Effect of melatonin on CMMCs and faecal pellet propulsion

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Acronyms and Abbreviations

- AANAT: aralkylamine N-acetyltransferase
- ACh: acetylcholine
- AH: after-hyperpolarising
- AJ: adherens junction
- ATP: adenosine triphosphate
- CFTR: cystic fibrosis transmembrane conductance regulator
- CGRP: calcitonin gene related peptide
- CMMCs: colonic migrating motor complexes
- CNS: central nervous system
- DC: distal colon
- EC: enterochromaffin
- EFS: electric field stimulation
- ENS: enteric nervous system
- EPSP: excitatory post-synaptic potential
- GI: gastrointestinal
- HIOMT: hydroxyindole-O-methyltransferase
- ICC: interstitial cells of Cajal
- IEC: intestinal epithelial cells
- IHC: immunohistochemistry
- IPAN: intrinsic primary afferent neuron
- JAM: junctional adhesion molecules
- L-NNA: N-nitro-l-arginine
- NANC: non-adrenergic, non-cholinergic
- NKCC: Na-K-Cl cotransporter
- NO: nitric oxide
- PACAP: pituitary adenyl cyclase activating peptide
- PC: proximal colon
- PRP: pattern-recognition receptor
- SP: substance P
- TJ: tight junction
- VIP: vasoactive intestinal peptide
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Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

3rd June 2016
Key Findings:

Melatonin presence was detected in the murine colon mucosa for the first time. The enzymes responsible for the synthesis of melatonin were also found to be present in the mucosa and in the same vicinity as EC cells. So despite inconclusive proof that melatonin is in EC cells, it can now be said that melatonin is locally synthesised in the mucosal layer of the murine colon. A positive correlation was also identified between the number of EC cells present and the level of melatonin along the length of the murine colon.

In terms of functionality, melatonin has been shown to affect different motility patterns presented in the mouse colon in different ways. Melatonin enhances serotonin-induced and EFS-induced contractions. These enhanced contractions were mediated by tachykinins in the serotonin response and cholinergic pathways in the EFS response. Melatonin effects on EFS are mainly mediated by MT₁ receptor while both MT₁ and MT₂ receptors are involved in mediating its effects on serotonin-evoked responses. Melatonin was also shown to improve the velocity of fecal pellet propulsion. However, melatonin decreases the rate of CMMCs.

Abstract

Melatonin is an indoleamine hormone that also serves as a neurotransmitter. It is naturally synthesised from tryptophan (Wurtman et al., 1964). Melatonin is well known to play a key role in the regulation of sleep patterns. However, little is known about its role in the colon. Studies in the 1970s showed that melatonin was present in the intestinal mucosa of rats and humans (Bubenik et al., 1977; Raikhlin et al., 1975), but provided no specific location of the mucosal source of melatonin. In 2008, using electrochemical methods, it was shown that melatonin is present and released from the ileum mucosa (Patel et al., 2008). There have also been limited studies on the functional role of melatonin in the intestinal tract. While some studies indicated that melatonin directs contraction, others indicate that it influences relaxation; and others suggest melatonin acts as an antagonist of
mucosal serotonin (Bubenik et al., 1989). The aim of this study is to understand the localisation of colonic melatonin and its role in governing colonic motility in the mouse.

For the first time in mice, the synthesis enzymes, AANAT and HIOMT, are identified in the colon; and in the vicinity of EC cells in the mucosal layer via immunohistochemistry. This suggests that melatonin is synthesised within the EC cells. The locations of EC cells and melatonin expression were in close proximity to one another. Melatonin was discovered to accumulate in higher levels in the murine proximal colon as compared to the distal colon, and this is related to the number of EC cells.

Functional studies presented here involve a novel in-depth look into the mechanisms governing melatonin effects on various colonic motility patterns. Melatonin shows a net positive effect on contractile activity triggered by both serotonin and EFS. This is building on top of past observational knowledge about the dual effects of melatonin. Previous studies have not made a deeper investigation into the mechanisms of melatonin activity in the presence of melatonin and external stimuli (EFS). In these induced motility tones, the effects appear to be mainly mediated through the upregulation of the contractile response. These effects are mediated by neuronal signalling pathways and not by the underlying musculature. Melatonin was discovered to have the opposite effect on the propagation of spontaneous CMMCs. It decreases the magnitude of spontaneous CMMC responses in the proximal colon, decreasing the velocity of propagation of CMMC waves down the length of the colon. However, in fecal pellet distention induced propulsion, melatonin has been shown to boost colonic transit times, promoting motility. Melatonin receptors are shown to be involved in both processes.

Our current results indicate that there is a significant role for melatonin in the colon in terms of motility regulation, and that it has the potential to be an important therapeutic target within the bowel.
Introduction

1 Gastrointestinal Tract

1.1 Anatomy of the GI tract

In mammals, the gastrointestinal (GI) tract consists of many components; from the stomach to the intestines, and is divided into the upper and lower gastrointestinal tracts. The enteric nervous system (ENS) is embedded the myenteric and submucosal layers of the GI tract as a mesh of neuronal plexi; and it is independent of the central nervous system (CNS) and autonomous nervous system since it regulates its own reflex activity (Furness, 2005).

The anatomical structure of the murine GI tract is presented in figure 1.1A. The small intestine consists of the jejunum, duodenum, and ileum. The components are coated by a continuous columnar epithelium arranged in tubular glands and the crypts of Lieberkühn, the structures surrounding the bases of the villi. The intestinal surface area decreases from the proximal to the distal end of the GI tract (Kararli, 1995). The large intestine is subdivided into the appendix, caecum, proximal colon, distal colon, rectum and anal canal. The caecum, which is located upstream from the ileocecal junctions, gave rise to the appendix. In Figure 1.1B, the lower GI tract is shown, where area of interest (proximal and distal colon) for the current study is shown in more representative detail.
Figure 1.1A: The schematic drawing of the murine lower GI tract. 1.1B; Realistic diagram of the morphological features of the murine colon. (Treuting et al., 2012)

Figure 1.2 shows a cross section of the mammalian intestinal wall. The mucosal layer is made up of an epithelial layer and the lamina propria, which supports the tissue underneath. The barrier between it and the submucosal layer is the muscularis mucosa. The submucosa consists of connective tissue and larger blood and lymph vessels. The submucosal plexus, which constitutes part of the ENS, is also found in the submucosal layer. Underlying the submucosa is an inner circular muscle layer and an outer longitudinal muscle layer. The myenteric plexus is situated between the two muscle layers, and is also part of the ENS. The muscular layers work in synchronisation to bring about intestinal motility patterns. The serosa, furthest from the lumen, is a continuation of the peritoneal membrane.
The colonic mucosa of the large intestine does not present well-defined villi and brush-border enzymes required for food digestion and absorption, but it has similar micro-projections to increase surface area for ion adsorption and secretion. It has a decreased affinity towards polar compounds due to the presence of tight junctions. Despite some structural differences on the luminal side, the large intestine presents the same functions as the ileum, including ion transport, and effecting neural and hormonal responses based on luminal stimuli (Karkali, 1995). The most important function of the colonic mucosal layer is the absorption of fluids and electrolytes (Furness, 2005). The colon has a larger diameter than the small intestine, and the contents of its lumen are much more viscous. In the distal colon, solid faecal matter is produced and temporarily stored.

Next, it is important to consider the vascular anatomy of the intestinal system. As shown in Figure 1.2, the intestinal walls are richly supplied with blood to allow it to carry out its functions. Each villus contains blood capillaries and elongated, blind-ended vessels (lacteals) that branch out to give lymphatic vessels and are central to its absorption functions (Friend et al., 1992).

Intramural pathways mainly supply the components of the large intestine. The proximal colon is supplied by the ileocolic, right and middle colic arteries all of which split from the superior mesenteric artery. The distal colon is supplied by the
left colic and sigmoid arteries, which are branches of the inferior mesenteric artery. These vessels disperse into channels that are fewer and less complex compared to the villi in the small intestine (Geboes et al., 2001). There is widespread vasculature in the submucosa both longitudinally and circumferentially. Arterioles from the submucosal plexus go through the muscularis mucosa and then spread out to form a web of capillaries. These capillaries rise up around the crypts and glands and reach the mucosal surface where they arrange in a honeycomb pattern around the gland openings, immediately underneath the surface epithelium (Geboes et al., 2001). The muscular layers contain capillaries branched from the submucosal plexus and are supplemented by larger arteries originating from the serosa and sub-serosa. The venous pattern largely follows the arterial supply.

In the murine colon, longitudinal and circular muscle layers are arranged orthogonal to one another as groups of densely packed smooth muscle cells. In the human colon, densely packed bands of longitudinal muscle called taeniae coli can be found. The taeniae coli contract lengthwise to produce the haustra, the bulges in the colon. In certain areas specialized physiological features have developed, such as sphincters (Kararli, 1995). A large number of different cell types including nerves and capillaries, intestinal cells, collagen fibres, intramuscular connective tissue, macrophages and mast cells populate the space between muscle layers. The uni-nucleated and spindle shaped smooth muscle cells of the intestinal tract can shorten by up to 75% of their original length and can hold tension for extended periods without fatigue (Friend et al., 1992).

1.2 Enteric nervous system

The enteric nervous system of the gastrointestinal tract is formed of a number of interconnected networks, or plexi, of neurons and enteric glial cells. In the colon, most enteric neurons are found in two networks of ganglia, namely the myenteric plexus and the submucosal plexus (Kottegoda et al., 1969; Furness, 2005). It is an autonomous system from the central and peripheral nervous system, mediating reflex activity independently. The axons of these nerve cells innervate other
ganglia and the tissues of the digestive organs, such as the muscle layers and the mucosa. The myenteric plexus is a layer of neuronal axons and small ganglia that reside between the longitudinal and inner circular muscle layers of the intestines (Gershon et al., 2004). The submucosal plexus is ganglionated network present in the small and large intestines.

The colon and ENS is mostly isolated but it contains important links to the central nervous system. The pelvic afferents innervate the large intestines and perform extra functions on top of the ones mediated by vagal afferents. These include regulating innocuous and noxious sensations. Splanchnic afferents are made up of low-threshold mucosal and stretch-sensitive afferents, as well as high-threshold neurons located in the serosal/mesenteric layers (Kararli, 1995). Gastrointestinal afferents may be sensitized or inhibited by chemical mediators released from several cell types.

Another morphological feature of the intestinal system is the interstitial cells of Cajal (ICC). There are 3 different subtypes as shown in Table 1.1. They can be classified by their morphology. Type 1 ICCs are the least like smooth muscle cells, type 2 are intermediate, and type 3 are the most like smooth muscle cells. The distribution in the colon presents a more homogenous pattern of subtypes that is seen throughout the length of the region. Each ICC subtype is mostly found in its own tissue layer, in the submucosal plexus, myenteric plexus and intramuscular layer respectively (Komuro, 2006). ICCs interact with smooth muscle cells in the longitudinal and circular muscle layers for generating rhythmicity (with ACh, VIP, and NO receptors) (Sakakibara et al., 2011).

<table>
<thead>
<tr>
<th>ICC type</th>
<th>Basal lamina</th>
<th>Caveolae</th>
<th>Gap junctions</th>
<th>Intermediate filaments</th>
<th>Mitochondria</th>
<th>Nerve contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Type 2</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Type 3</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

*Table 1.1: Three types of ICCs classified by their ultrastructural features. (-) indicates absent, (+/-) few and (+) present. (Komuro, 2006)*
The neurotransmitters used in the ENS are similar to those present in the CNS and autonomic nervous system. These include ACh, serotonin, tachykinins, melatonin, and NO. Melatonin and its interactions with the various other neurotransmitters are of particular interest in this investigation. There are many different neuron types in the ENS (Figure 1.3), including motor neurons to the circular and longitudinal muscles, intrinsic arterioles and epithelium, various interneurons, and possibly intrinsic sensory (primary afferent) neurons (Furness et al., 1998).

Excitatory transmission in motor neurons has been shown to possess a prominent muscarinic component to ACh (Borody et al., 1985). Nonetheless, there is residual excitation mediated primarily by release of tachykinins. Motor neurons in the ENS have been shown to present both the synthesizing enzyme for ACh (choline acetyltransferase) and tachykinins (Borody et al., 1985; Galligan et al., 1986). In the ENS, the transmission from inhibitory neurons is mediated by non-adrenergic, non-cholinergic neurons; these are also known as purinergic neurons, since purine nucleotides serve as their transmitters (Burnstock, 1972). These enteric inhibitory motor neurons also predominantly contain nitric oxide synthase and release NO (Sanders and Ward, 1992; Stark and Szurszewski, 1992). However, it is not the only transmitter (Makhlouf and Grider, 1993 and Furness et al., 1995b) involved in inhibitory signalling. The other inhibitory neurotransmitters include ATP (Burnstock, 1972 and Crist et al., 1992), VIP (Fahrenkrug, 1979), PACAP (Jin et al., 1994 and McConalogue et al., 1995) and carbon monoxide (Rattan and Chakder, 1993).

In the interneurons, which are responsible for neuro-neuronal transmission in the ENS, there are two non-cholinergic fast EPSPs, one mediated by ATP and the other by 5-HT (Lepard et al., 1997 and Zhou and Galligan, 1999). ATP is involved in a descending pathway, and might help to regulate motility through local reflexes (Lepard et al., 1997 and Johnson et al., 1999). Serotonin transmission in the interneurons is mainly involved in secretomotor reflexes (Furness et al., 1999).

Sensory neurons release calcitonin gene-regulated peptide (CGRP), substance P (SP), and ACh to interneurons. Interneurons release ACh and SP orally to excitatory motoneurons while ACh is released aborally to inhibitory motor neurons (Sakakibara et al., 2011). Excitatory motoneurons release ACh and SP to smooth
muscle cells while inhibitory motorneurons release NO, vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), and adenosine triphosphate (ATP) to smooth muscle cells (Furness et al., 2000; Sakakibara et al., 2011).

The IPANs are AH neurons. This refers to a specific neural property in which Ca$^{2+}$ carries a component of the action potential, and a delayed and prolonged after-hyperpolarising (AH) potential, the AHP, follows the action potential (Furness et al., 1998). Interneurons and motor neurons are S neurons, most of which lack these features, but receive large amplitude fast EPSPs, that are generally not observed in AH neurons. IPANs are mucosal chemosensors and mechanoreceptors; they are also responsive to stretch stimuli (Kunze et al., 1998 and Kunze et al., 1999). IPANs respond tonically to tension generated by muscle contraction, and phasically to the onset of tension, or to direct distortion of their processes. (Figure 1.3).

![Figure 1.3: Enteric neural circuitry relevant to peristaltic reflex. Following stretch stimuli, 5-HT is released from EC cells to intrinsic primary sensory neurons (with 5-HT3 and 5-HT4 receptors). Sensory neurons release calcitonin gene-regulated peptide (CGRP), substance (SP), and acetylcholine (ACh) to interneurons. Interneurons release ACh and SP orally to excitatory motorneurons while ACh is released aborally to inhibitory motorneurons. Excitatory motorneurons release ACh and SP to smooth muscle cells while inhibitory motorneurons release nitric oxide (NO), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), and adenosine triphosphate (ATP) to smooth muscle cells. Interstitial cells of Cajal (ICC) interact with smooth muscle cells for generating rhythmicity (with Ach, VIP, and NO receptors). (Sakakibara et al., 2011)
1.3 General functions of the colon

1.3.1 Ion transport for homeostasis

The small intestine is the main site for ion transport, but ion transport still occurs within the colon. The main ions transported are sodium, potassium, calcium, phosphate, and chloride ions. Motor neurons originating from the submucosal plexus, as well as a small proportion of myenteric neurons that innervate the mucosal epithelium, release neurotransmitters that cause Cl⁻ and H₂O secretion (Dawson, 1991; Greger et al., 1996). Through this process, enteric secretory motor neurons have an indispensable role in the regulation of fluid homeostasis. This happens at least partially by counteracting the continuous underlying absorption of electrolytes (Moynes et al., 2014).

Under non-stimulated conditions, apical K⁺ and Cl⁻ channels on the epithelial cells are active and lead to the secretion of KCl, which is balanced by increased Na⁺, K⁺, and Cl⁻ entry via the basolateral NKCC protein (Dawson, 1991). The CFTR channel on epithelial cells has been identified as the primary source for apical Cl⁻ efflux in the colon (Briel et al., 1998). It is found in abundance and is activated in a similar way to the NKCC protein (Greger et al., 1996). It is activated by protein kinase A and other second messenger pathways. In addition, it has also been shown to be activated by protein kinase C, calcium/calmodulin-dependent kinase, and a cGMP-dependent kinase (Kunzelmann et al., 1994; Kunzelmann et al., 1997). There are also calcium activated Cl⁻ channels that are also involved in Cl⁻ efflux from the colon. They are activated by increasing levels of calcium at the intracellular face of the channel (Cliff et al., 1990; Kunzelmann et al., 1992). cAMP levels have been implicated in regulation of Cl⁻ secretion. As the level of cAMP increases, CFTR and NKCC are activated and deactivated in a coordinated manner leading to net efflux of Cl⁻. This results in a rapid onset of hyper-secretion and inhibition of the absorptive components of the colon (Yun et al., 1997).

In the colon, K⁺ efflux is mediated via apical membrane and basolateral membrane K⁺ channels (Warth et al., 2000). Basolateral K⁺ channels maintain a hyperpolarising membrane voltage and hence, driving force for Cl⁻ secretion and
Na\(^+\) absorption. They are activated either by cAMP or increases in intracellular Ca\(^{2+}\) (Loo et al., 1989; Mall et al., 1998).

To maintain a slightly alkaline lumen pH, the colon can also secrete HCO\(_3^-\) in addition to active KCl secretion from epithelial cells. In guinea pigs, rabbits, and rats, this is done via electrogenic efflux, Cl/HCO\(_3^-\) exchange, and short chain fatty acid/bicarbonate exchange (Kunzelmann, 2002).

The colon is the main site for NaCl absorption in the gastrointestinal tract. NHE3, found on the apical membranes of colonic epithelial cells, is the predominant sodium-hydrogen exchanger involved in active Na\(^+\) transport across the murine colon by coupling the export of H\(^+\) with the import of Na\(^+\) (Melvin et al., 1999). NHE3 is unregulated under Na\(^+\) depletion in proximal colon, but attenuated in the distal colon. In the absence of NHE3 activity, an amiloride-sensitive electroneutral process partially makes up for the decrease of Na\(^+\) absorption. On the other hand, the absence of NHE2 activity in the large intestines of NHE2 knockout mice does not have a significant effect on the overall rate of net Na\(^+\) absorption (Gawenis et al., 2002).

Active K\(^+\) absorption occurs in both the small and large intestine of mice through the H\(^+\)-K\(^+\)-ATPase, which exchanges a K\(^+\) ion in for a H\(^+\) ion out in an electroneutral way by coupling the process to the dephosphorylation of ATP to ADP (Aizman et al., 1998). Potassium is actively involved in the functions of the enteric nervous system in the repolarisation of cell membranes via voltage-gated channels, due to the efflux of K\(^+\) ions through the K\(^+\) channel protein.

Calcium is required for skeletal development as well as neural signalling, especially in motor neurons. In mammals, Ca\(^{2+}\) absorption occurs via paracellular movement and transcellular transport (through the colonic epithelial cells as opposed to between; passing both the apical membrane and basolateral membrane). Vitamin D is needed in both non-saturable (passive diffusion) and saturable (facilitated and active transport) pathways. In new-born rodents, absorption is primarily though paracellular and transcellular non-saturable diffusion across all regions of the intestine. This pathway is independent of vitamin D levels. However, in adult animals, this is replaced by both vitamin D-dependent and –independent saturable active transport, which is linked to an increase in transcellular Ca\(^{2+}\) absorption.
efficiency (Toverud et al., 1986). This allows for transport of Ca\(^{2+}\) into the cell against a steep electrochemical gradient using Ca\(^{2+}\)-ATPase at the basolateral membrane of colonic epithelial cells (Fukushima et al., 1998).

In conclusion, ion transport is a complicated coordinated effort involving many transporters, exchangers, pumps and regulatory proteins in the large intestines. In young mammals especially, the colon has a significant role in the absorption of water and ions and homeostatic control of normal fluid and electrolyte compositions.

### 1.3.2 Barrier and immune function of colon

The colon forms a physical and biochemical barrier to commensal and pathogenic microorganisms. Together with the rest of the GI tract, it forms one of the largest integral barriers between the internal and external environments. The intestinal epithelial barrier is maintained by complex protein-protein networks that form desmosomes, AJs and TJs (Farquher et al., 1963). This is mediated by IECs that sense and respond to microbial stimuli, reinforcing their barrier function and participating in the coordination of appropriate immune responses, ranging from tolerance to anti-pathogen immunity (Podolsky et al., 1999).

The barrier is maintained by the expression of AJs and TJs, including cadherins, claudins, occludin and JAM proteins, which seal together adjacent cells and provide cytoskeletal anchorage (Laukoetter et al., 2006). Alterations of TJ protein formation and distribution or destabilisation of the TJ complexes can result in intestinal epithelial barrier dysfunction.

In mice, it has been discovered that Claudin-1 deficient mice die within one day of birth due to significant trans-epidermal water loss (Furuse et al., 2002). The level of claudin-6 expression in the colonic epidermis is also crucial as transgenic overexpression led to disrupted tight junction formation and increased epithelial permeability (Turksen et al., 2002). These provide corroborative data supporting the importance of claudins in the regulation of barrier function.
JAM deficient mice have been shown to be more susceptible to chemical-induced colitis, due to increased mucosal permeability, resulting in enhanced dextran flux and decreased transepithelial resistance. Dextran sodium sulphate administration to JAM-deficient mice induced more severe colonic injury as compared to wild-type animals (Laukoetter et al., 2007). This shows the importance of JAM in the formation and assembly of TJs in intestinal epithelial cells.

The IECs act as frontline sensors for microbial encounters and integrate commensal bacteria-derived signals into antimicrobial and immunoregulatory responses (Abreu, 2010; Broquet et al., 2011). This is crucial for maintenance of barrier and immunoregulatory functions in the colon. IECs express PRRs that enable them to act as dynamic sensors of the microbial environment and as active participants in the directing of mucosal immune cell responses (Chieppa et al., 2011). These include Toll-like receptors, NOD-like receptors and RIG-I-like receptors (Elinev et al., 2013). Both PRRs and commensal microorganisms are important for the protection against intestinal inflammation and repair of epithelial damage.

The integrity of tight junctions and transepithelial permeability are regulated by commensal microbial signals, including Toll-like receptor 2-dependent redistribution of the TJ proteins to apical cell–cell contacts (Cario et al., 2004). Thus, the ability of IECs to sense their microbial surroundings has an integral role in regulating their barrier function. Toll-like receptor deficient and broad spectrum antibiotic-treated mice (commensal bacteria-derived signals blocked) are more susceptible to dextran sodium sulphate induced colitis due to decreased epithelial homeostasis and repair (Medzhitov et al., 2004). IEC-intrinsic toll-like receptor signalling is also involved in the expression of cytoprotective heat-shock proteins, epidermal growth factor receptor ligands (Brandl et al., 2010), and trefoil factor 3 (promotes cell survival and repair) (Podolsky et al., 2009), and the enhanced integrity of apical TJ complexes (Cario et al., 2004).

To maintain the immune regulatory function of the colon, IECs produce various immunoregulatory signals essential for activating immune cells, limiting steady-state inflammation and directing appropriate innate and adaptive immune cell responses against pathogens and commensal bacteria (He et al., 2007; Atarashi
et al., 2011). The production of the cytokines thymic stromal lymphopoietin (Rimoldi et al., 2005), transforming growth factor-β (Zeuthen et al., 2008) and Interleukin-25 (Zaph et al., 2007) and the B cell-stimulating factors a proliferation-inducing ligand and B cell-activating factor (Xu et al., 2007) by IECs is promoted by commensal bacteria via PRR signalling. IECs also play an important part in the induction of T helper cells and innate lymphoid cells (Sawa et al., 2011).

The maturation of naive B cells into mature IgA-secreting plasma cells occurs via heavy chain class-switch recombination (Macpherson et al., 2004). IEC-derived signals help to promote IgA class switching through the production of NO, Interleukin-10 and retinoic acid in conjunction with transforming growth factor β signalling (Mora et al., 2006; Mora et al., 2008).

IECs directly influence mature T cells in terms of functional maintenance and survival in the lamina propria (Yu et al., 2006). Immune homeostasis at the intestinal barrier is maintained by bidirectional interactions between intraepithelial lymphocytes and IECs (Cheroutre et al., 2011; Edelblum et al., 2012). In mice, IECs help to refine the population of CD8+ TRM cells in favour of high-affinity precursors that possess a more efficient memory response to secondary mucosal challenge (Huang et al., 2011).

1.3.3 Secretory function for immunoprotection

Some IECs are specialised for secretory functions, such as enteroendocrine cells, goblet cells and Paneth cells. Enteroendocrine cells secrete various hormone regulators of digestive function (Kim et al., 2010). Goblet cells and Paneth cells help maintain a physical and biochemical barrier to microbial contact with the colonic epithelial cells and immune cells, by secreting mucins and antimicrobial proteins into the lumen, respectively (Gallo et al., 2012).

The first line of defence against microbial infection is the highly glycosylated mucins secreted by goblet cells. Mucin 2 is involved in the organisation of the mucosal layer of the colon (Johansson et al., 2008). Mice deficient in mucin 2 production spontaneously develop colitis and have an increase probability of
developing inflammation-induced colorectal cancers (Velcich et al., 2002). Goblet cells also secrete trefoil factor 3 and resistin-like molecule-β. Trefoil factor 3 contributes to mucus structural integrity by crosslinking mucin and acting as an epithelium repair promoter (Taupin et al., 2000). It also increases resistance of IECs to apoptosis (Dignass et al., 1994). Resistin-like molecule-β upregulates mucin 2 secretion, as well as mediating macrophage and adaptive T cell responses during inflammation (Artis et al., 2004).

Antimicrobial peptides help to regulate both commensal and pathogenic bacteria and limit bacterial resistance to antimicrobial responses (Gallo et al., 2002; Mukherjee et al., 2014). These are secreted by various IECs such as enterocytes (C-type lectin regenerating islet-derived protein) and Paneth cells (cryptidins, cathelicidins and lysozymes). Antimicrobial peptides disrupt highly conserved and essential features of common bacteria, such as surface membranes, which are targeted by pore-forming defensins and cathelicidins, and Gram-positive cell wall peptidoglycans, which are targeted by C-type lectins (Bevins et al., 2011).

1.3.4 Motility patterns of the intestinal tract

Muscle contractions in the intestinal system are important for mixing the contents of the lumen, movement of the intestinal contents at a speed that is optimum for efficient absorption and evacuation of waste products from the anus. There are two main motility patterns, peristalsis and the migrating motor complexes (MMCs).

Rhythmic contractions and relaxations in the colon are mediated through the muscles of the intestinal system that are driven by chemical transmission of various neurotransmitters and downstream signalling molecules (Smith et al., 1998).

Myogenic, hormonal and neural factors control motility in the intestinal tract. These consist of interactions between the parasympathetic and sympathetic nervous systems, local enteric neurons and hormones. The muscle layers propagate contractions as a response to neuronal or hormonal signals. Cooperation between circular and longitudinal muscle layers occurs via a feedback relay that allows the
propagation of slow contraction waves in the smooth muscle along the entire intestinal tract (Smith et al., 1998; Furness, 2005). The speed of propagation of the peristaltic wave is dependent on the region of the GI tract, species, and experimental conditions.

Motility rate was found to be much slower in the large intestine compared to the murine small intestine (Kararli, 1995); this was especially true in the proximal colon. In humans, the luminal contents experience slow, largely non-propulsive wave patterns in the proximal colon, allowing for a holding function in this region. In the distal colon however, the motility pattern was strong and propulsive for extended periods (Friend et al., 1992). The slow feeding in of content from the proximal colon maintained a prolonged dosage form in this region. Export of fecal matter to the rectum for excretion was triggered by food uptake that resulted in sudden, strong peristaltic waves through the distal colon.

The other main type of motility pattern is the migrating motor complex (Figure 1.4). This is not triggered by the movement of luminal contents but occurs spontaneously in the background. Colonic migrating motor complexes (CMMCs) were described as spontaneous contractions occurring continuously in the background at regular intervals, which were followed by periods of relative quiescence (Fida et al., 1997). CMMCs had been recorded in various species: humans, dogs, cats, mice, rats, guinea pigs (Stevens, 1978; Kozma, 1974; Anderson, 1970; Karkarli, 1995); with the likely function of enforcing slow wave contractions as well as physically mixing of the colonic contents. Various methods including external suction electrodes, force transducers, and intracellular electrophysiological studies had been used to record CMMCs in the mouse colon depending on the origin of contractile activity being investigated.
The pattern involved regular bursts of membrane potential oscillations (2 Hz) superimposed on slow depolarisations (Furness, 2005). In mice, as shown in Figure 1.4, the CMMCs occurred at a rate of around one every 3 minutes, sometimes following a transient hyperpolarization, and alternated with periods of relative quiescence (Fida et al., 1997). These unidirectional contraction patterns are triggered by the enteric nervous system (acetylcholine induced oscillations in membrane potential (Bywater et al., 1989, 1998; Lyster et al., 1993, 1995). with nitric oxide involved in the maintenance of the quiescence period between CMMCs.

1.4 Pathways governing colonic motility patterns

1.4.1 Excitatory neurotransmission pathways

In the mammalian colon, the local enteric circuits within the myenteric plexus mediate the characteristic peristaltic motility pattern, with ascending excitation and descending inhibition of circular muscle achieving a wave of propagation.
The nerve endings of myenteric IPANs, which project into muscle layers, are specialised to detect mechanosensations from the distension and stretch of smooth muscle cells (Grider, 1994). The mucosal epithelium initiates the release of serotonin as a response to mucosal distortion (Bubenik et al., 1989); and downstream submucosal sensory neurons that synapse with myenteric sensory neurons release secondary messenger neurotransmitters CGRP, tachykinins and acetylcholine (Grider and Jin, 1994).

There is then further release of excitatory transmitters: acetylcholine and tachykinins by the stimulated myenteric sensory neurons (Figure 1.5). This causes adjacent myenteric motor neurons, interneurons and other sensory neurons to transmit either fast or slow excitatory post-synaptic potentials (EPSPs), or both (Harrington et al., 2010). This excitation drives the motor neurons to release their neurotransmitters; both excitatory and inhibitory motor neurons subsequently

Figure 1.5: Representation of functional organization and neurochemistry of enteric neurons. ACh: acetylcholine; SP: substance P (tachykinin); NO: Nitric oxide; VIP: Vasoactive intestinal peptide; ATP: Adenosine triphosphate. (Benarroch, 2007).
release a variety of neurotransmitters onto smooth muscle cells to induce muscle contraction (depolarisation of excitatory neurons and hyperpolarization of inhibitory neurons).

### 1.4.1.1 ATP as excitatory neurotransmitter

ATP and its exogenous analogue adenosine 5′-O-2-thiodiphosphate (ADPβS) have been shown to cause contractile events in the longitudinal muscle of murine distal colon, through the activation of P2Y receptors. P2Y receptors are coupled to Gs protein signalling pathways that lead to an increase in intracellular calcium, and subsequently induce muscular contractions (Zizzo et al., 2008).

In the lower bowel regions of the gut, ATP triggers excitation responses and it is involved in the non-cholinergic, non-tachykininergic component of excitatory responses originating from enteric neurons (Zagorodnyuk and Maggi 1998; Zhang and Paterson 2005; Zizzo et al., 2007b). Hence, ATP can produce classical contractile responses mediated by P2X receptors, although there is evidence to suggest some P2Y receptors which are located on cholinergic nerve endings or on smooth muscle, contributing to upstream contraction in both guinea pig and mouse intestinal preparations.

This is particularly true in the mouse distal colon, where P2Y receptor stimulation located on the longitudinal muscle mediates the excitatory neurotransmission. ATP can either act directly at post-junctional location or indirectly through the activation of cholinergic neurons (Zizzo et al., 2007). In this location of interest, ATP signalling ultimately results in the release of Ca^{2+} from intracellular Ca^{2+} stores, causing muscular contraction. As expected this response was not significantly decreased in Ca^{2+}-free medium, but it was reduced to undetectable levels after the depletion of intracellular calcium stores (Zizzo et al., 2008).

Purinergic neurotransmitters such as ATP can have dual effects. P2Y receptor activation by ATP usually induces muscular inhibitory responses. Numerous studies have proven that P2Y_1 receptors mediate the purinergic component of non-adrenergic, non-cholinergic muscular inhibition, which will be discussed in
further in the next section (De Man et al., 2003; El Mahmoudy et al., 2006; Farre et al., 2006; Gallego et al., 2006, 2008). Additionally, other P2 receptor subtypes may be also be involved in muscle relaxation (Zizzo et al., 2007).

In summary, at least in the murine distal colon, ATP recruits a unique P2Y receptor, which then activates differential signal pathways leading to intracellular Ca²⁺ increase in the longitudinal muscle layer.

1.4.1.2 Tachykinins as excitatory neurotransmitter

Mammalian tachykinins are a family of neuropeptides; the most important members are substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). They all possess the conserved carboxyl-terminal amino-acid sequence Phe-X-Gly-Leu-Met-amide. This common structural feature determines their relative affinity for the various tachykinin receptors. All the tachykinins have the ability to cause fast contraction of smooth muscle in the gastrointestinal tract (Holzer et al., 2001).

All the 3 tachykinins are derived from 2 preprotachykinin (PPT) genes. Both SP and NKA are encoded by the PPT-A (or PPT-I) gene, while the PPT-B (or PPT-II) gene sequence transcribes to NKB only (Holkfelt et al., 2001). Because PPT-A is the predominant gene found in the gut, SP and NKA are the main tachykinins expressed in this organ system, whereas NKB is much more difficult to detect in the bowel.

A majority of the tachykinins detected in the GI tract are in the cells of enteric nervous system. Presence of SP is detected in enterochromaffin and immune cells of the gastrointestinal mucosal layer (Holzer et al., 2001). More significantly, tachykinins are found to be extensively co-localised with the enzyme choline acetyltransferase.

Substantial evidence has linked SP presence to several classes of enteric neurons that differ in their morphology, chemical coding, projection and function. In the rodent intestine, SP is usually found in the intrinsic primary afferent neurones (IPANs) from the myenteric and submucosal plexus. In addition, it is also detected
within the myenteric plexus, in ascending interneurons and excitatory motor neurones connected to the circular and longitudinal muscles (Furness, 2000; Holzer et al., 1998).

Tachykinin transmission is responsible for the communication between IPANs. They form a network of neurons of self-reinforcing signals. Tachykinins are also involved in neurotransmission between IPANs and interneurons in excitatory neuromuscular transmission (Costa et al., 2000). Although in this aspect, they are secondary to acetylcholine, the principal transmitter. Nonetheless, they are still important participants of the signalling pathways at neuronal and neuromuscular junctions in the gastrointestinal tract.

Tachykinins mediate many of the physiological functions of the intestinal system and play an indispensable role in the integrated response of the nervous system to various stimuli in the colon. These include functional motility responses such as regulation of the peristaltic wave activity (Holzer and Holzer-Petsche, 1997). Tachykinins have the dual functions of either stimulation or inhibition of gastrointestinal motility, through their signalling within interneurons that allow for an assimilation of both excitatory and inhibitory stimuli (Holzer and Holzer-Petsche, 1997, 2001).

Tachykinins bind to specific receptors, designated as tachykinin NK1, NK2 and NK3 receptors; each receptor has a different ranking of affinity for the different tachykinins (Maggi, 2000). Through the GTP-binding proteins that the receptors are coupled to, downstream signalling pathways are initiated, which utilise the phospholipase C/phosphoinositide system (Holzer et al., 2001).

NK1 and NK2 receptors have been shown to be the main receptors mediating the peristaltic activity of the mouse colon. This has been shown to co-exist at the same time with cholinergic signalling (Deiteren et al., 2011). This overturned the previous hypothesis that modulation of peristalsis by tachykinins only takes place in the inhibition of acetylcholine neurotransmission (Furness, 2000; Holzer et al., 1998; Holzer and Holzer-Petsche, 1997a; Holzer and Maggi, 1994). This suggests that tachykinins actually have a more significant contributing role to peristalsis in the murine colon, despite being secondary to cholinergic neurotransmission. Note
that in the rabbit colon and in the human oesophagus and intestines, this excitation is primarily mediated by only NK2 receptors (Holzer et al., 2001).

Unlike NK1 and NK2 receptors, NK3 receptor distribution showed a regional difference as it was expressed more in the distal compared to the proximal colon. Hence, the effect of stimulating NK3 receptors leads to a stronger excitatory response in the distal colon (Deiteren et al., 2011). A similar case of regional differences in NK3 receptor distribution was previously observed for the murine rectum compared to the rest of the colon. However, no comparisons were made for the proximal compared to distal colon.

In conclusion, tachykinins provide a complementary signalling system to ACh during the neural activation of gastrointestinal muscle contractions during peristalsis. In the murine intestinal system, this involves the NK1 and NK2 receptors. In the distal colon of the mouse, this activation also includes NK3 receptors, which play a contributing role to the formation of the peristaltic wave.

1.4.1.3 Acetylcholine as excitatory neurotransmitter

Acetylcholine released from enteric neurons plays the most important role in the regulation of gastrointestinal motility among all the excitatory neurotransmitters. It is the main mediator of all excitatory responses. The targets for acetylcholine are the muscarinic receptors located on enteric neurons and muscle cells (Kottegoda et al., 1969; Bubenik et al., 1986; Levey et al., 1993). Nicotinic receptors are another type of receptor mediating cholinergic transmission in the ENS. These receptors are ligand-gated ion channels and are primarily involved in inter-neuronal signalling instead of muscular contraction responses (Caulfield and Birdsal, 1998).

From molecular cloning studies, five receptor subtypes (M1–M5) have been identified (Levey, 1993) and 2-3 subtypes have been known to be expressed simultaneously at a single organ (Caulfield and Birdsall, 1998; Eglen et al., 1996). The presence of all 5 muscarinic receptors has been detected along the gastrointestinal tract, on the various enteric neurons and muscle cells. However,
the main receptor subtypes expressed on muscle cells that mediate acetylcholine-induced contractions are the M2 and M3 subtypes (Eglen, 2001; Eglen et al., 1996; Ehlert et al., 1997; Sawyer and Ehlert, 1998).

The M2 receptor subtype has also been identified on enteric cholinergic nerves, which regulates acetylcholine release (Coulson et al., 2002; Harrington et al., 2010; Vizi et al., 1989). M2 receptors are additionally expressed on muscle, nerve endings and interstitial cells of Cajal (ICC) in the deep muscular plexus and intramuscular layers. On the other hand, M3 receptors have been found on muscle cells, myenteric neurons and ICC in the myenteric plexus.

The M1 receptor is also expressed on myenteric nerves, as evident from immunohistochemical studies (Dietrich and Kilbinger, 1995). It has the dual function of regulating acetylcholine release, (Harrington et al., 2007, 2010) as well as NO release from inhibitory nerves (Iversen et al., 1997; Wiklund et al., 1993).

The focus in this section will be on the M2 and M3 receptors as they are the most common muscarinic receptor subtypes expressed in the gastrointestinal tract (Eglen, 2001; Eglen et al., 1996; Ehlert et al., 1997; Levey, 1993). In muscarinic receptor-deficient mice, functional studies in the stomach and ileum have shown that both M2 and M3 receptors have important roles in muscarinic acetylcholine-induced contraction of circular and longitudinal muscle layers (Kitazawa et al., 2007; Unno et al., 2005). Similar results have been found in the colon, contraction responses to acetylcholine are mediated by both M2 and M3 receptors; and it helps to promote propulsive motility in the colon and defecation. The M1 receptor has also been found to promote an antagonistic response to motility stimuli through its interactions with enteric nitrergic nerves in this particular region of the murine gastrointestinal tract. In M2 and M3 receptor-knockout mice acetylcholine-induced contractions were eliminated, demonstrating that intestinal contraction occurs via a synergistic pathway that requires the activation of both subtypes, unlike in the stomach (Sakamoto et al., 2007).

The function of the muscarinic receptor on the enteric muscle cells is to mediate the direct effect of acetylcholine on muscle reflexes and distention, resulting in a cholinergic-evoked muscle contraction (Tonini et al., 1981). Even though there are fewer M3 receptors expressed on smooth muscle cells compared to M2 receptors,
it is the dominant receptor in muscle contraction regulation (Matsui et al., 2002; Uchiyama and Chess-Williams, 2004). M2 and M3 receptors operate through different signalling mechanisms within the same target muscular layer (Sakamoto et al., 2007).

M2 subtype activation results in downstream inhibition of adenylate cyclase, while the M3 subtype triggers phosphoinositide hydrolysis, resulting in calcium mobilisation and the contractile response (Griffin and Ehlert, 1992). Depolarising ion fluxes are induced in the motor neurons simultaneously in response to acetylcholine binding. Cholinergic inhibition of adenylate cyclase, mediated by M2 receptor activation, resulted in decreased levels of cAMP. Hence, acetylcholine binding allowed for basal relaxation signals, which are mediated by an increase in cAMP levels, to be switched off, resulting in the net neural signal for contraction to occur (Kondo et al., 2011).

On the other hand, M3 receptor activation is directly involved in the downstream initiation of the tonic contractile phase. It increases the levels of inositol-trisphosphate synthesis, depleting the intracellular Ca\(^{2+}\) store, and therefore triggering Ca\(^{2+}\) entry from the voltage-dependent Ca\(^{2+}\) channel and tonic contraction (Sakamoto et al., 2007; Unno et al., 2005). M3 receptors also play a regulatory role on the responses triggered by M2 receptor activation. As depolarisation occurs as a result of M2 receptors initiating the opening of cationic channels, M3 receptors have been shown to regulate the activity of these cation channels (Bolton and Zholos, 1997).

Taken together, these results showed that non-sustained colonic contraction induced by acetylcholine is a cooperative response between the M1, M2 and M3 receptors. It is a 2-step process comprising smooth muscle contraction (M2 and M3 receptors) and suppressed inhibitory nitricergic relaxation (M1 receptor) to achieve the desired net balance response. Even within the smooth muscle contraction phase there are dual effects induced by muscarinic receptors; a direct M3 receptor-mediated contraction via hydrolysis of phosphoinositide and an indirect M2 receptor-mediated inhibition of the relaxation, also known as offsetting relaxation. In conclusion, the M3 receptor is especially important to colonic propulsion in the murine colon.
1.4.2 Inhibitory neurotransmission pathways

In the original study by Okasora and Okamoto (1986) and Wood et al. (1986), the circular muscle of mouse colon demonstrated inhibitory junction potentials (IJPs), which are induced by NANC nerve activation leading to smooth muscle relaxation. These IJPs are characterised by an initial fast hyperpolarization (fast IJP) followed by a slower, longer-lasting hyperpolarization (slow IJP) of smaller amplitude, which is nitrergic in nature (Stark et al., 1993; Kishi et al., 1996; Xue et al., 1999). As seen in figure 3.1, this has effects on downstream NO and ATP mediated signalling for relaxation.

1.4.2.1 Fast Inhibitory junction potential

Endogenous ATP and ADPβS, which are both P2Y purinoceptor ligands, are responsible for fast IJP in circular muscle from murine colon. They exert their effects through Ca\(^{2+}\) - activated K\(^+\) channels. K\(^+\) channels with properties consistent with small conductance K\(^+\) channels are expressed in the murine colon. These are similar to those found in other gastrointestinal muscle and they are involved in ATP induced membrane hyperpolarisation (Koh et al., 1997). Treatment with exogenous ATP increased muscular hyperpolarisation, while treatment with apamin (blocker of subtypes of small and intermediate conductance Ca\(^{2+}\)-activated K\(^+\) channels) almost totally inhibited the fast IJP evoked by both EFS and ATP application (Serio et al., 2002).

P2X receptors on the other hand, do not appear to be crucial to fast IJP generation in mice despite being prominent in the guinea-pig colon (Zagorodnyuk et al., 1996). P2X receptors, are nonetheless, present in the distal colon of the mouse. However, the application of \(\alpha, \beta\)-methylATP (an analogue of ATP that targets P2X receptors) for a sustained period of time failed to produce the fast IJP (Serio et al., 2002).
Another regulatory neurotransmitter is PACAP (pituitary adenylate cyclase-activating peptide), which plays a minor role and is highly dependent upon the neural action potential and ATP activation of P2Y receptors. PACAP has been shown to be a neuromodulator of the inhibitory pathway. It upregulates ATP release from inhibitory neurons and acts on the apamin-sensitive Ca\(^{2+}\) -dependent K\(^+\) channels at the post-junctional level. This increases the magnitude of the muscular relaxation response to ATP (Rattan and Chakder, 1997; Fox-Threlkeld et al., 1999). Even spontaneous IJPs were sensitive to a PACAP receptor antagonist, demonstrating that PACAP plays an enabling role in tonic relaxation (Ekblad et al., 2000).

In summary, ATP is the main neurotransmitter that regulates both the spontaneously occurring IJPs and the fast IJP presented in the circular muscle layer of the murine colon, with a minor modulation from PACAP. Apamin-sensitive Ca\(^{2+}\) -dependent K\(^+\) channels are the downstream target of P2Y mediated ATP neurosignalling, resulting in the hyperpolarisation of the cell membrane. P2Y or P2X receptors antagonists both have a negative effect on evoked fast IJP, but P2Y is the crucial receptor for tonic relaxation. Lastly, the fact that LNNA treatment did not affect ATP-hyperpolarisation rules out a possible interplay between ATP and NO.

1.4.2.2 Slow Inhibitory junction potential

Nitric oxide is the primary neurotransmitter released by inhibitory nerves projecting onto the longitudinal muscle of mouse colon. Nitric oxide influences 2 separate downstream pathways: guanylate cyclase stimulation and opening of Ca\(^{2+}\) -dependent K\(^+\) channels that work in parallel to produce slow IJP (Smith et al., 1998; Serio et al, 2002).

Nitric oxide induced effects are predominantly mediated through the guanosine 3’, 5’ -cyclic monophosphate (cGMP) synthesis pathway. NO does not have a specific receptor; instead it diffuses freely through the membranes of ENS cells to exert its effects. Its main target is the soluble NO-sensitive guanylyl cyclase (NO-GC). NO
activation results in the production of the intracellular messenger cGMP (Iversen et al., 1997; Wiklund et al., 1993). Other downstream phosphorylation of cellular proteins, such as IRAG (Inositol-3-phosphate receptor associated cyclic GMP kinase substrate) and a calcium-ATPase on the endoplasmic reticulum, help to transmit the NO signal for induced relaxation of the smooth muscle layer (Smith et al., 1998).

Some of these effects are triggered by the exposure of ion channels to NO, which can produce a range of effects including increased K+ conductance (results in hyperpolarisation of the cell membrane) and tyrosine kinases activation (further protein phosphorylation). Immunohistochemical and release studies have also indicated that the M1 acetylcholine receptor is involved in NO signalling. It is found in myenteric nerves and regulates NO release (Dietrich and Kilbinger, 1995; Harrington et al., 2007, 2010).

NO is produced by the enzyme nitric oxide synthase (NOS). In mice, NOS-containing neurons are present in the smooth muscle layers throughout the gastrointestinal tract. In mice, NO is released endogenously by enteric inhibitory nerves or it can be released by drugs such as sodium nitroprusside. Either way, the slow IJP is caused by the activation of apamin-sensitive Ca2+-dependent K+ channels, much like fast IJP (Kondo et al., 2011). Nonetheless, the production of cGMP still plays a huge role, as treatment with the inhibitor of soluble guanylyl cyclase antagonised both nerve stimulation and sodium nitroprusside application induced relaxation.

The effect that NO has on slow IJP has been demonstrated in various experiments. N-nitro-l-arginine (LNNA) is the antagonistic form of the natural substrate l-arginine for NOS. ODQ is a potent and selective inhibitor for NO-sensitive guanylyl cyclase. Treatment with LNNA and ODQ relieved endotoxin induced delay in intestinal transit in lipopolysaccharides induced colitis (De winter et al., 2002). This shows that the inhibition of slow IJP allows for excitation contractile responses to become dominant again.

There are 3 forms of NOS: the endothelial isoform (eNOS), neuronal isoform (nNOS) and the inducible isoform (iNOS). The inducible and neuronal isoform primarily contribute to the production of NO in the ENS. Inhibition of the inducible
form especially had the greatest effect on restoring motility in colitis (De winter et al., 2002).

NO-GC is the most important messenger in the NO induced relaxation signalling pathway. There is an absence of NO-induced relaxation of GI smooth muscle in Mice that lack NO-GC. NO-GC knock-outs had an increase in gut transit time, resulting in motility dysfunction. The relative location of NO-GC is also crucial to its function. However, recent studies have also shown that NO-induced relaxation decreased only slightly in mice that express no NO-GC in smooth muscle specifically and there was no change in whole-gut transit time compared to wild type mice (Gruenberg et al., 2011). Similar to the phenotype of total GC knockout mice, these knockout mice present a lack of NO responsiveness of vascular smooth muscle and hypertension. However, they still demonstrate a wild type response with regards to whole gut transit time and NO/EFS-induced relaxation unlike the total GC-KO mice. In total GC-KO mice, both pharmacologically applied and endogenously released NO led to relaxation of the mouse colon preparations.

Taken together, this data suggests that NO-GC is the only messenger in the ENS for conveying nitrergic signalling responsible for generation of the slow IJP. NO-GC expression in smooth muscle is surprisingly not crucial for the relaxation component of peristalsis, but this might just be due to the upregulation and compensation from fast IJP (Groneberg et al., 2011). Moreover, NO-GC expression has also been reported in ICC and fibroblast-like cells (Furness, 2000). Hence, an involvement of one or both of these cell types in nitrergic relaxation is plausible.

1.5 Colonic migrating motor complexes

Colonic migrating motor complexes (CMMCs) are spontaneous cyclical contractions in the colonic smooth muscular layer that propagate over significant distances in the colon. They originate and are regulated by the enteric nervous system and have been shown to aid in the propulsion of colonic contents (Fida et
al., 1997). CMMCs have been demonstrated in various mammals, including both the murine (our study model) and human colon (Barnes et al., 2014).

CMMCs are characterised by a hyperpolarization, followed by fast oscillations (slow waves) with action potentials superimposed on a slow depolarisation wave (Dickson et al., 2010). These periodic contractions occurred spontaneously without the need for intraluminal stimulation. The main functional role of the CMMC contractions in the mouse colon is supplementing solid faecal pellets propulsion; it was demonstrated \textit{in vitro} that the start of each CMMC was linked to the subsequent progression of an artificial pellet down short distances in the colon (Spencer, 2001). The velocity of the propagation wave was found to be approximately $3.9\pm0.6$ mm/s in the murine colon, in the C57BL/6 strain (Bush et al., 2000). There was usually an interval between CMMCs ($3.52\pm0.31$min) and the duration of the contractions was shorter ($30.7\pm3.6$min).

The ENS is deeply involved in this process. CMMCs are found to be dependent on cholinergic neurosignalling. They were blocked by administration of hexamethonium (nicotinic nACh receptor antagonist) and attenuated by atropine (muscarinic ACh receptor antagonist). Exposure of the mouse colon to TTX also increased the resting tone of the tissue (Bush et al., 2000). This showed the presence of tonic inhibition and the significance of its regulatory role in this bowel region. TTX has also been shown to increase spiking activity in the smooth muscle (Bywater et al., 1997).

The fact that atropine attenuated, but did not completely get rid of CMMCs in the large intestine, proved that ACh and excitatory motor neurons are integral to the mechanism of the contractile portion of CMMCs. However, the frequency of CMMC activity was not mediated by muscarinic ACh receptors and all the cells that express it, including enteric neurons, muscle cells and ICCs (Spencer et al., 1998a). Therefore, there must be additional excitatory neurotransmitters or further removal of tonic inhibition that is involved in muscular contractions associated with CMMC activity in the colon.

Even within the colon, there were observable differences in the characteristics of the CMMC contractions recorded from the proximal, mid and distal regions. The contractions in the proximal and mid-colon consisted of a slow contraction superimposed with smaller rapid contractions. These rapid contractions occurred
at a frequency similar to electrical slow waves recorded using intracellular microelectrodes (Fida et al., 1997). Hence, these contractions may be dependent upon basal slow wave activity. This pattern of superimposed contractions was not detectable consistently in the distal colon, and was mostly not observed (Zagorodnyuk and Spencer, 2011).

Despite the fact that they are spontaneous occurrences, CMMCs occur at significantly lower frequencies or are absent completely when the colon is free of luminal contents. A recent study by Barnes et al. (2014) showed that past in vitro studies on whole segments of empty murine colon always presented CMMCs at high frequencies because conventional recording techniques all indirectly stimulated the colon. CMMCs usually absent, or at very low frequencies in an empty colon. However, if faecal content is present or if in vitro techniques are used that stimulate the intestine, their frequency increases significantly.

There has been speculation that the regulatory mechanisms for CMMC generation and frequency must lie within the myenteric plexus or the muscularis, since removal of the mucosa and submucosal plexus does not prevent their generation; but there are also studies that suggest that with the mucosa CMMC occur (Smith et al., 2010; Keating and Spencer, 2010; Zagorodnyuk and Spencer, 2011; Spencer et al., 2013).

In conclusion, the generation cycle of CMMCs has multiple distinct steps. During the intervals between CMMCs in the mouse colon, the membrane potential of the circular muscle layer was maintained under tonic inhibition. This is done with the underlying release of inhibitory neurotransmitter. As the CMMC propagates down the colon, there are two crucial processes that occur. Firstly, there is release of ACh from cholinergic motor neurons into the circular muscle. Next, there is presynaptic cessation inhibitory neurotransmitters release (disinhibition). The latter effect is responsible for the slow membrane depolarization of the smooth muscle layer during each CMMC cycle (Spencer, 2001).
1.5.1 Neurotransmitters responsible for the regulation of CMMCs

Various ENS neurotransmitters have been shown to be involved in the regulation and generation of CMMCs. The tonic inhibitory control appears to be the result of the release of nitric oxide and purines from inhibitory motor neurons to the circular muscle layer (Bywater et al., 1997, Lyster et al., 1995, Powell et al., 2001, Spencer et al., 1998). A withdrawal of the tonic inhibitory signalling (disinhibition) had been suggested to be mostly responsible for CMMC initiation (Bayguinov et al., 2009). Other studies using tension recordings, have proposed that, rather than disinhibition, CMMCs are generated predominantly by the release of both acetylcholine and tachykinins from excitatory motor nerves instead (Brierley et al., 2001). It has been suggested that the mouse strains used might be to blame for the difference in results (C57BL/6 vs. Swiss). However, it is more likely that excitatory neurotransmitters do not only affect the contractile responses directly but also interrupt neuronal transmission suppressing CMMCs (Brierley et al., 2001).

1.5.1.1 Role of Nitric oxide on CMMCs

The mouse colon was suggested to be maintained in a state of tonic inhibition through the neuronal release of NO and other inhibitory neurotransmitters (Spencer et al., 1998). Both the excitability of the latent pacemakers and also the neural transmission that governs the frequency of propagation of CMMCs are inhibited by nitric oxide released from the myenteric plexus (Bayguinov et al., 2009). NO has be shown to regulate neurotransmission in descending and ascending interneurons, allowing an interval to be maintained between waves of CMMC propagation. However, in mice treated with a nitric oxide synthase endogenous substrate (L-arginine), there seemed to be a resumption of CMMCs after continued exposure (Dickson et al., 2010). This meant that alternative processes cannot be ignored.

Blocking NO production and signalling changed the frequency and amplitude of CMMCs. In short exposures to N-nitro-l-arginine (LNNA; NOS inhibitor), the
interval between CMMCs was decreased by 55% (from approximately 3 min in control). NOS inhibitor presence was also linked to a shift in migration direction of CMMCs, such that CMMCs migrated orally instead of aborally (Powell, 2011). Treatment with L-arginine in increasing concentrations resulted in increased suppression of CMMCs for the first 15 minutes, after which higher frequency contractions (10–15 min⁻¹) of variable amplitudes presented. These appeared mostly in the proximal region of the colon and they only lasted for a short amount of time. Subsequently, CMMC cycles were observed to restart. Hence, it can be said NO plays an important role in suppressing the frequency of migrating contractions in the isolated mouse colon (Powell, 2011).

These interim CMMC contractions of high frequency and short-duration, in the presence of L-arginine, are not observed in the distal colon (Dickson et al., 2010). In the proximal colon, there appears to be a switching mechanism between two motor patterns in the mouse colon; from non-propulsive high-frequency twitches to propulsive migrating contractions interweaved with periods of relative quiescence (Powell et al., 2011). This agrees with the proposal that CMMC migration is mediated by an excitation neural wave that propagates along the myenteric plexus, while the quiescence period is enforced with tonic signalling of inhibitory motor neurones, which release inhibitory neurotransmitters like NO into the circular muscle (Dickson et al., 2010). The inhibitory motor neurones also stem from the myenteric plexus of the colon, and their NO release has inhibitory effects both upstream and downstream.

However, there is an interesting scenario to consider. In a study of NOS knockout mice, there was surprisingly no significant difference in the frequency of CMMCs. In wild-type mice, the presence of the NOS inhibitor LNNA caused a marked increase in CMMC frequency as well as a depolarisation of the circular muscle layer (Spencer et al., 2013). Compared to controls, there was no depolarisation of the resting membrane potential of circular muscle cells in NOS knockout mice. There was also no significant difference in the amplitude of the slow depolarization phase underlying CMMCs between knockout and wild-type offspring. These results show that the major characteristics of CMMCs and their electrical correlates in NOS knockout mice are still indistinguishable from wild-type mice, casting a shadow of doubt about the exclusivity of NO control of CMMCs.
In summary, NO plays an essential role in suppressing the frequency of migrating contractions in the mouse colon through its effects on smooth muscle tone (hyperpolarising the muscle cells to allow the start of a new CMMC). The proximal colon presents more CMMC activity than the mid-distal colon (Fida et al., 1997). The continued application of l-arginine in experiments should be given consideration as it does not seem to be effective for long periods. Also, the fact that NOS knockout mice demonstrated no overall difference in the propagation of CMMCs suggests that there is substantial compensation through upregulation of other neurotransmitters, receptors, ion channels, or signalling pathways possible.

1.5.1.2 Role of excitatory neurotransmitters on CMMCs

Excitatory neurotransmission plays a role in the pacemaker activity during the CMMCs. Excitatory neurotransmitters create a synchronised interaction between neurons and interstitial cells of Cajal networks to generate the CMMC. The generation of CMMCs is mediated by the activation of both myenteric and submucosal pacemaker cells that release acetylcholine and tachykinins from excitatory motor neurons (Heredia et al., 2009). It was previously shown by Brierley et al. (2001) and Gourcerol et al. (2009) that the M2 and M3 muscarinic receptors on cholinergic nerves are involved and in migrating motor complexes.

Tachykinins bind to NK1, NK2 or NK3 receptors. In the presence of NK1 or NK2 receptor antagonists, RP 67580 and MEN 10376 respectively, faecal pellet propulsion along the murine and guinea pig colons appeared to be completely inhibited (Dickson et al., 2010). Tachykinin antagonists have no effect on the resting membrane potential of the muscular layers. This suggests that ongoing tonic inhibition of the circular muscle was maintained, linking tachykinin signalling to NO signalling.

In addition, NK2 receptor expression was reduced in slow-transit constipation highlighting an important role for tachykinins in generating CMMCs (Powell et al., 2001). A decrease in the number of pacemaker ICC has also been observed in
slow-transit constipation (Lee et al., 2005). It has been suggested that this might compromise neural coupling to the muscle during the CMMC. Hence, CMMC initiation should not be considered as just a neural occurrence, but instead be viewed as a synergistic interaction between neural and muscle networks. Excitatory neurotransmitters such as acetylcholine and tachykinins are crucial in mediating this phasic pattern of CMMCs. (Fida et al., 1997; Brierley et al., 2001)

1.5.1.3 Role of Serotonin on CMMCs

There are some disagreements about the role of serotonin in regulation of CMMCs. Some studies have shown that mucosal serotonin is not involved in the generation of CMMCs, but other studies say that it plays an important part in propagation of the wave activity.

In whole colon preparations with mucosa and submucosal plexus removed, the frequency and amplitude of spontaneous CMMCs was not significantly different, between groups treated with reserpine or without (Spencer et al., 2013). Resperine is a molecule that depletes serotonin (locally synthesised in the ENS) to undetectable levels. This suggested that both the mucosal and enteric serotonin are not required for CMMCs. Serotonin had no effect on the frequency of CMMCs when added to intact mucosal preparations (Keating et al., 2010), suggesting that neurons projecting into the mucosa are not important for CMMC generation. Serotonin only regulates the firing of the intrinsic pacemaker and pattern generator, which is located in the myenteric plexus (Keating et al., 2010)

Treatment with ondansetron (5-HT3 receptor antagonist) was equally or even more efficient at reducing CMMC frequency in mucosa and submucosal plexus-free preparations, compared with control preparations. This showed that even if 5-HT3 receptors were involved in the process, the ones in the mucosa were not essential. These results are consistent with a previous study that showed CMMCs prevailed after the mucosa, submucosa, and submucosal plexus were removed from the colon sample (Keating et al., 2010). Also, ondansetron inhibited CMMC frequency despite the absence of endogenous serotonin (Keating et al., 2010). However, in
this case, CMMCs actually occurred at lower frequency compared to the control preparation. Nonetheless, it was suggested that reduction of CMMC frequency by 5-HT3 receptor blockades was done through dampening fast synaptic transmission at 5-HT3 receptors within the myenteric plexus.

Surprisingly, reserpine pre-exposure had no effect on the efficacy of ondansetron in CMMC inhibition (Spencer et al., 2013). All these experiments show that neither endogenous serotonin in enteric neurons nor the mucosa is required for the spontaneous CMMC generation or propagation. Moreover, ondansetron seemed to be having negative effects on CMMC frequency, which were not mediated via the blockade of 5-HT signalling in the mucosa, submucosal plexus and myenteric neurons (Martin-Cano et al., 2013; Spencer et al., 2013).

However, other studies suggest that 5-HT is an important regulator of CMMCs. Tryptophan hydroxylation 1 is the essential enzyme necessary for the serotonin synthesis from dietary tryptophan in enterochromaffin cells in the mucosa. The effects of mucosal 5-HT in regulating CMMCs and faecal pellet propulsion were examined in tryptophan hydroxylation 1 knockout (TPH1KO) mice. Compared to wild type mice, propagation of small diameter pellets was inhibited in the TPH1KO, while larger pellets were seen to pass normally through the colon. These knockout mice exhibited no mucosal reflexes that allowed for propagation of smaller pellets that do not stretch the colon significantly (Smith et al., 2010; Heredia at al., 2013). There were also reduced responses to intraluminal distension and only larger faecal pellets were propelled, suggesting that overreliance upon stretch reflexes alone.

So unlike the other studies, there is evidence to support the important role of serotonin release from the mucosa in non-stretch reflexes and for normal colonic propulsion. There is an observable dysfunction in the regulation of colonic motility in mice lacking serotonin in the ENS even though this is not obvious in the integrative measurement of the total spontaneous CMMC frequency in the isolated colon. This difference might be due to the fact that integrative nature of tension measurements does not give insight to the nature of underlying reflexes or motor patterns, as well as the lack of endogenous colon stimuli.
1.6 Melatonin

1.6.1 General functions of melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is a highly lipophilic molecule and it can diffuse freely through the plasma membranes. Thus, it can potentially have various effects within the cell even in the absence of its designated membrane receptors, through activation of nuclear receptors (Motilva et al., 2001).

Melatonin is both a hormone and a neurotransmitter (Arendt, 1988; Bubenik, 1980; Cardinali, 1980; Carpentieri, 2012) and as a result has widespread and interlinked effect on cells. Melatonin was first identified as a hormone from the pineal gland that regulates circadian rhythm (Quay, 1964). Melatonin is produced in varying amounts during the night and day. Melatonin secretions peak during the night time. Melatonin is produced from serotonin (5-hydroxytryptamine (5-HT) as an indoleamine hormone (Raikhlin et al., 1975).

As a pineal hormone, melatonin triggers both acute neuronal inhibition and phase-shifting in the suprachiasmatic nuclei (SCN) in the brain. The SCN is located in the hypothalamus and controls the circadian cycles and influences physiological and behavioural rhythms occurring over a 24-hour period, such as the sleep/wake cycle. It is the master clock in the mammalian brain (Klein et al. 1991). Light and darkness stimuli reset the circadian rhythm driven by the SCN to the 24-hour day. This reset ensures that biological processes are occurring in the correct temporal order in relation to each other.

Since the late 1970s it has also been identified to be present in the GI tract; however its precise location and function are still not well understood. In humans, it was first detected in the appendix, followed by the mucosal layer in the general gastrointestinal system (Raikhlin and Kvetnoy, 1975). Other investigations have confirmed the presence of high concentrations of melatonin in both the gastric and duodenal mucosa. Melatonin is also secreted in large amounts into the bile (Messner et al., 1999). Following release melatonin has been calculated to have a short half-life in the range 30–50 min (Carpentieri et al., 2012) and hence needs to
be continuously synthesised as required. Melatonin has not been identified in the colon, and in particular, the mouse.

Melatonin influences a variety of cellular functions in the gastrointestinal tract. Melatonin serves as a hormone when secreted into the hepatic portal vein as well as a paracrine signalling molecule on mucosal surfaces in the gastrointestinal tract including the stomach, small intestine and colon (Konturek et al., 2007). It is involved in regulating motor function, the microcirculation and proliferation of mucosal cells in the gastrointestinal tract, on top of its more general functions in regulating biorhythmicity, as an antioxidant and a regulator of the immune response (Bubenik, 2008).

Melatonin had been shown to trigger gastroprotective compound synthesis as well via receptor-mediated mechanisms; it increases the activity of nitric oxide (NO) synthase (NOS)-NO and cyclooxygenase (COX)-prostaglandin E (2) (PGE (2)) systems resulting in the increase of mucosal blood flow and mucosal integrity in ulcers, lesions and colitis states (Konturek et al, 2006; Konturek et al, 2010; Terry, 2009; Stebelova et al., 2010). It has also been demonstrated that melatonin has a role in regulating colonic motility and sensation (Lu et al., 2005; 2009). Clinical doses of melatonin helped in pain relief in the abdominal region of patients who suffer from irritable bowel syndrome (IBS), allowing them less interrupted sleep (Song et al., 2005). Melatonin has also been shown to decrease the inhibitory response of non-adrenergic, non-cholinergic (NANC) neurons and therefore has the potential to regulate motility (Storr et al., 2002).

The role of melatonin in the gastrointestinal system remains an area for further research. Despite all these observed effects, the mechanisms of melatonin’s actions and the pathways it influences are not definitively understood in particular regions of the GI tract like the colon.
1.6.2 Biosynthesis and metabolism of Melatonin

Most of the circulating melatonin is synthesised in the pineal gland, but production has also been detected in the gastrointestinal tract, where production is suspected to be in mucosal epithelial cells (Bubenik, 1980; Raikhlin et al., 1975). Besides the two sites where higher production is observed, melatonin production has also been detected in mast cells, natural killer cells, eosinophilic leukocytes, platelets, endothelial cells and other non-endocrine tissues (Arendt, 1988; Bubenik, 1980; Cardinali, 1980; Carpentieri, 2012).

Melatonin synthesis in the pineal gland is influenced by the light/dark cycle, while its day-time blood concentrations are attenuated but sustained mainly due to its release from the gastrointestinal tract (Quay et al., 1964; Bubenik et al., 1980). However, in the gastrointestinal tract, melatonin production is affected by food ingestion patterns, and it is postulated that it is under the control of a positive feedback loop related to the amount of tryptophan (Weissbach et al., 1960; Wurtman et al., 1964). In the gastrointestinal tract melatonin production occurs even during the day, particularly following food intake. It had been demonstrated that there was melatonin in the mucosal layers during fasting, which then increases in dose-dependent manner both in intact and pinealectomised animals after oral application of tryptophan, the starting point of melatonin synthesis (Bubenik, 2002; Weissbach et al., 1960).

Figure 1.6 shows the biosynthesis process of melatonin. Tryptophan, an essential amino acid is the starting point of melatonin biosynthesis. As seen in Figure 1.6, it is converted by the enzyme tryptophan hydroxylase to 5-hydroxytryptophan (Step 1). Serotonin (5-hydroxytryptamine) was formed in the next step through the action of aromatic amino acid decarboxylase (Step 2). Melatonin catabolism was achieved through 2 enzymatic steps from serotonin (Steps 3 & 4). The first was the N-acetylation by aralkylamine N-acetyltransferase (AANAT) to yield N-acetylserotonin. The next step in the production of melatonin production is catalysed by Acetylserotonin O-methyltransferase (HIOMT), which transfers a methyl group from S-adenosylmethionine to the 5-hydroxy group of N-acetylserotonin to give melatonin (Mrnka et al., 2008). The study of these
enzymatic activities in the GI tract has been done in goldfish, but not rodents yet. The variation in enzymatic activity for HIOMT in goldfish hindgut was not significant with time. However, AANAT appeared to be under physiological regulation, with a significant increase in activity observed at night in general (Nisembaum et al., 2013; Velarde et al., 2010). In the rodent pineal gland where the enzymes were detected originally, the rate-limiting step of melatonin synthesis was the acetylation of serotonin, which was catalysed by AANAT (Cardinali et al., 1998; Boutin et al., 2005). HIOMT methylation activity was not as pronounced in the pineal gland as it was in the gastrointestinal tract (Verlade et al., 2010).

Melatonin degradation pathways have been investigated widely and 3 major ones have been identified: the first one identified was hepatic degradation and the metabolite was 6-hydroxymelatonin (Reiter, 1991; Facciola et al., 2001). The second was kynuric degradation to give \( N1\)-acetyl-\( N2\)-formyl-5-methoxykynuramine (AFMK) (Slominski et al., 2008; Hirata et al., 1974; Tan et al., 2007; Hardeland et al., 1993). The last is the indolic degradation pathway that results in 5-methoxyindole acetic acid (5-MIAA) or 5-methoxytryptophol (5-MTOL) as metabolites (Grace et al., 1991; Rogawski et al., 1979). Melatonin metabolism involves the liver and various CYP P_{450} enzymes were shown to be involved, including CYP1A1, CYP1A2, and CYP1B1. They were involved in the production of 6-hydroxymelatonin from melatonin (Ma et al., 2005). 6-hydroxymelatonin could then be further processed via conjugation with glucuronides or sulphates before being excreted from the body in urine (Arendt, 1988).
Melatonin could also be consumed in other mechanisms that do not act through receptors. It could be a scavenger molecule for reactive nitrogen and oxygen species (Gomez-Moreno et al., 2010). These reactive oxidants, such as hydroxyl radical (HO\(^{-}\)), hydrogen peroxide (H\(_2\)O\(_2\)) and nitric oxide (NO\(^{-}\)) combine with melatonin to form irreversible products, consuming melatonin which is then
released as a terminal antioxidant (Galano et al., 2011; Tan et al., 2001; Slominski et al., 2012).

1.7 Location of melatonin in the GI tract

The intestine is both a source and accumulation point for melatonin; its high concentration is attributed to suspected local synthesis in EC cells, which is triggered by food intake (Stebelova et al., 2010; Bubenik, 2002; Konturek et al., 2007). Intestinal melatonin is present in the extracellular media between layers of tissue, the gastrointestinal lumen and is secreted into the circulatory system. As melatonin is highly lipophilic, can diffuse through biological membranes (Matheus et al., 2010). Melatonin is a transient molecule with a short half-life, and it exerts its effects through various mechanisms such as the endocrine, paracrine, neurocrine and autocrine signalling systems.

Attempts have been made in the past to identify melatonin and its source of synthesis in the GI tract. These techniques include western blot, transcript analysis, immunohistochemistry/radioimmunology and binding column studies (all of which will be discussed in detail below). However, although these techniques were able to localise the presence of melatonin to the mucosal epithelium, the precise localisation remains inconclusive. Specifically, melatonin was localised to the mucosa of the small intestine using immunohistochemistry in rats and guinea pigs (IHC) (Bubenik et al., 1977). Melatonin labelling was also identified in the enteroendocrine cells, but not specifically EC cells, in human appendix mucosa tissue cultures (Raikhlin and Kvetnoy, 1975). In the rat colon, immunofluorescence studies also confirmed the presence of melatonin in the mucosa (Holloway et al., 1980).

Other studies have looked at the distribution of its receptors for insight into where it is located based on functionality. In the gastrointestinal tract, MT₁ melatonin receptors have been found on myenteric plexus neurons in the rat colon, suggesting enteric neurons could be responsive to melatonin (Soták et al., 2006).
The studies conducted to localize melatonin have provided evidence for the location in the mucosal epithelium in various regions of the GI tract in different species, but not in the murine colon. There have also been studies that have shown the presence of melatonin receptors and enzymes involves in the synthesis of melatonin in various regions of the intestinal wall. Overall, it is unclear on where specific production of intestinal melatonin occurs and no knowledge is available about this from the mouse colon.

1.7.1 An evaluation of past immunohistochemical studies

The mucosa of the stomach, duodenum and the hepatobiliary system, which were part of the duodenal cluster unit, all contain high levels of melatonin (Konturek et al., 2007). Melatonin was localised to the mucosa of the rat intestine using immunohistochemistry (IHC) (Bubenik et al., 1977). In the rat colon, immunofluorescence studies also confirmed the presence of melatonin in the mucosa (Holloway et al., 1980). Within isolated cell cultures of the human gut mucosa, melatonin is observed in the nucleus, microsomes, mitochondria and cytoplasm (Kvetnoy et al., 2002). Using immunohistochemical analysis, studies were able to link the mucosa, the site of EC cells, to melatonin presence (Raikhlin and Kvetnoy, 1975). These studies all support that gastrointestinal melatonin is most likely to be generated from the mucosal epithelium, but the precise cellular location is still unclear.

From the original immunohistochemistry results in Figure 1.7A melatonin was first shown to be localised to the mucosal layer of the gastrointestinal tract. There was a scattering of epithelium cells that showed labelling for melatonin, which would support that melatonin is discretely stored and not freely diffusible. The localisation of melatonin was shown to be correlated to that of EC cell serotonin, as shown in Figure 1.7B. However, this study does not fully indicate if melatonin was present in EC cells (Bubenik et al., 1977).
Systemic administration of melatonin to rats saw, its accumulation in the stomach and the colon (Bubenik, 1980). From the original results shown in Figure 1.8, it was concluded that the highest binding levels were detected in the mucosa and intestinal villi (Lee et al., 1995; Bubenik, 2002). This supports the presence of melatonin receptors on the mucosa. The majority of the studies investigating the location of intestinal melatonin have been either from rat colon or isolated cell cultures of the human gut mucosa. The few studies that have used mice have focused on melatonin expression in the small intestine (duodenum and ileum). Therefore, melatonin expression in the mouse colon has not been examined.
Figure 1.8: The immunohistochemical localisation of melatonin to the mucosal layer of the rat small intestine (A): control (B): after systematically administration of melatonin (Bubenik et al., 1980). Blue arrows indicate the mucosal layer.

Looking at past immunohistochemical studies (Bubenik et al., 1977; Bubenik et al., 1980; Raikhlin and Kvetnoy, 1975), it could be concluded that melatonin labelling was at much higher levels than could be accounted for solely by localisation and storage in EC cells. These studies also failed to adequately prove (Figure 1.8B) that the melatonin was truly co-localised with EC cells, as there was no specific antibody/marker used to visualise EC cells. This does not prove a definite causal relationship between EC cells and the synthesis of melatonin, despite providing strong hints for the direction of further investigation.

1.7.2 Localisation by non-immunohistochemical methods

There have been a number of indirect studies that have examined the expression patterns of melatonin. These studies have examined the presence of molecules associated with melatonin and its known signalling pathways. These have particularly focused on its biological receptors: MT₁ and MT₂. As part of the superfamily of G-protein coupled receptors, both MT₁ and MT₂ have been shown to possess 7α helical transmembrane domains and activate G protein signalling pathways (Hall et al., 1999). Melatonin has been shown to bind to a receptor site
composed of helices V, VI, and VII (Navajas et al., 1996). Both MT₁ and MT₂ receptors can undergo dimerization in both the homo and hetero configurations as confirmed in bioluminescence resonance energy transfer (BRET) experiments (Ayoub et al., 2002). It was more common to observe MT₁ homodimers and MT₁–MT₂ heterodimers, which form at similar rates. MT₂ homodimers were 3–4 times less likely to be produced compared to the heterodimers (Ayoub et al., 2004). These differences allow for them to be distinguished from one another and interpreted in methods as described below.

1.7.2.1 Binding column studies

Melatonin receptors come from the same family and follow a hyperbolic binding curve with a specific binding site that could be saturated (Audinot et al., 2008). The MT₁ and MT₂ receptors expressed very high binding affinity for melatonin (in the sub-nanomolar ranges) and they were coupled to G-proteins. MT₃ had a relatively lower affinity for melatonin (approximately 10nM) in animals (e.g. hamster) in which it was found (Audinot et al., 2008).

Within the setting of the gastrointestinal system in mice and rats, lower binding levels were observed in the oral mucosa and oesophagus whereas higher binding levels were observed in the stomach, duodenum, jejunum and ileum as well as in distal colon (Poon et al., 1997). In duck jejunum and colon, maximum binding occurred in the mucosa and intestinal villi (Lee et al., 1995). Further studies also confirmed the presence of melatonin receptors in the gastrointestinal tract. The greatest numbers of melatonin receptors were detected in the jejunum and colon mucosal layers (Pontoire et al., 1993; Lee and Pang, 1993; Poon et al., 1996). Although melatonin production did not present a circadian rhythm, melatonin receptor expression in the gastrointestinal tract seemed to vary with photo luminosity; the highest levels of expression were observed at night at 21:00 in humans cell lines (Pandi-Perumal et al., 2008). This could be linked to meal times regulated by appetite cues, in which melatonin has been known to play a part.

In the context of mice, all that is known from binding column studies is that the presence of melatonin receptors is detected in the distal colon.
1.7.2.2 Transcript analysis

Using transcription mRNA-PCR analysis, MT$_1$ expression was observed to be strongest in the both epithelial and subepithelial layers in the duodenum, decreasing in the jejunum and ileum, before increasing in levels again in the distal colon (Stebelova et al., 2010). In the rat intestine, Stebelova et al. showed that there was no correlation to the circadian cycle for MT$_1$ transcription, and interestingly there was more expression detected in the subepithelial part of tissues studied in comparison to the epithelial part. The distribution of MT$_1$ and MT$_2$ receptors were confirmed by another study; the colon presents the highest density for both receptors (Sotak et al., 2006).

For the MT$_2$ receptor the highest level of mRNA expression was detected in the colon and stomach region, followed by the duodenum and pancreas (Sotak et al., 2006). mRNA expression for MT$_2$ was mainly observed in the muscularis mucosae and in the muscularis layer in the stomach. The submucosa presented less expression and there was no expression observed in the mucosa layer. The expression pattern was slightly different in other areas including the colon and duodenum. There was still expression in the mucosal and in the muscular layers but there was also positive labelling in the villi of the mucosa layer (Stebelova et al., 2010). The high density of MT$_2$ melatonin receptors in the circular and longitudinal muscle layers of colon suggests and that melatonin plays a part in intestinal motility.

From transcript analysis, it can be concluded that both MT$_1$ and MT$_2$ receptors are expressed at the highest levels in the rat colon. There have been no similar studies in the mouse but this hints at potential levels expected in the murine colon, and hence the colon being a target for melatonin actions.

1.7.2.3 Western blot analysis

In the gastrointestinal tract, receptors for melatonin have been detected in almost
all mucosal membranes. The lowest number of receptors is expressed in the oesophagus and mouth, while the greatest number of receptors was found in the jejunum and colon (Sjoblom et al., 2003). As seen with the previous techniques, there were 2 types of receptors found in the rat and murine gastrointestinal tract: MT₁ and MT₂. Another less common subtype, MT₃ has been detected in the guinea pig colon (Mrnka et al., 2008), and also in the intestine of hamsters, although none has yet been found in the human and murine gastrointestinal tract (Slominski et al., 2012). A separate study using western blot analysis on rat pancreas, stomach, duodenum and colon tissue samples also confirmed the colon as expressing the highest levels of MT₂ receptors (Chen et al., 2011). Overall, MT₂ receptors were found in greater numbers and they were also expressed on EC cells in the intestines (Sjoblom et al., 2003).

In the murine colon, it can be concluded that MT₁ and MT₂ receptors are the main subtypes identified and MT₂ receptors appear to be more common.

1.8 Functions of melatonin in the colon

1.8.1 Cell Proliferation

Melatonin has been suspected to have anti-proliferation effects since pinealectomy, which involved the removal of a major source of melatonin synthesis, induced proliferation in cells from various tissues and organs including those in the gut (Kvetnøy et al., 2002). For a period of at least 6 months after the operation, the cells of the mucosa in the colon continued to display high rates of proliferation. This represented a significant period in the life span (21 months) of rats used in the experiment. Endocrine, paracrine and neurocrine processes mediated this effect on proliferation (Farriol et al., 2000). In a separate study on a mouse colon cancer cell line, melatonin was able to halt cell division and this effect was shown to involve the MT₂ receptor, via an association with the RZR/ROR α receptor in the nucleus (Winczyk et al., 2002). This was confirmed by the reversal
of the effect when the RZR/ROR a receptor antagonist, CGP 55644, was added (Pandi-Perumal et al., 2008).

1.8.2 Ion regulation

Another function demonstrated in several studies was the ability of melatonin to regulate intestinal ion transport (Bubenik, 2002). Melatonin has been suspected to be involved in the regulation of ion transport for ions such as bicarbonates (HCO₃⁻) and chlorides (Cl⁻). In the intestines melatonin regulation of ion transport has been widely studied (Bubenik, 2002). Additionally, melatonin was shown to raise levels of calcium within enterocytes in the duodenum in humans and rats, in a mechanism mediated by MT2 receptors (Sjöblom et al., 2003). Melatonin is also involved in facilitating chloride ion secretion in the colon (Chan et al., 1998).

After vagal and sympathetic stimulation, melatonin was secreted into the lumen of the duodenum and increased duodenal bicarbonate levels (Sjöblom et al., 2001). This was largely beneficial to the mucosa during ulceration and inflammation. The same effect was also noticed in the colon mucosa; the stimulation of bicarbonate ion release by locally produced melatonin was important during colitis (Kvetnoy et al., 2002).

Further evidence of melatonin in action in affecting ion transport was its effect on short circuit currents (Iₛₜ). Picomolar concentrations of melatonin introduced from both the mucosal and muscular layers of the rat distal colon resulted in an increase the short circuit current in the colon either through an increase in cAMP or though effects on enteric neurons (Mrnka et al. 2008).

1.8.3 Anti-inflammatory protection and free radical scavenging

Melatonin had been shown to interact with the immune system (Maestroni, 1998; Mei et al., 2002). Melatonin exerts protective functions in the lower bowel in various pathological conditions, such as colitis in rats (Pentney and Bubenik,
Melatonin can activate antioxidant enzymes indirectly even at relatively low concentrations in the gastrointestinal tract (Nosalova et al., 2007). It can also affect blood vessel constriction and relaxation to regulate the supply of blood to the affected part of the gastrointestinal tract (Reiter et al., 2001). Indeed, recent studies have shown the presence of melatonin receptors (MT1, MT2 and MT3) in rodent, porcine and human colon blood vessels (Bandyopadhyay et al., 2000). Although the precise mechanism by which melatonin affects the blood vessels is unclear, it has been observed that blood flow in the mucosa increased and motility decreased during inflammation with melatonin treatment (Stebelova et al., 2010; Carpentieri et al., 2012). This increase in mucosal blood flow protected against ischemia triggered damage and aids reperfusion, acting as a direct defence mechanism for the colon epithelium.

Various studies in the gastrointestinal tract have demonstrated that melatonin has an inhibitory effect on the nitric oxide signalling pathway (Cuzzocrea et al., 2001). During colitis, nitric oxide synthase production in the mouse colonic neurons is increased. In the presence of melatonin NO signalling was decreased, reducing the severity of the inflammation (Mei et al., 2005). In irritable bowel syndrome, patients demonstrate a heightened visceral sensitivity. Melatonin exacerbated visceral hypersensitivity, resulting in painful sensations even during normal levels of intestinal motility (Chojnacki et al., 2013).

Melatonin has been shown to have antioxidative properties. Ignoring unrealistic claims based on non-physiological levels of melatonin, there had been numerous studies demonstrating protective effects of melatonin in cell culture and in vivo (Hardeland, 2005; Poeggeler et al., 2002; Srinivasan et al., 2005). Melatonin acts as an effective antioxidant, scavenging free radicals in the gastrointestinal tract, accounting for its potent anti-inflammatory actions. It has been shown to act as a scavenger on its own without the need for other free radical acceptors, which makes it highly efficient (Motilva et al., 2001). Melatonin has been proposed as both a treatment and prevention regime for various pathophysologies in the digestive system. These include ulcers found throughout the mucosal surfaces, including the colon (Nosalova et al., 2007).
Previous studies have demonstrated that melatonin is capable of reversing oxidative stress-induced changes in gastrointestinal smooth muscle function (Gomez et al., 2010). Oxidative stress, as measured by levels of hydrogen peroxide, was twice as high in the proximal colon compared to the distal colon. It is believed this is due to the proximity of the proximal colon to the caecum and the constant exposure to oxidized particles of food, toxins, as well as reactive metabolites produced by various bacterial microflora (Pascua et al., 2011). Melatonin treatment increased levels of the antioxidant enzyme catalase the colonic smooth muscle, which reduced ageing induced increases in hydrogen peroxide levels. In addition melatonin also decreased the expression of NF-kB and cyclooxygenase 2 (COX-2) and normalised apoptotic activity suggestive of colonic functional recovery. Although the effects were more significant in the proximal colon, these benefits were detected in both the proximal and distal colon; this correlated to the higher levels of oxidative damage and apoptotic markers found in the proximal colon (Chen et al., 2011). On top of increased levels of hydrogen peroxide levels, other signs of oxidative imbalance with age include lipid peroxidation and reduction in catalase activity. The effects were enhanced by pinealectomy. In the aged colon, the muscle layers displayed increased expression of pro-inflammatory enzymes (COX-2) as well as activated isoforms of caspases 3 and 9 (Stebelova et al., 2010). Melatonin treatment suppressed these effects, limiting oxidative damage. In summary, oxidative imbalance and the triggering of pro-apoptotic and pro-inflammatory processes by age could be reversed by melatonin therapy in the colon (Carpentieri et al., 2012).

In conclusion, melatonin has roles in many anti-inflammatory pathways in the intestinal system. These include its effects on anti-inflammatory and inflammatory molecules, and antioxidant properties.

1.8.4 Role in regulating motility patterns

Melatonin can affect the transit velocity of luminal contents in the lower GI tract. During periods of fasting, increased levels of melatonin were secreted. The effect of this was to dampen peristalsis in the small intestines, indirectly allowing more
time for absorption of nutrients into the body during fasting conditions (Bubenik, 2008). Melatonin has also been shown to play a part in the autonomous control of gut motility (Merle et al., 2000).

Studies in murine gut muscular layer showed that melatonin in small doses (2mg intraperitoneal injection) caused relaxation and hence promoted colonic motility by allowing luminal contents to move along in waves (Bubenik and Dhanvantari, 1989). The ability of melatonin to relax smooth muscle was widespread throughout the gastrointestinal tract, especially in the presence gastric and intestinal ulcers, via non-noradrenergic, non-cholinergic transmitters such as ATP, NO and tachykinins (Nosalova et al., 2007). Other studies have confirmed that these relaxant effects of melatonin occur through the activation of MT receptors since luzindole (MT₁ and MT₂ receptor antagonist) neutralised the effects. In addition the authors demonstrated that melatonin exhibited both contractile and relaxation effects on the lower bowel; at low doses, melatonin increased the rate of intestinal transit, whereas higher doses had the opposite effect (Bubenik and Dhanvantari, 1989).

In motility disorders such as irritable bowel syndrome, melatonin has been shown to improve transit times in constipation-prone colon (Bubenik, 2008). Melatonin has also been shown to mitigate the effects of age in old animals with respect to gastrointestinal mobility. It has been demonstrated to restore the normal contraction rhythm to the detrusor muscle, via regulation of the levels of calcium ions, polarity in mitochondria, neuromuscular junctions and oxidative stress (Pozo et al., 2010).

Melatonin is also linked to the depolarisation response to trigger motility by influencing electron transport. A partial depolarisation of the mitochondria resting potential at rest occurred with age; this resulted in a desensitised depolarisation in response to stimuli in the cell. Unlike in younger cells, the mitochondrial enzyme ATP synthase remains mostly in resting mode instead of catalysing reverse reactions in old mice. This change was negated by melatonin treatment (Martin-Cano et al., 2013). Murine colon strip contractions, both stimulated and spontaneous, decreased with age as a result of glycolysis becoming the main energy source.
driving force behind contractions instead of mitochondria, which were far more energy efficient. However, these functional changes were reversed by melatonin treatment (Merle et al., 2000). Melatonin upregulates the ATP synthase activity in intestinal tissues (Storr et al., 2002).

In addition to the colon, melatonin helped restore myogenic contractile and neuromuscular function in the gall bladder after treatment for 4 weeks (2.5 mg/kg/day) in aged mice. Contraction response to calcium ions influx and electric field stimulation was completely recovered and efferent and sensitive innervation also showed improvement, despite no change to the ability of calcium ion regulation (Gomez et al., 2010). This suggested that contractile proteins and calcium ion sensitisation of the neuromuscular junction mediated the recovery of normal contractility.

The dual effects of melatonin in modulating intestinal motility and promoting powerful antioxidant and anti-inflammatory activity were essential in diseases such as irritable bowel syndrome (Storr et al., 2002). Evidence has shown its inhibition of motor activity of smooth muscle by a myriad of mechanisms: stimulating particular receptors directly, regulating cellular calcium and potassium ion exchange channels, and stimulating the visceral nervous system indirectly. Melatonin is a competitive inhibitor of receptors for nicotinic acetylcholine in submucosal plexus neurons (Chojnacki et al., 2013). Melatonin triggered an increment of cholecystokinin release as well as activating its receptors CCK1R/CCK2R in murine colon, resulting in the stimulation of vagal nerve afferent fibres. This caused a dual effect on motility; at low concentrations, melatonin hastened intestinal transit time, at higher concentrations, the effect was reversed.

1.8.5 Interactions with intestinal serotonin

Melatonin has previously been shown to regulate 5-HT signalling in the intestines of the GI tract (Bubenik, 2008). Specifically, its effects appear to be to antagonise the action actions of 5-HT a pro-motility agent. This has been shown to take place via 2 different signalling pathways. Melatonin can block 5HT3 serotonin receptors
as well as acting through its own MT2 receptor to physiologically and pharmacologically antagonize the effects of serotonin (Bubenik et al., 1989; Thor et al., 2007).

High levels of serotonin have previously been shown to cause spasmic contractions of the both the small intestines and colon (Bulbring et al., 1959). However, subsequent administration of melatonin reversed the spastic state and allowed for the renewal of peristalsis. MT2 receptors were responsible for the relaxation effect on the rat intestinal smooth muscle (Stebelová, 2010). Unlike serotonin, melatonin secretion was not triggered through any advanced mechanosensory stimuli. This suggested that melatonin was a functional antagonist of serotonin and that it consequently had an inhibitory role in the gastrointestinal system (Patel et al., 2008). Melatonin did not react to the same stimuli for release even though it was also predominantly produced in EC cells like serotonin. Nonetheless, they were both present in the same time in the mucosal layer of the intestines, which provides circumstantial proof that they interact as antagonists since the effect of one was not magnified by the other.

Local increments in serotonin levels would promote gut motility whereas increases in melatonin levels would aid its relaxation (Harlow and Weekley, 1986; Thor et al., 2007). The effects of melatonin on inhibiting serotonin were dependent on doses, for both short-term and long-term melatonin exposure. Nonetheless its effects were reversible and no permanent tissue damage occurred, serotonin effects on muscle layer was restored after 30 minutes of melatonin withdrawal despite previous exposure for a day (Matheus et al., 2010).

Melatonin also regulated serotonin activity indirectly through its interactions with serotonin re-uptake transporter (SERT). Melatonin was shown to reduce both the Vmax and Km of the serotonin transporter although it had no effect on its expression or trafficking of the membrane. This inhibition of SERT was not dependent on melatonin acting via either MT1 or MT2 receptors This suggested that melatonin binds directly to the SERT protein probably via an allosteric citalopram-sensitive site on SERT (Matheus et al., 2010). SERT from brain neural membranes and platelet surfaces had demonstrated at least 2 different interaction sites on the protein. There was a binding site for serotonin reuptake with a high
binding affinity and an allosteric site for regulation molecules such as melatonin that had a lower binding affinity (Martin-Cano et al., 2013). Melatonin binding in the allosteric site had a negative effect on both the capacity and the affinity of SERT.

In summary, melatonin has varying effects on motility dependent upon the interactions with serotonin and its concentration in the intestines. It can increase relaxation and inhibitory signalling, but it has also shown to restore neuromuscular function in cases where ionic balance seemed to be impaired and promote normal contractility. To understand the dual effects of melatonin, more studies will have to be done.

1.9 Hypothesis

The hypothesis of this study was that melatonin is produced and released by EC cells in the murine colon and it is capable of regulating colonic motility via the activation of melatonin receptors.

1.10 Aims

The aim of this project is to provide an understanding of the relative location of melatonin and its synthesis enzymes in the context of the murine colon, as well as the functional effects of melatonin in regulating the different motility patterns such as neurotransmitter induced (serotonin-induced and EFS-induced) responses, fecal pellet transit, and spontaneous migration complexes. This action might be antagonistic to serotonin response, and melatonin might cause both contractile and relaxation effects on the lower murine bowel.
1.11 Objectives

All studies were carried out using colon tissue samples obtained from C57Bl/6J mice. Immunohistochemical (IHC) studies were conducted for localisation of melatonin, its synthesis enzymes, and serotonin. This is to investigate if melatonin synthesis occurs in the enterochromaffin (EC) cells as speculated. Functional bioassays were carried out to study how varying concentrations of melatonin influenced serotonin and electrical field stimulated (EFS) induced colonic contractions. Further ex vivo experiments were done to investigate the role of melatonin in influencing colonic migrating motor complexes (CMMCs) and fecal pellet propulsion.
Immunohistochemical studies into the localisation and synthesis of melatonin

2 Location and origin of gastrointestinal melatonin

Melatonin levels vary in different regions of the mammalian gastrointestinal tract. Melatonin is present in high concentrations in the pineal gland, and the gastrointestinal tract, with the latter organ system containing melatonin levels that are 400 times greater than that seen in the pineal gland (Ekmekcioglu, 2006). It is inferred that the GI tract has the ability to synthesise, store and secrete melatonin independently of the circulatory system, and that all the substrates and enzymes necessary for melatonin’s synthesis are present within the GI tract. (Reiter and Tan, 2003). The main site of synthesis of melatonin in the GI tract is speculated to be the EC cells (Raikhlin and Kvetnoy, 1975). Immunohistochemical studies in rats have shown that the rectum and colon contain the highest levels of melatonin whilst the lowest levels were observed in the ileum and jejunum. This variation in levels in different regions was consistent with the levels of melatonin binding sites in a number of mammalian species, such as rabbit, mouse, rat and human (Poon et al., 1997; Chen et al., 2011).

In order to fully understand the role of melatonin in the GI tract, it is important to trace its site of synthesis within the bowel wall.

2.1 Melatonin biosynthesis and targets for immunolabelling

Since melatonin is synthesised in 2 enzymatic steps from serotonin (Figure 1.7), it has been proposed that melatonin is most likely to be produced in the same vicinity as serotonin (Raikhlin et al., 1975). More recently, studies have also proposed that melatonin was produced by the EC cells (Konturek et al., 2007). Using thin-layer chromatography and immunohistochemical analysis, Raikhlin and Kvetnoy (1975) were able to show the presence of melatonin in enteroendocrine cells, but not specifically the EC cells, in human appendix. The various studies
have indicated that melatonin is located in mucosal epithelial cells; however there is no clear evidence of its location to the EC cells, which are the main source of mucosal serotonin.

Both enzymes involved in the synthesis of melatonin, aralkylamine N-acetyltransferase (AANAT) and hydroxyindole-O-methyltransferase (HIOMT) are expressed in the intestinal wall of goldfish (Slominski et al., 2012). Further studies confirmed that HIOMT and AANAT were both expressed in the GI tract of the rat as demonstrated via the expression of mRNA – via polymerase chain reaction studies (Bubenik, 2008). The 2 enzymes had also been detected in gastrointestinal tract mucosal layer using immunoblotting studies; supporting the theory that melatonin was most likely synthesized from serotonin (Carpentieri et al., 2012). Moreover, there could be 10 to 100 times greater melatonin levels in the intestine than in serum (Bubenik, 2008) suggesting that melatonin is not simply diffusing from the blood into the GI tract tissue. Despite this work the cellular localization of these enzymes is currently unknown.

2.2 Summary of previous studies and basis for current work

As mentioned in the introduction, recent melatonin localisation studies (last 15 years) have mostly focused on where melatonin binds along the gastrointestinal tract. These have focused on the location of melatonin receptors as evidence of the presence of melatonin. Using indirect localisation studies of melatonin, especially receptor-based investigations (Audinot et al., 2008; Lee et al., 1995; Pontoire et al., 1993; Lee and Pang, 1993; Poon et al., 1996), have their limitations due to the differences in regulation of melatonin receptor expression and melatonin levels. Hence, an examination of the localisation of melatonin receptors can provide a good idea of melatonin localisation based on where melatonin might be acting, but not a certain indication of its levels at any given point in time. Labelling receptors also does not provide information regarding the source of melatonin.
Therefore, there is a need for effective direct localisation of melatonin as well as investigation into its relative levels in various regions of the lower gastrointestinal tract. It is also important to investigate the common assumption that EC cells are the sites of melatonin synthesis. It is now possible to immuno-label for melatonin synthesis enzymes, and the identification of these within the murine colon would provide additional evidence for the local synthesis of melatonin.

2.3 Hypothesis

My hypothesis is that melatonin and the enzymes necessary for its synthesis are localised to the mucosal epithelium (specifically EC cells) of the murine colon.

2.4 Aims

The aim of this study is to locate melatonin and its synthesis enzymes in the mucosal murine colon using immunohistochemistry, and subsequently compare them to the location of EC cells.
2.5 Methods

2.5.1 Colon Harvesting

Male C57BL/6J mice were obtained from Harlan UK at 8 weeks of age and kept under a conventional housing system. Animals were maintained at 20.0 ± 2 °C, 50 ± 5% humidity and fed on a maintenance diet (R&M diet no1 SDS expanded diet) until required for use in experiments. They were maintained at normal circadian light cycles and had free access to food and water. Three-month-old animals were stunned, and killed using Schedule 1 method (cervical dislocation). The whole colon was harvested and placed in ice-cold oxygenated (95% O₂ and 5% CO₂) Krebs’ buffer solution, pH 7.4 (117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃ (all from Fisher Scientific), and 11 mM glucose (from Sigma Aldrich)). The faecal pellets were removed by flushing with ice-cold Krebs’ buffer and the intestine preparation is subsequently washed 3X with more Krebs’ buffer. The oral end of the colon is determined as the section following the caecum structure, and the anal end is determined to be to 2cm from the rectal exit.

2.5.2 Fixation

The colon was cut into two sections (proximal and distal) and cut open longitudinally along the mesenteric border. The oral end of the proximal colon and the anal most end of the distal colon were used for immunohistochemistry. The tissue was stretched and pinned out on a Sylgard-lined plate and Zamboni’s fixative (2% v/v formaldehyde, 0.2% v/v picric acid in PBS; both from Sigma-Aldrich) was added until the tissues were covered completely. Fixation occurred overnight at 4°C. After which the pins were removed and the fixed tissues were washed three times in PBS for five minutes each time. After washing, 30% w/v sucrose (15g sucrose, 50ml PBS) solution was added to the fixed tissue and left for two days at 4 °C. Sucrose offered cryoprotection for the tissues, helping to avoid crystal formation when freezing; and it also preserved cell morphology. The
tissue sections were then removed from sucrose solution and washed with PBS for three times for five minutes each time before being embedded in OCT matrix (Fisher Scientific UK) over dry ice at -20 °C. Tissues were then stored at -80 °C until required.

2.5.3 Immunolabelling

The 8μM cryosections, cut with a microtome were attached onto Superfrost™ Plus slides (Thermo Scientific). When cryosectioning, the OCT matrix blocks were warmed from -80 °C to -20°C beforehand, and subsequently maintained at -20°C. Freezing at -80 °C rubberizes the OCT resulting in lost plasticity, and therefore freezing could create a barrier between the tissue and OCT due to the Leidenfrost effect that could subsequently cause wobbling during sectioning. Sectioning at -80 °C would therefore lead to inconsistencies in the thickness of the sections, hence the need to bring the temperature up to -20 °C.

The sections were then incubated in 2M hydrochloric acid for 1 hour at room temperature for antigen retrieval. Formaldehyde based fixation forms protein cross-links that mask antigenic sites in specimens, thereby giving weak or false negative staining. A hydrochloric acid solution is designed to break the protein cross-links, therefore unmask the antigens and epitopes in formalin-fixed and then incubated with 10% goat serum to block non-specific binding or the secondary antibody (10% goat serum, 1% BSA, 0.05% sodium azide in PBS) for 2 hours at room temperature; goat serum was used as the secondary antibody was raised in goats.

Various primary antibodies were used in these experiments: (1) rabbit polyclonal antibody against aralkylamine N-acetyltransferase (AANAT) (Santa Cruz Biotechnology: FL-207); (2) rabbit polyclonal antibody against melatonin (Abcam: ab35137, AbDSerotec: 0100-0203); (3) rabbit polyclonal antibody against hydroxyindole O-methyltransferase (HIOMT) (antibodies-online: ABIN873275); (4) rabbit polyclonal antibody against serotonin (Immunostar: 20080). In order to optimize the dilution of the primary antibodies a range of different dilutions were
trialled. These were (1) anti-AANAT: 1:200, 1:400, 1:600; (2) anti-melatonin: 1:100, 1:200, 1:400, 1:800; (3) anti-HIOMT: 1:200, 1:400, 1:600; (4) anti-serotonin: 1:2000, 1:4000, 1:8000. All primary antibodies were diluted in an antibody diluent solution (0.2% BSA in PBS). The sections on slides were incubated in 200μl primary antibody, in wells bound by a wax boundary, overnight at 4°C.

Negative controls were set up for each of these experiments by omitting the primary antibody. This demonstrates that any fluorescence observed after adding the secondary antibody was due to specific binding of the secondary antibody with the primary antibody.

Positive controls were set up with the use of primary antibody pre-incubated overnight at 4°C with the immunogen. This should inactivate the antibody and the sample sections should show little or no staining. This pre-absorbed antibody was then incubated with sample slides in place of the primary antibody alone. For anti-serotonin antibody, staining was completely eliminated by pre-treatment of the diluted antibody with 10 μM serotonin. For anti-melatonin antibody, staining was completely eliminated by pre-treatment of the diluted antibody with 10 μM melatonin. For anti-HIOMT and anti-AANAT antibodies, staining was eliminated when incubated with the conjugated synthetic peptides provided by the antibody suppliers.

To test for primary antibody specificity for melatonin in particular, rabbit host antibodies from 2 different suppliers were used. This approach was utilised as no knockout mice models for melatonin are available and the ability to study tissues where melatonin is not expressed is difficult due to fact melatonin is present systemically through vascular circulation. When looking at the responses from 2 primary antibodies from varying supplied, the resulting immunofluorescence developed using the same Alexa 488 secondary antibody is shown in Figure 2.1. They present similar labelling patterns in the distal colon suggesting that specificity is similar.
Figure 2.1: Demonstration of fluorescent signal intensity in distal colon using anti-melatonin primary antibody from 2 different sources. Distal sections viewed at 200X magnification and visualised with Alexa488 secondary antibody (A) AbdSerotec 1:200 (B) Abcam 1:200

The following day, the slides, including the controls, were washed in PBS and the sections incubated with the secondary antibody; Alexa 488 goat anti-rabbit antibody (1:400, Molecular Probes) for 2 hours at room temperature as suggested in specification sheet. Sections were again washed with PBS, and then were mounted with DAPIShield mounting medium (Vector Labs).

2.5.4 Confocal Imaging

Confocal image collection was done within 1-2 days of secondary antibody incubation to minimise photo-fading effects. In the meantime they were stored in the dark at 4°C. The Leica TCS SP5 Confocal Laser Scanning Microscope [CLSM] was used. The slides are mounted in VETASHIELD Mounting Medium with DAPI (Vector Laboratories) to reduce photo-bleaching of fluorescence. It was equipped with a DMI6000 inverted microscope and images could be taken at 100x, 200x and 400x magnification. The images are single optical sections.

The green fluorescence of the secondary antibody Alexa 488 did not remain steady with time and repeated scanning and the consequential decrease in
fluorescence intensity was a significant variable for this experiment, specifically as quantification of the labelling intensity was the needed. The laser (488 nm) and UV (405 nm) power used on the confocal microscope was fixed at 25%. This minimised signal loss but also compromised detection. To compensate for this the gain was set at 750-760mV. The images were collected at 100x, 200x and 400x magnification. Images were processed using the Leica LAS-AF software.

2.5.5 EC cell count

The number of putative EC cells was identified using rabbit anti-serotonin antibody 1:8000) in each of 3 sections from the same animal, and a total of 3 different animals are used (n=3). EC cells are known to store serotonin, and identification using this technique is an accepted way of quantifying them. They were counted by observation at 400x fields of view for each colon sample, and the average number of EC cells per field view was calculated.

2.5.6 Data Analysis

2.5.6.1 Intensity analysis

There were 2 types of intensity analysis done: whole sample intensity and area comparison. The area of interest was selected using a drawing/selection tool that fit it best. Freedom selection is used for whole sample intensity analysis (e.g. when comparing between proximal and distal colon samples) while box selection is used for intensity trace of difference layers of the sample. The box selection tool is used for comparing the intensity of the signals between the longitudinal muscle + myenteric plexus (LMMP) layer and the mucosa (e.g. Figure 2.2B). As seen in Figure 2.2A, the resulting selection area can vary greatly and hence it needs to be normalised. The intensity of the signal is calculated using the area, integrated density and mean grey value readings.
Figure 2.2: Demonstration of fluorescent signal intensity calculation on Image J. (A) Yellow box indicates selection of area of interest on a sample 100X proximal colon image for intra-region based analysis. (B) Freeform selection creates yellow border highlighting area of interest on a sample 100X distal colon image for inter-region based analysis.

A region next to the region of interest that had no fluorescence was selected to be the background. The size did not have to be consistent with the region of interest. The process was repeated for all the images acquired (6 samples from each animal, n=4; paired t-test).

The corrected total cell fluorescence (CTCF) was calculated in Excel. CTCF = Integrated Density – (Area of selected region X Mean fluorescence of background reading).

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\text{(Mean fluorescence Intensity/area) – Background Fluorescence/area}
\]

2.5.6.2 Line scan intensity

Line scan intensity allows for tracking of melatonin signal intensity, and hence its deduced levels, over different layers of the colon. 6 line scans were used per image, n=4; paired t-test. A plot profile is generated from the raw values of pixel
intensity that intersects with the line (e.g. figure 2.4). The scan is typically conducted from the outer connective tissue layer (Figure 2.3A & 2.3B, Point A) to the mucosal layer (Figure 2.3A & 2.3B, Point B).

Figure 2.3: Demonstration of line scan intensity calculation on Image J. Yellow line indicates slice of tissue being scanned from A to B direction. (A) On a sample proximal colon 100x image. (B) On a sample distal colon 100x image.
2.6 Results

2.6.1 Localisation of melatonin in the gut wall

Using distal colon sections from one single 3-month-old animal, all 4 primary antibodies were tested at a range of concentrations to identify the optimum concentration to use. It was concluded that the optimum dilutions for the antibodies to be used in the actual experiments were as follows: (1) anti-serotonin: 1:8000 (2) anti-melatonin: 1:200; (3) anti-HIOMT: 1:600; and (4) anti-AANAT: 1:600.

Having optimized the primary antibody concentrations, the next step was to identify the localisation of melatonin in the different layers of the colon. Using proximal and distal colon tissue sections from 4 different 3-month-old animals, the variation in melatonin levels across tissue sections was determined (using the method described in 2.5.6).

Figures 2.4 and 2.5 shows the relative intensity of the melatonin signal in different layers of the colon as crossed by the line (A to B). Figure 2.10 shows the proximal colon while Figure 2.11 shows the distal colon. The highest signal intensity (>60 units; standard error of the mean (SEM) =8.662) appeared towards the mucosal end (B), while there is also significant amounts (up to 40 units; SEM=5.738) near the muscular layers. The layers in between present very low melatonin signal intensity (<20 units).

From Figures 2.4 and 2.5, it could be deduced the distribution of melatonin signalling in the distal and proximal colon respectively in this experiment; where the highest concentration, and hence most intense signals, was detected in the subepithelial and epithelial layers of the both the proximal and distal colon.
Figure 2.4: Levels of melatonin in the proximal colon. Line scan intensity reading for longitudinal section of proximal colon labelled, from point A (muscular layer) to point B (mucosal layer), for melatonin and visualised using Alexa 488.

Figure 2.5: Line scan intensity reading for longitudinal section of distal colon labelled, from point A (muscular layer) to point B (mucosal layer), for melatonin and visualised using Alexa 488.

From the population analysis of signal strength in different regions (figure 2.6), there is significantly more melatonin accumulated in the mucosa area than the LMMP in both the proximal and distal colon. When the intensity of the signal of selected regions (using box method as described in 2.5.6.1) was quantified, it is
shown that there were higher levels of melatonin in the mucosa for both the proximal (Figure 2.6A) and distal (Figure 2.6B) colon. In both cases the (p value<0.05 for n=4).

Figure 2.6: Melatonin levels in LMMP layer and mucosa layer, in proximal and distal colon sections as quantified by fluorescence signal intensity (n=4). Values expressed as mean ± SEM. (A) Proximal colon (*: p value<0.05= 0.023; paired t-test). (B) Distal (*: p value<0.05=0.026; paired t-test).

2.6.2 Melatonin levels varied in different colonic regions

Having identified the layer expressing the most melatonin in the colon, it was important to compare how expression changed between the different regions of the colon. Using proximal and distal sections from 4 different 3-month-old animals, melatonin levels in the whole section proximal and distal colon were compared and analysed.

As demonstrated in Figure 2.7A, there was significantly more melatonin present in the proximal when compared to the distal colon (p value < 0.01, n=4). The proximal colon (average CTCF=250 units) demonstrated had almost twice the level of melatonin compared to the distal colon (average CTCF= 130 units).
The next step was to look at difference in EC cell numbers in the different regions. EC cells contain serotonin which is a precursor of melatonin, and their presence has been reported in the vicinity of melatonin labelling in the past. EC cell numbers could account for the difference in melatonin levels between the proximal and distal colon.

As shown Figure 2.7B, the number of EC cells/400x field of view was found to be 12.00 ± 0.5092 in proximal and 5.222 ± 0.5879 in distal colon. There were twice as many EC cells observed in the proximal colon compared to the distal colon (p-value < 0.001, n=4). The number of EC cells that was seen per crypt in this experiment is approximately 0.7 cells.

Figure 2.7: (A) Melatonin levels in proximal and distal colon sections as quantified by fluorescence signal intensity (n=4). Values expressed as mean ± SEM (**: p value<0.01= 0.0027; paired t-test). (B) Enterochromaffin (EC) cell numbers in 400X field of view (n=3). Values expressed as mean ± SEM. (**: p value<.001= 0.0009; paired t-test).

2.6.3 Immunolabelling of melatonin and EC cells by serial sections

In order to examine whether melatonin expression was present in the EC cells, double labelling experiments on serial sections from both the proximal and distal colon were performed. Studies were carried out on sections from 3 different 3-month-old animals.
Figure 2.8 shows proximal colon samples while Figure 2.9 shows distal colon samples. In Figure 2.8 and 2.9, the blue arrows show the sites of positive fluorescence labelling for serotonin (top row) and melatonin (bottom row). The sections being compared are serial sections from the same tissue sample in the same animal.

It can be concluded from figures 2.8 and 2.9 that direct overlap of EC cell location and melatonin presentation was not clearly evident, although they were in close proximity. The most intense signal levels were found to be along the mucosal surface closest to the lumen.

In figure 2.8 and 2.9, melatonin was shown to be present in many more cells compared to serotonin. There appears to be more cells labelled with melatonin as compared to serotonin.

![Figure 2.8: Immunohistochemistry for serotonin and melatonin in longitudinal sections of murine proximal colon as visualised using Alexa 488 at 400X magnification. Serotonin 1:8000 in different animals: mouse 1 – 3 (A) to (C); Melatonin 1:200 in corresponding sections for mouse 1 – 3 (D) to (F).](image-url)
animals: mouse 1 – 3 (D) to (F). Blue arrows show positive (A) to (C) serotonin labeling (D) to (F) melatonin labeling

Figure 2.9: Immunohistochemistry for serotonin and melatonin in longitudinal sections of murine distal colon as visualised using Alexa 488 at 400X magnification. Serotonin 1:8000 in different animals: mouse 1 – 3 (A) to (C); Melatonin 1:200 in corresponding animals: mouse 1 – 3 (D) to (F). Blue arrows show positive (A) to (C) serotonin labeling (D) to (F) melatonin labeling
2.6.4 Immunolabelling of melatonin synthesis enzymes by serial sections

In this experiment, the aim was to prove that the 2 enzymes (AANAT and HIOMT) involved in the final 2 steps of melatonin synthesis were localised in the EC cells via immunocytochemistry. Using serial proximal and distal sections from 3 different 3-month-old animals, AANAT and HIOMT immunolabelling was tested to explore this.

Figure 2.10 and 2.11 shows the localisation of melatonin synthesis enzymes, AANAT and HIOMT, in the proximal and distal colon, respectively. The blue arrows show the sites of positive fluorescence labelling for serotonin (marker of EC cells) in the top row and the 2 different enzymes in the bottom. The sections being compared are from the same tissue sample in the same animal, and two serial sections are being highlighted in the image frames. The labelling was specific, and shows a clear overlap in the localisation between the serotonin labelling and labelling associated with the melatonin synthesis enzymes. From Figures 2.10 and 2.11, we could conclude that both AANAT and HIOMT are localised to the mucosal layer and within the vicinity of EC cells in the mouse colon. It therefore appears that the melatonin observed in the murine colon is generated locally within colonic mucosa.
Figure 2.10: Serotonin, HIOMT and AANAT in longitudinal sections of murine proximal colon as visualised using Alexa 488 at 400X magnification. Serotonin A) 1:200, Serotonin B) 1:200, HIOMT C) 1:600; AANAT D) 1:600. Blue arrows show positive serotonin labeling (A&B), HIOMT labeling (C) and AANAT labeling (D).
Figure 2.11: Serotonin, HIOMT and AANAT in longitudinal sections of murine distal colon as visualised using Alexa 488 at 400X magnification. Serotonin A) 1:200, Serotonin B) 1:200, HIOMT C) 1:600; AANAT D) 1:600. Blue arrows show positive serotonin labeling (A&B), HIOMT labeling (C) and AANAT labeling (D).
2.7 Discussion

To ensure that the results seen are reliable, the specificity of the anti-melatonin antibody must be tested. Absorption based positive control was chosen because melatonin, the primary molecule of interest, is released into the bloodstream from the pineal gland too and it is difficult to find tissue that is free of melatonin in the mice used. The specificity of this anti-melatonin antibodies used have also been proven in previous papers by Schallreuter et al. (2012) in human epidermal melanocytes and keratinocytes cell cultures and Söderquist et al. (2015) in human GI tract tissue samples including the large intestines and colon.

The current immunohistochemical studies indicate for the first time 2 key findings about the expression of melatonin in the murine colon. First of all, it is most abundant in the mucosal layer of the colon, although there are significant amounts observed in the LMMP. Secondly, there is more melatonin present in the proximal colon when compared to the distal colon. Additionally, both enzymes responsible for the formation of melatonin are present in the mucosal layer and in close proximity of EC cells.

2.7.1 Where is melatonin located in the murine colon

Anti-melatonin labelling showed that the highest concentrations were observed in the subepithelial and epithelial layers of both the proximal and distal colon. This has been suspected in the past with the fact that, within the colon itself, there was a greater number of MT$_1$ receptors in the epithelial and subepithelial layers (Witt-Enderby et al., 2003).

A high level of labelling is also seen in the muscle layers in current studies. MT$_2$ receptor distribution was slightly different to MT$_1$ and there was the highest concentration of them in the stomach, duodenum and colon. Within these tissues, the localisation pattern of MT$_2$ receptors was conserved (Konturek et al., 2007; Stebelova 2010). There was the highest immunolabelling in the muscularis mucosae and in the muscularis layer and there appeared to be very few of them in
the mucosa. This would explain the bright spots of intensity in the submucosal muscle layers of both the murine proximal and distal colon, which was previously not expected.

Melatonin was shown to be present in higher levels in the mucosal layers than deeper areas further away from the lumen. This agrees with previous studies that show the presence of melatonin in the mucosa in rat colon and murine small intestine (Holloway et al., 1980; Bubenik et al, 1980; Lee et al., 1995). The localisation of melatonin receptors was closely related to the distribution of melatonin itself in the gastrointestinal tract, and as there were more MT<sub>1</sub> receptors than MT<sub>2</sub> receptors in the colon (Kvetnoy 2002), it was reasonable to observe that the signal intensity was relatively greater in the mucosa than in the submucosal and muscle layers.

There was significantly more melatonin present in the proximal colon compared to the distal colon. This direct comparison is a novel finding in the murine colon. One possible explanation for this finding could be due to the differences in the luminal contents in these different regions of the bowel. The proximal colon is continually exposed to oxidized particles of food, toxins, and reactive metabolites produced by various bacterial microflora as a result of its close location to the caecum and this is different when compared to the distal colon (Pascua et al., 2011). Hence, levels of the pro-oxidant, hydrogen peroxide in the proximal colon are more than twice those in the distal colon.

This corresponded to the relative amount of melatonin quantified from the proximal and distal colon from these experiments. This could be due to the fact that melatonin and its effects were in higher demand in the proximal region, providing a functional implication for varying levels in different colonic regions in the mouse. As melatonin has previously been shown to have anti-oxidant properties the higher levels observed in the proximal colon in this study may well represent a protective mechanism to offset the high levels of oxidative stress seen in this region of the bowel. Indeed, melatonin treatment has been shown to increase catalase levels in the colonic smooth muscle, thereby reducing hydrogen peroxide levels as well as decreasing the expression of NF-jB and cyclooxygenase 2 (COX-2) and
normalising apoptotic activity; all of which were essential for continued functional colonic activity (Chen et al., 2011; Stebelova et al., 2010).

2.7.2 Is there any relationship between EC cell numbers and melatonin levels

Previous studies have inferred that melatonin expression was at least in part localized to the EC cells. In mice it had been evaluated that 12 ± 4 % of cells contained serotonin (the precursor of melatonin), the percentage was raised to 84 ± 6% when only EC cells were considered (Schäfermayer et al., 2004). Therefore, if this is the case in the mouse colon melatonin levels would be expected to be higher in regions that contained more EC cells.

In this study, the number of EC cells was found to be twice as numerous in the proximal colon compared to the distal colon. The fact that melatonin levels in the proximal colon are approximately twice those seen in the distal colon is consistent with the hypothesis that EC cells could be the main source of melatonin synthesis.

Previously, the number of EC cells present has been evaluated. However, they had not been directly compared with the amount of melatonin in non-disease conditions until this experiment. In guinea pigs, the normal expression of EC cells was found to be 6 ± 2/mm, which was then raised twofold to 14 ± 2/mm during inflammation (Linden et al., 2003).

Links between the number of EC cells and the amount of melatonin present was previously suggested in induced colitis experiments. An experiment of similar methods was also done in a murine model with induced-colitis, where the number of mucosal crypts and serotonin positive EC cells was simply counted in nonadjacent 400x fields of view, such that the average number of EC cells per crypt and crypt per millimetre of colon was compared (Betrand et al., 2009). This method was similar to the one used to calculate the EC cell count in this experiment, but the number of crypts were not counted in this case because they stayed comparatively constant and they only increased during times of inflammation and oxidative stress. It corresponds to our results that each crypt presented 1-2 EC cells under normal conditions.
2.7.3 Are EC cells a location for melatonin

Anti-melatonin labelling was inconclusive in determining whether the EC cells were a source of melatonin synthesis despite the observation that high levels of melatonin were present in the mucosa the site of the EC cells and EC cell number was proportional to the intensity of melatonin labelling. This is mainly due to difficulties in double staining. Previous studies have looked to try to link EC cells with melatonin synthesis using a range of techniques including immunohistochemical methods such as Coon’s indirect immunofluorescent method, immuno-peroxidase and antiserum for melatonin, as well as thin-layer chromatography and electrosensing experiments (Kvetnoy et al., 2002). In all these investigations in the gastrointestinal tract of mammals (humans, dogs, rats and mice), there was a strong correlation between the areas where EC cells were found and the expression of melatonin (Konturek et al., 2007), however these studies were also unable to directly relate melatonin location to the EC cell.

After melatonin synthesis was suggested to take place in EC cells (Kvetnoy 2002; Lee and Pang, 1993; Poon et al., 1996), there were 3 main concerns about the identification of melatonin in this context. Firstly, was melatonin found in the same layer in the gastrointestinal mucosa as the EC cells? Secondly, could synthesis be localised to these cells? And finally, could melatonin be stored in these EC cells? EC cells had been shown to contain traces of melatonin, which was not surprising considering serotonin was the substrate for melatonin production (Bubenik, 2002).

The current study shows little direct overlap of EC cell location with melatonin expression, although they were in close proximity. This could be the result of melatonin being a highly lipophilic molecule, which does not require any transporter-aided relocation (Raikhlin and Kvetnoy, 1975); hence it diffused away instantaneously. This result also suggested that unlike serotonin, which was stored locally in the EC cells (Pontoire et al., 1993), melatonin was synthesised on demand. Melatonin produced in the gastrointestinal tract was released locally into the lumen (Reiter and Tan, 2003; Bubenik, 2002) and this corresponded to where the most intense signal levels were found in the current study. The lack of a corresponding clearance protein for melatonin, with a function analogous to SERT
for serotonin, could be a reason why a diffuse accumulation of melatonin was detected. It was not distinctly associated with a certain location and it was not efficiently removed from the gastrointestinal mucosal layer, leading to its accumulation.

Indeed, the same experiments it could be further observed that there was a more widespread distribution for melatonin in the colon in comparison to discreet signals for serotonin and EC cells. This can be explained by evidence in its metabolism and utilisation. As demonstrated from previous research, melatonin metabolism does not take place within the gastrointestinal tract, but instead involves the liver and various CYP P450 enzymes (Reiter, 1991; Facciola et al., 2001).

2.7.4 Are EC cells the site of melatonin synthesis

Similarly, it was impossible to conclusively localise melatonin synthesis enzymes (HIOMT and AANAT) to the EC cells. Current results suggest these enzymes are potentially in and around the EC cells, and definitely in the mucosal layer. But serial sections make it difficult to make precise conclusions. Localisation of melatonin synthesis enzymes to the mucosa is nonetheless encouraging, considering there was no direct overlap between melatonin expression and the EC cells. Previous indirect evidence has suggested that the number of EC cells and HIOMT expression followed the same pattern of expression during an inflammation response (Shen et al., 2008; Chojnacki et al., 2013).

From the immunolabelling experiments conducted, the enzymes responsible for the synthesis of melatonin are probably in the EC cells, but it can only be conclusively said to be in putative cells in the mucosal layer. The lack of clear evidence for melatonin also localising to the EC cells is probably due to the lipophilic, freely diffusible nature of melatonin once it has been synthesized. Production of melatonin was shown to be occurring in the EC cells of the colon. Previously, both enzymes involved in the production of melatonin have been identified in the gastrointestinal mucosa; more specifically HIOMT had been detected in the EC cells of the duodenum but not in the colon, which was the
tissue of interest in this study (Kvetnoy et al., 2002).

2.8 Conclusion

From these experiments it can be concluded that melatonin accumulated in higher levels in the murine proximal colon as compared to the distal colon, and this was related to the number of EC cells in the region. Within the colonic tissue itself, the most intense melatonin signals were detected in the sub-epithelial and epithelial layers of the both the proximal and distal colon. There was also significant immunolabelling in the muscularis mucosae and in the muscularis layer. The enzymes AANAT and HIOMT involved in the synthesis of melatonin were identified in the vicinity of EC cells, and in the mucosal layer of colonic tissue. Although the location of EC cells and melatonin expression was not directly related they were in close proximity to one another and this discrepancy is probably explained by the freely diffusible nature of melatonin and limitations of the experimental approach.
Functional bioassays on the effects of melatonin on colonic motility

3 Motility in the murine colon

Colonic motility is mediated through the neurons in the myenteric plexus and submucosal plexus through the smooth muscle layers, interacting with the pacemaker activity of interstitial cells of Cajal (Huizinga and Lammers 2009; Sanders et al. 2012). Various excitatory and inhibitory neurotransmitters are released from the motor neurons that innervate the circular and longitudinal muscle layers (Moynes et al., 2014). Acetylcholine (ACh) and substance P primarily trigger contraction, while vasoactive intestinal peptide (VIP), nitric oxide (NO), and purines regulate relaxation; some neurotransmitters such as ATP have dual functions (Furness 2005; Sanders 1998). Enteric nervous system (ENS) regulation of motility within the gastrointestinal tract allows the digestive system to meet the metabolic and nutrient absorption requirements of the body. In this chapter we will look at 2 ways of stimulating components of motility that propel solid and fluid contents through the colon: serotonin dosage and EFS; and investigate the effects that serotonin and melatonin are known to have on motility.

3.1 Role of serotonin and melatonin in the colonic contractions regulation

3.1.1 Serotonin

Serotonin is a major neurotransmitter found in various cells of the ENS. It has been detected in interneurons that project in the descending direction within the myenteric plexus and IPANs (Foxx Orenstein et al., 1996). However, the biggest store of serotonin comes from a non-neuronal source; the enterochromaffin cells of the mucosa present the most serotonin in the mouse intestine.

Early studies have shown that application of serotonin on the mucosa was able to initiate a peristaltic reflex (Bulbring et al., 1965). Serotonin is released from the
intestinal mucosa as a response to an increase in intraluminal pressure caused by fluids and faecal matter. Mucosal stimulation and muscle stretch have been shown to trigger simultaneous release of serotonin and calcitonin gene-related peptide (CGRP) into the immediate locale where stimulation was applied. This results in characteristic ascending contraction and descending relaxation of circular muscle (Foxx Orenstein et al., 1996).

More recent studies have corroborated these findings and proven that serotonin can be secreted from enterochromaffin cells to activate sensory neurons that relay both motor and secretory reflexes (Grider et al., 2003, Keating et al., 2010).

Once serotonin is released, local enteric nervous reflexes relay the signal to begin secretion of other excitatory neurotransmitters such as acetylcholine, ATP and tachykinins to generate propulsive motility (Grider et al., 2003). However, when serotonin is directly injected into the colonic tissue, it has been shown to inhibit the peristaltic reflex (Thor et al., 2007). This shows that there must be an inhibitory-feedback mechanism involved to regulating peristaltic contraction initiation. These serotoninergic neurons play a crucial role in regulating the balance between contractile and relaxant activity in the smooth muscle of the gastrointestinal tract.

The mouse proximal colon was shown to have the greatest binding affinity for 5-HT$_3$ receptor agonists, suggesting that the proximal colon is more susceptible to serotonin effects than the distal colon (Chetty et al., 2006). This also explains why contractions, compared to the internal standard, were generally higher in the proximal compared to the distal colon. Nonetheless, functional 5-HT$_3$ receptors are expressed in nerves along the entire length of the mouse intestinal tract. The 5-HT$_3$ receptor mediated uptake of serotonin and inactivation processes in the mouse colon are linked to both the inhibitory nitrergic and non-tachykinin excitatory pathways (Furness, 2000).
3.1.2 Melatonin

Melatonin was shown to play a part in the autonomous control of gut motility, predominantly through its effects on serotonin signalling processes. Transit time of faecal pellets and fluids through the colonic region is linked to various physiological disorders such as constipation, incontinence and irritable bowel syndrome (Merle et al., 2000). Serotonin had been shown to cause spasmic contraction of the intestines, whereas melatonin exposure reversed the spasm state and allowed for the renewal of peristaltic waves (Bubenik et al., 2002).

While the exact mechanism of this effect is not known in the colon, this was regulated in the gastric region and in vascular smooth muscle by controlling of calcium and potassium ion influxes into cells (Thor et al., 2007). Melatonin also exerted its effects on neuromuscular junctions in the intestinal system by inhibiting nicotinic acetylcholine receptors on the submucosal nervous plexus (Pozo et al., 2010).

Melatonin has been shown to reinforce the cyclic pattern of CMMCs while inhibiting the occurrence of irregular spike activity (Martin-Cano et al., 2010). Small doses of melatonin improve the rate of intestinal transit in mice. Higher doses antagonised this stimulatory effect of lower doses of melatonin. It inhibits intestinal motility through interactions with serotonin receptors in sympathetic neurons (Thor et al., 2007). Melatonin also offers protection against free radical oxidation and stimulates mucosal renewal and prostaglandin release to promote motility (Furness, 2005).

In motility disorders such as irritable bowel syndrome, melatonin has been linked to an improvement in transition times in constipation-prone colon (Bubenik, 2008). Melatonin had also been shown to mitigate the effects of age in old animals with respect to gastrointestinal mobility. It had been demonstrated to restore a normal contraction rhythm to the lower bowel, via regulation of the levels of calcium ions, polarity in mitochondria, neuromuscular junctions and oxidative stress in epithelial cells (Pozo et al., 2010). This is via calcium ion channels and calcium ion gated potassium ion channels, without affecting the intracellular mitochondrial stores of calcium ions in muscle cells. Despite all these observations, not much is known
about the mechanisms in which melatonin produces these effects in the gastrointestinal tract.

3.2 Hypothesis

The hypothesis here is that melatonin influenced colonic motility through its ability to modulate neurosignalling processes, mediated by melatonin receptors.

3.3 Aims

This study aims to elucidate the mechanisms with which melatonin exerts its effects on colonic contractions, though its effects on serotonin signalling processes as well as neurotransmitter molecules released by EFS.
3.4 Methods

3.4.1 Drugs

Drug concentrations are expressed as final bath concentrations. Muscarinic receptor antagonist: Scopolamine (Sigma-Aldrich; 1μM); NK₁ receptor antagonist: RP 67580 (Tocris; 2μM); NK₂ receptor antagonist: GR 159897 (Tocris; 1μM); NOS inhibitor: L-Nitro-arginine (Sigma-Aldrich; LNNA) (1μM); P2Y₁ receptor antagonist: MRS 2500 (Tocris; 1 μM); MT₁ and MT₂ receptor antagonist: Luzindole (Sigma-Aldrich; 1 μM); MT₂ receptor antagonist: 4-P-PDOT (Tocris; 1 μM); Voltage gated Na⁺ channel blocker: TTX (Abcam; 1μM) Depolarising agent: KCl (Fisher Scientific; 30mM).

Stock solutions of RP 67580 and LNNA were dissolved in 100% DMSO (Sigma-Aldrich) and further diluted in Krebs Buffer. The final volume of DMSO in the organ bath did not exceed 0.05% and had no effect on peristaltic contractions.

3.4.2 Colon Preparation

3 month old male C57BL/6J mice were stunned and dispatched by means of cervical decapitation. Mice reach maturity at 3 months of age. The whole colon was harvested, approximately 2 cm proximal to the anus and placed in ice cold oxygenated (95% O₂ and 5% CO₂) Krebs’ buffer solution, pH 7.4 (117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM, NaH₂PO₄, 25 mM NaHCO₃, and 11 mM glucose). The whole colon was cleaned of fatty tissues and gently flushed with Krebs’ buffer to clear the lumen of intestinal contents. The caecum was removed and the remaining colon was divided into a proximal and distal segment at the halfway point.
3.4.3 Measurement of colonic longitudinal smooth muscle contractions

The distal end of each full thickness segment was suspended in an organ and contractions were monitored by attaching the free end of the tissue to an isometric force transducer (ADInstruments). The signal was amplified using a bridge amplifier (ADInstruments).

Mechanical activity was digitised at 10mV/10Hz, visualised, recorded and analysed on a personal computer using the LabChart7 system (ADInstruments). The colon strips were mounted under an initial resting tension equivalent to 4.90 mN load and allowed to equilibrate for 30 min in warm (37 °C), oxygenated (95% O₂ and 5% CO₂) Krebs buffer, with solution changes every 10 min. At the beginning of each experiment, the preparations were challenged with 100 μM ACh for 1 min every 10 minutes until reproducible responses were obtained.

Electrical field stimulation (EFS) was performed with a pair of external platinum wire electrodes attached to the organ holder and connected to a square wave stimulator. Test response was done once at 60 V at 5 Hz of 0.3 ms pulse width for 30 s. 60V was chosen after testing voltages from 20 V to 120V for recovery times and ability to elicit response in colonic tissue. 60 V also gave a reproducible sub-maximal response which allowed the effects of drugs that reduce contractile activity and those that increase it to both be observed in the same preparation.

Trains of stimuli (0.3 ms duration, 0.1, 0.3, 1, 3, 10, 30 Hz at constant 60 V) were delivered for 30 s at 5 min intervals to construct a frequency response curve.

Serotonin dose response curves were obtained by exposure to 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, and 100 μM serotonin for 1 min at 10 min intervals. Differential pulse voltammetry studies have shown that 3-month-old mice had concentrations of 5-HT of 4.9 ± 1.0μm in the colon (Bertrand et al., 2010).

A second set of EFS and 5-HT applications were carried out in the presence of the antagonists (detailed in 2.1) and/or melatonin. Both antagonists and melatonin incubations were carried out for 20 min in the organ bath before data for EFS/serotonin dose response was collected. Melatonin has a short half-life and...
application needs to be repeated every 30min (but without 20min initial incubation) for the relevant experiments.

### 3.4.4 Data Analysis

Analysis of the tension recordings was performed using LabChart 5 software (ADInstruments). Peristaltic activity was assessed for each colonic segment by quantifying the Area under Curve (AUC) for each exposure period (30 seconds for EFS and 1min for serotonin) and an equivalent period of time before and after EFS or serotonin application. Results are shown as mean ± S.E.M. Each experiment was conducted in 6 proximal and 6 distal segments unless otherwise mentioned. Whenever possible, one proximal and one distal segment from the same animal were used.

For tension data, non-linear regression trend lines were fitted for an inhibitor/agonist dose response curve (GraphPad Prism). The population means were compared using two-way ANOVA with repeated measures (GraphPad Prism) and by Bonferroni's multiple comparison tests after combined drug/receptor antagonist treatment. The data obtained was normalised to the weight of the tissue. P<0.05 was taken as being significant.
3.5 Results

3.5.1 Effects of melatonin on resting muscle contraction activity

Firstly, it was important to establish the effect that the application of melatonin alone had on the isolated mouse colon. Melatonin dose response curves were obtained by exposure to 100nM, 300nM, 1 μM, 3 μM, 10 μM, 30 μM, and 100 μM melatonin for 1 min at 10min intervals. Physiological concentration of melatonin is $5.6 \pm 1.9 \mu \text{M}$ in the colon (Bertrand et al., 2010), and the range tested covers minute increases, realistic physiological concentrations as well as over-saturation levels.

Figure 3.1A and 3.1B show representative traces for proximal and distal colon respectively. These traces were overlaid snapshots taken at 0.3 μM, 3 μM and 30 μM MEL to represent a wide range of concentrations. From these traces, it is obvious that there is no observable difference in the activity levels of the colonic tissue (exposure time period indicated by the grey bar). Figure 3.1A and 3.1B also shows the general trend that the proximal colon demonstrates a higher level of spontaneous activity than the distal colon.

Figures 3.1C and 3.1D show dose response curves for melatonin in proximal and distal colon, respectively. There was no significant difference for increasing concentrations of melatonin on the activity of the distal and proximal colon (proximal $R^2$ value=0.020, distal not converged). The values stayed closed to the baseline 0. There was no correlation between increasing concentrations of melatonin and motility effects (both contraction and relaxation activities).
Figure 3.1: Response of proximal and distal colon tissue exposed to melatonin alone. (A) and (B): Representative traces from bioassay recording. The grey bar indicates the exposure period to melatonin (60 seconds). (C) and (D): Changes in response tension as concentration of melatonin increases from 100nM to 30μM. Values expressed as mean ± SEM. (n=6, 3 month old animals).
3.5.2 Effects of melatonin on serotonin-evoked responses.

Melatonin was shown to have no effect when applied alone to both the proximal and distal colonic tissue. As melatonin has previously been shown to alter the responses to serotonin, the effects of melatonin on serotonin-evoked responses were investigated in the presence of antagonists designed to either block the contractile response or the relaxation responses.

Figures 3.2A, B, C and 3.2D, E, F show representative snapshots of the raw tension trace for proximal and distal colon, respectively. In the presence of serotonin, the response triggered consisted of initial fast relaxation follow by a sustained contraction. From Figures 3.2A and D, melatonin appeared to be enhancing the contraction component. From these snapshots it can be seen that the effect of inhibitors on the nature of the peristaltic activity in the colon. Figures 3.2B and E clearly demonstrates that after treatment with excitatory neurotransmitter antagonists (scopolamine, RP67580, GR159897), the response is instead dominated by the slow relaxation in both regions, although in the distal colon there is a slow recovery contractile response towards the end of the period. The reverse is true is Figures 3.2C and F, where exposure to inhibitory neurotransmitter antagonists (MRS2500 and L-NNA) has led to a response that is predominantly contractile. This is most pronounced in the distal colon where the contraction response kept increasing in tension until the physical washing out of serotonin (Fig 3.2F).
Figure 3.2: Melatonin response of proximal and distal colon tissue exposed to 10 μM serotonin. Representative traces from bioassay recording. The grey bar indicates the exposure period to serotonin (60 seconds). Changes in Proximal (A, B, C) and Distal (D, E, F) response tension where (B and E): treatment with contraction antagonists scopolamine, RP67580, GR159897 and (C and F): treatment with relaxation antagonists MRS2500 and L-NNA.
Figure 3.3A, B and C shows the proximal colon response while Figure 3.3C, D and E shows the distal colon responses after exposure to 100nM, 300nM, 1 μM, 3 μM, 10 μM, 30 μM, and 100μM serotonin. Controls were done in presence of antagonists for positive control to facilitate the gathering of new baseline comparison level. Addition of serotonin leads to a concentration-dependent net contractile response in both the proximal and distal colon (Figure 3.3A and B). Melatonin had no significant effect on the serotonin evoked responses in the proximal colon but at 5 μM caused a significant increase in the magnitude of contractions seen in the distal colon (p<0.01).

It is not immediately obvious what is causing this. This increase could be due to an inhibition of the relaxation component or an increase in the contractile component of the 5-HT response. To test this, the experiment was repeated in the presence of drugs designed to selectively block either the contractile or the relaxation portions of the response. The control in this case is the serotonin dose response after incubation with drugs. When scopolamine (muscarinic acetylcholine receptor antagonist), RP67580 (NK₁ receptor antagonist) and GR159897 (NK₂ receptor antagonist) were added to the organ bath, it can be seen in Figure 3.3E that melatonin is able to elicit the contraction response at higher concentrations of serotonin, suggesting that melatonin might be capable of inhibiting a component of the relaxation (30 μM, p<0.05). This is in spite of the presence of antagonists for receptors of excitatory neurotransmitters, so perhaps melatonin acts through other contraction promoting pathways (besides those mediated by muscarinic ACh and NK₁ and NK₂). Melatonin had no effect when applied in the presence of drugs designed to block the relaxation component (Figure 3.3F).
Figure 3.3: Melatonin response of proximal and distal colon tissue exposed to increasing concentrations of serotonin. Changes in Proximal (A, B, C) and Distal (D, E, F) response tension as concentration of serotonin increases from 100nM to 30μM where (B and E): treatment with contraction antagonists scopolomine (S), RP67580 (RP), GR159897(GR) and (C and F): treatment with relaxation antagonists MRS2500 and L-NNA. Values expressed as mean ± SEM.(n=6, 3 month old animals). †p<0.05, † † p<0.01 for 5 μM MEL response vs. control, †p<0.05 for S+RP+GR vs. 5 μM MEL+S+RP+GR.
To differentiate between melatonin effects on the various neurotransmitters in the context of excitatory pathways, further experiments were carried out in which the tachykinin component was blocked. Similarly, to investigate melatonin effects on various signalling pathways in the relaxation response, further experiments were carried out in which only the NO component was blocked.

Figure 3.4: Melatonin response of proximal and distal colon tissue exposed to increasing concentrations of serotonin. Changes in Proximal (A, B) and Distal (C, D) tension response as concentration of serotonin increases from 100nM to 30μM where (A and C): treatment with contraction antagonists GR159897 (GR) and RP67580(RP) and (B and D): treatment with relaxation antagonist L-NNA. Values expressed as mean ± SEM. (n=4, 3 month old animals). *p<0.05, ** p<0.01 for 5 μM MEL response vs. control. †p<0.05, ††p<0.01 for MEL+L-NNA vs. control. ‡p<0.05, ‡‡p<0.01, ‡‡‡p<0.001 for 5 μM MEL vs. MEL+GR159897+RP67580.
In these experiments, the drugs are added after the tissues had already been incubated in 5 μM MEL. Controls were not done with antagonists. It is a simple experiment just to observe the effects of adding one type of antagonist would have on the net effect.

As expected, there was no significant difference in the proximal colon (Figures 3.4A and B). The area of interest for this study is distal colon. In the distal colon, addition of tachykinin antagonist GR159897 and RP67580 removed the contraction promoting effect of melatonin (Figure 3.4C). The red line (MEL+S+RP+GR) dropped below the black (control) because it was now blocking the intrinsic tachykinins already present within the tissue. Addition of NOS inhibitor L-NNA to the distal colon had no effect on the serotonin-evoked response in the presence of melatonin, as there was no difference between the red and grey lines (Figure 3.4D). There was no effect of melatonin through NO pathway in 5HT response.

3.5.3 Melatonin effects on electric field stimulation (EFS)

Next, the effect of melatonin EFS-evoked response was determined. EFS induced neurotransmitter release (both excitatory and inhibitory) from within the smooth muscle layers and mucosa. This setup allows for observation into how melatonin interacts with a range of ENS neurotransmitters. Hence, this investigation was set up with contraction antagonists and relaxation antagonists to investigate the effect of their interactions.
Figure 3.5: Melatonin response of proximal and distal colon tissue exposed to 10 Hz 60V EFS. Representative traces from bioassay recording. The gray bar indicates the exposure period to EFS (30 seconds). Changes in Proximal (A, B, C) and Distal (D, E, F) response tension where (B and E): treatment with contraction antagonists scopolamine, RP67580, GR159897 and (C and F): treatment with relaxation antagonists MRS2500 and L-NNA.
Figures 3.5A, B, C and 3.5D, E, F show representative snapshots of the raw tension trace for proximal and distal colon respectively. 10Hz stimulation induced a relaxation response that decreased the basal tension and abolished peristaltic contractions. The EFS effect is seen to go from contraction dominated to relaxation dominated as the frequency of EFS increases. In figures 3.5B and E, we can see that application of excitatory neurotransmitter antagonists (scopolamine, RP67580, GR159897) removes the contractile component of the response leaving a pure relaxation. In Figure 3.5C and 3.5F, blockade of the relaxant component with L-NNA and MRS2500 increased the magnitude of the initial fast contraction component of the response to EFS in both proximal and distal colon preparations, but failed to completely block the relaxation component. This is probably due to the contribution from VIP signalling pathways.

Figure 3.6A, B and C shows the proximal colon response while Figure 3.6C, D and E shows the distal colon response following periods of stimuli (0.3 ms duration, 0.1, 0.3, 1, 3, 10, 30 Hz at constant 60V) delivered for 30 s at 5 min intervals, to construct frequency response curves. It shows that EFS at lower frequencies (<1Hz) caused a net contractile response, while at higher frequencies the response is mainly dominated by relaxation (net response is a relaxation). In the control vs. increasing melatonin concentration experiments, there was no significant change in the predominantly relaxation response in the distal colon. But there was a significant switch to a contractile response seen in the proximal colon (p<0.05, n=6 for 5 μM MEL response vs. control) after incubation with melatonin at 1 μM and 5 μM for 20minutes (3.6A and 3.6D). This response appears to be directly correlated to the concentration of melatonin (p<0.005 for 1 μM MEL vs. 5 μM MEL response). Melatonin increases the response of the proximal colon to EFS, and this effect appears to be due to a potentiation of the contractile component; when contraction-signaling pathways are blocked, in 3.7B, effects of melatonin are negated (Figures 3.7B and E).

When MRS2500 and L-NNA were added to the organ bath, it can be seen in Figure 3.6C that contraction-promoting effects of melatonin persevered. (10 Hz and 30 Hz, p<0.05). Melatonin appears not to be acting through relaxation pathways.
Figure 3.6: Melatonin response of proximal and distal colon tissue exposed to increasing frequency of EFS. Changes in Proximal (A, B, C) and Distal (D,E,F) response tension as frequency of EFS increases from 0.1Hz to 30Hz where (B and E): treatment with contraction antagonists scopolamine, RP67580, GR159897 and (C and F): treatment with relaxation antagonists MRS2500 and L-NNA. Values expressed as mean ± SEM. (n=6, 3 month old animals). *p<0.05 is 1 μM MEL vs. control, †p<0.05, ††† p<0.005 for 5 μM MEL response vs. control. ‡‡‡p<0.005 1 μM MEL vs. 5 μM MEL. †p<0.05 for MRS2500+L-NNA vs. 5 μM MEL+MRS2500+L-NNA.
To differentiate between melatonin effects on various neurotransmitters involved in contractile response, the effects of only blocking tachykinin signalling is investigated. Similarly, to investigate melatonin effects on various signalling pathways in the relaxation response, further experiments in which only the NO component is blocked.

Figure 3: Melatonin response of proximal and distal colon tissue exposed to increasing frequency of EFS. Changes in Proximal (A, B) and Distal (C, D) response tension as Frequency of EFS increases from 0.1 Hz to 30 Hz where (A and C): treatment with contraction antagonists GR159897 (GR) and RP67580 (RP) and (B and D): treatment with relaxation antagonist L-NNA. Values expressed as mean ± SEM. (n=4, 3 month old animals). *p<0.05, ** p<0.01 for 5 μM MEL response vs. control. †p<0.05, ††p<0.01 for MEL+L-NNA vs. control. ‡p<0.05 is 5 μM MEL vs. MEL+L-NNA.
Controls were not done with antagonists. It was a simple experiment just to observe the effects of adding one type of antagonist would have on the net effect. From Figure 3.7C and D, it can be seen that there was no observable difference in the distal colon as expected. In Figure 3.7A we can see that melatonin is not doing anything significant to NO signaling. If it were, the grey line would resemble the red line more. From Figure 3.7B, it can be inferred that melatonin was mainly acting through ACh pathway to promote contractile activity. The red line is not significantly different from the grey line. Blocking of tachykinins signaling had no significant dampening effect on melatonin effects. However, there was still some contribution by tachykinins, as the red line was still observably lower than the grey.

3.5.4 Understanding the origin of melatonin effects.

From the previous experiments, melatonin is capable of increasing the excitability of both regions of the colon depending on the stimulus used. The next set of experiments examined the contribution that MT$_1$ and MT$_2$ receptors played in these responses.

After separate incubation with 1 μM 4-P-PDOT and 1 μM luzindole for 20 minutes, periods of electric field stimuli (0.3 ms duration, 0.1, 0.3, 1, 3, 10, 30 Hz at constant 60V) were delivered for 30 s at 5 min intervals to construct frequency response curves as shown in Figure 3.8C. Serotonin dose response curves in Figure 3.8D were obtained by exposure to 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, and 100 μM serotonin for 1 min at 10 min intervals. For the EFS experiments, it was only carried out on the proximal colon, as that was where significant effects of melatonin were observed (Figures 3.3A and 3.3C). Similarly, for the serotonin dose response experiments, it was only done on distal colon samples as melatonin only demonstrated a significant effect in that region (Figures 3.6D and 3.6E). After exposure to each drug, the tissues were washed 3 times and allowed to equilibrate back to the starting baseline activity level.
From Figures 3.8A and 3.8C, it can be deduced that there was a bias for MT<sub>1</sub> receptor to convey the effects of melatonin on EFS. Luzindole (pink line) has blocked all melatonin effects, whereas the application of 4-P-PDOT (red line) has not significantly disrupted this effect.

Figures 3.8B and 3.8D showed that there might be a slight preference for the MT<sub>2</sub> receptor mediated pathway, although MT<sub>1</sub> receptors seem to be contributing too when melatonin exerts its effects on serotonin response. When MT<sub>2</sub> receptor
antagonist: 4-P-PDOT (1 μM) was applied, there was a significant difference (p<0.05) between that response and the one observed in 5 μM melatonin (figure 3.8D).

3.5.5 Melatonin effects on muscle response

Seeing the effects on melatonin on contraction and relaxation responses, it might be easy to assume that all its effects are based on interactions with excitatory and inhibitory neurotransmitters. However, it is important to check if melatonin has any direct effect on the myocyte itself. Tetrodotoxin (TTX), a puffer fish toxin, has been shown to be effective for pharmacological denervation of neuromuscular preparations.

Figure 3.9A and C shows the proximal colon response while Figure 3.9B and D shows the distal colon response. Exposure to KCl (30mM) after a 20 minute incubation of TTX allows for generation of the myogenic contraction. From Figure 3.9, it can be concluded that the presence of melatonin (5 μM) affected neither the nature nor the magnitude of the myogenic response in a significant way. Hence, it can be deduced that all the effects of melatonin seen in the previous experiments here are mediated by its effects on neural transmission.
Figure 3.9: Response of proximal and distal colon tissue exposed to TTX and KCl in the presence of melatonin. (A) and (B): Representative traces from bioassay recording. The gray bar indicates the exposure period KCl (60 seconds). (C) and (D): Changes in response tension in the presence of 5μM melatonin. Values expressed as mean ± SEM. (n=3, 3 month old animals)
3.6 Discussion

Through these experiments, it can be concluded that melatonin has a net positive effect on peristaltic contractile activity, triggered by both serotonin and EFS. These effects are mediated by neuronal signalling pathways and not by effects on the underlying musculature. Melatonin was shown to have no significant effect on its own and thus was mainly involved in the modulation and regulation of other neurotransmission responses. Additionally, the MT$_2$ receptor seems to be crucial in allowing melatonin to have its effects.

Propulsion of intraluminal content through the gastrointestinal tract is achieved by peristalsis, a distinct motor pattern that is constituted by ascending excitatory and descending inhibitory reflexes (Bayliss and Starling, 1899). The effects of melatonin on these mechanisms have been long speculated but the exact mechanism through which this occurs is unknown. Functional bioassays were carried out to study how varying concentrations of melatonin influenced serotonin induced and electrical field stimulated contractions.

3.6.1 Melatonin effects on serotonin response

The current investigation showed that melatonin had a significant excitatory effect on serotonin signalling in the distal but not the proximal colon. Melatonin treatment has previously been shown to increase the positive excitatory role of the mucosa in the colon of aged animals by increasing the progression of the contractile wave (Martin-Cano et al., 2010). This is in agreement with what was observed in Figures 3.4D. Before elucidating the role of various excitatory receptors in this effect, we first characterised serotonin-evoked response in the mouse proximal and distal colon. Our results in Figure 3.3A and 3.3D demonstrate that serotonin-induced responses are of greater amplitude in the distal compared to the proximal colon. This is probably in keeping with the absorption of fluids across the colon, rendering more solid stools as water content decreases, and conceivably, requiring more vigorous contractions to propagate colonic content distally (Deiteren et al., 2011).
More 5-HT3 receptors have also been identified in the proximal than distal colon (Foxx Orenstein et al., 1996). Hence, it can be deduced that in the proximal colon, the results in Figures 3.3A, B and C, are predominantly caused by the 5-HT3 receptor-mediated serotonin contraction response. Presence of a small amount of melatonin (1 μM and 5 μM) does not make a difference. This suggests that the mechanisms by which serotonin evoke contractions is different in proximal compared to distal colon since presence of melatonin is able to have an incremental effect on the contraction response in the distal colon.

From figure 3.3 D, E and F, it can be deduced that melatonin treatment caused an increase in serotonin-induced contraction in the distal colon. After blocking contraction pathways in 3.4E, melatonin is able to elicit the contraction response at higher concentrations of serotonin, suggesting that melatonin was either inhibiting a component of the relaxation, or acting through other contraction promoting pathways besides those mediated by muscarinic ACh and NK₁ and NK₂, perhaps via nicotinic Ach receptors or NK₃ receptors.

The pathways mediated by melatonin is made clear in figure 3.4C, addition of tachykinin antagonists GR159897 and RP67580 removes the contraction promoting effect of melatonin, proving that tachykinins are essential for the promotion of contractile activity as an effect of melatonin. Tachykinins exert their effect primarily through the NK1 and NK2 receptor subtypes. Interestingly, their contribution to peristaltic activity shows an increasing proximal-to-distal gradient (Deiteren et al., 2011). Melatonin’s contribution to serotonin induced contractions follows the same pattern where it is only significant in the proximal colon, hinting at a probable more dominant NK2 pathway pathway for its effects.

Furthermore, Deiteren et al. also showed in mice that blockade of NK1 receptors reduced the peristaltic pressure amplitude in the proximal and distal colon while the interval was not significantly altered. However, the blockade of NK2 receptors reduced the peristaltic pressure amplitude and interval in the distal colon. This provides a fitting explanation for the novel finding presented here of melatonin effects on serotonin response in the murine distal colon.

Furthermore, tachykinins can both stimulate and inhibit GI motility, the net response depending on the type and site of tachykinin receptors that are activated.
It is important to note that under physiological conditions in vivo; however, tachykinin receptor antagonists have little effect on GI motor performance (Holzer et al., 1997). Only when the overwhelming cholinergic component of the neural activation of smooth muscle is blocked does blockade of NK1 or NK2 receptors impair propulsive motility in the gut. This is in agreement with the theory that melatonin is antagonistic to acetylcholine signaling in the murine colon, but it can nonetheless still promote contraction.

An alternative explanation is that melatonin might be helping to boost the effect of serotonin-induced contractile effect by increasing the amount of serotonin available. In a recent study on human colon epithelial cells, it has been shown that melatonin has an inhibitory effect on SERT, via its catalytic effect on the allosteric citalopram-sensitive site (Matheus et al., 2010).

3.6.2 Melatonin effects on EFS

This study has shown that melatonin treatment in the proximal colon caused an increase in evoked contractions as a response to EFS, while there is no significant effect in the distal colon. At higher frequencies, electrical field stimulation under non-adrenergic, non-cholinergic conditions evoked muscular relaxation occasionally followed, at the higher stimulus frequencies, by rebound contractions (Serio et al., 2002). This agrees with the general trend observed in figures 3.5 and 3.6. EFS elicits a transient hyperpolarization of gastrointestinal smooth muscle cells accompanied by smooth muscle relaxation (Serio et al., 2002). This is consistent with the results seen in figure 3.5.

From figure 3.6A, it can be concluded that melatonin treatment caused an increase in EFS-induced contractions in the proximal colon, and that this was due to an upregulation of the contractile response. In figure 3.6B, it can be seen that melatonin presence has no effect on EFS response in the presence of excitatory neurotransmitters antagonists. However, melatonin still causes significant contraction in the presence of only inhibitory neurotransmitter antagonists. Hence,
Melatonin must be not acting through those pathways, and it can be deduced to be promoting the excitation component of the EFS response.

From 3.7B, it can be deduced that melatonin is mainly acting through ACh pathway to promote contractile activity. Electric field stimulation works based on the depolarisation of neurons. It allows the secretory effects of locally-released neurotransmitters to be dissociated from other stimuli. Most neurons contain ACh and will release it in EFS as it is the principal neurotransmitter. Additionally, past studies have shown that EFS-induced colonic peristalsis in mice is mediated mainly through acetylcholine neurotransmission and is additionally regulated by endogenous tachykinins (Holzer et al., 1997, Saban et al., 1999, Kondo et al., 2011). This supports our findings in figure 3.7B, where the blocking of tachykinins signaling had no significant dampening effect on melatonin effects.

M2 and M3 receptors are the dominant muscarinic acetylcholine receptor subtypes expressed in the gastrointestinal tract (Eglen, 2001; Eglen et al., 1996; Ehlert et al., 1997; Levey, 1993). M2 and M3 are present on the cell membrane of muscle cells. Many studies, both functional and anatomical, show that the majority of mAChR on murine intestinal muscle are M2r (70–80%) and M3r (20–30%), with no measurable quantities of M1 receptors (Gabriel, 1990; Giraldo et al., 1988; Gomez et al., 1992; Iino and Nojyo, 2006; Ladinsky et al., 1988). Functional studies using muscarinic receptor-deficient mice have indicated important roles of both M2 and M3 receptors in muscarinic agonist-induced contraction of the ileum and colon (Kitazawa et al., 2007; Unno et al., 2005).

Ahmed et al. (2013) showed that in rabbit colon, melatonin caused rapid increase in cytosolic Ca^{2+} in single smooth muscle cells, and induced rapid contractions. This melatonin-induced P_{i} hydrolysis and subsequent contraction were blocked by the non-selective MT_{1}/MT_{2} antagonist luzindole, but not by a selective MT_{2} antagonist 4P-PDOT. This proved that rabbit gastric smooth muscle cells express melatonin MT_{1} receptors coupled to G_{q}. Activation of these receptors causes stimulation of P_{i} hydrolysis and increase in cytosolic Ca^{2+} and elicits muscle contraction. This is significant because this is how the M3 receptor for ACh works to contract muscles as previously discussed (Sakamoto et al., 2007; Unno et al., 2005).
In terms of the partial contribution of tachykinins in 3.7A, we know that tachykinins are involved in neurotransmission between IPANs and interneurons (Costa et al., 2000). They are involved in the excitatory neuromuscular transmission; perhaps they could upregulate ACh release from interneurons.

Furthermore, NK2 receptors appear to be the dominant receptor through which EFS induced release of tachykinins causes smooth muscle contraction. In human colons, application of NK2 receptor agonists induced contractions, while selective NK2 antagonists were capable of almost completely blocking EFS-induced colonic contractions (Giuliani et al., 1991; Nakamura et al., 2011). Deiteren et al. (2011) have shown that NK1 and NK2 receptor-mediated peristaltic activity in the mouse colon co-exist at the same time with cholinergic signaling. This overturned the previous hypothesis that modulation of peristalsis by tachykinins only takes place in the inhibition of acetylcholine neurotransmission (Furness, 2000; Holzer et al., 1998; Holzer and Holzer-Petsche, 1997a; Holzer and Maggi, 1994). This suggests that tachykinins actually have a more significant contributing role to peristalsis in the murine colon, despite being secondary to cholinergic neurotransmission.

3.6.3 Role of MT1 vs. MT2 receptor in motility

Some drugs and synthetic ligands were selective antagonists for the receptors (Figure 29). 4-phenyl-2-propionamidotetralin (4P-PDOT) is specific for the MT2 receptor. Luzindole is a non-specific inhibitor of both. From the experimental results shown in figure 3.8, it can be concluded that the MT2 plays a more significant role in mediating melatonin effects on colonic motility. There is a significant difference observed between 1 μM 4-P-PDOT vs. 5 μM MEL but not so between 1 μM 4-P-PDOT and control.

Previous studies have shown that the highest level of MT2 mRNA expression was detected in the colon and stomach, followed by the duodenum and pancreas (Sotak et al., 2006). In the colon there was expression in the muscularis mucosae and in the muscularis layers, but there was also positive labelling in the villi of the mucosa layer (Stebelova et al., 2010). The high density of MT2 melatonin receptors in the circular and longitudinal muscle layers of colon suggests and that
melatonin plays a part in intestinal motility. A separate study using western blot analysis on murine pancreas, stomach, duodenum and colon tissue samples also confirmed the colon as expressing the highest levels of MT$_2$ (Chen et al., 2011). Between MT$_1$ and MT$_2$, MT$_2$ receptors were found in greater numbers and they were also expressed in EC cells in the intestines (Sjoblom et al., 2003). All these observations support the results obtained here that suggests melatonin effects in the colon are mainly mediated through the MT$_2$ receptor.

In addition, the contribution from MT$_1$ receptors cannot be completely ignored. In a recent study in rabbit colon, it is demonstrated melatonin acts through MT$_1$ receptors to cause an increase of intracellular IP$_3$-dependent Ca$^{2+}$ and hence, instigate smooth muscle contraction.

### 3.7 Conclusion

These initial results indicate that there is a significant role of melatonin in the colon and that it has the potential to be an important therapeutic target within the bowel. These effects are mediated by neuronal signalling pathways and not by the underlying musculature. Melatonin has a net positive effect on contractile activity, triggered by both serotonin and EFS. This work builds on past observational knowledge about the dual effects of melatonin on serotonin. The current investigation shows that melatonin had a significant effect on serotonin signalling in the distal but not the proximal colon. Melatonin treatment caused an increase in EFS-induced contraction in the proximal colon. In both cases, the effects appear to be mainly mediated through the up-regulation of the contractile response, tachykinin signalling in the case of serotonin dose response and ACh signalling in the case of EFS.
Effect of melatonin on CMMCs and faecal pellet propulsion

4 Colonic Transit Time

The colon is responsible for fecal pellet motility and aids in defecation regulation (Brann et al., 1976). The colonic transit time describes the velocity of pellet passage through the colon. Heredia et al. (2009) showed that pellet propulsion along the murine colon is initiated by CMMCs. Our molecule of interest, melatonin, is known to have an effect on this. It has been shown that aged mice present lower levels of motility, both spontaneous CMMCs and induced, and that melatonin treatment seems to aid in returning motility levels (Martin-Cano et al., 2013).

Neuronal signalling within the colon most likely contributes to the regulation of colonic transit time. There are various studies that have demonstrated the contribution of the mucosa (Barnes et al., 2014), serotonin signalling (Spencer et al., 2013; Heredia et al., 2009) and the submucosal plexus (Martin-Cano et al., 2013) to rates of colonic motility. Since melatonin has been shown to have an effect on all these fronts, it might have a role to play in colonic transit time. In previous research in the isolated rat duodenum, melatonin is found to antagonise the excitatory response to serotonin (Quastel & Rahamimoff, 1965). In fact, in the duodenum, the stomach (Fioretti et al., 1972), the ileum (Bubenik, 1986) and the colon (Harlow & Weekley, 1986) of the rat, melatonin causes a reduction of the tone and of spontaneous phasic motility. There were also instances where melatonin is shown to promote colonic motility in diseased states such as during constipation, both in humans (Schiller, 2002; Wisker, 2010), and guinea pigs (Wade, 2002).
4.1 Role of Melatonin in CMMC regulation and fecal pellet propulsion

Intestinal melatonin is locally produced and released in EC cells (chapter 1), and diffuses into the layers of intestinal tissue, the lumen and into blood circulation. As previously discussed, melatonin has no problem with diffusion through biological membranes as it is highly lipophilic (Matheus et al., 2010). MT$_1$ receptors have been found on myenteric plexus neurons in the rat colon, suggesting enteric neurons were responsive to melatonin (Soták et al., 2006). Melatonin decreased the inhibitory response of non-adrenergic, non-cholinergic (NANC) neurons (Storr et al., 2002), which supports our previous experimental findings that melatonin promotes contractile responses. On the other hand, little is known about the influence of melatonin on CMMCs and these experiments will test if melatonin has similar effects on this motility tone.

Although current knowledge in the mechanisms of melatonin effects in physiological and pathophysiological colonic functions is limited, melatonin has long been observed to influence colonic motility regulation (Bubenik, 2008; Lu et al., 2005; Lu et al., 2009). However there does not seem to be a consensus in its effects in different species of animal models, even those within the rodent family.

In C57BL/6 mice, melatonin has shown to increase colonic motility (Diss et al., 2013) and additionally regulate rhythmic changes in gastrointestinal motility (Lee and Pang, 1993). The same has been observed in guinea pig colon smooth muscle preparations, where melatonin has been show to produce contractile effects (Lucchelli et al., 1997). In rat colon, however, melatonin treatment has been shown to promote relaxation instead (Harlow and Weekley; 1986). There seemed to be a concentration dependent effect in previous studies in rats, where exogenous melatonin treatment increased intestinal motility in small doses, but large doses decreased transit time (Drago et al., 2002; Thor et al., 2007).

Whilst the exact mechanism by which melatonin regulates motility is unclear, past studies have suggested blockade of nicotinic channels and interaction with Ca$^{2+}$ activated K$^+$ channels (Storr et al., 2000). There is also been propositions that
melatonin acts as an antagonist against serotonin (Bubenik, 2002). In rats, melatonin has been shown to inhibit gastric transit time through interaction with 5-HT₃ receptors (Kasimay et al., 2005).

To date limited studies have been conducted to investigate the role melatonin has on CMMCs in mice. Melatonin has been shown to inhibit the action of irregular spiking activity (ISA) and reinforce the phasic pattern of CMMC in rats (Merle et al., 2000). Studies on mice demonstrated that melatonin treatment recovered CMMC patterns that was impaired in the proximal colon, and suggested that the mucosa was integral to this effect (Martin-Cano et al., 2013).

4.2 Hypothesis

The role of melatonin on various stimulated colonic activity (serotonin dosage and EFS) has been investigated in the previous chapter, and it has been shown to promote motility. The hypothesis is that it will have the same effect on both colonic transit time and CMMCs.

4.3 Aims

The aim of is to understand the role melatonin plays in regulating the generation of CMMCs and faecal pellet propulsion in terms of colonic transit time.
4.4 Methods

4.4.1 Colon harvesting

3-month-old male C57BL/6J mice were stunned and dispatched by means of cervical decapitation. The whole colon was harvested, approximately 2 cm proximal to the anus and placed in ice cold oxygenated (95% O₂ and 5% CO₂) Krebs’ buffer solution, pH 7.4 (117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM, NaH₂PO₄, 25 mM NaHCO₃, and 11 mM glucose). The whole colon was cleaned of fatty tissues and gently flushed with Krebs’ buffer to clear the lumen of intestinal contents.

4.4.2 Recording CMMCs

To monitor CMMC waves from proximal to distal sections, transducers are placed along the length of the colon to monitor muscle tension and contractile activity in order to produce CMMC traces. Cotton was threaded through a layer of colon wall at the proximal and distal end of the colon (approximately 1 cm in) and tied at the ends to form a loop. The preparation was then transferred to a Sylgard-lined flow bath where warm oxygenated Krebs solution was constantly being recycled. The colon was held in place by a thin rod that was placed through the lumen of the colon and pinned to the Sylgard-lined bath at both ends. Cotton loops were subsequently hung up onto transducers in order to mount the tissue at a fixed tension. The preparation was exposed to flowing Krebs buffer to allow for acclimatisation for 20 minutes prior to commencement of recording.

4.4.3 Drugs

Drug concentrations are expressed as final bath concentrations. Melatonin (Sigma-Aldrich: 1 µM) and MT₁ and MT₂ receptor antagonist: Luzindole (Sigma-Aldrich; 1 µM, 5 µM); MT₂ receptor antagonist: 4-P-PDOT (Tocris; 1 µM); Stock
solutions of 4-P-PDOT and Luzindole were dissolved in 100% DMSO and further diluted in Krebs Buffer. The final volume of DMSO in the organ bath did not exceed 0.05% and had no effect on peristaltic contractions.

Tissue was exposed to melatonin after gathering a control response, then MEL+ 4-P-PDOT, subsequently MEL+1µM luzindole and finally MEL+5µM luzindole and the responses were measured after each treatment. Each tissue sample was perfused for 20min before readings were taken.

**4.4.4 Recording Faecal Pellet Propulsion**

The whole colon was loosely pinned in a Sylgard-lined flow bath. The isolated colon was constantly perfused in oxygenated Krebs’ buffer solution at 37 ± 1 °C at a flow rate of 8 mL min⁻¹. 30 minutes was allowed for the spontaneous evacuation of the natural faecal pellets. However, if this was not achieved during this time frame, the faecal pellets were gently removed from the isolated colon by perfusing the lumen of the colon with warmed Krebs’ buffer. The colon was then left to stabilize for 15 min, prior to recordings. Measurements were carried out using an epoxy-coated artificial faecal pellet. For each age group, a fecal pellet that was the average size/ shape for each age group was utilized. The artificial faecal pellet was inserted into the proximal end of the colon using a glass capillary tube. The pellet was monitored using a video camera and tracked using Ethovision tracking software. Each pellet was tracked for 20 minutes and 3 repetitions were carried out for each condition.

**4.4.5 Data Analysis**

Prism software was utilised to statistically assess experimental data and a one-way ANOVA test, presented as mean ± SEM. CMMC peak amplitude was measured via the subtraction of the baseline value from the maximum point on the contractile response (in order to find the magnitude of contraction). Area was analysed by obtaining the integral below the peak. Duration values were found by
measuring the length of the baseline from the start of the incline to the end of the decline of the CMMC propagation. Frequency was calculated by counting the number of CMMCs occurring within a 30-minute interval. Measurement of velocity was taken by dividing the distance between the two transducers by the time between successive CMMC peaks recorded by the proximal and distal transducers (see trace below)

![Figure 4.1: Using Chart7 to analyse tension traces for peak-to-peak interval values. A: proximal colon B: distal colon.](image)

Faecal pellet motility was measured using the Ethovision tracking software (Ethovision XT vs7), where the distance moved every 2 min over a 14 min period was recorded for each trial. The population means were analysed using two-way ANOVA (GraphPad Prism) and significant differences were determined using posthoc Tukey tests. All data were presented as mean ± SEM, and n represents the number of animals used for each experiment.
4.5 Results

4.5.1 Melatonin effects on CMMCs

Colons were exposed to 1µM melatonin for 20 minutes. After this colons were additionally perfused with 0.1µM 4-P-PDOT, 1µM luzindole and finally 5µM luzindole sequentially; and a minimum 5 replicates of CMMC responses was recorded.

Figure 4.2: (A): Representative traces shown above illustrate CMMCs from various experimental data sets; P is representative of proximal and D of distal colon. (B): Representative traces from control treatment with 1µM luzindole only.

From figure 4.2A, melatonin is shown to decrease the amplitude of the CMMC response in the murine colon. This was more obvious in the proximal than the
distal colon. Once melatonin receptor antagonists were added, this effect was actually further amplified in both sections, with now a complete wipe-out of detectable spontaneous activity in the distal colon. This is unexpected, as the antagonists appear to be potentiating the effects of melatonin. From figure 4.2B, luzindole is shown to have a dampening effect on CMMCs even on its own.

Figure 4.3 demonstrates that in the proximal colon, there is a significant reduction in amplitude of the CMMC upon the addition of 1µM melatonin (p<0.005). This indicates that melatonin is effectively suppressing CMMC amplitude and contractile activity within the proximal section. Further drug treatment with MT₂ selective receptor antagonist 0.1µM 4-P-PDOT produced no significant change in the amplitude compared to melatonin treatment alone. This suggests that the melatonin effects seen in the proximal colon are not mediated by MT₂ receptors. On the other hand, treatment with luzindole shows a concentration dependent reduction of CMMC amplitude (1µM luzindole: p<0.005; 5µM luzindole:...
p<0.001). This suggests that melatonin might not actually be suppressing CMMCs at all, since the removal of melatonin influence by blocking both the receptors suppressed the amplitude even further. Whatever the overall effect of melatonin, this seems mostly mediated by my MT₁ receptors in the proximal region, and more significantly, that endogenous melatonin.

Figure 4.3(B) illustrates that in the distal colon, despite a slight reduction the mean, the suppression of CMMCs by melatonin is insignificant compared to the control. This seems to suggest that the distal colon is less sensitive to the levels of exogenous melatonin introduced. However, 1µM luzindole is still able to cause a significant reduction in CMMC activity amplitude. The effect of melatonin appears to be enhancement of the amplitude. The fact that luzindole elicit a change in CMMC pattern (complete suppression as seen in Figure 1 and p<0.005 for 1µM Luzindole in Figure 4.3B), shows that the basal level of melatonin present in the distal colon still plays a regulatory role in CMMC propagation. Melatonin might well play an excitatory role in regulation of CMMCs in the distal region. But since amplitude is already maximal, supplementation with exogenous melatonin has no significant effect.

Figure 4.4: Population data (n=5) from proximal colon depicting velocity and drug treatment as variables. Mel equates to 1µM melatonin, Mel + P equates to 1µM melatonin and 0.1µM 4-P-PDOT, Mel + P + 1L equates to 1µM melatonin, 0.1µM 4-P-PDOT and 1µM luzindole, Mel + P + 5L equates to 1µM melatonin, 0.1µM 4-P-PDOT and 5µM luzindole. ◊ denotes the relationship between 0.1µM 4-P-PDOT and 1µM luzindole (◊ p=0.0002). ◊◊ Between 0.1µM 4-P-PDOT and 5µM luzindole (◊◊ p=0.0002).
As shown in figure 4.4, the addition of melatonin led to a small decrease in velocity of CMMC propagation, though not statistically significant. However, there is a significant reduction in contraction velocity when 1µM and 5µM luzindole are added. This suggests that in the proximal colon, melatonin receptor pathways are involved in mediating propagation of CMMCs. P-DOT appears to have reversed the effects of melatonin, whereas luzindole is slowing propagation of CMMCs. This might suggest a consolidation of 2 different effects by the 2 different melatonin receptors. Melatonin acts via MT$_1$ receptors to slow velocity, and MT$_2$ receptors to speed up velocity. In the presence of luzindole, the MT$_2$ effects are dominant.

![Figure 4.5](image.png)

*Figure 4.5: Population data from proximal and distal colon (n=5), with duration of response and drug treatment as variables. Mel equates to 1µM melatonin, Mel + P equates to 1µM melatonin and 0.1µM 4-P-PDOT, Mel + P + 1L equates to 1µM melatonin, 0.1µM 4-P-PDOT and 1µM luzindole."

As seen in figure 4.5, there is slight increase in duration of the CMMC responses in both the proximal and distal colon, but these are not statistically significant. There is no observable or significant difference in area and frequency of CMMCs in both proximal and distal colon after melatonin treatment. There is also no statistically significant effect after addition of 4-P-PDOT or luzindole to the frequency and duration.
4.5.2 Melatonin effects on fecal pellet propulsion

The motility profile of an artificial faecal pellet through an isolated whole colon with respect to time is recorded. In aged mice, melatonin has been shown to increase CMMC propagation (Martin-Cano et al., 2013). However, no experimentation has been done on normal, adult 3 month old mice as well as aged mice in terms of colonic transit time. Would it still present the same expected promotion of motility? Measurements were taken every 2 minutes.

Figure 4.6: Influence of endogenous melatonin on fecal motility. (A): Representative traces of fecal pellet motility before/after addition of melatonin are shown for 3 month old animals. White dot on the pellet indicates the point tracked during recordings, and the linear trace shown indicates the movement of the fecal pellet from the oral to the anal end of the colon. The black bar indicates the duration for which the population data was obtained between multiple animals. (B): The overall data for 3 month old animals is shown, where the movement of the pellet over 14 min at 2 min intervals is shown. Data shown as mean ± SEM, n = 5, *p < 0.05 and **p < 0.001 control vs 1 μM luzindole, †p < 0.05, ††p < 0.01, and †††p < 0.001 control vs 1 μM melatonin.

Figure 4.6(A) shows representative traces of the motility profile of artificial faecal pellets through the colons of 3 month old animals. In the control trace, the pellet progresses through the length of the colon in bouts of stepwise movements every 5 minutes and the pellet is usually expelled within 15 minutes.

Melatonin changes the movement pattern of the pellet through the length of the colon, from sudden bouts of movement to a smoother, continuous transition. After
addition of luzindole, the colonic transit rate slowed significantly to even below control levels. This shows that the promotion of motility by melatonin is mediated by MT1/2 receptor pathways. Furthermore, exogenous melatonin supplement does not lead to desensitisation; as it is still possible for pellets to move at a quicker rate through the length of the colon.

The overall analysis of all the traces is shown for 3 month animals in Figure 4.6B. It shows a significant decrease in colonic transit times of the faecal pellet (p < 0.001, n = 5). The pellets are expelled before 10 minutes is up. Specifically, in 3-month-old animals, the pellets moved further after 6 (p < 0.05, n=5) and 8min (p<0.001, n=5) when compared to the control traces. Luzindole treatment, on the other hand, significantly increased the colonic transit time of the pellets, with pellets moving less than 1cm along the colon even after 15minutes.

In summary, melatonin decreases pellet transit rates in the murine colon and these effects are directly mediated by melatonin MT1/2 receptors.
4.6 Discussion

4.6.1 Effects of melatonin on CMMCs

Current experimental results show that melatonin decreased the amplitude of CMMCs within the proximal colon but had no significant effect in the distal colon. The duration of the response also appears to increase in both proximal and distal colon. In both regions, treatment with MT2 receptor antagonist 4-P-PDOT did recover the amplitudes of CMMCs in the tissues compared to before melatonin treatment. Luzindole treatment, unexpectedly, led to a diminishment of amplitude in the proximal colon in comparison to melatonin treatment. It also led to a decrease in the propagation velocity of CMMCs. Luzindole at increasing concentrations (5µM) led to a total suppression of CMMCs in the proximal colon and distal colon.

In this case, melatonin appears to play a role in promoting CMMCs at endogenous levels, but with further supplementation, it appears to have the reverse effect, especially in the proximal colon, decreasing the amplitude of the CMMC wave. Luzindole could also have other partial agonist effects targeting different pathways that are CMMC process specific (e.g. pacemaker ICC).

The effect of melatonin on CMMCs in healthy, adult mice has not been studied before. These current traces demonstrate melatonin to decrease CMMC amplitude in the proximal colon, contrary to data from other studies postulating melatonin to act as a prokinetic agent to drive colonic motility (Merle et al., 2000; Thor et al., 2007). Nonetheless, it is important to remember that CMMCs represent a different motility pattern; it is spontaneous and not a key pattern that moves the fecal pellet. No concrete evidence exists to show that CMMCs directly cause fecal pellet movement, only as a supplementary modulation (Spencer, 2001), and not every tone would be regulated the same way by melatonin or use the same signaling mechanisms. In the rat colon, melatonin treatment alone also decreased the amplitude of spontaneous contractions and did not change the frequency as well, which is similar to what is seen here (Tan et al., 2013). Melatonin has also been shown to decrease tone and amplitude of spontaneous smooth muscle
contractions in rat ileum by 20% (Bubenik et al., 1977; Harlow and Weekley, 1986) with no influence on contraction frequency.

However, although a reduction in CMMC amplitude was seen, CMMCs still propagated. And looking at the traces it appears that melatonin changed the type of contraction profile, this was previously not studied in the murine colon. There is a significant decrease in amplitude but there is also an observable increase in duration. It is uncertain whether melatonin exhibits its action through suppression of muscle contraction or promotion of relaxation.

Previous experiments on rats have reported that melatonin can both inhibit contraction and increase relaxation in the intestines (Storr et al., 2002; Drago et al., 2002; Merle et al., 2000). Melatonin has been shown to have negative effects on NO production and NO signaling. During colitis, nitric oxide synthase production in the mouse colonic neurons was suppressed by melatonin, decreasing the amount of signaling nitric oxide present in an inflamed state (Mei et al., 2005). It is associated with a down-regulation of iNOS expression (De Filippis et al., 2007). It has been speculated that N-acetyl-5-methoxykynuramine, a main melatonin metabolite, rather than melatonin itself may down-regulate iNOS, as also suggest previously for nNOS. And this blocking of NO signaling can have an effect on CMMCs. Blocking NO production and signaling has been shown to increase CMMC frequency but decreasing their amplitudes in the proximal region of the mouse colon, with initial increased suppression of CMMCs followed by higher frequency contractions of variable amplitudes (Powell et al., 2011).

In mice colon, melatonin also demonstrates a mucosal effect related to the inhibitory nitrergic signalling and other inhibitory enteric nerves, as suggested by the effects of neuronal blockers (Martin-Cano et al., 2013). The same study also concluded another point that melatonin had no effects the frequency of the spontaneous activity, which agrees with the results seen here.

In support of contraction suppression theory, melatonin has been shown to inhibit smooth muscle contraction in the stomach, ileum, and colon of rats (Bubenik, 1986). However, another recent study in rabbit colon demonstrated melatonin to
act through MT$_1$ receptors to cause an increase of intracellular IP$_3$-dependent Ca$^{2+}$ and hence, instigate smooth muscle contraction (Ahmed et al., 2013). However, contraction in that study was defined as ‘decrease in basal cell length in response to melatonin’ as opposed to area under curve or amplitude of tension. The differences in species and experimental methods might account for the conflicting data.

The role of melatonin on influencing the acetylcholine and tachykinins signalling in the context of motility in the murine gastrointestinal tract has not been previously investigated. In other species however, melatonin was shown to have an inhibitory effect on acetylcholine signalling. The addition of melatonin induced a concentration-dependent attenuation of acetylcholine-induced contractile response in the isolated intestines of goldfish (Velarde et al., 2009). Melatonin has also been shown to inhibit the fast EPSPs by directly and specifically blocking the acetylcholine receptors on the submucosal plexus in the guinea pig (Barajas-López et al., 1996). Since the generation of CMMCs is mediated by the activation of both myenteric and submucosal pacemaker cells that release acetylcholine and tachykinins from excitatory motor neurons (Heredia et al., 2009), melatonin could possibly be having negative effects on acetylcholine and tachykinins signaling in the context of spontaneous motility tones like CMMCs.

In rat colon, melatonin has demonstrated that it inhibited the nicotinic receptors in the sub mucous plexus to regulate the cholinergic transmission, and interacted with small conductance K$^+$-channels to relax the contraction caused by activation of ACh and 5-HT (Barajas-López et al., 1996; Storr et al., 2000). This might be the predominant case in the absence of exogenous 5HT dosage and EFS.

In terms of explaining the mechanism of inhibitory action of melatonin on colonic contraction, there have been theories that point at the involvement of the calmodulin/CaMKII system and voltage-dependent calcium channels (Han et al., 2012). Apart from interactions with membrane receptors MT$_1$ and MT$_2$, melatonin has been shown to interact with intracellular proteins such as calmodulin because of its lipophilic permeability.
Melatonin may be influencing CMMCs in the murine colon through a non-neuronal component as well. In murine colonic smooth muscle, melatonin can interact with Ca\(^{2+}\)-activated calmodulin with high affinity and prevent it from activating myosin light-chain kinase, leading to decreased muscle contraction (Bayguinov et al., 2011). In rat colon, melatonin has been shown to inhibit contraction induced by high K\(^+\) (KCl pre-treatment), which is attributed to the influx of Ca\(^{2+}\) (Tan et al., 2013). This suggested that melatonin could interact with calcium channels and inhibit the influx of calcium. This mechanism is corroborated by similar experiments in the rat ileum, where melatonin produced a concentration-dependent inhibition of muscle contraction, which was not blocked by tetrodotoxin (blocks Na\(^+\) channels and hence neuronal response). These results show that melatonin has a proven effect on Ca\(^{2+}\)-activated, K\(^+\) channels, which are also involved in ATP signalling (Reyes-Vazquez et al., 1997). This is also suggested in murine colon as in the presence of tetrodotoxin, the CMMCs persisted in both the proximal and distal colon (Martin-Cano et al., 2013).

There have also been theories that link the Ca\(^{2+}\) channels to NO signalling in terms of melatonin effects. Membrane depolarisation reduction and the subsequent absence of increased muscle tone caused by the addition of melatonin results in the reduced possibility of voltage operated Ca\(^{2+}\) channels opening. This incomplete prevention of inhibitory nerve activity results in small amounts of NO being released onto muscle layers to cause relaxation (Fida et al., 1997). NO has been shown to be involved in maintaining a degree of muscle relaxation between motility complexes in mice (Spencer et al., 2013). In vivo, melatonin has also been found to attenuate sympathetic tone by direct activation of melatonin receptors (Poeggeler et al., 1993) and increasing NO availability in the murine colon (Heredia et al., 2009)
4.6.2 Lack of melatonin effects in the distal colon

There is no statistically significant reduction in amplitude in the distal region following the addition of melatonin in the current experiments. Other studies have shown melatonin leads to a reduction in colonic transit time of faecal pellets in the distal region, which aids colonic motility (Diss et al., 2013). This might suggest that melatonin is affecting a different motility tone, such as peristalsis, which is more prevalent within the distal colon.

It the traces obtained, it is quite clear that CMMCs are more predominant within the proximal region, with less activity recorded within the distal region. These results agree with what is seen in rat colons (Bubenik, 1986; Harlow and Weekley, 1986). General absence or decreased abundance of CMMCs could account for the lack of melatonin action in the distal colon. Previous studies have also shown that the CMMC period in the proximal colon to be considerably longer than in the mid or distal (Fida et al., 1997).

4.6.3 Role of melatonin receptors in regulating CMMCs

To establish whether the effects of melatonin mediated directly through their specific receptors, 4-P-PDOT and luzindole were added to the organ baths. The non-selective MT$_{1/2}$ receptor antagonist, luzindole, reduced CMMC amplitude relative to melatonin in the proximal colon, as shown in figure 2. The data suggests that melatonin effects on CMMCs are influenced by MT$_{1/2}$ receptors pathways, but not directly as expected. Blocking the receptors should have reversed the melatonin effect, but instead the response has been amplified.

It may suggest that there is another action of the melatonin receptors, which is allosteric or dependent upon the melatonin concentration. And that these pathways are prone to desensitisation.

It is also possible that the MT$_1$ receptors and MT$_2$ receptors have different roles. When selective MT$_2$ receptor antagonist 4-P-PDOT is added, there is an absence of significant reduction in amplitude of response compared to melatonin treatment.
Previous experiments in rabbit intestinal smooth muscle have found melatonin-induced contraction to be blocked by luzindole but not by 4-P-PDOT (Ahmed et al., 2013), proving the role of MT\textsubscript{1} receptors in regulating motility. Activation of downstream receptors causes stimulation of inositol phosphate hydrolysis and increases in cytosolic Ca\textsuperscript{2+} and elicits muscle contraction (Ahmed et al., 2013).

4.6.4 Influence of melatonin on propagation velocity

A decrease in velocity of propagation was observed upon the addition of luzindole in comparison to 4-P-PDOT. Suppression of velocity and therefore CMMC propagation was effected. This might be due to the fact that melatonin affects the time taken for muscle tone to revert to baseline level, as seen from the slight increase in duration of CMMC response. This is corroborated by other past experiments, which have also displayed melatonin to cause a variable net velocity decrease, essentially indicating that complexes took longer to migrate over a specified distance (Spencer et al., 2013). The velocity of CMMC propagation has been seldom studied in the past, apart from studies into their frequency, which seems to suggest it is dependent on the amount of luminal content present (Barnes et al., 2014) and that the mucosa is involved in tuning of the colonic motility (Pozo et al., 2010; Martin-Cano et al., 2013).

4.6.5 Role of melatonin in fecal pellet propulsion

In current investigations of the effects of melatonin on colonic motility, it suggests that melatonin is a pro-kinetic agent in the mouse colon. Additionally, it appears that melatonin receptors are involved in such regulation.

There have been numerous studies into the antagonistic role of melatonin with respect to serotonin, and shows that it inhibits mobility in general in the GI tract (Bubenik et al., 1994; Kojima et al., 2011; Gershon, 2004). Serotonin has long been recognized as a contractile/excitation modulator of colonic motility (Gershon, 2004; Gershon, 1991), whereas the role of melatonin has been less clear (Thor et
al., 2007, Bubenik et al., 2008). But in the mouse colon, at least, in our current experiments, melatonin has been shown to promote motility both in conjunction with serotonin and EFS stimulation (previous chapter).

Besides promoting the rate of transit in the colon, melatonin treatment also appears to alter the tone of the transit from stepwise movement into a smoother transit throughout the colon. The involvement of melatonin receptors is evident but its interaction with serotonin cannot be directly determined in this setup. Nonetheless, similar findings in goldfish have been described before where increasing concentrations of melatonin affected the contractility of the GI tract (Velarde et al., 2010).

What we have shown here is that melatonin is able to reduce colonic transit times. This is building on top of previous knowledge that melatonin promotes motility in aged animals, while at the same time confirming that this effect is not limited to CMMCs (Martin-Cano et al., 2013). Both MT₁ and MT₂ receptors might be involved in this effect as luzindole increased transit times seen in the colon preparations.

Melatonin has been shown to accelerate intestinal transit in rabbit smooth muscle cells (Drago et al., 2002). There have been no other direct studies, besides into CMMC, into the effects of melatonin on murine colon motility. However, previous studies into interactions between melatonin and serotonin have shown that daily melatonin supplementation in mice would reduce the levels of serotonin produced and increase transit time (Bertrand et al., 2010). This leads us to believe that melatonin must be acting through other pathways in this instance where it is promoting contractile activity. This brings to mind previously discussed mechanisms involving ACh nicotinic receptor pathways, Ca²⁺ induced K⁺ channels, and NO signalling (Ahmed et al., 2013; Barajas-López et al., 1996; Storr et al., 2000). In humans, melatonin does improve physical symptoms of patients with IBS induced constipation (Lu et al., 2005), but unexpectedly it increased colonic transit time (Lu et al., 2009).

Alternatively there might be an explanation in the different ways melatonin is administered in different studies. Orally given melatonin in both mice (Bertrand et al., 2010) and humans (Lu et al., 2009) might not be indicative of the actual functional concentration of melatonin when it reaches the area of interest. In mice,
at least, it has been shown that orally administered melatonin lead to a decrease in colonic levels of serotonin and melatonin (Bertrand et al., 2010). The current experiments, however, skips pass the problem of administration and focuses on the effects of a localized, direct rise in exogenous melatonin levels. If melatonin were to be utilised as a viable treatment option for various GI motility disorders in the future, a more targeted method of delivering it to the affected tissues must be devised.

4.6.6 Mechanisms of melatonin action

As explained previously, both the MT₁ and MT₂ receptors appear to be involved in colonic transit time, and especially the MT₁ receptor in regulating CMMCs. Does this agree with what we know in other animal models?

In the guinea pig, melatonin is shown to increase contractile activity in the proximal colon, in a similar manner to what is demonstrated here. The mechanism did not involve any of the serotonin HT₁/₂/₃/₄ receptors, while suggesting a role for the MT₂ receptor (Lucchelli et al., 1997). This agrees with our findings here that melatonin need not always be an antagonist of serotonin and that it did not modify serotonin-induced contractions in the colon.

In fact, melatonin might be helping to boost the effect of serotonin-induced contractile effect in other ways. In a recent study on human colon epithelial cells, it has been shown that melatonin has an inhibitory effect on SERT (Matheus et al., 2010). Melatonin was shown to modulate SERT activity via its catalytic effect on the allosteric citalopram-sensitive site in SERT. This results in an increase in serotonin availability in the colon. The inhibition of SERT from an allosteric site has also been previously studied with respect to serotonin desensitisation from long-term exposure (Iceta et al., 2008). The melatonin promotion of serotonin effects seen here may be partly mediated by similar mechanisms.
4.7 Conclusion

In summary, these experiments have demonstrated that melatonin has 2 separate, seemingly antagonistic, effects on CMMCs. It decreases the magnitude of spontaneous CMMC responses in the proximal colon, decreasing the velocity of propagation of CMMC waves down the length of the colon. However, blocking melatonin receptors lead to a suppression of CMMCs completely. This suggests that the effect of melatonin is dependent on the concentration; at lower endogenous levels, it promotes CMMCs while extra supplementation leads to desensitisation and the opposite effect.

In pellet distention induced peristalsis, melatonin has been shown to boost colonic transit times, promoting motility. Melatonin receptors are shown to be involved in both processes, with a distinct role for MT₂ receptors in the promotional role on CMMCs.
Conclusion

5 Initial aims

In summary, there is a need for effective direct localisation of melatonin as well as investigation into its relative levels in various regions of the lower gastrointestinal tract. It is also important to prove definitively the link between EC cells and melatonin synthesis.

One of the most important roles of melatonin in the gastrointestinal tract is based on its effects on colonic motility; its antagonistic actions to serotonin in the physiological environment as well as alleviations of motility diseases symptoms. This study aims to elucidate the mechanisms with which melatonin exerts its effects and its interactions with other neurotransmitters in the colon, including serotonin, acetylcholine, tachykinins, ATP and nitric oxide.

5.1 Novel contributions and findings

Melatonin is in the mucosa of the colon, which has not been shown in the mouse before, and also that it is most likely present in EC cells due to close proximity of the melatonin. Melatonin presence was not always correlated to the location of EC cells, but rather the enzymes that synthesise melatonin are found to in the vicinity of EC cells in the mucosal layer. Furthermore, there are more EC cells in regions where there are higher levels of melatonin.

Within the colonic tissue itself, the highest melatonin levels were detected in the subepithelial and epithelial layers of the both the proximal and distal colon. There was also significant immunolabelling in the muscular layer. Secondly, there is more melatonin present in the proximal compared to the distal colon, confirming what was previously found in binding column studies using melatonin receptors. Just relying on melatonin receptor studies is insufficient for the complete functional picture. There was no correlation between expression and food intake stimulus of the melatonin receptors. Hence, the changes in the function in the gastrointestinal
tract in response to food intake were most likely mediated by changes in melatonin level alone not receptor numbers. Hence, an examination of the localisation of melatonin receptors can provide a good idea of melatonin localisation based on its effects, but not a certain indication of its levels at any given point in time.

The novel angle presented here is a direct measurement of melatonin levels. Melatonin immunohistochemistry in the gut has been done in the past. In general, however, melatonin has not been clearly and directly visualized nor located in the mouse colon. The older studies are from rats, or isolated cell cultures of the human gut mucosa. And even when mice were the subjects of investigation, it is the small intestine (duodenum and ileum). There can be variations in animal species used and hence the amount labelled.

It also provides additional proof for an important hypothesis based on older studies. Melatonin was first localised to the mucosal layer of the gastrointestinal tract, then the EC cells were found in same mucosal layer as the melatonin. This, however, does not prove a definite causal relationship between EC cells and the synthesis of melatonin, despite providing strong hints for the direction of further investigation. Here in this study, significant location overlap (in the mucosal epithelium) of the synthesis enzymes and EC cells is presented using direct IHC.

Recent studies from 2008-2012 have shown that both of the suspected crucial enzymes involved in producing melatonin were expressed in the intestinal wall. The current investigation is building on top of this knowledge. Using the comparison of the relative location of melatonin and its synthesis enzymes, with that of serotonin, allow for a definitive identification of EC cells and inferences to be made regarding the generally assumed role that melatonin plays in EC cell signalling. This has not been done before in immunohistochemistry.

From the bioassay studies, it is discovered that melatonin has a net positive effect on contractile activity, triggered by both serotonin dosage and EFS. This is building on top of past observational knowledge about the dual effects of melatonin on serotonin. The current investigation shows that melatonin had a significant effect on serotonin signalling in the distal but not the proximal colon. This is in keeping with the function of fluids absorption across the colon, rendering
more solid stools as water content decreases, and conceivably, requiring more vigorous contractions to propagate colonic content distally.

Previous studies have not made a deeper investigation into the mechanism of melatonin’s activity in the presence of melatonin and external stimuli (EFS). They have reported the net effect but this study has looked into the separate components of peristaltic activity. Does melatonin actually promote contraction or does it inhibit relaxation?

Melatonin treatment caused an increase in serotonin-induced contraction in the distal colon, where the effects appear to be mainly mediated through the promotion of excitatory tachykinin signalling pathways to make the contractile response more pronounced. Various studies in the gastrointestinal tract have demonstrated that tachykinins has a regulatory effect on the excitatory signalling pathway, but not its interaction and effects with melatonin in the context of serotonin signalling.

The role of melatonin on influencing the acetylcholine and tachykinins signalling in the context of motility in the murine gastrointestinal tract has not been previously investigated. From the study with antagonists, it can be concluded that melatonin treatment caused an increase in EFS-induced contraction in the proximal colon, where the effects appear to be mainly mediated through the up-regulation of the ACh contractile response.

The melatonin effect on the acetylcholine component seems to have a larger contribution to this, compared to its effects on tachykinin signalling. This agrees with previous research that colonic peristalsis in mice is mediated mainly through acetylcholine neurotransmission and is additionally regulated by endogenous tachykinins. Now the results prove this is true for EFS-induced motility too.

Additionally, these effects are mediated by neuronal signalling pathways and not by the underlying musculature. It might be easy to assume that all its effects are based on interactions with excitatory and inhibitory neurotransmitters. However, it is important to check if melatonin has any effect on the myocyte itself. And the current TTX study results confirm that this is not the case, and melatonin is indeed affecting neurotransmitter pathways instead.
Current experiment results show that melatonin decreased the amplitude of CMMCs within the proximal colon. It also appears to increase the duration of the response in both proximal and distal colon. Luzindole treatment led to a significant diminishment of amplitude in the proximal colon in comparison to melatonin treatment. All these seem to suggest that melatonin has a concentration dependent effect on CMMCs. When its influence is removed completely, CMMC propagation suffers. So melatonin has pro-kinetic properties. But when presented in higher levels, in the proximal colon, it has an inhibitory effect on CMMCs. Or alternatively, there is a contribution of influence on CMMCs by melatonin antagonists.

On the other hand, melatonin speeds up colonic transit time of artificial fecal pellets, and changes the motility profile from a stepwise movement to a smooth transition. This is building on previously observed effects about the duality of melatonin effects on motility, and determines the effects in various motility tones.

In conclusion, melatonin has a mostly pro-kinetic effect on colon motility in the mouse, affecting both spontaneous and induced tones of motility.

5.2 Limitations and Future work

Melatonin is always going to be a freely diffusible molecule that is hard to localise at its origin. It is not stored like serotonin. The best that can be done is to improve the likelihood that the inference is representative of the true situation.

So for now, all that can be said is that the enzymes to produce melatonin are most likely found in the EC cells, and that is already better proof than was previously available to say that melatonin is definitively produced in EC cells.

There is also an issue in antibody availability leading to ICH slides not being co-localisations, only serial sections for comparisons. Alignment might not be the best due to the size of structures within the cytoplasm and the constraints of the thickness of the sections.
In addition, in bioassay experiments, not an exhaustive list of excitatory and inhibitory pathways was blocked with antagonists. In this study, we used NK₁ and NK₂ receptor antagonists for tachykinins, but not NK₃. When investigating the effects of inhibitory signalling, the effects of vasoactive intestinal peptide (VIP) were also ignored. Nicotinic receptors for acetylcholine can also be found on neurons in the colon too, but not blocked by any specific antagonists. This leads to a residual relaxation effect in some experimental setups even in the presence of L-NNA and MRS2500.

In the context of the colonic transit time experiments, the involvement of melatonin receptors is evident but its interaction with serotonin or any other neurotransmitters cannot be directly determined in this setup.

Nonetheless, what is done is based on the best available knowledge on motility and the significance of various neuro-signalling pathways involved in the murine colon. For future work, freed from the limitation of time, most of these limitations could be overcome.

In a way, all the research into melatonin function up to the present day had presented each new biological target of melatonin with a specific role in the wider signalling pathway map. Melatonin has widespread and interlinked effects on cells as well as other neuro-signalling molecules. Due to its properties melatonin could be considered as a preventative or possible treatment of digestive disorders such as IBS, childhood colic, colorectal cancer, gastric ulcer and ulcerative colitis. Melatonin treatment increased the positive excitatory role of the mucosa in the proximal colon of aged animals; it increased the progression of the contractile wave. This is in agreement with what was observed, and the knowledge garnered here will add to the understanding of how this mechanism works and the design of derivative drugs.
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