Patterns of motor activity in the isolated large intestine of mice

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Kyra Jay Barnes

BMedSci/BSci (Hons)

Discipline of Human Physiology

Flinders Medical Science and Technology

Centre for Neuroscience

School of medicine, Flinders University

Adelaide, South Australia

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Contents

Summary

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

In Chapter 2, experiments revealed the importance of study design, and how motility patterns can change characteristics depending on the nature of the *in vitro* recording methods. Chapter 2 demonstrates that traditional recording methods for measuring colonic migrating motor complexes (CMMCs) *in vitro* can potently modify their frequency, velocity and extent of propagation. It is suggested that these altered properties are likely due to circumferential stretch and/or pinching (stimulation) of the colonic wall. Recording these motor patterns without using traditional *in vitro* techniques reveals that CMMC frequency and velocity is significantly lower from previously reported literature.

In Chapter 3, the characteristics of CMMCs were studied in a strain of mice that mimic the condition Hirschsprung's disease in humans. These mice, named lethal spotted (ls/ls) mice lack the EDN3 gene, which is the same gene, when mutated in humans, that is directly responsible for the onset of colorectal aganglionosis and Hirschsprung's disease. In our hands, when raised on a C57BL6 strain, these mice surprisingly had been found to predominantly live a normal lifespan, and are able to expel pellets without developing lethal megacolon. Interestingly it was found that CMMCs did occur in these mutant mice, and the length of the aganglionic segments were not as prominent as previously described by other laboratories which had used mice of a different strain of origin (where the

mutation was fatal at a young age). We discuss the significance of the background strain of origin and length of aganglionosis in the lethality of the Hirschprung's model mice.

The experiments conducted in this thesis have led to a greater insight into the mechanisms underlying colonic motility in mouse large intestine.

Declaration

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

Kyra Jay Barnes, March 2015

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Peer-reviewed Research Journal publications arising from this work

Barnes, KJ, Beckett, EA, Brookes, SJ, Sia, TC, Spencer, NJ (2014), Control of intrinsic pacemaker frequency and velocity of colonic migrating motor complexes in mouse. *Frontiers in Neuroscience*, vol. 8, no. 96, pp. 1-8.

Barnes, KJ & Spencer NJ, (2015), Can colonic migrating motor complexes occur in mice lacking the endothelin-3 gene? *Clinical and Experimental Pharmacology and Physiology*, vol. 42, no. 5, pp. 485-495

Conference proceedings

2015 Australasian NeuroGastroenterology & Motility Association Inc (ANGMA) Conference, Adelaide, SA

- "Do colonic migrating motor complexes (CMMCs) occur in mice lacking the EDN3 gene? " (Poster #27)

2014 Australian Neuroscience Society Annual Conference, Adelaide, SA

- "Mechanisms underlying the generation of colonic migrating motor complexes: do they occur in an empty intestine? " (Poster #WED-002)

1.1 Anatomy and function of the colon

The gastrointestinal tract includes the oral cavity, oesophagus, stomach, small intestine (made up of the duodenum, jejunum, and ileum), caecum, colon, rectum and anus. The colon is further divided into ascending, transverse, descending and sigmoid colon. The proximal colon is a term used to refer to the ascending and transverse sections of colon, and distal colon refers to the descending, sigmoid, rectosigmoid junction and rectum. One of the most obvious differences between the proximal and distal colon is the state of the luminal content. In the proximal colon, content is usually in liquid or semi-solid form, however by the time it reaches the distal colon, it would be expected to be solid.

Ingested matter which is propelled from the small intestine into the colon has been digested and the majority of absorption of energy and other nutrients has already occurred. The main purpose of the colon is to then absorb water, Na^+ and other electrolytes, and to expel waste. Within the colon there is a large population of micro-organisms, which are responsible for most of the remaining breakdown of material before it is removed as waste [47].

Variation between mammals in the structure of parts of the tract is influenced by type and frequency of food intake, digestion time, and also the size and shape of the animal [97]. Mouse models are often used as a valuable research tool to investigate mechanisms underlying colonic motility in normal physiology and disease. This is because animal models, such as the mouse, provide excellent representatives of the neural circuitry and neurochemical coding of other large mammals, such as humans. Despite these similarities, there are also differences. The human left colon, which begins at the midline of the transverse colon, includes descending colon, rectum and ends at the external anal orifice. This is comparable to the mid and distal regions of mouse colon. In relative terms, the

mouse caecum, however, is much larger than that of the human, representing up to one third of the large intestine, containing high numbers of bacteria for fermentation of food products. Two other main differences of minor consequence are presence of an appendix in humans, which mice lack, and the fact the submucosa in a mouse is very thin throughout the gastrointestinal tract [155].

Over the past decade, the mouse colon has emerged as a supreme model for recording colonic activity *in vitro* [26,34,35,55,134,150]. This is because the small length of the large bowel means that the entire colon can be removed from mouse without interrupting the enteric neural pathways and allows cyclical propagating motor patterns to be preserved *in vitro*.

1.2 Wall of colon

The colon wall is comprised of several distinct anatomical layers which, among other functions, facilitate nerve conduction and muscle contraction to propel ingested luminal contents. The innermost layer, closest to the lumen, is the mucosa, which itself is made up of three layers, which mainly function in absorption and secretion. The innermost of the mucosal layers is the epithelial layer, followed by the lamina propria, and then the muscularis mucosae (a thin smooth muscle layer). The submucosa is the second layer, which contains the submucous plexus (or Meissner's plexus), consisting predominantly of enteric neurones, sympathetic and parasympathetic fires, extrinsic spinal afferents blood and lymphatic vessels. Lying adjacent to the submucosa is the circular smooth muscle layer, which is responsible for causing segmenting-type and propulsive contractions, of myogenic and neurogenic origin. The myenteric plexus (or Auerbach's plexus) then lies adjacent to the circular muscle which consists of an independent network of ganglia largely responsible for neurogenic contractions of the gut wall. The myenteric plexus also relays

neural signals from the sympathetic and parasympathetic nervous systems. Then a layer of smooth longitudinal muscle is present to allow for peristaltic type contractions. The final layer is the serosa, which covers the longitudinal muscle layers and also the inner circular muscle.

1.3 Enteric Nervous System

Motility and secretory functions in the small and large bowel that are neurogenic in origin are coordinated almost exclusively by the enteric nervous system. There are two main branching nerve plexuses in the wall of the gut. These are known as the myenteric plexus, sometimes referred to as Auerbach's plexus, and the submucous plexus, sometimes referred to as Meissner's plexus.

The gastrointestinal tract is unique in that it contains its own population of intrinsic sensory neurons. These are independent of the vagal and spinal afferents. Vagal afferents have their cell bodies in nodose and jugular ganglia and are largely non-noiciceptive sensory nerves [130]. In contrast, spinal afferents with cell bodies in dorsal root ganglia (DRG) are the major sensory neuron responsible for pain perception in mammals [139]

1.3.1 Myenteric Plexus

The myenteric plexus (located between circular and longitudinal muscles layers) in the gut wall, is a continuous network of nerve fibres and interconnected ganglia that extend across the full circumference of the bowel and along the full length of the GI-tract, including parts of the oesophagus. It receives a rich neural innervation from the parasympathetic and sympathetic nervous system which can modulate contractions and relaxations in the surrounding smooth muscle layers. The network also contains interneurons and intrinsic sensory neurons [59].

1.3.2 Submucous Plexus

The submucous plexus lies between the submucosa and circular muscle layers of the gut, and is mainly involved in absorption and secretion [59]. Found within this network are enteric neurons, spinal afferents, and parasympathetic and sympathetic efferent fibres, however the nerve bundles of the submucous plexus are much finer than those seen in the myenteric plexus.

1.4 Different morphological types of myenteric neurons

There have been many functional studies which demonstrate there are several physiologically different types of neurons in the enteric nervous system of mammal [82,120]. The main divisions being those which inhibit and excite motor neurons to the muscle (i.e. inhibitory and excitatory motor neurons), vasomotor neurons, secretomotor neurons, interneurones and sensory neurones [61]. The first description of the different morphologies of myenteric neuron was made by Dogiel [50], who proposed three categories (I, II and III) of neuron classification. Type I (Figure 1A) are flatter, with broad, flat dendrites which did not extend far from the body, and a single axon. Dogiel type II (Figure 1B) neurons are described as having oval shaped bodies, with many long axes and some shorter processes. Dogiel type III (Figure 1C) neurons have shorter dendrites than type II, which become thinner and branch as they move away from the cell body [59].

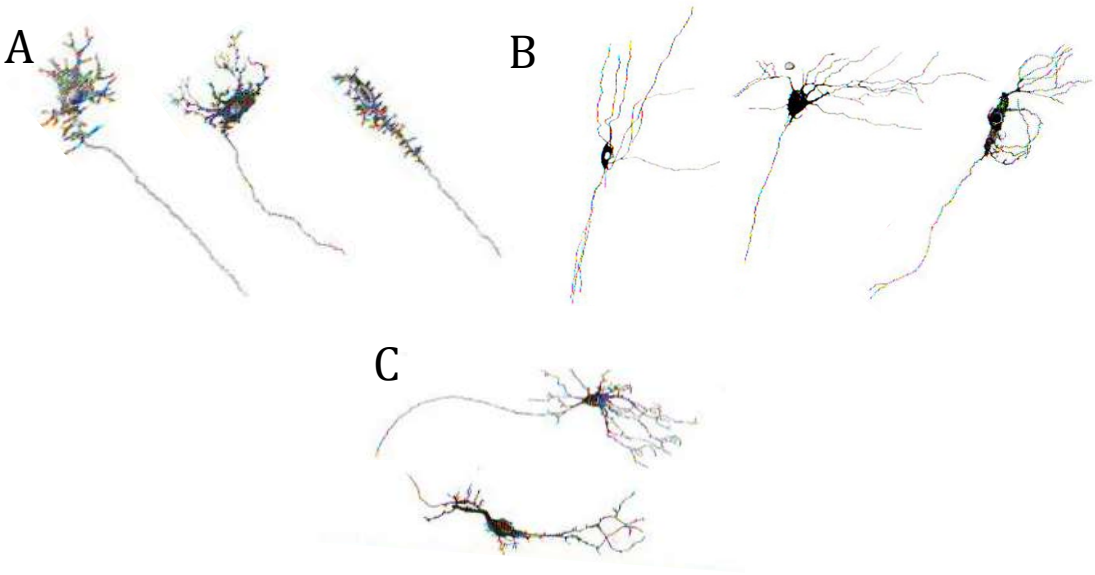


Figure 1. (modified from [59]) Dogiel classified neurons as drawn by Dogiel in 1899. A, Examples of Dogiel type I neurons taken from myenteric plexus of guinea-pig small intestine. B, Dogiel type II neurons, first drawing from the myenteric plexus of human small intestine, and second two from myenteric plexus of guinea-pig small intestine. C, Dogiel type III neurons from myenteric plexus of guinea-pig large intestine.

As electrophysiological techniques were developed and improved, neurons were able to be classified using this method. Nishi & North [120] first classified neurons into type 1 and 2 on the basis of their firing properties, followed closely by Hirst *et al.* [82] who are more widely quoted for their division of myenteric neurons of the guinea pig duodenum into two groups based on synaptic inputs and the after-hyperpolarisation characteristics following an action potential. These two groups were given the names S cells and AH cells. S cells had a large synaptic input, and were able to fire multiple, prominent, fast excitatory synaptic potentials (fESPS) when stimulated with maintained depolarising current pulses. AH cells had a prolonged after-hyperpolarisation after an action potential was fired in response to a depolarising current, and they lacked fESPS and the synaptic input which the S cells displayed.

Development of intracellular dye injections allowed for electrophysiological properties to be correlated with earlier morphology classification [21]. Multiple studies [20,96,98] further extended the work of Hodgkiss & Lees [83] to show that S neurons were predominantly uniaxonal, with smaller cell bodies, and several short lamellar processes. In contrast, AH neurones were almost all multiaxonal, with large, smooth cell bodies and short processes. These descriptions matched almost perfectly the morphological descriptions by Dogiel [50], and so it was concluded that S neurons were in fact Dogiel type I neurons, and AH neurons were Dogiel type II.

1.5 Mediators of Excitatory post-synaptic potentials

1.5.1 Acetylcholine

Acetylcholine (ACh) is the primary excitatory neurotransmitter in the enteric nervous system, which seems to be remarkably well conserved in this role, across all mammalian species [59]. ACh is synthesised from the two precursors acetyl-CoA and choline inside the neural cell body and terminal axon. Acetyl-CoA is the major product of protein and lipid catabolism in the body and choline is obtained from breakdown of choline containing phospholipids, or intracellular uptake [162]. After production, it is stored in vesicles inside the nerve varicosities before being released in response to a nerve impulse, via exocytosis from enteric nerves which innervate both the longitudinal and circular muscles of the intestine [72]. The release of ACh from neurons in the gastrointestinal tract was first discovered by Dale and Feldberg [46] who found that stimulation of the vagus nerve caused release of acetylcholine into the stomach. ACh was later deemed the primary excitatory transmitter after it was shown that excitatory neuromuscular transmission could be blocked by muscarinic antagonists such as atropine, and also, excitatory muscle transmission is increased by agents that block the acetylcholine digesting enzyme, acetylcholinesterase (AChE) [1,124]. On a larger scale, this means that antagonists of

muscle acetylcholine receptors are able to block muscle contraction during the peristaltic reflex, and also halt motility and propulsion of content [57]. There has been some evidence for failure of muscarinic antagonists to block transmission *in vivo* [2,19], however given these studies were done in a complete functioning body system, it follows that there would be quicker metabolism of the muscarinic antagonists meaning they were unable to have the usual strong blocking effect on the action of acetylcholine.

1.5.2 Tachykinins

Tachykinins are a family of endogenous peptides which act as excitatory neurotransmitters in the mammalian gastrointestinal tract. The very first of the tachykinins to be discovered was the peptide Substance P [157] and this remained the sole known transmitter of this family until about 1985, after which more began to be identified (such as neurokinin A and neurokinin B), and since this time they have all been collectively referred to as tachykinins [107]. The expression of their G-protein coupled receptors, NK1, NK2 and NK3 [125] is extensive in the GI tract. Here the peptides are able to bind to the receptors to modulate enteric secretomotor functions, inflammatory and immune responses and visceral sensitivity [94].

It has been shown that tachykinin NK2 receptors play an important part in the non-adrenergic, non-cholinergic excitatory transmission for the intestinal smooth muscle [107]. Tachykinins are often co-localised with ACh [125]. Whilst ACh is the primary excitatory neurotransmitter in the gut, tachykinins have been found to be very significant in terms of atropine-resistant peristalsis. It has been shown that antagonists of NK1 and NK2 receptors were able to block peristalsis in the presence of atropine (a muscarinic antagonist) [111].

1.5.3 Serotonin

Serotonin, or 5-Hydroxytryptamine (5-HT) is a monoamine neurotransmitter. It is synthesized predominantly in enterochromaffin cells (EC cell; a type of enteroendocrine cell) in the intestinal mucosa. Much smaller quantities of 5-HT are synthesized by enteric neurons, about 1% of neurons in the ENS [44,67]. 5-HT is believed to be used as a signalling molecule in many systems including the brain and the periphery, however, in comparison to how much is present in the body, the brain contains relatively little compared to the gut [165]. In fact, EC cells in the gut produce around 95% of the entire body's serotonin.

Originally, it was proposed in the laboratory of Professor Edith Büllbring [28] that serotonin played a major role in the initiation of intestinal peristalsis. This was based on a number of circumstantial findings that included the fact that high concentrations of endogenous serotonin were released upon stimulation of the mucosa and that endogenous 5-HT release was temporally correlated with the initiation of peristaltic contractions. Based on these observations, it seemed logical conclusion that 5-HT was indeed involved in the generation of peristalsis. The following decades of study only seemed to confirm the initial conclusions that serotonin was vital for peristalsis. Experiments showed that the application of agonists and antagonists of specific 5-HT receptors could potently inhibit peristalsis, and the fact that there are many serotonin receptors present in the intestinal walls, all seemed to support Büllbring's initial finding. However, recent studies have provided a spectacular revision of the hypothesis proposed by Büllbring and colleagues. New studies now show that if serotonin release from the mucosa was prevented, by removing the entire mucosa, peristalsis still occurred [148].

A more recent paper by Li *et al*. [108] used knockout mice which had been genetically modified to remove the rate limiting enzyme for mucosal serotonin synthesis. These mice

were found to be the same as wild type for all parameters measured, including gastric emptying, intestinal transit and colonic motility. This suggested that the role mucosal serotonin plays in GI motility must be very small if any at all.

1.5.4 ATP

ATP is able to act as both an excitatory and inhibitory transmitter in the enteric nervous system[61]. It can be released from neurones as an excitatory neuro-neuronal transmitter and can be released from damaged tissue in an unregulated manner, or it can be released in a controlled fashion from a non-neuronal origin to activate sensory neurons [170]. ATP, which is structurally classified as a purine, is involved in what colloquially is known as purinergic neurotransmission. It acts predominantly on P2X receptors (a ligand-gated ion channel) and P2Y (metabotropic) receptors in the gut [64] playing a major role in different gut activities [33]. Whether ATP acts as an excitatory or inhibitory neurotransmitter in the gut depends on the receptor and its location. P2X receptors found on longitudinal smooth muscle, have an excitatory role, as they do when found on the neurons (both pre- and postsynaptically). P2Y receptors on the circular smooth muscle have an inhibitory function, however the same receptor type on pre- and postsynaptic neurons has a mainly excitatory function [18].

Evidence suggests that the action of ATP on P2X receptors on myenteric neurons is responsible for fast excitatory post synaptic potentials (fEPSPs). This was demonstrated by the inhibition of hexamethonium-resistant fast EPSPs by P2 receptor antagonists in the guinea pig ileum [63]. It has also been suggested that slow EPSPs are blocked by a purine receptor agonist [22], and other research has suggested that the exact receptor was most likely P2Y [68,99].

There is also much evidence to support the occurrence of inhibitory transmission mediated by ATP on P2Y receptors on circular muscle in the gastrointestinal system. There have

been studies showing that inhibitory transmission to circular muscle is mediated by ATP, acting in concert with nitric oxide (an inhibitory transmitter, discussed in detail later) and neuropeptides such as vasoactive intestinal peptide (VIP) [45]. Studies in mouse colon have supported this [140]. The notion that ATP underlies the fast inhibitory junction potential (IJP) in mouse colon circular muscle is supported by mice in which the P2Y1 receptor is genetically ablated, but the slow IJP was preserved [62]. Apamin, a drug which suppresses ATP-mediated inhibitory mechanisms by blocking apamin-sensitive potassium channels, can be used to demonstrate the inhibitory function of ATP in normal peristalsis. When apamin was administered to block ATP-mediated inhibitory transmission, even though it would be expected that the descending inhibitory reflex would be blocked (resulting in blocking of peristalsis), peristalsis was increased unless both apamin and a NOS inhibitor (blocks nitric oxide synthesis) were administered concurrently [87,160]. The apamin-sensitive and NO-mediated components of the relaxation in peristalsis are both independent of each other and additive [12].

1.5 Non adrenergic, non cholinergic inhibitory neurotransmission (NANC)

Inhibitory neurons of the gut are neither adrenergic (using adrenaline/noradrenaline), nor cholinergic (using acetylcholine) [110]. This was discovered with the use of drugs which act to block sympathetic and cholinergic neurotransmission, but it was found that the inhibitory neuronal activity in the gut remained unaffected. This was a large discovery because up until this time, these two major pathways were thought to control all neurotransmission in the gut [43]. Another discovery to further support the NANC transmission findings was that inhibitory junction potentials persisted even when atropine, which blocks muscarinic transmission, and guanethidine (which blocks sympathetic transmission) were present [32]. These findings ignited an extensive series of experiments to identify that nature of the inhibitory neurotransmitters. It was found that NANC

inhibitory transmission was indeed present in various regions throughout the GI tract and in other species including rat, guinea-pig and rabbit [31,58].

1.6.1 Nitric Oxide (NO)

Nitric oxide is one of the major inhibitory neurotransmitters in the gastrointestinal tract. Its primary function in the gut is to relax smooth muscle. It is a unique neurotransmitter because it is made as it is required (as opposed to being manufactured and stored in vesicles until needed) by the enzyme nitric oxide synthase (NOS). It is made from L-arginine, and exists in its steady state in gaseous form, which is quite unusual for a neurochemical transmitter. The function of NOS as an inhibitory transmitter has been demonstrated through studies showing that it is present in inhibitory neurons in the GI tract, and that inhibitory neurotransmission is decreased (increased excitability) in different areas of the GI tract when drugs are used to block its production [168].

In mouse distal colon, NO is one of the major NANC inhibitory transmitters in the longitudinal muscle layer, and has recently also been confirmed as being one of the main NANC inhibitory transmitters in the circular muscle layer [121]. Also, inhibitory junction potentials in the circular muscle layer of mouse colon involve NO [140]. NO will be studied and discussed in greater detail in the experimental chapters of this thesis, discussed later.

1.6.2 Vasoactive intestinal peptide (VIP)

VIP is an amino acid peptide found in motor neurons that inhibit the muscle in the intestine. It is one of the multiple transmitters of the inhibitory neurons, meaning if one is deficient, the others can help to compensate, as demonstrated earlier between ATP and NO. VIP antiserum is able to stop neutrally induced relaxation in the gut [6,9] and it has been used to show that neurons releasing VIP are involved in the regulation of descending

relaxation [70]. VIP is also able to be found using immunohistochemical techniques [27] and myenteric neurons containing VIP have also been found to project from the intestines to the prevertebral sympathetic ganglia, and may be involved in intestino-intestinal reflexes [42] (the reflex that stops intestinal activity elsewhere when a part of the gut becomes overdistended).

1.7 Other transmitters

1.7.1 CGRP

CGRP is an endogenous peptide that is a potent vasodilator [163], and commonly synthesised in sensory neurons of dorsal root ganglion (DRG). It is found all through the spinal cords of species including man, marmoset, horse, pig, cat, guinea pig, mouse, rat and frog [69]. It is also present in many neurons throughout the intestines of mammals, where it has many important functions, such as its role in regulation of blood flow (gastric and mesenteric) and control of gastric acid secretion. It is able to have an effect on intestinal motility [86], demonstrated by studies showing that it has an effect on smooth muscle cells, causing a decrease in motility of the gut [10,100,112]. CGRP is also able to stimulate enteric neurons to release excitatory transmitters such as acetylcholine and other inhibitory mediators [85,114], which have shown to act together in the guinea-pig ileum longitudinal muscle to cause biphasic changes in tone and contractility [11].

1.8 Sympathetic Innervation of the gastrointestinal tract

The extrinsic innervation of the gastrointestinal system comes from the thoracolumbar region of the spine, and is mediated mainly via noradrenaline. The fibres from this region of the spine form connections with three main prevertebral ganglia, and paravertebral ganglia. The three prevertebral sympathetic ganglia involved are the celiac, superior mesenteric ganglion (SMG), and inferior mesenteric ganglion (IMG), all of which are

located close to the abdominal aorta and have synaptic inputs from enteric sensory afferents in the colon [36]. These ganglia not only innervate the gastrointestinal tract, but also provide input into other visceral organs. The celiac ganglion provides input into the lower oesophageal sphincter [14,92], and also, along with the SMG, provides input to the stomach, small intestine, caecum, proximal large intestine, liver, pancreas and kidneys [65,171]. The IMG sends input to the distal colon, interal anal sphincter and rectum [59,109]. Neurons which project from the IMG to the colon are thought to primarily inhibit motility [36]. Cells in the celiac, SMG and IMG not only provide input, but also receive signals from colonic mechanoreceptors. The celiac ganglion receives input from mechanosensory neurons in the orad colon, and, conversely, the IMG receives input from these neurons in the caudad colon. The SMG, which is physically located in between the other two prevertebral sympathetic ganglia collects information from both the orad and caudad colon [152].

1.9 Parasympathetic Innervation of the gastrointestinal tract

The parasympathetic innervation to the upper and mid GI-tract is primarily provided by the vagus nerve. The vagal efferent fibres are located in two nuclei in the brainstem, the dorsomotor vagal nucleus and the nucleus ambiguous. The pelvic nerves are also responsible for providing some efferent parasympathetic innervation from the sacral spinal cord. The distal regions of the colon and rectum are innervated by the sacral nerve roots which come from the lumbosacral spinal cord. It is generally thought that the effect of the parasympathetic stimulation on the gut is excitatory, causing an increase in gastrointestinal motility. The vagus nerve plays a major role sensory transduction from the periphery to central nervous system.

1.10 Different Motility Patterns in the Large Intestine

1.10.1 Peristalsis

Peristalsis is a coordinated contraction and relaxation of the smooth muscle layers in the GI-tract that propel contents primarily in an aboral direction. The smooth muscles ahead of the bolus relax to allow movement through the tube, while the muscles layer behind the bolus contract leading to propulsion of contents aborally. The peristaltic reflex was first described by Bayliss and Starling [15] in the small intestine of the dog, where they described the motor pattern which was dependent on the local nervous system, and able to propel a bolus using the above described muscle contraction and relaxations. Since then, the peristaltic reflex has been described in numerous mammalian species in both the small and large intestine.

It is now well known that for peristalsis to occur, intrinsic ascending excitatory neural pathways must be activated, in concert with descending inhibitory pathways [160]. The peristaltic motor pattern has been well demonstrated in numerous *in vitro* experiments from a variety of mammals [29,41,145]. One of the early studies to fully demonstrate peristalsis in vitro was an experiment showing propulsion of a pellet in guinea colon by Costa & Furness [41]. They showed that a bolus inserted into the guinea pig colon was able to be propelled in an aboral direction in response to the stretch (distension) induced by the bolus, and the subsequent activation of the excitatory and inhibitory pathways. This reliable phenomenon has since been repeated in countless studies to further investigate the pathways, mechanisms and neurotransmitters responsible for gut propulsion. In the colon of mice, calcitonin gene related peptide (CGRP) has been shown to be released from intrinsic sensory neurons in the large intestine and this may play a major role in activating the intrinsic circuits underlying peristalsis [71]

1.10.2 Colonic migrating motor complexes

The colonic migrating motor complex (CMMC) is a cyclical propagating neurogenic contraction that traverses significant distances (typically greater than half the length of the colon) along the large intestine. The CMMC has been shown to occur in a wide variety of mammals, including human [74,149], rat [54], dog ([138], and mouse [25,26,34,35,40,55,133,150,164]. As with other regular motor patterns, it is assumed the generation of CMMCs would be controlled by a pacemaker cell, just like the SA and AV nodes responsible for the generation of a heart muscle contraction. However, the pacemaker cell/pattern generator responsible for the initiation of this cyclical motor pattern is not fully clear. What is clear is that the pattern generator must lie in the myenteric plexus and/or muscularis externa since studies have clearly shown that CMMCs persist when the mucosa and submucous plexus are surgically dissected from the colon [101,169].

Intracellular electrical recordings have been made from circular smooth muscle cells the electrical pattern underlying CMMCs, termed the myoelectric complex (MC) is able to be recorded from circular muscle [37,140]. These recordings have shown that there are two parts to the generation of each MC. One of these components is the rapid oscillation of the membrane potential, and the other is an underlying slow membrane depolarisation. The rapid oscillations can be abolished by the addition of atropine or hyoscine (muscarinic receptor antagonists), indicating they are cholinergic in origin, in contrast to the slow depolarisation which was not affected by these antagonists [37].

Both the rapid oscillations and slow depolarisations are abolished by a sodium channel blocker (such as hexamethonium or tetrodotoxin) showing that they are both neurally mediated.

Both the MC and CMMC are under tonic neurogenic inhibition by ongoing release of nitric oxide (NO) from inhibitory motor neurons. This was demonstrated when tetrodotoxin (TTX) and L-NA (to block NO production) induced a similar level of membrane depolarisation [140-143]. The decrease in tonic inhibition after a NO synthesis inhibitor also caused a substantial increase in CMMC frequency, as NO is normally responsible for maintaining this frequency at a reduced rate [144].

Studies on CMMCs in the human colon have been difficult until relatively recently when samples of human colon became more readily accessible and able to be studied *ex vivo* [150]. Indeed, very short segments (up to 4cm) had been studied by various groups [39,103] with only limited conclusions able to be drawn about the behaviour of the whole colon based on a very small sample. Small segments of bowel are unfortunate, because the integrity of enteric interneurons is compromised, which is now known to be essential for generating and propagating CMMCs in mammals [147]. In more recent times, experiments at Flinders University, in conjunction with Flinders Medical Centre, have been made from significant lengths of human colon *in vitro*. These specimens have recorded rhythmic colonic motor complexes in both “healthy” and slow transit specimens. The frequencies of CMMCs were significantly higher in the *ex vivo* experiments than those completed *in vivo* manometry recordings [149], suggesting the extrinsic neural innervation may be rather important for modulating the CMMC frequency.

Studies investigating CMMC activity have usually been made from whole segments of empty colon suspended in an oxygenated bath of krebs. The common feature amongst all of these studies is that some form of mechanical stimulation was always applied to the gut in order to record the contractions occurring [26,34,128,133,150]. These studies, which showed that CMMCs occurred consistently with a regular interval in an empty colon, allowed the conclusion to be drawn that the complexes did not require content in order to

occur. In terms of efficiency of energy expenditure, there was no clear explanation as to why the colon would coordinate these large contractions (requiring a large amount of energy) if there was no content to propel. It was suspected that perhaps the external recording apparatus being applied to the gut wall was perhaps inducing the CMMCs in an empty colon. The main types of external stimulus being applied to the gut wall in past *in vitro* experiments were in the form of metal spring clips, which pinched the bowel [34,55,150] or distension of both the oral and anal ends of the gut to mount the colon and secure in the organ bath [26,34,128,150]. The other factor which may have induced CMMCs when they would not have otherwise been present is the resting tension/stretch which is applied to the colonic wall in order to obtain mechanical recordings.

1.10.3 Mass movements

Mass movements are propulsive contractions that traverse large segments of colon and are thought to propel content rapidly over these large distances. They were first reported by Hertz [80], and shortly after, reported by Holznecht [88] who recorded these large contractions only twice in over 1000 human studies. It wasn't until the late 1980s when manometric techniques were developed for full 24 hour studies in humans that the high amplitude contractions which propagated at about 1cm/sec were able to be recorded more frequently [116].

1.10.4 High amplitude propagating contractions

High amplitude propagating contractions (HAPC) are able to move colonic contents long distances, often leading to defecation [13]. They can occur spontaneously in response to specific agonists or luminal distenstion of the colonic wall. Most of the HAPCs are initiated in the proximal colon, and typically do not propagate further than the mid colon, and less than 5% propagate all the way to the rectum [7]. They have been shown to occur more

frequently just after waking up, are more frequent during the day and as one might predict, they do increase in frequency after meals [116]. Interestingly in patients with slow transit constipation, these HAPC have been show to decrease [49,75] , and conversely in patients with diarrhoea predominant irritable bowel syndrome (IBS-d) the contractions are increased [77]. This may provide an explanation for the abnormal colonic transit in people with these conditions.

1.11 Myogenic factors in gastrointestinal motility

1.11.1 Smooth muscles in the gastrointestinal tract

Smooth muscle cells are responsible for generating contractile activity of the musculature along the entire gastrointestinal tract except for the upper part of the oesophagus, which is skeletal muscle. Smooth muscles do not work in quite the same way as skeletal muscle, in that the activity seen in the gut is coordinated by an internal pacemaker, so smooth muscle contraction in the gut is driven intrinsically by this. The pacemaker responsible for this is the interstitial cells of Cajal (ICC) which will be discussed in detail below. The membrane potentials of each individual cell depolarise and repolarise in a phasic manner, and each time a depolarisation reaches threshold potential, a contraction will occur. At each point along the gastrointestinal tract, the contractions can vary in frequency and amplitude. These contractions which occur repeatedly, with quite a long build-up phase and contraction process are called slow waves [24,122] and are thought to be regulated by the interstitial cells of Cajal (ICC).

1.11.2 Interstitial cells of Cajal (ICC)

There are multiple classes of Interstitial cells of Cajal (ICC) in the gastrointestinal tract. ICC at the level of the myenteric plexus (ICC-MY) have been confirmed to be the intestinal pacemaker cells that underlie electrical rhythmicity [48] and are responsible for the phasic

myogenic contractions of smooth muscles in the gut wall [153]. These cells generate electrical activity which causes slow waves [93,154], which then cause phasic contractions to occur. They act in a manner similar to the sino-atrial node in the heart, generating electrical rhythmicity.

These cells were named after Santiago Ramon y Cajal, who first discussed these interstitial cells in 1889. Cajal focussed much attention on these cells during his career. However, it is still debated as to exactly who "discovered" the cells first, because the techniques were not available at the time to properly characterise them and clearly claim the discovery. It was observed by Keith in 1914 and 1915, the presence of cells which were connected with muscle cells, but had processes similar to ganglion cells. He postulated that they were similar to the nodal tissue in the heart, which we now know to be true [102].

It was only towards the end of the last century that patch clamp studies were able to show that if smooth muscle cells were studied in isolation, slow waves did not occur [53]. It was also shown that when ICC's were isolated, the muscle depolarised spontaneously in much the same way as slow waves occur [106].

There are three main groups of ICC which help to regulate gastrointestinal motility and are named according to location. ICC in the myenteric plexus are ICC_{MY}, those in the circular muscle layer (intramuscular) are ICC_{IM}, and those in the submucous plexus are called ICC_{SM}. [61]. All of these cells couple via gap junctions with other ICCs and also with the smooth muscle cells, which enables signal conduction.

A major step forwards in characterising the classes of ICC in the gut wall, was the discovery that ICCs express the tyrosine kinase receptor, Kit [93]. This allowed for an antibody against Kit (c-Kit) to be designed which would allow staining for the location of these cells in tissue.

Using the methods of staining for ICC using c-Kit, it was found that in Hirschsprung's disease (discussed earlier), that the networks of these cells were disrupted in the aganglionic regions of the gut [156], which would provide an explanation for the lack of spontaneous contractile activity in patients with Hirschsprung's disease, and further confirm the role and importance of ICC. Another report however, showed no difference between the networks of ICC in aganglionic compared to ganglionic segments of colon [89], instead concluding that there are natural regional differences even in healthy human colon tissue. This was confirmed by Ward *et al.* [159], who showed that the distribution of ICC in ls/ls mouse colon was no different to that of a wild-type mouse, even though the electrical activity was altered in the ls/ls mouse colonic smooth muscle.

The intramuscular ICC have also been shown to respond to neural inputs. There are receptors for both nitric oxide [166] and tachnykinins [127] on the ICCs, and it has been demonstrated that excitatory neural input can increase the amplitude of slow waves. This leads to an increase in calcium influx into the smooth muscles and subsequent stronger contractions [79].

1.12 Motility of Small Intestine

There are two major distinct motor patterns present in the small intestine of mammals. These are known as segmentation or segmental-type contractions, which are a phasic type of mixing movement, and peristaltic or propagating neurogenic contractions. These propagate aborally for short distances [72,73]. The movement of small intestine in many species is dependent on whether the animal is in a fed or fasted state. During the fasted state in some species, a migrating myoelectric complex (MMC) develops which is a pattern of electrical response activity. The MMC was first reported and described in dogs by Szurszewski [151]. There were four distinct phases of the MMC recognised, Phase I which

is relatively inactive (lasting 30-60 minutes), II showing irregular contractions (30-60 minutes), III which is intense contractile activity (4-7 minutes) and IV which was a final phase of silent behaviour. The origin of these motor patterns was shown to be mediated intrinsically via cholinergic transmission, and could be modified to some degree by extrinsic nerves or hormones [137].

When animals were fed a meal, the MMC pattern disappears and is replaced by segmentation patterns long the whole length of small intestine, which are thought to aid in mixing of digestive enzymes and juices [151]. Whilst the MMC was observed in some species only during fasting, and was disrupted by feeding, it was found to occur in ruminants at all times regardless of whether they were feeding or not [135,136]. A similar finding was made for guinea-pig by Nakajima *et al.* [115], where an *in vivo* study showed that MMCs were present in both fed and fasted states.

1.13 Motor activities recorded from the isolated mouse colon

Colonic migrating motor complexes (CMMC) are a neurally driven motor pattern which was first reported by Wood in [164] using extracellular electrodes to occur reliably every two minutes. Since they propagate over distances, it is thought that their main function is to propel intraluminal content; however this is not yet fully established. Since the landmark study by Wood [164] was conducted in the piebald-lethal mouse, the CMMC has been reported by others in the piebald lethal mouse strain [37,55,56] and also in other strains of mice including C57BL/6 [34,35], and C57BL/10snJ [113]. It was long accepted that CMMCs occurred at a relatively constant frequency of one complex every 1-3 minutes [26,34,35,55,134,150]. This seemed rather high for an empty segment of bowel, if the primary function of CMMCs is to propel content. The high CMMC frequency that occurs in an empty colon irrespective of the presence of intraluminal content, seems to contradict

the normal priority of the body to conserve energy. This thesis will further explore the frequency of these CMMCs and whether certain *in vitro* experimental techniques have led to a misrepresentation of actual intrinsic frequency and propagation velocities that occur in truly unstimulated segments of bowel.

1.14 In vitro techniques for measuring colon activity

1.14.1 Spatiotemporal mapping

One of the commonly used modern techniques for measuring colonic motility is the use of a video system which converts colonic movements into a spatiotemporal map. It was developed in the late 1990s [17,78] and is an elegant way to represent the changes in diameter of the bowel wall at a given point in time. It involves taking a short video (usually 0-10minutes in length) of the intestine in an organ bath, after which, each time point in the video can be converted into greyscale pixel representation of the entire colon, black representing the largest diameter and white the smallest diameter, with varying shades of grey in between. Each time frame within the video was treated this same way to create a diameter map of the colon over the time of the video. Figure 2 below is from Hennig *et al.*[78]. It shows how a frame of the video in (A) was first converted to a black and white only image in (B), and just above (B), how this was then converted to a single line of greyscale pixels corresponding to smaller diameters of the gut (white) and larger diameters (darker grey/black). (C) Shows how these pixelated representations of diameter for various points in time on the video are then converted to a whole map, with time increasing on the horizontal axis, and colon oral end on the left and anal end on the right. The white dotted line in (C) is to show where the line in panel (B) has been taken from.

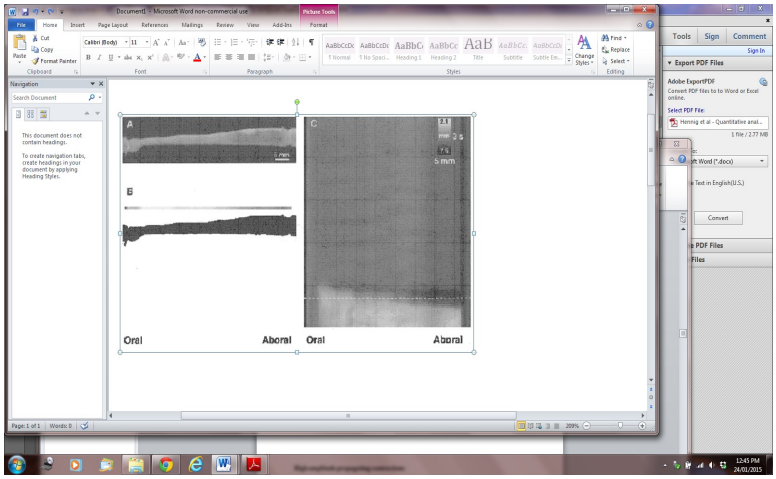


Figure 2. Construction of a diameter map (D-map). A, still frame of time point in video of live colon. B, Conversion of image to black and white, and above, into greyscale representation of diameter of black and white colon. C, Complete D-map with time=0 at the top and increasing horizontally. White dotted line to show where strip in B is taken from.

1.15 Hirschsprung's Disease

Hirschsprung's disease was first described in 1888 by Harald Hirschsprung who had two young patients, both male; die as a result of chronic severe constipation and bowel obstruction [81]. This condition is caused by the absence of myenteric and submucosal ganglia in the distal most portion of the large bowel and rectum [66,118,167]. Unfortunately, Hirschsprung's disease is common, affecting approximately 1:5000 human live births, and is usually diagnosed in the first 48 hours after birth. If there is a delayed passing (more than 24 hours after birth) of meconium (first stool of an infant), further tests will be conducted. Patients can be diagnosed later in life, either in childhood or occasionally in adulthood, with severe constipation and chronic abdominal distension [123].

Hirschsprung's disease occurs more often in males with a male:female ratio of 4:1, and is much more common in patients with Down's syndrome [76] and other congential abnormalities. The rectum and the distal colon are usually the only portions of bowel affected, but in very severe cases it has been reported that the small intestine is also affected [131]. There are four variants which have been described in humans: total colonic aganglionosis (3-8% of cases); total intestinal Hirschsprungs'(involving the entire bowel) [119]; ultra-short segment (only involving the distal rectum and anus) [117]; and suspended Hirschsprungs'(a portion of colon is aganglionic above the normal distal segment).

This condition arises because neuroblast cells (neuron precursors during embryonic development) have failed to migrate in weeks 5-12 of the gestation period [167]. The absence of ganglia in the colon, referred to as aganglionosis, impairs neurogenic propulsive motor patterns which results in a build-up of colonic content, often causing bowel obstruction (called megacolon, shown in figure 3). If the condition is left untreated, megacolon can occur which can result in death from acute enterocolitis (inflammation of the digestive tract). The treatment for this disease is surgical intervention to resect the affected piece of aganglionic tissue and rejoin the normal ganglionic segment to the anus.

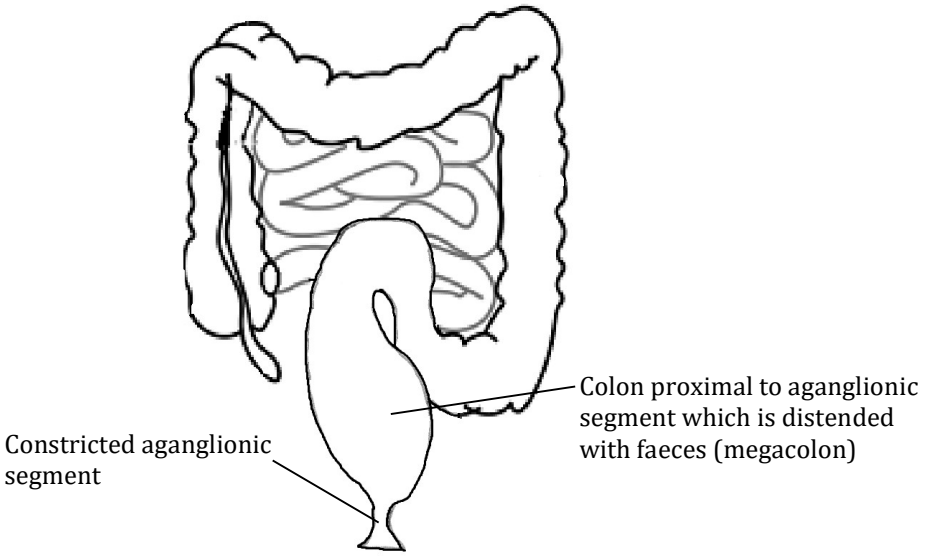


Figure 3. Diagrammatic representation of the likely consequences of colorectal aganglionosis. The aganglionic segment becomes constricted, which blocks passing of faeces, which then builds up in the area immediately proximal to the aganglionosis. This usually causes megacolon, which is commonly treated by removal of the aganglionic constricted segment of colon.

There are a number of different genes that can cause colorectal aganglionosis, some of which include, RET, GDNF, EDNRB, SOX10, and Edn3 [3,16,54,126]. The two major genetic mutations leading to Hirschsprungs' disease in human are RET and EDNRB. The RET signalling pathway involves RET, tyrosine kinase transmembrane receptor, and glial cell line-derived neurotrophic factor (GDNF) as the ligand. Other proteins are also required to allow GDNF to activate RET, and there are various growth factor ligands of RET in addition to GDNF, which have been identified, specific combinations of which are required for the correct development of central and peripheral neurons. The endothelin signalling pathway involves EDNRB and EDNRA, both of which are G-protein coupled receptors that allow signals to be passed through the binding of the endothelin proteins (EDN1, 2 and 3) [95]. Another mutation, much less frequently associated with only Hirschsprungs'

disease (is usually only responsible for megacolon when other disease states are also present) in comparison to the other pathway mutations, is the *SOX10* gene. A homozygous mutation of this gene is lethal in the embryonic stages [105].

It is fortunate that mouse models for all of these genetic mutations have been developed for the specific study of mechanisms underlying Hirschsprung's disease. It is particularly encouraging that the same genetic mutations in mice and humans can often lead to similar phenotypes in humans. These models have been widely used to gain a better understanding of the condition, precisely how they manifest their phenotype; and what might be responsible for variation between affected patients.

The exact mechanism by which Hirschprungs' disease develops at the embryonic stage begins with the migration of neuroblast cells described earlier. The enteric nervous system is primarily formed by these vagal neural crest cell migrating to colonise the gut [30]. Neural crest cells proceed to the foregut around embryonic day (E) 9-9.5 in mice, and at this point are called enteric neural crest-derived cells which are precursors to the enteric nerve and glial cells [66,118,167]. They move rostral to caudal and colonise the whole gut by E15 [51]. The correct movement of these precursors, and consequently the full development of an enteric nervous system is dependent on signalling pathways described earlier, one of which is through the effect of endothelin 3 polypeptide (ET-3) on the endothelin receptor B (EDNRB; a G-protein coupled receptor) [16]. Mutations in genes encoding either the ET-3 ligand or EDNRB receptor can result in varying degrees of aganglionosis in the gut, and also affect epidermal melanocyte development, giving a characteristic spotted coat colour in the mouse [90]. Mutations in the endothelin signalling pathway produce mice which provide a powerful model of human Hirschsprung's disease, since mutations in endothelin signalling also cause colorectal aganglionosis in humans [5].

It is important to note however, that less than 5% of human patients with Hirshsprung's disease have mutations in endothelin signalling pathways [23].

A missense mutation in exon 3 of the Edn3 gene produces a mice strain called *lethal spotted (ls/ls).* Mice which are homozygous for this mutation have spotted piebaldism with only 20-30% of their coat pigmented [90] due to the effect of the malfunctioning endothelin pathway on development of epidermal melanocytes [16]. These *ls/ls* mice have short-segment aganglionosis due to the absence of the ET-3 ligand. Humans with mutations in the same gene also develop short-segment aganglionosis [52,126].

In shorter segment aganglionosis, CMMCs are still able to occur which allow for faecal pellets to still be expelled, and a normal lifespan to be achieved [132]. In mice, it has been found that the degree of aganglionosis depends heavily on the genetic mutation causing the effect, and the background strain of the mouse which has been used [38,158], as discussed further in chapter 3.

A study by Roberts *et al.* [134] on the *ls/ls* mice (mutation in Edn3 gene), showed that CMMC s were absent, which is expected for these mice due to the fact that these particular mutant animals had ~70% aganglionosis along the length of the whole colon. The study presented in Chapter 3 of this thesis shows that despite lacking the same gene, *ls/ls* mice can still generate rhythmic coordinated CMMCs. However, *ls/ls* mice studied in the current thesis consisted of considerably shorter lengths of aganglionosis. This will be discussed in detail later.

Other studies using the *piebald lethal (sl/sl)* strain of mouse, which has a mutation in the endothelin B receptor gene (*ednrb*), have shown that the homozygotes with this mutation typically die within 4-6 weeks after birth [25,104,161,164], however the heterozygotes may live as long as a wild-type mouse [104,161]. It has been suggested that the piebald

heterozygotes which live a normal lifespan, do have some degree of colorectal aganglionosis, but typically do not develop megacolon due to the shorter segment of aganglionosis and the presence of CMMCs [132] .

CHAPTER 2: Factors controlling the intrinsic pacemaker frequency and velocity of colonic migrating motor complexes in mouse

2.1 ABSTRACT

The mechanisms that control the frequency and propagation velocity of colonic migrating motor complexes (CMMCs) in mammals are poorly understood. Previous in vitro studies on whole mouse colon have shown that CMMCs occur frequently (~every 1-3 mins) when the colon is devoid of all fecal content. Consequently, these studies have concluded that the generation of CMMCs and the frequency with which they occur does not require the presence of fecal content in the lumen. However, in these studies, stimuli have always been unavoidably applied to these empty colonic preparations, facilitating recordings of CMMC activity. We tested whether CMMCs still occur in empty whole colonic preparations, but when conventional recording methods are not used. To test this, we used video imaging, but did not utilize standard recording methods. In whole isolated colons containing multiple endogenous fecal pellets, CMMCs occurred frequently (2.2±0.1/min) and propagated at 2.16±0.2mm/sec. Surprisingly, when these preparations had expelled all content, CMMCs were absent in 11/24 preparations. In the remaining preparations, CMMCs occurred rarely (0.16±0.02/min) and at reduced velocities (0.75 ± 0.75 mm/sec), with reduced extent of propagation. When conventional recording techniques were then applied to these empty preparations, CMMC frequency significantly increased, as did the extent of propagation and velocity. We show that in contrast to popular belief, CMMCs either do not occur when the colon is free of luminal contents and they occur at significantly lower frequencies. The results of this series of experiments led

to the belief that previous *in vitro* studies on empty segments of whole mouse colon have consistently demonstrated CMMCs at high frequencies because conventional recording techniques stimulate the colon. The experiments described below reveal that CMMCs are normally absent, or infrequent in an empty colon, but their frequency increases substantially when fecal content is present, or, if in vitro techniques are used that stimulate the intestine.

2.2 INTRODUCTION

Colonic migrating motor complexes (CMMCs) are cyclical contractions of the colonic smooth muscle that propagate over significant distances of large intestine; are dependent upon the enteric nervous system; and are thought to aid in the propulsion of colonic contents. CMMCs have been reported to occur in a variety of mammals, including the human colon both *in vivo* [74] and *in vitro* [40,149]. They also occur reliably in the isolated colon of rat [54] and mouse [25,26,34,35,40,55,133,150,164]. Over the past decade, the mouse colon has proved an ideal model species for recording CMMC activity, at least *in vitro*, because CMMCs occur consistently and rhythmically in the isolated whole colon [26,34,55,133,150]. In isolated whole mouse colon, CMMCs occur every 1-3minutes [26,35,55,134,150]. Although the pacemaker cell(s) responsible for CMMC generation have not been identified, it is clear that the pattern generator underlying CMMC generation and frequency must lie within the myenteric plexus and/or muscularis, since removal of the mucosa and submucosal plexus does not block their generation [101,150,169]. At present, the functional role of CMMCs remains unclear, but it is likely they provide a propulsive force facilitating movement of intraluminal content.

To date, *in vitro* mechanical recordings of mouse CMMC activity have been made from empty segments of whole colon, where the luminal contents have been flushed free [26,34,128,133,150]. It has been presumed that because CMMCs occur reliably and frequently from these empty preparations, that CMMC generation is determined independently of the presence of fecal contents. However, in all these previous studies, some form of external stimulus has always been applied to the gut wall, in order for mechanical recordings of CMMC activity to be made [26,35,55,134,150]. We have suspected that these mechanical recordings may themselves have induced CMMCs in the absence of luminal contents, giving the false impression that CMMCs occur frequently in empty segment of whole colon. The types of *in vitro* stimulation that have been applied to the colon to facilitate mechanical recordings in the past have been in the form of metal spring clips (used to pinch the bowel) see e.g [34,55,150], or simply by distending the oral and anal cut ends of the colon, caused by mounting the preparation into and organ bath [26,34,40,128,150]. Also, all previous mechanical recordings have been made with a degree of resting stretch or tension applied to the colonic wall. This made us speculate that these stimuli may have inadvertently induced, or modified CMMC characteristics in empty segments of large bowel.

In this chapter, a major aim was to determine whether the characteristics of CMMCs reported in previous studies may be modified, or induced, by the recording apparatus that has been traditionally used to detect this motor pattern *in vitro*. To address this question, we have placed the entire isolated mouse colon into an organ bath, but avoided using a conventional organ bath that stimulates the oral and anal ends of the preparation; and also without using any conventional mechanical clips that pinch the bowel, enabling mechanical recordings to be made. Instead of these methods, we

have used video imaging of colonic wall movements, which does not require any contact with the bowel itself. This series of experiments reveals the unexpected observation that CMMCs occur rarely, or not at all, in isolated whole segments of mouse colon that lack luminal contents; and when conventional recording methods are not utilized.

2.3 METHODS

2.3.1 Preparation of Tissues

Adult mice between 30-120 days of age were euthanized by inhalation overdose of isofluorane followed by exsanguination, in a manner approved by the Animal Welfare Committee of Flinders University. The entire colon was removed and placed in a petri dish filled with warm (25˚C - 30˚C) Krebs solution constantly bubbled with carbogen gas (95% O_2/ 5% CO_2). The mesentery was carefully trimmed free and the entire colon, containing natural pellets, was placed in an organ bath at 35.5 ˚C ± 0.5˚C. The whole colon was anchored with a single stainless steel pin (<700µm diameter) at the oral and anal ends which did not interfere with movement of pellets in the lumen. The time taken for removal of colon from animal to the time at which the colon was anchored in the organ bath was <5 minutes.

2.3.2 Video recordings of colonic motility in lumen-free and stimulated segments of isolated intact whole mouse colon

The typical duration of video recordings of expulsion of faecal pellets was up to 30 mins. After recording the expulsion of natural pellets, the colon was gently flushed with 5 ml warm Krebs, and left to equilibrate for 30 mins. This procedure was necessary since not all colonic preparations had fully expelled pellets within the 30 minutes of video recordings. To maintain consistency for comparison between

preparations, it was necessary to fully flush all preparations with warm Krebs to ensure they were all free of luminal contents.

The empty colon was anchored with a single pin at each oral and anal end (see A, preparation 1 Fig.1), and three ten minute video recordings were made. The colon was then either 'stimulated' with conventional clips (usually used to make tension recordings) attached to the external surface of the colon, at the oral and anal ends (See B, preparation 2, Fig.1), or by anchoring the colon in a fluid infusion bath with cotton thread (See C, preparation 3, Fig.1). Ten minute duration video recordings were made of the stimulated colon. A fourth type of 'stimulation' was applied to the colon, in the form of stainless steel micro hooks that were attached through the serosa and musculature of the colon (See D, preparation 4, Fig.1), which were connected to force transducers to measure contractions.

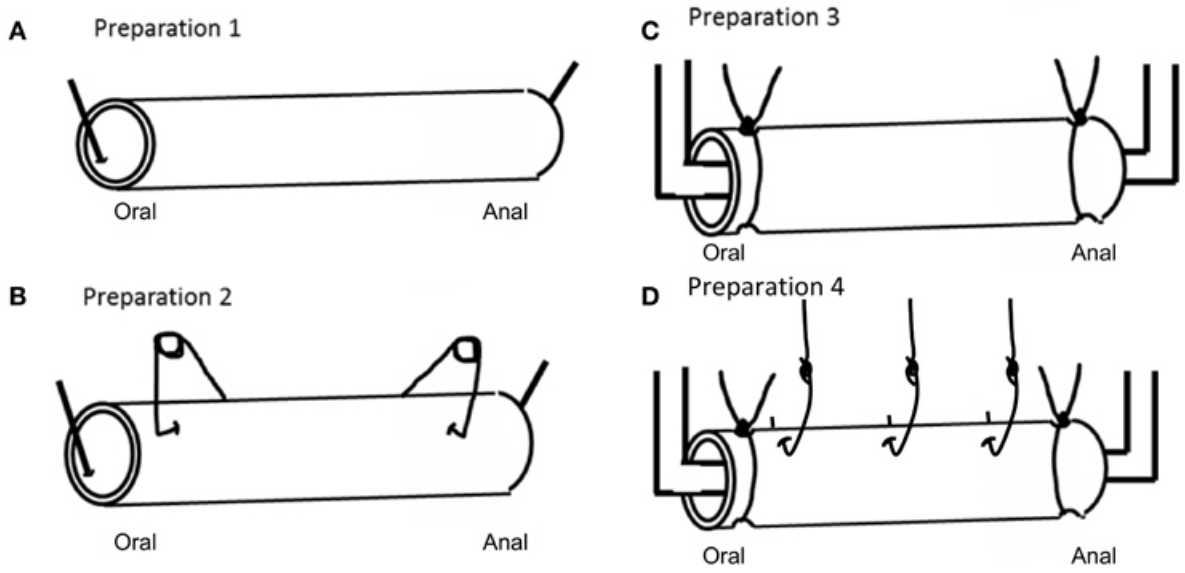


Figure 4. Shows the different types of preparations used to record CMMC mechanical activity in isolated whole segments of mouse colon. A, preparation 1 is a novel preparation, where video recordings are made from the empty whole colon, with only a single pin inserted at proximal and distal end to anchor the preparation to

the colon. No force transducer clips pinch the bowel. B, shows preparation 2, where the colon has force transducer clips attached to the serosal surface, but no resting tension is applied to the colon. C, preparation 3, shows the colon tied to an infusion bath with metal clips to pinch the bowel and 1gm resting tension applied to the colon, via suture thread. D, preparation 4 shows the oral and anal ends of the colon tied (as in preparation 3), but this preparation is also pierced with hooks and attached to force transducer under 1gm resting tension.

2.3.3 Video imaging of CMMCs and generation of spatio-temporal maps

As previously discussed in chapter 1.14.1, colonic wall movements can be represented in the form of a spatiotemporal map. The contractions of the circular muscle of the gut wall were recorded using the Gastrointestinal Motility Monitoring system (GIMM; Med-Associates Inc., Saint Albans, VT, USA). The colon was illuminated from beneath and a digital video camera was used to record the diameter changes in the colon wall. Spatiotemporal maps of the changes in the circumferential diameter (D-maps) were constructed from the digital videos. This was performed using software from the GIMM which converts the video image to a silhouette enabling the number of pixels (shadow caused by colon) to be calculated at each point along the colon, and then represented in the D-map as a greyscale, displaying which regions of the colon were undergoing a diameter change (in mm).

2.3.4 Mechanical recordings of circular muscle contractility during CMMCs

The force generated during each CMMC contraction was recorded using independent isometric transducers (Grass FT-03C; Grass, Quincy, M.A., U.S.A) connected via fine suture to custom made pre-amplifiers (Biomedical engineering, Flinders University) and then to a Powerlab (model: 4/30; AD Instruments, Bella Vista,

N.S.W, Australia). Labchart version 6.0 (AD Instruments, Australia) was used for acquisition and analysis of data. To record circular muscle contractility, approximately 1gm resting tension was applied to preparations of colon at three sites, one site in the proximal colon, one in the mid region and one in the distal colon (as represented by figure 4D). In a second type of preparation, we attached stainless steel spring clips to the bowel wall in the proximal and distal colon, as is represented by Figure 4B. In these preparations, no resting tension was applied to the circular muscle. The aim of this type of preparation was to determine whether the act of simply pinching the colon wall, without any imposed resting tension was sufficient to change CMMC properties.

2.3.5 Drugs and Solutions

The Krebs solution used contained (in mM): NaCl. 118; KCl. 4.7; $NaHPO_4.2H_2O$. 1.0; $NaHCO_3$. 25; $MgCl.6H_2O$. 1.2; D-Glucose. 11; $CaCl_2.2H_2O$. 2.5. Hexamethonium bromide was purchased from Sigma Chemical Co. St. Louis. Mo. U.S.A.

2.3.6 Measurements and Statistics

Spatio-temporal D (diameter)-maps generated from video recordings were used to calculate the number of CMMCs over a ten minute period, including the extent of propagation and velocity (mm/sec) of each contractions. Velocity was calculated using the scale produced with each map (showing distance (mm) and time(s)) to determine the time taken (s) for the full contraction to take place, and over what distance of the colon (mm) it occurred. Data in the results section are presented as means ± S.E.M. The use of "N" in the results section refers to the number of animals

on which observations were made. Statistical analysis of results was conducted using Student's paired *t* tests. P values < 0.05 were taken as being statistically significant.

2.4 RESULTS

2.4.1 General Observations

The entire colon was removed from 24 C57BL/6 control mice. Each preparation was found to have, a mean of 4 discrete fecal pellets within the colonic lumen (range of 1-7, N=24). Once the whole colon, containing pellets had been placed into the organ bath at 35-36^{o}C, it was found that in 5 of these 24 preparations all pellets were naturally expelled within 30 minutes. Three preparations did not expel any pellets from the lumen and in the remaining preparations, some, but not all pellets were propelled along the length of the large intestine, until expulsion from the lumen occurred. All of the 24 preparations contained at least 1 natural pellet at the time of removal of the preparation from the animal. Irrespective of whether all pellets had been naturally expelled from each preparation, all preparations were gently flushed with approximately 5 ml warm Krebs solution.

2.4.2 Expulsion of natural faecal pellets

Using the preparation described in Figure 4A, video imaging was performed on isolated whole colonic preparations that contained multiple natural endogenous fecal pellets. Under these conditions, the whole isolated mouse colon generated CMMCs at an average rate of 2.2 ± 0.1 min^{-1}, where each contraction was initiated in the proximal colon and propelled anally (N = 24). In most cases, each CMMC contraction propagated over fixed pellets; and did not lead to substantial aboral movement of any single pellet along the colon. Figure 5 shows spatiotemporal maps

generated from 4 separate colonic preparations. Figure 5F shows CMMCs that propagate over fixed pellets, but no substantial movement or expulsion of any pellets occurs. It was found that CMMCs traversed an average of 97.4 ± 0.7 % of the length of the colon. The velocity of these individual CMMCs was 2.2 ± 0.2mm/sec (N=24). Hexamethonium (250μM) immediately prevented propulsion of all faecal pellets (N=6), confirming the dependence of enteric synaptic potentials in the generation of each CMMC.

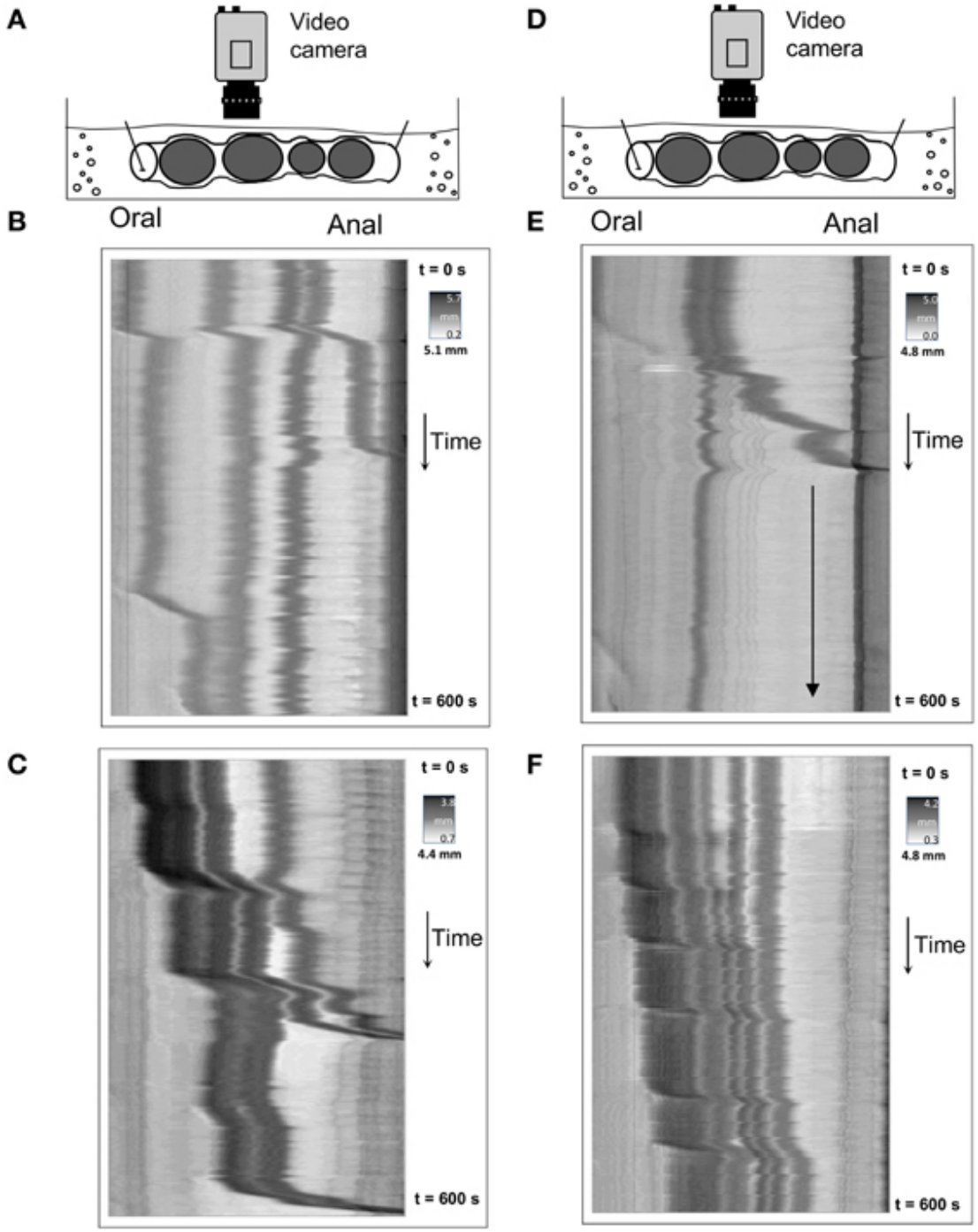


Figure 5 Spatiotemporal maps of colonic circumferential wall diameter in 4 separate mice, showing propulsion of natural pellets in an aboral direction along the full length of isolated colon. A and D are diagrammatic representations of the preparations of colon that contain endogenous fecal pellets and were used to record changes in circumferential diameter by a video camera mounted directly above the preparation. These were then converted to the D-maps as shown in B, C, E & F.

CMMCs occur which induce pellet movement, with some pellets being propelled significant distances aborally. E shows that after a single pellet has been expelled, the colon is empty and becomes relatively silent (indicated from the point at the downward arrow on the map).

2.4.3 Characteristics of CMMCs in empty segments of mouse colon

In the same preparations that demonstrated consistent CMMCs when multiple fecal pellets were present, to our surprise, we were particularly interested in whether CMMCs would still occur when these same preparations were devoid of any fecal content. It was found, that in these empty preparations CMMCs either did not occur, (11/24 preparations) shown in 3E and 3F, or, occurred at a significantly reduced frequency (mean frequency: 0.16 ± 0.02 min^{-1}; N=13) as shown by figure 6B and 6C. Also, interestingly, the extent of propagation of individual CMMCs was significantly decreased in empty preparations, to $48.2 \pm 9.3\%$ of the length of colon. The velocity of these CMMCs also was significantly slower than in full colonic preparations (mean velocity: 0.7 ± 0.1 $mm.s^{-1}$; $P<0.05$; N=13). To test whether CMMCs in an empty intestine were neurogenic in origin, we again applied hexamethonium to the organ bath. In 4/4 empty preparations HEX (250μM) abolished all propulsive CMMCs under these conditions.

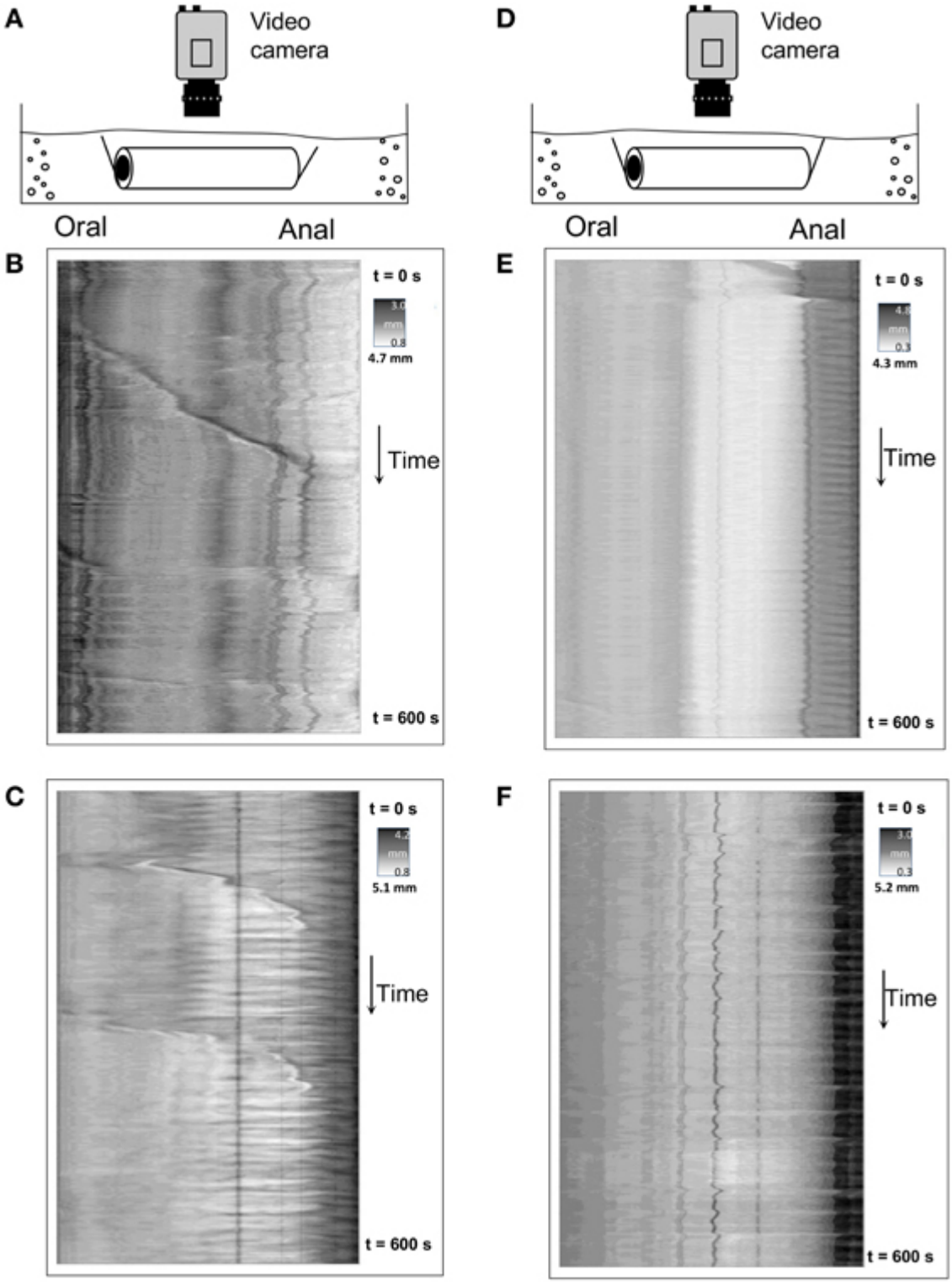


Figure 6 Spatiotemporal maps of colonic wall diameter showing CMMCs in 4 separate empty colonic preparations. A and D are diagrammatic representations of the empty preparations, with the video camera mounted directly above each preparation. B and C show infrequent CMMCs contractions propagating at a slower velocity, that typically do not propagate over the full length of colon. E shows the

absence of rhythmic contractions in an empty colon. F has no obvious CMMCs present.

2.3.4 Do conventional recording techniques modify CMMC frequency and their characteristics of propagation?

Since it was found that CMMCs occurred either significantly less frequently, or not at all, in empty segments of colon (and when conventional mechanical recording techniques were not employed), we were then interested in whether conventional isometric mechanical recording techniques (using force transducers) and an imposed degree of resting tension on the circular muscle, may induce the CMMC motor pattern. To do this, we used different preparations #2, 3, and 4 in Figure 4. Firstly, when empty preparations were stimulated by attaching tension recording clips at two sites along the colon (as in Figure 4B), the frequency of CMMCs and their extent of propagation did not change, however the velocity of propagation significantly increased to 2.15 ± 0.5 $mm.s^{-1}$ (N=5). In a separate cohort of experiments, we cannulated the oral and anal ends of the empty segments of colon after mounted into an organ bath (see Fig.1C). In these experiments, it was found that the frequency of CMMCs remained similar, as did the extent of propagation. However the velocity of CMMCs significantly increased to 2.6 ± 0.68 $mm.s^{-1}$. Another cohort of empty preparations were then studied where the preparations were mounted into the organ bath with oral and anal ends cannulated with suture thread, and then metal hooks attached to the colon for mechanical recordings where 1gm resting tension was applied (as in Fig.4D). In these experiments, the frequency of CMMCs significantly increased to (0.43 ± 0.05 min^{-1}), as did their extent of propagation (87 ± 6.3%) and propagation velocity (4.3 ± 0.7 $mm.sec^{-1}$) in comparison to empty preparations. This implies that the tension and resulting stretching applied to the colonic wall during this recording procedure is a key factor to induce changes in CMMC characteristics.

This notion is best exemplified in Figure 7, where CMMCs are observed expelling natural pellets (Fig. 7A & B), then the relatively quiescent behaviour is observed in the same preparation in the empty state (Fig. 7D). Finally, a pronounced increase in CMMC frequency occurs (Fig.7F) when resting tension is increased by the hooks (Fig. 7E). In 11/11 preparations where CMMCs were evoked by any of the conventional techniques described above using clips or hooks, all CMMC activity was abolished in the presence of HEX (250μM).

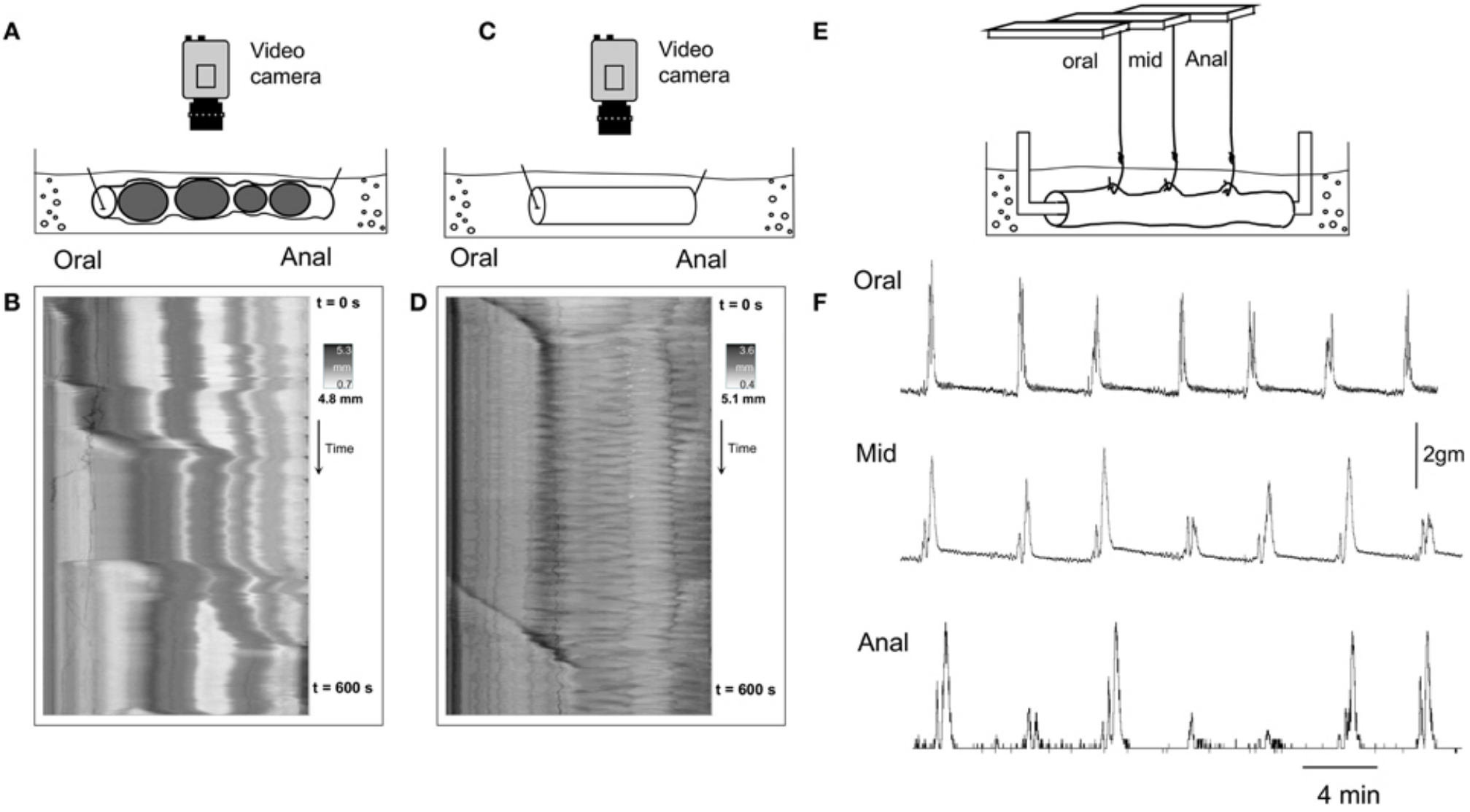

A
Video camera
Oral
Anal
B
t = 0 s
5.3
mm
0.7
4.8 mm
Time
t = 600 s
C
Video camera
Oral
Anal
D
t = 0 s
3.6
mm
0.4
5.1 mm
Time
t = 600 s
E
oral
mid
Anal
F
Oral
Mid
2gm
Anal
4 min

Figure 7 B, D and F are three separate recordings, from the same segment of whole mouse colon, using different recording methodologies. A shows a diagrammatic representation of the preparation from which video recordings were made from a whole colon that contained multiple fecal pellets. B shows the D-map from the same preparation, with CMMCs present that propel a number of pellets aborally. C, shows the preparation from which video recordings were made from the same segment of colon, but devoid of all fecal pellets. D shows a D-map from the same preparation of colon as in panel B, but the colon is now devoid of all fecal pellets. CMMCs are considerably less frequent. E shows a diagram of the preparation but now when isometric force transducers are attached to the circular muscle at three sites along the colon, with 1gm resting tension imposed. F shows that under these recording conditions, CMMCs are now regularly recorded which propagated from oral, to mid, to distal colon.

2.5 DISCUSSION

The major finding of the current study was the unexpected observation that CMMCs occur infrequently, or not at all, in empty segments of whole isolated mouse colon; provided that conventional recording methods were not used. Also, this study revealed that in empty preparations of colon, CMMCs were found to propagate over short distances of bowel and at significantly reduced velocities. This is a major observation which is in direct contrast to the current belief that CMMCs occur frequently in empty segments of isolated whole mouse large intestine, and that they do not require any form of stimulation for their generation. It is important to reconcile the methodological differences between this study and those used in previous studies and why these differences lead to such pronounced changes in CMMC characteristics.

2.5.1 How do the new findings in this study compare with previous studies?

Many studies have consistently demonstrated that CMMCs can be regularly recorded from isolated segments of whole mouse colon that lack any fecal content [26,55,128,134,146,150]. These studies have repeatedly shown that CMMCs occur in empty segments of colon, occurring approximately every 1-3 min; and are dependent upon the enteric nervous system for their generation [26,40,55,129,134,146] The presumption has been therefore that the presence of luminal content (fecal pellets) is not required for CMMC generation. Whilst there has been no doubt that these previous studies lacked luminal contents, there is also no doubt that some form of stimuli has been applied to the colon to enable recordings of CMMC activity. This

has usually been in the form of isometric force transducers that imposed a degree of stretch on the colon and mechanical clips that pinch the intestine, or, simply by mounting the oral and anal ends of the intestine into the organ bath to infuse intraluminal contents. The major difference between the current study and all previous reports of CMMC activity is that in this study we did not use clips to pinch the bowel, nor impose any resting tension on the musculature, nor mount the oral and anal ends of the colon in a conventional recording system for infusing intraluminal fluid. Instead of mounting the colon into an organ bath using conventional anchor points at the oral and anal ends, we used a single pin to anchor the oral and anal ends of the colon to the base of the sylgard lined organ bath. This minimized external stimulation of the preparation.

2.5.2 How do CMMC frequencies differ between preparations that contain multiple fecal pellets and preparations free of luminal contents?

In this study, we observed that in whole colons that contained multiple fecal pellets, CMMCs occurred frequently, with a mean interval of ~30 seconds (2.2 ± 0.1 CMMC.min^{-1}). However, when the same segments of colon were flushed free of all luminal content, CMMCs either did not occur, or, if they were present, occurred significantly less frequently (mean: 0.16 ± 0.02 min^{-1}). It would be a reasonable assumption that the frequency of occurrence of CMMCs would be higher when content is present in the lumen to facilitate the evacuation of content from the colon, and conversely lower in frequency, or absent, when the colon is free of content. In hindsight, it is now clear to us that the reason why CMMCs occurred in our previous

studies at relatively high frequencies (~1-3 minutes), is because the colonic preparations had been stimulated by the methodology used to record CMMCs from these segments of bowel see: [26,55,128,134,146,150]. It is known that the mechanisms by which stretch on the luminal wall increases CMMC frequency is not dependent upon the mucosa or submucosal plexus because circumferential stretch still increases CMMC frequency when the mucosa and submucosal plexus is removed [101,150,169].

2.5.3 Comparison of the extent of propagation of CMMCs in segments of isolated whole colon containing or lacking endogenous fecal content

We were particularly interested in whether the extent of propagation of CMMCs in preparations of colon containing endogenous fecal pellets would show similar characteristics as CMMCs recorded from the same segments of colon when devoid of fecal content. It was found that in whole colons that contained endogenous pellets, CMMCs propagated along the full length of colon. In contrast, in the same preparations of colon that had expelled all content, the extent of propagation of CMMCs decreased to 48% of their original length. In only 1 of 24 empty preparations was a single CMMC recorded that propagated the full length along the colon (proximal to mid to distal). This is in contrast with our previous findings that showed CMMCs propagate along the full length of colon in empty segments of colon, when mechanical clips were used to pinch the bowel enabling tension recordings to be made. Taken together, the presence of spring clips, or hooks that pinch or stimulate the bowel and the imposed resting tension leads to substantial

increases in the extent of propagation of CMMCs along the colon. A previous study by Roberts and colleagues demonstrated that cannulating the mouse colon in an organ bath did not significantly alter CMMC frequency [133]. This is consistent with our study. We found that only when the isometric force transducers were also attached to the colon and 1gm resting tension applied was there a significant increase in CMMC frequency (See Fig.5A).

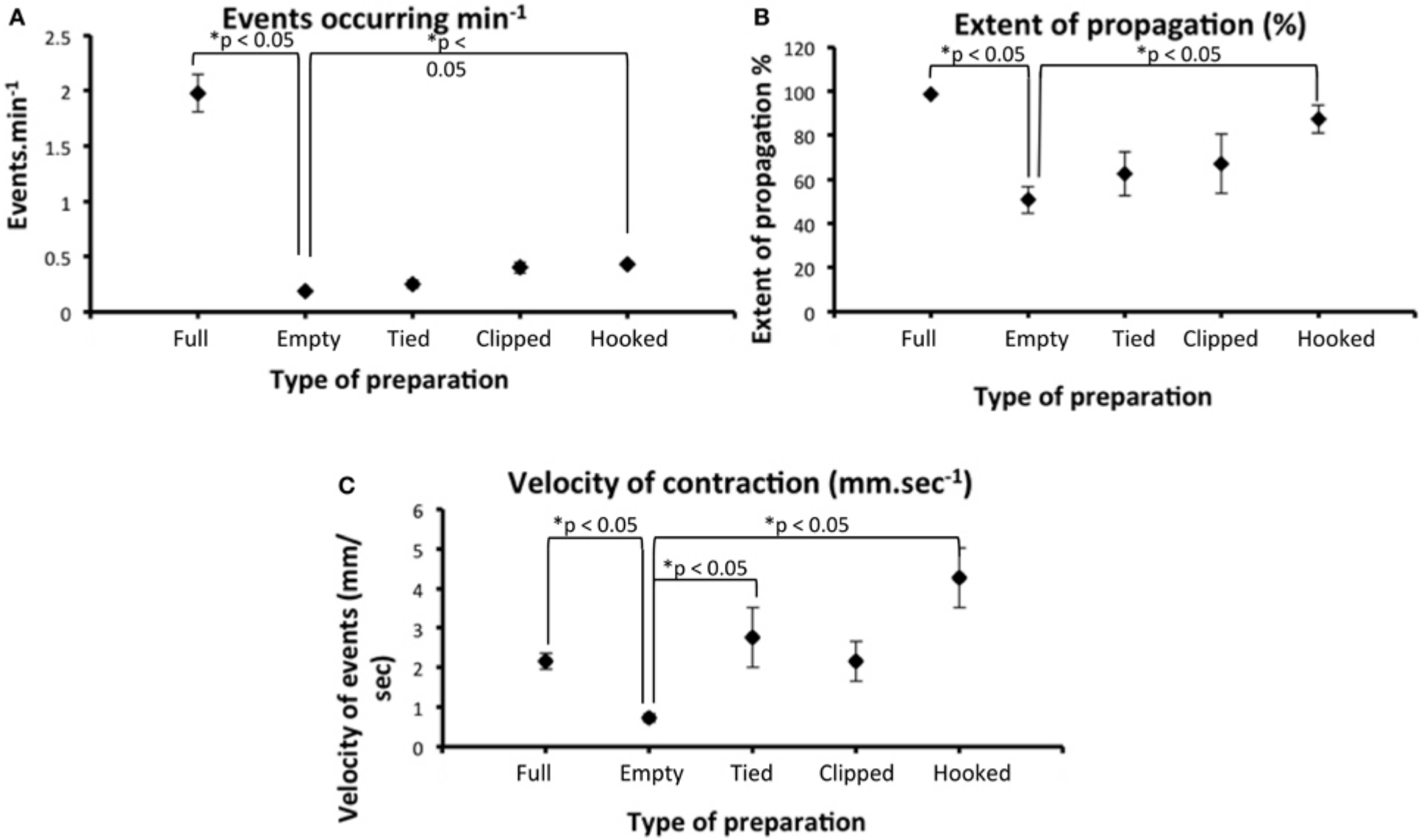

A
Events occurring min-1
*p < 0.05
*p < 0.05
Events.min-1
2.5
2
1.5
1
0.5
0
Full
Empty
Tied
Clipped
Hooked
Type of preparation
B
Extent of propagation (%)
*p < 0.05
*p < 0.05
Extent of propagation %
120
100
80
60
40
20
0
Full
Empty
Tied
Clipped
Hooked
Type of preparation
C
Velocity of contraction (mm.sec-1)
*p < 0.05
*p < 0.05
*p < 0.05
Velocity of events (mm/ sec)
6
5
4
3
2
1
0
Full
Empty
Tied
Clipped
Hooked
Type of preparation

Figure 8 There was a statistically significant reduction in the number of CMMCs that occurred per minute in full segments of colon (that lacked luminal contents) compared with the same segments that contained multiple endogenous pellets. A, empty preparations which had hooks applied and 1gm tension imposed had a significant increase in CMMC frequency (compare Empty data set with Hooked data). "Tied" refers to when the colon is cannulated at the oral and anal ends with suture thread and "clipped" refers to when the preparations have stainless steel clips to pinch the bowel wall (e.g Fig.1B). B, the extent of propagation of individual CMMCs was significantly increased preparations were hooked and resting tension imposed, compared with the same preparations empty. C – There was a statistically significant reduction in the propagation velocity of CMMCs between full and empty preparations. When these same preparations were then stimulated with one of the three conventional techniques, CMMC velocity significantly increased in all cases.

2.6 CONCLUSION

The findings of this study reveal that in the isolated whole mouse colon, CMMCs occur rarely, or not at all, when the colon is devoid of all natural fecal pellets and provided it is not stimulated by conventional recording methods. This study also shows that the frequency, propagation velocity and extent of propagation of CMMCs along the colon is significantly increased when stretch is applied to the colon, by multiple fecal pellets. We suggest that CMMC frequency, velocity and extent of propagation increase when contents are present in the lumen to facilitate evacuation from the large intestine. This provides important insight into how special consideration should be given towards the method used to record motor activity from the isolated gut wall. Until now, it has been assumed that CMMCs occurred reliably approximately every 3 minutes, regardless of content [149]. This study has shown this not to be the case, and therefore will affect every future mechanical recording study.

CHAPTER 3: Characteristics of colonic migrating motor complexes (CMMCs) in mice with colorectal aganglionosis - mutations of the EDN3 gene

3.1 ABSTRACT

In previous studies, mutant mice lacking the endothelin-3 (END3) gene which were raised on a 129SL background strain have been shown to have ~70% colonic aganglionosis, lack CMMCs and are lethal within 12 days postpartum. In contrast, EDN3 mutant mice raised and maintained on a C57BL6 background strain (known as lethal-spotted, ls/ls mice) can live for much longer periods. Experiments in this chapter are designed to investigate whether CMMC generation is preserved in these mice also lacking the EDN3 gene. The aim of this study was to determine whether CMMCs exist in ls/ls mouse colon; and if so, whether their existence and frequency is related to the length of aganglionosis. Spatio-temporal mapping and mechanical recordings of colonic wall movements were made from isolated whole colons obtained from wild type and ls/ls mice. Although ls/ls mice had megacolon, they still generated CMMCs in the ganglionic segment; which on some occasions could propagate short distances into the aganglionic region. There was large variability in the length of aganglionosis, which showed no correlation with the existence or frequency of CMMCs. Interestingly, CMMC propagation velocity was slower in ls/ls mice when evoked by intraluminal fluid. A myogenic motor pattern was identified in the aganglionic region that was maintained under tonic inhibition. Results in this chapter reveal that despite megacolon, ls/ls mice still generate CMMCs in the ganglionic region. These offspring have sufficient propulsive motility in the ganglionic segment to live a normal murine lifespan and rarely die of bowel obstruction.

3.2 INTRODUCTION

Colonic migrating motor complexes are cyclical neurogenic contractions of the large intestine that propagate over significant lengths of bowel [26,34,54,55,133,164] and facilitate the propulsion of colonic content. The mechanisms that underlie CMMC generation are critically dependent upon the enteric nervous system, since nerve conduction antagonists, or hexamethonium prevent their generation [26,101,149]. It is also known that the pattern generator responsible for the cyclical generation of CMMCs lies within the myenteric plexus and/or muscularis externa, because they still occur when the mucosa and submucous plexus are removed from the colon [101,169]. Electrophysiological recordings from the circular muscle have shown that CMMCs involve synchronised firing of enteric cholinergic interneurons and motor neurons that generate rapid cholinergic oscillations and action potentials in smooth muscle membrane potential [147]. Understanding how CMMCs are impaired in mice lacking a variety of genes that are known to induce aganglionosis provides a powerful model of human Hirschsprung's disease and colonic motility deficits. An elegant study by Roberts *et al.* [134] demonstrated that CMMCs were absent in homozygous EDN3 mutant mice. These mice were raised on a 129SL strain, but then subsequently backcrossed for 10 generations to a C57BL/6 strain. These mice had ~70% aganglionosis, lacked CMMCs and were lethal prior to day 12 postpartum [134]. Interestingly, mice with a homozygous mutation for the endothelin-B receptor (ENDRB) gene, known as (piebald-lethal mice) also have black and white coat spotting piebaldism and also completely lack CMMCs, but still have an intact (albeit reduced) myenteric plexus up to the terminal aganglionic segment which is approximately 20mm in length. Piebald-lethal mice also develop severe megacolon and are usually lethal within 12 weeks of birth [132] . Interestingly, heterozygote

littermates of the Piebald-lethal strain rarely die of megacolon, still generate CMMCs but have a shorter aganglionic segment compared to the homozygote littermates [132] . What remains unclear is how and why mutations in endothelin signalling lead to different lengths of aganglionosis and the degree of impairment to colonic motility. Understanding what causes differences in the lengths of aganglionosis can be a major determinant of the functional consequences to the lifespan of the mammal.

The enteric nervous system is primarily formed by vagal neural crest cell migration colonising the gut [30]. Neural crest cells proceed to the foregut around embryonic day (E) 9-9.5 in mice, and at this point are called enteric neural crest-derived cells which are precursors to the enteric nerve and glial cells [66,118,167]. These move rostral to caudal and colonise the whole gut by E15 [51]. The correct movement of these precursors, and consequently the full development of an enteric nervous system is dependent on several signalling pathways, one of which is through the effect of endothelin 3 polypeptide on the endothelin receptor B (EDNRB; a G-protein coupled receptor) [16]. Importantly, endothelin-3 synthesis from the precursor requires endothelin converting enzyme 1 (ECE1). Mutations in genes required for development of either the ET-3 ligand or EDNRB receptor can result in varying degrees of aganglionosis in the gut, and also affect epidermal melanocyte development, giving a characteristic spotted coat colour [90]. Mutations in the endothelin signalling pathway produce mice which provide a powerful model of human Hirschsprung's disease, since mutations in endothelin signalling also cause colorectal aganglionosis in humans [5]

Hirschsprung's disease occurs in 1:5000 human live births, and is caused by a lack of enteric neurons in the distal portion of the colon [66,118,167]. The absence of

ganglia in the colorectum, known as aganglionosis, leads to impaired neurogenic propulsive motor patterns, causing a build-up of colonic content, often leading to bowel obstruction termed megacolon. There are a number of different genes that can cause colorectal aganglionosis, some of which include, RET, GDNF, EDNRB, SOX10, and EDN3 [4,16,52,126].

Studies have shown that a missense mutation of exon 3 in the EDN3 gene produces a strain of mice which are given the name *lethal spotted* (ls/ls). Mice homozygous for this mutation also have spotted piebaldism with only 20-30% of body surface pigmented [90] as a result of the effect on epidermal melanocytes [16]. These mice have short-segment aganglionosis as a result of the absence of the ET-3 ligand. Humans with mutations in the same gene also develop short-segment aganglionosis [52,84].

In this study, we have investigated the colonic motor activity of homozygous ls/ls mutant mice in vitro. The aim was to determine whether CMMCs can occur in the ganglionic segment of ls/ls mice (raised on a C57BL/6 background) and if so, whether there is a correlation between CMMC frequency and the length of colorectal aganglionosis.

3.3 METHODS

Adult control C57BL/6 mice 30-180 days of age were euthanized by inhalation overdose of isofluorane followed by exsanguination, in a manner approved by the Animal Welfare Committee of Flinders University. Female and male littermates with a homozygous mutation of the EDN3 gene (Stock #000262; Strain name: LS/LeJ) were purchased from The Jackson Laboratory, Maine, 04609, USA. These rodents, commonly referred to as lethal spotted (ls/ls) mice were raised and maintained on a

C57BL6 strain. Only homozygote (male and female) offspring were generated by breeding age-matched male and female homozygotes. All ls/ls mice were studied within the first 30-150 days of age and were readily identified by their black and white spotting coat colour (figure 9G).

3.3.1 Tissue preparation

Following euthanasia, a midline laparotomy was made and the entire colon removed, then placed in a petri dish containing warm (25˚C - 30˚C) Krebs solution that was constantly bubbled with carbogen gas (95% O_2/ 5% CO_2). The mesentery was carefully trimmed free and the entire colon, containing natural pellets, was placed in an organ bath at 35 ± 1˚C. The whole colon was anchored with a single stainless steel pin (<700μm diameter) at the oral and anal ends which did not interfere with movement of pellets in the lumen. The time taken for removal of the colon from animal to the time at which the colon was anchored in the organ bath was typically <5 minutes.

3.3.2 Mechanical recordings of circular muscle contractility during CMMCs

We recorded the force generated during each CMMC contraction, using independent isometric recording transducers (Grass (FT-03C; Grass, Quincy, M.A., U.S.A) connected via fine suture. Each force transducer was connected to three custom made preamplifiers (Biomedical engineering, Flinders University) and then to a Powerlab (model: 4/30; AD Instruments, Bella Vista, N.S.W, Australia). Labchart version 6.0 (AD Instruments, Australia) was used for acquisition and analysis of data. To record circular muscle contractility 1gm of resting tension was applied to preparations of colon at three sites, one site in the proximal colon, one in the mid region and one in the distal colon (Figure 7E).

3.3.3 Video imaging of CMMCs and generation of spatio-temporal maps

As discussed in Section 1.14.1, propagating contractions of the circular muscle underlying each CMMC were recorded using the Gastrointestinal Motility Monitoring system (GIMM; Med-Associates Inc., Saint Albans, VT, USA). The colon was illuminated from beneath and a digital video camera was used to record the diameter changes in the colon wall. Spatiotemporal maps of the changes in the circumferential diameter (D-maps) were constructed from digital video recordings, whereby GIMM software converts the video image to a silhouette enabling the number of pixels (shadow caused by colon) to be calculated at each point along the colon, and then represented in the D-map as a greyscale, displaying which regions of the colon were undergoing a diameter change (in mm). A D-map is shown with the distance of colon on the horizontal axis (oral end on left, anal end on right), and time on the vertical axis. The darker the area is on the map, the larger the diameter of the colon was at that point in time. A dark line as though drawn with a marker usually represents a natural pellet in the colon, and these can be seen moving along the length and sometimes being ejected at the anal end. Figure 9C shows where a pellet is moving, and also demonstrates what a CMMC movement looks like on the D-map. The typical duration of video recordings of expulsion of natural faecal pellets was 30 minutes. After expulsion/movement of natural pellets had been recorded, the colon was gently flushed with 5 ml warm Krebs, and left to equilibrate for 30 mins. This procedure was necessary since most colon preparations did not fully expel all natural faecal pellets within the 30 minutes of video recordings. To maintain consistency, preparations that did fully empty were also flushed with warm Krebs. The empty colon was attached with a single pin at both the oral and anal ends and a 10 minute video recording of colonic wall movements (with regards to intraluminal movement

of fecal pellets) was made. The colon was then mounted in a fluid infusion bath anchored at the oral and anal ends with cotton thread. Ten minute video recordings were made of this preparation with fluid infused at a rate of 3 mL/min. The D-maps produced by the infusion recordings were used to calculate CMMC frequency and velocity.

3.3.4 Immunohistochemistry

Whole colonic specimens were fixed overnight in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2 at room temperature. Preparations were then cleared in dimethylsulfoxide (DMSO), followed by rinsing in phosphate buffered saline (PBS, 0.15 M NaCl, pH 7.2), before having the mucosa and some circular muscle removed by dissection. These sections were placed in blocking solution (10mg/ml bovine serum albumin (BSA), normal donkey serum (NDS) and PBS & triton) and then stained overnight for calcitonin gene related peptide (CGRP; IHC 6006, Peninsula Laboratories, IgG raised in rabbit), at a concentration of 1:1600. CGRP was used to stain Dogiel type II intrinsic neurons, and potently label extrinsic nerves [60]. Donkey anti-rabbit CY3 was used as a secondary antibody (catalogue: 711-165-152, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at a concentration of 1:200.

3.3.5 Drugs and Solutions

The Krebs solution used contained (in mM): NaCl. 118; KCl. 4.7; NaHPO4.2H2O. 1.0; NaHCO3. 25; MgCl.6H2O. 1.2; D-Glucose. 11; CaCl2.2H2O. 2.5. Hexamethonium bromide and L-Nitro-L-Arginine (L-NA) were purchased from Sigma Chemical Co. St. Louis. Mo. U.S.A.

3.3.6 Measurements and Statistics

Spatio-temporal D (diameter)-maps, which have been used for over a decade [17,78], were generated from video recordings were used to calculate the number of CMMCs over a ten-minute period, including the extent of propagation and velocity (mm/sec) of each contraction. Velocity of fecal pellet propulsion along the colon was calculated using the scale produced with each map that revealed the distance (mm) and time (s), to determine the time taken (s) for full CMMC contractions to propagate over a known full length of colon (mm). The numbers of ganglia were counted as one straight interconnected direct line across the circumference of the colon. In the proximal colon, the number of ganglia were calculated within the oral 20mm of colon, and for the distal colon calculated within the distal 15mm (precise distances measured for each animal). At the mid colon, the numbers of ganglia were calculated approximately 35-40mm from the anus. The length of aganglionosis was measured from the anal sphincter to the first ganglia along the colon. The first intact circumferential row of myenteric ganglia was recognised as the first complete row of ganglia across the colon. Data in the results section are presented as means ± S.E.M. The use of "n" in the results section refers to the number of animals on which observations were made. Statistical analysis of results was conducted using Student's paired t tests and unpaired t tests where applicable. P values < 0.05 were taken as being statistically significant.

3.4 RESULTS

3.4.1 General Observations

Following euthanasia, the entire colon was removed from a total of 52 mice, which included 20 C57BL/6 mice and 32 ls/ls mice. It was found that despite all ls/ls mice having some degree of megacolon and impaction (figure 9H), ls/ls homozygote offspring rarely died of bowel obstruction or colonic perforation. Upon removal from the animal, control C57BL/6 mice were found to contain a mean number of 3.3 ± 0.5 natural pellets (n=17), which was a statistically significantly less than the mean of 6.5 ± 0.3 natural pellets (n=29) found in ls/ls mice ($p < 0.01$, figure 10A). Although, there was no significant difference between the two types of mice with regards to the number of natural pellets that were expelled within the first thirty minutes (control preparations expelled a mean of 1.9 ± 0.41 pellets n=10, and ls/ls colonic preparations expelled a mean of 1.1 ± 0.2 pellets n=15, figure 10A).

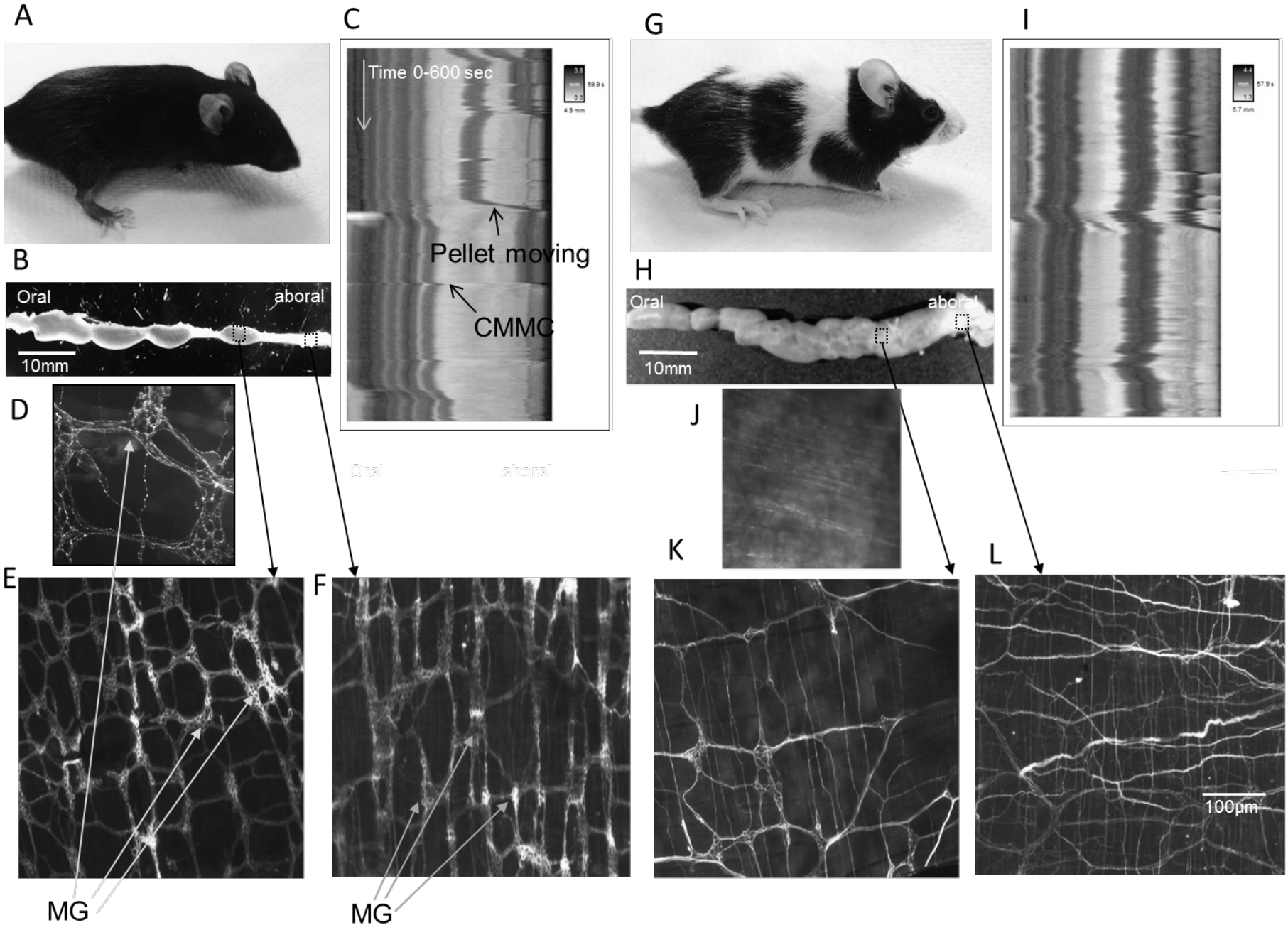
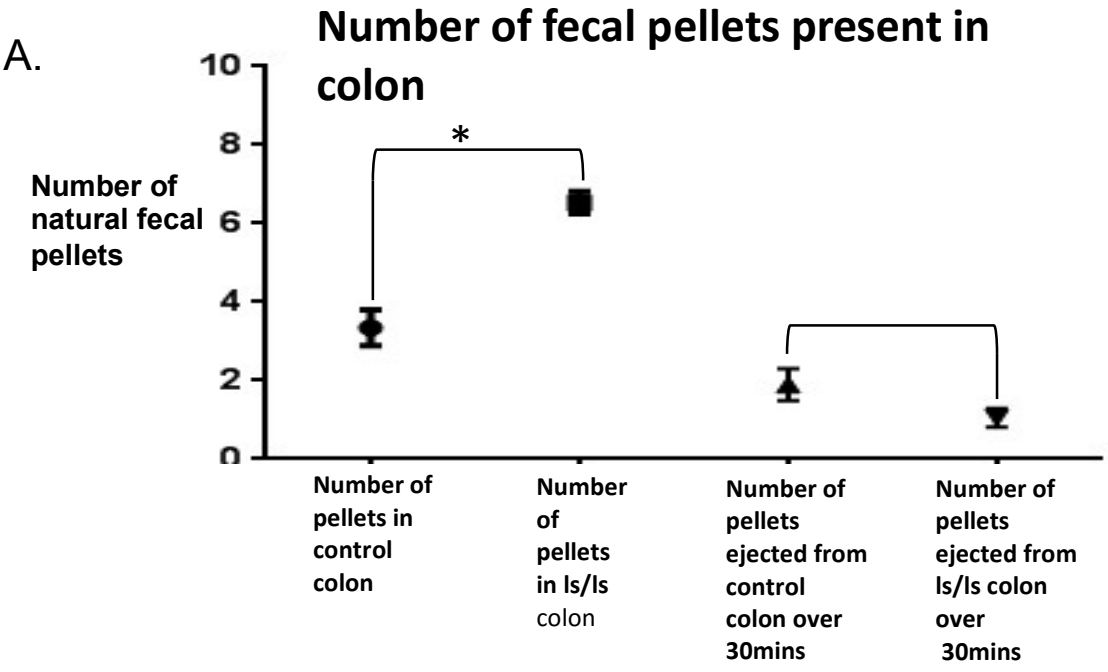

A
B
Oral
aboral
10mm
C
Time 0-600 sec
Pellet moving
CMMC
D
E
F
MG
MG
G
H
Oral
aboral
10mm
I
J
K
L
100μm

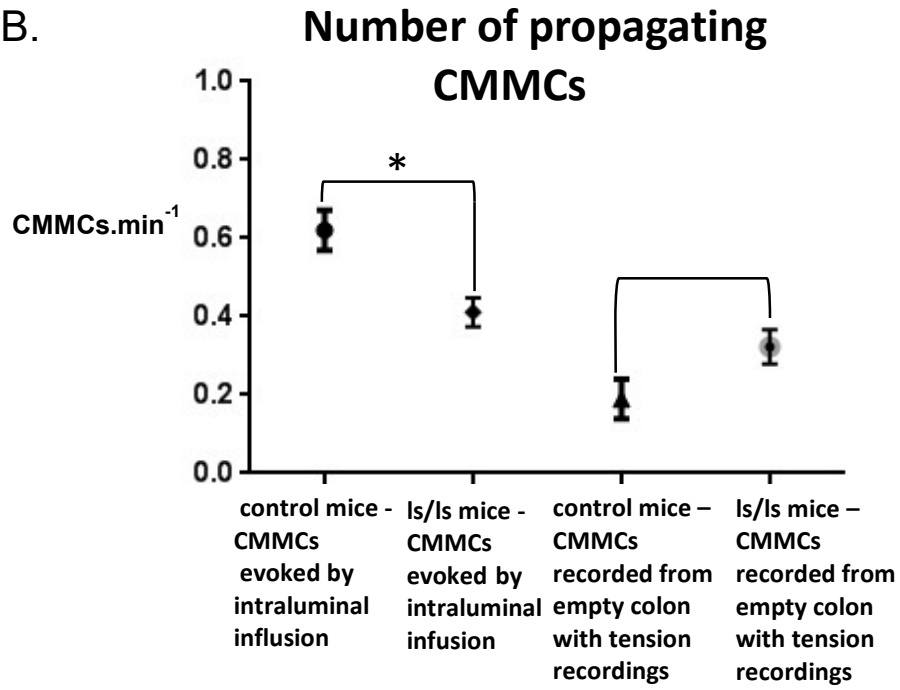
Figure 9.

A, wild type C57BL/6 mouse. B, a full length colon with endogenous pellets and no evidence of impaction or megacolon. C, spatio-temporal map showing natural ejection of fecal pellets. Time scale starts at T=0 sec (top of map) and increases in time are represented downwards toward the bottom of the map. CMMCs are represented by the horizontal lines (whitened regions) across map indicating whole colon movement (see arrows). D. A close up of a myenteric ganglia stained for CGRP. E & F, show an intact myenteric plexus with myenteric ganglia (MG) within 20mm and 3mm of the anal sphincter, as revealed by CGRP immunoreactivity. G, homozygote ls/ls mouse confirmed by spotted coat colour. H, photomicrograph showing megacolon and severely impacted fecal content in an adult mouse approximately 1 year of age. I, Spatio-temporal map from ls/ls mouse whole isolated colon showing that pellets can be ejected and CMMCs can be recorded.J. Close up of a section of aganlionosis showing no myenteric ganglia present (compare to what can be seen in D). K, transition zone of myenteric ganglia and extrinsic nerve fibres without enteric ganglia at a distance of approximately 15mm from the anal sphincter. L, absence of myenteric ganglia within 10mm of the anal sphincter, as revealed by only the presence of dense extrinsic nerve fibres.

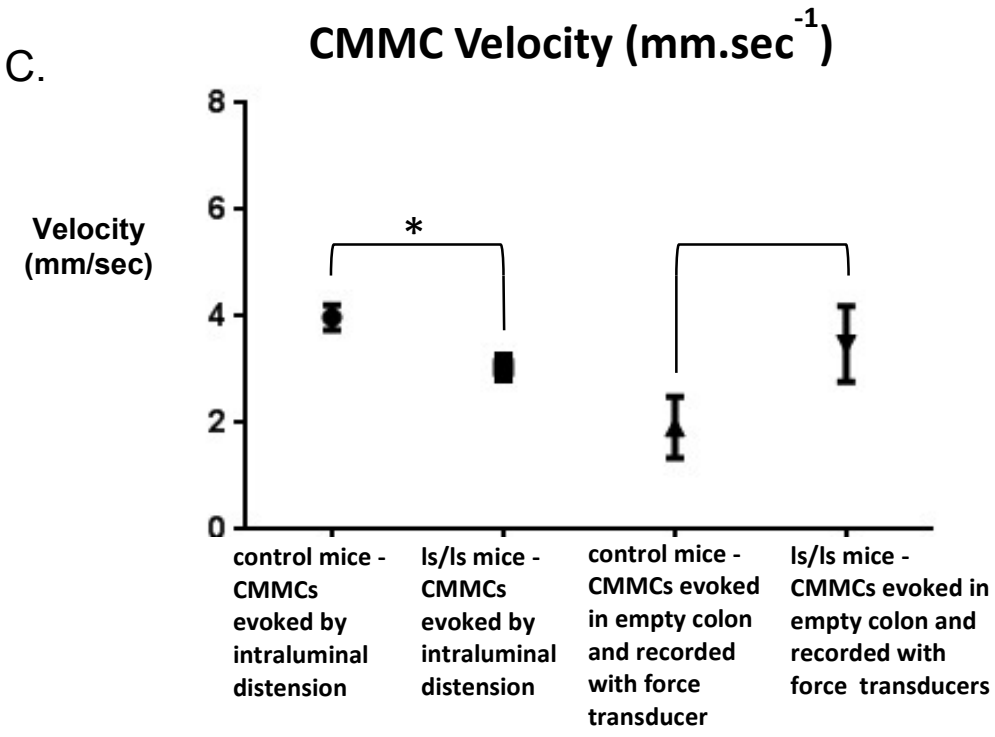
A.


Number of fecal pellets present in colon
Number of natural fecal pellets
10
8
6
4
2
0
*
Number of pellets in control colon
Number of pellets in ls/ls colon
Number of pellets ejected from control colon over 30mins
Number of pellets ejected from ls/ls colon over 30mins

B.


Number of propagating CMMCs
CMMCs.min-1
1.0
0.8
0.6
0.4
0.2
0.0
*
control mice - CMMCs evoked by intraluminal influsion
ls/ls mice - CMMCs evoked by intraluminal infusion
control mice – CMMCs recorded from empty colon with tension recordings
ls/ls mice – CMMCs recorded from empty colon with tension recordings

C.


CMMC Velocity (mm.sec-1)
Velocity (mm/sec)
8
6
4
2
0
*
control mice - CMMCs evoked by intraluminal distension
ls/ls mice - CMMCs evoked by intraluminal distension
control mice - CMMCs evoked in empty colon and recorded with force transducer
ls/ls mice - CMMCs evoked in empty colon and recorded with force transducers

D.

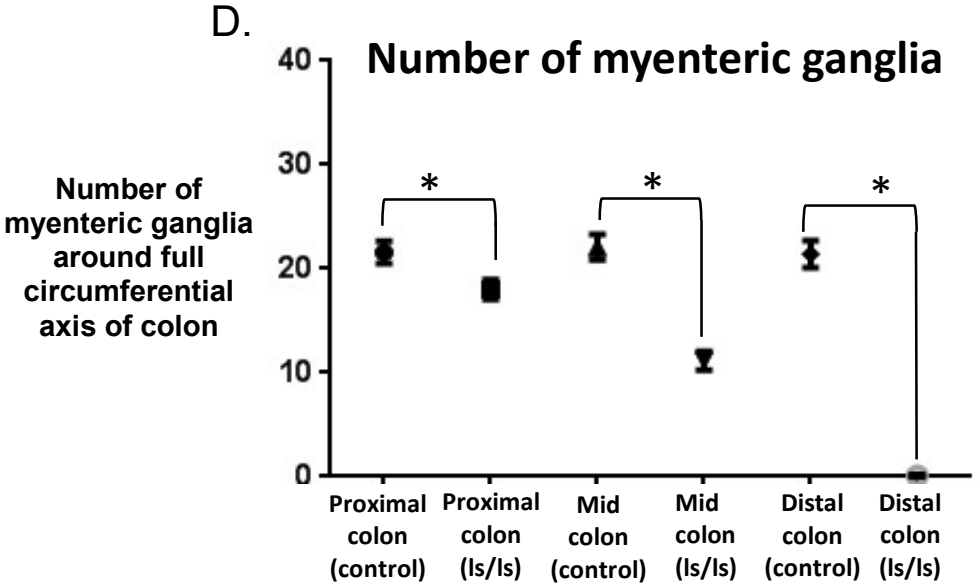
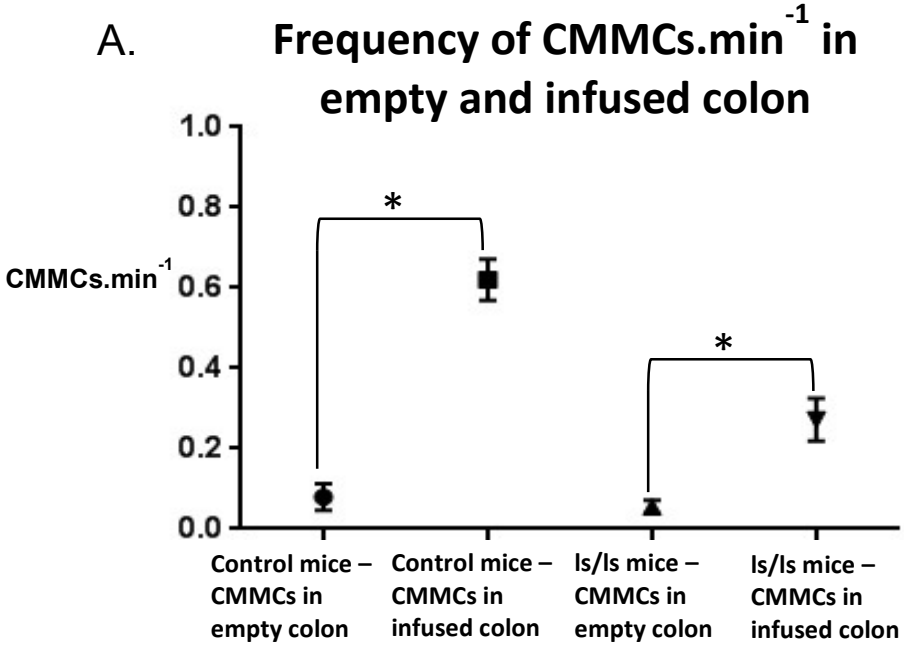

Number of myenteric ganglia
Number of myenteric ganglia around full circumferential axis of colon
40
30
20
10
0
*
*
*
Proximal colon (control)
Proximal colon (ls/ls)
Mid colon (control)
Mid colon (ls/ls)
Distal colon (control)
Distal colon (ls/ls)

Figure 10. Anatomical and physiological differences between ls/ls and wild type mice. A, significantly more fecal pellets present in the large intestine of ls/ls mice compared with controls at the time of euthanasia. No difference in the number of natural pellets ejected from the colon over 30 minutes. B, when intraluminal fluid (35^0C Krebs solution) was infused at the same rate into the proximal region of isolated control and ls/ls colonic preparations, there were significantly less CMMCs than mice that express EDN3. CMMC frequency measured using isometric force transducer showed no significant difference in CMMC frequencies between ls/ls and wild type mice. C, The velocity of CMMCs was significantly slower when evoked by slow influsion of intraluminal fluid. No difference in the velocity of CMMCs when recorded by using isometric force transducers maintained under 1gm resting tension. D, significantly fewer myenteric ganglia in the proximal and mid colon and myenteric ganglia were absent in the distal colon of ls/ls mice compared to controls.

3.4.2 Effects of intraluminal distension on colonic motor activity in control and ls/ls mice

In 10 C57BL/6 control mice, the proximal colon was slowly infused with warm Krebs solution to induce luminal distension, whilst colonic motility was characterised. In all control mice (n=10) propagating CMMCs were recorded from the proximal to distal colon at an average frequency of 0.6 ± 0.05 CMMCs per minute (figure 10B), and with a velocity of 4.0 ± 0.2 mm/sec (figure 10C). Eighteen ls/ls mice preparations were studied. One had no CMMC activity, while in 17/18 of the remaining preparations, CMMCs were recorded. Of these 17 preparations, 12 displayed regular CMMCs that propagated from proximal to distal colon at an average frequency of 0.4 ± 0.04 CMMCs per minute. This was significantly less (33.3%) than CMMC frequency in control mice 0.6 ± 0.05 CMMCs.min^{-1} ($p = 0.01$; figure 10). CMMCs in ls/ls mice had an average velocity of 3.0 ± 0.2 mm/sec, which

was significantly less (25% less) than the CMMC velocity in control mice (n=12) 4.0 ± 0.2 mm/sec (p = 0.01). Further analysis demonstrated that infusion of fluid (and therefore distension of the colon) caused the control colons to have an increase in CMMC frequency and velocity that was significantly higher than the increases seen in the ls/ls mouse colons in response to infusion. Figure 11A & 11B show the increases seen for each.



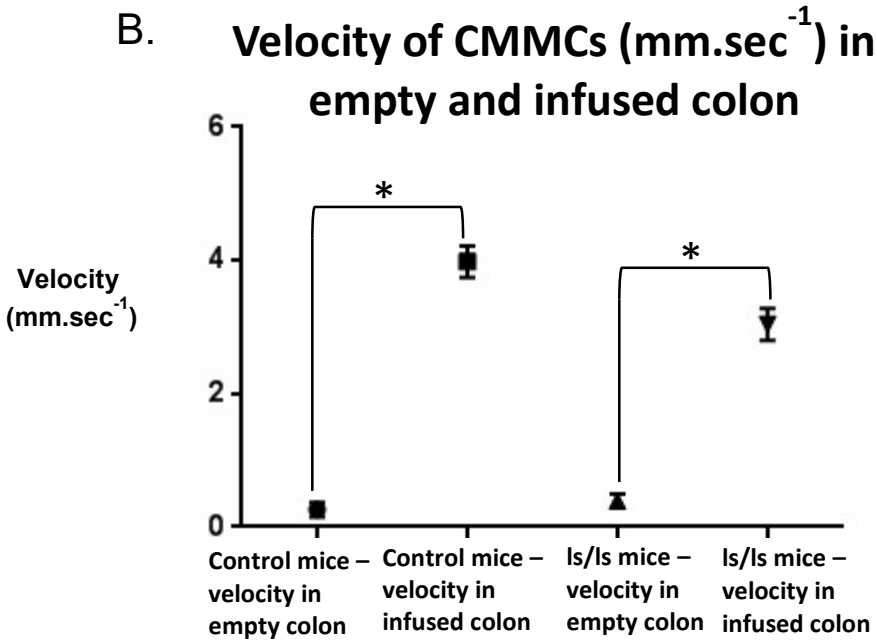


Figure 11. A, In control mouse colon, CMMC frequency was increased by infusion of Krebs. The frequency of CMMCs in an infused colon was 775% that of the frequency of CMMCs in an empty colon. The frequency of CMMCs in ls/ls mouse was also increased by infusion, where it was 490% that of the frequency in empty colon. 3B, In control mouse colon, CMMC velocity was increased to 915% of the empty value, by infusion of Krebs. The velocity of CMMCs in ls/ls mouse colon was also increased to be 517% that of an empty colon.

3.4.3 Mechanical recordings from wild type and ls/ls mouse colon

Eight control full length colonic preparations were mounted in the organ bath with isometric force transducers set to 1g resting tension (Figure 4D). Figure 7E shows the type of proximal, mid and distal recording that was taken from this set up and Figure 9K shows the lack of ganglia in the distal portion of the gut. It was found that

in 7 out of 8 control mice CMMCs were consistently generated which propagated the full length of colon with a mean frequency of 0.2 ± 0.05 per minute, and a mean velocity of 1.9 ± 0.6 mm/sec (n=7) (figure 11). We then tested ls/ls mice under the same recording conditions. It was found that 11 out of 13 ls/ls mice generated CMMCs, and surprisingly, in 5 of these animals, CMMCs were actually found to propagate short distances into the aganglionic segment of distal colon. However, under no circumstances were CMMCs ever initiated in the aganglionic segment. In the remaining 6 animals, CMMCs were present in the proximal and mid sections, but only phasic contractions were observed in the distal colon. The mean CMMC frequency was 0.32 ± 0.04 per minute; at a velocity of 3.5 ± 0.7 mm/sec. Neither the frequency of CMMCs nor the velocity with which they propagated along the colon were significantly different between control or ls/ls mice.

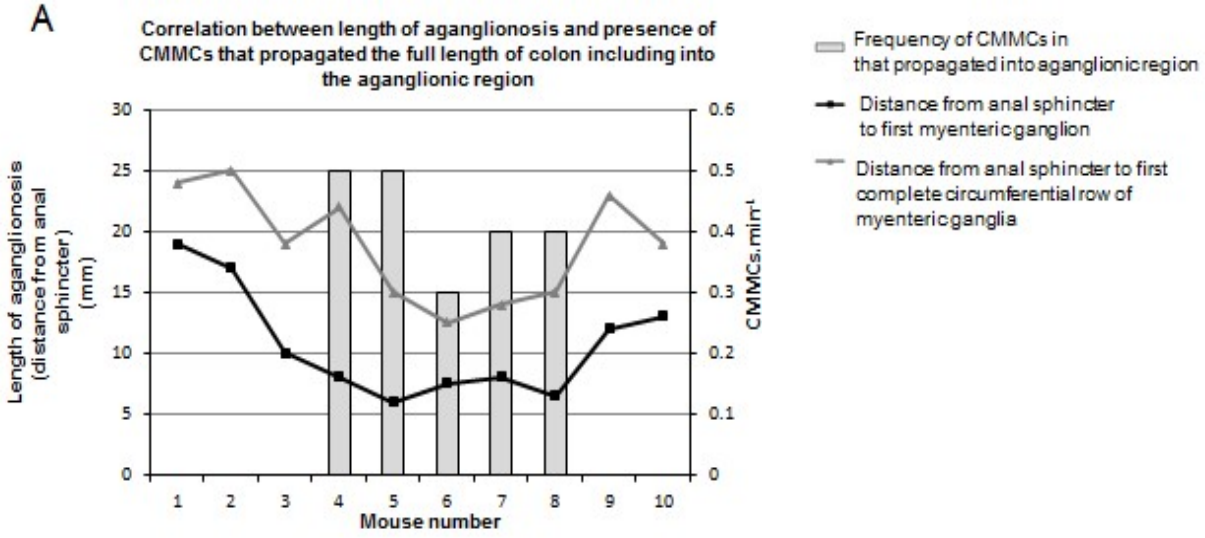
3.4.4 Immunohistochemical staining of myenteric ganglia

The presence of an intact network of myenteric ganglia along the full length colon was determined by staining preparations for CGRP (figure 9), which reliably labelled internodal strands and allowed us to ascertain the transition zone and aganglionic region far more robustly than with standard neuronal markers such as PGP9.5, which only label cell bodies. In twelve control full length colonic preparations and eighteen ls/ls colons, CGRP immunoreactivity was determined to characterize the number of myenteric ganglia that developed around the full circumference of the colon (colon cut longitudinally and flattened, and ganglia counted from one side to the other. Full length colons obtained from control mice had a mean number of 21.6 ± 1.1 ganglia around the circumference of proximal colon, 22.1 ± 1.2 ganglia around the circumference of mid colon, and 21.4 ± 1.3 ganglia around the circumference of distal colon. Ls/ls mice had a significantly reduced number of myenteric ganglia for

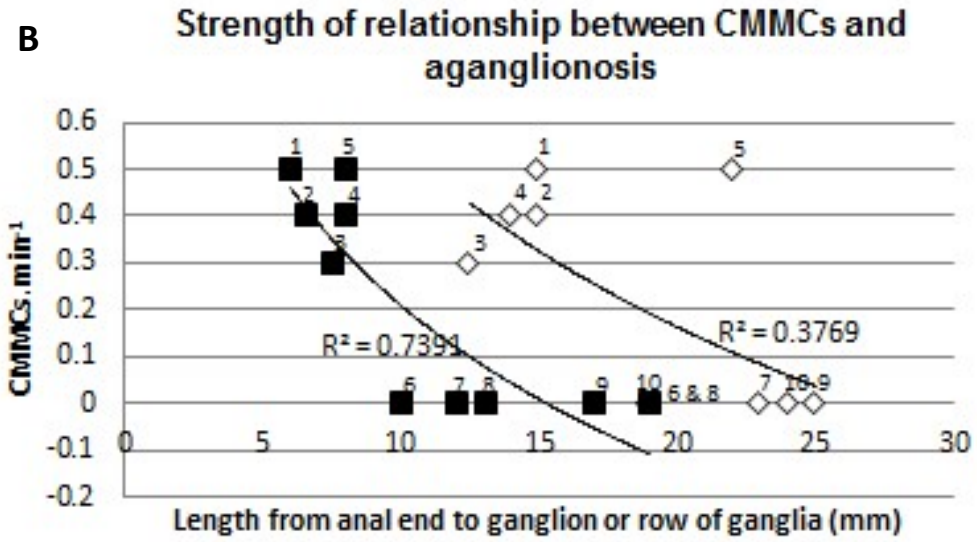
all three measured areas (proximal, mid and distal colon) with a mean number of 18 ± 0.9 ganglia around the circumference of proximal colon, 11.2 ± 0.9 ganglia around the circumference of mid colon, and 0.1 ± 0.1 ganglia around the circumference of distal colon. Most colonic preparations obtained from ls/ls mice were devoid of ganglia in the distal terminal colorectum. The mean distance from the anal sphincter to the first identified myenteric ganglion was 10.9 ± 1.3 mm, while the mean distance from the anal sphincter to the first complete row of myenteric ganglia (that were connected via internodal strands) was 18.7 ± 1.4 mm. Figure 12 also reveals that there was no significant difference between the mean lengths of aganglionosis in ls/ls mice where CMMCs were found to invade the aganglionic region (5 of 13 mice) compared with ls/ls mice where CMMCs were present in the ganglionic segment only.

In 5 ls/ls mice (mice 4-8 graph 1, figure 12A) CMMCs were found to propagate into the aganglionic region (within 3.5mm of the anal sphincter). In this cohort, mouse 6 has its first complete circumferential row of myenteric ganglia 13mm from anal sphincter and in mouse 4 the first complete circumferential row of myenteric ganglia occurred 22mm from the anal sphincter. Both ls/ls mice show CMMCs could invade within 3.5mm of the anal sphincter. A direct comparison of distance to first row of myenteric ganglia (mm) and CMMC frequency showed that there is no linear relationship between the two (R^2= 0.379). However, a curve fitted to the data to correlate between CMMC frequency and distance from anal end to first myenteric ganglion showed a stronger relationship (R^2 = 0.7391). The relationship between CMMC frequency and distance to first complete circumferential row of ganglia was weak (R^2 = 0.3769).

A


Correlation between length of aganglionosis and presence of CMMCs that propagated the full length of colon including into the aganglionic region
Length of aganglionosis (distance from anal sphincter) (mm)
CMMCs.min-1
Mouse number
Frequency of CMMCs in that propagated into aganglionic region
Distance from anal sphincter to first myenteric ganglion
Distance from anal sphincter to first complete circumferential row of myenteric ganglia

B


Strength of relationship between CMMCs and aganglionosis
CMMCs.min-1
R² = 0.7391
R² = 0.3769
Length from anal end to ganglion or row of ganglia (mm)

C

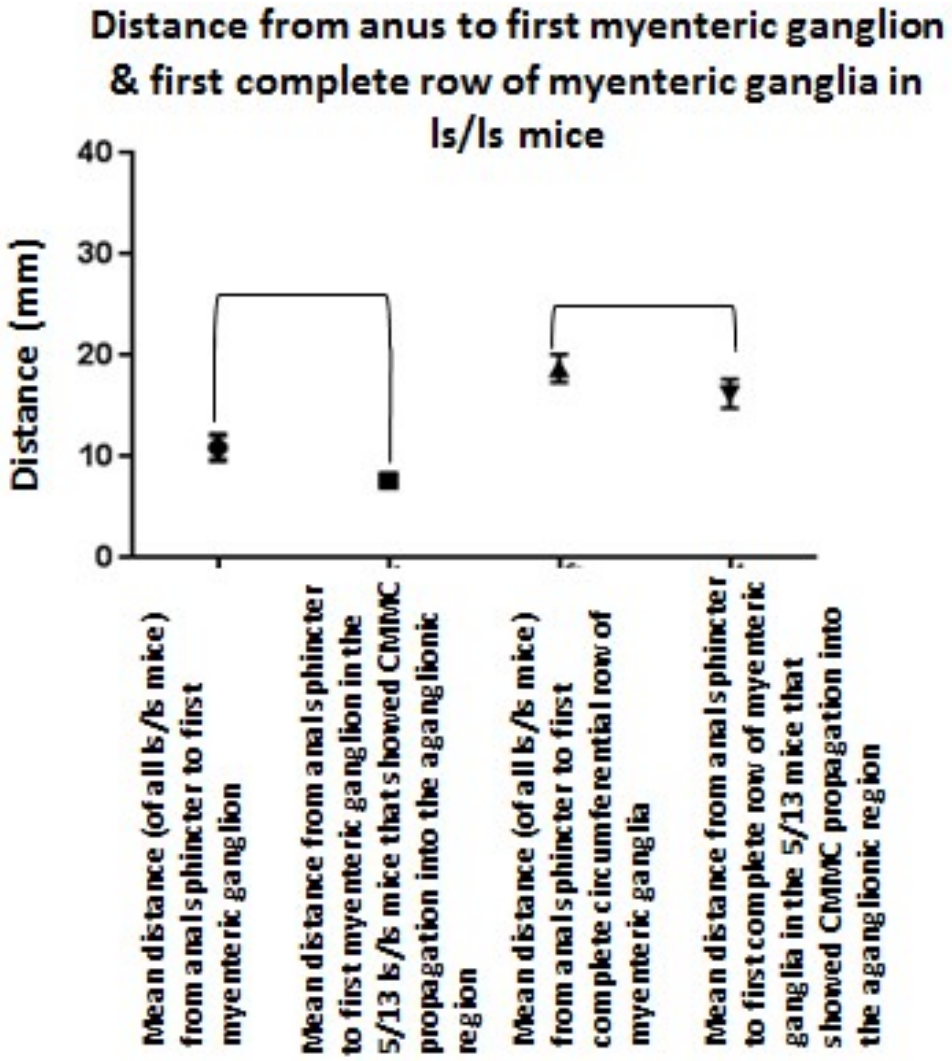


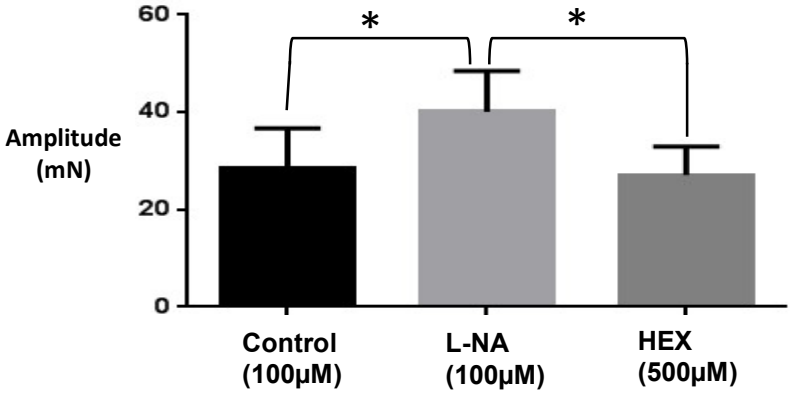
Figure 12. Graphical correlation between the extent of colorectal aganglionosis and the presence or absence of CMMCs in ls/ls mice. B, The individual results of 10 ls/ls mice studied, whereby the length of aganglionosis is directly compared with the presence of CMMCs, their frequency of occurrence, and the distance from the anal sphincter to the first myenteric ganglion and first complete circumferential row of myenteric ganglia. Lower graph compares distance to first row of ganglia against CMMC frequency. The R^2 value of 0.379 reveals a poor linear relationship, and hence both characteristics seem independent of each other. B, squares = Length to first ganglion and, diamonds = length to first circumferential row of ganglia. Graph shows loose correlation between length of total aganglionosis (distance to first

ganglion) and CMMC frequency. CMMC frequency decreases as this length increases. C, Graphical representation of the relative differences between the lengths of aganglionosis in ls/ls mice in which CMMCs were found to invade the aganglionic segment compared with ls/ls mice where CMMCs failed to invade this region

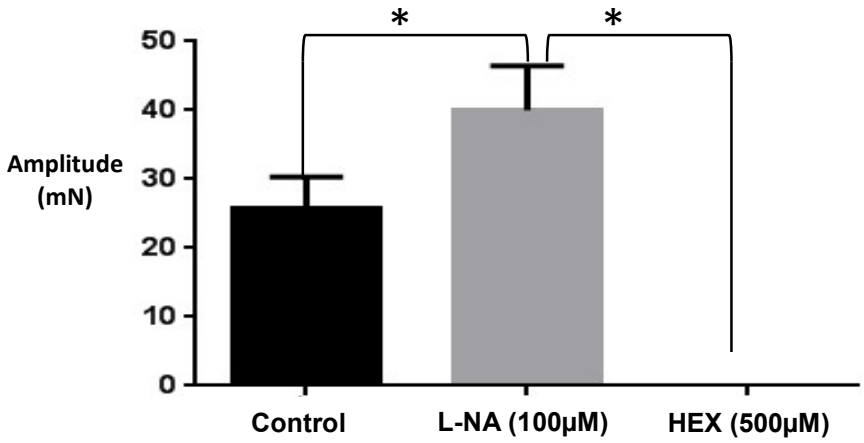
3.4.5 Effects of nitric oxide synthase blockade on colonic motility in control and ls/ls mouse colon

L-NA (100µM) was applied to test the role of endogenous nitric oxide production in the generation of the different motor patterns recorded from wild type and ls/ls whole colon preparations. It was found that L-NA consistently (9/9 preparations) increased the amplitude of CMMC contractions (figure 13), and increased CMMC frequency. However, in no preparation did L-NA induce a full CMMC when CMMCs were absent from the commencement of the experiment. CMMCs present in the distal portion (3.5mm from anal end) of the gut were increased from mean amplitude of 25.8 ± 4.5 mN to 40.0 ± 6.4 mN (55% increase; figure 13B) (p=0.02) with the addition of 100µM L-NA but the frequency of CMMCs in the distal portion of the colon was not significantly increased (0.42.min^{-1} to 0.63.min^{-1}; fig 6D). CMMC activity was abolished by the addition of 250µM HEX (100% decrease) (figure 13B). Phasic contractions increased from a mean amplitude of 28.4 ± 8.3 mN to 40.1 ± 8.4 mN (45.3% increase) in 100µM L-NA (p = 0.0002), which was then decreased to 27.1 ± 5.9 mN in 250µM HEX (32.4% decrease) (P = 0.01) (Figure 13A). The frequency of phasic contractions was increased from 1.7.min^{-1} to 2.1.min^{-1} (24% increase; p = 0.002) and decreased to 1.8.min^{-1} (14% decrease; p = 0.049) by the addition of HEX (figure 13C).

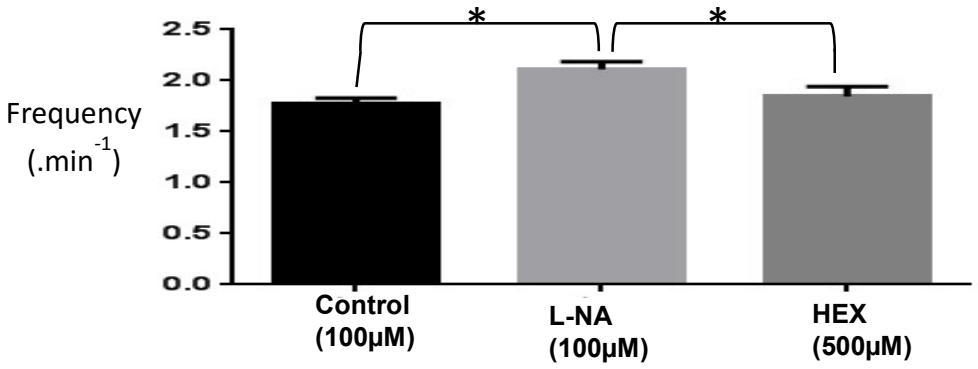
A. **Effect of L-NA and HEX on amplitude of phasic contractions in aganglionic region of ls/ls mice**



B. **Effect of L-NA and HEX on amplitude of CMMCs recorded from aganglionic region of ls/ls mice**



C. **Effect of L-NA and HEX on frequency of phasic contractions in aganglionic region of Is/Is mice**



D. **Effect of L-NA and HEX on frequency of CMMCs in aganglionic region of Is/Is mice**

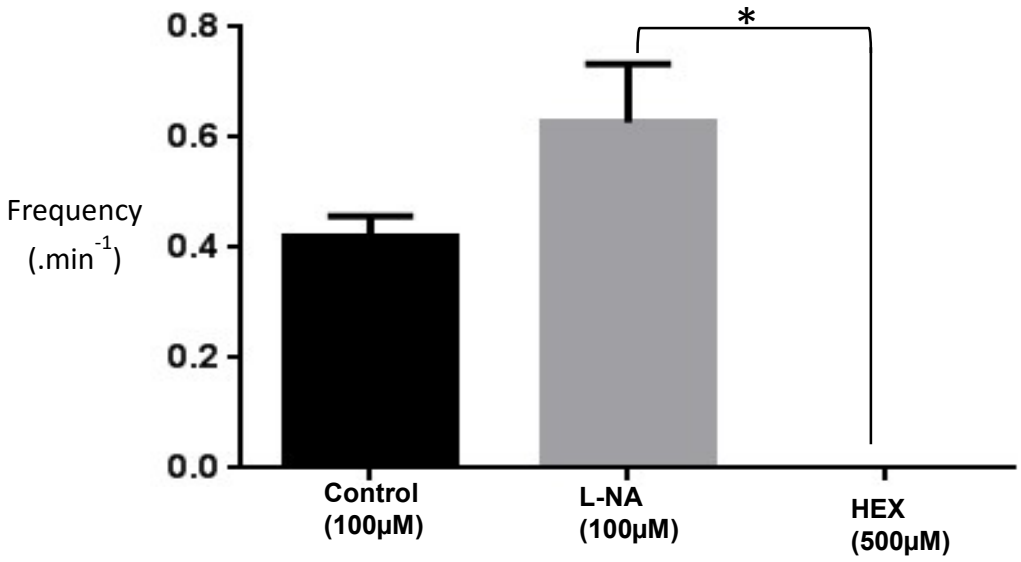


Figure 13. Graphical representation of the effect of L-NA and HEX on amplitude and frequency of phasic contractions and CMMCs. A, Effects of L-NA and hexamethonium on phasic contractions recorded from the aganglionic region. L-NA significantly increased the amplitude of phasic contractions in the aganglionic region and this increase in amplitude was prevented by hexamethonium. However, hexamethonium did not abolish phasic contractions, confirming they were of myogenic origin and occurred independently of activity in myenteric ganglia. B, Effects of L-NA and HEX on CMMCs. Blockade of nitric oxide synthesis with L-NA significantly increased CMMC amplitude in the aganglionic region. Subsequent addition of HEX then abolished CMMCs in the aganglionic region. C, Effect of L-NA and HEX on the frequency of phasic contractions in the aganglionic region of the mouse colon. L-NA significantly increased the frequency of phasic contractions, followed by a significant decrease in frequency by HEX. D, Effect of L-NA and HEX on the frequency of CMMCs in the aganglionic region of the mouse colon. L-NA did not significantly increase the frequency, but did abolish, and therefore significantly decrease the frequency of CMMCs.

3.5 DISCUSSION

There were at least two major findings of the current series of experiments. One was that CMMC generation and propagation could be preserved in the ganglionic segment of mice that lack the EDN3 gene; and on occasions these neurogenic contractions could propagate short distances into the aganglionic segment. Secondly, we found that whilst the length of aganglionosis varied considerably between different ls/ls mice, this variability had no relationship to the intrinsic CMMC pacemaker frequency. These findings are important because other comprehensive

studies [134] have shown convincingly that mice raised on other background strains of mice have substantial lengths of aganglionosis, completely lack CMMCs; and are lethal within the first 2 weeks of birth. In contrast, our study shows that CMMCs do occur in the isolated whole mouse colon of homozygous ls/ls raised and maintained on a C67BL strain, and that they can sufficiently expel natural fecal pellets for a normal murine life span. This is in contrast to common perception that ls/ls mice usually die of megacolon within the first 3-4 weeks. In fact, we found homozygous EDN3 mice were rarely lethal soon after birth, which means that colonic motility in these offspring must be sufficient to expel enough fecal content to avoid perforation or total bowel obstruction.

3.5.1 Why is the strain of origin important?

The findings of these experiments are important to reconcile with other studies which have convincingly demonstrated that motility in homozygote EDN3 mice, which were raised on a different strain (129SL), were found to completely lack CMMCs, have ~70% aganglionosis along the colon and are lethal prior to P12 [134]. As demonstrated in a study on *Sox10Dom* strain mice by Walters *et al* 2010 [158], the genetic background can have a large influence on the degree of aganglionosis. This could provide an explanation for the varying degrees of severity of Hirschsprung's disease observed in the human population.

The lethal spotted EDN3 mutant mice we obtained and used in this series of experiments were purchased as homozygotes from Jackson laboratories and the colony was maintained on a C57BL strain. In these mice, we found that animals did have a prominent aganglionic colorectum, consistent with other studies; and there were significantly more fecal pellets in the colon compared with wild type C57BL/6

mice. But, we found these mice reliably generated CMMCs and could adequately expel fecal pellets to sustain life, even with megacolon. On rare occasions, an ls/ls mouse did have to be euthanized or died of megacolon, but this was uncommon. In the study by Roberts *et al.* 2008 [134]; the EDN3 mice were purchased as heterozygotes from the Jackson laboratory, but were raised on a 129S background strain then subsequently backcrossed for 10 generations onto a C57Bl/6 background. Although the same gene was genetically ablated in both these types of mice, clearly there were major phenotypic differences. One might presume that after 10 generations of backcrossing to a C57BL6 strain, the mice used in the study of Roberts et al. 2008 would be comparable to the ls/ls mice we studied that were raised on a C57BL strain. Since the two types of mice had very different phenotypes, we are tempted to speculate that the major phenotypic differences we have detected between ls/ls mice in our study and the EDN3 homozygote mice used in the Roberts study maybe due to the strain of origin of the mice. Indeed, Cantrell *et al.* 2004 [38] demonstrated that the degree of aganglionosis is dependent on the interactions between specific genes arising from different back crossings.

3.5.2 How and why does colonic motility differ so much between ENDRB homozygote and EDN3 homozygote mice ?

Although the ET-3 polypeptide is the endogenous ligand for the EDNRB receptor, mice with a homozygote mutation of either EDN3 or EDNRB have major differences in colonic motility. In a previous study, we found that EDNRB homozygote offspring are lethal within the first 12 weeks of birth, develop severe megacolon and completely lack CMMCs even in the ganglionic region [132] . In contrast, in the current study, ls/ls mice rarely died of megacolon and commonly live a normal life span (1-2 years), breed successfully as homozygotes and still generate CMMCs

reliably in the ganglionic region which can sometimes weakly propagate into the aganglionic region (5 of 13 preparations). The reason for these differences is not entirely clear, but may be due to the mean length of aganglionosis between the different mice. The mean length of aganglionosis in EDNRB homozygotes was 20mm [132] which is to a larger degree than the mean length of aganglionosis (10.9mm) in EDN3 homozygotes revealed in this study. Although, in the current study, some ls/ls mice had an aganglionic segment that was ~20mm in length and were still found to generate CMMCs. The less severe phenotype of EDN3 mutants (compared to those with a mutation in the gene encoding EDNRB) could be due to the presence of two other ligands (ET1 and ET2) which also bind to EDNRB, and may partially compensate for the loss of ET3.

3.5.3 How do CMMCs propagate into the aganglionic region?

An important observation of the current study was that when mechanical recordings were made 3.5 mm from the anal sphincter it was found that 5/13 mice still showed CMMCs that propagated short distances into the aganglionic region, despite the fact that the mean aganglionic length in *these* mice was 7.7 ± 0.6 mm, and the first complete row of myenteric ganglia was at 16.3 ± 1.4mm. It is important to realize that CMMCs cannot be generated in an aganglionic region as it is known they require the enteric nervous system [26,101], but some CMMCs were able to propagate into this region. It is likely that CMMC contraction could propagate short distances into the ganglionic region due to the fact that descending interneurons can synapse onto motor neurones in the transition zone. Single motor neurones have been shown to project up to 4mm in the mouse colon (oral and anal) [147]. This would mean that that neurotransmitter could potentially be released some millimetres into the ganglionic region, even though there are no myenteric neuronal cell bodies here.

The relationship between CMMC frequency and distance from anal end to first myenteric ganglion (12B) showed that there was some correlation between the two; the longer the aganglionic segment was, the less CMMCs.min^{-1} that were generated (R^2 = 0.7391). The relationship between CMMC frequency and distance to first complete circumferential row of ganglia was weak (R^2 = 0.3769), however this is most likely due to the outlier figure of a mouse with 22mm distance to the first connected row of ganglia which still had CMMCs. The presence of CMMCs in this outlier point may be due to the relatively shorter total aganglioinic segment (distance to first ganglion 8mm) in comparison to the other mouse colons which had CMMCs absent and a much longer segment of gut with total absence of ganglia. In general, it seems that colons with a distance of 10mm or more to the first ganglion are unable to have CMMCs propagate into the distal portion. Figure 9 shows the degree of aganglionosis 9mm from the anal end, and also a spatiotemporal map of some CMMC activity. CMMCs were still weakly recorded, in addition to phasic contractions. L-NA increased the frequency and amplitude of CMMCs in all regions. Hexamethonium abolished CMMCs, but not the myogenic phasic contractions. The only explanation we have for this observation is that in this small proportion of animals that displayed this result, some enteric cholinergic and nitrergic motor nerve fibres project into the aganglionic region, but whose cell bodies are located rostrally in the ganglionated region. This is supported by the fact that hexamethonium abolished residual CMMCs that propagated into the aganglionic region, and that the NO inhibitor L-NA was able to increase the amplitude of CMMCs in this region. In rare preparations where L-NA had increased the amplitude of phasic contractions, but HEX was subsequently unable to decrease the effect of L-NA, this suggested that the increase in activity was driven by inhibition of nitrergic inhibitory neural inputs

arising from the ganglionic region. In further support of this idea, Roberts' and colleagues [134] showed that nitrergic nerve fibres innervate the aganglionic region of ET3 mutant mice. It seems possible that using L-NA to increase the amplitude of phasic contractions in the aganglionic region could increase motility in the gut (but not induce propagating CMMCs). However, it is unclear whether or not this would have any benefit clinically, due to the fact it would not help with initiating propagating CMMCs which propel content, and it may exacerbate megacolon by preventing evacuation of fecal content.

As discussed earlier, CMMC generation and propagation is critically dependent upon enteric nerves [142,147]. It is therefore not surprising that mice with a substantial lack of enteric ganglia also lack CMMCs along the whole colon. A good example is the EDN3 homozygote mice studied by [134] which lacked ~70% of myenteric ganglia and also completely lacked CMMCs. However, interestingly, in a previous study, we found that homozygote EDNRB mice (piebald-lethal) contained myenteric ganglia along the proximal to distal colon, albeit significantly reduced in number, and had a prominent ~20mm aganglionic segment, but these mice never generated CMMCs [132] . The reason for the total abolition of CMMCs in these mice was unclear. The observation that ls/ls homozygote mice in the current study still showed CMMCs along the colon and were viable for a full murine life span, but ENDRB homzogyotes were lethal and lacked CMMCs, suggests that the absence of CMMCs may be a better predictor for the development of severe megacolon and lethality in mice.

3.6 CONCLUSION

Our findings show that CMMC generation and propagation is preserved in ls/ls homozygote mice lacking the EDN3 gene. The preservation of this motor pattern is likely to be responsible for the sufficient expulsion of colonic content to avoid bowel obstruction or perforation and likely explains the fact that these mice rarely die at a premature age. An important outcome from this series of experiments was to show that the strain of origin of mouse, in combination with the gene of interest can have a significant impact on the phenotype. In this case, the severity of aganglionic deficit was changed by involvement of different background strains in the breeding line. This is an important consideration for future experiments, that the strain/s of mice involved at any stage in the breeding of a colony can potentially alter the phenotypic characteristics.

CHAPTER 4: Discussion

A major aim of this thesis was to investigate the control mechanisms that underlie the generation and frequency of occurrence of cyclical colonic migrating motor complexes (CMMCs) in isolated mouse large intestine. Specifically, the series of experiments described above reveal how *in vitro* recording conditions and intraluminal fecal contents modify CMMC characteristics. These findings have important ramifications for future studies that use isometric force transducers and other *in vitro* recording techniques that inadvertently stimulate the gut, whilst recordings of gut motility are made. Then, in the second part of this thesis, the characteristics of CMMCs were investigated in lethal spotted (ls/ls) mutant mice, with colorectal aganglionosis. The major aim was to provide important new insights into how the extent of colorectal aganglionosis modifies the characteristics of CMMC generation and propagation and how important considerations should be given to the strain of mouse origin when comparing the extent of colorectal aganglionosis that develops following mutation of the same gene (EDN3) between different strains of mice.

In Chapter 2, the characteristics of CMMCs were investigated in empty segments of intact whole mouse colon *in vitro*. The key difference between previous studies and the experiments carried out in Chapter 2 was that the empty segments of colon investigated in this study lacked any overt external stimuli applied to the colonic wall, such as conventional tension transducers. As far as we are aware, this experiment had not been performed previously. To our surprise, under these recording conditions, a major finding was that, CMMCs occurred rarely or not at all in empty segments of isolated whole mouse colon, provided the specimens lacked applied resting tension to the musculature. Regularly occurring CMMCs were only recorded when conventional

mechanical recording clips were used to pinch the muscularis externa and when ~1gm resting tension was applied to the colonic wall. It has been demonstrated in many previous studies that CMMCs are recorded regularly, approximately every 1-3minutes, from empty segments of mouse colon when the entire colon is devoid of fecal content [26,55,128,134,146,150]. These previous experiments have led to the assumption that the presence of fecal pellets in the colonic lumen are not required for CMMC initiation and propagation. However, these previous studies have used conventional mechanical recording techniques in some form which applied stretch or stimulation to the colonic wall. I propose in this thesis that these previous recording conditions act as a major stimulus to induce CMMCs and maintain their frequency at a higher than would be expected rate [8]. Unavoidably, all forms of isometric mechanical recordings from isolated segments of smooth muscle involve some sort of pinching, stretching or puncturing of the gut, or a combination of these stimuli.

Luminal distension which is normally induced by fecal content, can be mimicked by application of local distention directly to the colon wall. Results presented in chapter 2 of this thesis show that when the isolated mouse colon is mounted on recording apparatus at oral and anal ends (as in “preparation 3” of figure 4) this methodology is able to reliably generate CMMCs at a frequency consistent with previous literature, i.e. a CMMC interval of ~1-3 mins [55,133,146]. The velocity of these CMMCs is also consistent with these studies. Another technique used in this thesis to record colonic motility in involved a single pin anchoring each end of the mouse colon to the organ bath whilst video recordings were made from above. Under these conditions (to minimise stimulation of the bowel) we revealed a decrease in the frequency of CMMCs, or, often they were not recorded; and a decrease in the velocity of the CMMCs was apparent. A single pin anchoring method is suggested to cause less

stimulation/distension of the colon wall, and therefore is less likely to replicate the presence of fecal content, and in our hands less likely to induce CMMC generation and propagation.

Previous studies most commonly used techniques such as isometric force transducers, which involve piercing the colon wall with a hook, and applying stretch, so that force changes can be measured as a CMMC travels along the colon. Mechanical clips have also been used in a similar manner [55] however these do not typically pierce the colon, but do impose significant stimulation on the colonic musculature, especially when ~1gm resting tension is applied to the muscle layers. The other common recording method involved mounting both the oral and anal ends of the colon into an organ bath to facilitate infusion of intraluminal fluid. The colon was anchored to each end of the organ bath by firmly suturing the musculature with thread at either end, over a segment of tubing.

The study design used in this thesis involved first measuring activity of the colon with natural pellets, using spatiotemporal mapping, to ensure integrity of the neural pathways in the colon. The colon was then gently flushed free of content and left to equilibrate for a set period of time, before CMMC frequency was measured from the "empty" colon. These recordings were made when only a single pin was used to anchor the colon at oral and anal ends. This same colon was then "stimulated" with one of the three traditional recording methods, to characterise potential changes in CMMC frequency or velocity.

It is acknowledged that the piercing of the colon with even a single pin at either end is still applying some form of stimulus to the bowel. However this was the least invasive way of measuring CMMC activity that we could devise. It certainly avoided application

of resting tension and avoided application of circumferential stretch to the colon. Studies have shown that circumferential stretch of the colonic wall increases CMMC frequency [101,150,169], and these studies also showed that stretch-induced increases in CMMC frequency do not require the mucosa or submucosal plexus [101,150,169].

An important finding of the results of this thesis revealed that CMMC frequency and velocity significantly and substantially increased when natural faecal pellets were present compared with an empty colon, or even a colon which was 'stimulated' by recording techniques which apply some form of stretch or pinch. It has been shown previously that circumferential stretch applied to the colon is able to increase the frequency of CMMCs. These stretch induced CMMCs are able to occur without the presence of the mucosa, indicating that neurons in the submucosal plexus, or release of neurotransmitters from the mucosa are not required for their generation. The mechanoreceptors which are responsible for sensing the stretch and initiating a CMMC must lie in the myenteric ganglia and/or muscularis externa, with the intrinsic sensory neurons being located in the myenteric plexus [148].

From a philosophical point of view, it seemed illogical that an organ, like the colon would "want" to generate CMMCs so frequently in an empty state. This would require expenditure of considerable energy regardless of the presence of content – a concept that runs against the normal convention of the body to conserve energy where possible. This is why the results presented here seem to provide an explanation as to why the other studies obtained the high frequency of CMMCs that infact, the colon does conserve energy by not initiating costly CMMCs when the gut is truly empty.

These findings not only reveal that CMMCs do not always occur in a regular pattern regardless of content, but they also highlight the need for extreme care when designing

studies and interpreting results. It seems that the fact that these particular recording techniques which were widely used, were not questioned as to the level of stimulus they were creating, and whether the recording of regular CMMCs could indeed be an artefact of the apparatus.

These findings will contribute to the literature in such a way that future study design should, and hopefully will, be modified to minimise stimulus caused by measuring equipment. Failure to modify this would risk recording of results which were not true to the *in vivo* state, which is the usual goal of conducting an *in vitro* experiment, to have it mimic the *in vivo* as closely as possible. The results will also help to guide future discoveries into what governs colonic motility patterns; it is just another small piece of the puzzle.

Chapter 3 describes the finding that the mechanisms underlying CMMC generation are preserved in lethal spotted (ls/ls) mice, which lack the EDN3 gene; and they are able to propagate into the aganglionic region of colon. It was shown by Roberts *et al.* 2008 [134] that lethal spotted mice lack CMMCs, however these mice had an aganglionic segment of around 70% of the colon, and commonly died before they reached 12 days old. The mice used in the Roberts *et al.* (2008) study in particular, were raised on a background strain of 129SL before being back crossed for ten generations on a C57Bl/6 strain. The mice we worked with were raised solely on a C57Bl/6, which appears to have affected the degree of aganglionosis. It is fascinating that such pronounced differences in the extent of aganglionosis and the presence or absence of CMMCs can occur in mice lacking the same gene, but raised on different strains of mice.

The mice we studied had an average length of aganglionosis that occupied 14% of the colon. It is understandable then, when comparing the vast differences in aganglionosis,

that our study showed that these particular ls/ls mice had CMMCs present, and the mice in the study by Roberts *et al.* (2008) did not contain these motor patterns. The mice used in our study, were mostly able to live a normal murine lifespan, and able to expel faecal pellets throughout this time, avoiding potentially fatal megacolon. Some mice (as shown in pictures in chapter 3) did suffer from megacolon, but this was most often not fatal, at least not prior to 180 days of age which was the maximum age which the mice were euthanized for study. Some other mice which we did not use for study, but were of the same genotype, were able to live for up to two years of age. Although this study has shown that 14% aganglionosis is most often not fatal, it is understandable that many of these mice may be in some degree of discomfort if they are suffering from a decreased ability to expel pellets, much like the discomfort experienced by humans with bowel disorders such as constipation predominant irritable bowel syndrome (IBS-C).

The question was raised, why would the same mutation in the EDN3 gene be fatal in some mice early on in life (like in the study by [134]), and not affect others so severely? Other studies [38,158] have shown that the genetic background of the mice can have a large impact on how a gene will impact the degree of aganglionosis. The differing genetic backgrounds used between this study and the one by Roberts *et al* (2008)[134], although it was back crossed onto the same mice strain, could account for the large differences seen in aganglionosis and CMMC generation.

Interestingly, and against what one might predict, the degree of aganglionosis in the mice we studied did not have a direct correlation to the frequency or velocity of CMMCs. Some mice with a large degree of aganglionosis were still able to generate CMMCs, some of which even propagated into the aganglionic region. Conversely, CMMCs were not observed in some mice with a relatively short aganglionic segment.

The fact that CMMCs were able to propagate at all into the aganglionic region is in itself, highly interesting. Although it is not known exactly what initiates one of these propagating complexes, it is known that they require the enteric nervous system to occur. For them to continue past a region lacking ganglia is quite surprising. We propose that this could have occurred due to the presence of descending interneurons which are able to synapse onto motor neurones in the transition zone. It has been shown by others [147] that motor neurones are able to project up to 4mm either orally or anally in the mouse colon. Potentially, this could result in neurotransmitter being released millimetres into the aganglionic region (even though this is devoid of myenteric cell bodies). In general, CMMCs were not generated at all in colons which had a greater degree than 10mm aganglionosis.

The relationship between the frequency of CMMCs and the length of total aganglionosis (length to first ganglia) was shown to be somewhat correlated. Increasing length of total aganglionosis was related to a decrease in CMMC frequency. The relationship between CMMC frequency and distance to first circumferential row of ganglia was weak. The first circumferential row of ganglia is an important parameter, because this is where the network of nerves first becomes complete. There can be sporadically located ganglia up until this point, usually starting with just a single ganglion then progressively increasing to the full network, as the degree of aganglionosis decreases. This relationship was weak mainly due to an outlier which showed that CMMCs were generated in one mouse in which there was a 22mm distance from internal anal sphincter to the first detectable ganglion. This mouse however, did have a very short total aganglionic segment (that is, the distance to the very first ganglia), which could explain why it was still able to generate CMMCs despite the longer distance to the first complete network of ganglia. Further research

into elucidating exactly how CMMCs were able to propagate into the aganglionic region would be useful, as would development of some methods to increase the propagation distance.

Despite these relationships, it seems clear that the length of aganglionosis is not always the best predictor of whether or not an animal (at least mouse) will suffer from megacolon, or whether their normal lifespan will be altered. The fact that there were CMMCs present in many of the mice studied here which were easily able to survive past 3-4 weeks old and pass faecal pellets naturally, indicates that the presence of CMMCs is likely to be a better predictor of survival and /or possible development of megacolon.

Experiments in this thesis have revealed that whilst CMMCs were never initiated in an aganglionic segment, it is possible for the CMMC contraction to propagate short distances into this region -lacking enteric ganglia and in many cases, give the appearance as a normally propagating CMMC. Our explanation for this is likely due to the fact that some long descending inhibitory and excitatory motor neurons can project <5mm anally. This could mean transmitter is functionally released into the aganglionic region, from the neighbouring ganglionic segment. This provides an explanation as to why some mice with variable lengths of colorectal aganglionosis are still able to live a normal life span. These studies also further re-inforce the literature that background genetics of a mouse will contribute to the phenotype of this genetic condition. Even if the mouse has been further backcrossed, it may not be identical to those which have always been bred from the same strain.

The large variation in length of aganglionosis was identified between each lethal spotted mouse, lacking EDN3 expression. Hirschprung's disease in humans, as

described in chapter 1.15 is also well known to have major variability in the lengths of aganglionosis.

A newborn human baby is not investigated for Hirschsprung's diseases unless cause for concern is raised over their ability to pass meconium, or significant gastrointestinal difficulties are encountered in their first few years of life. Given the large degree of aganglionic variation demonstrated by studies presented in this thesis, it seems plausible that some humans may have a lesser number of myenteric ganglia in their colon, which could cause intermittent problems in transit, but perhaps never severe enough so that they would suffer from life threatening megacolon.

Recently, some exciting new research [91] has shown that transplanted neural progenitor cells can generate functional enteric neurons in the postnatal bowel. This technique offers a very exciting possibility that aganglionic segments may one day develop a fully functional enteric nervous system, and not require resection as they do currently.

The information presented in this thesis contributes to increased understanding of colonic motility and pathology in mice.

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