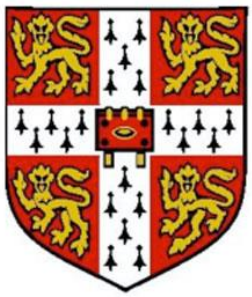

Expression and Function of Galanin in Colonic Sensory Neurones

Dissertation Submitted for the Degree of Doctor of Philosophy

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I. Preface

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. All work presented was carried out at the Department of Pharmacology, University of Cambridge between September 2016 and October 2019.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

Part of the work presented has been submitted as a pre-print to bioRxiv:

Taylor, T.S., Konda, P, John, S.S., Bulmer, D.C., Hockley, J.R.F and Smith, E.S. Galanin inhibits visceral afferent responses to noxious mechanical and inflammatory stimuli. *bioRxiv*, doi: 10.1101/746586

It does not exceed the prescribed word limit of 60,000 words.

II. Acknowledgements

I would like to thank my parents for their continual motivation, encouragement, and their relentless patience, all of which have allowed me to pursue my studies. I would also like to thank my partner, Chris, for the emotional support and endless hugs. Thanks for supporting me to follow my dreams and putting up with my tantrums. I would further like to thank Dr Christian Rudolph for all the opportunities to work in his lab and discover my passion for research which led me to apply to Cambridge.

The Department of Pharmacology has been a friendly and supportive environment to work in and I consider myself very lucky that over the last 3 years I have had the pleasure to work with and learn from a number of talented scientists. I am greatly indebted to my supervisor, Dr Ewan St. John Smith for his guidance and support throughout my studies and his endless enthusiasm in the preparation of this thesis. I would also like to thank all the members of the Smith lab especially Dr James Hockley, Dr Gerard Callejo, Sampurna Chakrabati, Dr Yavuz Kulaberoglu, and Luke Pattison. I am also very grateful to Dr David Bulmer for all his advice and guidance.

Finally, I am also grateful to the Cambridge Trust for awarding me the Vice Chancellor's Award that supported my studies and to the Rosetrees Trust whose funding supported this research.

III. Abbreviations

5-HT	5-hydroxytryptamine
5-HTR	5-hydroxytryptamine receptors
ANOVA	Analysis of variance
ASIC	Acid-sensing ion channel
ATP	Adenosine triphosphate
BK	Bradykinin
BSA	Bovine serum albumin
CaCC	Calcium-activated chloride channel
CD	Crohn's disease
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CRD	Colorectal distension
Ct	Cycle threshold
CTAB	Cetyl trimethylammonium bromide
DAI	Disease activity index
DH	Dorsal horn
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglion
DSS	Dextran sulphate sodium
EC_{50}	Half-maximal effective concentration
ECL	Enterochromaffin-like cell
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycolbistetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENaC	Epithelial sodium channel
ENS	Enteric nervous system
FB	Fast Blue

GALP	Galanin-like peptide
GalR1	Galanin receptor 1
GalR2	Galanin receptor 2
GalR3	Galanin receptor 3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GI	Gastrointestinal
GIRK	G protein-coupled inwardly-rectifying potassium channels
GMAP	Galanin-message-associated peptide
GPCR	G-protein coupled receptors
H&E	Haematoxylin and eosin
HABP	Hyaluronic Acid Binding Protein
HRP	Horseradish peroxidase
IB4	Isolectin B4
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IBS-C	Irritable bowel syndrome with constipation
IBS-D	Irritable bowel syndrome with diarrhoea
IC_{50}	Half-maximal inhibitory concentration
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-10	Interleukin 10
IMG	Inferior mesenteric ganglia
I.P.	Intraperitoneal
IS	Inflammatory soup
KO	Knock-out
LDCV	Large dense core vesicles
LS	Lumbosacral
LSN	Lumbar splanchnic nerve
MPO	Myeloperoxidase

mRNA	Messenger ribonucleic acid
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG	Nodose ganglia
NGF	Nerve growth factor
NPY	Neuropeptide Y
NSAIDs	Non-steroidal anti-inflammatory drugs
P2x7	Purinoceptor 7
PAR_2	Proteinase-activated receptor 2
PBS	Phosphate-buffered saline
PG	Pelvic ganglion
PGE_2	Prostaglandin E2
PGMP	Progalanin-message peptide
Piezo2	Piezo-type mechanosensitive ion channel component 2
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PN	Pelvic nerve
PNS	Peripheral nervous system
qPCR	Quantitative polymerase chain reaction
SD	Standard deviation
SEM	Standard error mean
siRNA	Small interfering Ribonucleic Acid
SMG	Superior mesenteric ganglion
SNRI	Serotonin-noradrenaline reuptake Inhibitor
SSRI	Selective serotonin reuptake inhibitors
TCA	Tricyclic antidepressants
TG	Trigeminal ganglion
TL	Thoracolumbar
TMB	3,3',5,5'-Tetramethylbenzidine
TNBS	2,4,6-trinitrobenzene sulfonic acid

TNFα	Tumour necrosis factor alpha
TRPA1	Transient receptor potential ankyrin 1
TRPV1	Transient receptor potential vanilloid 1
TTX-R	Tetrodotoxin resistant
UC	Ulcerative colitis
uORF	Upstream open reading frame
VMR	Visceromotor response

IV. Summary

The gastrointestinal (GI) tract is innervated by both the enteric nervous system and the sensory nervous system, and it is the latter that is responsible for giving rise to conscious sensations arising from the GI tract, such as pain. In particular, the distal colon is a key source of visceral pain in both inflammatory bowel disease (e.g. ulcerative colitis and Crohn's disease) and irritable bowel syndrome. In both conditions, pain relief is complicated by the GI side effects of commonly prescribed analgesics, such as non-steroidal anti-inflammatory drugs and opioids. Therefore, furthering knowledge about how sensory innervation of the GI tract is modulated in health and disease has the potential to identify new therapeutic avenues. Galanin is a neuropeptide that has various functions within the central and peripheral nervous systems, e.g. regulation of feeding and modulation of nociceptive pathways respectively. Galanin has been previously demonstrated to modulate the mechanosensitivity of vagal sensory afferents innervating the upper GI tract, but nothing is known about its role in the distal colon, i.e. the lower GI tract. Using mice, I therefore aimed to determine if galanin also modulates the lumbar splanchnic nerve (LSN), which innervates the distal colon, in both healthy and inflamed (hypersensitive) conditions. Using *ex vivo* LSN electrophysiological recordings I found that galanin dose-dependently inhibits LSN responses to mechanical stimuli and the mechanical hypersensitivity induced by acutely applied inflammatory mediators. Using galanin receptor agonists (GalR1: M671, GalR2: spexin), I identified that GalR1 mediates the inhibitory effects on LSN mechanosensitivity. Using a mouse model of colitis, I found that the LSN was hypersensitive to mechanical stimuli, but that galanin no longer produced any inhibitory effect. Immunohistochemistry experiments using thoracolumbar (T13-L1) and lumbosacral (L6-S1) dorsal root ganglia (DRG) retrograde labelled from the colon demonstrated that galanin is primarily expressed by putative nociceptors, but that no major expression changes occur during colitis. In summary, I have demonstrated that galanin inhibits LSN mechanosensitivity and inflammatory mediators induced mechanohypersensitivity, but that this inhibition is lost in an *in vivo* model. This work highlights the potential for targeting the galaninergic system to treat GI pain.

V. Table of Contents

1. Introduction **12**
1.1. Visceral nociception 13
1.2. Anatomy of GI tract innervation and spinal thoracolumbar pathways 14
1.3. The impact of chronic visceral nociception in chronic bowel disease 18
1.4. Modulation of colonic afferent excitability 23
1.5. Neuropeptides 28
1.6. Galanin 28
1.7. Galanin peptide family 29
1.8. Galanin receptor signalling 30
1.9. Role of galanin in peripheral inflammatory pain 35
1.10. Role of galanin in GI tract sensory afferent neurones 35
1.11. Principle aims 40

2. Materials and methods **41**
2.1. Whole-nerve electrophysiological recordings from mouse LSN 41
2.1.1. Ex vivo mouse LSN afferent preparations 41
2.1.2. Electrophysiological protocols for characterization of LSN afferent properties 44
2.1.3. Analysis of electrophysiological recordings 47
2.2. Immunohistochemistry on retrograde labelled dorsal root ganglion neurones 48
2.2.1. Retrograde labelling of colonic sensory neurones 48
2.2.2. Immunohistochemistry 48
2.2.3. Imaging and quantification 51
2.3. Chemically induced colitis models in mice 51
2.3.1. Trinitrobenzenesulfonic acid (TNBS) model of colitis 51
2.3.2. Dextran sulfate sodium salts (DSS) model of colitis 52
2.3.3. Disease activity index (DAI) for assessing colitis 52
2.3.4. Histology: H&E with alcian blue staining 53
2.3.5. Myeloperoxidase assay (MPO) assay 54
2.3.6. Biotinylated hyaluronan binding protein (HABP) staining 55
2.4. Quantifying levels of galanin in distal colon tissue 55
2.4.1. Tissue extract sample preparation 55
2.4.2. Galanin ELISA 56
2.5. Single Cell qPCR of colon-innervating DRG neurones 56
2.5.1. Primary culture and cell picking 56
2.5.2. Single-cell qPCR 57
2.6. Statistics 58

3. Galanin inhibits the lumbar splanchnic nerve afferent response to mechanical stimuli and inflammatory mediators **59**
3.1. Introduction 60

3.1.1. Sensation evoked by mechanical activation of colonic afferents 60
3.1.2. Experimental stimulation 61
3.1.3. Colonic mechanosensitivity and mechanotransduction 62
3.1.4. Galanin and inflammatory mediators 64
3.2. Aims 65
3.3. Results 66
3.3.1. Galanin is present in the distal colon and likely involved in nociceptive signalling 67
3.3.2. Galanin inhibits mechanically evoked neuronal excitation in LSN sensory afferent neurones 68
3.3.3. Galanin inhibits mechanically evoked neuronal excitation via GalR1 72
3.3.4. Galanin Inhibits chemically evoked neuronal excitation in LSN afferent neurones 76
3.4. Discussion 81

4. Validation of an inducible mouse model of colitis 84
4.1. Introduction 85
4.1.1. Murine models of inflammatory bowel disease 85
4.1.2. Chemically-induced models of colitis – TNBS 87
4.1.3. Chemically-induced models of colitis – DSS 88
4.2. Aims 89
4.3. Results 90
4.3.1. TNBS-induced colitis fails to produce robust and reproducible intestinal inflammation. 90
4.3.2. DSS-induced colitis produced robust intestinal inflammation 96
4.3.3. Myeloperoxidase (MPO) levels are not altered in mouse colons by DSS-treatment. 102
4.4. Discussion 104

5. Galanin does not inhibit DSS-induced mechanical hypersensitivity of the lumbar splanchnic nerve 106
5.1. Introduction 107
5.1.1. Galanin in the peripheral nervous system 107
5.1.2. Neurochemical characterisation of visceral sensory neurones 108
5.1.3. Changes in galanin expression in peripheral inflammation 109
5.2. Aims 110
5.3. Results 111
5.3.1. Validation of colonic Fast Blue labelling of DRG neurones 111
5.3.2. Expression of galanin is unchanged by acute DSS-induced colitis 115
5.3.3. Galanin is predominantly expressed in putative nociceptors 117
5.3.4. DSS induces mechanical hypersensitivity in the LSN 131
5.3.5. Galanin does not inhibit DSS-induced mechanical hypersensitivity 132

5.4. Discussion 134

6. Conclusions and future directions 136
6.1. Summary 137
6.2. Overview of results 137
6.3. Galanin signalling in colonic sensory neurones 138
6.4. Role of galanin in inflammatory pain pathways 142
6.5. Future work 143
6.6. Conclusion 145

7. Appendices 146
7.1. Antibody optimisation 148
7.2. Single cell qPCR 151

8. Bibliography 155

VI. List of Figures and Tables

1-1. Neuroanatomy of the colon 16
1-2. Mechanisms modulating excitability of visceral afferent endings 24
1-3. Organisation of the galanin and GALP genes 31
1-4. GalR transduction mechanisms 34
1-5. Galanin receptor expression in colonic DRG neurones 37
2-1. Schematic of electrophysiological recording technique 43
2-2. Protocols used for LSN recordings 45
2-3. Parameters measured from a response to phasic distention. 47
3-1. Expression of galanin in the spinal cord and distal colon 67
3-2. Repeated phasic distensions produce a robust LSN response that is inhibited by galanin 69
3-3. Dose-dependent effect of galanin on LSN mechanosensitivity 68
3-4. Effects of GalR agonists on LSN mechanosensitivity in multi-unit recordings 73
3-5. Effects of the GalR2 antagonist M871 on mechanosensitivity in multi-unit LSN recording 75
3-6. Effects of galanin and IS on LSN response to ramp distension 77
3-7. Effects of galanin and IS on LSN response to phasic distension 79
3-8. GalR1 coexpression against GIRK1 82
4-1. First pilot study of TNBS-induced colitis 91
4-2. Second pilot study of TNBS-induced colitis 93
4-3. Third pilot study of TNBS-induced colitis 95
4-4. Initial pilot study of 3% DSS induced colitis 97
4-5. DSS induces weight loss and macroscopic changes to the colon 99
4-6. DSS induces changes in colon histology 101

4-7. MPO activity analysis 103
5-1. Validation of colonic FB labelling of DRG neurones. 112
5-2. Comparison of FB-positive colonic sensory neurones isolated from healthy and DSS-treated mice. 114
5-3. Galanin expression in colonic sensory neurones and whole colon. 116
5-4. Coexpression of galanin and Gfrα3 in colon-innervating DRG sensory neurones. 119
5-5. Coexpression of galanin with TRPV1 in colon-innervating DRG sensory neurones 122
5-6. Coexpression of galanin with CGRP colon-innervating DRG sensory neurones. 125
5-7. Coexpression of galanin with TrkA colon-innervating DRG sensory neurones 128
5-8. Co-expression of galanin with TrkC colon-innervating extrinsic neurones 130
5-9. DSS-treated mice display LSN hypersensitivity 131
5-10. Galanin does not inhibit DSS-induced LSN hypersensitivity 133
6-1. Schematic of the potential signalling pathway activated by galanin in colonic afferents 141
7-1. Goat anti-CGRP primary antibody optimisation 147
7-2. Goat anti-Gfrα3 primary antibody optimisation 148
7-3. Goat anti-TrkC primary antibody optimisation 148
7-4. Guinea-pig anti-TRPV1 primary antibody optimisation 149
7-5. Goat anti-TrkA primary antibody optimisation 149
7-6. Rabbit anti-Galanin primary antibody optimisation 150
7-7. A schematic of workflow depicting single cell qPCR 152

Table 1. List of primary antibodies used 50
Table 2. List of secondary antibodies used 50
Table 3. Disease activity index (DAI) scoring system 53
Table 4. Histopathological assessment of colon sections 54
Table 5. IPC values obtained for GAPDH 153
Table 6. Sample of GAPDH Ct values from one healthy mouse 154

Chapter 1 – Introduction

1.1 Visceral nociception

One of the essential functions of the nervous system is to provide information about the threat or occurrence of injury and this function is mediated by nociception, the neural process of encoding noxious stimuli. Nociception normally only occurs at temperatures, pressures and other stimuli that can potentially damage tissues and the process is mediated by nociceptors, a specialized class of primary afferents that respond to these intense, noxious stimuli (Dubin and Patapoutian, 2010). Like other primary somatosensory afferents, nociceptors are pseudounipolar, the cell body is located in the dorsal root ganglion (DRG, body) or trigeminal ganglion (TG, head) and each cell sends a peripheral axon to innervate a peripheral structure and a central axon to synapse with second-order neurones in the dorsal horn (DH) of the spinal cord (Dubin and Patapoutian, 2010). Noxious stimuli are transduced by nociceptors and signals are transmitted through spinal pathways to areas of the brain where signals are integrated and perceived. Arrival of nociceptive signals in the spinal cord can also trigger spinal reflexes in which signals from the spinal cord cause activation of appropriate flexor muscles and inactivation of extensor muscles to withdraw the limb from noxious stimulus (Clarke and Harris, 2004).

Sensory modalities for somatic sensation include temperature, touch, pain, prickle, wetness, and itch sensations that are mediated via receptors specific to different stimuli, although some receptors are themselves polymodal. By contrast, visceral conscious sensations are far more limited than somatic sensations as demonstrated by Bentley (Bentley and Smithwick, 1940) who used balloon distention of the jejunum in a conscious man to show that distention of the gastrointestinal (GI) tract causes pain, but that other mechanical stimuli (cutting, pinching, and local stretching) failed to produce a painful sensation. Visceral pain is poorly graduated and localised, often with the conscious sensation manifesting as referred pain (pain perceived at a different location to the site of noxious stimuli). In the colon, this is thought to be due to the relatively low density of afferent innervation, lack of anatomical specification (such as encapsulation), and widespread distribution of afferent pathways (Knowles and Aziz, 2009; Sikandar and Dickenson, 2012). During tissue damage, mediators are released both from damaged cells and from immune cells involved in the inflammatory response. Nociceptors can be sensitized by these inflammatory mediators due to both

post-translational modification of ion channels and alteration in receptor expression, processes that result in lower threshold for activation and/or greater responsiveness to stimuli, overall causing an increase in neuronal excitability (Bhave and Gereau, 2004; Okuse, 2007). A specific example of colonic sensory neurone sensitisation was shown by Jones, in which, the application of an inflammatory soup (bradykinin, adenosine triphosphate (ATP), histamine, prostaglandin E2, and noradrenaline) to receptive fields of the colon caused nociceptor hyperexcitability (Jones, 2005).

As well as changes in visceral sensation associated with inflammation becoming better understood, the complex relationship between the gut microbiota and visceral pain is an emerging topic. Changes in the microbiota have been linked to immunological, neurological and inflammatory changes that are the hallmark signs in chronic pain conditions (see Section 1.3, Conte et al., 2006; Frank et al., 2007, Shankar et al., 2015; Simrén, 2014). Reported changes in the microbiota include reduced levels of *Bifidobacterum* and *Lactobacillus*, as well as an increased Firmicutes to Bacteriodetes ratio (Jeffery et al., 2012). However, whether these changes are causative of disease pathology, or a response to an alternative initiating factor is inconclusive. Interestingly, preclinical studies have shown that mice raised in sterile conditions with no GI bacteria have a blunted response to inflammatory pain caused by carrageenan injection to the hind-paw (Amaral et al., 2008). A finding which thus implicates commensal bacteria as having an important role in the development of inflammatory hyperalgesia. Moreover, antibiotic-induced reduction of gut microbiota in mice also results in animals displaying a reduced visceral pain response to intracolonic application of capsaicin (Aguilera et al., 2015). However, in rats, early exposure to antibiotics increases susceptibility to colonic hypersensitivity in adulthood (O'Mahony et al., 2014) and thus overall, the role of gut microbiota in visceral pain is certainly complex and not yet fully understood.

1.2 Anatomy of GI tract innervation and spinal thoracolumbar pathways

Afferent neurones innervating the gut relay signals to a wide range of laminae (I, II, V, and X) of the spinal cord dorsal horn (Grundy et al., 2006). Here, second order neurones transmit the message to the brain via spinoreticular, spinohypothalamic,

spinomesencephalic and spinothalamic pathways to the thalamic and parabrachial structures in the brain (Fukudo, 2013; W Jänig and McLachlan, 1987; Knowles and Aziz, 2009). All pathways are in the anterolateral quadrant of the spinal cord with the spinoreticular, spinohypothalamic, and spinomesencephalic pathways mainly involved in autonomic reflexes and the spinothalamic pathway involved in conscious sensation. Hollow organs can sense varying intensity of stimuli, from smooth muscle contraction to increasing intraluminal pressure (De Winter et al., 2016). Noxious activation of afferent pathways innervating the viscera can result in reflex behaviours, such as nausea and variation in heart rate, as well as contributing to conscious sensations like pain. Sensory afferents innervating the colon convey a wide range of conscious sensations including bloating, urgency, fullness and pain (De Winter et al., 2016; Knowles and Aziz, 2009).

The GI tract is innervated by both intrinsic and extrinsic pathways. The intrinsic pathway is located within the enteric nervous system (ENS) and mediates reflexes controlling secretion, blood flow and motility, independently of the central nervous system (CNS; Blackshaw et al., 2007). Transmission of sensory information from visceral organs to the CNS is mediated by extrinsic innervation of the GI tract by spinal thoracolumbar (TL; corresponds to lumbar splanchnic nerve, LSN), spinal lumbosacral (LS; corresponds to pelvic nerve) and vagal pathways. With respect to innervation of those areas of interest in inflammatory bowel disease (IBD), the vagal pathway innervates a wide variety of visceral organs, but makes only minimal contribution to sensory transduction from the distal colon (Berthoud et al., 1990; Berthoud and Neuhuber, 2000; Spencer et al., 2016). By contrast, the spinal TL pathway innervates the oesophagus, stomach, small intestine and colon, while the spinal LS pathways innervate the mid colon through to the rectum. Unlike vagal innervation of the GI tract, both TL and LS spinal pathways innervate regions associated with pain in IBD. The TL pathway includes afferents whose cell bodies are located in the lower thoracic and upper lumbar DRG (T10-L1), whereas afferents of the LS pathway have cell bodies in the lower lumbar and upper sacral DRG (L6-S1) (Christianson et al., 2006a; Figure. 1-1). Overall, in mice, the proportion of TL and LS DRG neurones that project to the colon is 2.9% and 2.3% respectively with DRG T13 and L1 containing the highest number of colon-innervating neurones (Brierley et al., 2008; Robinson et al., 2004).

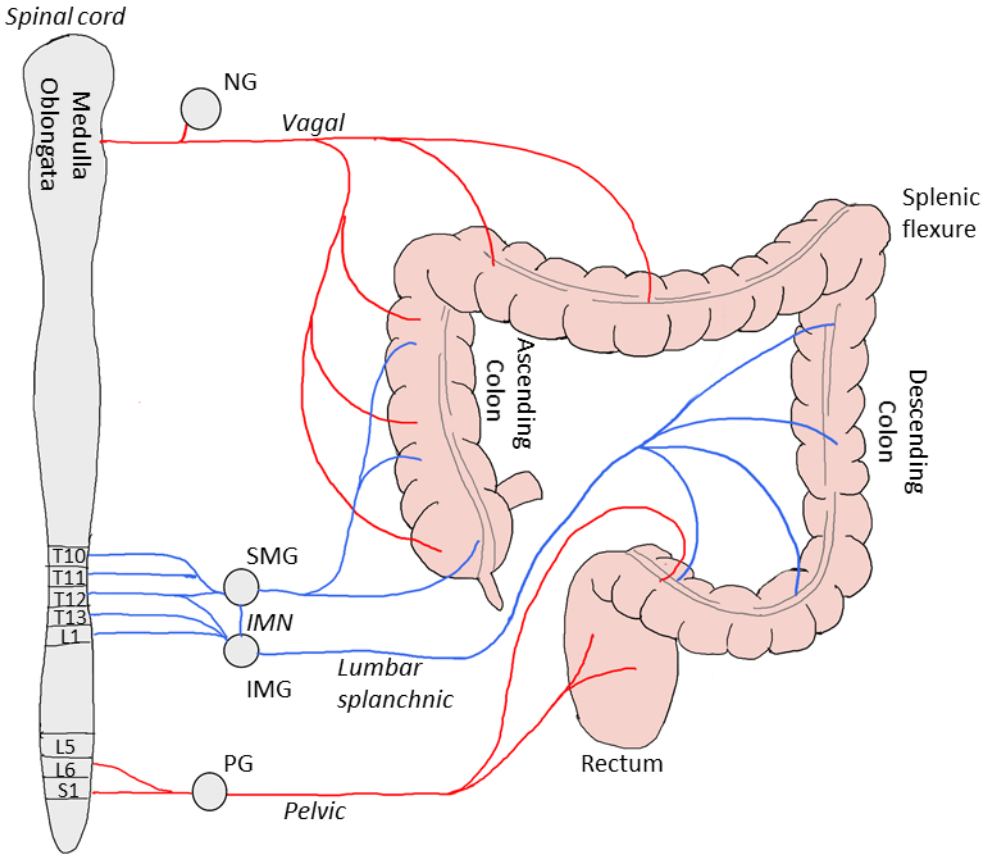


Figure 1-1. Neuroanatomy of the colon

The three main sympathetic (blue) and parasympathetic (red) pathways innervating the colon are shown; vagal, splanchnic and pelvic nerves. The vagal afferents originate from cell bodies of the nodose ganglia (NG) and project to the medulla oblongata. The vagal nerve innervates the proximal colon. Thoracolumbar afferents originate from DRG neurones (T10-L1) and project to the dorsal horn of the spinal cord. Colonic thoracolumbar afferents innervate the more proximal region of the colon via the least splanchnic nerve and the descending colon via the lumbar splanchnic nerve (LSN). Colonic thoracolumbar afferents pass through the superior mesenteric ganglia (SMG), and inferior mesenteric ganglia (IMG). Lumbosacral afferents originate in DRG neurones (L6 and S1) and, like thoracolumbar afferents, project to the dorsal horn of the spinal cord. Lumbosacral afferents innervate the distal colon and the rectum and pass through the pelvic ganglia (PG).

In the GI tract, afferent fibres can be characterised based on the location of their receptive fields and their sensitivity to different mechanical stimuli (these include von Frey hair probe, distention, circular stretch, and mucosal stroking). Through such mechanical characterisation, five categories of colonic sensory neurones have been identified: muscular-mucosal, muscular, mucosal, mesenteric, and serosal (Feng and Gebhart, 2011) and it is the mesenteric and serosal groups that are thought to be primarily involved in colonic nociception (Brierley et al., 2004). It is also worth noting that there is a sub-population of sensory afferents called silent afferents that under physiological conditions are insensitive to mechanical stimuli, but can become sensitized and respond to mechanical stimuli during inflammation, and thus contribute to the hypersensitivity of visceral pain (Brookes et al., 2013; Feng and Gebhart, 2011). The proportion of the different afferent types differs between TL and LS pathways, such that the LSN contains mucosal (4%), muscular (10%), serosal (36%) and mesenteric (50%) afferent classes, whereas, the pelvic nerve contains muscular/mucosal (23%), serosal (33%), mucosal (23%), and muscular (21%) afferent classes (Brierley et al., 2004). The serosal and mesenteric afferent subtypes are of particular importance in terms of transducing pain and are mainly present in the LSN (Brierley et al., 2004; Hughes et al., 2009), and thus this thesis focuses on LSN function, which I will now discuss in more detail.

The mesenteric afferent neurones are associated with blood vessels innervating the GI tract, such that in the colon about 83% are associated with mesenteric arteries, 13% with mesenteric veins and 3% are located in the mesentery but away from blood vessels (Song et al., 2009) and it is the neurones closely associated with blood vessels that respond to higher intensities of circular stretch and distention compared to muscular afferents (Hughes et al., 2009; Jänig, 1996). Moreover, mesentery afferents can be subcategorised into serosal and mesenteric afferents mainly based on their location, such that mesenteric afferents are located mainly on mesenteric blood vessels and serosal afferents are associated with intramural blood vessels; although named serosal afferents, this is somewhat misleading as there are no afferent endings located in the serosal layer of the colon (Brookes et al., 2013).

The serosal and mesenteric afferents make up the majority of the LSN innervating the colon and their expression of a range of receptors enables responses to a wide range of chemical stimuli, including: prostaglandins, 5-HT, bradykinin, and glutamate (Blackshaw and Gebhart, 2002). These afferents also express receptors for certain inflammatory cytokines, such as interleukin 6 (IL-6) and interleukin 1 beta (IL-1β), and therefore can respond to cytokine signalling in inflammation (Andratsch et al., 2009; Binshtok et al., 2008). Inflammatory mediators, such as nerve growth factor (NGF), can induce mechanosensitivity (via disinhibition of the mechanically-gated ion channel, Piezo2; Prato et al., 2017) in a small subpopulation of afferents that under control conditions are insensitive to mechanical stimuli and this mechanism could be therapeutically important in treating IBD and/or irritable bowel syndrome (IBS) (Feng and Gebhart, 2011).

Thirdly, colonic muscular afferents make up a small proportion of mechanosensory afferents in the LSN and respond to low intensity stretch stimulation. However, due to their responsiveness to mechanical noxious stimuli, it has been suggested that colonic muscular afferents have a role in nociception as wide dynamic range fibres (Brierley et al., 2004).

Lastly, mucosal afferents account for the smallest proportion of afferents in the LSN. They are clustered in the lower region of the distal colon and show adapting responses to low-threshold mucosal stroking and probing but are insensitive to circular stretch stimuli. This suggests a role in either providing fine mucosal input to reflexes controlling motility and/or refining the quality of perceived stimuli (Brierley et al., 2004).

1.3 The impact of chronic visceral nociception in chronic bowel disease

Acute somatic pain is an important component of the body's defence system by eliciting reflex and avoidance behaviours to protect the body from a hostile external environment (Woolf, 1995). However, visceral pain does not provide the same form of detect and protect mechanism. In some cases, visceral pain can be an indication of a

serious condition (such as appendicitis), but oddly, visceral pain cannot be evoked in every visceral organ (stimulation of solid organs, such as the liver and kidneys does not evoke visceral pain) and is not always linked to overt visceral injury (Cervero and Laird, 2004). For example, individuals with functional GI disorders, such as IBS, exhibit abdominal pain, discomfort, and altered bowel habits, but the underlying pathophysiological mechanisms are still poorly understood (Al Omran and Aziz, 2014). However, there are a group of chronic inflammatory disorders in which dysregulation of this protective function can lead to morbidity and the presence of chronic visceral pain reduces the patient's quality of life. A study in 2014 showed that 87% of IBD patients experienced abdominal pain at least once a day during a flare up and 62% reported abdominal pain at least once a week between flare ups (i.e. when in remission). The study also found that 46% of respondents reported abdominal pain leading to absence from work and that 40% reported being woken from sleep as a result of abdominal pain (Lönnfors et al., 2014). In another study, 20% of IBD patients reported that pain interfered with their daily functions (IsHak et al., 2017).

The clinical presentation of IBD includes diarrhoea, abdominal pain, bleeding, and fatigue with abdominal pain rating highly with regard to the impact of IBD on a patient's quality of life (Mowat et al., 2011; Singh et al., 2011; Arnott et al., 2012). However, IBD is not just a single disease, but rather a term used to encompass Crohn's disease (CD) and ulcerative colitis (UC), both of which are chronic, relapsing, inflammatory colon disorders. Overall, IBD is estimated to affect around 1.5 million Americans (with an incidence of 19.2 per 100,000 for UC and 20.2 per 100,000 for CD), 2.2 million Europeans (with an incidence of 24.3 per 100,000 for UC and 12.7 per 100,000 for CD), and several hundred thousand more worldwide (Ananthakrishnan, 2015; Molodecky et al., 2012). The peak incidence occurs between the ages of 20 and 40 years of age, but it has also been suggested that IBD has a bimodal incidence with a second peak occurring between the ages of 60 and 70 years of age (Loftus, 2004; Molodecky et al., 2012). The incidence is similar between men and women but is influenced by ethnicity. In countries considered to have high incidence, the risk is 3-fold higher for the Jewish population, especially Ashkenazi Jews, whereas a low prevalence is seen in African American and Hispanic populations (Mahid et al., 2008; Reddy and Burakoff, 2003). The cause of IBD is idiopathic and generally results from

multiple genetic mutations causing an acquired immunological response to certain commensal enteric bacteria (Kostic et al., 2014; Morgan et al., 2012). The onset of the disorder is thought to be triggered by an environmental event, such as infection, or the prolonged use of nonsteroidal anti-inflammatory drugs (NSAIDs; Berg et al., 2002). An initial infection can cause acute inflammation, which in a genetically susceptible host leads to reduced immunoregulation and, therefore, chronic inflammation (Sator, 2006). During “acute flares”, nociceptive sensory pathways can become sensitized by inflammation leading to visceral hypersensitivity (the increased responsiveness of the gut to stimulation) and this can persist in periods of remission from inflammatory flare ups for a proportion of patients (Bielefeldt et al., 2009).

Another common chronic disorder in which visceral pain has a negative impact on the patient’s quality of life is IBS, which has a prevalence of 22% in the UK (Gwee, 2005) and is thought to affect 10-15% of the population in developed countries (Card et al., 2014). A prevailing feature of IBS is transient noxious events leading to long-lasting sensitisation of the neuronal pain circuit, similar to the visceral hypersensitivity previously described for IBD (Barbara et al., 2011). The key difference between IBS and IBD is the aetiology of the symptoms. For IBD, the symptoms result from overt mucosal inflammation, whereas, the diagnosis of IBS is symptomatic with no obvious inflammation being observed and it is considered a functional disorder, i.e. no underlying disease causes the symptoms (Naliboff et al., 2012).

For IBD, early phase pharmacological treatment with antibiotics, corticosteroids, anti-TNFα therapy or immunomodulators has been shown to reduce inflammation and, as a result, visceral pain. However, there is still a large subset of patients that experience discomfort and pain even in the presence of reduced inflammation suggesting that visceral pain is discontinuous with the disease activity (Bernstein, 2014). Current treatments to manage abdominal pain in IBS and IBD are limited either by their effectiveness or by their adverse effects and the range of treatments available for the management of visceral pain is diverse, which likely reflects the complexity of visceral pain mechanisms in IBS and IBD (Szigethy, 2018).

One set of drugs that can be used to control visceral pain are NSAIDs that act by reducing prostaglandin (mediators known to promote inflammation) production by inhibiting the enzyme that synthesizes them, cyclooxygenases (COX). Although NSAIDs effectively reduce abdominal pain in the short term, there is evidence linking their use with an increased relapse frequency and therefore exacerbating IBD (Kefalakes et al., 2009). Long-term use of non-selective NSAIDs has been shown to produce GI toxicity in the form of ulcerations, perforation and diverticulitis in the distal colon (Lanas et al., 2006). NSAIDs that act through selective inhibition of COX-2, such as celecoxib and etoricoxib, are often used due to their improved GI profile. However, there has been controversy over the safety of COX-2 inhibitors, with Rofecoxib having been withdrawn due to cardiovascular side effects (Miao et al., 2009; Biancone et al., 2003). Recently, it has been shown that current COX-2 inhibitors are no worse than other NSAIDs with regard to cardiovascular toxicity (such as ibuprofen or naproxen; Nissen et al., 2017), thus, so long as the patient has no underlying cardiovascular risks they are generally used for chronic conditions. The anti-spasmodic drug hyoscyamine is a non-selective acetylcholine muscarinic receptor inhibitor, which acts to cause the intestinal smooth muscle to relax and thus reduce spasms associated with colonic pain. However, such anti-spasmodic drugs also reduce GI motility, which can lead to constipation and bowel occlusion, and thus themselves induce pain. Anti-spasmodics have been shown to be effective in IBS patients (Ford et al., 2008) but do not show as much effectiveness in IBD patients, although remissive patients or those with mild chronic pain may still find antispasmodics effective. Opioids, such as tramadol and morphine, are also used to treat severe cases of chronic pain in IBD patients act by antagonising opioid receptors. Although opioids reduce activity of the pain pathway at various points, their use is also associated with a range of adverse effects including: nausea, vomiting, reduced gut motility leading to constipation (of particular concern when treating IBD/IBS patients), and being highly addictive leading to substance abuse (Dasgupta et al., 2018; Mowat et al., 2011).

Another class of drugs that have been extensively used for treatment of IBS related abdominal pain are antidepressants (Drossman et al., 2009). Besides their positive effects on an individual's mood (depression is a common comorbidity of chronic pain), antidepressants are also useful for patients with functional abdominal pain without

depression due to their central pain-modulatory action (Morgan et al., 2005). Targeting serotonin (selective serotonin reuptake inhibitors; SSRIs) and noradrenaline (serotonin-noradrenaline reuptake inhibitors; SNRIs) has been shown to have potential in treating IBD chronic pain (Grover and Drossman, 2011; Mikocka-Walus et al., 2007). Although there is relatively little data supporting SSRI treatment for abdominal pain, fluoxetine has been shown to reduce abdominal pain in non-depressed IBS patients with colonic hypersensitivity (Kuiken et al., 2003). Considering the central roles of serotonin and noradrenaline in the descending modulation of pain, SNRIs seem like good candidates for treating chronic visceral pain. Indeed, SNRIs have been shown to increase the sensory threshold of mice in response to colorectal balloon distension (Chial et al., 2003). However, like all drugs, SNRIs do cause some side effects including: nausea, palpitations, sweating and disrupting sleep (Brennan et al., 2009). Like SNRIs, tricyclic antidepressants (TCAs) also inhibit both serotonin and noradrenaline reuptake, but they also have other functions, including inhibition of voltage-gated ion channels, opioid receptor activation and possible neuroimmune anti-inflammatory effects (Dharmshaktu et al., 2012). In addition, TCAs also inhibit muscarinic acetylcholine receptors and histamine receptors leading to antimuscarinic and antihistaminic side effects (e.g. drowsiness, xerostomia, and palpitations). A large study by Drossman et al., involving 216 women with IBS, showed numerical trends, but not statistically significant decrease in ISB-associated pain, from a 12-week treatment with the TCA desipramine, a major drawback being the 25% dropout rate due to side effects, although those patients that stayed to the end of the study did experience a significant benefit of desipramine compared to placebo (Drossman et al., 2003).

Serotonin is a key neurotransmitter in the GI tract that stimulates the release of other neurotransmitters to influence peristalsis and water secretion, as well as acting directly on visceral sensory nerves to cause pain (Crowell, 2004). Therefore, directly targeting mechanisms through which serotonin acts on the GI tract is an attractive therapeutic target, as demonstrated by alosetron, a serotonin type 3 (5-HTR3) receptor antagonist, being shown to relieve symptoms of diarrhoea predominant IBS (IBS-D) patients (Quartero et al., 2005). However, alosetron has also been associated with side effects of severe constipation and ischemic colitis. Tegaserod is a serotonin type 4 (5-HTR4)

receptor antagonist that has been shown to reduce abdominal pain, bloating and constipation in constipation predominant IBS (IBS-C) patients (Layer et al., 2007). It is well tolerated but can have the adverse effect of diarrhoea. The success of alosetron and tegaserod demonstrates that modulation of colonic sensory afferent signalling provides good potential for the development of future novel treatments for visceral pain associated with IBD and IBS.

1.4 Modulation of colonic afferent excitability

The frequency and intensity of action potentials transmitted from peripheral nerve endings to the spinal cord is the mechanism by which noxious stimuli are perceived. Inhibition of mechanosensitivity in colonic afferents has been demonstrated as an effective therapeutic approach in the treatment of visceral pain for some patients. For example, rectal application of lidocaine jelly (coating the first 4 cm of the rectum) treats pain associated with IBS in a subset of patients (Verne et al., 2005, 2003). Therefore, pharmacological modulation of visceral afferent excitability offers an attractive therapeutic avenue, but through using more selective compounds than lidocaine, which simply switches off all nerve function and is thus not a practical long-term solution.

Under physiological conditions neurones have a resting membrane potential of approximately -60 mV, which is maintained by the regulated movement of ions across the plasma membrane. A variety of ion channels and receptors have the potential to change the membrane potential leading to the generation of action potentials through depolarisation of the membrane. Visceral sensory neurones are heterogenous with regard to their expression profile, thus endowing them with differing sensitivity to various stimuli. Neuronal sensitivity to different stimuli is also prone to modulation, especially during inflammation when a plethora of mediators are released (Brierley et al., 2005a; Brierley et al., 2005b; Feng et al., 2012; Hughes et al., 2009). Experimental animal models of colitis are also associated with colonic afferent sensitisation and hypersensitivity to colonic distension (Feng et al., 2012; Hughes et al., 2009), and thus provide a good experimental model of investigating the molecular mechanisms that

drive changes in colonic afferent excitability in chronic visceral pain conditions, such as IBD.

To understand how any novel treatment might modulate visceral afferent excitability, the different noxious stimuli that can excite and sensitise afferent terminals in the GI tract all need to be considered as they all play a role in the pathogenesis of IBD-associated pain (Fig. 1-2). The noxious stimuli involved in IBD include direct noxious mechanical stimulation (such as that arising from occlusion or distension of the colon) and inflammatory mediators (such as ATP, histamine, bradykinin etc.).

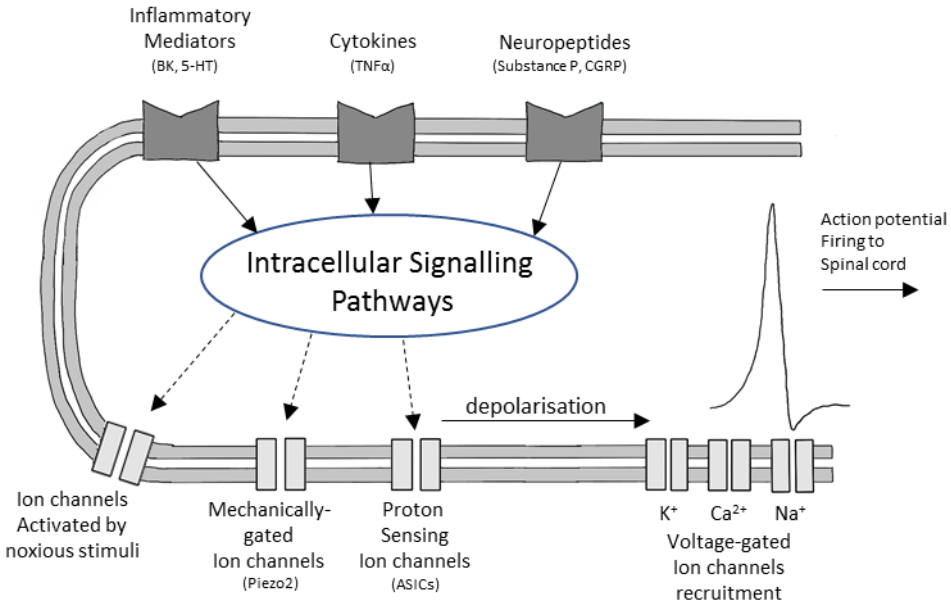


Figure 1-2. Mechanisms modulating excitability of visceral afferent endings

Following release of inflammatory mediators and tissue acidosis, several receptors and proton sensing ion channels are activated leading to intracellular changes which act to increase neuronal excitability by depolarizing the membrane (predominantly through cation influx), recruiting voltage-gated ion channels (such as Na_Vs) and leading to action potential generation. GPCRs and cytokine receptors for inflammatory mediators and neuropeptides activate intracellular signalling pathways leading to posttranslational changes and ultimately sensitisation of other ion channels (indicated by dotted arrows). BK – bradykinin, 5-HT – 5-hydroxytryptamine, TNFα – tumour necrosis factor alpha, CGRP – calcitonin gene-related peptide, ASIC – acid-sensing ion channel. Modified from Schaible et al. (2011).

Transduction of mechanical stimuli is vital for conveying GI sensations and is the primary cause of colonic pain. Multiple mechanisms have been implicated in the transduction of mechanical stimuli including direct activation of mechanically-gated ion channels, such as Piezo2 (Brierley, 2010; Prato et al., 2017). In addition to Piezo2, other ion channels have been implicated in visceral mechanosensation. For example, acid-sensing ion channels (ASICs) are part of the epithelial Na^+ channel (ENaC) family of cation channels and are activated by protons, but also appear to modulate responses to mechanical stimuli (Omerbašić et al., 2015), although ASICs are not themselves mechanotransducers (Drew et al., 2004). ASIC1a, ASIC2, and ASIC3 have all been associated with mechanosensitivity in the colon through the use of knockout (KO) mice, as will now be discussed. ASIC1a is expressed by 30% of TL colonic afferents (Hughes et al., 2007) and ASIC1a KO mice show increased mechanosensitivity in LSN afferents (Page, 2005a). ASIC2 is expressed in 50% of colonic thoracolumbar DRG neurones and in a similar manner to ASIC1a KO mice, ASIC2 KO mice display increased mechanosensitivity in serosal LSN afferents, although, mesenteric LSN afferents remained unaffected (Page, 2005a). Lastly, ASIC3 is expressed by approximately 75% of colonic thoracolumbar DRG neurones (Hughes et al., 2007) and by contrast to the phenotypes of ASIC1a and ASIC2 KO mice, ASIC3 KO mice showed reduced mechanosensation in mesenteric and serosal LSN afferents and muscular/mucosal pelvic afferents (Bielefeldt and Davis, 2008; Page, 2005a). Furthermore, using *in vivo* experiments, as opposed to the *ex vivo* electrophysiological recording mentioned above, ASIC3 KO mice also showed a significant reduction in the visceromotor response (VMR; electromyogram (EMG) response) to colorectal distension (CRD) (Jones, 2005). Sensitivity to protons means that during tissue acidosis, as occurs in in inflammation, ASICs contribute to altering basal excitability thereby sensitising afferents. Overall, the contribution of ASICs to specific aspects of visceral mechanotransduction and afferent sensitisation influences the encoding of noxious mechanical stimuli.

The Piezo family of cation channels consists of 2 members in mammals, Piezo1 and Piezo2, both of which are very large membrane proteins containing between 24-36 transmembrane segments. Piezos may be relevant to colonic mechanosensitivity as they have been shown to be rapidly-adapting mechanosensitivity transducers in DRG

neurones and are currently the only known mechanotransducer (Coste et al., 2010). Piezo2 KO mice have reduced touch and mechanical hypersensitivity during inflammation (Murthy et al., 2018; Ranade et al., 2014). Intriguingly, Piezo2 is expressed in just under 50% of all sensory neurones and its expression profile in colonic sensory neurones (Hockley et al., 2019) suggests that it plays a key role in colonic mechanosensitivity, although this remains to be tested.

Transient receptor potential (TRP) channels are predominantly non-selective cation channels that transduce a range of mechanical, thermal and chemical stimuli. TRP channels also undergo significant post-translational modification and thus can contribute to the hypersensitivity that follows inflammation. There are seven sub-families of the TRP superfamily (TRPC, TRPV, TRPA, TRPM, TRPN, TRPP and TRPML), some of which have been associated with visceral mechanosensitivity, e.g. TRPV1, TRPV4 and TRPA1 (Brierley et al., 2008, 2009; Hughes et al., 2007). TRPV1 is expressed by 80% of TL and 50-60% LS colonic DRG neurones (Christianson et al., 2006b; Robinson et al., 2004) and pelvic nerve sensory afferents isolated from TRPV1 KO mice show reduced mechanosensitivity (Brierley et al., 2008). However, TRPV1 is not directly mechanically gated and therefore it likely influences mechanosensation indirectly by affecting neuronal excitability. TRPV4 also plays a role in mechanosensation, such that deletion of TRPV4 or application of a TRPV4 antagonist reduces afferent mechanosensitivity in the colon (Brierley et al., 2008). Overall, the reduction in afferent mechanosensitivity observed in electrophysiology experiments on isolated colon-nerve preparations from TRPV4 KO mice correlates with observations *in vivo*, such that the VMR to CRD is also reduced in TRPV4 KO mice and in mice in which TRPV4 has been knocked down using siRNA (Brierley et al., 2008; Cenac et al., 2008). Lastly, TRPA1 also contributes to the mechanosensitivity of serosal, mesenteric and mucosal, but not stretch sensitive afferents in LSN and PN (Brierley et al., 2009). TRPA1 is expressed by approximately 50% of both TL and LS colonic afferents and both TRPA1 KO mice and rats treated with TRPA1 antisense oligonucleotides to knock down TRPA1 expression show a reduced VMR to CRD (Brierley et al., 2009; Yang et al., 2008). Moreover, in a model of bradykinin-induced colonic hypersensitivity, wildtype mice showed an increase in mechanosensitivity after bradykinin application, whereas TRPA1 KO mice showed no

change in mechanosensitivity after bradykinin application, suggesting that TRPA1 has perhaps a more significant role in sensitisation and mechanical hypersensitivity (Brierley et al., 2009)

The algogenic mediator, ATP, has also been suggested to be relevant to visceral nociceptor activation during colonic distension. This is because in hollow organs mechanical stress has been suggested to trigger the release of ATP from the epithelium, which in turn activates nociceptors (Burnstock, 2009). This could be a particularly prominent pathway during colonic inflammation as ATP release is enhanced during colitis. Moreover, ATP release has been proposed to regulate the inflammatory response through P2X7 receptor activation and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling in sensory neurones (Shinoda et al., 2009; Wan et al., 2016).

Visceral hypersensitivity to CRD has been seen in both IBS and IBD. Colons in individuals with IBD display overt inflammation, whereas those in individuals with IBS do not, however, in both cases mediators have been identified that are specific to the disease condition. In IBD, these are pro-inflammatory mediators, such as IL-10, TNF-α, and histamine (Gasche et al., 2000; Song et al., 2014). In IBS, mediators of hypersensitivity were first identified as being present by using supernatants made from colon biopsies of IBS patients that induced hypersensitivity when applied to mouse colon (Cenac et al., 2007). Recently, 5-oxoETE has been identified as a mediator in IBS-C that contributes to hypersensitivity without causing tissue inflammation (Bautzova et al., 2018). This shows that a mixture of mediators released from within the GI tract has the capability to sensitise colonic afferents and it has also been seen that the combination of inflammatory mediators released might differ between acute and chronically inflamed tissues (Feghali and Wright, 1997).

1.5 Neuropeptides

Neuropeptides are small neurotransmitter peptide molecules that are involved in a wide range of functions including analgesia (e.g. enkephalins) and nociception (e.g. substance P). The difference between neuropeptides and peptide hormones is the cell types they are released from and that respond to the molecule. Neuropeptides are released from neuronal cells and bind to receptors expressed by a selective population of neurones, whereas peptide hormones are released by neuroendocrine cells and travel via the blood to peripheral tissues (Fricker, 2012). Neuropeptides are co-released with small molecule neurotransmitters; however, unlike many small molecule neurotransmitters, neuropeptides are not recycled back into the cell, but are broken down by peptidases.

Further differences exist between small molecule neurotransmitters and neuropeptides. Firstly, small molecule neurotransmitters are usually synthesised in the nerve terminal from precursors, whereas neuropeptides are encoded by genes and synthesised as large precursor or preprocursor peptides in the cell soma and made bioactive by enzymatic cleavage either in the Golgi apparatus or vesicles. Preprocursors also general contain signal peptides which are short peptides found at the N-terminus that function to prompt translocation of the protein. Furthermore, whereas small molecule neurotransmitters containing vesicles are docked at the active zone and only require small changes in intracellular Ca^{2+} to neurotransmitter release, neuropeptides are stored in large dense core vesicles (LDCV), which are only released during high frequency firing that causes large, sustained increases in intracellular Ca^{2+}. One neuropeptide of particular interest with regard to GI function is galanin.

1.6 Galanin

Galanin is a neuropeptide that is expressed in many different parts of the body, including extrinsic nerves innervating the distal colon and other cell types in the colon, including epithelial cells and enterochromaffin cells (Lang et al., 2014). The broad expression of galanin in the colon suggests that it could potentially influence the excitability of colonic sensory afferents. Galanin is a 29/30-amino acid peptide (mouse/human) discovered by the Mutt lab, which isolated galanin from porcine

intestinal extracts (Tatemoto et al., 1983). The human *GAL* gene is located on chromosome 11q13.2, spans 6.6 Kb, contains 6 exons and produces a 124-amino acid long precursor protein (preprogalanin) that undergoes post-translational modification in which it is proteolytically cleaved to produce galanin (Kofler et al., 1996; Rökaeus and Brownstein, 1986) (Figure. 1-2A). Preprogalanin contains the signalling peptide, progalanin-message peptide (PGMP), galanin message-associated peptide (GMAP) and galanin (Yamamoto et al., 2014). This is discussed further in the next section.

In adult mice, galanin has a widespread distribution in both the CNS and peripheral nervous system (PNS) supporting the hypothesis that it plays a role in many physiological functions (see section 1.8). In the CNS of mice, it is highly expressed in the hypothalamus and brainstem with relatively low expression in the olfactory bulb, septal nuclei, thalamus, and parabrachial and spinal trigeminal nuclei (Cheung et al., 2001). In the PNS, galanin-like immunoreactivity has been observed in both the myenteric plexus and submucous plexus of the stomach, duodenum, ileum and colon with galanin positive fibres detected in the circular muscle layer, lamina muscularis mucosae and the lamina propria (Rökaeus et al., 1984). In the GI tract, galanin is expressed most highly in the duodenum tissue with lower levels of expression in the stomach and the colon tissue (Kaplan et al., 1988).

1.7 Galanin peptide family

The *GAL* gene protein product is proteolytically cleaved into galanin and the 59-amino acid peptide GMAP (Figure. 1-3A). The function of GMAP remains unclear, but it has been suggested to have a role in modifying nociception in the spinal cord and has also been reported to have antifungal activity (Rauch et al., 2007).

Another protein in the galanin family, but one that is encoded by a different gene, is galanin-like protein (GALP; Fig 1-2B). Originally described as a putative endogenous ligand of galanin receptor 2 (GalR2), GALP shares some homology (amino acids 9-21) with the first 13 amino acids of galanin and an overall homology of 43% of galanin. GALP has high affinity for GalR2 and is thought to have a role in energy homeostasis and reproduction (Lawrence and Fraley, 2010). GALP is also expressed in the mucosa

of the GI tract and is thought to play a role in stimulating feeding behaviour (Mensah et al., 2017). A splice variant of the GALP gene, which omits exon 3 causing a frame shift, also exists and is called alarin. Like GALP, alarin has been suggested to stimulate feeding behaviour, as well as influencing luteinizing hormone secretion. However, unlike GALP, the lack of homology of alarin to galanin means that it does not bind to any galanin receptor, but as yet no receptor for alarin has been identified (Boughton et al., 2010; Santic et al., 2006).

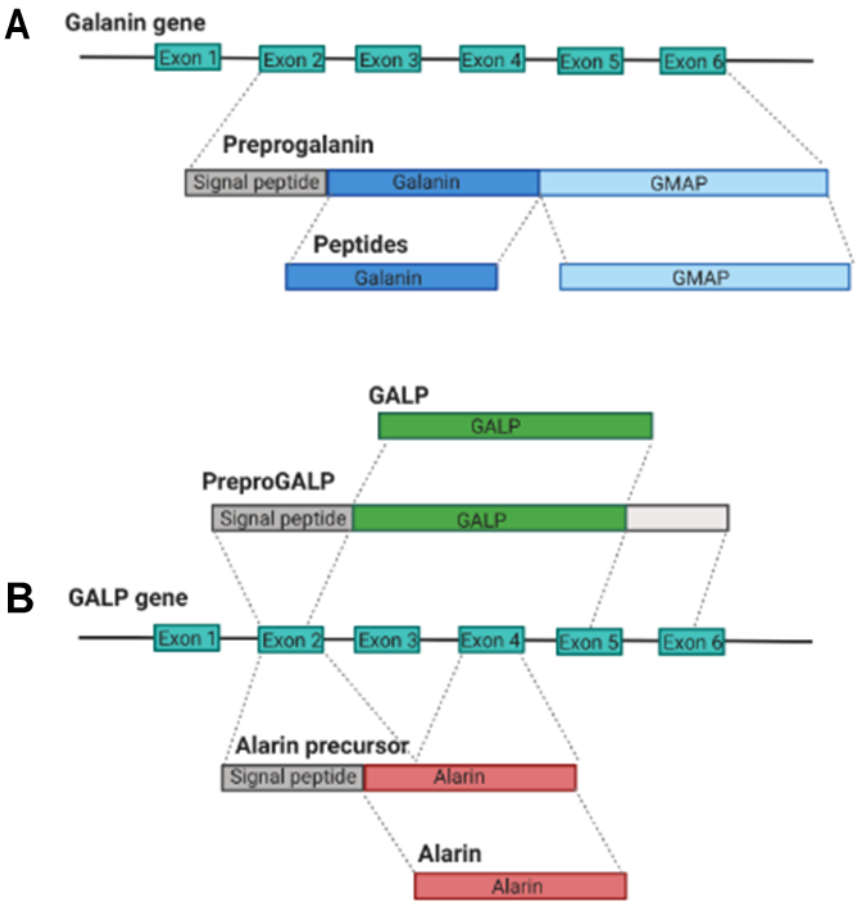


Figure 1-3. Organisation of the galanin and GALP genes

The first exon of the mouse galanin gene encodes the 5′-untranslated regions of the preprogalanin mRNA, exon 2 encodes the signalling peptide, exon 3 encodes the first 13 residues of galanin with exon 4 and 5 encoding the remaining 16 residues, as well as most of galanin message associated protein (GMAP), leaving exon 6 to encode the remaining section of GMAP and the 3′-polyadenylation sequence. For the GALP gene, exon 1 is non-coding and exons 2-6 encode preproGALP. The section of homology with galanin is between residues 9 – 21 and is the same as the first 13 residues of galanin. Exons 2 – 5 encode the GALP protein and post-translational splicing of GALP leads to a frame shift and the exclusion of exon 3, resulting in the alarin precursor. Precursors contain signalling peptides at their N-terminus that prompt their translocation in the cell and are cleaved into the final proteins by endopeptidases. Adapted from Picciotto et al., 2008

1.8 Galanin receptor signalling

Galanin is widely expressed in the CNS and PNS of many species (Meister et al., 1990; Pérez et al., 2001) and carries out its role through binding to three G protein-coupled receptors (GalR1, GalR2, and GalR3; Branchek et al., 2000, 1998), which have overlapping expression patterns in the CNS and the PNS (Freimann et al., 2015). Binding of galanin to its receptors has the potential to trigger signalling via multiple pathways depending upon which G proteins are coupled to the receptors. For example, GalR1 and GalR3 are mostly G_i-coupled resulting in inhibition of adenylate cyclase and the opening of G protein-coupled inwardly rectifying K^+ (GIRK) channels, which leads to neuronal hyperpolarization. These actions are mediated by $G_{i/o}$ α-subunits and βγ-subunit heterodimer G-proteins. By contrast, GalR2 is predominantly G_q-coupled leading to release of intracellular Ca^{2+} and the stimulation of protein kinase C (PKC), events associated with neuronal excitation (Freimann et al., 2015) (Figure. 1-4).

With regard to nociception, activation of GalR1/3 would be expected to produce anti-nociceptive effects, whereas GalR2 activation would cause pro-nociceptive effects. Behavioural and electrophysiological studies support this theory, such that GalR1 KO mice showed increased hyperalgesia after hind-paw tissue injury and inflammation (Malkmus et al., 2005). In addition, intraplanar injection of the GalR2 agonist AR-M1896 potentiated capsaicin-induced inflammatory pain, an effect blocked by a PKC inhibitor or mimicked by a PKC activator (Jimenez-Andrade et al., 2005).

GalR1 is abundantly expressed in olfactory structures, amygdala, thalamus, hypothalamus and laminae I - III of the spinal cord and thus has the potential to contribute to a variety of physiological processes. The complexity of galaninergic signalling is further enhanced by the fact that galanin receptors can form dimers. GalR1, for example, can form both homodimers and heterodimers with other galanin receptors as well as with other GPCRs such as 5-hydroxytryptamine (5-HT) receptors, neuropeptide Y (NPY) receptors, and dopamine D1-like receptors (Fuxe et al., 1998; Lang et al., 2014; Wirz et al., 2005). These heterodimers may provide one molecular mechanism for galanin peptides to modulate the function of different neuronal

networks in the CNS. There is evidence of GalR1-5-HT1A heterodimer existence in the limbic system of rats, and as such it has been suggested that modulation of the galaninergic system could provide a new avenue to the treatment of depression (Borroto-Escuela et al., 2010; Fuxe et al., 1998).

Unlike GalR1, the expression of GalR2 does not fluctuate during development (Burazin et al., 2000). However, like GalR1, GalR2 forms heterodimers with other galanin receptors (Fuxe et al., 2012) and non-galanin receptors, such as the NPY Y1-receptor (NPYY1), which has been observed in the amygdala of rats and involved in anxiolytic behaviour (Narváez et al., 2018). GalR2 is highly expressed in the hippocampus, particularly in the dentate gyrus, and the mammillary nuclei of the hypothalamus. Of more relevance to the current study, GalR2 has also been shown to be expressed in small and intermediate primary sensory neurones in DRGs (Brumovsky et al., 2006). GalR2 signals through multiple classes of G proteins activating many different intracellular pathways. For example, GalR2 largely signals via $G_{q/11}$-type proteins which activate phospholipase C (PLC) causing Ca^{2+} release from intracellular stores, activating PKC and opening of Ca^{2+} activated chloride channels (CaCC) (Fathi et al., 1998). GalR2 has also been shown to couple to $G_{12/13}$-type proteins causing activation of Rho A (small GTPase) (Wittau et al., 2000). In addition to its ability to couple to different G proteins, galanin has also been hypothesised to have a biphasic concentration-dependent action when acting at GalR2, such that GalR2 switches from G_q (low galanin concentration) to G_i (high galanin concentration) signalling, but this has yet to be confirmed (Hulse et al., 2012; Malin and Molliver, 2010).

In comparison to GalR1 and GalR2, GalR3 is still poorly defined, possibly due to the absence of a transfected cell line expressing sufficient GalR3 protein to functionally characterise its signalling, even though of course such studies do not necessarily recapitulate what happens *in vivo* (Robinson et al., 2013). However, in spite of expression problems GalR3 has been suggested to couple to $G_{i/o}$ in a similar manner to Gal1 (Smith et al., 1998).

When comparing galanin's affinity for its different receptors, radioligand binding analysis using [^{125}I]-galanin, has shown galanin to have the highest affinity for GalR1 (9.84 pK_d), followed by GalR2 (9.53 pK_d), and with the lowest affinity for GalR3 (9.01 pK_d) (Smith et al., 1998). Galanin's affinity for GalR heterodimers and heterodimers with 5-HT receptors remains unknown.

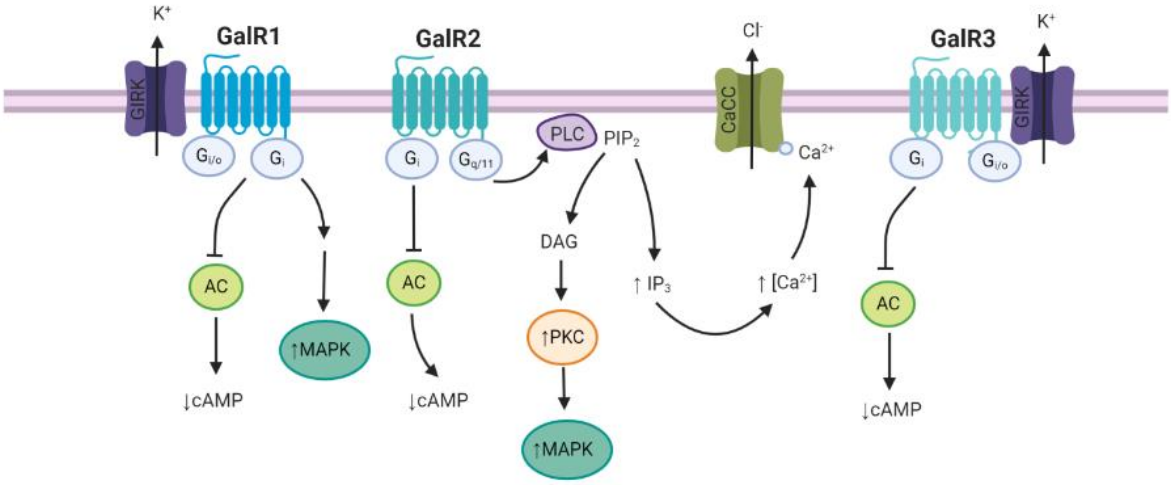


Figure 1-4. GalR transduction mechanisms

Both GalR1 and GalR3 predominantly signal via $G_{i/o}$ resulting in hyperpolarisation through activation of GIRKs and in addition inhibition of AC takes place. AC inhibition can also occur via GalR2, although GalR2 signals predominantly through $G_{q/11}$ causing stimulation of PLC, which cleaves PIP_2 to form DAG and IP_3, the latter causing a release of Ca^{2+} from intracellular stores. As well as causing direct depolarisation, the increase in intracellular Ca^{2+} concentration causes activation of CaCC, an efflux of Cl^- and depolarisation. Lastly, it has also been shown that PKC activation via DAG leads to activation of MAPK. PIP_2 - phosphatidylinositol bisphosphate, GIRKs - G protein-coupled inwardly-rectifying potassium channels, AC - adenylyl cyclase, PLC - phospholipase C, IP_3 - inositol trisphosphate, CaCC - Ca^{2+} activated chloride channels, PKC – protein kinase C, DAG - diacylglycerol, MAPK - mitogen-activated protein kinase

1.9 Role of galanin in peripheral inflammatory pain

There is evidence to suggest that during inflammation the expression of galanin mRNA is increased, mainly in the acute inflammatory phase. For example, in the uroepithelium of rats with cyclophosphamide-induced cystitis (Girard et al., 2008). However, inflammation appears to lead to lower galanin peptide levels despite upregulated mRNA expression in inflamed tissues, as was seen in arthritic ankle joints (Qinyang et al., 2004) and eczematous skin (El-Nour et al., 2004). By contrast, GalR expression has been more consistently reported to be upregulated in inflammatory conditions. For example, elevated GalR1 expression is observed in peripheral tissues in multiple experimental inflammatory models (Marrero et al., 2000; McDonald et al., 2007; Saban et al., 2002).

In DRG neurones, in a peripheral model of inflammatory pain (hind-paw injection of carrageenan), it has been shown that galanin mRNA levels decrease in the DRG neurones, but simultaneously increase in the dorsal horn of the spinal cord (Zhang et al., 1998). In the same model, like galanin, GalR1 mRNA is also downregulated in DRG, but GalR2 mRNA shows increased expression (Sten Shi et al., 1997; Xu et al., 1996). However, in a model of chronic arthritis (via hind-paw adjuvant injections), galanin mRNA initially displays a decrease in expression, similar to the previous studies, but then in the later stages, e.g. after 21 days, its expression increases (Calzà et al., 2000, 1998), which could suggest that galanin plays a greater role in chronic nociception compared to acute.

1.10 Role of galanin in GI tract sensory afferent neurones

Generally, galanin is co-expressed with a variety of small molecule neurotransmitters including acetylcholine, GABA, serotonin, glutamate, and dopamine, as well as numerous other neuropeptides including calcitonin gene-related peptide (CGRP), vasopressin, substance P, and NPY (Freimann et al., 2015; Webling et al., 2012). Since its discovery, many neuronal and non-neuronal roles have been suggested for galanin, including roles associated with metabolic and osmotic homeostasis (Landry et al., 2000), immunity (Lang and Kofler, 2011), endocrine function (Sundkvist et al., 1992), reproduction (Rossmanith et al., 1996), cognition (Kinney et al., 2002) and

cancer (Kwiatkowski et al., 2016). This study will focus specifically on the role of galanin in nociception.

In a recent study using single-cell RNA-sequencing on colon-innervating DRG sensory neurones (identified through retrograde tracing of the distal colon), we demonstrated that galanin and GalR1-3 are expressed by both TL (representing LSN innervation of the distal colon) and LS (representing PN innervation of the distal colon) DRG populations (Fig. 1-5; Hockley et al., 2019). In the study, 314 colonic sensory afferent neurones were arranged by unbiased clustering into 7 subtypes based upon their gene expression. With the exception of 2 neurones, the TL population (DRG T10-L1, n = 159) was split into 5 groups, whereas the LS population (L5-S2, n = 155) was split into 7 groups (2 almost exclusively consisting of LS neurones, i.e. n = 121/123). The five subgroups consisting of neurones from both TL and LS were defined with the prefix “m” for mixed. The five mixed groups consisted of mNeurofilament a (mNFa) and mNeurofilament b (mNFb), typically associated with myelinated neurones. Then mNon-peptidergic (mNP) consisting of non-peptidergic, putative nociceptors. Finally, mPeptidergic a (mPEPa) and mPeptidergic b (mPEPb), two putative peptidergic nociceptor groups. The two LS exclusive groups are defined with the prefix “p” for pelvic nerve: pNeuroFilament (pNF, likely mechanosensory) and pPeptidergic (pPEP, likely nociceptive). The pNF group is similar to mNFa/mNFb and pPEP is similar to mPEPa/mPEPb, however each group possesses a distinct pattern of expression suggesting functional disparity between the classes of neurones. When examining the expression of galanin in more detail, it is expressed predominantly in mPEPb and pPEP, both groups of peptidergic nociceptors. GalR1 is also expressed in mPEPb and mPEPa subgroups of peptidergic nociceptors, overlapping with galanin expression. GalR2 is expressed at a much lower level and almost exclusively in mPEPb, whereas GalR3 is also expressed at a much lower level but is present in all subgroups.

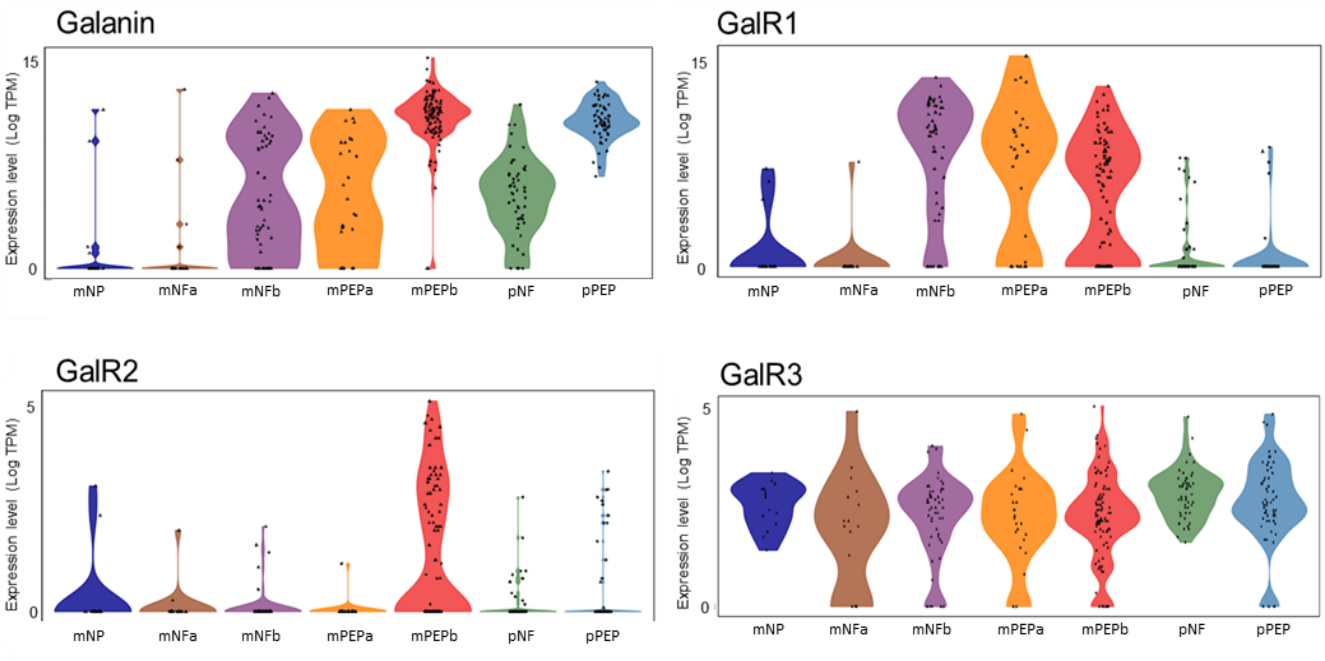


Figure 1-5. Galanin receptor expression in colonic DRG neurones.

Expression profile of galanin (top left), GalR1 (top right), GalR2 (bottom left), and GalR3 (bottom right) within 314 colonic sensory neurones grouped into 7 neuronal subtypes based upon clustering described by Hockley et al. (2019). Each black dot represents a single colonic sensory neurone isolated from TL or LS DRG. A probability density for each subtype is also displayed and coloured by subtype (mNP = dark blue, mNFa = brown, mNFb = purple, mPEPa = orange, mPEPb = red, pNF = green, and pPEP = light blue). Expression values are displayed in Transcript-Per-Million (Log[TPM]). Galanin is expressed predominantly in two peptidergic populations associated with nociception (mPEPb and pPEP), GalR1 is present in one of the peptidergic nociceptive groups that galanin is also present in (mPEPb), as well as two others (mNFb and mPEPa), GalR2 is expressed at a much lower level, but mainly in mPEPb, whereas GalR3 is present at low levels in all groups.

Galanin is also expressed by non-neuronal cell types in the colon including macrophages and fibroblasts, (Koller et al., 2019; Yamamoto et al., 2014). Similar to galanin, GalR1 is also expressed in non-neuronal cells, including epithelial cells and smooth muscle cells, which demonstrates galanin's role in GI motility (Lorimer and Benya, 1996). GalR1 and GalR2 have also been shown to be expressed in enterochromaffin-like (ECL) cells where galanin has an inhibitory effect on histamine release from ECL cells (Zeng et al., 1998).

In a study looking at the nociceptive flexor reflex model of decerebrated rats after intrathecal application, galanin has been shown to produce a biphasic dose-dependent effect on nociception through activation of GalR1 and GalR2 (Xu et al., 2000). There are also multiple studies showing under both normal conditions and in inflammation, that the effects of exogenous galanin are predominantly inhibitory (Hua et al., 2004; Xu et al., 2000). With the development of the GalR1 selective agonist, M617 (Lundström et al., 2005), and the GalR2 antagonist, M871 (Sollenberg et al., 2006), it has been suggested that spinal interneurons in the dorsal horn expressing GalR1 could mediate some of the inhibitory anti-nociceptive actions of galanin. Results gained through the use of GalR1 KO mice show that after partial sciatic nerve injury there is shortened latency in response to nocifensive behaviours on a hot plate compared to wildtype animals, i.e. greater thermal hyperalgesia (Malkmus et al., 2005), but by contrast there was no significant change in mechanosensation (Blakeman et al., 2003). This suggests no difference to acute nociception, but an increase in hyperalgesia after tissue damage and inflammation (Blakeman et al., 2003; Malkmus et al., 2005). By contrast to GalR1 KO mice, GalR2 KO mice did not significantly differ in phenotype to wildtype mice across a range of measures including: behaviour, reproductive physiology, feeding and body weight regulation, and seizure susceptibility, i.e. GalR2 appears not to have a dominant role in nociception (Gottsch et al., 2005; Shi et al., 2006). It is also thought that the galaninergic system plays a role in regulation of the activity of muscle in the GI tract and modulation of mucosal secretive process, as well as regulating the sensitivity of sensory neurones (Brown et al., 1990).

The role of galanin in GI afferent fibre excitability has been demonstrated in the upper GI tract where a galanin-mediated reduction in mechanosensitivity has been observed in gastro-oesophageal vagal afferents; however, a minority of neurones also shown enhanced mechanosensitivity (Page et al., 2007). In these studies GalR1 activation was shown to underpin the reduction of neuronal excitability observed and GalR2 activation was demonstrated as mediating the increase neuronal excitability; interestingly, although galanin inhibited the function of more fibres than it enhanced, similar GalR1 and GalR2 expression profiles were observed when analysing mRNA levels in vagal sensory ganglia (Page et al., 2007). One explanation for the disparity

in GalR expression and the effects of galanin in gastro-oesophageal vagal afferents is the presence of six upstream open reading frames (uORF) in mouse GalR2 mRNA, which inhibit expression of the primary ORF leading to less GalR2 protein synthesis, i.e. the mRNA level does not necessarily correlate with the protein expression level (Kerr et al., 2015). In contrast with the observed roles of GalR1 and GalR2, no evidence for GalR3 function in GI afferents has been produced, even though it is expressed in vagal sensory ganglia (Page et al., 2007). Indeed, in a previous study it was shown in GalR1 KO mice that GalR3 did not contribute to any effect in the upper GI tract (Page et al., 2007).

Although the main source of pain in many chronic conditions affecting the GI tract originates in the colon, e.g. in IBD and IBS, to date, the role of galaninergic signalling in colorectum nociception is unknown. Moreover, because single-cell RNA-sequencing data demonstrates co-expression of GalR1 with receptors for certain inflammatory mediators (e.g. bradykinin and 5-HT) and ion channels such as TRPV1 that are modulated by inflammatory mediators (Hockley et al., 2019), it is possible that galanin could inhibit mechanical hypersensitivity induced by inflammatory mediators and plays a role in *in vivo* models of acute colitis.

1.11 Principle aims

Based on the literature expression profiles (Fig. 1-5), I hypothesised that galanin would inhibit LSN activity predominantly through GalR1 receptor, similar to what has been shown in gastro-oesophageal vagal afferents (Page et al., 2005b). Therefore, the aims of this thesis were to:

- Determine galanin's role in modulation of LSN activity to mechanical stimuli
- Determine the contribution of different GalRs to the effects produced by galanin
- Determine the ability of galanin to modulate acute LSN mechanical hypersensitivity
- Compare the role of galanin in modulating LSN activity between healthy and inflamed colons
- Determine if galanin and/or GalR expression changes in colonic afferents in colitis.

Chapter 2: Materials and Methods

This research was conducted under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB). All experiments were carried out under Project Licences 70/7705 and P7EBFC1B1 by myself unless otherwise stated and those conducted on animals were only conducted by those holding a Home Office Personal Licence.

2.1 Whole-nerve electrophysiological recordings from mouse LSN

2.1.1 Ex vivo mouse LSN afferent preparations

This preparation was conducted broadly as described previously (Brierley et al., 2004). In brief, adult (8-25 weeks) C57BL/6J male and female mice (Envigo) were killed by cervical dislocation and exsanguination, and the distal colon (from the splenic flexure to rectum) with associated LSN removed. To remove the LSN, the skeletal muscle and neurovascular bundles were cut from the spinal column while maintaining LSN innervation of the colon. Faecal material was gently flushed from the colon with Krebs buffer and the colon tied to either end of a cannula, using fine thread (polyester, Gutermann) and serosally superfused (7 mL min^{-1}) with carboxygenated Krebs buffer (95% O_2, 5% CO_2). The input port was connected to a syringe pump (Harvard apparatus), this was used for continuous intraluminal perfusion of Krebs (200µL min^{-1}; Fig. 2-1A and B). The Krebs buffer (in mM: 124 NaCl, 4.8 KCl, 1.3 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4 \cdot 7H_2O$, 11.1 glucose, and 25 $NaHCO_3$) was supplemented with indomethacin (3 µM, non-selective cyclo-oxygenase inhibitor, to block prostanoid production), nifedipine (10 µM, voltage gated calcium channel blocker) and atropine (10 µM, non-selective muscarinic receptor antagonist) to block smooth muscle contraction. The bath was maintained at 32-34°C. The LSN inferior and superior mesenteric ganglia were identified at the point of the iliac bifurcation and the superior mesenteric ganglia was isolated from the abdominal aorta and cleaned of surrounding connective tissue. Suction electrode recordings of multiunit activity were made between the two ganglia on one of the two intermesenteric nerves.

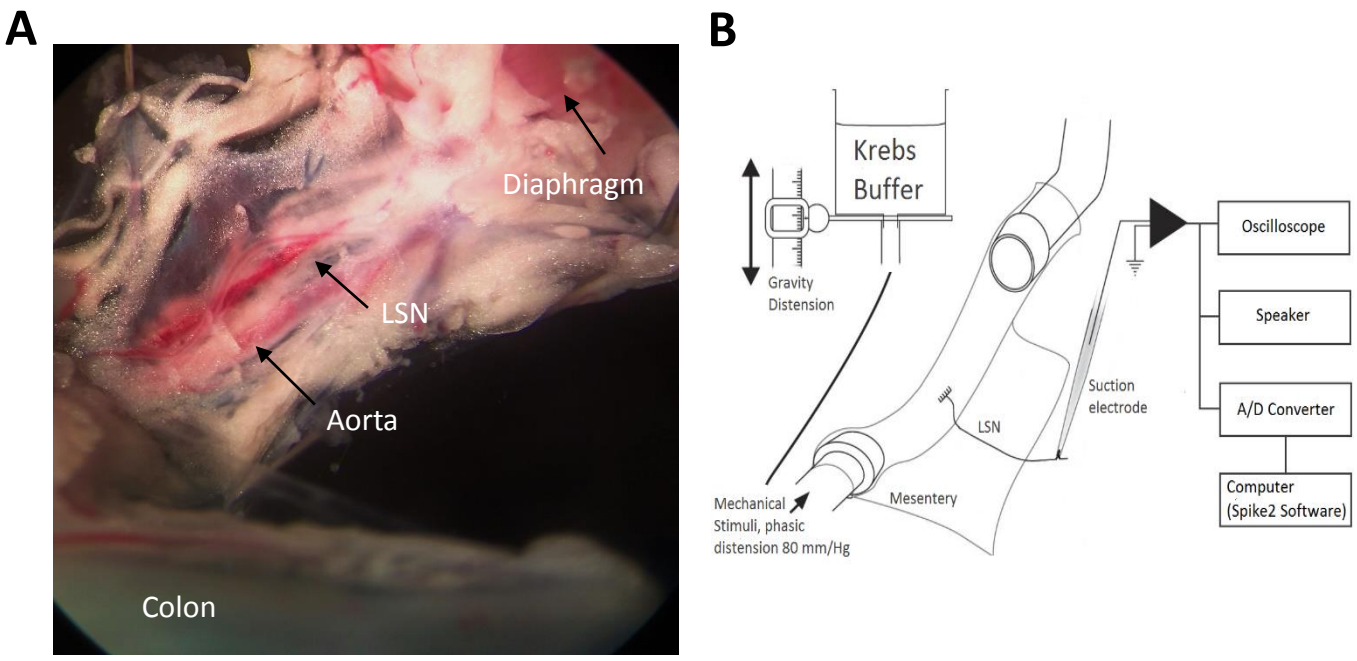


Figure 2-1 – Schematic of electrophysiological recording technique

(A) Photo of dissection pinned out in sylgard-lined chamber. In the mesentery, the LSN can be seen in the aortic neurovascular bundle. (B) Whole-nerve recording of LSN multiunit activity. The colon is cannulated and intraluminally perfused in the physiological (oral to aboral) direction. The LSN was cleared of surrounding connective tissue and whole-nerve suction electrode recordings were made (B - adapted from Brierley et al., 2018).

2.1.2 Electrophysiological protocols for characterization of LSN afferent properties

Borosilicate glass suction electrodes were used to record multi-unit activity from whole LSN. Signals were amplified, band pass filtered (gain 5Kz; 100-1300 Hz; Neurolog, Digitimer Ltd, UK), and digitally filtered for 50 Hz noise (Humbug, Quest Scientific, Canada). Traces were digitized at 20 kHz (micro1401; Cambridge Electronic Design, UK), and action potential firing counts were determined using a threshold of twice the background noise (usually around 100 μV). All signals were displayed on a PC using Spike 2 software. The baseline pressure was set up at 2-3 mmHg and the preparation was left for approximately 30 minutes until a stable baseline was observed before initiating the experimental protocols.

The distention protocol used to investigate mechanosensitivity consisted firstly of five phasic distensions to establish the baseline response to distension. Each distension raised the intraluminal pressure from 0 to 80 mmHg within two seconds, a pressure that has been shown to activate both mechanoreceptors and nociceptors in LSN recordings, with higher pressures evoking pain behaviour in rodents (Hughes et al., 2009; Ness and Gebhart, 1988). Galanin (20 mL) was serosally perfused via bath application between distensions four and five, followed by a further eight distensions to allow for the maximal drug effect to be observed (Fig. 2-2A). A ramp distension protocol was used to investigate the activity of different neuronal populations and for this the luminal outflow cannula was blocked and the subsequent increase in pressure was observed until the desired maximum of 80 mmHg was reached (typically 3-4 minutes), at which point the luminal outflow was re-opened. The protocol used to investigate the effects of an inflammatory soup on LSN activity included an initial ramp distension and 3 phasic distensions, followed by intraluminal perfusion of an inflammatory soup that has previously been shown to induce mechanical hypersensitivity (10 μM histamine, 10 μM prostaglandin E_2, 10 μM 5HT, 1 μM bradykinin, and 1 mM ATP; Su and Gebhart, 1998) for 20 minutes prior to and during subsequent ramp and phasic distensions (Fig. 2-2B).

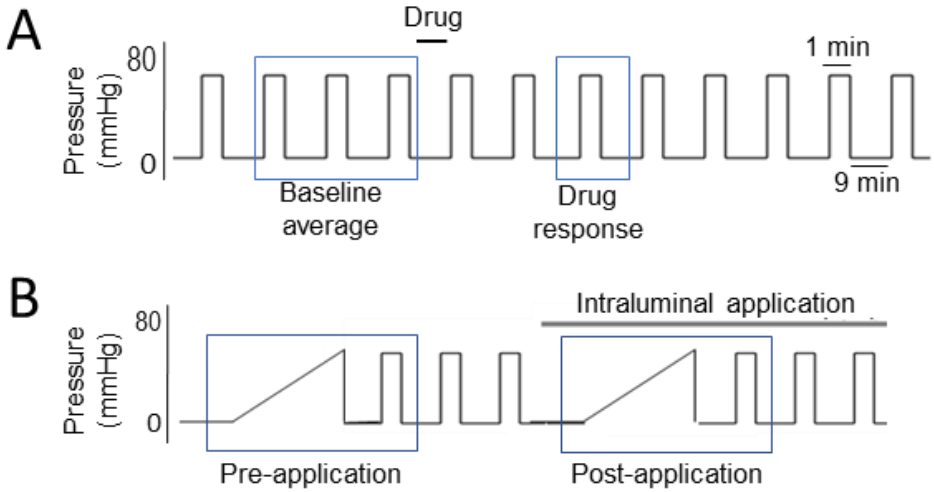


Figure 2-2 – Protocols used for LSN recordings.

(A) The mechanosensitivity protocol consisting of 12 phasic distensions to 80 mmHg for 1 minute at 9 minute intervals. Galanin or vehicle application (20 mL) after a baseline response to distension has been established, typically after the fourth distension. (B) Inflammatory soup protocol consisting of ramp and phasic distensions from 0 to 80 mmHg, before and during intraluminal perfusion of inflammatory soup (pretreated 20 mins before ramp and distensions).

2.1.3 Analysis of electrophysiological recordings

To establish the most appropriate time point to measure the effect of galanin, recordings using a variety of galanin concentrations were analysed to determine when the effect of galanin was at its greatest. From this analysis, the maximal effect of galanin upon phasic distension was observed in response to the third distension after galanin application and thus this response was routinely measured and compared to the baseline response to phasic distension (average of second to fourth distension).

A phasic distension of 80 mmHg leads to an increase in LSN activity, which peaks, drops by ~65% and then stays at a plateau that is maintained throughout the remainder of the distension. The parameters measured during each distension are shown in Fig. 2-3. Peak changes and time profiles of LSN activity were determined by subtracting baseline firing (average over 60 seconds before distention) from increases in LSN activity following distension. The effect of galanin at each concentration was measured by the change in LSN activity before and after galanin application using Student's t-test. During phasic distensions, peak firing was defined as the maximal firing rate observed during distensions, which usually occurred within the first 15 seconds. The response to phasic distention can also be split into two distinctive phases. Phase I is the initial peak in LSN activity in response to the dynamic change in pressure, which lasts the first 15 seconds of the distension. Phase II is the plateau activity seen during the subsequent 45 seconds. By contrast, ramp distention leads to a slow and steady increase in activity until the end of the distention. For ramp distension analysis, the activity was measured at every 5 mmHg and changes in LSN activity were compared at each interval between groups using a repeated measures two-way ANOVA with Sidak's post-hoc test.

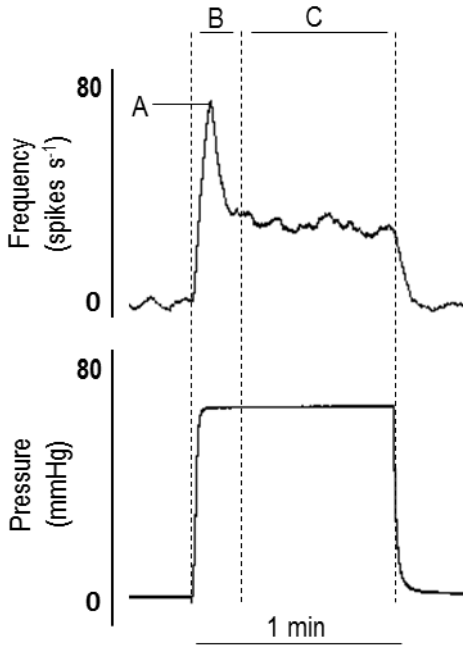


Figure 2-3. Parameters measured from a response to phasic distention.

Peak firing is determined by the maximal firing frequency observed in the first 15 seconds (A). The response to distention was split into two phases from which the average rate is calculated, phase 1 (seconds 1-15 of the distention; B) which represents the spike in activity due to the change in pressure and phase 2 (seconds 16-60 of the distention; C) representing the stable plateau stage of the response.

2.2 Immunohistochemistry on retrograde labelled dorsal root ganglion neurones

2.2.1 Retrograde labelling of colonic sensory neurones

The procedure for retrograde labelling of colonic sensory neurones was conducted as previously described (Hockley et al., 2019). In brief, C57BL/6J mice were anaesthetized with isoflurane (4% induction and 1-2% maintenance) before shaving their abdomen and then a midline laparotomy (~1.5 cm incision) performed to reveal the distal colon. A piece of sterile suture thread was passed under the colon to further secure the injection site on the distal colon. Five injections of 0.2 µL Fast Blue (2% in saline, Polysciences GmbH, Germany) were made into the wall of the distal colon using a glass needle at a rate of 0.4 µL min^{-1} using a microinfusion pump (Harvard Apparatus). After the abdominal cavity was flushed with saline to remove any excess Fast Blue dye, the muscle and skin layers were sutured and secured using 4-6 Michel clips. Postoperative care and analgesia (buprenorphine 0.05-0.1 mg kg^{-1}) was provided and a glucose enriched, soft diet provided, with regular checks of body weight. After a minimum of three days, animals were killed using sodium pentobarbital (200 mg kg^{-1} intraperitoneal (i.p.) injection) and transcardially perfused with phosphate buffered saline (PBS) followed by paraformaldehyde (4% in PBS; pH 7.4). Dorsal root ganglia (DRG; T13 – L1) were removed and further fixed in 4% paraformaldehyde for 30 minutes at 4 °C before cryoprotection in 30% sucrose overnight at 4 °C. The tissue was then embedded in Shandon M-1 Embedding Matrix (Thermo Fisher Scientific), snap frozen in liquid nitrogen, and stored at -80 °C until needed. Cryostat (Leica, CM3000; Nussloch) sections (12 µm) were collated across 10 slides (Superfrost Plus, Thermo Fisher Scientific) for each DRG. Surgery was performed by Dr James Hockley and I acted as a surgical assistant.

2.2.2 Immunohistochemistry

DRG sections or wholemount preparations were washed with PBS (twice for 2 minutes) and then blocked using antibody diluent (10% donkey serum, 5% bovine serum albumin (BSA) and 0.2% Triton X-100 in 0.1 M PBS) for one hour. In cases where a mouse primary antibody was used, an additional block for one hour at room temperature using mouse IgG blocking reagent (Vector Laboratories) was carried out. This was followed by overnight incubation at 4 °C with the appropriate primary

antibodies (Table 1). The sections were then washed three times for five minutes with PBS and then incubated for 2 hours at room temperature with the appropriate fluorophore conjugated secondary antibodies and/or isolectin B4 (IB4) from *Griffonia simplicifolia*-Alexafluor-488 (2.5 µg mL^{-1}; Cat #: I21411, Invitrogen, UK), a marker for non-peptidergic, small diameter, C-fibre sensory neurones. No labelling was observed in control experiments where the primary antibody was excluded or in the presence of a blocking peptide (galanin, Cat #: 2696, Tocris) for the anti-galanin antibody (see section 7.1).

Primary Antibody	Conc.	Company	Catalogue #	RRID
Rabbit anti-galanin	1:1000	Theodorsson Lab	Kind gift	-
Guinea pig anti-TRPV1	1:1000	Alomone Labs	AGP-118	AB_2721813
Goat anti-Gfrα3	1:300	R&D Systems	AF2645	AB_2110295
Goat anti-CGRP	1:500	Abcam	AB36001	AB_725807
Goat anti-trkA	1:1000	R&D Systems	AF1056	AB_2283049
Goat anti-trkC	1:500	R&D Systems	AF1404	AB_2155412

Table 1 – List of primary antibodies used, RRID – Research Resource Identifiers

Secondary Antibody	Conc.	Company	Catalogue #	RRID
Donkey anti-rabbit IgG-Alexafluor-488	1:1000	Invitrogen	A-21206	AB_2535792
Donkey anti-rabbit IgG-Alexafluor-568	1:1000	Invitrogen	A10042	AB_2534017
Donkey anti-goat IgG-Alexafluor-568	1:1000	Invitrogen	A-11057	AB_2534104
Donkey anti-guinea pig IgG-Alexafluor-488	1:1000	Jackson Immuno Research	706-165-148	AB_2340460

Table 2 – List of secondary antibodies used, RRID – Research Resource Identifiers

2.2.3 Imaging and quantification

Sections were imaged using an Olympus microscope (BX51) with QImaging camera (Surrey, Canada) and the relative intensities of the DRG neurones after immunostaining were measured using ImageJ 1.51n (NIH, USA). The mean background intensity was subtracted to control for variability in illumination between images. Percentages of relative intensities were determined by comparison with the least (0%) and most (100%) intensely labelled cells for each section. Relative intensities were calculated as a percentage by subtracting the relative intensity of the darkest neuronal profile (a) from the relative intensity of the cell of interest (b) and comparing this to the relative intensity of the brightest neuronal profile (c) with the relative intensity of the darkest neuronal profile subtracted. To summarise, the relative intensity of a cell as a percentage = (b – a)/(c – a) (Fang et al., 2002). Cells with intensity values greater than the mean intensity of the darkest neuronal profiles from all the sections plus five times its standard deviation (SD) were considered positively labelled.

2.3 Chemically induced colitis models in mice

2.3.1 Trinitrobenzenesulfonic acid (TNBS) model of colitis

C57BL/6J mice of either sex (8-12 weeks old) were weighed two days prior to the start of the procedure with their weight and stool content/consistency (see 1.3.3) being monitored daily throughout the experiment, which lasted 3 days. Based on a paradigm described previously (Wirtz et al., 2007), mice were firstly anaesthetised with ketamine (100 mg kg^{-1}) and Xylazine (10 mg kg^{-1}) and TNBS (Sigma, cat #: P2297 [0.85 mg, 1.9 mg, and 3.8 mg]) dissolved in ethanol (concentrations used were 30%, 40%, and 50%) was administered intracolonically to induce colitis. 100 µL of a TNBS mixture was instilled using a sufficiently lubricated (petroleum jelly) Luer Stubs (22 gauge; blue; LS22S Linton Instruments) attached to a 100 µL Hamilton syringe using 5 cm of PE-50 tubing (BTPE-50 Linton Instruments) and inserted approximately 4 cm internally from the anal verge; control mice received an ethanol-only solution at the appropriate concentration. The mouse was then maintained in a head down orientation for a further 2 minutes to prevent expulsion of the fluid and promote even distribution.

2.3.2 Dextran sulfate sodium salts (DSS) model of colitis

C57BL/6J mice of either sex (8-12 weeks old) were weighed two days prior to the procedure with their weight and stool content/consistency (see 1.3.3) being monitored daily throughout the treatment. Based on a paradigm described previously (Chassaing et al., 2014), 3% DSS supplemented drinking water was administered with control mice receiving the same drinking water without DSS. In the pilot studies, the mice received DSS treated water for 5 days after which it was replaced with normal drinking water for a further 3 days, but based on the severity of weight loss by day 8 the decision was made to reduce the period on normal drinking water post-DSS to 2 days instead of 3 . On day 7 the mice were killed and relevant tissue samples and measurements were obtained (Manicassamy and Manoharan, 2014; Wirtz et al., 2007). The experimenter was blinded during the experiment, drinking water being labelled A and B, being unblinded after results were analysed.

2.3.3 Disease activity index (DAI) for assessing colitis

Oral administration of DSS or intracolonic instillation of TNBS leads to the development of clinical signs of colonic inflammation including weight loss, diarrhoea, faecal bleeding and infiltration of granulocytes. The DAI score (Table 3) was used to evaluate the onset and extent of disease using a previously established scoring system (Manicassamy and Manorhan, 2014).

Score	Weight Loss	Stool Consistency	Blood in Stool
0	None	Normal	Normal
1	1 – 5 %		
2	5 – 10 %	Very Soft	Slight bleeding
3	10 – 15 %		
4	> 15 %	Watery diarrhoea	Gross bleeding

Table 3 – Disease activity index (DAI) scoring system (Manicassamy and Manorhan, 2014).

2.3.4 Histology: H&E with alcian blue staining

An approximately 1 cm section of colon was removed ~4 cm internally from the rectum, fixed in 4% paraformaldehyde for 4 hours and cryoprotected in 30% sucrose overnight. The tissue was then embedded in O.C.T. (VWR Q-path Chemicals), snap frozen in liquid nitrogen, and stored at -80 °C until needed. Cryostat sections (20 µm) were collected and stored at -20 °C until needed.

Slides were washed in tap water for 2 minutes before staining for 5 minutes with haematoxylin (1:2 dilution with tap water; Sigma). Slides were then washed in tap water for 3 minutes, followed by 0.3% HCl in ethanol for 30 seconds and then immediately washed in tap water for a further 2 minutes, before being incubated in tap water until the tissue developed a deep blue appearance. Slides were then stained with alcian blue (1% W/V in 3% acetic acid; Polysciences Inc) for 10 minutes and washed in tap water for 2 minutes before being immersed in 100% ethanol for 30 seconds. Slides were then stained with eosin (Acros Organics) for 90 seconds, washed in tap water for 1 minute and then dehydrated in 100% ethanol for 30 seconds followed by 70% ethanol for 30 seconds. Slides were then cleared using Histoclear (National Diagnostics) for 30 seconds before mounting coverslips with mowiol mounting media. Imaging was carried out using a NanoZoomer S60 Digital slide scanner (Hamamatsu). Histopathological scoring was done based on inflammation, crypt damage and ulceration (Table 4; Ren et al., 2011).

Score	Inflammation	Crypt Damage	Ulceration
0	None	Crypts intact	None
1	Increased number of granulocytes in lamina propria	Loss of basal one third of crypt	1-2 foci of ulceration
2	Confluence of inflammatory cells extending to submucosa	Loss of basal two thirds of crypt	3-4 foci of ulceration
3	Transmural extent of infiltrate	Entire crypt loss	Confluent or extensive erosion
4	-	Change in epithelial surface with erosion	-
5	-	Confluent erosion	-

Table 4 – Histopathological assessment of colon sections. Scoring for all 3 criteria produces a maximum score of 11 (Ren et al., 2011).

2.3.5 Myeloperoxidase assay (MPO) assay

Colon tissue (40 mg) was extracted ~4 cm internally from the rectum and washed in PBS by vortexing for 30 seconds before centrifuging at 13,500 g for 5 minutes at 4 °C. The supernatant was discarded, and the pellet homogenized using a sonic dismembrator (Fisher Scientific), followed by two 10 minute freeze/thaw cycles to lyse cell membranes. The samples were then sonicated in 500 µL CTAB buffer (50 mM hexadecetyltrimethylammonium bromide, CTAB, Sigma) in 50 mM potassium phosphate buffer at pH 6.0) for 90 seconds followed by centrifugation at 13,500 g for 5 minutes at 4 °C. In addition to colon samples, hind-paw tissue samples from animals that had been administered intra-plantar complete Freund's adjuvant in a separate study in the lab conducted by Dr Gerard Callejo, were used as a positive control.

MPO assays were performed in duplicates on 96 well microtiter plates. In each well, 10 µL of sample supernatant was combined with 80 µL 0.75 mM H_2O_2 (Sigma) and 110 µL TMB solution (2.9 mM 3,3′,5,5′-Tetramethylbenzidine, TMB, in 14.5% DMSO, Sigma) and 150 mM sodium phosphate buffer at pH 5.4). Optical density was then immediately read at 605 nm for 10 minutes at 30 second intervals using a CLARIOstar plate reader (BMG Labtech). CTAB buffer was added instead of sample for the negative control and the positive control used horseradish peroxidase (Vector Laboratories, SA-5704).

2.3.6 Biotinylated hyaluronan binding protein (HABP) staining

Colon samples collected from the distal portion region of the colon (~4 cm internally from the rectum) were post fixed in 4% PFA for 1 hour at 4 °C and incubated overnight at 4 °C in 30% sucrose for cryoprotection. The colon samples were then embedded in Shandon M-1 Embedding Matrix (Thermo Fisher Scientific), frozen using liquid nitrogen, and stored at -80 °C. Embedded samples were cut using a Leica Cryostat (CM3000; Nussloch, Germany) into 20 µm sections and mounted on glass slides (Superfrost plus, Thermo Fisher Scientific). The slides were washed twice with PBS-Tween before being blocked with an antibody diluent solution (0.2% Triton X-100 and 5% bovine serum albumin in PBS) for one hour at room temperature. Biotinylated hyaluronan binding protein (amsbio, Cat #: AMS.HKD-BC41 [1:200]) was incubated on the slides at 4 °C overnight.

On the second day, sections were washed three times in PBS-Tween and incubated for two hours at room temperature with Alexafluor 488 conjugated streptavidin (Invitrogen, Cat #: S11223 [1:1000]). Slides are then washed three more times with PBS-Tween before being mounted and imaged using an Olympus BX51 microscope (Tokyo, Japan) and QImaging camera (Surrey, Canada). All sections were imaged with the same exposure time (200 ms) and an excitation wavelength of 488 nm with the same brightness and contrast adjustments being made to all images in ImageJ. Negative controls (slides not incubated with the streptavidin conjugate) did not show any fluorescence.

2.4 Quantifying levels of galanin in distal colon tissue

2.4.1 Tissue extract sample preparation

Dissection of tissue was carried out on ice as quickly as possible to prevent degradation of galanin by proteases. Colon tissue (30 mg) ~4 cm internally from the rectum was dissected out and placed in 1.5 mL microfuge tubes, which were then immersed in liquid nitrogen for quick freezing. The tissue samples were then stored at -80 °C until needed.

For 30 mg of tissue, 1.8 mL of complete extraction buffer (100 mM Tris, 150 mM NaCl, 1 mM triethylene glycol diamine tetra-acetic acid (EGTA), 1 mM ethylene diamine tetraacetic acid (EDTA), 1% Trition X-100, 0.5% sodium deoxycholate and 1 mM

phenylmethane sulfonyl fluoride (PMSF)) was added and the sample was homogenized using a sonic dismembrator (Fisher Scientific). Samples were then placed on a shaker for constant agitation for 2 hours at 4 °C, before being centrifuged for 20 minutes at 13,500 g at 4 °C. The supernatants were then aliquoted and stored at -80 °C until needed.

2.4.2 Galanin ELISA

An ELISA was used (Peninsula Laboratories International, Cat #: S-1208; detection range 0.01 – 10 ng/ml^{-1}) to establish the galanin concentration in samples of colon tissue. In brief, 50 µL of sample or standard (provided with the kit) and 25 µL of antiserum was added to each well and incubated at room temperature for 1 hour. 25 µL of the biotinylated tracer was added to each well and incubated at room temperature for 2 hours. The plate was then washed 5 times with enzyme immunoassay (EIA) buffer before adding 100 µL of streptavidin horseradish peroxidase (HRP) to each well and further incubating for 1 hour at room temperature. The plate was then washed another 5 times with EIA buffer, then 100 µL of TMB solution was added to each well and incubated at room temperature for a further 1 hour. The reaction was terminated by adding 100 µL of 2 N HCl to each well and the optical density was read (using CLARIOstar Monochromator Multimode Microplate Reader, BMG Labtech) at an absorbance of 450 nm within 10 minutes of stopping the reaction. The concentration of galanin was determined according to the manufacturer's instructions relative to the standard curve provided.

2.5 Single Cell qPCR of colon-innervating DRG neurones

2.5.1 Primary culture and cell picking

After retrograde labelling of afferents innervating the distal colon (as described in 2.2.1), colon-innervating TL (T13-L1) and LS (L6-S1) DRG were dissected and dissociated as two separate cultures using previously published protocols (Hockley et al., 2016). Dissected DRG were trimmed of axons and connective tissue before being incubated in Lebovitz L-15 Glutamax (Thermo Fisher Scientific, UK) medium containing 1 mg mL^{-1} collagenase (Sigma-Aldrich, UK) and 6 mg mL^{-1} BSA at 37 °C for 15 minutes. This was followed by a 30 minute incubation at 37 °C with L-15 media containing 1 mg mL^{-1} trypsin (Sigma-Aldrich) and 6 mg mL^{-1} BSA. The DRG were then gently triturated with a 1 mL Gilson pipette before collecting, after brief centrifugation

at 1000 rpm, of dissociated cell-containing supernatant. Trituration and centrifugation cycles were then repeated five times. Collected neurones were then plated onto poly-D-lysine-coated coverslips (BD Biosciences, UK) and incubated at 37 °C in L-15 media containing 2 % penicillin/streptomycin, 24 mM $NaHCO_3$, 38 mM glucose and 10 % foetal bovine serum.

Fast Blue positive colonic sensory neurones were detected by 365 nm fluorescence illumination (Cairn Research, UK) and individual neurones were manually collected using a micromanipulator controlled (PatchStar, Scientifica, UK) pulled glass pipette on an adapted inverted Olympus microscope. Neurones were visually assessed prior to collecting and only those not associated with satellite glia cells and free from debris were captured and photographed (DCC1545M, ThorLabs Inc) for cell size analysis. A maximum of 20 neurones per culture, per mouse was collected within 8 hours of plating to minimise the potential for changes in gene expression. A small volume of bath solution was also collected at the end of collecting samples for each culture to provide negative controls as these were subjected to exactly the same protocols as samples containing isolated individual neurones. Labelled neurones were selected randomly.

2.5.2 Single-cell qPCR

Primary cultures of colon-innervating TL and LS neurones were collected as described above. Each cell was collected into a tube containing 5 µL CellDirect 2x reaction buffer (Invitrogen, UK), 2.5 µL 0.2x primer-probe mix, 0.1 µL SUPERase-in (Ambion, TX, USA), 1.2 µL TE buffer (Applichem, Germany) and 0.2 µL Superscript III Reverse Transcriptase-Platinum Taq mix (Invitrogen, UK) and immediately frozen on dry ice. Reverse transcription and preamplification of cDNA was done by thermal cycling (50 °C for 30 minutes, 95 °C for 2 minutes, then 24 cycles of 95 °C for 15 seconds, 60 °C for 4 minutes). Samples were diluted 1:5 in TE buffer and TaqMan qPCR assays were run for each gene of interest (TaqMan assay ID: Galanin-00439056, GalR1-00433515, GalR2-00726392, GAPDH-99999915, TRPV1-01246302 and Gfra3-00494589; Applied Biosystems) using the following cycling protocol of 50 °C for 2 minutes, 95 °C for 10 minutes then 40 cycles of 95 °C for 15 seconds, 60 °C for 1 minute. All single-cell RT-PCR products should express glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as a reference gene and bath control samples were

negative controls. As described above, an image of each cell was taken for post-hoc cell size analysis. Relative expression of marker genes were normalised to GAPDH quantification cycles (CT) using ΔΔCT. In total, 240 colonic sensory neurones were collected (60 from healthy TL, 60 from healthy LS, 60 from DSS-treated TL and 60 from DSS-treated LS) from 6 mice (male, 8-12 weeks, 3 healthy and 3 treated with DSS).

2.6 Statistics

All statistical analyses were performed using GraphPad Prism 6. Half-maximal inhibitory concentration (IC_{50}) values were derived by a sigmoidal dose-response (variable slope) curve using GraphPad Prism software. For ramp distensions, a repeated measures two-way ANOVA with Sidak's post-hoc test was used. Both basal firing and phasic distension data were analysed using a repeated measures one-way ANOVA with Tukey's post-hoc test. For the validation of the DSS model, DSS data were compared to control data using Student's t-test. Statistical significance was set at $P < 0.05$. Data are presented as mean ± SEM, N = number of animals, and n = number of cells.

Chapter 3 – Galanin inhibits the lumbar splanchnic nerve afferent response to mechanical stimuli and inflammatory mediators

3.1 Introduction

3.1.1 Sensation evoked by mechanical activation of colonic afferents

Mechanosensitivity of visceral afferents is central to the relay of conscious sensation in response to bowel distension. One example would be the pain arising following distention around a faecal bolus in a fully or partially occluded bowel (Brookes et al., 2013). The sensitivity of visceral afferents to mechanical stimuli can be modulated by inflammatory mediators, as demonstrated by the visceral hypersensitivity to barostat balloon distension observed in irritable bowel syndrome (IBS) patients (Mertz et al., 1995).

Pain signalling from the GI tract is relayed by primary afferent neurones to the brain via multiple pathways, including the spinomesencephalic, spinohypothalamic, spinoreticular, and spinothalamic tracts (Almeida et al., 2004). The spinothalamic tract, with thalamic projections to the insula, anterior cingulate cortex and somatosensory cortex mediates conscious sensation (Almeida et al., 2004). The spinomesencephalic pathways provide intensity and localisation of stimulus, as well as motivational-affective pain behaviours and integration into higher motor responses. The other pathways activate autonomic responses to visceral sensory input (Almeida et al., 2004; Palecek and Willis, 2003).

In humans, 15 – 40 mmHg pressure in the upper colon (>16 cm internally of the external anal sphincter) induces pain (Ray and Neill, 1947). This pain is abolished following a procedure in which part of the sympathetic nerve trunk in the thoracolumbar region is destroyed and can provide pain relief by blocking signalling of sympathetic nociceptors in the lumbar region (Bentley and Smithwick, 1940; Ray and Neill, 1947). Pain induced by distention of the distal colon (≤16 cm internal from the external anal sphincter) is however unaffected by sympathectomy, further demonstrating dual innervation of the GI tract by the LSN and PN (Ray and Neill, 1947).

Various stimuli can be used to activate visceral pain pathways. These include *in vivo* slow ramp distensions, rapid phasic distensions by balloon or fluid filling the bowel, chemically induced contractions, and *ex vivo* blunt probe, or circumferential stretch and stroke around distinct receptive fields of afferent fibres (Ray and Neill, 1947; Brierley et al., 2004). These methodologies have enabled the successful identification of various mechanistic and biochemical pathways relevant to visceral pain. It is also important to understand the rationale and limitations behind their uses when interrogating visceral afferent function.

3.1.2 Experimental stimulation

In rodents, *in vivo* activation of the pelvic pain pathway using colorectal distension (CRD) can be monitored using pseudoaffective reflexes and the visceromotor response (VMR) (Ness et al., 1990). A typical protocol would consist of a series of balloon inflations within the colon to a set intracolonic pressure distensions (15, 30, 45, and 75 mmHg) at 5-10 minute intervals (Shinoda et al., 2009). Responses to CRD initially decrease, due to tachyphylaxis, but then stabilise after 4-6 distensions, enabling the effects of intervention (e.g. pharmacological compound administration) to be investigated (Kamp et al., 2003; Sivarao et al., 2007).

VMR involves both ascending and descending pathways of the brainstem, although it does not require higher order processing (Ness and Gebhart, 1988). In mice, the response itself is initiated by the LS sensory pathway innervating the colon (i.e. pelvic nerve), as demonstrated by severing of the LSN (TL sensory pathway) having little effect on VMR following CRD (Kyloh et al., 2011). However, following inflammation, the TL pathway has also been shown to contribute to the VMR (Traub, 2000). These findings are consistent with observations seen in humans where bilateral sympathectomy (T7 – L3) does not affect the VMR to CRD within 16 cm of sphincter, showing that the response is mediated by pelvic pathway (Ray and Neill, 1947). By contrast, CRD in the colon above 16 cm from the sphincter is mediated by the TL pathway (Ray and Neill, 1947). In summary, VMR during CRD poorly models TL activation and associated behaviours.

Different pressures used for CRD can elicit different sensations in humans, low pressures evoking urge (8 mmHg) and high pressures evoking pain (16 mmHg) (Kwan et al., 2002). Comparably, relaxation of the anal sphincter occurs at 13 mmHg in rats and VMR at 22 mmHg (Ness and Gebhart 1988). From *in vivo* S1 afferent fibre (PN) recordings and jejunal afferents, rapid phasic distensions evoke a greater nerve discharge than slow ramp distension to the same pressure (Booth et al., 2007; Sengupta and Gebhart, 1994). This demonstrates that the experience of pain is dependent on temporal properties of the stimulus and not just the magnitude of stimulation.

A different approach to studying pain is measuring afferent firing to noxious stimuli in *ex vivo* preparations, which can be conducted in both rodent and human tissue (Hockley et al., 2018). These include recordings from the PN and LSN innervating the distal colon as either a tubular or flat sheet (opened along the mesenteric border) preparation. Examples of these preparations include: intracellular recordings from L6 DRG neurones combining the characterisation of high and low threshold mechanosensors with subsequent immunohistochemistry (Malin et al., 2009), extracellular recordings from LSN and pelvic afferent fibres characterising the role of $Na_V1.9$ in visceral pain (Hockley et al., 2014), and chemically-induced contractions of a mouse colon to model the occlusion observed in human bowel diseases (Zagorodnyuk et al., 2012).

3.1.3 Colonic mechanosensitivity and mechanotransduction

Several experimental models have been used to further our understanding of the molecular mechanisms responsible for mechanotransduction at afferent terminals of colonic sensory neurones. A number of ion channels have been implicated in visceral mechanosensation, including TRPV4, Piezo2, TRPA1 and ASICs (Coste et al., 2010; Harrington et al., 2018). Moreover, numerous receptors and ion channels that are not themselves mechanosensitive can modulate afferent mechanosensitivity, either through intracellular signalling pathways (e.g. GPCRs like angiotensin II type I, bradykinin receptor B2 receptors, or protease-activated receptor 2 (PAR_2)) (Coelho et al., 2002), or through modulating electrical excitability

(Hockley et al., 2014; Peiris et al., 2017). Voltage-gated K^+ ion channels also contribute to membrane excitability and play a key role in action potential repolarisation meaning that they strongly regulate neuronal firing rate. Two currents in particular play an important role, the transient I_A current and the sustained rectifying I_K current (Cobbett et al., 1989).

Many of the receptors and ion channels mentioned are brought into play during inflammation, such that inflammatory mediators bind to receptors and activate secondary messenger pathways that act to both regulate baseline activity and the response to mechanical stimuli due to the sensitising effects that they have on mechanically sensitive ion channels and/or those ion channels regulating membrane excitability. An example of this would be the release of ATP as a consequence of tissue damage leading to the activation of P2X receptors and other purinoceptors causing an increase in intestinal afferent mechanosensation (Kirkup et al., 1999; Wynn et al., 2003). During inflammation afferent hypersensitivity is also affected by coordinated reduction in I_A and I_K currents and an increase in tetrodotoxin-resistant (TTX-R) Na^+ currents leading to increased neuronal excitability (Beyak et al., 2004; Dang et al., 2004; Stewart et al., 2003).

Multiple inflammatory mediators are released in inflamed tissues and act synergistically to sensitise afferent endings. Whilst one mediator (for example bradykinin) could cause sensitisation, an inflammatory soup (IS) is used to better mimic the mix of mediators present in inflammatory conditions (Shinoda et al., 2009). For this reason, an IS consisting of bradykinin, ATP, PGE_2, 5-HT, and histamine is often used to induce a robust afferent hypersensitivity to mechanical stimulus and direct stimulation of afferents (Su and Gebhart, 1998). The IS is used to investigate how afferent function is influenced by inflammation and also characterise ‘silent’ afferents, those afferents that are mechanically insensitive, but become responsive to mechanical stimuli following exposure to chemical and inflammatory mediators (Brierley et al 2005a, Brierley et al 2005b). Intracolonic application of IS increases resting activity in approximately 60% of fibres and also reduces the activation thresholds for a subset of high-threshold fibres and

sensitises silent afferents (Feng and Gebhart 2011), thus correlating with the hyperalgesia and allodynia experienced by those individuals with inflammatory pain. The use of an IS also produces *in vivo* hypersensitivity to CRD following TNBS treatment (a model of colitis), which correlates with afferent hypersensitivity in subsequent *ex vivo* afferent analysis (Kiyatkin et al., 2013).

3.1.4 Galanin and inflammatory mediators

The expression of galanin and its receptors is altered throughout pain pathways in experimental inflammatory conditions and therefore it is likely to have functional implications for modulating inflammatory pain. For example, in DRG sensory neurones galanin levels are reduced following hind-paw injection of carrageenan, but at the same time galanin expression is increased in the dorsal horn (Ji et al., 1995; Zhang et al., 1998). In the same model, GalR1 is transiently downregulated and GalR2 is upregulated in DRG neurones (Sten Shi et al., 1997; Xu et al., 1996). To date, the expression of galanin and its receptors in colonic afferent terminals, and how these change under inflammatory conditions, remains unclear.

Functionally, galanin modulates mechanosensitivity in gastro-oesophageal vagal afferents in a predominantly inhibitory manner in healthy mice most likely through GalR1 activation with GalR2 playing a minor role in potentiation (Page et al., 2007, 2005b). However, the role of galanin in peripheral pain signalling remains unclear. Galanin has been reported to have pro-nociceptive effects via GalR2 and anti-nociceptive effects through GalR1 when capsaicin is injected into the hind-paw of mice (Jimenez-Andrade et al., 2006, 2004). Based on previous studies (Page et al., 2007, 2005b) and the comparative expression profiles of GalRs in colonic sensory neurones (Hockley et al., 2019), I hypothesized that galanin would inhibit LSN mechanosensitivity and, furthermore, that galanin would show antinociceptive effects in the presence of inflammatory mediators.

3.2 Aims

1. To investigate the effects of galanin on the response of LSN afferents to mechanical distension in an *ex vivo*, intact distal colon preparation.

2. To determine the contribution of GalR1 and GalR2 to the any effect mediated by galanin on LSN function.

3. To investigate the effect of an IS on LSN activity and how this is modulated by galanin.

3.3 Results

3.3.1 Galanin is present in the distal colon and likely involved in nociceptive signalling

TRPV1 and IB4 are markers of two major classes of nociceptors, peptidergic (TRPV1) and non-peptidergic (IB4). TRPV1 expressing nociceptors project predominantly to laminae I and IB4-stained nociceptors project to laminae II (Snider and McMahon, 1998). When examining sections of mouse lumber spinal cord it was observed that galanin immunofluorescence was present in neurones in the dorsal horn of the spinal cord and shows signs of co-localisation mainly with TRPV1 and, to a lesser extent, IB4, suggesting that galanin is expressed at nerve terminals in neurones involved in nociception (Fig 3-1B and C).

In transverse sections of the distal colon, galanin immunofluorescence was seen in multiple layers throughout the colon, but particularly in the circular muscle layer (Fig. 3-1E). Previous work has demonstrated that galanin is expressed in multiple cell types of the colon including macrophages and epithelial cells (Koller et al., 2019). The high level of galanin expression between the muscle layers suggests that galanin is expressed by fibres of the myenteric plexus. A detailed analysis of galanin expression in colonic sensory DRG neurones was also conducted (see Section 5.3.2).

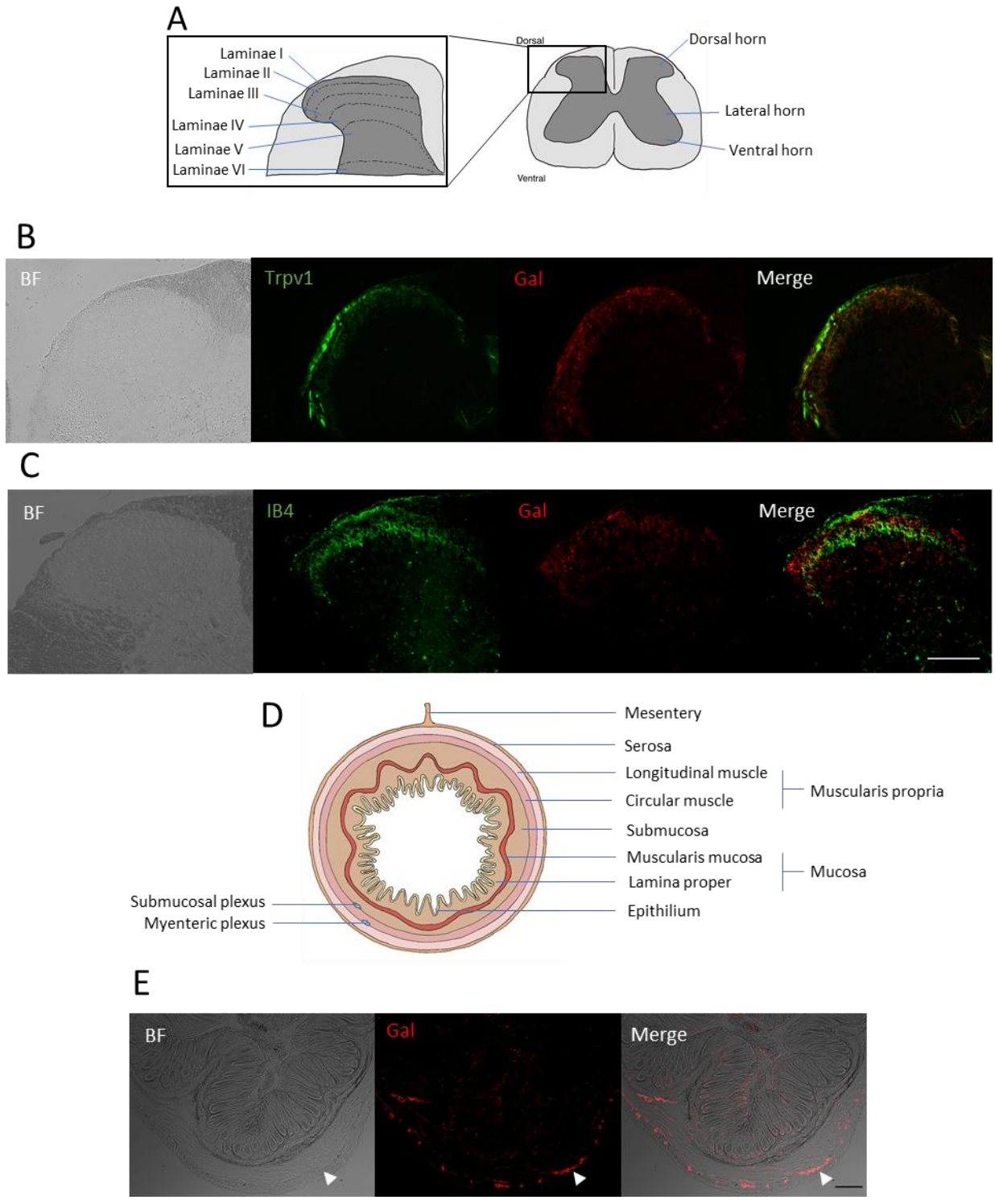


Figure 3-1. Expression of galanin in the spinal cord and distal colon.

(A) Diagram of transverse lumbar spinal cord sections with enlargement and detail of the dorsal horn (black box), outlining the Rexed laminae. In the spinal cord dorsal horn, galanin shows high coexpression with TRPV1 (marker of laminae I; B) and low colocalisation with IB4 (marker of laminae II; C) using IHC. (D) Diagram of a transverse section of colon showing location of myenteric plexus. Galanin is particularly localised between the muscle layers of the colon (indicated with white arrowhead) suggesting localisation in the myenteric plexus (E).

3.3.2 Galanin inhibits mechanically evoked neuronal excitation in LSN sensory afferent neurones

To determine the effects of galanin on peripheral terminals of colonic sensory neurones, I used whole-nerve, *ex vivo* electrophysiological recordings of the LSN with intact tubular colonic preparations that enable control over intraluminal pressure. The LSN is particularly relevant in the transduction of conscious sensation from the colon, including pain signalling, as it has been shown to be predominantly composed of nociceptors (Brierley et al., 2004; Hughes et al., 2009). Using this *ex vivo* preparation, phasic distension of the colon to a noxious pressure (80 mmHg; Ness and Gebhart, 1991) led to a robust peak phase of increased afferent firing, followed by a slight decrease and a sustained phase of firing for the rest of the 1 minute distension (Fig. 3-2 and see Fig. 2-3 for details of analysis). Responses to repeated distensions, following 9 minute intervals, decreased for the first 3-4 distensions until stabilising for subsequent distensions (Fig. 3-2B). This response remained stable for the remainder of the protocol (Fig. 3-2A).

Once a stable response had been established (e.g. after 3 distensions), galanin was applied by 20 mL bath application and the effects of the application on subsequent responses to mechanical distention were observed. In initial experiments, the peak effect of galanin application was observed to occur approximately 20-30 minutes after bath superfusion during the third post-galanin distension before washing off (Fig. 3-2C). Therefore, the third post-galanin distention timepoint was used to measure the maximum effect of galanin application in subsequent experiments.

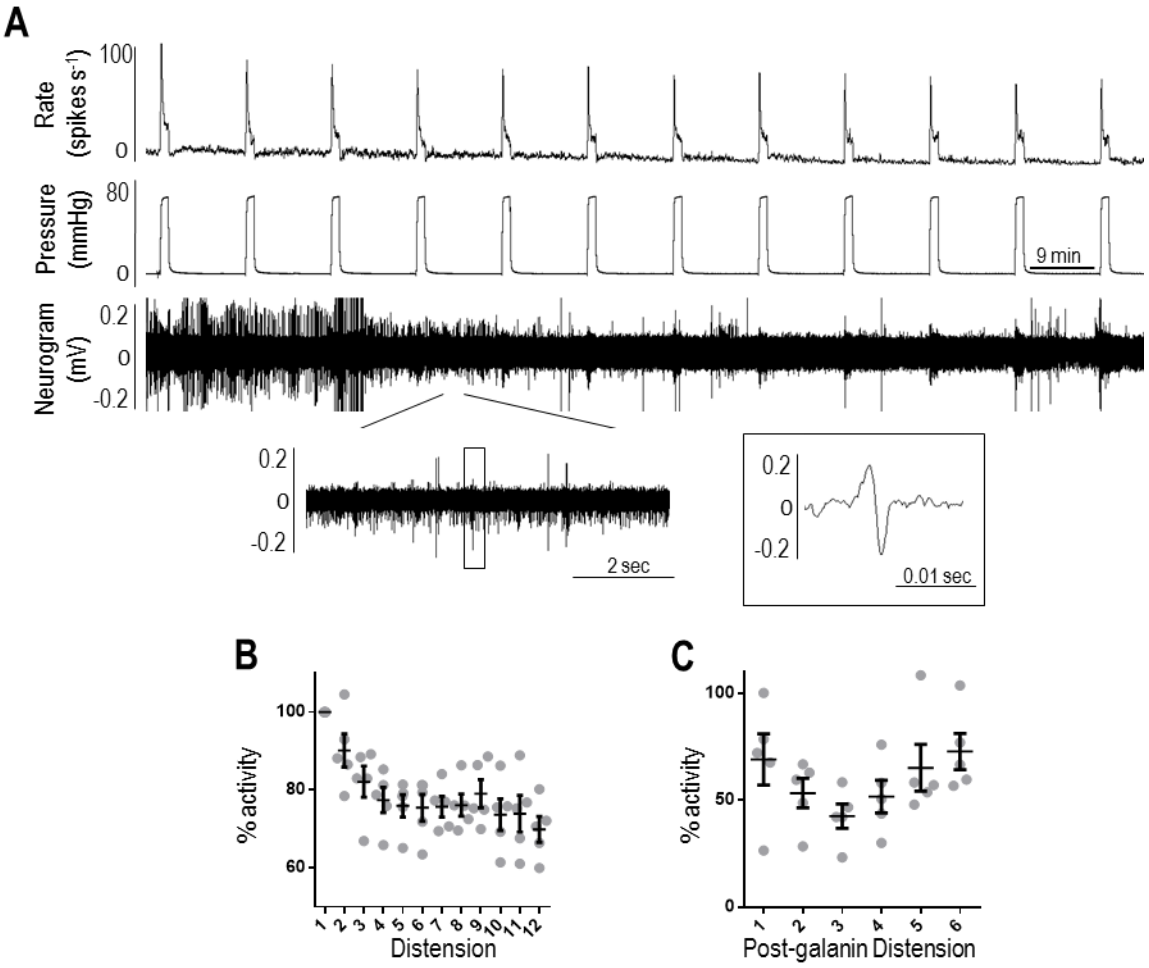


Figure 3-2. Repeated phasic distensions produce a robust LSN response that is inhibited by galanin

(A) Example raw trace and rate histogram of the colonic LSN response to repeated rapid phasic intraluminal distension (0 to 80 mmHg), showing a robust LSN response to a repeated noxious mechanical stimulus. Below, expanded sections of basal activity (left) and a single action potential has been expanded further (black box, right). (B) Percentage activity from the first distension of peak firing frequency over the first 12 distensions showing the initial decrease in response that eventually stabilises around distension 3-4. (C) Percentage peak firing frequency of the first 6 post-galanin (20 mL, 500 nM) distensions compared to the control response established pre-galanin. N = 5

Using a range of galanin concentrations (1 nM, 10 nM, 100 nM, 500 nM, and 1 μM), it was observed that galanin inhibited LSN activity in a dose-dependent manner, with the peak effect of galanin occurring at 500 nM (Fig. 3-3A). For example, peak firing frequency (53.3 ± 6.4 spikes/s vs 25.2 ± 2.6 spikes/s, N = 5, P = 0.0008, unpaired t-test), phase I average firing frequency (average of initial 15 seconds of 1 minute phasic distension: 30.3 ± 2.8 spikes/s vs 12.8 ± 1.1 spikes/s, N = 5, P = 0.0005, unpaired t-test) and phase II average firing frequency (average of last 45 seconds of 1 minute phasic distention: 18.6 ± 3.1 spikes/s vs 9.3 ± 2.1 spikes/s, N = 5, P = 0.013, unpaired t-test) were all significantly decreased by 500 nM galanin. Additionally, galanin also significantly inhibited spontaneous firing, with 500 nM galanin reducing basal spontaneous activity by a ~70% (1 minute average preceding phasic distension: 3.9 ± 0.7 spikes/s vs 1.2 ± 0.5 spikes/s, N = 5, P = 0.0276, unpaired t-test). All inhibitory effects were dose-dependent having an IC_{50} of 153.7 nM for basal nerve activity (Fig. 3-3Bi), an IC_{50} 65.7 nM for peak firing in response to phasic distension (Fig. 3-3Bii), an IC_{50} 84.8 nM for phase I average firing in response to phasic distension (Fig. 3-3Biii) and an IC_{50} of 112.7 nM for phase II average firing in response to phasic distension (Fig. 3-3Biv).

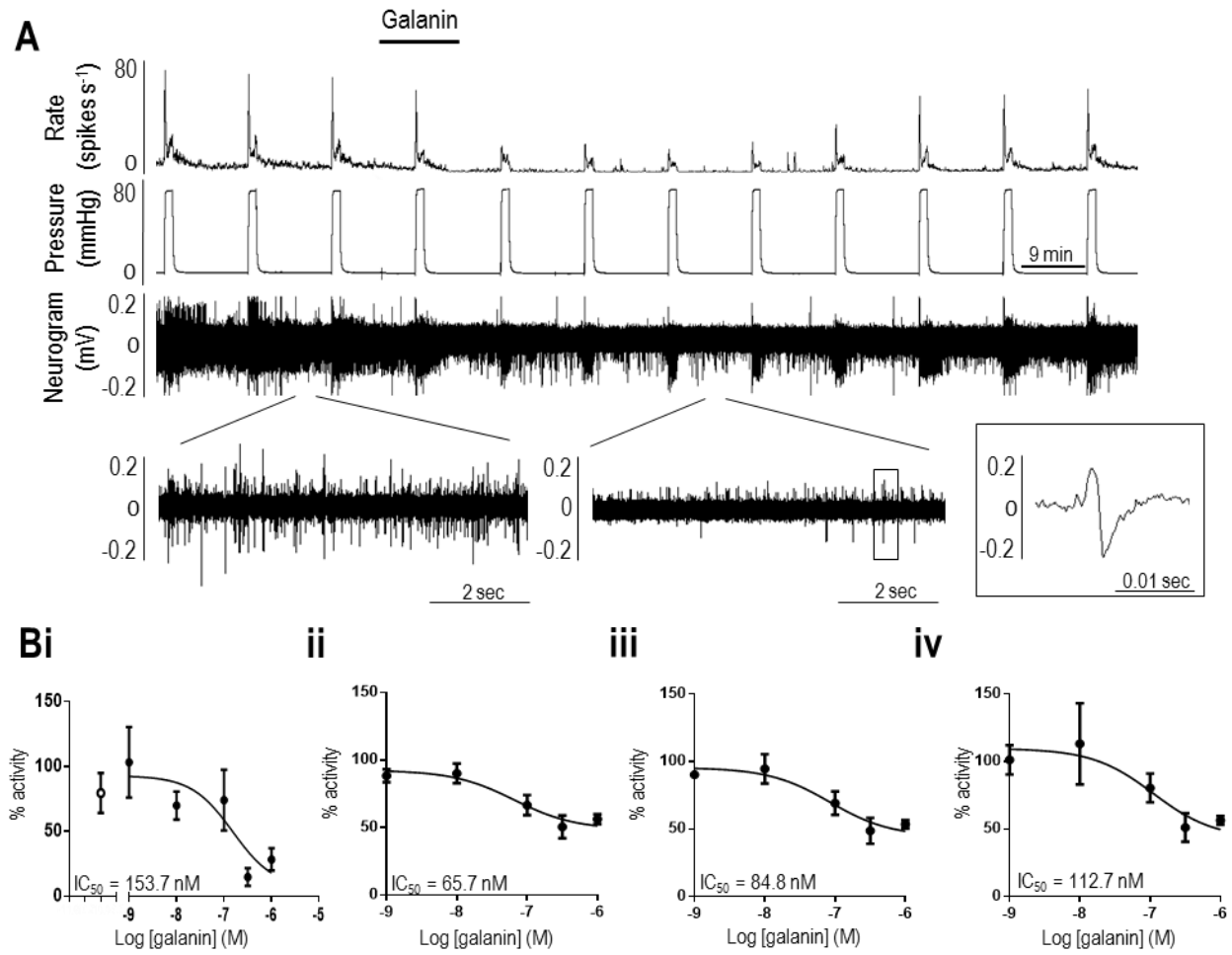


Figure 3-3. Dose-dependent effect of galanin on LSN mechanosensitivity

(A) Example raw trace and rate histogram of the colonic LSN response to repeated rapid phasic intraluminal distension (0 to 80 mmHg), showing reversible inhibition of the response by galanin. Below, expanded sections of basal activity before (left) and after (right) galanin application and a single action potential has been expanded further (black box). Galanin (500 nM, 20 mL) bath application indicated by the black bar above the rate histogram. (Bi) Basal nerve activity is dose-dependently inhibited by galanin (white circle indicating vehicle) as are the peak (ii), phase I average (iii) and phase II average (iv) firing frequencies; N = 5 for 1 nM, 10 nM, 500 nM and 1000 μM concentration, and N = 6 for 100 nM.

3.3.3 Galanin inhibits mechanically evoked neuronal excitation via GalR1

Next, I investigated the effect of GalR agonists on LSN activity to determine the involvement of different GalRs in the effects produced by galanin, using the same protocol as conducted with galanin (Fig. 3-4A). As discussed in Section 1.6, GalR1 signalling is predominantly G_i mediated leading to hyperpolarisation and therefore reduced neuronal excitability (Fuxe et al., 1998; Smith et al., 1998).

The synthetic peptide M617 (galanin(1-13)-Gln(14)-bradykinin(2-9)-amide) is a selective agonist of GalR1 (Lundstrom et al., 2005; Sollenberg et al., 2010) and it significantly inhibited the LSN response to phasic distention for peak (Fig. 3-4Bi; P = 0.0015, N = 4, paired t-test), phase I average (Fig. 3-4Bii; P = 0.0002 , N = 4, paired t-test) and phase II average (Fig. 3-4Biii; P = 0.0382, N = 4, paired t-test) firing frequency. The inhibitory effects of M671 were of a similar magnitude to those induced by galanin, e.g. M671 attenuated peak firing frequency by 41.4 ± 4.3%, phase I average firing frequency by 44.5 ± 5.9% and phase II average firing frequency by 39.8 ± 6.8%, similar to 500 nM galanin which attenuated peak firing frequency by 49.4 ± 8.5%, phase I average firing frequency by 51.2 ± 9.6% and phase II average firing frequency by 48.8 ± 10.6% (galanin N = 5, M617 N = 4).

Spexin is an endogenous neuropeptide expressed in various nervous and endocrine tissues with relatively little known about its function, but it has been proposed to modulate cardiac (increased arterial pressure and decreased heart rate) and renal function (decreased urine flow rate), as well as having anti-nociceptive activity in mice (Toll et al., 2012). Unlike galanin, which has very similar affinity for all 3 GalRs, spexin has been reported to act as an agonist of GalR2/3 (Spexin EC_{50} for GalR2 = 161 nM, EC_{50} for GalR3 = 626 nM), but not GalR1 (Kim et al., 2014; Lv et al., 2019). Following application of spexin, no significant change in peak (Fig. 3-4Ci; P = 0.245, N = 4, paired t-test), phase I average (Fig. 3-4Cii; P = 0.741, N = 4, paired t-test) or phase II average (Fig. 3-4Ciii; P = 0.218, N = 4, paired t-test) firing frequency was observed.

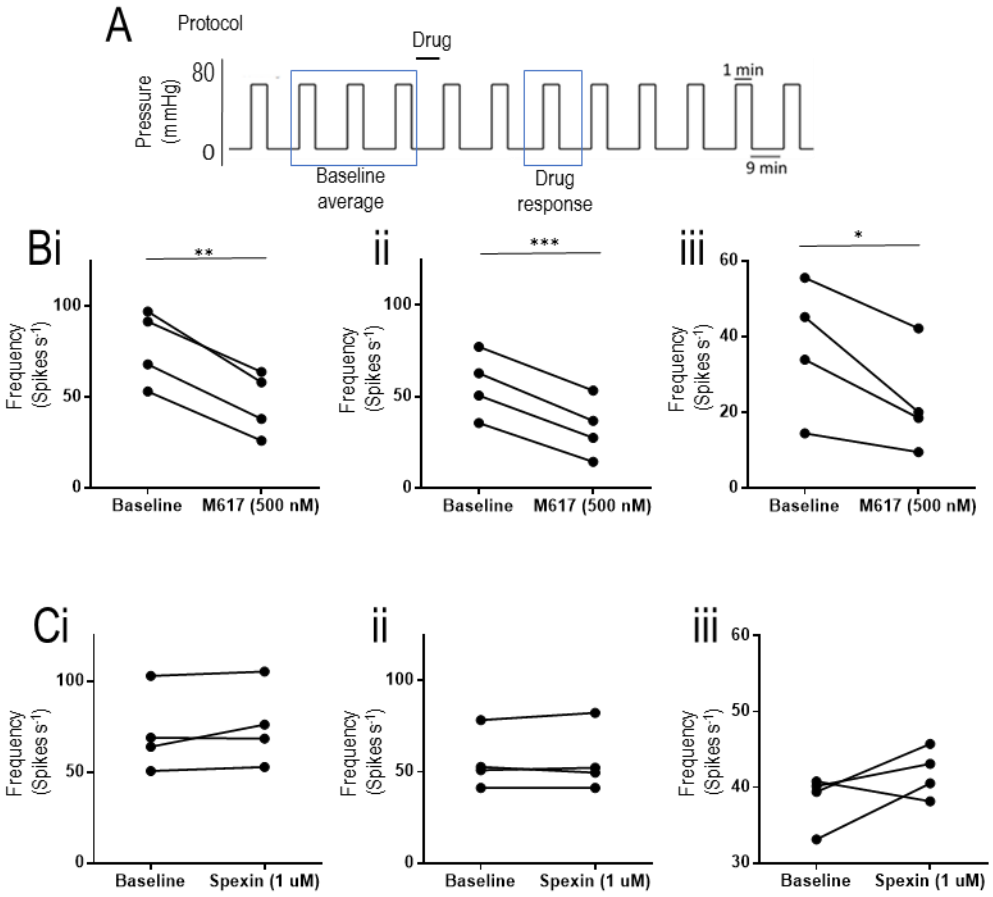


Figure 3-4. Effects of GalR agonists on LSN mechanosensitivity in multi-unit recordings

(A) Schematic of pressure protocol used for LSN electrophysiological recordings. Distensions used for analysis indicated with blue box, drug application indicated by black bar. M671 (GalR1 agonist) significantly attenuates firing frequency in both peak (Bi; P = 0.0015, N = 4, paired t-test), phase I average (Bii; P = 0.0002, N = 4, paired t-test) and phase II average (Biii; P = 0.0382, N = 4, paired t-test) firing frequency. Spexin (GalR2/3 agonist) does not significantly modulate the peak (Ci), phase I average (Cii), or phase II average (Ciii) firing frequency in response to a distension pressure of 80 mmHg.

Next, the effects of a GalR2 antagonist on LSN activity was investigated to further determine the potential role of GalR2 in the overall inhibitory effect of galanin. The peptide M871 (galanin(2-13)–Glu–His–(Pro)3–(Ala–Leu)2–Ala amide) is a selective antagonist of GalR2/3 (with lower affinity for GalR3; GalR2 K_i = 13 nM, GalR3 K_i = >10 μM) (Sollenberg et al., 2010, 2006) and thus it is possible that if galanin usually modulates all 3 GalRs that blockade of GalR2/3 might reveal a greater inhibition of LSN activity to suggest a tonic role for GalR2. An initial application of a low concentration of galanin (10 nM) superfused into the bath gave us a control response. After a washout period, another application of galanin (10 nM), this time mixed with M871 (1 μM) was given. By comparing the response to the first galanin application to the response from the second application, the effects M871 could be determined (Fig. 3-5A).

The initial application of galanin did not significantly reduce peak firing (Fig. 3-5Bi), phase I average firing (Fig. 3-5Bii), or phase II average firing (Fig. 3-5Biii) as expected from previous experiments (Fig. 3-3B). If GalR2 is significantly contributing to the whole-nerve response, adding M871 with a low dose of galanin might counteract the excitatory effect of any GalR2 activation and thus potentially reveal a significant inhibition of the LSN response to phasic distension at only 10 nM galanin. However, application of galanin + M871 did not significantly change the LSN response to phasic distention in peak (Fig. 3-5Bi; P = 0.92, N = 3, repeated measures one-way ANOVA with Tukey's test), phase I average (Fig. 3-5Bii; P = 0.93 , N = 3, repeated measures one-way ANOVA with Tukey's test) or phase II average (Fig. 3-5Biii; P = 0.86, N = 3, repeated measures one-way ANOVA with Tukey's test) firing frequency. These results support the lack of response from spexin and suggest that GalR2 does not significantly contribute to galanin's inhibitory effect on LSN mechanosensitivity in the healthy intact colon.

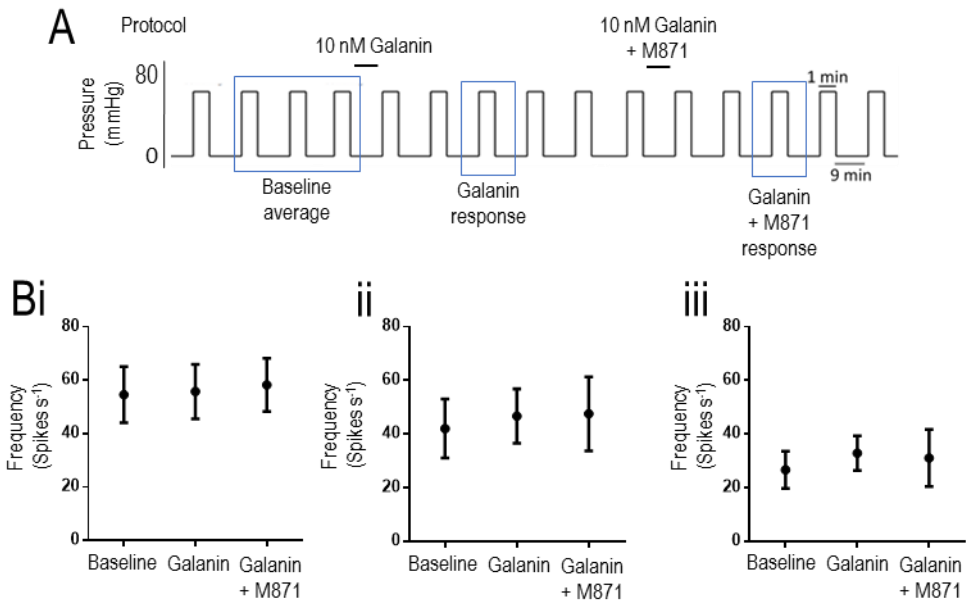


Figure 3-5. Effects of the GalR2 antagonist M871 on mechanosensitivity in multi-unit LSN recording

(A) Schematic of pressure protocol used for the electrophysiological recordings. Distensions used for analysis indicated with blue box, drug applications indicated by black bar. M871 (1 μM) did not significantly change firing frequency in peak (Bi), phase I average (Bii) and phase II average (Biii) firing frequency, N = 3.

3.3.4 Galanin Inhibits chemically evoked neuronal excitation in LSN afferent neurones

I next investigated the role of galanin in the afferent response to inflammatory mediators. To do this, whole-nerve recordings were made as before. A mixture of inflammatory mediators, an inflammatory soup (IS), as used previously (Hockley et al., 2014; Su and Gebhart, 1998), was perfused intraluminally (Fig. 3-6A). Application of the IS (consisting of 1 mM ATP, 10 μM histamine, 10 μM PGE_2, 1 μM bradykinin, and 10 μM serotonin) was initiated after the third phasic distention and continued for the duration of the experiment (Fig. 3-6B). In another group, 500 nM of galanin was intraluminally perfused (Fig. 3-6C) and in a final group a mixture of both IS and 500 nM of galanin was intraluminally perfused (Fig. 3-6D). These three groups along with a control group were compared to determine the effects of galanin on IS-mediated excitation and sensitisation of LSN activity.

The basal LSN activity significantly increased by 77.9 ± 13.4% ($P < 0.001$, N = 6, one-way ANOVA with Tukey's test) following application of IS. As expected from previous experiments, galanin significantly reduced the basal activity of LSN by 64.5 ± 7.5 % ($P < 0.001$, N = 6, one-way ANOVA with Tukey's test). A combination of IS and galanin did not significantly change the basal firing from the control group (Fig. 3-6Fi) suggesting that they cancel each other out.

To further investigate colonic hypersensitivity caused by IS application and the effects of galanin upon this hypersensitivity, a slow ramp distention up to a noxious pressure of 80 mmHg was used, pressure up to 20 mmHg is considered innocuous (Ness and Gebhart 1988; Fig 3-6A). As others have reported, there was a linear correlation between whole-nerve activity and pressure, reaching a maximum firing rate of 23.99 ± 4.69 spikes s^{-1} (N = 6) in the control group. As expected, IS produced an increased maximum firing rate to 31.45 ± 4.48 spikes s^{-1} (Fig. 3-6B; N = 6), whereas galanin inhibited the maximum rate to 14 ± 5.69 spikes s^{-1} (Fig. 3-6C; N = 6). The combination of IS and galanin lead to a maximum firing rate of 27.43 ± 7.33 spikes s^{-1} that was not significantly different to control (Fig. 3-6D; N = 6).

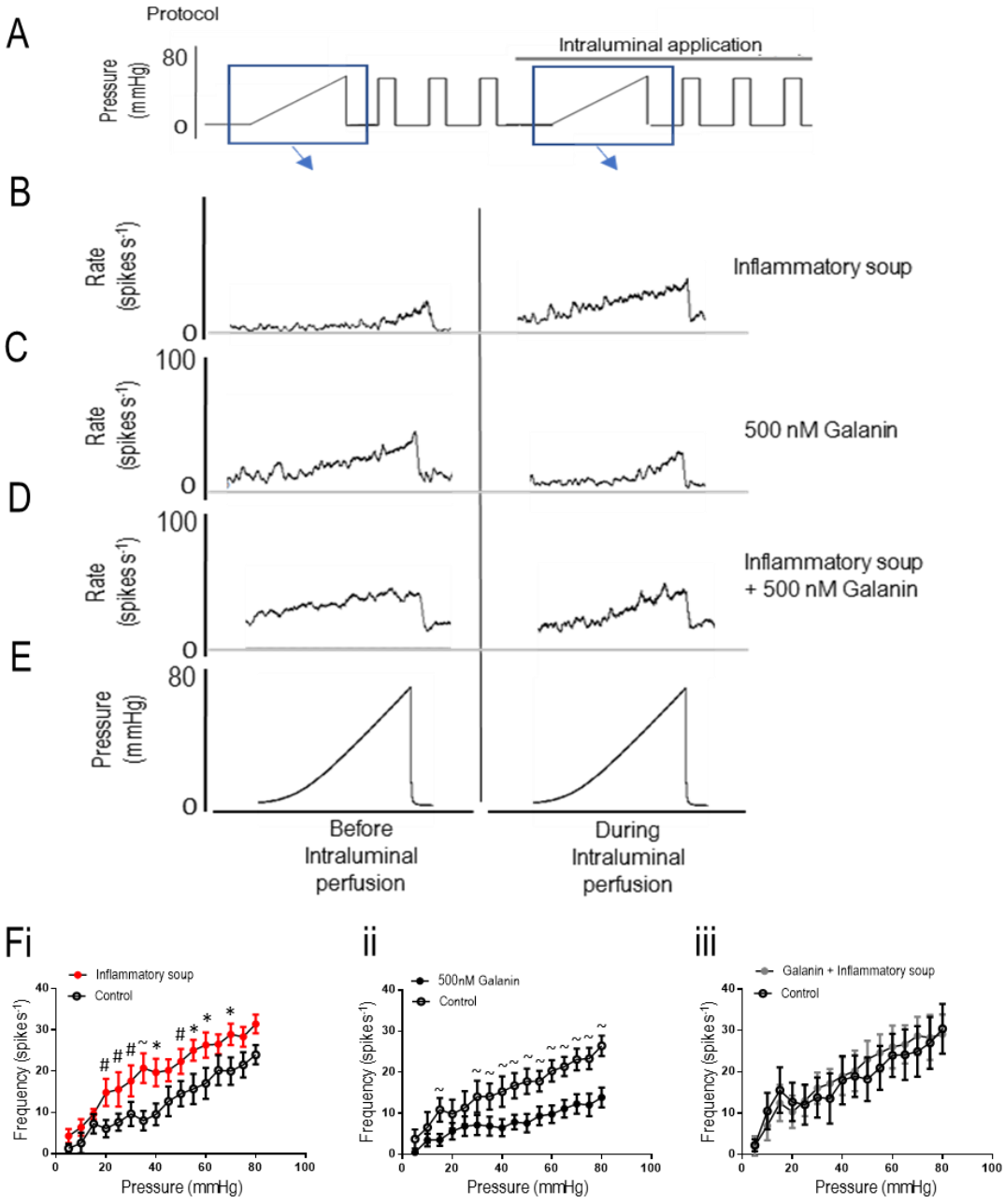
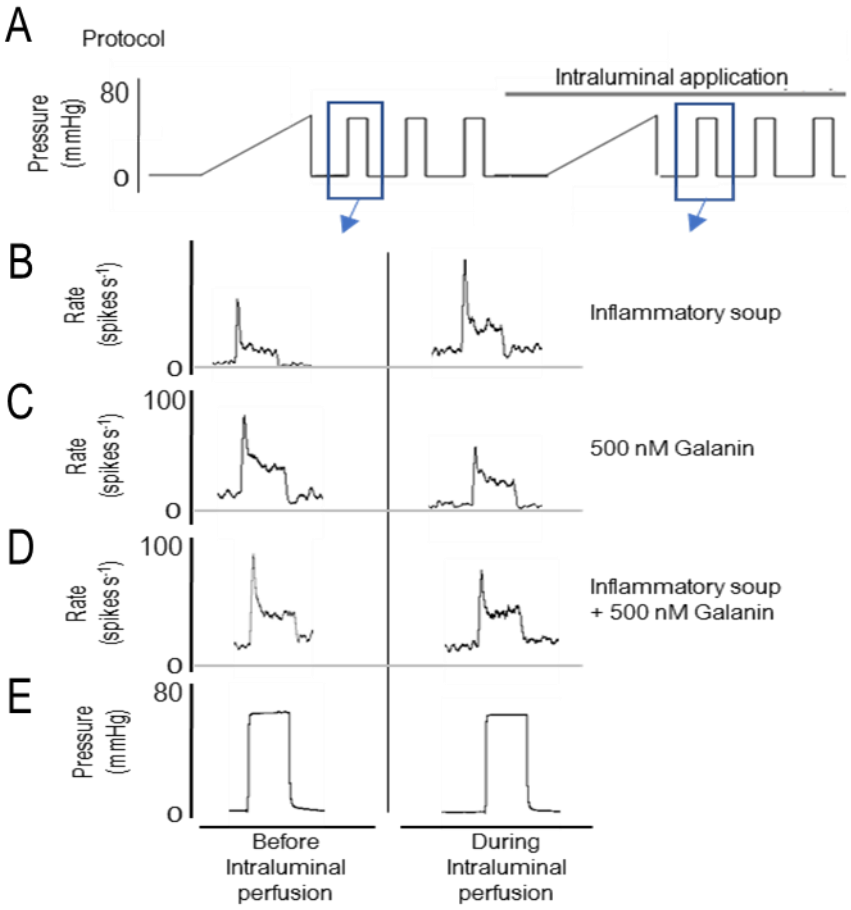


Figure 3-6. Effects of galanin and IS on LSN response to ramp distension

(A) Schematic of pressure protocol used in the multi-unit recordings. Ramp distensions demonstrated below indicated with blue box, intraluminal application of drug indicated by black bar above the protocol. (B) Example raw trace of ramp distension before and after the intraluminal application of IS, (C) galanin (500 nM), (D) and a combination of IS and galanin (500 nM) with (E) sample pressure histograms below. (F) Response profiles to ramp distension before and during intraluminal application of IS (Fi), 500 nM galanin (Fii) or a combination (Fiii) (two-way repeated-measures ANOVA with Sidak's post-hoc test, N = 6). Significance indicated by # = $P < 0.05$, * = $P < 0.01$, ~ = $P < 0.001$

When examining responses to phasic distention, IS significantly increased the peak firing frequency (37.6 ± 13.7 %, $P < 0.05$, N = 6, one-way ANOVA with Tukey's test) whereas galanin produced a significant decrease in peak firing in response to distension (41.4 ± 2.9 %, $P < 0.01$, N = 6, one-way ANOVA with Tukey's post-hoc test). Intraluminal co-application of IS and galanin did not produce a significant change in peak afferent firing in response to phasic distension compared to control preparations (Fig. 3-7Fii). A similar pattern was observed for phase I average firing frequency (IS increased by 33 ± 13.1 %, $P < 0.05$, N = 6, one-way ANOVA with Tukey's post-hoc test and galanin decreased by 36.3 ± 2.6 %, $P < 0.01$, N = 6, one-way ANOVA with Tukey's post-hoc test) with intraluminal co-application of IS and galanin not producing a significant change in phase I average firing in response to phasic distension (Fig. 3-7Fiii). IS application also significantly increased phase II average firing (48.8 ± 14.7 %, $P < 0.05$, N = 6, one-way ANOVA with Tukey's post-hoc test), however galanin did not produce a significant inhibition of sustained firing frequency. IS-induced mechanical hypersensitivity was lost when IS was combined with 500 nM galanin (Fig. 3-7Fiv), indicating that galanin may directly prevent the development of mechanical hypersensitivity of LSN afferents induced by IS.


A
Protocol
Intraluminal application
Pressure (mmHg)
80
0
B
Rate (spikes s-1)
Inflammatory soup
C
100
500 nM Galanin
D
Inflammatory soup + 500 nM Galanin
E
Before Intraluminal perfusion
During Intraluminal perfusion

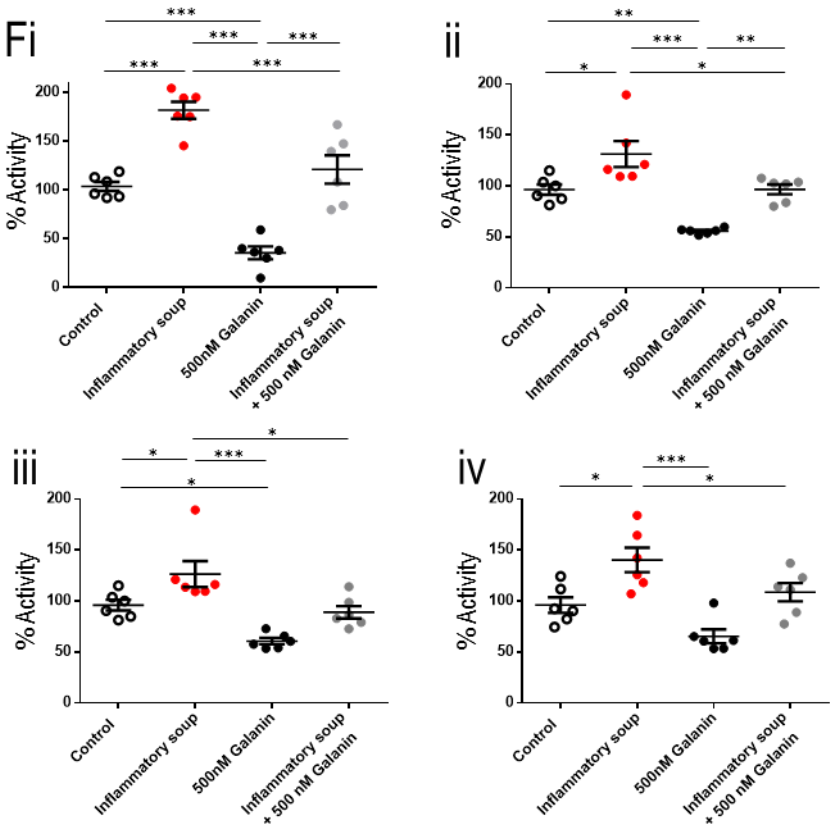

Fi
ii
iii
iv
% Activity
Control
Inflammatory soup
500nM Galanin
Inflammatory soup + 500 nM Galanin

Figure 3-7. Effects of galanin and IS on LSN response to phasic distension

(A) Schematic of pressure protocol used in the multi-unit recordings. Phasic distensions demonstrated below indicated with blue box, intraluminal application of drug indicated by black bar above the protocol. (B) example raw trace of phasic distension before and after the intraluminal application of IS (C) galanin (500 nM), (D) and a combination of IS and galanin (500 nM) with (E) sample pressure histograms below. (Fi) Changes in the basal nerve activity after intraluminal application of either IS, galanin, or a combination. Changes in peak (Fii), phase I average (Fiii), and phase II average (Fiv) firing frequency in response to phasic distension. Significant differences between groups tested by one-way ANOVA with Tukey's post-hoc test, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ (N = 6).

3.4 Discussion

Galanin is a neuropeptide with antinociceptive effects in both the central and peripheral nervous systems (Jimenez-Andrade et al., 2005; Liu and Hökfelt, 2002; Wiesenfeld-Hallin et al., 2005) and here I make the first report of the actions of galanin on colonic sensory activity. The data presented demonstrate potent inhibition of LSN responses to noxious mechanical stimulation and the mechanical hypersensitivity induced by an IS. Activation of GalR1 resulted in similar levels of inhibition to a comparable concentration of galanin, whereas neither a GalR2 agonist nor a GalR2 antagonist produced any significant effect. Combined, these results suggest that galanin acts predominantly via the inhibitory GalR1 to inhibit LSN responses to mechanical activation. In conclusion, I have discovered that galanin has a role in modulation of extrinsic sensory afferents in the distal colon. This fits alongside the previously demonstrated inhibitory roles of galanin in the upper GI tract extrinsically and intrinsically (control of gut secretion and motility) and parallels the function of galanin in somatic sensory innervation (Flatters et al., 2003; Heppelmann et al., 2000; Page et al., 2005b). Overall, these inhibitory effects suggest that GalR1 could be a potential target for treating pain arising from the colon.

Multi-unit recordings of LSN activity show that not only does galanin impact baseline firing, but that it can also inhibit responses evoked by distension of the distal colon to noxious pressures and the mechanical hypersensitivity induced by addition of inflammatory stimuli. This suggests the coupling of GalR1 to integral regulators of neuronal excitability. GalR1 predominantly couples to G_i (Branchek et al., 2000), therefore, one possibility is that through activation of GIRK1 by activated G-protein βγ-subunits neurones being hyperpolarised. This hypothesis is supported by the fact that GalR1 is co-expressed with GIRK1 in TL neurones, but little to no co-expression with other GIRKs (Hockley et al., 2019; Fig. 3-8). Multiple populations of sensory afferents innervate the distal colon with differing sensitivities to mechanical stimuli (e.g. stretch, stroke and von Frey hair probing of their receptive fields) (Brierley et al., 2004). These groups were not characterised in this study, although an inhibitory effect of galanin was observed across the full range of distension pressures from physiological through to noxious (i.e. 0 – 80 mmHg). This, alongside the multimodal effects of galanin of afferent firing to differing stimuli, infers GalR1 expression across multiple afferent

subtypes, further supported by mRNA expression in 3 of the 7 subtypes of colonic afferents (Hockley et al., 2019; Fig. 1-5).

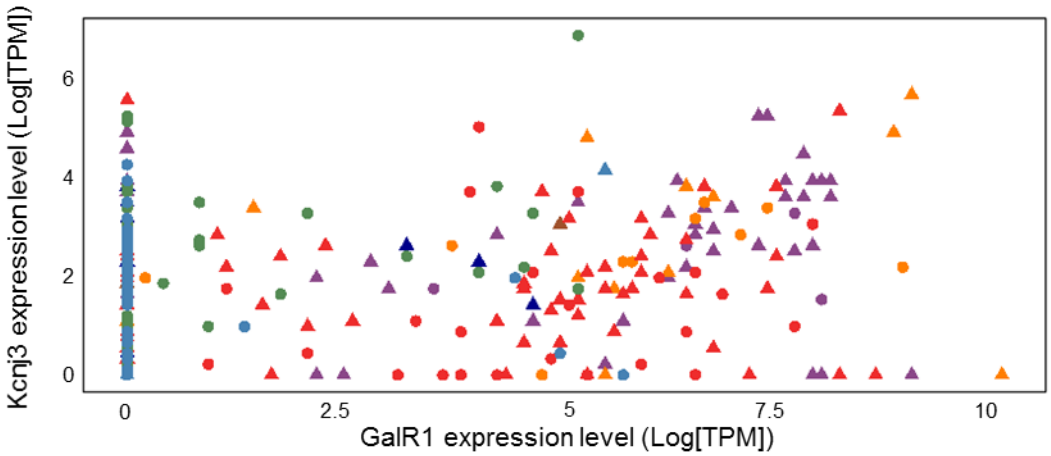


Figure 3-8. GalR1 coexpression against GIRK1

Kcnj3 is the gene for GIRK1 which coexpresses with the GalR1 gene. Each triangle or circle represents a single colonic sensory neurone isolated from either TL DRG (triangle) or LS DRG (circle). The colours represent the different neuronal subtypes identified by Hockley et al. which are mNP (dark blue), mNFa (brown), mNFb (purple), mPEPa (orange), mPEPb (red), pNF (green), and pPEP (light blue). GalR1 and GIRK1 appear to be coexpressed particularly in mPEPa and mPEPb subtypes which are peptidergic nociceptive neurones. Expression is presented in Transcript-Per-Million (Log[TPM]).

The effects of galanin are mediated by three different receptors that activate multiple second messenger pathways with the potential to modulate neuronal activity. GalR1 and GalR3 are positively coupled to GIRKs giving rise to hyperpolarization. The data presented shows that GalR1 agonist (M617) produced a similar level of inhibition as a comparable concentration of galanin. This fits with a previous study using single unit electrophysiological recordings of vagal afferents in mice showing a lack of any residual effects in GalR1 KO mice, results indicating that GalR3 did not contribute to the inhibitory effect seen with galanin (Page et al., 2007). This is further supported by the fact that SNAP37889 (GalR3 selective antagonist) failed to reverse the inhibitory effects of galanin (Page et al., 2007; Swanson et al., 2005). All three mRNA transcripts are expressed in TL (LSN) DRG neurones with GalR2 and GalR3 expression being lower than GalR1 and GalR2, only being expressed in 1 of 5 groups (Hockley et al.,

2019). Based on the widespread distribution of GalR1 mRNA in the central and peripheral nervous systems, it has been proposed that GalR1 mediates many of the inhibitory actions of galanin in nociception (Blakeman et al., 2003; Liu and Hökfelt, 2002). In this study I tested this hypothesis by examining the effects of M617 (GalR1 agonist) on colonic afferents and found potent inhibitory effects, similar to those of galanin, thus further supporting an antinociceptive role of galanin mediated by GalR1. GalR2 likely mediates excitatory effects of galanin as observed in the vagal innervated upper GI tract (Page et al., 2005b). My data shows that GalR2 has little to no contribution to the effect of galanin on LSN activity as the neuropeptide spexin (GalR2 agonist) produced no significant change in mechanosensitivity of the LSN.

As seen in previous studies, intraluminal application of an IS produced robust LSN activity and sensitised the response to mechanical stimuli (Su and Gebhart, 1998). Multiple mediators are released during inflammation and act synergistically to sensitise afferents, making the use of a single inflammatory mediator less likely to sufficiently recapitulate the effects of inflammation in the mouse model. Overall, my results demonstrate galanin's inhibitory effect on the mechanosensitivity of healthy LSN and galanin's ability to reduce IS-induced hypersensitivity. The previous study by Hockley et al. shows GalR1 does colocalise with bradykinin receptor B2, Serotonin receptor 5-HT3, Histamine receptor H2 and H3, and prostaglandin E2 receptor EP1 and EP4. Colocalisation of GalR1 with these receptors is particularly prominent in peptidergic nociceptors (mPEPa and mPEPb; Hockley et al., 2019).

In summary, I have found that galanin inhibits LSN activity predominantly through the action of the GalR1 receptor. Galanin has also been shown to reduce IS-induced hypersensitivity of the LSN supported by the previous study showing coexpression of galanin with various receptors of inflammatory mediators (Hockley et al, 2019), further supporting an anti-nociceptive role of galanin in the colon.

Chapter 4 – Validation of an inducible mouse model of colitis

4.1 Introduction

4.1.1 Murine models of inflammatory bowel disease

Inflammatory bowel disease (IBD) is characterised by chronic inflammation of the GI tract. Onset usually occurs in patients <30 years old and is characterised by a chronic relapsing-remitting course (Cosnes et al., 2011; Sawczenko and Sandhu, 2003). The clinical appearance of IBD is heterogenous and can be largely broken down into Crohn's disease (CD) and ulcerative colitis (UC), which are differentiated by their manifestation and also hypothesised pathogenesis. Whereas UC is primarily seen in the mucosal and submucosal layers of the colon, CD often presents with transmural and discontinuous patches of inflammation that can also affect other regions of the GI tract, e.g. the terminal ileum (Baumgart and Sandborn, 2012; Danese and Romano, 2011). The aetiology of both CD and UC is still not fully understood, however, genetic screening and epidemiologic studies implicate a combination of environmental factors, inherited susceptibility and altered immune responses as the cause (Ananthakrishnan, 2015; de Souza et al., 2017).

Due to their short gestation period, manageable size, genetic tractability and ease with which their environment can be manipulated, mice provide a versatile biological system to study disorders such as IBD, both with regard to their pathogenesis and potential treatment. Currently, there is no single animal model that fully recapitulates all the pathogenic features of IBD, likely owing to its complicated nature and our limited understanding of the mechanisms that drive IBD itself. Each model has its advantages and disadvantages for translating to human IBD and some of the main models will now be discussed.

The complexity of IBD in humans is mirrored by the number of animal models for IBD (discussed below) and the transgenic mouse strains, which exhibit IBD-like alterations, for example IL10 KO (Kühn et al., 1993; Strober et al., 2002; Wirtz et al., 2017). Studying these animal models of colitis has provided essential tools to understanding disease mechanisms in both CD and UC. For example, results from preclinical studies using mouse models of colitis (i.e. IL-10 KO) highlighted the therapeutic benefit of anti-TNFα monoclonal antibodies in reducing colonic

inflammation (Gratz et al., 2002) which have since been successfully utilised clinically in treating IBD (Vavricka et al., 2017), Moreover, analysis of mouse colitis models has also led to advancement of the understanding of the immunoregulatory processes in the gut, resulting in the current hypothesis that an immune response against components of the host's GI tract microbiota is involved in the initiation and perpetuation of IBD (Neurath, 2014; Saleh and Elson, 2011).

Previously established colitis models can be generally divided into five categories dependent on their mode of induction. Firstly, there are specific mouse strains that can be bred to develop spontaneous forms of intestinal inflammation, e.g. the P1/Yit strain undergoes spontaneous enteric inflammation and a pathogenesis similar to human IBD (Matsumoto et al., 1998). Next, mice can be genetically modified to produce an IBD-like phenotype, for example knocking out IL-10 in mice leads to spontaneous development of colitis (Kuhn et al., 1993). The next category is antigen-specific experimental models of colitis, such as introduction of *Helicobacter hepaticus* into the stomach of immunodeficient mice, which leads to the development of chronic colitis that is similar to that observed in humans with IBD (Kullberg et al., 1998). A further distinct category involves modulating the cellular component of the inflammatory response, for example transferring $CD4^{+}CD45RB^{high}$ T cells from healthy mice to a syngeneic recipient that lacks T and B cells results in colitis 5-8 weeks following transfer (Powrie et al., 1994). The final category is chemically-induced models of colitis that introduce a direct insult to the gut to generate the pathology, two widely used examples being intracolonic administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS; Son et al., 1998) and orally administered dextran sulphate sodium (DSS; Okayasu et al., 1990).

The onset and severity of colitis in most mouse models is variable and can depend on environmental factors, for example diet, housing conditions, and specified pathogen free environment influencing the microbiota of the mice (Mähler and Leiter, 2002). Some transgenic models ($IL10^{-/-}$; Kuhn et al., 1993) can take months to manifest symptoms of colitis, rendering them impractical for high throughput studies, such as drug screening studies, that need a fast, reproducible model. The dissection of the

pain signalling pathways relevant to colitis, enables pharmacological testing of agents and identification of future therapeutic avenues. Chemically-induced colitis models, such as DSS and TNBS, are widely used in these types of studies due to their ease of reproducibility, similarity of pathogenesis, and lack of need for expensive mice and reagents (Axelsson et al., 1998; Crespo et al., 1999). The study of colitis in mice through chemically-induced epithelial barrier injury induces an IBD-like phenotype by exposing the host defence to the microbiota. Intestinal bacteria act as a buffer between the external environment and the host. Dynamic changes to bacterial colonies occur not only between individuals, but also in the change from health to disease (Manichanh et al., 2012). Indeed, changes in intestinal bacteria diversity have been demonstrated in patients with CD and UC compared to healthy samples (Ott, 2004).

4.1.2 Chemically-induced models of colitis – TNBS

Trinitrobenzene sulfonic acid (TNBS) is a haptenizing molecule that binds to endogenous or microbiota-derived proteins making them immunogenic to the host (Elson et al., 1996). TNBS is administered intracolonically in ethanol, the ethanol being essential for providing access for TNBS to the epithelial barrier, impairing the barrier function and permitting the translocation of haptenated luminal proteins into the tissue. Exposure to autologous haptenated antigenic material stimulates the development of a delayed-type hypersensitivity immune response and it has been suggested that the haptenization of luminal antigens by TNBS inhibits tolerance to the mouse's own microflora. Moreover, the TNBS model is associated with increased numbers of activated T-cells and involvement of innate immune mechanisms as signs of mucosal acute inflammation can be observed in TNBS treated lymphopenic mice as also occurs in IBD (Fiorucci et al., 2002; Geremia et al., 2014).

In the TNBS model, weight loss, bloody diarrhoea and colonic shortening all occur alongside histopathological transmural inflammation within the colon being observed, which is associated with an influx of macrophages and lymphocytes (Manicassamy and Manoharan, 2014). These features demonstrate that TNBS-induced colitis is a good experimental model for the study of colitis in CD, because it results in Th1

response to proinflammatory cytokine release (Fichtner-Feigl, 2005). However, in the literature many different protocols have been reported, e.g. different ethanol/TNBS concentrations (te Velde et al., 2006), and there are also strain differences in response (e.g. mild and spontaneous resolution in C57BL/6J mice vs. highly susceptible SJL/J mice; te Velde et al., 2006). Furthermore, factors outside of experimental design, such as the presence of T-cell activating bacterial strains in the animal facility, can also greatly impact the variability of the study (Wirtz et al., 2017).

4.1.3 Chemically-induced models of colitis – DSS

Dextran Sulphate Sodium (DSS) is one of the oldest methods for inducing colitis, which is done by addition of a complex polymer of glucose and chlorosulphonic acid to drinking water. The onset and severity of DSS-induced disease is influenced by the molecular weight of DSS used, mouse strain, and concentration of the solution (usually between 1 and 5%; Wirtz et al., 2007). This model can be used to induce acute or chronic forms of colitis by alternating animals on and off DSS containing water, which simulates the relapsing and remitting aspects of certain GI conditions.

DSS affects the colonic mucosa with the most marked disease severity being observed in the distal colon. Clinical signs include diarrhoea and rectal bleeding, weight loss, and colonic shortening and histological examination reveals mucosal haemorrhage, ulceration, superficial inflammation, goblet cell loss, crypt distortion and abscesses (Okayasu et al., 1990). Acute tissue injury is accompanied by infiltration of neutrophils, macrophages and lymphocytic cells, and although the exact mechanism by which DSS triggers the immune response is not completely understood, it is believed to cause toxicity to epithelial cells, which leads to impaired barrier integrity and permeability defects. It has been suggested that DSS associates with medium chain length fatty acids present in the colon, which are then absorbed and processed in epithelial cells leading to their death (Laroui et al., 2012). The amelioration of DSS-induced colitis following the administration of antibiotics demonstrates that bacteria and microbial antigens are an important factor in DSS-induced colitis pathogenesis.

4.2 Aims

The previous chapter demonstrates that galanin inhibits colonic afferent mechanosensitivity and that it can also inhibit the hypersensitivity induced by acute application of inflammatory mediators. To further investigate the role of galanin under inflammatory conditions in the distal colon, similar to that seen in IBD, an animal model of colitis is needed to examine both the expressional and functional changes of galanin in colonic afferents.

Therefore, the aims of this chapter were to:

- Establish an optimised protocol for a robust and reproducible murine model of colitis.
- Fully validate the effects of the chosen model of colitis.

4.3 Results

4.3.1 TNBS-induced colitis fails to produce robust and reproducible intestinal inflammation.

The first model used was TNBS as it one of the most established inducible models of colitis in the literature. The initial pilot study used a very small number of mice for each group (N = 2), a low concentration of ethanol (30%) and three concentrations of TNBS to cover the range seen in literature. The aim was to establish an acute model of colitis with 100% survival and then repeat the experiment in a larger cohort with the same conditions that produced the best result from this pilot.

Healthy C57BL/6J mice were anaesthetised and TNBS/ethanol, or ethanol alone, was intracolonically administered. Mice were assessed and weighed daily, and then killed on day three with measurements made and tissue samples collected from the 4 groups. The 3.85 mg TNBS group were the only group to show any observable weight loss trend with the peak occurring on day 2 (13.9 ± 3.4 % weight loss, N = 2; Fig. 4-1A). All groups showed an increase in the disease activity index (DAI) from one day after induction, however only the TNBS groups shown an increase in DAI on day 3, by which time the control group symptoms resolved (Fig. 4-1B). The colon lengths and macroscopic score did not consistently change in any treatment group (Fig. 4-1C and D). Colon wet weight to length ratio did appear to be increase in both the 1.95 mg and 3.85 mg TNBS groups (but not the 0.85 mg group), which is indicative of the colon thickening observed in inflammation (Antoniou et al., 2016; Fig. 4-1E). H&E with alcian blue staining showed very limited indication of inflammation (1.95 mg TNBS group shown as an example; Fig. 4-1F), with crypt architecture still intact and no signs of ulceration or immune cell infiltration. In summary, the results from this first pilot suggested that the inflammation induced was too mild to produce consistent results.

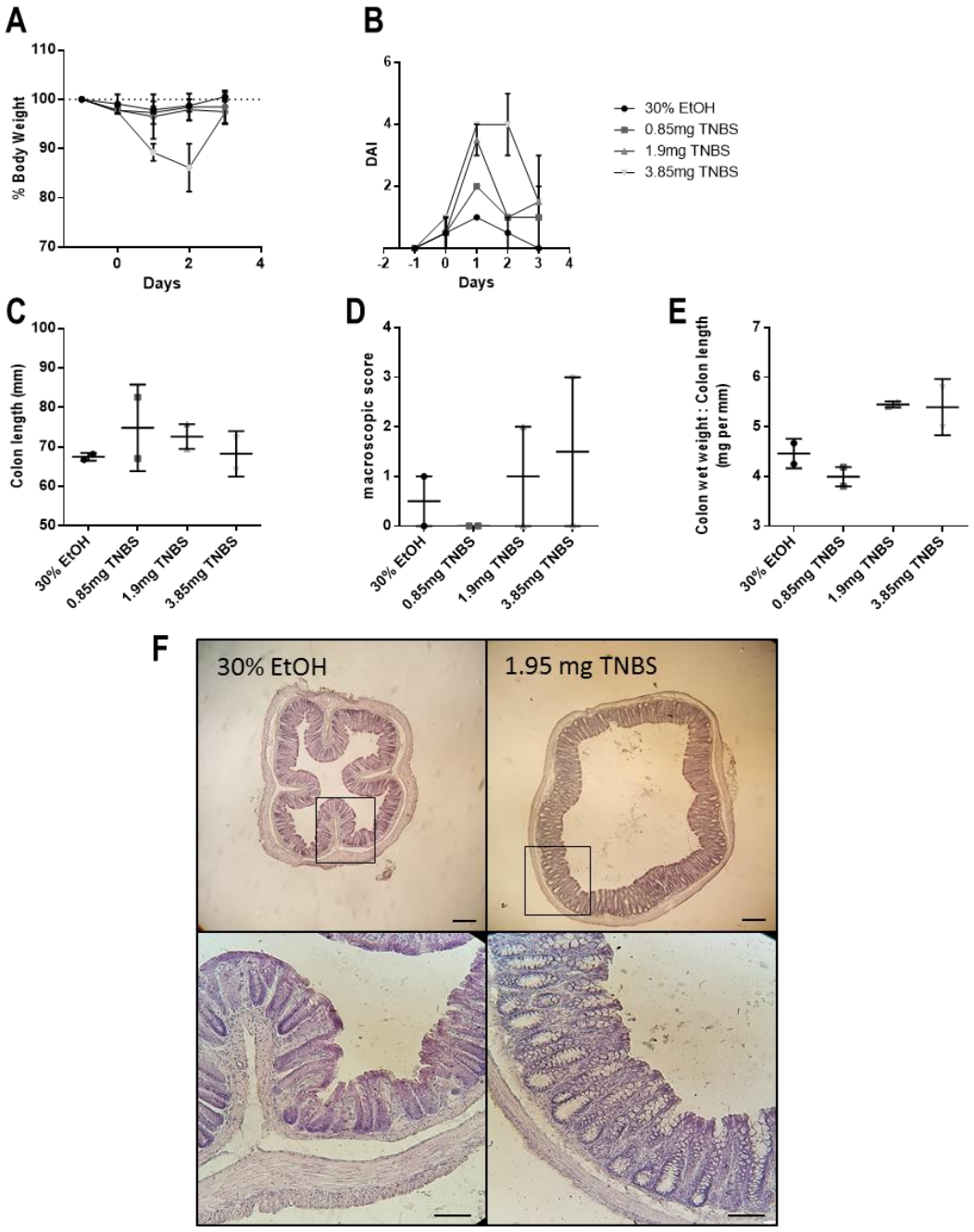


Figure 4-1. First pilot study of TNBS-induced colitis

(A) Body weight of 3.85 mg TNBS treated mice was reduced compared to untreated controls. (B) Disease activity index (DAI) is increased in colitis-induced mice. (C) Colon length is similar throughout all groups. (D) Macroscopic score is based on visual assessment of ulceration and hyperaemia of the colon and is not consistently different between control and TNBS groups. (E) Colon weight to length ratio increased in higher concentrations (1.95 mg and 3.85 mg) colitis-induced mice. (F) H&E with alcian blue staining of colonic tissue. Areas defined by black boxes are magnified in the lower images. Crypt structure remains intact with little sign of infiltration of immune cells in 1.95 mg group; scale bar for top images is 300 μm and for bottom images 100 μm. N = 2 for all groups.

To further optimise the model, a second pilot study using 40% ethanol and only the higher doses of TNBS (1.9 mg and 3.85 mg) was carried out, again using a small group size for initial characterisation (N = 2); one mouse in the 3.85 mg TNBS group was killed on day one due to overly severe DAI and weight loss leaving N = 1 for the 3.85 mg TNBS group.

As before, mice were assessed and weighed daily, before being killed on day three. None of the groups showed consistent weight loss over the three days (Fig 4-2A) and, as seen previously, all groups had an initial increase in DAI on day 1 (Fig 4-2B). However, in contrast to the initial study using 30% ethanol, all groups had a low DAI score on day 3, including the ethanol control group, i.e. no clear difference between TNBS and ethanol only groups was observed (Fig. 4-2B). In addition, the colon length and macroscopic score did not appear to consistently change in 1.95 mg TNBS group, but the 3.85 mg TNBS mouse showed a reduction in colon length and a small increase in macroscopic score (Fig. 4-2C and D). Furthermore, the colon wet weight to length ratio appeared to be slightly decreased in 1.95 mg and 3.85 mg TNBS treated mice, which is not consistent with the colonic thickening that is associated with inflammation (Fig. 4-2E). Lastly, H&E with alcian blue staining did show signs of inflammation in mice from the 1.95 mg TNBS group, with crypt architecture heavily disrupted in places and clear signs of mucosal ulceration and immune cell infiltration in both the mucosal and submucosal layers (Fig. 4-2F).

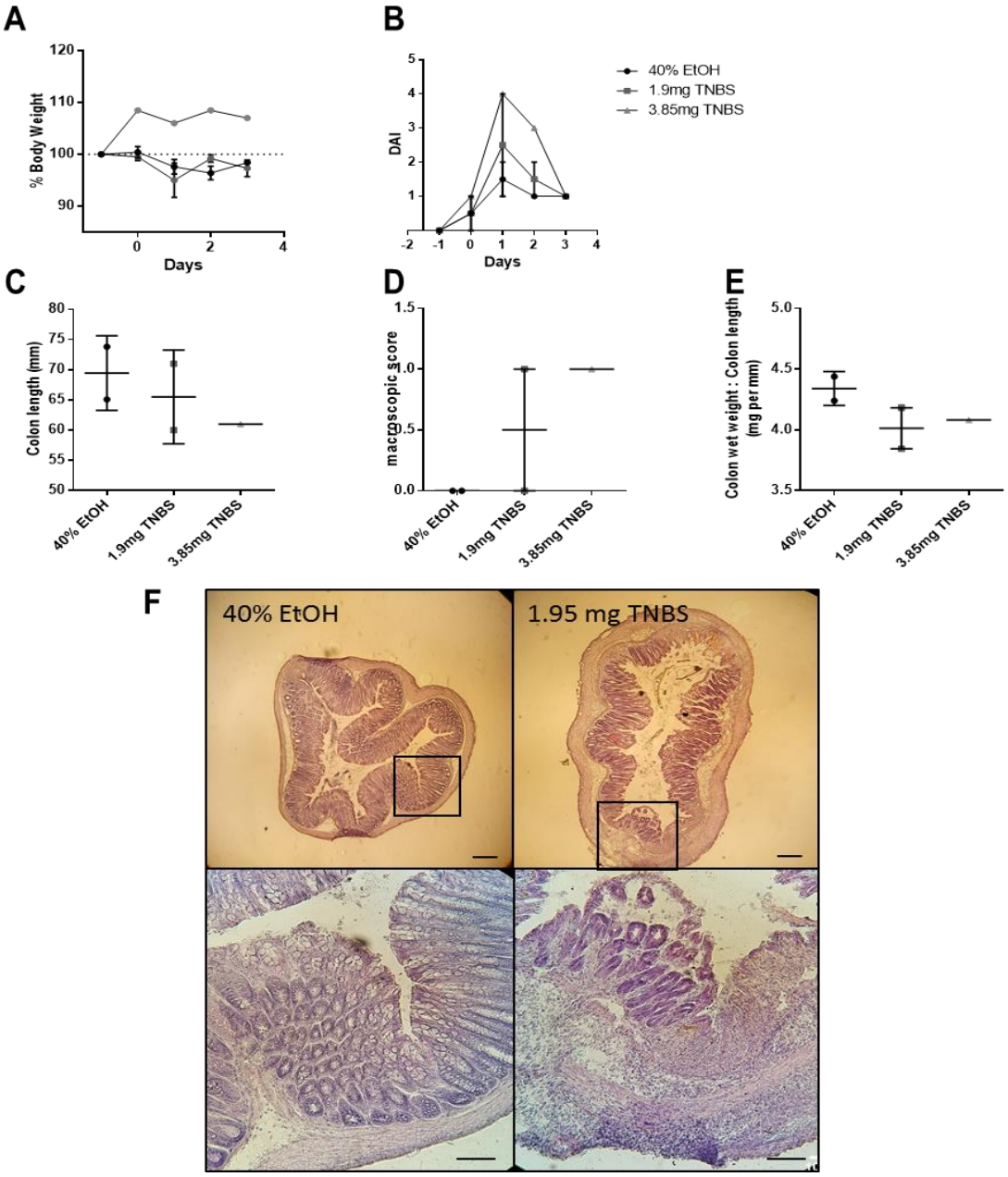


Figure 4-2. Second pilot study of TNBS-induced colitis

(A) Body weight of 3.85 mg TNBS mice increased compared to ethanol control, but 1.95 mg TNBS showed no difference. (B) DAI increases in TNBS-treated mice, but also ethanol control mice. (C) Colon length is not consistently different between control and TNBS-treated mice. (D) The macroscopic score was increased in the 3.85 mg TNBS mouse but was not consistently different between the 1.9 mg TNBS group and ethanol control group (E) Colon weight to length ratio was not consistently different between the ethanol control group and TNBS groups. (F) H&E with alcian blue staining of colonic tissue. In 1.95 mg TNBS mice, there was active inflammation and crypt or surface epithelial damage compared to untreated controls. Areas defined by black boxes are magnified in the lower images; scale bar for top images is 300 μm and for bottom images 100 μm. 40% ethanol and 1.95 mg TNBS N = 2, 3.8 mg TNBS N = 1.

The results from the second pilot were less conclusive of whether intestinal inflammation was developing in a robust manner or not and thus in a final attempt to optimise the model a third pilot was conducted using 50% ethanol and focusing on 1.9 mg TNBS group due to the death of 1 mouse in the 40% ethanol / 3.85 mg condition. The third pilot study also had a higher number of replicates to add power to the observations (50% ethanol control N = 6, 1.95 mg TNBS N = 5).

As per the previous pilot studies, the mice were assessed and weighed daily and killed on day three. Compared to the 50% ethanol control group, the 1.9 mg TNBS group did show significant weight loss (100.5 ± 1.4 % vs 90.9 ± 4.5 % starting weight, $P < 0.01$, two-way ANOVA with Sidak's post-hoc test; Fig. 4-3A). As observed in previous pilot studies, both the control group and the TNBS group showed an increased DAI score on day 1, but here the 1.95 mg TNBS group was significantly increased compared to the control group on day 3 (0.5 ± 0.2 vs 3 ± 1.35, $P < 0.01$, two-way ANOVA with Sidak's test; Fig. 4-3B). Whereas the colon lengths did not significantly change between the 1.95 mg TNBS and control groups (Fig. 4-3C), the macroscopic score did significantly increase in the 1.95 mg TNBS group (0.2 ± 0.1 vs 1.8 ± 0.6, $P < 0.05$, unpaired t-test; Fig. 4-3D) and the colon wet weight to length ratio was also significantly increased in the 1.95 mg TNBS group (4.3 ± 0.2 vs 5.6 ± 0.4, $P < 0.05$, unpaired t-test; Fig. 4-3E). Lastly, H&E with alcian blue staining did show signs of inflammation in the 1.95 mg TNBS group, with crypt architecture heavily disrupted in most places and clear signs of mucosal ulceration and immune cell infiltration in the mucosal layer (Fig. 4-3F).

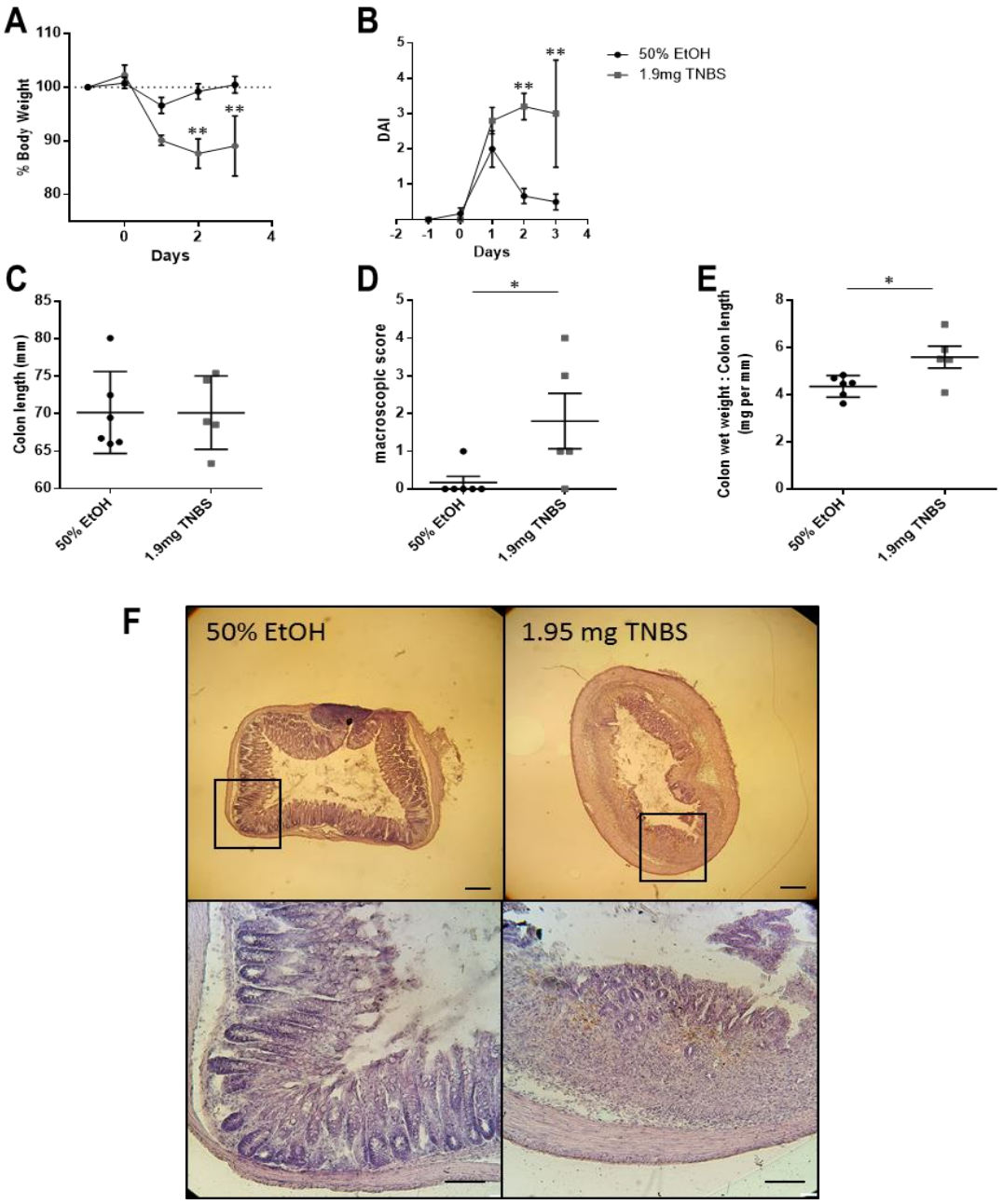


Figure 4-3. Third pilot study of TNBS-induced colitis

(A) Body weight of TNBS treated mice was significantly reduced compared to untreated controls (** = P < 0.01, two-way repeated-measures ANOVA with Sidak's post-hoc test). (B) DAI significantly increases in colitis-induced mice (** = P < 0.01, two-way repeated-measures ANOVA with Sidak's post-hoc test). (C) Colon length was not significantly different between ethanol control and TNBS group. (D) Macroscopic score was significantly increased in TNBS treated mice (* = P < 0.05, unpaired t-test). (E) Colon weight to length ratio was significantly increased in TNBS mice (* = P < 0.05, unpaired t-test). (F) H&E with alcian blue staining of colonic tissue. In TNBS mice, there was active inflammation with infiltration of immune cells and crypt, or surface epithelial damage compared to untreated controls. Areas defined by black boxes are magnified in the lower images; scale bar for top images is 300 μm and for bottom images 100 μm. 50% ethanol N = 6, 1.95 mg TNBS N = 5.

4.3.2 DSS-induced colitis produced robust and reproducible intestinal inflammation.

Due to the inconsistencies between the results of the three pilot studies for the TNBS model of colitis, an alternative model of inducible colitis was trialled. Oral administration of dextran sulphate sodium (DSS) via drinking water is widely reported to induce colitis (Manicassamy and Manoharan, 2014; Wirtz et al., 2007). An initial pilot study was conducted similar to previous TNBS pilot studies, with a relatively small number of mice in each group to avoid large scale unforeseen complications arising.

Healthy C57BL/6J mice were given either 3% DSS in their drinking water (N = 5) or normal drinking water (N = 4) for 5 days, followed by normal drinking water for an additional 3 days. Every animal had their symptoms assessed and was weighed daily. The animals were killed on day 8 with measurements and tissue samples collected from the two groups. There was significant weight loss in the DSS group compared to the control group (day 8: 89.2 ± 2.5% vs 107.2 ± 0.9% starting weight, $P < 0.001$, two-way ANOVA with Sidak's post-hoc test; Fig. 4-4A) and a significant increase in DAI score (day 8: 2 ± 0.5 vs 0, $P < 0.001$, two-way ANOVA with Sidak's post-hoc test; Fig. 4-4B). Colon length was also significantly decreased in the DSS group compared to the control group (66.9 ± 1.4 mm vs 87.9 ± 3.7 mm, $P < 0.01$, unpaired t-test; Fig. 4-4C), although there was no significant change in the colon wet weight to colon length ratio (Fig. 4-4E), suggesting there was colon shrinkage, which is associated with intestinal inflammation (Okayasu et al., 1990), but no thickening of the colon. There was no significant change in macroscopic score between the DSS and control groups.

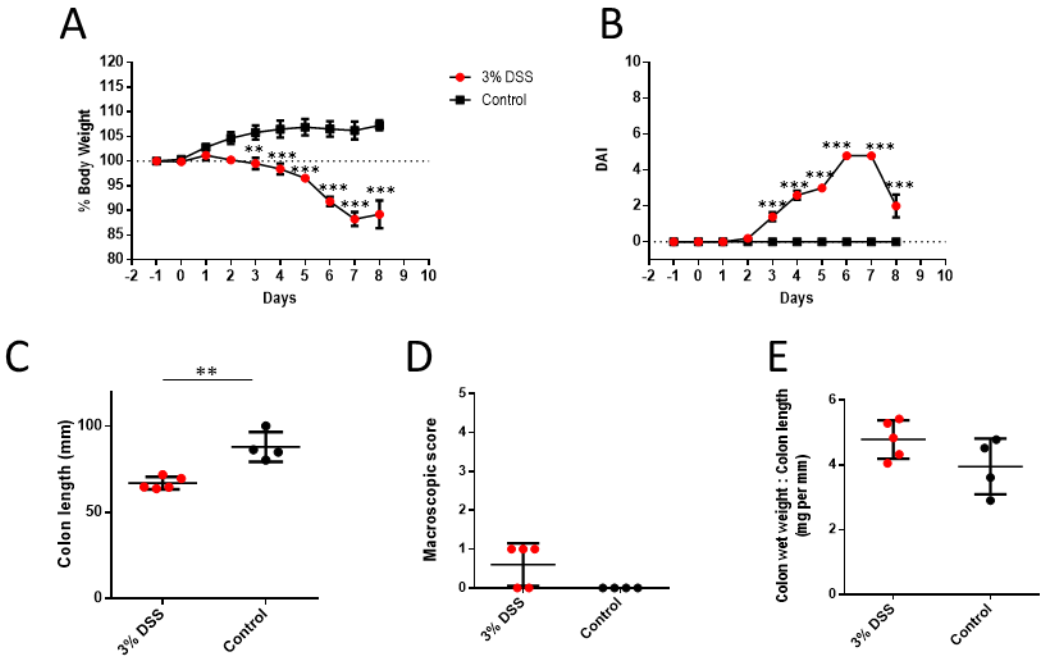


Figure 4-4. Initial pilot study of 3% DSS induced colitis

(A) Body weight of DSS treated mice was significantly reduced compared to untreated controls (** = P < 0.01, *** = P < 0.001, two-way ANOVA with Sidak's post-hoc test). (B) DAI significantly increased in colitis-induced mice (*** = P < 0.001, two-way ANOVA with Sidak's post-hoc test). (D) Colon length was significantly reduced in DSS mice (** = P < 0.001, unpaired t-test). (E) Macroscopic score did not significantly change between control and DSS groups (P = 0.06, unpaired t-test). (F) Colon weight to length ratio did not significantly change between control and DSS groups (P = 0.129, unpaired t-test). N = 5 for 3% DSS group, N = 4 for control group.

With the pilot study looking promising, to fully validate the DSS model a full study with 3% DSS and control groups of N = 10 was conducted. The peak of observed symptoms in the initial pilot study showed a peak at day 7 and so for the full validation study day 7 was chosen as the end point. Healthy C57BL/6J mice were given either 3% DSS in their drinking water for 5 days or normal drinking water, followed by normal drinking water for an additional 2 days, and weighed and assessed daily.

The DSS mice showed significant weight loss by day 4 compared with the untreated mice (day 7: 82.1 ± 1.9% vs 103.9 ± 3.6 % starting weight, $P < 0.001$, two-way ANOVA with Sidak's post-hoc test; Fig. 4-5A). DSS mice also showed a significant increase in DAI score (day 7: 9 ± 0.1 vs 0 DAI score, $P < 0.001$, two-way ANOVA with Sidak's post-hoc test; Fig. 4-5B). There was also significant damage observed macroscopically in DSS treated mice compared to control (1.5 ± 0.2 vs 0 macroscopic score, $P < 0.001$, unpaired t-test; Fig. 4-5C). In addition, the colon length was significantly decreased in DSS mice compared to control mice (46.2 ± 1.4 mm vs 69.8 ± 1.7 mm, $P < 0.001$, unpaired t-test; Fig. 4-5D and E) and the colon wet weight to length ratio also significantly increased (7.6 ± 0.3 mg/mm vs 4.3 ± 0.2 mg/mm, $P < 0.001$, unpaired t-test; Fig. 4-5F). The shortening of the colon and increased weight to length ratio suggest thickening of the colon wall, which has previously been reported in the DSS model of colitis (Marrero et al., 2000; Sánchez-Fidalgo et al., 2012).

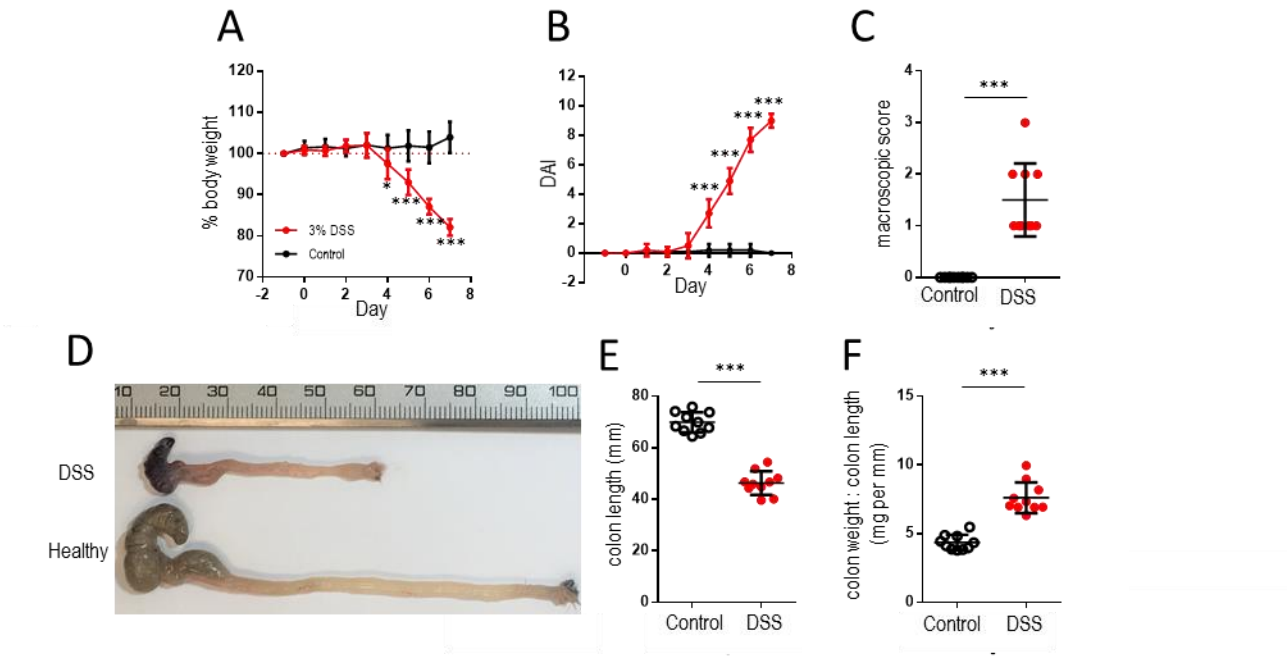


Figure 4-5. DSS induces weight loss and macroscopic changes to the colon

(A) Body weight of DSS treated mice was significantly reduced compared to untreated controls (* = P < 0.05, *** = P < 0.001, two-way repeated-measures ANOVA with Sidak's post-hoc test). (B) DAI significantly increases in colitis-induced mice (*** = P < 0.001, two-way repeated-measures ANOVA with Sidak's post-hoc test). DAI = disease activity index; assessment of inflammation by clinical parameters. (C) Macroscopic score is based on visual assessment of ulceration and hyperaemia of the colon (P < 0.0001, paired t-test). (D) Images of healthy and colitis-induced colons from caecum (left side) to anus (right side). (E) Colon length was significantly reduced in DSS mice (P < 0.0001, paired t-test). (F) Colon weight to length ratio was significantly increased in DSS mice (P < 0.001, paired t-test). N = 10 for both groups.

H&E and alcian blue staining used to identify crypt architecture, the presence of goblet cells and identify immune cells. Using a histopathological scoring system based on the presence of inflammatory cells, crypt damage and ulceration (Ren et al., 2011), significant damage was also observed in DSS treated mice compared to healthy mice (Fig. 4-6A and C, 6.8 ± 0.7 vs 0.5 ± 0.2 histology score, $P < 0.001$, unpaired t-test). Furthermore, as others have observed (Kessler et al., 2008), we also found that the extracellular matrix polysaccharide hyaluronan (HA) disappeared from the colon epithelium during DSS-induced colitis with deposits occurring in the subepithelial layers (Fig. 4-6B). Further evidence of development of colitis was obtained by measuring the muscular layer of the colon, which was found to be significantly thicker in the DSS group compared to healthy group (174.9 ± 10.1 μm vs 81.6 ± 3.5 μm, $P < 0.001$, unpaired t-test; Fig. 4-6D).

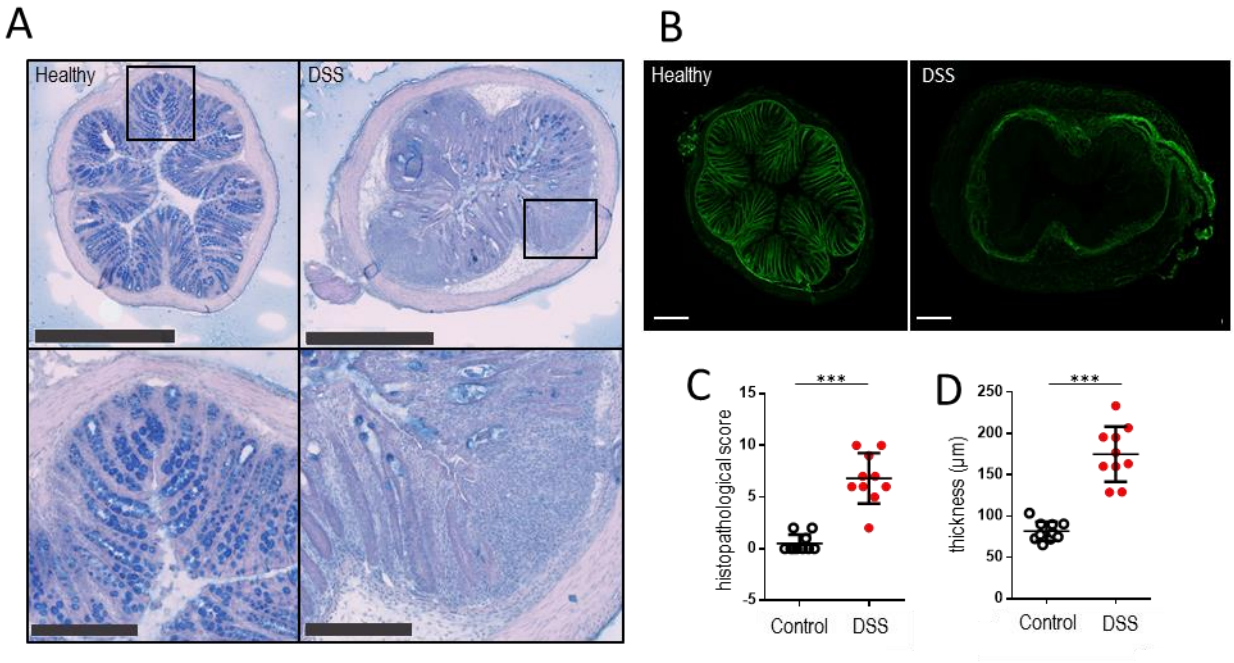


Figure 4-6. DSS induces changes in colon histology

(A) H&E with alcian blue staining of colonic tissue. In DSS mice, there was active inflammation, and crypt or surface epithelial damage compared to untreated controls. Areas defined by black boxes are magnified in the lower images; scale bar for top images is 1 mm and for bottom images 250 µm. (B) Changes in hyaluronic acid binding protein (HABP; green) arrangement and distribution in colitis-induced and healthy colons. Scale bar is 500 µm. (C) Histology score defined previously (more detail in section 2.3.4) significantly increases in DSS mice ($P < 0.0001$, $N = 10$, paired t-test). (I) Colonic muscle layer significantly thicker in DSS mice ($P < 0.0001$, $N = 10$, paired t-test).

4.3.3 Myeloperoxidase (MPO) levels are not altered in mouse colons by DSS-treatment.

Myeloperoxidase (MPO) is part of the haem peroxidase superfamily and is found in certain immune cells including neutrophils, monocytes, and some tissue macrophages. Colonic MPO activity is a marker of tissue damage and is a measure of neutrophil infiltration (Masoodi et al., 2011; Palmen et al., 1995). For optimising the protocol, the time course of the reaction between the tissue supernatants for healthy tissue, inflamed tissue (complete Freund's adjuvant, CFA hind paw model, performed by Dr Gerard Callejo) and a positive control (horseradish peroxidase; HRP) was tested with results of the reaction mixtures taken every 30 seconds (Fig 4-7A). From this, the peak of the reaction was at the 20th reading with clear differences between the three groups, which was then used for future experiments.

As expected, the samples from the CFA treated hind paws showed significantly increased MPO activity compared to the samples from healthy mice (1.2 ± 0.1 vs 0.1 ± 0.1 U mg^{-1}, $P < 0.001$, one-way ANOVA with Tukey's test; Fig. 4-7B). However, colon samples from both models of chemically-induced colitis (1.95 mg TNBS / 50% ethanol and 3% DSS) did not have significantly different MPO activity to either healthy colonic tissue or 50% ethanol control groups, suggesting there is little change in the levels of neutrophils in these models of colitis across the time course used in this study.

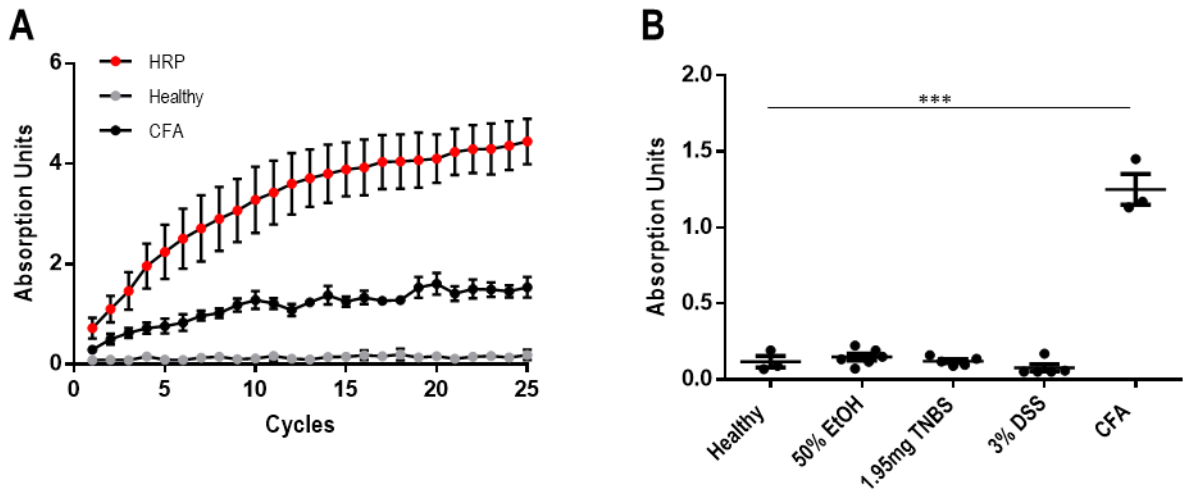


Figure 4-7. MPO activity analysis

(A) Time course of reaction between supernatants and reaction mixtures with readings taken every 30 seconds. (B) MPO activity in colon tissues from 1.95 mg TNBS / 50% ethanol and 3% DSS administered mice on day 3 and 7 respectively with their control groups. Hind paw tissue from a mouse treated with CFA was used as a positive control of an inflammatory model previously established in the lab. Healthy and CFA samples N = 3, 1.95 mg TNBS / 50% ethanol and 3% DSS samples N = 5, and 50% ethanol control samples N = 6

4.4 Discussion

The cause of IBD is not clear, but there is evidence of dysregulated mucosal immune responses, abnormal cytokine production, and intestinal tissue damage (Bouma and Strober, 2003; Hibi and Ogata, 2006; Strober et al., 2002). Experimental colitis models in mice have proved useful in studying pathogenesis of IBD and also in the study of colitic pain in general (Eijkelkamp et al., 2007; Larsson et al., 2006). There are a number of mouse models that exhibit histological and/or immunological characteristics of human IBD meaning that I needed to establish one to investigate the function of galanin in an *in vivo* setting. As discussed in Section 4.1, models established through genetic modifications to increase susceptibility to intestinal inflammation are time-consuming, although are good models for studying the role of particular signal transduction pathways (Engelhardt and Grimbacher, 2014; Wirtz and Neurath, 2005). I chose to establish a chemically-induced model of colitis due to the relatively rapid onset and ability to use wildtype mice. Although these models are not novel, pilot studies and optimisation was needed to establish a reliable model of colitis as this was new to the lab.

To study the effects of galanin in inflammatory pain, the TNBS model was first chosen as this has been previously reported as a robust and reproducible model of colitis. However, a lot of variation in methodology has been reported for the TNBS model, including mouse strain, age, number of TNBS administrations, concentration of TNBS/ethanol and study end points (Abad et al., 2005; Coccia, 2005). As the three initial experiments conducted in this study showed, the response was variable and fits with previous reports of TNBS resistance in C57BL/6J mice (Antoniou et al., 2016), especially compared to Balb/c and SJL/J strains (te Velde et al., 2006). A presensitization step (topical application of TNBS solution to a patch of skin 7 days before intracolonic application) with TNBS has been shown to increase the rate of successfully inducing inflammation (even in C57BL/6J mice; Wirtz et al., 2007), which would be a potential further option to explore, particularly if CD-like transmural inflammation is the pathology being investigated (Wirtz et al., 2017). As this study focussed more on colitis in general, rather than a particular pathway, switching to the DSS-induced model seemed more appropriate to rapidly establish a reproducible animal model. 3% DSS, administered for 5 days, induced marked colitis in C57BL/6J

mice as observed in previous studies (Manicassamy and Manoharan, 2014; Perše and Cerar, 2012). In this study, DSS treated mice showed a significant reduction in weight and an increased DAI score. Dissection of the colons also showed significant reduction in colon length, increased wet weight to length ratio, increased macroscopic score, and, histologically, more signs of mucosal inflammation. Importantly, not only were all the measurements consistent with development of colitis in DSS treated mice, the results from the DSS treated mice also showed significantly less variability than the TNBS treated mice.

There are several factors outside of the experimental design that can have a large impact on the disease manifestation including; genetic heterogeneity of mouse inbred strains and the local microbiota of the animal facility (Bleich and Fox, 2015); it could be that one of these factors played a role in the negative MPO assay. Therefore, initial pilot studies were important to determine the optimal dosages and housing conditions (Mähler and Leiter, 2002). Sex-specific differences in susceptibility to experimental colitis exists and was taken into consideration, an example being that for DSS-induced colitis, male mice have a tendency to have increased susceptibility than female mice (Wirtz et al., 2017) and hence for this reason, studies in both models used only male mice.

In summary, these results demonstrate that I established a robust and reproducible mouse model of colitis and based on these results I used the DSS model for investigating the ability of galanin to modulate LSN function in inflammatory conditions.

Chapter 5 – Galanin does not inhibit DSS-induced mechanical hypersensitivity of the lumbar splanchnic nerve

5.1 Introduction

5.1.1 Galanin in the peripheral nervous system

Galanin has widespread expression throughout the central (CNS) and peripheral nervous systems (PNS), as discussed in detail in Section 1.6. Galanin immunoreactivity and mRNA have been characterised in the CNS of the mouse (Cheung et al., 2001; Perez et al., 2001) and results show that galanin is coexpressed with an array of neurotransmitters (Jacobowitz et al., 2004). In addition to the numerous physiological functions of galanin in the CNS (e.g. its roles in feeding behaviour, energy homeostasis, osmotic regulation and water intake; reviewed in Lang et al., 2014), galanin has also been implicated in functional regulation of the PNS (Page et al., 2005b). For example, galanin is present in DRG neurones and has been shown to participate in the control of pain processing in the spinal cord (Liu and Hokfelt, 2002). Galanin also modulates neurotransmission in peripheral organs, such as the heart (Smith-White et al., 2003) and GI tract (Sarnelli et al., 2004).

Galanin was originally isolated from the small intestine (Tatemoto et al., 1983) leading to the investigation of its role in the GI tract. Galanin is widely expressed throughout the GI tract with galanin-expressing enteric neurones and sensory afferents projecting to all layers of the wall of the GI tract (Melander et al., 1985). More recent analysis demonstrated that galanin is expressed in many different cell types in the colon including macrophages, epithelial cells and colonic sensory afferents (Hockley et al., 2019; Koller et al., 2019). Within the sensory afferents, galanin is predominantly expressed in two of the seven groups that have been identified through single-cell RNA-sequencing of retrograde labelled colonic sensory afferents, one of which represents peptidergic nociceptors and thus supports a potential role for galanin in nociceptive signalling in the distal colon (Hockley et al., 2019). Galanin has also been shown to have physiological roles in the GI tract including inhibition of gastric acid secretion (Kisfalvi et al., 2000), inhibiting pancreatic peptide release (Herzig et al., 1993; Lindskog et al., 1995), and modulation of GI motility (Umer et al., 2005).

5.1.2 Neurochemical characterisation of visceral sensory neurones

The expression of known nociceptive protein markers in visceral afferents can be used to characterise galanin expressing neurones and further support the functional effects seen with exogenous galanin. In line with the focus of this thesis, the emphasis of the discussion here will be on spinal colonic innervation. Retrograde labelling studies of the rodent colon suggest that 3 – 15% of DRG (T8-L1, L6-S1) neurones project to the colon, with a bimodal distribution amongst spinal cord levels: one at spinal cord level T8-L1 in mouse (T8-L2 in rat) and the other comprising L6-S1 in mouse (also L6-S1 in rat) (Brierley et al., 2008; Christianson et al., 2006a; Hockley et al., 2019; Jänig and McLachlan, 1987; Perry and Lawson, 1998). The TL pathway corresponds to afferent fibres of the LSN and the LS pathway that of afferent fibres tracking with the PN.

Whilst thickly myelinated Aα/β-fibres are rare in viscerally projecting populations, thinly myelinated Aδ-fibres make up a significant proportion of the visceral afferent population, estimated at 19-26% of visceral afferents (Christianson et al., 2006a; Perry and Lawson, 1998), the rest being unmyelinated C-fibres. A recent study by Hockley et al., has used single-cell RNA-sequencing to categorise colonic afferents based on their transcriptomic profiles, directly linking the different subtypes to their inferred function (Hockley et al., 2019). From this study, it was seen that the receptor for neurotrophin-3, TrkC. shows almost no expression in the subtypes linked to nociceptive function (mPEPb and pPEP), suggesting that colonic afferents expressing TrkC have a non-nociceptive function; indeed, TrkC is considered a marker of proprioceptive neurones (Snider, 1994) and so its absence of expression in putative nociceptors and relatively low expression overall is to be expected in colonic sensory neurones. Both TRPV1 and Gfrα3 are expressed in nociceptive subtypes (Hockley et al., 2019). In a previous study supporting this, visceral afferent nociceptors were further characterised into high-firing frequency (HF) and low-firing frequency (LF) populations based on differential responses to distension (Malin et al., 2009). Gfrα3 being shown to be a good marker for the HF nociceptor population and TRPV1 for the LF nociceptive population.

5.1.3 Changes in galanin expression in peripheral inflammation

The expression of galanin and its receptors is altered throughout pain circuits in experimental inflammation conditions, which suggests that the function of galanin may also differ from health to disease. For example, galanin expression decreases in DRG neurons, but increases in DH neurons in response to hind-paw injections of the inflammatory stimulus carrageenan (Ji et al., 1995; Zhang et al., 1998). Moreover, the galanin concentration is reported to increase in DRG neurones in models of chemically-induced ileitis in pigs (Pidsudko et al., 2003) and cystitis in rats (Callsen-Cencic and Mense, 1997), although a similar study in rats reported no significant change (Zvarova and Vizzard, 2006). The galanin concentration also increases after noxious colorectal distension (in the absence of inflammation) (Lu et al., 2005) and in chronic diverticular disease (Simpson et al., 2008), indicative of a potential role in visceral, as well as, somatic pain modulation.

In summary, galanin mRNA has been shown to be present in nociceptive neuronal subtypes in both the LSN and PN. However, previous studies show contradicting results over changes in galanin expression in DRG neurones during peripheral inflammation. Despite this, galanin expression has been shown to increase in colonic tissue after noxious mechanical stimuli and in chronic inflammatory conditions further supporting a potential role for galanin in nociceptive signalling.

5.2 Aims

Results in Chapter 3 demonstrate that under physiological conditions galanin modulates LSN afferent function with an overall inhibitory effect on LSN mechanosensitivity. Galanin was also found to reduce the mechanical hypersensitivity induced by intraluminal application of an IS. From results in Chapter 4, it is clear that a robust model of colonic inflammation has been established.

Therefore, the aims of this chapter were to;

- Determine the expression of galanin in colon-innervating DRG neurones using immunohistochemistry.
- Examine whether colonic sensory neurone galanin expression changes in the DSS model of colonic inflammation.
- Investigate how colonic inflammation affects activity of the LSN and how this is modulated by galanin.

5.3 Results

5.3.1 Validation of colonic Fast Blue labelling of DRG neurones

The specificity of Fast Blue (FB) uptake by TL and LS colon innervating afferents following injections of FB into the distal colon wall was quantitatively investigated. Following transcardial perfusion with fixative (4% PFA), DRG from mice were removed from T, L and S regions: T7 – T13, L1 – L6, and S1 – S3. During surgery, injection sites were made primarily within the regions of the distal colon likely innervated by the LSN. Once dissected, individual DRG from FB-labelled mice were imaged using fluorescent microscopy to confirm uptake of FB into cells (Fig. 5-1). In DRG T9 – L1 and L5 – S1, fluorescent cells could be seen under UV illumination, with maximal frequency observed in T13, L1, L6 and S1 for lumbosacral. Importantly, none or very few retrograde-labelled cells (<5 cells/DRG) were observed in DRG T7, L3, L4, S2 and S3 (Fig. 5-1). This is consistent with the expression pattern observed in previous studies and provides confirmation that off-target labelling of non-colon innervating neurones is unlikely to have occurred (Christianson et al., 2006a; Robinson et al., 2004). As such, no alterations to surgical procedures (e.g. position or number of injection sites, concentration or quantity of FB injected) were made throughout the rest of the study. Due to the high numbers of FB-labelled cells observed, DRG T13 and L1 are used to represent colonic afferents in the LSN pathways and L6 and S1 are used to represent afferents of the PN.

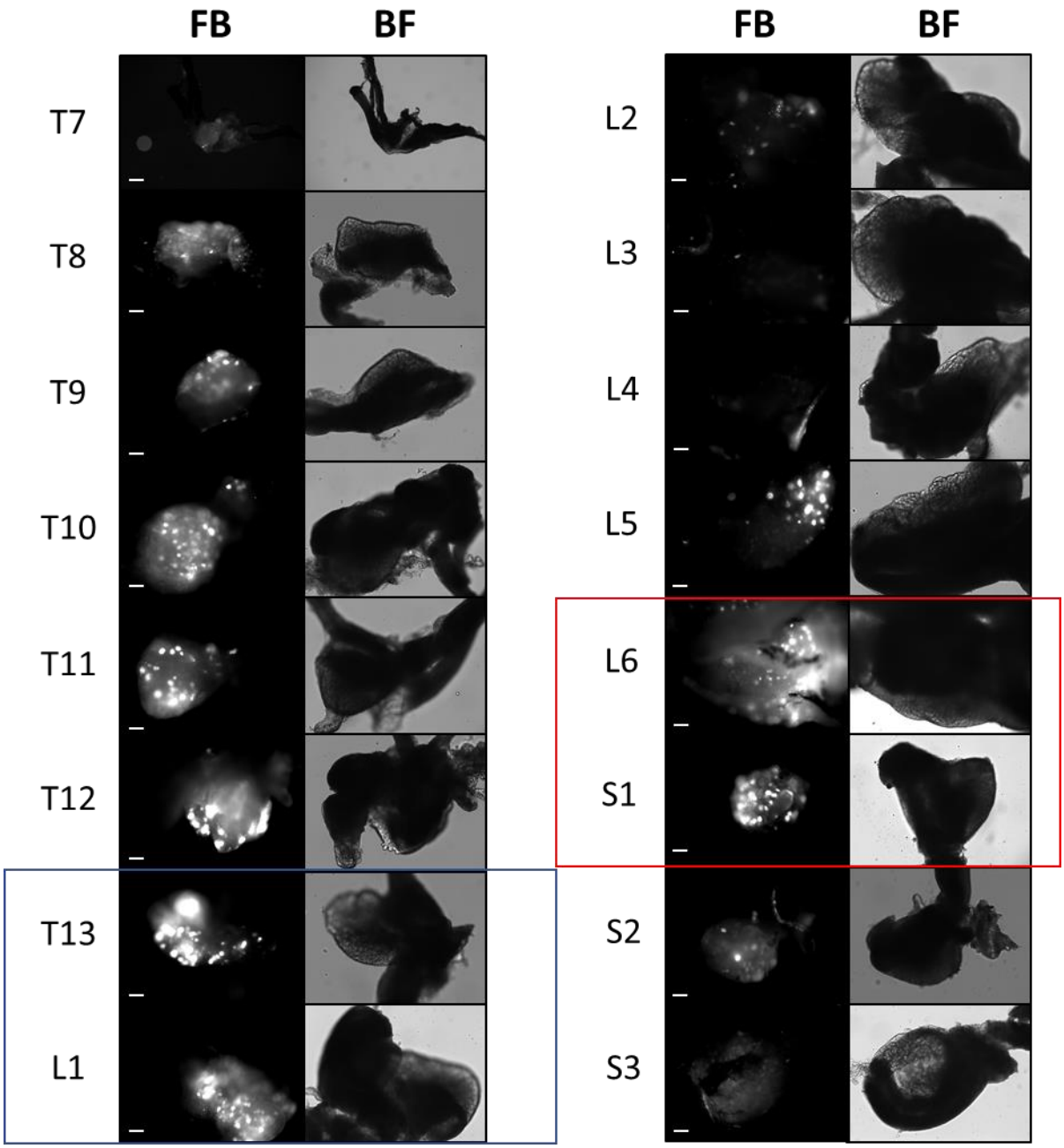


Figure 5-1. Validation of colonic FB labelling of DRG neurones.

FB injections were made into the distal colon wall of the mouse and FB underwent retrograde transport to DRG. Representative bright-field (BF) and fluorescent images of whole DRG from FB-labelled mice. DRG from T13 and L1 (blue box) contain high numbers of FB-labelled cells, corresponding to the LSN afferent pathway, while DRG L6 and S1 (red box) also contain high numbers of FB-labelled cells, corresponding to the PN afferent pathway. L3 and L4 contain none or very few (<5 cells/DRG) FB-labelled cells. Scale bars 100 μm.

In later studies, DSS-treated mice were used to investigate the functional role of galanin in visceral afferents during inflammation. Therefore, in order to confirm that DSS treatment was not altering the extent of colonic innervation by extrinsic afferent fibres, comparison of the FB+ DRG neurone profiles from healthy and DSS-treated mice was made on DRG sections (Fig. 5-2A). Following comparable surgical procedures, the extent of FB labelling of TL and LS DRG from healthy and DSS-treated mice did not significantly differ (two-way ANOVA, $P = 0.86$, Fig. 5-2B). Furthermore, a size-frequency analysis showed no significant change in the general distribution (two-way ANOVA, $P = 0.76$; Fig. 5-2C).

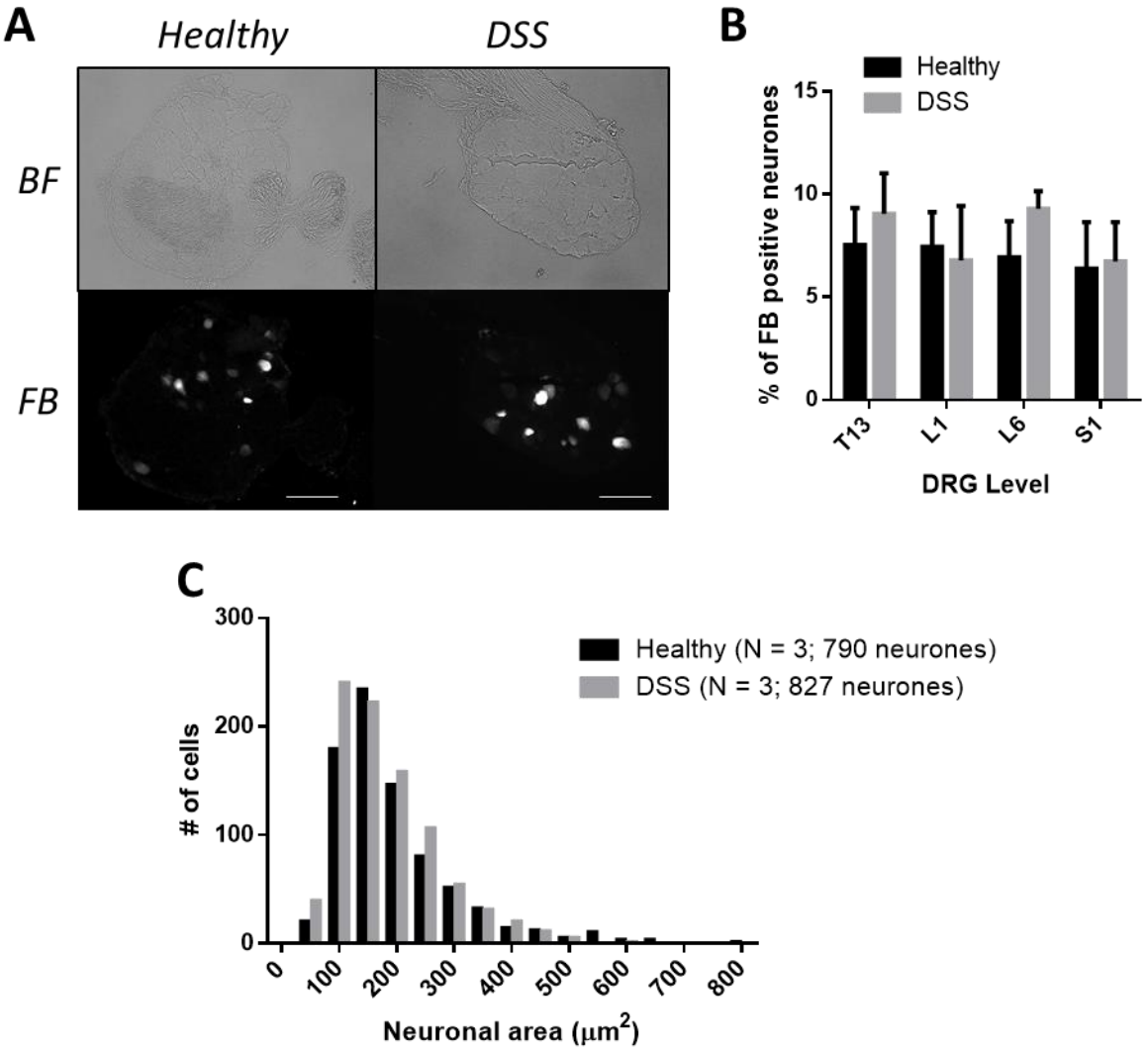


Figure 5-2. Comparison of FB-positive colonic sensory neurones isolated from healthy and DSS-treated mice.

Comparison of the total number of colon-innervating FB labelled neurones in DRG isolated from healthy and DSS-treated mice. (A) Representative brightfield and fluorescent images of healthy and DSS-treated DRG sections showing FB labelling of neurones. Scale bars 100 μm. (B) FB-positive neurones as a percentage of the total number of neurones (N = 3 mice for both groups, n = 790 for healthy mice and n = 827 for DSS mice). (C) Mean frequency of cross-sectional area of profiles (in 50 μm^2 divisions) from healthy and DSS-treated mice.

5.3.2 Expression of galanin is unchanged by acute DSS-induced colitis

In general, neuropeptides are preferentially released when a neurone undergoes high frequency firing (Lundberg, 1996). Thus, the presence of galanin in nociceptors would suggest that it is released during nociceptive signalling to modulate the activity of neurones expressing GalR. Firstly, we examined the protein expression of galanin in mouse colon-innervating TL and LS DRG neurones using immunohistochemistry (IHC, Fig. 5-3A). An anti-galanin antibody (Theodorsson and Rugarn, 2000) that produced no specific staining in control experiments was used. One control experiment included the primary antibody pre-incubated with galanin peptide and another experiment in which only the secondary antibody was used also showed no fluorescence (Section 7.1).

The expression of galanin was determined in healthy mice compared to those that had undergone DSS-induced colitis. A similar proportion of galanin expressing neurones was observed in DSS-induced colitis mice compared to healthy mice (10.9 ± 2.7% vs 5.5 ± 4% TL DRG neurones and 16.5 ± 3.5% vs 7.7 ± 0.5% LS DRG neurones (N = 3)). Moreover, a similar proportion of galanin expressing, FB+ neurones was found in DSS-induced colitis mice (22.7 ± 4.7% vs 16.6 ± 4% TL DRG neurones and 21.7 ± 1.9% vs 18.7 ± 5.5% LS DRG neurones (N = 3)) compared to healthy mice (Fig. 5-3C), suggesting that galanin expression in colon-innervating DRG neurones is not altered by DSS-induced colitis. In agreement with this result, an ELISA measuring the galanin concentration in extracts from whole colon segments showed that the galanin concentration did not significantly differ between healthy and DSS treated mice (0.4 ± 0.2 ng mL^{-1} vs 0.6 ± 0.3 ng mL^{-1}; Fig. 5-3D).

In healthy mice, there was also no significant difference between the mean neuronal area of FB+ and FB+ galanin+ colonic DRG neurones (FB+: 233.7 ± 9.4 μm^2 vs FB+ galanin+: 212.1 ± 6.6 μm^2, unpaired t-test; Fig. 5-3E). By contrast, in DSS-treated mice, the mean neuronal area of FB+ galanin+ cells was significantly smaller than that of the FB+ population (FB+ galanin+: 192.1 ± 4.5 μm^2 vs FB+: 215.3 ± 4.4 μm^2, P = 0.033, unpaired t-test; Fig. 5-3F).

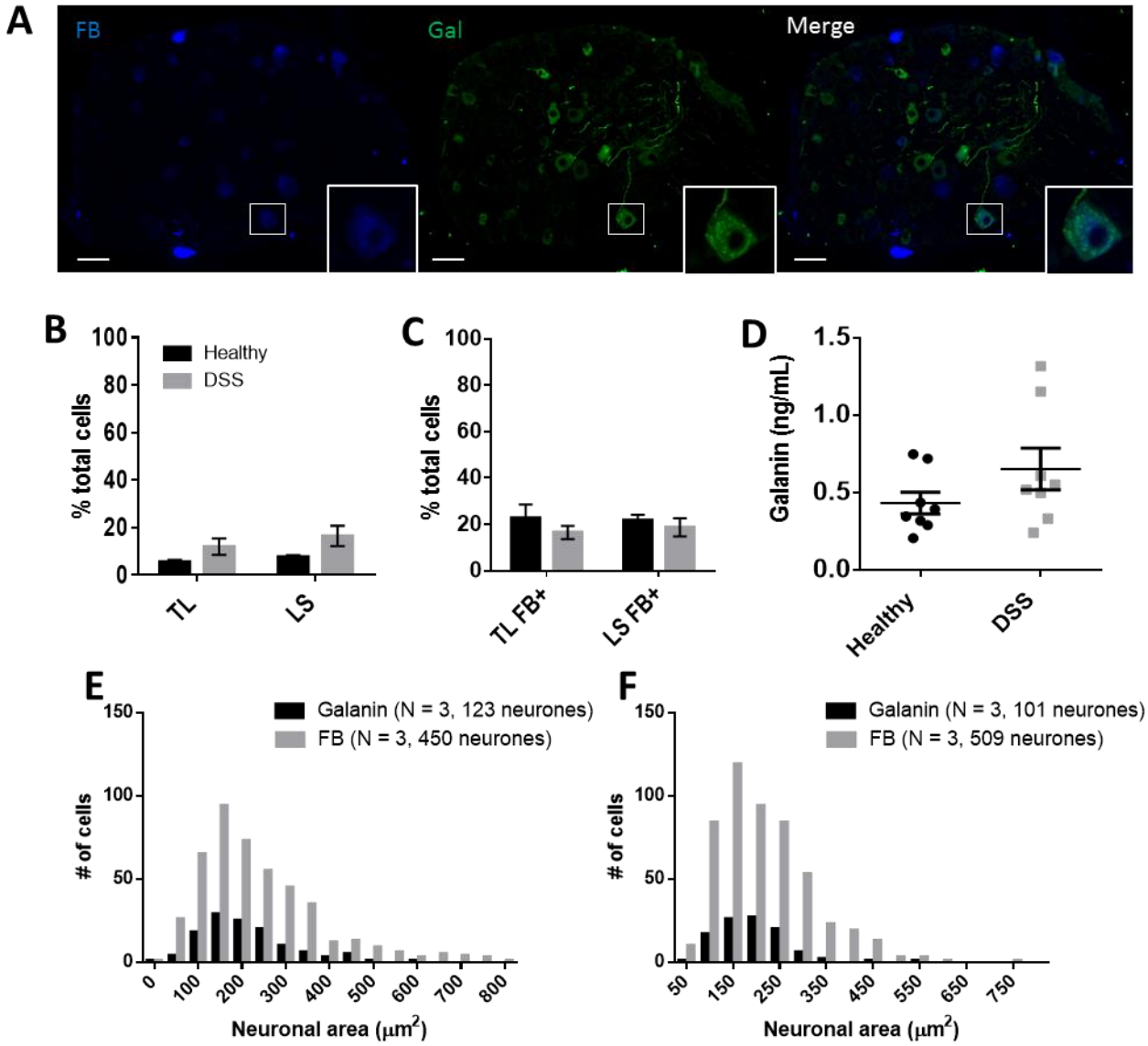


Figure 5-3. Galanin expression in colonic sensory neurones and whole colon.
(A) Representative images of FB labelled neurones following FB colon injections and galanin immunofluorescence, scale bar is 50 μm; magnified example of co-localisation shown in the inset. No significant change in the number of galanin positive neurones is seen between healthy and DSS-treated mice in the TL and LS populations in the whole DRG (B) or colon-innervating DRG neurones (C) (N = 3, unpaired t-test). (D) Galanin ELISA shows no significant change in galanin concentration in colon tissue between healthy and DSS-treated mice (N = 8, unpaired t-test). Cross-sectional area histogram of FB+ (grey bars) and galanin positive (black bars) neurones from healthy (E) and DSS-treated (F) mice (N = 3).

5.3.3 Galanin is predominantly expressed in putative nociceptors

To characterise the populations of sensory neurones expressing galanin, co-staining was performed with antibodies against a number of growth factor receptors and nociceptor markers, including Gfrα3 (high-threshold mechanoreceptors), TRPV1 (nociceptors), CGRP (peptidergic neurones), TrkA (putative nociceptors), and TrkC (non-nociceptive neurones). Gfrα3 expression was shown previously to correlate with high-threshold stretch sensitive afferent fibres in the PN (Malin et al., 2009) and we observed high co-expression of galanin and Gfrα3 in DRG neurones (i.e. ≥90% of galanin+ neurones co-stained for galanin and Gfrα3; Fig. 5-4A, B and C). In DRG neurones, Gfrα3 staining was observed in 36.4 ± 2.6% of TL DRG neurones (N = 3, n = 1516) and 33.5 ± 2.8% of LS DRG neurones (N = 3, n = 850) and stained 97.4 ± 2.1% of TL galanin+ DRG neurones (N = 3, n = 75) and 81.2 ± 8.7% of LS galanin+ DRG neurones (N = 3, n = 66)(Fig.5-4B). Gfrα3 also stained 43.3 ± 4.3% of TL (N = 3, n = 407) and 48.3 ± 7.8% of LS (N = 3, n = 225) colonic neurones (i.e. FB+) and stained 90.6 ± 0.7% of TL (N = 3, n = 71) and 97.5 ± 1.3% of LS (N = 3, n = 48) of galanin positive colonic neurones, highlighting the expression of galanin in a putative nociceptive population (Fig. 5-4C).

A significant reduction was observed in the number of TL DRG neurones expressing Gfrα3 in DSS-treated mice compared to healthy mice (36.4 ± 2.6% of healthy TL DRG neurones vs 11.1 ± 2.7% of DSS TL DRG neurones (DSS: N = 3, n = 608), $P < 0.05$, two-way ANOVA with Sidak's post-hoc test), but no such difference was observed in the LS DRG population (33.5 ± 2.8% of healthy LS DRG neurones vs 20.9 ± 4.6% of DSS LS DRG neurones (DSS: N = 3, n = 1062)) or in galanin stained DRG neurones (97.4 ± 2.1% of healthy TL DRG neurones vs 91.6 ± 6.2% of DSS TL DRG neurones (DSS: N = 3, n = 52), 81.2 ± 8.7% of healthy LS DRG neurones vs 90.2 ± 5% of DSS LS DRG neurones (DSS: N = 3, n = 72); Fig. 5-4B). In colonic afferents, there was also no significant difference in the number of neurones expressing Gfrα3 between DSS-treated mice and healthy mice (43.3 ± 4.3% of healthy TL DRG neurones vs 27.2 ± 4.5% of DSS TL DRG neurones (DSS: N = 3, n = 251) 48.3 ± 7.8% of healthy LS DRG neurones vs 34.5 ± 7.1% of DSS LS DRG neurones (DSS: N = 3, n = 434) in DSS; Fig. 5-4C). No significant difference in galanin and Gfrα3 coexpression was observed between healthy and DSS treated mice (90.6 ± 0.7% of healthy TL DRG

neurones vs 72.2 ± 10.7% of DSS TL DRG neurones (DSS: N = 3, n = 38), 97.5 ± 1.3% of healthy LS DRG neurones vs 90.2 ± 8.6% of DSS LS DRG neurones (DSS: N = 3, n = 59); Fig. 5-4C).

In healthy mice, the mean neuronal area of FB-labelled Gfrα3+ DRG neurones was significantly smaller compared to FB-labelled DRG neurones (FB+: 240.3 ± 5.7 μm^2 vs FB+ Gfrα3+: 185.7 ± 6.3 μm^2, $P < 0.0001$, unpaired t-test). Similar to healthy mice, in DSS-treated mice the mean neuronal area of FB+ Gfrα3+ DRG neurones was also significantly smaller than that of FB+ population (FB+: 219.4 ± 3.9 μm^2 vs FB+ Gfrα3+: 195.1 ± 4.8 μm^2, $P = 0.001$, unpaired t-test). This shows Gfrα3 is expressed in smaller diameter DRG neurones in healthy mice and this does not change in DSS-treated mice.

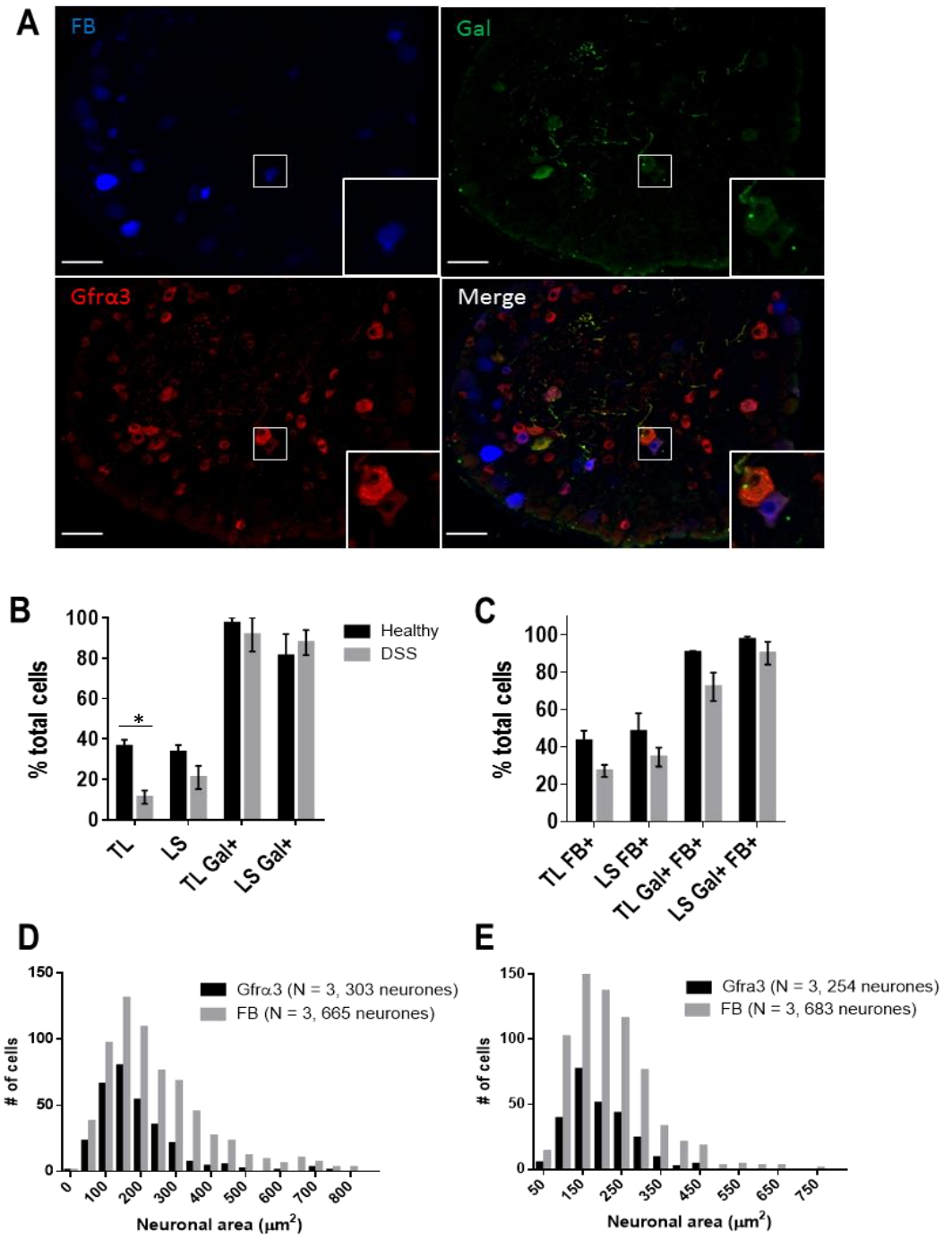


Figure 5-4. Coexpression of galanin and Gfrα3 in colon-innervating DRG sensory neurones.

(A) Representative images of FB staining representing colon-innervating DRG neurones, galanin and Gfrα3 immunofluorescence, scale bar is 50 μm; example of coexpression shown in the inset. No significant change in the galanin+ neuronal population is seen between healthy and DSS-treated mice in the TL and LS populations in the whole DRG (B) or colon-innervating DRG neurones (C) (N = 3, unpaired t-test). Cross-sectional area histogram of FB+ (grey bars) and Gfrα3+ (black bars) neuronal profiles from healthy (D) and DSS-treated (E) mice (N = 3).

TRPV1 can be broadly considered as a nociceptive marker, and it is also expressed in a low firing frequency population of mechanically sensitive afferents that play an important role in the development of inflammatory hyperalgesia (Malin et al., 2009). TRPV1 was expressed by 59.1 ± 6.2% of TL DRG neurones (N = 3, n = 786) and 49.7 ± 1.3% of LS DRG neurones (N = 3, n = 1421) and 96 ± 1.6% of TL galanin+ DRG neurones (N = 3, n = 58) and 94 ± 2.9% of LS (N = 3, n = 136) galanin+ DRG neurones (Fig. 5-5B). TRPV1 was also expressed in 48.7 ± 1.9% of TL DRG neurones (N = 3, n = 380), 56.5 ± 6.4% of LS DRG neurones (N = 3, n = 240) with a high proportion of galanin stained colonic afferents (97 ± 1.4% of TL DRG neurones (N = 3, n = 48), 90.6 ± 6.4% of LS DRG neurones (N = 3, n = 53); Fig. 5-5C) suggesting that galanin is expressed in those neurones involved in inflammatory hyperalgesia.

No significant difference in the number of TRPV1 expressing DRG neurones was observed between healthy and DSS conditions (59.1 ± 6.2% of healthy TL DRG neurones vs 60.1 ± 5.8% of DSS TL DRG neurones (DSS: N = 3, n = 914) and 49.7 ± 1.3% of healthy LS DRG neurones vs 57.2 ± 5.9% of DSS LS DRG neurones (DSS: N = 2, n = 917)) or the in the number of galanin+, TRPV1 expressing DRG neurones between healthy and DSS conditions (96.0 ± 1.6% of healthy TL DRG neurones vs 94.4 ± 3.3% of DSS TL DRG neurones (N = 3, n = 104) and 94.0 ± 2.9% of healthy LS DRG neurones vs 95.8 ± 1.1% of DSS LS DRG neurones (DSS: N = 2, n = 163); Fig. 5-5B). In colonic afferents, there was also no significant difference in the proportion of TRPV1 expressing neurones between healthy and DSS conditions (48.7 ± 1.9% of healthy TL DRG neurones vs 46.3 ± 4.8% of DSS TL DRG neurones (DSS: N = 3, n = 406) and 56.5 ± 6.4% of healthy LS DRG neurones vs 65.5 ± 0.8% of DSS LS DRG neurones (DSS: N = 2, n = 365)). There was also no significant difference in the proportion of galanin and TRPV1 co-expressing FB labelled neurones between healthy and DSS conditions (97.0 ± 1.4 % of healthy TL DRG neurones vs 94.1 ± 3.9% of DSS TL DRG neurones (N = 3, n = 55), 90.6 ± 6.4% of healthy LS DRG neurones vs 98.8 ± 0.8% of DSS LS DRG neurones (N = 2, n = 73); Fig. 5-5C).

In healthy mice, the mean neuronal area of FB-labelled TRPV1+ DRG neurones is significantly smaller than the FB-labelled DRG neurone population (FB+: 205.6 ± 5.1

μm^2 vs FB+ TRPV1+: 175.3 ± 5.2 μm^2, P < 0.0001, unpaired t-test; Fig. 5-5D). In DSS-treated mice the mean neuronal area of FB+ TRPV1+ neurones was also significantly smaller than that of the general FB+ population (FB+: 173.1 ± 4.4 μm^2 vs FB+ TRPV1+: 147.7 ± 4.1 μm^2, P < 0.0001, unpaired t-test; Fig. 5-5E). This shows TRPV1 is mainly expressed in small diameter neurones (i.e. putative nociceptors) and that this does not change under inflammatory conditions.

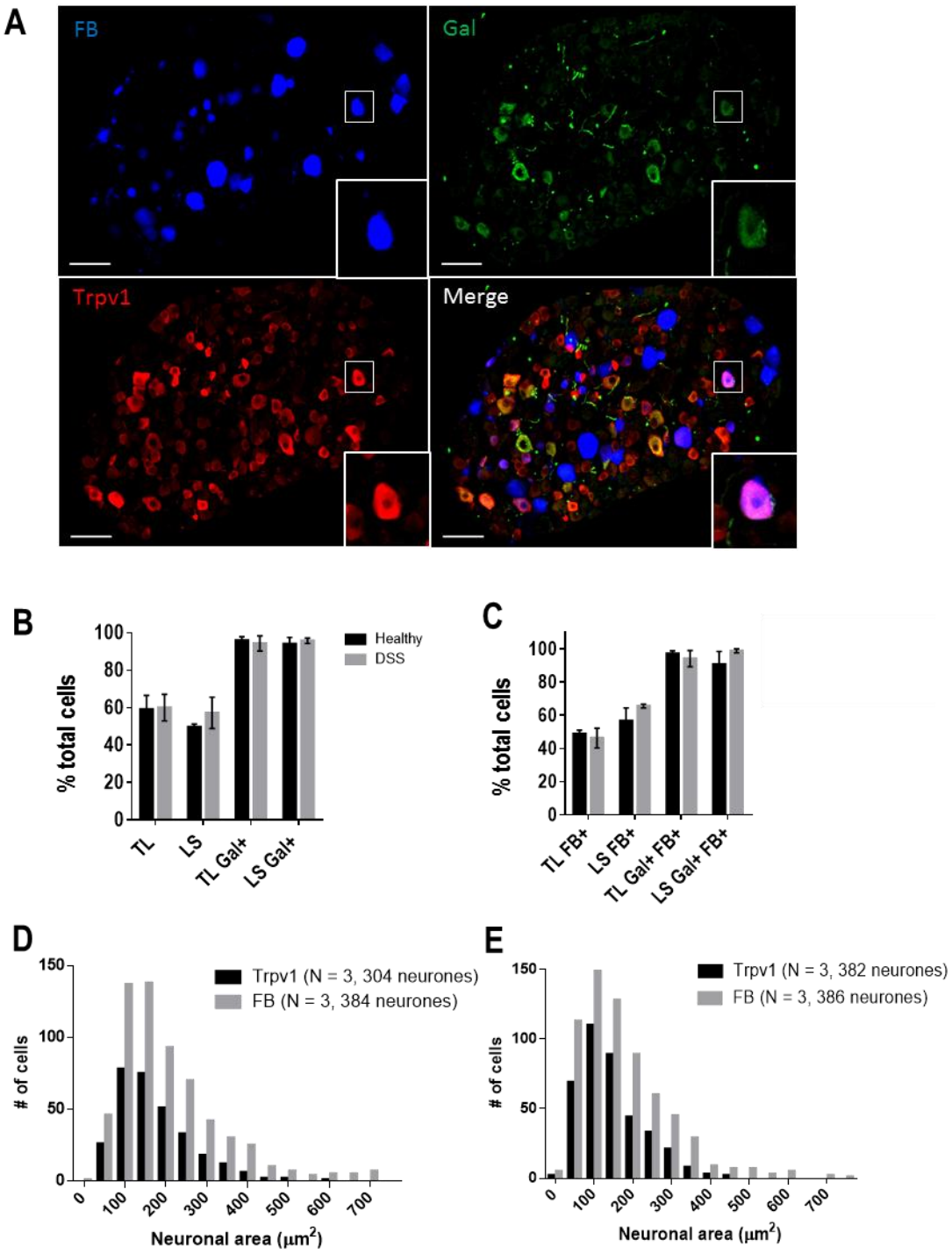


Figure 5-5. Coexpression of galanin with TRPV1 in colon-innervating DRG sensory neurones

(A) representative images of FB staining representing colon-innervating DRG neurones, galanin and TRPV1 immunofluorescence. Example of co-localisation shown in the inset. Scale bar is 50 µm. No significant change in the galanin positive neuronal population is seen between healthy and DSS-treated mice in the TL and LS populations in the whole DRG (B) or in colon-innervating DRG neurones (C) (N = 3, unpaired t-test). Cross-sectional area histogram of FB+ (grey bars) and TRPV1+ (black bars) neuronal profiles from healthy (D) and DSS-treated (E) mice (N = 3).

CGRP was used as a marker of peptidergic neurones. In DRG neurones, CGRP was expressed in 41.9 ± 2.4% of TL DRG neurones (N = 3, n = 363) and in 42.4 ± 2.7% of LS DRG neurones (N = 3, n = 561), similar to what has been previously reported (Qiao and Grider, 2009). CGRP also displayed high coexpression with galanin in DRG neurones (86.5 ± 3.8% of TL DRG neurones (N = 3, n = 47), 89.3 ± 6.7% of LS DRG neurones (N = 3, n = 89); Fig. 5-6C). In colonic afferents, CGRP was expressed in 33.3 ± 6.5% of TL DRG neurones (N = 3, n = 603) and in 27.8 ± 5.6% of LS DRG neurones (N = 3, n = 829), and also displayed high coexpression with galanin in colonic afferents (82.1 ± 9.4% of TL DRG neurones (N = 3, n = 51), 77.8 ± 9.1% of LS DRG neurones (N = 3, n = 72); Fig. 5-6B).

When comparing healthy and colitic mice, no significant difference was observed between healthy and DSS-treated mice in the number of CGRP expressing DRG neurones (41.9 ± 2.4% of healthy TL DRG neurones vs. 33.2 ± 10.6% of DSS TL DRG neurones (DSS: N = 3, n = 736) and 42.4 ± 2.7% of healthy LS DRG neurones vs. 39.3 ± 15.1% DSS LS DRG neurones (DSS: N = 2, n = 617)), or in the number of CGRP expressing galanin positive DRG neurones (86.5 ± 3.8% of healthy TL DRG neurones vs. 89.6 ± 5.6% DSS TL DRG neurones (DSS: N = 3, n = 53) and 89.3 ± 6.7% of healthy LS DRG neurones vs. 81.2 ± 10.7% of DSS LS DRG neurones (DSS: N = 2, n = 108); Fig. 5-6B). Expression of CGRP in colonic DRG neurones also showed no significant difference in DSS-treated mice compared to healthy mice (33.3 ± 6.5% of healthy TL DRG neurones vs. 50.5 ± 5.1% of DSS TL DRG neurones (DSS: N = 3, n = 349), 27.8 ± 5.6% of healthy LS DRG neurones vs. 45.2 ± 5.6% of DSS LS DRG neurones (DSS: N = 2, n = 156); Fig. 5-6C) and no change in coexpression with galanin was observed (82.7 ± 1.9% TL DRG neurones (DSS: N = 3, n = 44), 86.6 ± 0.6% LS DRG neurones (DSS: N = 2, n = 25); Fig. 5-6C).

In healthy mice, there was no significant difference in the mean neuronal area between FB-labelled DRG neurones and FB-labelled CGRP-positive DRG neurones (FB+: 221.6 ± 6.5 μm^2 vs FB+ CGRP+: 192.1 ± 7.4 μm^2, $P < 0.062$, unpaired t-test; Fig. 5-5D). There was also no significant difference in the profile area mean of FB+ CGRP expressing neurone compared to the FB+ DRG neurone population in DSS mice (FB+:

208.7 ± 6.8 μm^2 vs FB+ CGRP+: 209.7 ± 9.5 μm^2, $P < 0.94$, unpaired t-test; Fig. 5-5E).

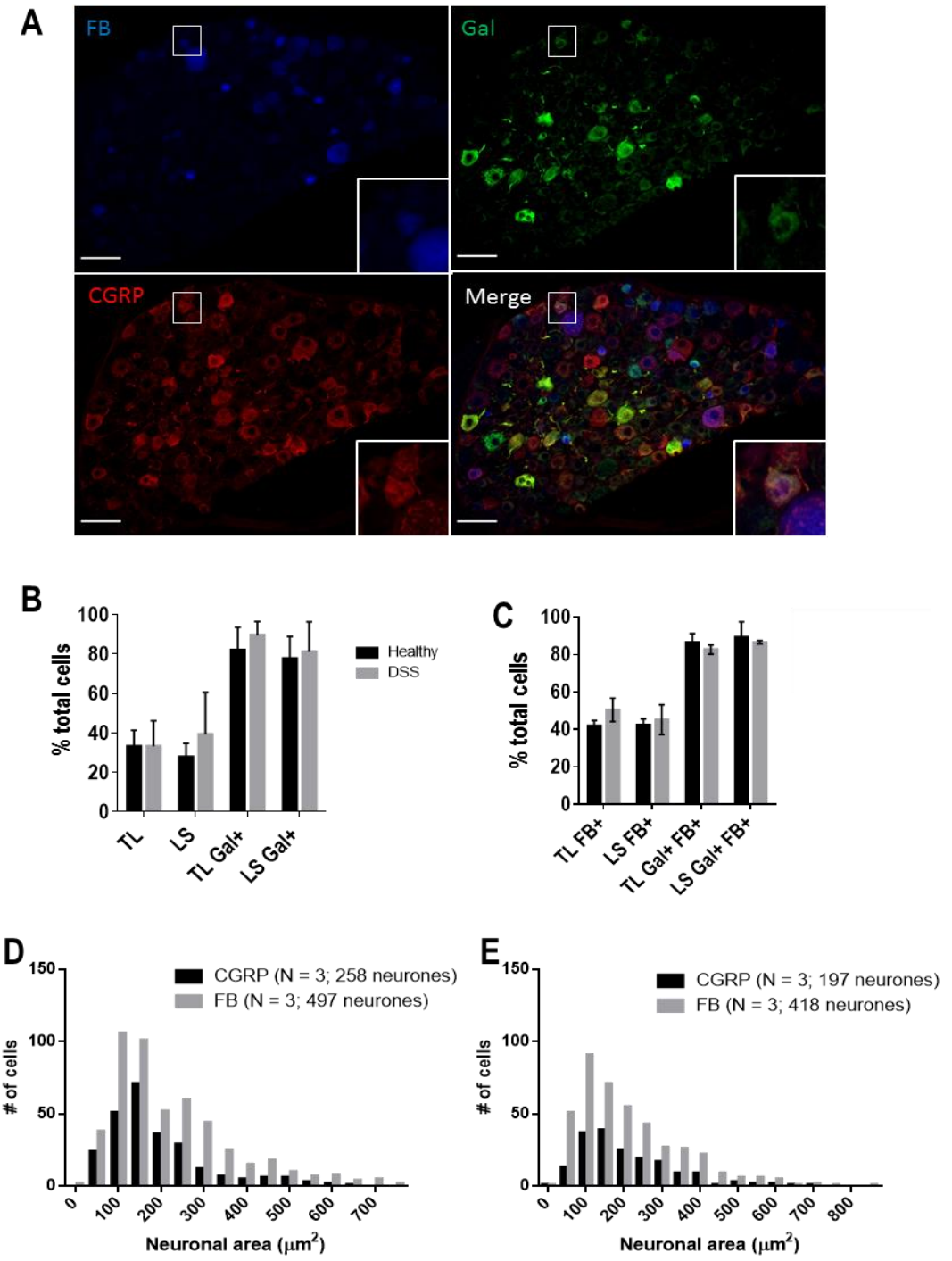


Figure 5-6. Coexpression of galanin with CGRP colon-innervating DRG sensory neurones.

(A) representative images of Fast Blue (FB) staining representing colon-projecting DRG neurones, galanin and CGRP immunofluorescence. Example of coexpression shown in the inset. Scale bar is 50 μm. No significant change in galanin positive neuronal population is seen between healthy and DSS-treated mice in the thoracolumbar (TL) and lumbosacral (LS) populations in the whole DRG (B) or colon-projecting DRG neurones (C) (N = 3, unpaired t-test). Cross-sectional area histogram of FB+ (grey bars) and CGRP+ (black bars) neuronal profiles from healthy (D) and DSS-treated (E) mice (N = 3).

TrkA was used as a second marker for peptidergic putative nociceptors (Gold and Gebhart, 2010). In DRG neurones, TrkA was expressed by 42.9 ± 2.3% of TL DRG neurones (N = 3, n = 1042) and 43.5 ± 2.8% of LS DRG neurones (N = 3, n = 887) and when examining galanin expressing DRG neurones, TrkA was expressed by 81.1 ± 7.7% of TL DRG neurones (N = 3, n = 61) and 84.4 ± 3.7% of LS DRG neurones (N = 3, n = 122) (Fig. 5-7B). TrkA was also expressed by 67.9 ± 3.2% of TL DRG neurones (N = 3, n = 466) and 49.3 ± 4.4% of LS DRG neurones (N = 3, n = 289). TrkA was also highly coexpressed with galanin in 75.5 ± 6.7% of TL DRG neurones (N = 3, n = 112) and 55.6 ± 2.5% of LS DRG neurones (N = 3, n = 90) (Fig. 5-7C), further supporting the hypothesis that galanin plays a role in nociceptive signalling.

No significant difference in the number of TrkA expressing DRG neurones was observed between healthy and DSS-treated mice (42.9 ± 2.3% of healthy TL DRG neurones vs 34.5 ± 8.2% TL DRG neurones (N = 3, n = 933) and 43.5 ± 2.8% of healthy LS DRG neurones vs 41.0 ± 3.7% of DSS LS DRG neurones (DSS: N = 2, n = 1316)), however, in DSS treated mice, coexpression with galanin significantly reduced in the LS DRG neurones population (84.4 ± 3.7% of healthy LS DRG neurones vs 37.4 ± 2.9% of DSS LS DRG neurones (DSS: N = 3, n = 97), $P < 0.001$, two-way ANOVA with Sidak's post-hoc test) but not the TL DRG neurones population (81.1 ± 7.7% of healthy TL DRG neurones vs 91.9 ± 3.9% of DSS TL DRG neurones (DSS: N = 3, n = 52); Fig. 5-7B). In colonic DRG neurones, again, there was no significant difference between healthy and DSS mice (67.9 ± 3.2% of healthy TL DRG neurones vs 57.4 ± 8.3% of DSS TL DRG neurones (DSS: N = 3, n = 213), 49.3 ± 4.4% of healthy LS DRG neurones vs 56.4 ± 6.8 % of DSS LS DRG neurones (DSS: N = 3, n = 387); Fig. 5-7C). However, interestingly, the coexpression with galanin significantly increased in the LS DRG neurones population (55.6 ± 2.5% of healthy LS DRG neurones vs 90.4 ± 3.9% of DSS LS DRG neurones (DSS: N = 3, n = 66), $P < 0.01$, two-way ANOVA with Sidak's post-hoc test), but not the TL population (75.5 ± 6.7% of healthy TL DRG neurones vs 72.5 ± 4.3% of DSS TL DRG neurones (N = 3, n = 38); Fig. 5-7C).

In healthy mice, there was no significant difference in the profile area means of DRG neurones between the FB+ population and FB+ TrkA+ expressing population (FB+: 236.6 ± 6.5 μm^2 vs FB+ TrkA+: 256.8 ± 7.8 μm^2, P = 0.062, unpaired t-test; Fig. 5-7D). There was also no significant difference in the mean neuronal area of DRG neurones between FB+ TrkA expressing and the FB+ population in DSS mice (FB+: 213.5 ± 4.1 μm^2 vs FB+ TrkA+: 223.6 ± 6.1 μm^2, P = 0.16, unpaired t-test; Fig. 5-7E).

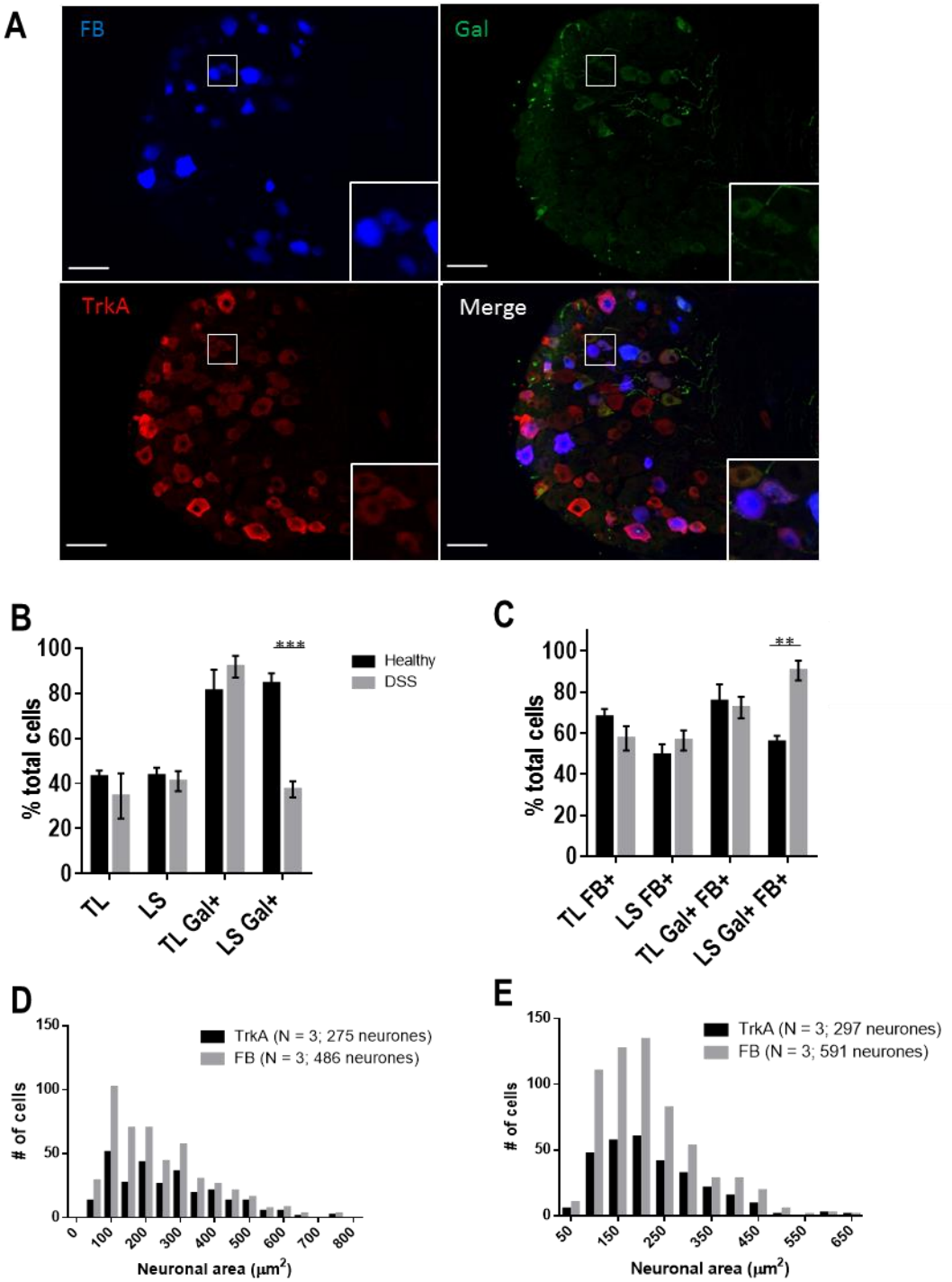


Figure 5-7. Coexpression of galanin with TrkA colon-innervating DRG sensory neurones.

(A) Representative images of FB staining representing colon-innervating DRG neurones, galanin and TrkA immunofluorescence. Example of co-localisation shown in the inset. Scale bar is 50 μm. No significant change in the galanin positive neuronal population is seen between healthy and DSS-treated mice in the TL and LS populations in the whole DRG (B) or colon-projecting DRG neurones (C) (N = 3, unpaired t-test). Cross-sectional area histogram of FB+ (grey bars) and TrkA+ (black bars) neurones from healthy (D) and DSS-treated (E) mice (N = 3).

Lastly, TrkC was used as a marker of non-nociceptive neurones (Stephens et al., 2005) and therefore, as expected, showed low expression in colonic afferent populations (8.3 ± 1.9% of TL DRG neurones (N = 3, n = 345), 1.5 ± 0.9% of LS DRG neurones (N = 3, n = 295)) and no coexpression with galanin in either TL or LS populations (0% TL DRG neurones (N = 3, n = 58) and LS DRG neurones (N = 3, n = 62) (Fig. 5-8B). In DRG neurones in general, TrkC was expressed by 11.9 ± 1.2% of TL DRG neurones (N = 3, n = 1189) and 11.6 ± 1.9% of LS DRG neurones (N = 3, n = 856) and showed no coexpression with galanin in TL DRG neurones (N = 3, n = 66) or LS DRG neurones (N = 3, n = 65) DRG neurones (Fig. 5-7B).

No significant difference was observed between healthy and DSS-treated mice in the number of TrkC expressing DRG neurones (11.9 ± 1.2% of healthy TL DRG neurones vs 12.5 ± 4.6% of DSS TL DRG neurones (DSS: N = 3, n = 535) and 11.6 ± 1.9% of healthy LS DRG neurones vs 18.8 ± 4.9% DSS LS DRG neurones (DSS: N = 2, n = 515)) or galanin+ DRG neurones (0% of healthy TL DRG neurones vs 6.9 ± 0.9% DSS TL DRG neurones (DSS: N = 3, n = 41) and 0% of healthy LS DRG neurones vs 0% of DSS LS DRG neurones (DSS: N = 3, n = 39); Fig. 5-8B). TrkC expression in colonic afferents also showed no significant change when comparing DRG neurones from healthy mice with those from DSS mice (8.3 ± 1.9 % of healthy TL DRG neurones vs 11.1 ± 2.3% of DSS TL DRG neurones (DSS: N = 3, n = 140), 1.5 ± 0.9% of healthy LS DRG neurones vs 1.8 ± 0.75% of DSS LS DRG neurones (DSS: N = 3, n = 323) in DSS; Fig. 5-8C), nor was any change in the coexpression with galanin observed (0% of healthy TL DRG neurones vs 5.9 ± 2.4% of DSS TL DRG neurones (DSS: N = 3, n = 40), 0% of healthy LS DRG neurones vs 3.2 ± 1.4% of LS DRG neurones (N = 3, n = 73); Fig. 5-8C).

In healthy mice, the mean neuronal area of FB-labelled TrkC+ DRG neurones was significantly larger than FB-labelled DRG neurones (FB+: 159.3 ± 4.1 μm^2 vs FB+ TrkC+: 265.8 ± 21.8 μm^2, $P < 0.0001$, unpaired t-test; Fig. 5-8D). In DSS-treated mice, the mean neuronal area of FB-labelled TrkC+ neurones was also significantly larger than that of FB-labelled DRG population (FB+: 215.5 ± 6.3 μm^2 vs FB+ TrkC+: 347.7 ± 28.4 μm^2, $P < 0.0001$, unpaired t-test; Fig. 5-8E). This shows TrkC is expressed in larger diameter DRG neurones that are less likely to be involved in nociception.

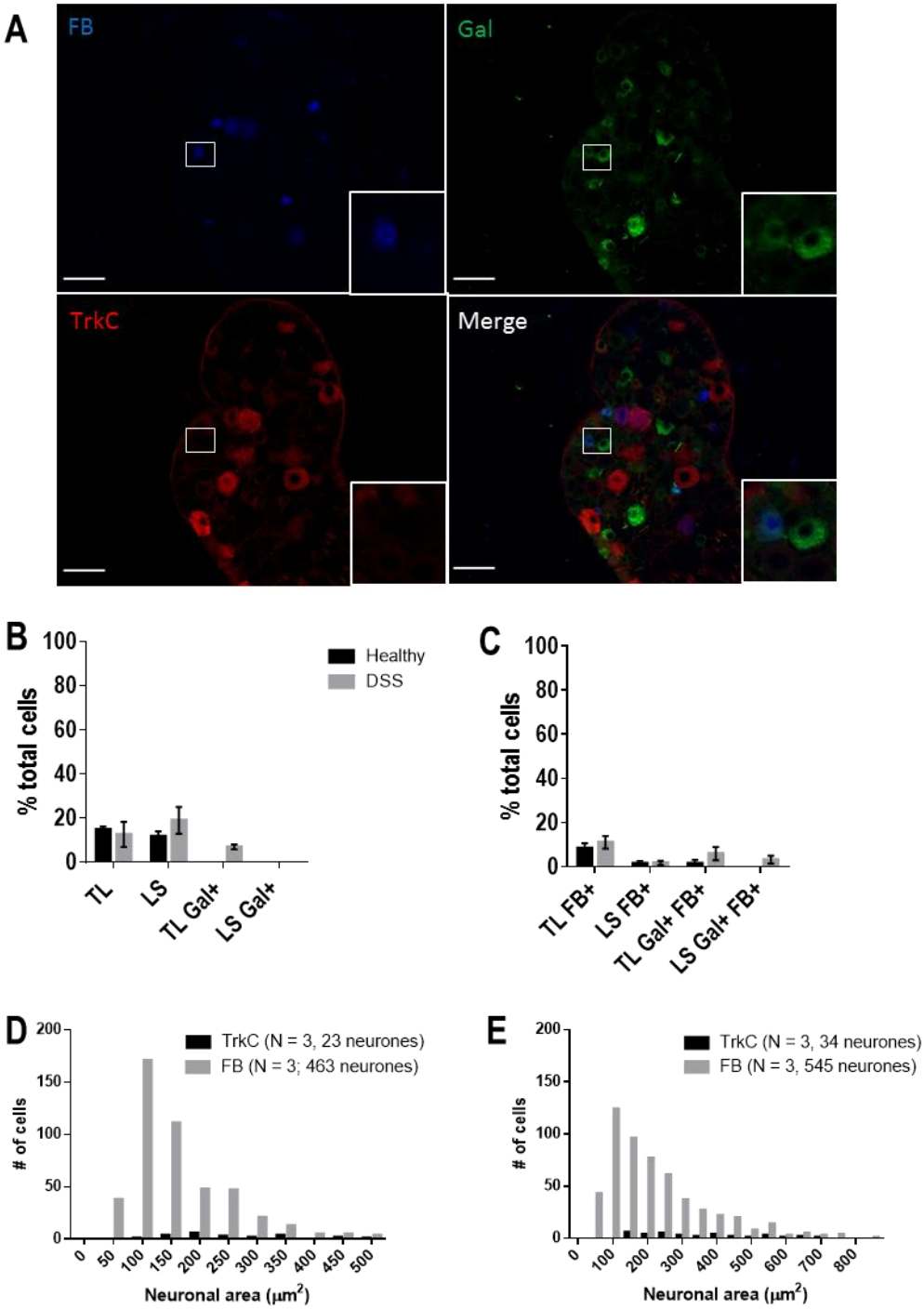


Figure 5-8. Co-expression of galanin with TrkC colon-innervating extrinsic neurones.
(A) representative images of FB staining representing colon-innervating DRG neurones, galanin and TrkC immunofluorescence. Example of coexpression shown in the inset. Scale bar is 50 μm. No significant change in galanin positive neuronal population was seen between healthy and DSS-treated mice in the thoracolumbar (TL) and lumbosacral (LS) populations in the whole DRG (B) or colon-innervating DRG neurones (C) (N = 3, unpaired t-test). Cross-sectional area histogram of FB+ (grey bars) and TrkC+ (black bars) neuronal areas from healthy (D) and DSS-treated (E) mice (N = 3).

5.3.4 DSS induces mechanical hypersensitivity in the LSN

Using a ramp distention from 0 to 80 mmHg, we observed that the LSN isolated from DSS treated mice produced a greater response at non-nociceptive pressure, but not at nociceptive pressure, compared to the LSN from healthy mice (20 mmHg: 6.9 ± 5.0 spikes s^{-1} vs 19.8 ± 4.7 spikes s^{-1}, P = 0.0184, N = 6, unpaired t-test, and 80 mmHg: 34.7 ± 8.1 spikes s^{-1} vs 39.7 ± 3.2 spikes s^{-1}, P = 0.137, N = 6, unpaired t-test, Fig. 5-10A and B). In addition, the basal firing of LSN from DSS treated mice was significantly greater than that of healthy mice (3.9 ± 1.4 spikes s^{-1} vs 28.4 ± 13.4 spikes s^{-1}, P = 0.012, N = 6, unpaired t-test). These data suggest that DSS induced a state of hypersensitivity in the LSN.

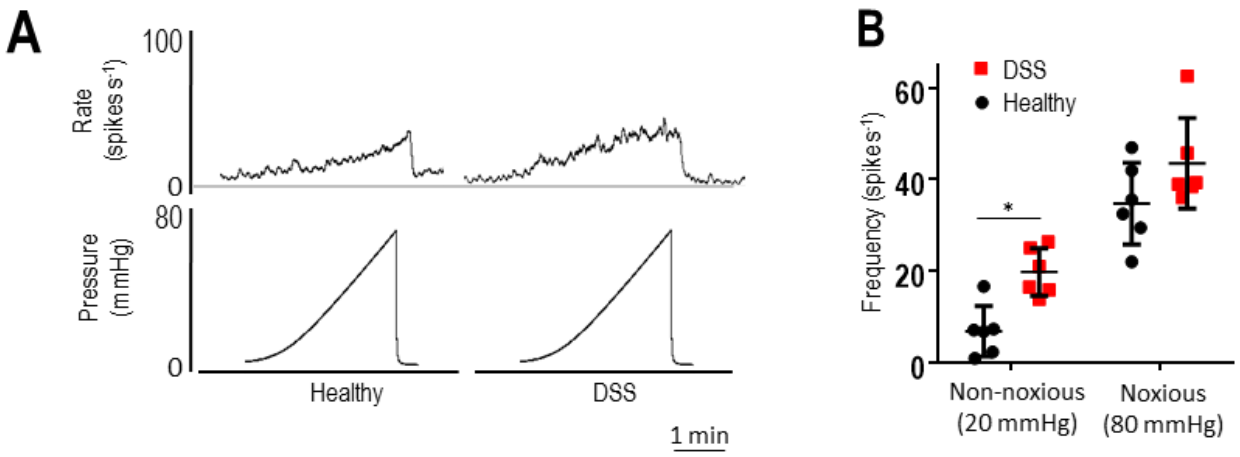


Figure 5-9. DSS-treated mice display LSN hypersensitivity

(A) Representative frequency histograms of ramp distensions, 0 – 80 mmHg in colons isolated from healthy and DSS-treated mice. (B) DSS increases the LSN response to non-noxious (20 mmHg), but not noxious (80 mmHg) pressures; unpaired t-test between groups *P < 0.05.

5.3.5 Galanin does not inhibit DSS-induced mechanical hypersensitivity

Finally, we investigated if the inhibition of LSN activity by galanin observed in healthy mice (Fig. 3-2) was maintained in the LSN isolated from mice undergoing DSS-induced colonic inflammation. As observed previously, here in a second cohort of mice, 500 nM galanin inhibited basal nerve firing (3.9 ± 1.4 spikes s^{-1} vs 0.6 ± 0.4 spikes s^{-1}, P = 0.017, N = 5, paired t-test; Fig. 5-11B), the peak firing frequency (53.3 ± 12.8 spikes s^{-1} vs 25.2 ± 5.2 spikes s^{-1}, P = 0.015, N = 5, paired t-test; Fig. 5-11C), phase I average firing frequency (31.8 ± 2.9 spikes s^{-1} vs 12.5 ± 1.9 spikes s^{-1}, P = 0.021 , N = 5, paired t-test; Fig. 5-11D) and phase II average firing frequency (18.6 ± 6.0 spikes s^{-1} vs 9.3 ± 4.3 spikes s^{-1}, P = 0.032, N = 5, paired t-test; Fig. 5-11E) in response to a phasic distension to 80 mmHg. The activity of the LSN between healthy and DSS-treated mice suggested the LSN in DSS-treated mice was sensitised with increased hypersensitivity to mechanical distension. The LSN basal activity in DSS-treated mice was significantly greater than LSN basal activity in healthy mice (3.9 ± 1.4 spikes s^{-1} vs 28.4 ± 13.6 spikes s^{-1}, P = 0.0053, N = 5 for healthy and N = 6 for DSS-treated, unpaired t-test; Fig. 5-11B and F). Neither the peak firing frequency (53.3 ± 12.8 spikes s^{-1} vs 65.7 ± 16.6 spikes s^{-1}, P = 0.275, N = 5 for healthy and N = 6 for DSS-treated, unpaired t-test; Fig. 5-11C and G) nor phase I average firing frequency (31.8 ± 2.9 spikes s^{-1} vs 46.3 ± 5.9 spikes s^{-1}, P = 0.116, N = 5 for healthy and N = 6 for DSS-treated, unpaired t-test; Fig. 5-11D and H) is significantly different between healthy and DSS-treated mice. However, phase II firing frequency (18.6 ± 6.0 spikes s^{-1} vs 34.1 ± 9.9 spikes s^{-1}, P = 0.037, N = 5 for healthy and N = 6 for DSS-treated, unpaired t-test; Fig. 5-11E and I) is significantly greater in DSS-treated mice compared to healthy mice.

However, when measuring LSN activity in nerves isolated from DSS mice, galanin had no significant effect upon basal nerve firing (28.4 ± 13.6 spikes s^{-1} vs 26.6 ± 12.1 spikes s^{-1}, P = 0.62, N = 6, paired t-test; Fig. 5-11F), nor was any effect of galanin observed on the peak firing frequency (65.7 ± 16.6 spikes s^{-1} vs 66.6 ± 22.3 spikes s^{-1}, P = 0.81, N = 6, paired t-test; Fig. 5-11G), phase I average firing frequency (46.3 ± 5.9 spikes s^{-1} vs 51.2 ± 8.8 spikes s^{-1}, P = 0.24, N = 6, paired t-test; Fig. 5-11H) or phase II average firing frequency (34.1 ± 9.9 spikes s^{-1} vs 41.9 ± 14.0 spikes s^{-1}, P = 0.54, N = 6, paired t-test; Fig. 5-11I) induced by a phasic distension to 80 mmHg.

These results indicate that the inhibitory action of galanin upon LSN activity observed in the LSN when isolated from healthy mice is lost in tissue isolated from mice with acute colitis.

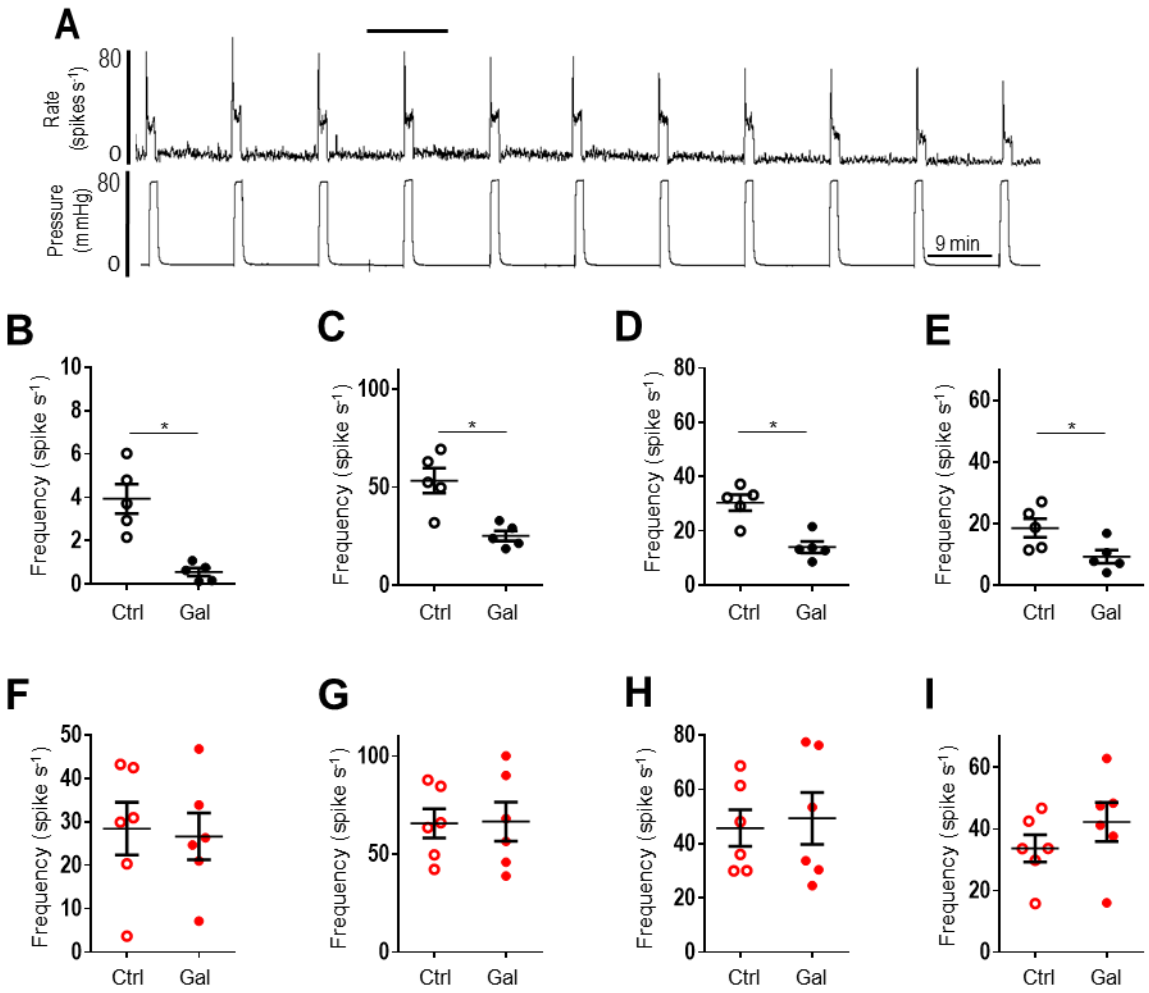


Figure 5-10. Galanin does not inhibit DSS-induced LSN hypersensitivity

(A) Example frequency histogram of the LSN response to repeated phasic intraluminal distension (0 – 80 mmHg), showing that the reversible inhibition by galanin of LSN mechanosensitivity in healthy mice (Fig. 3-2) is lost in DSS-treated mice; black bar – 500 nM galanin application. 500 nM galanin inhibits baseline (B), peak (C) phase I (D) and phase II (E) responses of the LSN to phasic distension of the colon to 80 mmHg in healthy mice, but has no such effect on baseline (F), peak (G), phase I (H) or phase II (I) responses of the LSN to phasic distension of the colon to 80 mmHg when the colon has been isolated from DSS treated mice (paired t-test, N = 5 for healthy and N = 6 for DSS).

5.4 Discussion

In a previous study, galanin mRNA was shown to be present in putative nociceptive populations (mPEPb and pPEP) (Hockley et al., 2019). The results shown here support this RNA-sequencing data by demonstrating at a protein level that galanin is expressed in likely nociceptive populations of colonic afferents, as evidenced through co-localisation with multiple nociceptive markers: TRPV1, TrkA and Gfrα3. We found the level of expression of these markers in healthy whole DRG was similar to previously published results: TRPV1 (Christianson et al., 2006a), TrkA (Qiao and Grider, 2009), and Gfrα3 (Baudet et al., 2000). Previous work has also showed that galanin displays low coexpression with TrkC, a marker for non-nociceptive neurones (Ogihara et al., 2016). There is a low number of non-nociceptive neurones innervating the distal colon (Sengupta et al., 1990; Sengupta and Gebhart, 1994), but of the small number of TrkC positive neurones observed in the current study, galanin showed little coexpression, suggesting that galanin has little to no function within this population. Coupled with galanin's high coexpression with nociceptive markers, these data suggest that galanin is expressed predominantly in a nociceptive colonic DRG neurone population. Here it was also observed that the number of galanin expressing neurones in this nociceptive population does not significantly increase in our model of acute inflammation, nor did the galanin concentration in whole colon extracts, which takes into account that galanin is expressed by multiple cell types in the distal colon in addition to sensory neurones, including enterochromaffin cells and fibroblasts (Schäfermeyer et al., 2004; Yamamoto et al., 2014). These data align with those of a previous study, which showed that galanin was upregulated in models of neuropathic pain (sciatic nerve transection), but not inflammatory pain (hind-paw injection of 4% carrageenan; Zhang et al., 1998).

However, the pattern of galanin expression and inflammation is not necessarily so straightforward, such that it has been observed that galanin mRNA and peptide levels do change in some acute (Jimenez-Andrade et al., 2006) and chronic (Butler et al., 1992) models of inflammation. For example, following hind-paw injection of carrageenan in rats, an ipsilateral decrease in galanin levels was observed 5 days later, followed by an increase in galanin levels above control levels after 21 days

(Calzà et al., 1998). This could suggest that galanin levels may increase in a chronic model of colitis. Also, following hind-paw injection of carrageenan in rats, GalR1 mRNA expression in DRG neurones (L4 and L5) has been shown to decrease (Xu et al., 1996), whereas GalR2 mRNA expression is transiently upregulated (Sten Shi et al., 1997). In this study, galanin was observed to lose its inhibitory modulatory effect on the LSN in DSS mice compared to healthy (Fig.5-11) and thus a similar down regulation of GalR1 in colonic sensory neurones to that observed by Xu et al. in the carrageenan model could account for the loss of galanin's inhibitory effect.

With regard to the lack of inhibitory effect observed by galanin on LSN activity from DSS mice, a further point to consider is that multiple inflammatory mediators will be released from the colon tissue during DSS-induced colitis (Spiller and Major, 2016), which would have their effects on a broad range of afferents. Therefore, any effect of galanin on the whole-nerve response may simply be overcome by the overall level of sensitisation, across a broad range of nerve fibres. The absence of reliable tools to investigate GalR protein levels and the validity of current antibodies being uncertain (Lu and Bartfai, 2009) makes quantifying the protein level of GalR in colonic afferents a significant challenge. Single cell qPCR of colonic retrograde labelled DRG neurones, isolated from both healthy and DSS-treated mice was utilised to discern if there was any change in the levels of galanin receptor mRNA during inflammation, however, the initial study produced unreliable results, which are outlined in Section 7.2, but this would still be an interesting area of further investigation in future studies and this, alongside further explanations for the findings in this chapter, are discussed in the future work section in Chapter 6.

Chapter 6 – Conclusions and Future Directions

6.1 Summary

Visceral sensory neurones are responsive to a wide range of both chemical and mechanical stimuli in physiological and pathophysiological states (Hockley et al., 2018; Brierley et al., 2018). The inflammation observed in IBD causes visceral hypersensitivity, such that physiological bowel movements produce pain. Identification of regulators of visceral afferent excitability represents an attractive target in developing novel analgesics to manage IBD pain and other visceral pain conditions. When considering future therapeutic avenues for visceral pain, modulation of galaninergic signalling is a potential target due to prior demonstration of galanin being a potent regulator of gastro-oesophageal afferent excitability (Page et al., 2005b), but to date there has been no published study that extensively investigates galaninergic signalling in the distal colon.

6.2 Overview of results

Using immunohistochemistry, I identified that galanin is present in approximately 20% of colon innervating sensory neurones (a result confirming published scRNA-seq data; Hockley et al., 2019), which is significantly higher than galanin's expression in the whole DRG neurone population (approximately 5%) suggesting a key role for galanin in colon-innervating afferents. The data presented also demonstrate high coexpression of galanin with markers of nociceptive populations (e.g. TRPV1 and TrkA) further suggesting a potential role for galanin in the modulation of visceral nociception.

The impact of galanin on mechanosensitivity in healthy mice was investigated using whole-nerve electrophysiological recordings of LSN activity in a tubular, *ex vivo* colon preparation, which enables the physiologically relevant method of activating LSN sensory neurones by colonic distension. Both phasic and ramp distension to noxious pressure were used to determine the impact of galanin on both low- and high-threshold sensory neurones. The data obtained demonstrate that galanin modulates colonic afferent excitability and responsiveness to innocuous mechanical and noxious chemical and mechanical stimuli. Specifically, the data show a potent, dose-dependent inhibition of LSN responses to innocuous and noxious mechanical stimuli under control conditions and in a state of hypersensitivity evoked by application of inflammatory mediators. Pharmacological experiments further suggested that GalR1

mediates the inhibitory effect of galanin and that there is no obvious role for GalR2 in colonic sensory neurones, and, as discussed in Chapter 1, previous studies have yet to show that GalR3 produces any modulating role in GI-innervating afferents (SNAP 37889; Page et al., 2005b; Swanson et al., 2005). The data from this study thus fit alongside galanin's reported predominantly inhibitory role in the upper GI tract (Page et al., 2005b, 2007) and parallels the inhibitory function of galanin in somatic sensory innervation (Flatters et al., 2003; Heppelmann et al., 2000). Overall, these results highlight the potential anti-nociceptive effects of galanin in the distal colon.

Multiple mediators are released during tissue damage and inflammation that activate and sensitise the afferent endings and thus likely contribute to chronic visceral pain (Hockley et al., 2018; Brierley et al., 2018). I thus investigated the effects of galanin in the presence of an IS, consisting of mediators known to be released from stressed or damaged cells and shown to cause visceral afferent sensitisation that is associated with IBD in humans (Grundy, 2004). The data obtained show that galanin reduces the LSN hypersensitivity induced by these mediators when applied intraluminally to a healthy mouse colon. Therefore, these results suggest that under certain circumstances that galanin may be beneficial in counteracting inflammation-induced visceral hypersensitivity.

The impact of disease-derived inflammatory mediators was further investigated by using a mouse model of acute colonic inflammation. The data presented demonstrate that the DSS model of chemically-induced colitis produces robust and reliable colonic inflammation in C57BL/6J mice, as others have shown (Cooper et al., 1993; Munyaka et al., 2016; Solomon et al., 2010). The data presented further show that the LSN from mice treated with DSS display hypersensitivity to colonic distension and an increased LSN basal firing rate, but that galanin no longer has any inhibitory effect on this hypersensitivity.

6.3 Galanin signalling in colonic sensory neurones

Galanin is expressed by multiple cell types in the distal colon including enterochromaffin cells, fibroblasts (Schäfermeyer et al., 2004; Yamamoto et al., 2014), and, as shown here, a subset of colonic sensory neurones. Galanin has been implicated as a modulator of numerous activities in the GI tract including regulation of

neurotransmitter release, motility and secretion (Benya et al., 1999; Sternini et al., 2004). With regard to nociception in the GI tract, galanin has been shown to modulate mechanosensitivity of gastro-oesophageal vagal afferents with predominantly inhibitory actions on individual fibres via GalR1 (Page et al., 2005b, 2007). The data presented here builds on this by demonstrating that galanin also inhibits LSN mechanosensitivity and likely does so though GalR1 activation.

Mechanical distension of the colon is capable of producing pain in humans and nociceptive behaviour in animals (Gebhart, 2004; Ness et al., 1990) and thus colonic distension is a useful tool when examining hyperexcitability in models of colonic inflammation. Multi-unit recordings presented in this thesis show the overall influence of galanin on LSN activity, but the effect of galanin on specific afferent populations (defined either by transcriptomics or activity; Hockley et al., 2019; Brookes et al., 2013) remains unknown and should be a future research objective. It is seen that from multi-unit recordings of LSN activity, not only does galanin inhibit baseline firing, but it also inhibits mechanically-evoked responses to distension of the distal colon to noxious pressures and the hypersensitivity following addition of inflammatory stimuli. This suggests that GalRs, specifically in the case of the LSN GalR1, function as integral regulators of neuronal excitability. Single-unit recordings could distinguish the type of mechanosensitive afferent (e.g. stretch, stroke and von Frey hair probing of their receptive fields; Brierley et al., 2004) and whether or not they are equally inhibited by galanin. The fact that the inhibitory effect of galanin was observed across the full range of distension pressures from physiological through to noxious (i.e. 0 – 80 mmHg) suggests that galanin is not only expressed by high-threshold nociceptors, but likely also wide dynamic range (WDR) afferents. When examining galanin expression, both colonic sensory neurone RNA-sequencing and immunohistochemistry demonstrate that galanin is expressed in putative nociceptors (Hockley et al., 2019). The observation that galanin modulates mechanosensitivity in the LSN and that it is contained within sensory neurones innervating the colon could indicate a potential autoregulatory role of galanin, in addition to the role of galanin from other sources.

With regard to GalR1, single-cell RNA-sequencing of colonic sensory neurones indicates that GalR1 is predominantly expressed in neurones expressing nociceptor markers, such as TRPV1 and the bradykinin B2 receptor, as well as in a population of neurones expressing the mechanosensitive ion channel Piezo2 (i.e. putative low-threshold mechanosensory afferents), an expression pattern that likely explains the inhibitory impact of galanin on LSN activity across a wide range of pressures. The GalR1 receptor is coupled to GIRKs giving rise to hyperpolarization (Smith et al., 1998; Walker et al., 1997), an effect that would account for the inhibitory activity of galanin observed in this study. This conclusion is further supported by data demonstrating that inhibition of LSN activity was also produced by the GalR1 agonist M617, but not the GalR2 agonist spexin. Further work to reinforce this would be to use a non-peptide GalR1 antagonist potentially to show reversal of galanin's inhibitory effect in healthy mice, however a non-peptide GalR1 antagonist is currently unavailable. GalR1 KO mice (used in previous studies; (Jacoby et al., 2002; Malkmus et al., 2005) could also potentially be used, such that a loss of the inhibitory effects of galanin in healthy mice in GalR1 KO mice would indicate the critical role of GalR1 in mediating the effects observed in this study.

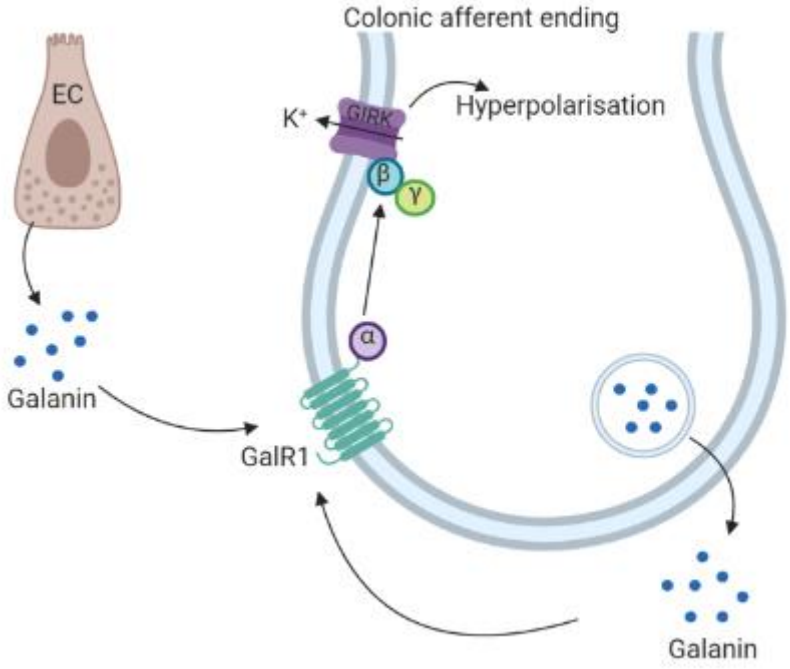


Figure 6-1. Schematic of the potential signalling pathway activated by galanin in colonic afferents

Once galanin is released from colonic sensory neurones or other cell types in the colon (such as enterochromaffin cells – EC) it can bind to GalR1, the β/γ subunit of the dissociated G protein then activates GIRK channels. This causes K^+ efflux leading to hyperpolarisation of the colonic sensory afferent and consequently reduces neuronal activity.

6.4 Role of galanin in inflammatory pain pathways

As observed in previous studies (Hockley et al., 2018; Brierley et al., 2018), the data presented here also demonstrate that intraluminal application of an IS produced robust LSN hyperexcitability to multiple noxious mechanical stimuli. Part of this hyperexcitability likely results from recruitment of 'silent' or mechanically insensitive afferents in both the LSN and PN (Feng and Gebhart, 2011), potentially through disinhibition of Piezo2 (Prato et al., 2017). The ability of galanin to reverse the mechanically hypersensitivity induced by the IS correlates with the fact that a variety of inflammatory mediator receptors are present in colonic sensory neurones that also express GalR1 (Hockley et al., 2019). This finding could be further expanded upon by investigating the effects of individual components of IS or different mediators. One example being unpublished data from the Smith lab (Katie Baker) shows that it might be interesting to investigate the effects of galanin on the hypersensitivity induced by TNFα and/or NGF. Interestingly, by contrast to its effects upon IS-induced hypersensitivity, galanin was not able to reduce the mechanical hypersensitivity present in LSN isolated from colitic mice that had been treated with DSS. Inflammation in the colon has the potential for increased enzymatic activity which could lead to an increase in the rate galanin is broken down, which could contribute to the reduced effects seen with galanin in DSS-treated mice at a comparable dose to healthy mice. A higher concentration of galanin could be used to overcome any enzymatic galanin breakdown and thus reproduce the inhibitory effects seen in healthy mice.

Why is it that galanin counteracts the acute effects of inflammatory mediators on LSN activity, but has no such effect on LSN activity in a mouse model of colitis? It has been observed that GalR expression is altered in certain models of inflammation and hence differential receptor expression could lead to galanin no longer exerting an inhibitory effect. For example, following hind-paw injection of carrageenan in rats, GalR1 mRNA expression in DRG neurones (L4 and L5) decreases (Xu et al., 1996) and therefore it is possible that a similar decrease in GalR1 expression occurs in the DSS model and hence galanin is no longer able to inhibit mechanically-evoked LSN activity. Alternatively, there could be an increase in the expression of excitatory GalR2. The absence of reliable tools to investigate GalR protein levels and the validity of current antibodies being uncertain (Lu and Bartfai, 2009) makes quantifying the protein level

of GalRs in colonic afferents a significant challenge. However, there are also alternative explanations for the lack of measurable galanin activity. For example, multiple inflammatory mediators are released from inflamed colon tissue (Spiller and Major, 2016), which would act upon a broad range of afferents, i.e. both GalR1+ve and GalR1-ve afferents, and thus the mechanical hypersensitivity observed in LSN isolated from DSS treated mice is likely at least partially mediated via GalR1-ve afferents. Therefore, any effect of galanin on the whole-nerve response may simply be overcome by the overall level of sensitisation. A further explanation would be that the coupling of GalR1 is altered in inflammation due to altered expression of G proteins and/or that the signalling of galanin at GalR1 becomes biased towards different pathways.

6.5 Further work

As described above, there are a number of studies that could be conducted to extend and consolidate the data presented here and further determine the mechanisms by which galanin inhibits LSN mechanosensitivity and IS-induced LSN hypersensitivity. Further investigation into the impact of galanin on different afferent subtypes innervating the distal colon could further define the role of galanin in visceral nociception. These experiments would involve electrophysiological recordings using a flat-sheet preparation where the colon is opened along the antimesenteric border and pinned mucosal side up to expose receptive fields and enable single-unit recordings to interrogate which subtypes are affected by galanin (Brierley et al., 2004). Of the 4 afferent subtypes identified functionally, the serosal and mesenteric fibres are believed to represent major populations of visceral nociceptors (Feng and Gebhart, 2011) and therefore I hypothesise that galanin will inhibit mechanosensitivity in both serosal and mesenteric afferent subtypes.

As previously discussed, the loss of galanin's inhibitory effect in the acute model of colonic inflammation could be partially due to changes in GalR1 expression. Although the initial SC-qPCR study (see Section 7.2) yielded inconclusive results, with further optimisation this technique could help address whether or not galanin receptor expression in colonic sensory afferents changes between healthy and acute inflammatory conditions. However, such analysis only examines mRNA levels and

another approach would be to examine GalR expression using immunohistochemistry. Currently, there is an absence of reliable tools to investigate GalR protein levels and the validity of current antibodies is uncertain (previous unsuccessful attempts, data not shown; Lu and Bartfai, 2009). However, knock-in mice expressing fluorescently tagged GalR1 and GalR2 have been generated and provide the potential to examine some GalR expression at a protein level (Kerr et al., 2015). Furthermore, to determine the involvement of different GalRs in modulating afferent sensitivity in acute inflammation, GalR specific agonists (as demonstrated in Chapter 3 using healthy mice) could be used. As shown in Chapter 3, the GalR1 agonist (M617) demonstrated a similar level of inhibition to galanin, whereas the GalR2 agonist (spexin) showed no significant effect on mechanosensitivity. If repeated in DSS-treated mice the results could establish if there is a functional change in the functional contribution of GalR1 and GalR2 to LSN activity. I would hypothesise that M617 would lose its inhibitory effect seen in healthy mice in a similar way to galanin's loss of effect in an acute model of colitis. Ideally, using non-peptide agonists would be preferable as they would be resistant to enzymatic breakdown.

Previously published studies have shown that although galanin is downregulated in acute peripheral inflammation it is then later upregulated suggesting a greater role for galanin in chronic inflammation (Calza et al., 1998, 2000). The DSS model utilised in this study focused only on the acute inflammatory state. However, using cycles of DSS administration, at lower concentrations, provides a more chronic model of colonic inflammation where it is possible to examine LSN function in relapse and remission as occurs in humans with IBD (Wirtz et al., 2007). Using the same multi-unit electrophysiological recordings as presented here, it would thus be possible to determine whether the inhibitory effect of galanin returns during periods of remission from overt inflammation, further supporting the hypothesis of galanin having a more prominent role in chronic inflammatory conditions.

Finally, to further investigate whether galanin or its receptors are a valid therapeutic target for treating IBD-associated visceral pain, an *in vivo* model of colitis could be used. Intracolonic application of galanin, a GalR1 agonist (M617), or ideally a small molecule, non-peptide GalR1 agonist, will provide insight to any changes in pain-

related behaviours. Results obtained could also be built upon by using different GalR KO mice (Drexel et al., 2018; Malkmus et al., 2005).

6.5 Conclusions

In conclusion, the data presented in this thesis show a new role for galanin in the modulation of LSN function in the distal colon: galanin inhibits LSN mechanosensitivity and acute mechanical hypersensitivity induced by an IS. Future work should elucidate more detail on the mechanisms underpinning why galanin is unable to exert any inhibition on LSN mechanical hypersensitivity following prolonged (*in vivo*) inflammation. Nevertheless, overall, the data suggest that targeting GalR1 could provide a new route for treating visceral pain under specific circumstances and support further investigation.

Chapter 7: Appendices

7.1 Antibody Optimisation

The quality of staining in immunohistochemistry is influenced by the primary antibody concentration, the diluent used, the incubation temperature and time. These variables may need to be optimized for each antibody and sample in order to achieve specific staining with minimal background. Usually, the antibody concentration is varied while maintaining a constant incubation time and temperature in order to optimize staining. Incubation times can be lengthened to ensure antibody penetrates tissues and combined with lower temperatures to promote specific binding. The optimal incubation time and temperatures are reported in section 2.2.2. Results for testing for optimal concentration shown here with secondary-antibody-only controls to show the staining is the result of the primary antibody only. In the case of galanin, as a control, the primary antibody was also incubated with 1 µM galanin peptide. If the antibody is specific it will bind only to the galanin and not to the tissue, resulting in no staining.

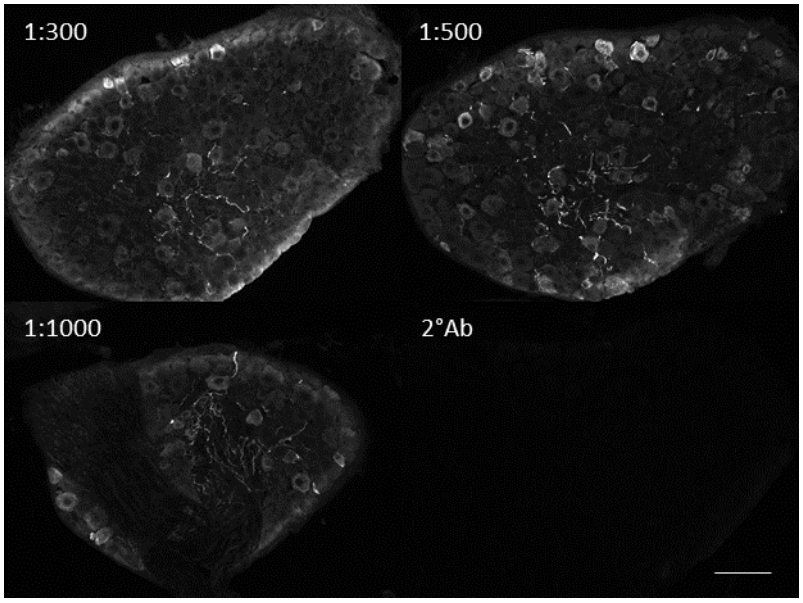


Figure 7-1. Goat anti-CGRP primary antibody optimisation

Goat anti-CGRP primary antibody with donkey anti-goat IgG-Alexafluor 488 tested at dilutions: 1:300 (top left), 1:500 (top right), 1:1000 (bottom left), and secondary only control (2°Ab; bottom right). Scale bar 50 µm.

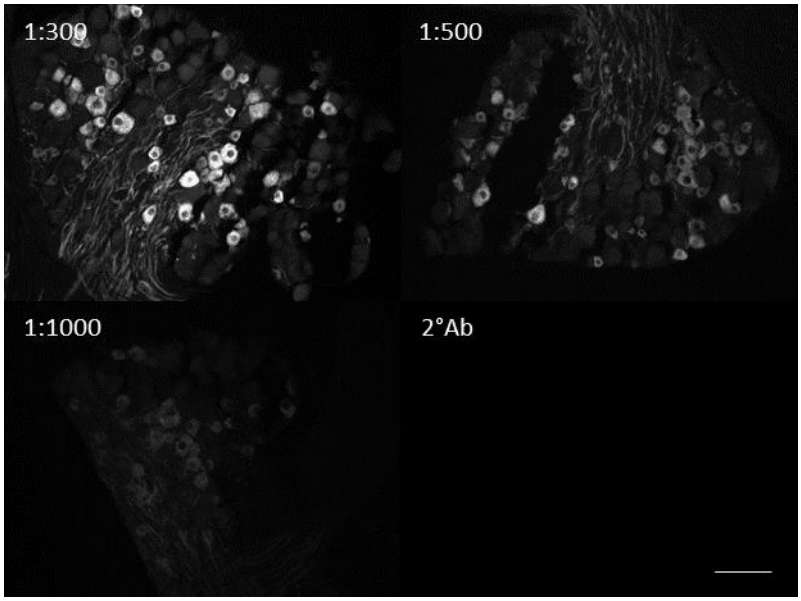


Figure 7-2. Goat anti-Gfrα3 primary antibody optimisation

Goat anti-Gfrα3 primary antibody with donkey anti-goat IgG-Alexafluor 488 tested at dilutions: 1:300 (top left), 1:500 (top right), 1:1000 (bottom left), and secondary only control (2°Ab; bottom right). Scale bar 50 µm.

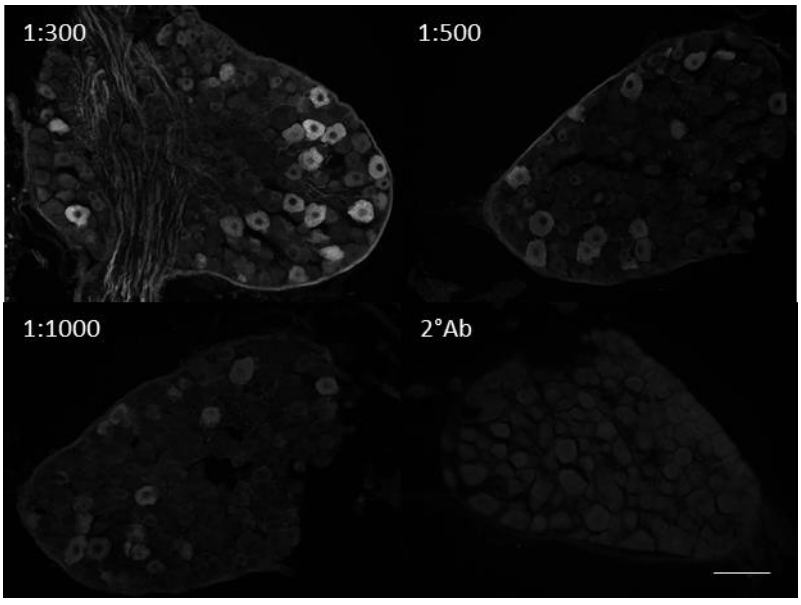


Figure 7-3. Goat anti-TrkC primary antibody optimisation

Goat anti-TrkC primary antibody with donkey anti-goat IgG-Alexafluor 488 tested at dilutions: 1:300 (top left), 1:500 (top right), 1:1000 (bottom left), and secondary only control (2°Ab; bottom right). Scale bar 50 µm.

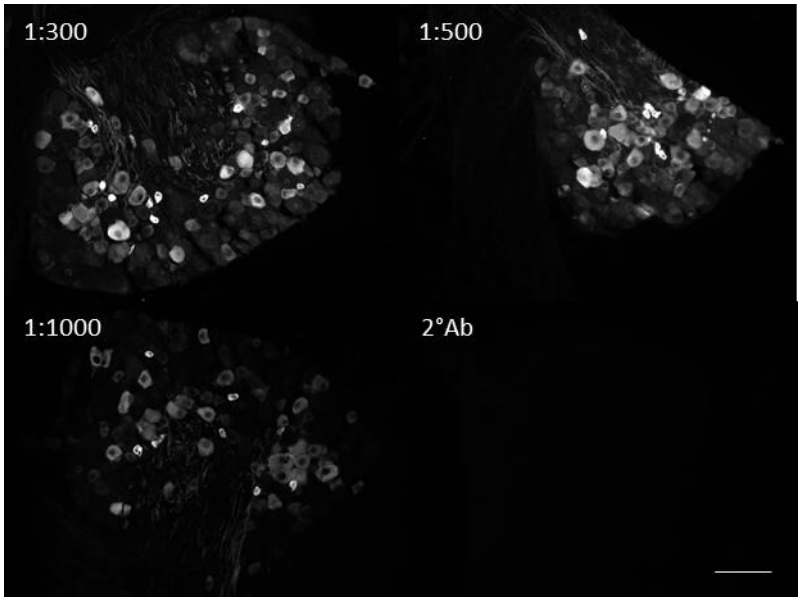


Figure 7-4. Guinea-pig anti-TRPV1 primary antibody optimisation

Guinea-pig anti-TRPV1 primary antibody with donkey anti-Guinea-pig IgG-Alexafluor 488 tested at dilutions: 1:300 (top left), 1:500 (top right), 1:1000 (bottom left), and secondary only control (2°Ab; bottom right). Scale bar 50 µm.

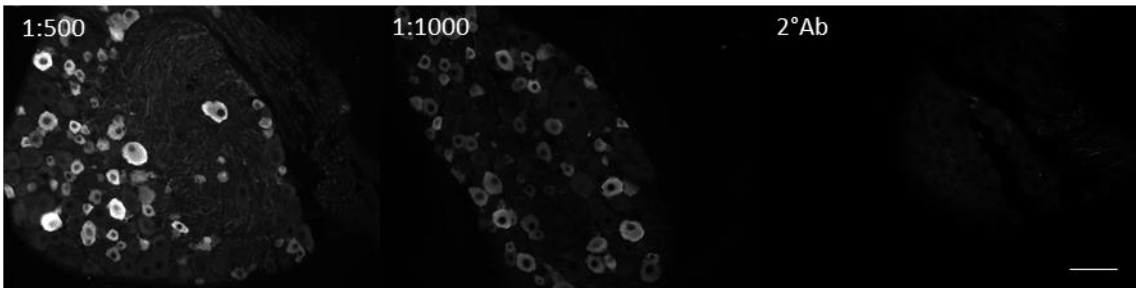


Figure 7-5. Goat anti-TrkA primary antibody optimisation

Goat anti-TrkA primary antibody with donkey anti-Guinea-pig IgG-Alexafluor 488 tested at dilutions: 1:500 (left), 1:1000 (middle), and secondary only control (2°Ab; right). Scale bar 50 µm.

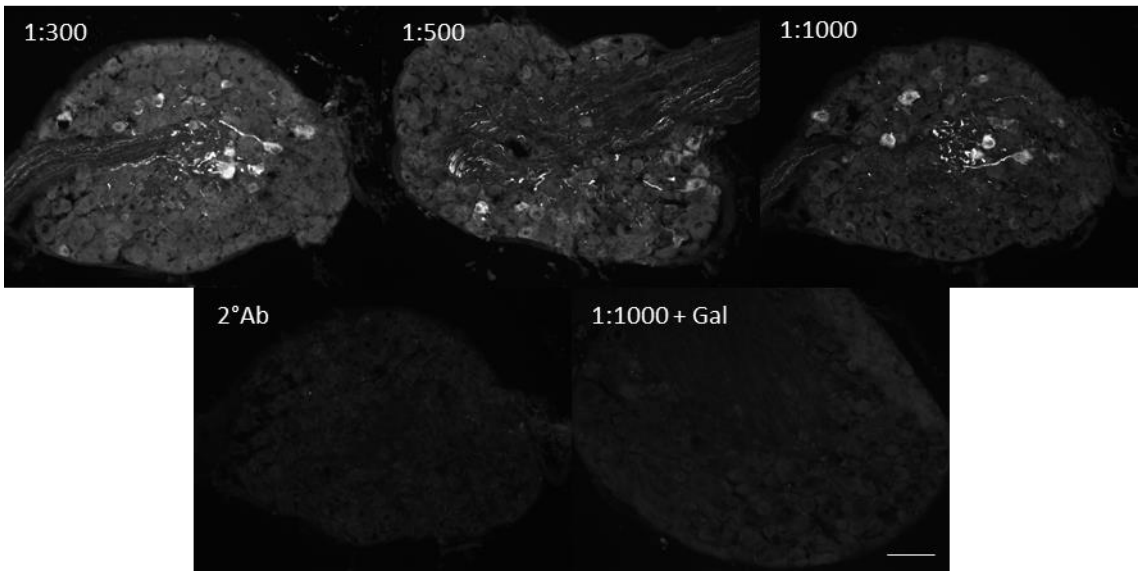


Figure 7-6. Rabbit anti-Galanin primary antibody optimisation

Rabbit anti-Galanin primary antibody with donkey anti-rabbit IgG-Alexafluor 568 tested at dilutions: 1:300 (top left), 1:500 (top middle), 1:1000 (top right), secondary only control (2°Ab; bottom left) and 1:1000 + galanin peptide (bottom right). Scale bar 50 µm.

7.2 Single cell qPCR

To further determine galanin's mechanism of action on LSN activity under inflammatory conditions the change in galanin receptor levels between healthy and inflammatory conditions (induced by DSS treatment) was investigated. As there are currently no reliable tools to investigate galanin receptor protein levels (as mentioned in section 6.3) an approach to determine general increases or decreases in galanin receptor mRNA expression in colonic afferents was used. Single cell qPCR is a method that involves the isolation of single cells (in this case retrograde labelled DRG neurones) and, with the use of probe conjugated primers, monitors the amplification of a targeted DNA molecule (Fig 7-7). This technique can be used in a quantitative or semi-quantitative manner to either calculate the amount of starting copies of mRNA in the original sample or determine if the original sample has above/below certain amounts of copies of the DNA relative to a reference gene. In this experiment I used semi-quantitative single cell qPCR to determine the changes in mRNA levels of galanin and its receptors relative to a commonly used reference gene (Glyceraldehyde 3-phosphate dehydrogenase (GAPDH); White et al., 2011; Li et al., 2016; Hockley et al., 2019) in both healthy and inflammatory conditions. Based on previous studies of galanin receptor levels in peripheral neurones and my functional electrophysiological data I hypothesised that GalR1 would be downregulated or GalR2 would be upregulated in inflammatory conditions.

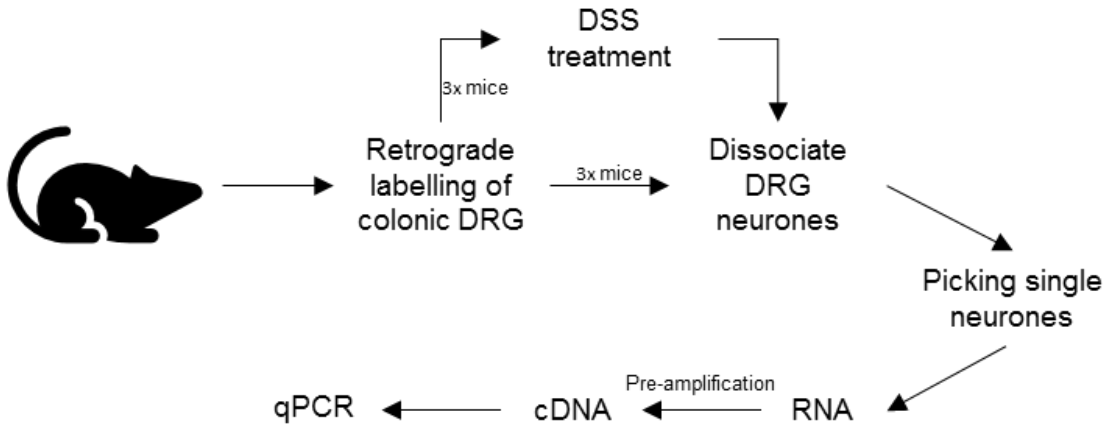


Figure 7-7. A schematic of workflow depicting single cell qPCR

C57BL/6J mice had colonic afferents retrograde labelled with FB before 3 mice underwent DSS treatment to induce colitis and 3 mice remained on standard drinking water. Both groups then had their TL (T13 & L1) and LS (L6 & S1) DRGs dissected and dissociated. FB positive neurones were then isolated through the process of cell-picking. Targeted mRNA of genes of interest were then pre-amplified and converted to cDNA ready for qPCR.

As described in section 2.4, six male C57BL/6J mice between the ages of 8-12 weeks underwent retrograde labelling of colonic afferents. Then 3 of the 6 mice were treated with DSS to induce colitis while the remaining three remained on normal drinking water. From each mouse 20 FB-labelled DRG neurones were isolated from TL DRG (T13 & L1) and 20 from LS (L6 & S1) giving a total of 60 cells for each condition (healthy TL and LS DRG neurones, DSS TL and LS DRG neurones). Negative controls included bath samples (PBS being perfused whilst DRG neurone isolation occurred), and “no-template” controls (wells containing all PCR components apart from the sample DNA) which all came out negative. GAPDH was used as the reference gene due to being widely used in previous literature (Hockley et al., 2019; Li et al., 2016; White et al., 2011). As the experiment was run across 15 95-well plates, interplate calibrators (IPC) were used to remove the plate to plate variability. The IPC was cDNA extracted from whole DRGs as these should contain mRNA from all genes of interest.

The outcome of the experiment was unfortunately inconclusive due to high variability within the IPCs and also the gene of reference, GAPDH. The summation of variability meant no robust conclusions could be drawn from the data. The IPC calibration is a

calculation procedure to detect and remove inter-plate variation. Identical samples of DNA are used in the different plates and the difference in ct values can be used to calculate the correction factor to correct the readings from that plate removing inter-plate variability (Hellemans et al., 2007). For this experiment, mRNA extracted from whole DRGs (T9 - L6) and converted to cDNA was used and the results are shown in Table 5 below. Correction factors above 0.5 or below -0.5 are usually considered high, thus the range of values obtained suggests the IPC may not have worked on all plates.

	Interplate Calibrator Average Ct value	***Correction factor***
Plate 1	18.06	-5.48
Plate 2	19.01	-4.53
Plate 3	17.67	-5.87
Plate 4	23.55	0.01
Plate 5	35	11.45
Plate 6	22.10	-1.44
Plate 7	25.21	1.67
Plate 8	24.67	1.13
Plate 9	26.31	2.76
Plate 10	23.35	-0.19
Plate 11	22.14	-1.40
Plate 12	24.69	1.14
Plate 13	24.18	0.63
Plate 14	24.05	0.50
Plate 15	23.17	-0.36

Table 5 – IPC values obtained for GAPDH

Reference genes are expressed in all cells under normal and patho-physiological conditions. GAPDH plays an important role in glycolysis and is a commonly used housekeeping gene used in gene expression studies (Hockley et al., 2019). The expression of GAPDH in the 240 cells collected in this study seemed to vary greatly (Table 6) and in many cases produced higher Ct values than the gene of interest.

	GAPDH Ct Values
Healthy 1	26.66
Healthy 2	17.16
Healthy 3	13.58
Healthy 4	17.79
Healthy 5	20.41
Healthy 6	17.65
Healthy 7	23.80
Healthy 8	21.16
Healthy 9	19.39
Healthy 10	17.98
Healthy 11	27.34
Healthy 12	26.79
Healthy 13	27.72
Healthy 14	26.94
Healthy 15	29.69
Healthy 16	22.54
Healthy 17	35.75
Healthy 18	26.62
Healthy 19	27.16
Healthy 20	18.92

Table 6 – Sample of GAPDH Ct values from one healthy mouse.

The variation seen in IPC and reference gene values could be the result of inexperience with the technique. Future attempts should incorporate multiple reference genes (such as 18S rRNA; Kuchipudi et al., 2012), further optimisation of DRG dissociation and isolation, and qPCR preparation.

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