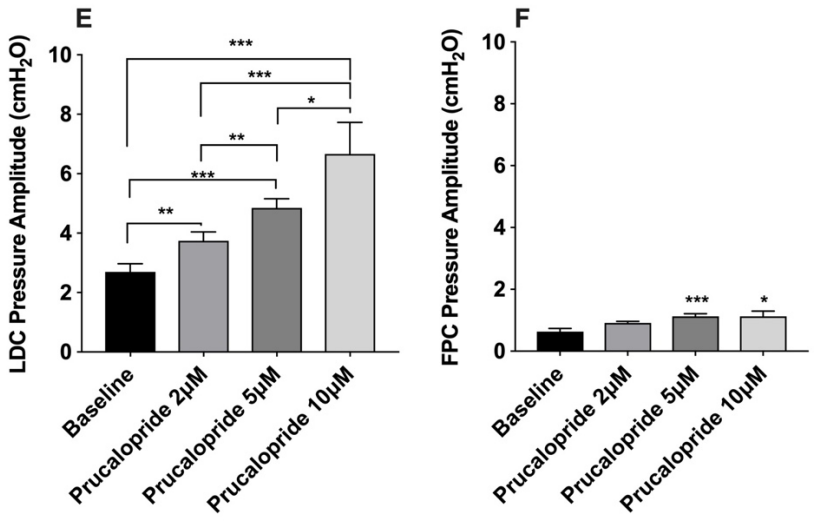
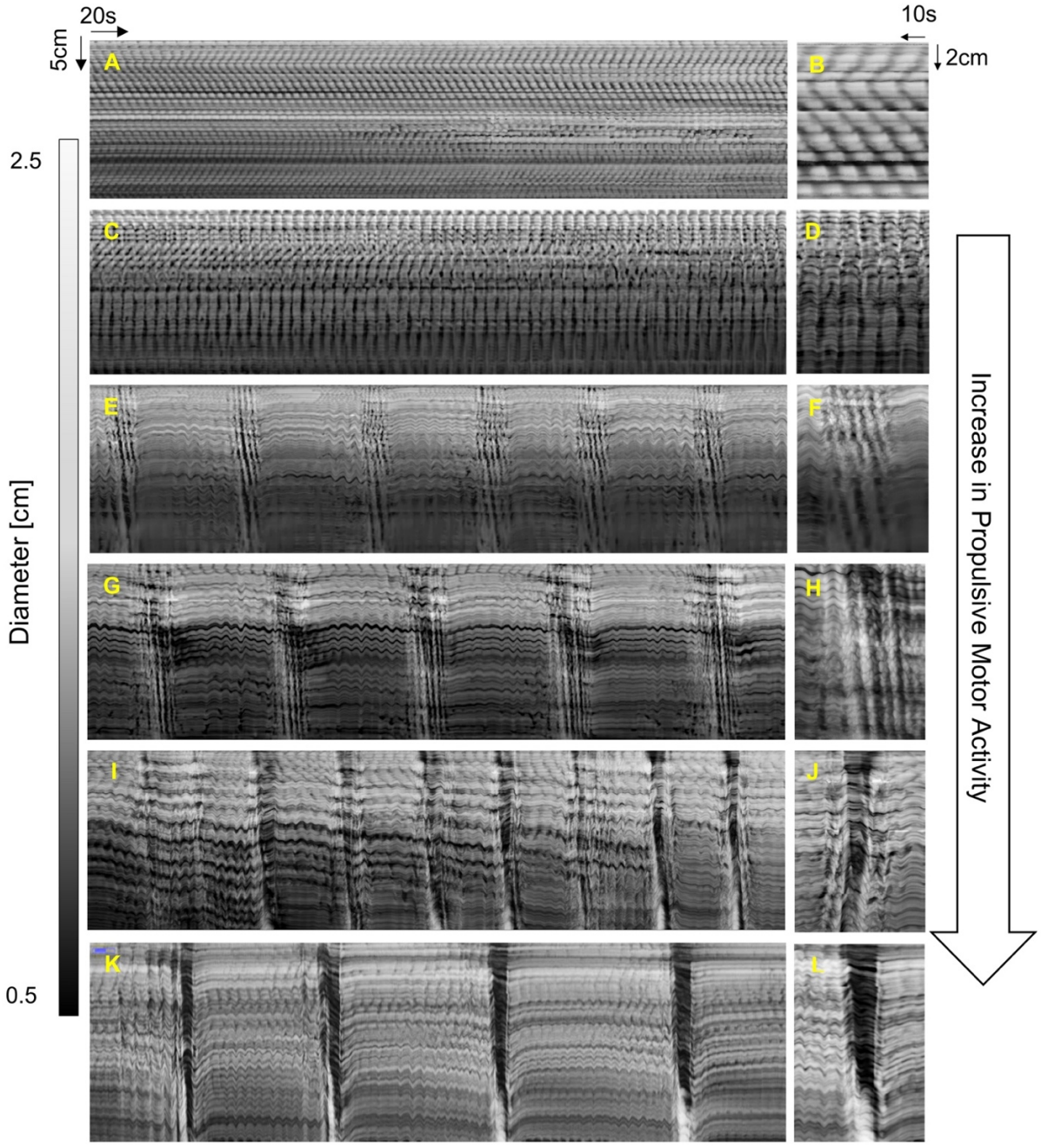


Figure 5. Intraluminal Pressure Patterns as a Consequence of Forceful Contractions in Response to Intraluminal Prucalopride

(A) Intraluminal pressure profile and D-map of two LDCs (28 and 34.5 cmH_2O) in response to intraluminal prucalopride 10μM, (B) Intraluminal pressure profile and D-map of clusters of forceful FPCs in response to intraluminal prucalopride 5μM.(C) D-map and pressure profile of one cluster comprising of 4 FPCs developed in response to intraluminal prucalopride 5μM. The most forceful FPC (shown with red arrow) corresponding to 1.47 cm of contraction amplitude, is correlated with the highest increase in intraluminal pressure in the cluster (11.6 cmH_2O) and the least forceful FPC (shown with red arrow) corresponding to 1.12cm contraction amplitude is correlated with the lowest increase in intraluminal pressure inside the cluster (10.4 cmH_2O). (D) Intraluminal pressure profile and D-map of individual FPCs at baseline. The white line in all maps shows the position of the catheter. (E) The effect of prucalopride on intraluminal pressure amplitude associated with LDCs. (F) The effect of prucalopride on intraluminal pressure amplitude associated with FPCs ($^{*}P < 0.05$ $^{**}P < 0.01$, $^{***}P< 0.001$).


20s
10s
5cm
2cm
A
B
C
D
E
F
G
H
I
J
K
L
2.5
0.5
Diameter [cm]
Increase in Propulsive Motor Activity

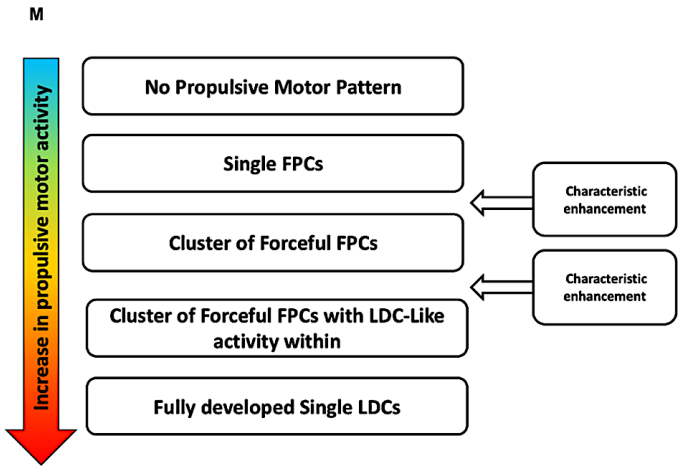


Figure 6. Spatiotemporal Diameter Maps Show Levels of Propulsive Activity from the Lowest (A,B) to the highest (K,L) with Each Level Manifesting a More Forceful Motor Pattern

Figures are taken from 5 different colons in response to intraluminal prucalopride and 5-HT (A,B) Ripples are present while no FPCs or LDCs are observed. (C,D) Single FPCs with lower force compared with FPCs in cluster (5-HT 2μM) (E,F) Clusters of forceful FPCs (Prucalopride 2μM) (G,H) Gradual development of LDC-like activity within FPC clusters (Prucalopride 5μM) (I,J) Gradual formation of full LDCs within FPC clusters (Prucalopride 10μM). (K,L) Formation of fully propagating forceful individual LDCs (Prucalopride 10μM). (M) Graphic representation of graded excitation. "Characteristic enhancement" refers to enhancement of various parameters of the motor pattern without changing it to another motor pattern.

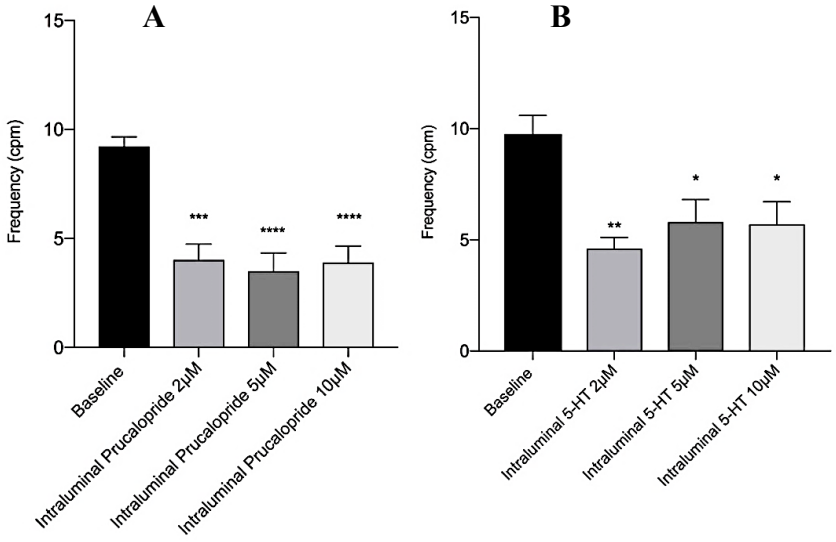


Figure 7. Change in Ripple Frequency after Administration of Intraluminal Prucalopride and Exogenous 5-HT

(A) Ripple frequency decreased significantly after intraluminal prucalopride in all concentrations. (B) Ripple frequency decreased significantly after intraluminal 5-HT in all concentrations. * compared to baseline conditions ($^{*}P< 0.05$, $^{**}P < 0.01$, $^{***}P< 0.001$, $^{****}P < 0.0001$).

CHAPTER 3- EFFECTS OF INTRALUMINAL PRUCALOPRIDE ON COLONIC MOTOR ACTIVITY IN THE PRESENCE OF A BULK OF STOOL IN THE COLON SIMULATED BY AN INFLATED RAPID BAROSTAT BAG

INTRODUCTION

In chapter 2, it was demonstrated that intraluminal prucalopride results in strong prokinetic effects in the 3-taeniated and 2-taeniated rabbit colon. However, this was measured in an empty colon constantly perfused with solutions. In this chapter, effects of intraluminal prucalopride is evaluated in the presence of simulated fecal impaction. A significant number of constipated patients develop large fecal bulk in a part of colon. Fecal impaction is defined as a large mass of feces at any intestinal level that cannot be evacuated spontaneously (Serrano Falcon et al., 2016). Fecal impaction is usually the result of severe chronic constipation. The elderly represents the main risk group for fecal impaction although children and patients with neuropsychiatric disease also manifest this problem (Serrano Falcon et al., 2016; Obokhare, 2012). Fecal impaction causes increase in intraluminal pressure which may lead to ulcer and colonic perforation while sustained dilation of the colon can result in megacolon (Serrano Falcon et al., 2016). It may also cause mechanical obstruction of the colon and constant compression of nerves and vascular structures. Additionally, the continuous contact of the fecal bulk with the colonic wall results in increase of mucus secretion and has been shown to cause prolonged relaxation of the internal anal sphincter in children (Serrano Falcon et al., 2016).

Although fecal impaction usually develops in the rectum, accumulation of fecal matter can also happen in the right colon. The term “proximal constipation” was previously used to refer to fecal matter being built-up in the right colon (Serrano Falcon et al., 2016). The etiologic factors responsible for fecal impaction are similar to those responsible for constipation. Fecal impaction is seen as an acute complication of chronic and untreated

constipation (Obakhare, 2012). Factors contributing to the development of fecal impaction are colonic hypomotility and inadequate fiber and water intake (Obakhare, 2012). Paradoxically, laxative abuse is associated with constipation and fecal impaction. The laxative-dependent patient is usually unable to produce normal propulsive motor patterns in response to colonic distention and progressively requires higher doses to achieve a bowel movement (Creason, 2000). Symptoms such as reduced frequency of defecation, bloating, cramping and sensation of incomplete defecation accompany this problem which is usually diagnosed using medical imaging techniques such as computed tomography or CT scanning (James et al., 2018).

The main objective of this chapter is to investigate the effects of intraluminal prucalopride on propulsive motor patterns in the presence of fecal matter in the colon. The large fecal bulk is simulated by a modified barostat bag. The bag volume can be adjusted, and the same volume of bag inflation can be sustained for a long period of time to replicate a situation in which a large bulbous of fecal matter is accumulated in the colon. The effects of distention as a luminal stimulant that evokes propulsive motor activity is not overlooked in this study. Distention evokes propulsive contractions possibly via 5-HT release from EC cells and mechanical stimulation of stretch-sensitive IPANs. Balloon-distension techniques have been used for a long time in clinical studies of the GI tract. The barostat bag was designed as a device to record rectal compliance and to determine sensory and pain thresholds and tension of the organs. Tension and strain are of principal interest because these parameters give information about the GI tract elastic properties. Abnormal rectal function due to increased distensibility or compliance might be an underlying mechanism of therapy-resistant constipation (Van den Berg et al., 2001; Gregersen et al., 2006). The basic principle of the barostat is to maintain a constant pressure within the bag in the lumen even though muscular contractions and relaxations occur. To maintain a constant pressure, the barostat aspirates air into the bag during contractions and injects air during relaxation. Nowadays, many distension studies use a polyethylene bag rather than a latex balloon since

latex balloons resist inflation and thus, show a rapid increase in intra-balloon pressure with small volumes of distension (Gregersen et al., 2006).

Intraluminal distension elicits peristaltic contractions in the human small intestine (Bassotti et al., 1994) and balloon distention of the colon has been associated with initiation of both high and low amplitude propagating pressure waves in humans (Bassotti et al., 1994; Chen et al., 2018). Proximal balloon distension also increased the number of SPWs associated with gas expulsion and evoked proximal HAPWs that turned into SPWs in the human colon (Chen et al., 2018). Liem et al., (2010), demonstrated that proximal balloon distention of the colon stimulates propulsive contractions in children as well. Nevertheless, there are also other studies evaluating the effects of colonic distension that have failed to elicit propulsive contractions, suggesting that distension does not necessarily determine initiation of colonic propagated activity (Bassotti et al, 1994). In this study, the effect of barostat bag inflation on colonic motor patterns is first assessed before application of intraluminal prucalopride to account for the potential effects of colonic distention on propulsive motor activity at baseline conditions. Later, the effects of intraluminal perfusion of prucalopride on motor patterns in the presence of the inflated bag are evaluated.

MATERIALS AND METHODS

Animals and tissue preparation: The same procedure was followed as described in chapter 2. New Zealand white rabbits (N=11) were sacrificed (Male =10, female =1, body weight of 1.9 to 3 Kg) with 2ml sodium pentobarbital per 4.5 kg body weight through ear vein injection. Natural pellets in the rabbit colon were measured at 6 ± 1.05 mm (N=6, n=10). Average length of the colon was measured at 22 ± 0.9 cm (N=11).

Experimental setup: The organ bath setup was similar to chapter 2 with addition of a rapid barostat (Mui Scientific, ON Canada). A bag with the maximum volume of 9 ml, and the size of 35mm × 25mm was made out of polyethylene gloves and was fixed around the

barostat catheter (Mui Scientific, ON Canada). To cannulate the colon and simultaneously fix the barostat catheter in the middle section of the tissue, a 6cm long needle of gauge 18 was fixed around the barostat catheter using two plastic collars. It was then extended with a polyethylene tube (ID 1.57 mm, OD 2.08mm) for proper drainage as bag inflation could block the outflow of the solutions.

The barostat catheter was inserted in the colon from the distal end and was fixed in a manner that the bag would be placed in the middle section of the tissue at camera channel 6. The other end of the catheter was attached to the rapid barostat. Before starting the experiments, the tissue compliance was measured separately in each experiment (N=11). After positioning the bag inside the colon, it was allowed to be filled until the maximum possible pressure corresponding to the maximum degree of tissue distensibility was reached. The barostat automatically deflates the bag as soon as it reaches a certain volume that corresponds to the pressure resulting from the maximum tissue distention. This maximum pressure was determined to be 58 ± 2.00 mmHg at bag volume 7ml (N=11). The aim of this study was to sustain bag inflation for a relatively long period of time (20 minutes) without automatic bag deflation during this period. Therefore, the bag was inflated to the maximum volume of 6ml to distend the colon to near maximal degree without any automatic deflation.

Slow perfusion rate of ~1 ml/min was used to avoid luminal distention while optimizing the survival of the epithelial cells. The perfusion rate was kept constant during the experiment to ensure that the propulsive motility was induced by the drug and the bag inflation. After a 30-minute acclimatization, motility recordings were initiated. After 20 minutes of baseline recording, the bag was inflated to 6ml equivalent to the average intraluminal pressure of 41.5 ± 0.92 mmHg (N=11). This would distend the colon to 1.76 ± 3.2 times of its baseline diameter (N=11). The bag was kept inflated for 20 minutes then it was deflated. 20 minutes after bag deflation, recording of intraluminal prucalopride 10μM took place for 20 minutes which was followed by another 20-minute bag inflation session in the presence of intraluminal prucalopride.

Mapping and analysis: Spatiotemporal maps were created using ImageJ plugins. Changes in colonic diameter associated with LDCs, single FPCs and FPC clusters were measured as explained in chapter 2.

Statistics: Data is presented as mean ± SEM. Significant difference was determined by Wilcoxon, or t-test as stated in table footnotes using Prism 8 software (GraphPad, United States), $P < 0.05$ was considered significant.

Test compounds: Prucalopride (4-Amino-5-chloro-2,3-dihydro-N-[1-(3-methoxypropyl)-4-piperidinyl]-7-benzofurancarboxamide) was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in DMSO.

RESULTS

Effects of Bag Inflation on LDCs and FPCs at Baseline

At baseline conditions before bag inflation, single FPCs at the frequency of 1.00 cpm with contraction amplitude of 0.56 ± 0.01 cm, cluster of FPCs at the frequency of 0.67 ± 0.18 cpm with contraction amplitude of 0.68 ± 0.01 cm and LDCs at the frequency of 0.01± 0.01 cpm with contraction amplitude of 0.78 ± 0.01 cm were observed (Table 8). Bag inflation resulted in immediate development of more forceful propulsive motor patterns due to distention. During the period of bag inflation, the frequency of single FPCs (the least forceful motor patterns), significantly *decreased* while the frequency of LDCs (the most forceful motor patterns) significantly *increased* consistent with the scale of propulsive motor activity discussed in chapter 2 (Table 8). Contraction amplitude and duration of motor patterns did not change significantly. However, all motor patterns stopped propagating as soon as they collided with the inflated bag and neither FPCs nor LDCs passed over it (Table 8, Fig 8,9).

Table 8. Comparison of Motor Patterns Before and During Bag Inflation at Baseline Conditions

	Baseline before bag inflation			Baseline during bag inflation		
	LDC (N=11, n=13)	**FPC cluster (N=11, n=170)**	**Single FPC (N=11, n=28)**	**LDC stopped at the inflated bag (N=11, n=40)**	**FPC cluster stopped at the inflated bag (N=11, n=175)**	**Single FPC stopped at the inflated bag (N=11, n=2)**
Contraction amplitude (cm)	0.78 ± 0.01	0.68 ± 0.01	0.56 ± 0.01	0.76 ± 0.02	0.72 ± 0.01	0.50 ± 0.03
Velocity (cm s $^{-1}$)	1.6 ± 0.05	6.5 ± 0.36	6.00 ± 0.83	1.67 ± 0.12	4.02 ± 0.14	6.5 ± 0.07
Duration (s)	9.70 ± 0.30	3.38 ± 0.11	2.27 ± 0.20	6.52 ± 0.38	2.9 ± 0.07	2.00 ± 0.01
Frequency (cpm)	0.01 ± 0.01	0.67 ± 0.18	1.00	0.31 *	1.30 ± 0.50 *	0.50 *
% of propagation along the colon	91.74 ± 1.42	77.21 ± 0.95	52.4 ± 2.41	46.00 ± 1.14 *	48.64 ± 0.91*	61.3 ± 0.37
Propagation length (cm)	15.32 ± 0.31	16.40 ± 0.27	10 ± 0.46	9.40 ± 0.26 *	9.56 ± 0.20 *	11.70 ± 0.07

Values are mean ± SEM. Significant difference determined by Wilcoxon test
* compared to baseline conditions before bag inflation (*P< 0.05).

Effects of Intraluminal Prucalopride on Colonic Motor Patterns During Bag Inflation

Consistent with chapter 2, intraluminal prucalopride 10μM induced forceful LDCs that propagated fully along the colon at the frequency of 0.51 ± 0.14 and contraction amplitude of 1.15 ± 0.05 (N=11, n=48) (Table 9). After bag inflation, two groups of LDCs were observed. Some LDCs did not continue propagating and stopped as soon as they collided with the inflated bag similar to the LDCs observed during the bag inflation session at baseline. The other group of LDCs did in fact continue propagating along the whole colon despite colliding with the inflated bag and they managed to pass over it. In total, 60.5% of LDCs evoked by intraluminal prucalopride passed over the bag.

The difference between these two groups of LDCs was that the LDCs that continued propagating in spite of the inflated bag, were significantly higher in contraction amplitude and duration (Table 9, Fig 10,11). Therefore, they were forceful and long lasting enough to pass over the inflated bag. The fully propagating LDCs that managed to pass over the bag were very similar in characteristics to the LDCs developed in response to the intraluminal prucalopride before bag inflation. However, the LDCs that stopped at the inflated bag showed characteristics more similar to the LDCs generated in response to bag inflation at baseline conditions (Table 8,9). The frequency of total LDCs did not change significantly before and during the bag inflation in the presence of intraluminal prucalopride.

Table 9. Comparison of LDCs in Response to Intraluminal Prucalopride Before and During Bag Inflation

	Intraluminal prucalopride 10µM before bag inflation (N=11, n=48)	**Intraluminal prucalopride 10µM during bag inflation**	
		LDCs passed down over the inflated bag (N=11, n=43)	**LDCs stopped at the inflated bag (N=11, n=28)**
Contraction amplitude (cm)	1.15 ± 0.05****	1.25 ± 0.04****	0.80 ± 0.02
Propagation length (cm)	18.2 ± 0.48****	17.02 ± 0.50****	10.44 ± 0.65
% of propagation along the colon	88.05 ± 1.51****	80.40 ± 3.20****	52.30 ± 1.62
Velocity (cm s $^{-1}$)	1.54 ± 0.07	1.84 ± 0.12	1.55 ± 0.11
Duration (s)	12.60 ± 0.44***	10.44 ± 0.51***	7.61 ± 0.55
Frequency (cpm)	0.51 ± 0.14	0.30 ± 0.07	0.22 ± 0.05

Values are mean ± SEM. Significant difference determined by unpaired t-test. * compared to LDCs stopped at the inflated bag (***P< 0.001, ****P < 0.0001).

Consistent with results from chapter 2, no single FPCs were observed after administration of intraluminal prucalopride 10µM. Contraction amplitude of FPC clusters increased and their frequency significantly *decreased* compared with baseline conditions as LDCs increased in frequency (Table 8,10) which was compatible with the various levels of excitation model. Identical to both FPCs and LDCs at baseline conditions, almost all FPCs failed to propagate fully along the colon after bag inflation. Therefore, the degree of

propagation along the colon was significantly lower compared to FPCs developed in response to intraluminal prucalopride 10μM before bag inflation. Nevertheless, only two FPCs propagated over the inflated bag. These two FPCs were significantly higher in contraction amplitude compared with the rest of FPCs that stopped propagating when collided with the bag (Table 10). The frequency of FPCs that did not pass down over the inflated bag after the administration of intraluminal prucalopride was significantly higher (Table 10). In total, 92% of drug-induced FPCs failed to pass over the inflated bag.

Table 10. Comparison of FPC clusters in response to intraluminal prucalopride before and during bag inflation

	Intraluminal prucalopride 10μM before bag inflation (N=11, n=22)	**Intraluminal prucalopride 10μM during bag inflation**	
		FPCs passed over the inflated bag (N=11, n=2)	**FPCs stopped at the inflated bag (N=11, n=22)**
Contraction amplitude (cm)	0.88 ± 0.02	0.91 ± 0.06 *	0.78 ± 0.02
Propagation length (cm)	15.54 ± 1.01	13.23 ± 2.26	9.5 ± 0.44
% of propagation along the colon	71 ± 4.50****	58 ± 9.90	45.16 ± 1.80
Velocity (cm s $^{-1}$)	3.40 ± 0.33	5.40 ± 0.30	5.00 ± 0.35
Duration (s)	5 ± 0.31	3 ± 0.73	2.42 ± 0.13
Frequency (cpm)	0.31 ± 0.24	0.1	1.46 $^{\#}$

Values are mean ± SEM. Significant difference determined by unpaired t-test, confidence interval at 95% for intraluminal prucalopride before bag inflation and FPCs propagating over the inflated bag. *compared to FPCs stopped at the inflated bag (*P< 0.05, ****P < 0.0001). $^{\#}$ compared to FPCs propagated in spite of the inflated bag ($^{\#}$P< 0.05).

Comparing the contraction amplitude of total LDCs evoked during bag inflation at baseline vs. in the presence of intraluminal prucalopride 10μM revealed that LDCs in response to the drug had a significantly higher contraction amplitude. This comparison included all LDCs evoked during bag inflation, both the LDCs that passed over the bag and the ones that did not (Fig 10F,G).

FIGURES

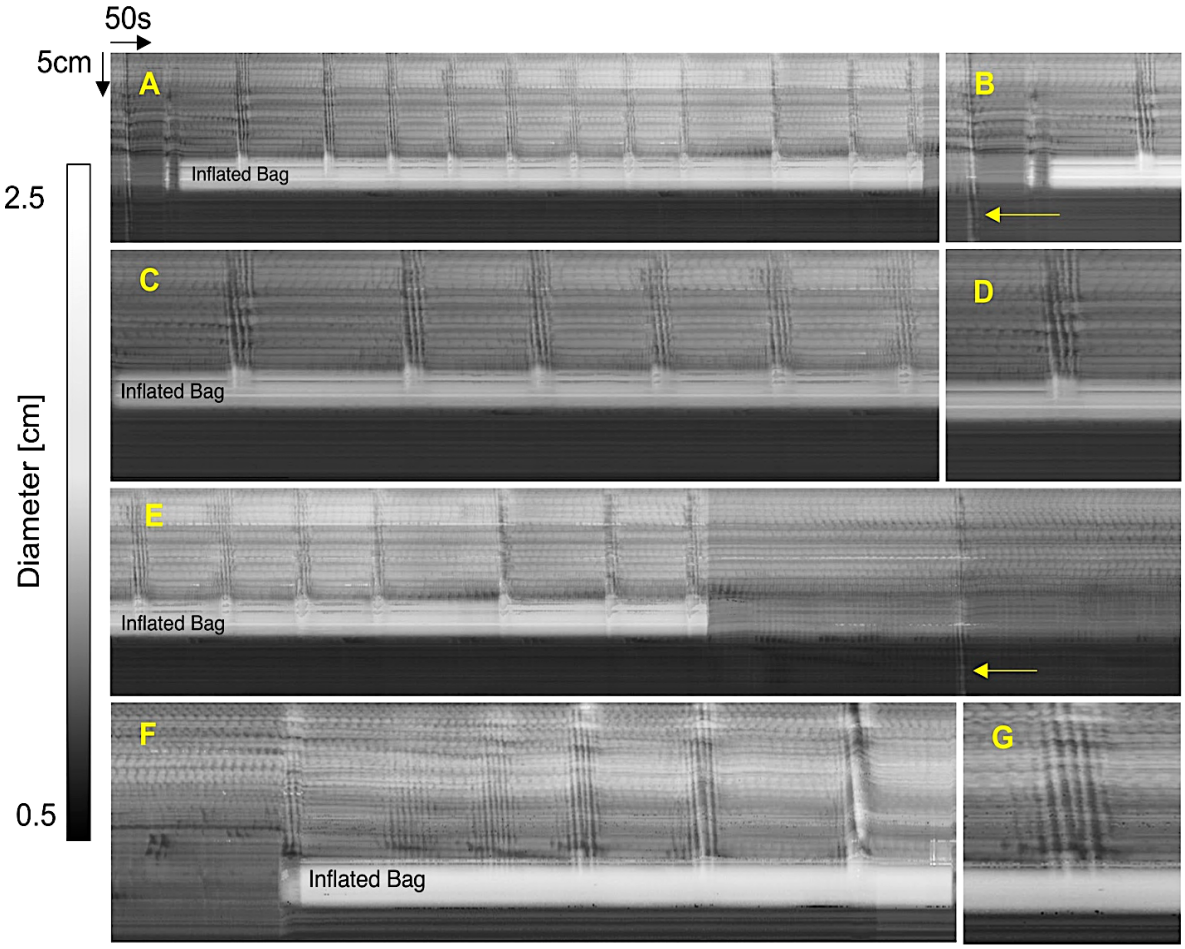


Figure 8. FPC clusters Don't Pass Over the Inflated Bag at Baseline

Experiment 1, (A,B) 20 minutes recording of sustained bag inflation at baseline. FPC clusters before bag inflation propagate fully. FPC clusters of higher contraction amplitudes developed in response to bag inflation. FPC clusters during bag inflation session did not pass over the inflated bag. (C,D) Closer caption of (A). (E) Baseline recording after bag deflation. Arrow shows an FPC cluster propagating fully along the colon.

Experiment 2, (F,G) Gradual development of more forceful FPC clusters after bag inflation none of which passed over the inflated bag.

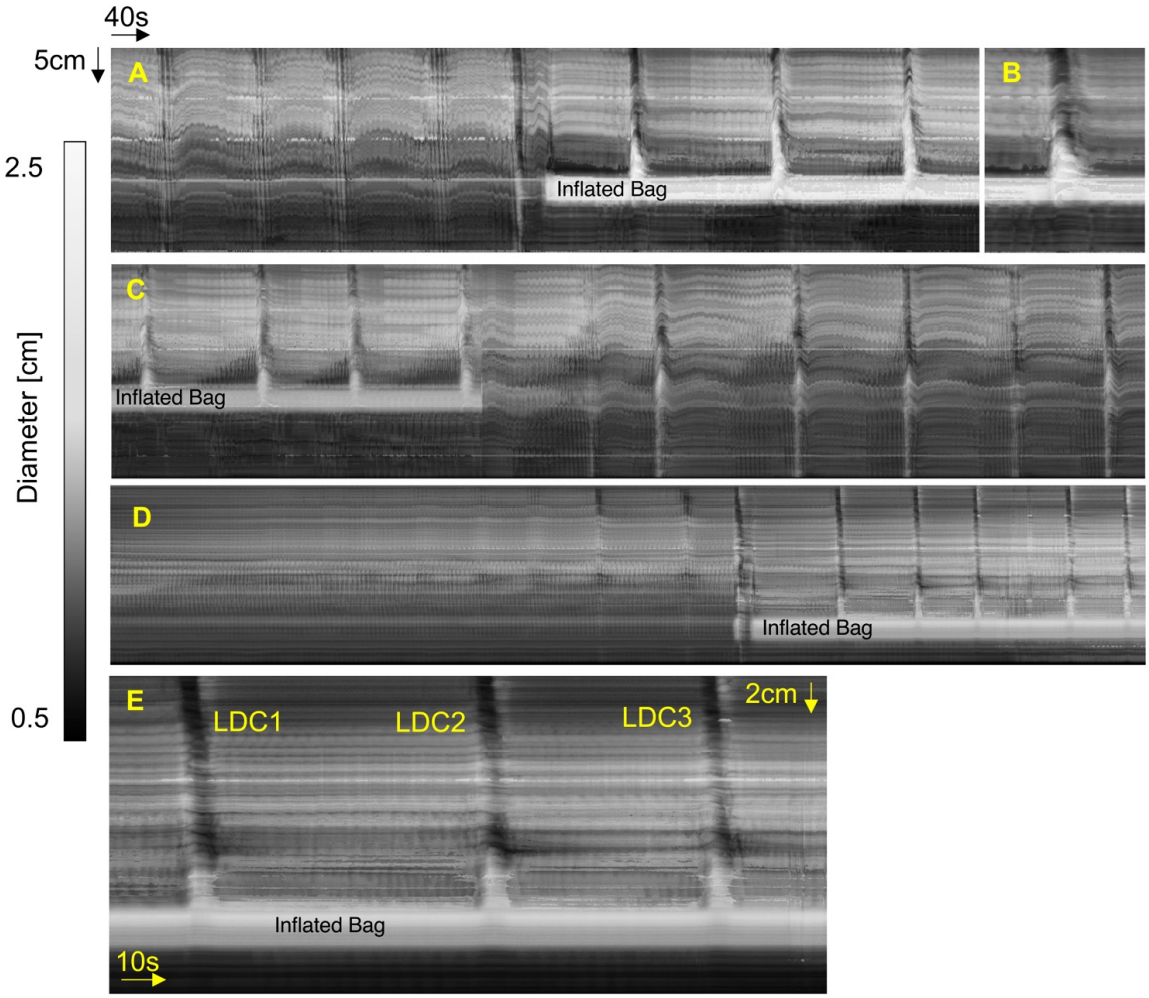


Figure 9. LDCs Don't Pass Over the Inflated Bag at Baseline

Experiment 1, (A,B) FPC clusters were present before bag inflation at baseline and propagated fully. LDCs developed in response to bag inflation at baseline but did not pass over the inflated bag. (C) After bag deflation LDCs continued to propagate fully along the colon.

Experiment 2, (D) No propulsive motor pattern is present at baseline only myogenic activity is observed. LCDs develop in response to bag inflation, but they do not pass over the inflated bag. (E) close caption of three LDCs from (D). LDC 1 with contraction amplitude of 0.76 cm, LDC2 with contraction amplitude of 0.71 cm and LDC3 with contraction amplitude of 0.66 cm.

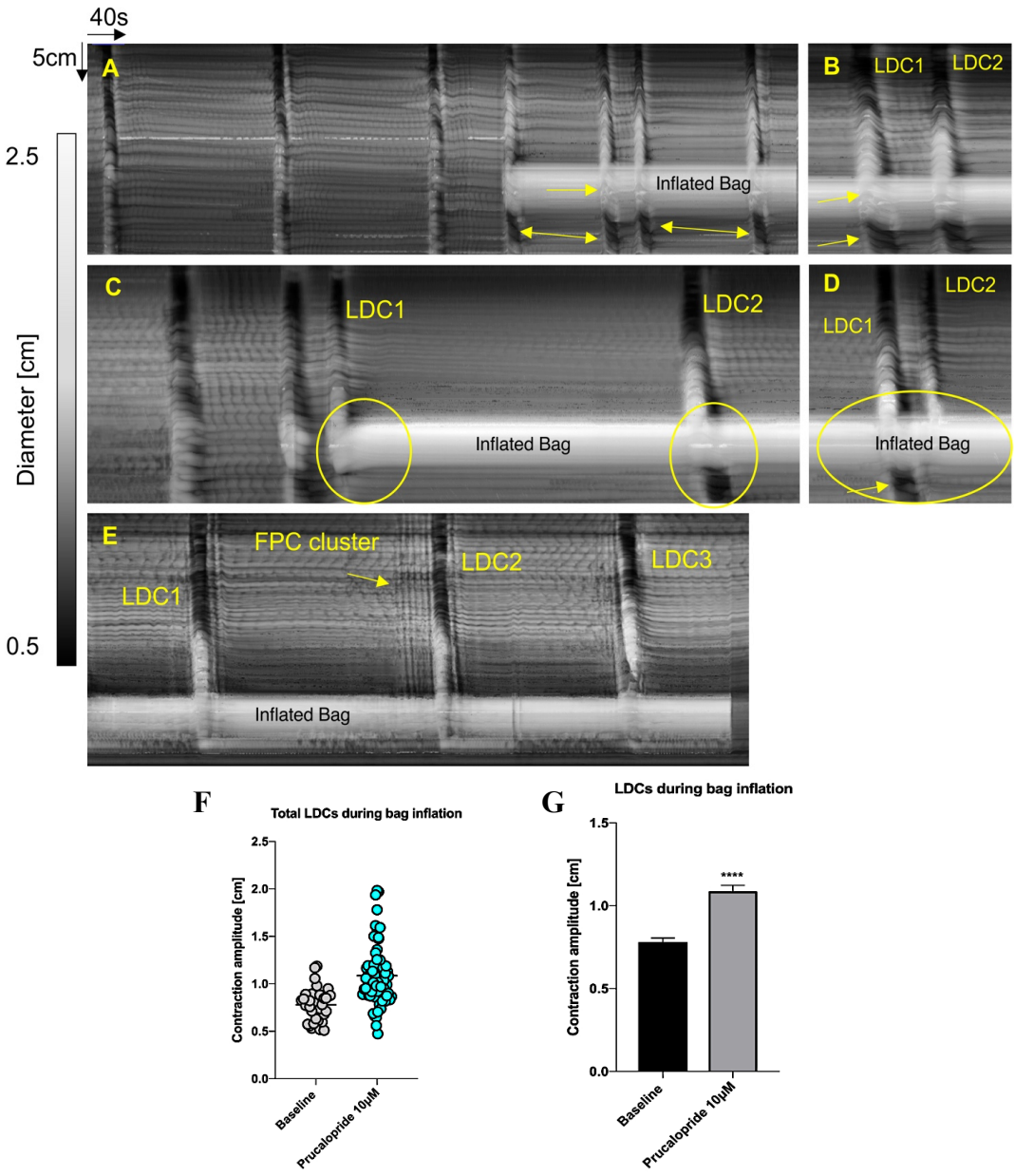


Figure 10. LDCs in Response to Intraluminal Prucalopride 10μM Before and During Bag Inflation. LDCs of Higher Contraction Amplitude Pass Over the Inflated Bag

Experiment 1, (A) LDCs passed over the inflated bag at the presence of intraluminal prucalopride 10μM. Yellow arrows show LDCs propagating over the inflated bag. (B) Shows close caption of two LDCs from (A). LDC1 with contraction amplitude of 1.36 cm and LDC 2 with contraction amplitude of 1.12 cm passed over the bag.

Experiment 2, (C) LDCs with lower contraction amplitude did not pass over the inflated bag. LDC 1 with contraction amplitude of 0.83 cm did not pass over the inflated bag. LDC 2 with contraction amplitude of 1.2 cm passed over the inflated bag. (D) Shows two adjacent LDCs with different contraction amplitudes from the same experiment. LDC 1 with contraction amplitude of 1.18 cm passed over the inflated bag with some

delay. LDC 2 with contraction amplitude of 0.85 cm did not. Experiment 3, (E) Low amplitude LDCs and FPC clusters did not pass over the inflated bag. LDC 1 with contraction amplitude of 0.76 cm, LDC 2 with contraction amplitude of 0.70 cm and LDC 3 with contraction amplitude of 0.68 cm. FPC cluster consisting of five FPCs with contraction amplitude of 0.56, 0.53, 0.50, 0.46, 0.44 cm from left to right failed to pass over the inflated bag. (F,G) Comparison of total LDCs evoked in response to intraluminal prucalopride 10μM vs. baseline during bag inflation (**** P<0.0001).

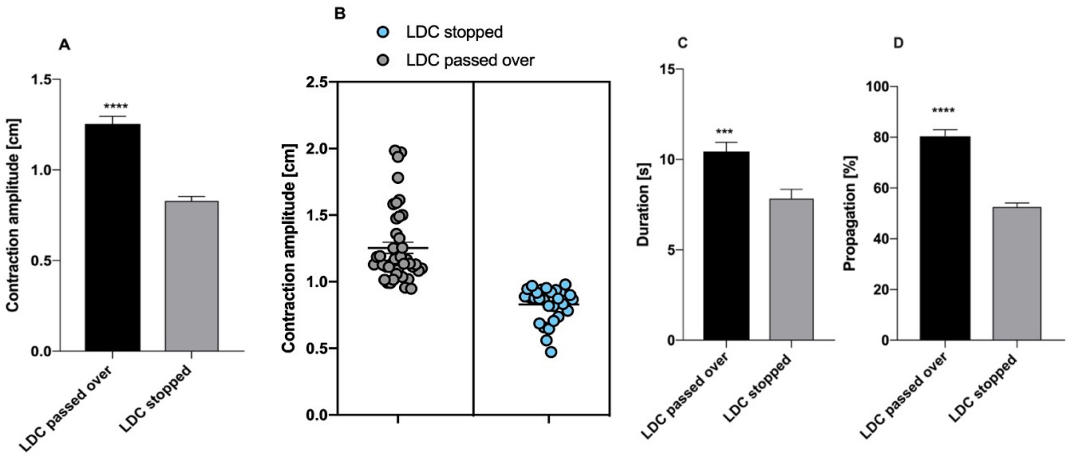


Figure 11. Comparison of Characteristics of LDCs in Response to Intraluminal Prucalopride 10μM Before and During Bag Inflation

(A,B) Contraction amplitude of LDCs that passed over the inflated bag was significantly higher than the LDCs that did not. (C) Duration of LDCs that passed over the inflated bag was significantly higher than the LDCs that did not. (D) LDCs that passed over the inflated bag propagated along the whole colon with significantly higher degree of propagation (***P < 0.001, ****P< 0.0001).

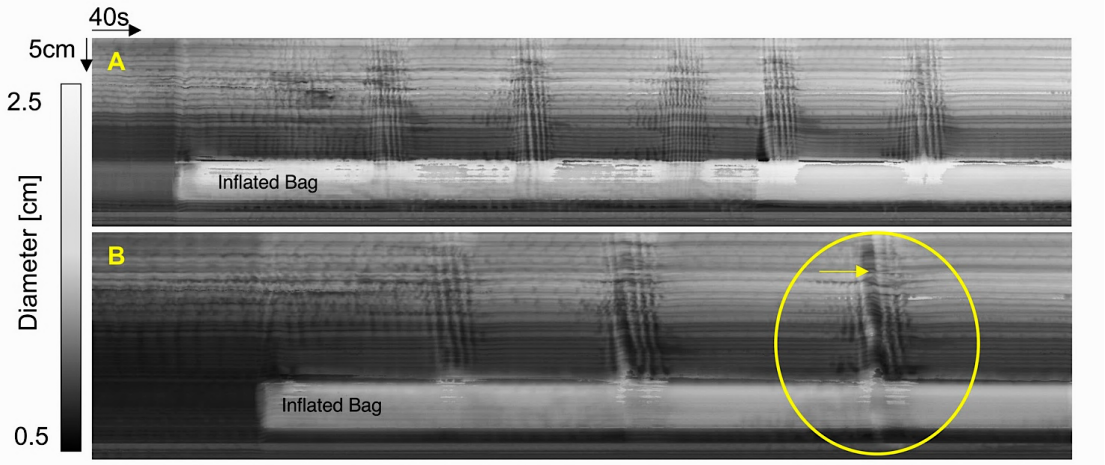


Figure 12. Cluster of FPCs Don't Pass Over the Inflated Bag in the Presence of Intraluminal Prucalopride 10μM

Experiment 1, (A) FPC clusters at the presence of intraluminal prucalopride 10μM failed to pass over the inflated bag. Experiment 2, (B) FPC cluster with LDC-like activity developing within the cluster failed to pass over the bag. Yellow arrow shows the LDC-like activity with contraction amplitude of 0.71 cm inside the FPC cluster.

SECTION II

HUMAN STUDIES USING HIGH RESOULTION COLONIC MANOMETRY

CHAPTER 4- COLO-ANAL MOTOR COORDINATION AND CHARACTERIZATION OF SIMULTANEOUS PRESSURE WAVES AS BIOMARKERS FOR COLONIC MOTILITY

INTRODUCTION

HIGH AMPLITUDE PROPAGATING PRESSURE WAVES IN THE HUMAN COLON

While most colonic motor activity in the human colon is non-propulsive and segmental, high amplitude propagating pressure waves (HAPW) can transfer colonic contents over long distances and are associated with defecation (Bharucha, 2012; Chen et al., 2018). Most HAPWs begin in the cecum of ascending colon and propagate in the anal direction. However, these proximal pressure waves do not generally propagate beyond the mid-colon and only around %5 of them reach the rectum (Bharucha, 2012).

Traditionally, the presence of HAPWs has been identified as a marker of colonic neuromuscular integrity (Liem et al., 2010; Chen & Huizinga, 2018). Manometry testing in adults has shown that these motor patterns increase significantly in number after a meal and upon awakening. In addition to occurring spontaneously and in response to colonic distention, HAPWs can be induced by pharmacological agents which are perhaps a more potent stimuli than colonic distention. Intraluminal stimuli such as glycerol, bisacodyl, oleic acid, and bile acids can elicit HAPW in adult humans (Bharucha, 2012).

It is possible that several stimuli including hormonal release, mucosal secretion and luminal distention are responsible for “initiating” HAPWs (Liem et al., 2010). Studies of colonic motility in adults have shown that chronically constipated patients exhibit fewer HAPWs accompanied by a decreased frequency of urge to defecate while HAPWs are increased in patients with IBS-D. This can indicate that one of the pathophysiological mechanisms for

chronic constipation might be decreased propulsive motility of the colon while propulsive motility increases in IBS-D (Liem et al., 2010; Bharucha, 2012). Nevertheless, King et al., (2009), reported normal frequency of HAPWs during 24-hour manometry testing in children with slow transit constipation, and hypothesized that the presence of HAPWs alone is not sufficient to establish normal colonic motility (King et al., 2009; Liem et al., 2010).

A low dose of the nitric oxide inhibitor N(G)-monomethyl-L-arginine (L-NMMA), which inactivates nitric oxide and the nitrergic transmission, can increase the frequency of colonic HAPWs (Bharucha, 2012). In addition to nitrergic nerves, extrinsic sympathetic input also inhibits colonic motility and HAPW. The adrenergic α_2-antagonist yohimbine induced colonic HAPW in healthy subjects (Malcolm & Camilleri, 2000; Bharucha, 2012). HAPWs were also more frequent in a canine model of colonic extrinsic denervation (Leelakusolvong et al., 2003) and in patients with autonomic neuropathy and diarrhea (Bharucha, 2012). However, the specific colonic neurophysiological mechanisms responsible for generating HAPWs are not yet completely understood (Bharucha, 2012).

COLO-ANAL MOTOR COORDINATION (THE COLO-ANAL REFLEX)

As discussed in chapter 1, the exact mechanism by which internal anal sphincter (IAS) relaxation takes place in coordination with propulsive motor activity of the colon remains insufficiently known (Rodriguez et al., 2012). Similar to lower esophageal sphincter relaxation which takes place at the onset of esophageal peristalsis, anal sphincter relaxation can occur at the onset of the HPAW (Rodriguez et al., 2012) or precede the arrival of the HAPW in the recto-sigmoid colon (Bharucha, 2019). Activation of intra-colonic descending inhibitory pathways probably explains anal sphincter relaxation before the HAPW arrives at the rectosigmoid colon (Bharucah, 2019).

Internal anal sphincter relaxation in constipated children was associated with HAPWs migrating in the proximal and distal colon (Rodriguez et al., 2012). Both Malcolm et al., (2000) and Rodriguez et al., (2012) documented IAS relaxation associated with HAPWs

and suggested that anal sphincter relaxation is a neurally mediated reflex rather than a mere phenomenon associated with rectal distention as is noted with the recto-anal inhibitory reflex (RAIR, Rodriguez et al., 2012; Malcolm et al., 2000). Rodriguez et al., (2012) also reported that a third of anal sphincter relaxations occurred while the HPAW was still in the right colon. They also observed that the percentage of IAS relaxation associated with HAPWs was significantly higher than the relaxations elicited by balloon distention (RAIR) during anorectal manometry and the duration of HAPW associated relaxations was significantly longer. This indicates the importance of the coordination between HAPW events and anal sphincter relaxation in defecation by allowing more transit of stool in the anal canal compared to the shorter IAS relaxation seen with the anorectal manometry which is possibly more related to the inflation reflex (Rodriguez et al., 2012). The fact that HAPWs propagating into the rectum were associated with higher percentage and longer duration of IAS relaxation compared with the ones that only partially propagated, can further support the role of HAPWs in eliciting internal anal sphincter relaxation to facilitate normal defecation (Rodriguez et al., 2012).

In this chapter, the association of motor patterns with anal sphincter relaxation as an important aspect of propulsive motor activity of the colon is quantified to provide a basis for better understanding of the defecation reflex. Before fully analyzing the colo-anal motor coordination, it is necessary to characterize the simultaneous pressure wave (SPW) as a common, yet, mainly ignored motor pattern that was also observed to be associated with anal sphincter relaxation in this study.

CHARACTERIZATION OF THE SIMULTANEOUS PRESSURE WAVE (SPW)

Patients with functional bowel disorders have abnormal bowel movements and/or outlet obstruction and often complain about bloating (Burri et al., 2014; Malagelada et al., 2017). Some studies have shown that abdominal bloating is the most bothersome symptom for such patients (Kanazawa et al., 2016). Patients with bloating have normal colonic accommodation of gas loads and ileocecal continence but impaired clearance of gas from

the colon (Hernando-Harder et al., 2010). Patients with poor handling of gas, also show poor handling of semi–liquid contents (Serra et al., 2010). Impaired gas clearance appears to involve tonic contractions of the small intestine (Tremolaterra et al., 2006; Serra et al., 2010) and abnormal motor activity of the colon (Malagelada et al., 2017). Proof of concept studies show a positive effect of prokinetics on gas related symptoms (Serra et al., 2001; Caldarella et al., 2002) which suggests that gut motor activity determines gas transit, and that bloating is caused by a motility disorder; however, the precise mechanisms underlying gas transit have not been established.

Clinical colonic manometry studies focus on bisacodyl and/or meal-induced motor patterns (Camilleri et al., 2008; Rao et al., 2011; Mugie et al., 2013; Dinning et al., 2016), in particular HAPWs; but there has been little or no emphasis on the study of specific gas-associated motor patterns. Colonic manometry is mostly focused on the identification of a potential "inert" colon, usually defined as the absence of HAPWs during a colon function test, and when inert colon is diagnosed, surgery is usually contemplated (Chen & Huizinga, 2018).

There are, however, many uncertainties related to such a conclusion. First, healthy volunteers may not have HAPWs in a 24-hour period (Cook et al., 2000; Rao et al., 2001; Dinning et al., 2010); hence, whether absence of HAPW definitely indicates an abnormal colonic activity is questionable. It is not yet clear if slow transit is explained by an absence of HAPWs (Bharucha, 2012). It is also uncertain if the colon can be deemed normal if HAPWs are induced only by bisacodyl, since bisacodyl may induce HAPWs whereas physiological stimuli might not (Liem et al., 2010; Bharucha, 2012).

These uncertainties and the understanding that colonic motility is often analyzed in a cursory manner, may be a reason why colonic motility tests are not often performed in adults; and in children they add even more ambiguity to the outcome. Hence, if a colonic motility test is to assess the full capabilities of the colon, including motor patterns associated with gas evacuation, it needs to expand its scope. This possibility seems at the

horizon with the application of HRCM. Dinning has highlighted the marked increase in understanding of motor patterns with high resolution manometry and the danger of interpreting motor patterns using low-resolution manometry (Dinning et al., 2013).

Simultaneous pressure waves (SPWs) are pressure transients that occur simultaneously at all sensors that record them. Rao et al., (2001) identified the SPW as a common motor pattern but it was largely ignored in subsequent studies due to the uncertainty about it being related to abdominal pressure changes. A report was released on SPWs using high-resolution manometry with a solid-state catheter incorporating 36 sensors at 1 cm spacing covering the descending colon down to the anal sphincter (Chen et al., 2017). Corsetti et al., (2017) also reported the phenomenon using a catheter with 40 solid state sensors at 2.5cm spacing and called them “pan-colonic pressurizations”.

The present study characterized SPWs for the first time using high-resolution manometry with 84 sensors at 1 cm spacing throughout the entire colon including the anal sphincters. In order to fully understand the physiological importance of SPWs, a protocol was designed with the following stimuli; a 1000 kcal fat-rich meal, luminal balloon distention and luminal bisacodyl administration. Furthermore, the colonic motor patterns are presented in three-dimensional format to facilitate visual interpretation.

The National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers, 2001). Here, the SPW is characterized as such kind of biomarker, including what role it plays under normal physiological conditions and in response to pharmacological intervention. This comprehensive characterization of SPWs in healthy subjects by HRCM may provide significant clinical value to evaluate pathophysiology in patients with colonic dysmotility and abdominal bloating. It also serves

as a foundation for analysis of the effects of intraluminal prucalopride using HRCM (chapter 5).

MATERIALS AND METHODS

Study Subjects

Seventeen healthy volunteers (age: 20–54 years; 6 females) were recruited through local advertising. All participants gave written informed consent and all procedures were approved by the Hamilton Integrated Research Ethics Board (HiREB). Exclusion criteria were abdominal surgery, hepatic, kidney or cardiac diseases, connective tissue disorders, central nervous system disorders, thyroid diseases, prostate diseases or malignancies. All subjects had normal stool consistency and normal bowel frequency, between 1 every 3 days and 3 per day. None had defecation difficulty, and none were taking medication that might influence bowel movements.

High-Resolution Colonic Manometry

High-resolution colonic manometry was performed on a custom-made platform (Medical Measurement Systems (MMS); Laborie, Toronto, ON, Canada). An 84-sensor water-perfused catheter was designed (Mui Scientific, Mississauga, ON, Canada) that included two 10-cm long balloons between sensors 10 and 11 and 40 and 41, with sensor 1 placed in the ascending colon. In four volunteers, a separate rectal balloon was used. The catheter was inserted with minimal sedation (fentanyl i.v. 50–100 mcg and midazolam i.v. 2–5 mg) with the assistance of a colonoscope after a bowel cleaning procedure using an inert osmotic laxative (PEG-Lyte, Pendopharm, Montreal, QC, Canada), but no use of stimulant laxatives such as bisacodyl. For the bowel cleaning procedure, 3L of PEG (70 g/L) was taken between 4 and 6 pm the day before the procedure, with more water consumed if needed to have all solids removed. The next morning, 1L was taken at 4 am. The tip of the catheter

was clipped to the mucosa via a fish line, a few centimeters distal to the cecum. The catheter was made of 100% silicon. After use, an extensive approved cleaning procedure was executed followed by sterilization. A disposable dual lumen stomach tube (3.3 mm × 91 cm; Salem SumpTM, Covidien Ilc, United States) was placed in the rectum for passive liquid drainage. All subjects were in the supine position during the entire recording, except during meal intake. Subjects were instructed to report all events such as gas or liquid expulsion, bowel movements, pain, and discomfort. Subjects were asked not to promote or prevent gas or liquid expulsion by increasing abdominal pressure or contracting the external anal sphincter if an urge arose. All body movements such as changing body position, talking, coughing, laughing, and urination were noted immediately into the data acquisition files.

Protocol

A 90 min recording of baseline activity was started 30 mins after the colonoscope was withdrawn. Afterwards, the response to a 5 min balloon distention at the proximal colon and rectum was investigated. The proximal balloon was distended with 250–400 ml air while the severity of abdominal discomfort reached to 6–7 on a 10-point symptom scale, then responses were recorded during and 20 min after the deflation of the balloon. Thereafter, a meal was given (500g of organic vanilla yogurt fortified with organic milk fat (Mapleton Organics, Moorefield, ON, Canada), to reach 800 –1000 kcal depending on the volume consumed. Its effect was observed for a minimum of 90 min. Lastly, the effects of 10mg of bisacodyl (Dulcolax; Boehringer Ingelheim, Sanofi Canada, Laval, QC, Canada) in the proximal colon via the catheter or in rectum were observed for 30 min; the bisacodyl suspension was made in saline by crushing 2 tablets, 5 mg each, with a pestle and mortar for 5 minutes.

Water-Perfusion

The catheter delivered 0.1 ml/min sterile water via each sensor to a total of 0.57 L per hour; the water pressure was calibrated at 1000 mbar. A drainage tube (OD 3.3 mm) was placed in the rectum to allow passive outflow of excess water. In this way, 1–2 L water was diverted without notice by the subjects; the remainder of the water was expelled by colon motor activities or absorbed. Intraluminal pressure in between motor patterns did not change during the 6–8-h session, hence, the water inflow did not cause passive tonic pressure changes.

Identified Motor Patterns

1. Simultaneous pressure waves (SPWs) are pressure transients that occur simultaneously at all sensors that record them, as identified by Rao et al., (2001) and De Schryver et al., (2003), and further defined by us (Chen et al., 2014, 2017). They have also been called simultaneous contractions (Rao et al., 2004) or pan-colonic pressurizations (Corsetti et al., 2017). The term contraction was avoided since contraction was not measured and pressure is not equivalent to contraction (Chen et al., 2016; Quan et al., 2017).

2. High amplitude propagating pressure waves (HAPWs) are defined as transient increases in pressure of more than 75 mmHg that propagate almost always in anal direction. They are also called high amplitude propagating contractions or sequences (Bharucha, 2012).

3. High amplitude propagating pressure waves followed by simultaneous pressure waves (HAPW-SPWs). This frequently occurring motor pattern is described in this study. An HAPW starts in the proximal colon and promptly switches to an SPW at the transverse or descending colon; hence, the SPW within this motor pattern is not pan-colonic.

4. Haustral boundaries are rhythmic or continuous pressure increases that occur in single sensors without activity in adjacent sensors; they occurred in multiple sensors about 5cm apart (Chen et al., 2017; Quan et al., 2017). These pressure increases define the boundaries of haustra (Bharucha & Brookes, 2012).

5. Haustral activity was an activity that occurred in 4–5 consecutive sensors but not immediately outside these sensors, suggesting it to be activity within a haustrum (Chen et al., 2017; Quan et al., 2017).

6. Anal sphincter activity and anal sphincter relaxation, either spontaneously or as part of a motor pattern.

Visual Identification and Quantitative Analysis of Motor Patterns

All data were acquired using the software developed by Medical Measurement Systems (MMS; Laborie, Toronto, ON, Canada), and analyzed using programs developed by Sean Pasrons by Image J (National Institutes of Health, Bethesda, MD, United States) and MATLAB (Mathworks, Natick, MA, United States). After a motor pattern was identified visually in Image J, a rectangle was placed around the SPW, or a HAPW was encircled free hand. In high-resolution manometry, with an acquisition rate of 10/s, an average SPW has 10.080 data points when all sensors record it. A typical HAPW has many more. These data were used to calculate the average amplitude, duration and length in Image J. MATLAB was used to generate the 3D images. This method is substantially different from measurements using low-resolution manometry where only a few points along the motor pattern are considered. This also indicates that normal values have to be re-evaluated in HRCM when compared to low resolution manometry. A positive gastrocolic reflex was defined as an increase in motor patterns, compared to baseline in response to a meal (Snape et al., 1980).

Analysis of Anal Sphincter Activities

When voluntary external anal sphincter contractions occurred, it was not possible to quantify relaxation. When the internal anal sphincter showed rhythmic pressure activity, and the SPW coincided with the "relaxation phase" of the rhythmicity, the relaxation induced by the SPW could not be assessed accurately. Due to the oscillatory nature of the anal sphincter pressure, relaxation was quantified when it reached higher than 25% of the average pressure value recorded in a 3-minute period before the relaxation. The period of relaxation started with the first sign of relaxation and ended with return to its baseline pressure. In the middle of this period, complete anal sphincter relaxation could be achieved.

Symptom Correlations and Artifacts

Subjects were instructed to report all symptoms, such as abdominal pain, passing gas or liquids per rectum, abdominal bloating, rectal urgency, urinary urgency. They were also instructed not to withhold or resist gas expulsion or liquid outflow. Artifacts were caused by body movement or coughing. Talking without body movement did not cause artifacts. Such artifacts were immediately written in the acquisition files and excluded from the analysis.

Statistical Analysis

The present study was designed to record the baseline colonic motor activity, which was followed by sessions with different stimuli. It is a descriptive study to document the features of normal SPW activity in healthy volunteers and the association of motor patterns with anal sphincter relaxation. The stimuli were given consecutively, therefore, the responses to stimuli may have been influenced by the remaining activity of the previous intervention with the exception of the first stimulation (proximal balloon distention). The responses to stimuli were described and compared to baseline activity qualitatively and quantitatively. Data are given as mean ± SD. Significance was determined by one-way ANOVA with Dunnett's post-test using Prism 7 software (GraphPad, United States), $P < 0.05$ was

considered significant. For SPWs with and without gas/liquid expulsion and potential differences between male and female subjects, the statistical significance was determined using t-test (Prism 7).

Representation of Balloons on Maps

The position of the balloons in all figures is identified by a white line. The white line represents a gap of 10cm where no data were recorded. The length of the colon covered by the sensors as indicated in the figures is the true length, with the balloon sections considered.

RESULTS

Association of Motor Patterns with Anal Sphincter Relaxation (The Colo-anal Reflex)

At baseline, SPWs appeared along the entire length of the colon with an average amplitude of 12.1± 8.5 mmHg, ranging from 5 to 38.4 mmHg. Their duration ranged from 2 to 58 s, average 13.8 ± 9.1 s (Table 12). SPWs could emerge at the termination of a proximal HAPW in the mid colon (Fig 13,14), this pattern was termed HAPW-SPWs. At baseline, the SPW component of the HAPW-SPW had an average amplitude of 20.2 ± 10.2 mmHg compared to pan-colonic SPWs at 12.1 ± 8.5 mmHg, and an average duration of 18.0 ±12.3 s, associated with a proximal HAPW with an average amplitude of 105.4 ± 49.7 mmHg (Fig 15, Table 12). This study involved 6 females and 11 males, a total of 241 SPWs were observed in females and 547 in males. The total occurrence of SPWs was not significantly different per subject in males (49.7 ±10.7) vs. females (40.2 ± 5.7) ($P = 0.54$). The average amplitude, however, was significantly lower in females with 12.5 ± 6.3 mmHg for females and 17.0 ±11.0 mmHg for males ($P < 0.0001$). Whether or not an HAPW was followed by

a SPW was not predictable based on the HAPW amplitude. Figure 16 shows two very similar proximal HAPWs, but only one was followed by a SPW.

The anal sphincter showed intrinsic rhythmicity. In 12 subjects, highly rhythmic internal anal sphincter pressure transients occurred at 1.22 ± 0.26 cpm, oscillating between 62.2 ± 8.2 and 90.5 ± 12.0 mmHg (Fig 17). 67% of pan-colonic SPWs (124 out of 185) were associated with measurable anal sphincter relaxation (Table 11, Fig 18), whereas 88% of HAPW-SPWs (51 out of 58) were associated with measurable anal sphincter relaxation. Anal sphincter relaxation most often occurred the moment the SPW propagated into the anal canal, but it sometimes started with a delay and after the SPW ended. Meal and bisacodyl induced significantly higher degrees of SPW associated anal sphincter relaxation compared to baseline in both pan-colonic SPWs and HAPW-SPWs (Fig 18, Table 11). HAPW associated anal sphincter relaxation showed significantly higher degree and duration of relaxation in response to bisadodyl (Table 11, Fig 18).

These are average values of relaxation but in many cases a short period of complete anal sphincter relaxation was achieved (Fig 13,14). The duration of anal sphincter relaxation associated with SPWs was 15.6 ± 5.8 s in baseline, 11.8 ± 4.2 s during the meal session ($P < 0.01$ compared to baseline) and 14.8 ± 6.0 s at the presence of bisacodyl. The average amplitude of SPWs associated with anal sphincter relaxation was 21.0 ± 8.8 mmHg (n=124) which was significantly higher compared to 10.0 ± 6.1 mmHg (n=61) without quantifiable anal sphincter relaxation. Nevertheless, the amplitude of SPWs that were associated with anal sphincter relaxation and gas or liquid expulsion spanned the entire repertoire of amplitudes and durations (Fig 15).

When subjects reported gas expulsion, this was always associated with the occurrence of an SPW or an HAPW-SPW. Low amplitude SPWs (<30 mmHg) and associated anal sphincter relaxations were not noticed by the subjects, except when gas or liquid expulsion occurred. Higher amplitude SPWs (>35 mmHg) could be associated with urge of

defecation. Occasionally, subjects wanted to prevent outflow and tightened the external anal sphincter as shown in Figure 13D. An HAPW that was not followed by a SPW, was often not associated with anal sphincter relaxation (Fig 19). Anal sphincter relaxation did occur with 47% of HAPWs (Table 11); the relaxation usually started when the HAPW progressed into the sigmoid colon. An HAPW that did not reach the distal colon was rarely associated with anal sphincter relaxation.

SPWs in Response to a Meal

After a 1000 kcal meal, the number of SPWs increased markedly from 3.2 ± 0.5 in the last 30 minutes at baseline to 6.4 ± 0.8 in the first 30 minutes after a meal (Fig 20). The amplitude of gas expulsion-related SPWs increased significantly compared to baseline (29.4 ± 12.6 vs. 12.6 ± 12 mmHg) and compared to SPWs without gas expulsion evoked postprandially (15.3 ± 8.4 mmHg) (Table 12, Fig 15). The duration of SPWs did not show a significant change (Table 12).

The Rhythmicity of SPWs

Pan-colonic SPWs occurred in a rhythmic fashion on 26 occasions at an average frequency of 1.77 ± 0.77 cpm, ranging from 0.32 to 4.37 cpm. Furthermore, 13 of the rhythmic SPW activities were observed in response to the meal where the average frequency was 2.05 ± 0.90 cpm (Fig 21). Figure 21B shows an example where rhythmic SPWs increase in amplitude with time, accompanied by increasing anal sphincter relaxation. A second rhythmicity was observed within SPWs. This rhythmicity became apparent when the SPW was not of uniform amplitude. In those instances, SPWs were observed to consist of clusters of very narrow, high frequency SPWs at 24 ± 2 cpm (n = 5; Fig 22).

Table 11. Anal sphincter Relaxation Associated with SPWs and HAPWs in 17 Healthy Subjects

	Motor pattern mean amplitude (mmHg ± SD)	Anal sphincter mean amplitude before relaxation (mmHg ± SD)	Anal sphincter mean amplitude during relaxation (mmHg ± SD)	% of Anal sphincter relaxation	Duration of Anal sphincter relaxation (s)
SPW (n= 124), 67% of total (n=185) associated with anal sphincter relaxation					
Baseline (n=52)	18 ± 7.7	60.2 ± 20.6	31.25 ± 12.5	48.0 ± 8.8	15.6 ± 5.8
Meal (n= 44)	21.5± 9.5	65.6 ± 19.7	27.52 ± 9.16	57.2 ± 10.7 **	11.8 ± 4.2**
Bisacodyl (n= 24)	25.3± 6.7 **	75.6 ± 19.9	25.9 ± 8.4	66.0 ± 7.1****	14.8 ± 6.0
Balloon (n=4)	24.5 ± 14.7	76.6 ± 16.2	42.8 ± 22.2	48.8 ± 12.2	12.3 ± 7.0
SPW in HAPW-SPW (n= 51), 87.9 % of total (n=58) associated with anal sphincter relaxation					
Baseline (n=16)	21.8 ± 6.1	70.8 ± 18.4	31.2 ± 13	55.8 ± 11.6	19.6 ± 7.7
Meal (n=16)	24.4 ± 7.1	84.5 ± 29.5	30.3 ± 12.6	63.4 ± 9.2 *	18 ± 9.3
Bisacodyl (n= 15)	29.4 ± 12	83 ± 25.2	24.3 ± 9.6	70.6 ± 7.1***	16 ± 7.55
Balloon (n=4)	16.4 ± 16	72.7 ± 6.40	26.6 ± 16	62.8 ± 16.6	21.2 ± 3
HAPW (n= 20), 46.5% of total (n=43) associated with anal sphincter relaxation					
Baseline (n=6)	119.4 ± 37.2	72.0 ± 18.6	35.9 ± 12.3	50.2 ± 10.4	20.3 ± 7.5
Meal (n= 3)	120.3 ± 16.8	65.7 ± 12.7	29.2 ± 9	56.0 ± 5.3	6.3 ± 4.1*
Bisacodyl (n=8)	207.0 ± 51.0 **	82.2 ± 11.0	27.2 ± 10.4	67.3 ± 10.2 *	22.6 ± 10.8
Balloon (n=3)	129.0 ± 33.5	86.6 ± 19.0	29.2 ± 9.0	46.1 ± 11.3	9.6 ± 8.9 *

Values are mean ± SD. Significance was determined by one-way ANOVA with Dunnett's post-test. All compared with their baseline conditions ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

Table 12. Quantification of SPW Activity in 17 Healthy Subjects

	Total SPWs	SPWs with gas expulsion	HAPWs	HAPW-SPWs (HAPW data)	HAPW-SPWs (SPW data)
Baseline (90 mins)					
Occurrence	N = 17 n = 245	N=8 n=15	N = 5 n = 15	N = 7 n = 28	N = 7 n = 37
Amplitude (mmHg)	12.1 ± 7.3 5 – 38.4	20.8 ± 4.3 ††† 7.1 – 31.0	146.6 ± 49.4 74.8– 270.9	105.4 ± 49.7 34.3 – 206.2	20.21±10.2# 2.9 – 38.4
Duration (s)	13.8 ± 9.1 2 – 58	10.8 ± 4.6 6 – 25	78.6 ± 30.2 37 – 78	52.4 ± 21.7 16 – 120	18.0 ± 12.3 4.7 – 38
Proximal Balloon (20 mins)					
Occurrence	N = 13 n = 42	N=5 n=15	N = 7 n = 21	N = 11 n = 19	N = 11 n = 20
Amplitude (mmHg)	15.3 ± 7.4 5 – 49.6	26.6 ± 10.1 †††† 9.6 – 45.0	150.5 ± 36.2 95.3 – 203.8	105.3 ± 63.8 27.4 – 236.9	15.5 ± 8.0 3.9 – 32.0
Duration (s)	10.3 ± 8.0 2 – 36	8.1 ± 2.5 3 – 12	98.1 ± 46.9 44 – 219	67.8 ± 28.2 * 24.2 – 124	12.0 ± 7.7 5 – 30.1
Meal (90 mins)					
Occurrence	N = 17 n = 282	N=9 n=34	N = 5 n = 7	N = 10 n = 36	N = 10 n = 32
Amplitude (mmHg)	15.3 ± 8.4 5.1 – 47.2	29.4 ± 12.6 */ †††† 2.1 - 66.3	154.5 ± 69.8 63.8 – 146.0	95.6 ± 35 31.3 – 176.5	25.2 ± 13.4# 6.7 – 61.3
Duration (s)	10.8 ± 7.1 2 – 57.3	9.3 ± 6.8 2 – 40	67.2 ± 24.9 46 – 106	45.4 ± 23.3 12 – 96	15.6 ± 8.1 4 – 35
Bisacodyl (30 mins)					
Occurrence	N = 15 n = 219	N=8 n=12	N = 12 n = 37	N = 13 n = 73	N = 13 n = 67
Amplitude (mmHg)	19.7 ± 9.9 **** 3 – 57.5	29.0 ± 6.0 */ †††† 18.4 – 36.8	284.4 ± 50.9 *** 88.8 – 322.5	132.3 ± 50.9* 47.2 – 225.6	21.6 ± 13.1# 5 – 57.5
Duration (s)	10.1 ± 9.3 2 – 57	13.1 ± 4.7 4 – 20	77.6 ± 38.6 15 – 168	67.8 ± 37.3 * 14 – 216	15.1 ± 11.2 2 – 57

Values are average ± SD and range; except occurrence. * comparison with their baseline conditions (*P < 0.05, ***P < 0.001, ****P < 0.0001). † comparison between SPWs associated with gas expulsion and total SPWs without gas expulsion at baseline (†P <0.05, ††† P<0.001, ††††P < 0.0001). # comparison between SPWs in HAPW-SPWs with total SPWs at baseline (#P <0.05).

SPWs Occurred Independent of Haustral Boundaries and Isolated HAPWs

An often-occurring motor pattern showed irregular or rhythmic pressure transients observed at single censors about 5cm apart (Fig 21A), likely representing haustral

boundaries (Chen et al., 2017; Quan et al., 2017). When rhythmic, the dominant frequency was ~3 cpm. When SPWs traversed these haustral boundary pressure transients, their amplitudes summated. Hence, SPWs did not abolish the haustral boundaries (Fig 21A).

SPWs in Response to Proximal Balloon Distension

Proximal balloon distension did not, on average, increase the number of SPWs per unit of time compared to baseline, but the number of SPWs associated with gas expulsion markedly increased. Furthermore, the amplitude of SPWs accompanied by gas expulsion was also significantly increased compared to SPWs without gas expulsion (Table 12). Proximal balloon distension evoked HAPWs as well as proximal HAPWs that turned into SPWs (Fig 13, Table 12).

SPWs in Response to Bisacodyl Delivered in the Proximal or Descending Colon

Luminal administration of bisacodyl (10 mg suspension) in the proximal colon or descending colon evoked HAPWs and HAPW-SPWs that started in the proximal colon with associated gas or liquid expulsion. Amplitudes of HAPWs, HAPW-SPWs and SPWs with gas expulsion were markedly higher than that of baseline (Table 12). SPWs associated with gas expulsion after bisacodyl had significantly higher amplitudes than those that were not associated with gas, but their duration did not differ significantly (Table12, Fig15).

SPWs in Response to Rectal Stimulation

Rectal balloon distention evoked significantly higher numbers of SPWs (10.5 ± 1.2) in a 20-min period (N = 4) compared to 3.5 ± 1.1 at baseline over the same time period ($P < 0.001$), as well as increasing the number of proximal HAPW- SPWs (1.5 ± 1.2 vs. 0.3 ± 0.5; $P < 0.01$). The average SPW amplitude increased from 5.6 ± 1.6 to 11.1 ± 2.3 mmHg ($P < 0.05$). In Figure 23, a SPW is shown which consists of a cluster of high frequency SPWs with one reaching an amplitude of 55 mmHg associated with a strong urge to

defecate and followed by expulsion of the balloon. Rectal bisacodyl (10 mg suspension, measured over 30 min periods; n = 6) evoked an increase in the number of SPWs from 1.2 ± 0.5 at baseline to 4.8 ± 1.3 after bisacodyl (N= 4; $P < 0.01$, at an amplitude of 13.2 ± 5.2. mmHg). HAPW-SPWs increased from 0.2 ± 0.1 during baseline to 4.7 ± 1.2 in the presence of bisacodyl ($P < 0.001$) in a 30-min period, at an amplitude of 9.5 ± 2.3 mmHg. The effects started 5–8 min after bisacodyl administration (Fig 24).

FIGURES

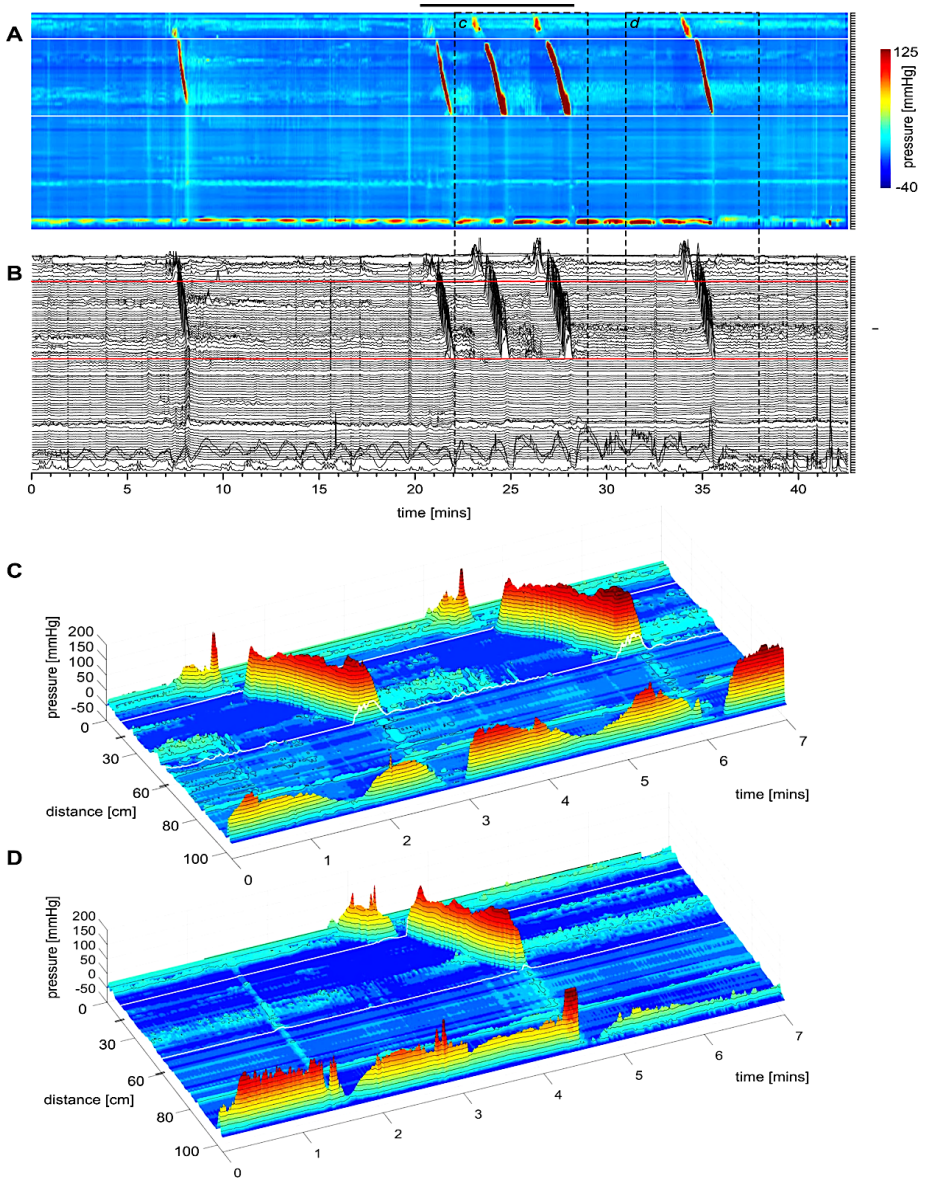


Figure 13. A HAPW that develops into a SPW in response to proximal balloon distention (HAPW-SPW)

The position of a balloon is indicated by a white line. This means that there are 10 cm gaps in this recording at the two white lines. Hence, a recording of 80 sensors will have a total reach of 100 cm. In (A,B), the dashes at the right side of the figure represent the position of the sensors (80 sensors, spaced 1 cm apart). The top section of the figures shows the proximal colon. In (C,D) on the distance axis, 0 is the location of the most proximal sensor. (A) The time period of proximal balloon distention by 240 ml air is indicated by the black line above (A). The balloon is positioned at the proximal white line. Proximal HAPWs developed into SPWs (generating HAPW-SPWs) associated with anal sphincter relaxation. Extensive haustral activity (see Chen et al., 2017; Quan et al., 2017) is seen between 30 and 35 cm following each HAPW. (B) Same as (A), showing

the actual pressure traces. (C) Section of (A) between the first two vertical dashed lines show two HAPW-SPWs that are followed by anal sphincter relaxation. Note that the HAPW evoked by the balloon starts at the more proximal sensors. (D) Section of (A) between the 3rd and 4th vertical dashed lines. A low amplitude SPW with anal sphincter relaxation is seen at 1.8 cm. This is followed by an HAPW-SPW and anal sphincter relaxation; the relaxation is preceded by a brief voluntary external anal sphincter contraction. The subject was asked a minute after the motor pattern occurred whether or not the sphincter was voluntarily squeezed. The first HAPW-SPW during balloon distention was associated with bloating, the second with a feeling of urgency. The HAPW-SPW that occurred ~ 5 min after balloon distention was associated with urgency. The SPW was not associated with gas or liquid expulsion.

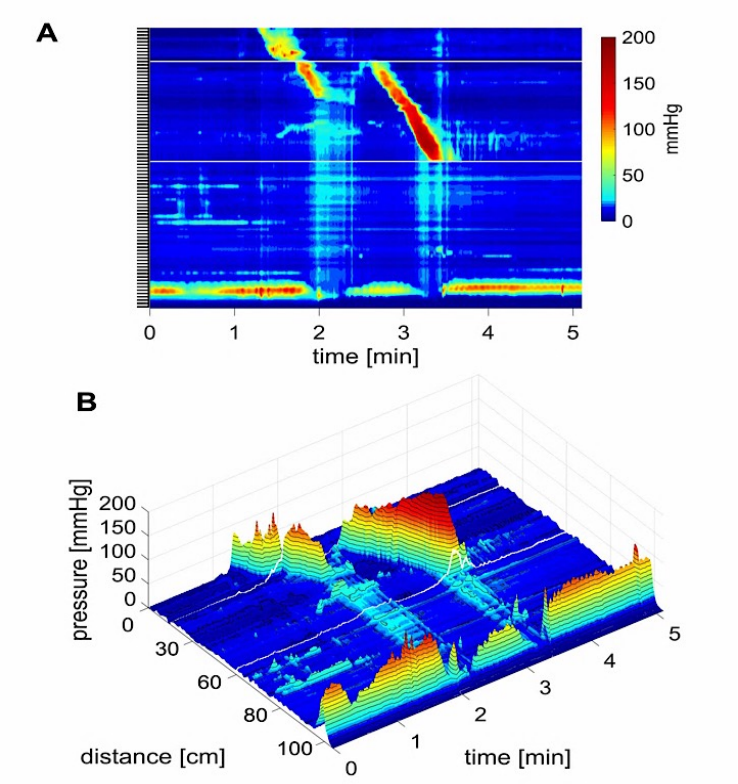


Figure 14. Two proximal High Amplitude Pressure Waves (HAPWs) that develop into SPWs (HAPW-SPWs in response to a meal)

(A) HAPWs that turn into SPWs with full anal sphincter relaxation. The image shows activity in 100 cm of the colon (position of 84 sensors are shown at the left side of the figure). The activity was observed 12 min after meal intake. The first HAPW-SPW was associated with liquid outflow, the second with liquid and gas expulsion. (B) 3D representation of (A).

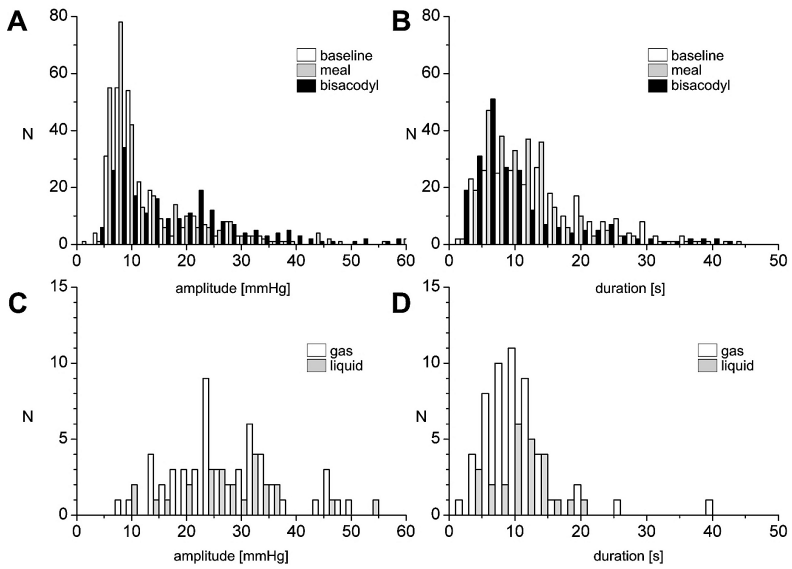


Figure 15. Amplitude and duration distribution of SPWs and their relationship to gas or liquid expulsion

(A,B) Number of occurrences of SPWs, binned according to amplitudes (A) or duration (B), observed in 17 subjects, before and after a meal and in response to bisacodyl. (C,D) Number of occurrences of SPW amplitudes (C) and durations (D) for SPWs associated with gas or liquid expulsion under all conditions.

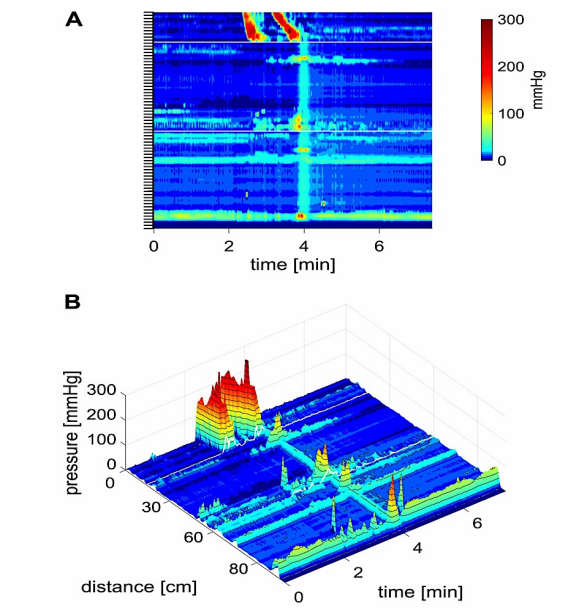


Figure 16. A proximal HAPW and an HAPW-SPW in response to meal intake

(A) Two very similar proximal HAPWs appeared in response to a meal, but only one developed into an SPW. The SPW was accompanied by external sphincter contraction and by sphincter relaxation and gas expulsion.

The SPW started at the end of the HAPW, hence it was not pan-colonic. The first HAPW was not associated with gas expulsion. Position of the pressure sensors is shown on the left side. (B) 3D rendering of (A). The 0 cm position is in the proximal colon.

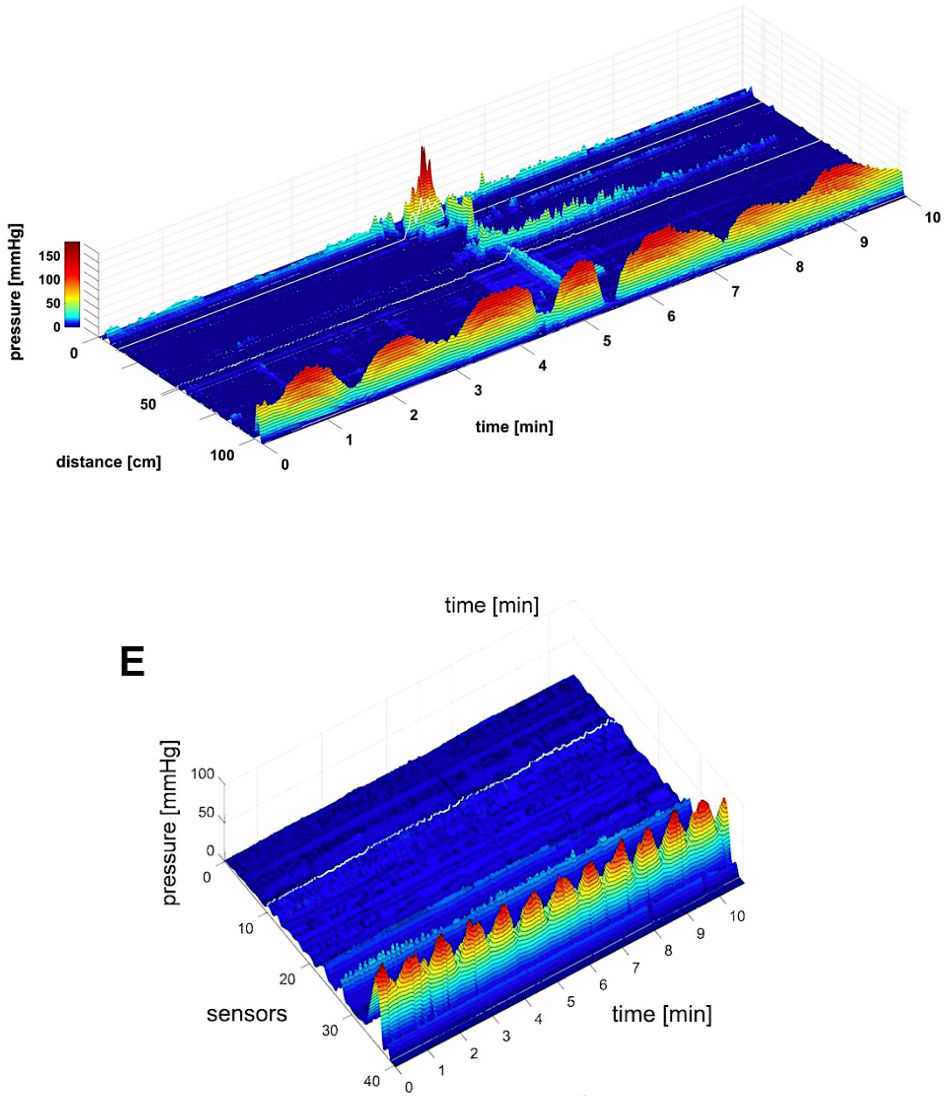


Figure 17. Rhythmic activity of the internal anal sphincter

(A) Rhythmic activity of the internal anal sphincter at 0.7 cpm. In addition, a short proximal HAPW appears followed by an SPW and full anal sphincter relaxation. This activity was recorded 80 min after meal intake. (B) Low amplitude colonic activity during baseline with rhythmic anal sphincter activity at 1.2 cycles per min.

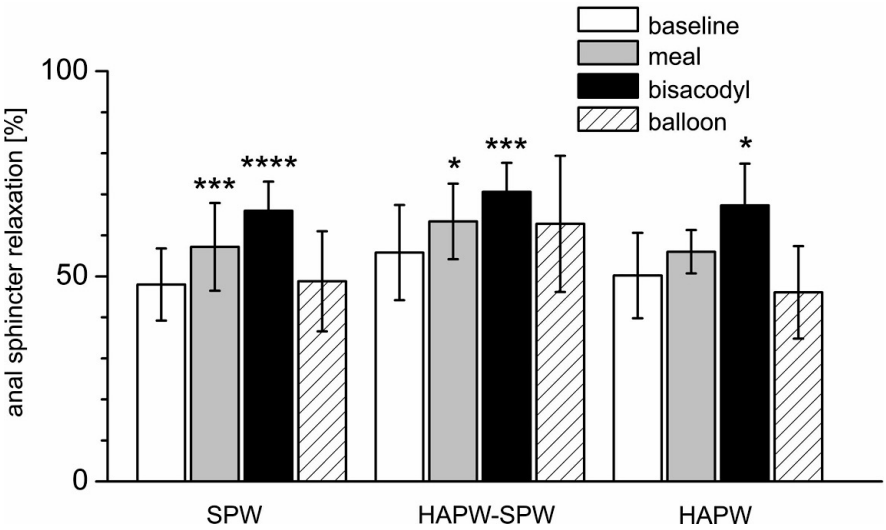


Figure 18. Relaxation of the anal sphincters associated with different motor patterns and in different conditions

Shown is the relaxation of the anal sphincter as a percentage of the anal sphincter pressure just prior to the motor patterns indicated on the X axis ($^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

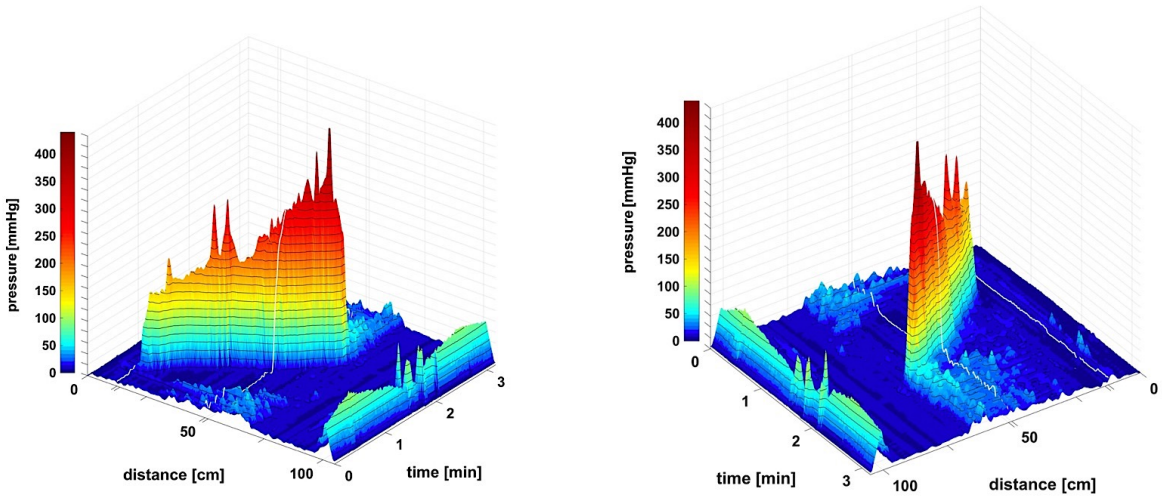


Figure 19. An HAPW that was not followed by a SPW and was not associated with anal sphincter relaxation

Most HAPWs that are associated with anal sphincter relaxation terminate into an SPW that enters the anal canal. In this subject, an HAPW that terminated suddenly and was not followed by an SPW and did not evoke anal sphincter relaxation. This motor pattern occurred 4 min into a proximal balloon distention (240 ml air). The motor pattern started distal to the balloon distention.

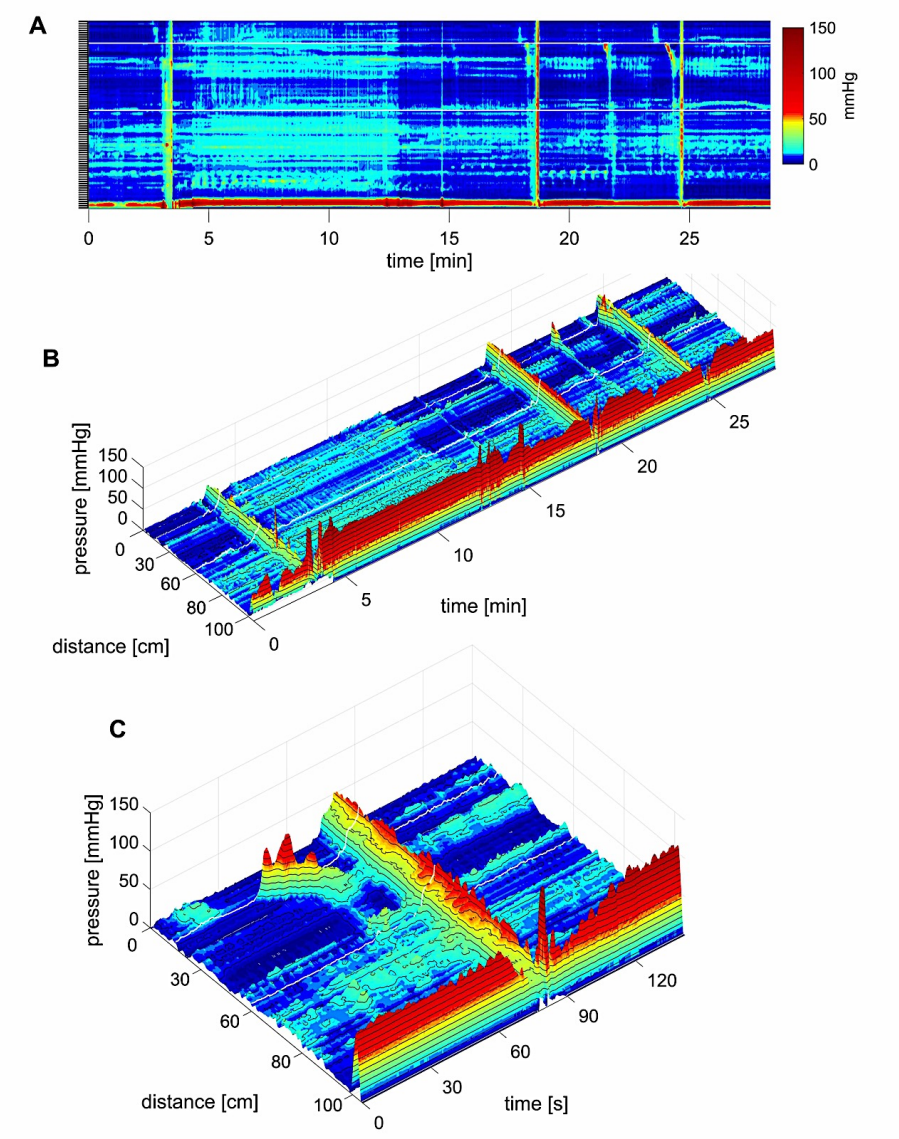


Figure 20. Simultaneous Pressure Waves in response to meal intake

(A) SPWs that span the entire colon (102 cm). The lighter area, between ~ 4 and 12 min, is the time period where the subject sits up causing an increase in intraluminal pressure and takes in the meal. In this subject, a meal did not induce HAPWs but relatively high amplitude SPWs associated with liquid expulsion. An SPW appeared just before (anticipating the meal) and two following the meal. Anal sphincter relaxations were part of the SPW activity and the SPWs were consistently preceded by relatively low amplitude proximal propagating pressure waves. In addition, a lot of segmenting activity occurred. No pressure waves appeared in the time period between the end of balloon distention and the start of meal intake, which was 20 min, except for the one just 1 min before meal intake. All three SPWs were associated with liquid outflow. (B) A 3D rendition of (A). (C) 3D image of an SPW.

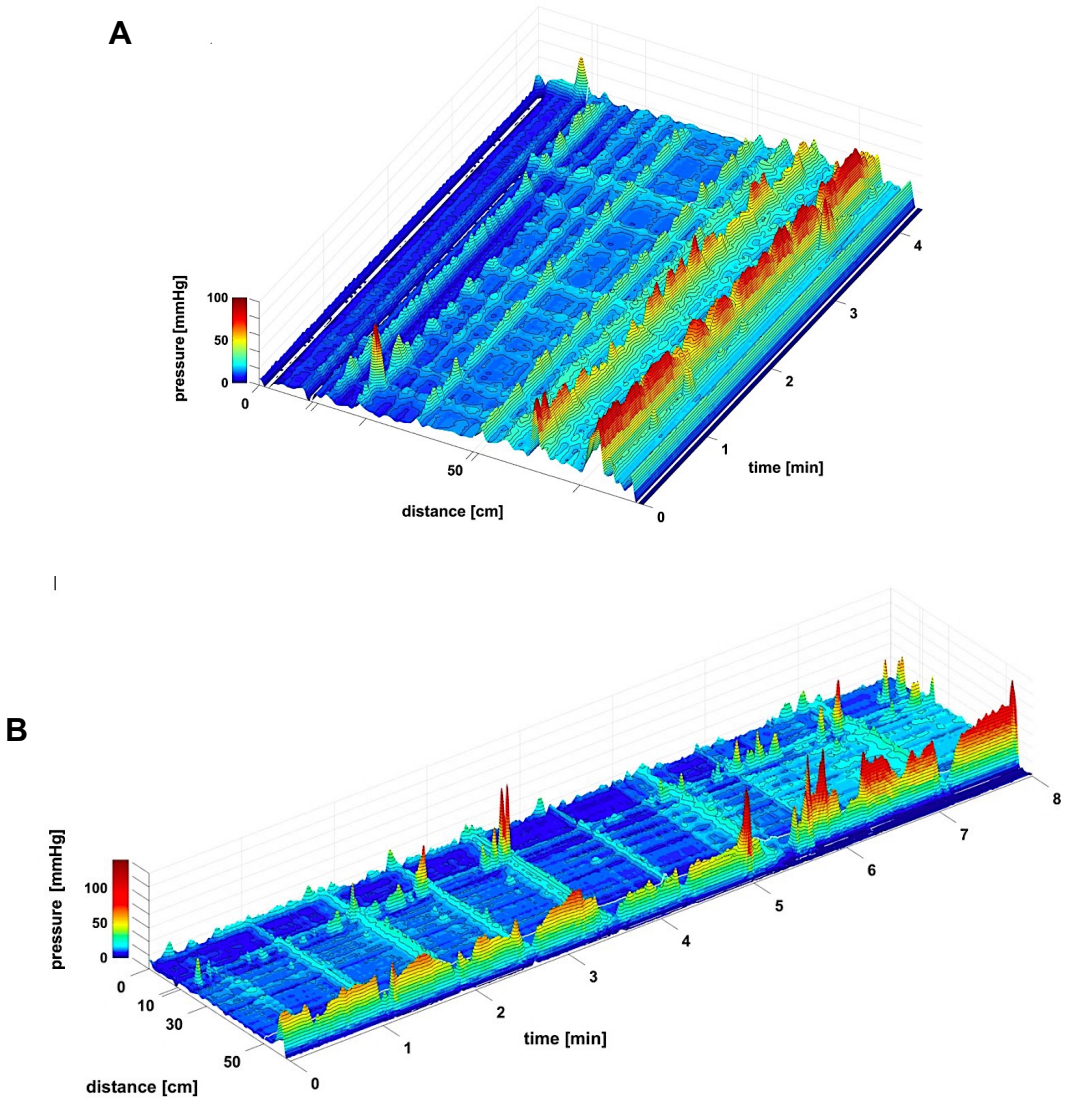


Figure 21. Rhythmic SPWs after meal intake

(A) Note that SPWs start at sensor 11, hence not in the proximal colon. The SPWs did not obliterate ongoing haustral boundary activities. In vivo, this likely means that under such conditions, gas can pass but stool flow will be restricted by the haustral boundaries. A functional sphincter is prominent, 12 cm above the anal sphincters. The response was observed 44 min after meal intake. There was no gas or liquid outflow. (B) Rhythmic SPWs with anal sphincter relaxation. Note that increasing SPW amplitudes are associated with increasing anal sphincter relaxations. This response was observed 150 min after meal intake. There was gas expulsion with the SPW at 3.5 min, and liquid outflow with the SPWs at 5.5 and 7.4 min.

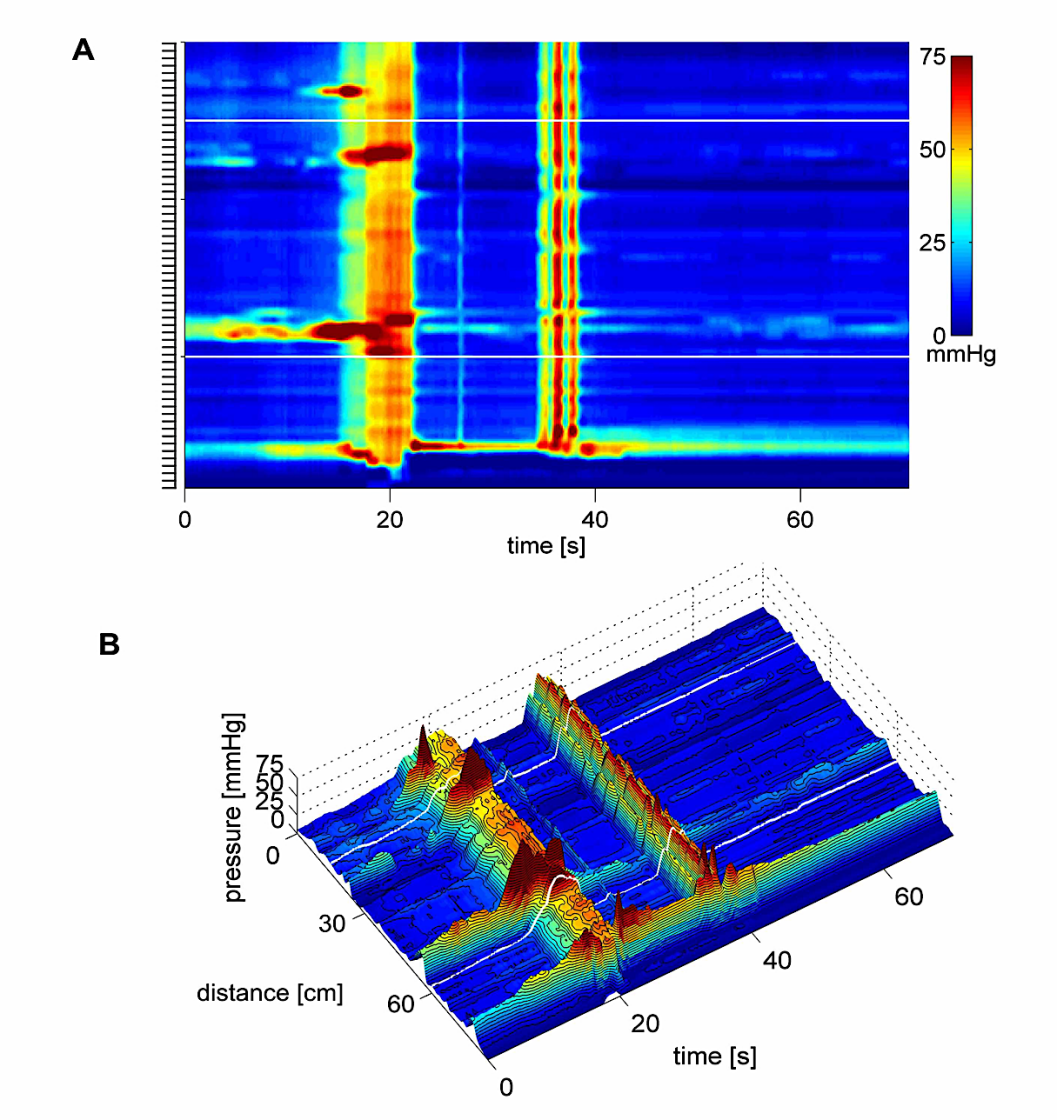


Figure 22. SPWs consist of multiple high frequency pressure waves

(A) Two SPWs appear in response to the meal. They can be seen as consisting of clusters of high frequency multiple pressure waves, suggesting that they may be caused by a cluster of rapidly propagating circular muscle contractions as was shown to be the case in the rabbit colon (Quan et al., 2017). (B) 3D image of (A).

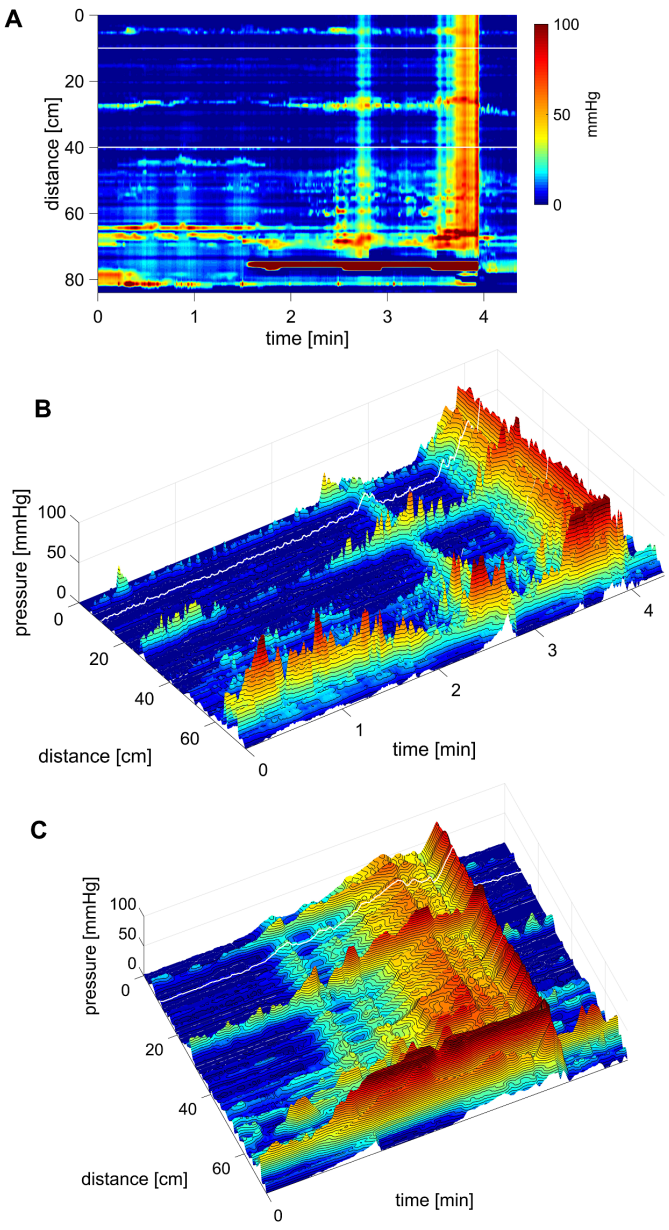


Figure 23. A high amplitude SPW associated with balloon expulsion

(A) Two SPWs appear in response to rectal balloon distention; the dark area above the anal sphincter shows the pressure in the rectum induced by the balloon. The first SPW was associated with liquid outflow. The second SPW consists of a cluster of high frequency multiple pressure waves, the last wave at 55 mmHg is followed by relaxation of the sphincter and it expels the balloon. Preceding the balloon expulsion, the SPW was associated with pain and urge. (B) 3D image of (A), the balloon distention and anal sphincter activity was removed to make the SPW better visible. (C) Close up of the second SPW as shown in (B) where its composition of multiple pressure waves is clearly visible.

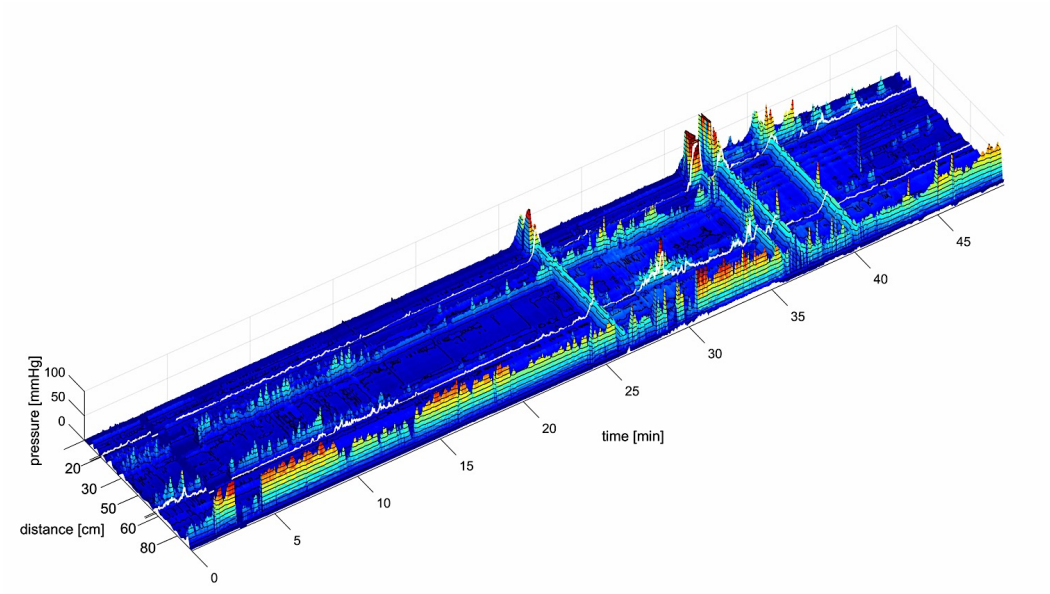


Figure 24. High amplitude SPWs in response to rectal bisacodyl

A 10 mg bisacodyl suspension was injected in the rectum at 16 min. Between 3 and 4 min, data acquisition was halted due to refilling of water reservoirs. This subject produced normal HAPWs in response to balloon distention (not shown). The average amplitude of the SPWs in response to bisacodyl was 32.2 mmHg. All 4 SPWs were associated with liquid outflow and the middle two SPWs with gas expulsion as well.

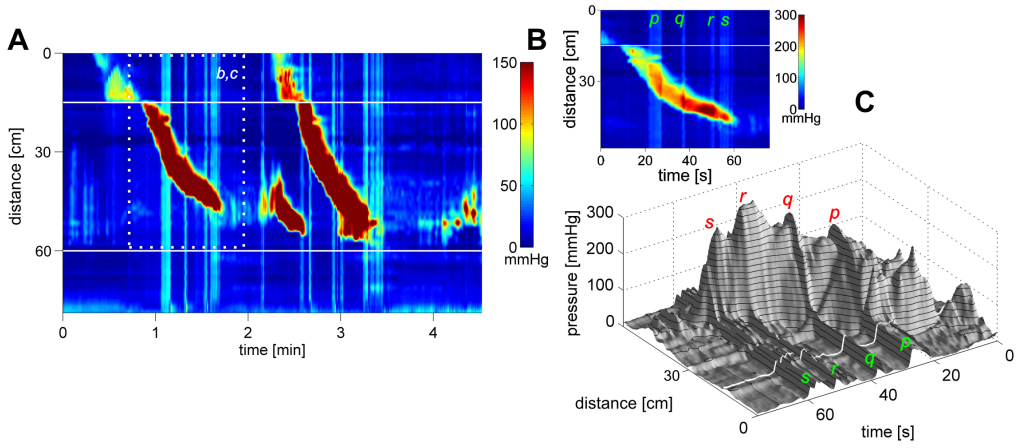


Figure 25. Independent HAPWs and SPWs show addition of pressure

(A) Concomitant occurrence of HAPWs and SPWs. The anal sphincter pressure is not shown. (B) A closeup of one of the HAPWs with unrelated SPWs. (C) The 3D graph of (A) shows that the pressures of the HAPW and the SPWs add up. This activity started 8 min after injecting 20 mg bisacodyl suspension in the descending colon. There was no outflow felt.

CHAPTER 5 – A CASE STUDY OF PROKINETIC ACTIONS OF INTRALUMINAL PRUCALOPRIDE IN THE HUMAN COLON AND ITS EFFECT ON THE BON CELLS

INTRODUCTION

In chapters 2 and 3 it was demonstrated that intraluminal prucalopride increases colonic peristaltic activity in the whole proximal and mid rabbit colon in a dose dependent and antagonist sensitive manner. It was also elucidated that luminal prucalopride exerts its strong prokinetic effects even in the presence of impaction simulated by an inflated barostat bag that distended the colonic wall almost twice its original size for a sustained period of time.

In chapter 4, the colo-anal reflex as an integral part of defecation was studied in 17 healthy subjects using HRCM. SPWs were characterized as prominent motor patterns in the human colon associated with anal sphincter relaxation and gas/liquid or balloon expulsion. Therefore, in addition to HAPW associated colo-anal reflex which has been studied previously (Rodriquez et al., 2012; Malcolm et al., 2000; Sintusek et al., 2018), SPW associated colo-anal reflex was also quantified and characterized. Together, these studies provided the necessary foundation for better understanding and improved analysis of the effects of intraluminal prucalopride in humans using HRCM.

Clinical trials have provided evidence that prucalopride when taken orally, reduces colonic transit times, increases stool frequency and decreases straining in humans (Camilleri et al., 2010; Bassotti & Villanacci, 2009; Bouras et al., 1999; Tack & Corsetti, 2013). Prucalopride can be effective in treating constipation and it may have potential for treatment of other gastrointestinal disorders such as IBS-C and visceral hypersensitivity (Tack & Corsetti 2013; Hoffman et al., 2012). As outlined in chapter 1, the mechanism of action of oral prucalopride is via the neuronal 5-HT_4R pathway. The hypothesis of the

present study is that similar to the rabbit animal model, intraluminal prucalopride enhances propulsive motor activity in the human colon via the epithelial 5-HT_4R pathway. Hence, it can be an effective prokinetic agent for various sub-types of constipation since constipation is a GI motility/secretion disorder in which EC-cell-derived 5-HT has a key role (Galligan, 2017).

Besides evidence from ex vivo animal, tissue and manometry studies, freshly isolated mammalian EC cells and the EC cell models such as BON cells, have been shown to promote 5-HT release via cAMP signaling pathway (Hoffman et al., 2012; Tran et al., 2004). BON cells are human carcinoid cells that secrete 5-HT and various peptides. Different receptors have been described in BON cells; such as acetylcholine, 5-HT, isoproterenol, and somatostatin receptors. It has also been reported that low levels of 5-HT were secreted by BON cells stimulated mechanically or by increase of D-glucose concentration (Tran et al., 2004). Literature suggests that at least four different functional receptors (muscarinic, cholinergic, serotonergic, p-adrenergic, and somatostatin) exist on BON cells and acetylcholine (Ach) can stimulate the release of 5-HT from BON cells (Parekh et al., 1994). In this study, the effects of prucalopride and exogenous 5-HT on 5-HT outflow from BON-1 cells as models for human EC cells are also evaluated.

MATHERIALS AND METHODS

High Resolution Colonic Manometry (HRCM)

High-resolution colonic manometry was performed in a healthy volunteer (F, 24 years old) as described in chapter 4 using a custom-made platform (Medical Measurement Systems (MMS); Laborie, Toronto, ON, Canada). An 84-sensor water-perfused 105 cm long catheter was designed (Mui Scientific, Mississauga, ON, Canada). The spacing between sensor 1-28 was 1.5 cm while the spacing between the rest of the sensors was 1cm. The catheter included one 10-cm long balloon between sensors 7 and 8 with sensor 1 placed in

the ascending colon. The protocol was as described in chapter 4 except that 2mg prucalopride suspended in 20ml sterile water was administrated in the lumen. Drug administration was followed by more water to flush the drug delivery catheter which makes it difficult to calculate the exact drug concentration once it is delivered in the lumen due to the fact that it contains water from the water perfused catheter. The participant gave written informed consent and all procedures were approved by the Hamilton Integrated Research Ethics Board (HiREB).

BON cells

To measure 5-HT outflow in response to prucalopride and exogenous 5-HT, BON-1 cells (RRID: CVCL_3985) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were seeded at 200,000 cells per well in 24-well plates. In the first experiment they were incubated for only 24 hours, in the subsequent experiments they were incubated for 2, 24, and 48 hours at 37°C in the complete growth medium. The medium was replaced with serum-free medium and treated with the appropriate concentration of prucalopride, GR113808, 5-HT and sodium butyrate (Sigma-Aldrich, Oakville, Canada). All drugs were prepared as explained in chapter 2. Following the treatment, the cell supernatants were collected and stored at -80ºC for further analysis. An ELISA (Serotonin EIA, Beckman Coulter, Inc.) was used to measure serotonin levels. To check for cell proliferation in response to higher concentrations of the drug, cell counting was done using trypan blue.

Quantification and Statistical analysis

Quantification and analysis of motor patterns and anal sphincter activities was done following the same procedure as described in chapter 4. Responses to stimuli were described and compared to baseline activity and activity after meal. Data are given as mean ± SEM. Significance was determined by ANOVA with multiple comparisons as mentioned in the table footnotes using Prism 8 software (GraphPad, United States), $P < 0.05$ was

considered significant. Percentage of relaxation was calculated by comparing the mean amplitude of the anal sphincter in a 3-minute period before relaxation and the whole period of relaxation as described in chapter 4.

Identified Motor Patterns

The focus of this case study was on propulsive motor patterns and their coordination with the internal anal sphincter relaxation. Therefore, the following motor patterns were studied as described in chapter 4:

1. Simultaneous pressure waves (SPWs)
2. High amplitude propagating pressure waves (HAPWs)
3. High amplitude propagating pressure waves followed by simultaneous pressure waves (HAPW-SPWs).
4. Anal sphincter activity and relaxation associated with motor patterns.

RESULTS

Effects of Intraluminal Prucalopride on Propulsive Motor Patterns

Propulsive motor patterns were rare at baseline and 90 minutes after meal intake. HAPWs were not present neither at baseline nor postprandially while SPWs were observed at baseline (n=1) and after meal intake (n=3). Administration of intraluminal prucalopride in the proximal colon significantly increased the total occurrence of all propulsive motor patterns compared with both baseline and after meal intake (Table 13, Fig 26). It also significantly increased the frequency of HAPWs, SPWs and HAPW-SPWs (Table 14, Fig 26). Moreover, intraluminal prucalopride resulted in significant increase of SPW amplitude compared with both baseline and 90 minutes after meal intake (Table 14).

At baseline, a SPW with mean amplitude of 8.71 mmHg, maximum pressure of 24 mmHg and duration of 3.7 s was observed which was pan-colonic but disjointed and it was not associated with any gas or liquid expulsion. After meal, no HAPWs were evoked. Pan-colonic SPWs were observed with mean amplitude of 7.15 ± 0.8 mmHg ranging between 6.6 – 8 mmHg and duration of 12.2 ± 2.5 s that entered the rectum but were not associated with gas nor liquid expulsion (Table 14).

HAPWs in response to intraluminal prucalopride (n=4) started in the proximal colon ranging between 104 –173 mmHg in amplitude and 23 –112 s in duration (Table 14). They propagated with an average velocity of 0.55 ± 0.4 cm s^{-1} and did not penetrate into the rectum; however, they could enter the left colon. 1/4 HAPWs was associated with urge to defecate (Fig 28).

The HAPW component of HAPW-SPWs ranged between 110–125 mmHg in amplitude and propagated with the velocity of 0.52 ± 0.01 cm s^{-1} along the colon (n=2). They stopped propagating in the mid colon and were followed by a SPW which entered the rectum with average amplitude of 18.42 ± 9.8 s ranging between 11.4 – 25 mmHg with the duration of 6 ± 2 s. Both HAPW-SPWs were associated with liquid expulsion and urge to defecate and one HAPW-SPW was associated with nausea (Fig 26, 27B). Pan- colonic SPWs were evoked with an average amplitude of 13 ± 6.4 mmHg ranging between 7.3 – 24 mmHg with the average duration of 6.2 ± 2 s (n=5, Table 14).

Table 13. Total Incidents of Propulsive Motor Patterns

	Baseline	Meal	Intraluminal Prucalopride
SPW	1	3	5
HAPW	0	0	4
HAPW-SPW	0	0	2
Total	1	3	$11^{*/\#}$

Values are mean ± SEM. Significance was determined using one-way ANOVA with multiple comparison, *compared to baseline (* P< 0.05), $^{\#}$ compared to meal ($^{\#}$ P <0.05).

Table 14. Characteristics of Motor Patterns in Response to Intraluminal Prucalopride

	HAPW	SPW	HAPW in HAPW-SPW	SPW in HAPW-SPW
Baseline (90 mins)				
Frequency (cpm)	0 (n=0)	0.01 (n=1)	0 (n=0)	0 (n=0)
Amplitude (mmHg)	NA	8.71	NA	NA
Duration (s)	NA	3.75	NA	NA
Velocity (cm/s)	NA	NA	NA	NA
Meal (90 mins)				
Frequency(cpm)	0 (n=0)	0.03 (n= 3)	0 (n=0)	0 (n=0)
Amplitude (mmHg)	NA	7.15 ± 0.8 6.6 – 8	NA	NA
Duration (s)	NA	12.2 ± 2.5 10 – 15	NA	NA
Velocity (cm s^{-1})	NA	NA	NA	NA
Intraluminal Prucalopride 2 mg (60 mins)				
Frequency(cpm)	0.07 $^{*/\#}$ (n= 4)	0.07 * (n=5)	0.03 $^{*/\#}$ (n= 2)	0.03$^{*/\#}$ (n= 2)
Amplitude (mmHg)	146.5 ± 29.5 104 – 173	13 ± 6.4 $^{*/\#}$ 7.3 – 24	117.5 ± 11 110 – 125	18.42 ± 9.8 11.4 – 25
Duration (s)	61.5 ± 37 23 – 112	6.2 ± 2 2.1 – 13.7	31 ± 4.4 28 – 34	6 ± 2 4.6 – 7.5
Velocity (cm s^{-1})	0.55 ± 0.4 0.2 – 1.15	NA	0.52 ± 0.01 0.5 – 0.53	NA

Values are mean ± SEM. Significance was determined using one-way ANOVA with multiple comparisons, *compared to baseline (* $P< 0.05$), $^{\#}$ compared to meal ($^{\#}$ $P <0.05$).

Association of Motor Patterns with Anal Sphincter Relaxation (The Colo-anal Reflex)

The anal sphincter showed intrinsic rhythmicity which occurred at 5.5 cpm. Internal anal sphincter (IAS) relaxation could occur without full external anal sphincter relaxation (i.e. comparing the proximal and distal part of the anal canal, Fig 27). Motor patterns were sometimes associated with urge to defecate and at times accompanied by external anal sphincter contraction. (Table 15, Fig 28).

2/4 HAPWs with the mean amplitude of 154.3 ± 1.6 were associated with significant internal anal sphincter relaxation. The amplitude of the IAS before relaxation ranged

between the maximum pressure of 55 ± 5.5 mmHg and the minimum pressure of 9.2 ± 3.3 mmHg with the mean amplitude of 29.2 ± 0.4 mmHg. During the relaxation phase which lasted 29 ± 9.5 s on average, the mean amplitude of the IAS decreased to 14 ± 0.2 mmHg with maximum pressure of 47 ± 4 mmHg and minimum pressure of 3.6 ± 1.4 mmHg, yielding 52% relaxation (Table 15).

1/2 HAPW-SPWs was associated with 68% IAS relaxation. The HAPW component stopped propagating in the right colon where the SPW component started. Amplitude of the internal anal sphincter before relaxation was 63.5 mmHg ranging between the maximum amplitude of 163 mmHg and the minimum pressure of 6 mmHg. During relaxation, the minimum amplitude did not change; however, the maximum amplitude decreased to 70 mmHg. The relaxation took place at the onset of SPW when the HAPW terminated in the right colon (Table 15).

A SPW-cluster consisting of 4 pan-colonic SPWs was associated with significant IAS relaxation. Internal anal sphincter did not recover in between the four SPWs. Hence, relaxation persisted the whole duration of the SPW-cluster. Before relaxation, the mean amplitude of the sphincter was 31.6 mmHg with maximum and minimum amplitudes of 56 and 15.4 mmHg respectively. During the relaxation phase, IAS amplitude decreased to 13.1 mmHg with maximum pressure of 45 and the minimum pressure of 3.6 mmHg resulting in 60% relaxation. 1/5 pan-colonic SPWs overlapped with an HAPW; therefore, anal sphincter relaxation could not be reliably attributed to either of the motor patterns.

The SPW observed at baseline was associated with 69% IAS relaxation while SPWs evoked postprandially were associated with 87% relaxation on average. Comparing the degree of IAS relaxation associated with SPWs during baseline, meal and intraluminal prucalopride did not show any significant difference.

Table 15. Anal Sphincter Relaxation Associated with Motor Patterns

	Motor pattern amplitude (mmHg)	Internal anal sphincter amplitude before relaxation (mmHg)	Internal anal sphincter amplitude during relaxation (mmHg)	% of relaxation	Duration of relaxation (s)	Time of occurrence
HAPW						
Intraluminal prucalopride (n=2)	154.3 ± 1.6 153 – 156	29.2 ± 0.4 9.2 – 55	14 ± 0.2 3.6 – 47	52	29 ± 9.5	When HAPWs entered the left colon
SPW						
Intraluminal prucalopride (n=4)	10 ± 1.1 7.3 – 12.4	31.6 15.4 – 56	13.1 3.6 – 45	60	13.3	At the onset of SPW-cluster
Baseline (n=1)	8.7 3.7 – 24	27.7 14 – 46	11.3 5 – 23	69	10.8	At the onset of SPW
Meal (n=3)	7.15 ± 0.8 6.6 – 8	26 ± 4.4 5.3 – 57.3	7.7 ± 8 2 – 20.3	87	15.6 ± 3.3	At the onset of SPW
HAPW-SPW (HAPW data)						
Intraluminal prucalopride (n=1)	125.3 29 – 343	63.5 6 – 163	20.5 6 – 70	68	18.3	At the onset of SPW
HAPW-SPW (SPW data)						
Intraluminal prucalopride (n=1)	11.5 5 – 32	63.5 6 – 163	20.5 6 – 70	68	18.3	At the onset of SPW

Values are mean ± SEM.

BON cells

Results of the first experiment at 24 hours of incubation revealed that prucalopride 5μM triggers a significant 5-HT outflow from BON-1 cells compared with untreated cells but not at concentrations of 0.2 and 1μM (Fig 29A). Untreated cells at 24 hours released 39.5 ± 1.7 nM of 5-HT which increased significantly to 68 ± 5.5 nM in response to prucalopride 5μM. To mimic the physiological conditions at which prucalopride is administrated, the drug was also added in the presence of butyrate. Butyrate alone resulted in the release of

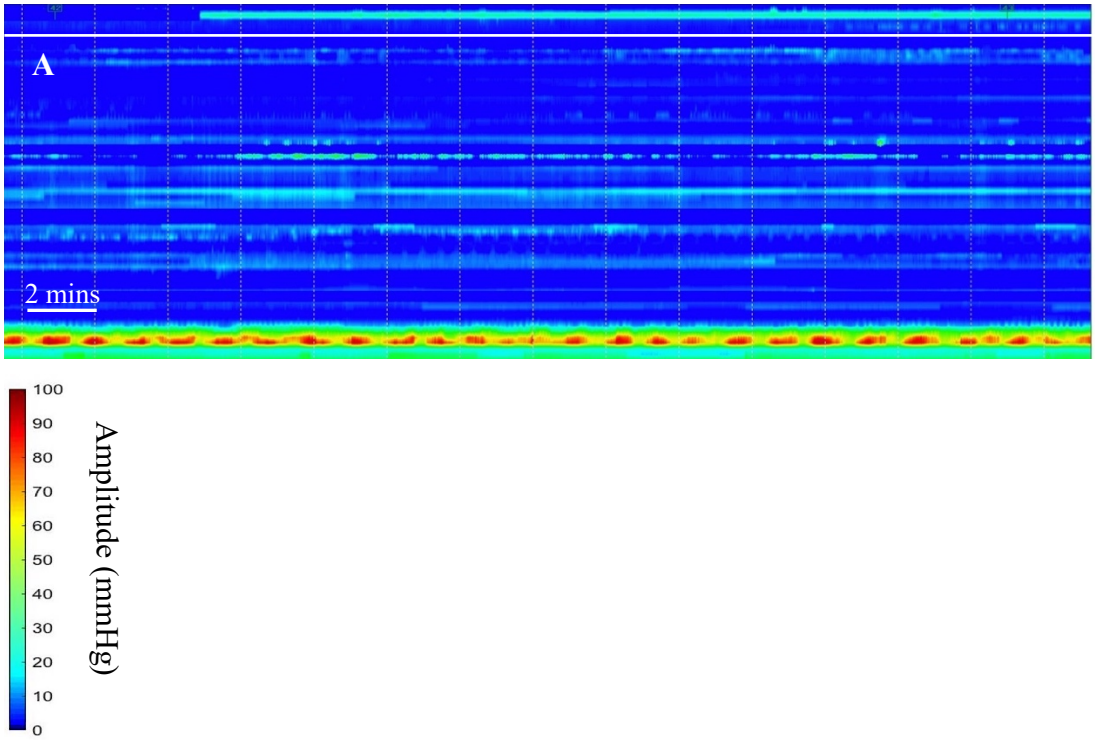
140 ± 7.7 nM 5-HT and 146 ± 5 nM of 5-HT together with prucalopride 5μM. The response to prucalopride at the presence of butyrate was not significantly different compared to when butyrate was added alone. Exogenous 5-HT at 1μM and 10μM resulted in 866 ± 96 nM and 6597 ± 778 nM 5-HT outflow respectively (considering that 10^3 and 10^4 nM was exogenous 5-HT, Fig 29B) which was significantly higher than untreated cells and prucalopride at all concentrations.

Next, 5-HT outflow was measured in a time dependant manner in the absence and presence of the 5-HT_4R antagonist GR113808. Application of prucalopride 1 and 5μM at 2 hours did not result in any significant increase in 5-HT outflow compared with untreated cells (18.8 ± 2.7 nM, Fig 30A). At 24 and 48 hours prucalopride 5μM markedly increased 5-HT outflow in an antagonist sensitive manner (Fig 30B,C). Prucalopride 1μM resulted in the increase of 5-HT outflow from 34 ± 0.9 nM at 2 hours to 44.5 ± 2.5 nM at 24 hours and 92 ± 20 at 48 hours although the results did not reach statistical significance. Prucalopride 5μM stimulated the release of 37 ± 4.3 nM 5-HT at 2 hours which increased significantly to 71 ± 2 nM at 24 hours and to 241 ± 23.4 nM at 48 hours (Fig 30).

Prucalopride 10μM at all time points resulted in an exponential surge in the 5-HT release yielding 11453 ± 1238 nM 5-HT at 2 hours (compared with 18.8 ± 2.7 nM at control conditions, 2 hours) and 13755 ± 452 nM at 24 hours (compared to 38 ± 2.6 nM at control conditions, 24 hours) and 23067 ± 1367 nM at 48 hours (compared to 79.2 ± 7.2 nM at control conditions, 48 hours). This response was clearly time dependant but was not blocked by the antagonist.

Cell counting using trypan blue staining revealed that prucalopride10μM, increased the number of cells significantly from 1.4×10^5 to 4×10^5 while prucalopride 5μM did not (Fig 31C). Hence, cell proliferation could account for the exponential increase in 5-HT outflow observed in previous experiments.

FIGURES



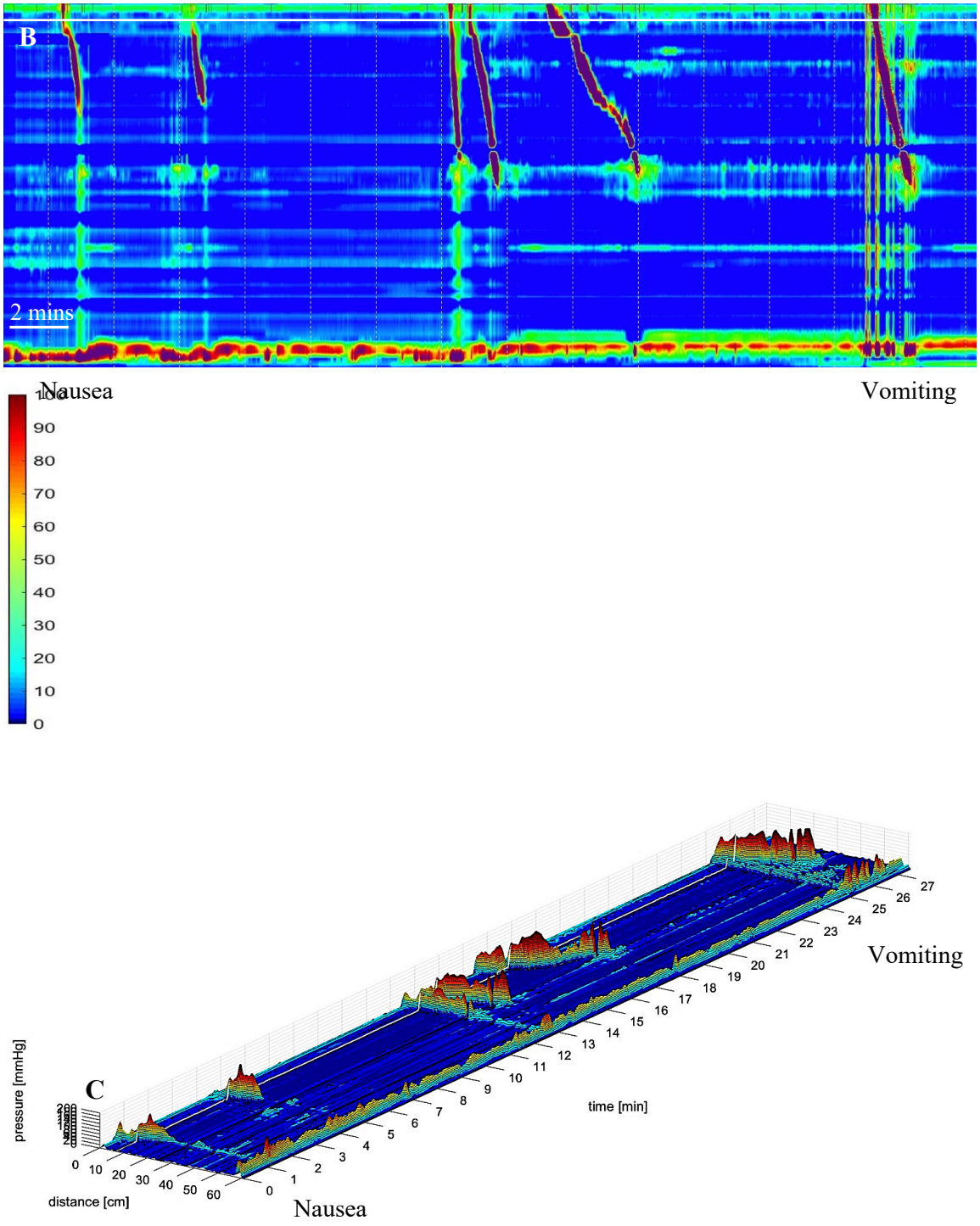


Figure 26. Comparison of ~ 30 minutes of baseline and intraluminal prucalopride sessions showing the effects of the intraluminal drug

(A) 31 minutes baseline with no major propulsive motor patterns. (B,C) 28 minutes of intraluminal prucalopride showing various propulsive motor patterns in 2-D and 3-D. Motor patterns could be associated with nausea and vomiting. The white line shows the position of the balloon.

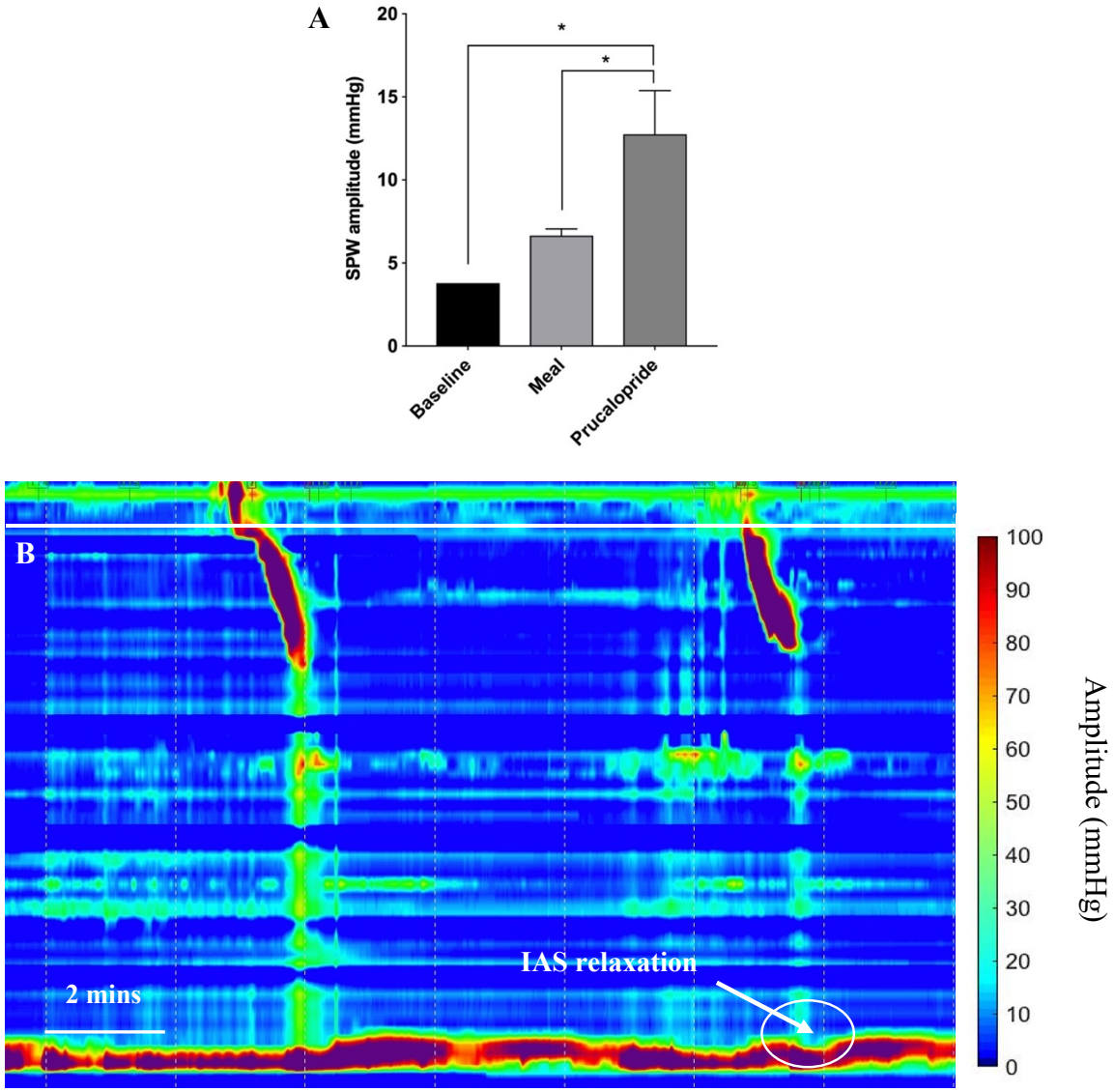


Figure 27. SPW amplitudes in different conditions and two HAPW-SPWs evoked by intraluminal prucalopride

(A) Comparison of SPW mean amplitudes in response to intraluminal prucalopride, baseline and meal (90 mins before prucalopride), *compared to baseline (* $P < 0.05$). (B) Two HAPW-SPWs evoked in response to intraluminal prucalopride. The second HAPW-SPW is associated with internal anal sphincter relaxation which occurred without full external anal sphincter relaxation although it yielded some relaxation. Both were associated with liquid expulsion and urge to defecate and the first HAPW-SPW from left was associated with nausea. The white line shows the position of the balloon.

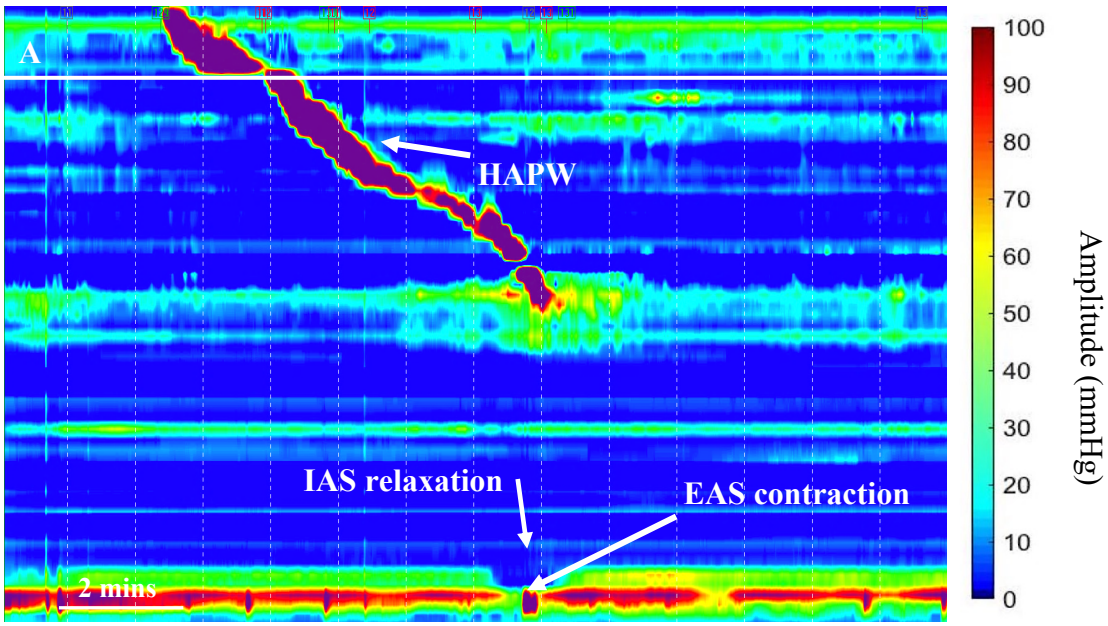


Figure 28. An HAPW evoked by intraluminal prucalopride

An HAPW with mean amplitude of 156 mmHg evoked by intraluminal prucalopride propagating with a velocity of 0.2 cm s^{-1}, is associated with internal anal sphincter relaxation and strong urge to defecate which likely prompted the subject to contract her external anal sphincter to avoid outflow. The white line shows the position of the balloon.

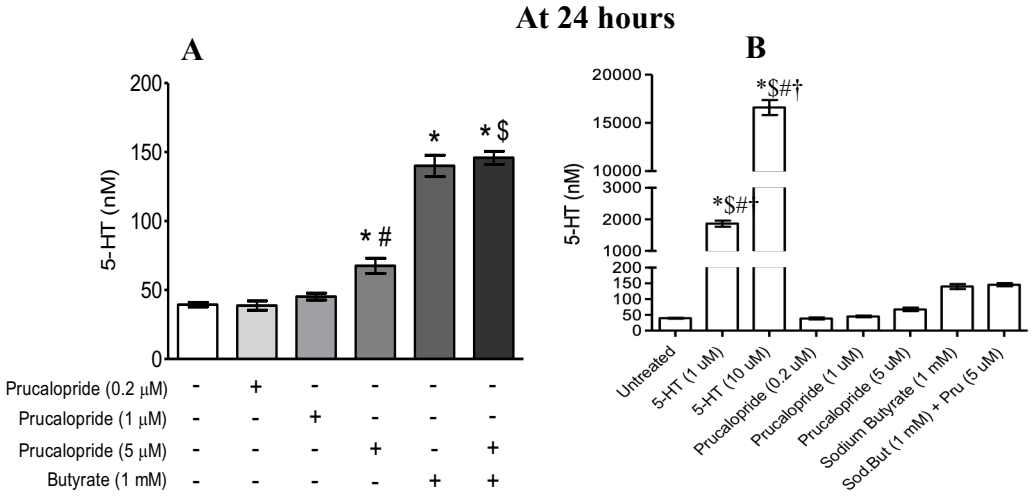


Figure 29. Prucalopride and exogenous 5-HT stimulated 5-HT outflow from BON cells

(A) Prucalopride at 5μM significantly increased 5-HT outflow from the BON cells. *compared to untreated cells ($^{*}P < 0.05$), $^{\#}$ compared to prucalopride 0.2μM ($^{\#}P < 0.05$), † compared to prucalopride 1μM ($^{\dagger}P<0.05$), $^{\$}$ compared to prucalopride 5μM ($^{\$}P < 0.05$), (B) 5-HT outflow in response to the exogenous 5-HT at 1μM and 10μM compared with 3 concentrations of prucalopride, butyrate and prucalopride at the presence of butyrate.

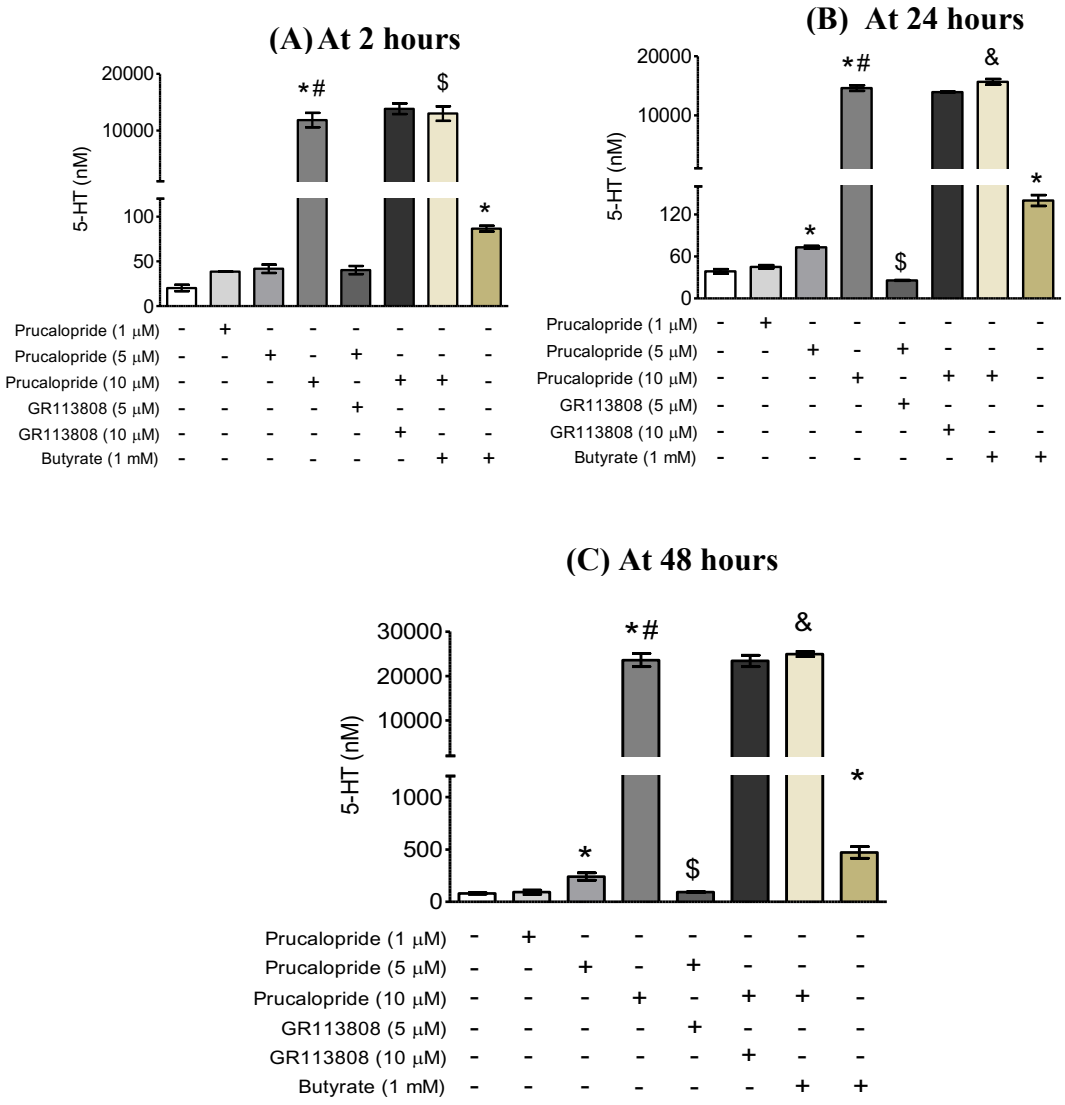


Figure 30. 5-HT outflow from BON cells in response to prucalopride occurred in a time-dependent and antagonist sensitive manner

A) 5-HT outflow in response to the test compounds at 2 hours. B) 5-HT outflow in response to the test compounds at 24 hours, C) 5-HT outflow in response to the test compounds at 48 hours, * $P < 0.05$, compared to untreated cells, $^{\#}$ $P < 0.05$, compared to prucalopride (5μM), $^{\$}$ $P < 0.05$, compared to prucalopride (5μM), $^{\&}$ $P < 0.05$, compared to sodium butyrate (1mM).

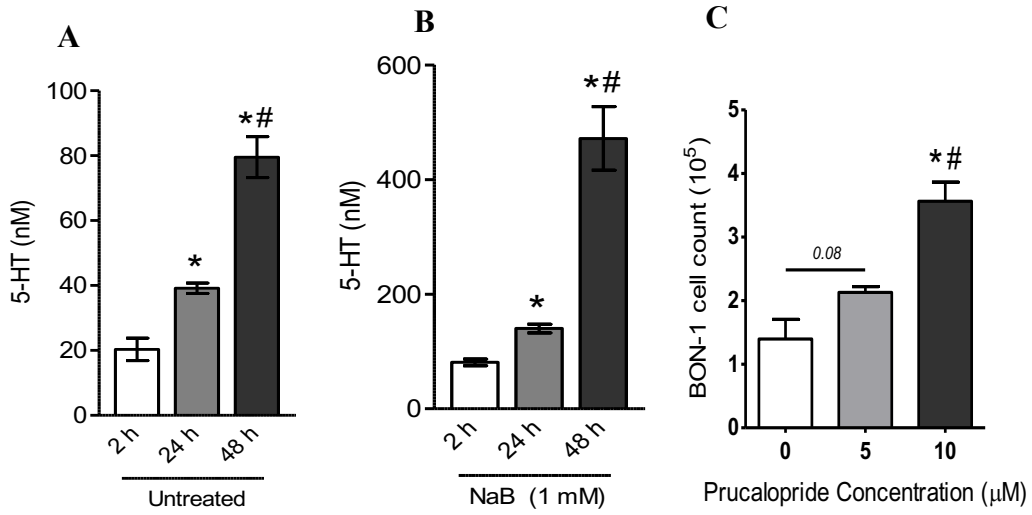


Figure 31. 5-HT outflow in three different time points and cell count

A) untreated cells, B) In response to sodium butyrate (1mM) $^{*}P < 0.05$, compared to 2 hours, $^{\#}P < 0.05$, compared to 24 hours. C) Cell count in the presence of prucalopride 5 and 10 μM, $^{*}P<0.05$ compared to control, $^{\#}P<0.05$ compared to 5μM.

CHAPTER 6- DISCUSSION

EFFECTS OF ACTIVATING LUMINAL 5-HT$_4$ RECEPTORS IN THE RABBIT COLON

The present study shows that intraluminal prucalopride and intraluminal exogenous 5-HT, acting on luminal 5-HT$_4$Rs, evoke the colonic motor complex in all its manifestations. Luminal prucalopride increased propulsive colonic motor activity in the proximal 3-taeniated and mid 2-taeniated rabbit colon in a dose dependent and antagonist sensitive manner. This occurred via evoking forceful propulsive motor patterns and/or increasing the contraction amplitude, intraluminal pressure associated with the motor patterns, as well as the velocity and degree of propagation of the motor patterns along the colon. Moreover, the frequency of motor patterns with higher contraction amplitudes markedly increased while the frequency of less forceful motor patterns manifesting lower contraction amplitudes, significantly decreased.

These findings are consistent with previous studies that demonstrated that activation of luminal 5-HT$_4$Rs in the guinea pig colon stimulates the release of 5-HT into the interstitial space of the lamina propria to activate the sensory neurons which subsequently triggers neural circuitries in the myenteric plexus and results in propulsive motor activity (Hoffman et al., 2012). Results are also consistent with a study that demonstrated stimulation of luminal 5-HT$_4$ receptors lowers the distention threshold required for propulsive contractions in the guinea pig ileum (Gwynne et al., 2014). Although prucalopride and 5-HT were given locally, full pan-colonic motor complexes were generated that started oral to the site where prucalopride was administrated. This is because the IPANs activate a neuronal circuitry that governs the entire colon and it does not randomly activate the myenteric nerves. This neuronal circuitry that programs the pan-colonic motor patterns also involves glial cells and ICC (Smith et al., 2014).

Studies have previously shown that distention-induced LDCs as well as distention induced-clustering of FPCs have a neurogenic component as they are blocked by TTX suggesting their dependence on neural circuitry at the myenteric plexus (Quan et al., 2017; Chen et al., 2016). Thus, the most likely mechanism of action of intraluminal prucalopride in the rabbit colon is that activation of luminal 5-HT_4 receptors results in the release of 5-HT from enterochromaffin cells which in turn stimulates 5-HT receptors on intrinsic primary afferent (IPAN) neurons. This derives the neural circuitry of the myenteric plexus to initiate contractions. It has also been proposed that luminally applied 5-HT may facilitate propulsive reflexes via activation of 5-HT_4Rs and subsequent release of a tachykinin acting at NK_3 receptors on myenteric AH neurons (Gwynne et al., 2014). Since intraluminal prucalopride mimicked the effects of exogenous intraluminal 5-HT and both were inhibited by the 5-HT_4R antagonist GR113808, prucalopride likely acted on receptors for luminal endogenous 5-HT.

In addition, this study shows that enhancing colonic motor activity occurs through various levels of excitation beginning with single erratic FPCs followed by different manifestations of the colonic motor complex. This includes clusters of FPCs with higher amplitudes followed by development of LDCs within the clusters, the gradual disappearance of FPCs and finally the emergence of a pattern of rhythmic forceful LDCs as the highest level of excitation observed in the rabbit colon. Each level of excitation manifests a specific motor pattern configuration associated with a degree of pressure development (chapter 2, Fig 5,6).

Data revealed that change in the motor pattern configuration from single FPCs to fully developed LDCs occurred in a dose-dependent manner. In both intraluminal prucalopride and intraluminal exogenous 5-HT experiments increase in drug concentration resulted in either enhancement of the characteristics of the motor patterns or total change of the motor pattern from a less forceful complex to a more forceful one (chapter 2, Fig 6). Moreover, single FPCs at baseline conditions were significantly lower in contraction amplitude compared with FPC clusters (chapter 2, Fig 3D) indicating that FPC clusters are inherently

a more forceful motor pattern compared with single FPCs. Additionally, with increase in drug concentration, contraction amplitude of FPC clusters increased while their frequency significantly decreased compared with baseline conditions. This was accompanied by development of LDCs within the FPC clusters and increase in LDC frequency. Both FPC clusters and LDCs are propulsive motor patterns that propel content to the anal direction. However, LDCs are the most forceful manifestation of the colonic motor complex corresponding to the highest contraction amplitude, and intraluminal pressure (chapter 2,3) and the highest amount of outflow (Chen et al., 2016). Therefore, the gradual development of propulsive motor patterns from less forceful complexes to the most forceful ones (chapter 2, Fig 6) occurring with increase in drug concentration, demonstrates that several important motor patterns precede LDCs; and LDCs are not the only manifestation of the colonic motor complex. FPC clusters also develop in response to the prokinetic drug; though in a lower level of excitation.

Manometry is currently used clinically as a diagnostic tool for detecting colonic motor dysfunction. The LDC in the rabbit colon has similar characteristics to the HAPW in the human colon (Chen et al., 2017) as it is the most forceful propulsive motor pattern. The FPCs are likely the equivalent of rapidly propagating circular muscle contractions; a cluster of fast propagating contractions that produces the SPW in humans (Chen et al., 2018). Supporting evidence for this interpretation is provided by a previous study in the rabbit colon where spatiotemporal mapping of video-recorded motor patterns was correlated with pressure waves obtained by simultaneously executed high-resolution colonic manometry (Quan et al., 2017). Quan et al., (2017) demonstrated that there is a positive correlation between contraction and intraluminal pressure in the rabbit colon. Correlating intraluminal pressure maps measured in this study with contraction D-maps associated with observed motor patterns revealed similar results (chapter 2). All motor patterns were associated with increase in intraluminal pressure; even single erratic FPCs. The higher the contraction amplitude, the higher the increase in intraluminal pressure associated with motor patterns.

Hence, LDCs evoked by higher concentrations of intraluminal prucalopride and exogenous 5-HT resulted in the highest intraluminal pressure amplitude (chapter 2, Fig 5).

In current manometry tests, absence of HAPWs or failure to evoke this motor pattern in response to stimuli such as a meal or bisacodyl is often considered as a biomarker for abnormal colonic motility in humans. The HAPW is assessed because it is believed to be essential for satisfactory defecation (Chen & Huizinga, 2018). When patients are unable to generate HAPWs, they are diagnosed to have "inert colon". Nevertheless, evaluating only HAPWs without considering other motor pattern complexes does not constitute a full assessment of colonic motility. The present study shows that a 5-HT_4R agonist acting from the lumen can evoke several propulsive contraction complexes that are associated with pressure development which lends support for the assessment of other motor patterns besides HAPWs in the human colon.

Luminal prucalopride and luminal exogenous 5-HT both reduced ripple frequency in between the colonic motor complexes, in particular in its most excited form, the LDCs. This is likely because of inhibition of smooth muscle cells and ICC by nitrergic nerves which occurs in between the colonic motor complexes as observed by Smith and colleagues in the mouse colon (Smith et al., 2015). The dose independent decrease in ripple frequency indicates that prucalopride and 5-HT did not have a direct effect on the ripples; however, the reduction in ripple frequency was inhibited by the antagonist. Therefore, the inhibition of ripple frequency was mediated by its action on the luminal 5-HT_4Rs. Both LDCs and FPC clustering are neurogenic and TTX sensitive (Chen et al., 2016) while ripples are non-propulsive and their frequency is unaffected by Hexamethonium (Lentle et al., 2008) and TTX (Chen et al., 2016). The ripples are a unique circular muscle activity (Lentle et al., 2008; Chen et al., 2015) and the pacemaker of the ripples is likely originating in the ICC of the sub-muscular plexus (ICC-SMP) consistent with other animal studies in which the omnipresent colonic slow-wave activity was governed by ICC-SMP (Chen et al., 2016).

As identified by Ramon and Cajal, ICC are embedded into the enteric neural circuitry (Chen et al., 2015). Some neural pathways may go to the musculature exclusively via ICC, and the gap junction contacts between ICC and smooth muscle cells make the ICC ideal to convey information from the nerves to smooth muscle cells (Chen et al., 2015). Stimulant-dependent pace maker activity has been observed in the stomach and guinea pig small intestine (Chen et al., 2015) which indicates that ICC pacemaker activity can be dependent on the activity of the enteric nervous system. The enteric AH neurons have been documented to modulate the ICC activity (Zhu et al., 2014). AH neurons were demonstrated to increase the pacemaker frequency of local ICC-MP (ICC associated with the myenteric plexus) via increasing the frequency of ICC calcium transients. Evoking new pacemakers distal to the proximal lead pacemaker will initiate both retrograde and antegrade propulsion causing back and forth movements that disrupt peristalsis and promote absorption (Zhu et al., 2014). Therefore, promoting propulsion as observed in this study via activating luminal 5-HT_4Rs, is accompanied by diminish in myogenic ripple activity to avoid disruption in peristalsis.

Haustration was annihilated by hexamethonium (Lentle et al., 2008) and TTX (Lentle et al., 2008; Chen et al., 2015) hence, haustral boundary contractions are propagating motor patterns with neurogenic component (Chen et al., 2015). Despite the neurogenic nature of haustral boundary contractions, they do not produce significant intraluminal pressure (Quan et al., 2017), do not propel content down the colon (Hanman et el., 2019) and are not propulsive (Chen et al., 2016). They are suggested to be governed by a constant pacemaker at ~ 0.5 cpm (Chen el al., 2015). The integrated action of the propagating haustral boundary contractions and the ripples, creates the classical segmentation motor pattern (Chen et al., 2015; Hanman et al., 2019). Segmentation is a well-understood mixing movement that divides the chyme into separated portions and facilitates absorption of nutrients. It involves moving the chyme in both directions (Huizinga et al., 2014). Therefore, haustration slows down the progression of chyme along the colon.

Segmentation can be activated by luminal infusion of fatty acids and mucosal 5-HT which is probably involved in the production of this motor pattern (Ellis et al., 2013). In an ex vivo guinea pig small intestine assay, segmentation was evoked by addition of the SERT inhibitor, fluoxetine, and segmentation patterns initiated by fatty acids or fluoxetine were inhibited by antagonists of the 5-HT_3 or 5-HT_4 receptors, as well as by cholecystokinin (CCK) receptor antagonists. Therefore, both 5-HT and CCK could be involved in the regulation of segmentation motor patterns (Ellis et al., 2013; Mawe & Hoffman, 2013).

Alvarez was the first scientist to find out that the frequency of rhythmic segmenting contractions occurred at the frequency of a myogenic pacemaker (Huizinga & Chen, 2014), suggesting a firm relationship between slow waves and segmentation. Spontaneous segmentation is associated with a waxing and waning of the slow wave activity that can occur prominently in the presence of nerve conduction block (Huizinga et al., 2014). Of course, this does not negate the integral role of neural activity in generating segmentation motor pattern. Slow waves themselves do not provide forceful contractile activity. Generating contractions depends on excitatory neural stimulation to bring the slow waves propagated from ICCs into the musculature, above the threshold for calcium channel activation (Huizinga et al., 2014). Thus, neural activity is an active component of the segmentation motor pattern.

In this study, the frequency of HBCs significantly decreased after the administration of both prucalopride and exogenous 5-HT inside the lumen; however, this was accompanied by significant increase in propulsive motor activity. Moreover, similar to the ripples, reduction in HBC frequency was inhibited by the antagonist indicating that it was luminal 5-HT_4R-mediated. The velocity of HBCs, however, remained unaffected after both prucalopride and 5-HT administration. Therefore, the decrease in HBC frequency is likely due to significant increase in propulsive motor activity via luminal 5-HT_4R activation and release of 5-HT which subjects the musculature to extensive neurogenic control to propel content down the colon. HBCs slow this process down due to the fact that they create haustra to promote

absorption of nutrients. Therefore, HBCs were diminished to avoid disruption of propulsive contractions.

This study also provides evidence that endogenous luminal 5-HT may play a physiological role in regulation of colonic motility in the rabbit colon. Luminal exogenous 5-HT evoked powerful propulsive activity via epithelial 5-HT_4Rs in the colonic mucosa. Luminal administration of exogenous 5-HT has been shown to stimulate motility, increase in the fecal pellet output and acceleration of colonic transit in rats (Fukumoto et al, 2003; Tsukamoto et al, 2007). In addition to the lamina propria, 5-HT can be released into the intestinal lumen in response to mechanical stimulation, vagal nerve stimulation, luminal acidification, food intake, signals from gut microbiota and in response to an increase of intraluminal pressure and restraint stress of the rat proximal colon (Lund et al., 2018; Tsukamoto et al., 2007; Hata et al., 2017). Furthermore, free biologically active 5-HT can be generated by luminal microbiota via deconjugation of glucuronide-conjugated 5-HT (Hata et al., 2017) and indigenous spore-forming bacteria from the mouse and human microbiota promote 5-HT biosynthesis from colonic EC cells by upregulating TPH1 expression in response to various biochemical substances of bacterial origin (Yano et al., 2015). Thus, 5-HT released into the colonic lumen may play an important role in mediating normal colonic peristalsis and stress-induced stimulation of colonic motility (Tsukamoto et al., 2007).

It has been proposed that released 5-HT in the lumen is carried to the distal colon with feces to stimulate colonic motility and transit (Tsukamoto et al., 2007). Stress-induced stimulation of the distal colonic motility was significantly attenuated by the luminal administration of ondansetron, indicating a need for further research into the role of luminal 5-HT_3 receptors (Tsukamoto et al., 2007). It seems possible that similar to the small intestine, the threshold for distention to induce peristalsis in the colon is influenced by luminal factors (Gwynne et al., 2014). Hence, the possibility exists that in some constipated patients who manifest lack of colonic peristalsis, the absence of propulsive motor patterns

may not be because of inability to generate peristaltic contractions but insufficient levels of 5-HT and/or luminal 5-HT_4 receptors. At baseline conditions, an effect of the 5-HT_4R antagonist on the ongoing colonic motor complex was not observed in this study. However, under these ex vivo experimental conditions there was no agonist present in the colon as the lumen had to be constantly perfused with the Krebs solution. Thus, presenting a 5-HT_4R antagonist in vivo may well affect motor activity in the presence of luminal 5-HT.

In chapter 3 it was demonstrated that intraluminal prucalopride results in strong prokinetic activity even during sustained simulated fecal impaction; that is, in the presence of a bulk of content stretching the rabbit colon to almost double (1.76 ± 3.2) its baseline diameter. Fecal impaction, which is a common occurrence in chronic constipation, causes increase in intraluminal pressure and hence, leads to ischemic phenomena (Grinvalsky et al.,1959). Ischemic phenomena give rise to a local inflammatory reaction and necrosis leading to ulcers and a possible subsequent perforation (Serrano Falcon et al., 2016, Craft & Prahlow, 2011). Moreover, the disruption of colonic transit caused by impaction may result in sustained increase in gut wall distensibility and dilation which often co-exists with chronic constipation and megacolon. The continuous contact between fecal matter and colonic wall may also cause mucous membrane irritation and a resulting increase in mucous secretion which can cause abnormal prolonged internal anal sphincter relaxation (Serrano Falcon et al., 2016).

Currently, treatment options for fecal impaction include manual des-impaction, stool softeners and rectal enemas when the mass is palpable in the rectum; and oral lavage with polyethylene glycol solutions for proximal fecal masses (Joels et al., 1994; Serrano Falcon et al., 2016). Laparotomy may be indicated if medical therapies are not effective (Zhao & Ke, 2010). Paradoxically, laxative abuse is associated with constipation and fecal impaction (Obokhare, 2012). As discussed in chapter 3, laxative-dependent patients are usually unable to produce normal propulsive motor patterns in response to colonic distention and progressively require higher doses to achieve a bowel movement (Creason, 2000).

Although fecal impaction can occur in all ages and types of patients (Hussain et al., 2014; Zhao & Ke, 2010), it is a more common GI problem in the elderly (Hussain et al., 2014; Serrano Falcon et al., 2016). Early identification and treatment can minimize discomfort and potential risk for aspiration, ulceration, or perforation (Obokhare, 2012). The aims of management are to relieve symptoms, clear out colon, and restore normal bowel habit but the most important regimen is to evacuate feces as soon as possible to reduce the risk for ulcer and perforation (Zhao & Ke, 2010).

In the present study, intraluminal prucalopride evoked LDCs with significantly high contraction amplitudes and duration which were forceful enough to pass over the simulated accumulated fecal mass (fixed inflated bag) and continue propagating along the entire length of the colon. Although bag inflation alone resulted in initiation of strong propulsive motor patterns due to distention even before drug administration, these distention-induced LDCs were not forceful enough to pass over the large inflated bag and stopped propagating as soon as they collided with it. The contraction amplitude and duration of these distention-induced LDCs were significantly lower compared to the LDCs generated by intraluminal prucalopride suggesting that prucalopride results in strong prokinetic effects even when there is content or impaction in the colon.

Since the LDCs evoked by prucalopride passed over the bag and continued propagating along the entire length of the colon, they would most probably be able to push it to the anal direction if the bag was not fixed. Cluster of FPCs were not strong enough to pass over the inflated bag neither at baseline conditions nor after the administration of prucalopride in the lumen. This is consistent with the level of excitation model in which FPC clusters were demonstrated to be a less forceful manifestation of the colonic motor complex compared to LDCs (chapter 2, Fig 6). The only two FPC clusters that did pass over the bag in the presence of intraluminal prucalopride, were similar to LDCs in contraction amplitude and were immediately followed by single LDCs indicating that the colon was most probably in the midst of shifting from a lower level of excitation to the highest.

At baseline conditions, distention-induced motor patterns stopped propagating as soon as they collided with the inflated bag and the segment of the colon positioned after the inflated bag did not generate any propulsive contractions. A possible explanation for this phenomenon could be the inhibitory effects of the nitrergic nerves. Contractile activity of the colon is tuned by several excitatory and inhibitory factors. Colonic inhibitory signal transduction is mainly mediated by the neurotransmitter nitric oxide (Beck et al., 2019). Beck et al., (2019) have recently shown that nitric oxide receptor in smooth muscle cells and ICC are required for normal peristaltic activity in mice. The nitric oxide receptor in both ICC and smooth muscle cells modulated the frequency of LDCs while the nitric oxide receptor in smooth muscle cells adjusted propulsive efficiency by regulating force of contraction of the LDCs (Beck et al., 2019). Additionally, the existence of polarized reflex pathways that appear to generate ascending excitation (depolarization) and descending inhibition (hyperpolarization) along the small intestine, which is consistent with the Bayliss & Sterling's law of the intestine, have been reported by various researchers (Spencer et al., 1999). Therefore, it is possible that descending inhibition via nitric oxide receptors is responsible for the inhibition of the propagation of these motor patterns in the rabbit colon. Nitric oxide receptor blockers can be used in the future to further evaluate this phenomenon.

It is possible that in patients suffering from fecal impaction, colonic distention by accumulated stool in the descending colon activates sympathetic neurons to prevent the ongoing propulsive activity resulting in preventing urge to defecate. Therefore, the impaction itself can exacerbate constipation by preventing defecation through sympathetic inhibition (Hanman et al., 2018). This inhibition can be overcome by strong activation of propulsive motor patterns which also has a parasympathetic component. Thus, activation of luminal 5-HT_4 receptors with intraluminal perfusion of prucalopride to evoke strong propulsive motor patterns that propagate along the whole colon could help in preventing fecal impaction.

In summary, rabbit studies indicate that luminal 5-HT_4Rs and endogenous 5-HT mediate colonic peristalsis and activation of these receptors via administration of luminally acting prokinetics such as intraluminal prucalopride, can result in strong propulsive motility. These studies support further investigation into incorporating 5-HT_4R agonists in colon-targeted drug delivery systems with the aim of applying such drugs to the colonic mucosa to treat dysmotility in patients with constipation while minimizing the adverse side effects associated with their systemic absorption (Mawe & Hoffman 2013; Amidon et al., 2015). The present rabbit studies also highlight the effect of luminal 5-HT in regulation of colonic motor function. Since luminal 5-HT itself showed similar prokinetic properties compared to prucalopride, the use of 5-HT instead of prucalopride is worth investigating. It may also be possible to adjust luminal 5-HT through diet or modifying 5-HT producing microbiota, in particular when patients with constipation might have reduced luminal 5-HT.

COLO-ANAL MOTOR COORDINATION AND SPWS IN THE HUMAN COLON

Results in chapter 4 show colonic motor patterns in healthy volunteers collected via an 84-sensor water-perfused catheter (1cm spacing) that permitted balloon distention and bisacodyl stimulation in the proximal and distal colon. This catheter allowed for a significantly higher resolution and intraluminal drug administration. SPWs were characterized in detail and both HAPW associated and SPW associated colo-anal reflexes were fully assessed. This HRCM study provided the basis for improved understanding and analysis of the prokinetic actions of intraluminal prucalopride in humans in the future experiments.

Simultaneous pressure waves were more or less ignored for a long time because pan-colonic pressure changes can be caused by general abdominal pressure increases and in some studies such events were filtered out as artifacts (Wiklendt et al., 2013). Abdominal pressure changes due to coughing, straining or changing of body position, do in fact induce simultaneous pressure changes, but these activities are usually associated with anal sphincter contraction, not with anal sphincter relaxation (Chen et al., 2017). They are also

associated with abdominal muscular action potentials; whereas SPWs are not (Corsetti et al., 2017). It is, of course, critical that these artifacts are recognized and filtered out during a colon assessment by HRCM.

SPWs have been previously defined as pressure waves that occur simultaneously at 3 or more sensors (Rao et al., 2001); the sensors were six strain gages with variable distances from 7 to 15 cm. The average amplitude was around 55 mmHg and the duration from 6 to 14 s. They increased upon awakening and after a meal (Rao et al., 2001). Initial high-resolution manometry studies suggested the SPWs to occur simultaneously throughout the descending colon (Chen et al., 2017) or to be pan-colonic (Corsetti et al., 2017). Using higher resolutions, SPWs of lower amplitudes were also identified. Simultaneous pressurizations were noted in a pediatric population (4–15 years) associated with constipation but were not found in children who were deemed to have normal colon function (Giorgio et al., 2013).

In this study, pan-colonic SPWs, and SPWs that follow proximal high-amplitude propagating pressure waves (HAPW-SPWs), were the dominant propulsive motor patterns observed in healthy participants from the age 20; and they had a strong association with anal sphincter relaxation and gas and/or liquid expulsion. The SPWs that often followed a proximal HAPW (HAPW-SPW), could start in the transverse colon (chapter 4, Fig 21A) and end proximal to the rectum. The HAPW-SPW pattern can also be seen in published low-resolution manometry recordings, although the SPW development was usually not mentioned (e.g., Bassotti et al.,1999).

Wessel et al., (2016) reported a motor pattern in children with intractable constipation that appears similar to SPWs. This motor pattern was called “long single propagating motor pattern” which traveled rapidly across all of the recording sites that spanned the descending and sigmoid colon. Their amplitude was similar to observed SPW amplitudes in this study at 16.2 ± 8.3 mmHg. This pattern occurred in one child rhythmically at 1.2 cpm, similar to patterns reported in the present study. A discrepancy is the average propagation velocity

which was 2.7 ± 0.7 cm s^{-1} pre-prandially (Wessel et al., 2016). Here, SPW propagation velocity could not be calculated because the SPWs occurred simultaneously or they were too fast. Reports on the "long single propagating motor patterns" observed in healthy adults differ from SPWs observed in this study in their pre-prandial amplitude which was at 49.7 ± 16.5 mmHg (Dinning et al., 2014). The velocity reported for those motor patterns was at 1.8 ± 1.2 cm s^{-1} which is more similar to the propagation velocity of HAPWs at 0.4 – 2.8 cm s^{-1} (Rao et al., 2004; Liem et al., 2012).

In the present study, the SPW amplitudes during baseline ranged between 5 to 38 mmHg and between 5 to 47 mmHg postprandially. The amplitude of SPWs increased significantly after meal intake while their occurrences did not. Nevertheless, the amplitude of gas-related SPWs increased significantly postprandially. Meal-induced SPWs indicate that the SPW is part of the gastrocolic reflex. In a previous HRCM study, comparison between healthy volunteers and constipated patients revealed no statistically significant difference in the occurrence of SPWs after a meal (Chen et al., 2017). This could likely be due to the fact that here a 1000kcal meal was used; whereas the previous study used only a 320kcal meal (Chen et al., 2017).

The observation that SPWs can occur in a highly rhythmic manner, suggests that networks of ICC might determine a part of their pattern (Quan et al., 2017). This could indicate that neurogenic and myogenic mechanisms overlap and work in concert to create the motor pattern (Huizinga & Lammers, 2009; Costa et al., 2013). The number of pan-colonic SPWs that were evoked by balloon distention or bisacodyl was likely influenced by the number of HAPWs that were initiated since they did not often occur simultaneously. However, when they did occur simultaneously, the amplitudes of HAPWs and SPWs summated, indicating their independence (chapter 4, Fig 25).

Although SPWs were not always associated with gas or liquid expulsion, gas expulsion was always accompanied by a SPW or a HAPW-SPW. Additionally, the amplitude of

SPWs associated with gas expulsion were significantly higher in all conditions compared to the ones with no associated gas release (chapter 4, Table 12). This observation suggests that the SPW could be a biomarker for gas transit. It has been previously demonstrated that poor handling of gas in IBS patients is a consequence of dysmotility (Serra et al., 2010), and the present study suggests that this may be related to poor sensitivity to evoke SPWs or poor development of SPWs. Spontaneous gas expulsions have been associated with 18 ± 3 mmHg propagating contractions in a previous study using an 8-sensor catheter with 12cm spacing (Bassotti et al., 1996). These contractions were assumed to be similar to mass movements, but the tracings suggest that there was an HAPW in the descending colon that switched to a SPW in the distal colon followed by anal sphincter relaxation which fits the HAPW-SPW pattern observed in this study.

Mass movements of colonic content involve inhibition of the haustra in the rabbit (Ehrlein et al., 1982) and human colon (Holzknecht, 1969) allowing smooth passage of stool. In the first rabbit study in this thesis (chapter 2), similar results were observed and haustral boundary contractions decreased significantly to facilitate peristaltic movements. In this HRCM study, haustral boundary pressure transients remained unaffected in the presence of SPWs (chapter 4, Fig 21), which likely allows the passage of gas while keeping stool inside the haustra. This is indeed observed with in vivo X-ray imaging in the rabbit (Ehrlein et al., 1982) and in humans where semiliquid content remains inside the haustra (Ritchie, 1968, 1971; Ritchie et al., 1971).

Additionally, SPWs were associated with liquid outflow using a water-perfused catheter. SPWs could reach high amplitudes (> 50 mmHg) in response to rectal balloon distention (chapter 4, Fig 23) and were associated with urge to defecate and expulsion of the balloon. This suggests that SPWs may be involved in the process of normal defecation, consistent with previous observations during natural stool evacuation (Bampton et al., 2000). Natural stool evacuation was associated with simultaneous pressurization of the colon, although some or all of the simultaneous pressurizations might have been due to straining. However,

HAPWs occurred well before evacuation and appeared to be associated with moving content into the rectum and urge to defecate (Bampton et al., 2000).

As mentioned in previous chapters, the factors determining the initiation of a proximal HAPW or a proximal SPW are not yet fully clear. In a study investigating the possible relationships between ileal and colonic motor events, it was found that only 9% of cecal colon propagating contractions were clearly linked to an ileal motor event (Dinning et al., 1999). This observation may indicate that the initiation of the SPW and the HAPW-SPW occurs in the most proximal part of the colon.

Based on a previous study in the rabbit colon (Quan et al., 2017) and the present rabbit studies (chapters 2,3), it can be hypothesized that the motor pattern underlying the SPW is a composite propulsive motor pattern. In the rabbit colon, SPWs are generated by a cluster of fast propagating contractions (FPCs) at 15–26 cpm (Quan et al., 2017). In the human colon, SPWs are composed of a cluster of multiple narrow SPWs at ~ 25 cpm. This was visible when the different narrow SPWs had varying amplitudes. It may well be that all SPWs are a composite but that pressure waves of similar amplitude merge to present as a single pressure wave. If SPWs originate from fast propagating circumferential muscle contractions, it will explain their propulsive nature. This notion is supported by the fact that fast propagating contractions are a normal feature of the human colon as shown by X-rays (Ritchie, 1968; Hertz & Newton, 1913) and by the presence of very fast propagating bursts of action potentials recorded intraluminally (Bueno et al., 1980). Sarna et al., (1981) observed clusters of electrical oscillations at ~ 28 cpm when recording from the human colon in vivo, with serosal electrodes which is similar to the frequency of the narrow high frequency pressure waves observed in this study as shown in chapter 4, figures 22,23. As mentioned previously, rabbit experiments have shown that the SPW is generated by myogenic FPCs which are likely controlled by interstitial cells of Cajal associated with the myenteric plexus (ICC-MP) (Lentle et al., 2008) but the organization of these fast propagating contractions into rhythmic clusters or the formation of FPC clusters, is

neurogenic (Chen et al., 2016; Quan et al., 2017). The situation may be the same in the human colon which has a rich network of ICC-MP (Rumessen et al., 2009) and fast propagation can easily be facilitated by an ICC network (Wei et al., 2017).

When the rectum was stimulated by balloon distention or bisacodyl in this study, SPWs starting in the proximal colon were generated. This could involve extrinsic autonomic nerves. Evidence for spinal nerve involvement was obtained by Kock et al., (1972) when bisacodyl was administered in the rectum in patients with a bladder substituted by a section of the sigmoid colon. In these patients, contractions appeared in the substitute bladder. In the rectum, both IMA (intramuscular array) and IGLE (interganglionic laminar endings) mechanoreceptors (Spencer et al., 2008) detect tension, stretch or ganglia deformation (Blackshaw & Gebhart, 2002; Brierley et al., 2004; Lynn et al., 2005). It is possible that similar to the distal colon, a proportion of these mechano-sensors may have multiple receptive fields which integrate mechanical and chemical information within the rectum (Berthoud et al., 2001). This information will travel to the sacral defecation center and from there to several brain stem centers (Stiens et al., 1998; Berthoud et al., 2001) including the locus coeruleus–Barrington's nucleus complex, the nucleus tractus solitarius (NTS) and the insula (Maggi et al., 1988; Nagano et al., 2004; Martínez et al., 2006; Larsson et al., 2012). Then, the dorsal motor nucleus of the vagus could be stimulated and vagal nerves may initiate activity in the proximal colon where the signal ends within intramuscular arrays that include intramuscular ICC (Takaki et al., 1987; Wang & Powley, 2000; Komuro, 2006; Wang et al., 2014; Powley et al., 2016; Wei et al., 2017).

The function of the colo-anal reflex has been debated. There are suggestions that it might be coordinated with colonic contractions to release flatus through the anal canal (Sintusek et al., 2018) Given the significantly longer duration of the colo-anal reflex in comparison with the recto-anal inhibitory reflex (RAIR), the former probably plays an important physiological role allowing more transit of stools through the anal canal (Rodriguez et al., 2012). High amplitude pressure waves may be associated with anal sphincter relaxation as

has been shown in the present and previous studies (Malcolm & Camilleri, 2000; Rodriguez et al., 2012; Sintusek et al., 2018). However, in previous studies, the presence of SPWs as a continuation of the HAPW was not contemplated. Besides HAPW associated colo-anal reflex, SPWs were also found to be associated with anal sphincter relaxation in this study.

Here, anal sphincter relaxations associated with HAPWs never occurred prior to the onset of HAPWs. In studies where this was suggested such as Malcolm & Camilleri (2000), sensors were not placed in the most proximal colon. Hence, it is likely that the time and location of HAPW initiation could not be detected precisely. Since an 84-sensor catheter was used in this study and the first sensors were positioned in the proximal colon, it was possible to observe the whole HAPW, and hence, the timing of anal sphincter relaxation associated with the HAPW could be detected with higher precision. Some HAPWs were followed by a SPW (HAPW-SPW) and 87.9% of them were associated with significant anal sphincter relaxation (chapter 4, Table 11). The relaxation was mostly associated with the onset of the SPWs when the HAPW terminated in the right colon and usually lasted the entire duration of the SPW which suggests the existence of a colo-anal reflex independent from the recto-anal inhibitory reflex (Sintusek et al., 2018; Rodriguez et al., 2012; Malcolm et al., 2000).

It is unlikely that the SPW that follows an HAPW is due to passive pressure build up in the part of the colon distal to the HAPW because in more than 50% of the HAPWs studied here, the concomitant SPW and anal sphincter relaxation did not occur. Moreover, as previously noted, passive increase in intraluminal pressure does not generate anal sphincter relaxation but anal sphincter contraction accompanied by intraluminal pressure increase due to artifacts such as body movements or coughing.

Decreased anal pressure can lead to fecal incontinence, while elevated anal pressure is observed in patients with an anal fissure due to hypercontractility of the IAS. ICC have been identified in the IAS of mouse, monkey, and humans with immunohistochemical

techniques and in the dog with electron microscopy. There are significant differences in the morphology and localization of ICC in the IAS versus the colon. ICC associated with the myenteric plexus (ICC-MP), intramuscular ICC (ICC-IM) and submucosal ICC (ICC-SM) are all present in the rectum. However, the density of ICC-MP and ICC-SM declines in the aboral direction leaving only ICC-IM in the distal IAS. Interestingly, the distal IAS is also where the largest and highest frequency slow wave activity occurs (Keef & Cobine, 2019).

Association of SPWs and HAPWs with anal sphincter relaxation suggests a neuro-myogenic control. The neurogenic program underlying the motor pattern is likely similar to esophageal contraction associated with lower esophageal sphincter relaxation (Goyal & Paterson, 2011). Therefore, consistent with Sintusek et al., (2018), this study indicates that anal sphincter relaxation is likely a neurally mediated reflex rather than a mere phenomenon associated with rectal distention as is noted with the recto-anal inhibitory reflex. The anal sphincter tone is primarily generated by the internal anal sphincter musculature (Rattan, 2005; Keed & Cobine, 2019) and controlled by slow wave activity of ICC-IM that is conducted into internal anal sphincter smooth muscle cells. This slow wave activity is responsible for orchestrating the rhythmic contractile activity (Cobine et al., 2017), as observed in the present study.

The IAS is innervated by both excitatory and inhibitory motor neurons that can profoundly affect smooth muscle contraction and relaxation (Keef & Cobine, 2019). Autonomic nerves supply the internal anal sphincter via the inferior rectal branches of the pelvic plexus (Kinugasa et al., 2014) with sympathetic nerves being dominant. Although sympathetic innervation may not significantly contribute to basal tone, it can cause anal sphincter contraction through direct innervation of smooth muscle cells via alpha-1 receptors (Frenckner & Ihre, 1976; Glavind et al., 1997; Rattan, 2005). The human IAS most likely receives both sympathetic excitatory and inhibitory innervation from the presacral hypogastric nerves that contain both excitatory and inhibitory neurons (Carlstedt et al.,

1988). The external sphincter contributes to anal sphincter tone and the mechanism of continence, in part by a sacral reflex (Broens et al., 2013).

The relaxation of the anal sphincter in response to a SPW could be complete, resulting in the absence of any pressure gradient between rectum and the external environment. This could involve inhibition of the IAS by enteric nitrergic nerves (De Lorijn et al., 2005) with the sensory arm of the rectal reflex that causes anal sphincter relaxation mediated by ICC (De Lorijn et al., 2005). Nitrergic nerves have also been demonstrated to play a role in the relaxation of the IAS following rectal distension (Keef & Cobine, 2019). Inhibition of the IAS could also be facilitated by activation of inhibitory nerves with vasoactive intestinal peptide (VIP) or carbon monoxide as transmitter (Rattan, 2005). Inhibition may further be facilitated by parasympathetic activation of myenteric inhibitory nerves to the IAS (Gonella et al., 1987). Relaxation of the external anal sphincter can be achieved through decrease in the discharge frequency of sacral motor neurons to the external anal sphincter that contribute to anal tone (Gonella et al., 1987). Cell bodies of the pudendal nerve fibers to the external sphincter are in the Onuf's nucleus and these nerves can be affected by parasympathetic nerves, likely via interneurons, from the sacral defecation center (Roppolo et al., 1985; Kihira et al., 1997).

In a nutshell, this study suggests that SPW is a prominent colonic-rectal motor pattern evoked with meal, bisacodyl and rectal distention. It can propagate into the anal canal and can be associated with anal sphincter relaxation and gas expulsion in healthy subjects. Therefore, the SPW has the potential to become a biomarker for evaluating motor patterns that facilitate gas transit as well as the gastrocolic reflex and extrinsic neural responses to rectal stimulation, with potential diagnostic values in patients with colonic dysmotility and bloating. SPWs are also associated with expulsion of liquid and a high amplitude SPW can expel a balloon. Hence, SPWs may also be associated with stool expulsion. Additionally, SPWs can be initiated at the termination of a proximal HAPW or begin in the transverse colon without being preceded by an HAPW.

HRCM can reveal the ability of the colonic musculature to generate many distinct motor patterns likely associated with distinct functions. It has the capability of showing intactness or absence of motor patterns associated with neural reflexes in response to distention, meal or chemical stimuli. Consistent with Keef & Cobine (2019) and Sintusek et al., (2018), this study also suggests that the coordinated IAS relaxation with propulsive motor patterns is the result of an independent neurally controlled colo-anal reflex. Hence, this study lends support for inclusion of SPWs and documentation of the colo-anal reflex as an important part of the manometric assessment of the colon.

EFFECTS OF INTRALUMINAL PRUCALOPRIDE IN THE HUMAN COLON

In chapter 5, results from a case study of the effects of intraluminal prucalopride in a healthy volunteer were reported and propulsive motor patterns as well as the colo-anal reflexes were analyzed. This case study demonstrated that similar to the rabbit animal model, luminal administration of prucalopride in the proximal human colon evokes powerful propulsive motor patterns and/or enhances the amplitude of propulsive motor patterns compared to both the baseline conditions and after meal intake.

In chapter 2, it was demonstrated that the colonic motor complex is manifested in different motor pattern configurations depending on the level of excitation and intraluminal prucalopride and intraluminal exogenous 5-HT evoked not only the most forceful propulsive contractions (LDC in the rabbit colon) but also other propulsive motor patterns associated with pressure development. Similarly, in this case study, different types of propulsive motor activity including HAPWs, SPWs and HAPW-SPWs were observed which were associated with internal anal sphincter relaxation. These results not only show the effect of the intraluminal drug but also lend further support for inclusion of the colo-anal reflex and SPWs into manometry assessments.

Prolonged manometry studies have demonstrated that adults with slow transit constipation have fewer spontaneous HAPWs than healthy subjects (Dinning et al, 2010; Rao et al.,

2004; Bharucha, 2012; Haggar et al., 2003). This was accompanied by a decreased frequency of urge to defecate (Liem et al., 2010) indicating that one of the pathophysiological mechanisms for constipation might be decrease in the frequency of HAPWs in the colon. In addition to fewer HAPWs, the spatiotemporal organization of the motor patterns may also be disturbed in constipation (Bharucha, 2012; Dinning et al., 2010). Specifically, constipated patients did not have the normally observed increase in frequency and amplitude of HAPWs before defecation (Bharucha, 2012). On the other hand, some patients with diarrhea-predominant irritable bowel syndrome (IBS-D) have been observed to generate more HAPWs compared with healthy subjects (Hasler et al., 2011; Bharucha et al., 2009).

There are, however, inconsistencies in the literature. For instance, short-duration (not prolonged) colonic manometry identified fewer postprandial HAPWs in slow but not normal transit constipation (Bharucha, 2012) while some healthy subjects did not generate any HAPWs over a 24-hour period (Dinning et al., 2010). Additionally, normal frequency of HAPWs were observed during 24-hour manometry testing in children with slow transit constipation (King et al., 2009). Despite such inconsistencies, data collectively suggests that HAPWs are important for propagating colonic content and they are an integral motor pattern in the defecation reflex. In this case study, the occurrence of HAPWs increased significantly in response to intraluminal prucalopride and HAPWs were at times associated with urge to defecate and significant anal sphincter relaxation. This further supports the role of HAPWs in defecation and indicates the involvement of 5-HT in generating these motor patterns which is consistent with previous studies that evaluated the effects of oral prucalopride in humans (De Shryver et al., 2002).

It is likely that intraluminal prucalopride induces propulsive motor patterns in humans by a sequence of events similar to mechanisms responsible for initiating colonic motor complexes in the rabbit animal model as presented in this study. 5-HT release from EC cells results in activation of mucosal endings of IPANs and subsequently the submucosal

AH neurons. IPANs and some vagal and pelvic afferent endings come into close proximity to the mucosal epithelium (Vanner et al., 2016; Mawe & Hoffman, 2013) which exposes them to chemicals absorbed across the mucosal epithelium or released from enteroendocrine cells whose apical membrane is exposed to luminal content such as the EC cells. As discussed in chapter 1, this arrangement is similar to the relationship between taste buds and gustatory mucosal afferents in the mouth and the same G-protein-coupled receptors found in the mouth are also expressed within the GI tract (Vanner et al., 2016). Afterwards, propagation of excitation along the colon occurs through inter-neuronal pathways which converge onto other AH neurons, and finally excitation of myenteric ICCs which activate longitudinal and circular muscles. When excitatory motor neurons are active, contractions can summate, giving rise to powerful, pan-colonic contractions that may propagate substantial distances along the colon (Bharucha, 2012).

Furthermore, luminal prucalopride markedly increased the frequency and amplitude of pan-colonic SPWs compared with both baseline and after meal intake. Pan-colonic SPWs were associated with significant prolonged IAS relaxation although no gas or liquid expulsion was reported. In chapter 4, it was demonstrated that SPWs are a propulsive motor pattern as they could be associated with gas, balloon and liquid expulsion. They were also associated with significant IAS relaxation in both HRCM studies (chapter 4,5). The fact that a 5-HT_4R agonist affected SPW amplitudes and evoked their initiation indicates that 5-HT could be involved in generation of SPWs. Administration of a 5-HT_4R antagonist in the lumen in future studies could assist in understanding of the role of epithelial 5-HT_4R-mediated generation of HAPWs and SPWs in humans.

Results from BON cell studies further support the above interpretation. Data showed that prucalopride (5μM) evoked significant 5-HT outflow from human EC model cells which was inhibited by a 5-HT_4R antagonist (chapter 5, Fig 29,30). Since prucalopride is a highly selective 5-HT_4 receptor agonist, it can be concluded that 5-HT_4Rs are expressed on the surface of these cells and their activation results in the release of 5-HT. In addition, data

showed that prucalopride in high concentrations (10μM) results in cell proliferation (Fig 31), although this response was not antagonist sensitive. Previous studies have shown a significant antagonist-sensitive increase in the rate of cell migration in human epithelial colorectal adenocarcinoma cells treated with tegaserod (Spohn et al., 2016). Further experiments are required to determine the effects of prucalopride on cell migration and proliferation. However, these results support the main hypothesis of the present study as they indicate a significant antagonist-sensitive 5-HT outflow induced by prucalopride.

In conclusion, this case study showed that intraluminal prucalopride strongly enhances peristaltic activity in the human colon, very similar to bisacodyl, the most effective prokinetic known thus far. The prokinetic effects of intraluminal prucalopride in the human colon mimicked the results observed in the rabbit animal model. Similar to the rabbit, results suggest that endogenous 5-HT may be an important regulator of colonic motility. The strong peristaltic activity observed in this experiment after prucalopride administration, and the induction of vomiting which is a known effect of mucosal 5-HT receptor activation in the small bowel (Spiller, 2008), is also similar to the effect of luminal bisacodyl given in the proximal colon or rectum (Chen et al., 2018; Bharucha, 2012).

All in all, this thesis project suggests that prucalopride incorporated in colon-specific delivery systems has the potential to become the preferred prokinetic for the treatment of constipation. As mentioned in chapter 1, newer colon targeted drug delivery systems use polysaccharide coatings that can be degraded only by the microorganisms in the colon resulting in increased delivery success. Nevertheless, with the advancement of nanotechnology, various forms of nanoparticle formulations for colon delivery have been investigated (Zhang et al.,2017) which could replace all other types of colon-specific delivery systems in the future. This can be especially beneficial for the aging adults. The elderly are a high-risk population that manifest the highest incidences of chronic dysmotility and fecal impaction while having associated renal and cardiovascular co-

morbidities (Sajid et al., 2016). Therefore, they could particularly benefit from colon-specific delivery systems of prokinetics such as prucalopride.

LIMITATIONS

In drawing conclusions from this project, the following limitations must be considered. Ex vivo animal models are limited in their ability to be extrapolated to in vivo physiological conditions. Although efforts were made to simulate the physiological conditions as closely as possible, in ex vivo studies, the lumen is constantly perfused with solutions which is vastly different from the in vivo luminal microenvironment. Although the HRCM human case study showed that prucalopride works similarly in vivo, it is still required to increase the sample size to confirm the results.

Colonic motility is a complex process that includes several layers of neural and hormonal control from the colon up to the central nervous system (Andrews & Storr, 2011); however, since the extrinsic nerves are severed in an ex vivo set up, it is not possible to study the contribution of extrinsic nerves to the control of colonic motility which is another limitation of ex vivo studies.

Additionally, when the drug is administrated in physiological conditions, the colon is usually not empty; hence, the effects of the tested drugs could be different in vivo compared to ex vivo animal models. To tackle this issue, an inflated barostat bag was used in this study to simulate fecal impaction as a common symptom in severe constipation. Using a rapid barsotat bag, we were able to adjust the volume of the bag and measure the pressure it exerts on the tissue wall to better mimic a large bulk of content in the colon. This helped us in predicting the effects of the drug when it is released from oral CDDs in the lumen of patients suffering from severe constipation. A limitation of this set up was that the bag had to be fixed because similar to all ex vivo studies, the colon needed to be cannulated before being mounted in the organ bath. Hence, the bag could not be moved by colonic propulsive motor activities. The fact that the high amplitude LDCs induced by luminal prucalopride

passed over the bag suggests that it would most probably be pushed to the anal direction by such strong propulsive contractions if it was not fixed. However, a different ex vivo set up could be contemplated for future studies to allow an expellable bag.

With respect to human studies, one of the limitations of HRCM experiments is that various stimuli were given consecutively, hence, responses to stimuli may have been influenced by the remaining activity of the previous intervention. This could be avoided by reducing the number of interventions and/or allowing more time in between the stimuli in future experiments. Regarding the HRCM case study of the effects of intraluminal prucalopride, since powerful prokinetic results were observed in this healthy volunteer, the next step would be increasing the sample size to first, determine the effects of the drug in healthy volunteers and then in patients.

Patients need to be controlled for different types of constipations although the difficulty in assessing symptoms exists in all studies involving functional disorders. Recruiting volunteers and patients from diverse age groups and both sexes can help in better determining the possible gender-dependent and age-dependent effects of the drug. The prevalence of constipation increases significantly with age (Bitar, 2003; Gandell et al., 2013). The reduced GI contractile activity may be due to the effects of aging on smooth muscles. In colonic smooth muscle cells from aging rats, a decrease in calcium and potassium channel currents is observed that affects the initiation of contractions (Bitar, 2003). The age-dependent changes in the smooth muscle function have been observed both in the cholinergic neurotransmission and the response of smooth muscle to acetylcholine (Bitar, 2003). As discussed above, since the elderly are a high-risk population that manifest the highest incidences of chronic dysmotility while having associated renal and cardiovascular co-morbidities (Sajid et al., 2016), intraluminal prucalopride can be especially beneficial for the aging adults. Hence, including the elderly in future HRCM studies of the effects of intraluminal prucalopride is essential.

FUTURE DIRECTIONS

Extrinsic Innervation

A next avenue to explore following this project, could be evaluating the effects of intraluminal prucalopride on extrinsic nerves. Although the GI tract possesses intrinsic neural plexuses that allow a significant degree of autonomy over GI functions, the CNS provides extrinsic neural inputs that regulate these functions (Browning & Travagli, 2014). Provided that extrinsic sensory nerve endings exist in mucosa (Mawe & Hoffman, 2013) and they also possess 5-HT receptors (Schikowski et al., 2002), mucosal release of 5-HT could eventually activate extrinsic afferents. The involvement of 5-HT_4Rs in the modulation of extrinsic afferent sensitivity of the intestinal wall has been previously studied in the cat rectum using tegaserod. Results indicated that tegaserod had an inhibitory effect on intramural mechanoreceptors in the cat rectum which was in line with the observation that tegaserod relieves the sensory symptoms of patients suffering from IBS (Schikowski et al., 2002).

The parasympathetic innervation of the colon plays a significant role in regulating propulsive colonic motility, particularly prior to defecation (Browning & Travagli, 2014). Damage to parasympathetic nerves, or their denervation, results in dysregulated colonic motility and constipation. However, over time colonic motility patterns may be restored suggesting that the role of parasympathetic inputs is mainly to enhance and modulate intrinsic motility rather than initiating colonic functions (Browning & Travagli, 2014). Vagal primary afferent neurons create synapses exactly where EC-cell-derived 5-HT acts on IPANs (Bellono et al., 2017). Therefore, it is likely that when 5-HT is released in the lamina propria, it acts on vagal afferent neurons as well. Moreover, luminal application of exogenous 5-HT can attenuate vagal-dependent vesico-motor reflex in response to colorectal distension in rats (Zhang et al., 2011).

In vivo, distension of a hollow organ like the colon will inevitably activate 5-HT release and it will potentially diffuse to stretch-receptive endings in close proximity (Schikowski et al., 2002). Provided these endings also possess 5-HT_4R-binding sites, mucosal stimuli could eventually modify the sensitivity of extrinsic intramural afferents (Schikowski et al., 2002). Moreover, Hoffman et al., (2012) reported that intracolonic infusion of tegaserod or naronapride reduced the visceromotor response in a dose-dependent manner and the antinociceptive responses to tegaserod and naronapride were blocked by a 5-HT_4R antagonist. Collectively, these findings showed that exposure of the colonic mucosa to 5-HT_4R agonists alleviates visceral hypersensitivity in rats (Hoffman et al., 2012). Such results provide a rationale for the therapeutic use of agents acting at 5-HT_4Rs on extrinsic sensory neurones in humans.

Epithelial Protective Effects

Besides prokinetic effects, activation of epithelial 5-HT_4Rs has been shown to mediate "epithelial protective effects" via a variety of mechanisms; including maintenance of the epithelial barrier through cell proliferation and migration, and also by increasing resistance to epithelial apoptosis induced by oxidative stress (Spohn et al., 2016). A critical aspect of epithelial healing is enhanced epithelial cell migration (Heath, 1996). Epithelial healing is important in IBD ulcerative colitis and Crohn's disease which manifest leakiness in the cell membranes of epithelial cells (Gibson et al., 1988). Tegaserod enema in the distal colon of a mouse model of colitis, reduced the permeability of the gut. Additionally, a significant antagonist-sensitive increase in the rate of cell migration in human epithelial colorectal adenocarcinoma cells treated with tegaserod was observed (Spohn et al., 2016). In the present study, data showed that prucalopride in high concentrations results in BON cell proliferation, but this response was not antagonist sensitive. Further experiments are required to determine the effects of prucalopride on cell migration and proliferation. Unlike tegaserod which is a partial 5-HT_4R agonist, prucalopride is a highly selective 5-HT_4R agonist; therefore, its epithelial protective effects are worth investigating.

ENS Neurogeneration and Protection

Another avenue to explore following this project is the role of 5-HT in neurogeneration and protection of the ENS. Slow transit constipation could be due to enteric nervous system abnormalities. A decrease of enteric neural elements (neurons and/or neurofilaments) in both the submucosa and myenteric plexus has been reported in several studies evaluating patients with slow transit constipation which may partially be due to apoptotic phenomena (Bassotti & Villanacci, 2006). 5-HT has been shown to have a role in neural development and regulation of neurite growth in several species including invertebrates (e.g. Helisoma snail), lower vertebrates (e.g. gold fish) and mammals (e.g. rats; Trakhtenberg & Goldberg, 2012). Studies have demonstrated that 5-HT_7 and 5-HT_{1A} receptors are involved in the neurite growth. 5-HT_7 receptor has been shown to be involved in axonal regeneration in C. elegans (Sobrido-Cameán et al., 2018). Several experiments have revealed that 5-HT_{1A} receptors are highly expressed in motor neuron progenitor cells of the adult zebrafish spinal cord (Barreiro-Iglesias et al., 2015).

Liu et al., (2009) demonstrated that the number of enteric neurons increased significantly in wild type mice (WT) four months after birth but not in transgenic mice lacking 5-HT_4Rs (KO). In vitro, 5-HT_4R agonists increased enteric neuronal development and survival; decreased apoptosis and activated CREB which is a cAMP response element-binding protein. In vivo, in WT but not KO mice, 5-HT_4R agonists induced bromodeoxyuridine incorporation into cells that expressed markers of neurons or neural precursors, hence, they demonstrated that 5-HT_4Rs have a physiological role in ENS neuroprotection and neurogenesis. However, prucalopride was never tested. In light of such findings, it is likely that activation of 5-HT_4Rs with prucalopride could assist in neurogeneration and neuroprotection in the human ENS which is worth examining.

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