The Role of Enterochromaffin Cells in Gut Motility and their Interactions with Extrinsic Sensory Nerves

by

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Thesis
Submitted to Flinders University
for the degree of

Doctor of Philosophy (PhD)

College of Medicine and Public Health

30th March, 2022

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Nomenclature

5-HT	5-Hydroxytryptamine, serotonin
5-HTP	5-hydroxytryptophan
5-HTR	5-HT/serotonin receptor
AAAD	aromatic-L-amino-acid decarboxylase
Abx	antibiotic
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
bHLH	basic helix-loop-helix
BMP	Bone morphogenic protein
Ca^{2+}	Calcium
CCK	Cholecystokinin
CGRP	Calcitonin gene-related peptide
CMMC	Colonic migrating motor complex
CNS	Central nervous system
DE	Differential expression
DRG	Dorsal root ganglion
DTA	Diptheria toxin
EC cell	Enterochromaffin cell
EEC	Enteroendocrine cell
ELISA	Eenzyme-linked immunosorbent assay
ENS	Enteric nervous system
Fz	Frizzled
Gap43	Growth associated protein 43
GAST	Gastrin
GF	Germ-free
GI	Gastrointestinal

GHRL	Ghrelin
GIP	Gastric inhibitory polypeptide
GLP-1/2	Glucagon-like peptide-1/2
GO	Gene ontology
HFD	High fat diet
IBS	Irritable bowel syndrome
ICC	Interstitial cells of Cajal
IPAN	Intrinsic primary afferent neuron
ISX-9	Isoxazole9
KO	Knockout
Leu-enk	leucine-enkephalin
$LGR5^+$	leucine-rich-repeat-containing G-protein-coupled receptor5
Met-enk	methionine-enkephalin
n	Number of biological replicates
N	Number of technical replicates
Na^+	Sodium
NGF	Nerve growth factor
Ngn3	Neurogenin-3
NK	Neurokinin
NPY	Neuropeptide Gamma
ns	not significant
NTS	Neurotensin
PACAP	pituitary adenylate cyclase activating peptide
PFA	paraformaldehyde
PNS	Peripheral nervous system
PYY	Peptide YY
rabG	rabies G glycoprotein
Rfx6	Regulatory factor X6

rpm	rotations per minutes
SCFA	Short chain fatty acid
scRNAseq	single cell RNA sequencing
SCT	Secretin
s.c	subcutaneous
SERT	Serotonin reuptake transporter
SEM	Standard error of the mean
Sema	Semaphorin
SFRP1	Secreted frizzled-related protein 1
SGLT1	Sodium/glucose transporter 1
siRNA	small interfering RNA
SMC	Smooth muscle cell
SSRI	Selective serotonin reuptake inhibitor
TA	Transit amplifying
TBS	Tris-buffered saline
TK	Tachykinin
Tph1/2	Tryptophan hydroxylase isoform 1/2
$Tph1/2^{-/-}$	Tph1/2 knockout mouse
Tph1 DTA	$Tph1^{CreERT2/+}$;$Rosa26^{DTA/+}$
Tx	Tamoxifen
UTP	Uridine triphosphate
VIP	Vasoactive intestinal peptide
VGLUT1	Vesicular glutamate transporter 1 protein
VMAT1/2	Vesicle monoamine transporter 1/2

Thesis Summary

Enterochromaffin (EC) cells are a specialised enteroendocrine cell (EEC) type scattered throughout the gastrointestinal (GI) epithelium, responsible for the production of 90-95% of the body's serotonin. Serotonin is a pleiotropic bioamine, with a wide range of functions throughout the body, both centrally and peripherally. Serotonin is important in the sleep-wake cycle, control of mood, anxiety and depression, GI motility, platelet aggregation, bone density regulation, and throughout many aspects of metabolism.

Within the GI tract, serotonin has long been known to play a role in GI motility, although the importance of serotonin in gut motility has been argued within the field for years. Initial evidence suggested that serotonin was essential in driving gut motility, however more recent studies demonstrate that GI motility still occurs in mice lacking EC cell serotonin *ex vivo*. EC cell knockout mice were created, however, come with complications, including developmental changes in GI tract morphology. As such, the field has been lacking an adequate conditional knockout model to conclusively define the importance of EC cell-derived serotonin in GI motility.

EC cells are important luminal sensory cells that can detect mechanical stimuli within the GI tract, a function of EC cells which has long been known, however the mechanisms involved are not well understood. The most recent evidence suggests the newly identified mechanosensory ion channel, Piezo2, is important in mouse mechanobiology, however this has not yet been characterized in human.

To facilitate the sensory functions of EECs, including EC cells, it has recently been demonstrated that EECs form functional synaptic-like connections with enteric neurons. These connections are thought to be facilitated by basal projections, termed neuropods, although neither the connections, nor neuropods, have been investigated of EC cells as yet.

The aims of this work were to conclusively define whether EC cell serotonin is essential in gut motility, and to investigate the mechanosensing capabilities of both mouse and human EC cells and whether EC cell mechanotransduction is facilitated through the mechanosensitive ion channel, Piezo2. This work also aimed to determine whether colonic EC cells make direct contact with sensory nerves in the intestinal wall, and contain basal projections, termed neuropods. Finally, this work aimed to investigate

the role for extrinsic afferents in driving the formation of EC cell neuropods in mouse colon.

This study utilized a specific, conditional knockdown mouse model approach, in which EC cells were specifically ablated post-development. As such, we have conclusively demonstrated that EC cell serotonin is not essential, but modulatory, in GI motility both *ex vivo* and *in vivo*. Further, we have provided significant evidence for the importance of Piezo2, the mechanosensing ion channel, in mouse and human EC cell mechanobiology. We provide novel evidence that cell Piezo2 is important in GI motility and demonstrate the novel finding that EC cell mechanosensitivity is lost with age in the human colon. We also characterize EC cell neuropods and establish for the first time the connection between EC cells and sensory innervating nerves to the mouse colon, also providing preliminary evidence for human EC cell neuropods and neuronal connections. As such, these findings increase the validity of work completed in mice for human applications and progress our understanding of the EEC-neuronal connections thought to underpin the gut-brain axis. Furthermore, we find that the removal of extrinsic spinal afferents to the mouse colon *in vivo* significantly reduces EC cell neuropod number and length, as well as EC cell numbers within a short timeframe, which replenish over time. As such we identify 13 protein:receptors pairs of interest from the bioinformatic analysis of genes coded for by CGRP nerve fibres and EC cells respectively.

Overall, this study has demonstrated the role for EC cell mechanosensitivity and serotonin release in GI motility, and the connections EC cells form with sensory afferents which might underpin the gut-brain axis, all of which has significant implications in regulating human health.

Publications throughout time of Candidature:

The following publications have arisen from work associated with this thesis:

1. **Jones, L. A**., Sun, E. W., Martin, A. M. & Keating, D. J. 2020. The ever-changing roles of serotonin. *Int J Biochem Cell Biol***,** 105776.

2. Wei, L., Singh, R., Ha, S. E., Martin, A. M., **Jones, L.**, Jin, B., et al.Ro, S. 2021. Serotonin deficiency is associated with delayed gastric emptying. *Gastroenterology*.

3. **Jones, L. A.**, Jin, B., Martin, A. M., Wei, L., et al., Ro, S., Keating, D. J. Under review. Age-dependent decline in human enterochromaffin cell mechanosensitivity and Piezo2 channel expression slows *in vivo* gut transit. *Gastroenterology*.

4. Martin, A. M., **Jones, L. A.**, Wei, L., Sanders, K., Spencer, N. J., Ro, S., Keating, D. J. Under review. Distinguishing the contributions of neuronal and mucosal serotonin in the regulation of colonic motility. *Neurogastroenterology & Motility.*

The following publications have arisen from work unrelated to this thesis:

1. Martin, A. M., **Jones, L. A**., Jessup, C. F., Sun, E. W. & Keating, D. J. 2020. Diet differentially regulates enterochromaffin cell serotonin content, density and nutrient sensitivity in the mouse small and large intestine. *Neurogastroenterology & Motility,* 32**,** e13869.

Declaration

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

Lauren Ashleigh Jones

2/10/2021

Acknowledgements:

I would like to thank my supervisors, Damien, Claire, and Nick for their induvial contributions to my candidature and this thesis. Damien has provided a supportive lab environment in which I have had the chance to develop a vast breadth of skills in all aspects of research. Damien has been a brilliant primary supervisor, providing invaluable guidance, knowledge, and above all he has been a wonderful source of inspiration. Claire has provided me with endless moral support, without whom I may not have completed this thesis. I am eternally grateful for all that she has done for me. Nick has been a useful resource, helpful in the development of many of my experimental studies and provided me with access to essential equipment.

I'd like to give a big shout out to Alyce, Emily, Mandy, Clare, Oahn and Dan, among other lab members and students that I have shared pipettes, chats and offices with throughout my time in the Keating lab. Alyce in particular has been the best office mate, travelling buddy, partner in crime, friend, and sounding board that I could have asked for - at many times she has been my rock and I am eternally grateful that our paths have crossed in the ways that they have.

I must also mention Lauren, Rochelle, Adam, Mel, and Lee, who have all provided numerous laughs, technical support, and comradery along the Level 6 corridor.

Various other people at Flinders have shown support throughout my candidature of which I would also like to thank, including Karin, Rainer, Dusan, Briony, and Pat. I'd like to particularly pay mention to Pat, who has taught me almost all that I know around tissue fixation and preparation, cryostat cutting (she's a wizard) and immunohistochemical techniques. She has also provided unwavering support, stimulating conversation and chocolate treats throughout my candidature, and as such I would consider her my (science) fairy godmother.

I must give a massive thanks to all of my friends in the 'Reno Lab' at the University of Nevada, including Jin, Brian 'obiwan', Rajan, Hannah, Sandra, See-eun, Professor Seungil Ro, and most significantly Lai Wei, who were all incredibly welcoming, supportive and hospitable to me during both of my visits. I hope to work with you all again one day.

I would like to acknowledge the contribution of the Australian Government Research Training Program Scholarship, the Department for Water and Agriculture, the Centre

for Neuroscience, and various other small grants that have made much of this work possible.

A massive thanks to all the Colorectal Surgeons, and to the patients who kindly consented to the collection of their tissue for use in the subsequent chapters, without whom important scientific and medical advances would fail to occur. You are highly important and should always be acknowledged. Similarly, I must acknowledge the animal house staff, and all of the mice sacrificed, without whom medical research in its current form would not be achievable.

I must thank my family, Mum, Dad, Ryan, Courtney, Tim, Chris, Leanne, and Grandma, who all love me and inspire me every day. I know that you will all share in this joy with me and breathe a massive sigh of relief that I've finally finished. Also, thanks for my 'science brain' mum and dad!

All friends, but particularly Tess and Lewis, who have been the most supportive best friends a person could possibly ask for - they've fed me, they've entertained me, they've been an endless shoulder to cry on. I love them and I couldn't have done it without them.

Finally, I would like to individually and personally thank Clare Peterson, the sole human being responsible for my sanity and the subsequent completion of this thesis. Without whom I would be nowhere. Thank you for sticking by me through the good times and the bad, for always celebrating my achievements, and for tirelessly reminding me of how far I have come. I adore you Clare and am eternally grateful for all that you are.

Chapter 1: Literature Review

1.1 Intestinal Epithelium

1.1.1 The function of the intestinal epithelium

The gut lining (or intestinal epithelium) is the single layer of cells that separate the contents of the intestinal lumen from the underlying tissue. The cells making up this barrier are replaced every 4-5 days, representing the highest turnover rate of any adult mammal tissue (Heath, 1996). These cells can be divided into five categories, including the pluripotent stem cells, paneth cells, goblet cells, enterocytes, and enteroendocrine cells (EECs). Each of these cell types have separate functions, including but not limited to absorption, digestion, and transport of electrolytes and water (Guzman et al., 2013).

The intestines are anatomically divided into different regions, the small intestine, made up of the duodenum, jejunum, and the ileum, and the large intestine, typically termed the colon, which can be divided into the ascending, transverse, descending, and sigmoid colon. The small intestinal lining is defined by large villi and crypts, which provide a significant surface area for absorption (De Santa Barbara et al., 2003). The principal function of the small intestine is digestion, and the subsequent absorption of nutrients (De Santa Barbara et al., 2003). Whereas the colon only contains crypts with the rest of the epithelial lining remaining as a flat surface (van der Flier and Clevers, 2009). The primary function of the colonic epithelium is to absorb water and salt (De Santa Barbara et al., 2003). The colon hosts the majority of the gut microbiome, the bacteria living inside the gut used to digest food and aid in host nutrient metabolism (Jandhyala et al., 2015).

1.1.2 Identification of epithelial stem cells

More than 30 years ago it was hypothesised that there must reside a stem cell population present at the base of intestinal crypts, however this population had long evaded scientists. A cycling cell placed at the very base of the crypts adjacent to the Paneth cells, were termed crypt base columnar cells at the time by Leblond and Cheng (Cheng, 1974). These cells are now known as $LGR5^+$ stem cells, as Clevers distinguished in 2007 (Barker et al., 2007a). These $LGR5^+$ cells fulfill the stem cell requirements, including that they are long lived, and have the ability of differentiating into any of the cell types found within the epithelium (Barker et al., 2007a). With between 4 and 6 stem cells per crypt (Barker et al., 2007a, Bjerknes and Cheng, 1999), these cells are continually cycling along the intestine, with some remaining quiescent in the colon (Barker et al., 2007a).

LGR5 stands for 'leucine-rich-repeat-containing G-protein-coupled receptor 5', although it has also previously been termed 'Gpr49' (Barker et al., 2007a). This gene encodes an orphan G-protein-coupled receptor that is characterised by a large leucine-rich extracellular domain (Hsu et al., 1998). However, the function of Lgr5 within physiology is still currently not known, particularly as its ligand remains elusive, although it is hypothesised to be involved in the Wnt signaling pathway (Barker et al., 2007a). $Lgr5^+$ stem cells cycle steadily to form transit-amplifying (TA) cells, which last approximately two days in the crypt, then divide multiple times before terminally differentiating into all lineages of the epithelium (Flier and Clevers, 2009). This process is aided through Wnt signaling, a highly conserved signaling system involved in cellular proliferation and differentiation (Mah et al., 2016). Other signaling involved, particularly further up in the crypts, include R-Spondin, EGF and Notch signaling (Spit et al., 2018), all essential growth signals required for the continuous self-renewing of the intestinal

epithelium. The different lineages include enterocytes, goblet cells, and EECs, which differentiate at the crypt-villus junction (see Figure 1.1) (Barker et al., 2008). These cells migrate up the villi in bands, with each villus receiving cells from various neighbouring crypts. The Paneth cell, however, remains at the base of the crypt (Barker et al., 2008). (Barker et al., 2007a)The high turnover from the proliferation and differentiation of these stem cells is offset by apoptosis of cells occurring at the tips of villi. Epithelial cells then typically traverse to the top of the villus in around three days, where the cells undergo spontaneous apoptosis, the cells are then shed from the villus into the intestinal lumen (Hall et al., 1994).

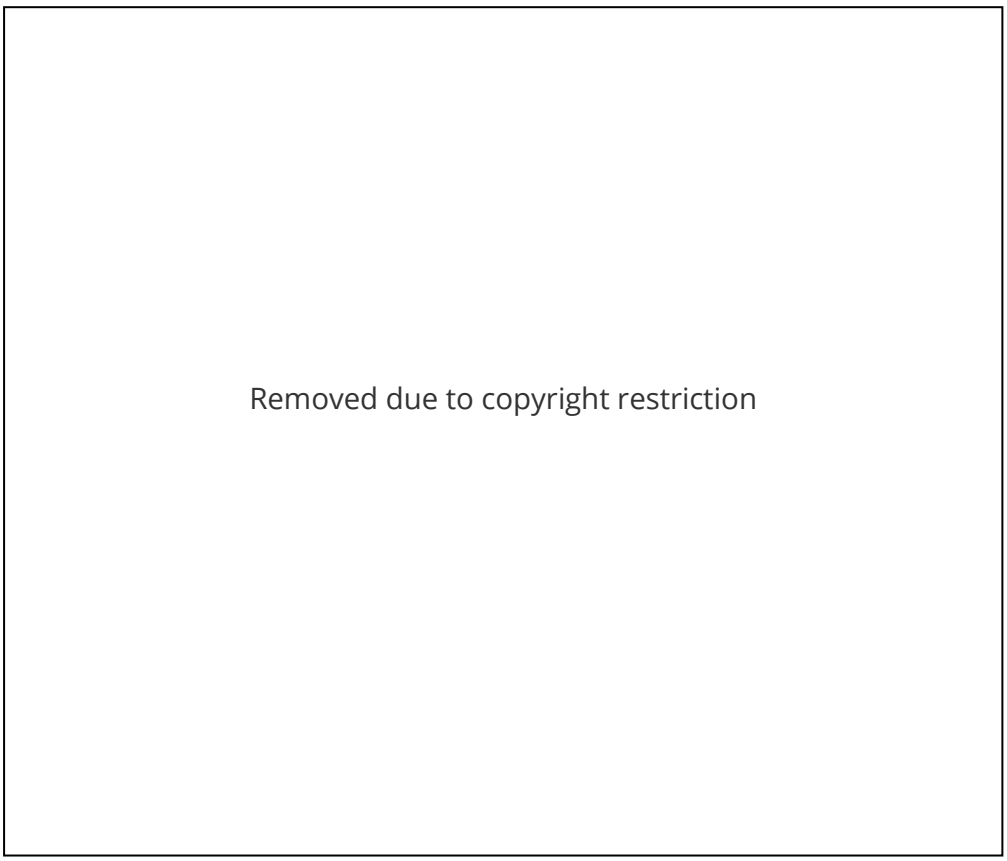

Figure 1.1: The differentiation of intestinal stem cells.

A schematic of the differentiation of intestinal stem cells, including an example cross-section of an intestinal crypt and villi, in which the proliferation and differentiation of epithelial cells are outlined. The trajectory from stem cell to transit amplifying (TA) cell, to the final differentiation of Paneth, Goblet, Enteroendocrine cell and Enterocyte. Adapted from (Ricci-Vitiani et al., 2008).

1.1.3 Gut epithelial cells

Goblet cells get their name from their morphology, pertaining to their goblet like shape. Goblet cells secrete protective mucins which form the 200 μm thick layer of mucous that lubricates and protects the walls of the intestine (Johansson et al., 2014), also involved in the movement and expulsion of gut contents, as well as the protection from dangerous contents such as chemical injury (Flier and Clevers, 2009). The proportion of goblet cells represents 5% of the small intestinal epithelial cells (Karam, 1999).

Paneth cells are unique from the rest of the differentiated cells of the intestinal epithelium, found only in the small intestine, and lasting approximately three weeks, far longer than any other terminally differentiated villus cell (Bjerknes and Cheng, 1981). Paneth cells exist towards the bottom of the crypts, adjacent to the $LGR5^{+}$ stem cells. They are the only epithelial cell type to migrate to the bottom of the crypt, after forming in the middle of the crypt (Bjerknes and Cheng, 1981). Paneth cells are considered part of the innate immune system, containing large secretory granules enclosing antimicrobials, lysozymes, and defensins among other immune factors (De Santa Barbara et al., 2003, Flier and Clevers, 2009). Although most importantly Paneth cells express EGF, Wnt3, Notch-ligand D114 and TGFα, all vital factors for stem cell maintenance and the proliferation and differentiation of gut epithelial cells (Sato et al., 2011).

Enterocytes, often termed columnar cells, are the most abundant cells making up the gut lining, totaling approximately 80% of all GI epithelial cells, and forming the basis of the structural integrity of the epithelium (De Santa Barbara et al., 2003). Enterocyte morphology is described as columnar, with apical microvilli which significantly increases the surface area, making them responsible for absorbing and transporting nutrients out of the lumen and into circulation (Flier and Clevers, 2009). These cells

are also largely involved in limiting the number of antigens reaching the epithelial surface. This is highly important as the luminal contents are filled with antigens, such as broken-down food, resident bacteria, and foreign micro-organisms (Snoeck et al., 2005). This is a difficult task, as the epithelium must be permeable for important factors such as nutrients and macromolecules essential for growth and development, which are of similar size to that of many antigens (Snoeck et al., 2005). Thus, enterocytes form tight junctions between the epithelial cells, which control the paracellular transport of antigens (Snoeck et al., 2005), as well as adopting and processing antigens to then present to immune cells (Snoeck et al., 2005).

1.1.4 Enteroendocrine cells

Enteroendocrine cells (EECs) are the main hormone secreting cells which line the gut, their secreted peptide hormones play a major role in the control of the body's metabolism. These cells are very low in number, making up less than 1% of the gut epithelium (Reimann and Gribble, 2013), and are scattered amongst the vast numbers of other more prominent cell types. This factor can make them hard to study, along with the inability for primary EECs to be maintained for long periods in cell culture. However, much research has gone into understanding the nature of different EECs, including the differences and similarities in the peptides they secrete (Habib et al., 2012, Haber et al., 2017, Adriaenssens et al., 2018, Gehart et al., 2019), and their location and densities along the GI tract (Martin et al., 2017a, Suzuki et al., 2018, Beumer et al., 2018, Gehart et al., 2019).

It is important to note that the literature is adapting to accept an enteroendocrine spectrum model, moving away from the enteroendocrine nomenclature previously established. Originally EECs were classified using a letter code based on their location and main secretory product. EECs were split into 8 specific subclasses, including cells

expressing secretin (SCT), cholecystokinin (CCK), proglucagon (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), somatostatin (SST), neurotensin (NTS), ghrelin (GHRL), and serotonin (5-HT), traditionally termed S, I, L, K, D, N, A and EC cells, respectively (Gribble and Reimann, 2016). However, studies using fluorescent reporter mice (Egerod et al., 2012, Habib et al., 2012) and single-cell transcriptomic data (Gehart et al., 2019) have demonstrated that a single EEC can secrete a multitude of varying hormones. Studies looking at the secreted factors from various EECs along the gut lining have shown that most EECs express a range of hormones (Diwakarla et al., 2017, Habib et al., 2012, Haber et al., 2017). For example, one EEC was found to express SCT, CCK, proglucagon and GHRL, and using this outdated terminology, could be labelled as SILA (Haber et al., 2017). Habib et al (2012) showed using microarray expression profiling comparisons that GLP-1 cells in the proximal small intestine are closer in expression profile with neighbouring GIP cells, than with GLP-1 cells from the colon (Habib et al., 2012). Similarly, it has recently been reported that EECs alter their characteristics, including their signaling and receptor expression profiles, along the crypt-villus axis (Beumer et al., 2018). Together, these recent advances reflect the adaptive enteroendocrine hormonal profile of cells which traverse the crypt-villus axis, supporting a potential for change in the nomenclature of EECs to better reflect the spectrum in which they lie.

EECs are polarised, with each cell containing an apical surface which faces the gut lumen, and a basal surface exposed to the circulating plasma (Noah et al., 2011), giving these cells the ability for sensing on both sides of the epithelial membrane. This polarisation is ideally suited for a sensory cell, as it can sample the luminal composition and respond by releasing hormones into circulation (Reimann and Gribble, 2013). EECs contain G-protein-coupled receptors and nutrient transporters in order to sense the luminal contents (Chimerel et al., 2017).

1.1.5 Enterochromaffin cells

(Ahlman and Nilsson, 2001)Enterochromaffin (EC) cells are the most abundant enteroendocrine cell (EEC), spanning all regions of the digestive tract (Sjölund et al., 1983). EC cells primarily secrete serotonin but have also been found to contain other hormones such as SCT, substance P and CCK (Diwakarla et al., 2017, Haber et al., 2017). EC cells can be activated by an array of stimuli including toxins, inflammatory cues, mechanical stimuli, and the gut microbiota (Bertrand and Bertrand, 2010, Yano et al., 2015, Williams et al., 2016). EC cells respond to such stimulants through the range of varying channels and receptors which they express, including voltage-gated sodium (Na^+) and calcium (Ca^{2+}) channels (Bellono et al., 2017), microbial metabolite sensors (Yano et al., 2015, Reigstad et al., 2014), and mechanosensitive ion channels (Wang et al., 2017, Alcaino et al., 2018). It was recently shown that EC cell depolarisation can induce serotonin release, indicating EC cells are electrically excitable (Bellono et al., 2017). These sensory transduction pathways stimulate Ca^{2+} channels to control serotonin release, primarily via P/Q-type voltage-gated Ca^{2+} channels (Bellono et al., 2017). Although recent evidence has indicated that different Ca^{2+} channels may be responsible for differing forms of stimulated serotonin release (Alcaino et al., 2020), such as T-type Ca^{2+} channels, CaV3.2 also known to be enriched in EC cells (Bellono et al., 2017).

1.1.6 Regulation of EEC differentiation

All intestinal epithelial cell types originate from $Lgr5^+$ crypt stem cells (Barker et al., 2007b), and are differentiated into various cell types including EECs, however our understanding of the factors which control EEC differentiation is incomplete (Piccand et al., 2019, Tsakmaki et al., 2020). The differentiation of varying EEC subtypes is guided through factors such as their location along the GI tract and height within the

crypt-villus axis, both of which dictate their exposure to signalling gradients from the stem cell niche, such as Wnt and bone morphogenic protein (Basak et al., 2017, Beumer et al., 2018). As cells begin to differentiate from stem cells at the bottom of crypts, Notch inhibition followed by Atoh1 expression shifts them into the secretory lineage (Zecchini et al., 2005a, Stanger et al., 2005, Fre et al., 2005). Atoh1 cells are then assigned to the endocrine lineage by expression of the basic helix-loop-helix (bHLH) transcription factor Neurogenin-3 (Ngn3) (Jenny et al., 2002a, Lee et al., 2002). Hence Ngn3 is expressed in all endocrine progenitors which give rise to EECs (Mellitzer et al., 2010a, Gehart et al., 2019). The expression of Ngn3 is then suppressed as cells migrate up the crypt-villus axis and terminally differentiate into distinct EEC subtypes (Piccand et al., 2019). A number of studies have used mouse lineage tracing to identify the regulatory transcriptional factors downstream of Ngn3, important in the regulation of specific subpopulations of EEC differentiation (Beucher et al., 2012, Du et al., 2012, Larsson et al., 1998, Terry et al., 2014, Ye and Kaestner, 2009, Gierl et al., 2006, Naya et al., 1997, Gehart et al., 2019). Transcriptional factors identified for certain EEC subtypes include NeuroD1 (SCT and CCK containing EECs) (Mutoh et al., 1997, Naya et al., 1997), Insm1 (substance P and NTS containing EECs) (Gradwohl et al., 2000), Nkx2.2 (CCK, GAST, GIP and SST containing EECs) (Desai et al., 2008), Pax4 (5-HT, SCT, GIP, PYY and CCK containing EECs) (Beucher et al., 2012, Larsson et al., 1998), Pax6, Foxa1 and Foxa2 (preproglucagon and its products GLP-1 and 2 containing EECs) (Ye and Kaestner, 2009, Larsson et al., 1998), Arx (GLP-1, GIP, CCK, SCT, GAST and GHRL containing EECs) (Beucher et al., 2012, Du et al., 2012), Islet-1 (GLP-1, GIP, CCK containing EECs) (Terry et al., 2014), and Lmx1A (5-HT containing EECs) (Gross et al., 2016).

Several investigations have demonstrated that EC cells branch off as a separate pool from endocrine progenitors to all other EECs earlier in the differentiation process

(Gehart et al., 2019, Piccand et al., 2019). As such, distinct transcriptional factors have been associated with the differentiation of EC cells specifically, including Nkx2-2 (Gross et al., 2016), Lmx1a (Gross et al., 2016), Mnx1, Atf6, Glis3, and Lhx1 (Piccand et al., 2019). Furthermore, the use of a 'Regulatory factor X6' (Rfx6) knockdown mouse demonstrated a role for this gene in Ngn3-positive enteroendocrine progenitors, as a specific reduction in EECs was seen in the intestines of Rfx6 knockdown mice (Piccand et al., 2019). Despite the overall reduction in EECs seen in Rfx6 mice, the majority of EC cell enriched genes were up regulated, including Tph1, ChgA, and Scn3a, as well as transcription factors Lmx1a and Glis3 (Piccand et al., 2019). Other genes specific to EC cell lineage were unaffected, suggesting Rfx6 controls only the expression of specific genes associated with EC lineages (Piccand et al., 2019). Separately, the use of a small molecule isoxazole9 (ISX-9), discovered in a chemical screen for drivers of neuronal differentiation (Schneider et al., 2008), was found to drive the differentiation of EC cells and I cells when applied to intestinal organoids (Tsakmaki et al., 2020). ISX-9 increased the expression of genes correlated with the EC progenitor pool (Rfx6, Fev, Prdm16 and Hmgn3), and decreased genes linked with the general EEC differentiation pool (Isl1, Arx and Cdkn1a) (Gehart et al., 2019). These results further highlighted the early separation of EC cells from other EECs and demonstrated a novel mechanism of ISX-9 to drive EC formation (Tsakmaki et al., 2020).

1.2 Serotonin

1.2.1 Synthesis and source

EC cells play a crucial role in both GI regulation and more broadly in peripheral metabolism and the gut-brain axis. EC cells express a range of gut hormones but are primarily noted for their secretion of serotonin, known to produce 90-95% of the body's peripheral serotonin (Erspamer, 1966). EC cell serotonin release is stimulated through multiple mechanisms, including chemical sensing and mechanical stimuli among others. Serotonin, or 5-Hydroxytryptamine (5-HT) is perhaps more commonly known as a neurotransmitter, with its link to mood, sleep and control of the circadian rhythm within the central nervous system (CNS). All forms of serotonin are sourced from tryptophan, an essential amino acid provided to the body only through diet (Le Floc'h et al., 2011). Tryptophan is absorbed from the gut lumen, where it is made available in the circulation (Fernstrom and Fernstrom, 2006), or to enter EC cells. Tryptophan can then cross the blood brain barrier and participate in serotonin synthesis in the CNS (Ruddick et al., 2006) or, as the majority of serotonin is produced, be processed into serotonin within EC cells lining the gut (Spiller, 2008). Tryptophan is converted into 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (Tph), known as the rate limiting enzyme in serotonin production (see Figure 1.2) (Walther and Bader, 2003). There are two isoforms of the Tph enzyme, Tph1 present in specific non-neuronal cell types (Walther et al., 2003), and Tph2, present in the central and enteric nervous system (Walther et al., 2003). The short lived 5HTP is then converted by aromatic-L-amino-acid decarboxylase (AAAD) to form serotonin (5-HT). Serotonin is synthesized in 2% of nerve cell bodies in the enteric nervous system (ENS) (Costa et al., 1996). Recently, a third enzyme, phenylalanine hydroxylase, has been identified to contribute to serotonin synthesis in the blood (Mordhorst et al., 2021). Phenylalanine hydroxylase

is reported to be responsible for the remaining 10% of serotonin found in mice lacking both Tph1 and Tph2 (Mordhorst et al., 2021).

Tryptophan can also take a different metabolic path, known as the kynurenine pathway, which does not result in the production of serotonin. The kynurenine pathway is actually the dominant physiological pathway for the processing of tryptophan (Schwarcz et al., 2012), making up about 95% of tryptophan metabolism (Tyce, 1990). Kynurenic acid can cross the blood brain barrier, involved in CNS synthesis of certain neuroactive metabolites (Ruddick et al., 2006).

Once synthesized serotonin can be stored in cells within vesicles for release, or taken up by platelets for storage (Humphrey and Jaques, 1954), where it can then participate in various physiological processes throughout the body. The transporter which typically facilitates the uptake of serotonin through the plasma membrane is SERT (Blakely et al., 1991). SERT is expressed by EC cells, other EECs, enterocytes and serotonergic neurons (Martel, 2006). Recently, a SERT-like transporter was identified on certain microbiota with similar sequence and structure to mammalian SERT, allowing certain bacterium to take-up and store serotonin (Fung et al., 2019).

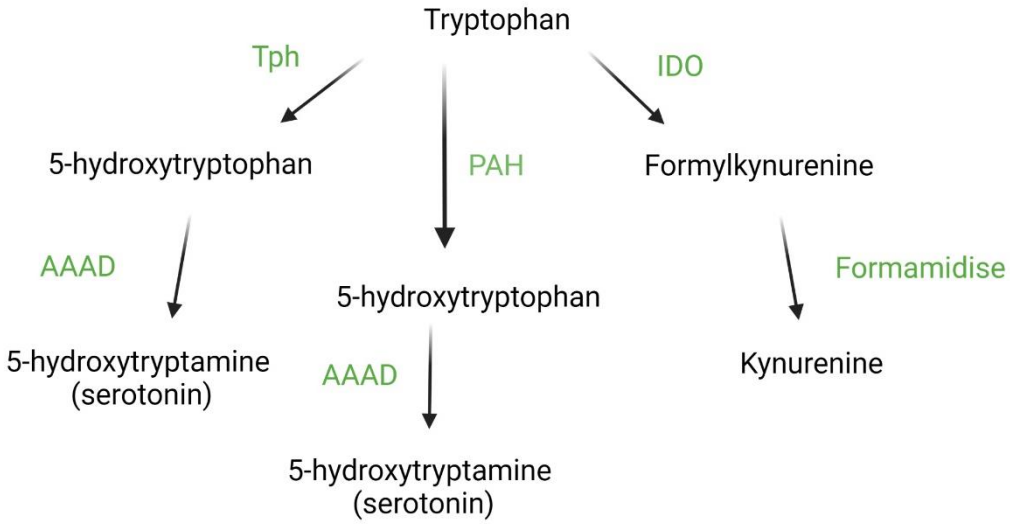

Figure 1.2: The three pathways of tryptophan metabolism.

Adapted from (Olson et al., 1960)

1.2.2 Serotonin receptors

The family of serotonin receptors is comprised of 14 distinct subtypes, grouped into 7 different classes (Nichols and Nichols, 2008), not including receptors generated through alternative splicing of single genes (Hoyer et al., 2002, Nichols and Nichols, 2008). All receptors, except one ligand-gated ion channel (the $5HT_3$ receptor), are G-protein coupled receptors (Nichols and Nichols, 2008). All subtypes can be found in the brain, and 5 receptor subtypes are expressed within the intestine (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, and 5-HT_7) (see Table 1) (Hoyer et al., 2002). The 5-HT_{2B} receptor is vital for life, as mice with the genetic knockout of this gene produce severe embryonic defects and do not survive (Nebigil et al., 2000, Nichols and Nichols, 2008). This is most likely due to the importance of this receptor in development, regulating the formation of essential organs such as the heart and brain (Nebigil et al., 2000, Choi et al., 1997) (Nebigil et al., 2001, Nichols and Nichols, 2008). Separately the 5-HT_4 receptor is linked with the development of the enteric nervous system, involved in neuroprotection within the gut (Liu et al., 2009, Bianco et al., 2016).

The most understood receptor subtypes within the gut are 5-HT_3 and 5-HT_4. 5-HT_4 receptors are found predominantly on presynaptic enteric neurons, while 5-HT_3 receptors are typically localized on sensory and myenteric nerves (Richardson et al., 1985, Johnson and Heinemann, 1995, Mawe et al., 1986). Both receptor subtypes are targeted via various drugs for the treatment of a number of motility disorders, including IBS (Goldberg et al., 1996, Gershon, 2004).

It is through these receptor subtypes that serotonin is known to function and undertake its vast number of roles throughout the body.

Table 1: Expression of serotonin receptor subtypes throughout the body.

Receptor family subtype	Primary Coupling Mechanism	Tissues in which receptor subtypes are expressed
1	$G_{i/o}$-coupled	Enteric Nervous System (ENS), spleen, neonatal kidney (Rosenbaum et al., 2007), and brainstem (Smith et al., 2002, Nichols and Nichols, 2008).
2	$G_{q/11}$ -coupled	ENS, Enterochromaffin (EC) cells, cardiovascular related tissues (Kaumann and Levy, 2006, Nagatomo et al., 2004), Dorsal Root Ganglion (Sommer, 2004, Van Steenwinckel et al., 2008), liver, kidneys, stomach, gut, lung, (Nichols and Nichols, 2008)and brain (Duxon et al., 1997, Choi et al., 1994, Clemett et al., 2000, Pasqualetti et al., 1999, Nichols and Nichols, 2008)
3	Ligand-gated Na^+/K^+ cation channel	ENS (Gershon, 2004), EC cells, peripheral nervous system (PNS), and central nervous system (CNS) (Nichols and Nichols, 2008, Parker et al., 1996, Ohuoha et al., 1994).
4	G_s-coupled	ENS, EC cells, CNS (Nichols and Nichols, 2008)
5	$G_{i/o}$-coupled	Limited to the CNS (Nichols and Nichols, 2008).
6	G_s-coupled	Almost exclusively expressed within the CNS (Nichols and Nichols, 2008).

7	G_s-coupled	ENS, Brain (Bickmeyer et al., 2002, Neumaier et al., 2001), Peripheral blood vessels and colon (Irving et al., 2007).

1.2.3 Roles of serotonin

The majority of work presented in the following section (2.3) is comprised of published work, from the following publications: Lauren A Jones et al (2020) “The ever-changing roles of serotonin.” *International Journal of Biochemistry and Cell Biology, 125*, [105776]. The relative author contributions to this work are as follows: LA Jones, and DJ Keating were involved in planning the content of the manuscript. LA Jones, and DJ Keating reviewed the literature. E Sun and AM Martin provided critical input and advice. LA Jones wrote the majority of the paper with minor contributions from DJ Keating, and all authors were involved in drafting the manuscript.

https://doi.org/10.1016/j.biocel.2020.105776

1.2.3.1 Traditional Roles of serotonin

Central serotonin is heavily implicated in important brain functions such as mood, sleep and appetite regulation (Martin et al., 2017b). Increased synaptic serotonin availability in the CNS is associated with elevated mood and reduced anxiety (Gershon and Tack, 2007), which forms the basis of several classes of widely used anti-depressants that prevent the removal of serotonin from synaptic space by blocking serotonin transporter (SERT). Serotonin was initially thought to drive peristalsis, as serotonin is released from the mucosa concomitantly with peristaltic contractions (Bulbring and Lin, 1958). However, EC cell-derived serotonin is not essential in the establishment of these contractions, as peristalsis is preserved, albeit less frequent, in experimental models that were physically or genetically devoid of EC-derived serotonin (Heredia et al., 2013, Keating and Spencer, 2010). The role for EC cell-derived serotonin in GI motility will be discussed in depth in section 3 of the current chapter. Serotonin has also been known to have a key role in platelet aggregation, and vasoconstriction as it was first identified 70 years ago (Rapport et al., 1948),

however more recently other roles for serotonin in the periphery have been further elucidated, including its role in metabolism.

1.2.3.2 Newly identified functions of peripheral serotonin in metabolic control

The physiological roles of gut-derived serotonin have more recently begun to move away from paracrine effects within the gut. Serotonin was reported to have a role in bone formation; however, these findings have not been supported by the work of other groups (Brommage et al., 2015, Cui et al., 2011, Yadav et al., 2009). A role for serotonin in bone degradation was indicated in mice in whichTph1 inhibitors were both a preventative tool and a treatment in mouse models of osteoporosis (Yadav et al., 2009). However, other groups found no correlation between peripheral serotonin levels and bone mass in mice, with replication of these aforementioned experiments reporting no improvement in bone mass with the use of Tph1 inhibitors or in $Tph1^{-/-}$ mice (Brommage et al., 2015, Cui et al., 2011). Hence leaving the role of serotonin in bone mass unclear and outlining the need for further research.

Increasing evidence now demonstrates a clear role of peripheral serotonin as a hormone capable of modulating peripheral metabolism (Martin et al., 2017b, Young et al., 2015). Similar to the classical fasting hormone glucagon, circulating serotonin is markedly elevated in response to fasting as a result of upregulation of intestinal Tph1 expression. During fasting, serotonin potently promotes lipolysis from adipocytes, which in turn provides substrates for hepatic gluconeogenesis, a process that is also markedly upregulated by serotonin (Sumara et al., 2012). On the other hand, gut-derived serotonin also effectively reduces energy expenditure by suppressing thermogenic processes such as the browning of white adipose tissue (Oh et al., 2015), and brown adipose tissue thermogenesis, through lowering the activation of hormone sensitive lipase, and reducing the expression of uncoupling protein 1 responsible for

thermogenesis (Crane et al., 2015). As such, gut-derived serotonin plays a key role in driving multiple physiological adaptations to nutrient deprivation by increasing short-term energy availability and promoting longer term energy conservation.

Paradoxically, circulating serotonin levels are also increased under obesogenic conditions. Elevated levels of circulating and gut-derived serotonin occur in obese humans (Young et al., 2018) and after a high fat diet in rodents (Le Beyec et al., 2014). Peripheral serotonin modulates glucose stimulated insulin secretion in islets (Zhang et al., 2017), and increased levels of peripheral serotonin from a high-fat diet cause hepatic steatosis through the increase of fat storage in the liver (Choi et al., 2018). Pharmacological or genetic reductions in gut serotonin protect against diet-induced obesity, hepatic steatosis and glucose intolerance (Choi et al., 2018, Crane et al., 2015, Oh et al., 2015, Sumara et al., 2012), illustrating a causative role of elevated gut-derived serotonin in driving metabolic dysfunctions. Both the density of EC cells and the expression of TPH1 are increased in human obesity (Young et al., 2018), however mechanisms driving such changes remain unknown. Recent evidence supports a causative role of gut microbiota in controlling gut serotonin levels and in host metabolism.

1.2.3.3 Intestinal bacteria regulate peripheral serotonin production

A complex microbial ecosystem exists along the length of the gastrointestinal tract (Scarpellini et al., 2015). These commensal bacteria, commonly known as the microbiota, and their genes and gene products (referred to as the gut microbiome), can influence host physiology in multiple ways. Germ-free (GF) mice that lack an endogenous microbiome from birth, or mice treated with antibiotics to deplete gut microbiota, both have substantially lower levels of Tph1 expression, mucosal serotonin content (Reigstad et al., 2014) and reduced numbers of EC cells compared to

conventionally raised mice (Yano et al., 2015). However, how gut microbiota signal to EC cells to regulate their numbers remains unknown.

EECs are known to act as sensory cells that are capable of responding to their local environment including ingested nutrients (Sun et al., 2017, Sun et al., 2018a), microbial metabolites (Lund et al., 2018), immune activation (Findeisen et al., 2019) and some medications (Bahne et al., 2018, Sun et al., 2019). Similarly, EC cells release serotonin in response to increased gut contractile force (Bertrand, 2006, Keating and Spencer, 2010), and ingested nutrients (Martin et al., 2017c, Martin et al., 2017d). EC cells also have the capacity to sense microbial metabolites, as they express short chain fatty acid receptors, Ffar2 and Ffar3 (Akiba et al., 2015, Martin et al., 2017c), toll-like receptors (Wang et al., 2019a) and a number of olfactory receptors (Bellono et al., 2017, Lund et al., 2018). Contrasting to their robust secretory response to high extracellular glucose (Lumsden et al., 2019, Martin et al., 2017d, Young et al., 2018, Zelkas et al., 2015), primary EC cells do not readily release serotonin upon acute exposure to short chain fatty acids, an abundant microbial metabolite (Martin et al., 2017d). Chronic exposure to short chain fatty acids, however, does increase Tph1 expression and serotonin biosynthesis in a human EC cell line (Reigstad et al., 2014) and other serotonin-secreting cell-lines (Yano et al., 2015). As such, it is likely that gut microbiota influences intestinal and circulating serotonin levels by altering the levels of different microbial metabolites within the gut lumen, which subsequently exert a modulatory effect on serotonin biosynthesis in EC cells. Whether true EC cells respond in such a way to microbial metabolites and to which metabolites they respond to remain to be ascertained.

1.2.3.4 Physiological implications of the serotonin-gut microbiome interaction in the gut

As serotonin is an important regulator of gut functions (Gershon and Tack, 2007), reduced intestinal serotonin observed in mice without a microbiota is anticipated to have direct functional consequences. Indeed, GF mice display abnormal ENS neuroanatomy (De Vadder et al., 2018) and altered intestinal motility patterns (Ge et al., 2017, Kashyap et al., 2013), which can be rectified upon microbiota colonization (Kashyap et al., 2013) in a serotonin-dependent manner (Yano et al., 2015). The fact that luminal infusion of the short chain fatty acid butyrate, a microbial metabolite, is able to restore altered gut motility in GF mice, an effect that is blunted in $Tph1^{-/-}$ mice, indicates gut microbiota likely regulates intestinal motility by acting directly on EC cells, rather than on serotonin-producing enteric neurons (Vincent et al., 2018). Nevertheless, intestinal microbiota also plays a crucial role in the development and maintenance of the serotonergic network within the ENS, which in turn, is essential for the development and survival for other enteric neuron populations. Serotonin is largely absent from neurons within the myenteric plexus of GF mice and microbiota recolonization can gradually restore serotonin neurons, which leads to differentiation and maturation of other enteric neurons through $5\text{-}HT_4$ receptors (De Vadder et al., 2018).

Furthermore, altering serotonin levels alters gut microbiota composition with down-stream implications for inflammation in the gut. It is known that pharmacological and genetic suppression of Tph1 in mice reduces the severity of experimental colitis by reducing macrophage infiltration and proinflammatory cytokine levels (Ghia et al., 2009). Faecal matter transfer from $Tph1^{+/-}$ mice into GF $Tph1^{-/-}$ mice increases the severity of pharmacologically induced colitis (Kwon et al., 2019). These findings

demonstrate a novel role of gut-derived serotonin in shaping the composition of gut microbiota in relation to the susceptibility to colitis and identify the serotonin–microbiota axis as a potential therapeutic target in inflammatory bowel disorders. Thus, there exists an interdependent relationship between gut serotonin and gut microbiota, and that this represents a previously undefined pathway that modulates numerous aspects of host physiology.

1.2.3.5 Implications of the serotonin-microbiome interaction beyond the gut

Most recently we (Martin et al., 2019), and others (Fung et al., 2019, Kwon et al., 2019, Mandić et al., 2019), have further demonstrated that the interactions of microbiota with gut serotonin is highly complex and bi-directional, with physiological implications beyond the gastrointestinal tract (Figure 1.3). Long term oral delivery of serotonin in mice alters the diversity of the microbiome and the levels of various families of spore-forming bacteria (Fung et al., 2019), a subset of microbe previously shown to increase host serotonin (Yano et al., 2015). This effect was blocked by the SERT inhibitor, fluoxetine, due to the fact that specific microbiota contain transporters for serotonin that can be inhibited by selective serotonin reuptake inhibitors (SSRI), a class of anti-depressants to which fluoxetine belongs to. Indeed, the authors reported that all four strains of Turicibacter sanguinis, which are closely linked with serotonin levels, expresses a serotonin transporter orthologous to mammalian SERT (Fung et al., 2019). This supports a bi-directional link between gut-derived serotonin and the microbiome and provides a mechanism to explain how host serotonin modulates the levels of only specific bacterial species. Related to this, it was recently reported that the oral delivery of SSRIs, including fluoxetine, signal via the gut-brain axis, mediated by vagus nerve activation, to execute their anti-depressive function (McVey Neufeld et al., 2019). It is known that EECs form direct connections with nerve endings

innervating the gut (Hunne et al., 2019, Kaelberer et al., 2018), and that quantities of serotonin secreted from EC cells are high enough to activate nearby afferent serotonin receptors (Raghupathi et al., 2013). Thus, anti-depressants targeting serotonin levels may have clinical effects through modulating serotonin availability from EC cells.

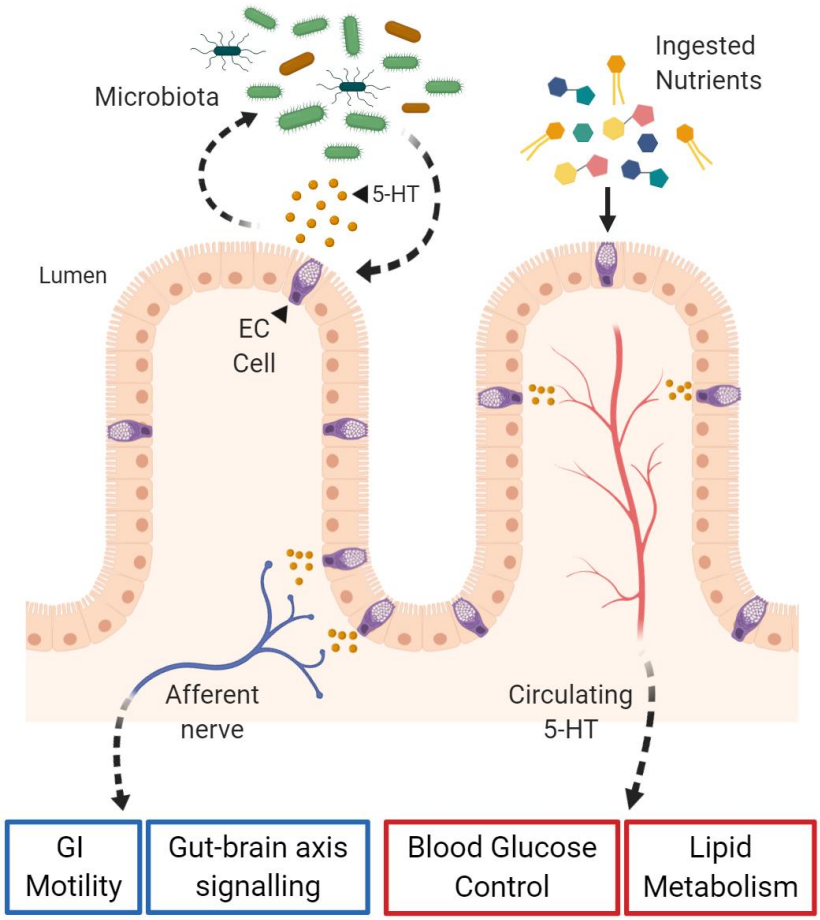

Figure 1.3: EC cells are sensory cells within the gut lumen which affect host physiology through the circulation and afferent pathways. Release of serotonin (5-HT) from EC cells (shown in purple) ignites a bi-directional relationship with gut microbiota, whereby serotonin increases colonisation of specific microbiome, and the microbiome increases the number of EC cells. Serotonin release can stimulate vagal afferents innervating the colon, to modulate intestinal motility and augment gastric emptying. On release, serotonin can also enter the circulation, and exert effects on glucose homeostasis and lipid metabolism.

Microbiome composition, or at least the presence of particular bacterial species, significantly impacts the likeliness of obesity in mice. In GF mice, T. sanguinis monocolonization in the small intestine significantly decreased serum triglyceride levels and altered expression of genes relating to lipid metabolism, effects that were neutralised by fluoxetine (Fung et al., 2019). Similarly, in mice monocolonised with Clostridium ramosum, another spore-forming bacteria bi-directionally linked with serotonin, along with an increase in EC cell numbers, these mice experienced a shift in the expression of genes relating to lipid transport and storage (Mandić et al., 2019). When fed a high fat diet (HFD), C.ramosum mice also experienced significant increase in Tph1 expression and a subsequent body weight gain, with increased WAT and blood glucose levels compared to GF mice (Mandić et al., 2019). Thus, gut microbiota affect host lipid metabolism through a serotonin-dependant mechanism.

Psychiatric drugs, including SSRIs, are associated with marked reductions in at least one bacterial species in humans (Hiemke and Hartter, 2000, Maier et al., 2018), indicating SSRIs may have effects through the microbiota-serotonin link in humans as they do in mice. Long-term use of SSRIs is also implicated with increased incidence of obesity and dysregulated metabolism (Lee et al., 2016). Hence these recent studies introduce a new paradigm where SSRIs affect serotonin-microbiota interactions and modify the gut-brain axis, resulting in their antidepressant actions, whilst subsequently altering metabolic function and lipid metabolism. Further studies will be needed in humans to verify the clinical relevance of these findings in rodents.

Interactions between microbiota and gut-derived serotonin have also recently been illustrated to modulate host glucose metabolism. Pharmacological inhibition of gut serotonin synthesis or $Tph1^{-/-}$ mice, with or without antibiotic (Abx)-associated microbiota depletion, were all found to have significant improvements on glucose

clearance (Martin et al., 2019). Importantly, there was no additive effect with both serotonin depletion and Abx-associated microbiota depletion compared to either treatment alone (Martin et al., 2019), implicating gut-derived serotonin as the link through which gut microbiota modulates host glucose homeostasis. The serotonin-microbiota axis may therefore be a new target in the treatment of various human health disorders both within and outside of the GI tract.

1.3 Serotonin and gut motility

1.3.1 Overview of gut motility

GI motility defines the contractile events which occur in the gut wall to push the luminal contents along the GI tract and towards the rectum. This key physiological phenomenon is understood at a functional level, but not yet at a cellular level. After a meal the bowel senses an increase in pressure within its lumen and responds with neurologically stimulated contractions. This involves propulsive waves of oral contractions and anal relaxation with respect to the bolus, hence driving the bolus towards the rectal end of the GI tract (see Figure 1.4) (Bayliss and Starling, 1899). This theory when first introduced was termed as ‘the law of the intestine’ (Bayliss and Starling, 1899), however is now referred to as the peristaltic reflex. The peristaltic reflex refers to the contractile process that occurs when a bolus stimulates a contraction of circular muscle orally and a relaxation of the circular muscle and contraction of the longitudinal muscle caudally (Brierley, 2016).

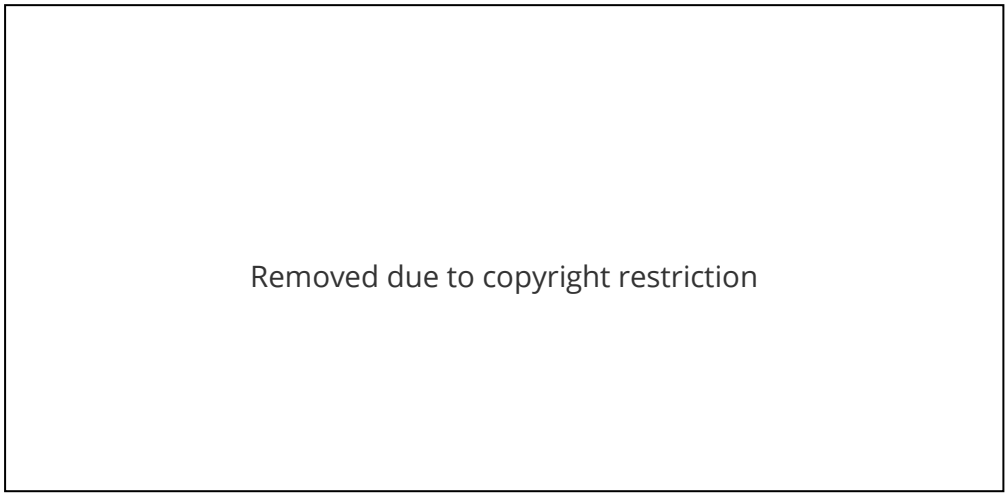

Figure 1.4: Schematic of the peristaltic reflex.

A depiction of the proximal contraction and distal relaxation that occurs in the peristaltic reflex to propel contents distally along the GI tract. Adapted from (Koeppen, 2009)

The gut also contains a rhythmic contractile cycle which occurs naturally, without stimulus, consisting of two key types of contraction. One type of contraction which underlies the natural rhythmic cycle is the myogenic rhythmic contraction. This is thought to be a natural periodical occurrence generated by pacemaker cells within the GI, the Interstitial Cells of Cajal (ICC) (Ward et al., 1999). The second type of contraction is an ongoing neurological action, with temporally synchronized activity between large pools of both excitatory and inhibitory neurons (Spencer et al., 2018). This ENS activation is highly synchronized, and involves large areas of myenteric neurons, all firing at ~2 Hz intervals (Spencer et al., 2018), preceding smooth muscle depolarization. Hence indicating a role for the enteric neurons in controlling rhythmic depolarizations in smooth muscle (Spencer et al., 2018). These are the first neuronal rhythmic firing patterns of the ENS to be described in detail, hence there is still a significant amount to be understood regarding the neurological control and mechanisms underlying GI motility. These neurogenic mediated cyclic rhythmic contractions are known as colonic migrating motor complexes (CMMCs), which are not dependent on content (Spencer et al., 2016). The coupling of myogenic and neurogenic rhythmic contractions forms propulsive movement initiated by a bolus (Spencer et al., 2018, Corsetti et al., 2019), defined as peristalsis.

This theory has been built on to encompass the continuous loop in which peristalsis occurs along the intestines, the neuromechanical loop hypothesis (Dinning et al., 2014, Spencer et al., 2016), in which distension evokes the polarised reflex (as described by Bayliss and Starling) is then continued along the colon as the bolus moves to activate a similar neural pathway further down the intestine (see Figure 1.5). Furthermore, this neuromechanical loop takes into consideration the effects of varying content consistency, shape and size of the luminal contents which have all been shown to affect the rate of peristalsis (Costa et al., 2015, Costa et al., 2021). This is

likely because the size of the content determines the size of the area, and hence number of sensory enteric neurons activated by the stimulus. Hence peristalsis is not a simple reflex as described by Baylis and Starling, but instead a series of polarized pathways which respond to mechanical factors, to form a dynamic functional loop that can adapt motility to deal effectively with differing contents or stimuli (Spencer et al., 2016). This neuromechanical loop is thought to work in synchrony with CMMCs, together underlying all propulsive movements which occur within the intestines (Spencer et al., 2016).

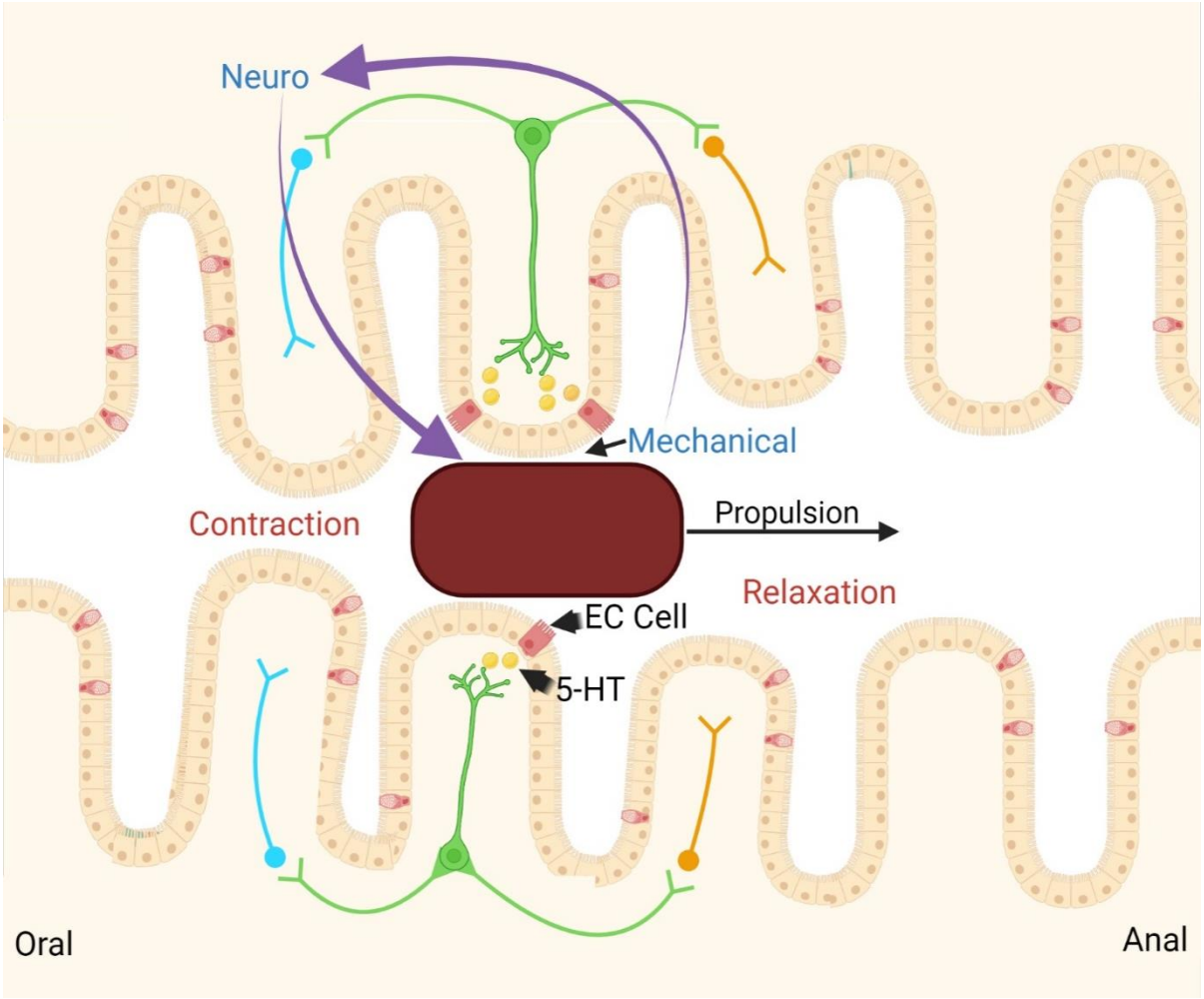

Figure 1.5: The proposed neuromechanical loop.

The mechanical stimulus of the bolus (‘Mechanical’, blue) activates EC cells (red, arrow), to release serotonin (‘5-HT’, arrow). As such, serotonin activates enteric neurons (‘Neuro’, blue;); including mucosal-projecting neurons (green and blue) and interneurons (green), within the gut wall, which causes a contraction (red) prior to the bolus, and a relaxation (red) of the muscles anally, with respect to the bolus. The bolus is then propelled along the GI tract, stimulating the same pathway, thereby defining the neuromechanical loop.

1.3.2 The role of EC cell derived serotonin in GI motility

Seminal studies completed in the 1950s noted that the release of serotonin coincides with GI contractile activity (Bulbring and Crema, 1958). As such, it was believed that luminal distension stimulated the release of serotonin from EC cells, to activate intrinsic sensory nerve endings in the mucosa and hence drive peristalsis (Foxx-Orenstein et al., 1996, Grider et al., 1996, Jin et al., 1999, Kadowaki et al., 1996). However, the role of serotonin in gut motility is still not fully understood, with opposing reports published detailing the extent of the role and importance of serotonin in the phenomenon (Smith and Gershon, 2015a, Spencer et al., 2015, Smith and Gershon, 2015b). One primary question the field is trying to understand is whether serotonin is essential for motility to occur, and what role it plays specifically in the process.

The application of exogenous serotonin, or 5-HT_3 and 5-HT_4 receptor agonists, potently stimulates peristalsis (Bulbring and Lin, 1958, Keating and Spencer, 2010, Spencer et al., 2011, Jin et al., 1999). We know that intrinsic sensory neurons contain endings which lie in close proximity to EC cells along the intestinal mucosa (Kunze et al., 1995a, Bertrand et al., 2000), and that serotonin activates intrinsic primary afferent neurons (IPANs) which drive motility (Bulbring and Crema, 1959) (Pan and Gershon, 2000, Heredia et al., 2009, Bayguinov et al., 2010). Therefore, it is hypothesised that serotonin acts on these nerve endings to have some form of modulatory effect on GI motility. Further, serotonin antagonists can inhibit peristalsis, along with CMMCs (Bush et al., 2001, Heredia et al., 2009, Grider et al., 1996). One study suggested that serotonin release from EC cells was essential for CMMCs, due to their absence in *ex vivo* colonic preparations with their mucosa removed (Heredia et al., 2009). Taken together, these data indicated that serotonin must play a pivotal role in peristalsis. However, this evidence was refuted as Keating and Spencer (2010) removed the

mucosa, submucosa and submucosal plexus from the entire mouse colon, and still recorded spontaneous, consistent CMMCs. The remaining cyclical migrating complexes and peristalsis (Keating and Spencer, 2010, Spencer et al., 2011), indicated that serotonin is not essential for gut motility but instead acts as a modulator. Fluid and pellets placed in intact preparations, and mucosa-free intact preparations still maintained peristalsis, albeit at significantly slower frequencies (Spencer et al., 2011).

Further experiments were conducted, using the same serotonin receptor antagonists as were found to inhibit peristalsis of intact preparations previously (Sia et al., 2013a), in mucosa-free colonic preparations, and found to have the same inhibitory effects on CMMCs and peristalsis (Barnes et al., 2014). This was a surprising result as the mucosa-free preparations, depleted of all endogenous serotonin, were expected to be unaffected by 5-HTR-selective antagonists on peristalsis. It has since been proposed that these antagonists are not acting in a serotonin dependent manner, but that the 5-HT_3 and 5-HT_4 receptors on which they act are constitutively active. This indicated that these receptors do not require their ligand, in this case serotonin, to stimulate intracellular signaling or activate the cells. Constitutive activity of both 5-HT_3 and 5-HT_4 receptors have been described (Hu and Peoples, 2008, Berthouze et al., 2005), which explains the result in which 5-HTR antagonists blocked peristalsis in serotonin depleted preps.

A Tph1 knockout mouse ($Tph1^{-/-}$) line was generated to better answer the question surrounding the role of serotonin in motility, this mouse line showed that EC-derived serotonin is not critical for motility as no effect on *in vivo* GI transit was demonstrated (Heredia et al., 2013). $Tph1^{-/-}$ were shown to have no difference in total GI transit time, gastric emptying, small intestinal transit and colonic transit compared with control mice (Li et al., 2011). *Ex vivo* propulsion of pellets was slower in $Tph1^{-/-}$ mice and CMMCs

still occurred in Tph1$^{-/-}$ mice, although they were significantly slower, and propagated significantly less often (Heredia et al., 2013). Such *in vitro* data, supports the notion that EC cell derived serotonin is a modulator of gut motility but that it is not essential for it to occur. However, this was not a perfect model as developmental issues were found within these mice, including changes in colonic anatomy. As such colons of Tph1$^{-/-}$ mice were hypertrophic, longer and with a larger internal diameter than that of Tph1$^{+/+}$ mice (Heredia et al., 2013). This significant change makes the comparison between normal mice and Tph1$^{-/-}$ in terms of GI motility and transit effectively impossible, and as such this model was not able to definitively answer the question of the importance of serotonin in GI motility.

In summary, the exact role of serotonin in GI motility still remains unclear. It has been shown through multiple studies that taking serotonin out of the system, through removal of mucosa, the use of pharmaceutical techniques, and genetic ablation of serotonin synthesis, that peristalsis and CMMCs remain intact. Therefore, it is suggested that serotonin is not essential for peristalsis but does modulate this physiological phenomenon. In order to definitively support this hypothesis, it seems a genetic model with complete removal of EC cell-derived serotonin in the gut but with no development effects would be ideal.

1.3.3 Neuronal serotonin in GI motility

The individual contribution of the two different serotonin sources (EC cells and enteric neurons) to motility and activation of the neural pathways in the ENS is an additional unknown in the field. Discrete quantities of serotonin are synthesised in 1-2% of Tph2 enteric neurons in the ENS (Costa et al., 1996), and is a neurotransmitter of myenteric interneurons (Gershon and Tack, 2007). As such, studies have attempted to determine whether neuronal serotonin might also play a role in the control of gut motility through

the use of Tph2 knockout ($Tph2^{-/-}$) mice (Li et al., 2011). These mice were found to have a reduced total GI transit time compared with controls; however, these mice also experience significant neuroanatomical and neurochemical differences in the ENS, which are likely to be the cause for the changes in transit time (Li et al., 2011). A following study tested preparations with the mucosa and submucosa removed, along with the treatment of reserpine, a blocker of the vesicular monoamine transporters (VMAT1 and VMAT2). VMAT2 transports serotonin from the cytoplasm into presynaptic vesicles ready for subsequent release (Yelin and Schuldiner, 2002). Thereby the blockade of VMAT2 by reserpine inhibits the uptake and removing both mucosal and enteric pools of serotonin, without the neuronal side effects of a Tph knockout mouse. Mice were injected with reserpine 24 h prior to euthanasia, to determine the effect of the loss of neuronal serotonin on CMMCs (Sia et al., 2013b). The study using these preparations showed no effect on distention-evoked peristalsis (Sia et al., 2013b) or CMMCs (Spencer et al., 2013) hence indicating no role for neuronal serotonin in motility. However, these preparations still undergo off-target effects with reserpine, and the disruption of physical integrity through the removal of mucosa. Not to mention, the removal of mucosa is not specific to EC cells. Hence, similar to EC cell-derived serotonin, the lack of an adequate model to test the role of neuronal serotonin in GI motility remains, hence the exact role of neuronal serotonin in GI motility is not yet fully understood.

1.4 Mechanosensing of EC cells

1.4.1 Evidence of EC cell mechanosensitivity

It is evident that in order for digestion to occur, the GI tract must respond to the presence of contents in the lumen and work to push the contents caudally. This is achieved through fluid secretion and peristalsis, however, the mechanism through which the gut epithelial layer senses and responds to mechanical stimulation is not currently understood within the field. EC cells themselves have long been recognized as mechanically sensitive (Bulbring and Crema, 1959), typically responding through the secretion of serotonin (Bulbring and Crema, 1959, Kim et al., 2001, Kim et al., 2007). This was first described by Edith Bulbring in 1959, who noted small amounts of serotonin released in response to mucosal pressure applied to guinea pig small bowel (Bulbring and Crema, 1959). As such, Bulbring and Crema (1959) proposed that the deformation of EC cells from peristalsis or general distention of the gut wall could be responsible for the release of serotonin. The same conclusion was made when an EC-like cell line, BON cells, released serotonin in response to mechanical stimulation (Kim et al., 2001). Furthermore, EC cells in whole-thickness preparations of guinea pig small intestine release serotonin in response to the shear stress of fluid caused by rotational shaking *ex vivo* (Kim et al., 2001). This occurred in the presence of a neuronal blocker, tetrodotoxin, to block any neuronally stimulated serotonin release. The release of serotonin was proportional to the level of shaking. Hence, it would appear that EC cells are capable of responding to a variety of mechanical forces. There are numerous kinds of mechanical stimuli which occur within the GI tract, including intraluminal pressure, shear stress, distension, deformation, touch and centrifugal forces during peristalsis (Linan-Rico et al., 2016). Amperometry studies on the guinea-pig ileum mucosa, revealed in real-time that the release of serotonin occurs

as a direct result of mucosal compression of EC cells within the mucosa (Bertrand, 2004). Hence it is apparent that EC cells are mechanosensitive, and likely respond to contractile events of the GI wall *in vivo* to modulate GI motility.

1.4.2 Previously hypothesised mechanisms of EC cell mechanobiology

Previously, a number of mechanisms have been identified to play a role in EC cell mechanosensitivity. These include G-protein coupled receptors (Kim et al., 2001), purinergic receptors P2Y and P2X (Kim et al., 2001, Christofi, 2008, Chin et al., 2011, Liñán-Rico et al., 2013), and caveolae (Kim et al., 2007). More specifically, mechanical stimulation of EC cells stimulates the release of nucleotides Adenosine triphosphate (ATP) and Uridine triphosphate (UTP) (Cooke et al., 2003, Christofi, 2008, Liñán-Rico et al., 2013, Linan-Rico et al., 2014), both commonly released from cells in response to mechanical stimuli (Linan-Rico et al., 2016). However, the role for these nucleotides in mechanosensory signaling remains unknown, particularly as purinergic receptors themselves are not mechanosensitive (Linan-Rico et al., 2016). It has been suggested that these are likely downstream of the primary mechanosensor (Liñán-Rico et al., 2013). Such an example has been identified in the bladder, in which ATP release occurs secondary to Piezo channel activation, to provide a positive feedback loop upon mechanical stimulation (Miyamoto et al., 2014a). Furthermore, ATP release has been associated with painful sensations in pathophysiological conditions, in which ATP activates EC cells, to signal to both enteric and visceral afferent pain pathways (Wynn et al., 2004, Burnstock, 2009). As such, it is important to note that a large proportion of EC cells have been identified which do not require ATP or other nucleotides for mechanotransduction (Liñán-Rico et al., 2013). Others have indicated that mechanosensory cells likely contain a range of mechanoreceptors to mediate mechanotransduction (Eyckmans et al., 2011a, Joshi et al., 2021), with the potential

for differing stimuli to be detected through different mechanosensory pathways (Cooke et al., 2003). However, it has since been suggested that Piezo2, a recently identified mechanosensitive ion channel, is the primary mechanosensor important for the mechanosensing capabilities of EC cells (Wang et al., 2017, Alcaino et al., 2018).

1.4.3 Piezo channels

The Piezo family of ion channels were discovered just 10 years ago, consisting of Piezo1 and Piezo2 (Coste et al., 2010, Coste et al., 2012). The Piezo channels are transmembrane proteins which form mechanosensitive ion channels selective for cations (Coste et al., 2012). The two channels confer distinct properties (Coste et al., 2010), differing in both their inactivation kinetics and their roles as mechanosensory channels. The time constant of inactivation of Piezo2 dependent mechanosensory currents, 7 ms, are half that of Piezo1, 16 ms (Coste et al., 2012, Ikeda et al., 2014). Piezo1 and Piezo2 also have unique structures (Taberner et al., 2019, Wang et al., 2019b, Lin et al., 2019), although they both form very large and complex proteins (Lin et al., 2019).

The Piezo family have been extensively investigated in recent years, demonstrating their key role in the development and function of a variety of sensory transducing mechanisms. Piezo1 has been shown to sense shear stress (Cox et al., 2016a, Lewis and Grandl, 2015, Syeda et al., 2016), cell volume changes (Retailleau et al., 2015a, Faucherre et al., 2014a, Miyamoto et al., 2014b, Cinar et al., 2015a) and cell crowding (Slattum and Rosenblatt, 2014). In particular, Piezo1 expression is observed in smooth muscle, red blood, vascular endothelial cells (Faucherre et al., 2014b, Cinar et al., 2015b, Retailleau et al., 2015b), bladder, colon, kidney, lung and skin, with low expression seen in dorsal root ganglion (DRG) (Coste et al., 2010). Piezo1 has been demonstrated to function in mechanotransduction during vascular development

(Ranade et al., 2014a), lymphatic development (Nonomura et al., 2018), red blood cell volume control (Zeng et al., 2018), blood pressure control (Wang et al., 2016), arterial remodeling (Retailleau et al., 2015a), stretch-activated channel activity in proximal convoluted tubule epithelial cells of the kidney (Peyronnet et al., 2013a), and tissue inflammation (Romac et al., 2018, Solis et al., 2019). Although there are conflicting reports surrounding the mechanosensing role of this channel in vascular architecture (Fang et al., 2019, Mehta et al., 2020), with the most recent data reports that Plexin D1 is the key mechanosensory channel in vascular endothelial cells, demonstrating no effect on force response when Piezo1 expression was knocked down (Mehta et al., 2020).

Piezo2, alternatively is important in general cellular mechanosensation, activated typically via membrane deformation and touch (Wu et al., 2017). Piezo2 is expressed in the bladder, colon, lung, and somatosensory neurons (Coste et al., 2010), Merkel cells of the skin (Ikeda et al., 2014), and in the kidney (Coste et al., 2010). Piezo2 is the key mechanosensory channel involved in light touch sensation of the skin (Ikeda et al., 2014, Ranade et al., 2014b, Woo et al., 2014, Chang et al., 2016), functioning in Merkel cells the primary epithelial cells on the surface of the skin. Merkel cells form serotonergic synapses with epithelial projecting DRG to transduce mechanosensory information (Ikeda et al., 2014, Chang et al., 2016). Furthermore, Piezo2 is involved in a number of other touch sensing mechanisms throughout the body, including in airway stretch and lung inflation (Nonomura et al., 2017), somatosensory touch (Ranade et al., 2014b), proprioception (Woo et al., 2015), visceral sensation (Yang et al., 2016), pain sensation (Basbaum et al., 2009, Lewin and Moshourab, 2004), and most recently within sensory neurons and urothelial cells controlling bladder sensing and the micturition reflex (Marshall et al., 2020).

Hence it is clear that Piezo1 and Piezo2 are important mechanosensors in a range of mechanosensory systems throughout the body. A number of these systems have been confirmed in human, through investigations of patients who exhibit gain-of-function and loss-of-function mutations in Piezo1 and Piezo2 proteins. Gain-of-function mutations in Piezo1 are linked with dehydrated hereditary xerocytosis, a rare genetic disease which is characterised by red blood cell dehydration with mild hemolysis (Albuisson et al., 2013). Whereas loss-of-function mutations in Piezo2 exhibit deficiencies in vibration detection, touch discrimination, joint proprioception (Chesler et al., 2016, Szczot et al., 2018), and a reduction in detection of low-threshold mechanical stimuli in pain (Szczot et al., 2018). Finally, these patients are also affected in bladder sensation, exhibiting negative impacts in bladder control and bladder-filling sensation (Marshall et al., 2020).

1.4.4 Evidence for mouse EC cell Piezo channels

Piezo2 has been identified in EC cells, in mice and human, demonstrated through both immunohistochemistry and gene expression profiling (Coste et al., 2010, Wang et al., 2017). Wang et al (2017) found that Piezo2 mRNA was expressed in human (jejunum) and mouse small bowel (ileum, duodenum, jejunum) mucosa, with ~90% of EC cells found to contain Piezo2 using immunohistochemistry on human jejunum (Wang et al., 2017). A separate study used lineage tracing, to show that Piezo2 is expressed in a subset of EECs, of which 80% were identified as EC cells (Alcaino et al., 2018). Alcaino (2018) reported updated numbers on Piezo2 expression in EC cells, with 85% Piezo2 positive EC cells in the small bowel, and ~80% in the large bowel (mouse) (Alcaino et al., 2018). Furthermore, super-resolution microscopy has demonstrated that Piezo2 is located in close proximity to serotonin vesicles within EC cells (Alcaino et al., 2018).

Functionally, a number of *in vitro* experiments, utilizing EC cell model QGP-1 cells, have demonstrated a role for Piezo2 in EC cell mechanosensitivity (Wang et al., 2017). Pressure-induced currents were blocked with the mechanosensitive ion channel antagonist, D-GsMTx4, in QGP-1 cells and HEK cells transfected with a human Piezo2 construct (Wang et al., 2017, Alcaino et al., 2018). Further, use of D-GsMTx4 or Piezo2 siRNA inhibited pressure induced serotonin release from mouse small bowel *ex vivo* (Wang et al., 2017). An EEC reporter mouse line (NeuroD1^{+} reporter mice) was created, in which a proportion of NeuroD1^{+} isolated cells were found to be EC cells (Alcaino et al., 2018). The shear force of a perfused liquid stimulated 58% of NeuroD1^{+} cultured cells, measured via calcium imaging, and caused an intracellular increase of Ca^{2+} of more than 200% (Alcaino et al., 2018). This response was inhibited using Ca^{2+}-free media, D-GsMTx4 and Piezo2 siRNA (Alcaino et al., 2018), demonstrating the importance of Piezo2 channels in EC cell mechanosensitivity. These results also highlighted this mechanosensory mechanism as Ca^{2+} dependent, hence indicating that Piezo2 stimulates serotonin release through an increase in intracellular Ca^{2+}. It is postulated that the exocytosis of serotonin vesicles is caused through large Ca^{2+} entry via both Piezo2 channels and voltage gated Ca^{2+} channels (Chang et al., 2016). Piezo2 activation in Merkel cells also causes an inward flux of cation currents (Woo et al., 2014), causing depolarization of the cell and activation of L-type Ca^{2+} channels (Ikeda et al., 2014). The opening of L-type Ca^{2+} channels allows a further influx of Ca^{2+}, postulated to stimulate exocytosis (Haeberle et al., 2004). Our lab has previously demonstrated Ca^{2+} influx as the main physiological trigger for serotonin release (Raghupathi et al., 2013), indicating EC cells may use the same Ca^{2+} mediated pathway in their mechanically stimulated serotonin release. A conditional GI epithelium-specific Piezo2 knockdown mouse model (Vil-cre;Piezo2$^{f/f}$) was created (Alcaino et al., 2018). Pressure-induced stimulation of intestinal mucosa from Vil-

Cre;Piezo2$^{f/f}$ mice was measured, in which a significant reduction in mechanically stimulated secretion was seen compared with controls (Alcaino et al., 2018). The effects on GI motility were not measured in this model, leaving a significant gap within the literature surrounding the role of EC cell Piezo2 in GI motility.

Although gut transit has been investigated in a gut specific Piezo1 inducible knockout (Villin-Piezo1$^{flox/flox}$) mouse model (Sugisawa et al., 2020), in which total gut transit time was significantly slower compared with controls, the initial presence and subsequent reduction in Piezo1 levels were not measured. Interestingly, both serum and duodenal serotonin levels were significantly reduced in Villin-Piezo1$^{flox/flox}$ mice, as well as Tph1 expression and the number of EC cells in the duodenum (Sugisawa et al., 2020). This investigation also highlighted that Piezo1 knockdown, in an EC cell model, did not influence mechanically stimulated serotonin secretion, while Piezo2 knockdown of the same model inhibited serotonin release (Sugisawa et al., 2020). Together, this data suggests that Piezo1 is involved in serotonin production, and hence it is likely that a reduction in EC cell serotonin levels caused the slower transit seen in Villin-Piezo1$^{flox/flox}$. Finally, a study conducted in Drosophila, found that Piezo KO flies ate and drank significantly more than controls, some to the point of overeating to death, they consumed more regardless of nutritional value (Wang et al., 2020). Indicating that flies feeding is based on mechanical cues from Piezo^{+} neurons in the gut, which sense gut distension and signal food consumption and satiety to halt overconsumption. It is important to note that flies contain just one Piezo channel type, and hence cannot be used as a direct comparison to mammals, although generally the endocrine and neuropeptide systems in Drosophila which control feeding are conserved in mammals (Jourjine, 2017, Wang et al., 2020).

1.5 Gut-brain axis

1.5.1 Sensory innervation of the gut

The intestines are a unique organ, in that they are innervated by an intrinsic nervous system (the enteric nervous system, ENS), and extrinsic neurons which arise primarily from either nodose ganglia or dorsal root ganglia (DRG) (Furness et al., 1999). The ENS is organised into two interconnected nerve plexuses, the myenteric plexus embedded between the circular and longitudinal muscle layers, and the submucosal plexus located just under the mucosa in the gut wall. Each plexus is filled with a heterogeneous population of neurons, of which approximately 20 varying subtypes of enteric neurons can be defined (Brookes and Costa, 2002, Furness, 2008), based on diverse neurochemical coding, function, morphology, and region of innervation. Amidst the 20 subtypes exist sensory neurons commonly termed intrinsic primary afferent neurons (IPANs) (Furness et al., 2014), which project into the mucosal epithelium in both the proximal and distal direction (Bertrand et al., 1998). IPANs innervate all layers of the gut wall, including the mucosa, in which they detect information of luminal content and the physical state of the gut (Furness et al., 2004a). IPANs then communicate with interneurons, motor neurons and other IPANs, to modulate functions such as gut motility, secretion, and blood flow (Furness et al., 2014).

The extrinsic innervation of the gut is derived from vagal neurons, spinal neurons, pre- and postganglionic sympathetic and parasympathetic neurons (Kang et al., 2021). Spinal afferents originate in DRG and project to the spinal cord and into the layers of the gut wall (Brookes et al., 2016), whereas vagal cell bodies are located in the nodose or jugular ganglia, projecting both centrally to the brainstem and into the gut wall (Brookes et al., 2016). The vagus provides both sensory and motor innervation to the

GI tract, with sensory afferents consisting of mucosal mechanoreceptors, chemoreceptors and tension receptors, which primarily innervate the oesophagus, stomach and small intestine (Furness et al., 2014). In the current study, we will focus on the extrinsic sensory innervation to the colon.

1.5.2 Extrinsic sensory innervation of the colon

The colon is primarily innervated extrinsically by neurons which arise in the DRG of the spinal cord (Furness et al., 1999). It is unclear to what proportion the vagus nerve also contributes to colonic extrinsic innervation although it is believed to be minimal (Altschuler et al., 1993, Furness et al., 2014, Herrity et al., 2014). Extrinsic sensory afferents of the colon can be subdivided based on the anatomical location in which their cell body lies, this includes splanchnic nerve cell bodies located within the thoracolumbar DRG, and pelvic afferents which originate from lumbosacral DRG (Grundy and Brierley, 2018) (see Figure 1.6). The majority of sensory afferents in the mouse colon are proposed to originate from lumbosacral DRG (Christianson et al., 2006, Tan et al., 2008). Furthermore, the pelvic and splanchnic thoracic and lumbar spinal cord innervate the proximal colon via the lumbar splanchnic nerve, while the lumbar splanchnic and sacral pelvic nerves both separately innervate the distal colon (Harrington et al., 2018).

Colonic sensory afferents are primarily responsible for mediating conscious sensations such as fullness, discomfort, bloating, urgency, and pain (Harrington et al., 2018, Brookes et al., 2013). Spinal afferents can be classed based on the layer in which they innervate within the GI wall, such as the mucosa (Yu et al., 2016), muscle (Yu et al., 2016, Jiang et al., 2011, McGuire et al., 2018), serosa (Yu et al., 2016, Jiang et al., 2011, Hockley et al., 2016, McGuire et al., 2018), and mesentery (Yu et al., 2016, Hockley et al., 2016, McGuire et al., 2018). A recent study identified seven

distinct classes of sensory neurons based on their genetic profiling derived from single-cell RNA sequencing (Hockley et al., 2019). Broadly, these groups include two distinct neurofilament classes both associated with myelinated DRG neurons; non-peptidergic nociceptors; three classes of peptidergic neurons expressing calcitonin gene-related peptide (*Calcb*) and substance P (*Tac1*), transient receptor potential cation channel A1 (*Trpa1*) and tyrosine hydroxylase (*Th*) respectively; and the final subtype, which expresses Piezo2 (Hockley et al., 2019). Distinct expression patterns were identified for 7 spinal afferent subtypes, which likely suggests functional differences between the classes of neurons that innervate the colon (Hockley et al., 2019).

Overall, we still have a limited understanding on how sensory projections into the GI tract sense information about luminal contents, and how these sensory connections are developed and integrated (Kang et al., 2021). However, the most recent evidence suggests the potential for a direct synaptic-like connection between EECs and neurons within the GI wall (Bohórquez et al., 2015, Kaelberer et al., 2018).

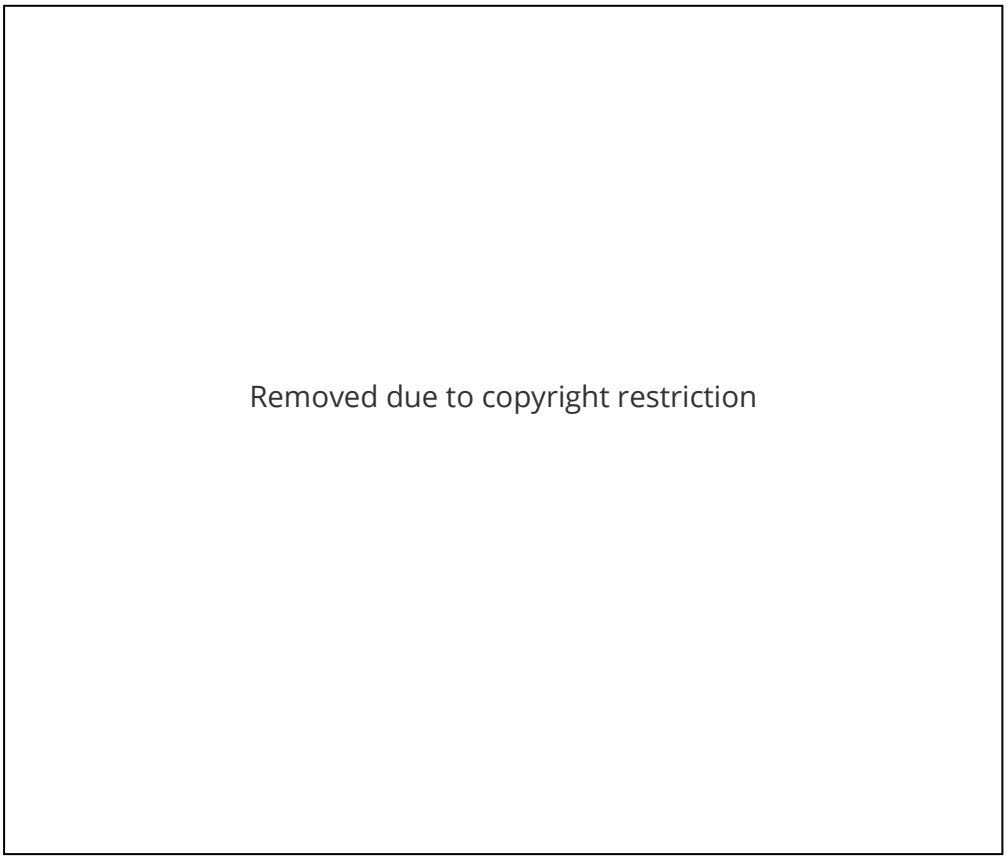

Figure 1.6: Extrinsic innervation of the GI tract.

Schematic diagram showing sympathetic (blue) and parasympathetic (pink) innervation to the GI tract. Adapted from (Uesaka et al., 2016).

1.5.3 Functional evidence of direct signalling in the gut-brain axis

The mechanism through which the CNS is informed of the sensory environment within the GI tract is an area of growing interest, with a myriad of recent studies demonstrating evidence that a fast direct line of communication exists between the gut and the brain, termed the gut-brain axis. As EECs are sensory cells which are on the front-line of the intestinal epithelium, they are responsible for a significant portion of collecting the sensory information within the GI lumen. EECs respond to a vast array of stimuli, including ingested nutrients (Ye et al., 2021), bacterial metabolites (Yano et al., 2015), and mechanical stimulation (Alcaino et al., 2018). In response to such stimuli, EECs release various hormones and neurotransmitters, through a Ca^{2+}-dependent mechanism (Furness et al., 2013), to facilitate sensory information to surrounding cell types. As such EECs are able to act locally to regulate GI motility, intestinal secretion, digestion, and the absorption of nutrients (Liddle, 2019). Furthermore, EECs transfer sensory information to extrinsic nerves which innervate the GI tract, a sensory connection thought to contribute to the gut-brain axis. Despite the important sensory role of EECs, the mechanism which facilitates this direct transmission of information onto extrinsic nerves is poorly understood (Bohórquez et al., 2015, Ye et al., 2021). It was long believed that EECs signalled to the CNS through the diffusion of hormones, in which hormones would be released into the circulation to be carried to receptors on distant organs (Wade and Westfall, 1985a, Okano-Matsumoto et al., 2011, Psichas et al., 2015). Whilst some evidence suggests EECs do not come into direct contact with blood vessels (Bohórquez et al., 2015), it is true that released hormones can act via diffusion into the circulatory system or in a paracrine fashion to neighbouring cells (Bertrand, 2009, Cummings and Overduin, 2007). However, in order for such a mechanism to facilitate this communication to the

CNS would take minutes to hours after the ingestion of a meal (Rehfeld, 1998), this does not align with the most recent evidence of the fast, direct communication which indicates that neurons in the hypothalamus respond to luminal contents within seconds (Beutler et al., 2017, Su et al., 2017). Such evidence suggests that the brain receives sensory information from the gut through fast neuronal signalling.

1.5.4 Evidence for direct EEC – neuronal connections

Due to the recent evidence of fast communication between EECs and neurons, several studies have highlighted the potential for EECs to form synaptic connections with neurons (Bohórquez et al., 2014, Bohórquez et al., 2015, Kaelberer et al., 2018). The most notable aspect of EECs which have been highlighted are basal processes often seen extending into the intestinal wall. These long processes were described up to 40 years ago on D cells (Larsson et al., 1979), and more recently on L cells (Lundberg et al., 1982, Karaki et al., 2006). Original evidence for basal processes on D cells, indicated that such processes were directed at neighbouring cell types, to release somatostatin from these pseudopod-like structures to act in a paracrine manner to target cells containing somatostatin receptors (Larsson et al., 1979). However, in 2010 the description of ‘pseudopod-like basal cell processes’ were described of I cells in the small intestine (Chandra et al., 2010), projected to facilitate direct synaptic-like connections with neurons. As such these processes have recently become a focal point for many in the field, termed neuropods by some in the literature (Bohórquez et al., 2011, Bohórquez et al., 2014, Bohórquez et al., 2015).

Neuropod anatomy differs between EEC subtypes, with some L cell neuropods extending up to 50 μm in length (Bohórquez et al., 2011), while I cell neuropods were usually less than 10 μm (Chandra et al., 2010). Although both L cell and I cells have been found to contain multiple neuropods (Chandra et al., 2010, Bohórquez et al.,

2011), or singular extensions which bifurcate in two (Chandra et al., 2010, Bohórquez et al., 2011). Investigation into neuropods of I cells were found to project significant distances from the cell body, with endings described as bulb-like swellings (Alumets et al., 1979, Chandra et al., 2010) which resemble synaptic boutons (Bohórquez et al., 2011, Bohórquez et al., 2014). Further investigation into neuropods has revealed that they contain both hormone containing large dense-core vesicles and small clear synaptic vesicles (Nilsson et al., 1991), with the highest concentration of secretory vesicles noted within neuropods (Bohórquez et al., 2014). The ends of neuropods also contain a significant distribution of mitochondria, hypothesised to maintain the secretion of vesicles from neuropods (Bohórquez et al., 2014). Neurofilaments, structural proteins typical of neuronal axons, have also been identified within these structures, extending from the cell body to the end of neuropods (Bohórquez et al., 2014). A number of classical pre-synaptic and post-synaptic markers, as well as synaptic adhesion genes have been identified in EECs, such as synapsin 1 (Bohórquez et al., 2015, Südhof, 2012, Kaelberer et al., 2018), piccolo, bassoon, MUNC13B, RIMS2, latrophilin 1, transsynaptic neurexin 2 (Südhof, 2012, Bohórquez et al., 2015), transsynaptic neuroligins 2 and 3, homer 3, and postsynaptic density 95 (Chen et al., 2008, Bohórquez et al., 2015), Efnb2, Lrrtm2, Lrrc4,and Nrxn2 (Kaelberer et al., 2018). Together, this evidence is postulated to suggest a physical connection, which facilitates direct synaptic-like communication, is formed between EEC neuropods and enteric nerves (Bohórquez et al., 2011, Bohórquez et al., 2014).

The proposal of a synaptic connection between EEC neuropods and neurons has been doubted by some (Bertrand, 2009, Koo et al., 2021), particularly due to the rapid turn-over of EECs typically thought to renew every 5 days. Such a short life for EECs has been noted as a particularly short window of time for such a synaptic connection to form, particularly as EECs are changing constantly moving up the crypt-villus axis

and thought to change in characteristics as they traverse this axis such as their hormonal profile and morphological shape (Bohórquez et al., 2011, Beumer et al., 2018). In opposition to these concerns, the most recent evidence on the life span of EECs has indicated that a proportion of cells can live significantly longer than originally thought, even up to 13 months for some EEC subtypes (Bohórquez et al., 2015, Wei et al., 2021). Such data suggests that a proportion of EECs have a comparable life span to that of other innervated epithelial sensory cells in other body systems, such as olfactory receptor neurons or taste cells (Hamamichi et al., 2006, Hinds et al., 1984).

Furthermore, it has been shown that various EEC subtypes make direct contact with neurons which innervate the mucosa of the ileum and colon (Bohórquez et al., 2015), and that such nerves appeared to penetrate the basal lamina to directly contact EEC neuropods (Bohórquez et al., 2015). The different neuronal subtypes which contacted L cells were investigated, it was found that 67.3% contacted PGP 9.5 nerves, with the majority being neurofilament medium nerves and calbindin positive nerves (Bohórquez et al., 2015). A portion of L cells were found to contact calcitonin gene-related peptide (CGRP) nerves; but none appeared to touch vasoactive intestinal peptide (VIP) nerves (Bohórquez et al., 2015). To further investigate the nature of such connections, EECs were co-cultured with isolated sensory neurons *in vitro*, neurons were found to extend a neurite towards the EEC within 12 hrs, followed by the EEC growing a cytoplasmic process toward the neuron to form a connection just one hour later (Bohórquez et al., 2015, Kaelberer et al., 2018). This connection lasted up to 88 hrs in co-culture. It is important to note that not all EECs formed connections with neurons in co-culture, typically just 15% of I cells were found to form contact with a neuronal fibre *in vitro* (Bohórquez et al., 2015).

Further *in vivo* experiments were conducted in which a GFP labelled rabies virus, known to only travel via monosynaptic connections, was used in Pyy-Cre–rabG mice to trace synaptic connections between L cells and their connecting neurons (Bohórquez et al., 2015, Kaelberer et al., 2018). Pyy-Cre–rabG mice are mice in which L cells are genetically modified to express the G glycoprotein necessary for transsynaptic spread of rabies (rabG), allowing L cells to be transduced and pass on the virus to any synaptically-connected cells (Bohórquez et al., 2015). Pyy-Cre–rabG mice were given a GFP labelled rabies virus via an enema into the distal colon, EECs and their connecting neurons were seen to be infected with the virus 7 days post-enema (Bohórquez et al., 2015). Hence demonstrating evidence for a functional synaptic-like connection between EECs and neurons in the colon. Furthermore, labelled fibres were seen in the DRG and vagal nodose ganglia, which indicated that EECs communicate with spinal afferents as well as vagal neurons (Bohórquez et al., 2015, Kaelberer et al., 2018). This study further analysed the brain regions in which EECs communicate, demonstrating tracing into the nucleus tractus solitarius of the brainstem (Bohórquez et al., 2015, Kaelberer et al., 2018).

EECs express two enzymes essential in dopamine synthesis, aromatic-L-amino-acid decarboxylase (AAAD) and tyrosine hydroxylase (Bohórquez et al., 2015), as well as the vesicular glutamate transporter 1 protein (VGLUT1) (Kaelberer et al., 2018). As vesicular glutamate is a neurotransmitter typical of other sensory epithelial cells, these results indicated that EECs might use glutamate in synaptic-like transmission. As such, the role for glutamate as a key neurotransmitter in I cells was investigated through co-culturing isolated, I cells with vagal neurons, in which an EEC stimulus was found to activate vagal neurons. The inhibition of the glutamate transporter blocked this response in connected vagal neurons (Kaelberer et al., 2018), indicating that glutamate mediates synaptic-like communication between EECs and neurons. More

specifically, these experiments demonstrated the role for glutamate as a fast mediator of communication with vagal neurons, occurring within milliseconds of EEC stimulation. Whilst also demonstrating that CCK release occurs at a slower rate, exhibiting a paracrine action on co-cultured vagal neurons (Kaelberer et al., 2018).

We know that sugars are readily sensed within the small intestine, however whether this stimulus is sensed directly by the vagus, or through EECs which transmit the signal to the vagus, has been unclear until recently (Williams et al., 2016). A recent investigation presented that EECs respond to luminal content, such as sugars, to then stimulate connecting neurons, such as the vagus nerve (Kaelberer et al., 2018). This was first demonstrated *in vitro*, in which isolated nodose neurons were tested via calcium imaging when treated with glucose, with no response seen in neurons (Kaelberer et al., 2018). HoweverHowever,, when vagal neurons were co-cultured with EECs, the same neurons were activated in response to the glucose stimulus {Kaelberer, 2018 #238), indicating that glucose activation of EECs is transmitted to synapsing neurons. This proposed system was tested *in vivo*, in which sugars were delivered via intraluminal transfusion directly into the duodenal lumen, while the vagal neurons were recorded via electrophysiology in real time (Kaelberer et al., 2018). In such experiments, sugars significantly increased the vagal firing rate, a response abolished with the severing of the vagus, or a glucose transporter (SGLT1) inhibitor (Kaelberer et al., 2018). As SGLT1 is expressed in EECs and not in vagal neurons, this data suggests that luminal stimuli are sensed via EECs, which transduce this signal to connecting vagal neurons (Kaelberer et al., 2018).

1.5.5 EC cell – neuronal connection

There is considerable data available on the morphology of EC cells along the GI tract of mouse, rat and human, although no reported investigations into EC cell neuropods,

or the connection between EC cells with neurons in the GI tract. In the rat colon, 80% of EC cells contain cytoplasmic processes (Kuramoto et al., 2007). Some EC cells are bipolar, with a long process running down the crypt, and a second process extending towards the lumen from the apical end (Kuramoto et al., 2007). Furthermore, the processes of EC cells have also been described to resemble axons, containing swollen bulges similar to nerve varicosities (Kuramoto et al., 2007). Like other EECs, EC cells have also been shown to express several presynaptic markers, such as synapsin, and postsynaptic markers, such as PSD-95, observed immediately adjacent to EC cells (Bellono et al., 2017). Although, these processes typically extended towards the submucosa, there has been no evidence to suggest that they cross the basement membrane into the lamina propria (Kuramoto et al., 2007). The limited knowledge on EC cell neural connections, has suggested that 5-HT_3R labelled mucosal afferents appear to contact the basolateral side of EC cells (Bellono et al., 2017).

A number of immunohistochemical studies have investigated the presence of basal processes on EC cells along the human GI tract, demonstrating their occurrence in the stomach, colon and rectum (Inokuchi et al., 1984, Bordi et al., 2000, Sjölund et al., 1983). Human fundic EC cells are described as pleomorphic (Inokuchi et al., 1984), noting long and sometimes multipolar cytoplasmic processes projecting in all directions (Inokuchi et al., 1984, Kusumoto et al., 1988). While other studies suggest that there are 3 distinct morphologies of human EC cells, ‘round and closed cells’, ‘typical flask-shaped open cells’, and ‘cells with multiple basal processes’ (Fakhry et al., 2019). Whilst a number of studies have described the anatomy of EC cells, and basal processes, there is no data which analyses the relationship between EC cell neuropods and nerves within the human gut.

1.5.6 What drives connections between EECs and neurons?

It is apparent that EECs and neurons within the GI tract make anatomical connections, as shown through *in vitro* and *in vivo* studies (Bohórquez et al., 2014, Bohórquez et al., 2015, Bellono et al., 2017, Kaelberer et al., 2018), however the mechanism which drives the formation of such connections is not currently understood. It has been proposed by some that chemoattractants are released from both EECs and neurons to drive the extension of neuropods and neurites to form a connection (Bohórquez et al., 2014, Liddle, 2019). Enteric glia are important in the development of enteric neurons (Bush et al., 1998), known to drive axonal growth through the release of neurotrophins (Van Landeghem et al., 2011, Neunlist et al., 2014). The first evidence of a relationship between enteric glial cells and the L cell neuropods was recently described (Bohórquez et al., 2014), thought to imply that enteric glia drive neuropod growth from EECs through release of neurotrophic factors (Bohórquez et al., 2014). L cells express several receptors for glial derived neurotrophins, including TrkA and glial-derived neurotrophic factor 3 (Kaplan et al., 1991, Klein et al., 1991), which are stimulated through the neurotrophins nerve growth factor (NGF) and artemin respectively. NGF and artemin are two established neurotrophins known to stimulate axonal growth in neurons (Yasuda et al., 1990, Baloh et al., 1998, Madduri et al., 2009). These two neurotrophins were used to treat intestinal organoids, both found to increase the number of EECs containing neuropods, as well as increasing the length of neuropods on average (Bohórquez et al., 2014).

It is apparent that the brain must communicate directly with EECs, based on the recent evidence that the CNS responds to luminal stimuli *in vitro* and *in vivo* within a matter of milliseconds. Here, we have described the recent advances in the field, in which EEC basal extensions, termed neuropods, are hypothesised to form direct synaptic-

like connections with innervating neurons. With the exception of D cells, the exact function of neuropods in EECs is not yet understood, although, they have been suggested to facilitate direct connections between EECs and the brain. There is a gap in the literature surrounding the presence of EC cell neuropods, and the factors which facilitate the connections between EECs and neurons within the GI tract.

1.6 Concluding Remarks

EC cell serotonin has numerous functions throughout the body, in both central and peripheral physiology. The exact role for EC cell-derived serotonin in GI motility, and the mechanisms used by these cells to sense mechanical stimulation, are not completely understood. This is particularly the case due to a lack of availability of adequate tools to fully elucidate this system *in vivo*. The most recent evidence suggests that Piezo2, the newly identified mechanosensory ion channel, is important for mouse EC cell mechanobiology, however this has yet to be deduced in human EC cells. Furthermore, our understanding of connections between EECs and enteric neurons is also gaining momentum, with evidence now suggesting that EECs may form direct synaptic-like connections with innervating neurons. The exact mechanisms which facilitate such connections, and the communication of EC cells with enteric neurons, are currently understudied warranting deeper investigation.

Further knowledge on the nature of EC cell-neuronal communication is required to shed light on the mechanisms which underpin the gut-brain axis and form a greater understanding of the circuitry with which EC cells mediate their function. Overall, the two key questions of how serotonin is involved in GI motility, and how EC cells communicate with neurons, remain as an important and clinically relevant questions to be answered.

1.7 Experimental aims and hypotheses

The broad aims of the current study were to:

1. Validate the new specific, inducible $Tph1^{CreERT2/+}$;$Rosa26^{DTA/+}$ mice, and use this model to determine the importance of EC cell-derived serotonin in gut motility.

2. Investigate the presence and function of the mechanosensing ion channel, Piezo2, in mouse and human EC cells.
3. Identify and characterize neuropods in colonic EC cells.
4. Identify whether EC cells contact sensory nerve fibres in the colonic wall and determine the role of extrinsic sensory afferents in the formation of EC cell neuropods.

The broad hypotheses of the current study were that:

1. The inducible and specific ablation of EC cells will slow, but not cease, gastrointestinal motility.

2. Piezo2 is an ion channel significantly involved in the mechanosensing function of human EC cells, with implications in GI motility.

3. Both mouse and human colonic EC cells contain neuropods, which have the potential to form neural connections via direct connections.

4. The removal of extrinsic sensory innervation will alter EC cell neuropod formation in the mouse distal colon.

CHAPTER 2: The role of enterochromaffin cell derived serotonin in colonic motility.

2. The role of enterochromaffin cell derived serotonin in colonic motility.

2.1 Introduction

Serotonin was first implicated in gastrointestinal motility 70 years ago when Bulbring and Lin described the presence of the hormone during peristalsis (Bulbring and Lin, 1958). Since then, the role of serotonin in gut contraction has not been conclusively defined. Initial studies described the presence of high concentrations of serotonin around the time of contractile events in the guinea pig small intestine (Bulbring and Crema, 1959) and hypothesized that EC cells respond to the distention caused by luminal contents, which stimulates the release of serotonin, initiating peristalsis. Hence, serotonin was suspected to be the key driver of intestinal motility and for many years it was believed that peristalsis would fail to initiate in its absence.

Early studies found that the application of exogenous serotonin potently stimulated peristalsis (Bulbring and Lin, 1958) and that serotonin antagonists inhibit peristalsis and other migrating motor complexes (Bush et al., 2001). Numerous studies have since reported conflicting evidence regarding the role of serotonin in motility. One study reported a lack of colonic migrating motor complexes (CMMCs) in mucosa-free colonic preparations (Heredia et al., 2009). This group concluded that serotonin is vital for motility to occur since mucosa-free colons lack EC cell serotonin. Evidence from our research group disputes this conclusion. We confirmed that serotonin is released from EC cells in close temporal association with peristalsis (Keating and Spencer, 2010), and that cyclic motor activity still occurs, albeit more slowly, in mucosa-free colons lacking EC cell serotonin (Keating and Spencer, 2010). Furthermore, serotonin receptor antagonists used on colonic preparations lacking mucosa, and hence also lacking EC cell serotonin, blocked CMMCs in these preparations (Barnes et al., 2014). This result opposes earlier conclusions that serotonin is critical for motility (Bush et al.,

2001), as it indicates these antagonists act in a manner independent of EC cell serotonin.

In contrast, more recent studies using the pharmacological inhibition of Tph1 and genetic Tph1 ablation ($Tph1^{-/-}$) mouse models found no difference in total gastrointestinal (GI) transit time compared with controls (Yadav et al., 2010, Li et al., 2011), indicating that a lack of EC cell serotonin has no effect on *in vivo* transit. However, in further work on $Tph1^{-/-}$ mice, less frequent CMMCs were observed (Heredia et al., 2013). This was contrary to earlier work from this group, as motility remained despite the lack of EC cell serotonin. Further studies found that $Tph1^{-/-}$ mice have significant differences in their GI morphology compared with controls (Heredia et al., 2013), which likely influenced gut motility.

The existence of a second pool of serotonin derived from Tph2, contained in neurons within the enteric nervous system (ENS) (Walther et al., 2003), adds complication to the investigation of the role of serotonin in motility. It has proven difficult to distinguish between the separate effects of these two pools of serotonin on motility. Experiments using reserpine, a drug which inhibits the neuronal uptake of serotonin through VMAT (a vesicular serotonin transporter), found no differences in mucosa-free CMMCs between reserpine treated or control mice (Sia et al., 2013b, Spencer et al., 2013). This result indicates enteric neuronal derived serotonin does not play a role in regulating CMMCs. Although Tph2 knockout ($Tph2^{-/-}$) mice were found to have a significant reduction in all *in vivo* measures of GI transit (Li et al., 2011), further studies revealed that $Tph2^{-/-}$ mice were developmentally different including a decrease in myenteric neuronal density and proportions of certain neuronal types in the ENS. These differences occurred as Tph2-derived serotonin is important in the neurodevelopment of the ENS (Li et al., 2011, De Vadder et al., 2018).

It has proven difficult to distinguish the importance of EC cell derived serotonin in the peristaltic reflex due to the lack of an adequate model. The various pharmacological, surgical dissection, and genetic approaches previously utilized to advance understanding in this area have lacked specificity and physiological relevance. The need for an inducible Tph1 knockdown mouse model is merited due to the following reasons: 1) pharmacological approaches have maintained the physiological integrity of tissue, but are often not specific to Tph1 derived serotonin, or not totally effective in reducing mucosal serotonin (Yadav et al., 2010, Martin et al., 2019). 2) Dissection methods in which the mucosa is removed from the colon is not specific to effects on EC cell serotonin and also impair the integrity of the GI tract and removes other important epithelial cell types, leaving the question of its physiological relevance (Keating and Spencer, 2010). 3) Constitutive mouse knockout models are severely impacted during development, including compensatory adjustments to intestinal morphology (Heredia et al., 2013) and impaired development of the ENS (Li et al., 2011), leaving potential cofounding factors in results gathered using these mice. Although each model has contributed to our understanding of the role of serotonin in this system, it is clear they each come with their own disadvantages. Thus, a superior model is needed to specifically knockdown EC cells post-development and conclusively define the role of Tph1 derived serotonin in motility.

To finally deduce the role of EC cell derived serotonin in motility and define the importance of the hormone in peristalsis, we used an inducible Tph1 knockdown mouse model $Tph1^{CreERT2/+}$;$Rosa26^{DTA/+}$ (referred to as Tph1 DTA mice in the following chapter) (Wei et al., 2021). In Tph1 DTA mice, tamoxifen injections promote the production of diphtheria toxin (DTA) only in cells expressing Tph1, causing cell death via its inhibition of protein synthesis (Collier, 1975). This mouse model will allow us to

specifically test the importance of EC cell serotonin in GI motility of developmentally normal mice. The following chapter contains results from *ex vivo* colonic experiments performed by me in the laboratory of Associate Professor Seungil Ro at the University of Nevada (Reno) during my time on site in this laboratory. The specific aims are to:

1. Confirm the reduction in mucosal serotonin in Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mice and test whether morphological changes occur in Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mouse GI tract;

2. Test whether motility changes are observed in Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ colon.

The overriding hypothesis of this chapter is that the inducible and specific ablation of EC cells will slow, but not cease, gastrointestinal motility.

2.2 Methods

2.2.1 Preparation of Mouse Tissues

$Tph1^{CreERT2/+}$;$Rosa26^{DTA/+}$ (Tph1 DTA) male mice with a C57BL/6 background, of 8-12 weeks old were used in the following chapter. Generation of this mouse line was described previously (Wei et al., 2021). A $Tph1^{CreERT2/+}$ mouse was (Wei et al., 2021) crossed with a $Rosa26^{DTA/+}$ mouse line (The Jackson Laboratory, B6.129P2-Gt(ROSA)26Sortm1(DTA)Lky/J, stock no. 009669), to create a $Tph1^{CreERT2/+}$;$Rosa26^{DTA/+}$ mouse model. Tph1 DTA mice were intraperitoneally injected with tamoxifen (Tx) (Sigma Aldrich, 1.0mg/20g body weight daily) for 5 consecutive days (Wei et al., 2021). The control groups were injected with the same volume of sunflower oil (Sigma Aldrich). The mice were then anesthetized through CO2 inhalation, followed by cervical dislocation in accordance with approval by the Institutional Animal Care and Use Committee at University of Nevada. A ventral midline incision was made to expose the peritoneal cavity, and the intestines were gently removed from the animal. For mechanical recordings, segments of mouse colon ~8 cm long were placed into beakers containing oxygenated Krebs solution ((in mM): NaCl, 118; KCl, 4.7; NaH_2PO_4, 1.0; $NaHCO_3$, 25; $MgCl_2$, 1.2; D- Glucose, 11; $CaCl_2$, 2.5) and bubbled with 95% O_2/5% CO_2.

2.2.2 Immunohistochemistry

Intestinal tissue was removed from mice and fixed with 4% paraformaldehyde (PFA) at 4°C for 20 minutes and left in 1 x Tris-buffered saline (TBS) at 4°C overnight. Samples were dehydrated in 20% sucrose in TBS at 4°C. Tissue was frozen in 1:1 optimum cutting temperature (Sakura)/20% sucrose in TBS using liquid nitrogen. Frozen tissue was cut into 8 µm-thick cryosections, then used for

immunohistochemistry experiments. Sections were blocked with 0.5% Triton X-114, 4% skim milk in TBS for 1 hour at room temperature, then washed with TBS twice for 10 minutes each. Primary antibodies directed for Tph1 (1:200, Rabbit anti-Tph1 IgG, Cat# ab52954, Abcam) was applied for 48 hours on a rocker at 4°C. Sections were washed with TBS twice for 10 minutes and then incubated with secondary antibodies conjugated to Alexa Fluor 488 (1:500, Goat Anti-Rabbit IgG H&L, Cat# ab150077, Abcam) for 2 hours at room temperature. The sections were washed 3 times with TBS for 10 minutes each, dried, and mounted with mounting medium containing DAPI (Wei et al., 2021).

2.2.3 Microscopy and Image Analysis

All images were taken using an Olympus Fluoview FV1000 confocal laser scanning microscope. Tph1 cell knockdown was analysed using ImageJ software (National Institutes of Health) (Wei et al., 2021).

2.2.4 Measuring colonic motility *ex vivo* via mechanical recordings

The colons were removed from mice and maintained in Krebs solution, as above (2.2.1). The colons were left to naturally expel faecal pellets in 37°C Krebs and after 10 minutes, the remaining faecal pellets were gently flushed out with 37°C Krebs. Each end of the colon was anchored into an organ bath using fine suture thread and microhooks. Three hooks were connected to three separate regions of the colon, which were attached to three force transducers with fine suture thread. A resting tension of 0.5 g was applied to all preparations. These force transducers were connected to three separate custom-made preamplifiers (Biomedical Engineering, Flinders University), all connected to a PowerLab 4/30 (AD Instruments, Castle Hill, NSW, Australia). Recordings were made and analysed using LabChart (version 8.0, ADInstruments).

2.2.5 Measuring contractions on a fixed pellet *ex vivo*

The colons were removed, prepared, and anchored as described above (2.2.4). Fresh faecal pellets were collected from mice, covered in superglue (Gorilla) and attached to fine suture thread. These pellets were inserted into the oral end of the colon, allowed to naturally propel along the colon until stopped halfway along. The other end of the suture thread was connected to a force transducer, measuring the forces placed on the fixed pellet by colonic contractions. These recordings were made using the same equipment as above (2.2.5).

2.2.6 *Ex vivo* total transit time

Colons were removed, prepared, and anchored as described above (2.2.4). Fresh faecal pellets were collected from mice and covered in superglue (Gorilla), then inserted into the oral end of the colon. Pellets were allowed to freely move along the colon with natural contractions, this was filmed, and the time taken for the pellet to transverse to the anal end of the colon was recorded as the *ex-vivo* total transit time.

2.2.7 Serotonin ELISA assay

Serotonin levels in serum, lysed mucosa, and lysed brain were measured using a serotonin ELISA (Serotonin Research ELISA, LDN, Germany, Cat# BA E-5900R). Mucosal samples were collected from the desired regions of the GI tract, and stored in RIPA buffer (Thermo-Fischer), and treated with serotonin stabilizer (1:1000, Stabilizer, BA E-5937, LDN, Germany). Whole blood samples were collected from mice and rested for 20 minutes at room temperature to allow the platelets to clot, samples were then centrifuged at 12,000 rpm for 10 minutes and the serum collected. Tissue samples were lysed using a homogenizer at 4°C. The homogenates were centrifuged at 12,000 rpm at 4°C for 20 min, and supernatants were collected. All

samples were stored at -80°C until analysis. Protein concentrations of mouse GI tissue samples were determined using the BCA assay (Thermo-Fisher). All samples were run in duplicate on the ELISA plate and normalized to protein concentrations. The ELISA was performed as per manufacturer's instructions and results analysed using a 4-parameter sigmoidal curve with GraphPad Prism (Version 9, GraphPad Software, Inc., San Diego, CA, USA).

2.2.8 Statistical analysis

Statistical analysis was performed by using GraphPad Prism. Results with two groups were tested for statistical differences using a two-tailed unpaired t-test, unless otherwise specified. Statistical analysis on results with multiple comparisons was tested using a one-way ANOVA with post-hoc Tukey's multiple comparisons test, unless otherwise specified. Differences were considered significant when $p<0.05$. All statistical results are reported as mean +/- standard error of the mean unless where otherwise stated. The number of animals used in each set of experiments is indicated as lower case 'n'. All non-significant differences ($p>0.05$) are reported as 'ns'.

2.3 Results

2.3.1 Ablation of EC cells and intestinal morphology in Tph1$^{CreERT2/+}$;Rosa$^{26DTA/+}$ mice

Our collaborators from the University of Nevada have developed an inducible mouse line, Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ (Wei et al., 2021), in which tamoxifen (Tx) treatment induces diphtheria toxin (DTA) activation specifically in Tph1 expressing cells. DTA expression inhibits cellular protein synthesis which leads to apoptotic death of the target cell, in this case EC cells. To confirm the phenotype of Tph1 DTA mice, intestinal tissue from oil-treated (control) mice and Tx-treated mice was collected and immunohistochemically stained for Tph1 (Figure 2.1A). These images confirm the loss of Tph1 containing cells in the Tx-treated Tph1 DTA mice compared with oil-treated controls (Figure 2.1A) (for quantification see (Wei et al., 2021)). We confirmed whether these mice experienced any intestinal morphological changes, as seen in previous Tph1 genetic knockout approaches (Heredia et al., 2013). No difference in intestinal length was seen in the small intestine, cecum or colon between control and Tx-treated mice (Figure 2.1B-D). Finally, to ensure the mouse model was specific for Tph1 producing cells and did not affect the levels of Tph2 derived serotonin, measurements were taken of serotonin levels in the brains and intestinal mucosa of control and Tx-treated Tph1 DTA mice by ELISA (Figure 2.2). There was no change in serotonin levels in the brain between groups but a significant decrease in mucosal serotonin of the Tx-treated mice compared with controls. These results support this model as an inducible and reliable EC cell depletion model, and hence a suitable model to determine the role of EC cell derived serotonin on gastrointestinal motility.

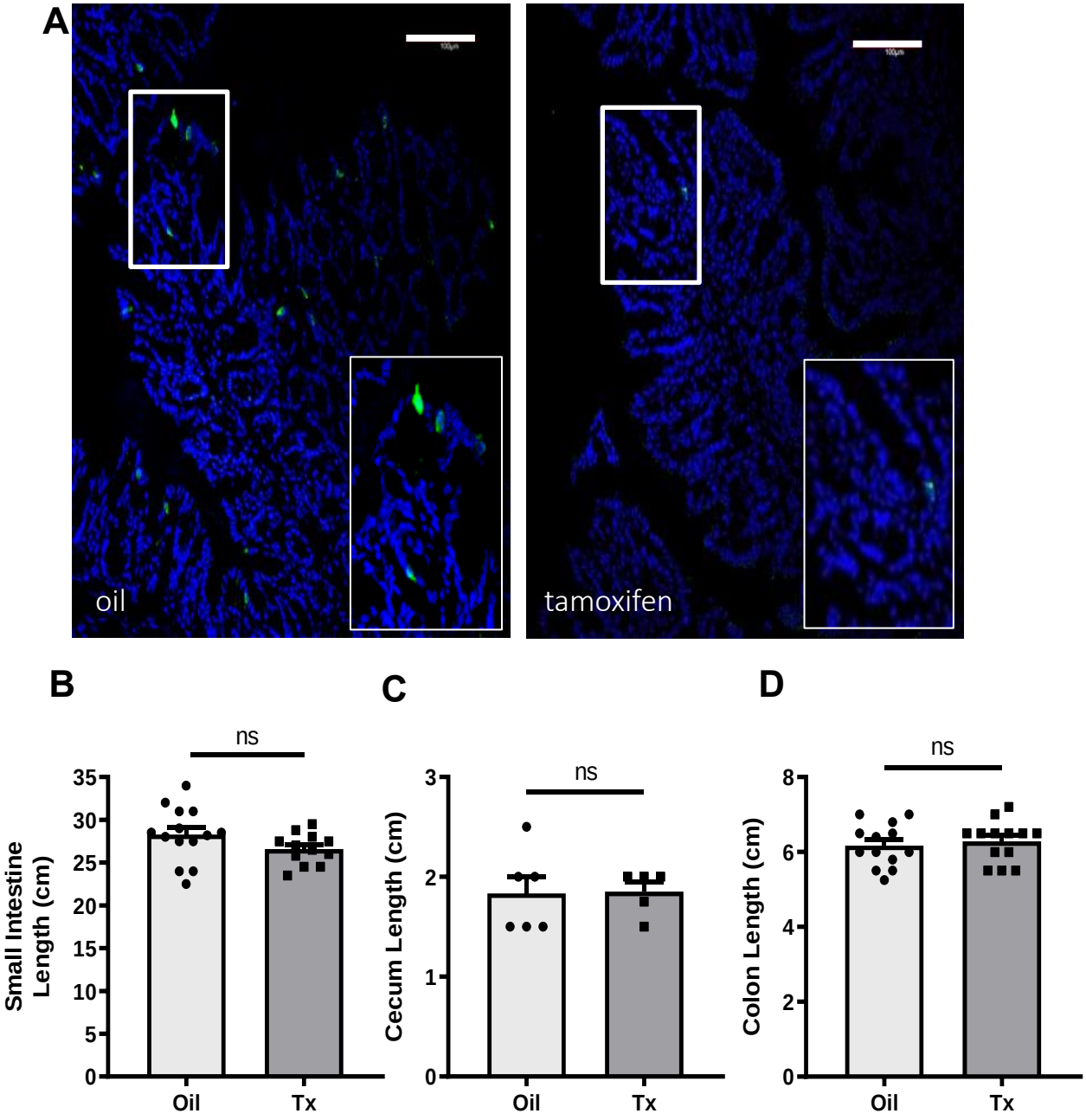

Figure 2.1: Confirmation of the Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mouse model.

(A) Tph1 (green) staining in Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mice colon after 5 days showing near complete ablation of Tph1^{+} cells in tamoxifen treated tissue compared with oil controls, enlarged examples inset in white boxes. Scale bar = 100 μm. (B) The lengths of Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ small intestine (n=14) (A), cecum (n=5) (B), and colon (C) after 5 days oil or tamoxifen treatment (n=14). Data are shown as mean ± SEM.

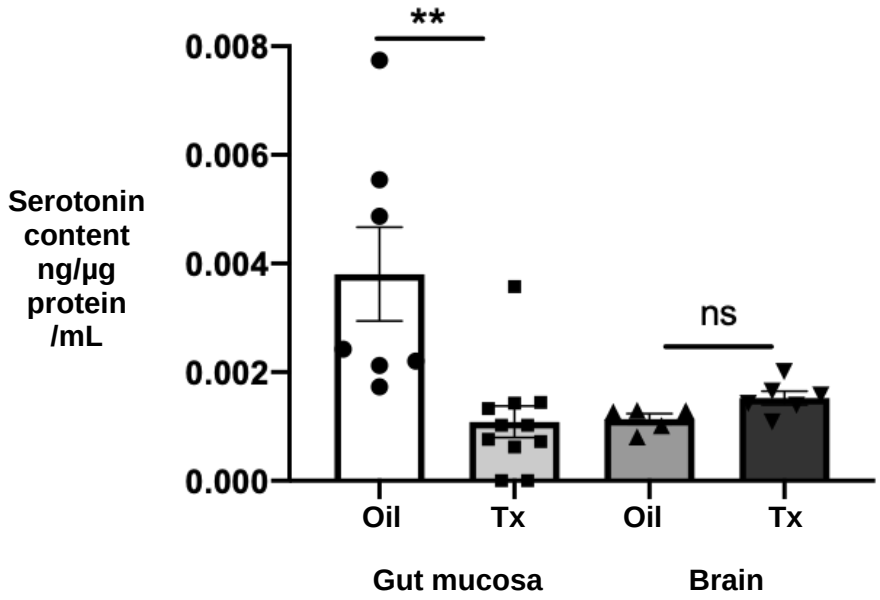

Figure 2.2: Confirmation of the specificity of EC cell serotonin knockdown in Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mice.

Serotonin content in the brain and intestinal mucosa confirmed the Tph1 specific knockout of serotonin production in EC cell ablated mice (Tx; n=11) compared with oil injected controls (n=7). Data are shown as mean ± SEM. **p < 0.01

2.3.2 *Ex vivo* colonic migrating motor complexes are less frequent but still present in Tph1$^{CreERT2/+}$;Rosa$^{26DTA/+}$ mice

To test the role of EC cell derived serotonin on *ex-vivo* colonic motility, the Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mouse line was used to evaluate colonic migrating motor complexes (CMMCs) in EC cell-depleted and control mice. CMMCs were measured from oil- and Tx-treated mice, using force transducers (see Method 2.2.5). The example traces shown in Figure 2.3 are representative of the change in characteristics of proximal CMMCs seen between control (oil-injected) and Tx-treated Tph1 DTA mice. There was a significant decrease between control (oil-injected) and Tx-treated proximal CMMCs in the frequency of contractions (Figure 2.4A), as well as the percentage of contractions which propagated down the colon from the proximal to the distal end (Figure 2.4B). The CMMC frequency was positively correlated with serum serotonin concentrations across both Tx-treated and oil-treated Tph1 DTA mice (Figure 2.5). To rule out the potential effects of tamoxifen on motility, Cre$^{-/-}$ mice were also tested after being given the same tamoxifen treatment regime as Tx-treated Tph1 DTA mice and analysed the CMMCs *ex vivo*. The Tx-treatment of C57BL/6 mice revealed no effect of tamoxifen on colonic motility compared with oil injected control mice in both frequency and propagation (Figure 2.4). These results indicate that EC cell derived serotonin is not essential for CMMC generation but has a role in the modulation of frequency and propagation of contractile events down the colon.

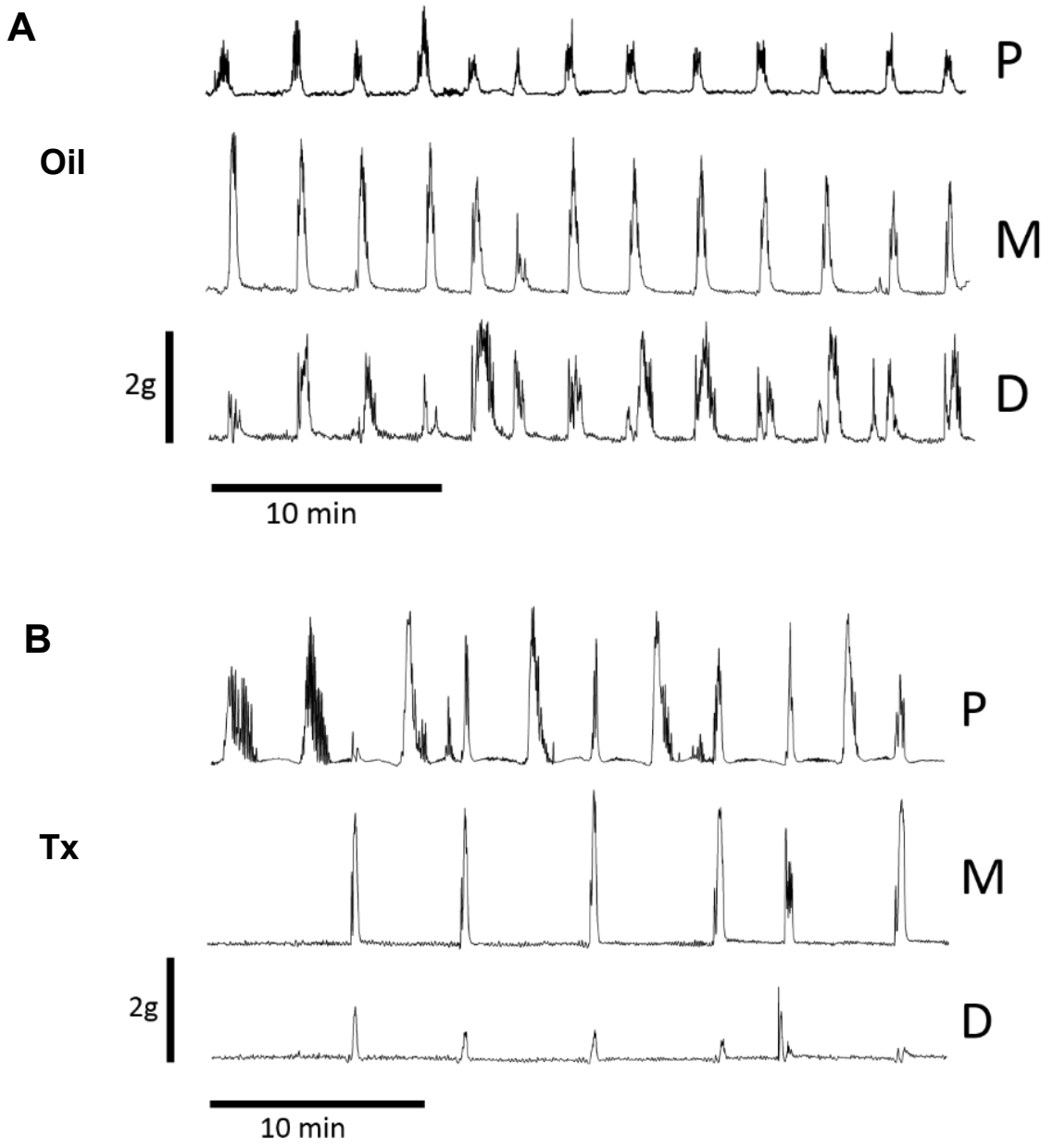

Figure 2.3: Changes in CMMCs in EC cell depleted Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mice.

Example traces of (A) oil-injected and (B) tamoxifen treated (Tx) Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ CMMCs recorded from the proximal (P), mid (M) and distal (D) region of the colon.

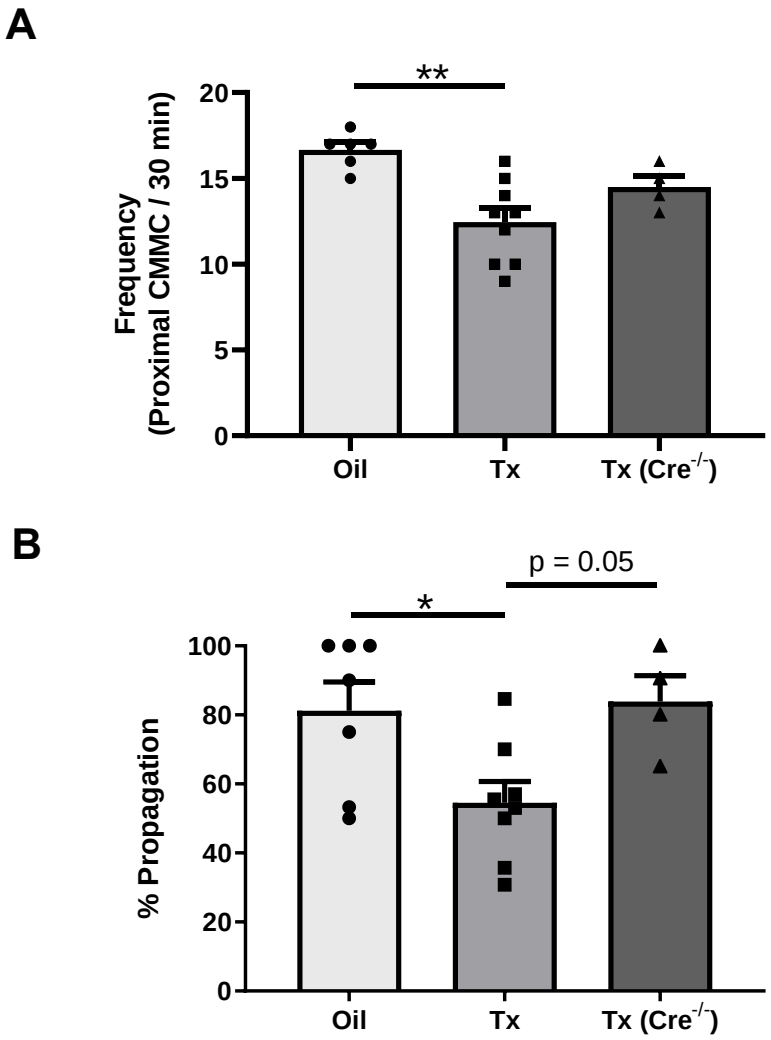

Figure 2.4: Changes in CMMCs in EC cell depleted Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mice.

(A) Frequency of proximal CMMCs from oil or tamoxifen injected Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mice, and tamoxifen injected Cre$^{-/-}$ mice. (B) The percentage of CMMCs which propagate from the proximal to distal colon of oil or tamoxifen injected Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mice, and tamoxifen injected Cre$^{-/-}$ (C57BL/6) mice. Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$

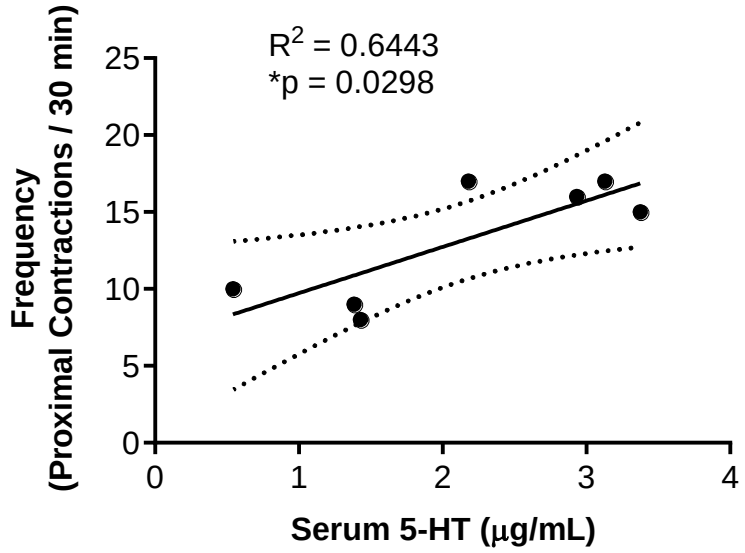

Figure 2.5: The frequency of CMMCs is correlated with serum serotonin levels.

The frequency of baseline proximal contractions of $Tph1^{CreERT2/+}$;$Rosa26^{DTA/+}$ mice plotted against serum serotonin (5-HT) levels. Data are shown as mean ± SEM. *p < 0.05

2.3.3 Contractions on a fixed pellet are reduced in force and frequency in Tph1$^{CreERT2/+}$;Rosa$^{26DTA/+}$ mice lacking serotonin

To measure the effect of EC cell derived serotonin on peristalsis, the force of contractions used on a natural pellet fixed in place was recorded using *ex vivo* colons from control and Tx-treated Tph1 DTA mice (Figure 2.6A). This allows the measurement of the peristaltic reflex stimulated by a natural bolus to propel it distally towards the rectum. The force of contraction (amplitude) was reduced in Tx-treated Tph1 DTA colons compared with controls (Figure 2.6C). The frequency of contractions was also decreased in Tph1 DTA mice compared with controls (Figure 2.6B). These results support the notion that EC cell derived serotonin is not required for peristalsis but does have a role in the strength and timing of the peristaltic reflex.

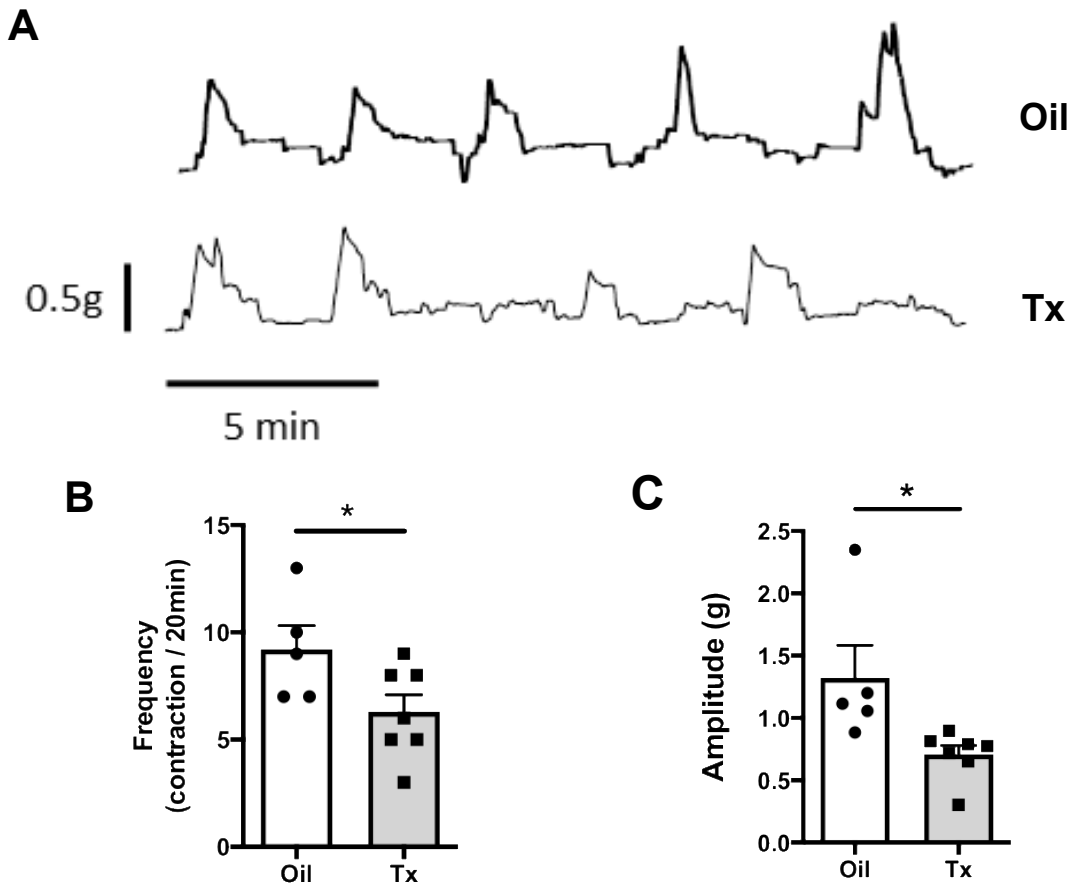

Figure 2.6: A lack of Tph1 causes a reduction in the frequency and amplitude of contractions on a fixed pellet *ex vivo*.

(A) example traces of oil-injected and tamoxifen treated Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ contractions on a fixed pellet. The baseline frequency (B) and amplitude (C) of fixed-pellet contractions *ex vivo* of control (oil injected) and tamoxifen injected (Tx) Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mice. Data are shown as mean ± SEM. *p < 0.05

2.3.4 *Ex vivo* colonic transit time is slower in EC cell depleted mice

The time taken for a natural pellet to transit the length of the colon was measured *ex vivo* in control and Tx-treated Tph1 DTA colons to deduce the effect of serotonin on peristalsis. The time taken for the pellet to move down the Tx-treated Tph1 DTA colons was on average 4 times ($p < 0.05$) longer than controls (Figure 2.7). The significantly longer time observed in the Tx-treated Tph1 DTA colonic transit time indicates that the lack of EC cell derived serotonin contributes significantly to the efficiency of peristalsis but does not inhibit the occurrence.

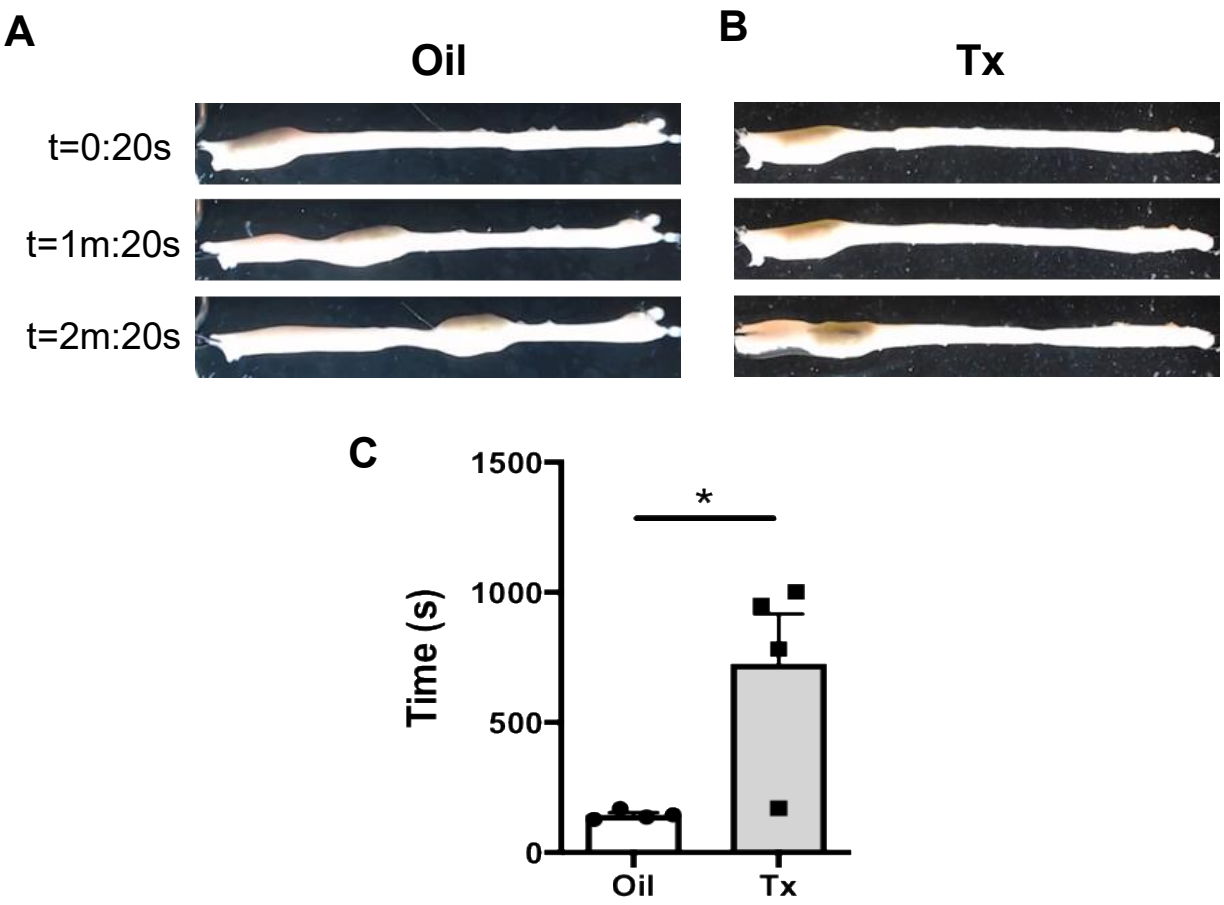

Figure 2.7: *Ex vivo* colonic transit is slower in mice lacking EC cell serotonin.

Representation of *ex vivo* colonic transit of a pellet along the colon of a (A) oil and (B) tamoxifen treated Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mouse at various time points (t). (C) *Ex vivo* transit time for complete pellet transit in oil vs tamoxifen treated Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mice *ex vivo*. Data are shown as mean ± SEM. *p < 0.05

2.3.5 Fluoxetine effect on GI motility of Tph1$^{CreERT2/+}$;Rosa$^{26DTA/+}$ mice

Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) routinely prescribed for anxiety and depression (Wong et al., 1974, Hillhouse and Porter, 2015). SSRIs function by blocking the serotonin transporter, SERT, from allowing the reabsorption of serotonin into SERT containing cells (Ross and Renyi, 1969). Thus, free serotonin accumulates outside of cells and is readily available to act on various serotonin receptors. The use of fluoxetine (2 µm, Sigma-Aldrich) on CMMCs in control C57BL/6 mouse colons *ex vivo* was trialled to determine the role of excess endogenous serotonin on motility. Preliminary work by our research group has found that while fluoxetine slows the frequency of CMMCs in an intact preparation, it has no effect when the mucosal layer (and therefore all EC cells) is removed by dissection (see Appendix Figure 1). Thus, suggesting fluoxetine must affect colonic motility through EC cell derived serotonin, with no role for neuronal serotonin in regulating GI motility. We wanted to confirm this by repeating this experiment in a superior model, such as the Tph1 DTA mouse model, with the benefit of a genetic approach to specifically remove EC cells thus not needing to remove the entire mucosal layer. CMMCs of intact *ex vivo* colons from control and Tx-treated Tph1 DTA mice at baseline and with fluoxetine (2 µM). Fluoxetine had similar effects on control Tph1 DTA mice as was found with mucosa-on preparations, in which a decrease in frequency is seen in CMMCs with treatment (see Figure 2.8A). Also, in accordance with initial results using mucosa-free colons we found that fluoxetine did not affect CMMC frequency in EC cell depleted mice. We additionally tested the effects of fluoxetine on fixed pellet experiments to test whether neuronal serotonin had an impact on the peristaltic reflex, and similarly found fluoxetine reduced the frequency of contractions on a natural pellet stimulus in control conditions but had no significant effect in Tx-treated Tph1 DTA on

the frequency of contractions of a fixed pellet (see Figure 2.8B). These results support the notion that fluoxetine affects colonic motility through EC cell derived serotonin and indicates that ENS-derived serotonin does not regulate motility.

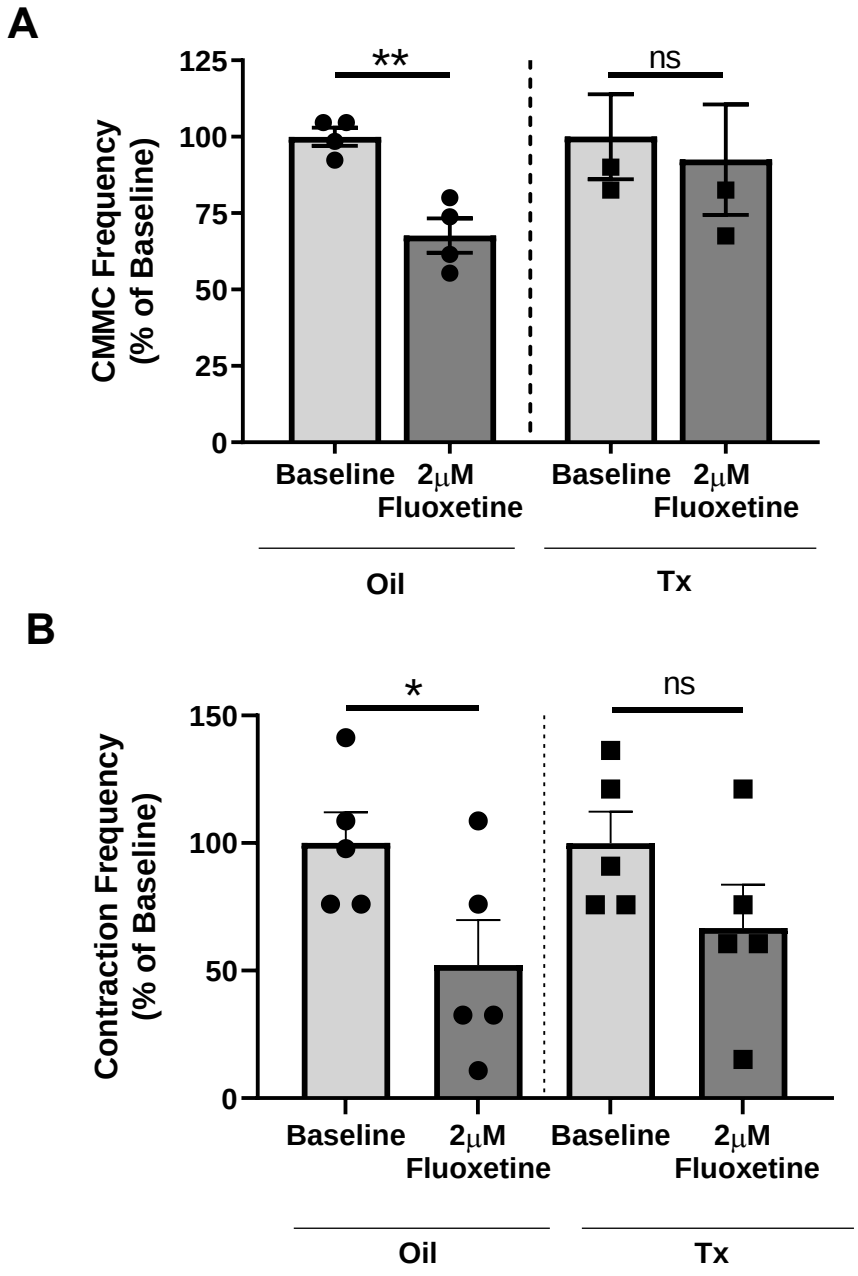

Figure 2.8: Fluoxetine works through EC cell serotonin, with no role for neuronal serotonin in motility.

The frequency of proximal CMMCs (A) and fixed-pellet contractions (B) measured from oil and tamoxifen injected Tph1$^{CreERT2/+}$;Rosa26$^{DTA/+}$ mice, with fluoxetine (2 μM) . Data are shown as mean ± SEM. *p < 0.05

2.4 Discussion

The precise role of EC cell derived serotonin in gastrointestinal (GI) motility has long been contested within the field. The suitability of the models used in research addressing this question has been a consistent issue. The aim of this body of work was to determine the importance of serotonin in colonic motility using a new specific and inducible mouse model $Tph1^{CreERT2/+}$;$Rosa26^{DTA/+}$ (Tph1 DTA), that specifically targets the ablation of EC cells. Determining the role of serotonin in motility will provide a better understanding of the mechanisms involved in normal GI motility. This is crucial considering the regulation of GI motility is reasoned essential for overall healthy GI function (Gershon, 2004). To elucidate the exact role of serotonin in this process will aid in highlighting key aspects of motility to target for the treatment of motility issues and thus, enable the better management of various motility disorders.

This study has validated the highly specific, inducible Tph1 depletion mouse model, Tph1CreERT2/+;Rosa26DTA/+, and verified its appropriateness as a tool in deducing the role of EC cell serotonin in motility. The lengths of all components of the GI tract were found to be unchanged in this inducible model indicating no obvious change in intestinal morphology. This is unlike differences seen previously in $Tph1^{-/-}$ intestines (Heredia et al., 2013). The inducible nature of the new model allows it to bypass the developmental effects on intestinal morphology seen in $Tph1^{-/-}$ mice. The size of faecal pellets appeared larger in Tph1 DTA mice (Wei et al., 2021), as was reported in $Tph1^{-/-}$ (Heredia et al., 2013), demonstrating that a lack of serotonin can impact the size of faecal pellets. This could be due to a reduced frequency of contractions seen in the Tph1 DTA mouse model. It has recently been confirmed in mice, as previously in guinea pig (Costa et al., 2015), that faecal pellets are formed at a functional colonic flexure between the proximal and distal colon (Costa et al., 2021). These motor

patterns are preserved down the entire length of the colon (Costa et al., 2015), and contractile activity forming large boluses have been shown to be less frequent (Ehrlein et al., 1983). Hence, the rate of GI motility impacts the size of faecal pellets. The contractions measured in our colonic preparations from Tph1 DTA mice were significantly slower. The less frequent contractions likely produced the larger faecal pellets seen from EC cell depleted mice. The consistency of content is another determination of pellet size (Ehrlein et al., 1983), with more fluid consistencies resulting in larger faecal pellets (Costa et al., 2015). The predominant absorption of water along the GI tract occurs in the SI, aided by a process known as segmentation (Huizinga et al., 2014). Segmentation is a series of contractions local to the small intestine, which mix content to stimulate nutrient and water absorption (Huizinga et al., 2014). Serotonin has been linked with segmentation, increasing the number of segmentation contractions after a nutrient meal (Siegle and Ehrlein, 1989). EC cell serotonin is also proposed to play an important role in the feedback loop which controls segmentation (Ellis et al., 2013). Furthermore, serotonin has been demonstrated to significantly enhance water absorption along the GI tract (Kisloff and Moore, 1976). Hence in our model, a lack of serotonin could contribute to a decrease in water absorption, and therefore more fluidic faecal content in the proximal content leading to larger faecal pellets. Overall, these results indicate that this inducible Tph1 DTA mouse model does not contain the developmental perturbances noted in the intestinal morphology of the previous Tph1$^{-/-}$ model. We believe that the inducible Tph1 DTA model is an advanced tool, which bypasses previous issues, to better deduce the role of serotonin in motility.

The specificity of this model was evident through both the depletion of Tph1 positive cells in Tx-treated Tph1 DTA mouse GI tissue, and the significant reduction in EC cell derived serotonin content in the GI mucosa. No difference was shown between the

levels of serotonin in control and Tx-induced Tph1 DTA mouse brains, consistent with previous Tph1$^{-/-}$ models (Walther et al., 2003), indicating our Tph1 DTA model had no effect on Tph2 derived serotonin levels, and further confirming the specificity of this model. The Tph1 DTA model did not produce a total knockout of mucosal serotonin, as there were remaining levels of the hormone post-tamoxifen treatment. The most likely explanation for this detection is that the Tx treatment of our model wasn't long enough to deplete all EC cell serotonin, hence a longer protocol might have further reduced serotonin levels. This is reflected in a recent study on the refinement of Tx injection protocols, which highlights several parameters which can affect the specificity and effectiveness of Cre-loxP recombination *in vivo*. Such parameters include the dose and duration of Tx, the level of CreERT2 expression and tissue type, as different tissues respond differently to Tx protocols (Jahn et al., 2018). Another possibility for the detection of remaining serotonin is it being taken up by other colonic epithelial cells containing the serotonin reuptake transporter (SERT) in the mucosal lining of Tph1 DTA mice. Enterocytes, as well as certain enteroendocrine cell types known to contain SERT (Wade et al., 1996, Chen et al., 1998) (Lund et al., 2020) could have taken up serotonin and potentially contributed to the remaining serotonin in the mucosa. Together, these postulations likely explain the residual serotonin present in the Tph1 DTA mucosa. Ultimately, EC cell serotonin was specifically reduced in this mouse model, hence indicating this model is ideal for testing the role of EC cell derived serotonin in motility.

A number of *ex vivo* colonic motility tests were performed in this investigation to determine the effect which a lack of EC cell serotonin has on the characteristics of colonic motility. Colonic motility has been described by numerous studies, using varying terms, animal models, and experimental tools (Corsetti et al., 2019). Here, we describe the effects of serotonin on CMMCs, cyclic contractions that propagate along

the colon regardless of the presence of content (Spencer et al., 2016). Although CMMCs can occur regardless of faecal content, distention of the bowel has been shown to increase CMMCs frequency (Zagorodnyuk and Spencer, 2011) indicating a relationship between the presence of content and CMMCs. The inducible knockdown of EC cell serotonin in our model was found to reduce both the frequency and propagation of CMMCs along the colon, indicating that CMMCs are not likely to depend on EC cell serotonin. This data supports previous findings where the removal of the mucosa, and hence EC cell serotonin, also decreased the frequency of CMMCs (Keating and Spencer, 2010). Similarly, a decrease in *ex vivo* CMMCs was also reported in Tph1$^{-/-}$ mice (Heredia et al., 2013). Serum serotonin levels were found to have a positive correlation with proximal CMMC frequency. As gut serotonin levels are known to substantially contribute to serum serotonin levels (Yano et al., 2015), this further highlights the relationship between serotonin and colonic motility. This infers that one role of EC cell derived serotonin is to modulate the frequency of cyclic contractions. Contractile events on a fixed pellet were also measured *ex vivo*, experiments that allow the unique collection of the size and frequency of contractions in direct response to a natural stimulus. The recordings from a fixed pellet are thought to reflect the *in vivo* peristaltic reflex (Bayliss and Starling, 1899, Heredia et al., 2013). In these fixed pellet experiments, it was clear that the force and number of contractions on a natural stimulus were reduced compared with controls. This reduction in amplitude has been previously noted in fixed faecal pellet contractions with the application of a 5-HT$_3$ receptor antagonist, ondansetron (Heredia et al., 2009). Furthermore, the peristaltic reflex of Tph1 DTA mice was tested *ex vivo* using a natural faecal pellet to further determine the role of serotonin in colonic motility. Peristalsis is defined as a propulsive movement initiated by a bolus, occurring due to the coupling of myogenic and neurogenic rhythmic contractions (Spencer et al., 2018, Corsetti et

al., 2019). The time taken for the pellet to freely transverse the length of the colon, known as the total colonic transit time, was significantly slower in Tph1 DTA preparations lacking serotonin. This result reflected previous findings, in which the introduction of a faecal pellet into mucosa-free colonic preparations still initiated peristalsis albeit significantly slower than intact preparations (Spencer et al., 2011). Together, all *ex vivo* measurements of contractile activity in the current study suggest that EC cell serotonin does not drive peristalsis, but rather has a role in modulating certain characteristics of these cyclic contractions. This links to previous real-time recordings of serotonin release from the colonic mucosa, in which serotonin was hypothesised to be released as a consequence of and not prior to contractions (Keating and Spencer, 2010). These findings support our hypothesis that serotonin is a modulatory molecule of peristalsis, and not the driving force.

Distension within the colon stimulates an oral ascending excitatory contraction and a descending inhibitory relaxation (Bayliss and Starling, 1899), facilitating the propulsion of a bolus along the colon. It has previously been hypothesised that the propulsion of content is accomplished through the combination of CMMCs and activation of the peristaltic reflex through distension, working together to form propulsive movements (Spencer et al., 2016). Both CMMCs and peristalsis are neural in origin, as they are both inhibited through the application of tetrodotoxin (Spencer et al., 2018). It has also been confirmed in this study, as supported by previous studies (Keating and Spencer, 2010, Spencer et al., 2011, Heredia et al., 2013), that serotonin is not essential for CMMCs or peristalsis to occur. With significantly reduced serotonin this Tph1 DTA model experiences a diminished rate of cyclic contractions, and slower pellet propulsion both *ex vivo* and *in vivo*, indicating a role for serotonin in the modulation of colonic motility. It is proposed that serotonin behaves as a signaling molecule, used by EC cells in a feedback loop controlling the rate of cyclic contractions and peristalsis.

In line with this theory, the addition of exogenous serotonin dramatically increases the rate of CMMCs (Bulbring and Lin, 1958) and initiates strong responses on neuronal excitability and transmitter release (Gershon, 2000). It is likely that EC cell serotonin plays a role in this feedback loop, as the release of serotonin from the mucosa stimulates downstream neural pathways via intrinsic primary afferent nerves (IPANs) (Spencer et al., 2016). A similar 'neuromechanical loop' has been hypothesized previously (Spencer et al., 2016), and this new data using Tph1 DTA mice provides further evidence supporting this hypothesis. EC cells are mechanically stimulated through colonic distention, mediated by their recently identified mechanosensing properties (Wang et al., 2017, Alcaino et al., 2018). Mechanically stimulated serotonin release signals to intrinsic mucosal afferents, including mechanosensitive myenteric and submucosal sensory neurons (Bertrand et al., 1997, Furness et al., 2004b), which contain serotonin receptors (Gershon, 2000, Gershon, 2004) and are responsive to serotonin (Bulbring and Crema, 1959, Pan and Gershon, 2000, Heredia et al., 2009). These IPANs then signal to both excitatory and inhibitory motor neurons, stimulating a neural-mediated contraction to push the bolus along the colon (Spencer et al., 2016). As the bolus advances, it continues to activate this pathway. Hence the positive feedback loop continues, whereby serotonin is released in response to mechanical stimuli, signaling to various afferents, which signal to both excitatory and inhibitory motor neurons perpetuating cyclic contractions and peristalsis, repeatedly continuing the loop which propagates along the colon and ultimately causes propulsion of content.

Importantly, *in vivo* tests were conducted on Tph1 DTA mice, with both colonic and total gastrointestinal transit time measured by our collaborators (Wei et al., 2021). All *in vivo* GI transit measures revealed a reduction in GI motility in EC cell depleted mice, confirming *ex vivo* results discussed above. This result contrasts previous findings

from both the pharmacological inhibition of Tph1 (Yadav et al., 2010) and Tph1$^{-/-}$ mice (Li et al., 2011), which saw no change in *in vivo* GI transit. One important note to make, is the significant difference in timeframes of EC cell depletion between pharmacological inhibition and Tx induced Tph1 knockdown. The timecourse for the Tph1 inhibitor, LP533401, used by Yadav et al is around 1 month (2010), which could be enough time for compensatory effects to kick in. Comparatively, the timecourse for Tx treatment is just 5 days. The use of LP533401 has previously decreased serum serotonin by 80% (Yadav et al., 2010). Our lab has unpublished data using LP533401 *in vivo*, where total GI transit time was also unchanged (see Appendix Figure 2). When we investigated the levels of serotonin remaining in the mucosa, only a ~60% decrease was found (see Appendix Figure 3), showing that the amount of mucosal serotonin still present likely facilitates motility as normal. Tph1$^{-/-}$ mice undergo adaptive compensation, as discussed earlier, which includes significantly larger intestines in these mice. CMMCs in gut from Tph1$^{-/-}$ mice are significantly slower than control (Heredia et al., 2013), likely due to the lack of serotonin release feeding into the neuromechanical loop. However, no change is seen in total GI transit time *in vivo* of these mice (Heredia et al., 2013), which suggests the morphological adaptions must be enough to overcome the lack of EC cell serotonin in *in vivo* motility. The Tph1 DTA model used in this study is a more elegant model, ruling out the need to use pharmacological tools (Yadav et al., 2010) and circumventing the compensatory adaptations seen in congenital KO colons (Heredia et al., 2013). This study clearly suggests that mice lacking EC cell serotonin have a significantly slower GI transit time, supporting our initial hypothesis that serotonin is not essential but has an important role in modulating motility.

To counter the potential confounding results of using tamoxifen (Tx) to switch on the Cre^{ERT2}/loxP system in our genetic approach to EC cell knockdown, we tested *in vivo*

motility of Tx injected $Cre^{-/-}$ C57BL/6J mice. Tx is an anticancer drug commonly used for the treatment of breast cancer (Osborne, 1998), reported to have frequent GI side effects in patients (Lee et al., 2005). Only a singular study has reported any effects of Tx on GI motility in the literature, suggesting Tx acts on ion channels in mouse colonic smooth muscle cells to decrease GI motility (Lee et al., 2005). However, this study only reported the effect of Tx on voltage dependent potassium channels in isolated mouse colonic smooth muscle cells, without demonstrating an effect directly on GI motility. Here we show no change in *ex vivo* CMMCs of Tx treated C57BL/6J mice in comparison to oil injected controls and conclude that Tx treatment does not have an impact in our results or on overall GI motility (Wei et al., 2021).

It is possible that other non Tph1 derived factors secreted from EC cells play a role in regulating motility. Enteroendocrine cells, including EC cells, contain a profile of secreted hormones (Haber et al., 2017). One such candidate from EC cells are the tachykinins (Roth and Gordon, 1990, Haber et al., 2017, Beumer et al., 2020), which includes the neuropeptides Substance P, neurokinin (NK) A, and NK B (Holzer and Holzer-Petsche, 1997). Tachykinins are postulated to play a role in regulating aspects of GI motility (Holzer and Holzer-Petsche, 1997, Deiteren et al., 2011, Dickson et al., 2010), acting via NK1 and NK2 receptors present in the longitudinal and circular muscle layers (Mulè et al., 2007, Jaafari et al., 2007). One study has tested the effects of each tachykinin through pharmacological means, testing the agonists and antagonists of each respective tachykinin receptor on the mouse colon (Deiteren et al., 2011). Stimulation of NK2 and NK3 receptors increased the amplitude of contractions in both the proximal and distal colon, where stimulation of the NK1R only enhanced the amplitude in the distal colon (Deiteren et al., 2011). Where the NK1R agonist increased the frequency of colonic motility in the distal colon, both the NK2R and NK3R prolonged the intervals of contractile events of the distal colon (Deiteren et

al., 2011). Alternatively, blocking these receptors led to a reduction of amplitude but no change in interval for NK1R, a dose-dependent inhibition of peristalsis in the distal colon for NK2R, and no effect on peristalsis from blocking the NK3R (Deiteren et al., 2011). Similar results were found in separate studies, which concluded a synergistic enhancement of peristalsis in mouse colon with stimulation of NK1 and NK2 receptors (Mulè et al., 2007) and the inhibition of peristalsis via NK2 or NK1 antagonists (Dickson et al., 2010). Overall, these results support the potential role for endogenous tachykinins in regulating GI motility, however, no direct evidence of the role of EEC derived tachykinins has yet been identified in the literature. The source of tachykinins' affecting motility could also be primary afferent neurons known to also contain these peptides (Matsumoto et al., 2009). Further studies are needed to better understand which pool of these peptides has a role in GI motility, and hence whether EC cell depletion could also impact tachykinin controlling GI motility. Such studies could utilize the $Tph1^{CreERT2/+}$ mouse line used in the current study, to cross with a Tachykinin1 floxed mouse line, to generate mice with the inducible specific knockdown of TK1 expression in EC cells. A model such as this would allow conclusive investigation into the role of EC cell derived substance P in *in vivo* and *in vitro* motility.

It is known that around 1-2% of neurons within the myenteric plexus contain serotonin (Costa et al., 1982, Wardell et al., 1994, Costa et al., 1996) and as such, it has been suggested that neuronal serotonin might also contribute to GI motility (Li et al., 2011, Smith and Gershon, 2015a). The role of neuronal serotonin has been another point of contention within the field, as outlined in multiple rebuttals from groups with differing opinions (Smith and Gershon, 2015a, Spencer et al., 2015, Smith and Gershon, 2015b). Smith et al (2015) suggested that neuronal serotonin is essential for peristalsis based primarily on results attained from $Tph2^{-/-}$ mice (Li et al., 2011). However, Spencer et al (2015) argue that there is no definitive evidence for neuronal serotonin

as a mediator of motility, highlighting the lack of evidence for serotonin as an active neurotransmitter in the mouse ENS (Furukawa et al., 1986, Nurgali et al., 2004). Furthermore, the significant developmental changes in the ENS of Tph2-/- mice (Li et al., 2011) reduce the feasibility of this mouse model for studies relating to the role of ENS serotonin and gut motility and are likely responsible for the slower transit times seen in these mice (Li et al., 2011). Hence, we wanted to use our EC cell serotonin depletion model coupled with pharmaceutical approaches to delineate the role of neuronal serotonin in GI motility. To do this we used a selective serotonin reuptake inhibitor (SSRI), Fluoxetine, to block the serotonin transporter, SERT. Consequently, the re-uptake of serotonin into SERT containing cells or neurons is inhibited (Ross and Renyi, 1969) and the released serotonin pools at synaptic terminals. As we are using EC cell depleted mice, the major source of serotonin acting at these synaptic terminals must therefore be neuronal. When fluoxetine was applied to our *ex vivo* colonic preparations, CMMCs in EC cell depleted colons were unaffected however CMMCs of oil treated Tph1 DTA control mice were reduced in frequency. The same results were seen when fluoxetine was applied to fixed pellet preparations, with a reduction in the frequency of control contractions and no change in Tph1 DTA contractions. This supports our previous data, in which fluoxetine slowed the frequency of contractile events in intact preparations but did not affect mucosa-free preparations (see Appendix Figure 1). This suggests fluoxetine effects colonic motility through EC cell derived serotonin and does not depend on serotonin from enteric neurons. Furthermore, this aligns with previous studies in which the inhibition of neuronal serotonin (through reserpine treatment) in EC cell serotonin depleted models (mucosa and submucosa free), saw no effect on CMMCs or peristalsis (Sia et al., 2013b, Spencer et al., 2013). Together these results support the notion that neuronal serotonin does not have a role in regulating GI motility.

One future direction arising from this work could include further investigation into the role of neuronal serotonin in motility, by use of a similar specific and inducible Tph2 floxed model. This would enable a conclusive deduction of the differing actions of neuronal serotonin in motility without effecting EC cell serotonin. The use of such a model would provide the same benefits as seen with the Tph1 DTA model discussed here, whilst also bypassing issues with previous pharmacological and knockout mouse models (Yadav et al., 2010, Li et al., 2011). As Tph2 neurons are cholinergic, removing these through a DTA model would slow transit, as cholinergic neurons are known to play a central role in motility (Okamoto et al., 2014). Hence the use of a floxed model, in which Tph2 expression is knocked down in the enteric nervous system, by the Wnt1 promoter specific to peripheral neural crest-derived neurons and glia (Wiese et al., 2013, Schriemer et al., 2016) would allow the inducible loss of Tph2 expression while cholinergic activation would remain unaffected. In particular, the use of such an inducible model would allow the conditional knockdown of Tph2 expression and hence circumvent the developmental differences seen in the neuroanatomy $Tph2^{-/-}$ ENS. Whilst the data depicted in the current study is reflective of a lack of a role of neuronal serotonin in gut motility, a model such as the one proposed would allow conclusive investigation into the role of neuronal serotonin in *in vivo* and *in vitro* motility in developmentally normal mice without the potential of off-target effects from pharmaceuticals.

The $Tph1^{CreERT2/+}$ mouse model is additionally beneficial in enabling the production of EC cell-restricted genetic manipulations, including single gene knockouts or knock-ins (Wei et al., 2021). To do this, the $Tph1^{CreERT2/+}$ model could be crossed with other floxed mouse models to investigate the roles of specific proteins within EC cells. This could help to further deduce the involvement of EC cell serotonin in motility, such as using this model to investigate the mechanical sensing properties of EC cells. The

identity of the specific protein involved in mouse EC cell mechanosensing has recently been identified as Piezo2 (Wang et al., 2017, Alcaino et al., 2018), however the role for such mechanisms in EC cell serotonin modulation of GI motility is yet to be investigated. The use of such a model could help to further delineate the mechanism through which EC cells sense distention or contraction to modulate motility.

The research completed within this chapter to distinguish the role of serotonin in GI motility is important for our overall understanding of GI motility in both health and disease. This knowledge enables insight into the mechanisms which are affected in motility disorders and allow the formation of more specific treatments for people affected by them. A recent study published by our group, with our collaborators, highlights the importance of serotonin in gastric emptying and demonstrates that patients with idiopathic gastroparesis have reduced levels of gastric serotonin (Wei et al., 2021). This study shows that treatment with exogenous serotonin corrects both delayed gastric emptying and slow transit constipation displayed in Tph1 DTA mice (Wei et al., 2021). Further down the GI tract, mucosal serotonin has also been implicated in the pathogenesis of inflammatory bowel syndrome (IBS) (Coates et al., 2004, Gershon, 2004, Linan-Rico et al., 2016, Vahora et al., 2020). IBS can have different phenotypes, including chronic constipation, diarrhea or mixed (Gershon, 2004) (Vahora et al., 2020), and significantly affects the quality of life for sufferers (Fukudo et al., 2016). Despite having a significant research base, the precise pathophysiology of IBS is still unclear (Sinagra et al., 2012). Various serotonergic agents acting on the $5HT_3$ and $5HT_4$ receptors are prescribed for IBS patients (Vahora et al., 2020). Treatment for the syndrome includes $5HT_4R$ agonists such as Prucalopride (Sinagra et al., 2017), Renzapride (Sinagra et al., 2017), Velusetrag (Sinagra et al., 2017), Tegaserod (Camilleri, 2018); and $5HT_3R$ antagonists such as Ramosetron (Sinagra et al., 2017, Camilleri, 2018, Fukudo et al., 2016, Qi et al., 2018),

Alosetron (Olden et al., 2019, Lacy et al., 2018), and Ondansetron (Gunn et al., 2019a, Cangemi and Lacy, 2019, Goldberg et al., 1996, Gunn et al., 2019b). The use of SSRIs is contested due to significant adverse effects and hence they are not readily prescribed for IBS (Lacy, 2016, Xie et al., 2015, Chen et al., 2017). Whilst such serotonergic agents have proven helpful in IBS treatment, they do not abolish all symptoms of the syndrome and typically involve significant side effects (Zheng et al., 2017). Hence, furthering our understanding of the role of EC cell serotonin in motility might allow for the development of pharmaceutical treatments which could act directly on EC cells to either stimulate or block the release of serotonin. Such treatment options could be designed to act specifically on EC cells and be non-absorbable insomuch that they would not cross the GI mucosal barrier. This would mean a significant reduction in off-target effects to improve the quality of life for those who suffer from such chronic disorders.

2.5 Conclusion

In this study we confirm the specificity and feasibility of a new inducible Tph1 knockdown mouse model, $Tph1^{CreERT2/+}$;$Rosa26^{DTA/+}$. We find that EC cell depleted mice experience a constipation-like phenotype, with a marked reduction in both *in vivo* and *ex vivo* motility. Furthermore, we conclude that EC cell serotonin is not essential in GI motility but modulatory, and that neuronal serotonin does not have a role in this process.

CHAPTER 3: The mechanosensing properties of enterochromaffin cells and their subsequent effect on colonic motility.

3. The mechanosensing properties of enterochromaffin cells and their subsequent effect on colonic motility.

3.1 Introduction

The ability for enterochromaffin (EC) cells to sense mechanical stimuli has long been known, with initial evidence published over 60 years ago that demonstrated mechanical stimulation of the intestinal mucosa triggers the release of serotonin (Bulbring and Crema, 1959). Bulbring et al (1959) saw an increase in serotonin levels during peristaltic contractions of the guinea pig ileum, which stimulated large debate surrounding the role of serotonin release in peristalsis (discussed in the previous chapter). Further studies have since demonstrated mechanically stimulated release of serotonin from EC cell-like human cell lines (BON cells and QGP-1 cells) (Kim et al., 2001, Liñán-Rico et al., 2013, Wang et al., 2017), and mouse and guinea pig colonic and small intestinal mucosa (Kim et al., 2001, Bertrand, 2004, Keating and Spencer, 2010, Alcaino et al., 2018). These studies each demonstrate that EC cells across species respond to varying types of mechanical force, such as fluid movements and mucosal compression, through the release of serotonin. EC cells are exposed to various forms of mechanical stimuli under physiological conditions, including mucosal compression, distention, flow shear stress, deformation, intraluminal pressure, and fluctuations in cell volume (Linan-Rico et al., 2016), although the exact mechanisms involved in EC cell mechanotransduction are not well understood. One remaining frontier in understanding EC cell mechanobiology is the study of mechanically stimulated serotonin release from human EC cells, and the role that such mechanisms have in the regulation of GI motility in all species. Early investigations reported the involvement of G-protein coupled receptors (Kim et al., 2001), purine receptors (Christofi, 2008, Liñán-Rico et al., 2013), and mechanosensing caveolae (Kim et al., 2007) as potential mediators of EC cell mechanotransduction. However more recent

studies have indicated the involvement of a newly identified mechanosensing ion channel, Piezo2, as the primary mechanotransduction protein in mouse EC cells (Wang et al., 2017, Alcaino et al., 2018).

The Piezo ion channel family were only recently identified as important mechanosensors (Coste et al., 2010), found to be responsible for mechanotransduction in a variety of cell types throughout the body (Xiao, 2020). The Piezo channels, consisting of Piezo1 and 2 (Coste et al., 2010), have differing properties, including their structure (Lin et al., 2019, Taberner et al., 2019, Wang et al., 2019b), function (Alcaino et al., 2017a, Taberner et al., 2019, Sugisawa et al., 2020, Wang et al., 2020), and their biophysical and gating properties such as their inactivation kinetics (Coste et al., 2010, Coste et al., 2012). Piezo2 is a transmembrane protein which forms a non-selective cationic ion channel, involved in light touch of the skin (Ikeda et al., 2014, Ranade et al., 2014b), sensing stretch in the lung (Nonomura et al., 2017), mediating the baroreceptor reflex (Zeng et al., 2018), and in bladder distention (Marshall et al., 2020). Phenotypic data from Piezo2 knockout mouse models for bladder sensation, touch perception, and nociception, have been confirmed by symptoms in humans with Piezo2 genetic mutations (Chesler et al., 2016, Szczot et al., 2018, Marshall et al., 2020).

The presence of the Piezo2 ion channel in EC cells has been reported in human jejunum, and quantified in mouse small intestine and colon (Wang et al., 2017, Alcaino et al., 2018). Evidence of the channel's functionality as a mechanosensory channel in EC cells has been shown with the QGP-1 cell line, and mouse intestinal EC cells (Wang et al., 2017, Alcaino et al., 2018), with use of the Piezo inhibitor D-GsMTx4 (Alcaino et al., 2017b, Wang et al., 2017, Alcaino et al., 2018) and knockdown of Piezo2 expression using small interfering RNA (siRNA) (Wang et al., 2017, Alcaino et al., 2018). These studies revealed that blocking Piezo2 resulted in a dramatic

reduction in EC cell activation in response to mechanical stimuli, inferring the importance of Piezo2 in EC cell mechanobiology.

Piezo2 produces an inward cationic current upon stimulation by a mechanical force, which depolarises the cell and activates calcium channels resulting in an influx of calcium (Chang et al., 2016). An influx of calcium is important for serotonin release from EC cells (Raghupathi et al., 2013). Studies using a gastrointestinal (GI) epithelium specific Piezo2 knockout (Vil-cre;Piezo2f/f) mouse model found a significantly reduced response to pressure-induced secretion in these mice (Alcaino et al., 2018), however this model was not specific to EC cells, nor was serotonin measured. In order to better understand the role of Piezo2 in EC cells a more specific model is required.

Further investigation is required to deduce the mechanisms in which EC cells of both mouse and human sense mechanical stimuli and respond, and to define the importance of Piezo2 in human EC cell mechanotransduction. The following chapter endeavours to provide evidence that human EC cells of both the small and large bowel also contain the mechanosensing ion channel Piezo2, and provide the very first demonstration that this channel is functional and important in the mechanotransduction abilities of human EC cells. I will do this through the use of various techniques previously used by our research group, including immunohistochemistry, the real time detection of serotonin release through carbon fibre amperometry, and serotonin ELISA kits. Finally, I aim to provide evidence that Piezo2 in EC cells has an important role in GI motility. In the previous chapter, I described the role of serotonin in GI motility, concluding that serotonin is released as a result of contractile events, and not as their driving force. This result inferred the role of mechanosensing in EC cells as an important factor in GI motility. I aim to provide evidence of this mechanism in this current chapter through studies involving human

and mouse intestinal tissue, with pharmacological techniques to block the Piezo channels and to delineate the importance of this channel in gut motility. The specific aims are to:

1. Quantify the co-localisation of Piezo2 in EC cells of human small and large bowel;
2. Test the importance of Piezo2 in the real-time release of serotonin from mechanical stimulation of mouse and human intestinal mucosa;
3. Determine the role of Piezo2 *ex vivo* in mouse colonic motility.

The overriding hypothesis of this chapter is that Piezo2 is an ion channel significantly involved in the mechanosensing function of human EC cells, with implications in GI motility.

3.2 Methods

3.2.1 Preparation of Mouse Tissues

C57 BL/6 male and female mice of 8-12 weeks were anesthetized with isoflurane (4%, IsoFlo) and euthanized humanely by cervical dislocation in accordance with approval by the Animal Welfare Committee of Flinders University (965-19). For all mice, a ventral midline incision was made to expose the peritoneal cavity, and the intestines were gently removed from the animal. Segments of mouse colon and ileum were used, these were placed into beakers containing oxygenated Krebs solution and bubbled with 95% O_2/5% CO_2 to keep tissue viable for hours after removal.

3.2.2 Human Intestinal Tissue

Human colonic and ileum samples were collected from the Flinders Medical Centre and Flinders Private Hospital surgical theatres with patient consent, as per the South Australian Human Research Ethics Committee (50.07). Fresh tissue was collected from cancer or stoma reversal bowel resections, the tissue well outside the perimeter of cancerous tissue was collected for use and considered as healthy tissue. The specimens underwent dissection of adipose, muscular and connective tissue for use in amperometry and immunohistochemistry. This human tissue protocol has been previously published by our lab (Sun et al., 2017). For all experimental work outside of immunohistochemistry, tissue was kept in oxygenated Krebs solution and bubbled with 95% O_2/5% CO_2 to keep the tissue viable until used in experiment. The complete information collected from the human specimens can be seen in Appendix Table 1-3, where the age, sex and region are listed for each patient.

3.2.3 Immunohistochemistry on Human Intestinal Sections

Freshly removed human ileum and colonic tissue was collected (as per 3.2.2) and maintained in oxygenated Krebs solution. Tissue was pinned out as far as could be stretched, with mucosa facing down, using micro pins in Sylgard (Sylgard 184 Silicone Elastomer Kit, Dow Silicones Corporation) coated dissection dishes. Tissue was fixed in 4% paraformaldehyde (PFA) at room temperature overnight. The next day the tissue was removed and placed in a 50 mL pot containing 4% PFA for several hours to ensure full penetration of fixation. Tissue was then washed 3 times in 1x PBS and left in PBS containing sodium azide (0.01%) until required for staining. A small segment from each sample was placed in 30% sucrose in PBS at 4°C overnight to cryoprotect. Tissue was then frozen in a cryomold, in optimal cutting temperature compound (Sakura Finetek) using liquid nitrogen. Tissue was cut transversely into 10 µm sections with a cryostat (Leica CM1850) at -20°C and mounted onto polyethylene glycol coated microscope slides for immunohistochemical staining. Sections were air dried for 30 minutes, then washed with 1x PBS for 10 minutes to remove excess OCT. Sections were blocked with 10% Donkey Serum (Cat# D9663, Sigma Aldrich) in PBS for 30 minutes, and then stained for 5-HT (1:2000, Goat anti-Serotonin IgG, Cat# 20079, Immunostar) and Piezo2 (1:400, Rabbit anti-Piezo2 IgG, NBP1-78624, Novus Biologicals) in 10% donkey serum in 1x PBS for 2 nights, at room temperature in a humidity box. Sections were washed 3 times 5 minutes in 1x PBS, before secondary antibodies were applied, Cy3 conjugated Donkey anti-Rabbit IgG (H+L) and Cy5 Donkey anti-Sheep IgG (H+L) (1:200, Jackson ImmunoResearch Laboratories) along with the nuclei marker DAPI (1:1,000, Cat# D9542, Sigma-Aldrich), in PBS for 2 hours at room temperature in a humidity box. Glass coverslips were then mounted with 100% buffered glycerol to

cover the sections on the slides, using clear nail varnish to seal, and kept at 4°C until viewed on the microscope.

3.2.4 Microscopy

Immunofluorescence of samples was viewed using an upright fluorescence microscope (Olympus Fluorescence AX70 Upright Microscope) and imaged using the Zeiss Axiocam 506 monochrome camera with Zen Blue software (Zen Blue Image Capture Software, ZEISS). The exposure time was kept constant for each image capture. Images were analyzed and cells were counted using Zen Blue software. High-resolution confocal images were taken using the LSM 880 high-resolution airyscan confocal microscope (ZEISS LSM880, Carl Zeiss, Sydney, Australia). Images were obtained using the 20x air objective in two channels: 561 nm and 633 nm on the LSM 880. Z-stacks were taken throughout the thickness of the tissue (~10 µm), using regular confocal mode. Images were collected using Zen Black software (Zen Black Image Capture Software, ZEISS).

3.2.5 Amperometry

The real time release of serotonin was measured using carbon fibre amperometry on the mucosa of both mouse and human colonic and ileal tissue after a mechanical stimulus was applied. This amperometry technique has been used and published by our lab previously (Raghupathi et al., 2013). Amperometry records serotonin oxidation using a carbon fibre electrode (ProCFE, Dagan Corporation, Minneapolis, MN, USA) voltage-clamped of +400 mV, at which serotonin will readily oxidise. Current due to serotonin oxidation was recorded using an EPC-10 amplifier and Pulse software (HEKA Electronic, Germany) with the current sampled at 10 kHz. For quantitative analysis files were converted to Axon Binary Files (ABF Utility, version 2.1,

Synaptosoft, USA) and analysed (Clampfit, version 10.7, pCLAMP, USA). Tissue was maintained in Krebs solution under constant bubbling with 95% O_2/5% CO_2. The carbon fibre electrode, which was slowly lowered down onto the mucosa to a depth of 100 µm using a digital motorised micromanipulator (MP-285, Sutter Instruments, USA) to mechanically stimulated the mucosa (Bertrand, 2006, Keating and Spencer, 2010). The peak response is recorded as the largest change in current from baseline. The steady state refers to the current 10 seconds after initial compression, also recorded as a change from baseline (see Figure 3.1).

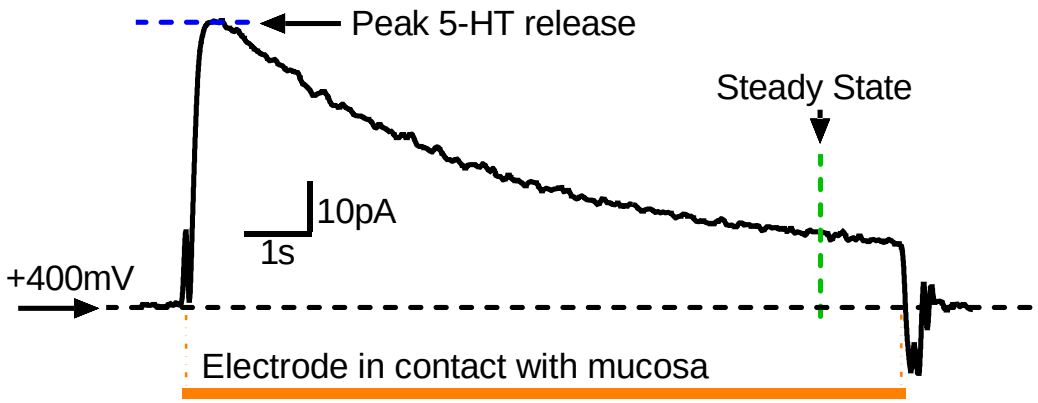

Figure 3.1: An example trace measuring the real-time release of serotonin as a result of mucosal compression.

3.2.6 Mechanical Orbital Shaking Experiments

To measure the stimulation of serotonin release via fluid mechanical stimulation of intestinal mucosa, segments of human colonic and ileal tissue were pinned out in 45 mm dissection dishes (Corning) lined with Sylgard (Sylgard 184 Silicone Elastomer Kit, Dow Silicones Corporation), in Krebs solution containing Fluoxetine (Sigma-Aldrich, 1 µM) and serotonin stabilizer (1%, Stabilizer, BA E-5937, LDN, Germany), pH 7.3-7.4. These dishes were then placed on an orbital shaker at varying RPM levels, 0, 200, 300 and 400 rpm for a period of 20 minutes. The supernatant was collected for serotonin release quantification, tissue samples were also collected, weighed, and lysed for serotonin total content quantification to normalize serotonin release. Serotonin quantification was measured using a serotonin ELISA kit (Serotonin Research ELISA, LDN, Germany, Cat# BA E-5900R). Samples from lysed human intestinal mucosa and secreted from mucosa secretion experiments were diluted at 1/1000 and 1/10 respectively and loaded onto the serotonin ELISA. The ELISA was performed as per manufacturer's instructions, and results analysed using a 4-parameter sigmoidal curve with GraphPad Prism (v9).

3.2.7 Colonic motility recordings

The recordings of CMMCs in this chapter were all measured from flat sheet colon preparations, in which the colons of mice were removed, cut open longitudinally along the mesenteric border and pinned down in organ baths with the mucosa facing up. The hooks were then attached to 3 separate regions along the colon on one side of the tissue longitudinally, correlating with the proximal, mid and distal regions. CMMCs were recorded as previously described (Method 2.2.4 of the previous chapter).

3.2.8 Drugs

GsMTx4 (ab141871, Abcam) was made up to 100 µM in distilled H_2O, and diluted further to 1, 5, and 10 µM in Krebs solution. Krebs' solution contained (in mM): NaCl, 118; KCl, 4.7; NaH_2PO_4, 1.0; $NaHCO_3$, 25; $MgCl_2$, 1.2; D- Glucose, 11; $CaCl_2$, 2.5 and was maintained via constant bubbling of 95% O_2/5% CO_2 gas.

3.2.9 Statistical analysis

Statistical analysis was performed as in previous method (2.2.8).

3.3 Results

3.3.1 Piezo2 in human EC cells of the ileum and colon.

Human intestinal sections were stained using antibodies raised against serotonin and Piezo2 to confirm their presence and quantify the co-expression of the ion channel in human EC cells in both ileum and colonic samples. Serotonin was used as a marker for EC cells. Piezo2 expression was found in some but not all EC cells in human ileum and colonic tissue (Figure 3.2A). Piezo2 expression was quantified and present in 62.2 ± 9.3% of cells which contained serotonin in the ileum (Figure 3.2B), and 33.9 ± 5.0% in the colon (Figure 3.2B). Conversely, 56.1 ± 4.0% of Piezo2 positive cells were serotonin positive in the human ileum, and 32.4 ± 6.7% in colon (Figure 3.2C). These results indicate that the proportion of EC cells containing Piezo2 is greater in human ileum than colon. Together these results confirm the presence of the mechanosensitive channel, Piezo2, in human small and large bowel.

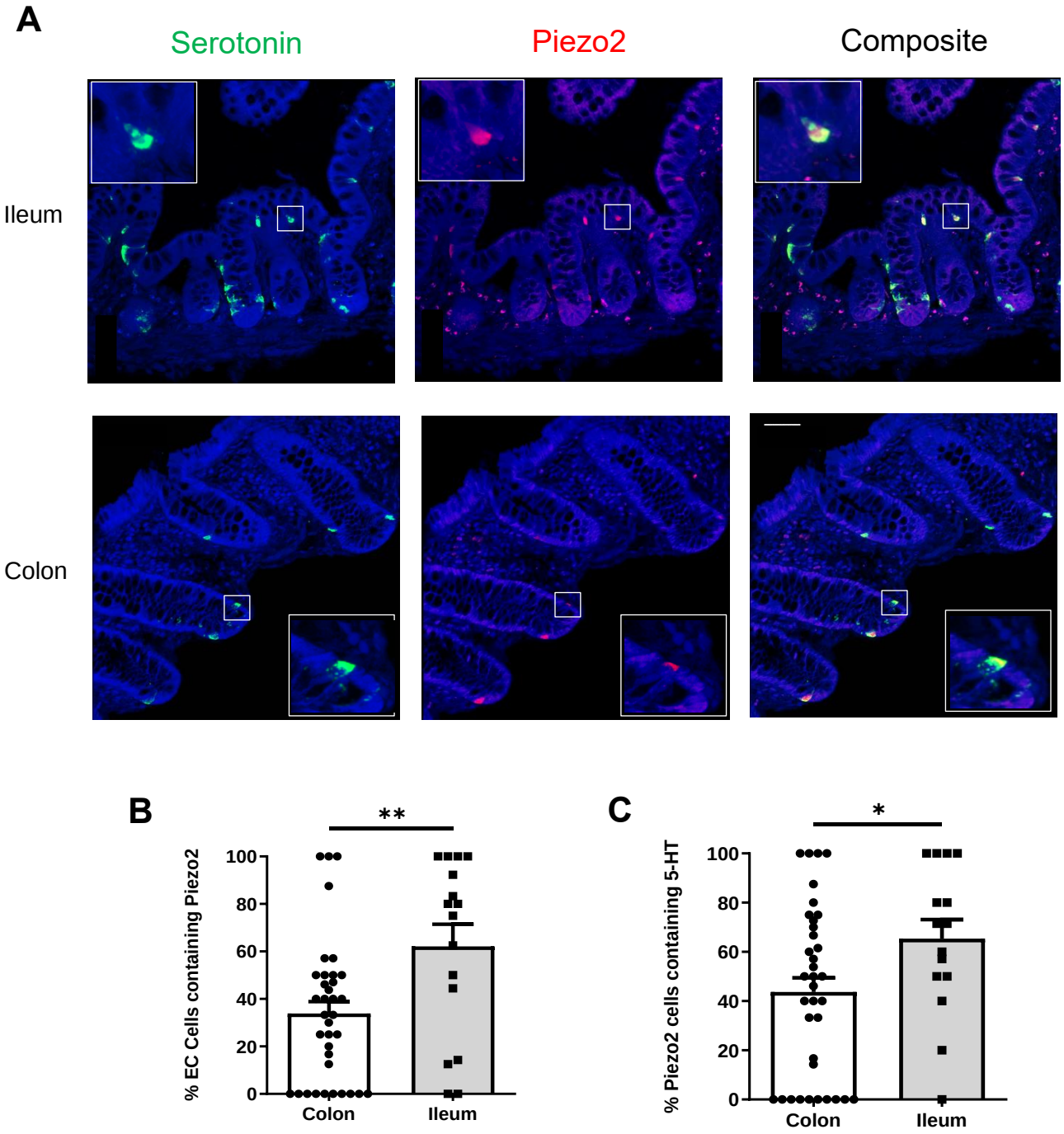

Figure 3.2: Human EC cells contain the mechanosensing ion channel Piezo2.

A) EC cells containing serotonin (green) and Piezo2 (red) co-stained in human ileum and colon, with DAPI (blue) as a cell nuclei marker. Scale bar = 50 μm. B) The percentage of EC cells which contained Piezo2 in human colon (n=16, N=36) and ileum (n=3, N=16). C) The percentage of Piezo2^{+} cells which contained serotonin. Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$

3.3.2 The role of Piezo channels in the release of serotonin from human EC cells of the ileum and colon.

Human GI tissue was collected from surgical theatres (as per method 3.2.2) and stimulated via mechanical compression, using a carbon fibre probe lowered into the mucosa to a depth of 100 µm (Keating and Spencer, 2010). In all preparations, the oxidation current increased significantly as the carbon fibre touched the mucosal epithelium (Figure 3.3-3.4), confirming that human EC cells release increased concentrations of serotonin in response to mechanical stimuli. This response was dampened by the addition of the Piezo inhibitor, GsMTx4 (5 µM) (Figure 3.3), indicating the mechanical sensing properties of EC cells are diminished by blocking Piezo channels. This effect was largely reversible upon wash-out of the drug (Figure 3.3). This process was repeated in human colonic samples, where the peak and steady state release also decreased in the presence of GsMTx4, only the peak difference reached statistical significance (see Figure 3.4). Overall, these results support the hypothesis that Piezo channels are important in the mechanosensing properties of human EC cells.

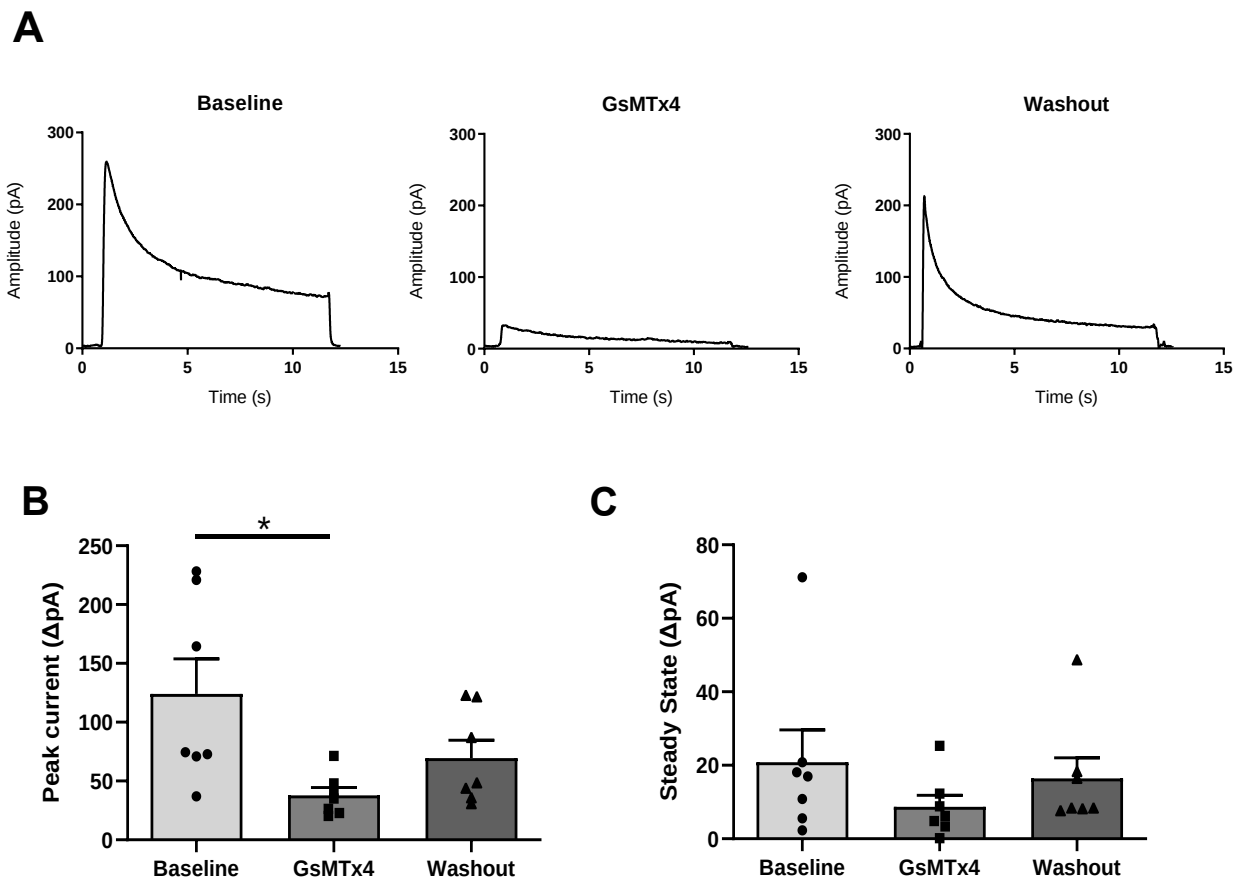

Figure 3.3: The inhibition of Piezo channels reduces mechanically stimulated serotonin release from human ileum.

A) Example traces of serotonin release from the mechanical stimulation of human ileum at baseline, with GsMTx4 (5 μM) and at washout. B) Peak release of serotonin from human ileum (n=7). C) Steady state serotonin release from human ileum (n=7). Data are shown as mean ± SEM. *$p < 0.05$

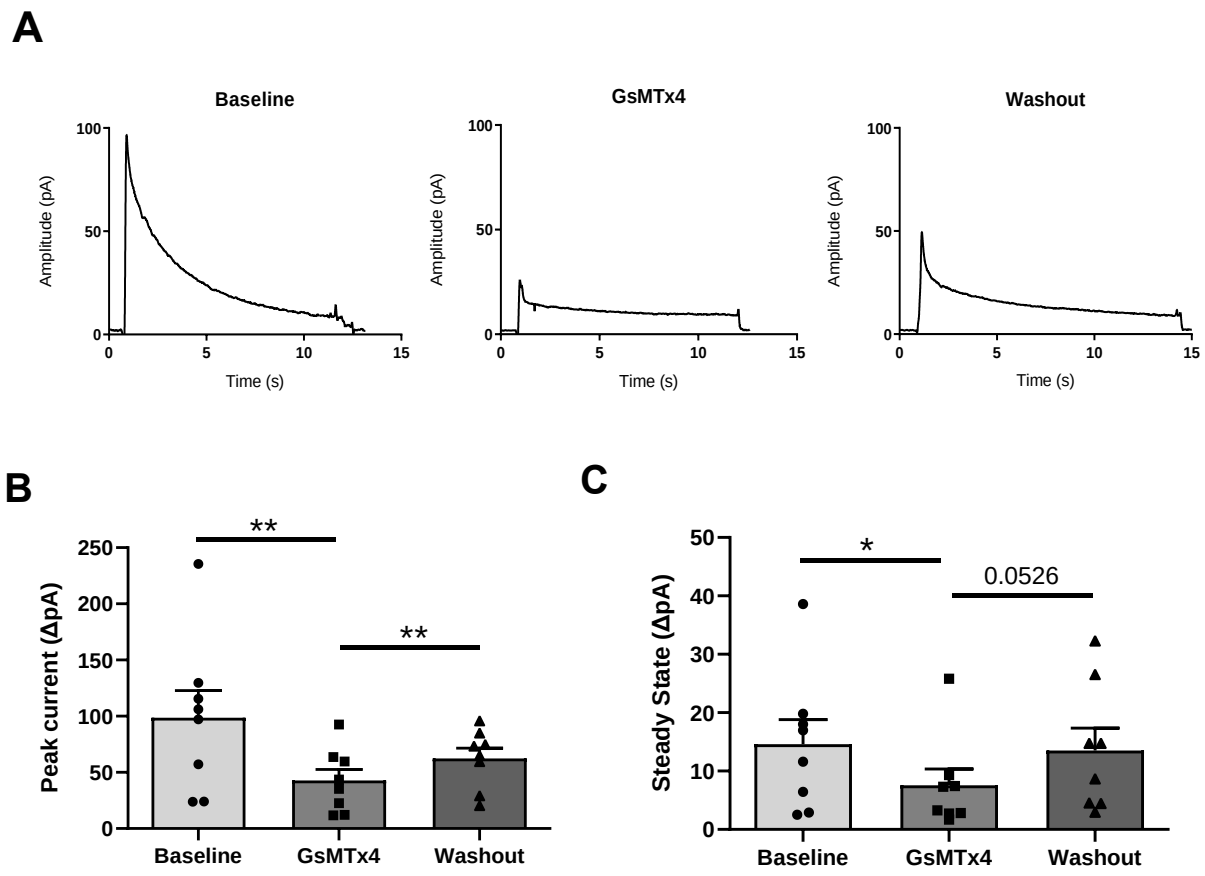

Figure 3.4: The inhibition of Piezo channels reduces mechanically stimulated serotonin release from human colon.

A) Example traces of serotonin release from the mechanical stimulation of human colon, measured at control, with GsMTx4 (5 μM), and after the washout of drug. B) Peak release of serotonin from human colon (n=8). C) Steady state of serotonin release from human colon (n=8). Data are shown as mean ± SEM. *p < 0.05

3.3.3 Measuring the role of Piezo channels in the release of serotonin, from mouse EC cells in the ileum and colon.

Mouse intestinal tissue was collected from control C57BL/6 mice as described previously (see method 3.2.1). A change in current representing serotonin release was measured using carbon fibre amperometry (as above), and segments of mouse ileum or colon were mechanically stimulated via mucosal compression. The current was recorded from mucosal stimulation at baseline, and with the Piezo inhibitor (GsMTx4, 5 μM) (Figure 3.5). As with human tissue, mouse ileum and colon showed a reduction in peak serotonin release in the presence of GsMTx4 (see Figure 3.5, 3.6), which returned to baseline with wash out. Overall, these results indicate the similarities between EC cell mechanosensitivity across species and provide further evidence for the functional role of Piezo channels in mouse EC cell mechanobiology.

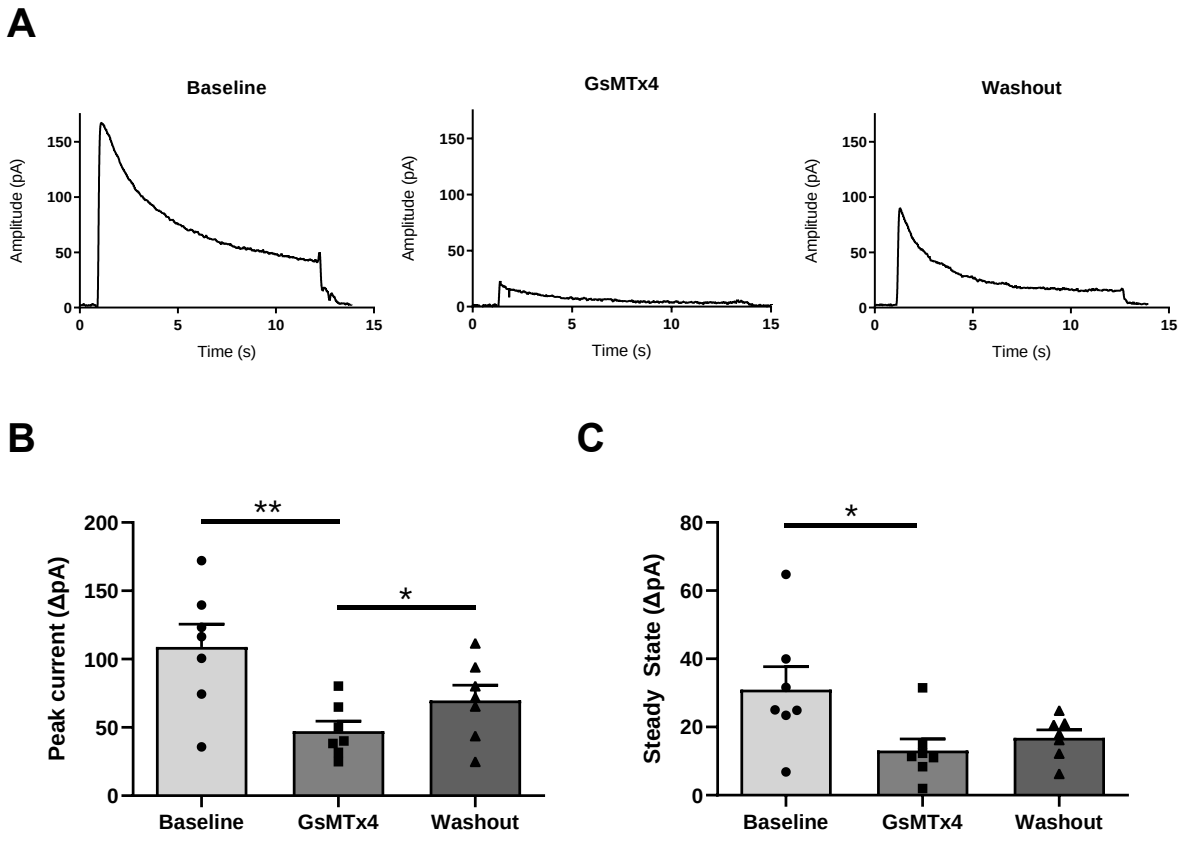

Figure 3.5: Serotonin release from mouse ileum in response to mucosal compression is blocked with Piezo inhibition.

A) Example traces of serotonin release from mechanical stimulation of mouse ileum, measured at baseline, with GsMTx4 (5 μM), and after the washout of drug. B) Peak release of serotonin from mouse ileum (n=8). C) Steady State of serotonin release from mouse ileum (n=8). Data are shown as mean ± SEM. *p < 0.05, **p < 0.01.

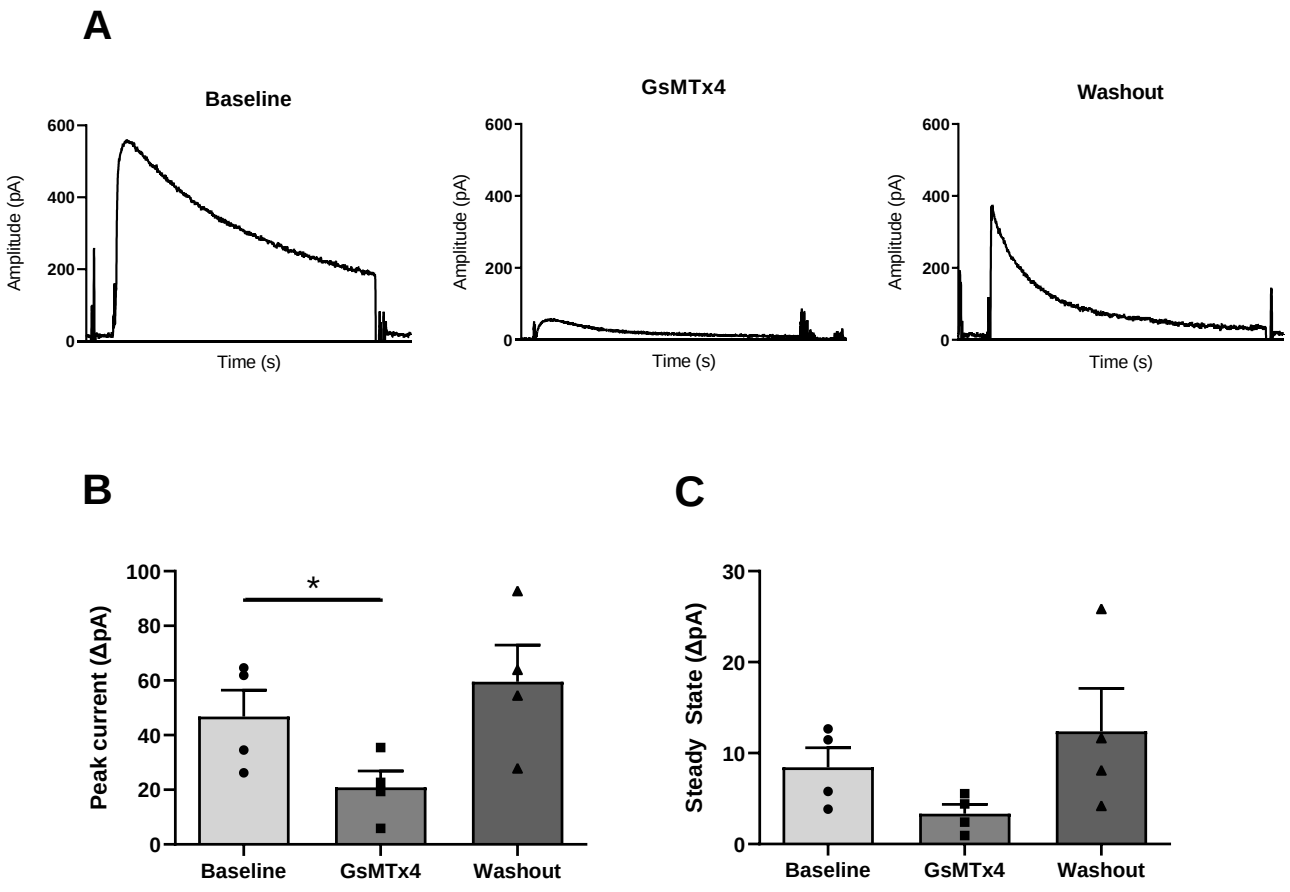

Figure 3.6: Serotonin release from mouse colon in response to mucosal compression is blocked with Piezo inhibition.

A) Example traces of serotonin release from mechanical stimulation of mouse colon, measured at control, with GsMTx4 (5 μM), and after the washout of drug. B) Peak release of serotonin from mouse colon (n=4). C) Steady state of serotonin release from mouse colon (n=4). Data are shown as mean ± SEM. *$p < 0.05$

3.3.4 Mechanical stimulation of EC cells using fluid movement, causes a dose-dependent release of serotonin.

EC cell serotonin release was measured from stimulation via fluid movement across the intestinal mucosa. Human ileum and colonic samples were incubated at varying degrees of orbital shaking (0, 200, 300, 400 rpm) for a 20-minute period, and the amount of serotonin released per sample was deduced. This result was normalised to the total serotonin content of each sample. A stimulation dependent release of serotonin was observed in the ileum, where the amount of serotonin release correlated with the amount of orbital shaking experienced (see Figure 3.7A). The same effect was not seen in human colonic samples, with no consistent release of serotonin measured as a result of stepwise increases in shaking (Figure 3.7B). This experiment was repeated on human ileum with the addition of the Piezo inhibitor, GsMTx4 (5 µM), which reduced serotonin release due to orbital shaking and fluid stimulation (see Figure 3.7C). These results indicate that fluid stimulation causes the release of serotonin from EC cells of the human small bowel, an effect which can be reduced by blocking Piezo channels.

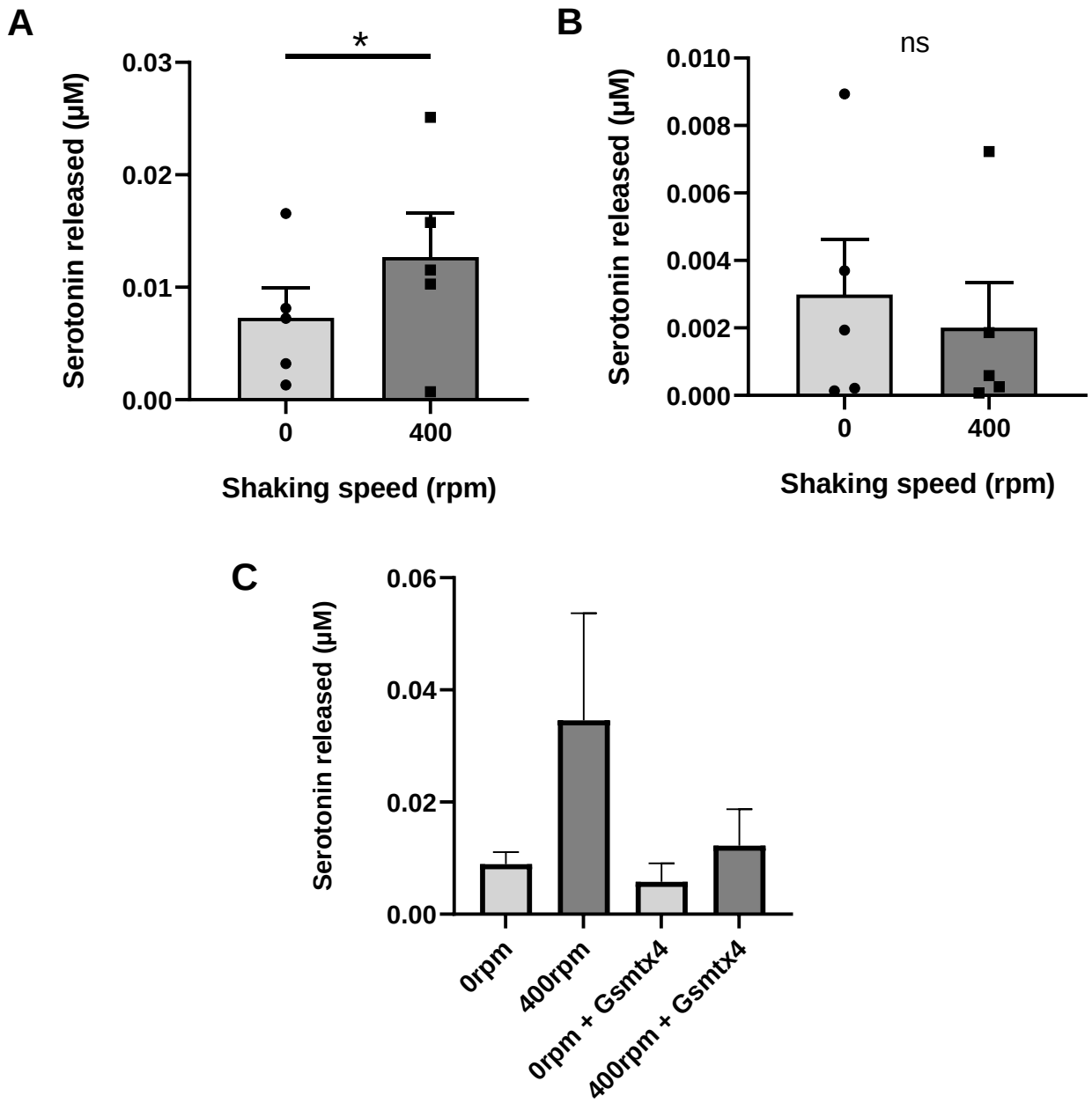

Figure 3.7: Serotonin release from human colon and ileum due to fluid shear stress.

Release of serotonin from (A) human ileum (n=4) and (B) human colon (n=5) from control (0 rpm) and mechanical stimulation via orbital shaker at 400 rpm. (C) Release of serotonin from human ileum with (n=3) and without (n=5) GsMTx4 (5 µM) on mechanical stimulation via orbital shaker at 0 and 400 rpm. Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001

3.3.5 Human and mouse ileal EC cells are more sensitive to mechanical stimuli than colonic EC cells.

Various methods of mechanical stimulation used in this chapter have indicated that serotonin release in response to a mechanical stimulus is lesser in the colon than the ileum. Amperometry recordings of mucosal compression of mouse and human ileum both resulted in a significantly greater release of serotonin that the colon (see Figure 3.8 A, B). These results were reanalysed to deduce the mechanical sensitivity, using peak/baseline. The mechanical sensitivity showed an almost significant ($p=0.05$) difference, with the ileum greater than colon (Figure 3.8C. Similar results were seen from serotonin release stimulated by fluid movement in ileum and colon, in which serotonin release was also greater from the human ileum compared with the colon. These results were confirmed when comparing paired human samples, in which human colon and ileum were collected at the same time from a patient undergoing a right hemicolectomy. This paired analysis confirmed that the ileum releases a greater amount of serotonin in response to a mechanical stimulus (see Figure 3.9A, B). Interestingly the total content of serotonin was analysed in colonic and ileal mucosa, with the colonic mucosa found to have a significantly greater total serotonin level than the ileal mucosa (Figure 3.9C). These results indicate that ileal EC cells are more mechanically sensitive than the colonic EC cells.

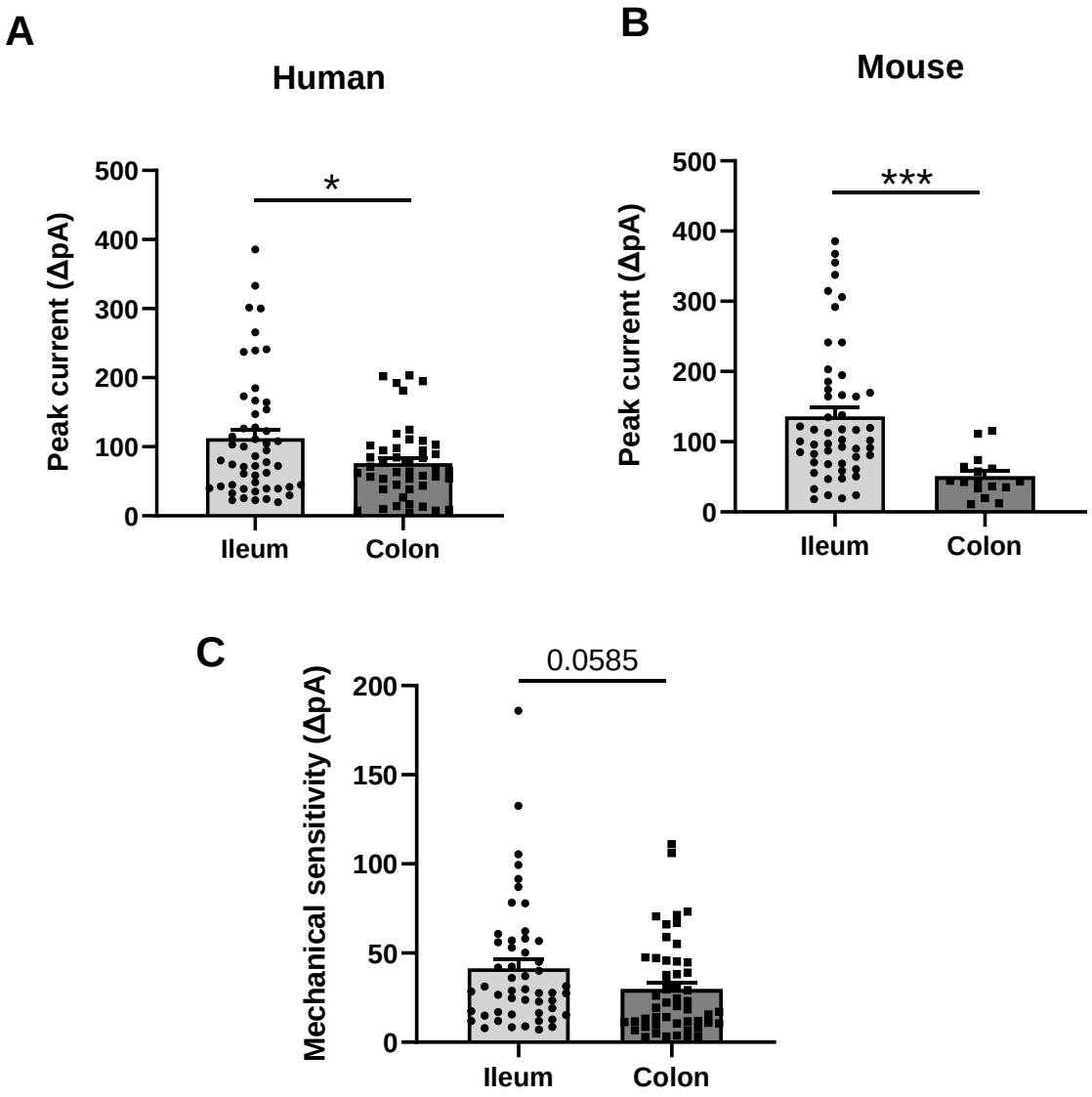

Figure 3.8: Ileal EC cells are more mechanosensitive than colonic EC cells.

Real-time serotonin release resulting from the mechanical compression of (A) human colon (n=8, N=45) and ileum (n=8, N=51) and (B) mouse colon (n=4 mice, N=16) and ileum (n=8 mice, N=52). C) The mechanical sensitivity of human ileum (n=8, N=51) and colonic (n=8, N=51) EC cells. Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001

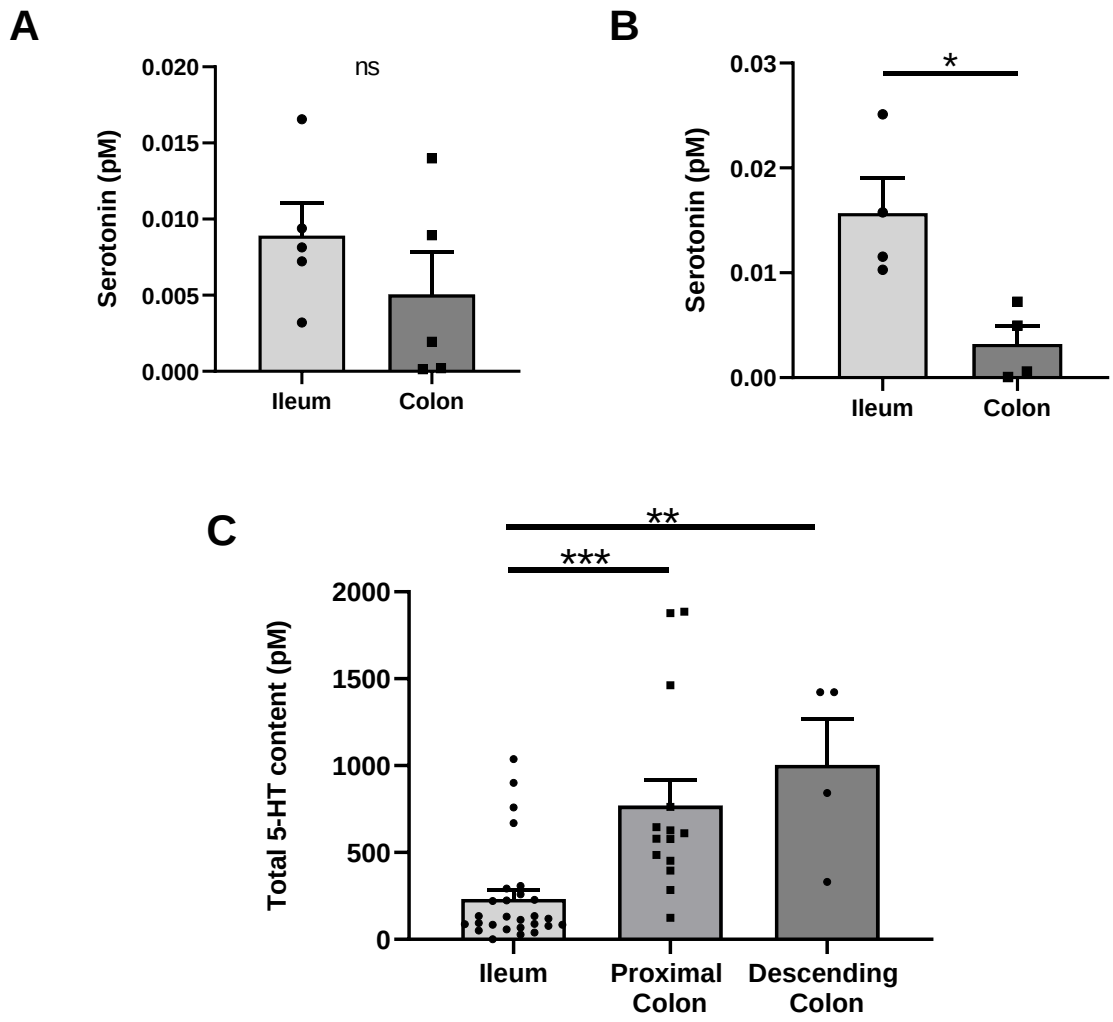

Figure 3.9: Serotonin content and release differs across the human GI tract.

(A) Baseline and (B) fluid shear stress stimulated release of serotonin from the human ileum and colon of the same patient (n=5). (C) Total serotonin content across separate regions of the human intestinal tract. Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001

3.3.6 Blocking the Piezo channels causes a dose dependent reduction in CMMCs.

To investigate the role of Piezo2 in colonic motility, CMMCs were measured from C57BL/6 mouse colonic preparations *ex vivo.* CMMCs were measured for 30 min at baseline, and in the presence of the Piezo channel antagonist (GsMTx4) at varying concentrations (1 μM, 5 μM, and 10 μM). An initial dose of 1 μM appeared to slow down CMMCs and cause a reduction in propagation (Figure 3.10), the addition of 5 μM GsMTx4 then stopped propagation altogether and appeared to slow CMMCs further (Figure 3.10). The final addition of 10 μM GsMTx4 caused an immediate abolition of all CMMCs (Figure 3.11). It could hence be seen that CMMCs had a proportional decrease in frequency in relation to the concentration of GsMTx4 indicating a dose-dependent relationship. The following experiments were therefore conducted at concentrations including 1 μM and 5 μM. The frequency of contractions slowed significantly with the use of GsMTx4 (5 μM) (see Figure 3.12), and the amplitude also reduced at 1 and 5 μM (Figure 3.12). These results indicate that the inhibition of Piezo channels causes a reduction in colonic contractile activity.

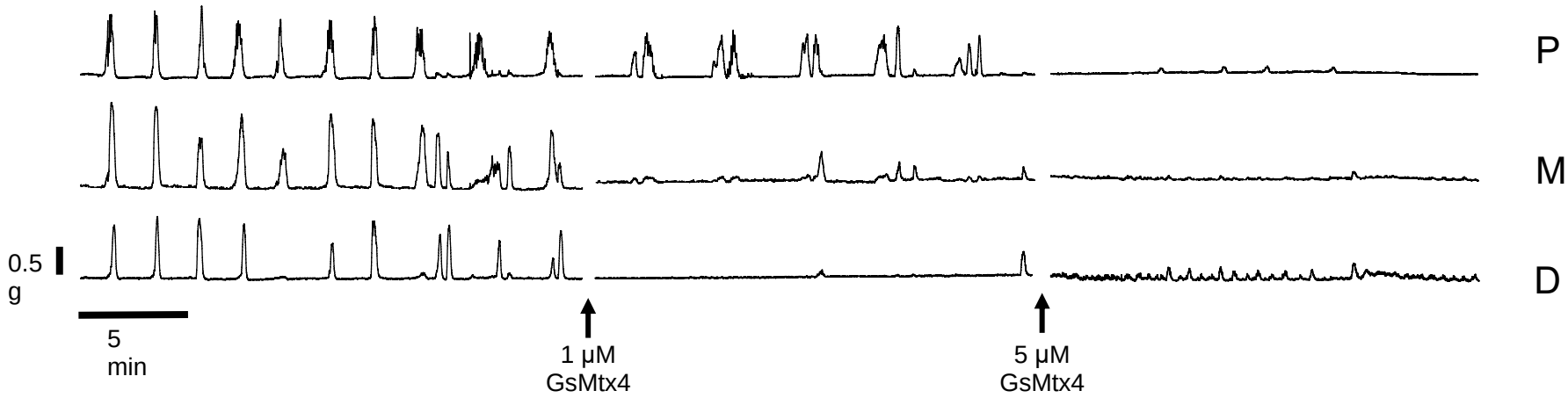

Figure 3.10: Inhibiting Piezo channels causes a dose dependant reduction in mouse CMMCs.

An example trace of *ex vivo* mouse CMMCs with GsMTx4 dose response at baseline, 1 µM, and 5 µM, measured from the proximal (P), mid (M), and distal (D) region.

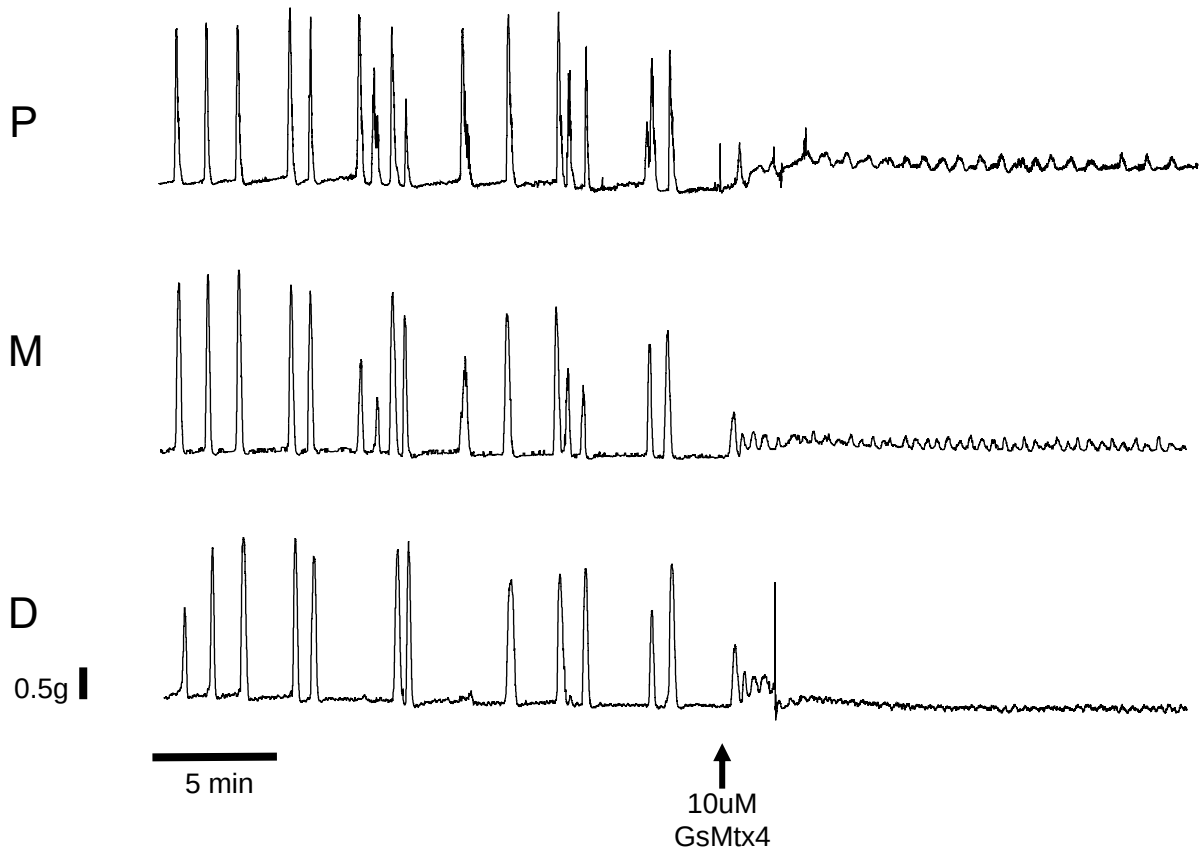

Figure 3.11: GsMTx4 dose response at 10 μM on mouse CMMC trace.

Example trace of CMMCs at baseline, and with GsMTx4 (10 μM), measured from the proximal (P), mid (M), and distal (D) region.

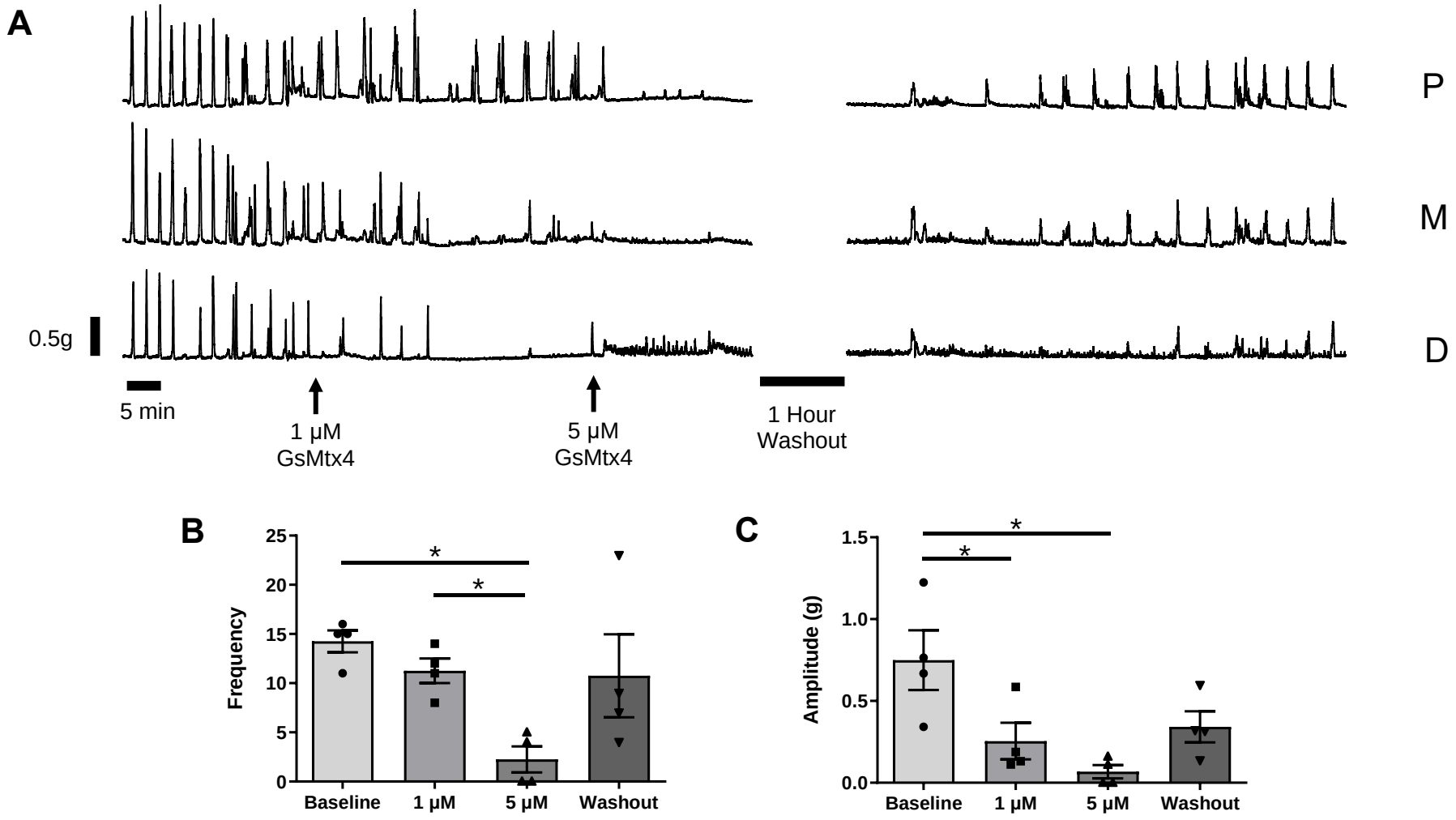
A
P
M
D
0.5g
5 min
1 μM
GsMtx4
5 μM
GsMtx4
1 Hour
Washout
B
Frequency
25
20
15
10
5
0
*
*
Baseline
1 μM
5 μM
Washout
C
Amplitude (g)
1.5
1.0
0.5
0.0
*
*
Baseline
1 μM
5 μM
Washout

Figure 3.12: Blocking the Piezo channels reduces mouse CMMCs *ex vivo*.

A) Example trace of CMMCs at baseline, with GsMTx4 (1 μM, 5 μM) and at wash out, measured from the proximal (P), mid (M), and distal (D) region. B) Average proximal frequency of CMMCs at baseline compared with GsMTx4 (n=4). C) Average amplitude of CMMCs at baseline compared with GsMTx4 (n=4). Data are shown as mean ± SEM. *$p < 0.05$

3.3.7 Blocking the Piezo channels in mucosa-free colon also reduces colonic motility.

A number of enteric neuronal subtypes are also mechanosensitive, including IPANs amongst other myenteric neurons (Kunze et al., 2000, Spencer and Smith, 2004, Mazzuoli and Schemann, 2009, Mazzuoli and Schemann, 2012, Williams et al., 2016), some of which have recently been identified to also contain Piezo2 (Drokhlyansky et al., 2020). Hence, we wanted to test the role of non-EC cell, or mucosal in general, Piezo2 in colonic contractile activity. CMMC recordings were repeated using mouse colonic samples with their mucosa removed, leaving just the muscle layers containing the myenteric plexus and submucosal plexus intact. These experiments would define the effect of the Piezo blocker, GsMTx4, on contractile activity of tissue lacking EC cells. The frequency of CMMCs in mucosa-free preparations was significantly reduced from intact preparations at baseline (see Figure 3.13A), as expected based on previous experiments from our research group (Keating and Spencer, 2010). When GsMTx4 (5 μM) was applied to the bath of mucosa-free preparations, CMMCs were completely abolished (see Figure 3.13). The frequency and amplitude of mucosa-free preparations was significantly reduced compared with control (Figure 3.13B, C). This data suggests that there are Piezo channels within the myenteric or submucosal plexuses, or the muscle layers of the colon which are also mechanically sensitive and may play a role in the formation of CMMCs.

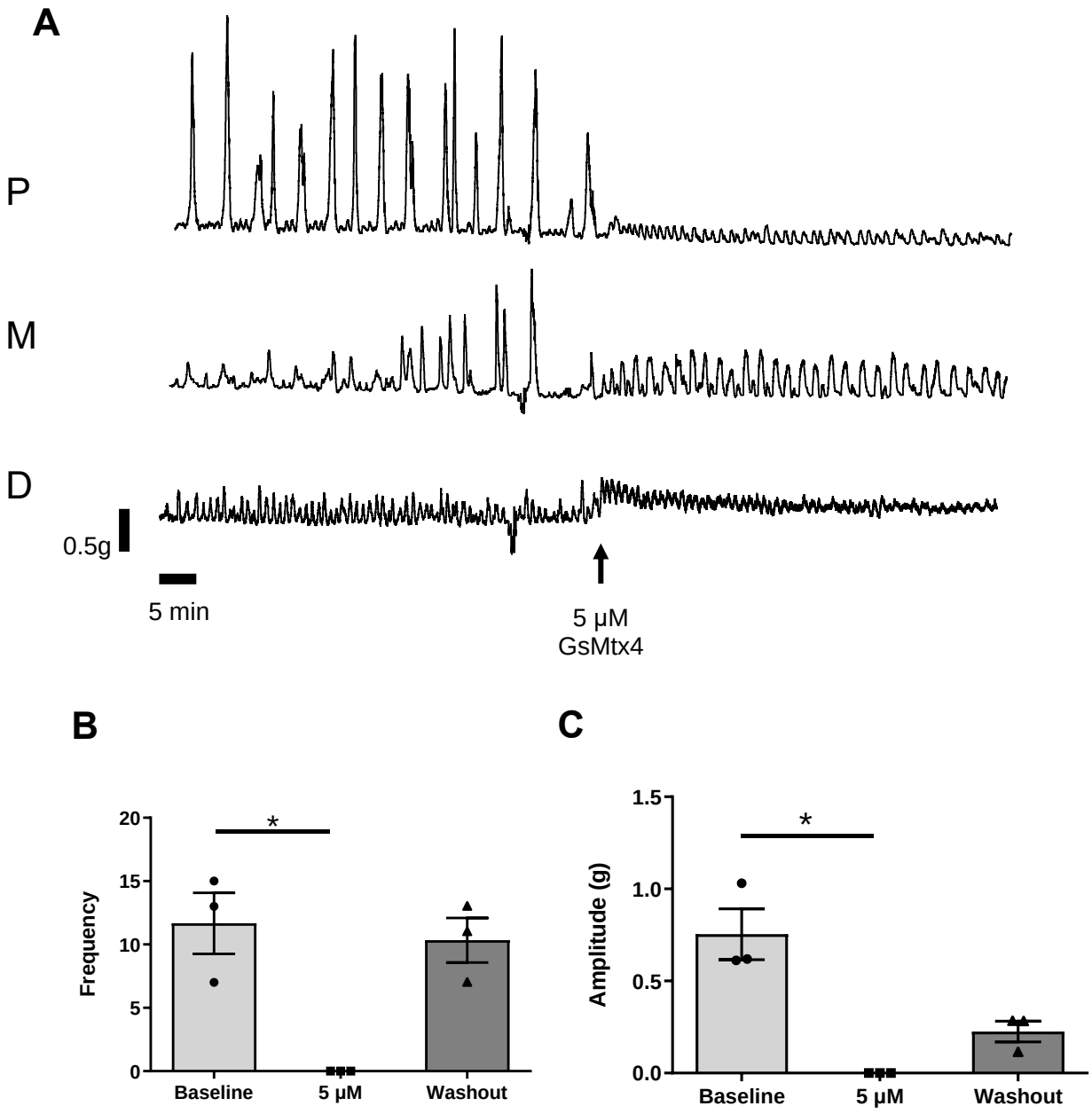

Figure 3.13: Piezo inhibition abolishes CMMCs in mucosa-free mouse colon.

A) Example trace of mucosa-free CMMCs at baseline, and with GsMTx4 (5 µM), measured from the proximal (P), mid (M), and distal (D) region. B) Average frequency of mucosa-free CMMCs at baseline compared with GsMTx4 (n=3). C) Average amplitude of mucosa-free CMMCs at baseline compared with GsMTx4 (n=3). Data are shown as mean ± SEM. *$p < 0.05$

3.3.8 EC cell mechanosensitivity changes with age and sex.

We noted that the co-localisation of Piezo2 in human colon was lower than that reported in mice (Alcaino et al., 2018), and observed that this co-localisation appeared lower in samples from aged human gut. A correlation analysis on the percentage of Piezo2 containing EC cells from immunohistochemical images was measured against age. This analysis revealed a reduction in the percentage of Piezo2 positive EC cells with age in the colon, as seen by the negative correlation (see Figure 3.14A). The same analysis was conducted on the amount of serotonin released in real-time from mechanical stimulation of human colonic EC cells against age, finding the same relationship (Figure 3.14B). The results of mechanical stimulus on male and female mouse colonic and ileal mucosa revealed that the real-time peak release of serotonin from female mice was greater than that of male mice in both the colon and ileum (see Figure 3.15A, C). The steady state release of female mice was also greater from the colon, although no differences were seen in ileum steady state release (Figure 3.15B, D). Overall, these results suggest that both age and sex impact the mechanosensing capabilities of EC cells.

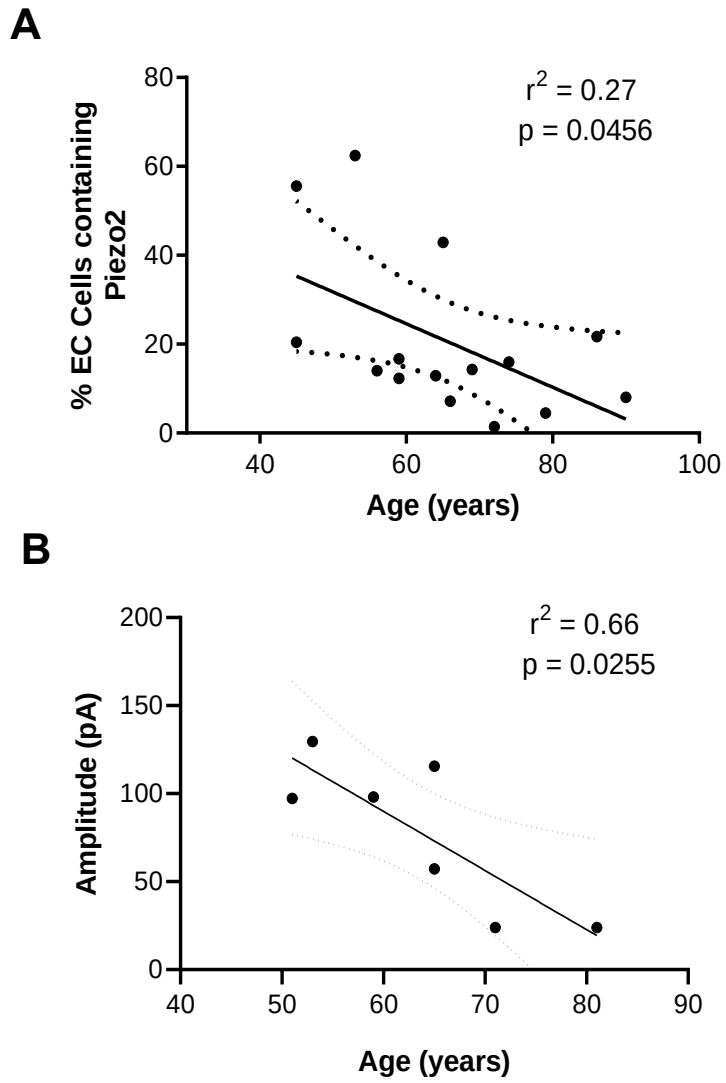

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Figure 3.14: Human EC cells lose mechanosensitivity with age.

A) The percentage of EC cells containing Piezo2 in human intestine is negatively correlated with age (n=15). B) The mechanically evoked release of serotonin is reduced with age (n=7). Data are shown as mean ± SEM. *p < 0.05

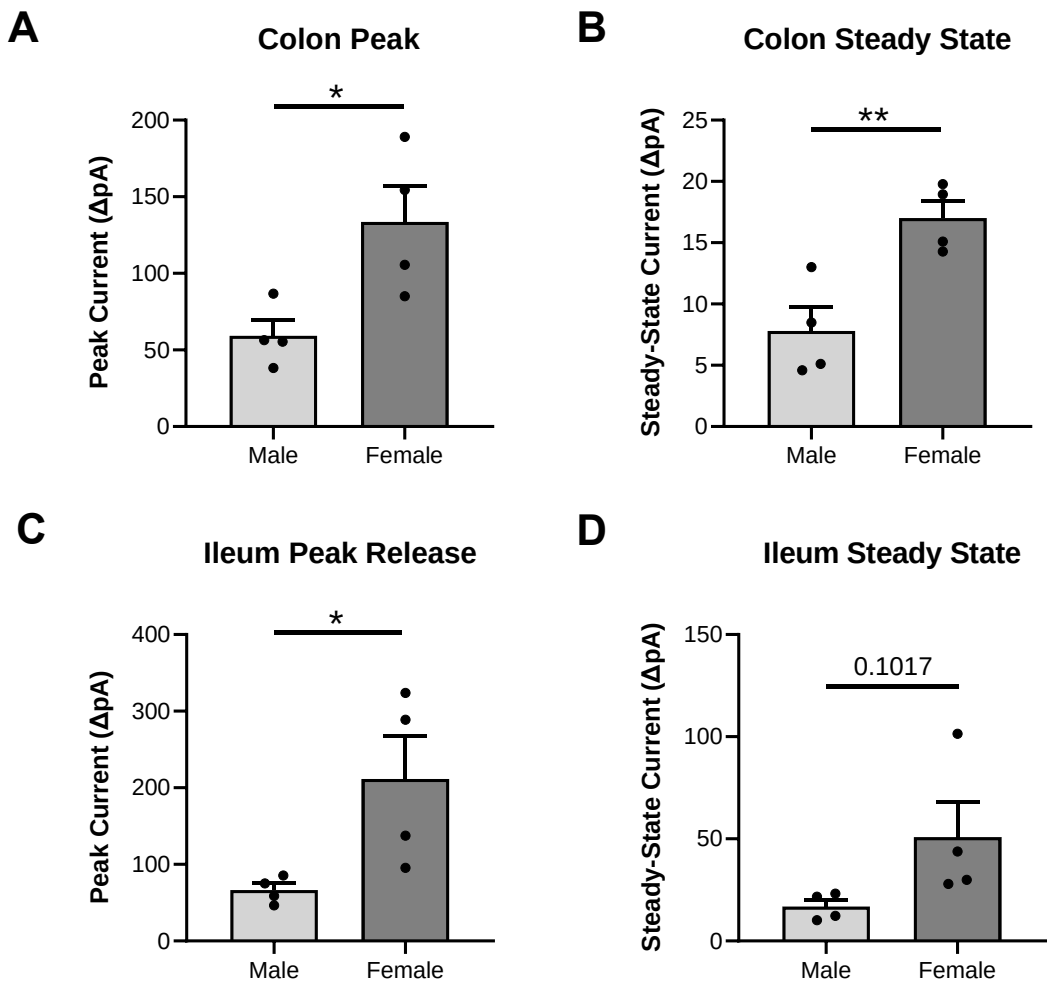

Figure 3.15: Sex differences in the mechanically evoked release of serotonin from mouse GI tissue.

A larger release of serotonin from the colon as measured by the (A) peak and (B) steady state release from the mouse colon, and from the (C) peak and (D) steady state release from mouse ileum. Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

3.4 Discussion

While EC cells from mouse and guinea pig small and large bowel have long been known to be mechanosensitive (Bertrand, 2004, Kim et al., 2001, Keating and Spencer, 2010), it had recently been hypothesised that Piezo2 functions as the main mechanosensory mechanism in mouse and human EC cell mechanobiology (Wang et al., 2017, Alcaino et al., 2018),. In the present study we quantify Piezo2 expression in human small and large bowel and provide the first evidence of the functionality and importance of Piezo2 in human EC cell mechanotransduction. Furthermore, we demonstrate a role for Piezo2 in colonic motility, and an association with a decline of expression of the mechanosensing channel in EC cells with age in humans.

Our immunohistochemical studies on human tissue are the first to reveal that both colonic and ileal EC cells contain the mechanosensing ion channel Piezo2, supporting previous evidence that human jejunum expresses the gene encoding for Piezo2 (Wang et al., 2017). We find a greater percentage of Piezo2 positive EC cells in human small bowel compared to colon, similar to previous findings in mice (Alcaino et al., 2018). A number of human EC cells do not contain Piezo2, a similar trend is seen in mice albeit the proportion of Piezo2 negative cells is smaller in mice (Wang et al., 2017, Alcaino et al., 2018). Both of these results indicate that there is a proportion of mouse and human EC cells which either contain another mechanosensor or are potentially not mechanically sensitive. One hypothesis proposed 50 years ago, suggested that enteroendocrine cells (EEC) can have different functions relating to differences in morphology. This study suggests that the apical process of open EECs detect chemical stimuli (Fujita and Kobayashi, 1971), while closed cells were thought to respond to mechanical stimuli (Fujita and Kobayashi, 1971, Kobayashi et al., 1971). This hypothesis is supported by recent evidence that suggests EC cells can be divided

into two distinct pools, with one EC cell pool remaining in the mucosa for up to 13 months (Wei et al., 2021), a new discovery which significantly differs from the previous dogma that EECs turnover within 4-5 days (Heath, 1996). Similarly, two separate pools containing Tph1, predicted to be two distinct EC cell subtypes, have been described from RNA sequencing studies performed on both mouse and human intestinal epithelial cells (Gehart et al., 2019, Beumer et al., 2020). Hence there are likely subtypes of EC cells found along the GI tract, which differ in their time of residence, morphologies and lumen facing characteristics. As highlighted by Fujita et al (1971), these differences in morphology might indicate that different EC cells may respond to different stimuli or facilitate different functions. Leading to the hypothesis that some EC cells are mechanically sensitive, while others may respond to other, non-mechanical stimuli hence potentially explaining the lack of Piezo2 in a proportion of EC cells. This hypothesis could be studied further in the future, through the isolation of EC cells using our previously published method (Martin et al., 2017a). In such an investigation, we could measure single cell mechanosensitivity of EC cells, and then analyse Piezo2 expression within these same cells to form pair analyses and deduce whether mechanosensitivity correlates with the presence of Piezo2.

There was a proportion of unidentified Piezo2 positive cells in the mucosal and submucosal layers which did not contain serotonin (see Appendix Figure 4). Whilst there are currently no other specially identified mechanosensitive intestinal epithelial cells in the literature (Beyder, 2019), past studies have indicated that other epithelial cells are mechanosensitive (Burnstock, 2009). Recently, an enteroendocrine lineage reporter mouse line (NeuroD1-cre; GCaMP5-tdTomato) was found to contain Piezo2 (Alcaino et al., 2018). In NeuroD1-cre; GCaMP5-tdTomato mice, cells containing the transcription factor involved in late stage enteroendocrine cell development, NeuroD1

(Haber et al., 2017), are labelled with red (tdTomato) fluorescent protein. NeuroD1 cells contained Piezo2 expression in enteroendocrine cells not containing serotonin, inferring that other enteroendocrine cells, aside from EC cells, contain Piezo2 and are likely mechanosensitive. Several cells in the muscularis mucosa and submucosal layers were also found to contain Piezo2. It is possible that these are smooth muscle cells (SMCs), such as Interstitial cells of Cajal (ICC), which are known to be mechanosensitive (Kraichely and Farrugia, 2007, Joshi et al., 2021). These cells act through a calcium-mediated mechanism (Farrugia et al., 1999, Joshi et al., 2021) similar to Piezo2 (Woo et al., 2014, Ikeda et al., 2014), however the presence and function of Piezo channels in GI SMCs remains unexplored (Joshi et al., 2021). It is likely that other intestinal epithelial cell types also contain Piezo2, however further studies are needed to identify these cell types and confirm their mechanosensitivity.

The current study suggests that the ion channel Piezo2 is important for mechanosensing in human EC cells, as human intestinal mucosa was shown to release serotonin as a direct response to mucosal compression in real-time. The release of serotonin typically occurred in less than a second from the point of contact, comparable to previous reports of mechanically stimulated release of serotonin from guinea pig small intestine and mouse colon (Bertrand, 2004, Keating and Spencer, 2010). The same response was seen in both human and mouse small and large bowel, with a fast peak response at the point of mucosal compression followed by a slower steady state release of serotonin. The peak or ‘fast’ release of serotonin from mucosal compression was seen from all samples regardless of species or location along the GI tract, followed by a steady state or ‘slow’ release lasting at least 10 seconds (the maximum time recorded per mechanical stimulus). The same release kinetics were described from mouse EC cells, in which there was a fast Piezo2-dependent

mechanosensitive release of serotonin, followed by a sustained phase lasting many seconds (Alcaino et al., 2018). This peak release could represent the readily releasable pool of serotonin vesicles in EC cells, while the longer steady state release of serotonin could represent the slower release of the reserve pool of serotonin vesicles. The steady state duration measured does not match the typical inactivation decay kinetics for Piezo2 of ~6-7 ms (Coste et al., 2012, Ikeda et al., 2014). In such a case, the peak response could represent the opening and closing of the Piezo2 channel, while the steady state release could be mediated through other mechanisms down-stream of Piezo2 which allow the slower release of the reserve pool of serotonergic vesicles. Potential downstream mechanisms likely involve a steady influx of Ca^{2+} resulting in the longer lasting release of serotonin, such a mechanism could be mediated through 'long-lasting' L-type Ca^{2+} channels important for EC cell serotonin release (Raghupathi et al., 2013). Previous reports by Alcaino et al (2018) describe intracellular Ca^{2+} increases seen in mouse EC cells as a result of intestinal membrane displacement, which lasts many seconds and is only limited by extracellular Ca^{2+} availability (Alcaino et al., 2018). It is common for cells to use Ca^{2+} signaling to control the size and length of response to receptor current (Beyder, 2019). This is similar to the Merkel cell, in which force activation of Piezo2 causes an inward cation current, that leads to the depolarization of the cell, stimulating L-type Ca^{2+} channels which allow a further influx of intracellular Ca^{2+} that presumably leads to exocytosis (Woo et al., 2014, Ikeda et al., 2014). Together, these findings suggest that both human and mouse EC cells augment Piezo2 responses to force into temporally controlled serotonin release. These results coupled with the confirmed expression of Piezo2 in human EC cells indicates that this channel is likely involved in the

mechanotransduction of human EC cells, as shown in mouse EC cells (Wang et al., 2017, Alcaino et al., 2018).

The real-time release of mechanically stimulated serotonin from human intestinal mucosa was significantly reduced when Piezo channels blocked pharmacologically with the inhibitor, GsMTx4, confirming a role for Piezo channels in human EC cell mechanotransduction. This inhibitor is a peptide known to block both Piezo1 and Piezo2 channels specifically (Coste et al., 2010, Bae et al., 2011, Gottlieb et al., 2011, Coste et al., 2012, Alcaino et al., 2017b, Wang et al., 2017, Alcaino et al., 2018, Gottlieb and Sachs, 2012), derived from the venom of the spider *Grammostola spatulate.* This inhibitor blocks cationic-selective stretch channels by increasing the threshold required for channel activation (Suchyna et al., 2000, Suchyna et al., 2004), hence favouring the closed state of both Piezo1 and Piezo2 channels (Gottlieb et al., 2011, Alcaino et al., 2017b). A second form of the inhibitor also exists, D-GsMTx4, simply an enantiomer of the naturally occurring GsMTx4 (Suchyna et al., 2004). Both forms of the inhibitor have been shown to have the same effectiveness in blocking Piezo channels (Suchyna et al., 2004) (Bae et al., 2011). In the current study, GsMTx4 caused a 30-45% reduction in the mechanically stimulated peak release of human and mouse colonic and ileal serotonin release, indicating that a Piezo channel is likely to be a significant mediator for mechanical transduction in human and mouse EC cells. This degree of reduction seen with GsMTx4 is comparable with previous reports, in which the same drug successfully blocked the mechanosensation in the QGP1 cell line, primary mouse EC cells, and HEK-293 cells transfected with human Piezo2 (Wang et al., 2017, Alcaino et al., 2017b, Alcaino et al., 2018). Wang et al (2017) reported an 89% inhibition of mechanosensitive inward currents in QGP-1 cells by D-GsMTx4. Similarly, Alcaino et al (2018) blocked pressure-invoked responses from

cultured mouse NeuroD1+ cells by 94.6%. Previous reports of total inhibition of mechanical response have only been achieved with the use of Piezo2 siRNA in primary mouse EC cells (Alcaino et al., 2018). This is expected as siRNA knockdown is a more targeted approach to silence the channel (Fire et al., 1998), hence more specific and effective than pharmaceutical approaches such as GsMTx4. The reported results with siRNA suggest that Piezo2 is the primary mechanosensory channel in mouse EC cells, hence indicating it is possible human EC cell Piezo2 is equally as important albeit further testing with Piezo2 siRNA or other approaches are necessary to conclusively deduce such information.

As GsMTx4 is known to block both Piezo1 and Piezo2 channels (Coste et al., 2010, Bae et al., 2011, Gottlieb et al., 2011, Coste et al., 2012, Alcaino et al., 2017b, Wang et al., 2017, Alcaino et al., 2018, Gottlieb and Sachs, 2012), we cannot conclusively state that Piezo2 is the primary mechanosensor involved in human EC cell mechanotransduction. It is also important to note that while recent findings have implicated Piezo2 as important in EC cell serotonin release (Wang et al., 2017, Alcaino et al., 2018), the presence or potential role of Piezo1 in EC cells has not been explored to date (Sugisawa et al., 2020). However, we hypothesize that Piezo2 is the primary mechanosensory protein in human EC cells for the following reasons:

1) The results from the current study reflect those found in primary mouse EC cells, in which D-GsMTx4 was found to significantly reduce EC cell activation and serotonin release (Alcaino et al., 2018), confirmed to be through Piezo2 through siRNA knockdown of Piezo2 in mouse EC cells which abolished EC cell mechanically stimulated response altogether (Alcaino et al., 2018).

2) The two channels are known to have distinct mechanosensing functions in different cell types throughout the body. This is evidenced by the literature, as Piezo1 is typically

involved in sensing shear stress and membrane tension (Cox et al., 2016b, Lewis and Grandl, 2015, Syeda et al., 2016), changes in cell volume (Peyronnet et al., 2013b, Faucherre et al., 2014b, Miyamoto et al., 2014a, Cinar et al., 2015b, Retailleau et al., 2015b, Martins et al., 2017) and cell crowding (Slattum and Rosenblatt, 2014). In the gut, Piezo1 has been shown to sense static forces such as cell crowding (Eisenhoffer et al., 2012) and cell migration (Yang et al., 2014) in the GI epithelium. Piezo2, however, is important in specialized mechanosensation such as sensing light skin touch (Ikeda et al., 2014, Ranade et al., 2014b), proprioception (Woo et al., 2015, Chesler et al., 2016), mechanical pain (Szczot et al., 2018) and stretch in airway (Nonomura et al., 2017) and bladder (Marshall et al., 2020).

It is likely that the mechanosensing response of EC cells is not solely mediated by Piezo2, particularly as the mechanical response of primary human and mouse EC cells is not entirely depleted when Piezo channels are pharmacologically inhibited or knocked down. In the current study, this was evident through amperometry studies in which the real-time release of serotonin was significantly reduced, but not abolished in the presence of GsMTx4. Similar results were previously reported from primary mouse EC cells, in which D-GsMTx4 did not completely eliminate the mechanically stimulated release of serotonin (Wang et al., 2017, Alcaino et al., 2018). Similarly, GI epithelial Piezo2 knockdown (Vil-cre;Piezo2$^{f/f}$) mice did not experience complete inhibition of pressure-induced secretion either (Alcaino et al., 2018), suggesting another mechanism contributes to this mechanically stimulated response. However, this Piezo2 conditional knockout mouse line was not specific to EC cells, and interestingly nor was serotonin release reported as a parameter in this study. Also of note, the mouse intestinal samples used in this study were full thickness, hence mechanosensory neurons and smooth muscle cells also likely impacted the results.

One explanation for the incomplete inhibition of mechanically stimulated serotonin release in the current study, is that EC cells may use different mechanosensory pathways to both detect and respond to different stimuli (Cooke et al., 2003) (Eyckmans et al., 2011b). Initial reports on EC cells found other mechanisms important in their mechanical sensing properties, including purinergic receptors for adenosine and nucleotides (ATP, ADP) (Cooke et al., 2003, Christofi, 2008) (Liñán-Rico et al., 2013), disruption of caveolae (Kim et al., 2007), and G-protein coupled receptors, such as P2Y (Kim et al., 2001, Christofi, 2008, Chin et al., 2011, Liñán-Rico et al., 2013). Mechanical stimulation causes the release of nucleotides, ATP and UTP, from various cell types, a process which also occurs from the epithelial lining of the intestine during peristalsis (Kim et al., 2001, Cooke et al., 2003). Caveolae also play a role in mechanosensitivity (Yu et al., 2006), and have been linked with mechanically stimulated ATP and serotonin release from the human EC cell model, BON cells (Kim et al., 2007). Similarly, mechanical stimuli such as mucosal stroking or stretch promote the release of ATP and adenosine to regulate mechanosensitive secretory reflexes in BON cells and the guinea pig colon (Cooke et al., 1999, Christofi et al., 2004). In the bladder epithelium, ATP release resulting from mechanical stimulation is linked with Piezo1, in which the mechanical activation of Piezo1 leads to Ca2+ influx and ATP release from urothelial cells (Miyamoto et al., 2014c). Whilst P2X channels are not mechanosensitive themselves, it is well documented that ATP is commonly released in response to the mechanical stimulation of different cellular systems (Guan et al., 2018), therefore it may provide a positive feedback loop upon mechanical stimulation potentially linked with Piezo channels as the mechanism recently shown in the bladder (Miyamoto et al., 2014c). As these described molecules have not been established as mechanosensitive (Beyder, 2019), it is likely that the primary mechanosensor in

human EC cells is Piezo2, and that other mechanisms are either involved in modulating, or downstream of Piezo activation.

Studies have indicated a role for many of the aforementioned mediators, including nucleotides, in modulating Piezo function. Specifically, UTP and ATP reduce the threshold for mechanically stimulation of peptidergic nociceptive DRG neurons, and hence enhance their mechanosensitivity (Lechner and Lewin, 2009). At the time of this study, Piezo channels had not yet been identified, however it has since been determined that Piezo channels are expressed in DRG and contribute to their mechanosensitivity (Ranade et al., 2014b, Shin et al., 9000). Importantly the desensitisation of Piezo2 containing sensory neurons by bradykinin is important in inflammatory and pathophysiological mechanical responses (Dubin et al., 2012). A similar pathway was depicted, in which the activation of G protein-coupled receptors also enhances mechanically activated Piezo2 channels and DRG neurons, interestingly this pathway was shown to act via serotonin receptors (Del Rosario et al., 2020). Under pathophysiological conditions of the gut, irregular distension associated with painful sensation simulates ATP release (Wynn et al., 2004, Burnstock, 2009). ATP activates EC cells, which trigger visceral afferent pain pathways (Wynn et al., 2004, Burnstock, 2009). However, not all EC cells require endogenous ATP or other nucleotides for mechanotransduction. This is demonstrated by indirect (Liñán-Rico et al., 2013), and direct pharmacological experiments (Wang et al., 2017, Alcaino et al., 2018) all completed on healthy tissue. Hence, we propose that mediators such as ATP and other nucleotides likely modulate Piezo channels, playing a role in hypersensitive or pathophysiological conditions, and that Piezo2 is the primary mechanism for EC cell mechanosensation in normal or healthy states.

EC cells are known to be subject to various forms of mechanical stimulation, including distention, stretch, deformation, and shear stress among others (Linan-Rico et al., 2016). In the current study, we find that both shear stress and mucosal compression stimulate mouse and human EC cell serotonin release. We replicated previous shear stress experiments completed by Kim et al (2001), in which human ileum and colon mucosa were mechanically stimulated via shaking of fluid over the tissue. A dose-dependent increase in shaking correlated with an increase in serotonin release from human ileum samples. The release of serotonin from human ileum samples was inhibited by GsMTx4 to a level similar to that of control (no shaking) samples, although this study was underpowered. This reflected previous reports, in which shear stress stimulated 58% of NeuroD1+ cultured cells measured via calcium imaging (Alcaino et al., 2018). These results were blocked or inhibited by calcium free media, Gadolinium (common mechanosensitive channel blocker), D-GsMTx4 and Piezo2 siRNA, indicating the importance of Piezo channels in the response. The colonic samples however were not stimulated in a uniform manner, this could be a result of underpowered results, or it could signify a difference in mechanosensing capabilities between the human small and large bowel. It is interesting that the colon did not appear to respond to fluid stimulation and begs the question whether this is a functional difference between the colon and small intestine. It is possible that the difference in morphology of the epithelium between these two regions of intestines has a role in their mechanosensing properties. The villi which occur in the small intestine have the potential to be more prone to sensing fluid movement, compared with colonic crypts. This would make sense as the small intestine is typically involved in water absorption along the GI tract, aided by the large surface area and a process known as segmentation (Huizinga et al., 2014). It has been demonstrated that content becomes

increasingly less fluid along the colon due to fluid absorption (Hukuhara and Neya, 1968, Costa et al., 2015), suggesting that most of the colon would not typically experience large volumes of fluid. For this reason, colonic EC cells likely require less sensitivity to fluid, and more specific response to other types of mechanical stimulation such as mucosal compression or colonic distention. It has been suggested previously that different stimuli activate different mechanosensory systems (Cooke et al., 2003, Linan-Rico et al., 2016).

Interestingly the degree of response to mechanical stimulation was greater from the small intestine of both mouse and human compared with colon. This correlates with the higher percentage of EC cells containing Piezo2 in the small intestine of both species and could further indicate different mechanosensing abilities between EC cells of the small and large bowel. One might think that a greater number of EC cells in the small intestine could cause such a result, particularly as it has been previously reported that the level of serotonin release is dependent on EC cell density (Raghupathi et al., 2013). However, the colon contains a greater number of EC cells (Martin et al., 2020a, Wei et al., 2021) and has a greater total content of serotonin, as shown in the current study, compared with the small intestine. This suggests that EC cells in the small intestine are more mechanically sensitive, hence EC cells of different regions of the GI tract may respond differently to mechanical stimuli. Previously published work by our research group reported changes in nutrient response and receptor profiles of EC cells between the small and large bowel (Martin et al., 2017d). A recent study demonstrated that colonic EC cells have smaller capacitances and faster fast inactivation time constants via electrophysiology (Strege et al., 2017). Hence these studies demonstrate differences in EC cells based on their location along the GI tract. Further investigation is needed to delineate the difference in

mechanosensing properties of EC cells between different regions of the GI tract, as EC cells are known to differ in characteristics based on their location.

EC cell derived serotonin has recently been shown to modulate GI motility (Wei et al., 2021). We wished to further understand whether Piezo2 is the mechanism by which EC cells sense distension to trigger serotonin secretion and thereby modulate colonic motility. The previously mentioned Piezo inhibitor, GsMTx4, was used on mouse CMMCs *ex vivo* and found to dramatically reduce the frequency of CMMCs. The drug was given at varying doses, where 1 and 5 μM also reduced the amplitude and propagation of CMMCs, and a dose of 10 μM ablated CMMCs entirely. This is the first demonstration that blocking Piezo2 channels has a negative impact on colonic motility. As expected, the colonic motility data shown here reflects that recorded from previous models lacking EC cell serotonin (Keating and Spencer, 2010, Spencer et al., 2011, Wei et al., 2021), in which the frequency and propagation of CMMCs were also reduced significantly. Together, this reflects the proposal that EC cells sense distention of the gut via Piezo2, and as a result release serotonin to further perpetuate motility and increase the frequency and strength of contractions. Thereby suggesting that the mechanism through which EC cells are mechanosensitive and contribute to the modulation of GI motility is through the mechanosensing ion channel Piezo2. A potential limitation of the current study is that contractile activity was measured from open-sheet preparations, hence the inhibitor is not applied just to the mucosa but can also reach the serosal side of the tissue. In which case there is potential for the inhibitor to act on smooth muscle cells and neurons within the myenteric and submucosal plexus. In future experiments it would be desirable to use intact preparations in which the drug is perfused through just the lumen, in which the area of application is limited to just the mucosa. Previous results reported that application of D-GsMTx4, to the

mucosal side of GI tissue in an Ussing chamber, blocked pressure-induced serotonin release by 43% (Wang et al., 2017). This is the first investigation into the role of Piezo2 in EC cell's role in CMMCs, which occurs likely through a reduction in serotonin release due to a decreased ability of EC cells to sense mechanical stimuli. This data supports the hypothesis that the mechanosensory protein, Piezo2, is responsible for EC cell's ability to sense mechanical stimuli to modulate motility.

CMMCS from mucosa-free mouse colonic preparations were measured with the Piezo inhibitor to test whether neuronal mechanotransduction, via Piezo2, has a role in colonic motility. The baseline frequency of mucosa-free CMMCs was significantly slower than that of intact preparations, in line with previously published results (Keating and Spencer, 2010). The inhibition of Piezo channels, with GsMTx4, on mucosa-free preparations completely abolished CMMCs. This result indicated that neurons within the myenteric or submucosal plexuses might also contain Piezo channels that contribute to the formation of CMMCs. Piezo2 is found in ~85% of DRG neurons (Coste et al., 2010, Ranade et al., 2014b, Eijkelkamp et al., 2013, Lou et al., 2013), and Piezo1 and 2 are expressed by several neuronal types of the mouse ENS (Drokhlyansky et al., 2020). Furthermore, enteric neurons are known to be mechanosensitive (Kunze et al., 2000, Zagorodnyuk and Spencer, 2011, Hibberd et al., 2012), and their response to mechanical stimulation has previously been identified to contribute to motility in the gut (Spencer et al., 2003). This suggests that there is likely a coupling of mechanosensation in the gut, between the mechanosensing EC cells and mechanosensing neurons in the myenteric or submucosal plexuses that contribute to the modulation of colonic contractile activity via Piezo2 mediated pathways. This is similar to other mechanosensing systems throughout the body, known to utilize Piezo2 as their main mechanotransducer, in which Piezo2 is

expressed and functional in both the epithelial cell type and synapsing neurons (Ranade et al., 2014b, Woo et al., 2014, Maksimovic et al., 2014, Nonomura et al., 2017, Marshall et al., 2020). Many of these studies have indicated a two-part signaling mechanism involving Piezo2, in which epithelial cell–neuronal sensory machinery shares the response to mechanical stimuli. This has been demonstrated through the deletion of Piezo2 in both epithelial and sensory neurons, such as the Merkel cell and sensory neurons (Ranade et al., 2014b). Similarly, we see the complete termination of CMMCs when GsMTx4 is applied to both the mucosal and serosal side of colonic preparations. Hence it would seem likely that a similar system is found in the gut, where EC cells, known to interact with mechanosensitive sensory neurons (Kunze et al., 2000), and mechanosensing neurons both contain Piezo2 as their primary mechanotransducer and have a role in the modulation of CMMCs.

Our collaborators created a specific Tph1 Piezo2 knockdown mouse model, $Tph1^{CreERT2/+}$;$Piezo2^{flox/flox}$, in which Piezo2 is knocked down from all Tph1 containing cells specifically. This model allows us to deduce the role of EC cell mechanically stimulated serotonin release in CMMCs without effecting potential confounding factors such as neuronal Piezo2. GI motility of $Tph1^{CreERT2/+}$;$Piezo2^{flox/flox}$ mice was measured *in vivo*, and mice lacking EC cell Piezo2 were found to have a significantly slower whole gut and colonic total transit time compared with control mice (see Appendix Figure 5). This is the first evidence to confirm the role for EC cell Piezo2 in modulating GI motility and supports our hypothesis that Piezo2 in EC cells is important in activating the release of EC cell derived serotonin to modulate colonic motility. A recent investigation on Piezo in *Drosophila*, described that Piezo knockout (KO) flies ate and drank significantly more than controls, some to the point of overeating to death (Wang et al., 2020). This indicates that flies feed based on mechanical cues from Piezo+

neurons in the gut, which sense gut distension and signal food consumption and satiety to halt overconsumption (Wang et al., 2020). A separate study described a slower gut transit time in a gut epithelia-specific Piezo1 knockdown (Villin-Piezo1$^{flox/flox}$) mouse line, although no evidence was provided to demonstrate the expression of Piezo1 in EC cells specifically or to confirm that the protein was knocked down from EC cells (Sugisawa et al., 2020). The study did describe a reduction in both serum and duodenum serotonin levels, Tph1 mRNA expression in the gut and a reduced number of serotonin positive cells in the duodenum of Piezo1 floxed mice (Sugisawa et al., 2020). Interestingly, it was reported from this research group, that Piezo1 knockdown did not influence serotonin secretion in response to cyclic stretch, in RIN14B cells, however Piezo2 knockdown did inhibit serotonin release to cyclic stretching (Sugisawa et al., 2020). Overall, the results from this study are inconclusive, and further work is needed to delineate the separate functions of Piezo1 and Piezo2 in EC cell mechanobiology and colonic motility. Original evidence suggested serotonin is released during peristalsis from the guinea pig ileum (Bulbring and Crema, 1959), which indicated the ability for EC cells to mechanically sense content via distention. The order in which serotonin release and peristalsis occurs has been debated within the field, with some groups suggesting that EC cell serotonin drives GI motility (Smith and Gershon, 2015a). However, the most recent evidence substantiates that peristalsis occurs without serotonin (Wei et al., 2021), indicating that serotonin is not the driver of peristalsis but rather is released consequently to perpetuate the neuromechanical loop (Spencer et al., 2016). This indicates that the mechanosensing properties of EC cells likely modulate the role that EC cells play in GI motility, a function mediated through Piezo2. Furthermore, additional investigation of

Tph1$^{CreERT2/+}$;Piezo2$^{flox/flox}$ mice will allow the deduction of the importance of neuronal Piezo2 in colonic motility.

The importance of functional mechanosensing in healthy GI function is evident as shown by abnormalities in GI mechanosensation linked with certain motility disorders, such as irritable bowel syndrome (IBS) (Yang et al., 2016) and constipation (Neshatian et al., 2015, Alcaino et al., 2017a). As outlined in the previous chapter, it is also known that serotonin is closely linked with a number of functional motility disorders {Gershon, 2004 #586; Galligan, 2007 #1132; Wei, 2021 #688}. The current study provides the first evidence of a relationship between EC cell mechanosensitivity and age, revealing a reduction in both the number of EC cells containing Piezo2 and EC cell mechanosensitivity in the colon with age. Similarly, female mice EC cells were found to be significantly more mechanosensitive than male EC cells in both the small and large bowel. As highlighted by the use of Tph1$^{CreERT2/+}$;Piezo2$^{flox/flox}$ mice, a reduction in EC cell mechanosensitivity leads to a constipation-like phenotype with the significant slowing of GI transit seen in these mice. This is interesting, as females are known to be affected by IBS at a significantly higher rate than males (Kim and Kim, 2018). It is also widely known that constipation affects the aging population at a significantly higher rate, with approximately 50% of adults over the age of 80 (Higgins and Johanson, 2004). However, the underlying cause of age-related constipation is still unknown (Gandell et al., 2013). Various studies report a reduction in the number of myenteric neurons in human and mouse (Sun et al., 2018b, Saffrey, 2013, Camilleri et al., 2000) as a contributing factor to age-related GI motility dysfunction. Other reports describe the importance of mechanosensitivity of smooth muscle cells, such as ICCs, in motility (Rao and Gershon, 2016, Joshi et al., 2021) and indicate a reduction in the number of ICCs in the aging colon (Gomez-Pinilla et al., 2011, Joshi

et al., 2021). However, age-related differences in GI anatomy and physiology are not deemed to be significant contributors to the development of constipation (Camilleri et al., 2000). The research from this chapter suggests that the reduction in EC cell mechanosensitivity in aging individuals might contribute to age-related constipation, however significant further investigation is required to assess this hypothesis. One suggestion is to assess EC cell mechanosensitivity and GI Piezo2 expression in colonic biopsies from elderly patients who experience age-related constipation, this would allow a deduction of the role of GI Piezo2 in age-related constipation specifically. Overall, this sheds light on a link between Piezo2 expression with age and sex, indicating potential new target for treatment in age-related constipation.

3.5 Conclusion

This study has provided the first evidence of Piezo2 functionality as a key mechanosensor in EC cells in the human small and large bowel. We have provided further evidence detailing the role of Piezo2 in mucosal differing forms of mechanical stimuli on mouse EC cell mechanosensitivity. Furthermore, the pharmacological inhibition of Piezo, in various experiments, was shown to significantly diminish mechanically stimulated serotonin release from both human and mouse gut. Evidence for a role of Piezo2 in colonic motility has also been reported for the first time, demonstrating EC cell Piezo2 is important in the modulation of GI motility. Finally, we note the link between EC cell mechanosensitivity with both age and sex.

CHAPTER 4: Enterochromaffin cells contain neuropods, and directly contact sensory nerve fibres in the mouse distal colon.

4. Enterochromaffin cells contain neuropods, and directly contact sensory nerve fibres in the mouse distal colon.

4.1 Introduction

The nerves within the gastrointestinal (GI) tract have long been known to sense the contents of the lumen and cause both local effects, as well as communicate sensory information to the brain via a mechanism termed the gut-brain axis. It was originally hypothesised that enteroendocrine cells (EECs) secrete hormones which act locally on adjacent cells, innervating nerves, or enter into the circulation by diffusion (Bertrand, 2009, Cummings and Overduin, 2007, Wade and Westfall, 1985b). However the most recent evidence indicates that neuronal responses to luminal contents occur within seconds (Beutler et al., 2017, Su et al., 2017), making diffusion (which takes hours to minutes (Rehfeld, 1998)) less likely to be the predominant mechanism. This finding sparked investigation into the mechanism of communication between EECs and nerves in the GI tract, with several groups suggesting a new hypothesis that EECs directly synapse with neurons innervating the GI mucosa (Bohórquez et al., 2015, Kaelberer et al., 2018). Thus, suggesting that synaptic connections may underpin the gut-brain axis, however further investigation is warranted to understand the nature of such connections.

The GI tract is unique as it is the only organ system which has its own intrinsic nervous system. The enteric nervous system (ENS) interacts with extrinsic innervation to mediate GI function but can also operate in complete neural circuits within the intestinal wall without input from the brain (Kunze et al., 1995b, Furness et al., 1995). The extrinsic innervation is part of the peripheral nervous system (PNS) and innervation is derived from distinct anatomical sources relative to different regions along the GI tract. The upper gut, pertaining primarily to the stomach and small

intestine, is primarily innervated by the vagus nerve (Powley et al., 1994, Berthoud et al., 1995, Berthoud et al., 1997). The vagus nerve is a nerve bundle which originates in the nodose and jugular ganglia and projects predominantly to the nucleus of the solitary tract (Norman and Bower, 1982). The distal gut, which largely refers to the colon and the rectum, is innervated principally by spinal neurons that originate in the spinal cord (Brookes et al., 2013, Brierley et al., 2018). Intrinsic and extrinsic efferent and afferent neural systems work together to coordinate various gut functions; however, it is the extrinsic sensory neurons of the GI tract that facilitate the direct connection between the GI tract and the central nervous system (CNS).

It has been hypothesised the basal processes (termed “neuropods” (Bohórquez et al., 2011)) extending from EECs act as neurite-like extensions to facilitate the newly appreciated direct communication between EECs and neurons. The discovery of neuropods was made by Bohorquez, who found that both CCK and PYY EECs appear to make direct contact with nerves that penetrate the basal lamina (Bohórquez et al., 2011, Bohórquez et al., 2015). These EECs express presynaptic and postsynaptic genes which encode proteins required for trans-synaptic signaling (Südhof, 2012, Chen et al., 2008), strengthening the case that these cells can form functional connections with neurons. Several *in vitro* studies have indicated that EECs are able to grow neuropods towards sensory neurons in culture (Bohórquez et al., 2015, Kaelberer et al., 2018), suggesting that neurons may secrete chemoattractants to stimulate and direct EEC neuropod growth (Bohórquez et al., 2015). *In vivo* experiments in which mice were given an enema of a fluorescently tagged Rabies virus, which only travels through monosynaptic connections, showed that connected EECs and enteric neurons transmitted the virus (Bohórquez et al., 2015, Kaelberer et

al., 2018). These results were the first evidence that EECs *in vivo* form direct functional connections with enteric neurons.

There have been many reports of EECs containing neuropods (Chandra et al., 2010, Bohórquez et al., 2011, Bohórquez et al., 2014, Bohórquez et al., 2015) however, despite the importance of EC cell-derived serotonin both within the gut and more broadly in the gut-brain axis (Jones et al., 2020), the presence and distribution of neuropods on enterochromaffin (EC) cells is currently missing within the literature. The morphology of EC cells has been described to contain neuropod-like structures (Sjölund et al., 1983, Kuramoto et al., 2007), however, these have not been thoroughly characterised. Whilst one group observed that EC cells are electrically excitable, express presynaptic genes and are located within close proximity to nerve fibres containing serotonin receptors (Bellono et al., 2017), the relationship between EC cells and nerves has not been quantified or well characterised to date.

This chapter will describe the characterisation of EC cell neuropods in the mouse and human colon. This characterisation includes investigation into the presence and distribution of neuropods in EC cells of the mouse distal colon, and analysis of the anatomy of EC cell neuropods. Further goals of this study to characterise EC cell neuropods, are to determine whether EC cells contact sensory nerves within the distal colon and quantify the nature of such connections. We also investigate human colonic tissue, to determine for the first time whether neuropods on human EECs contact nerves which innervate the colonic wall. The specific aims are to:

1. Identify and characterise neuropods in mouse colonic EC cells;
2. Determine whether EC cells contact sensory nerve fibres within the colonic wall;
3. Identify neuropods on EC cells in human colon.

The overriding hypothesis is that both mouse and human colonic EC cells contain neuropods, which have the potential to form neural connections via direct connections.

4.2 Methods

4.2.1 Mice Tissue

C57 BL/6 male mice of 8-12 weeks were anesthetized with isoflurane inhalation (4%, IsoFlo) and euthanized humanely by cervical dislocation in accordance with approval by the Animal Welfare Committee of Flinders University (965-19). As in previous methods (3.2.1) segments of mouse colon were collected and stored in beakers containing oxygenated Krebs solution bubbled with 95% O_2/5% CO_2 to keep tissue viable until use.

4.2.2 Human Tissue

Human colonic samples were collected from the Flinders Medical Centre and Flinders Private Hospital surgical theatres with patient consent, as per previous chapter method (3.2.2).

4.2.3 Immunostaining

Freshly removed colonic tissue was collected from mice and human surgical samples, pinned out as far as could be stretched, mucosa facing down, using micro pins in Sylguard (Dow) coated dissection dishes. Tissue was fixed within one hour of removal in 4% paraformaldehyde (PFA) at room temperature overnight. Whole thickness samples were washed 3 times in 1x PBS and left in PBS containing sodium azide (0.01%) at 4°C until required for immunofluorescence staining. Samples were blocked with 10% Donkey Serum (Sigma Aldrich) in PBS for 1 hour, and then treated with primary antibodies for; serotonin (1:2000, Goat anti-5-HT IgG, 20079, Immunostar); calcitonin gene-related peptide (CGRP) (1:2000, Rabbit anti-CGRP IgG, T-4032, Peninsula) and PGP9.5 (1:1500, 165622D, Cedarlane), in 10% donkey serum in 1x

PBS and left shaking for 3 nights at room temperature. Samples were washed 3 times 10 minutes with 1x PBS, before secondary antibodies were applied. Secondary antibodies used were Cy3 conjugated Donkey anti-Sheep IgG (H+L) and Cy5 Donkey anti-Rabbit IgG (H+L) (1:200, Jackson ImmunoResearch Laboratories) in PBS overnight at room temperature. Finally, the samples were washed with PBS 3 times for 10 minutes at room temperature while shaking, and then left in 100% buffered glycerol for 5 minutes before being mounted onto microscope slides. Samples were mounted onto polyethylene glycol coated slides using 100% buffered glycerol to completely cover the samples, glass coverslips were placed, and clear nail varnish was used to seal slides. Slides were maintained at 4°C until viewed using microscopy.

4.2.4 Microscopy

Microscopy and image capture for the following chapter was performed as in previous method (3.2.4).

4.2.5 Image analysis

Images were exported and analysed offline using the 3D/4D imaging analysis software Imaris (Version 8.4.1, Bitplane). Thresholds were set for each fluorescent channel and applied to all images within the same experimental batch. Surfaces were created for cells and neuronal fibres using Imaris in-built software. Surfaces were set to a 0.2-0.5 μm surface grain size. Surfaces of non-specific staining were filtered out based on fluorescence intensity, sphericity, and volume. The created surfaces were exported to quantify cell statistics, such as numbers of cells, cell volume, area, length, and fluorescence intensity. The Distance Transformation function was used to determine distances between two surfaces. Information such as the presence and length of neuropods, and the location of connection between EC cells and sensory nerve fibres

were all calculated manually. All EC cells fully within the perimeter of the image were counted. Cells were deemed to contain a neuropod if they contained an extension or process longer than 2 μm clearly extending from the cell body. The location of connection between EC cells and sensory nerves was determined through analysis in all three dimensions.

4.2.6 Statistical analysis

Statistical analysis was performed as in previous method (2.2.8). Additionally, the number of technical replicates in total used per experiments is indicated as uppercase ‘N’.

4.3 Results

4.3.1 Optimisation of imaging and analysis method.

Wholemount tissue was stained for serotonin and calcitonin gene-related peptide (CGRP) to determine the characteristics of EC cells and to better understand the relationship between EC cells and the sensory innervation of the distal colon. Images taken using 2D epifluorescent microscopy can be informative (see Figure 4.1), however do not allow accurate interpretation of the 3D spatial relationship of the cells, and their connection with neural fibres. By using laser scanning confocal microscopy we were able to scan and image individual layers throughout the intestines, with significantly better resolution, and form 3D images (see Figure 4.2). Advanced analysis software (Bitplane's 'Imaris'), enabled us to gain intricate information from 3D reconstructed images. By applying a surface to each filter, fluorescence intensities can be used as parameters to reduce background and increase the accuracy of results through the analysis of specific immunofluorescence.

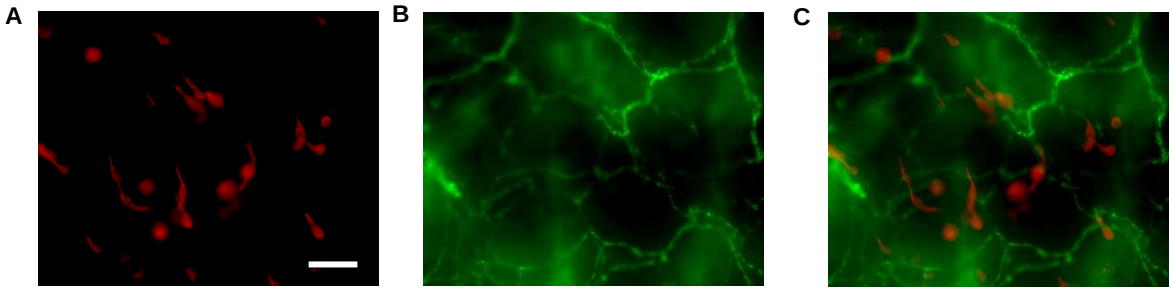

Figure 4.1: EC cells and CGRP labelled nerve fibres within the mouse distal colon imaged through 2D epifluorescence microscopy.

A) Serotonin (red) labelled EC cells. B) CGRP (green) labelled neuronal fibres. C) Composite image. Scale bar = 40 μm.

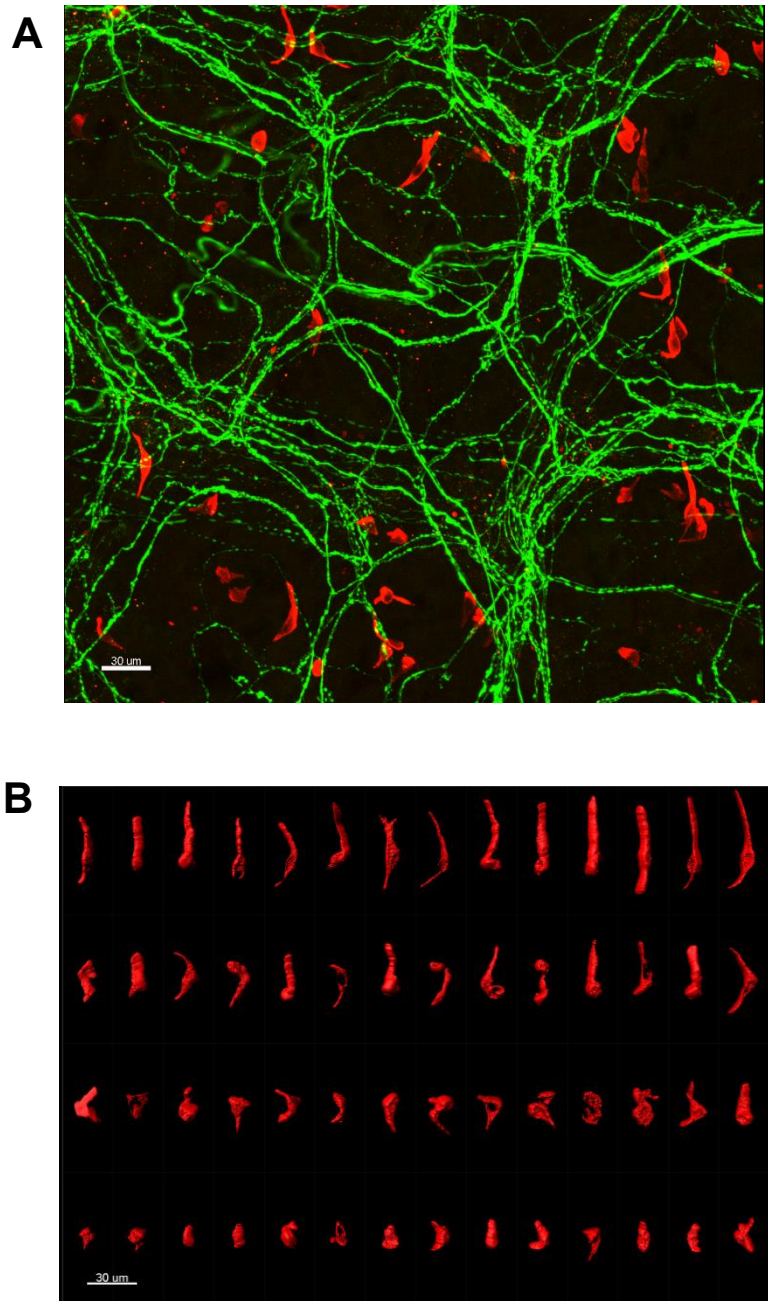

Figure 4.2: Rendered EC cells from confocal microscopy and 3D image analysis.

A) A z-projection of serotonin (red) stained EC cells and CGRP (green) stained neuronal fibres in the mouse distal colon. B) A gallery image of EC cells ordered by length from the z-projection in A. Scale bar = 30 μm.

4.3.2 Characteristics of EC cells in the mouse distal colon

Using 3D reconstruction, various characteristics of EC cells in mouse distal colon were ascertained. The average volume of EC cells in the distal colon was 618.1 ± 25.8 μm^3 (see Figure 4.3B). EC cells were analysed through all three planes, using 3D reconstructed models, and the presence of neuropods was determined. An EC cell was determined to contain a neuropod if the cell had a notable extension determined to be greater than 2 μm in length (see Figure 4.3A). It was found that 82.3 ± 2.4 % of EC cells contained neuropods in the mouse distal colon (n=4 mice, N=20 images) (see Figure 4.3C). Interestingly, some EC cells appeared to have multiple neuropods extruding from the cell body. The number of neuropods per cell was also investigated, it was found that 14.9 ± 2.1% had no neuropods, 55 ± 2.8% had 1 neuropod, 22.2 ± 2% had 2 neuropods and 0.2 ± 0.1% had 3 neuropods (see Figure 4.3E). The length of neuropods were manually measured for each individual cell using the software measuring tool. The length was measured in 3D, hence taking into account the protrusion of neuropods into different planes. The average length of neuropods on EC cells was 11.6 ± 0.3 μm (see Figure 4.3D).

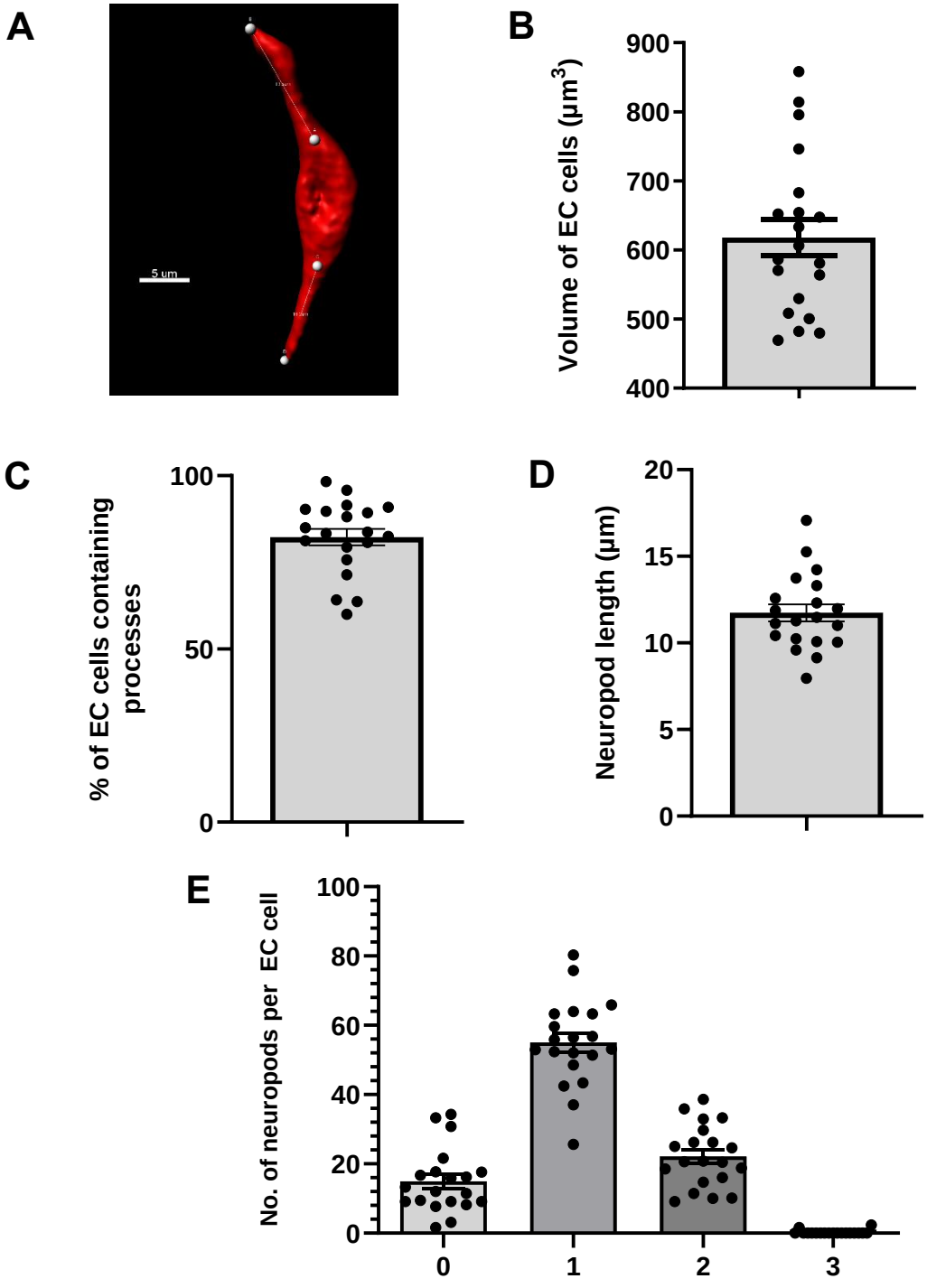

Figure 4.3: Mouse distal colonic EC cell characteristics.

A) Example image of an EC cell with the length of neuropods measured. B) The average volume of EC cells. C) The percentage of EC cells which contain neuropods. D) The average length of EC cell neuropods. E) The number of neuropods per EC cell (n=4 mice, N=20 images). Data are shown as mean ± SEM.

4.3.3 Spatial relationship between CGRP fibres and EC cells in the distal colon.

We were interested to determine whether EC cell neuropods came in close contact with sensory nerve fibres of the distal colon. Mouse distal colon was immunolabelled for serotonin and CGRP (a common marker for sensory nerve fibres found within this region of the GI tract) and imaged using confocal microscopy. The sensory nerve fibres stained with CGRP were apparent, and it was clear at first glance that some EC cells come into contact with CGRP nerves within the GI wall (see Figure 4.4). The distance between EC cells and CGRP neuronal fibres within all 3 dimensions of the mouse distal colon was measured and used to derive a 3D heat map based on CGRP staining intensity (see Figure 4.5). This data was determined across all samples, and the distances were sorted into bins of 2 μm increments, from which the frequency of varying distances were quantified, revealing that 64.1% of EC cells were within 0-2 μm with a CGRP fibre (see Figure 4.6). All EC cells were found to lie within a 0 – 15 μm range to the nearest CGRP fibre, and those which lied within the 0-2 μm bin were considered a close contact for the purposes of this study. We were then interested to determine the point at which CGRP fibres made contact with EC cells, as in some instances it appeared that the CGRP fibre was contacting the EC cell body (see Figure 4.7A). The location of connection between EC cells and CGRP neuronal fibres was manually determined for each identified point of connection in all samples. The regions identified were on the cell body, halfway along the neuropod, and at the end of the neuropod (see Figure 4.7). The location EC cells most regularly contacted CGRP fibres was at the cell body, totalling 57.7% of all connections (see Figure 4.7D). CGRP fibres were found to contact the end of the neuropod 27.6% of the time, and halfway along the neuropod 14.7% of the time (see Figure 4.7D). These results indicate that

EC cells contain neuropods of which a proportion are in contact with CGRP sensory nerve fibres.

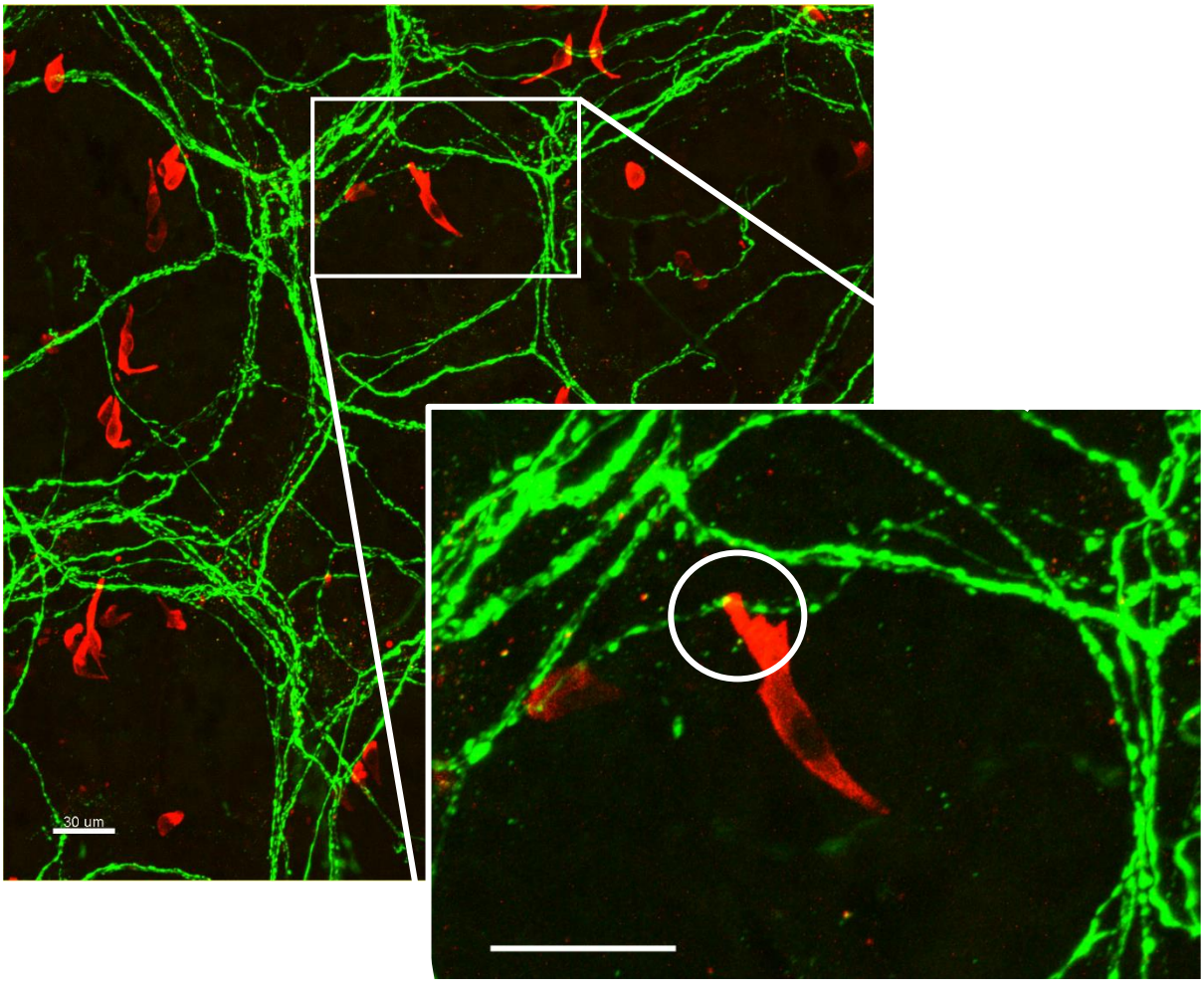

Figure 4.4: EC cells contact CGRP labelled sensory nerve fibres in the mouse distal colon.

A) Serotonin (red) labelled EC cell contacting a CGRP (green) labelled neuronal fibre. Scale bar = 30 μm.

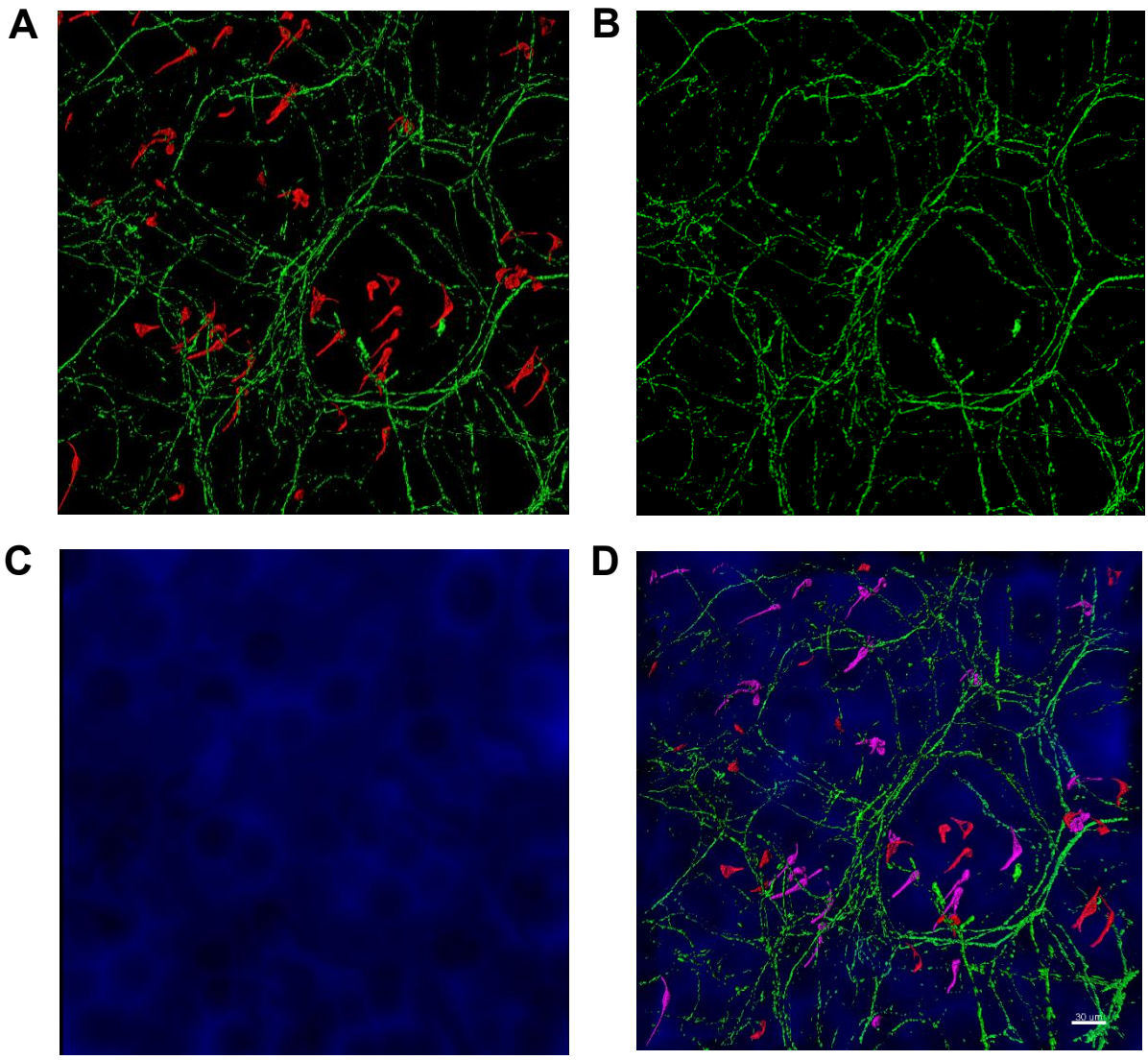

Figure 4.5: The creation of a CGRP 'heatmap' to determine the distance between EC cells and CGRP labeled sensory nerve fibres.

A) Serotonin (red) labelled EC cells and CGRP (green) labelled neuronal fibres. B) A surface is created to cover CGRP immunofluorescence and is rendered green. C) A new channel is created through the distance transformation function for the CGRP surface, in which a black-blue scale indicates distance from CGRP. D) All of the EC cells which contact the CGRP, as analysed through the new 'heatmap' channel, are now shown in pink. Scale bar = 30 µm

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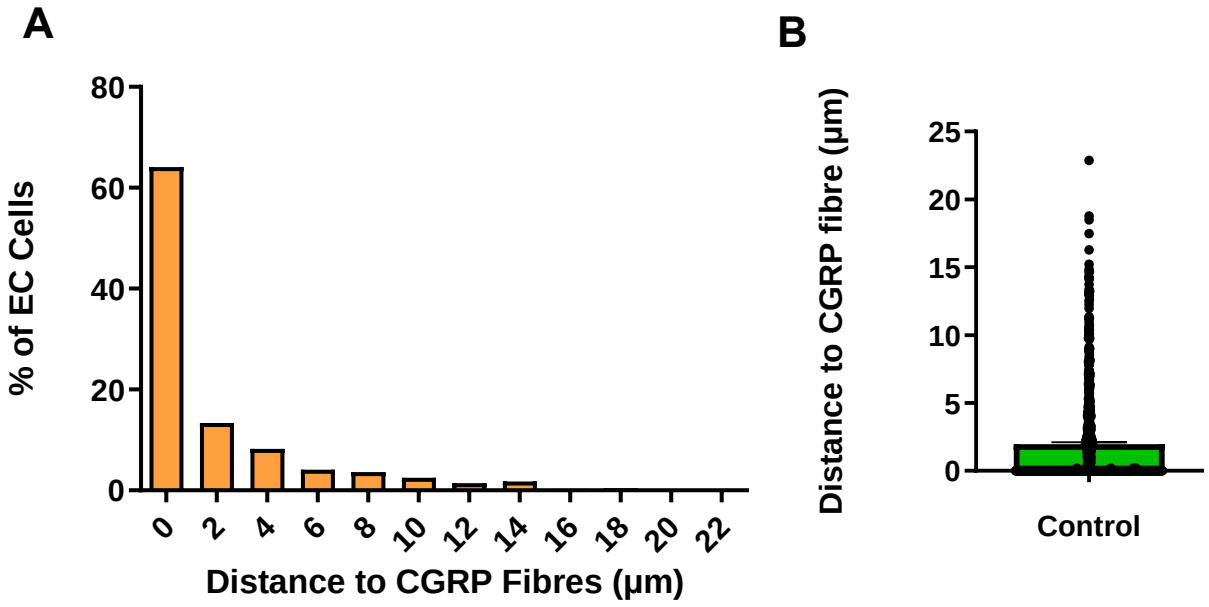

Figure 4.6: Most EC cells are touching CGRP nerve fibres.

A) The percentage of EC cells at 2 µm distance bins to CGRP nerve fibres within the mouse distal colon. B) The distance between EC cells and CGRP fibres (n=4 mice, N=20 images). Data are shown as mean ± SEM.

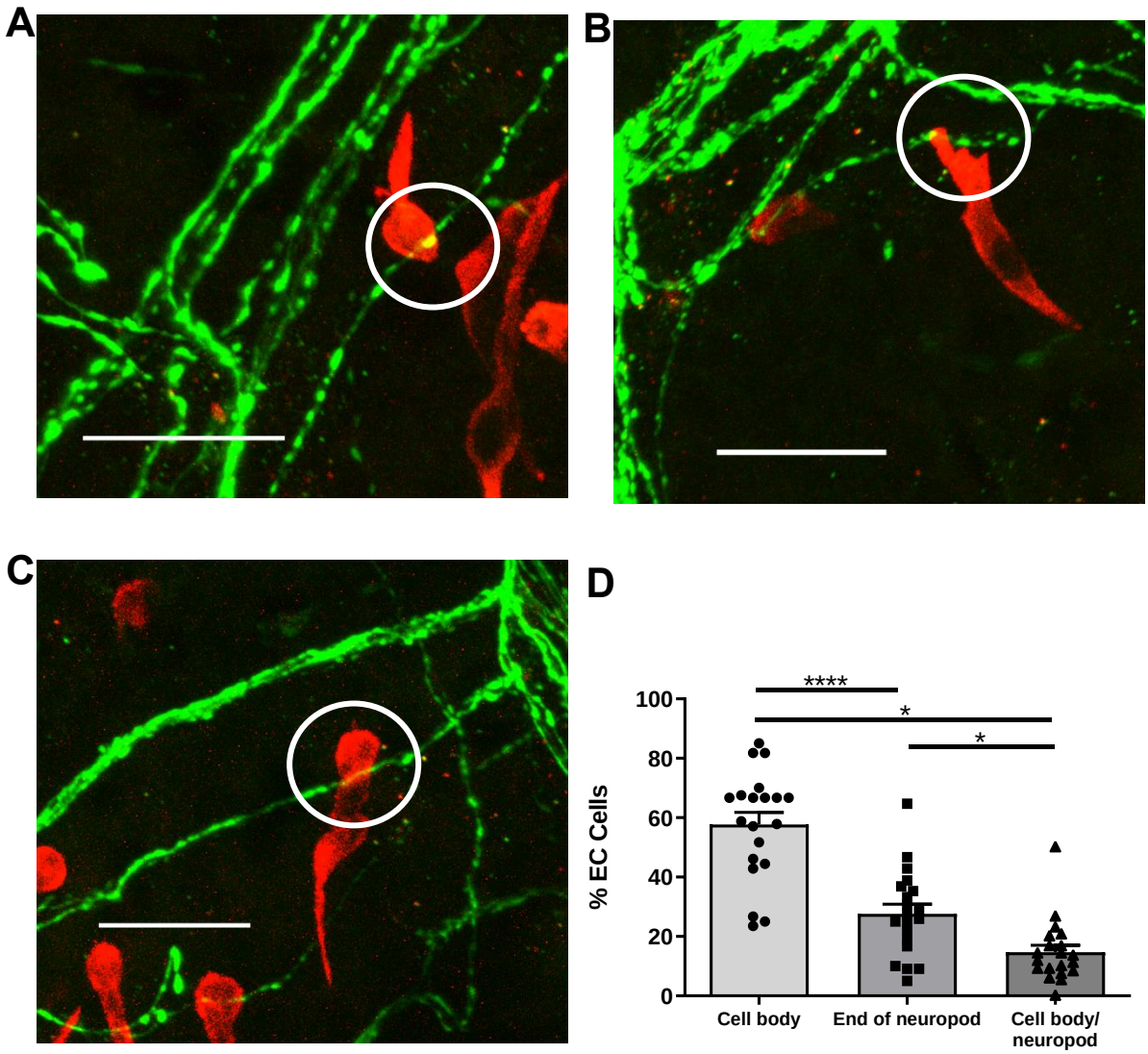

Figure 4.7: CGRP fibres predominantly contact EC cells at the cell body.

A CGRP fibre making contact with A) an EC cell body, B) the end of an EC cell neuropod, and C) EC cell along the neuropod, near the cell body. D) Quantification of the number of contacts which were defined as 'Cell Body', 'End of Neuropod' or 'Cell/body/neuropod' (n=4 mice, N=20 images). Scale bar = 30 μm. Data are shown as mean ± SEM. *=p<0.05, ****=p<0.0001.

4.3.5 EC cells in human colon contain neuropods and contact innervating nerve fibres.

Finally, we wanted to test whether human colonic EC cells also contain neuropods, and whether these cells also form connections with neuronal fibres in the human colon. One human colonic sample was collected and stained for serotonin and either CGRP or PGP9.5, a sensory nerve marker and a pan-neuronal marker respectively. The CGRP staining was not as successful in these thick human samples compared to mouse tissue, however PGP9.5 stain was clearly visible (see Figure 4.8) and hence was used for further quantification. Human colonic EC cells also appear to be in close proximity with neuronal fibres (see Figure 4.9A, B). The 3D analysis software was used to determine whether these EC cells are in contact with neuronal fibres in human gut. 19.4% of EC cells were found to be in contact with neuronal fibres within the intestinal wall (see Figure 4.9C). This is the first specific characterisation of these structures in human EECs.

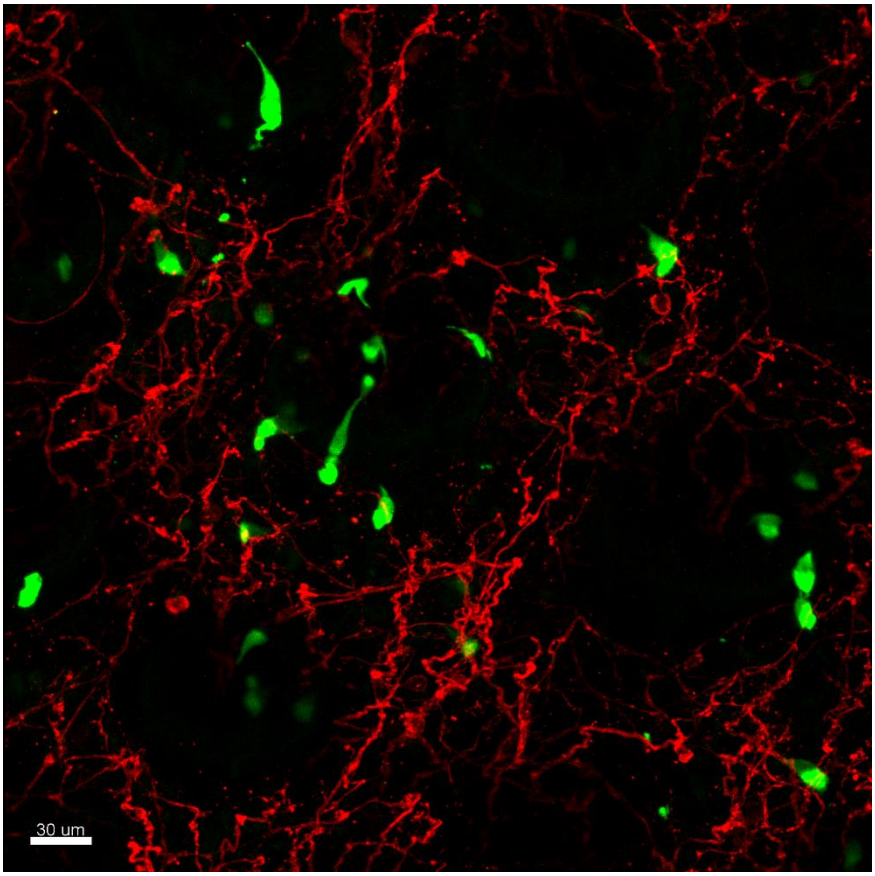

Figure 4.8: Colonic human EC cells also contain neuropods.

A) A z-projection of EC cells (green) and PGP95 labelled neuronal fibres (red) in the human colon. Scale bar = 30 μm.

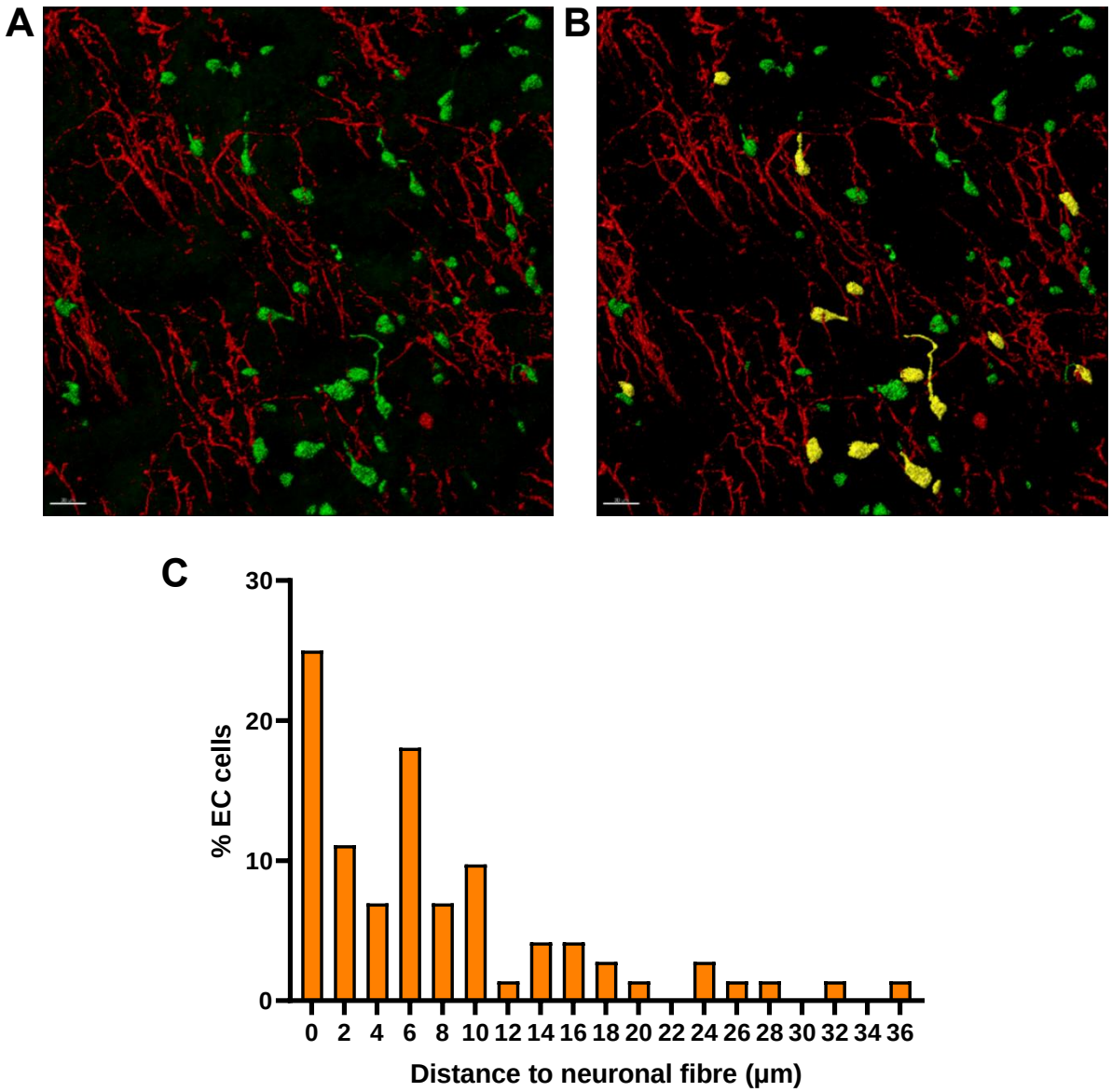

Figure 4.9: Colonic human EC cells also contact neuronal fibres.

A) an example z-projection of EC cells (green) contacting PGP95 labelled neuronal fibres (red) in the human colon (n=1). B) EC cells (yellow) make direct contact with PGP9.5 neurons. Scale bar = 30 µm. C) The percentage of EC cells at 2 µm distance bins to PGP-95 labelled nerve fibres within the human colon (n=1). Data are shown as mean ± SEM.

4.4 Discussion

The mechanism through which enteroendocrine cells (EECs) communicate directly with the neuronal innervation of the gastrointestinal (GI) tract is not currently known. In this chapter we examined how EC cells interact with sensory nerves which innervate the mouse distal colon. We were interested to see whether EC cells contain basal processes known as neuropods, reported in other types of EECs, and whether these neuropods form connections with sensory nerve fibres within the gut wall. To gain a better understanding of EC cell neuropods, mouse wholemount colonic tissue was immunohistochemically stained and imaged using confocal microscopy. 3D reconstruction and analysis were conducted to confirm the presence of neuropods on mouse EC cells and define their characteristics in the distal colon.

In this study the in-depth analysis of the spatial characteristics of EC cells revealed that 82% of EC cells in the mouse distal colon contain neuropods, supporting previous data obtained using thick sections of the rat distal colon which reported 80% of EC cells contained ‘cytoplasmic processes’ (Kuramoto et al., 2007). Here, we also show that numerous EC cells contain multiple neuropods which are seen in a wide range of lengths, the largest extending up to 37.5 µm. EC cells have previously been reported as bipolar (Kuramoto et al., 2007, Koo et al., 2021), with long neuropods running down the crypt, and second neuropods from the apical end of the cell extending towards the lumen (Kuramoto et al., 2007). The lengths of EC cell neuropods vary significantly in length, with many EC cells containing neuropods longer than their cell body, occasionally extending beyond 100 µm (Kuramoto et al., 2021, Koo et al., 2021). We found that 22.2% of EC cells had 2 neuropods, which is comparable to I cells of the small intestine (33% of which have 2 neuropods) (Chandra et al., 2010). Interestingly we observed some EC cells in the distal colon with neuropods which bifurcate (see

Appendix Figure 7), as seen previously in I cells (Chandra et al., 2010). EC cells in the small intestine are reported not to contain neuropods (Koo et al., 2021), contrasting data on other EEC subtypes in the small intestine such as I and L cells (Chandra et al., 2010, Bohórquez et al., 2011, Bohórquez et al., 2014, Bohórquez et al., 2015, Kaelberer et al., 2018). Other aspects of these cells differed including neuropod length, as neuropods of I cells of the small intestine are short, with the longest at 15 μm (Chandra et al., 2010) half the size of the longest neuropod on a colonic EC cell. L cells also have long neuropods in the small intestine (Bohórquez et al., 2011, Bohórquez et al., 2014), reaching up to 70 μm in length (Bohórquez et al., 2014), and differ in neuropod anatomy compared with colonic L cells which do not contain synaptic-like structures (Bohórquez et al., 2011). Hence, it can be seen that there are differences in the anatomy of EEC neuropods dependent on EEC subtype and region of the GI tract. The factors leading to these differences are unknown. Overall, it is clear that EC cells in the colon contain neuropods and that EEC morphology and neuropod characteristics differ between both EEC subtype and region of the GI tract.

We also provide the preliminary evidence of neuropods on human colonic EC cells, supporting initial reports from up to 30 years ago which describe these ‘cytoplasmic processes’ in human EC cells in different regions of the stomach (Inokuchi et al., 1984, Kusumoto et al., 1988), and colon (Sjölund et al., 1983). In the current study, EC cells contained neuropods of varying lengths in human colon. However more samples need to be examined to adequately quantify the number of human EC cells containing neuropods. Previous work on human GI tissue described EC cells with varying shapes, sizes and morphologies (Inokuchi et al., 1984), while another group observed 3 distinct morphologies of human EC cells in the oxyntic mucosa, including cells with multiple neuropods (Fakhry et al., 2019). It was also noted that EC cells contain long uni- and

sometimes multipolar neuropods which projected in many directions (Inokuchi et al., 1984, Kusumoto et al., 1988).

The use of 3D analysis has proven highly beneficial in the study of EEC morphology, in particular the anatomy of EEC neuropods, as shown through various studies over the last decade. Confocal and electron microscopy coupled with significant advances in 3D analysis software has enabled noteworthy developments in understanding the morphology and cellular interactions in many tissue types, including the identification of anatomical features of EECs in all 3 planes (Bohórquez et al., 2014). Such advancements allow the examination of spatial information of EECs and their relationships within the crypts of the GI tract, and with other cell types. Multiple studies have utilized thick sections of mouse and rat GI samples (60-120 μm (Kuramoto et al., 2007{Kuramoto, 2021 #712, Koo et al., 2021)), while we used wholemount GI samples, to identify EEC morphology and distribution. Thick sections or wholemount samples provide a better representation of cellular morphology compared to conventional thin sections (~10 μm), as they can reveal the existence of long neuropods and provide in-depth information on EECs in terms of morphology and position (Koo et al., 2021). This was shown in one study, in which the 3D reconstruction of thicker sections indicated an under-representation of I cells containing neuropods, due to their extension into the z-plane, which were invisible through 2D observation of a 5 μm thick section (Chandra et al., 2010). The use of 3D analysis also allows the determination and in-depth analysis of connections between different cell types, not possible when consulting 2D images.

One limitation in the current study, was the lack of a fluorescent marker to depict the orientation of the tissue, hence the polarity of EC cells within crypts was unable to be determined. This particularly reduced the ability to distinguish whether neuropods

originated from the basal or apical membrane of the cell, as well as the cell's position along the crypt-villus axis. In future the use of a nuclear stain, such as DAPI, would aid in delineating the overall structure of the tissue and hence the orientation of EC cells within crypts. This is an important limitation to note, as previous studies have classed neuropods as basal or apical through the identification of the polarity of EECs, hence in the current study we have been unable to do this, instead defining all extensions from EC cells as neuropods generally. Other studies have also used the polarity of the cell to determine characteristics such as the direction of the neuropod within the crypt (Chandra et al., 2010, Bohórquez et al., 2011); the length of neuropods comparatively between the direction from which they extend from the cell (Kuramoto et al., 2021); and the cell position number within the crypt (Bohórquez et al., 2011), such information can provide insight into characteristics such as the age, or level of differentiation of the EEC. These characteristics of neuropods are also important in the investigation into their function. Hence this was one limitation of the current study, however, considerable information can still be deduced through the co-staining of other immunohistochemical markers, particularly directed at other epithelial cells and neurons.

Since the discovery of EEC projections, they have been termed "basal processes" (Kobayashi et al., 1971), "pseudopod-like basal cellular" processes (Chandra et al., 2010), and most recently due to the postulated connection between the basal processes of EECs and neurons which innervate the intestines, these basal processes were given the name 'neuropods' (Bohórquez et al., 2011, Bohórquez et al., 2015, Kaelberer et al., 2018). Most recently they have been suggested to grow towards neurons to form direct connections, hence allowing the fast transfer of information between the EECs which sense the lumen, and the brain (Bohórquez et al., 2015,

Kaelberer et al., 2018). This has been demonstrated through immunohistochemical and confocal analysis, in which EECs directly contact nerves that penetrate the basal lamina to innervate the mucosa of the mouse ileum and colon (Bohórquez et al., 2015, Kaelberer et al., 2018).

Serotonin plays a significant role in modulating gut function, in particular GI motility, a mechanism known to be primarily controlled by neurons in the gut (Spencer et al., 2018). EC cell serotonin was originally thought to act on neurons in a paracrine manner, but recent studies have indicated a direct functional connection between EC cells and GI innervating neurons (Bellono et al., 2017). Here we show that 64.1% of EC cells appear to be within 0-2 μm from the nearest CGRP labelled nerve fibres in the mouse distal colon. Overall, 95% of EC cells were found to come within 10 μm from the nearest CGRP labelled sensory nerve fibre, a distance at which previous studies have indicated EC cell released serotonin is in high enough concentrations to activate nearby serotonin receptors (Raghupathi et al., 2013). CGRP is a known marker of sensory neurons in the GI tract, known to label both intrinsic and extrinsic sensory neurons, with 90% of sensory mucosal endings in the colon being $CGRP^{+}$ (Spencer et al., 2014). Extrinsic CGRP labelled sensory neurons typically have a role in sensing temperature, itch, and pain (McCoy et al., 2013), while both extrinsic and intrinsic forms are thought to play a role in GI motility (Furness et al., 2004b). Others have reported that two-thirds of EECs synapse with nearby nerves innervating the intestinal mucosa (Bohórquez et al., 2015), although only two common EEC subtypes were analysed, which does not represent all EECs. Further studies found that L cell neuropods in the colon contacted 67.3% of PGP9.5 (a pan-neuronal marker (Otsuki et al., 2004)) labelled nerve fibres (Bohórquez et al., 2015). These same cells were also specifically found to contact CGRP nerves in the colon, although these

connections were not quantified (Bohórquez et al., 2015). Other studies on EC cells found that the basolateral side made contact with 5-HT_3 receptor ($5HT_3R$) expressing nerve fibres in intestinal tissue collected from $5HT_3R$ reporter mice (Bellono et al., 2017). $5HT_3R$ is expressed by intrinsic neurons of the intestines, as well as a subset of extrinsic sensory nerve fibres (Tecott et al., 1995). One future direction from the current study, is to use 5-HTR reporter mice to determine the frequency at which neurons containing the serotonin receptor are in close contact with EC cells. This tissue could also be immunohistochemically stained and analysed for other neuronal markers which commonly innervate the intestinal mucosa to determine neuronal circuitry in which EC cells are involved within the gut wall, and hence gain a better understanding of the mechanisms through which serotonin might act.

Interestingly, a recent study reported an opposing conclusion on the relationship between EC cells and neurons, declaring that colonic EC cells do not form consistent close relationships with any nerve fibres, including CGRP, tachykinin (TK) or vasoactive intestinal polypeptide (VIP) nerve fibres (Koo et al., 2021). This study did note that some EC cells of the proximal colon, which contained both serotonin and TK, were in close proximity to TK containing nerve fibres (Koo et al., 2021). However, the method used to study this, and the definition used to determine a ‘close contact’ was not mentioned in this study, and hence it is hard to compare their conclusions with what was found in the current study.

The current study is the first published record that human EECs contain neuropods which make contact with nerve fibres in the colon, as we provide the first evidence that EC cells of the human colon contact PGP 9.5 labelled neuronal fibres within the GI wall. This provides the first evidence that EECs of the human GI tract also have the capacity to make direct communication with nerves in the gut wall. Interestingly just

25% of human EC cells were found to be within 0-2 μm of the nearest nerve fibre, this is significantly lower than the ~60% of EC cells which come within 0-2 μm of the nearest nerve fibre in mice, however, is likely due to either significant underpowered results in human, or the differences in neuronal marker used between the two tissue types. Previous reports suggested that human EC cell neuropods extend to other epithelial cells (Sjölund et al., 1983). Whilst significant further investigation is required, it is apparent that some human EECs contain neuropods with the potential to form functional connections with neurons.

In the present study, every EC cell found to touch a CGRP nerve fibre was manually studied to determine the location at which the sensory fibre contacts the EC cell in mouse colon. The majority of contact points on EC cells from neuronal fibres was discovered to be on the cell body, rather than at the end of neuropods. This was an unexpected finding given the previous reports of the functional connection formed between these two cell types and does not align with the previous hypothesis that the primary function of neuropods is to grow towards neurons to form direct connections. Hence whilst the term ‘neuropod’ has been used recently, we believe that this is not an accurate description of these cells, particularly as only a proportion of cells contain neuropods, and most connections with neurons are not made at the point of neuropods. For consistency throughout this thesis, however, we will continue with the term neuropods.

Others have suggested that EECs contain swellings along their neuropods which resemble neuronal varicosities (Bohórquez et al., 2011, Kuramoto et al., 2021). Varicosities are known to facilitate the diffuse transmission of neurotransmitter release along neuronal axons but can also form the point of synaptic transmission (Zhang et al., 2011). This idea is supported by studies which found the expression of genes

encoding for presynaptic and postsynaptic proteins in EEC subtypes (Bohórquez et al., 2015, Kaelberer et al., 2018) including EC cells (Bellono et al., 2017); as well as the identification of genes which transcribe the glutamate transporter (vesicular glutamate transporter 1 protein) (Kaelberer et al., 2018) and enzymes which are essential for the synthesis of the neurotransmitter dopamine within some EEC subtypes (Bohórquez et al., 2015). Neuropods also contain neurofilaments, which resemble filaments present in axons (Bohórquez et al., 2014), suggesting that these basal structures are similar to axonal projections. Finally, when co-culturing EECs with sensory neurons, a connection is formed between the two cell types, as the EEC develops a process that extends towards the neuron (Bohórquez et al., 2015). Together, these data suggest that EEC neuropods function as synaptic-like structures which facilitate direct communication to innervating nerves. Taken together these results indicate that neurons make functional connection with EECs, however this might not be the direct function of neuropods. The use of immunohistochemical analysis to determine whether presynaptic and postsynaptic proteins are present within both EECs and on neuronal fibres at the points of contact would provide further evidence to indicate whether these interactions might facilitate functional connections.

The prospect that the connections between EC cells and neurons facilitate direct synaptic-like communication has been doubted by some research groups. Such researchers argue that neither EC cell neuropods nor nerves fibres generally cross the basal lamina (Kuramoto et al., 2007, Koo et al., 2021, Kuramoto et al., 2021), hence the likeliness of these cells forming synaptic connections seems questionable. Other studies have identified a complete lack of close contact between other EECs, such as I cells, and enteric or vagal sensory nerves in the rat small intestine (Berthoud and Patterson, 1996). Furthermore, the likelihood of such connections between EEC

cells, known to migrate along the crypt-villus axis in typically 4-5 days (Heath, 1996), and neurons innervating the GI tract is doubted by others (Bertrand, 2009). As this rapid turnover of EECs indicates only a short timeframe for the formation of such neuronal connections, hence reducing the feasibility of such connections. However, the most recent evidence on EEC lifespan is adapting, with recent studies suggesting that specific pools of EECs remain in the epithelium for up to 13 months, for EC cells (Wei et al., 2021), or up to 70 days for other EECs (Bohórquez et al., 2015, Tsubouchi and Leblond, 1979). This suggests that there might be pools of EECs which are long-lived and hence more likely to form functional connections with neurons in the intestinal wall. It would be interesting to co-stain intestinal tissue from EC cell reporter mice (Wei et al., 2021) to determine whether these long-lasting EC cells are the subgroup of cells that form connections with neurons.

A key to better understanding the function of these neuropods will be to identify the cell types to which they extend towards. Reports have suggested that EEC neuropods might form contact with other epithelial cells, including other EECs. This is indicated by the vast literature describing the location of neuropods within the mucosal lining, in which some neuropods are seen to extend away from the direction of the crypt (Bohórquez et al., 2011), in other cases they are shown to extend towards adjacent cells (Chandra et al., 2010, Kuramoto et al., 2021), and other times towards epithelial cells considerably further away (Bohórquez et al., 2011). Interestingly neuropods have been found to extend across 3 or more neighbouring cells (Chandra et al., 2010), even spanning up to 15 epithelial cells away. Neuropods also extend significant lengths, upwards of 100 μm (Koo et al., 2021), and in an intricate and complex manner by weaving between neighbouring cells (Bohórquez et al., 2011). Submucosally-directed neuropods are usually longer than apical-directed neuropods opening to the lumen

(Kuramoto et al., 2021), and often run along the bases of surrounding epithelial cells (Koo et al., 2021). These EEC neuropods have also been suggested to form an interface between epithelial cells and the lamina propria (Bohórquez et al., 2011). The complex anatomy and intricacies of EEC neuropods suggests that the direction and length to which they are formed is purposeful, however significant work is required in order to further understand this purpose.

D cells were the first EEC subtype described to contain a ‘basal process’ in the literature, since found to function through paracrine secretions onto other EEC subtypes (Larsson et al., 1979). In this system, D cell basal processes come into close contact with target cell types, G cells and parietal cells (Chandra et al., 2010), to form a circuit in which D cell basal processes are seen to innervate other cells, so that somatostatin could be delivered to a targeted single cell (Larsson et al., 1979). As mentioned above, other EEC subtypes, including L, I and EC cells, have been described to extend towards other epithelial cell types (Bohórquez et al., 2011, Sjölund et al., 1983), such as other EECs, or enterocytes (Chandra et al., 2010). Similarly, we have shown that EC cell neuropods also appear to project towards each other in the mouse colon (see Appendix Figure 8) however further analysis is required. Studies on human colon and rectum identified EC cells which extended neuropods towards adjacent epithelial cells (Sjölund et al., 1983). It is possible that EECs might release hormones in a more directed paracrine fashion onto specific cell types through neuropods. A very recent study identified that serotonin release from EC cells has a paracrine role in regulating L cell differentiation (Lund et al., 2020). These results suggest that basal processes on EC cells, and EECs more broadly, might facilitate interactions on surrounding epithelial cells, including other EECs, and nerve endings through either a synaptic connection or a paracrine manner.

It is possible that different subtupes of EC cells exist, which facilitate separate functions, hence explaining why only a portion of EC cell neuropods make contact with sensory nerve fibres. Support for the hypothesis that there are at least two separate pools of EC cells in the GI tract is growing within the literature, based on numerous recent studies. One pool of EC cells is known to be significantly longer lived (Wei et al., 2021), while the other pool is thought to turnover at the typical rate for EEC cell cycle. There is also evidence that EC cells can be divided into two distinct pools based on genotype, as two separate clusters of EC cells are seen as a result of genetic sequencing of the intestinal epithelium (Piccand et al., 2019, Gehart et al., 2019, Beumer et al., 2020). Similarly, EC cells are known to differ greatly in morphology, with some populations containing neuropods and others which do not, as in both the current study and previous studies (Kuramoto et al., 2007, Koo et al., 2021, Kuramoto et al., 2021). Such differences in EC cell morphology might suggest different functions of these cells. It has long been known that some EC cells, as well as other EECs, are open to the lumen, while other cells are closed off to the lumen (Koo et al., 2021), hence only a percentage of EC cells are able to detect luminal contents. This prompts questions such as how open and closed cells might differ in function. Both open and closed EC cells contain neuropods (Koo et al., 2021), with a greater abundance of EC cells containing long neuropods in the mouse distal colon compared to the proximal colon (Koo et al., 2021, Kuramoto et al., 2021). Open EC cells are the most common morphology seen along the colon (Kuramoto et al., 2021), although interestingly most EC cells with long neuropods were closed off from the lumen (Kuramoto et al., 2021). It is likely that the different morphologies seen in EC cells pertain to different roles that these cells are known to play as sensory cells, such as responding to nutrients (Bellono et al., 2017) and metabolites (Reigstad et al., 2014), mechanical stimuli

(Wang et al., 2017, Alcaino et al., 2018), and noxious stimuli (Latorre et al., 2016a). Furthermore, genetic subtypes have been identified, in which endocrine and non-endocrine genotypes have been described (Piccand et al., 2019, Tsakmaki et al., 2020). Recent evidence suggests that mechanosensitive and chemo-sensitive EC cells utilise separate signalling pathways (Alcaino et al., 2020). A similar hypothesis was postulated up to 50 years ago that open type cells extend their apical neuropod to reach the lumen to detect the chemical contents (Fujita and Kobayashi, 1971, Fujita, 1973) in order to relay back to neurons in the GI wall. While closed cells were thought to spread their neuropods over the basement membrane, opening them up to be easily distorted in response to mechanical pressure (Fujita and Kobayashi, 1971), hence responding to mechanical stimuli applied to the mucosal membrane (Kobayashi et al., 1971, Kuramoto et al., 2021). This hypothesis has not yet been disproven, and with two thirds of neuropod-containing EC cells found in the lower half of the crypt (Kuramoto et al., 2007), and most of the EC cells with long neuropods being of closed morphology (Kuramoto et al., 2021), the idea that closed cells could be mechanoreceptors is favoured. In further support of the idea of two different subtypes of EC cells, other EECs have been shown to display different forms of communication, such as I cells which have been shown to release CCK in a paracrine function, as well as use glutamate as a transmitter to activate vagal sensory nerves via synaptic connections (Kaelberer et al., 2018). It is also known that synaptic connections only cover a few hundred nanometres in length (Bohórquez et al., 2014) and hence these large and long neuropods likely facilitate both neural connections along the process, as well as hormonal release towards the tip in a paracrine fashion. It may depend on where EECs are along the crypt-villus axis, or which EEC subtype they differentiate into, as to whether these neuropods are functional as connections to neurons, or as

paracrine communicators which release hormones to other epithelial and EECs. It is likely that there are different subtypes of EC cells, which differ in their lifespan, morphology, and function, however further investigation is required to delineate these subtypes and characterise them individually.

Many studies have indicated that the function of these neuropods is to form connections with neurons which innervate the GI tract, to facilitate the direct communication between EECs which sense the luminal environment and extrinsic innervation which signals to the brain. Such a direct connection has been supported through two recent studies which demonstrate an inhibitory effect in hypothalamic neurons, known to control food intake, within seconds nutrients entering the duodenum (Beutler et al., 2017, Su et al., 2017). More specifically to EC cells, GPR65-containing vagal afferents, largely present in the small intestinal mucosa, have been shown to be serotonin sensitive (Williams et al., 2016). GPR65 neurons are responsive to meal associated stimuli in the intestinal lumen (Williams et al., 2016), hence it is likely that direct EC cell-neuronal connections may facilitate such a direct transfer of information. Similarly, it is known that EC cell serotonin acts within the gut-brain axis to mediate chemotherapy-induced nausea (Blackshaw and Grundy, 1993). It is also likely that such connections could facilitate communication between EC cells and intrinsic neurons, to facilitate their function in the modulation of GI motility as a direct functional connection between EC cells and intrinsic neurons labelled with serotonin receptors was recently revealed (Bellono et al., 2017). Such connections are likely to facilitate the neuromechanical loop in which EC cells signal to intrinsic sensory neurons to modulate gut motility (Spencer et al., 2016). Hence, there are many functions for which direct connection between EC cells and nerves within the gut-wall, with implications both within the GI tract and more broadly the gut-brain axis.

4.5 Conclusion

In the current study we have found that more than half of EC cells contain neuropods in the mouse distal colon and provide the first evidence that EC cells form direct contact with sensory afferents in mouse colon and nerve fibres in the human colon. We also note, however, that such connections primarily occur at the cell body, and not at the point of neuropods as was hypothesised based on the initial descriptions of neuropods. Overall, we demonstrate the EC cells do come into direct contact with sensory neurons in the gut wall, however we open up a range of further questions surrounding such the function of neuropods.

CHAPTER 5: Characterization of the connection between enterochromaffin cells and spinal sensory neurons in the mouse colon.

5. Characterization of the connection between enterochromaffin cells and spinal sensory neurons in the mouse colon.

5.1 Introduction

Enteroendocrine cells (EEC) are important in a range of physiological functions including in intestinal physiology, peripheral metabolism and the gut-brain axis (Martin et al., 2020b, Latorre et al., 2016b, Gribble and Reimann, 2016, Jones et al., 2020). The connection between EECs and enteric neurons, which facilitate the gut-brain axis, is of emerging interest due to the significant list of physiological functions in which the gut-brain axis has been implicated (Agirman and Hsiao, 2021). While the connections between EECs and enteric neurons are not well understood, it has recently been hypothesised that they form functional synaptic-like connections to communicate sensory information (Bohórquez et al., 2015). The existence of such connections is clear as described in the previous chapter, as well as by numerous other studies (Bohórquez et al., 2011, Bohórquez et al., 2015, Kaelberer et al., 2018), however there is a poor understanding of the factors which drive the formation of such connections.

The primary extrinsic innervation of the mouse colon originates in the spinal column, with the entirety of spinal afferent nerve cell bodies encompassed in the dorsal root ganglion (DRG) (Brierley et al., 2018). The mouse distal colon, specifically, is thought to be predominantly innervated by extrinsic afferents originating from levels L6-S1 DRG (Spencer et al., 2020). Initial in vitro work on the morphology of neuropods indicates that both EEC neuropods and neuronal extensions grow towards each other (Bohórquez et al., 2015), suggesting that there are likely chemoattractants expressed by both cell types guiding the formation of connections. Such chemoattractants could include neurotrophic factors, known to encourage neuronal growth and guide axons of DRG neurons *ex vivo* (Yasuda et al., 1990, Madduri et al., 2009), which also form

chemical gradients that stimulate and guide the formation of neuropods in EECs (Bohórquez et al., 2014). Further *in vitro* work utilising intestinal organoids has demonstrated nerve growth factor (NGF) as an important neurotrophin in driving neuropod formation of EECs (Bohórquez et al., 2014). However, the cellular source and relevance of NGF signalling *in vivo*, is currently unknown. Enteric glia have also been proposed to play a role in promoting enteric neuronal and axonal development via the secretion of neurotrophic factors (Bush et al., 1998, Bohórquez et al., 2014), however this work is only in its infancy. Overall, the factors driving the formation of EEC-neural connections warrant further study.

Similarly, little is known about the factors that control the fate of locally residing intestinal stem cells, which give rise to five terminally differentiated epithelial cell types: enterocytes, paneth, goblet, tuft and EECs. Recent lineage tracing studies have described transcriptional factors that contribute to the differentiation of these epithelial cells, such as those that inhibit Notch signaling and upregulate Atoh1 expression associated with the secretory cell lineage (Zecchini et al., 2005b, Stanger et al., 2005, Fre et al., 2005). Notably EEC differentiation is also favoured through neurogenin-3 (Ngn3) expression (Jenny et al., 2002a, Lee et al., 2002). In addition, EECs adapt their hormonal profile based on their position along the crypt-villus axis (Beumer et al., 2018), a process mediated by bone morphogenic protein (BMP) signaling (Beumer et al., 2018). Other non-genetic factors can also alter intestinal epithelial cell fate, including the microbiome. Germ-free mice have significant increases in the number of EECs (as per Chromogranin A expression), enterochromaffin (EC) cells, K cells and L cells (Modasia et al., 2020) which are restored to baseline after colonization with certain bacterial strains (Modasia et al., 2020). In some circumstances particular cell types can be favoured, such as in mice on a high fat diet (HFD), in which colonic EC

cell density is markedly increased (Martin et al., 2020a). This is reflected in obese human gut, also shown to contain significantly higher densities of EC cells compared to controls (Young et al., 2018). Few studies have considered a role for enteric neurons in the control of epithelial cell proliferation. A small number of studies have provided evidence that mucosal afferents can regulate intestinal stem cell proliferation (Lundgren et al., 2011, Peck et al., 2017), with one such study indicating the potential role for calcitonin gene-related peptide (CGRP) and substance P in such a capacity (Lundgren et al., 2011). Other neuronal subtypes, such as vasoactive intestinal peptide (VIP), have also been shown to influence intestinal stem cell fate, favouring differentiation into goblet cells (Schwerdtfeger and Tobet, 2020). Despite a significant amount of ongoing research, the precise mechanisms and factors that control intestinal stem cell fate remain incompletely understood.

In this chapter we address the numerous gaps in understanding of the factors which control EEC fate and the formation of EEC-neuronal connections. Due to the close relationship between EC cells and CGRP neuronal fibres, with ~64% of EC cells making these connections, we were interested to determine the effect of which the removal of extrinsic sensory fibres would have on EC cells. The following chapter aims to test the role of extrinsic sensory innervation of the mouse distal colon specifically on EC cells and their neuropod characteristics. To do this we utilise a surgical mouse model, in which L6-S1 DRG are ablated, hence lesioning the majority of extrinsic sensory innervation of the distal colon *in vivo*. Confocal microscopy analysis on the colonic tissue from DRG-ablated and control mice was conducted to deduce the role of extrinsic sensory innervation on the formation of EC cell neuropods and EC cells. The specific aims were to:

1. Determine whether the removal of extrinsic spinal sensory innervation of the colon affects the number of EC cells and characteristics of their neuropods;
2. Ascertain the effect of this surgical removal of extrinsic innervation on EC cells over time;
3. Identify chemoattractants which might contribute to the formation of EC cell neuropods.

The overriding hypothesis of this chapter is that the removal of extrinsic sensory innervation alters EC cell neuropod formation and the density of EC cells in the mouse distal colon.

5.2 Methods

5.2.1 Mice Tissue and DRG Removal Surgery

Male C57 BL/6 mice between 12-21 weeks old were used in this study. The following surgery was conducted in accordance with Flinders University Animal Welfare Committee, approval number 933/16. Mice were individually housed for 6 days prior to lesion surgery. At commencement of surgery, mice were anaesthetised using inhalation isoflurane – induction 3% in 1L O_2 and maintained at 1.5-2% in 1L O_2. The dorsal surface was cleaned with sterile alcohol (70%)/chlorhexidine (0.5) (Bayer). Opsite film was applied to the surgical site and a 1.5 cm incision is made into the skin. A bilateral incision is then made into the muscle along the spinal column, and the vertical bone dissected to expose L5-S1 DRG, at no point was the spinal cord exposed. Using super fine 55 forceps (Dumont by FST) the dorsal nerves were lesioned and the DRG detached from the motor/ventral nerve and removed (see Figure 5.1). At completion, the wound was irrigated with sterile saline and muscle incision was closed using 6.0 absorbable polyglycholic acid suture (SilverGlide). Skin was closed using 6.0 non-absorbable nylon suture (SilverGlide) and treated with sterile alcohol (70%)/chlorhexidine (0.5). Mice received a 3-day course of analgesics, subcutaneous (s.c) 1mg/kg Buprenorphine (Temvet, Ilium) at time of surgery, and 1mg/kg Buprenorphine (Activis) in Nutella paste on days 2 and 3 post op. Mice received a single s.c. dose of antibiotic Enrofloxacin (Baytril), 10mg/kg at time of surgery. Mice were clinically monitored for 5 days post-surgery and maintenance monitored until time of euthanasia (day 7 or 21). At time of tissue collection, mice were anesthetized with isoflurane (4%, IsoFlo) and euthanized humanely by cervical dislocation. Colonic tissue was collected as in previous method (3.2.1) and stored in

a beaker containing oxygenated Krebs solution, bubbling with 95% O_2/5% CO_2 until use.

5.2.2 Immunostaining

Immunohistochemistry for the following chapter was performed as in previous chapter methods (4.2.3)

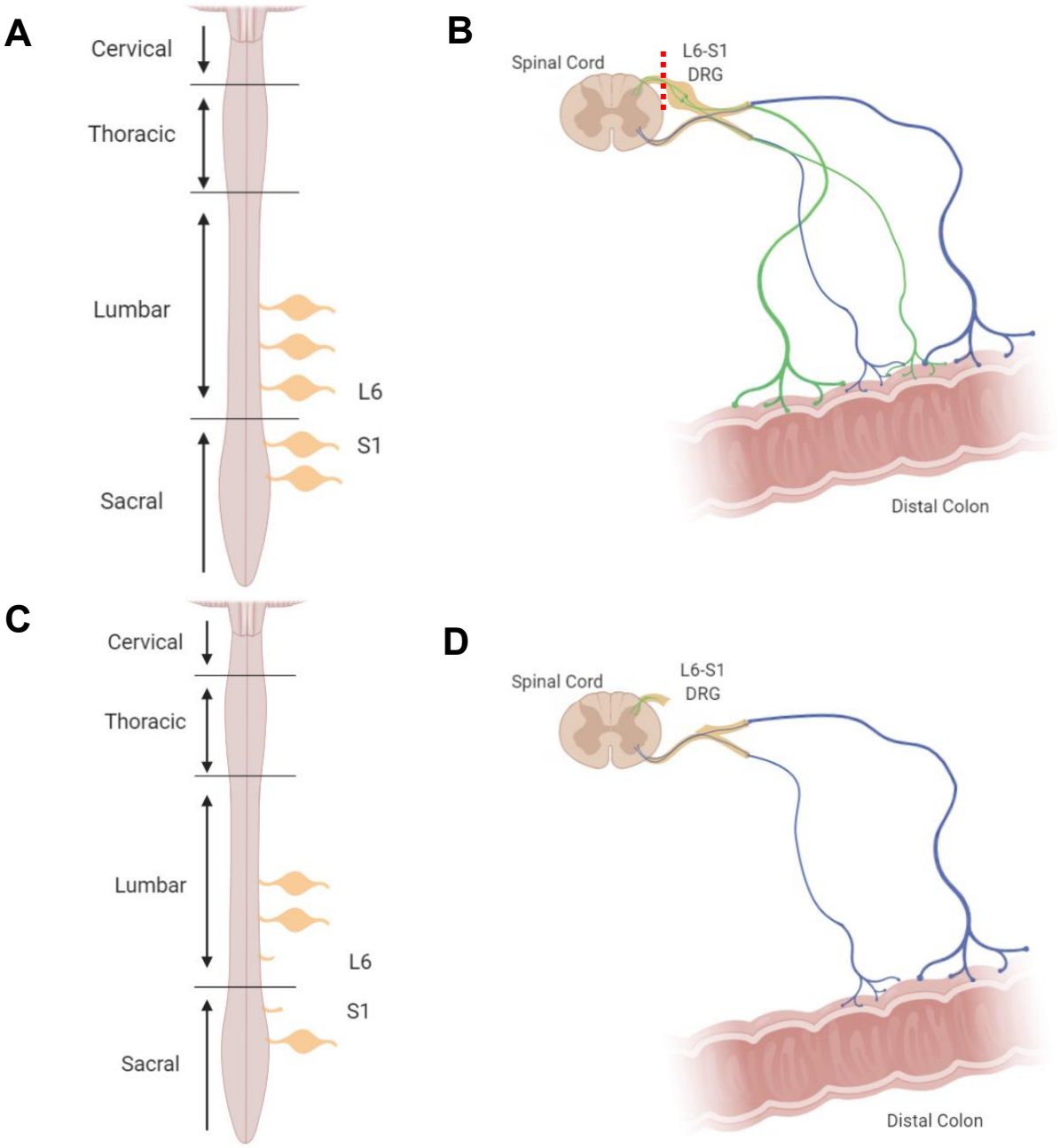

Figure 5.1: Schematic of the surgical method used to remove bilateral L6 and S1 DRG.

A) A schematic of the spinal cord, with a red-dotted line indicating the area of DRG which are cut, and B) the extrinsic innervation of the distal colon, with sensory (green) and efferent (blue) neural innervation. C) A schematic of the spinal cord, with the L6 and S1 DRG removed, and D) the resultant reduction in the extrinsic sensory innervation of the distal colon. Created with BioRender.com.

5.2.3 Microscopy

Microscopy and image capture for the following chapter was performed as described in previous methods (3.2.4).

5.2.4 Imaris

Imaris image analysis for the following chapter was performed as described in previous methods (4.2.5). Additionally, in this chapter the thickness of tissue was used to normalise the intensity of CGRP staining for analysis.

5.2.5 Bioinformatic analysis and Gene Sorting

Bioinformatics analysis was performed through systematic integration of two publicly available gene lists. The first list was derived from the analysis of mouse DRG sensory neurons in two mice reporter lines (Chiu et al., 2014), in which gene expression in 3 populations of non-overlapping somatosensory neuron classes were quantified using microarray. This initial list was filtered using the gene ontology (GO) term “protein secretion”, such that only expression from genes coding for secreted proteins were retained for downstream analyses. Further, focussing on the pairwise comparison conducted between $IB4^+$ and $IB4^-$ SNS-Cre lineage neurons, where differential expression (DE) was determined by the statistical criteria of fold-change >2, $p < 0.05$, we created a curated list of protein secretion genes more highly expressed in $IB4^-$ compared to $IB4^+$ SNS-Cre. The second list was derived from EC cells, using a GFP-tagged Chromogranin A reporter mouse small intestinal tissue (Bellono et al., 2017) in which gene expression was quantified through single cell RNA sequencing (scRNAseq). Briefly, this study collected RNA from GFP^+ and GFP^- cells, in which GFP^+ was a purified population of EC cells from organoids, and GFP^- consisted of all other intestinal epithelial cells. This list was interrogated for all known receptors of the

previously identified secreted proteins of interest. Further, using the data obtained by Bellono et al. (2017), we retained only receptors with a fold-change >1 and statistical significance $p< 0.05$ based on differential expression between GFP^+ and GFP^- cells.

5.2.6 Statistical analysis

Statistical analysis was performed as in previous method (4.2.6).

5.3 Results

5.3.1 Acute reduction in CGRP innervation of the distal colon after L6-S1 DRG removal surgery.

Confocal microscopy and 3D image analysis were used to confirm the reduction in extrinsic sensory innervation of the distal colon, post-removal of the DRG known to innervate this region (L6-S1). The reduction in sensory innervation was measured through CGRP immunofluorescence in images of the distal colon 7 days post-surgery, confirming a significant reduction in CGRP-labelled nerve fibres of DRG ablated mice (Figure 5.2A, B). This was quantified through analysis of the total fluorescence of CGRP staining in 3D re-constructed images, revealing a 52.7% reduction in CGRP fluorescence of DRG ablated colonic tissue (see Figure 5.2C).

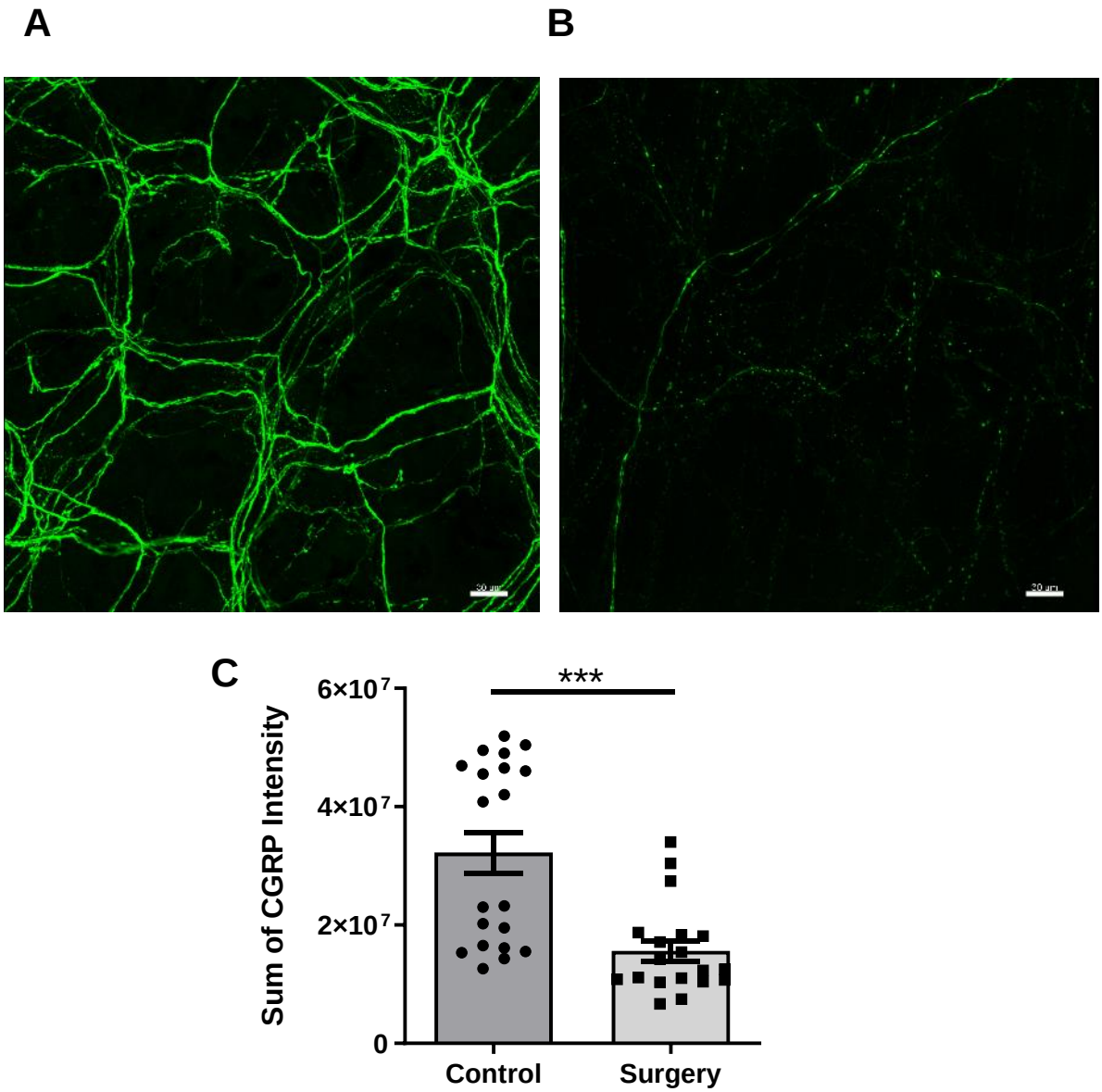

Figure 5.2: Reduced CGRP labelled sensory innervation in DRG ablation surgery mice.

An example z-projection of CGRP (green) labelled A) control mouse distal colon and B) mouse distal colon 7 days post-DRG removal surgery. Scale bar = 30 μm. C) The quantified immunofluorescent intensity of CGRP compared between control and post-DRG removal. Data are shown as mean ± SEM. **** $p < 0.0001$

5.3.2 Depleted CGRP spinal innervation and EC cell number post L6-S1 DRG removal.

EC cells were immunohistochemically labelled and quantified in control and DRG-ablated distal colonic tissue using epifluorescence and confocal microscopy. Images (n=5 per colon) were taken in a blinded manner from the most distal 1 cm of the colon (termed ‘distal cm’), and the next 1 cm of the distal colon more proximally (termed ‘proximal distal cm’), by two different researchers. These images were then quantified separately and in a blinded manner by the two researchers. The data from both researchers confirmed a significant reduction in EC cell number in the DRG-ablated colon compared with controls (see Figure 5.3). A similar proportion of reduction was noted from both researchers, with a 53% and 48% reduction reported of the most distal cm, and 37% and 33% reduction in the more proximal distal cm. These results were then re-analysed using 3D imaging analysis, which confirmed the reduction in EC cell number of the distal colon (see Figure 5.4), indicating a 52% reduction in EC cells in the distal colon of DRG-ablated mice (see Figure 5.5). This data demonstrates that a reduction in the extrinsic sensory innervation of the distal colon leads to the significant reduction in the number of EC cells in the mouse colon.

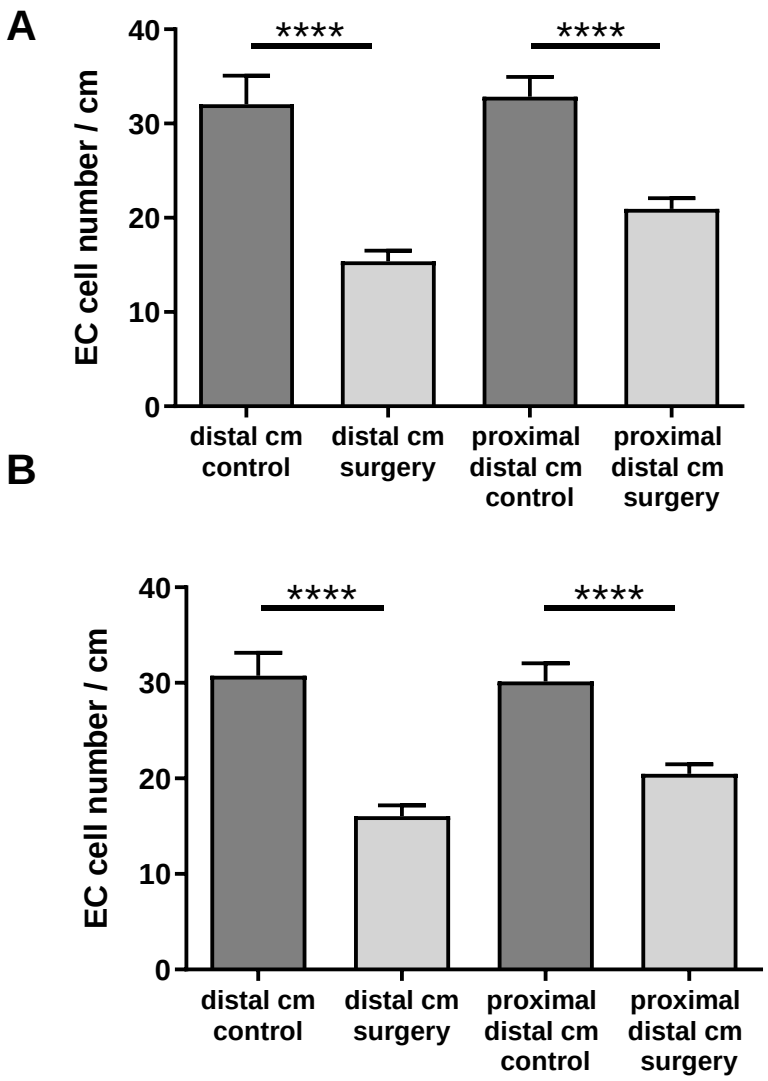

Figure 5.3: EC Cell numbers quantified from 2D epifluorescence imaging.

The number of EC cells counted by double blinded A) researcher one and B) researcher two (n=4, N=10). Data are shown as mean ± SEM. ****p < 0.0001.

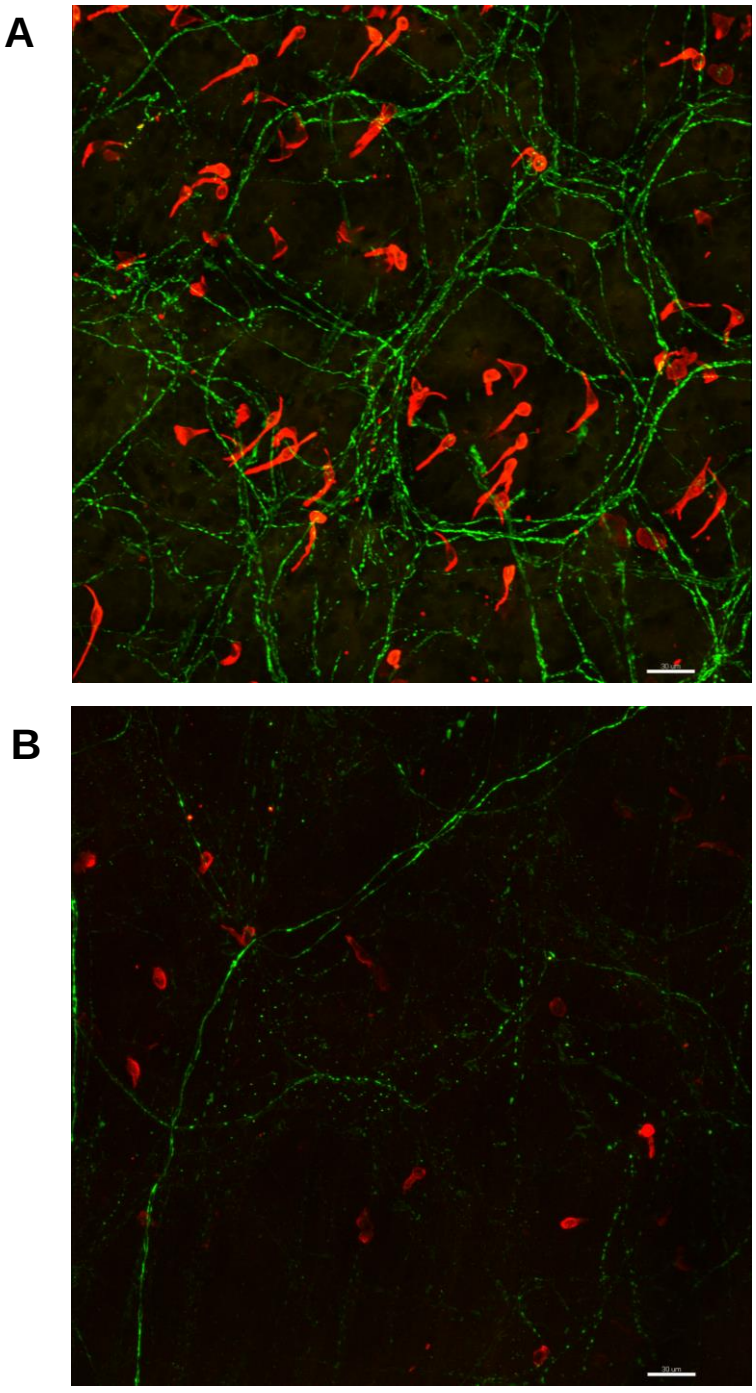

Figure 5.4: Fewer EC cells are observed in the distal mouse colon post-surgery.

Representative z-stack projections of EC cells labelled with serotonin (red) and CGRP-labelled nerve fibres (green) in the A) control distal colon, and the notable reduction of both in B) DRG-ablated mice. Scale bar = 30 μm.

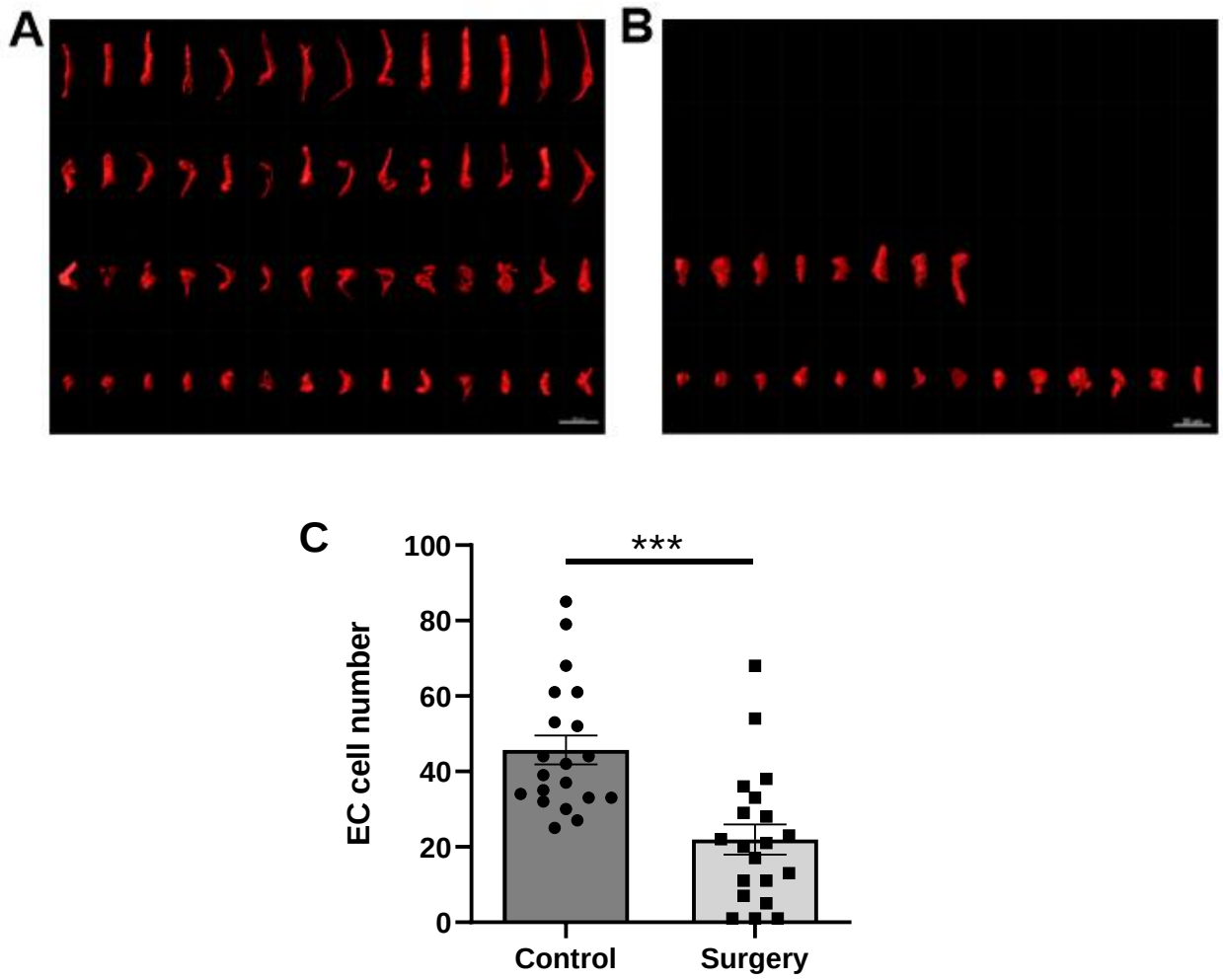

Figure 5.5: EC cells are dramatically reduced in mouse colon post-DRG removal.

Representative gallery images of the number of EC cells (stained for serotonin) counted in the distal colon of control (A) and surgery (B) mice. Scale bar = 30 μm. C) The number of EC cells counted in the distal colon of control and surgery mice (n=4; N=20). Data are shown as mean ± SEM. *p < 0.05, ***p < 0.001,

5.3.3 Changes in EC cell characteristics due to the removal of spinal afferent innervation.

Considering the differences in EC cell number, we were interested to see whether there were other cellular changes in the characteristics of the remaining EC cells. Using 3D reconstruction and analysis, the volume of EC cells was decreased in DRG-ablated distal colons by 17.7%, with an average volume of 508.6 μm^3 compared to 618.1 μm^3 in controls (see Figure 5.6A). In addition, there was a reduction in the proportion of EC cells containing neuropods, decreasing by 20.6% (see Figure 5.6B). The number of neuropods per EC cell was also quantified between DRG-ablated mice and controls. DRG-ablated mice were found to have a reduced number of multiple neuropods overall (see Figure 5.7). The number of EC cells with no neuropods was increased from 14.9% to 30.6% (see Figure 5.7C), as expected because this reflects the reduction in the proportion of EC cells containing neuropods. The proportion of EC cells with 2 neuropods was decreased by 72.8% in DRG-ablated mice, from 22.1% to 6% (see Figure 5.7C). The length of neuropods from cells with only single neuropods was reduced by ~1 μm on average in DRG-ablated colons compared with control (see Figure 5.6C). Hence it is clear that the removal of extrinsic sensory innervation significantly reduced the number, size and neuropod characteristics in EC cells.

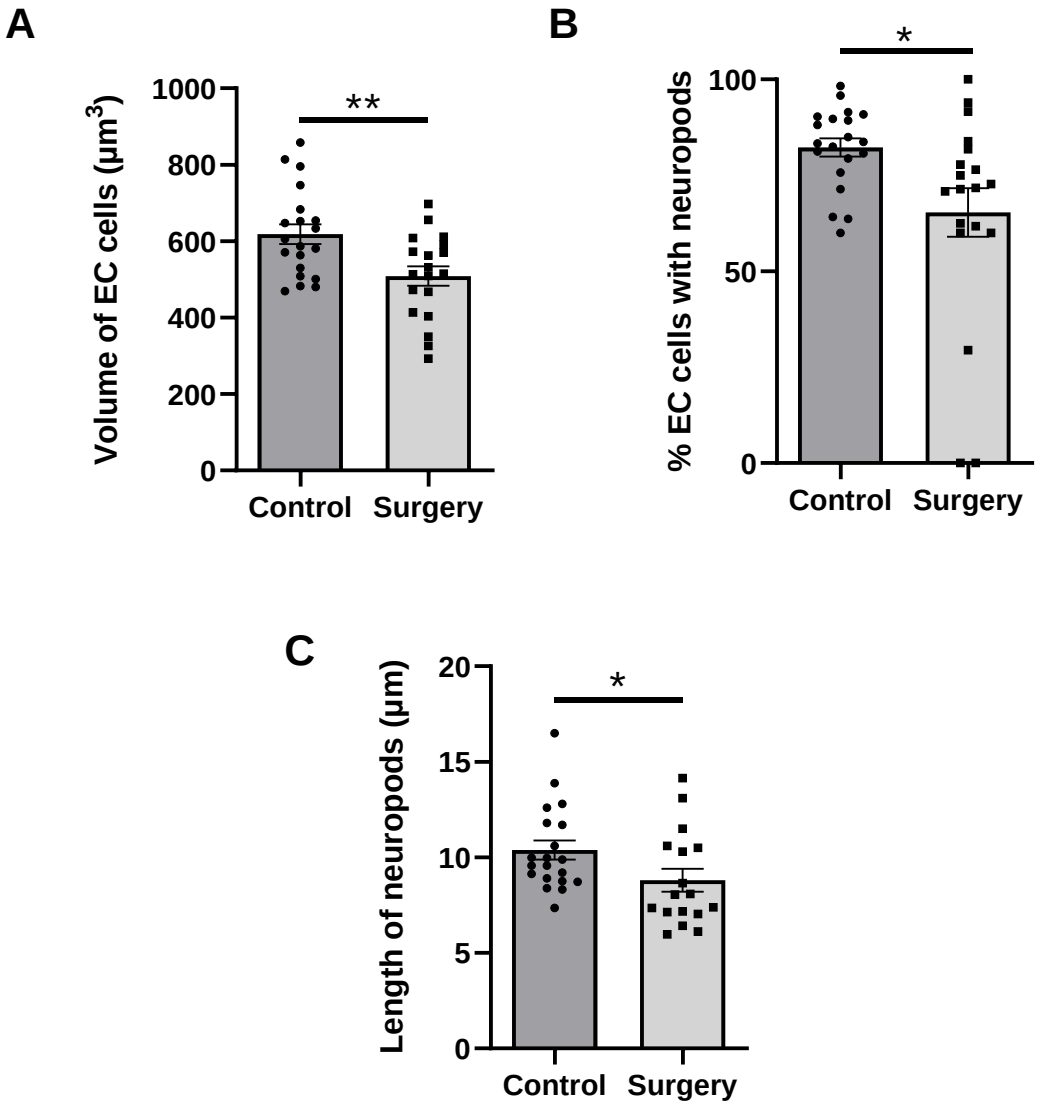

Figure 5.6: Characteristics of EC cell morphology change with the removal of extrinsic spinal innervation to the mouse colon.

A) The volume of EC cells measured in the distal colon of control and DRG-ablated mice. B) The percentage of EC cells containing neuropods. C) The median length of all singular neuropods. Data are shown as mean ± SEM. *=p<0.05, **=p<0.01

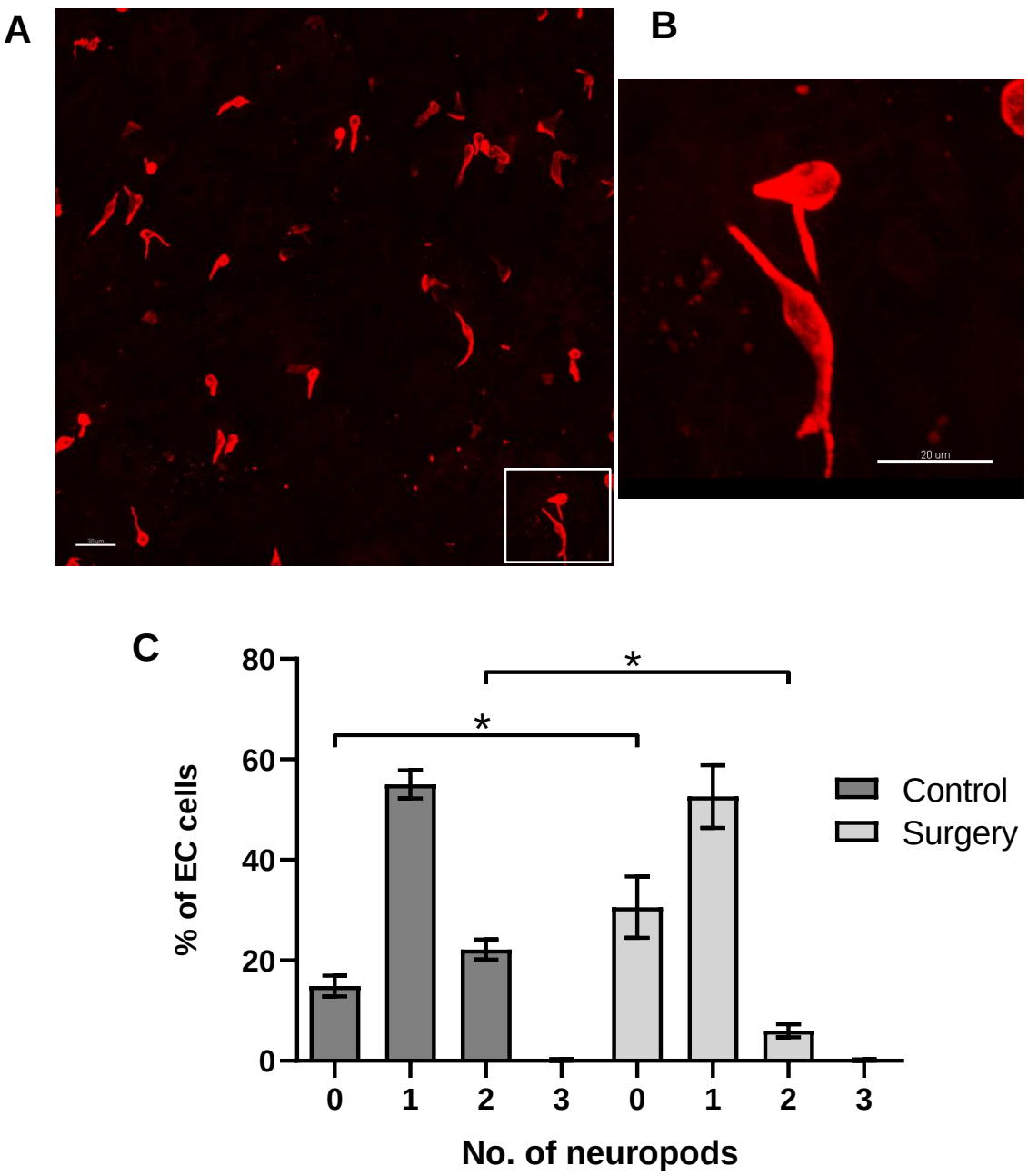

Figure 5.7: EC cells can contain multiple neuropods.

A) An example image of a region of control distal colon with an EC cell with multiple neuropods (in white box) and B) a close-up of this EC cell. Scale bar = 30 μm. C) The number of neuropods per EC cell, relative to cell number, in the distal colon of control and surgery mice. Data are shown as mean ± SEM. *=$p<0.05$

5.3.4 Changes in EC cells in the colon 3 weeks post DRG removal.

To examine the persistence of differences in CGRP labelled sensory nerve fibres and EC cell number over time DRG-ablated and control proximal and distal colonic tissue was analysed 21 days after surgery. A similar reduction in CGRP fluorescence was seen at 21 days post-surgery in the distal colon (see Figure 5.8), with a 43.1% reduction in total CGRP intensity (see Figure 5.9A). However, the number of EC cells was not different between control and DRG-ablated mouse distal colon at this longer time point (see Figure 5.9B). Interestingly, the total intensity of CGRP fluorescence in the proximal colon of surgery mice also showed a trend of reduction compared to controls, by 57.2% (see Figure 5.10, 5.11A). The number of EC cells is also decreased in the proximal colon of DRG-ablated mice (see Figure 5.11B), by 51%. This data suggests that separate regions of the colon are affected differently at distinct time points.

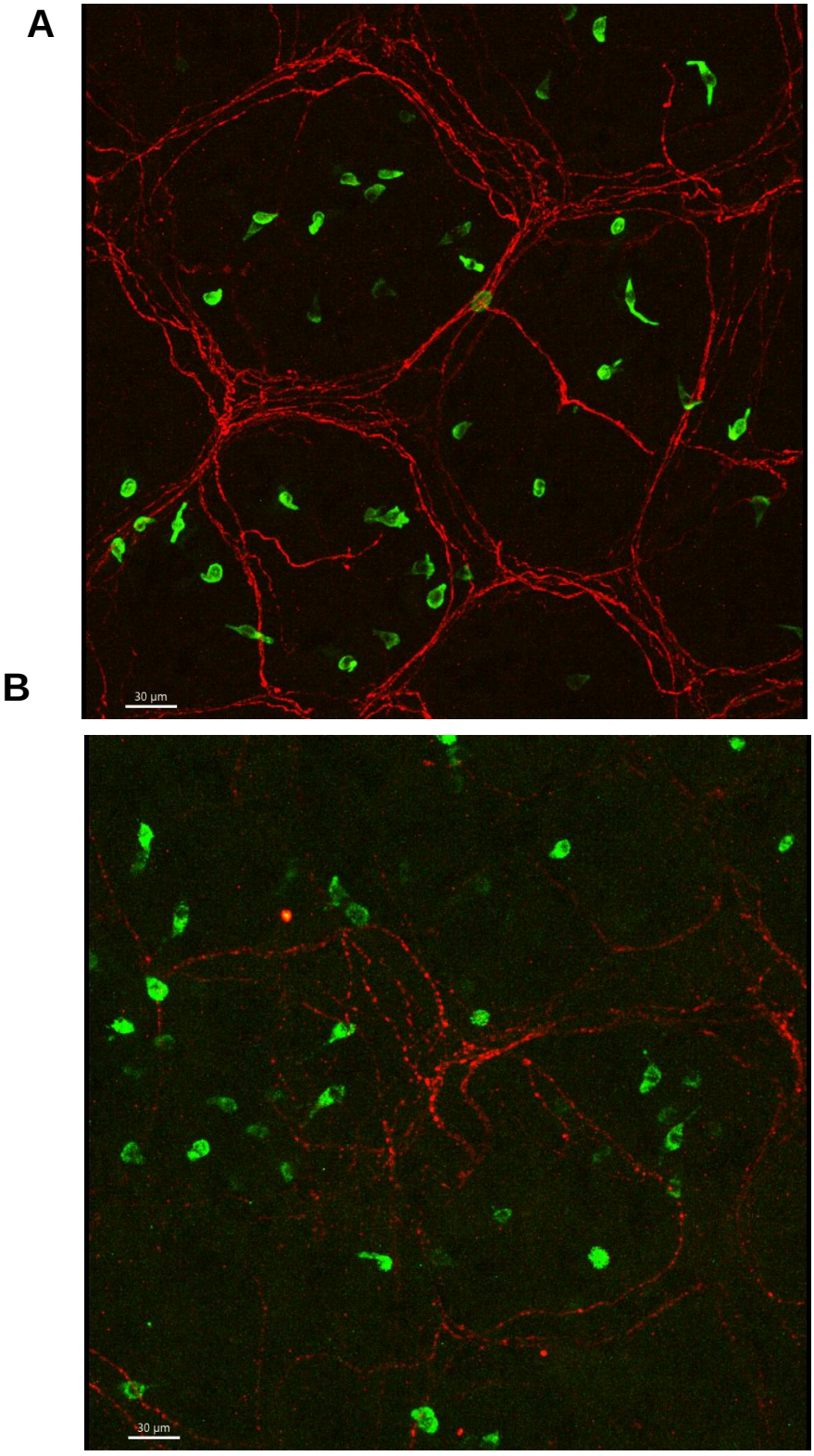

Figure 5.8: Representative images of the distal colon 3 weeks post-surgery.

Z-stack projection of A) control distal colon and B) surgical DRG-ablated colon, stained for serotonin (green) and CGRP (red). Scale bar = 30 µm.

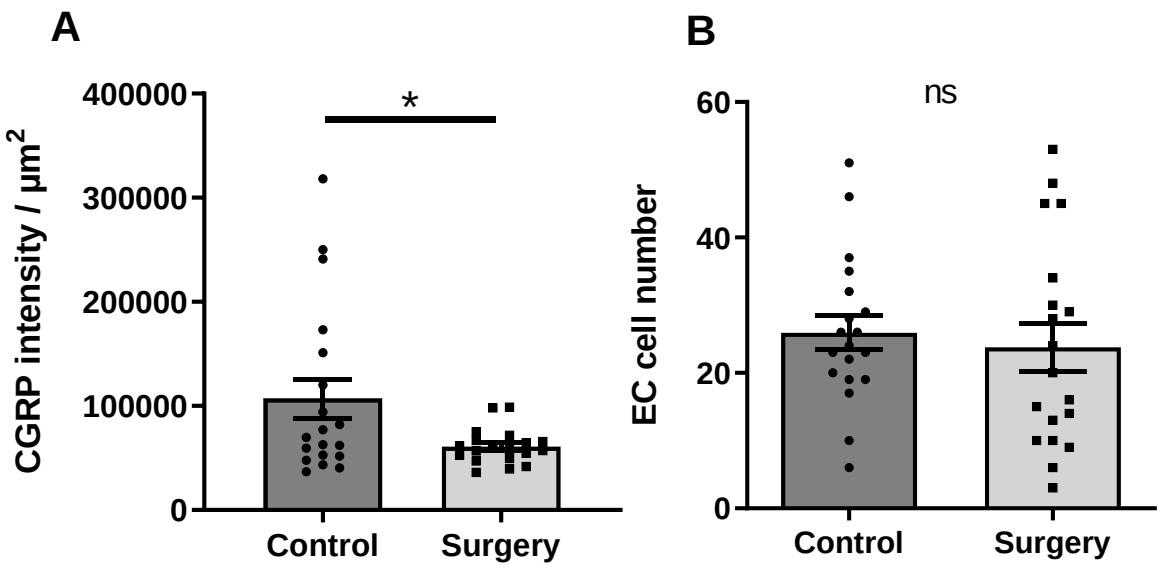

Figure 5.9: Time induced effect of removal of L6-S1 DRG on the distal colon 3 weeks post-surgery.

A) The total CGRP fluorescence intensity of the distal colon between control and DRG-ablated mice. B) The number of EC cells in the distal colon of control and DRG-ablated mice. Data are shown as mean ± SEM. *=$p<0.05$

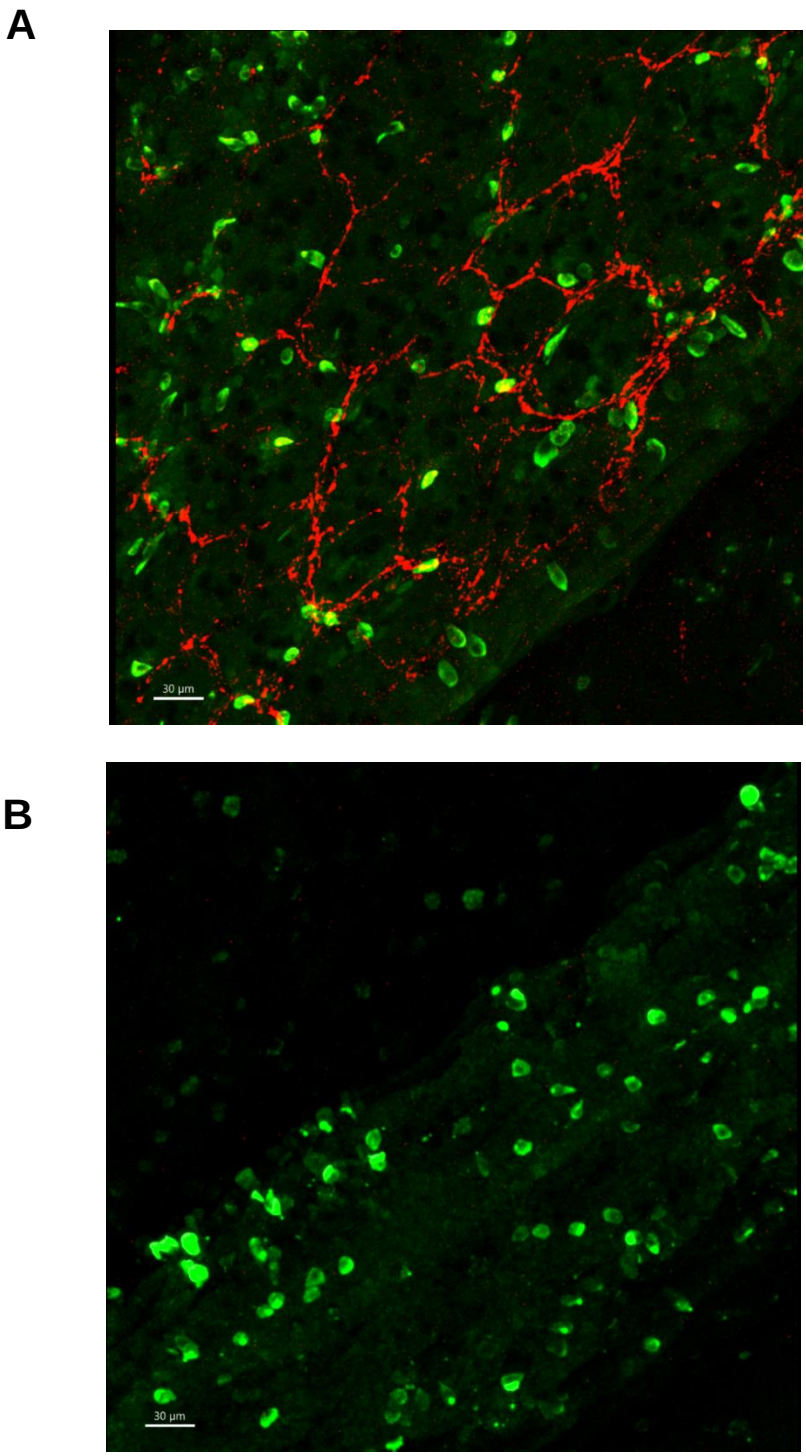

Figure 5.10: Representative images of the proximal colon 3 weeks post-surgery.

Z-stack projection of A) control proximal colon and B) surgical DRG-ablated colon, stained for serotonin (green) and CGRP (red). Scale bar = 30 µm.

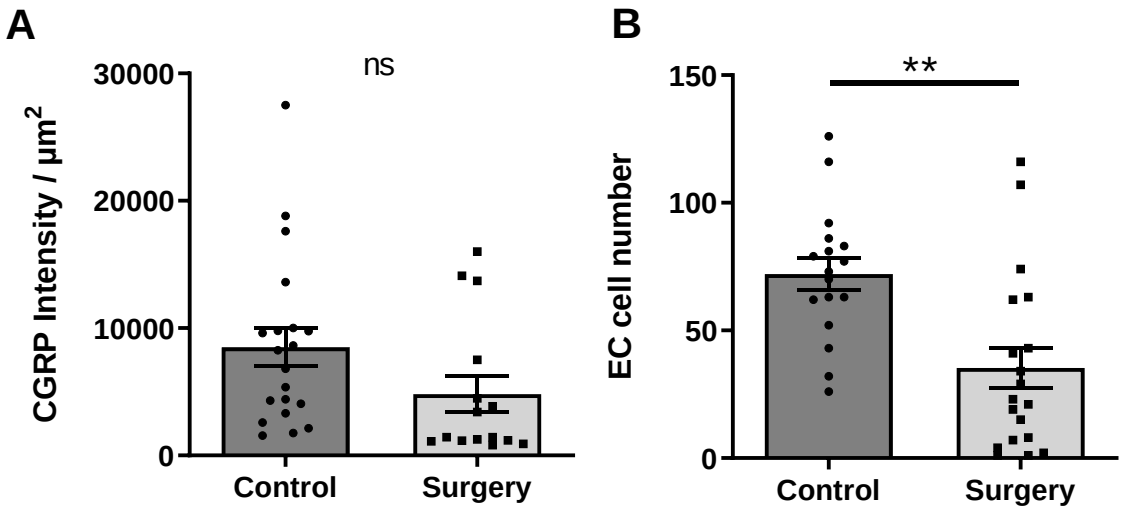

Figure 5.11: Time induced effect of the removal of L6-S1 DRG on the proximal colon 3 weeks post-surgery.

A) The total CGRP fluorescence intensity is unchanged in the proximal colon between control and DRG-ablated mice. B) The number of EC cells in the proximal colon is reduced in DRG-ablated mice 3 weeks post-surgery. Data are shown as mean ± SEM. **=p<0.01

5.3.5 Bioinformatic identification of secreted protein:receptor pairs in DRG sensory neurons and EC cells, respectively.

Due to the differences in EC cell density as a result of the removal of the extrinsic sensory innervation to the mouse distal colon, we were interested to determine whether potential chemoattractants secreted from CGRP-labelled sensory nerve fibres contain receptors on EC cells. To do this, we used publicly available data from scRNAseq, to identify the transcript expression of protein-coding genes associated with a subset of mice DRG neurons, $IB4^{-}$SNS-Cre/$TdTomato^{+}$ (Chiu et al., 2014). This pool of neurons is known to contain the complete population of CGRP sensory neurons which innervate the colon (Chiu et al., 2014). The $17{,}000^{+}$ genes sequenced from this population were investigated using gene ontology, and after a comprehensive literature search, 164 protein-encoding genes that transcribe for known secreted proteins were identified. The scRNAseq dataset used here was composed of 2 specific DRG cell types, $IB4^{+}$SNS-Cre/$TdTomato^{+}$ and $IB4^{-}$SNS-Cre/$TdTomato^{+}$ (as in method 5.2.5). Using results from the differential expression performed by Chiu et al (2014), the list of protein-coding genes from CGRP neurons was then evaluated based on expression and narrowed down to 42 genes of interest up-regulated in CGRP neurons.

To confirm whether these encoded proteins could be involved in driving EC cell neuropod outgrowth, or EC cell fate, a second literature search was performed to determine receptors for each protein of interest. This list of receptors was cross analysed with a list of genes expressed in EC cells (see Table 2), also from a publicly available scRNAseq data set (Bellono et al., 2017). Notably, of the 162 receptors of interest, 120 were encoded for by genes expressed in EC cells (Bellono et al., 2017). Finally, of the protein:receptor pairs identified, 13 genes were shown to be up

regulated in EC cells, compared to all other intestinal epithelial cell types, by differential expression performed by Bellono, et al (2017). This identified a unique set of 13 proteins of interest, where both the protein-coding gene for CGRP neuron secreted factors and the protein-coding gene for EC cell receptor were up-regulated (see Figures 5.12 and 5.13).

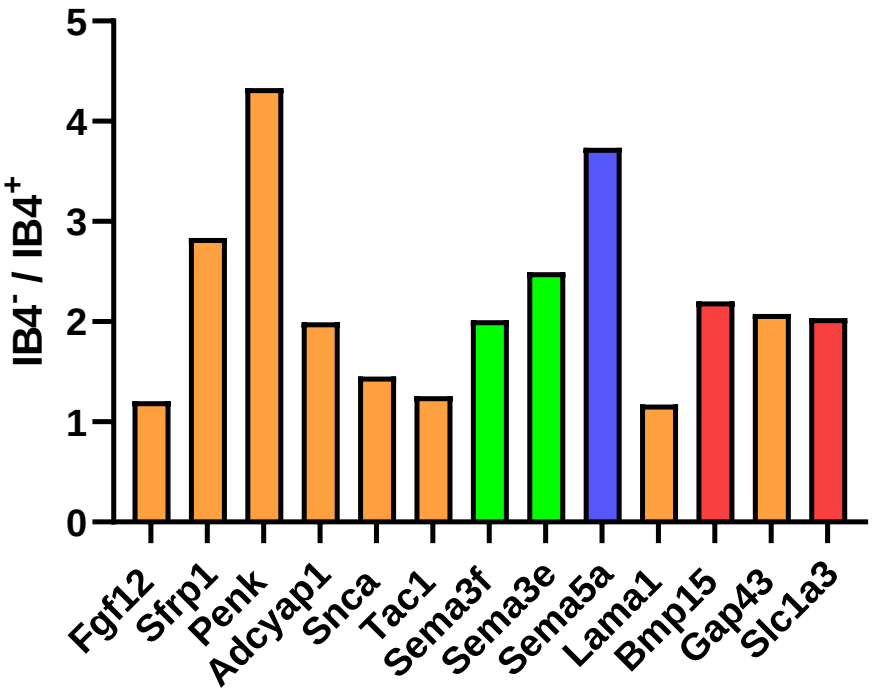

Figure 5.12: Genes expressed in CGRP cells which produce secretory products of interest.

The fold-change expression of the 13 protein coding genes of interest in the CGRP pool (IB4^{-}SNS-Cre/TdTomato^{+}) of DRG neurons relative to non-CGRP containing DRG neurons (IB4^{+}SNS-Cre/TdTomato^{+}). In which statistical significance of enrichment is indicated by colour, where orange indicates $p < 0.05$, green indicates $p < 0.01$, blue indicates $p < 0.001$, and red indicates $p = 0.05$.

Table 2: List of genes which produce secretory products matched with known receptors.

Protein of Interest		Receptor	
Gene Symbol	**Protein**	**Gene Symbol**	**Enriched in EC cells**
Fgf12	fibroblast growth factor 12	Fgfr1	Yes
Sfrp1	secreted frizzled-related protein 1	Fzd1 Wnt10a	Yes
Penk	[Met]enkephalin [Leu]enkephalin	Receptor not highly expressed in EC cells	No
Adcyap1	adenylate cyclase activating polypeptide 1	Receptor not highly expressed in EC cells	No
Snca	Alpha synuclein	Esr1	Yes
Sema3f	Semaphorin 3f	Plxnb3 Plxnd1 Nrp2 Itga4 Itga9 Itgal Itgb7	Yes
Sema3e	Semaphorin 3e		
Sema5a	Semaphorin 5a		
Lama1	laminin	Dagla	
Bmp15	bone morphogenetic protein 15	Receptor not highly expressed in EC cells	No
Gap43	growth associated protein 43	Receptor unknown	N/A
Slc1a3	solute carrier family 1 (glial high affinity glutamate transporter)	Grin2c Grik4 Grm4	Yes

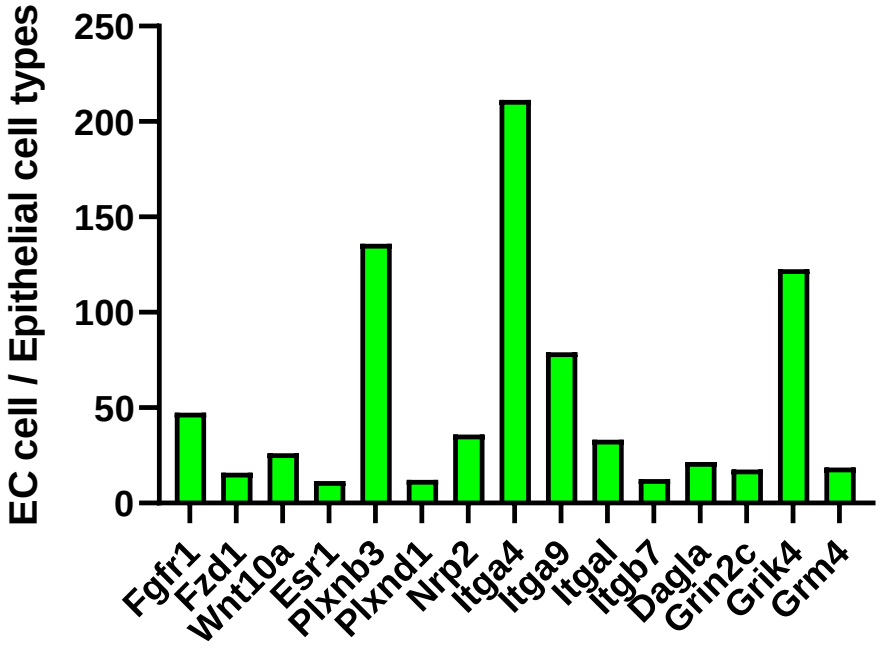

Figure 5.13: Receptor coding genes expressed in EC cells.

Fold change expression of receptors for secreted proteins of interest highly enriched in EC cells.

5.4 Discussion

A physical connection between EECs lining the GI epithelium and neuronal innervation to the GI tract was recently identified in mouse gut (Bohórquez et al., 2014, Bohórquez et al., 2015, Kaelberer et al., 2018), as discussed in depth in Chapter 4, however the mechanisms which drive these points of contact are not currently well understood. Here we use a surgical DRG-ablation mouse model, in which the extrinsic sensory innervation of the mouse distal colon is removed, to investigate the impact that extrinsic afferents have on EC cells. We investigated the implications of this de-innervation on EC cell density and EC cell neuropod characteristics over multiple time points, with the use of confocal microscopy, 3D reconstruction and advanced image analysis.

The removal of the extrinsic afferents resulted in a 50% reduction in total CGRP of the mouse distal colon, with remaining CGRP assumed to comprise intact intrinsic afferents. The removal of just the extrinsic innervation to the colon *in vivo* has not been achieved before, although similar experiments are routine in the upper gut, known as a vagotomy. A vagotomy is a surgery in which the primary extrinsic innervation to the small intestine, the vagus nerve, is severed to remove the primary extrinsic innervation to the stomach and small intestine among other organs. The separate contributions of intrinsic and extrinsic sensory innervation to the distal colon have proven difficult to distinguish, due to their similarities in chemical coding and genetic make-up, our unique DRG-ablation surgery model allows the identification of such contributions.

Interestingly, just 7 days after surgery there is a dramatic reduction in the number of EC cells in the distal colon, as measured through serotonin staining. This considerable loss of EC cells was surprising as we had anticipated a more significant effect from the surgical DRG-ablation on EC cell neuropod formation than on the density of cells

themselves. However, it is important to note that only serotonin fluorescence intensity was used to quantify EC cell numbers, not cell counts, hence it is possible that Tph1 expression was affected rather than the number of EC cells. A reduction in Tph1 expression would also result in a reduction of serotonin production, which could lead to the dramatic decrease in serotonin staining seen here in colonic tissue from DRG-ablation mice. Other circumstances have been shown to alter Tph1 expression, such as the absence of a microbiome which causes a reduction in colonic Tph1 in germ-free (GF) mice (Sjögren et al., 2012, Yano et al., 2015). In these mice, Tph1 can be restored by short chain fatty acids (SCFA) (Reigstad et al., 2014). To address this in future studies, samples from DRG-ablation mice should be co-stained with other EC cell markers not involved in serotonin production, such as VMAT1, Tac1 or Reg4 (Haber et al., 2017).

EC cell neuropod morphology was also affected as a result of the DRG-ablation surgery, specifically, the percentage of EC cells containing neuropods, neuropod length and the number of EC cells with multiple neuropods were all reduced in the distal colon of DRG-ablated mice. These results supported our hypothesis that CGRP fibers might secrete chemoattractants to promote their connection with EC cells and hence drive neuropod growth. A similar hypothesis was made by another group, suggesting that a chemical gradient is formed by the secretion of such chemical attractants, which drive these directional extensions of cellular processes (Liddle, 2019). Preliminary work by Bohorquez (2014) displayed a role for particular neurotrophins in driving EEC neuropod outgrowth. In this study, two growth factors, nerve growth factor (NGF) and artemin, both secreted from enteric glia and known to have a role in axonal growth were chosen (Yasuda et al., 1990, Baloh et al., 1998, Madduri et al., 2009). *In vitro* work showed that both NGF and artemin increased

neuropod formation and length of EECs in intestinal organoids (Bohórquez et al., 2014). This work was important in narrowing down factors which control EEC neuropod formation, and potentially drive EEC-neuronal interactions. However, such work is only preliminary and requires *in vivo* evidence to determine the source of such neurotrophins. Otherwise, there is a paucity of research investigating the factors driving EEC-neural connections. Our study, however, provides further indication that neurons might secrete signals that drive EEC neuropod growth.

A positive relationship between the number of EC cells and CGRP fibres was determined which indicates that CGRP fibres might play a role in determining epithelial cell fate, favouring EC cell differentiation from intestinal stem cells. There is some evidence of such a phenomenon occurring, in which mucosal afferents were found to impact intestinal stem cell proliferation (Lundgren et al., 2011, Peck et al., 2017). In these studies, markers for cell proliferation were investigated in the intestinal mucosa rather than specific epithelial cell markers (Lundgren et al., 2011). This study also suggested that this effect might have been mediated by CGRP or substance P (Lundgren et al., 2011). The effect of CGRP on proliferation, suggests that it would be interesting to determine if other EECs or epithelial subtypes are affected in our DRG-ablation mice. Interestingly, it was recently shown that vasoactive intestinal peptide (VIP), presumedly secreted from VIP neurons, modulates ileal goblet cell density in mice (Schwerdtfeger and Tobet, 2020). These studies provide evidence that secreted factors from enteric neurons could have an impact on intestinal stem cells and cell fate. Similarly, other factors have been shown to impact EC cell density, such as short chain fatty acids (SCFA's) shown to increased serotonin release and the number of Chromogranin A positive cells in organoids (Tsuruta et al., 2016). Other studies have shown that commensal bacteria increase EC cell density via SCFAs (Reigstad et al.,

2014, Yano et al., 2015). The reduction in EC cells observed in the current study could occur through an impact of these various factors on the stem cell niche, hence controlling differentiation into EC cells. Recently, transcriptional profiling on mouse EEC fate in real-time has investigated factors which control epithelial cell fate (Gehart et al., 2019), revealing that particular transcriptional factors are important in distinct epithelial lineages (Gehart et al., 2019), such as, Atoh1 expression (Zecchini et al., 2005a, Stanger et al., 2005, Fre et al., 2005) and neurogenin3 (Jenny et al., 2002b, Lee et al., 2002) important in secretory cell fate. The correlation between CGRP intensity and EC cells, together with this evidence increases the feasibility that CGRP neurons have the potential to drive EC cell differentiation.

As we hypothesise that CGRP fibres secrete factors which drive EC cell neuropod formation and regulate EC cell density or Tph1 expression we were interested to determine whether receptors for CGRP-derived guidance signals or neurotrophins are enriched in EC cells. We used bioinformatic analysis to identify genes coding secreted proteins in sensory neurons (Chiu et al., 2014) with receptors for these proteins on EC cells (Bellono et al., 2017), resulting in a shortlist of 13 ligands. Gene ontology determined ligands associated with axon guidance and synaptic formation. One limitation of this dataset was that the publicly available dataset for the CGRP-containing DRG neuronal pool was collected using microarray (Chiu et al., 2014), which is a targeted approach and may not have recorded all genes of interest in comparison to RNAseq which will find all genes above a non-zero transcript abundance. The other publicly available dataset, however, was produced through scRNAseq (Bellono et al., 2017), making it a more sensitive and less biased dataset. Another factor to consider is that this bioinformatic analysis was conducted on mature EC cells, which does not account for the possibility that secreted proteins from CGRP

nerve fibre could affect differentiation at the stem cell level. Hence future bioinformatic analysis should also look at receptors on the colonic stem cell niche, as it is important to delineate whether the effect of sensory neurons is on mature EC cells, Tph1 expression, or on stem cell fate.

The final list of secreted factors of interest includes pituitary adenylate cyclase activating peptide (PACAP), secreted frizzled related protein 1 (SFRP1), methionine-enkephalin (Met-enk), leucine-enkephalin (Leu-enk), Alpha-synuclein, Semaphorin 3f, 3e and 5a, bone morphogenetic protein 15 (BMP15), laminin, growth associated protein 43 (Gap43) and glutamate. Glutamate is an amino acid that was selected based on the expression of solute carrier family 1, a glial high affinity glutamate transporter, which indicates glutamate is transported by these neurons.

The final list of proteins were selected as potential factors of interest due to their known roles in neuronal development and axonal growth, as well as controlling cell fate. PACAP has a role in neuronal development and neurite outgrowth of sympathetic neuroblasts of the subcallosal cingulate gyrus in the central nervous system (CNS) (DiCicco-Bloom, 1996, Sherwood et al., 2000). PACAP and its receptor are notably expressed throughout the embryonic nervous system, suggested to function in regulating neural patterning and neurogenesis (Dicicco-Bloom et al., 1998).

SFRP1 is a secreted protein part of the secreted frizzled-related protein family (Kawano and Kypta, 2003). SFRP1 binds directly to Wnt, and Frizzled (Fz) proteins (Bafico et al., 1999, Kawano and Kypta, 2003) modulating Wnt signalling (Kawano and Kypta, 2003, Esteve et al., 2019), and also regulates Bmp and Notch (Esteve et al., 2019). Wnt, Bmp and Notch all have significant functions in controlling intestinal stem cell proliferation and cycling (Ricci-Vitiani et al., 2008). SFRP1 in the mouse

developing retina has a downstream role in Notch signaling, and retinal neurogenesis (Esteve et al., 2011, Esteve et al., 2019).

The naturally occurring endogenous opioids have been described to act as growth factors (Zagon and McLaughlin, 1984, Zagon et al., 1999), these peptides are cleaved from the preproenkephalin gene which we found to be highly expressed by CGRP nerve fibres in our bioinformatic analysis. Met-enk is an endogenous opioid peptide, sometimes referred to as the native opioid growth factor (Malendowicz et al., 2005). The peptide is found in several tissue types throughout the body (Tegeder and Geisslinger, 2004), and has roles in controlling cell proliferation and tissue organisation during embryogenesis (Zagon et al., 1999), (Malendowicz et al., 2005). While the other naturally occurring endogenous opioid, Leu-enk, is expressed in enteric nerves and EC cells (Alumets et al., 1978), the role for the peptide in this context has not been well investigated. As such, it was left in our final list of proteins of interest due to the requirement of further understanding of the peptide's role.

The function of Alpha-synuclein is also not currently well understood (Bendor et al., 2013), although the misfolding of the protein is linked with Parkinson's disease (Bendor et al., 2013). It was included as a protein of interest during the bioinformatical process due to the limited knowledge surrounding the protein's function.

Semaphorins were originally unearthed as axon guidance molecules in the CNS (Koncina E., 2007, Alamri et al., 2017), in particular the class 3 of semaphorins (Alamri et al., 2017). More specifically, Semaphorin 3f (Sema3F) has been shown to act as an axon guidance molecule, with implication for growth cones (Nakayama et al., 2018). Similarly, Semaphorin 3e (Sema3e) has been shown to function in axonal growth through its interactions with Plexin-D1 and NRP1 (Chauvet et al., 2007, Alamri et al., 2017)). This occurs in the spinal cord, in which spinal post-synaptic neurons release

Sema3E as a guidance cue to repel or attract incoming axons expressing Plexin-D1, dependant on NRP1 co-expression (Yoshida, 2012). In this instance, Sema-3E controls the specificity of monosynaptic sensory-motor connections, preventing inappropriate synapse formation (Pecho-Vrieseling et al., 2009, Alamri et al., 2017). The third type of Semaphorin highly expressed by CGRP neurons is Semaphorin 5a (Sema5a), known to act as a bifunctional axon guidance cue in the mammalian midbrain (Hilario et al., 2009).

Although no direct role for BMP15 has been indicated in the literature as yet, other BMP proteins are known to impact intestinal epithelial cell differentiation along the crypt-villus axis (Beumer et al., 2018).

Laminin is well known to stimulate axonal growth and play a prominent role in growth cone guidance (Edgar et al., 1994, McKerracher et al., 1996), as well as in axon guidance in development and regeneration (McKerracher et al., 1996, Turney and Bridgman, 2005). Laminin has been linked with neurite growth of sensory neurons in particular (Baron-Van Evercooren et al., 1982), and the guidance of sensory innervation *in vivo* (Sann et al., 2008). These studies highlight laminin as a prominent protein in sensory neuron growth, leading to its inclusion in the current list of proteins of interest.

GAP43, also known as Neuromodulin, is implicated as a protein significantly linked with neuronal growth and pathfinding (Aarts et al., 1998), shown to have specific roles in neurite elongation and synapse formation (Rosskothen-Kuhl and Illing, 2014).

Glutamate is typically known as a traditional neurotransmitter for its functions as an excitatory signaling molecule used significantly in the CNS (Watkins and Jane, 2006). Other roles for the amino acid have been identified, including its function in promoting new growth of functional spines in the developing cortex (Kwon and Sabatini, 2011).

Whilst glutamate is not a typical growth factor, the possibility of such a function within the enteric nervous system or contributing to EEC neuropod formation and growth cannot be ruled out. As described here, the known functions of these proteins indicate that they may contribute to neuropod outgrowth of EECs or potentially play a role in intestinal epithelial cell fate. For future studies, these proteins of interest could be tested on an intestinal organoid model to determine whether they contribute to neuropod outgrowth or EC cell density. If such pathways are identified, future experiments could utilise $Tph1^{Cre/+}$ mice (Wei et al., 2021) to knockdown receptors of interest in EC cells specifically to look *in vivo* at whether this influences neuropod development, serotonin and Tph1 expression, or EC cell numbers.

After 21 days post-surgery the amount of CGRP in the distal colon appeared to be less significantly affected, potentially indicating recovery in the system. One possible reason for this could be that the initial ablation of DRG caused such substantial denervation to the colon that it may have caused inflammation in the affected regions, although further evidence would be needed in order to substantiate such a hypothesis. This inflammation could have caused the significantly greater depletion in EC cell density seen initially 7 days post-surgery, as seen previously of EECs in GI inflammation (Lomax et al., 2006), a phenomenon which could have corrected itself by the 21-day time point, hence explaining the recovery of EC cells and CGRP-labelled nerve fibres that we see 3 weeks post-surgery. Interestingly along with the recovery of EC cell numbers in the distal colon, a subsequent reduction of CGRP and EC cell number in the proximal colon was also noted at the longer time point post-surgery. These observations might indicate that sensory neurons from the proximal colon could be migrating to the distal colon to replenish this severely affected region. Similar reinnervation after injury has been shown previously in zebrafish, in which migration

of existing precursor cells distinct from stem cells migrate to affected areas post injury (Vandestadt et al., 2019). Similarly, innervation from the ENS could also have occurred, a phenomenon which has been shown following denervation of the small intestine (Galligan et al., 1988, Jiang and Surprenant, 1992). Such a recovery mechanism might occur due to the importance of EECs along the gut wall. The importance of these cells has been outlined through mouse models, in which an important transcriptional factor for EEC differentiation, Ngn3, is lethal in knockout mice (Mellitzer et al., 2010b). It is also important to understand the mechanisms by which EECs differentiate, as EEC numbers have implications in a variety of human diseases, such as various gut disorders, diabetes, obesity, and even cognitive disorders including depression and anxiety (Tsakmaki et al., 2020). Hence to define the regulatory network which controls EEC differentiation has significant therapeutic potential.

The lack of a sham model in this study is a significant limitation, one which would help to decipher whether inflammation caused by the surgery might be a confounding factor in such results. Hence it is clear that in future studies using these experimental mice a number of extra important controls will be included, such as sham surgery mice and the use of proximal tissue from all time points as an internal control. The use of sham surgery mice are particularly important as surgical procedures themselves can cause confounding effects. Hence, the use of a sham model would confirm that the results we see in the current study are as a direct result of the removal of spinal afferent innervation to the gut, and not as a result of the surgical procedure itself.

The molecular pathways which guide the innervation of the mucosa and determine the anatomy of the myenteric and submucosal plexuses are also not entirely understood yet (Kang et al., 2021). A few studies have recently identified neurons as having a role

in the control of the epithelium and intestinal stem cell fate, and vice versa (Lai et al., 2020). Serotonin has been shown to play a role in enteric neural development, proposed to act as an axon guidance cue in the ENS {Park, 2019 #4556}(Vicenzi et al., 2020). This has been suggested by other researchers, who propose that chemo-attractants are released from both EECs and enteric neurons to promote neurite and neuropod growth to form EEC-neuronal connections (Liddle, 2019). Hence EC cells might secrete serotonin to attract neurons to innervate the mucosa and synapse with EECs, whilst neurons might secrete factors which control EEC cell differentiation and neuropod outgrowth. Recent work has shown that serotonin might act as a chemoattractant/directional cue in axonal outgrowth of DRG sensory neurons *in vitro* (Vicenzi et al., 2020). As such, low concentrations of serotonin were shown to attract sensory growth cones, while high concentrations were shown to stop growth cones. Such a gradient would make sense as a guidance cue, as the concentration of serotonin would start low, and get higher as the axon grows closer to EC cells. *In vivo* models, such as germ-free mice, have demonstrated both a reduction in EC cell serotonin and a reduction in enteric neurons, and the replenishment of enteric neurons with the addition of exogenous serotonin (De Vadder et al., 2018). As such, it has been suggested that serotonin may function as a mucosal growth factor, important as a developmental signal in the early postnatal mouse ENS {Park, 2019 #4556}. In future studies, it would be interesting to look at the ENS of $Tph1^{CreERT2/+}$;$Rosa26^{DTA/+}$ mice (Wei et al., 2021), in which a knockdown of EC cell serotonin can be induced in mature mice, to determine the role of EC cell serotonin on enteric neurogenesis and maintenance.

5.5 Conclusion:

This body of work has demonstrated, and characterised, for the first time the relationship between EC cells and extrinsic sensory neurons in the mouse distal colon. We show through the utilisation of a surgical DRG-ablation mouse model, that the removal of the extrinsic sensory innervation to the mouse distal colon has inhibitory effects on EC cell neuropods, and the number of EC cells themselves. This phenomenon appeared to recover over time, however more investigation is required. Finally, we highlight 13 protein:receptor pairs of interest, through bioinformatic analysis of CGRP and EC cell sequencing, which may mediate the role of CGRP-secreted proteins in EC cell formation.

CHAPTER 6: General Discussion

6. General Discussion

The overall aim of this body of work was to determine how serotonin is involved in gastrointestinal (GI) motility, and to characterise the connections between enterochromaffin (EC) cells and sensory neurons. Using a combination of primary mouse and human GI tissue, and mouse models with Tph1-specific genetic manipulations or novel DRG ablation surgery, this work specifically aimed to answer the following questions:

1. Is EC cell serotonin essential in gut motility?
2. Is Piezo2 the mechanosensitive ion channel responsible for human and mouse EC cell mechanotransduction?
3. Do EC cells make direct contact with sensory nerves in the intestinal wall?
4. What role do extrinsic afferents play in driving the formation of EC cell neuropods?

This chapter discusses how these questions have been addressed by this thesis, and highlights both contributions to the field and future directions from these studies.

6.1 Summary of results and implications

6.1.1 EC-cell derived serotonin is modulatory, but not essential in GI motility:

Significant investigation has taken place surrounding the importance of serotonin in GI motility, since the establishment of initial dogma suggested serotonin as the key driver of motility. Work within the last decade focusing on the importance of serotonin in motility has polarised research groups, with debate surrounding whether serotonin is essential in driving motility or is released in response to motility (Smith and Gershon, 2015a, Spencer et al., 2015, Smith and Gershon, 2015b). Previous work within this field has utilised dissected preparations, pharmacological approaches and knockout models, although each have been confounded with their own issues detracting from normal physiology (Keating and Spencer, 2010, Li et al., 2011, Heredia et al., 2013). Hence, there has been a distinct lack in technology, in which the need for a superior model to specifically knockdown EC cells post-development has left an overall gap in the literature around the explicit role of serotonin in motility. To address this, Chapter 2 of this thesis details the investigation of the role of EC cell derived serotonin in colonic motility utilising a novel mouse model in which the specific ablation of EC cells is induced in mature mice. In this study we confirm the specificity of ablation and normal gut development in the $Tph1^{CreERT2/+}$;$Rosa26^{DTA/+}$ mouse line, allowing us to conclusively investigate the role of EC cell derived serotonin in motility while bypassing many of the previous confounding issues. We demonstrate conclusively for the first time that contractile activity and peristalsis is still functional in adult mice lacking EC cell serotonin, indicating that EC cell serotonin is not essential for GI motility. Furthermore, Chapter 2 details the conclusive finding that EC cell depleted mice have significantly slower motility both *ex vivo* and *in vivo* compared with control mice, demonstrating that EC cell serotonin is modulatory, but not the key driver, of GI

motility. This work builds on previous reports by our research group on the role of EC cell serotonin as important, but not required, for gut motility.

Furthermore, the debate regarding the role of serotonin in GI motility extends to neuronal-derived serotonin, and whether this pool of serotonin is important for contractile activity. We addressed the role of neuronal serotonin in motility in Chapter 2, providing evidence that inhibiting serotonin re-uptake at neuronal synapses has no effect on motility in mice devoid of EC cells. Hence demonstrating that Tph2-derived serotonin is unlikely to have a role in modulating motility, supporting the conclusion drawn by previous investigation (Sia et al., 2013b, Spencer et al., 2013).

The research completed within this thesis conclusively distinguishes the role of serotonin in GI motility, an important addition to our overall understanding of GI motility in both health and disease. This new knowledge may now enable better insight into the mechanisms which are affected in various motility disorders, hence opening up potential new targets to direct treatments towards for such disorders. Altered gut serotonin levels have previously been implicated in a number of functional motility disorders, such as gastric emptying, slow transit constipation, and IBS (Gershon and Tack, 2007, Wei et al., 2021). Currently many of these are treated with serotonergic agents that reduce symptoms, but do not cure disease and typically come with significant side effects. The data presented in this thesis adds significantly to our understanding of the role of serotonin in motility which will allow for the development of pharmaceuticals to act directly on EC cells to either stimulate or block the release of serotonin to treat various motility disorders. The primary benefit of this work is that it identifies a target for future drug treatments, as non-absorbable oral drugs could be developed to directly target EC cells to better modulate motility in these patients. Such

specificity would significantly reduce off-target affects common with serotonergic acting drugs and improve the quality of life for those who suffer from such chronic disorders.

6.1.2 Piezo2, an important mechanosensitive channel in mouse and human EC cells, is important in GI motility:

EC cells have long been known to possess mechanosensing abilities, with early evidence published over 60 years ago demonstrating the release of serotonin from guinea pig intestine upon mucosal compression. Since these initial reports the mechanisms in which EC cells sense mechanical stimuli have been further investigated, with the most recent evidence demonstrating a role for the mechanosensing ion channel Piezo2 in mouse EC cell mechanobiology (Wang et al., 2017, Alcaino et al., 2018). However, to date no evidence for human EC cell mechanotransduction has been described. In Chapter 3 of this thesis, we provide the first evidence that the mechanosensing channel Piezo2 is present and important in human EC cell mechanotransduction. We demonstrate through carbon-fibre amperometry that the real-time release of serotonin is significantly reduced from human small and large bowel in the presence of a Piezo inhibitor, highlighting the role for Piezo2 in human EC cell mechanically evoked serotonin release for the first time. Throughout this study we use varied techniques to describe the release of serotonin in response to both mucosal compression and fluid movement and provide evidence which suggests differences between small and large bowel EC cell mechanosensitivity.

One key factor in the debate surrounding the importance of EC cell serotonin in motility is the order in which events serotonin release and gut contractions occur. Some

researchers suggest that serotonin release comes first to drive GI motility (Heredia et al., 2009, Smith and Gershon, 2015a), while others believe serotonin is released in response to distention or contraction to modulate this process (Keating and Spencer, 2010, Spencer et al., 2015). However, evidence provided in Chapter 2 demonstrates that contractile activity still occurs in EC cell depleted mice supporting the notion that EC cell serotonin is not required for contractile activity. We instead hypothesised that EC cell serotonin is released in response to motility. Results from Chapter 3 using an *ex vivo* model where we measure CMMCs in the presence of a Piezo channel inhibitor to demonstrate for the first time that EC cell Piezo2 is a significant modulator of GI motility. These results were confirmed by our collaborators using a novel *in vivo* model, in which mice with Tph1 cells lacking Piezo2 demonstrate a constipation-like phenotype. This data implicates Piezo2 as the key mechanosensitive ion channel in mouse and human EC cells which allow these cells to detect and respond to distention and contraction, stimulating serotonin release and modulating the rate of peristalsis. Hence, we provide the first conclusive evidence that Piezo2 mediated EC cell mechanosensation is important in modulating colonic motility. Put together, these observations suggest that serotonin release occurs as a result of motor activity rather than as a trigger for it, this role for serotonin is likely in the intrinsic feedback loops which modulates motility. These results further support the previous hypothesis of a positive feedback loop, whereby a bolus stimulates EC cell serotonin which signals to intrinsic neurons to increase the rate of contractions and propagate CMMCs, known as the ‘neuromechanical loop’. Here, we add EC cell Piezo2 to the neuromechanical loop, depicting the mechanically stimulated EC cell serotonin is implicated in this pathway and is important in modulating colonic motility (see Figure 6.1). Furthermore,

we suggest a potential role for neuronal Piezo2 in this loop and highlight the need for future investigation to delineate the exact circuitry involved.

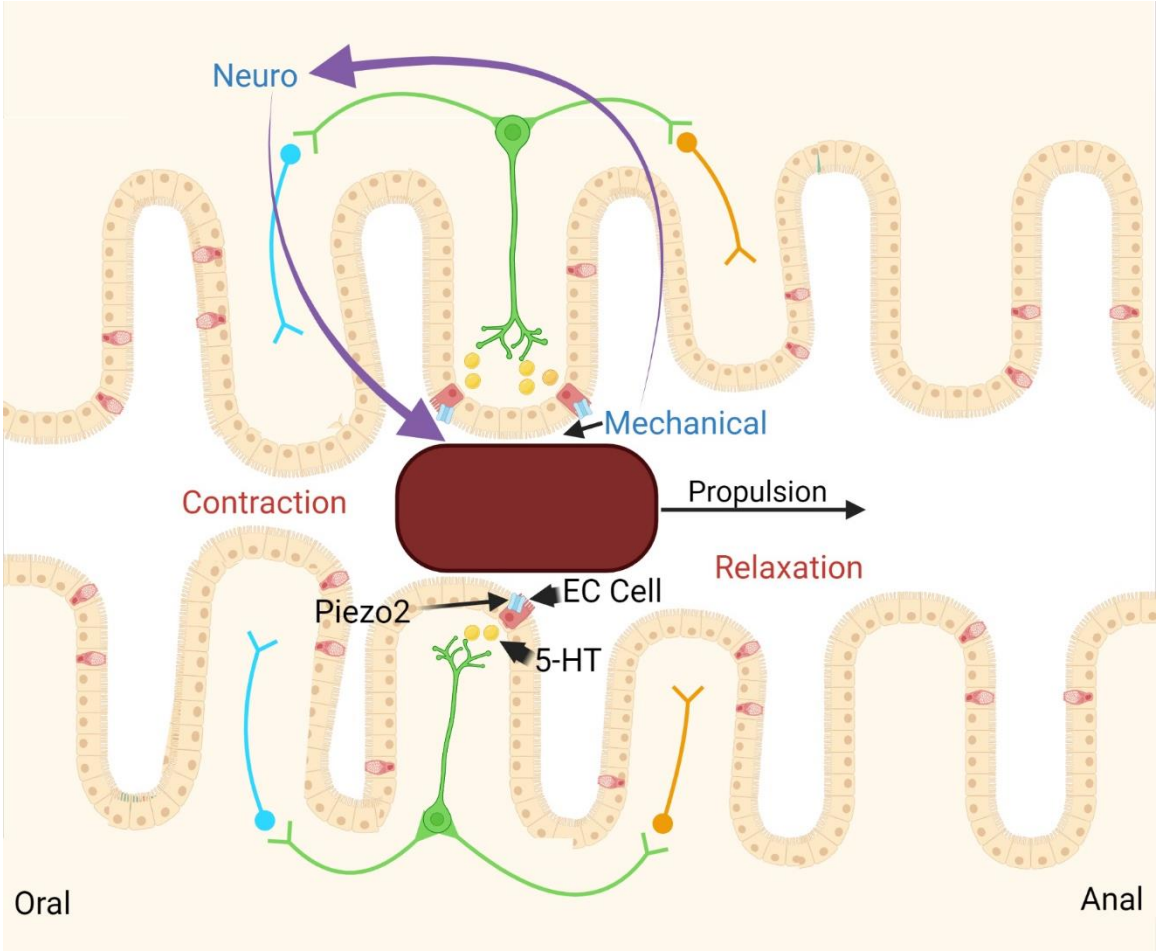

Figure 6.1: A schematic of the neuromechanical loop hypothesis including EC cell Piezo2.

The mechanical stimulus of the bolus (Mechanical, blue) activates Piezo2 (arrow) in EC cells (red, arrow), to release serotonin (5-HT, arrow). As such, serotonin activates enteric neurons (Neuro, blue) within the gut wall, which causes a contraction (red) prior to the bolus, and a relaxation (red) of the muscles anally, with respect to the bolus. The bolus is then propelled along the GI tract, stimulating the same pathway, thereby defining the neuromechanical loop.

The work is important in building our understanding surrounding the mechanisms involved in GI motility, particularly as functional mechanosensing is important for healthy GI function. Abnormalities in GI mechanosensation are also linked with diseases including motility disorders such as IBS and constipation (Alcaino et al., 2017a). In slow transit constipation, there is an issue in detecting or responding to a bolus, or distention (Dinning et al., 2015, Dinning et al., 2016) which indicates an issue in the mechanosensing of a stimulus/the luminal environment. This property highlights a potential role for Piezo2 in such motility disorders and opens up a key avenue for future investigation into the pathophysiology underlying slow transit constipation. This is particularly evident as we show here that the specific knockdown of EC cell Piezo2 leads to a slow transit constipation phenotype, similar to that in the Tph1 DTA mouse line (Wei et al., 2021), hence providing further evidence for the potential role of Piezo2 in such motility disorders. Another significant and novel finding from Chapter 3 is that EC cell mechanosensitivity is lost with age, a factor which might contribute to age-related motility issues such as chronic constipation common amongst the ageing population. Such evidence highlights a new avenue for future investigation into this common issue which could in the future lead to better understanding and treatments for age-related constipation. Hence, data in this thesis creates a building block for future work looking at the potential link between Piezo2 function with slow transit constipation and/or age-related constipation.

6.1.3 EC cells contain neuropods, some of which contact sensory neurons:

We wanted to characterise the connection between EC cells and sensory neurons in the colon to determine how EC cells might communicate with neurons to modulate peristalsis. Recent studies suggest a direct connection between enteroendocrine cells (EECs) and extrinsic sensory neurons of the upper gut via synaptic-like connections,

such connections are postulated to occur via anatomical extensions from EECs termed ‘neuropods’. Research in this area has focused on L and I cells of the small intestine (Chandra et al., 2010, Bohórquez et al., 2011, Bohórquez et al., 2015, Kaelberer et al., 2018), two EEC subtypes known to play a role in appetite and satiety signalling through communication with extrinsic nerves. There are currently no reports in the literature pertaining to EC cell neuropods, and whether these cells form connections with extrinsic or intrinsic afferents in the colon. We were interested to look at whether EC cells form direct connections to sensory nerves, a mechanism which might facilitate the control of GI motility or impact other central mechanisms. In Chapters 4 and 5 of this thesis we investigate for the first time the relationship between EC cells and CGRP-labelled sensory nerves within the intestinal wall of the colon and demonstrate the presence of neuropod-like processes on most EC cells in the mouse colon. We also demonstrate the presence of EC cell neuropods in human colonic tissue and the very first evidence of direct connections between mouse and human EC cells with colonic neurons. In Chapter 4, we provide evidence that more than half of all EC cells make direct contact with sensory afferents in the distal mouse colon, although upon closer investigation we determine that many of these connections lie at the EC cell body and not at neuropods. This data prompts many questions surrounding such connections, such as whether they are functional, whether they are facilitated by neuropods or another mechanism, and leaves uncertainty around the function of these anatomical processes. Furthermore, whether these connections occur within intrinsic or extrinsic pathways is likely to correlate with their function, EC cells which communicate with intrinsic sensory pathways are likely to modulate colonic contractile activity, whereas those which communicate with extrinsic neurons are likely to have implications in the gut-brain axis. The gut-brain axis has been implicated in a raft of

functions such as in appetite and satiety signalling, as well as numerous diseases, including obesity, psychiatric disorders, and neurodegenerative diseases such as Parkinson's disease (Cryan et al., 2019). Hence these connections are of great interest as they may play a significant role in gut-brain axis signalling, and hence also in health and disease throughout the body. Thus, a better understanding of the connections between EECs and neurons will have implications locally for GI motility, and better define the role for EECs in the gut-brain axis, and hence more broadly for health and disease impacted through the gut-brain axis.

6.1.4 Factors which might drive connections between EC cells and extrinsic sensory neurons:

There is currently very limited understanding of the factors which drive the connections between EECs and neurons in the gut wall, likely due to the novelty of this field. The only previous study to outline connections between EECs and neurons highlights a role for various growth factors in this process, demonstrating *in vitro* an increase in EEC neuropods in organoids treated with growth factors (Bohórquez et al., 2014). The nature of such connections, as discussed in depth in Chapters 4 and 5, is of significant importance in understanding the molecular mechanisms which underlie the gut-brain axis. Hence, it is also important to understand what drives such connections, assuming EEC-neural connections are functional, to further our understanding of the role EECs play in the gut-brain axis. In the current study we were interested to determine factors which might drive the connections between EC cells and CGRP-labelled sensory neurons in the mouse colon, using a novel mouse model in which extrinsic sensory neurons are removed from mice *in vivo.* This unique surgical model allows the ablation of sensory innervation to the distal colon specifically *in vivo*, via the highly specialised removal of particular DRG bilaterally. DRG-ablation mice allow us to delineate the

specific role that extrinsic sensory innervation has in driving EC cell neuropod formation and EC-neural connections. In Chapter 5, we used advanced confocal microscopy and 3D analysis software to characterise EC cell neuropods in DRG-ablated mice, to determine the effect of the removal of extrinsic sensory innervation on EC cell neuropods. Here, we quantify the significant reduction of CGRP-labelled nerves in the colons of these mice and see a dramatic reduction in the number of EC cells, an unexpected result. We also see a reduction in the number of EC cells which contain neuropods and the mean length of EC cell neuropods within the distal colon of these mice. These findings highlight the potential role for sensory neurons to impact EC cell numbers, and drive EC cell neuropod formation. Such results give us a greater insight into the potential relationship between EC cells and sensory innervation into the colon and open up a range of possibilities for future experiments into this area.

Due to the large impact seen on EC cell numbers in DRG-ablated mice, we used publicly available data sets to investigate potential factors released from CGRP nerves that may impact EC cell numbers and neuropod formation. Using a bioinformatic approach, we define 13 genes of interest expressed in CGRP-containing sensory neurons known to encode for proteins important in axonal growth and development, and controlling cell fate, all of which contain receptors encoded for by genes enriched in EC cells. Our resultant dataset provides a shortlist of factors which are supportive of evidence that chemoattractants from extrinsic sensory innervation contributes to the formation of EC cells and EC cell neuropods. Such work, as described in Chapter 4 and 5 of the current thesis, provides a valuable platform for future investigation into the mechanisms which underpin EEC-neuropod interactions and, more broadly, the gut-brain axis (discussed in the following section, ‘6.2 Future Directions’).

6.2 Future Directions

The current body of work has demonstrated a modulatory role for EC cell derived serotonin in GI motility, the mechanosensing properties of EC cells by Piezo2, and demonstrated the potential connection of EC cells with both intrinsic and extrinsic sensory neurons of the mouse and human colon. These findings may be built upon by future investigations into the serotonergic mechanisms underlying motility, including the role of neuronal serotonin and neuronal Piezo2 in the regulation of GI motility. Such investigations could entail the use of an inducible Tph2 floxed model, in which Tph2 expression is knocked down in the enteric nervous system by an enteric neuronal promotor, such as ubiquitin carboxy-terminal hydrolase L1 (Wiese et al., 2013). This would allow the inducible loss of Tph2 expression and hence neuronal serotonin, leaving Tph2 cholinergic neurons unaffected. Motility experiments on a Tph2 floxed model would bypass issues with previous pharmacological and knockout mouse models, such as developmental differences seen in the neuroanatomy of $Tph2^{-/-}$ ENS, hence aid in the conclusive delineation of neuronal serotonin on motility. Additional investigation of $Tph1^{CreERT2/+}$;$Piezo2^{flox/flox}$ mice could entail pharmacological inhibition of Piezo2 on these mice *ex vivo* to measure the effect on CMMCs, such a result would indicate the importance of neuronal Piezo2 in colonic motility.

Furthermore, mechanisms by which EC cells or EC cell serotonin is altered in motility disorders could be further deduced by the collection of biopsies from elderly patients with age-related chronic constipation or others suffering from chronic constipation to measure the levels of Piezo2 in their gut. Similarly, it would be interesting to determine the occurrence of constipation in human patients already known to have a loss-of-function mutation in their Piezo2 gene (Chesler et al., 2016, Marshall et al., 2020), or

generally what effects are seen in these patients' bowel movements. Together these studies will better define the mechanisms which are affected in various motility disorders, from which we can create more specific treatments to target in such disorders. This could include treatments which specifically target EC cells, directed towards proteins known to be exclusively expressed by EC cells along the GI tract, such proteins are already known to exist. These treatments could be designed as non-absorbable, hence with their specificity they would only act on EC cells and not produce off-target effects with other tissues outside of the gut. Further, there are a number of factors which can be altered surrounding drug manufacturing, such as pH, specific enzymes, reactive oxygen species (ROS), or a combination, which can allow the specific release to occur in the colon and hence bypass the upper gut (Teruel et al., 2020). As we understand that a reduction in EC cell serotonin leads to constipation, the use of such EC cell activating drugs could cause EC cell serotonin release, to mitigate constipation in patients with various motility disorders. Considerable further investigation would need to be undertaken in order to design such drugs; however, this is the promising future for more precise medicines.

EC cells are typically described as sensory cells, receptive to a range of provocations, including mechanical (Wang et al., 2017), chemical (Martin et al., 2017c), microbial (Ye et al., 2021), and noxious stimuli (Bellono et al., 2017). However, as highlighted in Chapter 3 and 4, there is growing evidence to suggest that subtypes of EC cells exist, in which different subtypes of EC cells may respond to differing stimuli or mediate different functions along the GI tract. EC cell subtypes have been distinguished through genetic profiling (Haber et al., 2017, Gehart et al., 2019, Beumer et al., 2020), distinct morphologies along the crypt-villus axis (Koo et al., 2021, Kuramoto et al., 2021), differing nutrient sensing capabilities along the GI tract (Martin et al., 2017d)

and varied life-cycles (some short-lived and others long-lasting (Wei et al., 2021)). In the current study, we highlight that a proportion of EC cells contain Piezo2, while others do not, and that a proportion of EC cells contact nerves, while others do not. As hypothesised by Fujita et al (1971), differences in EC cell morphology may indicate that some EC cells are mechanically sensitive while others may respond to chemical stimuli. As such, whether distinct subtypes of EC cells do in fact exist could be distinguished through a number of mechanisms. This includes but is not limited to, further analysis of the genetic profiles of EC cells, which could be analysed to distinguish whether EC cells can be separated out into pools of EC cells based on gene clustering. This could also be tested functionally, through sorting EC cells based on Piezo2^{+} expresison using fluorescence-activated cell sorting. As such Piezo2^{+} EC cells could be sorted from EC cells lacking Piezo2, these separate pools could then be tested with mechanical and chemical stimuli, and analysed in real-time using calcium imaging to determine whether separate subtypes of EC cells respond differently to different stimuli.

The burgeoning field of EEC-neuronal interactions is one with a wide range of questions which need investigation. Key unknowns within this field currently include the legitimacy of synaptic connections between EECs and neurons, whether such connections are feasible in an ever-changing environment such as the intestinal epithelial lining and finally the mechanisms which drive such connections. The novel surgical DRG-ablation mice described in Chapter 5 could be utilised further to delineate the contributions of extrinsic and intrinsic innervation to the colon, through comparison of 3D analysis of CGRP-labelled sensory innervation to the entire length of the colon, pre- and post-surgery. Additionally, these mice could be used to measure the proportion of EC cells which form connections with intrinsic and extrinsic nerves

respectively. This could be measured through 3D distance analysis, as seen in Chapters 4 and 5, and would need to be performed on tissue 3-weeks post-surgery to reduce confounding effects of the reduction of EC cells seen 1-week post-surgery. EC cells found to form connections with CGRP-labelled nerve fibres post-surgery would represent the percentage of EC cells which contact intrinsic sensory neurons and could be compared to the same analysis in Chapter 5 to delineate the proportions of EC cells which contact intrinsic and extrinsic afferents respectively.

To add to the data presented in this thesis, further immunofluorescent experiments could be performed on control colonic tissue to stain for indicators of functional synaptic connections, such as pre- and post-synaptic markers. Such an investigation could give evidence as to whether the points of contact between EC cells and sensory neurons have functional capacity. This key piece of information would be highly beneficial in our understanding of whether EECs can form functional synaptic-like connections with nerves within the gut wall. Separately, gut tissue from EC cell reporter mice, in which a pool of EC cells are long-lived (Wei et al., 2021), could be co-stained with CGRP and pre- and post-synaptic markers to determine whether long-lived EC cells preferentially make functional connections with sensory neurons compared to short-lived EC cells. This would provide further evidence surrounding the feasibility of such connections between EECs and neurons within the gut wall.

Finally, the 13 proteins of interest, highlighted in Chapter 5, could be tested on intestinal organoids to measure EC cell number and percentage of EC cells containing neuropods, building upon experiments performed previously (Bohórquez et al., 2014). If such investigations on organoids were successful in determining protein candidates which impact EC cell number and/or neuropod formation, the $Tph1^{CreERT2/+}$ mouse

model could be utilised to produce EC cell-restricted genetic manipulations, to knockdown receptors within EC cells specifically. Such single gene knockouts or knock-ins to investigate the roles of specific proteins within EC cell and neuropod formation *in vivo*. This could help to further our understanding of factors controlling EC cell differentiation and neuropod formation, and hence advance our knowledge surrounding the mechanisms which underlie the gut-brain axis.

6.3 Concluding remarks

The results in this study demonstrate that EC cells have a role in GI motility, a mechanism mediated by Piezo2, with evidence that EC cell mechanosensitivity and serotonin are modulatory but not essential for GI motility. Furthermore, we provide evidence which supports our hypothesis that neuronal-derived serotonin does not function in gut motility. This work also shows a reduction in EC cell mechanosensitivity and Piezo2 expression in human colon with age, highlighting the potential for disordered EC cell mechanosensitivity in age-related constipation. Together, this data presents EC cells as a potential target for future specialised treatments in functional motility disorders. Using advanced microscopy and 3D analysis, we characterise the presence and distribution of EC cell neuropods in mouse and human colon. We also investigate the connection between EC cells and sensory innervation and develop a better understanding of the frequency at which EC cells contact sensory nerves. We reveal that most EC cell-neural connections occur at the cell body, hence potentially undermining the term neuropod in description for EC cell cytoplasmic processes. Furthermore, through a novel surgical approach, the removal of extrinsic sensory innervation to the colon reveals a role for extrinsic sensory afferents in the development of EC cells and EC cell neuropods. These findings provide insight into

factors which might drive EC cell proliferation and neuropod formation, but also opens many new questions in the quest to better understand the communication between ECs and innervating nerves. Bioinformatic analysis of EC cells and sensory neurons highlights 13 protein:receptor pairs of interest, that might mediate the link between extrinsic afferents and EC cell differentiation or neuropod formation. With the inclusion of the results described in this thesis, the field is now poised to further investigate the connection between EC cells the gut neural innervation to better understand the circuitry in which EC cells modulate GI motility and facilitate functions within the gut-brain axis.

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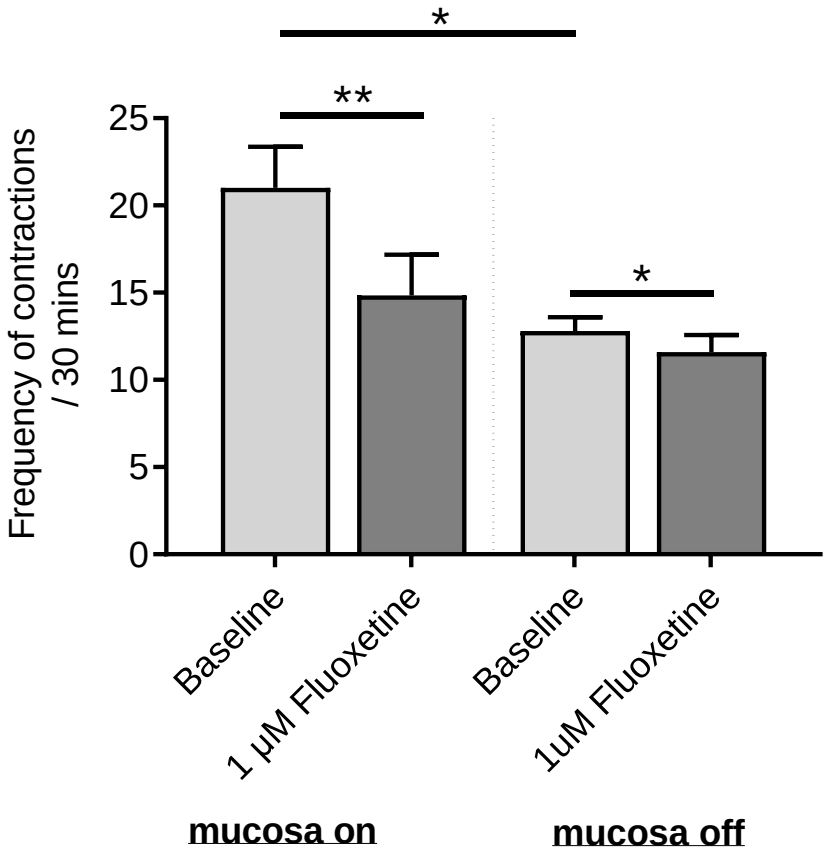
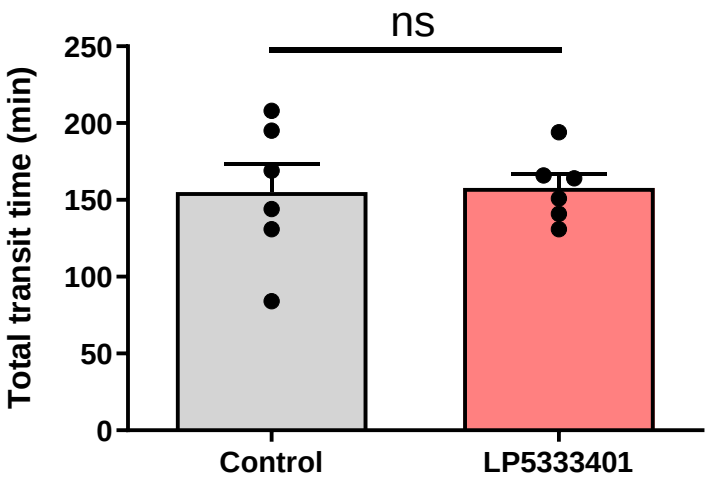
APPENDICES



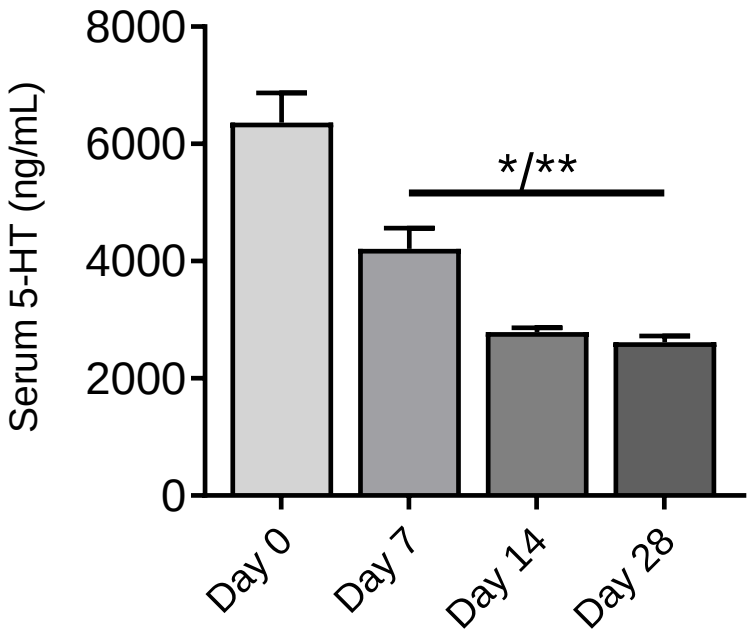
Appendix Figure 1: Fluoxetine reduces CMMC frequency in intact *ex vivo* mouse colonic preparations.

Fluoxetine reduces CMMC frequency significantly in intact colonic preparations *ex vivo* but has not effect on CMMC frequency of mucosa-free preparations. Data are shown as mean ± SEM. * = $p<0.05$, **=$p<0.01$.



Appendix Figure 2: Tph inhibitor treated mice have no change in GI transit *in vivo*.

No difference is seen in the total GI transit time of Tph inhibitor (LP5333401) treated mice *in vivo*. Data are shown as mean ± SEM.



Appendix Figure 3: A reduction in GI mucosal serotonin in Tph inhibitor treated mice.

A 50% reduction of GI mucosal serotonin is seen after 28days treatment with the Tph inhibitor, LP5333401, *in vivo*. Data are shown as mean ± SEM. *=p<0.05, **=p<0.01.

Appendix Table 1: Patient information for each human specimens used in immunohistochemical analysis.

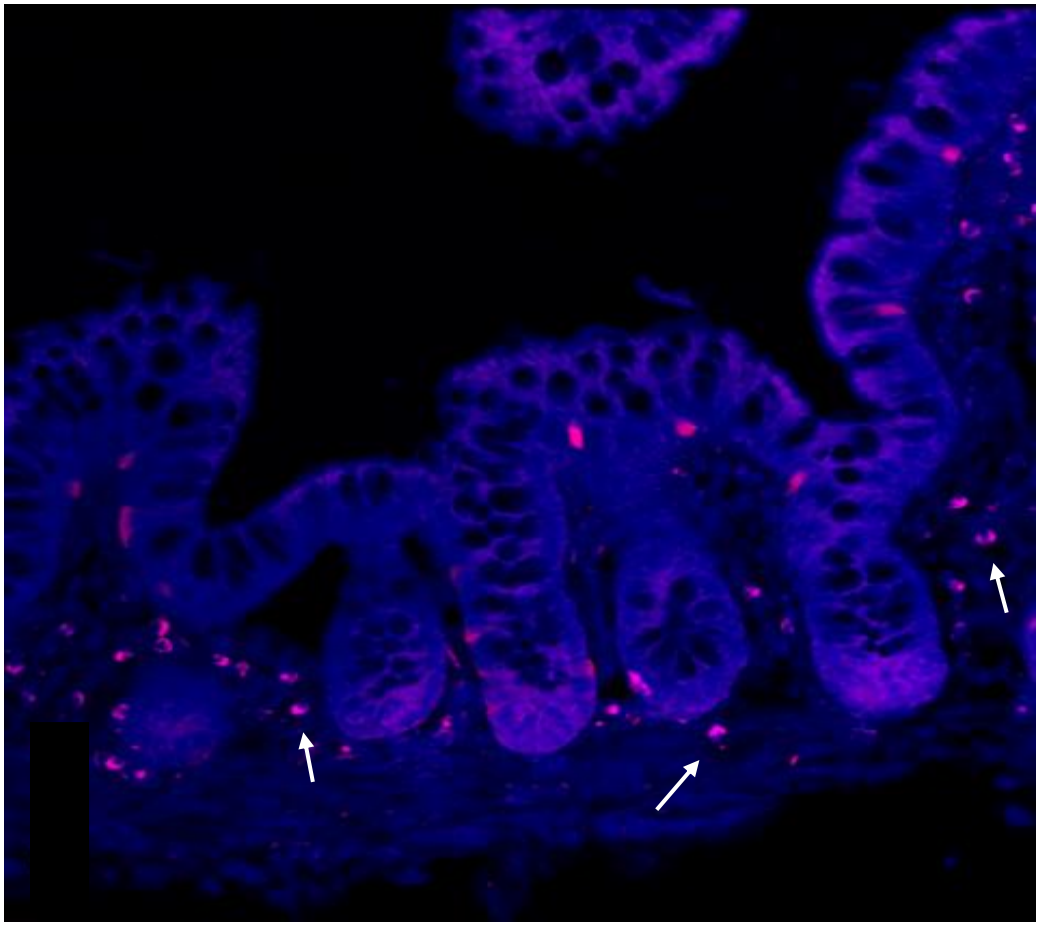
Patient ID	Age	Sex	Region
IP201	90	F	Ileum
IP160	41	F	Ileum
IP159	93	M	Ileum
CP174	59	f	Descending Colon
CP201	90	f	Ascending Colon
CP363	53	m	Sigmoid Colon
CP365	45	f	Descending Colon
CP370	71	f	Ascending Colon
CP371	65	f	Sigmoid Colon
CP360	86	M	Descending Colon

Appendix Table 2: Patient information for each human specimen used in amperometry analysis.

Patient ID	Age	Sex	Region	BMI
IP198	81	F	Ileum	27.9
IP200	70	F	Ileum	24.7
IP201	91	F	Ileum	30
IP202	71	F	Ileum	26.5
IP203	73	F	Ileum	30
IP227	59	M	Ileum	29.6
IP229	85	M	Ileum	-
CP381	81	F	Colon	27.9
CP383	70	F	Colon	24.7
CP384	63	M	Colon	38
CP385	91	F	Colon	30
CP386	71	F	Colon	26.5
CP387	73	F	Colon	30

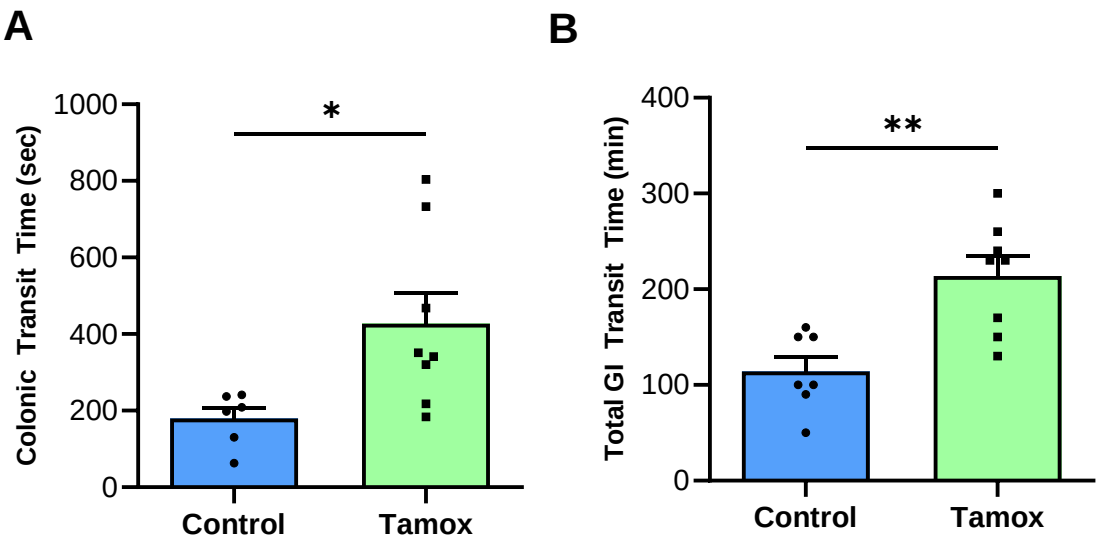
Appendix Table 3: Patient information for each human specimen used in orbital shaking mechanical stimulation experiments.

Patient ID	Age	Sex	BMI
IP198	81	F	27.9
IP200	70	F	24.7
IP201	91	F	30
IP202	71	F	26.5
IP203	73	F	30
IP227	59	M	29.6
IP229	85	M	-
CP381	81	F	27.9
CP383	70	F	24.7
CP384	63	M	38
CP385	91	F	30
CP386	71	F	26.5
CP387	73	F	30



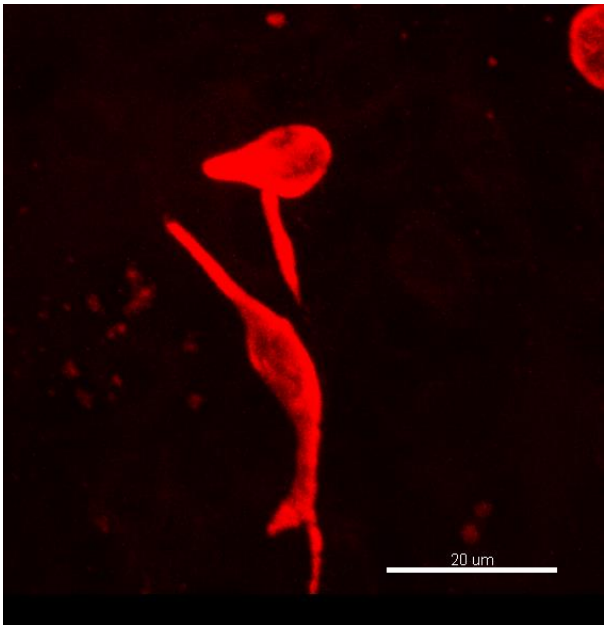
Appendix Figure 4: Example of unidentified Piezo2 positive cells outside of the mucosa.

An example image of human small intestine stained with Piezo2 (red) and DAPI (blue). White arrows depicting Piezo2^{+} cells outside or below the mucosa.



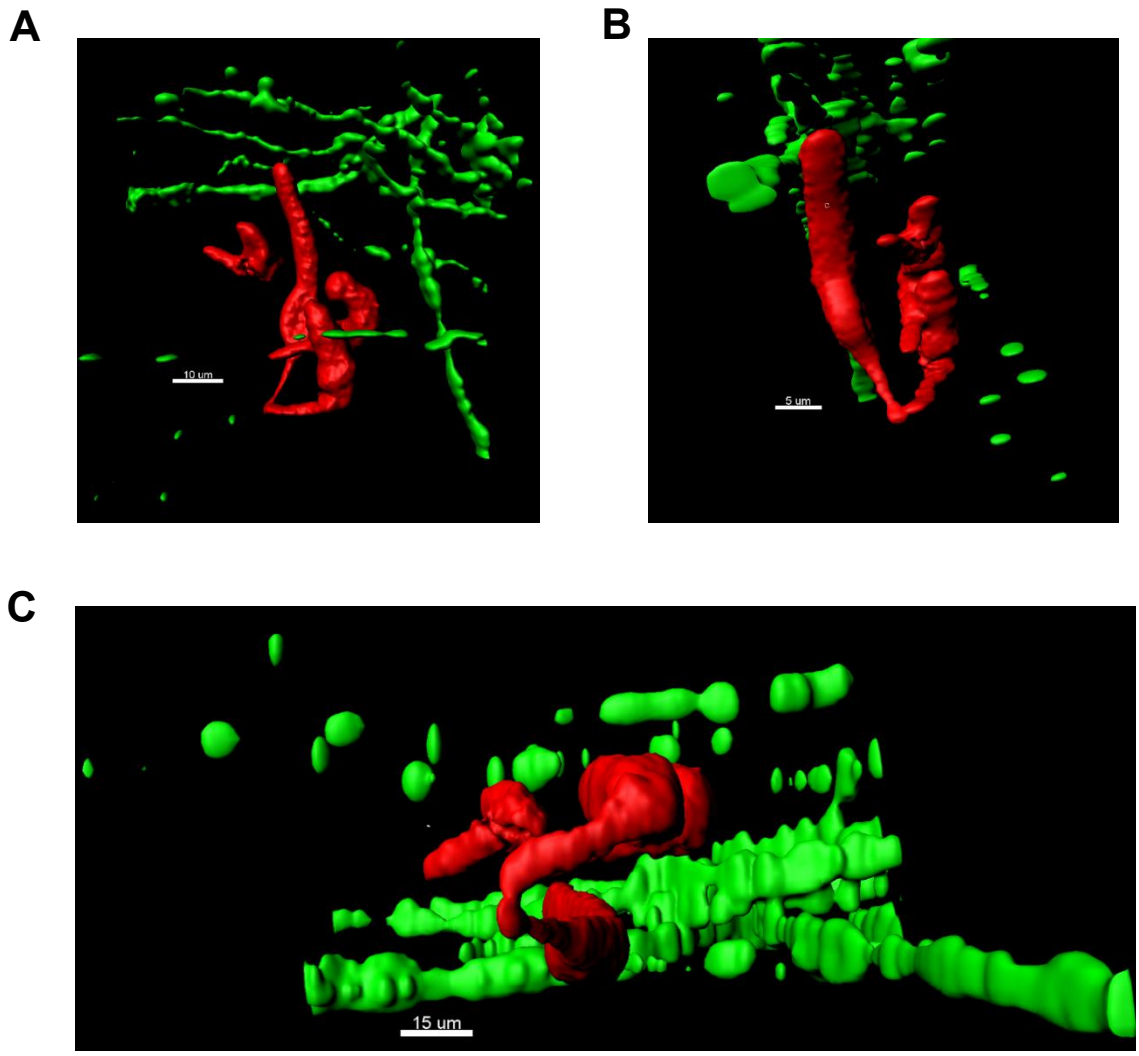
Appendix Figure 5: Tph1$^{CreERT2/+}$;Piezo2$^{flox/flox}$ mice have a reduced *in vivo* GI transit.

The (A) colonic transit time and (B) total GI transit times are significantly longer in Tx treated Tph1$^{CreERT2/+}$;Piezo2$^{flox/flox}$ mice in *vivo*. Data are shown as mean ± SEM. *=p<0.05, **=p<0.01



Appendix Figure 6: EC cell neuropod bifurcating into two.

An example image of an EC cell, labelled with serotonin (red), in which the neuropod bifurcates in two. Scale bar = 30 μm.



Appendix Figure 7: Example of two EC cell neuropods contacting each other.

Serotonin (red) labelled EC cells touching, from above (A), rotated to one side (B), and rotated to the other (C). Scale bar = 10 μm, 5 μm, and 15 μm respectively.