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

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

Simona Elisa Carbone

Bachelor of Medical Science, Bachelor of Science (Honours)

Discipline of Human Physiology

Flinders Medical Science and Technology

Centre for Neuroscience

School of Medicine, Flinders University

Adelaide, South Australia

August 2012

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

1.1 The anatomy of the gastrointestinal tract

The gastrointestinal tract is a continuous tube extending from the mouth to the anus with accessory organs to assist in digestion. The primary organs of the gastrointestinal tract constitute the *alimentary canal* and include the mouth, pharynx, oesophagus, stomach, small intestine and large intestine. Accessory elements include the teeth, tongue, salivary glands, liver, gall bladder and pancreas. Its design facilitates the break down of food, the absorption of nutrients and minerals, and the excretion of unnecessary or harmful substances (Widmaier et al., 2008).

1.1.1 The layers of the gastrointestinal tract

A general arrangement of the gastrointestinal wall is maintained throughout the tract from the oesophagus to the anus. This permits coordination of gastric motility thus coordinating movements along the tract. The *serosa* is the outermost layer. It is a serous membrane of areolar and connective tissue. The *tunica muscularis* or *muscularis externa* is a combination of inner (circular) and outer (longitudinal) muscle layers, as well as other cell types. The contents within the lumen are propagated along the tract by contractions and relaxations of these layers. The *mucosa* is the inner most layer of the tract. This layer consists of: an epithelial layer which directly contacts the luminal contents; a layer of connective tissue termed the *lamina propria* which also contains immune cells; and a thin layer of smooth muscle. The mucosa connects with the circular muscle by a connective tissue layer termed the *submucosa* which contain many large blood vessels and lymphatic vessels (Widmaier et al., 2008).

1.1.2 The enteric nervous system

Within the gastrointestinal wall is a network of neurons forming the *enteric nervous system*; a network that functions largely independently of the central nervous system. The submucosal plexus lies in the submucosa; the myenteric plexus beween the two

layers of muscularis. The enteric nervous system is involved in the control of the functions within the gastrointestinal tract and integrates parasympathetic and sympathetic input. The various ganglia in the two plexuses contain the cell bodies of enteric neurons. These types of neurons include primary afferent neurons, interneurons and motorneurons (Costa et al., 1998). Primary afferent neurons detect a range of mechanical and chemical stimuli. Interneurons integrate this information and provide input to motor neurons or secretomotor neurons. Motorneurons excite or inhibit smooth muscle cells. Enteric glia are also present in all ganglionated plexuses as well as all non-ganglionated plexuses and interconnecting nodal strands (Rühl, 2005). They outnumber enteric neurons, up to 4 times more in some instances (Bassotti et al., 2007). They resemble astrocytes from the central nervous system (Furness, 2006). Within the gut, glial cells are involved in neurotransmitter, immune and homeostatic functions, as well as providing mechanical support to the enteric nervous system (Bassotti et al., 2007).

1.2 Smooth muscle cells

Excluding the upper two thirds of the oesophagus, smooth muscle cells are the sole contractile unit in the tunica muscularis. In most regions of the gut, the longitudinal muscle layer is made up of a thin layer of cells. However in some species the colon has bands of densely arranged longitudinal muscle fibres form regions called *taenia coli*. The colon of smaller species is not taeniated; in the guinea pig only the caecum contain taenia; in the rabbit taenia are contained to the proximal colon and in the human three separate bands of taenia run along the outside of the ascending, transverse, descending and sigmoid colons (Bulbring, 1954, Ehrlein et al., 1982, Ehrlein et al., 1983). Contractions of the longitudinal muscle result in local shortening of the tract. The circular muscle layer is a thicker layer of cells. Contractions of circular smooth muscle cells constrict the lumen of the gut. This occlusion allows propulsion of luminal contents.

1.2.1 Morphology

Smooth muscle cells are spindle shaped structures with a high surface area to volume ratio. They are approximately 300-500µm long and 5µm wide. *Caveolae* are basket shaped invaginations that align along particular regions of smooth muscle cell to increase the surface area of the membrane by up to 75%. Caveolae are closely apposed to the sarcoplasmic reticulum, the storage site of calcium in smooth muscle cells. This arrangement suggests a role for caveolae in maintaing Ca^{2+} homeostasis in smooth muscle cells (Taggart, 2001). *Dense bands* are predominately comprised of actin and are the site of attachment for thin actin filaments. They are 1-2µm long and 0.2-0.4µm wide. Within the cytoplasm *dense bodies* link to dense bands by *intermediate filaments*. This arrangement forms the contractile unit of the smooth muscle cell (Bond and Somlyo, 1982). There are two forms of cell contacts between smooth muscle cells in the gastric wall: intermediate junctions and gap junctions (Henderson et al., 1971). *Intermediate junctions* arise where dense bodies from adjacent cells are juxtaposed. In this region cell membranes are separated by less than 30nm. *Gap junction* or *nexuses* are another form of junction between smooth muscle cells. These channel-like junctions are associated with closely apposed cell membranes with a gap of by less than 3nm. They permit the passage of certain metabolites, second messengers and ions between cells.

1.2.2 Contractile apparatus

The contractile apparatus of smooth muscle cells includes: thin actin filaments (5-7nm wide), thick myosin filaments (15nm wide) and intermediate desmin filaments (10nm). Actin filaments traverse the long axis of the smooth muscle cell, one end interacts with dense bodies the other free end folds into thick myosin filaments. Intermediate filaments attach to adhesion sites on the plasma membrane at one point, and at another point are fixed to dense bodies, thereby anchoring filaments to the cytoplasm (Bolton et al., 1999). Thick filaments are comprised of a pair of myosin heavy chains and two pairs of myosin light chains that are not covalently bound. The myosin heavy chains combine to form a helical core and at the end of each strand is a globular head. The myosin heads are the *cross bridge* complex of the contractile apparatus. Each head consists of two domains: a *motor domain* with a site for ATP hydrolysis and an actin

binding site; and a *neck domain* which acts as a lever allowing the thick filament to slide during muscle contraction (Craig and Woodhead, 2006). The myosin light chains make up the neck domain where an *essential light chain* is close to the motor domain and a *regulatory light chain* is important for muscle contraction (Trybus, 1994).

Smooth muscle cell contraction is regulated by the phosphorylation of the regulatory myosin light chain subunits via the *myosin light chain kinase* (Gunst and Zhang, 2008). Under physiological conditions, phosphorylation of this kinase is dependent on the internal concentrations of calcium. Calcium enters the cytoplasm of smooth muscle cells largely via voltage dependent calcium channels, the main type being $Ca_v1.2$ (Takashima, 2009). Release of calcium from sacroplasmic reticulum, through activation of IP_3 receptors can also contribute to cytoplasmic calcium concentrations (Berridge and Irvine, 1989). Increased intracellular calcium raises calcium/ calmodulin binding which activates the myosin light chain kinase (Tansey et al., 1992). Activation of the kinase results in phosphorylation of the myosin light chains.

The myosin globular heads are attached to actin. Phosphorylation of the regulatory myosin light chain results in a conformational change to the head, detaching myosin from actin and activating the ATPase in the motor domain. Hydrolysis of ATP provides energy to slide the actin and myosin filaments past each other so that myosin can reattach at another site further down the actin filament. It is this movement that results in muscle contraction (Adelstein 1983). Myosin light chain phosphatase modulates the activity of myosin light chain kinase. Dephosphorylation of the kinase decreases the cross bridge cycling and therefore results in a muscle relaxation (Takashima, 2009, Murphy, 1988)

1.2.3 Electrophysiology

Smooth muscle cells in the gastrointestinal tract have a negative resting membrane potential that varies from -80 to -45mV. This variation results from differences in the expression of ion channels between cells and the activity of Na^+/K^+ ATPase pump. As

in all other cells, the negative cytostolic resting membrane potential is also strongly determined by permeability to different ionic species. Potassium is the most dominant of the ions contributing to membrane potential. Non-selective cation conductances also provide a significant contribution, while sodium pump channels contribute a small amount (Sanders, 2008). Changes in membrane potential generally underlie contractile responses in the muscle via the activation of voltage dependent calcium channels causing an influx of calcium into the cells. Phasic contractions typically occur intermittently with periods of activity followed by periods of quiescence. Most phasic contractions are paced by depolarisations and repolarisations of the cell's membrane. This pattern of activity often depends on *slow waves,* which are oscillations in membrane potential.

Excitation-contraction coupling is closely associated with an influx of extracellular calcium through voltage dependent calcium channels. The probability of opening voltage dependent calcium channels increases during depolarisation. Since contraction of smooth muscle cells is initiated by an increase in cytosolic calcium concentrations, a contraction will only be generated once a cell membrane potential has depolarised beyond a *threshold potential* (Bauer and Sanders, 1985). In some instances slow waves generate a large enough depolarisation to stimulate a contraction, in other instances superimposed smooth action potentials, or *spikes* with slow waves are required. These spikes can be generated independently or from slow waves summating with excitatory junction potentials. Relative contributions of these mechanisms vary across regions of gut and between species. For example in the jejunum: canine circular muscle contraction can be generated by slow waves alone; in the opposum single spikes superimposed on slow waves can generate contraction; and in the human multiple spikes superimposed on slow waves result in contraction (Hara et al., 1986).

An important property of excitation-contraction coupling is that smooth muscle spikes generally do not occur in isolation: they must be timed with a slow wave. This is an important property for the formation of phasic contractions. In the canine antrum, transmural nerve stimulation between slow waves did not result in a contraction, but

applied simultaneously with slow waves large contractions resulted (Morgan et al., 1981).

It has been suggested that some smooth muscle cell contractions are not voltage sensitive and do not require extracellular calcium. In isolated gastric muscle cells from the guinea pig and human, the amplitude, latency and duration of contractions were not significantly different in normal versus calcium-free solutions (Bitar et al., 1986). I suggest contractions in these instances may have been driven by increases in cytosolic calcium from intracellular stores. However in experiments on whole-mount preparations, contractions in the absence of extracellular Ca^{2+} do not occur. In control solutions, as the membrane potential of canine gastric muscle cells depolarises during a slow wave, cytosolic calcium concentrations increase corresponding with an increase in muscle tension. Addition of nicardipine reduces the amplitude of the plateau potential during the slow wave, cytostolic calcium concentrations reduce as does the corresponding muscle contraction (Ozaki et al., 1991). Isolating smooth muscle cells is a damaging process and it may be that in preparing cells in this manner the sensitivity of the contractile apparatus to calcium is altered.

1.2.4 Ionic basis for the modulation of smooth muscle cell membrane potential

The expression of ion channels in smooth muscle cells varies between parts of the gastrointestinal tract. However, smooth muscle cells from almost all regions of the tract contain voltage dependent calcium channels, calcium activated potassium channels, delayed-rectifier potassium channels from the K_v1 family (particularly $K_{v1.5}$ and K_{v2}); and ATP dependent potassium channels (K_{ATP}). The conductance of these channels can be regulated by a range of mediators and second messengers.

The L-type channel, *$Ca_v1.2$,* is the predominant component of high threshold *voltage dependent calcium channel* in smooth muscle cells of the gut. These channels are associated with long lasting Ca^{2+} currents and an influx of Ca^{2+} ions normally results in membrane depolarisation. When these channels are blocked by dihydropyridines, such as nicardipine and nifedipine, their inhibition results in reduced spontaneous and

neurally evoked contractions (Zhang et al., 2006). L-type channels require depolarization to be activated. In the case on smooth muscle cells, they are typically activated during action potentials occurring simultaneously with slow waves. In patch clamp experiments of isolated smooth muscle cells, nifedipine sensitive inward currents were maximally activated when the membrane potential was depolarised to -5 to +10mV (Vogalis et al., 1991). *Ca_v3* channels producing T type , transient, Ca^{2+} currents, are also present in gastric smooth muscle cells however their physiological role is less clear.

Calcium activated potassium channels are abundant in smooth muscle cells in the gut. Opening of these channels is dependent on voltage, intracellular calcium concentrations and may be modulated by second messenger pathways. They are divided into three groups: large conductance channels (BK_{Ca}), intermediate conductance channels (IK_{Ca}) and small conductance channels (SK_{Ca}) (Farrugia, 1999).

BK_{Ca} channels have a conductance of approximately 80-250pS (Farrugia, 1999). The open probability is low at normal resting membrane potential and at resting cytoplasmic calcium levels. However, open probability increases when the membrane is depolarized or when intracellular calcium concentrations rise, resulting in an outward current (Vogalis and Sanders, 1991). In canine circular and longitudinal myocytes from the proximal colon outward currents are activated when cells are depolarised to 20mV (Carl et al., 1995). BK_{Ca} channels do not have an inactivation voltage. They are involved in the regulation of smooth muscle electrical activity, but do not necessarily influence resting membrane potential. In canine circular muscle, resting membrane potential, slow wave duration, and repolarisation of slow waves were unaffected by the BK_{Ca} channel blocker charybdotoxin (ChTX, (Carl et al., 1995).

SK_{Ca} have conductance of approximately 5-10pS, (Farrugia, 1999). These channels are more sensitive to Ca^{2+} than BK channels. Channel conductance is activated by ATP and inhibited by apamin (Vogalis and Goyal, 1997, Koh et al., 1997) IK_{Ca} have a conductance of approximately 40pS and are blocked by mM concentrations of extracellular TEA (Vogalis and Goyal, 1997) . Inhibiting IK_{Ca} and SK_{Ca} channels increases the membrane potential of smooth muscle cells (Spencer et al., 1998). SK_{Ca} are thought to play a role in the generation of inhibitory junction potential (IJPs). An IJP is a membrane hyperpolarisation resulting from neurotransmitter release from an inhibitory motorneuron. IJPs are inhibited by apamin (Niel et al., 1983), by P2 antagonists, pyridoxalphosphate-6-azophenyl-2',4'-disulphuric acid (PPADs), suramin (Zagorodnyuk and Maggi, 1994, Zagorodnyuk et al., 1996), and by specific $P2Y_1$ antagonists MRS 2179, MRS2279 and MRS2500 (Wang et al., 2007, Gallego et al., 2006, Gallego et al., 2008, Gallego et al., 2011). $P2Y_1$ knocked out mice fail to generate a fIJP in response to electrical field stimulation (Gallego et al., 2012). This suggests an interaction between purinergic P2, particularly $P2Y_1$ receptors and SK_{Ca} channels on the smooth muscle cell membrane.

K_{ATP} channels may be present in the smooth muscle cells; conductance of these channels increases as intracellular ATP concentrations decrease ((Farrugia, 1999).

Delayed rectifier K^+ channels open following a short delay after membrane depolarisation and are also slow to deactivate. They differ from the previously mentioned K^+ channels as Ca^{2+} does not activate them. They contribute to maintaining the resting membrane potential. Delayed rectifier K^+ channels are divided into 9 families based on the expression of their α subunits, not surprisingly these channels vary in response to pharmacological agents and in their characteristics along the gastrointestinal tract (Farrugia 1999).

Other types of channels involved in smooth muscle cells include: two-pore potassium channels, inward rectifiers, ether-a-go-go related channels, M-current channels and MinK channels (Sanders, 2008).

1.2.5 Calcium release from intracellular stores

Within smooth muscle cells, calcium is stored in the sacroplasmic reticulum and mitochondria. Inositol (1,4,5)- trisphosphate receptor operated calcium channels (IP_3R) are responsible for much of the release of calcium from the sarcoplasmic reticulum. Literature regarding calcium release involving ryanodine receptors is controversial in smooth muscle. The release of calcium by IP_3R is regulated by the production of IP_3 which depends on the activity of a variety of G-protein coupled receptors (Berridge and Irvine, 1989).

G proteins coupled receptors are gated by a number of ligands, and are comprised of an α and $\beta\gamma$ subunits. When a ligand binds to the G protein coupled receptor, the $\beta\gamma$ subunits dissociate from the G protein. On the α subunit, GTP replaces GDP, resulting in dissociation of the $G\alpha$ complex from the receptor. Both the activated $G\alpha$ and the $\beta\gamma$ subunits are able to influence other cellular proteins activity. In the case of IP_3 production, activation of an isoform of phospholipase C (PLC) is a target (Somlyo and Somlyo, 1994). In gastrointestinal smooth muscle, there are four isoforms of PLC (PLCβ1-4). PLCβ1 is activated by the alpha subunit $G_{q/11}$ from receptors such as cholecystokinin-A (CCK-A), muscarinic 3 (M3), neurokinin 1 and 2 (NK1 and NK2) or serotonin$_2$ (5-HT_2) (Sanders et al., 2012). PLC causes the formation of IP_3 via hydrolysis of phosphatidylinositol 4,5- bisphosphate (PIP_2). This reaction generates two second messengers; IP3 and diacylglycerol (DAG). Binding of IP_3 to IP_3R in the sacroplasmic reticulum allows the release of Ca^{2+} into the cytoplasm. DAG binds to and activates protein kinase C which regulates the function of many proteins by phosphorylation (Bolton et al., 1999).IP_3 as a stimulus for muscle contraction has been demonstrated in tracheal smooth muscle cells. Significant increases in IP_3 levels were measured in smooth muscle cells up to 500 ms after neural stimulation, however phophorylation of MLC and the generation of contraction increased after 500ms

(Miller-Hance et al., 1988). Also, suppressing hydrolysis of PIP_2 with the PLC antagonist U-73122 significantly reduced the amplitude of spontaneous contractions in strips of bladder smooth muscle (Tanaka et al., 2003).

The second messengers cAMP and cGMP activate cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) respectively to relax gastric smooth muscle cells (Bolton et al., 1999). Both PKA and PKG phosphorylate PLC and inhibit the production of IP_3, however there is some dispute as to whether these second messengers inhibit Ca^{2+} release by directly acting on the IP_3 receptor (Murthy, 2001). Activation of P2Y1 receptors by a purine (such as ATP, or ß-NAD), through $G_{q/11}$, leads to increased intracellular Ca^{2+} from intracellular stores, involving IP_3, DAG pathways as explained previously. However P2Y1 receptors are coupled to the Ca^{2+} activated K^+ channel SK3, and the increased intracellular Ca^{2+} concentration causes the cell to hyperpolarise. Nitric oxide (NO) released from inhibitory motorneurons activates guanylyl cyclase within the post junctional cell, thereby increasing cytoplasmic cGMP, which in turn activates protein kinase G (Sanders et al., 2012). These second messengers open K^+ channels to hyperpolarise the cell and activate myosin light chain phosphatase which limits the contraction apparatus. Neuropeptides, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP), through VPAC1 and VCAP1 activate G_s thus increase cAMP concentrations. This leads to increased intracellular Ca^{2+} concentrations which activate K^+ channels, and also may have affects on myosin light chain phosphatase (Hagen et al., 2006).

1.3 Slow waves

Many smooth muscle cells display oscillatory depolarisations and repolarisations of their membrane potential, which are generally called slow waves (Van Helden et al., 2010). When depolarisations reach a threshold, slow waves often cause a small amplitude contraction in smooth muscle cells. Rhythmical slow waves are purely myogenic in origin, as they persist in the presence of the Na channel blocker tetrodotoxin (TTX) (Nonomura et al., 1966, Bülbring and Tomita, 1967).

1.3.1 Electrophysiology

The frequency, amplitude and shape of slow waves vary between regions of the gut and across species. In the mouse, slow waves in the stomach occur at 3-5 cycles per minute (Dickens et al., 2001) compared to 30 cycles per minute in the ileum (Ward et al., 1994). Slow waves occur in the human colon at about 2-4 cycles per minute (Rae et al., 1998), whereas in the canine colon they occur at approximately 6 cycles per minute (Smith et al., 1987a).

The slow wave often commences with a rapid depolarisation or *upstroke phase* followed by a *plateau phase* where the membrane potential is maintained for several seconds. In circular muscle cells from the guinea pig gastric antrum the amplitudes for this depolarisation was 27-35mV and the rate of depolarisation were less than $0.2Vs^{-1}$ (Dickens et al., 1999). In some instances, a slight repolarisation proceeded the plateau phase. At the end of each slow wave, repolarisation returns the membrane potential back to levels which preceded the rapid depolarisation.

1.3.2 Ionic basis for slow waves

The inward current of some gastrointestinal smooth muscle slow waves is mediated by L-type Ca^{2+} channels, since transient inward currents recorded in these cells are largely inhibited by nifedipine (Ward and Sanders, 1992). However, slow waves can be recorded from smooth muscle cells in the presence of dihydropyridines (Ozaki et al., 1991, Suzuki and Hirst, 1999) although the duration of the plateau phase is limited. This suggests that the upstroke of the slow wave is initiated in another cell which is electrically coupled to the smooth muscle cell.

1.4 Interstitial cells of Cajal

Interstitial cells of Cajal (ICC) are pacemaker cells in the gastrointestinal tract that trigger the electrical oscillations in the smooth muscle membrane potential. Through electrical coupling with smooth muscle cells they contribute to motility patterns. ICC found within various layers of the gut have different functions: ICC-MY (or ICC-MP) are found between the circular and longitudinal muscle layers; ICC-SM lie on the submucosal surface of the circular muscle layer; ICC-IM are ICC that are intermingled within the muscle layers (intramuscular); within the septa separating muscle cell bundles are ICC-SEP; ICC-DMP have been identified in the small intestine near the submucosal border within the circular muscle layer and have a similar role to ICC-IM. Ultrastructural analysis, immunohistochemistry and functional analysis studies have all been used to study the functions of these cells. However definitive confirmation of these functions has largely relied on the use of transgenic and mutant mice.

1.4.1 ICC and the pacemaker region

The pacemaker region within the gastrointestinal tract is contained within the tunica muscularsis (Suzuki et al., 1986). Within the tunica muscularis are fibroblasts, enteric neurons, glial cells, immune cells, smooth muscle cells and ICC. Suzuki et al. (1986) identified the boundary between circular muscle and longitudinal muscle layers as the site of slow wave generation in open segments of feline jejunum using intracellular recordings. Slow waves were largest at this boundary and decreased in amplitude as impalements were made closer to the inner circular muscle layer. The amplitude of slow waves in the longitudinal muscle was also smaller than that recorded at the boundary (Suzuki et al., 1986). This highlighted slow waves were being generated by cells at the circular muscle- longitudinal muscle border.

In the colon however, slow waves are also generated by pacemaker cells near the submucosal edge (Durdle et al., 1983). Intracellular recordings from cross sections of canine colon have been used to record from circular smooth muscle cells at different depths through the layer. Slow wave amplitude was greatest in the cells closest to the submucosal border and decreased with distance from this region, however slow wave

duration remained constant. The removal of the submucosa and a third of the circular muscle cells abolished slow waves entirely (Durdle et al., 1983). Slow waves were recorded from isolated segments of submucosa from the feline proximal colon but were absent in isolated muscularis externa segments without the submuscoal border (DU and Conklin, 1989). This was also shown in the canine colon (Smith et al., 1987c). In the canine colon, the membrane potential of circular muscle cells located near the myenteric border oscillated at around 19 cycles per minute to form an activity pattern termed *myenteric potential oscillations* (Smith et al., 1987b). This pattern persisted when the submucosal pacemaker cells were removed, indicating that the pattern generator for this activity was located in the myenteric region. Slow waves, occurring at around 5 cycles per minute, were recorded from circular muscle cells located near the submucosal border (Smith et al., 1987b). These two electrical events summated, and a mixed electrical pattern could be recorded from smooth muscle cells in the middle of these two regions (Smith et al., 1987b). It is this mixed pattern that appears to be important for the control of phasic contractions in the circular muscle layer.

Based on anatomical and morphological studies, ICC were long suspected to be the pacemakers within the gastrointestinal tract (Thuneberg, 1982, Taylor et al., 1977, Faussone-Pellegrini et al., 1990). Electron microscopic studies have highlighted an association between smooth muscle cells, nerve endings and ICC (Faussone-Pellegrini and Cortesini, 1985, Gabella and Blundell, 1981). Although not quantified, observations by Faussone-Pellegrini and Cortesini suggested ICC were more frequently innervated by nerve fibres than were smooth muscles cells. If each muscle bundle were considered as an anatomical unit, ICCs, nerve endings and smooth muscle cells would be present in each unit. They hypothesised that ICC may be important in myogenic motility as ICC were more abundant in the lower oesophageal sphincter where myogenic activity predominates, versus the oesophageal body where motility is strongly modulated by neural activity. Several types of junctions between smooth muscle cells and ICC have been reported including: gap junctions; desmosomes and interlocking membranes (Faussone-Pellegrini and Cortesini, 1985, Gabella and Blundell, 1981, Taylor et al., 1977, Thuneberg, 1982). Together with the electrophysiological data, these features are

consistent with the idea that slow waves originated from one region of the gut wall and could easily propagate throughout the smooth muscle layers.

Intracellular recordings from ICCs at the submucosal border in canine colon demonstrated that slow waves could be recorded from ICC (Barajas-López et al., 1989), however this did not demonstrate that these cells generated the wave pattern. Removing ICC-MY from the feline jejunum by sharp dissection abolished slow waves in circular muscle cells (Suzuki et al., 1986). Methylene blue is a dye which accumulates in ICC. Illuminating stained cells causes damaging changes to mitochondria. In canine colon, methylene blue stained ICC became swollen following exposure to light, while nerves and smooth muscle cells were unaffected. Intracellular recordings demonstrated that the selective damage to ICC coincided with a loss of slow waves (Liu et al., 1994).

C-kit is the proto-oncogene encoding the tyrosine kinase receptor Kit. This receptor has an extracellular binding site for its ligand, Stem Cell Factor, and an intracellular tyrosine kinase domain. Stem Cell Factor binding results in autophosphorylation, thereby activating the kinase activity. *C-kit* is functionally involved in the development of some cell line lineages. Following intraperitoneal injection of ACK2, a *c-kit* neutralising body, slow waves were abolished in the distal ileum of few day old mice (Maeda et al., 1992). Similarly, addition of ACK2 to preparations of murine stomach in organ culture reduced the number of Kit-immunoreactive cells and corresponded with a loss of slow waves recorded from smooth muscle cells when compared to control (Ordög et al., 1999). ACK2 has been used for immunohistochemical demonstration of c-kit on ICC. Labelling of the ICC in the guinea pig small intestine is one of the earliest examples of this immunoreactivity (Komuro and Zhou, 1996) and it has been widely used in other preparations since.

The dominant white spotting locus (*W)* in mice corresponds to a *c-kit* mutation (Ward et al., 1994). Mutation of the gene for Stem Cell Factor, *Sl* also results in loss of ICCs. The gene for Stem Cell Factor is contained within the *Steel* (*Sl)* locus. Complete deletion of

the locus (*Sl/Sl*) results in death by anaemia, however *Sl/Sl*^{d} heterozygotes survive, with partial loss of ICC. The same is true for homozygous *W/W* versus heterozygotes *W/W*^{V}. Demonstrated with immunohistochemistry and electron microscopy, ICC-MY were absent in the small intestine of *W/W*^{v} mutants. Slow waves could not be recorded from smooth muscle cells in these preparations, however smooth muscle action potentials still occurred (Hulzinga et al., 1995, Ward et al., 1994). ICC-MY were also absent in the *Sl/Sl*^{d} murine ileum and jejunum. Electrical recordings from the circular muscle did not record slow waves but smooth muscle action potentials persisted in *Sl/Sl*^{d} muscle (Ward et al., 1995). These functions led to the conclusion that ICC-MY were the cells which generate pacemaker activity within the gut. However, the persistence of action potentials indicated neurons were still able to function in these mutants.

Kit signalling is pivotal in determining the cell lineage for the formation of ICC during embryogenesis. ICC-MY and slow waves are evident by E17 in embryonic mice (Beckett et al., 2006). Muscle strips from murine jejunum at P0, exposed to either ACK2 or imatinib mesylate (a tyrosine kinase inhibitor) show a loss of slow waves and immunohistochemistry revealed an absence of ICC-MY (Beckett et al., 2006). Removal of the antagonists resulted in detectable presence of ICC-MY and more prominent slow waves over 3-12 days. In comparison, in *W/W*^{V} mice ICC-MY and slow waves consistently failed to develop in culture (Beckett et al., 2006).

Supporting evidence that slow waves are generated from a myogenic source and do not require the ENS came from work conducted on glial derived neutrotrophic factor (GDNF) knock out mice. The gastrointestinal tract of these mice is mostly aganglionic. Slow waves persisted in ileal smooth muscle cells of GDNF-/- mice. Preparations were immunoreactive for the Kit protein, but not for the neural markers protein gene product (PGP) and glial fibrillary acidic protein (GFAP) (Ward et al., 1999). Therefore, the ENS is not required for the generation of ICC nor for the formation of the slow waves.

Dickens et al. (1999) unequivocally demonstrated that slow wave activity originates in ICC-MY by simultaneously recording from an ICC-MY and a circular smooth muscle cell in guinea pig stomach. The depolarisation in ICCs consistently preceded that of slow waves in smooth muscle, had a faster rate of rise and greater amplitude (Dickens et al., 1999). They called the depolarisation in ICCs '*driving potential.'* The authors also demonstrated electrical coupling between these cells, as injecting a current pulse into the ICC-MY resulted in an electrotonic shift in the membrane potential of the impaled circular muscle cell. This result is significant as it provided a mechanism by which the driving potential in the ICC-MY could change the resting potential of the circular muscle cell. Dickens et al. (1999) recorded a third type of activity from longitudinal muscle cells, different from 'driving potentials' in that it was smaller in amplitude and had a slower rate of depolarisation than circular muscle. They called the potential in longitudinal muscle *'follower potential'.* These three forms of activity have also been recorded in the murine stomach (Hirst, 2002b).

1.4.2 ICC and neurotransmission

Unlike ICC-MY, ICC-IM are dispersed amongst smooth muscle cells bundles, running parallel with them. In the small intestine, there is a similar group of ICC that cluster within the deep muscular plexus, ICC-DMP. These ICC receive ultrastructurally defined close synaptic junctions from enteric motor neurons and form gap junctions with smooth muscle cells (Daniel and Posey-Daniel, 1984, Yamamoto, 1977). The anatomical arrangement of ICC-IM, enteric motor neurons and smooth muscle cells suggests that the neuromuscular junction within the gastrointestinal wall may include ICC-IM as an element (Rumessen and Thuneberg, 1982). The distance between synaptic release sties from enteric motor neurons and the smooth muscle cells is probably too far for efficient transmission. However, enteric motor neuron axons run parallel with ICC-IM for several hundred micrometers (Ward and Sanders, 2006).

Nerve fibres immunoreactive for neural nitric oxide synthase (nNOS), vesicular acetylcholine transferase (vAchT), vasoactive intestinal peptide (VIP) or substance P in various combinations, are closely aligned with c-kit immunoreactive ICC-IM (Beckett,

2002, Horiguchi et al., 2003, Ward et al., 2000a). Synaptotagmin and SNAP 25 immunoreactive varicosities, highlighting synapse-associated proteins, in either cholinergic or nitrergic motor neurons are closely apposed to ICC-IM (Beckett et al., 2005). ICC-DMP in the guinea pig small intestine are also closely associated to nNOS and substance P immunoreactive nerve fibres (Toma et al., 1999, Wang et al., 1999). Nerve fibres immunoreactive for nNOS and vAchT in the human ileum are also closely associated to ICC-DMP (Wang et al., 2003).

ICC respond to both exogenous or endogenous neurotransmitters, and have the elements required for neuromuscular transmission. ICC express nitric oxide sensitive guanylate cyclase as evidenced by immunohistochemistry (Young et al., 1993). In response to exogenous NO or electrical nerve stimulation, ICC-IM in canine colon had increased cGMP immunoreactivity (Shuttleworth et al., 1993). Intracellular Ca^{2+} concentrations of freshly dispersed canine colonic ICC transiently increased on exposure to carbachol or substance P (Publicover et al., 1992). Multiple receptors for neurotransmitters involved in neuromuscular transmission in the gastrointestinal tract have been located on ICC-IM. RNA of muscarinic receptors (M2, M3,) VIP-1 and tachykinin receptors (NK1 and NK3) have been identified from freshly dispersed ICC-IM of the murine fundus (Epperson et al., 2000). RT-PCR has identified, $NK1_R$, M_2 and M_3, purinergic receptors ($P2Y_1$ and $P2Y_2$) and VIP receptor from manually harvested ICC-DMP of the murine small intestine (Chen et al., 2006).

W/W^{v} mice lack ICC-IM in the stomach, the lower oesophgeal sphincter and the pyloric sphincter. Single pulse electrical field stimulation of enteric nerves evoked excitatory junction potentials (EJPs) and inhibitory junction potentials (IJPs) in circular smooth muscle cells from the gastric fundus in wildtype mice. The IJP was rescued by L-Arginine, and therefore largely meditated by NO. Junction potentials were reduced in *W/W*^{v} mutant mice. However preparations from wild type and *W/W*^{V} mice, showed comparable depolarisations and increases in tone in response to exogenous acetylcholine therefore impaired mechanisms in the smooth muscle cell did not account for the loss of response. Immunohistochemistry confirmed that ICC-IM were absent in

the W/W^V fundus but enteric nerves were present and many could be labelled for VAChT (Ward et al., 2000a). Therefore, smooth muscle cells in W/W^V lacked a response to acetylcholine released from nerves. This suggests that acetylcholine released from enteric nerves may not reach smooth muscle cells but may act via ICC-IM which are then coupled to smooth muscle cells.

ICC-IM are also potentially involved in inhibitory transmission. Electrical field stimulation of a bundle of circular muscle cells from the gastric antrum of wildtype mice evoked an initial fast IJP, followed by a slow IJP, then a large depolarisation. The excitatory component was inhibited by atropine, the slow IJP was blocked by L-NA and subsequent addition of apamin reduced the amplitude of the fast IJP. Comparable recordings from W/W^V mice revealed only a fast IJP and no other responses to a repetitive stimulation. L-NA had no effect on the fast IJP, however apamin reduced its amplitude (Suzuki et al., 2003a). Therefore, both cholinergic and nitrergic neural inputs are mediated via ICC-IM although a purine (such as ATP, ß-NAD, ADP (Mutafova-Yambolieva et al., 2007, Crist et al., 1992)) from inhibitory motor neurons may still have some effect on muscle cells or another intermediary cell (Kurahashi et al., 2011, Vanderwinden et al., 2002b, Klemm and Lang, 2002).

While this evidence supports the idea that ICC-IM are an intermediary cell between nerve terminals and smooth muscle cells for some types of neuromuscular transmission, more direct evidence is needed. Concurrent recording of junction potentials in smooth muscle cells and ICC-IM from the same preparations, and then selectively inhibiting ICC-IM function would provide more direct evidence. The subsequent loss or persistence of junction potentials would confirm whether it does have a role.

ICC-IM are associated with an electrical pattern called *unitary potentials* (Hirst, 2002b). These are small amplitude fluctuations of membrane potential that can also be recorded from smooth muscle cells. Unitary potentials may be the basic pacemaker units produced by ICC which summate to form pacemaker potentials and trigger the slow

wave in muscle cells (Edwards et al., 1999). In ICC-IM , direct input from enteric motor neurons increases the likelihood of summation of unitary potentials. In wild type murine antrum, acetylcholine released from enteric neurons phase advanced slow waves, but not in preparations from W/W^V mice, which are devoid of ICC-IM (Beckett, 2003, Forrest et al., 2006). Slow waves can be recorded from smooth muscle cells in some regions which are devoid of ICC-MY but contain ICC-IM (Van Helden et al., 2010) such as the guinea pig corpus and in the murine gastric antrum in the lesser curvature region. However, the rate of the upstroke phase is slower in these preparations compared to those where the ICC-MY are present (Hashitani et al., 2005, Hirst, 2002b). ICC-IM are absent in the antrum of W/W^V mice however ICC-MY remain. The amplitude of slow waves in smooth muscle of these preparations is reduced, as is the duration of the plateau potential (Dickens et al., 2001). Therefore there appears to be a role for both ICC-MY and ICC-IM in the generation of the slow wave waveform (Van Helden et al., 2010) .

1.4.3 Generating spontaneous activity in ICC

The mechanisms by which ICC generate spontaneous activity is not fully understood. The initiation of slow-waves requires the release of calcium from intracellular stores through activation of IP_3 receptor-operated stores. 2-aminoethoxydiphenylborate (2-APB) which inhibits IP_3 receptors, significantly reduces the amplitude of spontaneous or evoked slow waves (Hirst, 2002a, Ward et al., 2000b). However it should be noted that 2-APB is also a gap junction blocker (Bai et al., 2006). This is supported by evidence that slow waves are absent in smooth muscle cells from the gastric antrum of mutant mice which lack IP_3 type 1 receptor (Suzuki et al., 2000). While IP_3 receptor operated stores are thought to be involved in the initiation of slow waves, the manner in which they effect depolarisation is uncertain.

A decrease in cytoplasmic Ca^{2+} generates a large, inward, non-selective cation current in ICC (Koh, 2002). Using a combination of intracellular recording and video fluorescence imaging in murine small intestine, Ward and colleagues demonstrated that fluctuations in mitochondrial Ca^{+} concentrations preceded the onset of the inward

current (Ward et al., 2000b). Agents that inhibit respiratory processes within the mitochondria blocked the inward current and mitochondrial Ca^{2+} concentrations. Slow wave activity from murine small intestine, guinea pig stomach and canine colon are also inhibited by these agents (Ward et al., 2000b).

Sanders and colleagues hypothesised that this non-selective cation channel may be responsible for the unitary potentials and plateau phase of slow waves (Sanders et al., 2006). They originally suggested the pacemaker activation results from the following sequence of mechanisms:

1) A small puff of Ca^{2+} is released into the cytoplasm from IP_3 operated sacroplasmic reticulum stores. This triggers Ca^{2+} uptake channels on the mitochondria to open, allowing Ca^{2+} to enter the organelle.

2) More Ca^{2+} than was released by the puff is taken up by the mitochondria, therefore the Ca^{2+} concentrations in this region of cytoplasm decrease below normal.

3) The reduced Ca^{2+} concentration activates non-selective cation conductance, evoking an inward current which depolarises the cell membrane. This component explains the unitary potentials

4) Membrane depolarisation activates voltage dependent Ca^{2+} channels resulting in an influx of Ca^{2+}. This action results in the secondary component of the slow wave. (Sanders et al., 2006)

This hypothesis was based on experiments conducted on cultured ICC cells and did not take into account evidence that a Ca^{2+} activated Cl^- conductance may be involved in the generation of slow waves, since these currents had not been readily recorded from cultured ICC (Takeda et al., 2008, Goto et al., 2004). However, pharmacologically blocking Cl^- channels either completely inhibits slow waves recorded from smooth muscle cells, (Hirst et al., 2002) or limits the duration of the plateau phase of the driving

potential in ICC-MY (Kito, 2003). It is thought that Ca^{2+} released from IP_3 stores may activate the Cl^- conductance. ANO1 had been identified as the Ca^{2+} activated Cl^- channel on the ICC membrane. The gene encoding ANO1 is the highest expressed gene in ICC. In fact, ANO1 is a specific immunohistochemical marker for labelling ICCs, more specific than c-Kit which also labels mast cells (Gomez-Pinilla et al., 2009). Electrophysiology experiments have identified a large conductance inward current in ICC with similar properties to inward currents recorded from ANO1 transfected HEK cells (Zhu et al., 2009). Isolated ICC from T*mem*16*a* knockout mice, which do not express ANO1, do not generate slow waves (Hwang et al., 2009). It would appear that ANO1 has a role in generating spontaneous activity in ICC. Further studies are needed to understand its interactions with Ca^{2+} influx from intracellular stores, changes in mitochondrial Ca^{2+} concentrations and non-selective cation channels, and how these interactions generate driving potentials in ICC.

1.4.4 Coordinating and propagating spontaneous activity of ICC

Each ICC can display spontaneous depolarisations yet their activity is normally coordinated to produce pacemaker rhythms which propagate in smooth muscle cells.

In intact specimens slow waves can propagate at 5-40mm/s in the muscularis externa. This propagation may depend on the coordinated release of intracellular calcium and calcium diffusion(Ward, 2004). Interestingly, exposing a segment of canine antrum to either a T and L type channel blocker, or to reduced extracellular calcium will block aboral propagation of spontaneous slow waves (Ward, 2004). Therefore, voltage dependent calcium entry may be required for slow wave propagation. It is thought that voltage strongly coordinates the release of calcium from these intracellular stores, where depolarisation enhances the activity of these stores (Imtiaz et al., 2010, van Helden and Imtiaz, 2003). For depolarisation to be a strong mechanism which synchonises the activity of intracellular stores, smooth muscle and ICC must be coupled to form a functional electrical syncytium, allowing rapid propagation of the depolarisation. The presence of gap junctions is believed to be significant in the coordination and propagation of slow waves along the gastrointestinal tract (Serio et al., 1991, Serio et al., 1990). The synchrony of slow waves is lost when gap junctions are

blocked pharmacological by agents such as 18ß glycyrrhetinic acid (van Helden and Imtiaz, 2003).

1.5 Cellular Junctions

Junctions between cells forming sheets or 3 dimensional structures are a necessary feature for multicellular organisms. Intercellular junctions may provide a barrier, in the case of endothelial and epithelial cells, between luminal content and the internal mileu. Junctions may also permit coordinated mechanical activity, for example in smooth muscle cells. Sheets of smooth muscle cells require physical attachments between cells as well as connections allowing the propagation of action potentials and the spread of metabolities. Five main types of physical attachments between cells have been described:

Adherens junctions are multi-protein clusters forming dense plaques along the surface between two adjacent cells (Cereijido, 2004) and are anchored by cadherins, a cell adhesion molecule (Ebnet, 2008). These cadherins extend within the cytoplasm to link and generally bind with caterins, which are protein structures located on the cytoskeleton (Ebnet, 2008). These junctions also consist of several extracellular domains which provide a means for binding with adjacent cells (Cereijido, 2004). Their function appears to be largely related to mechanical connection between cells.

Occluding junctions allow sheets of cells to form a barrier and thereby regulate diffusion of ions and molecules between compartments (Mater ad Balda 2003). In vertebrates they are also referred to as *tight junctions* or *zonula occludens.* Transmembrane protein adhesion molecules, the main type being claudins, are embedded in the membranes of adjacent cells allowing them to connect and seal the gap between cells. Gaps between tight junctions may give rise to ‘leaky epithelia’ where they effectively form low resistance pathways. This leakiness facilitates permeation of substances across membranes. The tightness of occluding junctions can be modulated by phosphorylation of the junction proteins (Cereijido, 2004). Tight junctions are common between epithelial cells, eg in the gut wall, and are typically located towards the apical end (Matter and Balda, 2003).

Desmosomes are sites where two adjacent cells are tightly bound, via close appositions which form electron dense plaques (Green and Simpson, 2007). The plaques attach to intermediate filaments within the cell and this provides the base for a strong scaffolding. This structure can resist large mechanical stresses and hence these junctions are typically noted in the epidermis and myocardium (Green and Simpson, 2007).

Signal relay junctions are structures underlying cellular communication which relay signals from one cell to an adjacent cell by releasing a chemical which is received via a receptor on the plasma membrane of the recipient cell. An example of this form of junction is a chemical synapse between neurons.

Channel-forming junctions provide a passage between two cells which link their cytosols. Multiple channel junctions within one location on a plasma membrane form a *gap junction* or *nexus.* Due to their involvement in smooth muscle cells of the gut, gap junctions that will be the main intracellular junction considered here.

1.6 Gap junctions

Gap junctions, sometimes known as *intercytoplasmic junctions*, link the cytoplasm of two adjacent cells. Multiple cells connected in this manner form a functional syncytium. Electron microscopy reveals regions of cell to cell contact with a characteristic pore providing cytoplasmic continuity between cells. This enables ions, second messengers and small metabolites to move between the cells without leaking into the extracellular fluid. These junctions contribute to the biochemical and electrical coupling involved in cellular processes such as: cell synchronisation; differentiation; cell growth; and for metabolic coordination of a vascular organs including the epidermis and lens (Meşe et al., 2007).

1.6.1 Morphology of gap junctions

Gap junctions comprise two hemichannels, or *connexons*, from two adjacent cells fusing together. Each connexon is comprised of six small proteins called connexins. Nicholson et al. (1987) demonstrated the presence of multiple ‘protein subunits’ before

the term connexin became standardised. Connexons were believed to be comprised of six identical protein subunits, however western blotting and immunohistochemistry have demonstrated not only that there are multiple subunits but that different subunits can be present in one connexon (Nicholson et al., 1987). It is now known that there are approximately 21 different connexins in humans and mice (Sáez et al., 2010), which vary in their conductance and permeability properties.

Channels can be *homotypic* meaning they are made of the same connexon, or *heterotypic* meaning they are made of two different connexons. Connexons can be *homomeric* meaning they are comprised of 6 copies of the same connexin, or *heteromeric* meaning they are composed of different connexins. The interaction of connexins in this manner determines the channel size and shape, thereby influencing the conductance and permeability properties of each channel (Kumar and Gilula, 1996).

Electron microscopy demonstrated connexons cluster together in a lattice-like arrangement. Freeze fracture studies have enabled gap junction morphology to be visualised in detail. Tissue is frozen and then cracked, producing a fracture plane. This occurs in a region of low mechanical resistance such as through the hydrophobic core of a lipid bilayer. A replica is produced from heavy metal deposition and strengthened by a carbon film (Lucio Benedetti et al., 2000). These techniques have demonstrated in different tissues that gap junctions are closely clustered together in a hexagonal arrangement (Revel et al., 1973, Baldwin, 1979). This may maximise the shared cytoplasm. The number of channels within a gap junction may vary over time as channels are assembled and degraded. Formation and degradation of these channels is strictly regulated as they have a half life of only a few hours (Meşe et al., 2007)

1.6.2 Channel formation

The synthesis of individual gap junction proteins occurs in the rough endoplasmic reticulum (Falk, 2000). In membrane-bound ribosomes, connexins are translated and become incorporated into the membrane of the endoplasmic recticulum (Martin and

Evans, 2004). When formed, each connexin is a monomer. From the endoplasmic reticulum the monomer is transferred to the Golgi apparatus and formed into an oligomer, the connexon (Salameh, 2006). The connexon is then transported via microtubule-based transport to the plasma membrane where it is incorporated into the cell membrane (Pfeffer and Rothman, 1987). These channels cluster together in gap junctional plaques. New channels are formed around the outer edge of the cluster, while old channels in its centre are removed (Meşe et al., 2007). The manner by which gap junction channels are targeted into a specific membrane site is not yet understood, however two mechanisms have been hypothesized. The channels are either directed to the exact site of the junctions; or they may be incorporated into the plasma membrane and then migrate laterally to the required site (Falk, 2000). Preliminary research suggests the latter option; this hypothesis is also supported by the presence of channels and closed hemichannels incorporated in plasma membranes of cultured cells away from junctional complexes (Falk, 2000). If the connexon does not come into contact with a hemichannel from an adjacent cell, then a non-junctional hemichannel is formed which typically remains closed. Alternatively, channels that are juxtaposed to a hemichannel in another cell can align with it, resulting in the formation of an intercellular channel (Schulz et al., 2007).

Each connexin subunit has four transmembrane domains, two extracellular loops, one intracellular loop, an intracellular N terminus and an intracellular C terminus (Bloomfield and Völgyi, 2009). The extracellular loops of adjacent connexons are believed to interact, enabling the two halves to form a complete channel (Falk, 2000). Calcium dependent adhesion molecules such as E cadherins facilitate the process of hemichannel fusion (Gourdie et al., 2006).

1.6.3 Degradation

For reasons not currently understood, gap junctions have a half life of a few hours, therefore they are constantly being renewed (Laird, 2006). The channels in the centre of the plaque become internalised within a vesicle which is endocytosed, leaving behind an ‘annular junction’. Once internalised the gap junction is thought to be degraded by

either lysosomal enzymes or proteosomal pathways (Jordan et al., 2001, Leithe and Rivedal, 2007). This process increases during conditions of stress and is associated with inflammatory responses (Chanson et al., 2005).

1.6.4 Gap junctions and metabolic coupling

The transfer of metabolites between cells is dependent on the connexins that constitute the gap junction. Metabolic coupling allows molecules to be transferred between cells and also permits metabolic coordination. Gap junction channels were originally thought to be non-specific, allowing all ions and metabolites less than 1kDa to passively diffuse between cytoplasms (Spray, 1996). While diffusion of small molecules and ions is permitted, these channels are selectively permeable to larger metabolites and secondary messengers (Axelsen et al., 2007). The connexins which constitute the connexons determine their permeability. Unpaired channels typically remain closed and thus prevent leakage of molecules, however in some instances hemichannels can open to allow the release of small molecules into the extracellular space. For example metabolic inhibition is an in vitro model of ischemia; in HeLa cells transfected with mouse Cx32, metabolic inhibition increased hemichannel permeability and resulted with an increase in intracellular Ca^{2+} concentration (Sánchez et al., 2009).

1.6.5 Gap junctions and electrical coupling

Gap junctions permit the propagation of electrical signals as ions, and hence charge, pass rapidly through the channels (Spray, 1996). This electrical synapse allows signals to transmit many times faster than chemical synapses (Lodish et al. 2004.) providing rapid signal propagation in excitatory tissues and electrical synchronisation of cells. In the heart this contributes to efficient cardiac output (Barr et al., 1965, Weidmann, 1952).

Gap junctions were first proposed to be involved in the propagation of a low resistance electrical signal by Weidmann in 1952. Using intracellular recording techniques, Weidmann (1952) recorded from single Purkinje fibres from excised strands of the

ventricular conductive system of juvenile goat. Current pulses were injected through one electrode and membrane potential responses were measured with a recording electrode placed at varying distances. Membrane potential responses were greatest when distance in from the stimulus was minimal, but a small response could still be recorded at almost 5mm. Since each fibre is made up of a series of cells, this demonstrated that current was able to flow between cells and therefore a low resistance pathway was present.

Demonstrating the flow of current between cells was a significant step in characterising electrical coupling, however it provides little information on the time course of coupling and ionic conductance. The development of patch-clamping allowed the properties of individual channels to be elucidated. On paired cells using dual whole-cell voltage clamp, junctional conductance can be calculated to measure the total current through the gap junction. Pairs of cardiac cells from 7 day chick embryos were used to measure junctional and non-junctional currents (Veenstra and DeHaan, 1986). Channel conductance was recorded at approximately 165 picoSiemens, and this conductance was found to be independent of voltage (Veenstra and DeHaan, 1986).

The properties of specific connexins, or combinations of connexins forming channels at this point had not been specifically tested. Developments in molecular biology allowed the electrical properties of cells transfected with specific connexin proteins to be analysed, so that the conductance of specific channel types could be measured (Ebihara et al., 1989).

1.6.6 Factors influencing gap junction conductance

As with all ion channels, gap junction conductance is influenced by a range of factors. In some cases, gap junctional conductance is voltage dependent. Using paired whole cell voltage clamp, small potential differences can be applied across these junctions - *transjunctional voltages.* Generally the polarity of transjunctional voltages is not important in affecting gap junctional conductance, but large voltage steps will decrease

junctional conductance compared to small steps. Dye coupling, which is used as an alternative indictor of gap junction coupling is also reduced with large changes in transjunctional voltage compared to small changes (Spray et al., 1979). Voltage sensing by gap junctions may depend on multiple voltage sensors on the connexins, however the mechanisms which lead to conformational change of the channels remain to be fully understood (Kumar and Gilula, 1996, Rozental et al., 2001).

Evidence suggests that all gap junctions are pH sensitive, with changes in intracellular pH modulating conductance. Extracellular acidity does not reduce gap junctional conductance (Spray and Bennett, 1985). Generally, increases in acidity decrease conductance; slight increases in alkalinity increase conductance. Spray et al. (1981) demonstrated this in the cleavage stage of blastomeres from amphibian and teleost embryos. Gap junctional conductance was almost abolished in acidic conditions and began to plateau at slightly alkaline conditions (Spray et al., 1981). However, in doublet guinea pig cardiac cells the same pH range used by Spray et al. (1981) had little influence on junctional conductance (Noma and Tsuboi, 1987). Much more acidic conditions were required to block gap junction conductance. Since pH dependence of gap junctional conductance varies between tissue types, researchers have assumed differential effects of pH on different connexins. The conductances of HeLa cells transfected with either Cx46 or Cx49 were influenced by a different pH range (Eckert, 2002). It was speculated that these differences arose from the variations in the cytoplasmic loop at the C terminus and the N terminus (Eckert et al. 2002). Gap junctions slowly transition from an opened state to closed by changes in intracellular pH, compared to changes in transjunctional voltage which evoke rapid transitions (Bukauskas and Peracchia, 1997)

The carboxy terminus and the internal loop regions of connexins contain multiple sites for phosphorylation (Martin and Evans, 2004). Phosphorylation is mediated by Ca^{2+}/ calmodulin-cAMP and cGMP dependent kinases. Each connexin, and therefore each channel, differs in its response to the protein kinases (Kwak et al., 1995b, Kwak et al., 1995a). In acinar cells, exposure to acetylcholine increased intracellular calcium and

dye coupling was significantly reduced. This effect was prevented when calcium stores were sequestered or when cells were incubated in calcium free culture medium (Chanson and Suter, 2001). Increases in intracellular calcium also decrease gap junction conductance between guinea pig cardiac cells (Noma and Tsuboi, 1987). This mechanism could provide a means of control of the spread of electrical excitability through tissues.

Neural activity in retina can modulate the activity of gap junctions (Bloomfield and Völgyi, 2009) (Bloomfield). The conductance of gap junctions between rod and cone photoreceptors in the eye is modulated by the release of dopamine. This is probably via changes in intracellular cAMP concentrations, thereby altering the activity of protein kinase A. Not only does this influence mean open conductance times, but it also affects junctional densities. Electrical coupling between horizontal cells in the eye is affected by nitric oxide (NO) acting via cGMP (Bloomfield and Völgyi, 2009).

A variety of pharmacological agents block gap junctions. However, they are mostly non-specific for the connexon channels. Targeting specific connexons may be difficult as access to the extracellular domains on the gap junction channel is limited by their arrangement. Long chain alcohols such as heptanol and octanol, and aldehydes such as formaldehyde and glutaraldehyde block gap junctions. Gap junctional conductance can be reversibly and dose dependently reduced in the presence of heptanol (Takens-Kwak et al., 1992). However, non-junctional currents are also inhibited, therefore heptanol does not act specifically at the gap junction (Takens-Kwak et al., 1992). As evidenced by their non-specific action, long chain alcohols may not act on specific sites on the connexins rather they affect interactions between lipids and channels in the membrane (Rozental et al., 2001).

18α-glycyrrhetinic acid and 18β-glycrrhetinic are other gap junction inhibitors. Carbenoxolone, a water soluble analogue of 18α-glycyrrhetinic acid, is a widely used gap junction inhibitor. In pairs of retinal cells, carbenoxolone (100μM) reduced the

voltage-response curve of gap junction conductance when compared with control in a dose depended manner (Vessey, 2004). However, calcium imaging demonstrated that the influx of calcium in retinal cells caused by depolarisation, was also reduced in the presence of 100μM carbenoxlone (Vessey, 2004). Thus glycyrrhetinic acids also affect other target/channels in the cells. As well as being gap junction blockers, glycyrrhetinic acids limit the conversion of cortisol to cortisone by inhibiting 11-beta hydroxysteroid dehydrogenase (Edwards et al., 1988, Monder et al., 1989). The mechanisms of action of glycyrrhetinic acids may involve phosphorylation of the connexins, affecting either channel formation or channel gating (Evans and Leybaert, 2007),.

Bai et al. (2006) demonstrated the sensitivity of connexins to 2-aminoethoxydiphenyl (2-APB). The gap junctional conductance of N2A cells (neuroblastma cell line) expressing either Cx26, Cx30, Cx36, Cx40 and Cx45 were reversibly inhibited by 2-APB at 20μM. Cells expressing Cx32, Cx43 and Cx46 were inhibited, but at higher concentrations of 2-APB (Bai et al. 2006). However 2-APB also inhibits the IP_3 receptor and is therefore a non-specific gap junction inhibitor (Maruyama et al., 1997). Very little is known about how 2-APB modulates gap junction permeability (Yang et al., 2011).

Engineered uncoupling agents have been made to target extracellular domains on the connexons in an effort to inhibit gap junctions. Antibodies raised to peptides derived from connexins (Becker et al., 1995) and connexin mimetic peptides have been shown to functionally block coupling. These agents bind to sites on the extracellular domains of the connexons and, in doing so, they inhibit channel formation. However, the molecular mechanisms of inhibition are not known. Examples of mimetic peptides include *Gap 26* and *Gap 27.* These short synthetic peptides possess the amino acid sequence for segments on extracellular loop I and II, respectively, of gap junctions composed of connexin 43. Gap 26 and 27 reversibly limit intercellular communication (Dora et al., 1999, Chaytor et al., 1997, Boitano and Evans, 2000). Mimetic peptides have a higher potency to inhibit gap junctions than the external loop domain antibodies, however neither type of agent is as efficacious as other gap junction blockers (Rozental

et al., 2001). However, their specificity for targeting gap junctions is a highly desirable feature.

1.6.7 Clinical significance of gap junctions

The absence of gap junctions or certain connexins has been associated with some diseases (Meşe et al., 2007). The secretion of certain hormones is affected by the presence or absence of gap junctions (Michon et al., 2005). A reduction in gap junction communication has been associated with an increase in tumor growth in some tissues (Salameh and Dhein, 2005). Heart arrhythmia may be caused by a decrease in gap junctional intercellular communication in some cases (Basso et al., 2012) and antiarrythmic peptides such as rotigaptide increase the intercellular conductance of gap junctions (Axelsen et al., 2007). Immunohistochemical experimentation has suggested a marked decrease of the levels of Cx43, in Hirschsprungs disease bowel compared with normal (Nemeth et al., 2000). In the brain electrical coupling may have a role in generation of seizures, however the mechanisms are unclear (Salameh and Dhein, 2005). The anticonvulsant effects of carbenoxolone have been demonstrated in many epilepsy models, both *in vivo* and *in vitro* (Connors, 2012).

1.6.8 An electrical syncytium in the gastrointestinal tract?

Simultaneous intracellular recordings from ICC-MY and circular smooth muscle cells reveal that the driving potential generated by ICC-MY precedes the slow wave (Dickens et al., 1999, Hirst, 2002a). This suggests that ICC and smooth muscle cells are electrically coupled, however the nature of this coupling needs to be more completely elucidated.

Electrical coupling is a method used to demonstrate functional coupling in the *muscularis externa*. Intracellular recordings with double impalement on whole mounts allows simultaneous recordings from pairs of cells from the same specimen. For example, one electrode can record from either a circular muscle or a longitudinal muscle cell, and the other can record from an ICC-MY or another circular muscle cell.

In guinea pig antrum, injecting current into one circular muscle generated a large electrotonic potential in the second circular muscle cell, indicating that circular muscle cells were strongly electrically coupled. Electrical coupling was less effective between ICC-MY and circular muscle, and longitudinal muscle and circular muscle had the weakest electrical coupling . Electrical coupling between circular and longitudinal muscle cells has been previously reported in the guinea pig ileum (Bywater and Taylor, 1986, Cousins et al., 2003).

Within the gastrointestinal tract, ultrastructural studies showed gap junctions between ICC, and ICC and smooth muscle cells. Lucifer Yellow injected into singular ICC-MY labelled coupled ICC (Belzer et al., 2002, Belzer et al., 2004). Dye coupling between ICC-MY and smooth muscle cells has not been seen in some studies (Belzer et al., 2002, Belzer et al., 2004), although it has been in others (Kobilo et al., 2003, Farraway et al., 1995). It is generally accepted that dye coupling results from gap junction coupling (Daniel and Wang, 1999). Dye molecules such as Lucifer Yellow and Neurobiotin have molecular weights over 300Da and unlike most ion channels, gap junctions have a very large pore allowing passage of molecules up to 1kDa. Manipulations that affect gap junction coupling also affect dye coupling. Gap junction blockers and decreasing intracellular pH reduce the number of dye coupled ICC and smooth muscle cells (Belzer et al., 2002, Belzer et al., 2004, Kobilo et al., 2003). Dye coupling increases with increased intracellular pH (Kobilo et al., 2003). A flaw of these studies is that the amount of dye injected into cells may vary with ranging current injection times. The time between injection and quantifying coupling is not also noted, therefore the amount of time allowing dye spread may also vary. This might contribute to differences between the percentage of cells dye coupled following injection of the dye.

Immunohistochemistry on gut from different species has identified Cx26, Cx32, Cx40, Cx 43 and Cx45 in the gut wall (Saez et al., 2003, Wang and Daniel, 2001, Seki and Komuro, 2001, Seki et al., 1998, Mikkelsen et al., 1993). In the guinea pig ileum, ultrastructural studies noted gap junctions between ICC-MY and the outer circular

muscle layer, but were not present between: ICC-MY and the inner circular muscle layer, ICC-DMP and inner circular muscle layer or between inner circular muscle cells. This was later confirmed with immunohistochemical labelling for Cx40, Cx43 and Cx 45 which showed sparse labelling in the inner circular muscle layer of the canine ileum (Wang and Daniel, 2001). However, intracellular recordings revealed that slow waves in circular muscle cells near the myenteric border or the deep muscular plexus were significantly reduced in amplitude and frequency by the gap junction blocker, octanol. Mechanically recorded contractions occurring at the same frequency as slow waves were also abolished by octanol. This suggests that gap junctions are required for the propagation of electrical activity, but are not numerous. It can be speculated that electron microscopy studies may have missed rare, small gap junctions. Daniel and colleagues hypothesised that gap junctions are not required for ICC to influence smooth muscle cells by assessing the influence of gap junction inhibitors on mechanical activity of canine colonic and ileal tissue (Daniel et al., 2001). TTX and $_{L}$-NOARG were administered to remove the influence of nerve activity. In colonic tissue, spontaneous mechanical activity persisted in the presence of carbenoxlone, however the amplitude and frequency of contractions decreased. In the ileum, the amplitude of contractions decreased, but the frequency increased with either carbenoxolone or 18 α glycyrrhetinic acid (Daniel et al., 2001). The decrease in amplitude suggests that gap junctions could be involved in the pacing mechanism, however the increase in frequency seems contradictory. It may be that uncoupling smooth muscle cells from the ICC resulted in uncoordinated activity patterns, therefore increasing the overall rate of spontaneous contractions. Intracellular recordings would be needed to confirm the effects of gap junction blockers on ICC pacing of smooth muscle cells.

Dye coupling between canine colonic longitudinal muscle cells is less extensive than between circular muscle cells (Farraway et al., 1995). Electrical coupling between longitudinal muscles, and between circular-longitudinal muscle cells has been demonstrated in the guinea pig ileum and gastric antrum. In two separate studies by Cousins and colleagues, injecting current into either a longitudinal muscle cell (Cousins et al., 1993) or a circular muscle cell resulted in an electrotonic potential in a neighbouring longitudinal muscle cell. While this is functional evidence of coupling

between longitudinal muscles and other cells in the external musculature, ultrastructural evidence of gap junction coupling is lacking. An electron microscopy study in the guinea pig ileum indicated that gap junctions are not present between longitudinal muscle cells and ICC-MY (Daniel et al., 1998, Cousins et al., 2003). A subsequent immunohistochemical study in the dog revealed an absence of labelling for Cx43, Cx40 and Cx45 in the longitudinal muscle of the lower oesophageal sphincter (LOS), the stomach antrum, the ileum and the colon (Wang and Daniel, 2001). Dense labelling for Cx43 was found in the circular muscle of the LOS, stomach and ileum; Cx40 staining was also prominent in the circular muscle of LOS and stomach. Indeed, in the same study by Cousins et al. (2003) that demonstrated electrical coupling between circular and longitudinal muscle cells in the guinea antrum, immunohistochemical labelling for Cx43, Cx40 and Cx45 was absent in the longtudinal muscle layer. Cx43 labelling in this layer was only present in regions which colocalised with c-Kit staining, thus labelling ICC. However, staining for Cx43 in the circular muscle layer, independent of c-Kit labelling, was present .

This growing body of evidence suggests that gap junctions are not present between neighbouring longitudinal muscle cells, longitudinal muscle cells and ICC-MY. Furthermore the suggestion that electrical coupling is not required for propagation of signalling through the musculature of the gut wall leads to the question: how are these cells coupled together? Ultrastructural studies have reported that longitudinal muscle cells form “peg and socket” arrangements with neighbouring cells (Daniel and Wang, 1999). Thuneberg and colleagues theorise that these peg and socket arrangements allow “stretch coupling” between cells. They demonstrated that contractions dominating in either the circular or longitudinal muscle layer correspond with an increase in peg and socket coupling in that layer. However, the number of gap junctions did not change (Thuneberg and Peters, 2001). The investigation of this mechanism may prove to be difficult as the molecular basis for peg and socket junctions is not known and therefore immunohistochemistry and molecular analysis is not currently possible. Indeed, Thuneberg and Peters (2001) demonstrated the difficulty of visualising this arrangement, which required a modified fixation method. There are also limitations in

functional analysis; functional imaging may be the best method suitable to demonstrate this arrangement.

1.7 Motility patterns of the gastrointestinal tract

Gastrointestinal motility is the result of interactions between slow waves, ICC, smooth muscle cells, gap junctions, input from the enteric nervous system and movement of content. The generation of specific motor patterns requires neural modulation from both intrinsic and extrinsic sources from sympathetic and parasympathetic nerves.

1.7.1 Muscle contractions

Simultaneous intracellular and mechanical recordings from the pig colon revealed that action potentials which coincide with slow waves generate an oscillatory mechanical activity occurring at the same frequency as the slow waves (Huizinga et al., 1983). In the mouse colon, intraluminal pressure waves recorded simultaneously with electrical recordings revealed 1:1 ratio of pressures waves with smooth muscle action potentials superimposed with slow-waves (Huizinga et al., 1998). The generation of smooth muscle action potentials does not necessarily require neural input since they can persist in tetrodotoxin (TTX) and atropine (muscarinic blocker) (Huizinga et al., 1998).

1.7.2 Peristalsis

Peristalsis is a wave of circular muscle contraction that causes the propulsion of contents within the intestine. In some cases, peristalsis requires neural input for initiation and propagation (Costa et al., 1998). However some investigators include propagating contractions of the circular muscle layer that lead to partial or total occlusion of the lumen (Huizinga and Lammers, 2008). With the latter definition, contractions occurring at slow wave frequency would be considered a form of peristaltic activity. An argument for their exclusion is that propagation is often limited to short distances when reliant on non-neural mechanisms (Kunze and Furness, 1999).

1.8 Peristaltic reflex and 'the law of the intestine'

Bayliss and Starling described the peristaltic activity in the dog small intestine (Bayliss and Starling, 1899), and the large intestine of the dog and rabbit (Bayliss and Starling, 1900). This activity consisted of an oral contraction and an aboral relaxation at the point of mechanical stimulation. This polarised activity was termed the *"law of the intestine"*. Although this response is considered a *peristaltic reflex*, the ability for the resulting contractions to propagate along the intestine as a result of neuro-mechanical feedback makes it a *motor pattern* (Costa et al., 2000, Brookes et al., 1999). Excitatory junction potentials (EJPs) are generated in circular smooth muscle cells located orally to a mechanical stimulus and inhibitory junctions potentials (IJPs) generated in aboral cells (Hirst and McKirdy, 1974, Smith et al., 1991). These peristaltic reflexes are neurogenically mediated and the neurons that mediate these polarised responses are contained within the myenteric plexus. These responses persisted when the submucosa was removed (Smith et al., 1990).

Mechanical stimuli that elicit this response in tubular preparations include distension of the gut wall by intraluminal pressure changes (Trendelenburg, 2006, Trendelenburg, 1917) or by a bolus or balloon (Bozler, 1949, Frigo and Lecchini, 1970). In flat sheet preparations, distension (Costa and Furness, 1976) or stroking the mucosa (Raiford and Mulinos, 1934, Smith and Furness, 1988) will generate similar responses. These reflexes can be mediated without input from extrinsic nerves, indicating that they are entirely dependent on the myenteric and submucosal plexuses. Intrinsic primary afferent neurons (IPANs), interneurons and motorneurons within these plexuses form the neural circuitry for this motor activity. The neurons use various combinations of neurotransmitters, and with the use of immunohistochemistry they have been classed based on this chemical coding.

1.8.1 Ascending excitatory pathway

It had long been established that cholinergic pathways (involving nicotinic and muscarinic receptors) and non-cholinergic pathways were involved in the ascending excitatory reflex (Paton and Zaimis, 1949, Holzer, 1989, Trendelenburg, 1917). However following an elegant study by Tonini and Costa (1990) the location of these receptors in this pathway was better understood. Segments of guinea pig small intestine were placed into a three way partitioned bath allowing the oral, intermediate and aboral regions to be pharmacologically isolated. The intestinal wall was distended by an inflated balloon. The addition of hyoscine (a muscarinic receptor anatagonist) to the oral chamber reduced reflex contractions to approximately 5% of controls, whereas little reduction occurred when hyoscine was applied in either the intermediate or aboral chambers (Tonini and Costa, 1990). This demonstrated that acetylcholine released from motorneurons was activating muscarinic receptors on the smooth muscle cells. However, the hyoscine-resistant component was sensitive to TTX, suggesting another excitatory transmitter was involved in the neuro-muscular transmission generating the response (Tonini et al., 1981). The addition of hexamethonium (nicotinic receptor antagonist) to the oral, intermediate and aboral chambers reduced contractions to approximately 25, 20 and 40% of control values respectively. This shows the participation of nicotinic receptors on the ascending interneuronal pathways and on enteric motorneurons (Tonini and Costa, 1990). The remaining contractile response indicated that, although acetylcholine acting on nicotinic receptors appeared to be an important neurotransmitter, another unknown excitatory transmitter was also involved.

There are three main tachykinin receptors NK1, NK2 and NK3 and these receptors are preferentially activated by substance P, neurokin A and neurokin B respectively. Tachykinins have long been shown to have spasmogenic effects on gastrointestinal smooth muscle (Trendelenburg, 2006, Barthó et al., 1982b, Barthó et al., 1982a, Trendelenburg, 1917), and to depolarise myenteric neurons (Katayama and North, 1978, Morita et al., 1980). However selective activation of each type of receptor allowed their location in the ascending excitatory reflex to be better understood. In the guinea pig ileum, selective activation of NK3 receptors results in an atropine and TTX-sensitive contraction, indicating that NK3 receptors are present on neurons (Maggi et al., 1990).

NK2 receptors are present on post-junctional sites (smooth muscle, ICC), since selective activation of NK2 receptors generates a TTX -insensitve contraction. Selective activation of NK1 receptors generates an atropine-insensitve contraction which is depressed by TTX indicating that these receptors are predominately located at postjunctional sites but may also be on neurons (Maggi et al., 1990). Neuro-neuronal transmission in ascending excitatory reflexes involves NK3 receptors on ascending interneurons and excitatory motorneurons, as excitatory junction potentials evoked in response to mucosal compression or stretching of the gut wall were inhibited by NK3 antagonists when administered close to the site of recording (Johnson et al., 1998, Johnson et al., 1996). Inhibitory junction potentials evoked by mucosal compression orally were inhibited by NK3 receptor antagonists (Johnson et al., 1998, Johnson et al., 1996) demonstrating a role for NK3 receptors on descending interneurons.

These functional studies demonstrating the involvement of cholinergic and tachykinergic pathways in the ascending excitatory reflex are complemented with visual evidence provided by labelling studies. Application of DiI to the circular muscle layer retrogradely labels cells projecting locally, oral and aboral to the site of the dye application (Brookes and Costa, 1990). This technique combined with immunohistochemistry allows circular muscle motor neurons to be specifically labelled and therefore their neurochemical coding can be identified. Only cells with somata aboral to the dye application site, and with projections directed orally displayed immunoreactivity for choline acetyltransferase (ChAT) (Brookes et al. 1991). Some of these cells with longer projections also displayed immunoreactivity for substance P providing further evidence that tachykinins and NK receptors have a role in excitatory transmission at the neuromuscular junction. Retrograde labelling from the longitudinal muscle plexus combined with immunohistochemistry demonstrated excitatory motor neurons projecting to the longitudinal muscle containing immunoreactivity for ChAT and SP (Costa et al., 1996, Brookes et al., 1992). Only one class of ascending interneuron exists in the guinea pig small intestine and these cells are immunoreactive for SP and ChAT (Brookes et al., 1997, Costa et al., 1996).

1.8.2 Descending pathways

In guinea pig ileum descending relaxation was not detected in response to mechanical stimulation but descending contraction occurred (Spencer et al., 1999). While mechanical recordings note a descending contraction, electrical recordings still demonstrate descending inhibitory activity (Smith and Furness, 1988). Therefore the descending pathways may activate both excitatory and inhibitory responses. In the guinea pig ileum, following intestinal distension circular and longitudinal muscle cells generated an inhibitory junction potential (IJP) about 1s after the stimulus and a prolonged excitatory junction potential (EJP) 3-7 sec after the stimulus (Hirst et al., 1975). The EJP was blocked by atropine, indicating it is mediated by cholinergic motorneurons. However, the distension-evoked IJPs were inhibited by tubocurarine, a nicotinic receptor antagonist (Hirst and McKirdy, 1974) which suggests a role for cholinergic transmission from descending interneurons onto inhibitory motoneurons. This has, however, been disputed by Bian and colleagues who found that transmission between descending interneurons and motor neurons, in the guinea pig ileum, is almost exclusively mediated by P2X receptors in descending inhibitory reflexes and not cholinergic receptors. The amplitudes of inhibitory junction potentials evoked by mucosal stroking or compression were reduced by P2 receptor antagonists suramin and PPADS, however further addition of hexamethonium had no effect on their amplitudes. PPADS had no measurable effect when the solution in the stimulation chamber (not the recording chamber) was modified to reduce synaptic transmission (low Ca^{2+}, high Mg^{2+}). This suggests that blocking P2X receptors must have affected transmission between descending interneurons and motorneurons (Bian et al., 2000a). The involvement of nicotinic receptors appears to vary with region, as similar experiments in the guinea pig and rat colon revealed IJPs were inhibited by hexamethonium (Bian et al., 2003, Bian et al., 2004).

1.8.2.1 Descending inhibitory pathway

While the ascending contraction provides a force large enough to propel the luminal content, the descending inhibition may facilitate its movement along the intestine. IJPs result from descending inhibition and can be generated by stroking the proximal segment of an opened guinea pig ileum preparation. The response consists of fast and

slow IJPs (Smith and Furness, 1988). The fast IJP is inhibited by apamin, the SK3 channel blocker, and also by P2Y receptor antagonists (Wang et al., 2007, Gallego, 2006). This suggests the SK3 channel is opened by activation of P2Y receptors by ATP (or another related compound (Hwang et al., 2011)). SK channels are voltage independent, small conductance, calcium-activated potassium channels. The channels are made up of 6 transmembrane domains, and cytosolic N and C termini, and their pores are relatively selective for K^+. While they are gated by submicromolar concentrations of intracellular Ca^{2+}, they do not contain an intrinsic binding domain for the ion; rather the pore forming subunits interact with calmodulin, a calcium binding protein (Adelman et al., 2012). P2Y receptors are metabotropic. These G protein coupled receptors are coupled to the $G_{q/11}$ subunit. Through stimulation of phospholipase C, hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) releases diacyl glycerol (DAG) and inositol triphosphate (IP3) (Burnstock, 2007). These second messengers activate mechanisms to increase intracellular Ca^{2+} concentrations, which thereby activate SK channels.

The remaining slow IJP suggests a role for other neurotransmitters at the neuromuscular junction between inhibitory neurons and smooth muscle cells (Wang et al., 2007, Gallego et al., 2006). Application of authentic NO or the NO donor, sodium nitroprusside (SNP) mimics sIJPs, which are suppressed by soluble guanylate cyclase inhibitors (Goyal and He, 1998). Spencer and Smith (2001) compared the involvement of NO and ATP in generating the descending inhibitory responses. Intracellular recordings were made from smooth muscle cells in open flat sheets of guinea pig colon; proximally was an intact tubular segment of intestine. The colon was distended using intraluminal balloon, thereby generating an IJP distally. Only the fast IJP in the circular muscle cells was blocked by apamin; longitudinal cells were unaffected. The remaining response was blocked by N^{ω}-nitro-L-arginine (L-NA, a nitric oxide synthase blocker) in both layers suggesting a role for NO (Spencer and Smith, 2001). Other neurotransmitters have been found to play a role in neuromuscular transmission in the descending inhibitory pathway. In the rat colon stretch activated the descending relaxation and with graded increases in stretch this response increased. The response significantly was reduced in the presence of vasoactive intestinal peptide (VIP)

antiserum at each level of stretch applied (Grider and Makhlouf, 1986). Interestingly, the velocity of pellet propulsion along a segment of guinea pig colon is dose dependently decreased upon exposure to a VIP antagonist, VIP(10-28) (Foxx-Orenstein and Grider, 1996). Retrograde labelling and immunohistochemistry in the guinea pig small intestine demonstrated the properties of descending neuronal pathways. Cell bodies labelled oral to the site of DiI application to the deep muscular plexus (with aborally directed projections) were often immunoreactive for VIP (Brookes et al., 1991). In the guinea pig small intestine, all NOS immunoreactive neurons are also VIP immunoreactive (Costa et al., 1992). In the descending inhibitory reflex, activation of inhibitory motorneurons by interneurons may not only be cholinergic but also by activation of P2X receptors by ATP (Johnson et al., 1999, Bian et al., 2000a) and NK1 receptors by tachykinins (Bian et al., 2000b, Johnson et al., 1998).

1.8.2.1 Descending excitatory pathway

The descending excitatory pathway has not been as extensively studied. In the guinea pig ileum, activation of the descending excitatory reflex by stroking the mucosa is inhibited by simultaneous application of hexamethonium and PPADs (Spencer et al., 2000). There is some suggestion that the descending inhibitory reflex may limit the descending excitatory reflex, since application of apamin (which abolishes fIJPs) increases the descending contractions triggered by mucosal stroking or radial distension (Spencer et al., 1999). Serotonin may also play a role in the descending excitatory reflex (Monro et al., 2002). In flat sheet preparations of guinea pig ileum, the descending excitatory contraction evoked by oral distension was attenuated in the presence of the $5HT_3$ receptor antagonists granisetron and ondansetron in the circular muscle layer but not the longitudinal muscle. This suggests a role for serotonin in the descending excitatory reflex and indicates that the longitudinal and circular muscle layers may be activated in different neural circuits/pathways.

The presence of both descending inhibitory and descending excitatory reflexes, together with roles for acetylcholine, serotonin and ATP suggest that descending reflex pathways are more complex than ascending pathways. In the guinea pig ileum both long and short inhibitory motorneurons to the circular muscle immunoreactive for NOS and VIP have been characterised. There are three, potentially four, groups of descending interneurons all of which are cholinergic: 1) 5-HT immunoreactive; 2) Somatostatin immunoreactive; 3) VIP immunoreactive 4) VIP and NOS immunoreactive (Costa et al., 1996).

1.8.3 Propagating response

Peristaltic contractions propagate aborally in open flat sheet preparations of guinea pig ileum and can be initiated by a slow circumferential stretch (Brookes et al., 1999). The latency between the stretch and the responding contraction increased at more distal points along the sheet, and corresponded with a decrease in amplitude. Intracellular recordings of circular muscle revealed that there was not only an increased latency between the stimulus and the first spike in aboral regions versus oral, but also a decreased number of generated spikes, a decrease in spike duration and a decrease in integrated spike bursts (Brookes et al., 1999). Shortening the longitudinal axis of the flat sheet decreased that amplitude of the peristaltic contractions in response to the stretch stimulus. A stretch induced contraction did not occur in preparations reduced to less than 10mm, this likely to be due to the absence of an ascending excitatory pathway, since excitatory motorneurons are less than 10mm long. This paper highlights that peristalsis can be systematically studied in flat sheet preparations, allowing the myogenic and neurogenic mechanisms that contribute to the motor patterns to be separately studied.

1.8.4 Occult reflex

Stretching a segment of colon longitudinally has been reported to evoke an *occult reflex*, where the peristaltic reflex evoked by circumferential stretch is abolished (Dickson et al., 2007). Adding L-NA, the nitric oxide synthase antagonist, abolished the occult reflex and allowed distension evoked reflexes to occur (Dickson et al., 2007). The role of this phenomenon in physiology is uncertain, as elongation of the intestine

may not always occur during filling.

1.9 Other forms of peristalsis

1.9.1 Migrating motor complex

The migrating motor complex (MMC) is a cyclical motor pattern that occurs in the stomach and small intestine (Deloose et al., 2012). This activity pattern was first described in the dog stomach by Boldyreff (1902). In this study it was noted that during periods of fasting, high amplitude contractions occurred during an active phase for around 20 minutes, this active phase was then followed by a rest phase of around 80 minutes (Deloose et al., 2012). Feeding interrupted the cyclic activity, however the pattern returned once the stomach was emptied. MMCs are reoccurring events that propagate from the stomach and along the small intestine over 1.5/2 hrs (Code and Marlett, 1975). In the dog, they propagate at different velocities along the length of the intestine from 3.5 to 6.2 cm/minute in the proximal small bowel, to 1.2 to 1.9 cm/minute in the distal section (Szurszewski, 1969). MMC are divided into 4 phases of activity: phase I is a period of quiescence; phase II is a series of seemingly disorganised contractions; phase III has a sudden onset and is a period of more intense activity (corresponding with the activity front in earlier studies); and phase IV is a rapid decrease of contractions. MMCs have been identified in the rabbit, sheep, pig, rat, guinea pig and human (Aeberhard et al., 1980, Grivel and Ruckebusch, 1972, Deloose et al., 2012, Galligan et al., 1986, Galligan et al., 1985). Propagation of MMCs is blocked by atropine and hexamethonium indicating a role for intrinsic, cholinergic motor neurons and interneurons in propagating the complex, however the pattern generator is still unknown (Sarna et al., 1981).

1.9.2 Colonic migrating motor complex

Sarna and colleagues were among the first to describe the *colonic migrating motor complexes* (CMMC) (Sarna, 1986). This motor complex had different characteristics to the migrating complexes recorded in the small intestine: in the dog colon long duration contractions occurred at 0.5-2 cycles per minute and this burst of activity was followed by a period of quiescence. Unlike the small bowel, CMMCs were not disrupted by a

meal; rather their frequency increased (Sarna, 1986). CMMCs migrated over at least half the length of the colon (Sarna, 1986). Electrical recordings associated these contractions with a burst of high frequency oscillations superimposed on slow depolarisations (Sarna, 1986). This aligned with previous electromyogram studies in the cat where bursts of electrical activity migrated along the length of the colon. These spike bursts lasted 1-2 minutes and occurred at 3-5 minute intervals (Christensen et al., 1974). A similar electrical pattern had been recorded in the isolated mouse colon using suction electrodes (Wood, 1973). However, Wood's study indicated that extrinsic input was not required for the electrical activity to persist and migrate along the colon and therefore CMMCs could be studied in *in vitro* experiments (Wood, 1973). In the isolated mouse colon, CMMCs are abolished by TTX and hexamethonium suggesting the complexes are neurogenic and involve cholinergic interneurons (Fida et al., 1997). The amplitude of the contractions is reduced by atropine (Fida et al., 1997) and intracellular recordings from circular muscle have demonstrated that atropine blocks the rapid electrical oscillations but not the slow depolarisations (Lyster et al., 1993). Therefore, cholinergic enteric motorneurons have a role in CMMC amplitude but not in their occurrence. There is evidence that nitrergic input controls the periods of quiescence and paces the frequency of contractions (Brierley et al., 2001, Fida et al., 1997). It is likely that the pattern generator for CMMCs is within the myenteric plexus since these complexes persist in with mouse colon after the removal of the mucosa and submucosa by sharp dissection (Keating and Spencer, 2010).

1.9.3 Giant migrating motor complex

In animal studies, the giant migrating motor complex has mainly been described in the dog. Giant migrating motor complexes (GMCs) are large amplitude, long duration contractions that are rapidly propulsive (Sarna, 2006). Recordings using strain gauge transducers implanted extraluminally on the distal canine colon, *in vivo*, demonstrated that GMCs frequently occur before defecation (Karaus and Sarna, 1987). Contractions migrate approximately 13cms along the length of the canine colon, which is about ¼ of its length (Sarna, 1991). In the dog frequent, cyclical GMCs only occur in the cecum at around 1 cycle per hour (Sarna et al., 1988). In comparison, GMCs in the small intestine and colon occur around 2-5 times per day (Sarna, 2006).

1.9.4 Propagating between muscle layers

Kottegoda (1969) postulated that when one layer of the muscularis contracts, the other relaxes due to reciprocal innervation of these layers (Kottegoda, 1969). This was based on a mechanical study where the author claimed that both spontaneous and induced contractions in one layer always corresponded to relaxation or quiescence in the other layer. However in this study relaxation was defined as the decrease in tension back to baseline following a rapid increase in tension, i.e. the end of the contraction (Kottegoda, 1969). It has since been shown by spatiotemporal mapping analysis (Hennig et al., 1999) and force transducer recordings (Spencer et al., 1999) that contractions in the longitudinal and circular muscle layers of the guinea pig ileum generally occur in synchrony.

1.10. Human studies

Understanding the cellular basis of gastrointestinal motility has been largely based on studies in animal models. However, it cannot be assumed that what occurs in the animal also occurs in the human. This next part of this review focuses on gastrointestinal motility studies in the human colon.

1.10.1 Immunohistochemistry: highlighting the neural pathways

The projections of motorneurons and interneurons that make up pathways within the gut wall have been studied directly. Retrograde labelling neurons from the deep muscular plexus demonstrated the length of motorneurons within human ileum and colon (Wattchow et al., 1995). In the colon, cell bodies were located up to 16mm oral to the site of DiI application. Motorneurons with ascending projections were labelled up to 11mm away, while circumferential projections spanned up to 10mm. DiI placed onto the myenteric plexus labelled cell bodies up to 68mm orally and 30mm aborally indicating descending interneurons may have longer axons. They were also more numerous than ascending interneurons (Wattchow et al., 1995).

By combining this retrograde labelling technique with immunohistochemistry, Wattchow and colleagues demonstrated the polarised distribution of inhibitory versus excitatory motor neurons in the human colon (Wattchow et al., 1997). Descending motor neurons immunoreactive for VIP had processes up to 17mm long. Filled cell bodies were mainly located oral to the DiI site indicating that the majority of axons were directed anally.. Labelled cell bodies of ascending excitatory motor neurons that had immunoreactivity for tachykinins, were predominantly labelled less than 10mm aboral from the site of DiI application indicating that these axons were projecting orally(Wattchow et al., 1997). Other studies demonstrated that almost all inhibitory motor neurons are immunoreactive for nitric oxide synthase (NOS) (Porter et al., 1997). The majority of circular muscle motor neurons with oral projections are immunoreactive for choline acetyltransferase, an enzyme involved in the synthesis of acetylcholine (ChAT) (Porter et al., 1996). A small percentage of ChAT immunoreactive motor neurons project aborally (Porter et al., 1997).

Similar polarised distributions were noted for interneuronal pathways. Interneurons with immunoreactivity for VIP were located up to 30mm oral to the DiI application site. Tachykinin containing interneurons were located up to 30mm aboral from the site of dye application (Wattchow et al., 1997). The immunoreactivity of neurons for VIP and tachykinins did not account for all retrogradely labelled neurons suggesting that there are other neurochemical classes within the myenteric plexus of the human colon. Orally directed interneurons predominantly contain ChAT (90%) but lack NOS (Porter et al., 2002a). Descending interneurons often contain NOS alone (46%), ChAT alone (20%) or a combination of NOS and ChAT (29%) (Porter et al., 2002a). The neurochemical coding of descending interneurons is as complex in the human as the guinea pig (Costa et al., 1996, Porter et al., 2002a). However, it would seem there are two classes of ascending interneurons (those with and without ChAT) compared to the guinea pig, which all contain ChAT (Costa et al., 1996). To further define these two classes of ascending interneurons in the human, a comparison of ascending interneurons with/without TK immunoreactivity may prove useful.

1.10.1 Spontaneous oscillatory activity in human colon

In combination with tension recordings, Huizinga et al. (1985) used suction electrodes, sucrose-gap method and microelectrodes to record the electrical activity of smooth muscle cells from small segments of human sigmoid colon. They demonstrated that various frequencies of electrical activity could be recorded from these cells. Circular muscle cells produced membrane potential oscillations at 4.5-60 cycle/min. These oscillations sometimes had superimposed spikes, associated with muscle contraction, similar to many preparations from laboratory animals. Prolonged contractions were associated with oscillations of membrane potential 12 cycles/min or faster (Huizinga et al., 1985). In the same study, an oscillatory mechanical activity separated by 1 minute intervals, and of much larger amplitudes than those generated by slow waves (approximately 6g versus 1g), was reported. These contractions corresponded with periods of intense spiking, recorded by suction electrodes (Huizinga et al., 1985, Huizinga and Waterfall, 1988a). Atropine (2 μM) failed to inhibit either the electrical or the mechanical activity in 7 of 14 preparations. Of the 7 where inhibition occurred, the electrical activity could be reinitiated by stretching the preparation. TTX (0.5μM) did not block these large contractions but spiking rate reduced (Huizinga et al., 1985). This result demonstrates a pattern of myogenic activity that differs from slow waves. CMMCs may be similar to the cyclical migrating contractions that occur in the guinea pig colon, but these complexes are TTX sensitive (D'Antona et al., 2001). In the pig and rat however, these TTX-sensitive contractions return after either stretching the preparation, or application of carbachol (Huizinga et al., 1983, Huizinga et al., 2011). It seems there is another myogenic pattern generator in the colon, oscillating at a slower frequency than slow waves. In the human, this myogenic mechanism may be large enough to produce contractions without neural input. In animal models however, a neural stimulus may be required for the myogenic pattern generator to generate contractions. Application of carbachol (cholinergic agonist), or stretching segments of colon increases the frequency of these contractions of in the human (Huizinga and Waterfall, 1988).

Intracellular recordings from segments of human colon demonstrated slow waves occurring at 2.5-4 cycles per minute (Rae et al., 1998). Their amplitude decreased with

distance from the submucosal border, suggesting that they may originate from ICCs in this region, rather than the myenteric plexus (Rae et al., 1998). This is comparable with findings in the dog colon (Smith et al., 1987a, Smith et al., 1987b). Mechanical recordings by Rae and colleagues revealed contractions occurring at a frequency attributable to slow waves, but also an additional slower pattern, comparable with that noted by Huizinga and colleagues. In dissociated preparations, this pattern occured at mean rates of: 0.9 cycles per minute in 36% of specimens with intact circular muscle specimens; 0.6 cycles per minute in 82% tissues with just the myenteric half of the circular muscle and 0.3 cycles per minute in 67% of tissues from the interior portion of the circular muscle layer (Rae et al., 1998). The electrical activity was not recorded at the same time as the mechanical activity for this motor pattern. However, the slow pattern was of greater amplitude in regions further away from the submucosa suggesting that it may not originate at the submucosal border. Another pattern of high frequency oscillations, occurring at 17-18 cycles per minute were noted in preparations with the myenteric plexus intact and resembled *"myenteric potential oscillations"* from the canine colon (Smith et al., 1987b).

Colonic manometry measures motor patterns in human subjects *in vivo*. Catheters with various types of pressure sensors record the changes in intraluminal pressures resulting from contractile activity. Previously, catheters typically had pressure sensors spaced at 7-10cm intervals, yielding low resolution recordings (Dinning et al., 2010a). However with developing technologies the resolution of these recordings is improving. Newly developed fibre optic catheters contain up to 72 sensors spaced at 1cm intervals (Arkwright et al., 2009). With this technology the propagation of contractions across regions of gut *in vivo* be can better determined. Various pressure waves have been recorded with colonic manometry. The majority of motor patterns recorded are non propagating sequences, occurring at different sites along the colon (Dinning et al., 2010b). Propagating pressure waves are events which migrate either anterogradely or retrogradely along the length of the colon. However the use of different catheters with differing recording resolutions, and different criteria for defining these contractions has led to inconsistent reporting on their characteristics (Dinning et al., 2010b). Dining and colleagues have reported the frequency of anterograde propagating sequences in control

patients averaged 65 events in 24 hours, while 17 events in 24 hours occurred propagated retrogradely (Dinning et al., 2009). Bursts of regular, phasic, pressure waves are sometimes regionally linked and the organisation of these colonic motor patterns may be an important diagnostic feature (Dinning et al., 2009). Another characteristic pressure wave, high amplitude propagating sequences, were far less frequent, migrating anterogradely and averaging 9 events in 24 hours in healthy controls (Dinning et al., 2009). These events compared to the *giant migrating motor complexes* and, as in the canine colon, they are commonly associated with defecation (Sarna, 2006). In published manometry studies, there is little mention of contractile events at frequencies corresponding with those recorded from segments of colon *in vitro*. The amplitude of these events may be too small to be noticed amongst other recorded patterns. The slow events recorded by manometry are not apparent in *in vitro* studies and this may be due to the removal of extrinsic and hormonal inputs *in vitro*, affecting their expression.

1.10.3 Neuromuscular transmission in the human colon

Acetylcholine is the major excitatory neurotransmitter released from enteric motorneurons to contract smooth muscle cells. Carbachol contracts colonic circular smooth muscle, and electrically induced contractions are reduced by atropine (Stanton et al., 2003). Acetylcholine in the presence of TTX, to limit the activity of neurons activated by acetylcholine on nicotinic receptors, also contracted human colonic smooth muscle (Aulí et al., 2008). Many studies on the neuromuscular transmission in human colon have focused on the role of tachykinins. Substance P and neurokinin A, non-selective agonists for NK_1 and NK_2 receptors, caused concentration dependent shortening of isolated, single smooth muscle cells from the human sigmoid colon (Cao, 2000). In segments of colon, electrically evoked contractions were almost abolished by a peptide NK_2 receptor antagonist, while NK_1 antagonist (FK888) had little influence (Cao, 2000). The amplitude of contractions evoked by 10μM substance P in the presence of TTX was reduced by 74% by NK_2 receptor antagonists, but only 20% by NK_1 receptor antagonists (Aulí et al., 2008). This suggests that NK_2 receptors may have more of a role in neuromuscular transmission in the human colon. There is evidence supporting this notion in the human ileum also (Maggi et al., 1992).

Some inhibitory motorneurons are purinergic. Electrically stimulated fast inhibitory junction potentials (fIJPs) recorded from human colonic smooth muscle cells, were inhibited $P2Y_1$ receptor antagonists MRS2179, MRS2279 and MRS2500 (Gallego et al., 2006, Gallego et al., 2008, Gallego et al., 2011). $P2Y_1$ receptors are thought to be activated by ATP or a related compound. Evidence from animal studies suggests this compound may be ß nicotinamide adenine dinucleotide (ß NAD) (Hwang et al., 2011). Circular muscle cells from segments of human colon were hyperpolarised by transient superfusion of $P2Y_1$ receptor agonists ADPßS10, MRS2365 and ß NAD as demonstrated by intracellular electrophysiology. Hyperpolarisation by ß NAD was smaller in amplitude than responses to ADPßS10 and MRS2365 (Gallego et al., 2011). The amplitude of spontaneous contractions was reduced by ß NAD, however $P2Y_1$ receptor antagonists failed to block this inhibition (Gallego et al., 2011). Therefore it seems that in the human colon, ß NAD may not be the endogenous mediator of purinergic neuromuscular transmission. In the guinea pig ileum, in the presence of apamin and atropine, high frequency electrical stimulation of inhibitory motorneurons generates a slow IJP in circular muscle cells (Bywater and Taylor, 1986, Niel et al., 1983). In the human colon, the sIJP is inhibited by the NO synthase inhibitor, L-NA. Nitric oxide donor, sodium nitroprusside, abolishes spontaneous contractions (Gallego et al., 2008). As in animal models, both nitrergic and purinergic inhibitory motorneurons have a role in neuromuscular transmission in the human colon.

While there is understanding of some of the mechanisms involved in neuromuscular transmission in the human colon, studies focused on neuro-neuronal transmission are lacking. Functional analysis of the neuro-neuronal networks that contribute to gastrointestinal motility need to take into consideration the lengths of various neural pathways as demonstrated by retrograde labelling and immunohistochemistry studies.

1.11 Summary

Gastrointestinal motility patterns are generated from the contractions and relaxations of smooth muscle cells. However following the setup of preparations and prior to the commencement of studies into gastrointestinal motility an ‘equilibration’ or ‘warm up’

period is usually allowed by most investigators. Smooth muscle cells are initially unresponsive to electrical or pharmacological stimulation in many preparations, they fail to generate spontaneous contractions and lack tone. Responses develop as preparations recover, following setup. While a period of recovery after dissection is often reported in the methods section, the mechanism which leads to the apparent loss of gastrointestinal motility during this period has not been previously considered. Something is occurring in this period that significantly reduces gastrointestinal motility. Using electrophysiology, tension recordings and immunohistochemistry I have characterised the changes in gastrointestinal physiology over this period to understand the mechanisms that may account for this apparent loss of responsiveness. Gap junctions give rise to an electrical syncytium between smooth muscle cells allowing electrical signals to rapidly propagate over the network and to generate cohesive motor patterns. One of the aims developed during my study was to demonstrate factors which influence the permeability of these channels thereby influencing the electrical properties of a segment of intestine. Motility patterns are also influenced by neural mechanisms. These patterns have been described in the animal model, however understanding the mechanisms that underlie motility in human gut are not well understood. The final aim of this study was to demonstrate the mechanisms which generate spontaneous and induced motor patterns in the human colon, *in vitro.*