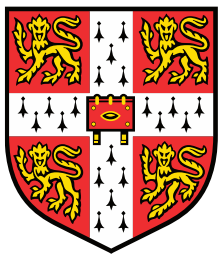
Investigating novel therapeutic targets for treatment of visceral pain



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This dissertation is submitted for the degree of Doctor of Philosophy

October 2022

I. Preface

This thesis is the result of my own work and includes nothing which is done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any work that has already been submitted or is being concurrently submitted for a degree, diploma or other qualification at the University of Cambridge or any other University or any other 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 of similar institution except as declared in the Preface and specified text.

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

II. Summary

Investigating novel therapeutic targets for treatment of visceral pain
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Abdominal pain is a common symptom of gastrointestinal disorders such as Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS). However, despite the many treatments for inflammation in IBD and disordered motility in IBS patients, few therapies target abdominal pain directly, and many commonly used analgesics are contraindicated due to gut related side effects. Consequently, the management of pain in IBD and IBS patients remains a significant clinical challenge.
The aim of this thesis is to evaluate novel therapeutic approaches to the treatment of visceral pain in gastrointestinal diseases by:

- Studying the effect of calcium activated potassium (K_{Ca}) channel openers on the activation of colonic afferents by prototypic algogenic stimuli.
- Examining the effect of inflammatory mediators (angiotensin II and matrix metalloproteinase 1) upregulated during colitis on colonic afferent activity.

Pre-treatment with NS 1619, an opener of BK_{Ca} (subtype of K_{Ca} channels) had no effect on the colonic afferent response to ATP. Similarly, the combined IK_{Ca}/SK_{Ca} opener SKA 31 had no effect on the response to ATP and bradykinin and colonic ramp distension. In contrast subsequent pre-treatment with the KCNQ channel opener retigabine inhibited the colonic afferent response to ATP, bradykinin and ramp distension. Furthermore, in a separate set of experiments application of NS 1619 and SKA 31 abolished ongoing peristaltic activity demonstrating the drugs were pharmacologically active at the concentration and routes of administration in our studies. Findings from this work indicate that KCNQ but not K_{Ca} channel openers may have therapeutic potential for the treatment of abdominal pain in gastrointestinal disease by inhibiting the activation of colonic nociceptors.

Next, we studied the pro-nociceptive potential of angiotensin II (Ang II) and matrix metalloproteinase-1 (MMP1) by examining their effect on colonic afferent activity and mobilisation of intracellular Ca^{2+} in sensory neurons isolated from dorsal root ganglia (DRGs).

Ang II elicited a robust increase in colonic afferent activity including fibres subsequently characterized as nociceptors by their co-sensitivity to noxious distension and the algogenic mediator capsaicin. This effect was inhibited by angiotensin AT_1 but not AT_2 receptor antagonists indicating that Ang II may contribute to the production of abdominal pain in IBD through the activation of AT_1 receptors.

MMP1 caused a marked increase in intracellular Ca^{2+} in DRG neurons classified as nociceptors by their co-sensitivity to capsaicin. This effect was blocked by pre-treatment with the protease activated receptor-1 (PAR_1) receptor antagonist SCH 79797. However, neither the application of MMP1 or the PAR_1 agonist, TRAP-6 produced a direct activation of colonic afferent activity despite expression of PAR_1 receptors in colonic projecting sensory neurons. Further studies are therefore warranted to understand the consequences of MMP1 mediated PAR_1 receptor activation on colonic sensory nerve activity and abdominal pain.

III. Acknowledgements

I would like to thank my supervisor, Dr David Bulmer, for all his support, guidance, and patience throughout my Ph.D.

I would also like to thank my family, especially my mother Nothando, and my sisters Nonhlanhla and Andile for their support and encouragement throughout my PhD. I am very grateful to my nieces and nephews (Angel, Lidiel, Alex, Lethabo, Noluthando, Lwandile, and Luna) who have been a constant source of joy. I would also like to thank my friends for all their support and encouragement. Angel, Carol, Cynthia, Eric, Jahalezansi, Learn, Methembe, Nancy, Nobesuthu, Nokukhanya, Nokuthaba, Roy M, Roy R, Sandile M, Sandile S, Thato, Thembelihle, Thubelihle, and Tinashe-I appreciate all your support.

I am very thankful to my mentors, Dr Heather Bean and Dr Nicole Basta, whose mentorship and guidance over the years have been invaluable.

I would also like to thank the entire Pharmacology department, especially the members of the Bulmer and Smith labs for their support over the years. A special thank you to Michelle Meng and Dr Toni Taylor, for all their help, support, and encouragement.

Lastly, I would like to thank the Gates Cambridge Scholarship for funding this PhD.

IV. Abbreviations

5HT	5-hydroxytryptamine (serotonin)
AA	Arachidonic acid
ACE	Angiotensin converting enzyme
AHP	Afterhyperpolarization
ANOVA	Analysis of variance
ARB	Angiotensin receptor blocker
ASICs	Acid sensing ion channels
AT_1	Angiotensin type 1 receptor
AT_2	Angiotensin type 2 receptor
ATP	Adenosine triphosphate
BK_{Ca}	Big conductance calcium activated potassium channels
CaM	Calmodulin
CaMBD	Calmodulin binding domain
cAMP	Cyclic adenosine monophosphate
CD	Crohn's disease
CMMCs	Colonic migrating motor complexes
CNS	Central nervous system
COX	Cyclooxygenase
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglion
DSS	Dextran sulfate sodium
EC_{50}	Half maximal effective concentration
ECCs	Enterochromaffin cells
ECM	Extracellular matrix
ECS	Extracellular solution
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetra acetic acid
ENS	Enteric nervous system
ERK	Extracellular signal-regulated kinase

FGID	Functional gastrointestinal disorder
GDP	Guanosine diphosphate
GI	Gastrointestinal
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
IBD	Inflammatory bowel diseases
IBMX	3-isobutyl-l-methyl-xanthine
IBS	Irritable bowel syndrome
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
IGLEs	Intraganglionic laminar endings
IK_{Ca}	Intermediate conductance calcium activated potassium channels
IL	Interleukin
IMA	Intramuscular array
IP_3	Inositol trisphosphate
IPAN	Intrinsic primary afferent neurons
K_{Ca}	Calcium activated potassium channels
KCl	Potassium chloride
K_V	Voltage gated potassium channels
LS	Lumbosacral
LSN	Lumbar splanchnic nerve
MAPK	Mitogen activated protein kinase
MCP	Monocyte-chemotactic protein
MMP	Matrix metalloproteinases
Na_V	Voltage gated sodium channel
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NSAIDs	Non-steroidal anti-inflammatory drugs
PAR	Protease activated receptor
PIP_2	Phosphatidylinositol bisphosphate
PI-IBS	Post infectious IBS
PKA	Protein kinase A
PKC	Protein Kinase C

PLC	Phospholipase C
PNS	Peripheral nervous system
RAS	Renin angiotensin system
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of the mean
SK_{Ca}	Small conductance calcium-activated potassium channels
SP	Substance P
SST	Somatostatin
TEER	Transepithelial electrical resistance
Th	T helper
TL	Thoracolumbar
TM	Transmembrane
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF	Tumour necrosis factor
TRAP-6	Thrombin receptor activator peptide 6
TRP	Transient receptor potential
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
VGCC	Voltage dependant calcium channel

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1 General Introduction

1.1 The unmet clinical need to treat visceral pain in gastrointestinal diseases

Visceral pain is a common symptom of organic gastrointestinal (GI) disorders such as Inflammatory Bowel Diseases (IBD) and the main diagnostic criterion of functional GI disorders such as irritable bowel syndrome (IBS) (Vanuytsel et al., 2014; Zeitz et al., 2016). Chronic visceral pain, such as that experienced by IBD and IBS patients markedly reduces a person's quality of life and interrupts psychosocial functioning thereby contributing significantly to the substantial socioeconomic cost associated with these conditions (Grundy et al., 2019; Schirbel et al., 2010).

IBD is a chronic autoimmune condition that is characterized by severe recurring inflammation of the gastrointestinal (GI) tract leading to recurrent diarrhea, abdominal pain, and weight loss (Pithadia & Jain, 2011). Pain is common during active phases of the disease with up to 70% of IBD patients reporting pain during flare and persists during remission for approximately 30% of patients (Bielefeldt et al., 2009; Zeitz et al., 2016). During inflammation, pain is driven by the stimulation of nociceptors in response to mediators released from the inflamed gut and persists following resolution of inflammation due to the continued sensitization of peripheral and central nociceptive pathways (Bielefeldt et al., 2009). Pain therefore making a significant contribution to disease morbidity in IBD patients during flare or remission (Schirbel et al., 2010).

In contrast to IBD patients, there is an absence of demonstrable structural or biochemical abnormalities in patients with IBS leading to its classification as a functional gastrointestinal disorder (FGID). IBS is diagnosed based on the persistent presentation of symptoms, the gold standard criterion for diagnosis being the current Rome IV criteria defined by the ROME committee as the presence of recurrent abdominal pain on average at least 1 day/week in the last 3 months associated with defecation, changes in appearance or stool frequency (Bai et al., 2017; Farmer & Aziz, 2009). Pain is the most common symptom experienced by IBS patients and is the main reason behind increased care seeking behaviours in IBS patients (Yu et al., 2021).

In 2017, it was estimated that there were 6.8 million cases of IBD cases globally, whereas IBS is more common and was estimated to affect approximately 11% of the global population (Alatab et al., 2020; Corsetti & Whorwell, 2017). While significant improvements have been

made in treating symptoms of IBS and IBD, management of abdominal pain remains a clinical challenge due to the absence of analgesic drugs which lack GI specific side effects and have efficacy for the treatment of abdominal pain. Many of the available treatment options for IBD such as aminosalicyclates, corticosteroids and immunomodulators mainly focus on reduction of inflammation for IBD whereas IBS treatments mainly target motility disturbances, constipation and diarrhoea, with only a few therapies aimed directly at diminishing abdominal pain (Cai et al., 2021; Ceuleers et al., 2016). Consequentially, only a small proportion of patients report complete relief of abdominal pain following symptomatic treatment. Therefore, there is urgent need to develop effective visceral analgesics for the treatment of pain in IBS and IBD.

1.2 Visceral hypersensitivity

Visceral hypersensitivity: defined as the increased perception of stimuli from the viscera that leads to heightened sensitivity and awareness of the physiological processes of the viscera is a characteristic of chronic pain and is thought to be the main mechanism underlying abdominal pain in IBS (Camilleri et al., 2001). Two major components of visceral hypersensitivity are allodynia and hyperalgesia. Hyperalgesia is an increased response to noxious stimuli and allodynia is a state in which previously innocuous stimuli are perceived as noxious (Figure 1) (Farzaei et al., 2016). Under normal circumstances, healthy individuals are unaware of functions of the gastrointestinal tract such as distensions and contractions, and when awareness is triggered, it is usually by sensations of hunger, fullness, or urge (Grundy et al., 2019). Intense stimuli are required to trigger sensations that are uncomfortable or painful. However, in disease states, low-intensity stimuli that would normally not be perceived are felt as painful and noxious (Bielefeldt et al., 2009).

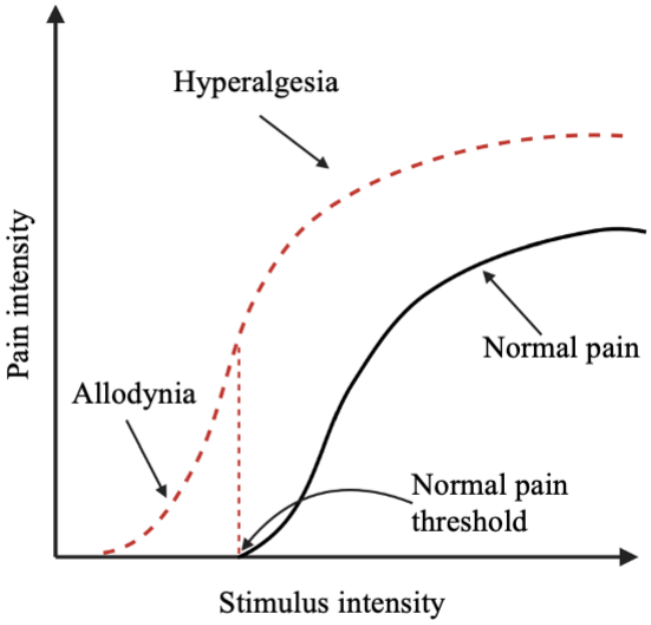


Figure 1. Changes in pain sensation in visceral hypersensitivity.

The black curve represents normal pain perception, and the red curve demonstrates hypersensitivity characterised by hyperalgesia (increased response to noxious stimuli) and allodynia (painful repones to normally innocuous stimuli).

Visceral hypersensitivity in IBS patients was first demonstrated by Ritchie et al. who showed that IBS patients had increased pain perception in response to balloon distension of the colon compared with healthy controls (Ritchie, 1973). Since then, several other studies have confirmed these results reporting that 33-90% of IBS patients present with reduced thresholds to pain or discomfort in response to rectal distension (Mertz, 2003). Visceral hypersensitivity has also been demonstrated following inflammation in chemically induced colitis in animal models. For example, TNBS induced colitis produced visceral hypersensitivity in rats reflected by increased sensitivity to colonic distension (Zhou et al., 2008). Additionally, prolonged stress and negative emotions, as is common in chronic pain patients also plays a role in visceral pain hypersensitivity. The role of stress on hypersensitivity has been demonstrated in animal models of IBS where inducing stress using foot shocks or maternal separation causes animals to develop visceral hypersensitivity to colonic distension (Coutinho et al., 2002; Stam et al., 1996). Surprisingly, there is contradicting information regarding visceral hypersensitivity in IBD patients. Visceral hypersensitivity has been proposed in patients with active UC and those in remission were reported to experience significantly increased perception of urge, discomfort, or pain than controls during pressure distensions (Rao et al., 1987; van Hoboken et al., 2011).

In contrast, other studies have found no evidence of visceral hypersensitivity in in colitis patients with UC patients found to have higher discomfort threshold compared with IBS patients and controls (Chang et al., 2000).

1.3 Peripheral sensitisation

Visceral hypersensitivity is a multifactorial process that involves the sensitization of peripheral and central nociceptive pathways (Fuentes & Christianson, 2016). Peripheral sensitization represents a reduction in the threshold and/ or increase in magnitude of responsiveness at the peripheral ends of sensory nerve fibres in response to mediators released by nociceptors and non-neuronal cells in response to tissue injury or inflammation (Gangadharan & Kuner, 2013). Inflammation is a complex interaction between damaged endothelial cells, white blood cells (monocytes, basophils, eosinophils and neutrophils), sympathetic afferents and primary sensory afferent which release inflammatory mediators that contribute to and adjust the immuno-inflammatory response (Abdulkhaleq et al., 2018; Bhave & Gereau, 2004).

Immune cells such as mast cells, T-cells, and neutrophils facilitate the production and release of inflammatory mediators such as proteases, ATP, prostaglandins, H^+, proinflammatory cytokines (tumour necrosis factor (TNF), interleukin (IL) 1β) and proinflammatory chemokines in response to tissue injury (Ji et al., 2014). These inflammatory mediators can influence the sensitivity of visceral afferents by direct activation of receptors present on nerve endings or by releasing algesic mediators from other cells and altering channel and receptor expression in visceral nociceptors (Brierley et al., 2005; McGuire et al., 2018). Seeing that peripheral sensitization plays a key role the development of chronic visceral pain, targeting key mediators and receptors on sensory afferents neurons is a key strategy that can be utilized to ameliorate sensation of visceral pain.

The mechanisms by which inflammatory mediators initiate signalling cascades that modify the function and expression of ion channels involved in nociception will be addressed later in the chapter.

1.4 Central sensitization

Prolonged noxious stimulation of primary sensory neurons by inflammatory mediators can promote excitability of the spinal cord and higher centre neurons that mediate nociceptive processing, in a process called central sensitization (Mayer & Gebhart, 1994; Moshiree et al., 2006). In inflammatory conditions, central sensitization persists after inflammation has resolved and contributes to the development of chronic pain (Ji et al., 2014). Changes in the spinal cord and dorsal horn can increase pain signals through increased synaptic transmission and/or decreased descending inhibitory modulation (Greenwood-Van Meerveld & Johnson, 2017).

For example, enhanced neuronal excitability results in increased release of neurotransmitters and neuromodulators such as glutamate, substance P and brain derived neurotrophic factor (BDNF). These neuromodulators activate ligand gated ion channels (NMDA-R -glutamate), metabotropic receptors (mGluR-glutamate, NK1-substance P) and tyrosine kinase receptors (TrkB-BDNF) on post synaptic membranes resulting in an increase in intracellular calcium and consequent activation of calcium dependant enzymes (protein kinase C, calcium calmodulin kinase, protein kinase A and tyrosine kinases). These kinases phosphorylate membrane bound receptors/ion channels such as NMDA resulting in increased responsiveness to synaptically released glutamate to evoke depolarizing current and increase neuronal output from the neurons (Woolf & Costigan, 1999).

Secondly, enhanced activity from the spinal cord can sensitize the cortical and limbic brain regions that respond to the emotional and physical components of pain thus directly promoting and maintaining chronic pain (Greenwood-Van Meerveld & Johnson, 2017). Additionally, changes in the pain modulating regions of the brain results in the loss of descending inhibition of ascending noxious signals as which has been demonstrated in chronic pain conditions (Ossipov et al., 2014).

1.5 Pain

The International Association of pain recently updated the definition of pain to ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage’ (Raja et al., 2020). The ability to sense pain is essential for survival and the inability to sense pain leads to repeated injuries and prevents normal healing. The importance of pain is made evident in individuals with congenital insensitivity to pain (a condition that is characterized by the inability to feel pain from birth) who are highly prone to burn related injuries and suffer from self-mutilating oral and finger lesions because of repeated self-biting (Basbaum et al., 2009; Nagasako et al., 2003). While acute pain is essential for survival and serves as a protection mechanism against repeated injury, chronic pain (pain that persists for extended periods, <3 months) is a clinical challenge that negatively impacts on a person’s quality of life and interrupts psychosocial functioning (Treede et al., 2015). Chronic visceral pain affects more than 20% of the global population and current clinical management options are inadequate (Grundy et al., 2019). Pain that persists long after acute injury has resolved outlives its purpose as a warning system and becomes chronic and debilitating (Basbaum et al., 2009). For example, in chronic pain conditions such as IBS where pain occurs without any demonstrable tissue damage or in IBD where pain can occur during remission, pain no longer serves its original purpose of preventing injury.

Pain sensation is mediated by the activation of nociceptors; a class of sensory nerve fibre or primary afferents that transduce and transmit painful or tissue damaging stimuli to the central nervous system (Basbaum et al., 2009; Yam et al., 2018). Nociceptors are typically unspecialized free nerve endings that are widely located in the skin, muscle, bone, and some major internal organs and are functionally used to detect potentially damaging chemical, mechanical and thermal stimuli (Yam et al., 2018). Based on anatomical and functional criteria, nociceptors can be classified into 2 major classes: small diameter unmyelinated C-fibres and medium diameter myelinated Aδ -fibres. C fibres which are polymodal and can be activated by mechanical, thermal, and chemical stimuli have a slow conduction velocity typically less than 1µm/s and mediate delayed, diffuse, and dull pain (Basbaum et al., 2009; Yam et al., 2018). Aδ -fibres which are activated by thermal and mechanical stimuli have faster conduction velocities due to their myelination and mediate rapid, acute, and sharp pain (Yam et al., 2018).

1.6 Characteristics of visceral pain

Visceral pain- originating from internal organs (thoracic, abdominal, and pelvic regions), is a common complaint from patients seeking medical attention. The prevalence of intermittent abdominal pain in adults is estimated to be 25% (Drewes et al., 2020). Visceral pain can be a result of direct injury or inflammation of the organ (e.g., IBD), blockage of ducts or tubes (e.g., kidney stones), or functional visceral disorders whereby no specific pathophysiology can be identified (e.g. IBS) (Robinson & Gebhart, 2008). Despite the prevalence of visceral pain, less is known about its mechanisms compared to somatic pain and most of what is known about pain is derived from studies of somatic and not visceral pain. Visceral pain differs from somatic pain in that it is not always linked to tissue injury. For example, visceral pain is a major complaint from patients with IBS, whose colons lack apparent organ damage or inflammation (Feng & Guo, 2020). Additionally, the severity of pain does not always reflect the severity of the condition causing pain. For example, gas, which causes no observable damage to organs can cause considerable pain whereases relatively mild pain or no pain may be present with severe and life-threatening conditions such as cancer of the colon (Al-Chaer & Traub, 2002).

Secondly, visceral pain is not evoked from all viscera. Visceral pain is usually evoked from hollow visceral organs such as the stomach, bladder and reproductive organs whereas solid visceral organs such as the lungs, pancreas, spleen, and liver lack the sensory innervation to sense pain. Therefore, tissue damage in these organs can go unnoticed until the late stage of severe disease (Feng & Guo, 2020). Additionally, stimuli that are known to evoke somatic pain such as cutting, pinching, burning, and piercing are typically not painful when applied to viscera in animals and conscious humans due to differences in the functional properties of the peripheral receptors that innervate certain visceral organs (Cervero & Laird, 1999). Lastly, visceral pain is often accompanied by motor and autonomic reflexes such as sweating, increase in blood pressure, nausea, and vomiting, a phenomenon that can be explained in part by colocalization of visceral primary afferents with sympathetic nerves and parasympathetic nerve fibres (Drewes et al., 2020).

Visceral pain is difficult to locate and diagnose for a several reasons: i) it is diffuse and poorly localised due to a low density of sensory innervation and extensive divergence of visceral input within the central nervous system (CNS) (Sikandar & Dickenson, 2012). Neuronal tracing methods established that visceral afferent fibres constitute 5-15% of the total afferent inflow to

the thoracolumbar spinal cord (Ness & Gebhart, 1990). Secondly, it is often referred to other locations due to convergence of visceral afferent fibres onto spinal cord neurons that are driven by somatic inputs (Cervero, 1994).

1.7 Innervation of the GI tract

The GI tract is composed of a series of layers including the inner mucosal layer composed of absorptive and secretory epithelial cells, the submucosal layer containing nerves, lymphatics, and connective tissue; the smooth muscle layer composed of longitudinal and circular smooth muscle; and the outer serosal layer. The neural innervation of the GI tract allows the gut to carry out its functions including the movement of contents along the GI tract, secretion of digestive enzymes, absorption of luminal contents, and excretion of waste. Neural control of the gut is regulated by the intrinsic and extrinsic nervous systems. (Greenwood-Van Meerveld et al., 2017).

1.7.1 The enteric nervous system

Intrinsic innervation of the GI tract is controlled by the enteric nervous system (ENS) which is the largest component of the autonomic nervous system. The ENS is contained within the gut wall and is uniquely able to control gastrointestinal secretor-motor functions independent of the CNS (Rao & Gershon, 2016). Enteric neurons are comprised of intrinsic primary afferent neurons (IPANs) that detect chemical and mechanical stimuli and communicate with interneurons and motor neurones (ascending and descending) to coordinate the different effector functions of the gut through actions on a broad range of cell types including smooth muscle, pacemaker cells, blood vessels, mucosal glands, and epithelia (Costa et al., 2000).
IPANs have their cell bodies located in either the myenteric (Auerbach's) plexus or submucosal (Meissner's) plexus. The myenteric plexus is located between the circular and longitudinal smooth muscle layers and regulates regulate GI motility, whereas the submucosal plexus is contained within the connective tissue beneath the mucosa and regulates secretory and absorptive functions of the GI epithelium, local blood flow, and neuroimmune function (Greenwood-Van Meerveld et al., 2017). The myenteric plexus extends from the upper oesophageal to the external anal sphincter whereas the submucosal plexus is restricted to the submucosa of the small and large intestine (Bernardazzi et al., 2016). Like the CNS, the enteric nervous system contains a broad array of excitatory and inhibitory transmitters. Excitatory mediators including acetylcholine and substance P stimulate smooth muscle contraction,

increase intestinal secretions, release enteric hormones and dilate blood vessels (Ghazi & Shanthi, 2010). Inhibitory mediators include ATP, nitric oxide (NO) and vasoactive intestinal peptide (VIP) (Fleming et al., 2020).

1.7.2 The extrinsic nervous system

Extrinsic primary afferents of the GI tract provide the anatomical connection with the CNS. Afferent fibres from the gut follow three clear pathways: the vagal, splanchnic and pelvic nerves. The peripheral endings of vagal and spinal afferents are located at various levels within the gut wall where they maintain a steady flow of afferent traffic to the CNS relating information on activity both within and outside the gut wall (Brookes et al., 2013). Vagal afferents have cell bodies located in the nodose and jugular ganglia located at the base of the skull whereas spinal afferents (splanchnic and pelvic) have cell bodies located in the thoracolumbar (TL) and lumbosacral (LS) dorsal root ganglia (DRG) located bilaterally adjacent to the spinal cord. Each region of the gut is innervated by two different extrinsic nerves with splanchnic nerves innervating the entire GI tract whereas vagal nerves innervate the upper (oesophagus, stomach, small intestine, and upper colon) and pelvic nerves innervate the lower (colorectum) GI tract (Figure 2). Thoracolumbar spinal afferents are associated with sympathetic efferent pathways whereas vagal and pelvic pathways are associated with parasympathetic efferents (Brookes et al., 2013).

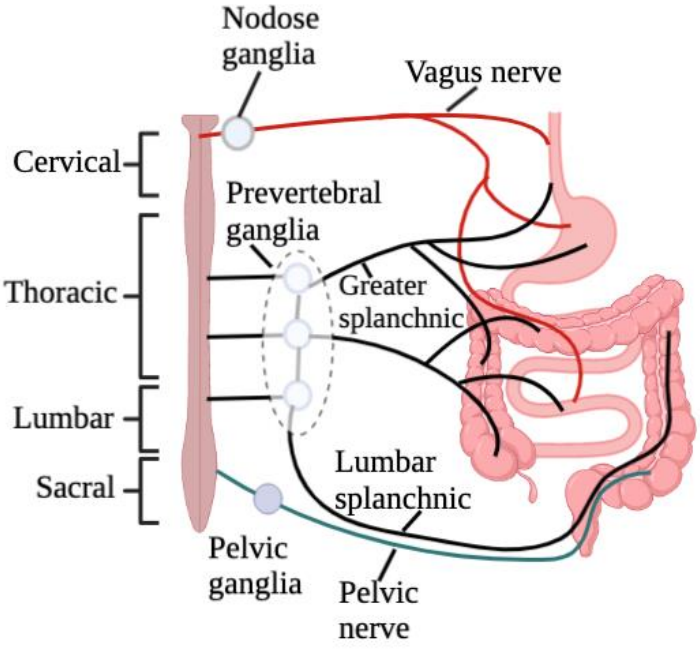


Figure 2. Schematic of the extrinsic innervation of the GI tract.

The three main pathways innervating the GI tract are shown: the vagal, splanchnic and pelvic. Vagal afferents have cell bodies located within the nodose/jugular ganglia whereas spinal afferents have cell bodies in multiple dorsal root ganglia. Vagal afferents innervate the upper GI tract and are absent in the distal colon and rectum. The proximal colon receives spinal innervation from the thoracic to the lumbar spinal cord via the lumbar splanchnic nerve and sacral pelvic nerves.

Spinal innervation of the small intestines spans from the thoracic to the lumbar spinal cord via the greater splanchnic nerves. The proximal colon receives spinal innervation from the thoracic to the lumbar spinal cord via the lumbar splanchnic nerves while the distal colon receives dual innervation from the lumbar splanchnic nerves and the sacral pelvic nerves. (Brierley et al., 2018; Harrington et al., 2018). The rectum receives innervation from the lumbosacral spinal cord via sacral pelvic nerves. Splanchnic afferents from the colorectum project onto the thoracolumbar segments T10-L2 in mice and rats, whereas pelvic afferents converge onto the lumbosacral segments L6-S2 (Harrington et al., 2018; Vermeulen et al., 2014).

1.7.3 Vagal afferents

Vagal afferents detect tension at physiological levels and are believed to serve a homeostatic function (i.e., the perception of hunger, fullness, satisfaction, bloating, nausea and urgency, and desire to defecate) (Alcaino et al., 2017; Harrington et al., 2019). Consistent with the hypothesis that vagal afferents are involved in physiological regulation, vagal muscle mechanoreceptors have been shown to have low thresholds of activation that reach maximal responses within physiological levels of distension (Grundy, 2004). Although vagal afferents do not signal pain, they are sensitive to many of the same noxious chemical mediators as spinal afferent nociceptors, therefore the activation of vagal afferents by painful stimuli results in some of the autonomic responses that accompany visceral pain (Bulmer & Roza, 2018). In support of this, one study showed that expression of nociceptive ion channels and receptors such as Nav1.8 and TRPV1 on vagal afferents (Kupari et al., 2019).

Based on the morphology of vagal afferents endings in the gut wall, vagal afferents are classified into three subtypes, intraganglionic lamina endings (IGLEs), intramuscular arrays (IMAs) and mucosal afferents (Berthoud & Powley, 1992). IGLEs are distributed throughout the GI tract and are found in the myenteric plexus and are thought to be the site for vagal mechaotransduction (Berthoud et al., 1997; Zagorodnyuk et al., 2001). IMAs are restricted to

the circular and longitudinal muscle layers of the stomach and sphincter regions and are responsive to stretch (Berthoud & Powley, 1992; Phillips & Powley, 2000; Wang & Powley, 2000). Mucosal afferents innervate three mucosal layer of the gut wall and penetrate the lamina propria (Wank & Neuhuber, 2001).

1.7.4 Spinal afferents

Spinal afferents (splanchnic and pelvic) project into discrete laminae of the superficial dorsal horn and make synaptic contacts with second order neurons within the dorsal horn of the spinal cord. Afferents innervating the distal colon terminate predominantly in the superficial dorsal horn laminae I and laminae V (Cervero, 1988). Spinal afferents have a high threshold of distension detection and can respond a wide range of stimuli extending to the noxious range such as pain, bloating, discomfort, and urgency (Alcaino et al., 2017; Brierley et al., 2018; Harrington et al., 2018). Splanchnic nerves were demonstrated to play an important role in the nociceptive pathway after unilateral splanchiotomy provided relief in chronic pancreatitis patients and blocking of splanchnic nerves with procaine yielded good results (Connolly & Richards, 1950).

In addition to pain, pelvic nerves transmit similar modalities of information as the vagal nerves such as urgency and desire to defecate. Pelvic nerves are specialized to detect circular stretch, the primary stimulus generated by low-intensity colorectal distension or stool passage (Vermeulen et al., 2014). Rectal IGLEs which are functionally similar to vagal IGLEs, located in myenteric ganglia are the transduction sites for low threshold mechanical activation in pelvic nerves (Lynn et al., 2003).

1.7.5 Classification of extrinsic afferents

Vagal and spinal afferents innervating the GI tract are classified into various subclasses depending on their mechanical responsiveness and mechanical activation thresholds, gene expression and their location within the layers of the GI tract (Harrington et al., 2019). In recent years, the understanding of the sites of termination of colonic afferents has evolved due to improved tracing and imaging studies. Based on the anatomical location of their nerve endings and functional characteristics visceral afferent innervating the human colorectum are classified into various subtypes that make up the pelvic and splanchnic pathways in varying proportions.

These subtypes include:

- mucosal afferents that are characterized by low activation thresholds and are highly sensitive to distortion of the colonic mucosal epithelium.
- muscular afferents that respond at low distension thresholds.
- muscular-mucosal afferents that respond to both mucosal distortion and circular stretch.
- vascular afferents that wrap around blood vessels in the mesentery and submucosa and respond to high-threshold stimuli and a variety of inflammatory and immune mediators
- serosal afferents that respond to high threshold stimuli.
- mechanically insensitive or silent afferents' that are further classified as chemically activated by inflammatory or immune mediators, or those that are mechanically sensitized or both (Brierley et al., 2018; Grundy et al., 2019).

The distribution and functional characteristics of these afferent subtypes are described in *Table 1* below.

Class	**Distribution**	**Stimuli**
Mucosal afferents	Rare in lumbar splanchnic and common in the pelvic nerve.	Low activation threshold and respond to light stroking or compression.
Muscular/Tension	Present in both the lumbar splanchnic and pelvic nerve.	Low distension threshold (<20 mmHg) or low intensity stretch stimuli (<3 g).
Muscular/Mucosal	Pelvic nerve.	Respond to circular stretch and mucosal distortion.
Vascular	Lumbar splanchnic nerve.	High threshold stimuli.
Serosal	Lumbar splanchnic/pelvic nerve.	High threshold, distension (>40 mmHg), and stretch (>9 g).
Silent/Mechanically insensitive	Lumbar splanchnic/pelvic nerve.	Initially mechanically insensitive but respond following exposure to chemicals.

Table 1. *Distribution of afferent subtypes within the lumbar splanchnic and pelvic nerve projecting to the human colorectum.*

Figure 3 below is an illustration of the classes of spinal afferents innervating the colorectum and their respective sites of termination.

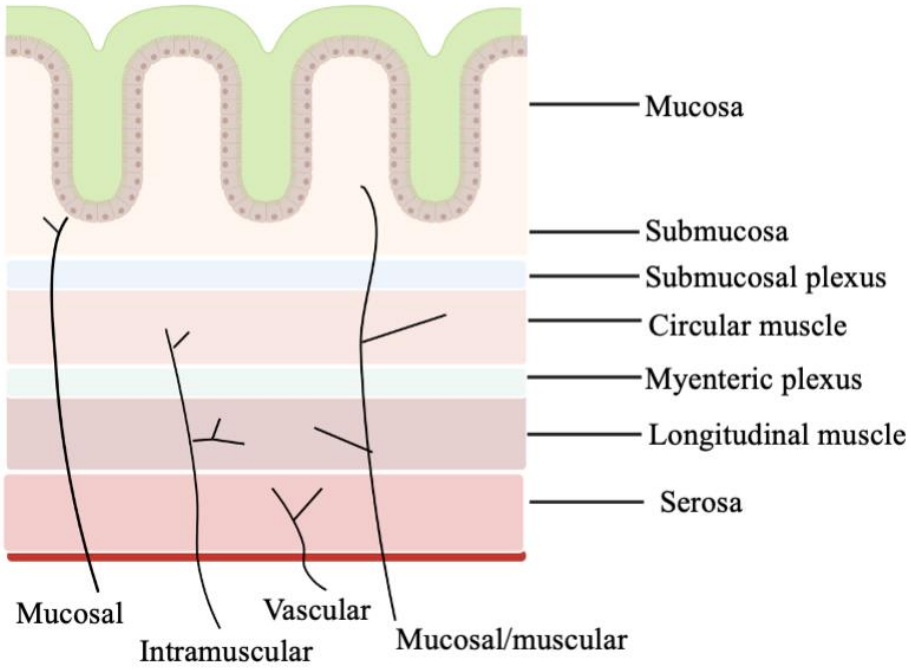


Figure 3. Spinal afferent termination in the GI tract.

Spinal afferents innervating the GI tract are classified based on their ability anatomical location and functional characteristics into muscular afferents, mucosal afferents, muscular/mucosal and vascular afferents.

1.7.6 Classification of sensory neurons innervating the colorectum using molecular markers

In addition to classification based on morphological and functional characteristics, visceral afferents can also be classified based on their molecular expression profiles. A study conducted in our group identified seven distinct colonic sensory neuron subtypes and discriminative markers in the thoracolumbar (T10-L1) and lumbosacral (L5-S2) pathways using single cell RNA sequencing of mouse colonic sensory neurons. Five main subtypes: mNeuroFilament-a (mNFa), mNeuroFilament-b (mNFb), mNonPeptidergic (mNP), mPeptidergic-a (mPEPa), mPeptidergic-b (mPEPb), were identified from thoracolumbar DRGs and lumbosacral DRGs. 2 additional subtypes: pNeuroFilament (pNF) and pPeptidergic (pPEP) were exclusively identified in the (Hockley et al., 2018). The mNP population shows expression of P2X3 and Mas-related GPR, member D, which have previously been associated with expression of non-peptidergic nociceptors. The two mixed peptidergic subtypes, mPEPa and mPEPb express calcitonin gene-related peptide (CGRP), substance P and TrkA and represent peptidergic

nociceptors (Hockley et al., 2018). Data from this study was published in an online database that allows visualization of gene expression split among the seven subtypes in colonic sensory neurons and is utilized to examine expression profiles of genes of interest in the current study.

1.8 Pain transmission from the GI tract

Spinal mesenteric and serosal nerve endings which make up the majority of splanchnic afferents mediate acute pain as occurs with significant distension or ischemia whereas mucosal afferents are thought to play a greater role after sensitization which occurs in inflammatory states (Knowles & Aziz, 2009; Vermeulen et al., 2014). Nociceptive signals from the GI tract are conveyed to the CNS by extrinsic afferent fibres. Pain from the rectum is mediated by pelvic pathways, whereases thoracolumbar spinal afferents mediate pain from more proximal regions of the gut (Brookes et al., 2013). The first synapse is in the superficial layers of the dorsal horn of the spinal cord. The nociceptive signal is then transmitted to the spinal cord and pain signals reach the brain via the spinothalamic tract and dorsal column. Within the brain, the signal is then relayed to cortical areas for localization and to limbic areas for the emotional component of the pain response (Figure 4) (Greenwood-Van Meerveld et al., 2017). Output from the cortical and limbic regions in response to pain then activates descending inhibitory circuitry within the brain stem that causes the release of inhibitory neurotransmitters within the dorsal horn and spinal cord (Vermeulen et al., 2014).

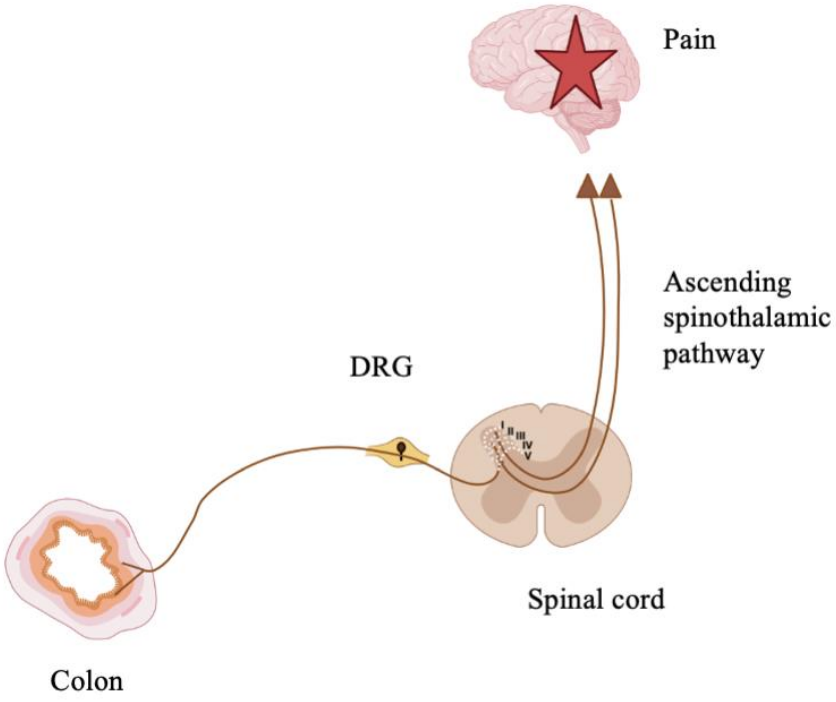


Figure 4. Pain signalling from the colorectum

Primary afferent nociceptors are responsible for conveying noxious information received to the projection neurons in the dorsal horn of the spinal cord where it is passed on to higher centres in the brain along the spinal cord.

1.9 Signal transduction in sensory neurons

Stimulus evoked action potentials generated by sensory nerves are initiated in response to changes in membrane potential brought about by the activation of specific receptors and ion channels expressed in peripheral nerve endings and propagate along axons of primary afferents to transmit information to the CNS (Waxman & Zamponi, 2014). Ion channels are central to the regulation and rapid change of electrical activity in neurons as they facilitate the movement of ions (predominantly Na^+, K^+, and Cl^-) between the extracellular fluid and interior of the neuron which in turn determines the electrical properties of the neuron such as the resting membrane potential, action potential generation, and neurotransmitter release (Raghavan et al., 2019; Waxman & Zamponi, 2014).

The resting membrane potential at sensory nerve endings is largely unknown, however the mean resting membrane typically recorded at the cell body located in the DRG is typically around -55 mV (the cell interior is negative relative to the exterior) demonstrating a propensity to depolarisation compared with central neurons or axons. The resting membrane potential is predominantly driven by differences in the permeability and electrochemical gradient for K^+ and Na^+ ions across the neuronal cell membrane. At rest, the membrane is more permeable to K^+ ions that are highly concentrated inside the cell than Na^+ ions which are more concentrated outside the cell membrane, leading to a greater efflux of K^+ compared with influx of Na^+ ions. Hence, the production of a negative membrane potential at equilibrium, which lies closer to the reversal potential of K^+ than Na^+ (Raghavan et al., 2019). Respective ionic gradients (and hence resting membrane potential) are maintained by the action of the Na^+, K^+ ATPase pump which utilizes ATP to move Na^+ and K^+ against their concentration gradient in an asymmetrical manner. For every molecule of ATP hydrolysed three Na^+ ions are moved out of the cell, and two K^+ ions into the cell (Fletcher, 2019; Raghavan et al., 2019; Yam et al., 2018).

Activation of receptors expressed in sensory nerve terminals such as transient receptor potential (TRP) channels, acid sensing ion channels (ASICs), serotonin (5-HT) receptors and P2X

receptors in response to various stimuli causes membrane depolarization due to influx of cations (Figure 5) (Yam et al., 2018).

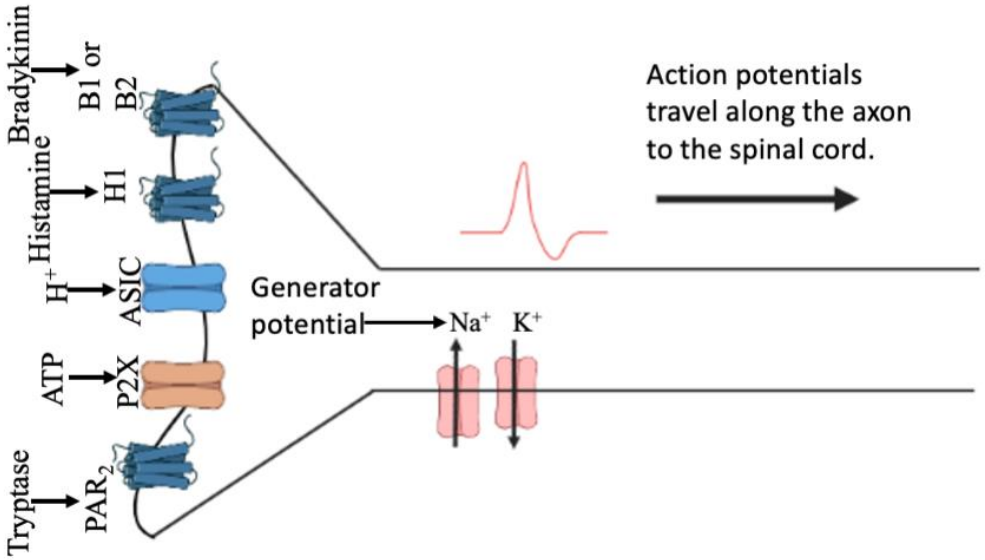


Figure 5. Peripheral nerve ending.

Activation of specific receptors and ion channels in peripheral nerve endings by chemical, mechanical or thermal stimuli results in the initiation of action potentials that propagate along the axons of primary afferents.

Voltage-gated ion channels with activation thresholds close to the resting membrane potential open in response to the generator potential to cause further depolarization of the membrane. An action potential is initiated once the degree of depolarization exceeds the threshold value. The voltage gated sodium channels Nav1.1, Nav1.6, Nav1.7, Nav1.8 and Nav1.9 are expressed in primary sensory neurons with Nav1.8 and Nav1.9 exclusively expressed in nociceptors and contribute to the action potential upstroke and action potential firing (Bennett et al., 2019). Membrane depolarisation also triggers the opening of voltage-activated K^+ channels, that allow the efflux of K^+ together with K^+ leak channels resulting in the repolarisation of the membrane. Membrane conductance to K^+ ions continue beyond the resting membrane potential, and this results in a membrane potential that is more negative than the baseline that lasts for a few milliseconds and is known as the afterhyperpolarisation (Raghavan et al., 2019). When the K^+ channels eventually close, the Na^+/K^+ ATPase pump restores the resting membrane potential (Yam et al., 2018). Action potentials propagate along the axon to synaptic nerve terminals located in the dorsal horn of the spinal cord which is organized into anatomically and electrophysiological distinct laminae with predominantly C-fibre visceral afferents innervating distal colon terminating in the superficial dorsal horn laminae I and laminae V (Harrington et al., 2019)

1.10 Ion channels involved in pain signalling

Nociceptors are equipped with ion channels that detect noxious or tissue damaging stimuli such as chemical agents (e.g. protons and inflammatory mediators including cytokines) and physical stimuli (e.g. temperature, and mechanical stress) (Bulmer & Roza, 2018). Examples of "pain transducing" channels include the ATP activated P2X3 channels, heat/capsaicin sensitive TRPV1, cold/redox-sensitive TRPA1, acid sensing ion channels ASICs, and serotonin (5-HT) receptors (Basbaum et al., 2009). Activation of these receptors results in membrane depolarization and if these are sufficient in magnitude trigger action potential firing that propagates along the axons of primary afferents to synaptic sites in the spinal cord dorsal horn. This mediates release of neurotransmitters such as glutamate and substance P which activate second-order neurons that project onto pain processing regions of the brain (Waxman & Zamponi, 2014).

Given the key role of ion channels on neuronal signaling, several ion channels that are involved in pain signaling such as voltage gated sodium channels (Nav) and TRP channels have been targeted as a therapeutic intervention in humans (Waxman & Zamponi, 2014). Examples of ion channel modulators that are used to reduce pain in pre-clinical and clinical settings include lidocaine and carbamazepine which block Navs and reduce the peak currents of Na^+ channels, reducing the excitability of neurons thus preventing or reducing the sensation of pain (Skerratt & West, 2015; Yang et al., 2020).

1.11 GI tract noxious mechanosensitivity

Stimuli that evoke somatic pain such as heat, cold, or inflammation are often inadequate to evoke colorectal visceral pain (Feng & Guo, 2020). Mechanical stimuli, however, have been shown to adequately evoke pain in the colorectum. Mechanosensitive cells are present in all layers of the gut, and these include epithelial enterochromaffin cells, intrinsic and extrinsic neurons, smooth muscle cells, and interstitial cells of Cajal (ICC) (Alcaino et al., 2017). Mechanosensitivity may arise through direct activation of mechanosensitive ion channels present in the afferent nerve terminals or though indirect activation of sensory afferents following the release of chemical mediators such as ATP which stimulate purinergic receptors present on afferent terminals (Grundy et al., 2006).

Sensory endings contain a variety of mechanosensitive ion channels that facilitate the conversion of stimulus energy into a change in membrane potential and subsequently action potentials. Local stress or strain around the afferent endings drives the opening of mechanosensitive ion channels that results in neuronal membrane depolarization and generation of action potentials (Feng & Guo, 2020). Ion channels that contribute directly to mechano-transduction of colorectal afferents include TRPV4, TRPC1, ASIC3, Piezo2, and three K_{2P} subtypes: TREK-1, TREK-2, and TRAAK (Feng & Guo, 2020). In the lumbar splanchnic afferents, ASIC1, ASIC3, TRPV4, and TRPA1 contribute to mesenteric mechanoreceptor function whereas ASIC1, ASIC2, ASIC3, TRPA1, and TRPV4 contribute to serosal mechanoreceptor function (Brierley, 2010). While the opening of mechanosensitive cation channels leads to depolarization and triggers action potential firing, mechanosensitive K^+ channels hyperpolarize the membrane potential and decrease the likelihood of an action potential (Ranade et al., 2015).

Alternatively, the release of transmitters in response to distension from other cell types can stimulate colonic afferents. For example, release of ATP by epithelial cells during mechanical distension has been well documented. ATP released during distention of epithelial cells lining tubes such as ureter and the gut acts on P2X3 and P2X2/3 receptors on subepithelial nerves to initiate impulses that are relayed via the spinal cord to pain centres in the brain (Wynn et al., 2003). Piezo2 is thought to be the main mechanotransducer in epithelial cells. Conditional GI epithelium Piezo2 knockout significantly decreases pressure induced epithelial secretion (Alcaino et al., 2018).

Visceral afferents can be classified into high and low threshold based on their sensitivity to mechanical stimuli. The high threshold fibres are activated by intense mechanical stimuli and display sensitivity to a range of noxious and algogenic chemical stimuli such as bradykinin or ATP and are thought to be polymodal nociceptors. 25% of mechanosensitive afferent fibers have a high threshold for response (>30 mmHg) and are thought to represent visceral nociceptors. Lower threshold fibres are thought to be non-nociceptive with important roles in the relay of phycological information (Bulmer & Roza, 2018).

1.12 G protein coupled receptors (GPCRs)

G-protein coupled receptors are the largest and most functionally diverse family of receptors consisting of 850 members in the human genome (Fredriksson et al., 2003; Schlyer & Horuk, 2006). Activation of GPCRs promotes intracellular signaling cascades and regulates numerous physiological and pathological processes including pain transmission. GPCRs are the primary target for over 30% of clinically used drugs due to their critical importance in many pathophysiological processes (Hopkins & Groom, 2002). GPCRs are activated by ligands such as ions, neurotransmitters, and by sensory agents such as photons, touch, taste and smell (Takezako et al., 2015).

GPCRs are characterized by a seven-membrane domain topology, an extracellular amino-terminus and an intracellular carboxyl-terminus and associate with heterotrimeric guanyl nucleotide-binding proteins (G-proteins). G-proteins are heterotrimers consisting of α (molecular mass=39-46 kDa), β (37 kDa), and γ (8 kDa) subunits (Hepler & Gilman, 1992). The α subunit has a high affinity binding site for guanine nucleotides (GDP or GTP). The GDP bound form of α is tightly bound to β- and γ- subunits which exist as a single unit. Ligand binding to the GPCR leads to activation of G proteins through exchange of GDP for GTP, resulting in the release of Gα from Gβγ complexes (Offermanns, 2003; Weis & Kobilka, 2018). Both Gα and Gβγ can act as downstream effectors which mediates production of second messenger molecules in the cells. G-protein signaling is turned off by the hydrolysis of GTP to GDP, rendering the Gα ineffective (Hamm, 1998). In humans there are 16 Gα that can be classified into four subfamilies according to their α subunit: G_i, G_s, $G_{12/13}$, and $G_{q/11}$ (Denise (Wootten et al., 2018).

The stimulation of G_q subfamily activates phospholipase C-β that catalyzes the hydrolysis of phosphatidylinositol bisphosphate (PIP_2), into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts as a second messenger to release Ca^{2+} into the cytoplasm from IP_3 regulated intracellular stores, while DAG acts as a second messenger that activates protein kinase C (PKC). Ca^{2+} and PKC participate in diverse signalling to evoke different cellular events. For example, increased intracellular calcium promotes neurotransmitter release at presynaptic terminals (Stone & Molliver, 2009). Stimulation of G_s subfamily activates adenyl cyclase which catalyzes the formation of cyclic AMP (cAMP) from ATP. Elevated cAMP results in activation of protein kinase A (PKA) and cyclic nucleotide-gated channels (Stone & Molliver,

2009). PKA mediates most of the cellular actions elicited by G_s activation such as proliferation and gene transcription (Tasken & Aandahl, 2004). In contrast, stimulation of G_i inhibits adenylyl cyclase activity, decreasing the production of cAMP (Syrovatkina et al., 2016).

1.13 The role of GPCRs in pain signalling

GPCRs are widely expressed on sensory nerves and many inflammatory mediators that contribute to nociceptor sensitization and therefore visceral hypersensitivity act via GPCRs making this class of receptors an important target for therapeutic intervention for treatment of visceral pain. GPCRs regulate pain transmission at multiple levels, with the ability to both stimulate and inhibit pain transmission (Gottesman-Katz et al., 2021). Drugs that activate inhibitory effects or block excitatory pathways through GPCR activation have shown considerable promise in treating visceral pain.

Binding of inflammatory mediators to their respective receptors augments pain sensation through the production of secondary messengers such as cAMP, PKA, and PKC that have been implicated in nociceptor sensitization. For example, PKC signalling initiated by inflammatory mediators such as bradykinin and NGF sensitizes nociceptors and produces visceral hypersensitivity through phosphorylation of nociceptive ion channels such as TRPV1. Consequently, knockout of PKCε has been demonstrated to reduce sensitization in response to inflammatory mediators (Khasar et al., 1999; McGuirk & Dolphin, 1992; Yao et al., 2005). Furthermore, transmitters that couple with G_q and or G_{11} such as the bradykinin receptor, B2 and the histamine H1 receptors inhibit M channels ($K_V7.2$-$K_V7.5$) resulting in excitation of neuronal fibres (Brown & Passmore, 2009; Du & Gamper, 2013). The M current activates in the range of the resting membrane potential and can be activated by PIP_2 whose depletion by PLC inhibits M channels resulting in increased excitability in neuronal membranes (H. L. Zhang et al., 2003).

Prostaglandin mediated sensitization of nociceptors occurs via the G_s coupled cAMP-PKA signalling pathways as demonstrated by the use of PKA inhibitors to block the sensitizing actions of prostaglandins (Bhave & Gereau, 2004). PKA activation has been demonstrated to enhance Na_V currents including $Na_V1.8$ which is preferentially expressed in nociceptors, thereby contributing to development of hyperalgesia (Vijayaragavan et al., 2004). In contrast, activation of G_i coupled receptors reduce cAMP levels and thereby attenuate the action of

prostaglandins providing an endogenous mechanism of pain control (Gottesman-Katz et al., 2021). G_i receptors mediate many of the inhibitory effects of neurotransmitters and almost all GPCR agonists that have an analgesic effect are coupled to G_i proteins. G_i modulate inwardly rectifying K^+ channels (K_{ir}) which are important in maintaining the resting membrane potential duration and excitability. Upon activation of G_i proteins, the Gβγ subunit directly binds and activates G protein coupled inward rectifying potassium channels (GIRK) following activation of GPCRs such muscarinic acetylcholine receptors, dopamine receptors and opioid receptors (Kano et al., 2019; Sadja et al., 2003). Additionally, G_i activation inhibits voltage dependent calcium channels resulting in reduced neurotransmitter release (Stone & Molliver, 2009).

GPCR signalling pathways also result in the activation of mitogen-activated protein kinases (MAPK) which modulate key signalling pathways including cell proliferation, differentiation and apoptosis and have been implicated in nociceptor sensitization (Goldsmith & Dhanasekaran, 2007). The MAPK family consists of 3 members: the extra cellular signal - regulated kinases (ERKs), c-Jun N-terminal kinase (JNK) and p-38 MAPK (Hucho & Levine, 2007). p38-MAPK and JNK are activated by proinflammatory cytokines e.g., TNF-α whereas eERK is activated by persistent and pathological stimuli including NGF, capsaicin, electrical stimulation, and nerve transection (Ji et al., 2009). Activation of MAPK leads to production of inflammatory cytokines and inhibition of all three MAPK pathways has been demonstrated to attenuate inflammatory and neuropathic pain in various animal models (Ji et al., 2009). Additionally, MAPK mediate transcriptional and posttranslational changes that result in upregulation of ion channels involved in nociception such as TRPV1 thereby contributing to pain hypersensitivity (Ji et al., 2002).

1.14 Pain in inflammatory bowel diseases

IBD consists of a broad spectrum of colletids the majority of whom are classified clinically based on the anatomical and histological features into the two main types of IBD, Crohn's disease (CD) and Ulcerative Colitis (UC). For example, CD is characterized by the presence of transmural inflammation, which can present throughout the digestive tract (although ileocecal and colonic sites are common), and classically presents with fatigue, prolonged diarrhoea, abdominal pain, weight loss, and fever (Mowat et al., 2011; Wallace et al., 2014). By contrast UC is characterized by the presence of mucosal inflammation that is restricted to the large bowel and presents with rectal bleeding, frequent passage of stool, mucus discharge from the rectum, and lower abdominal pain (Wallace et al., 2014). The diagnosis of IBD is confirmed by clinical evaluation and a combination of haematological, endoscopic, histological, or imaging based investigations (Mowat et al., 2011).

The chronic nature of IBD and the associated complications that arise due to the diseases result in frequent physician visits and hospitalizations that contribute significantly to the large socioeconomic cost of the conditions (Grundy et al., 2019). In the UK, the cost of IBD care to the NHS was estimated to be £ 1 billion in 2010 with these costs projected to increase due to the rising incidence of the condition (Ghosh & Premchand, 2015). Pain contributes to the decreased quality of life in IBD patients and leads to hospitalisation for pain management in a significant minority of patients (Schirbel et al., 2010). Symptoms of IBD commonly interfere with the patients' work habits resulting in reduced earning potential for patients. Additionally, the higher rates of anxiety, mood disorders and sleep disturbances associated with IBD interfere with patients' social activity and contribute the decreased quality of life in patients (Bakshi et al., 2021; Graff et al., 2009; Knowles et al., 2018).

Significant advances have been made in the treatment of Crohn's disease and Ulcerative Colitis over the past 2 decades precipitated by the introduction of biologic therapeutics which have been shown to be superior to conventional therapies in inducing sustained remission and mucosal healing (Louis, 2018). Therefore, despite the increasing prevalence of IBD worldwide, the rate of IBD related hospitalization, surgery and mortality are decreasing (Mehrmal et al., 2021). Additionally, biologics improve the quality of life for IBD patients and are associated with increased ability to perform in everyday and social activities (Holdam et al., 2016). However, most of the available treatment options mainly focus on the reduction of

inflammation with only a few therapies aimed directly at diminishing abdominal pain. Therefore, abdominal pain remains a challenge for a proportion of IBD patients whose pain does not resolve after inflammation. Treating abdominal pain is an ongoing challenge for gastroenterologists due to the absence of analgesic drugs that lack GI specific side effects and have efficacy for the treatment of abdominal pain (Greenwood-Van Meerveld et al., 2017).

The pathogenesis of IBD is complex and is hypothesized to consist of an interaction between genetic factors which contribute to disease susceptibility and immune and environmental factors which precipitate and perpetuate the disease (Danese et al., 2004). A meta-analysis combining genome-wide association studies (GWAS) identified over 240 gene loci associated with IBD (de Lange et al., 2017). Biological processes associated with IBD-specific loci include barrier function, epithelial restitution, microbial defence, innate immune regulation, reactive oxygen species generation, autophagy, regulation of adaptive immunity, endoplasmic reticulum stress, and metabolic pathways associated with cellular haemostasis (Ramos & Papadakis, 2019). Environmental factors including diet, smoking, antibiotic use, hygiene status, microbial exposures, and pollution have been implicated as potential risk factors for IBD (Ananthakrishnan et al., 2018). For example, the use of antibiotics during the early stages of life has been associated with an increased risk of IBD as microbiota plays a critical role in shaping immune cell development (Ramos & Papadakis, 2019).

IBD has historically been considered a disease of western nations, however, recent trends indicate increasing incidence in the developing world (Alatab et al., 2020). This increasing incidence has been attributed to environmental risk factors associated with industrialization and improved surveillance of IBD in developing countries due to advances in technology and improved access to health care (Kaplan, 2015). IBD can be diagnosed at any age, but the peak age for onset is between 15-30 years and occurs equally in both men and women (Hanauer, 2006). The prevalence of IBD also varies depending on ethnicity and race. Caucasians are the most affected by IBD whereases it is rare among African Americans, Hispanics and Asians. In the US, it is estimated that the prevalence of IBD is 1099/100 00 in whites, which is significantly higher than in Blacks (324/100 000) and Hispanics (383/100 000) (Nguyen et al., 2014). Although IBD affects the quality of life, the mortality rate of patients is similar to the normal population (Matricon, 2010).

1.15 The etiology of IBD

IBD develops in 2 stages. Firstly, the integrity of the intestinal barrier separating the lumen and mucosa is compromised allowing luminal contents to penetrate the epithelial mucosal barrier (Matricon, 2010). Secondly, both the innate and adaptive immune system responses are dysregulated. The innate immune system serves as the first line of defence and provides an immediate protective response against infections (Wallace et al., 2014). Cells of the innate immune system including dendritic cells, macrophages, innate lymphoid cells, and neutrophils act together to initiate inflammation. Neutrophils are early responders in the inflammatory process and play a key role in the inflammatory process by recruiting and activation of other white blood cells (e.g. macrophages) through production of chemokines and proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8 (Neurath, 2014). This leads to phagocytosis of the infected cells and microorganisms, antigen presentation, and activation of the adaptive immune system (Wallace et al., 2014). In IBD, the clearance of foreign material by the innate immune system is impaired resulting in a compensatory acquired immune response that leads to chronic inflammation (Matricon, 2010).

The adaptive immune system is comprised of lymphocytes (T and B cells) and contributes to disease progression in IBD patients as consequence of increased proinflammatory drive resultant from cytokine release by T helper (Th) cells and reduced anti-inflammatory activity by regulatory T cells (Treg) (Wallace et al., 2014). In IBD patients, there is an imbalance between Th and regulatory T cells. A dysregulated T cell response as in IBD leads to the onset of inflammation by the excessive release of cytokines and chemokines (Matricon, 2010). Th cells which are essential in the elimination of intracellular pathogens are formed from differentiation and activation of naïve cells (T0) into Th1, Th2 or Th17 cells. Th1 and Th17 are thought to drive the pathogenesis of CD whereas UC is predominantly driven by Th2 cells (Wallace et al., 2014). Th1 cells are stimulated by IL-2 and produce IL-2, IFN-γ and TNF-α (Bouma & Strober, 2003). Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 whereas Th17 cells are induced by IL-6, transforming growth factor β (TGF-β) expanded by IL-23 and are characterised by production of IL-17A, IL-17F, IL-21 and IL-22 (Bouma & Strober, 2003; Matricon, 2010). Treg cells produce anti-inflammatory cytokines including IL-10 and TGF- β.

1.16 IBD management

The goal of IBD therapy is to induce and maintain clinical remission. Conventional treatment for IBD is based on anti-inflammatory drugs that include biologics and small molecule drugs. Biologics are molecules including recombinant cytokines, monoclonal antibodies, and antagonists of cytokines and receptors that are involved in modulating inflammation during immune-mediated processes.

Examples of biologics that are currently used to manage IBD include several anti-TNF- α agents (e.g. infliximab, and adalimumab, certolizumab and golimumab), anti-integrins (e.g. vedolizumab and natalizumab) and IL-12 and IL-23 antagonists (ustekinumab) (Rawla et al., 2018). Biologics targeting TNF-α were the first ones to be used in biologic therapy and were shown to be effective in inducing and maintaining remission in IBD patients (Kim & Cheon, 2017). TNF-α is a proinflammatory cytokine that is produced by activated macrophages, monocytes, and T lymphocytes and plays an important role in the development of IBD, therefore a viable target for treating IBD (Gareb et al., 2020). However, despite its vital role in IBD, up to 40% of IBD patients do not respond to TNF inhibitors and 23-46% experience secondary loss of response 1 year after anti-TNF-α treatment (Ben-Horin et al., 2014). Vedolizumab is the most widely used anti-integrin therapy in IBD shown to be effective in inducing and maintaining remission in UC and CD with a favourable safety profile. Vedolizumab acts by preventing leukocyte migration to intestinal tissue, thereby alleviating local intestinal inflammation (Gubatan et al., 2021; Park & Jeen, 2018).

Small molecule drugs including oral corticosteroids (e.g., budesonide, prednisone) immunomodulators (e.g., azathioprine, 6-mercaptopurine,), and salicylates (e.g., 5-aminosalicylic acid (5-ASA) have been used to treat IBD since the 1950s, however, are associated with adverse side effects (Yeshi et al., 2020). Corticosteroids supress inflammation through inhibition of inflammatory mediators and proinflammatory transcription factors such as nuclear factor-kappa B (NF-κβ) and activator protein 1 (AP-1) (Riccardi et al., 2002). Corticosteroids are associated with adverse side effects including opportunistic infections, diabetes mellitus and hypertension. Aminosalicylates act by interfering with the metabolism arachidonic acid and production of cytokines. Side effects associated with 5-ASA are generally mild and include nausea and abdominal pain (Cai et al., 2021).

The gut microbiota plays a key role in pathogenesis of IBD, therefore, antibiotics such as metronidazole, ciprofloxacin, and clarithromycin have also been used alongside other medications for IBD to treat symptoms of IBD. Treatment with antibiotics has been demonstrated to be beneficial especially in CD. However, antibiotics are unsuitable for long term therapy as required in IBD as they are associated with side effects that cause intolerance and increasing antibiotic resistance (Pithadia & Jain, 2011). Nutritional interventions have been demonstrated to have a beneficial role in treating symptoms of IBD, particularly in children where studies have demonstrated that exclusive enteral nutrition induced clinical remission in the majority of patients, at a rate comparable with steroids (Swaminath et al., 2017). Additionally, use of probiotics has also been demonstrated to be beneficial in managing symptoms of IBD (Nitzan et al., 2016; Pithadia & Jain, 2011).

In cases where therapy fails, surgery is performed as a last choice of treatment options as it results in extensive loss of the small bowel and disability (Yeshi et al., 2020). It is estimated that more than 55% of IBD patients undergo surgery in their lifetime although in recent years, surgery rates have been decreasing (Dittrich et al., 2020; Lowe et al., 2020; Schirbel et al., 2010). The decrease in surgery rates is attributed to utilization of biologic therapies, earlier diagnosis and treatment and improved patient education (Dittrich et al., 2020).

1.17 Current management of pain in IBD

IBD treatments that target inflammation can alleviate inflammation related pain in most patients. However, abdominal pain persists in 20-50% of patients after inflammation has resolved thereby presenting as a challenge for clinicians (Zielinska et al., 2019). The continuous release of inflammatory mediators in IBD sensitizes peripheral nerve endings resulting in abdominal pain that occurs during disease flares and periods of remission. In IBD, the recurring inflammation and release of mucosal signalling molecules such as NGF as well as changes in ion channel expression (TRPV1/TRPA1) can result in the development of visceral hypersensitivity and chronic visceral pain (Zielinska et al., 2019). Increased of expression of ion channels involved in nociception such as TRPV1, P2RX3, and ASICs has been demonstrated in the inflamed human GI tract and has been shown to contribute to pain (Anand et al., 2007).

The pathogenesis of pain in IBD is complex and remains unclear but several pathological mechanisms including inflammation, bowel obstruction, psychological, psychosocial, neurological, and genetic factors have been attributed to the development of pain (Bielefeldt et al., 2009; Morrison et al., 2013). Conventional pain therapies such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioids have been used to treat pain in IBD but have been associated with GI related side effects.

NSAIDs are anti-inflammatory analgesics that act by blocking the production of prostaglandins through inhibition of cyclooxygenase (COX-1 and COX-2) enzymes and are commonly considered to be the first line of treatment to relieve pain and treat inflammation (Gunaydin & Bilge, 2018). However, they are linked to disease exacerbation in IBD patients demonstrated by the higher rate of NSAIDs use among patients who are admitted to the hospital for IBD therefore are unsuitable for treating pain (Feagins & Cryer, 2010). Prostaglandins are involved in maintaining the mucous-bicarbonate barrier, submucosal blood flow and modulation of the immune system of the colon, therefore reduced prostaglandin production due to inhibition of COX-1 have been implicated in early and frequent relapse of IBD (Russell, 2001). Additionally, NSAIDs alter intestinal barrier function and lead to increased permeability through interaction with phospholipids that constitute the brush border and depletion of ATP that influences intracellular integrity through control actin-myosin complexes (Bjarnason & Takeuchi, 2009; Lichtenberger et al., 2006).

Similarly, opioids which are primarily used to treat severe pain have also been associated with adverse gastrointestinal related effects such as constipation, incomplete evacuation, bloating and gastric reflux, therefore are unsuitable for IBD patients (Bakshi et al., 2021). Additionally, long term use of opioids is linked to addiction and abuse (Zielinska et al., 2019).
Lack of effective pain management in IBD patients has been associated with increased stress, anxiety, and depression (Srinath et al., 2014). Antidepressants that are commonly used to treat symptoms of depression and anxiety have been shown to improve abdominal pain without some of the GI related side effects associated with conventional analgesics (Bakshi et al., 2021). However, there are concerns about long term dependency, side effects, and withdrawal associated with the use of these drugs to treat pain. Psychotherapy or behavioural therapy has been suggested as an effective complementary therapy for pain that cannot be completely eradicated (Srinath et al., 2014).

1.18 Pain in irritable bowel diseases (IBS).

Recurrent abdominal pain is the main symptom of IBS which presents as one of four forms: IBS with constipation (IBS-C), IBS with diarrhoea (IBS-D), IBS mixed (IBS-M) and IBS unsubtyped (IBS-U) (Corsetti & Whorwell, 2017). The presentation and characteristics of abdominal pain vary by IBS subtype with abdominal pain reported to be more severe and generalized withing the IBS-C subtype compared with IBS-D of IBS-M subtypes (Shah et al., 2020). Despite the high prevalence of IBS, the underlying causes are still poorly understood. Several factors have been implicated in the pathophysiology of IBS symptoms including genetic disposition, diet, intestinal microbiota and mucosal low-grade inflammation (El-Salhy, 2015). A complex interaction between altered GI motility, visceral hypersensitivity, altered intestinal permeability, altered gut flora, low grade inflammation and disorders in gut brain-axis is implicated in the pathogenesis of pain in IBS (Nam et al., 2018; Vanuytsel et al., 2014).

While IBS is generally regarded as a functional disorder, studies in the recent years have shown the persistence of mucosal inflammation at the microscopic and mucosal level. Increased immune cells including T cells and mast cells has been reported in post infectious IBS (PI-IBS) which occurs in 4-36% of patients with infectious gastroenteritis (Spiller & Garsed, 2009). Consequently, patients with PI-IBS have increased inflammatory mediators including serotonin and cytokines compared with controls (Ng et al., 2018; Thabane & Marshall, 2009). Additionally, studies have shown that the amount of activated mast cells within the proximity of bowel enteric nerves correlate with the severity and frequency of abdominal painful sensations (El-Salhy, 2015). Activated mast cells release mediators such as histamine and proteases which have been shown to induce hypersensitivity in nociceptive neurons thus contributing to the generation of abdominal pain (Barbara et al., 2007).

Due to the complex, multimodal nature of IBS, there is no single treatment regarded as being universally applicable to the management of the disease and treatment often focuses on single symptom management (Corsetti & Whorwell, 2017). Current therapy for IBS includes antibiotics, antidepressants, probiotics, fiber supplements, 5HT3 antagonists and dietary interventions (Vanuytsel et al., 2014). Despite the many symptomatic treatments, less than one third of IBS patients are reported to be satisfied with the therapies used to treat their symptoms. Conventional pain therapies including NSAIDs, and narcotics are normally prescribed to

manage visceral pain in IBS patients but are associated with adverse side effects that outweigh the benefits and are therefore not recommended for treating pain in IBS (Corsetti & Whorwell, 2017).

Therefore, although pain is a prominent symptom of IBD and IBS, management of abdominal pain is evidently lacking. Hence, it is imperative to develop drugs that target peripheral organs and nerves to minimize off-target effects in IBD and IBS.

1.19 Thesis aims

Although great progress has been made in understanding visceral pain, a significant unmet clinical need still exists for the development of novel visceral analgesics with limited side effects. In this thesis I have explored the potential utility of several drug targets for the treatment of visceral pain which target different points in the cascade of events which trigger pain in disease states. These have been determined utilising the RNA sequencing data generated by our group showing gene expression in mouse colonic neurons and altered gene expression in colonic biopsy samples from IBD patients (CD & UC).

Based on these observations we elected to explore:

1. The effects of calcium-activated potassium (K_{Ca}) channel modulation on colonic afferent fibre activity.
2. The role of putative pro-nociceptive mediators (MMP1 and angiotensin II) identified by their significantly increased expression (or that of their precursor protein) in tissue from IBD patients and marked expression of their respective cognate receptors PAR_1 and AT_1 on mouse colonic neurons.

2 Methods

2.1 Animals

Experiments were performed using tissue from male C57B6 mice and female CD1 mice (10-14 weeks of age) euthanatized by exposure to increasing concentrations of CO_2 followed by cervical dislocation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 and following local ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

2.2 Electrophysiological recordings from mouse lumbar splanchnic nerve

Following euthanasia, the abdomen was exposed by laparotomy and the colorectum (from the splenic flexure to anus) and associated lumbar splanchnic nerves were carefully isolated and removed. Faecal matter was gently flushed out of the colorectum using Krebs buffer (in mM: 124 NaCl, 4.8 KCl, 1.3 NaH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11.1 D-glucose, and 2.5 $CaCl_2$) before the tissue was transferred to a recording bath in which the colorectum was cannulated at both ends. Tissue was luminally perfused (200 μl/min) and superfused serosally with carbogenated Krebs buffer at a rate of 7 ml/min. Krebs buffer was supplemented with nifedipine (10 μM) and atropine (10 μM) to block smooth muscle contraction and the bath temperature was maintained between 32-34 °C using an in-line heater (Warner instruments). Luminal pressure was measured via a buffer filled pressure transducer (Neurolog model NL 108) and maintained between 2-5 mmHg by an elevated end pressure.

The mesentery (containing the splanchnic nerves) and attached tissue were pinned out on the sylgard base of the tissue bath proximal to the colon using insect pins. Individual lumbar splanchnic nerve (LSN) bundles were dissected free of the main nerve trunk central to the pre-vertebral ganglia and an individual nerve bundle drawn into a borosilicate glass suction electrode to facilitate electrophysiological recordings (Figure 6). A silver chloride reference electrode (AgCl) was placed in the tissue bath and ongoing nerve activity was recorded using a differential amplifier (Neurolog head stage and AC pre amp- Neurolog model NL 104). Signals were amplified (gain 5K), bandpass filtered (100-1500 Hz), and digitally filtered for 50 Hz noise (Humbug; Quest Scientific, Canada). Raw traces were digitized at 20 kHz (micro1401; Cambridge Electronic Design) and the signals were displayed on a PC using Spike 2 software. Action potentials were counted online using Spike 2 software using a cut-off

threshold of twice the background noise (noise level typically 50 mV), and ongoing nerve discharge displayed as a rate histogram.

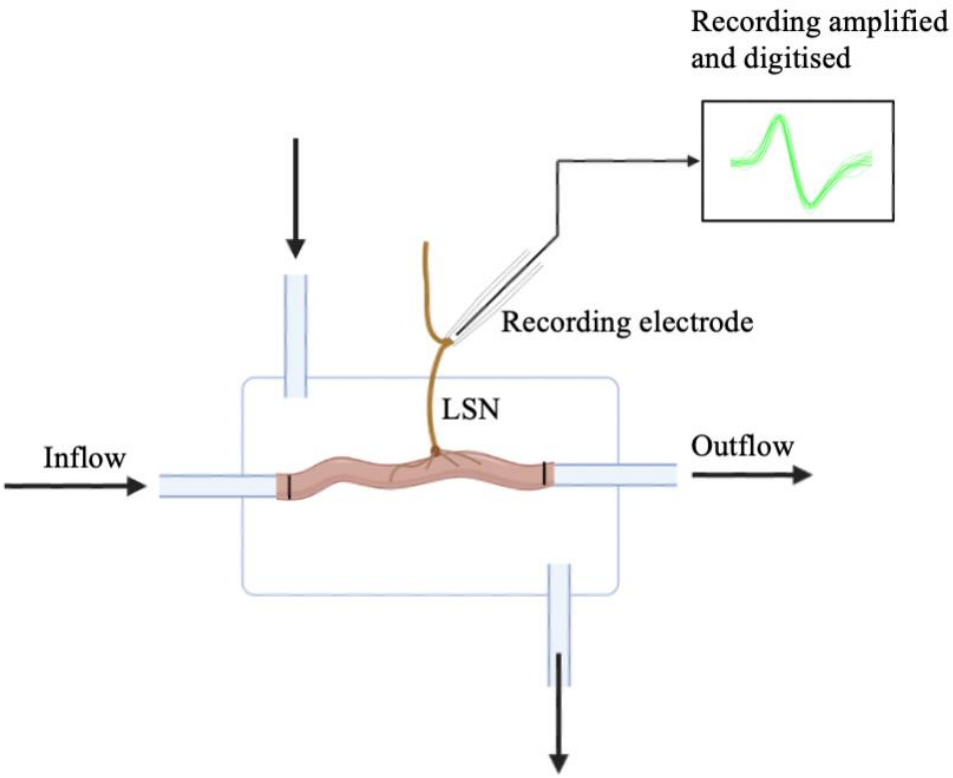


Figure 6. Schematic of electrophysiological recordings of lumbar splanchnic nerve (LSN) activity.

The colon was luminally perfused (200 µl/min) and superfused serosally with carbogenated Krebs buffer. Electrophysiological recordings were made from nerve bundle drawn into a borosilicate glass electrode.

Preparations were maintained for a minimum of 20 minutes once electrophysiological recordings with a signal-to-noise ratio sufficient to allow accurate spike counting preparations were acquired before the commencement of test protocols (please see individual chapters). For studies examining the effect of mediator challenge, tissue bath perfusion was switched from supplemented carbogenated Krebs buffer to a solution of the drug prepared in supplemented Krebs buffer. The drugs were serosally perfused via the inline heater and a washout period of 30 minutes was allowed between repeat drug applications. Pre-treatments were given in a solution of supplemented carbogenated Krebs buffer 7 minutes before administration of the drug. An example trace of LSN activity response to drug treatment (ATP) and the measurements derived from the profile is shown below (Figure 7).

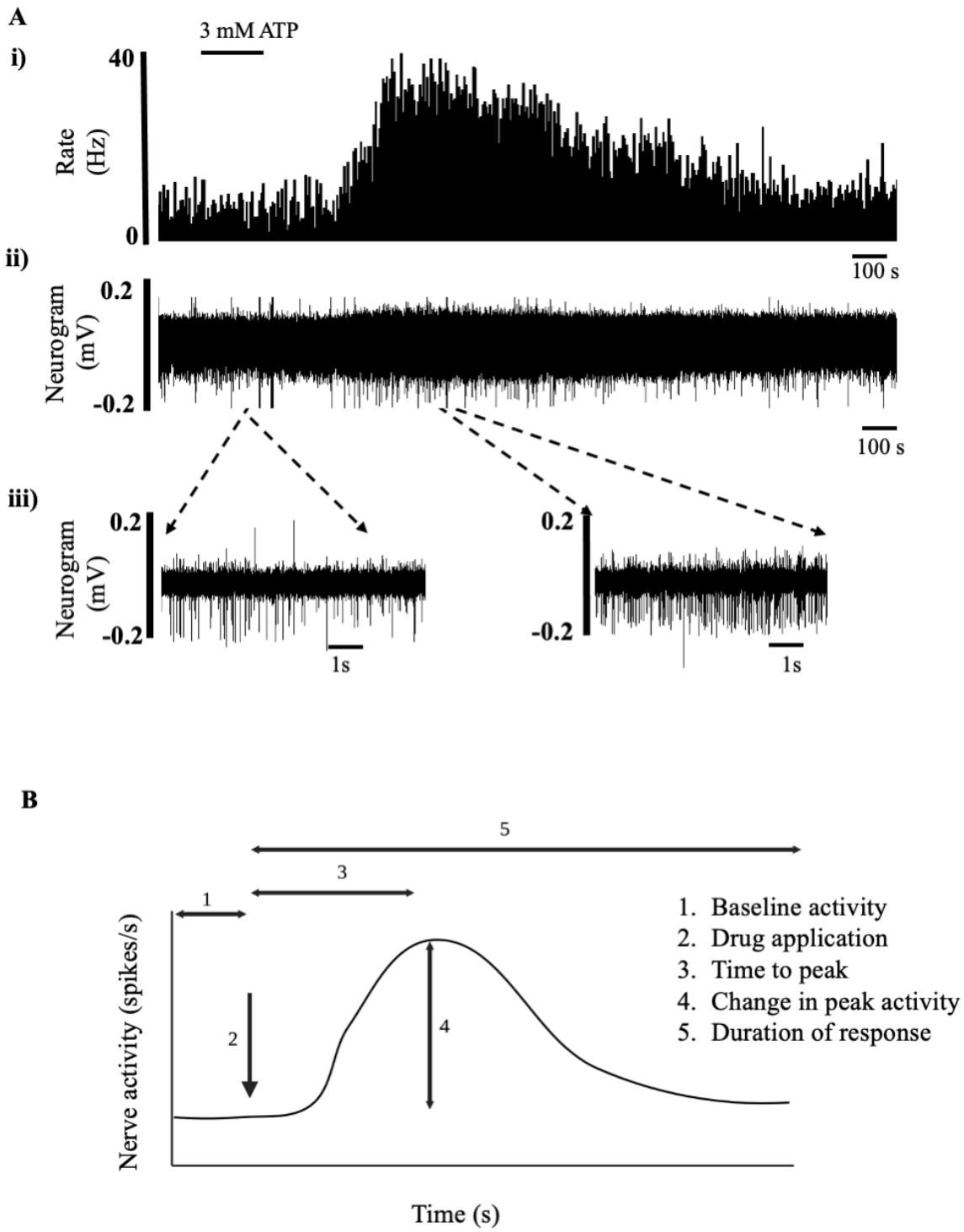


Figure 7. Example of the lumbar splanchnic response (LSN) to drug application (ATP).

(A) Representation of raw data showing (i) rate histogram (10 seconds bin width) and, (ii) neurogram of the LSN nerve response to ATP. (iii) shows the expanded trace at baseline activity and at peak activity. (B) Schematic of response profile showing (1) the timing and measurements of baseline activity, (2) drug application time point, (3) measurements of the time taken from drug entering the bath to activity reaching its peak and (4) the change in activity at peak which was determined by subtracting baseline activity from peak firing. Duration of the response (5) was determined as the time taken for nerve activity to return to baseline from onset of response.

2.2.1 Whole nerve distension studies

For whole nerve distension studies, slow ramp distensions were performed by blocking the luminal perfusion outflow of the cannulated colon to increase the pressure by 80 mmHg. This pressure has been shown to evoke pain behaviours in vivo in mice and robustly activate all known mechanoreceptors (Ness & Gebhart, 1988). It took an average of 3 minutes for the pressure to increase by 80 mmHg. Nerve activity immediately returned to baseline upon relief of the pressure. Repeat distensions were performed 15 minutes apart. In distension studies with the mediator challenge, the drug was administered between distensions 3 and 4 and subsequent distensions were performed after nerve activity returned to baseline. In studies to evaluate the effect of receptor or ion channel modulation on the LSN response to ramp distension, the drugs were given in a solution made up in Krebs buffer, 7 minutes before and during the ramp distension 4. Time matched controls in which the vehicle was administered in place of the drug were conducted for each set of experiments. An example of LSN response to ramp distension is shown below (Figure 8).

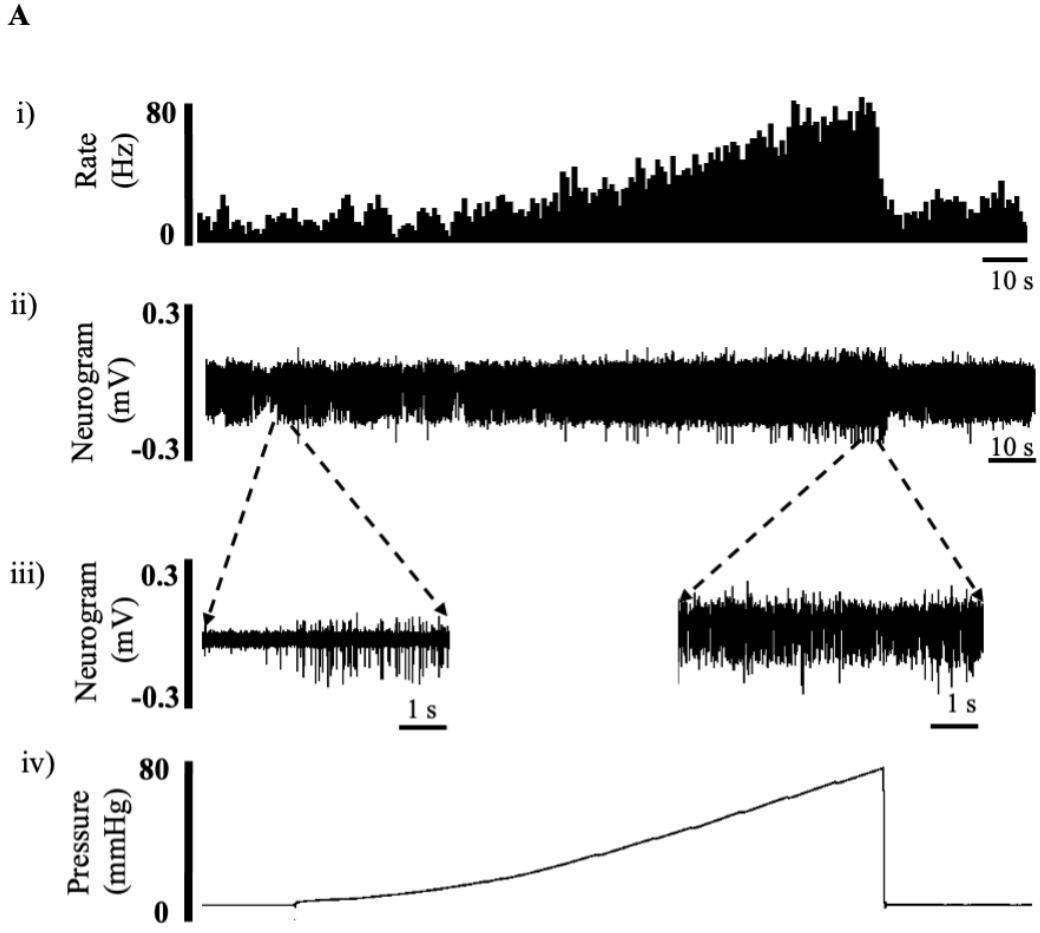


Figure 8. Example of whole nerve response to ramp distension.

(A) Representation of raw data showing (i) the rate histogram (10 seconds bin width), (ii) neurogram, (iii) expanded trace at baseline and at peak activity measured at 0 and 80 mmHg, respectively and (iv) the accompanying intraluminal pressure trace.

2.2.2 Analysis of electrophysiological recordings

Ongoing nerve discharge was analysed using a custom-made script in Spike 2 which calculated the mean firing rate at 1-minute intervals for duration of the experiment. For drug treatment studies, changes in nerve discharge were determined post-challenge by subtracting a baseline value derived from the mean of the values generated for 3 consecutive minute intervals before each challenge from the nerve discharge for each minute post-challenge. Data from individual experiments were subsequently averaged to generate a mean and SEM value of the change in nerve discharge over time following the challenge.

For ramp distension analysis, the baseline was determined by averaging nerve activity over 3 minutes before the distension. The change in nerve activity was determined by subtracting the baseline value from nerve discharge measured at every 5 mmHg increase in pressure. Data from individual experiments were averaged and changes in LSN were compared between groups.

All statistical analysis was conducted on Graphpad Prism 9. Changes in peak activity were analyzed using Student's t-test or one way ANOVA with Dunnett's or Tukey's multiple comparisons test as appropriate. Changes in the response profiles were analyzed using a two-way ANOVA with Dunnett's, Bonferroni's or Tukey multiple comparisons test as appropriate. Statistical significance was set at $p<0.05$ and data are displayed as mean ± SEM.

2.2.3 Few fibre recordings

To record nerve activity from few fibres of the LSN, the tissue was set up as previously described and the LSN teased to separate the fibres. Once electrophysiological recordings with a signal to noise ratio sufficient to allow accurate spike counting, the preparation was maintained for 15 minutes prior to commencement of the protocol.

2.2.4 Analysis of few fibre recordings

Analysis of nerve activity from few fibre recordings was performed using the waveform analysis function of the Spike 2.0 software based on previous methodologies (Richards et al., 1996). The recordings contained action potentials of different amplitudes and waveforms, some of which were distinct enough to be discriminated from each other as single units. Waveform templates were generated from spikes with amplitude greater than the set threshold (typically 100 mV) with a new template generated for least 8 similar spikes. Action potential waveforms were matched with the different templates and the number of units were identified per recording. Spike discrimination was confirmed manually by overlaying the templates and spikes for each unit. An example of one vs two distinct units responding to ramp distension (0-80 mmHg) with the accompanying action potential overlays is shown below (Figure 9).

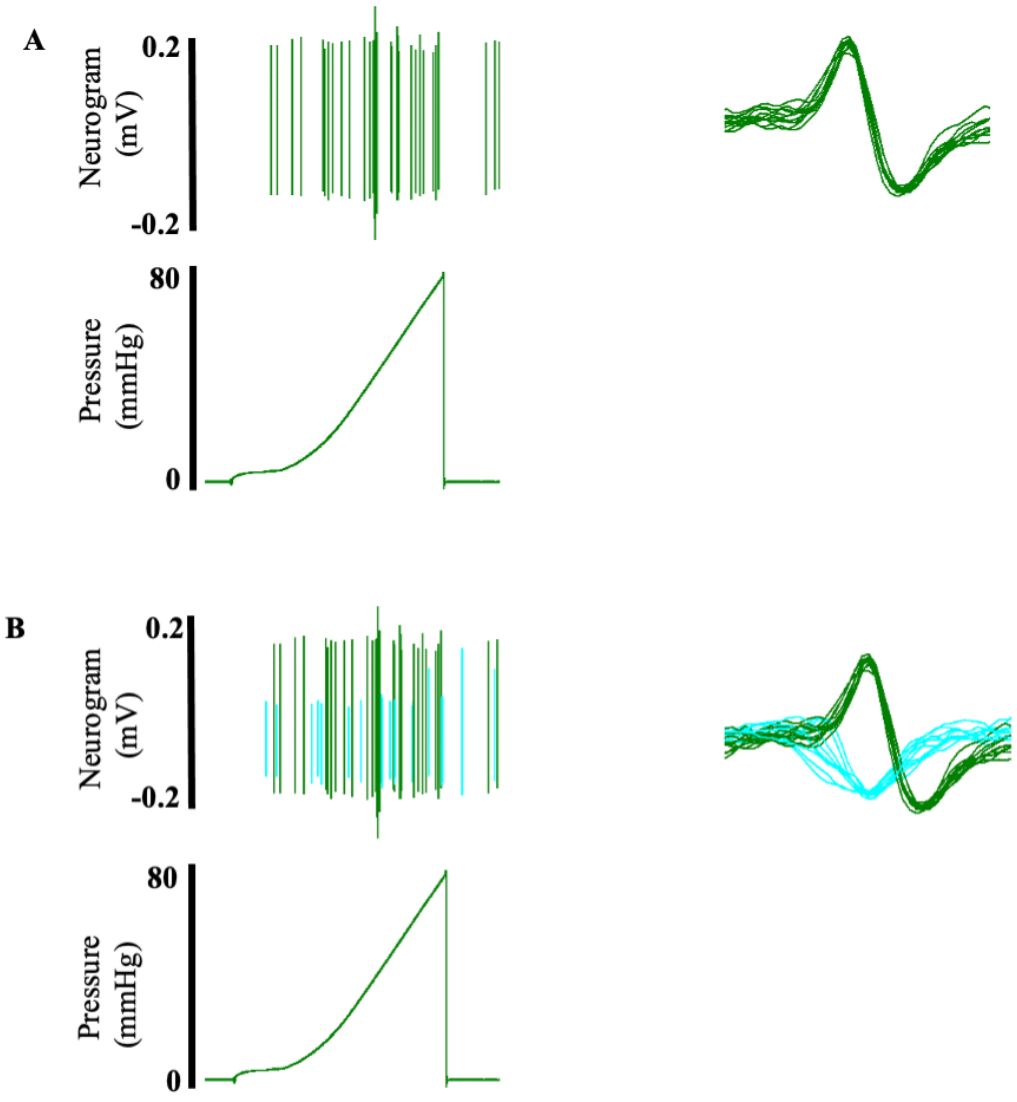


Figure 9. Illustration of single unit discrimination from few fibre recordings.

(A) Illustration of a single unit responding to increasing pressure with the accompanying overlay of action potentials. (B) Illustration of two distinct units responding to increasing pressure with the accompanying overlay of action potentials for each unit.

2.3 Colon motility recordings

Following euthanasia, the abdominal cavity was exposed by laparotomy and the colon was dissected free from anus to the caecum. Luminal content was removed by gentle manipulation with a cotton bud and flushing with Krebs buffer. The colon was then cannulated at both ends in a tissue bath that was constantly perfused with carbogenated Krebs buffer via an inline heater at a rate of 7 ml/min. The temperature of the bath was maintained between 32-34 °C.

Luminal pressure was measured by connecting a pressure transducer (Neurolog model NL 108) to the outflow. The output signal was amplified and digitized by a 1401 plus to a digital converter and recorded using spike 2 Software. Luminal pressure was raised to 5-10 mmHg, sufficient to trigger colonic migrating motor complexes (CMMCs), through the application of Krebs buffer and the inflow closed creating a sealed system. The tissue was allowed to stabilize for 30 minutes demonstrating CMMCs of reproducible frequency before any drug application. Drug treatments were given by switching tissue bath perfusion from supplemented carbogenated Krebs buffer to a solution of the drug prepared in supplemented Krebs buffer. An example trace showing spontaneous colonic contractions and the time measurements derived from the recording (Figure 10).

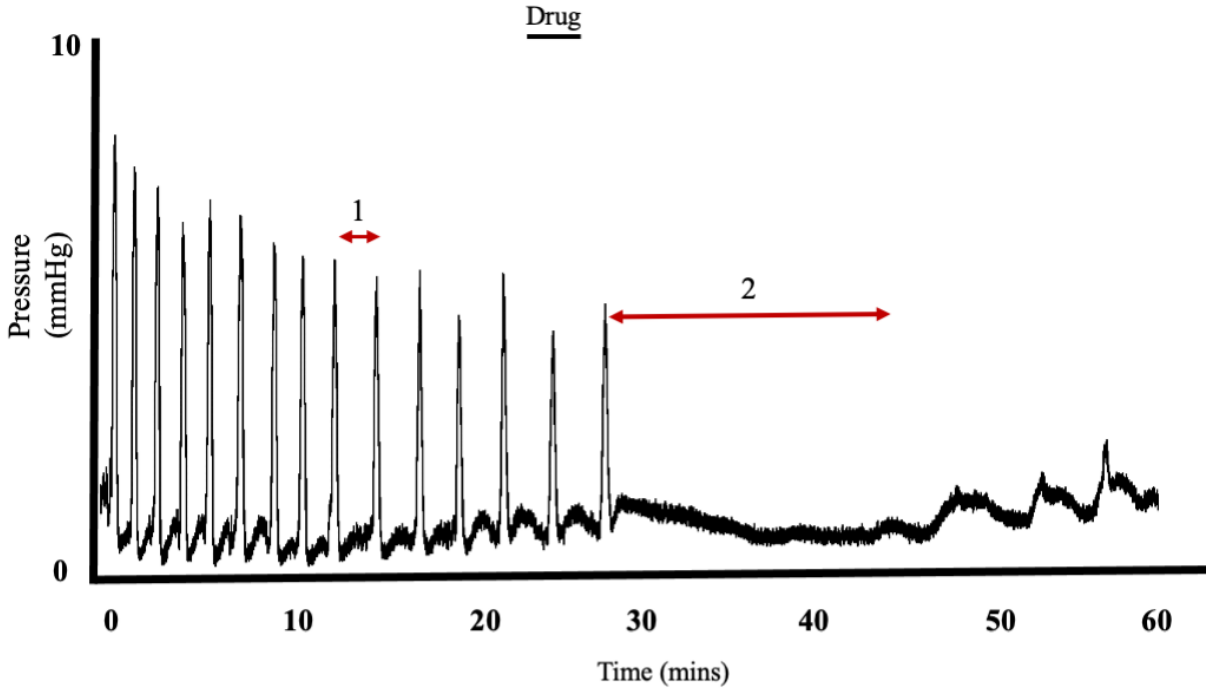


Figure 10. Example trace demonstrating spontaneous colonic motility before and after drug treatment.

Frequency of CMMCs was determined 30 minutes before and after drug treatment. Time between contractions was measured (1) before and (2) after drug treatment.

The frequency of CMMCs was calculated during the 30-minute period prior to addition of the drug and in the 30-minute period following drug administration. Similarly, the time between contractions was determined during the 30-minute period before drug administration and in the 30-minute period following drug administration. Data from individual experiments were subsequently averaged to generate a mean and ±SEM value of CMMCs frequency over time. The changes in frequency and time between contractions before and after addition of the drug were compared using a paired Student's t test.

2.4 Calcium imaging

2.4.1 Primary cell culture preparation

Following euthanasia, DRGs (T13-L6) were dissected, trimmed of connective tissue, and placed in ice-cold Lebovitz L-15 Glutamax media (Life Technologies) supplemented with 2.6% (v/v) $NaHCO_3$. The DRGs were then incubated in 3 ml collagenase solution (1 mg/ml collagenase type 1A (Sigma-Aldrich, UK) and 6 mg/ml BSA (Sigma-Aldrich, UK)) for 15 minutes at 37°C followed by 3 ml trypsin solution (1 mg/ml trypsin (Sigma-Aldrich, UK) and 6 mg/ml BSA) incubation for 30 minutes at 37°C. The L-15 media was replaced by 2 ml of Lebovitz L- 15 Glutamax media with additions containing 2 % penicillin/streptomycin, 24 mM $NaHCO_3$, 38 mM glucose, and 10 % fetal bovine serum (Sigma-Aldrich, UK). Mechanical dissociation of the DRGs was performed using a 1 ml pipette to gently triturate the DRGs 8 times followed by centrifugation at 1000 rpm for 10-15 seconds before collecting the dissociated cell containing supernatant. The trituration steps were repeated 5 times until 10 ml of the supernatant had been collected. The 10 ml supernatant was then centrifuged at 1000 rpm for 5 mins and the pellet resuspended in 50 µl/dish of L-15 media with additions. The cells were then plated in laminin-coated (20 µg/mL; Life Technologies) poly-D-lysine-coated 35 mm glass-bottom dishes (MatTek Corporation, USA) and incubated at 37°C for 4 hours before flooding with L-15 media with additions. All DRG neurons were incubated at 37°C overnight (12-16 hrs) before Ca^{2+} imaging experiments (Figure 11).

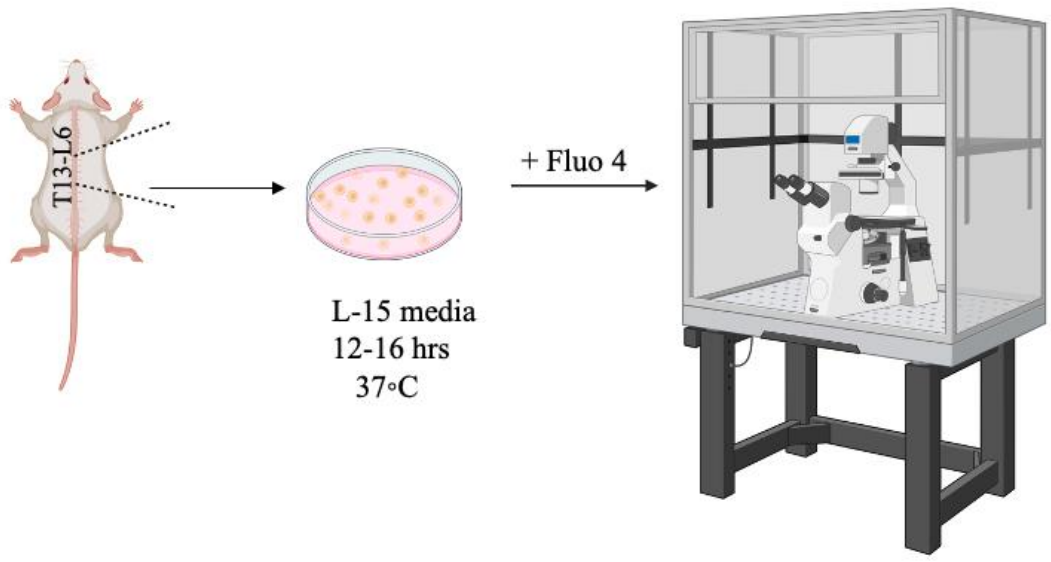


Figure 11. Schematic of calcium imaging procedure.

DRG neurons (T13-L6) were enzymatically dissociated and cultured overnight in L-15 media with additions. Neurons were loaded the calcium indicator Fluo-4 AM before imaging.

2.4.2 Calcium imaging protocol

Neurons were loaded with fluorescent Ca^{2+} indicator, Fluo-4 (10 μM, Thermo Fisher Scientific) in extracellular solution (ECS in mM;140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 4 glucose; adjusted to pH 7.4 with NaOH and 300-310 mOsm with sucrose) for 30 minutes at room temperature. The dishes were then washed with ECS and placed in an imaging chamber. Cells were imaged using a Nikon Eclipse Ti microscope at a 10x objective. Fluo-4 was excited using a 470 nm LED (Cairn Research, UK) and fluorescent images were acquired every second using a Zyla sCMOS camera (Andor, UK) and Micro-Manager software (v 1.4; National Institute of Health). KCl was employed as a positive control for these studies. Administration of KCl induces neuronal depolarization due to increase in K^+ ions that subsequently leads to Ca^{2+} influx through several channels including various voltage sensitive calcium channels and intracellular stores in the endoplasmic reticulum (Rienecker et al., 2020).

2.4.3 Calcium imaging analysis

Images were processed and analysed using Fiji/ImageJ (National Institute of Health, USA). Neurons were identified morphologically, and regions of interest (ROI) were drawn around each cell per dish. Mean gray values of the ROIs were obtained from ImageJ and then analyzed using custom written scripts in Rstudio to calculate change in Ca^{2+} influx. The program subtracted background fluorescence from each cell and normalized the drug response to the 50 mM KCl response (Fmax) to generate normalized intensity values (F/Fmax). The script generated a file with normalized intensity (F/Fmax) values for all cells that met the threshold (mean ± 5* stdev). The proportion of neurons responding to the drug and the magnitude of the responses were determined per dish. Only cells which showed a 10% increase in fluorescence during stimulation were deemed as responsive to the drug. Data from individual experiments were subsequently averaged to generate a mean and ±SEM value for the proportion of neurons responding to each drug treatment. The responding proportions and changes in the magnitude of the responses were compared using one-way ANOVA with Dunnett's multiple comparisons test for the various drug treatments.

2.5 Colonic permeability

2.5.1 Electrophysiological set up

The multichannel DVC 1000 Voltage/Current clamp apparatus (World Precision Instruments) was used to measure changes in epithelial tissue permeability. Each channel was connected to a preamplifier (World Precision Instruments) mounted in a vertical position close to the Ussing system. Two current and two voltage electrodes filled with 1.5% agar (w/v 3M KCl) were connected to each amplifier. Parafilm was placed between the chamber halves before the chamber halves were connected. The reservoirs and chambers were filled with Krebs solution oxygenated with 95 % O_2-5 % CO_2. Krebs buffer with the following composition was used in mM: NaCl, 118; KCl, 5.3; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$ 25; and glucose 11.0. The reservoirs above each chamber were water jacketed to warm Krebs buffer to 37°C with the bubbles from the gas mixture driving circulation of the buffer. All bubbles interfering with chamber circulation were removed. The voltage and fluid resistance were zeroed to eliminate electrical bias. After the zeroing was completed, the voltage clamp circuitry was set to standby mode and the intestinal preparation was mounted in the chamber with fresh superfusate.

2.5.2 Tissue preparation

Following euthanasia, the abdomen was exposed by laparotomy and the colon was dissected free from anus to the caecum. Sections from the distal colon were cut and the tissue segments opened longitudinally. The intestinal section (typically 1 cm in length) was pinned mucosal side down on a sylgard dish and the muscle layers overlying the epithelium were stripped away under a dissection microscope (Figure 12). The serosal and the longitudinal/circular muscular layers were stripped away to minimize rhythmic contractions that produce corresponding changes to the short circuit current (IS_C) and voltage potential (Vt). Additionally, the seromuscular layers have been shown to present a significant diffusion barrier to experimental drugs, nutrients and oxygen which reduces tissue viability (Clarke, 2009). The stripped intestinal segment was then mounted (Figure 12) on the Ussing chamber (window area, 0.2 cm^2). The mucosal side faced one chamber half and the serosal membrane is faced the other half, thus separating the solutions that bathe each chamber half. The chambers were then filled with fresh Krebs buffer and bubbles interfering with the chamber circulation removed. The drugs were injected on the mucosal (right-hand) side of the chamber.

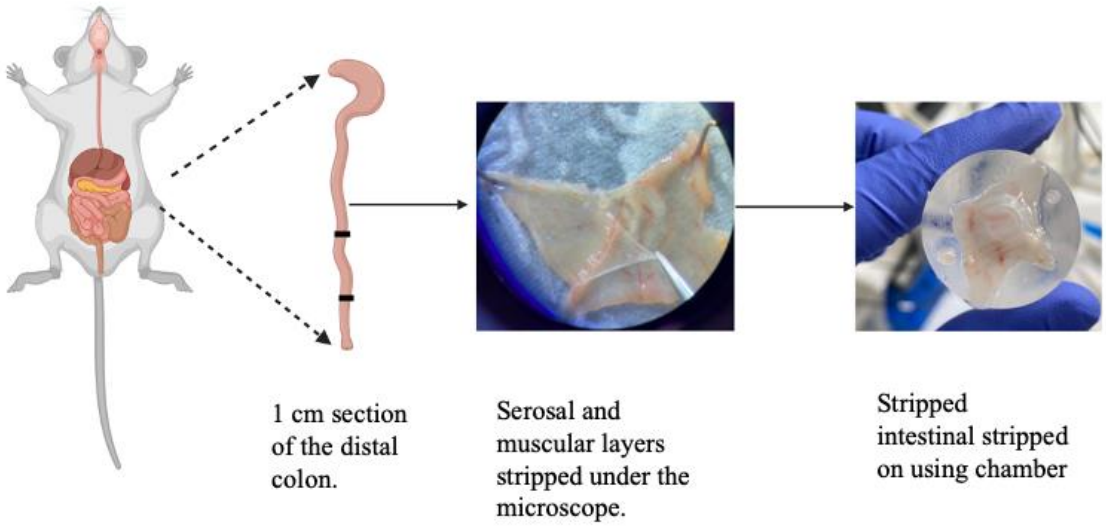


Figure 12. Tissue preparation for the Ussing Chamber set up.

Sections from the distal colon were cut and opened longitudinally. Seromuscular layers were stripped before the intestinal segment was mounted on the Ussing chamber.

2.5.3 Electrophysiological measurements

Intestinal epithelia tissue is polarized due to differential expression ion channels, pumps and transporters in the apical and basolateral membrane. Ion transport across the epithelium produces a potential difference (PD) across the epithelium. Using the Ussing system, the PD across the tissue is measured by two voltage electrodes placed near the tissue. Two current electrodes placed away from the tissue were used to inject a current that cancels out the voltage. The amount of current injected (ISc) is a measure of the net ion transport taking across the epithelium. The electrical resistance of the tissue in each chamber was determined by injecting 20 mV and recording the resulting change in IS_C. Ohm's law (V=IR) was used to determine the electrical resistance. The transepithelial electrical resistance (TEER) which is measurement of net flux of ions across the epithelium and demonstrates the integrity of the intestinal membrane was determined from the experiments. TEER values were determined by multiplying the area of the chamber covered by intestinal tissue (0.2 cm^2) with the resistance values found in the experiment.

$$TEER_{REPORTED} = R_{TISSUE}(\Omega) \times M_{AREA}\,(cm^2)$$

2.6 Drugs

The drugs were reconstituted in DMSO unless stated otherwise. Stock concentrations of atropine (100 mM, absolute ethanol), nifedipine (100 mM), Ang II (10 mM, H_2O), EMD6684 (10 mM), losartan (10 mM), valsartan (10 mM), PD123319 (10 mM), Bradykinin (1 mM), ATP (60 mM), SKA-31 (100 mM), NS1619 (100 mM), Retigabine (100 mM), TRAP-6 (1 mM) were prepared and stored at -20°C. MMP1 (10 µM, H_2O) was reconstituted and stored at -80°C. All compounds were diluted from frozen aliquots in Krebs buffer on the day of the experiment.

3 Calcium activated potassium channel modulation on colonic afferent activity

3.1 Potassium channels

Potassium channels are the largest and most diverse class of ion channels in the human genome, with 78 different genes encoding the principal pore-forming units (Ocana et al., 2004). Potassium channels control the transportation of potassium ions (K^+), which are more highly concentrated on the cytoplasmic than on the extracellular side of the plasma membrane, into and outside of cells. Potassium channels play crucial roles in both excitable and non-excitable cells regulating fundamental physiological processes that include cellular volume, hormone secretion, calcium signalling, membrane potential, and action potential firing (Brown et al., 2020). Based on structure and function, potassium channels are categorized into four main classes: (i) the voltage gated (K_V) potassium channels containing six transmembrane regions (S1-S6) with a single pore, (ii) the inwardly rectifying (K_{ir}) potassium channel containing two transmembrane regions and a single pore, (iii) the two-pore domain (K_{2P}) potassium channels containing four transmembrane with two pore regions and (iv) and the Ca^{2+} activated potassium (K_{Ca}) channels which are composed of six transmembrane domains (Ocana et al., 2004).

K_V channels are the largest gene family of potassium channels with 40 genes encoding the voltage-gated potassium channel pore forming subunits which are classified into 12 subfamilies (K_V1-K_V12) (Gutman et al., 2005). There are seven K_{ir} subfamilies that are grouped into four functional groups: classical K_{ir} channels that are constitutively open ($K_{ir}2.x$), G-protein gated K_{ir} channels ($K_{ir}3.x$), ATP sensitive K^+ channels ($K_{ir}6.x$) and K^+ transport channels ($K_{ir}1.x$, $K_{ir}4.x$, $K_{ir}5.x$, and $K_{ir}7.x$) (Hibino et al., 2010). The K_{2P} channel family is composed of 15 members which are grouped into seven subfamilies: TWIK, weak inward rectifying K^+ channel; TREK, TWIK related K^+ channels; TRESK, TWIK related spinal cord potassium channel; TASK, two-pore domain acid sensitive K^+ channels; TRAAK, two-pore domain related arachidonic acid activated K^+ channel; TALK, two pore domain alkaline activated K^+ channel and THIK, two-pore domain halothane inhibited K^+ channel (Feliciangeli et al., 2015). Calcium activated potassium (K_{Ca}) channels are classified into 3 subgroups: large, intermediate and small conductance calcium activated K^+ channels on the basis of channel conductance (Vergara et al., 1998).

Potassium channels are important in the regulation of resting membrane potential and action potential repolarization in neurons and other excitable cells (Tsantoulas & McMahon, 2014). In neuronal membranes, opening of K^+ channels facilitates a rapid K^+ efflux across the

membrane, thereby resulting in repolarization and hyperpolarization of the membrane. Therefore, the activity of K^+ channels can influence action potential threshold, waveform, and frequency making them a viable target for altering neuronal output (Tsantoulas & McMahon, 2014). The majority of K_V channels open when the membrane is depolarised and close when the membrane is hyperpolarized (Kuang et al., 2015). K_{ir} channels are unique in that they are blocked by Mg^{2+} ions and polyamines when the membrane is depolarised and open at hyperpolarisation when the blockers are released to conduct K^+ ions into the cell. Hence, they show little outward current during depolarization (Hibino et al., 2010). K_{2P} channels are usually constitutively open and act as the background 'leak' potassium channels that stabilize the negative resting membrane potential and counterbalance depolarization (Enyedi & Czirjak, 2010). The resting membrane potential is mainly stabilized by K_{2P} channels and K_V7 background conductance whereas the low threshold K_V1 and K_V4 channels filter out small depolarizations to limit the number of action potentials triggered. The high-threshold K_V3 channels open following the initiation of an action potential and limit action potential duration. K_{Ca} channels are opened during neuronal firing and play a role in repolarization and hyperpolarization of the membrane (Tsantoulas & McMahon, 2014). Figure 13 below summarizes the role of K^+ channels during action potential firing.

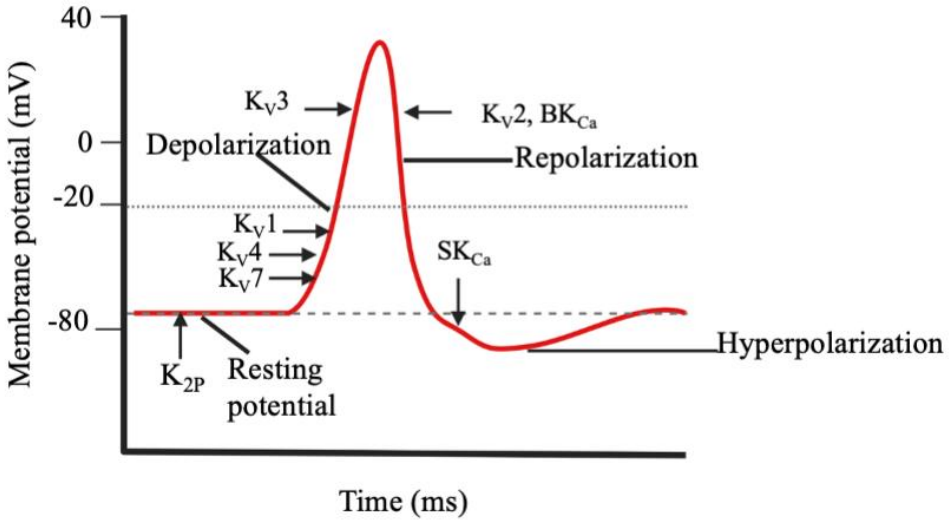


Figure 13. Potassium channel activation during action potential firing.

K_{2P} and K_V7 stabilize the resting membrane potential whereas low threshold K_V1 and K_V4 channels filter out small depolarizations to limit the number of action potentials triggered. K_V3 channels open following action potential initiation to limit action potential duration. K_V2, BK_{Ca} and SK_{Ca} channels contribute to the repolarization and after hyperpolarization (Tsantoulas & McMahon, 2014).

Altered function of K^+ channels is implicated in genetic and acquired diseases involving the changes in the functioning of neurons, smooth muscle cells and cardiac cells. Thereby, K^+ channel modulators are common medicines in various cardiac and neuronal conditions characterized by neuronal hyperexcitability. (Shieh et al., 2000). For example, the loss of function of several types of K^+ channels is implicated in epilepsy and K^+ channel openers including retigabine, a $K_V7.2$-7.5 channel opener are commonly used to treat symptoms of epilepsy (Gunthorpe et al., 2012). In cardiac tissue, various K^+ channels play key roles in regulating the heart rate by setting the resting membrane potential, amplitude and duration. Likewise, K^+ channels are commonly targeted to treat heart conditions. For example, ATP sensitive potassium channels (K_{ATP}), which are activated by Mg-adenosine diphosphate (ADP) and inactivated by increase in intracellular ATP, have been successfully targeted to treat hypertension (Pan et al., 2010).

Excitation of peripheral nerves is normally the first step in the generation of pain signals. Therefore, enhancement of K^+ channel activity to reduce cellular excitability of neuronal membranes provides a therapeutic strategy for treating pain. Opening of K^+ channels has been demonstrated to produce the antinociceptive effects of several analgesic drugs including agonists of many GPCRs such as opioid, $GABA_B$, muscarinic M_2, adenosine A and cannabinoid receptors (Ocana et al., 2004). Morphine, for example, is a commonly used analgesic that mediates its antinociceptive effects via an inhibitory G-protein, which inhibits cAMP formation and Ca^{2+} conductance and activates K^+ conductance via ATP sensitive K^+ channels to induce hyperpolarization in nociceptive cells (Ahmadi et al., 2014; Rodrigues & Duarte, 2000). Suppression or loss of function of various K^+ channels has been demonstrated to contribute to pain hypersensitivity in animal models and some pathological conditions. For example, mutations in the K_{2P} channel subtype, TRESK induces hyperexcitability in trigeminal neurons and is directly linked to migraines (Lafreniere et al., 2010).

Several studies have proposed a role for K_V7 channels in nociceptive pathways. K_V7 channels activate at subthreshold potentials and generate a steady outward voltage dependant current that contributes to the resting membrane potential (Brown & Passmore, 2009). Downregulation of K_V7 channels contributes to peripheral hypersensitivity of nociceptors and development of neuropathic pain (Abd-Elsayed et al., 2019). In contrast, K_V7 activators such as retigabine, reduce neuronal hyperexcitability and excitability and have shown promising results in treating various neuropathic conditions in animal studies (Djouhri et al., 2019; Dost et al., 2004).

Consequently, clinical trials have been conducted to determine the efficacy of retigabine to treat pain associated with post-herpetic neuralgia and disappointingly, the study failed to meet its primary efficacy endpoint (Passmore & Delmas, 2011).

Another study showed that K_V7 channels contribute to the sensitivity of visceral sensory fibres to noxious chemical and mechanical stimuli. Retigabine produced a marked inhibitory effect on the colonic afferent response to luminal distension and bradykinin in these studies suggesting K_V7 openers represent a viable therapeutic target for treating pain in GI diseases (Peiris et al., 2017). Building on this work, the current study aimed to evaluate the effects of K_{Ca} channel modulation on colonic afferents and to evaluate their potential role in altering the colonic afferent output.

3.2 Calcium-activated potassium (K_{Ca}) channels

Calcium activated potassium (K_{Ca}) channels play a key role in controlling cellular excitability by coupling the increase in intracellular Ca^{2+} concentration to hyperpolarization of the membrane potential (Kshatri et al., 2018). K_{Ca} channels are classified into three subfamilies based on their single channel conductance: big conductance (BK_{Ca} 200-300 pS), intermediate conductance (IK_{Ca} 32-39 pS), and small conductance (SK_{Ca}, 4-14 pS) (Kshatri et al., 2018). SK_{Ca} and IK_{Ca} channels are activated by Ca^{2+} concentrations between 300-700 nM whereas BK_{Ca} channels are activated by membrane depolarization (+20 mV) and elevation of intracellular Ca^{2+} concentration above 300 nM (Gueguinou et al., 2014). BK_{Ca} channels are ubiquitously expressed whereas SK_{Ca} channels are predominantly expressed in the nervous system and IK_{Ca} channels are distributed in the blood, epithelial cells, and some peripheral neurons (Kshatri et al., 2018).

In neurons, activation of K_{Ca} channels results in the efflux of K^+ ions that underlies the afterhyperpolarization (AHP) that follows bursts of action potentials. The AHP (a more negative resting potential compared to the resting one) lasts a couple of seconds and has 3 components: the fast afterhyperpolarization (fAHP), the medium AHP (mAHP), and the slow AHP (sAHP). The fAHP that typically lasts (1-10 s) immediately follows an action potential and is mediated by the activity of BK_{Ca} channels. The mAHP that is mediated by SK_{Ca} channels is activated rapidly following the action potential and decays over a time course of several hundred milliseconds (Sah & Faber, 2002). The AHP influences the excitability of neurons by

regulating the voltage trajectory between action potentials thus setting the frequency for action potential firing (Adelman et al., 2012).

3.3 BK_{Ca} channels

The BK_{Ca} channel is the most studied of the K_{Ca} channels and has many important physiological roles including regulation of neurotransmitter release, neuronal excitability, and smooth muscle tone (Bentzen et al., 2014). In neurons, membrane depolarization during an action potential, and Ca^{2+} entry through voltage-gated calcium (Ca_V) channels activates BK_{Ca} channels. Consequently, BK_{Ca} channels activate rapidly during the upstroke of the action potential and close rapidly following the return of the membrane to negative values (Sah & Davies, 2000). Activation of BK_{Ca} channels serves as negative feedback for Ca_V as BK_{Ca} channels help terminate the action potential and produce the fast afterhyperpolarization that results in the closing of Ca_V channels (Lee & Cui, 2010).

BK_{Ca} channels (previously known as Slo 1, $K_{Ca}1.1$, large conductance, and Maxi K_{Ca} channels) are formed by 4 pore-forming α subunits that contain 7 transmembrane spanning domains (S0-S6) (Figure 14). The α subunits are encoded by a single gene: *KCNMA1*.

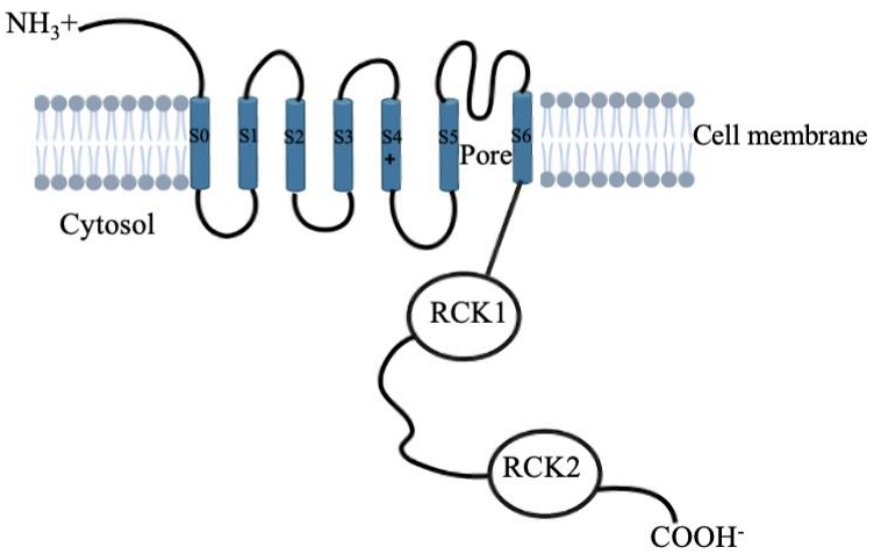


Figure 14. Schematic representation of the transmembrane topology of BK_{Ca} channel subunits.

Each subunit contains three main domains: voltage sensor domain (S0-S4), pore region formed by S5 and S6 segments and a C -terminal cytosolic region which functions as a Ca^{2+} sensor. The Ca^{2+} sensor domain is constituted by two domains (RCK1 and RCK2).

BK_{Ca} channels are sensitive to Ca^{2+} and changes in transmembrane voltage (Kaczmarek et al., 2017). The Ca^{2+} dependence of BK_{Ca} channels depends on the membrane potential. At resting membrane potentials (~-60 mV), the dissociation constant (Kd) for calcium is in the micromolar range, but in the nanomolar range at depolarized potentials (+20 to +40 mV) (Faber & Sah, 2003). The binding sites for Ca^{2+} ions are found in two regions of the extended cytoplasmic C-terminal termed the RCK1 and RCK2 (regulator of conductance for K^+ ions) domains. Eight RCK domains in each tetrameric BK_{Ca} channel together form a gating ring which opens upon the binding of Ca^{2+} (Gueguinou et al., 2014; Kaczmarek et al., 2017). BK_{Ca} channels can also open in the absence of Ca^{2+}, however, binding of Ca^{2+} enhances the probability of channel opening. The sensitivity of BK_{Ca} channels to membrane voltage is conferred by charged residues in the S2, S3, and S4 domains which move in response to changes in transmembrane voltage (Cui et al., 2009; Kaczmarek et al., 2017).

The Ca^{2+} sensitivity, pharmacological properties, and kinetic behaviour of BK_{Ca} channels varies in different tissues. Although there is a single gene that encodes the BK_{Ca} channels subunits, the diversity of the BK_{Ca} channels is achieved through alternative splicing of mRNAs and the association with auxiliary β (β1, β2, β3, and β4) and γ subunits. The binding of the BK_{Ca} channel to β and γ modifies the channel properties and pharmacology. For example, an association of the BK_{Ca} channel with one of four β subunits alters the voltage dependence,

activation rate, sensitivity to a wide range of drugs and determines whether the channel inactivates during sustained depolarization (Kaczmarek et al., 2017).

3.3.1 BK_{Ca} channel expression in neurons

Immunohistochemical studies have shown BK_{Ca} channel expression in DRG neurons. For example, one study showed that BK_{Ca} channels are preferentially expressed in small and medium-sized DRG neurons in mice. Additionally, double labeling immunostaining studies showed that 30.5% of BK_{Ca} channel immunoreactive cells expressed CGRP, a marker for peptidergic nociceptors, while 40.6% of BK_{Ca} channel immunoreactive cells bound the lectin IB4, a marker for nonpeptidergic neurons. This study also found that 92.8% of BK_{Ca} channel immunoreactive cells were positive for $Na_V1.8$, which is exclusively expressed in nociceptors. Together, this data suggests that BK_{Ca} channels are expressed in peripheral nociceptive neurons (Lu et al., 2014).

3.3.2 BK_{Ca} pharmacology

BK_{Ca} channels are blocked by low concentrations of tetraethylammonium (0.14 mM), the scorpion toxins charybdotoxin (2.9 nM) and iberiotoxin (1.7 nM), and mycotoxins paxilline and penitrem A, while they are activated by NS 1619 and NS 1608 (Sah & Faber, 2002; Smith, 2020). Blocking BK_{Ca} channels leads to depolarization and a subsequent increase in neuronal excitability whereas activation of BK_{Ca} channels suppresses action potential firing.

3.4 SK_{Ca} and IK_{Ca} channels

SK_{Ca} and IK_{Ca} channels exist as tetramers of subunits that consist of six transmembrane helices (S1-S6) and cytosolic N and C termini (Adelman et al., 2012). SK_{Ca} (SK1, SK2, and SK3) subunits are encoded by the genes *KCNN1*, *KCNN2*, and *KCNN3* respectively, whereas the IK_{Ca} subunits are encoded by the gene *KCNN4* (Christophersen & Wulff, 2015). The selectivity filter for K^+ ions and the pore of the channel are formed by the loop between the fifth and sixth (S5 and S6) transmembrane domains (Figure 15) (Gueguinou et al., 2014)

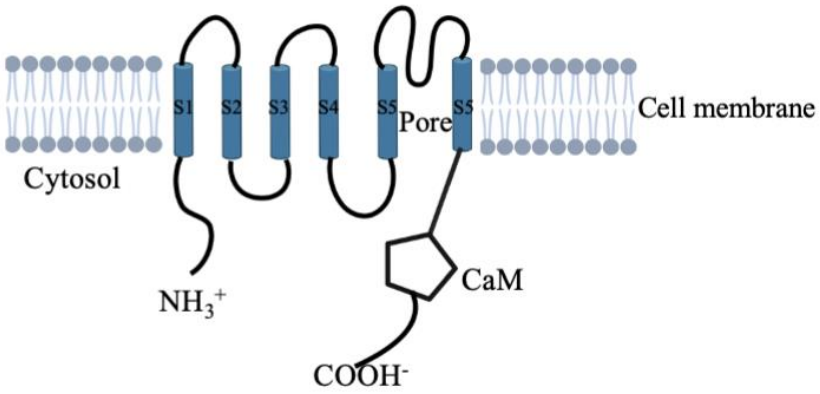


Figure 15. Schematic representation of the transmembrane topology of SK_{Ca}/IK_{Ca} channel subunits.

The pore formed between S5 and S6 segments is responsible for K^+ conductance. Calmodulin (CaM) that is constitutively associated with the calmodulin-binding domain (CaMBD) serves as the Ca^{2+} sensor.

SK_{Ca} and IK_{Ca} channels are activated through a mechanism that depends exclusively on calmodulin (CaM) which is tightly bound to the calmodulin-binding domain (CaMBD) in the C terminus of the channel (Christophersen & Wulff, 2015). CaM binds to the intracellular domain following the sixth transmembrane domain, with each subunit of the tetrameric channel binding one CaM. The binding of Ca^{2+} to the N lobes of CaM results in the structural rearrangement of CaM-CaMBD monomers pulling the bundle-crossed helices of the pore to open the channel (Kshatri et al., 2018). The reported EC_{50} values for Ca^{2+} range from 100 to 400 nM for IK_{Ca} channels and from 300 to 750 nM for SK_{Ca} channels. These K_{Ca} channels open at relatively negative membrane potentials when intracellular Ca^{2+} is raised in their immediate vicinity to hyperpolarize the membrane (Brown et al., 2020).

3.4.1 SK_{Ca} channel function

SK_{Ca} (SK1, SK2 and SK3) channels also termed $K_{Ca}2$ ($K_{Ca}2.1$, $K_{Ca}2.2$ and $K_{Ca}2.3$) regulate neuronal firing frequency and spike frequency generation by generating a Ca^{2+} dependent medium after afterhyperpolarization that typically lasts several hundreds of milliseconds (Adelman et al., 2012). The AHP limits the firing frequency of repetitive action potentials and protects the cell from deleterious effects of continuous activity (Kohler et al., 1996). Because of the role of SK_{Ca} channels on regulating neuronal excitability, SK_{Ca} activators have been

considered for the treatment of conditions that are characterized by hyperexcitability such as epilepsy and ataxia (Kaczmarek et al., 2017).

SK_{Ca} channels are activated by elevations in cytosolic Ca^{2+} from various sources: Ca^{2+} influx via voltage-gated calcium channels (VGCC) activated during an action potential, Ca^{2+} influx via Ca^{2+} permeable agonist gated ion channels such as NMDARs and nicotinic acetylcholine receptors (nAChRs), and Ca^{2+} release from intracellular Ca^{2+} stores by the generation of IP_3 via G-protein coupled receptors (Adelman et al., 2012). The activation of SK_{Ca} channels in neurons is largely due to the activation of VGCC channels that are activated during the upstroke of action potentials (Adelman et al., 2012).

3.4.2 SK_{Ca} channel expression in the nervous system/sensory neurons

SK_{Ca} channels are widely expressed in the nervous system. In the CNS, the activity of SK_{Ca} channels has rapid effects on intrinsic excitability and synaptic transmission as well as on long term changes that affect learning and memory formation (Adelman et al., 2012). In the peripheral nervous system, SK_{Ca} channels have been shown to be expressed in DRG neurons where they have been shown to play a role in nociception (Bahia et al., 2005; Brown et al., 2020).

3.4.3 SK_{Ca} pharmacology

SK_{Ca} channels are selectively blocked by the neurotoxin apamin, which is a component of bee venom, and are activated by 1-ethyl-2-benzimidazolinone (EBIO) by altering their Ca^{2+} sensitivity and opening probability (Sah & Faber, 2002). Most *KCNN* activators are positive gating modulators that act by shifting the Ca^{2+} activation curve towards a lower concentration of Ca^{2+} in a concentration-dependent manner thereby increasing Ca^{2+} affinity (Brown et al., 2020).

3.4.4 IK_{Ca} channel function

IK_{Ca} channels (also known as K_{Ca} 3.1) are structurally and functionally similar to SK_{Ca} channels but have a greater unitary conductance compared to SK_{Ca} channels. Like the SK_{Ca} channel, IK_{Ca} channels exist as tetramers of subunits that consist of six transmembrane helices (S1-S6; the pore region is formed by helices S5 and S6) and cytosolic N and C termini. Their sensitivity

to Ca^{2+} is determined by its association with CaM. The binding of Ca^{2+} to CaM results in conformational changes that are responsible for channel gating (Kshatri et al., 2018). IK_{Ca} channels are widely expressed throughout the body, present in various tissues including blood cells, epithelial cells, smooth muscle keratinocytes, and cells of the immune system such as T cells, B cells, mast cells and macrophages (Brown et al., 2020).

3.4.5 IK_{Ca} channels in the nervous system

IK_{Ca} channels were historically thought to be restricted to non-neuronal cells; however, several studies have demonstrated their expression and function in the nervous system. For example, IK_{Ca} channels have been shown to be expressed in enteric neurons, where they mediate the slow afterhyperpolarisations (Furness, 2006; Vogalis et al., 2002). Another study showed that IK_{Ca} channels are present in DRG neurons where they are preferentially expressed in small diameter neurons (Mongan et al., 2005). Recently, another study demonstrated the expression and function of IK_{Ca} channels in CA1 pyramidal cells where they mediate the slow afterhyperpolarization (King et al., 2015). Although expression of IK_{Ca} channels has been demonstrated in neurons, their roles remain unclear as some studies have demonstrated that they mediate the slow afterhyperpolarization whereas other studies illustrate otherwise (Kaczmarek et al., 2017).

3.4.6 IK_{Ca} channels in the immune system and IBD

IK_{Ca} channels play key roles in the immune system. Activation of IK_{Ca} channels in immune cells hyperpolarizes the membrane and facilitates Ca^{2+} entry via Ca^{2+} release-activated Ca^{2+} (CRAC) channels which is necessary for activation, proliferation and cytokine production (Feske et al., 2015). Consequentially, blockade of IK_{Ca} channels is beneficial in animal models of several autoimmune and inflammatory conditions including IBD. For example, inhibition of T-cell IK_{Ca} channels either through genetic deletion or by treatment with the IK_{Ca} blocker, TRAM-34 ameliorates T-cell mediated colitis (Di et al., 2010). On the other hand, reduced expression of IK_{Ca} channels contributes to the development of IBD symptoms. For example, reduced expression of basolateral IK_{Ca} channels is implicated in the pathogenesis of diarrhoea in UC (Al-Hazza et al., 2012). Secondly, reduced expression of IK_{Ca} channels in intrinsic primary neurons (IPANs) in the enteric nervous system of the colons of IBD patients results in increased contractility (Arnold et al., 2003). Lastly, orally ingested *Lactobacillus reuteri* increased the excitability of myenteric neurons though decreased afterhyperpolarisation caused

by a reduction in IK_{Ca} channels. These results were mimicked by the IK_{Ca} channel blockers TRAM-34 and clotrimazole (Kunze et al., 2009).

3.4.7 IK_{Ca} pharmacology

IK_{Ca} channels can be blocked by the peptide toxins charydtoxin and maurotoxin. Additionally, small synthetic molecules TRAM-34 and clotrimazole have been developed and are widely used as IK_{Ca} blockers. IK_{Ca} channels are activated by the benzimidazolone 1-EBIO, the oxime NS309, and the benzothiazole SKA 31 (Sforna et al., 2018).

3.5 The role of K_{Ca} channels on regulating neuronal excitability

Several studies have demonstrated that targeting K_{Ca} channels in neurons is an effective means of altering neuronal activity. Activating BK_{Ca} channels with NS 1619 increased the firing threshold and AHP and reduced the amplitude of action potentials thereby collectively resulting in suppression of action potential firing of small and medium-sized DRG neurons. In contrast, blocking the BK_{Ca} channels with iberiotoxin prolonged action potential duration and increased firing frequency (X. F. Zhang et al., 2003). Similarly, apamin the selective SK_{Ca} channel blocker, has been demonstrated to increase the intrinsic excitability and firing frequency in neurons, whereas SK_{Ca} activators enhance the magnitude of the medium AHP and slow down-firing rates (Adelman et al., 2012). Thereby, K_{Ca} channels serve as important targets for pharmacological manipulation in neurological disorders that involve abnormal neuronal firing patterns such as epilepsy, ataxia and alcohol use disorders (Kshatri et al., 2018).

3.6 K_{Ca} channels on pain signalling

Given the role of K_{Ca} channels in modulating neuronal excitability, their potential role in pain signalling has been evaluated in several studies. K_{Ca} channel expression has been demonstrated in DRG neurons where they have been implicated in pain signalling. For example, BK_{Ca} channels have been shown to be preferentially expressed in small and medium diameter DRG neurons in mice and have been shown to couple with TRPV1 (Wu et al., 2013). TRPV1 is a cation permeable ion channel with selectivity for divalent over monovalent cations ($Ca^{2+}>Na^{+}$ ions) and plays an essential role in pain signal generation and regulation. TRPV1 is activated by heat (>43 °C), protons (pH<5.5), and capsaicin to induce noxious pain (Wu et al., 2013).

Therefore, the coupling of TRPV1 and BK_{Ca} channels in neurons indicates BK_{Ca} channels may play a role in pain signaling.

Activating BK_{Ca} channels using NS 1619 ameliorated inflammatory pain behaviour in the formalin test and the zymosan model, confirming the functional contribution of BK_{Ca} channels at the level of primary nociceptive processing (Lu et al., 2014). Another study utilizing a model of neuropathic pain demonstrated that K_{Ca} currents decrease in axotomized medium sized DRG neurons which coincided with increased excitability of neurons demonstrated by increased action potential duration and repetitive firing during depolarization (Sarantopoulos et al., 2007). Lastly, although IK_{Ca} channels are poorly expressed in the nervous system, mice lacking IK_{Ca} demonstrated increased behavioural responses to noxious chemical stimuli such as formalin and capsaicin. The formalin induced nociceptive behaviour was also observed in wild type mice that were treated with the IK_{Ca} inhibitor, TRAM-34 demonstrating an inhibitory role of IK_{Ca} in the processing of noxious chemical stimuli (Lu et al., 2017).

3.7 K_{Ca} channel expression in gut innervating sensory neurons

Single-cell RNA sequencing studies conducted in our group all showed expression of *Kcnn1* and *Kcnma1* genes across all 7 neuronal populations in mouse DRG thoracolumbar and lumbosacral neurons but the highest expression in the mNP population which is associated with nociceptors. Additionally, Kcnma1 is highly expressed in the mPEPb subtype which is also associated with nociceptors. *Kcnn2* was highly expressed in the pNF population whereas *Kcnn3* was poorly expressed in all populations. *Kcnn4* was also poorly expressed in all populations except mNP (Figure 16) (Hockley et al., 2018).

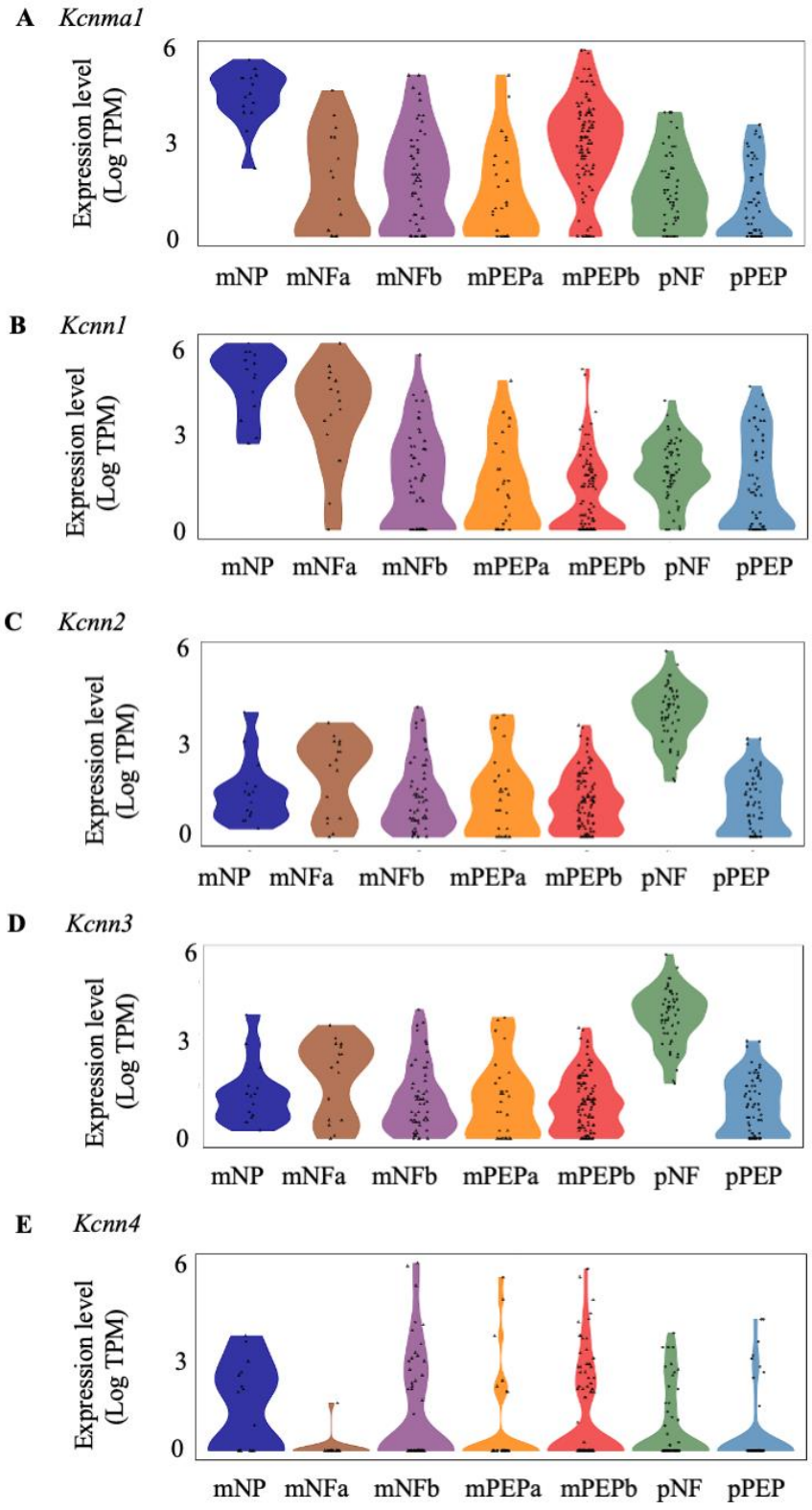


Figure 16. K_{Ca} channels expression in colon projecting DRG neurons.

Violin plots showing the expression of (A) Kcnma1, (B) Kcnn1, (C) Kcnn2, (D) Kcnn3, (E) Kcnn4 in the 7 neuronal populations identified in mouse thoracolumbar and lumbosacral neurons. Each black dot represents data from a single colonic sensory neuron isolated from either thoracolumbar (triangle) or lumbosacral (circle). The expression profile of each gene is

demonstrated in each of the subtypes distinguished by color (mNeuroFilament-a (mNFa), mNeuroFilament-b (mNFb), mNonPeptidergic (mNP), mPeptidergic-a (mPEPa), mPeptidergic-b (mPEPb), pNeuroFilament (pNF) and pPeptidergic (pPEP)). DRG. Expression values are expressed in Transcript-Per-Million (Log [TPM] (Hockley et al., 2018).

3.8 Choice of algogenic stimuli

3.8.1 ATP

ATP is an important extracellular signalling molecule that plays a crucial role in nociception and chronic pain. In the periphery, ATP can be released because of tissue injury, visceral distension or sympathetic activation and can excite nociceptive primary afferents (Chizh & Illes, 2001). The actions of ATP on neuronal signalling are complex and ATP can act via the activation of two classes of surface receptors: P2X (a family of ligand-gated ion channels) and P2Y (a family of G protein-coupled receptors) (Fredholm et al., 1994). Seven P2X (P2X1 to P2X7) and eight P2Y that are either grouped into G_q or $G_{i/o}$ coupled signalling have been cloned (Erb et al., 2006). P2X receptors are nonselective cation channels that mediate Na^+ influx, K^+ efflux and Ca^{2+} influx leading to depolarisation of the membrane. Depolarisation of the membrane results in the activation of VGCC thus causing accumulation of Ca^{2+} in the cytoplasm (Erb et al., 2006). P2X receptors are fast-acting ion channels and are responsible for the immediate response to ATP, whereas P2Y receptors act slower and are activated in addition to ATP by metabolites of ATP hydrolysis such as adenosine diphosphate (ADP) (Khakh, 2001). Therefore, in addition to activation of P2X receptors, administration of ATP in colonic afferents results in activation of P2Y and P1 receptors by ADP and adenosine, respectively. ATP, which is rapidly hydrolysed by cell surface enzymes to ADP and adenosine, is responsible for the immediate activation of colonic afferents whereas ADP and adenosine are thought to be responsible for the sustained response to ATP (Hockley et al., 2016).

The pathological role of extracellular ATP in the regulation of intestinal inflammation has been demonstrated. Levels of ATP are increased in experimental colitis compared with controls and blockade of ATP release in tissue ameliorates DSS-induced colitis (Wan et al., 2016). Additionally, administration of ATP exacerbates experimental colitis (Atarashi et al., 2008). Similarly, the role of P2X receptors in pain pathways has been well documented. Particular attention has been paid to the P2X3 receptor which is selectively expressed in a subset of predominantly nociceptive neurons (Chen et al., 1995; Ding et al., 2000). P2X3 homomers and P2X2/3 heteromers are predominantly localized in small diameter unmyelinated C-fibres and are thought to exert their effect by directly sensitizing C-fibres by membrane depolarization

and Ca^{2+} entry to facilitate pain transmission (Bernier et al., 2018). Both P2X (P2X2 and P2X3) and P2Y (P2Y1, P2Y2, and P2Y4) receptors are expressed on gut-projecting sensory neurons and have been shown to contribute to the transduction of visceral pain signals in the bowel (Hockley et al., 2016). Additionally, P2X3 is upregulated in inflammatory bowel disease (Yiangou et al., 2001)

In silico analysis of K_{Ca} channel expression revealed that *Kcnn1* and *Kcnma1* were mainly expressed in the mNonPeptidergic neuronal population of sensory nerves that also express P2X3, the main purinergic receptor present at sensory nerve terminals. This population of neurons is important in pain signalling (Burnstock, 2013). A strong co-expression between P2X3 and the K_{Ca} channels (SK_{Ca}.21 and BK_{Ca}) indicated that ATP would be an appropriate stimulus to use in nerve recording studies (Figure 17). Therefore, given the role of ATP in IBD progression and pain signalling from the GI tract, this study evaluates the therapeutic potential of activating K_{Ca} channels on the inhibition of nerve discharge in response to ATP.

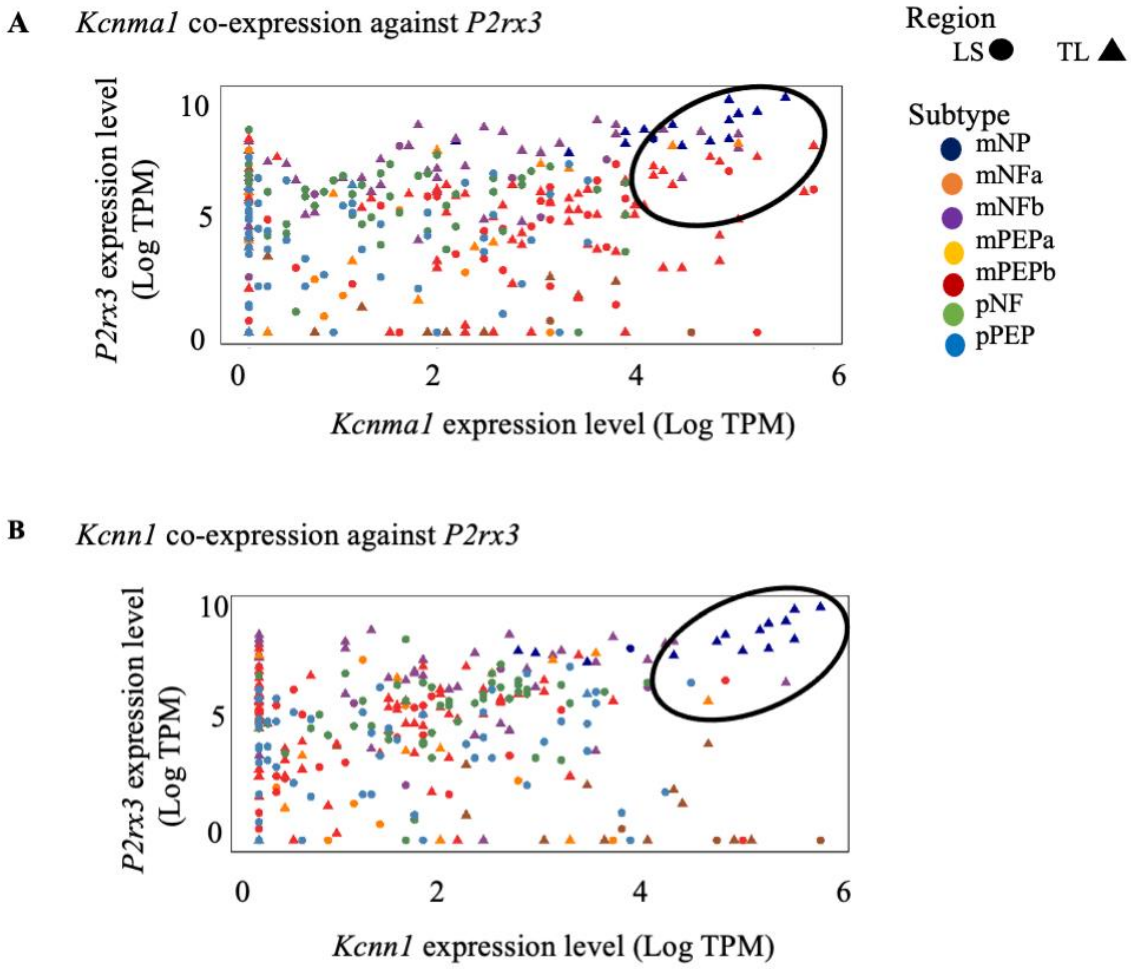


Figure 17. Co-expression of K_{Ca} channels (Kcnma1 and Kcnn1) with P2rx3.

(A) Kcnma1 against P2rx3 and (B) Kcnn1 against P2rx3 in mouse thoracolumbar and lumbosacral DRGs. Each dot represents data from a single colonic sensory neuron isolated from either thoracolumbar (triangle) or lumbosacral (circle). Expression values are expressed in Transcript-Per-Million (Log [TPM]. The subpopulation of neurons that highly co-express the KCa channel genes (Kcnma1 and Kcnn1) and P2rx3 is highlighted by the black oval.

3.8.2 Bradykinin

Bradykinin is an inflammatory algogenic (pain causing) mediator that binds to two G protein-coupled receptors B1 or B2 encoded by the genes *BDKRB1* and *BDKRB2*, respectively (Menke et al., 1994). Many of the effects of bradykinin including the acute activation of nociceptors and generation of pain are mediated by the B2 receptor which has a higher affinity for bradykinin compared to the B1 receptor. The B2 receptor is constitutively expressed whereas the B1 receptor is under-expressed in normal tissue and is increased in expression following tissue injury (Couture et al., 2001).

Bradykinin receptors have been localized to nociceptive pathways. B2 receptors are constitutively expressed on primary sensory nerve terminals and bradykinin can directly excite sensory neurons and sensitize other ion channels that are important in nociception such as TRPV1 and TRPA1 (Couture et al., 2001; Liu et al., 2010). Bradykinin interacts with its receptors to activate various signalling mechanisms through G_q and G_i proteins to activate phospholipase C (PLC) and phospholipase A (PLA). Activation of PLC results in the breakdown of PIP_2 into IP_3 and DAG. IP_3 induces Ca^{2+} release from the endoplasmic reticulum and DAG stimulates protein kinase C (PKC). Activation of PLA2 causes the release of arachidonic acid (AA) which is converted by cyclooxygenase (COX) and prostaglandin synthases to prostaglandin E2 (Wang et al., 2006). Bradykinin also inhibits the M current in nociceptors. M channels (Kv7 or KCNQ) are low threshold channels that are open at or near the resting membrane potential of neurons and play a key role in controlling resting membrane potential and threshold for action potential firing (Liu et al., 2010). Accordingly, M current inhibition depolarizes neurons and makes them more excitable whereas M current augmentation has the opposite effect. Additionally, bradykinin via the B2 receptor liberates Ca^{2+} from intracellular stores which can activate K_{Ca} channels resulting in a transient hyperpolarization before the depolarization phase of cell due to the inhibition of M channels (Higashida et al., 1986).

Evaluation of the B2 receptor gene *Bdkrb2* expression in the RNA sequencing database showed moderate co-expression of *Bdkrb2* with the BK_{Ca} and $SK_{Ca}2.1$ receptor genes *Kcnma1* and *Kcnn1*, respectively (Figure 18) (Hockley et al., 2018). Therefore, bradykinin was deemed as an appropriate stimulus for the present studies evaluating the therapeutic potential of K_{Ca} modulation on pain in GI pathologies.

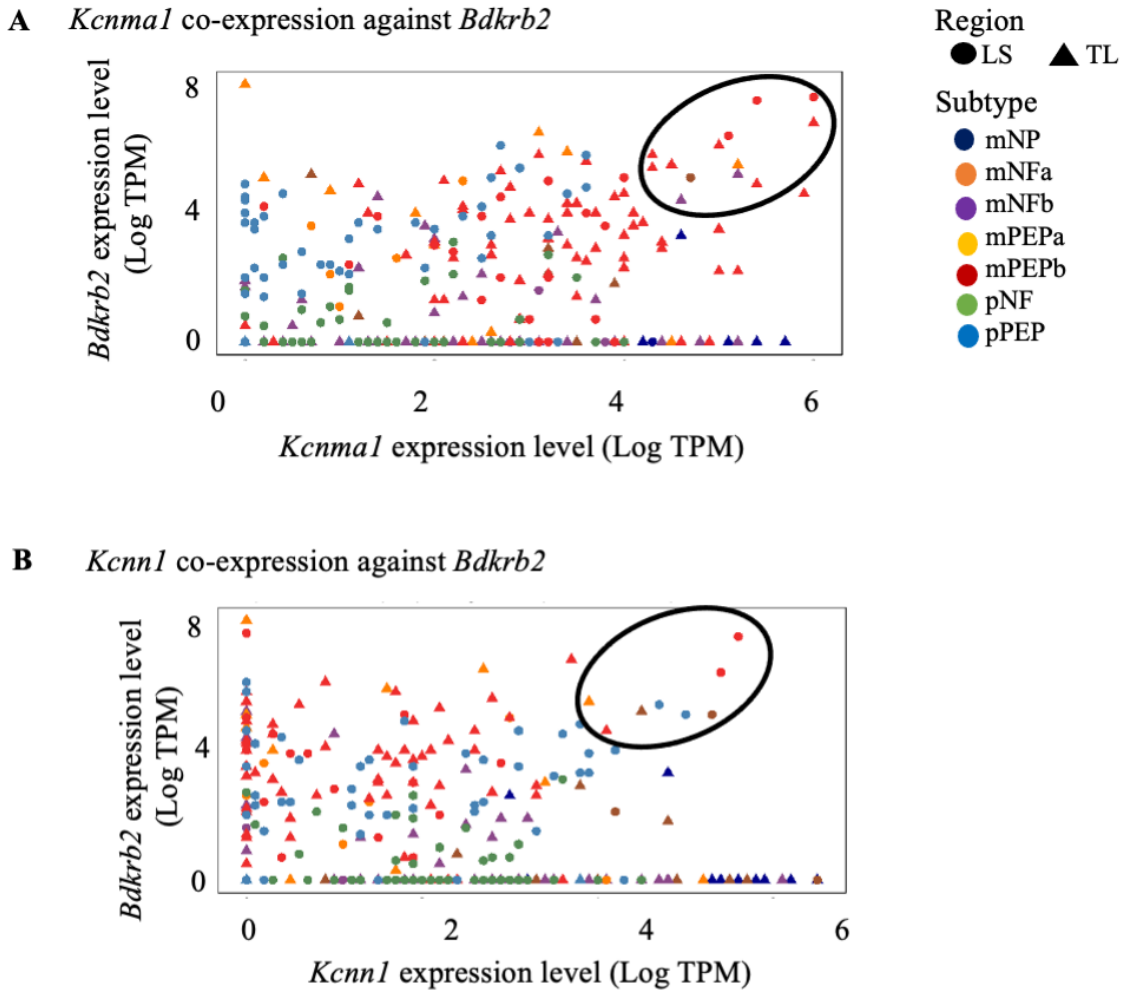


Figure 18. Co-expression of K_{Ca} channels (Kcnma1 and Kcnn1) with Bdkrb2.

(A) Kcnma1 against Bdkrb2 and (B) Kcnn1 against Bdkrb2 in mouse thoracolumbar and lumbosacral DRGs. Each dot represents data from a single colonic sensory neuron isolated from either thoracolumbar (triangle) or lumbosacral (circle). Expression values are expressed in Transcript-Per-Million (Log [TPM]). The subpopulation of neurons that highly co-express the KCa channel genes (Kcnma1 and Kcnn1) and Bdkrb2 is highlighted by the black oval.

3.9 GI tract motility

GI motility results from coordinated contractions of the muscular layers of the gut and is important in the mixing and movement of luminal contents along the GI tract. Small and large intestine motility is under multiple levels of control including the enteric nervous system (motor neurons, interneurons, and intrinsic sensory neurons), central nervous system, specialized cells named interstitial cells of Cajal (ICC), smooth muscle cells (SMCs), GI hormones, and paracrine agents (Sellers & Morton, 2014). Dysfunction of colonic motility results in altered bowel habits such as constipation and diarrhoea and intermittent abdominal cramping (Sarna, 2010). Changes in GI motility patterns have been demonstrated in IBD patients with both active and inactive inflammation and correlate with greater psychological morbidity and poorer quality of life (Bassotti et al., 2014).

Increase or decrease in intracellular Ca^{2+} plays an important role in the regulation of smooth muscle contraction. Activation of inward currents by excitatory agonists depolarize the membrane potential resulting in the activation of VGCC that result in Ca^{2+} influx to increase intracellular calcium and trigger contraction of smooth muscles (Somlyo & Somlyo, 1994). In contrast, the activation of outward currents or suppression of tonic inward currents stabilizes membrane potential or causes hyperpolarization resulting in reduced Ca^{2+} entry and a reduction of the open-state probability of VGCC thereby reducing contractions (Sanders, 2008; Somlyo & Somlyo, 1994). Therefore, inhibition of depolarizing currents or enhancements of repolarizing currents can be targeted for the relaxation of smooth muscle contraction (dela Pena et al., 2009). For example, activation of K^+ channels that hyperpolarize the membrane reduces contractility, whereas closure of these channels induces membrane depolarization and smooth muscle contraction (Curro, 2016). Thereby, compounds such as K^+ channel blockers and compounds increasing Ca^{2+} release or Ca^{2+} sensitivity are potentially effective therapeutic agents for GI dysmotility. Additionally, activation of receptors that result in an increase in Ca^{2+} by releasing Ca^{2+} from intracellular stores such as G_q proteins which increase intracellular Ca^{2+} through IP_3 also induce smooth muscle contraction (Bolton et al., 1999).

Under physiological conditions, the release of acetylcholine or substance P from the parasympathetic post-ganglionic nerve terminal innervating the smooth muscle inactivates K^+ channels causing depolarisation and then increasing Ca^{2+} ions influx into the cytoplasm. This Ca^{2+} release causes contraction of the circular muscle layer, followed by shortening of the longitudinal muscle (Radulovic et al., 2015). Calcium blockers can be used to block smooth

muscle contractility. In the present studies, nifedipine which inhibits L-type VGCC, and atropine, an antagonist of acetylcholine receptors were used to block smooth muscle contractility.

3.10 K_{Ca} channels on colonic contractility

K_{Ca} channels have been described in GI smooth muscle cells and ICCs where they contribute to repolarization and regulate cell excitability (Beyder & Farrugia, 2012). Activation of K_{Ca} channels has been shown to reduce contractility in GI tissue. For example, the BK_{Ca} opener LDD175 inhibited spontaneous contractions of the ileum, and this effect was attenuated by the BK_{Ca} channel blocker, iberiotoxin (dela Pena et al., 2009). In contrast, blocking the SK_{Ca} channel with apamin caused membrane depolarization and increased action potential firing thereby increasing contractions in colonic tissues (Maas & Denhertog, 1979). In the present study, we evaluated the effect of the K_{Ca} openers on colonic contractility to demonstrate activity of the openers in a system that known to be affected by K_{Ca} modulation.

3.11 K_{Ca} channel modulators

K_{Ca} channel subfamilies show distinct pharmacological properties. Significant work has been done to identify lead compounds that modulate K_{Ca} channels. The structure and EC_{50} of the K^+ channel openers used in the present study are shown in Table 2 below.

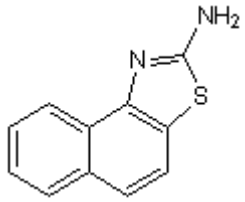
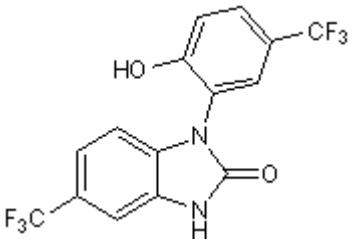
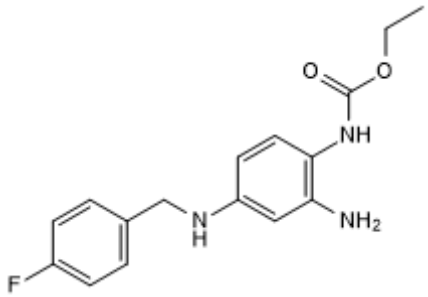
Drug	Target ion channel	Structure	EC_{50}
SKA 31	SK_{Ca} and IK_{Ca}		0.26 µM and 2.9 µM for IK_{Ca} and SK_{Ca}, respectively in murine endothelial cells (Sankaranarayanan et al., 2009).
NS 1619	BK_{Ca}		3.6 µM in human glioma LN229 cells (Debska et al., 2003).
Retigabine	K_V7		0.6 - 100 µM for $K_V7.1$ - $K_V7.5$ in CHO HEK293 cells (Blackburn-Munro et al., 2005).

Table 2. Potassium channel openers used in the present study.

3.12 Chapter aims

K_{Ca} channels are important regulators of neuronal excitability and are expressed sensory neurons innervating the colon. Activation of K^+ channels has been shown to have inhibitory effects on colonic nerve discharge as demonstrated by studies targeting KCNQ channels which showed that retigabine produces a marked inhibitory effect on colonic afferent response to luminal distension and bradykinin (Peiris et al., 2017). Therefore, the goal of this chapter is to evaluate the therapeutic potential of K_{Ca} channel activation in sensory neurons innervating the GI tract with retigabine serving as a positive control.

This chapter aims to:

1. Investigate the effects of calcium activated potassium (K_{Ca}) channel modulation on colonic afferent activity in response to chemical (ATP and bradykinin) and mechanical stimulus (slow ramp distension).
2. Demonstrate the effect of K_{Ca} channel modulation on colonic contractility.

3.13 Experimental protocols

3.13.1 ATP studies

Ex-vivo electrophysiological recordings were used to evaluate the functional effect of K_{Ca} channel modulation on lumbar splanchnic nerve (LSN) excitability, starting with the increase in LSN activity elicited by the algogenic mediator ATP. The studies with ATP were conducted using female CD1 mice.

Three applications of ATP were administered, each 30 minutes apart. The supplemented carbogenated Krebs was replaced with a 20 ml solution of 3 mM ATP prepared in supplemented Krebs buffer. The 2nd and 3rd applications were given following pre-treatment with either vehicle (0.1% DMSO) or drug treatment and in the continued presence of vehicle or drug treatment (Figure 19).

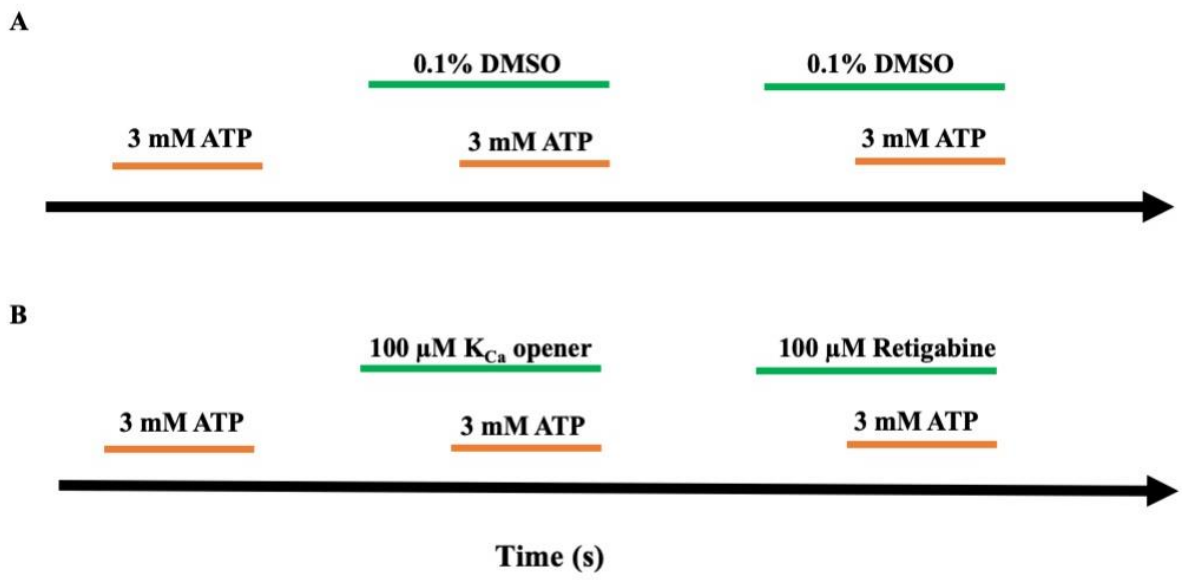


Figure 19. Schematic of the protocol used to evaluate the effect of K_{Ca} channel modulation on LSN activity in response to ATP application.

(A) Protocol used during control experiments to evaluate the effect of repeat application of ATP on nerve activity with DMSO treatments during the 2nd and 3rd ATP treatments. (B) The protocol used with the K_{Ca} openers, SKA 31 and NS 1619 given during the 2nd ATP application and retigabine, the K_V7 opener given during the 3rd ATP application.

3.13.2 Bradykinin studies

In control experiments, four applications of bradykinin were administered with vehicle treatment (0.1% DMSO) prior to and during the 3rd and 4th applications. To evaluate the effect of K_{Ca} channel modulation on the LSN response to bradykinin, SKA 31 was administered prior to and during the 3rd bradykinin application and retigabine given prior to and during the 4th application (Figure 20).

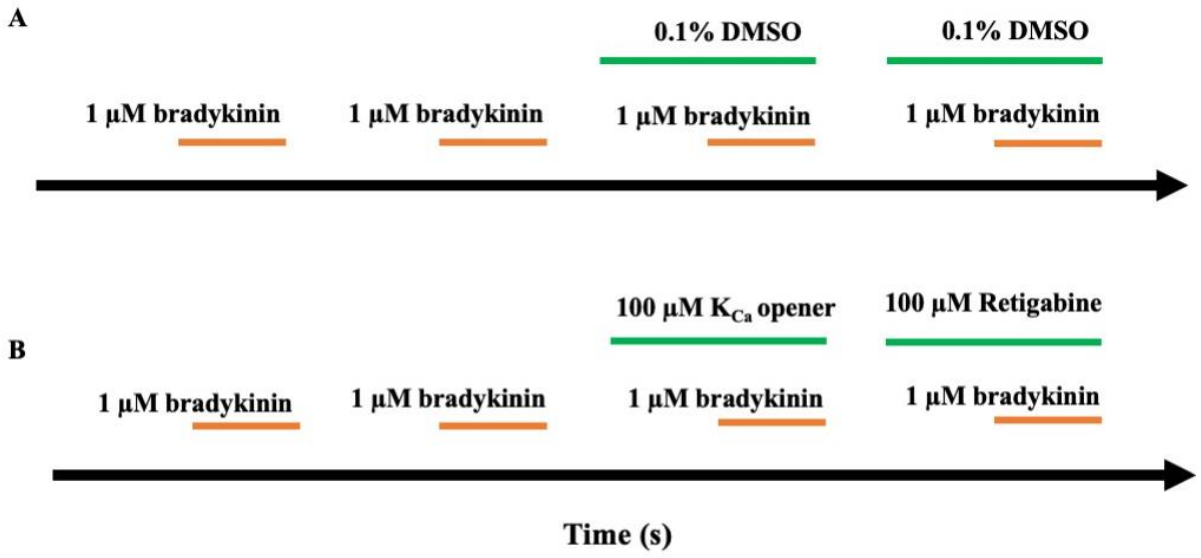


Figure 20. Schematic of the protocol used to evaluate the effect of K_{Ca} channel modulation on LSN activity in response to bradykinin application.

(A) Protocol used during control experiments to evaluate the effect of repeat application of bradykinin on nerve activity. (B) Protocol used with the K_{Ca} openers, SKA 31 given during the 3rd bradykinin application and retigabine, the K_V7 opener given during the 4th bradykinin application.

3.13.3 Ramp distension studies

Ramps were performed by blocking luminal flow of the cannulated colon to raise the pressure by 80 mmHg. 7 distensions, 15 minutes apart were performed in each experiment. In control experiments, the vehicle, 0.1% DMSO was given prior to and during distension 4 and 6. In experiments with the K^+ channel openers, the combined SK_{Ca}/IK_{Ca} opener, SKA 31 was given before and during distension 4 whereas the K_V7 opener, retigabine was given before and during distension 6. For pretreatment, supplemented Krebs buffer was replaced with a 50 ml solution

of the drug or vehicle (0.1% DMSO). Application of the drug or vehicle was continued in a 20 ml solution during the distension (Figure 21).

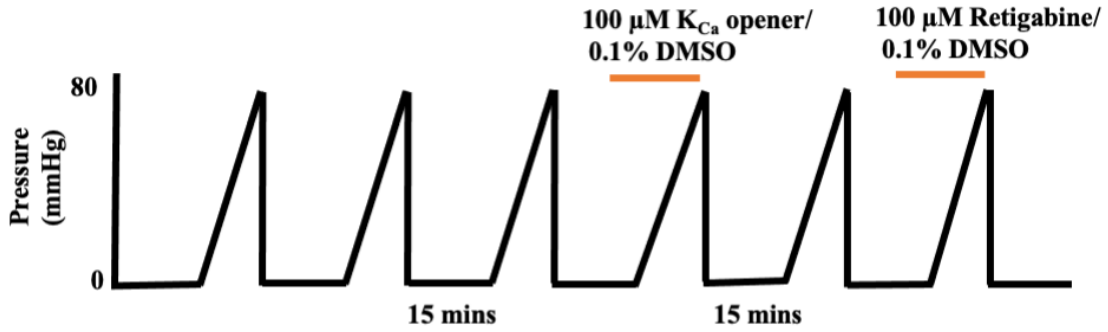


Figure 21. Schematic of the protocol used to evaluate the effect of K_{Ca} channel modulation on LSN activity in response to colonic ramp distension.

0.1% DMSO was given prior to and during distension 4 and 6 in control experiments. SKA 31 and retigabine were given prior to and during distension 4 and 6, respectively.

3.14 Results

3.14.1 K_{Ca} channel modulation on the LSN response to ATP

3.14.1.1 LSN response to repeat administration of ATP

To begin we first evaluated the stability of LSN responses to repeat application of ATP (3 mM) a concentration previously shown to elicit robust reproducible increases in mouse LSN activity (Hockley et al., 2016). Consistent with these observations, administration of 3 mM ATP produced a rapid robust increase in nerve activity (average peak firing frequency: 16.10±6.2 spikes/s) that was comparable in magnitude following repeat application (e.g.,14.28±4.7 spikes/s and 16.53±5.9 spikes/s during the 2nd and 3rd treatment, respectively, p= 0.61, repeated measures, one-way ANOVA with Dunnett's multiple comparisons test, N=6). The response to ATP gradually subsided in magnitude and nerve activity returned to baseline approximately 20 minutes after application. No significant difference was observed in the response profiles to ATP between the 1st and 2nd or 3rd application (p=0.85, two-way ANOVA with Dunnett's multiple comparisons test, N=6). The 2nd and 3rd applications of ATP given following pre-treatment with the vehicle (0.1% DMSO). The vehicle had no effect on spontaneous baseline activity and did not alter the response to ATP (Figure 22). These findings highlighting the utility of this experimental paradigm and suitability of the vehicle for study of the modulation of colonic afferent fibre chemosensitivity to K_{Ca} channel targeting drugs.

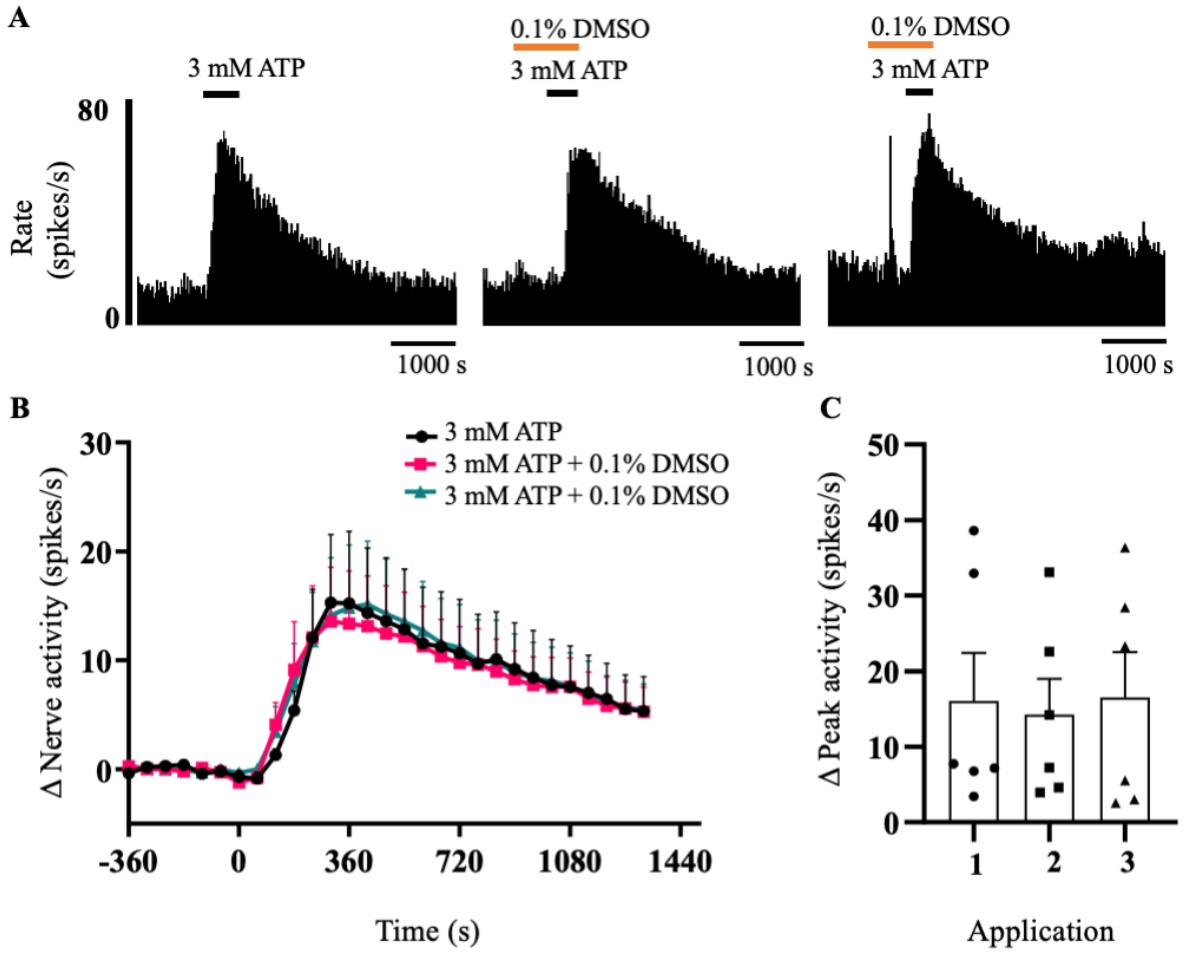


Figure 22. Effect of ATP on LSN activity.

(A) Example rate histogram of the LSN response to repeat application of ATP with the vehicle (0.1 % DMSO) administered prior and during the 2nd and 3rd application. Administration of ATP is indicated by the black bar whereas pre-treatment with DMSO is indicated by the orange bar. (B) Response profiles to repeat administration of ATP. (C) The average change in peak activity in response to the 3 ATP treatments. Data are shown as mean ±SEM.

3.14.1.2 LSN response to ATP following treatment with NS 1619 or SKA 31

Having established the stability of the experimental protocol for repeat ATP administration, we next investigated the effect of pre-treatment with either the BK_{Ca} opener, NS 1619 or the combined IK_{Ca}/SK_{Ca} opener SKA 31 on the LSN response to a 2nd application of ATP, and the effect of pre-treatment with retigabine a K_V7 opener on the LSN response to a 3rd application of ATP in separate studies. Surprisingly, pre-treatment with NS 1619 had no effect on the response profile (p=0.4, two-way ANOVA with Bonferroni's multiple comparisons test, N=5) or the peak increase in LSN activity to the 2nd ATP application of ATP (normalized peak activity: p=0.07, one-way ANOVA with Bonferroni's multiple comparisons test, N=5) compared with control experiments. In contrast, pre-treatment with retigabine significantly reduced the response profile as expected ($p<0.001$, two-way ANOVA with Bonferroni's multiple comparisons test, N=5) and the peak increase in LSN activity to the 3rd application of ATP (normalized peak activity, p=0.0033, one-way ANOVA with Bonferroni's multiple comparisons test, N=5). Additionally, I evaluated the duration of the response to ATP in the different treatments by calculating the time taken for nerve activity to return to baseline after ATP application in the presence or absence of the K^+ channel openers. Treatment with NS 1619 did not alter the duration of the response to ATP compared with the 1st application whereas retigabine reduced duration of the response (i.e. time taken to return to baseline: ATP,1584± 324.6 s; ATP + NS 1619, 1260± 327 s, p=0.59; ATP+ retigabine, 804± 130.9 s, p=0.05, repeated measures one-way ANOVA with Dunnett's multiple comparisons test, N=5) (Figure 23).

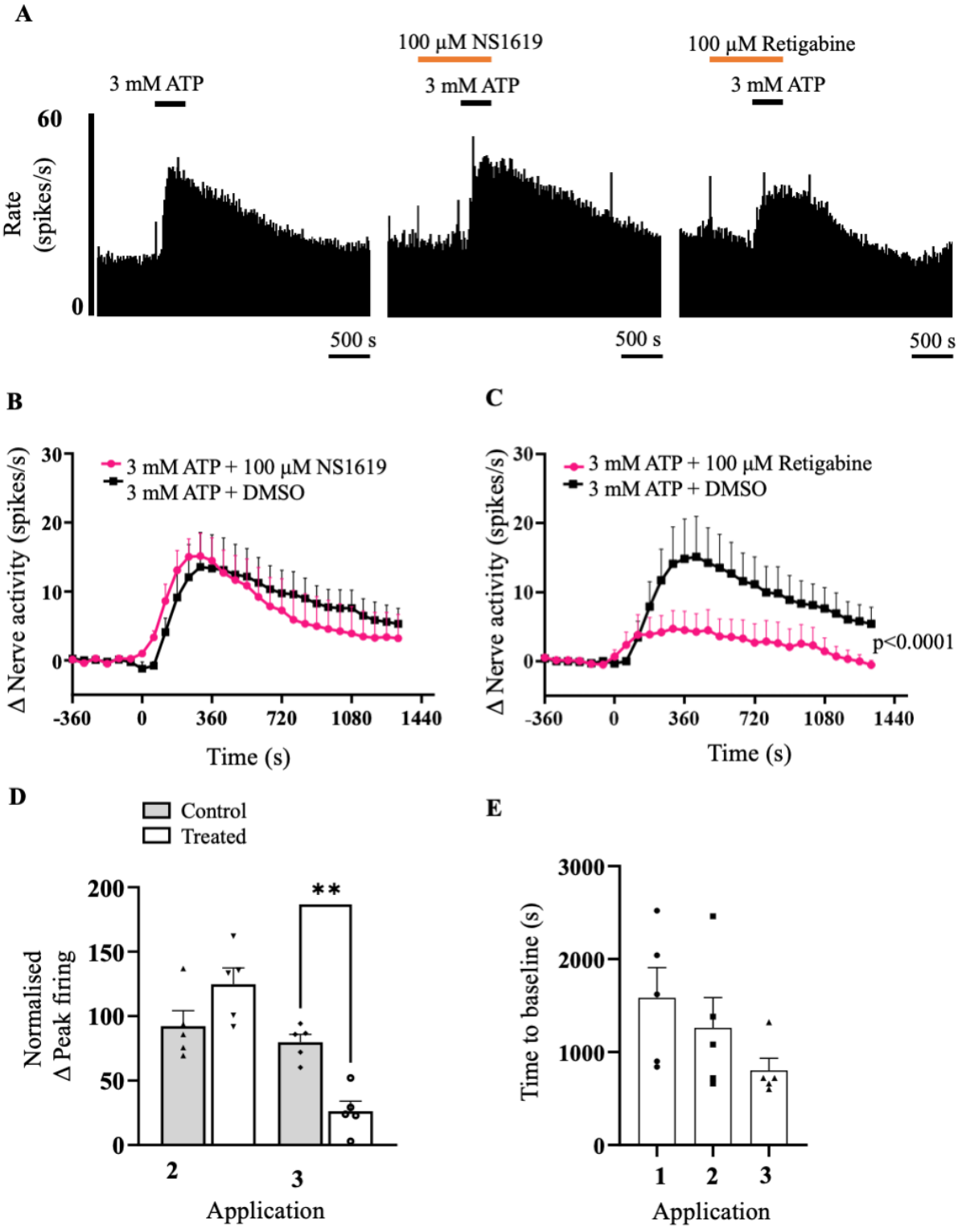


Figure 23. Effect of the BK_{Ca} opener on the LSN response to ATP.

(A) Example rate histogram of the LSN response to ATP with NS 1619 and retigabine treatment given prior to and during the 2nd and 3rd application of ATP, respectively. Addition of ATP is indicated by the black bar and addition of NS 1619 and retigabine by the orange bar. (B) Response profiles showing the overall LSN response to ATP following treatment with NS 1619

and (C) retigabine, compared with control experiments treated with DMSO. (D) The average change in peak activity in response to repeat ATP with NS 1619 administered prior to and during the 2nd application and with retigabine prior to and during the 3rd application, compared with control experiments treated with DMSO during the 2nd and 3rd application, respectively. (E) Time taken for nerve activity to return to baseline during the 3 applications (N=4). (p < 0.05, ** p < 0.01). Data are shown as mean ±SEM.*

Pre-treatment with SKA 31 also had no effect on the peak increase in LSN activity to application of ATP (normalized peak firing: p=0.13 one-way ANOVA with Bonferroni's multiple comparisons test, N=6) or the overall response profile (p=0.88, two-way ANOVA with Bonferroni's multiple comparisons test) compared with DMSO treated controls. Again, pre-treatment with the positive control retigabine produced a significant reduction in the response profile (p=0.0002, two-way ANOVA with Bonferroni's multiple comparisons test, N=4-5) compared with DMSO treated controls. In experiments with SKA 31, the duration of the response to ATP was reduced although not significantly, by pre-treatment with SKA 31 and retigabine (i.e. time taken to return to baseline: ATP,1350±196.7 s; ATP + SKA 31, 975±135 s, p=0.073; ATP+ retigabine, 975±221.0 s, p=0.19, repeated measures one-way ANOVA with Dunnett's multiple comparisons test, N=4) (Figure 24).

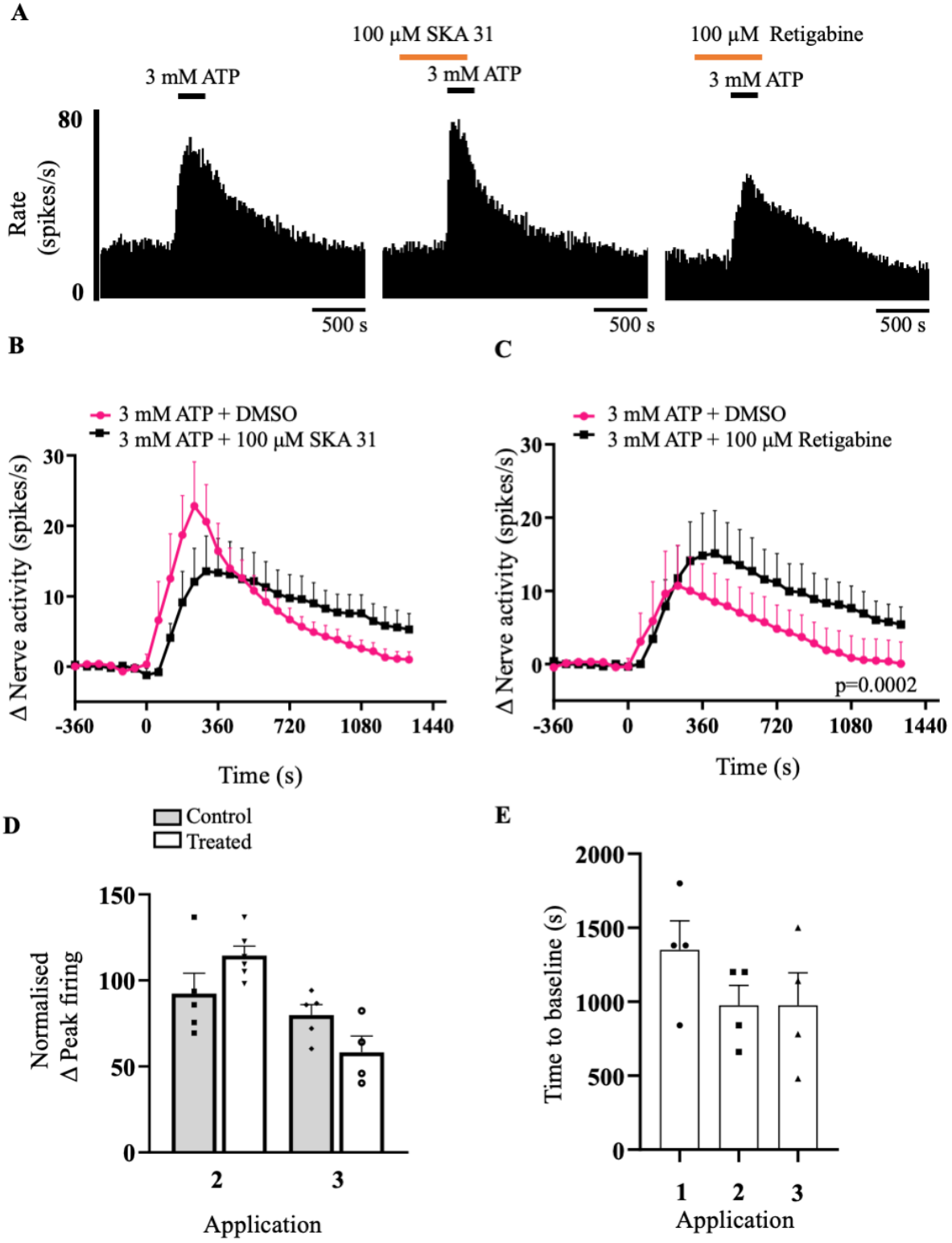


Figure 24. Effect of the IK_{Ca}/SK_{Ca} opener on LSN response to ATP.

(A) Example rate histogram of the LSN response to ATP with SKA 31 and retigabine treatment given prior to and during the 2nd and 3rd application of ATP, respectively. Addition of ATP is indicated by the black bar and addition of SKA 31 or retigabine are indicated by the orange

bar. (B) The response profiles showing the overall response to ATP following treatment with SKA 31 and (C) retigabine compared with DMSO treated controls. (D) The average change in normalized peak activity in response to repeat ATP application following treatment with SKA 31 and retigabine compared with DMSO treated controls. (E) Time taken for nerve activity to return to baseline following treatment with SKA 31 and retigabine (N=4). ($p < 0.05$). Data are shown as mean ±SEM.*

3.14.2 K_{Ca} channel modulation on the LSN response to bradykinin

3.14.2.1 LSN response to repeat bradykinin

Since the BK_{Ca} opener, NS 1619 and the combined IK_{Ca}/SK_{Ca} opener had no inhibitory effect on the LSN response to ATP, we decided to employ a different stimulus, bradykinin, with a different mechanism of afferent activation from ATP.

Initial experiments began by examining the effect of repeat application of bradykinin to colonic nerve activity. 1 μM bradykinin was used as previous studies done in the lab showed this concentration robustly excites colonic afferents in ex vivo electrophysiological experiments (Peiris et al., 2017). Sequential application of bradykinin (1 μM) resulted in a robust increase in afferent firing (peak: 22.23±6.9 spikes/s, 14.14±5.3 spikes/s, 11.53±4.2 spikes/s and 10.25±3.8 spikes/s in response to the 1st, 2nd, 3rd and 4th application to 1 μM bradykinin, respectively, *N*=5). The response to bradykinin gradually subsided in magnitude over a period of 20 minutes. There was a desensitization in response to the 2nd application of bradykinin resulting in reduced colonic nerve output compared with the response to the 1st application (p=0.0128, two-way ANOVA with Tukey's multiple comparisons *N*=6). However, response profiles and peak nerve activity to the 3rd and 4th application were consistent and comparable with the 2nd application (overall response : *p*= 0.073, two-way ANOVA with Dunnett's multiple comparisons *N*=6 and peak activity: *p*=0.2, repeated measures one-way ANOVA, with Tukey's multiple comparisons test, *N*=5), therefore enabling the investigation of the effect of the IK_{Ca}/SK_{Ca} opener, SKA 31 on the 3rd application and the effect of retigabine on the 4th application. 0.1% DMSO administered before and during the 3rd and 4th applications of bradykinin had no effect on baseline activity or colonic afferent discharge, confirming the vehicle has no effect on colonic afferent activity (Figure 25).

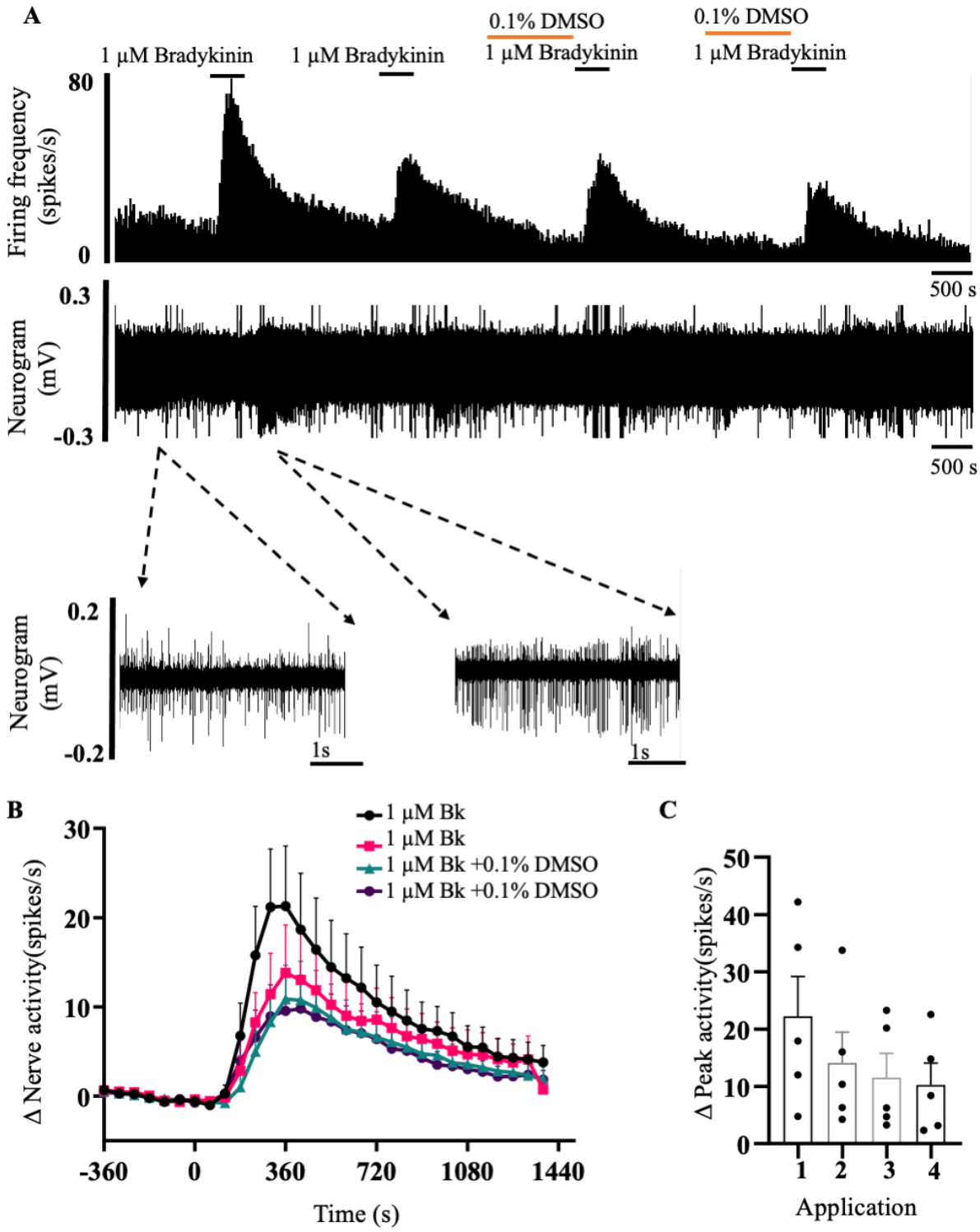


Figure 25. Effect of bradykinin on LSN activity.

(A) Example of raw data showing (i) rate histogram and (ii) neurogram of the LSN nerve response to repeat application of bradykinin with vehicle treatment (0.1% DMSO) during the 3rd and 4th application. (iii) The expanded trace at baseline activity and at peak activity in

response to bradykinin. Addition of bradykinin is indicated by the black bar and addition of DMSO by the orange bar. (B) Response profiles showing the overall LSN response to repeat administration of bradykinin. (C) Bar graph showing the average change in peak activity in response to repeat bradykinin application. Data are shown as mean ±SEM.

3.14.2.2 LSN response to bradykinin following treatment with SKA 31

To evaluate the effect of the IK_{Ca}/SK_{Ca} opener on LSN activity in response to bradykinin, 100 µM SKA 31 was administered prior to and during the 3rd application of 1 µM bradykinin and 100 µM retigabine was administered prior to and during the 4th application of bradykinin. Application of bradykinin resulted in an increase in colonic nerve activity (peak:18.4±2.7 spikes/s, 13.29±3.0 spikes/s and 4.2±1.6 spikes/s in response to the 2nd ,3rd and 4th application, respectively). Both the overall response and change in peak activity were not altered by pre-treatment with SKA 31 (overall response: p=0.45, two-way ANOVA with Bonferroni's multiple comparisons test, N=5-6 and normalized peak activity: p>0.99, one–way ANOVA with Bonferroni's multiple comparisons test, N=6). In contrast, pre-treatment with retigabine significantly reduced colonic nerve response to bradykinin (normalized change in average peak activity, p=0.0054, one-way ANOVA with Bonferroni's multiple comparisons test, N=6) compared with DMSO controls. The duration of the response to bradykinin was reduced by treatment with both SKA 31 and retigabine (time to baseline: bradykinin, 1380±108.4 s; bradykinin + SKA 31, 1010±135.4 s, p=0.043; bradykinin + retigabine 560±48.2 s, p=0.0001) (Figure 26).

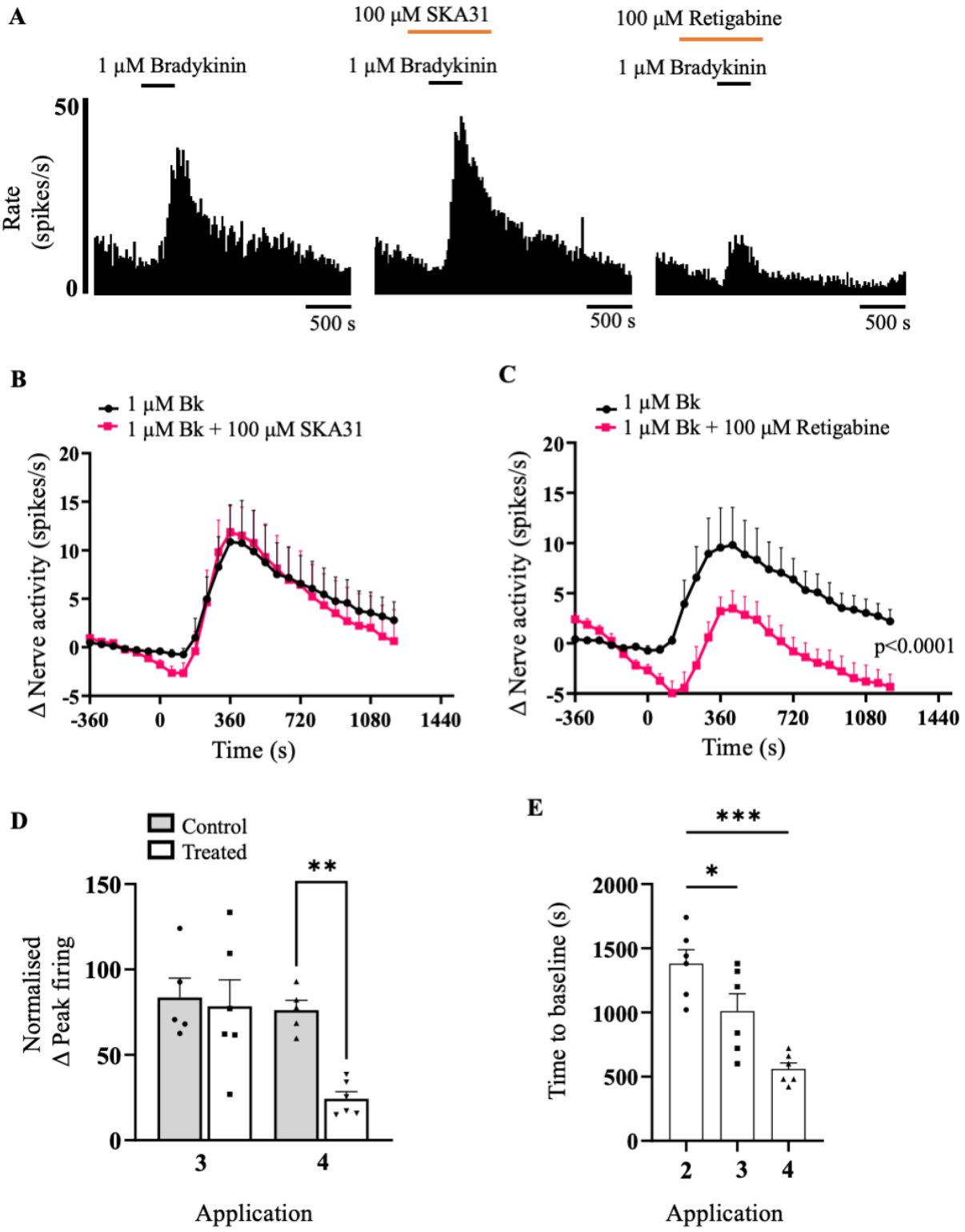


Figure 26. Effect of the IK_{Ca}/SK_{Ca} opener on the LSN response to bradykinin.

(A) Representation of raw data showing the rate histogram of colonic nerve response to repeat application of bradykinin, with SKA 31 and retigabine treatment prior to and during the 3rd and 4th applications, respectively. Addition of bradykinin is indicated by the black bar and

*addition SKA 31 and retigabine by the orange bar. (B) Response profiles showing the overall LSN response to bradykinin with SKA 31 given during the 3rd application and (C) retigabine during the 4th application. (D) Average change in peak activity in response to repeat administration of bradykinin with SKA 31 and retigabine or DMSO administered prior to and during the 3rd and 4th applications, respectively. (E) Bar graph showing the average change in peak activity in response to repeat bradykinin application. (** p < 0.01). Data are shown as mean ±SEM*

3.14.3 Effect of NS 1619, SKA 31 and retigabine on baseline nerve activity

Since there was no inhibitory effect in afferent activity with both the IK_{Ca}/SK_{Ca} opener in response to ATP and bradykinin or the BK_{Ca} opener, NS 1619 in response to ATP, additional analysis was conducted to evaluate the effect of NS 1619, SKA 31 and retigabine on spontaneous baseline activity prior to treatment with ATP or bradykinin. Changes in nerve discharge were determined post treatment with the K^+ channel openers or the vehicle, DMSO, by subtracting a baseline value derived from the mean of the values generated for 3 consecutive minute intervals prior to each treatment from the nerve discharge for each minute following treatment.

Administration of NS 1619 had no effect on baseline activity compared with time matched controls with the vehicle DMSO, (p=0.14, two-way ANOVA with Bonferroni's multiple comparisons test, N=4-5). In contrast, both SKA 31 and retigabine significantly lowered baseline activity in comparison with the time matched DMSO controls (SKA 31: p=0.0036, two-way ANOVA with Bonferroni's multiple comparisons test, N=5-6 and retigabine: p<0.0001, two-way ANOVA with Bonferroni's multiple comparisons test, N=5-6) (Figure 27).

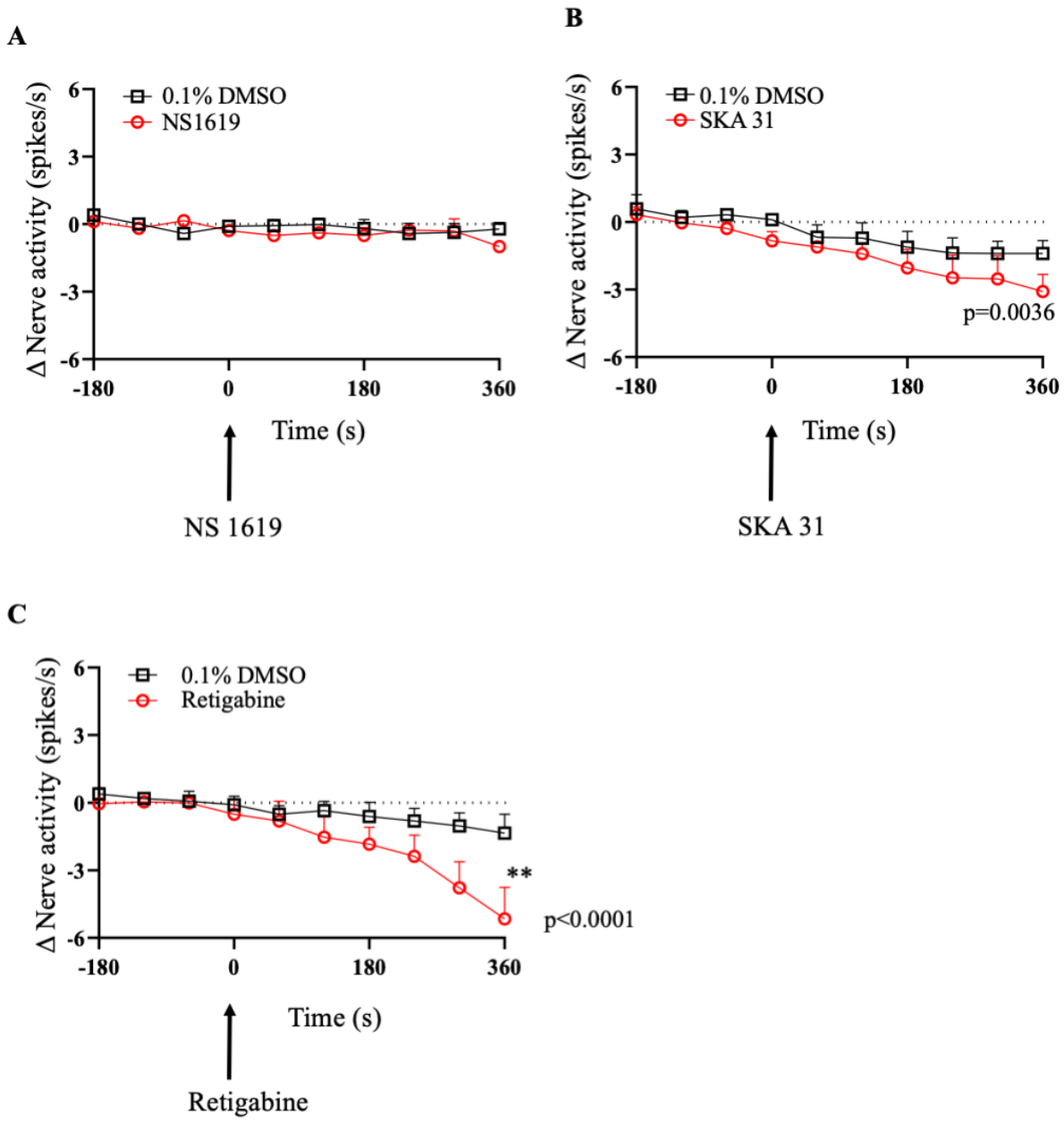


Figure 27. Effect of NS 1619, SKA 31 and retigabine on spontaneous baseline nerve firing.

Average response profiles illustrating the effects of (A) NS 1619 (B) SKA 31 and (C) retigabine on LSN baseline activity in comparison with the time matched DMSO controls. Addition of NS 1619, SKA 31, retigabine, or DMSO is at t=0. SKA 31: p=0.0036, two-way ANOVA with Bonferroni's multiple comparisons test, N=5-6 and retigabine: p<0.0001, two-way ANOVA with Bonferroni's multiple comparisons test, N=5-6). Data are shown as mean ±SEM.

3.14.4 LSN response to repeat slow ramp colonic distension

Finally, the effect of K_{Ca} openers was examined on the afferent response to grade application of mechanical stimulation by ramp colorectal distension. Preliminary studies were conducted to evaluate the effect of repeat ramp distensions on colonic afferent activity. Five ramp distensions that were 15 minutes apart were performed by blocking the luminal perfusion outflow of the canulated colon to increase luminal pressure by 80 mmHg. Ramp distensions up to 80 mmHg intraluminal pressure evoked whole nerve responses with a linear correlation between afferent activity and pressure. At peak firing, nerve activity increased by 29.5±2.7 spikes/s, 25.6±2.43 spikes/s, 24.43±2.45 spikes/s, 23.75±2.75 spikes/s and 21.7±2.85 spikes/s in response to distension 1, 2, 3, 4 and 5, respectively. Repeat distension resulted in reduced nerve activity in subsequent responses with the responses stabilizing by distension 3. The responses to distension 3 and 4 were compared and there were no significant differences in the average peak change in nerve activity (p= 0.63, paired t test, N=5) or the average response profile (p=0.46, two-way ANOVA with Bonferroni's multiple comparisons test, N=5) (Figure 28). Therefore, moving forward, drug treatments were administered from distension 3 onwards.

Additionally, colonic compliance, defined as the ability of the of the colon to expand (volume change) in response to pressure was evaluated in these preliminary studies. The volume of fluid infused to produce each pressure was plotted against the pressure. There were no changes in the compliance across the 5 distensions (p=0.28, two-way ANOVA with Tukey's multiple comparisons test, N=5) (Figure 28).

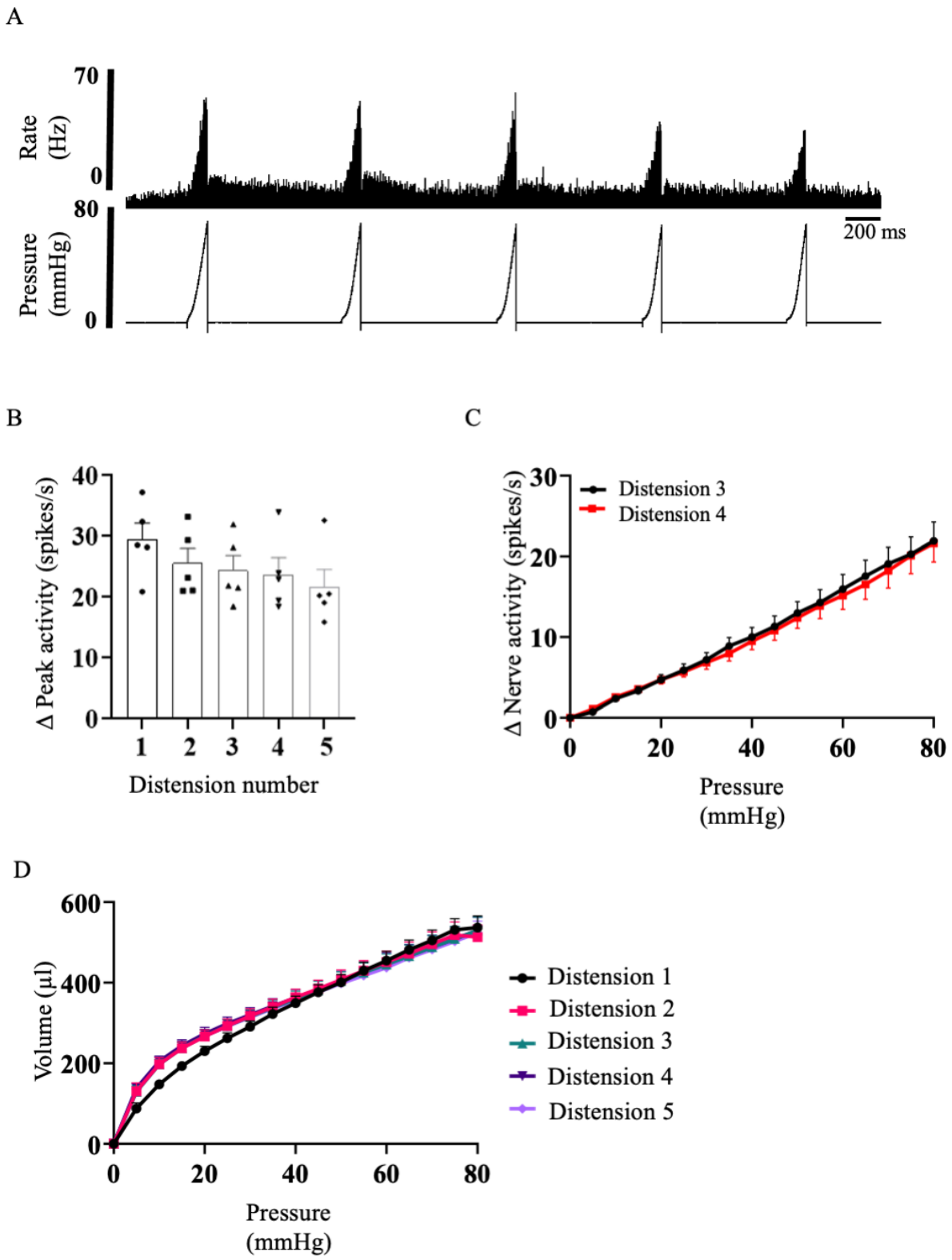


Figure 28. Example of LSN response to ramp distension.

(A) Example rate histogram of the LSN response to repeat slow ramp distensions (0-80 mmHg) and the accompanying pressure trace. (B) Bar graph showing the average peak firing in response to sequential (x5) slow ramp distension (N=5). (C) Response profiles showing the overall LSN response to distension 3 and 4. (D) Colonic compliance across the 5 distensions. Data are shown as mean ±SEM.

3.14.4.1 K_{Ca} modulation on the LSN response to ramp distension

Control studies were performed by administering the vehicle (0.1% DMSO) between the 3rd and 4th distension and between the 5th and 6th distension. The ramp distensions resulted in a pressure dependent increase in colonic nerve activity (for example, distension 1 average peak activity=45.82±8.2 spikes/s, *N*=6) which quickly returned to baseline activity following removal of stimulus. Repeated distension resulted in reduced peak activity in succeeding distensions. However, the responses stabilized by the 3rd distension enabling the effects of drug administration on colonic activity in response to ramp distension to be evaluated for the remainder of the protocol (Figure 29).

To assess the effect of the K^+ openers on colonic nerve response to ramp distension, the IK_{Ca}/SK_{Ca} opener, SKA 31 was given prior to and during the 4th distension, whereas retigabine, the K_V7 opener was administered prior to and during the 6th distension. The responses were normalized to the response to D3 and D5 in each of the experiments and the % change in the response during D4 and D6, respectively, plotted. The peak activity in response to colonic distension was unaltered by the treatment with 100 µM SKA 31 compared with the response to the 4th distension in DMSO treated experiments (normalized peak activity: p=0.77, one-way ANOVA with Bonferroni's multiple comparisons test, *N*=6). However, treatment with 100 µM retigabine significantly reduced the overall response (p<0.0001, two-way ANOVA with Bonferroni's multiple comparisons test, *N*=5 animals) and peak activity in response to distension 6 compared with DMSO treated experiments (normalised peak activity: p<0.0001, one-way ANOVA with Bonferroni's multiple comparisons test, *N*=5) (Figure 29).

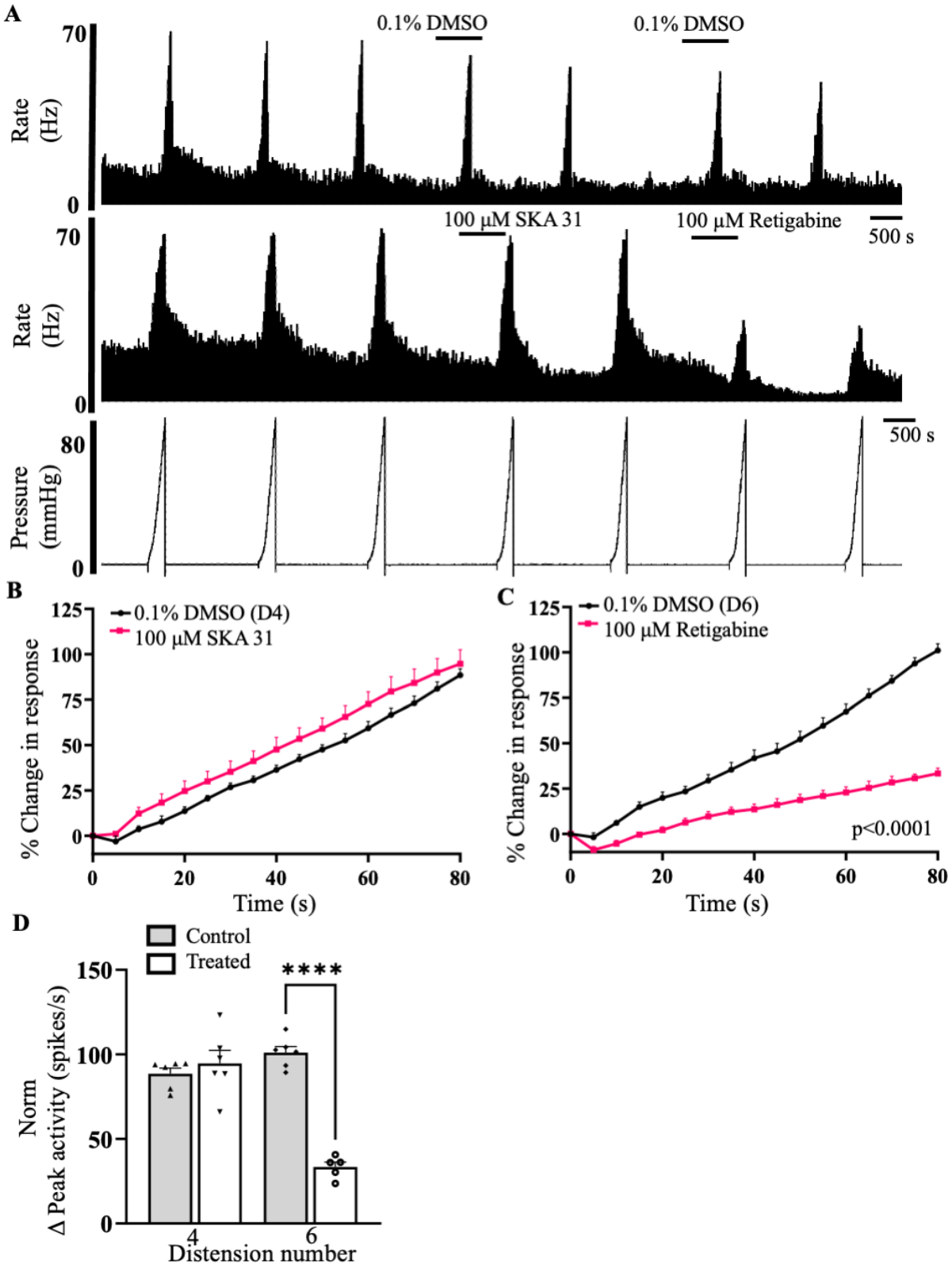


Figure 29. The effect of the IK_{Ca}/SK_{Ca} opener on the LSN response to ramp distension.

(A) Example rate histogram of the LSN response to repeat slow ramp distensions (0-80 mmHg) with (i) 0.1% DMSO treatment prior to and during distension 4 and distension 6 in control experiments and (ii) SKA 31 and retigabine treatments prior to and during distension 4 and

distension 6, respectively. (iii) The accompanying intraluminal pressure trace. (B) The normalized overall response profiles to ramp distension with SKA 31 and DMSO. (C) The normalized overall response profiles to ramp distension with retigabine and DMSO. (D) Bar graph showing the normalized average peak firing in response to sequential ramp distension in DMSO treated experiments compared with SKA 31 or retigabine treated experiments. ($p < 0.05$). Data are shown as mean ±SEM.*

3.14.5 K_{Ca} channel modulation on colonic contractions.

Since no effects were observed on LSN activity in response to ATP, bradykinin, and ramp distension with K_{Ca} channel modulation, we examined the effects of NS 1619 and SKA 31 on spontaneous colonic motility, to confirm the drugs were acting at their respective channels in the gut. Activation of K_{Ca} channels produces membrane hyperpolarization that reduces smooth muscle cell contractility by limiting Ca^{2+} influx through VGCC. We hypothesized that administration of the K^+ channel openers would hyperpolarize smooth muscle cells thus inhibiting gut contractility.

As expected, NS 1619 significantly decreased spontaneous colonic migrating motor complexes from an average of 5.27 ± 0.53 mHz to 1.94 ± 0.48 mHz (p=0.0007, paired t test, N=4). The time between contractions increased following treatment with NS 1619, although not significantly (140.8 ± 24.9 s vs 464.2 ± 128.4 s, p=0.063, paired t test, N=4) (Figure 30).

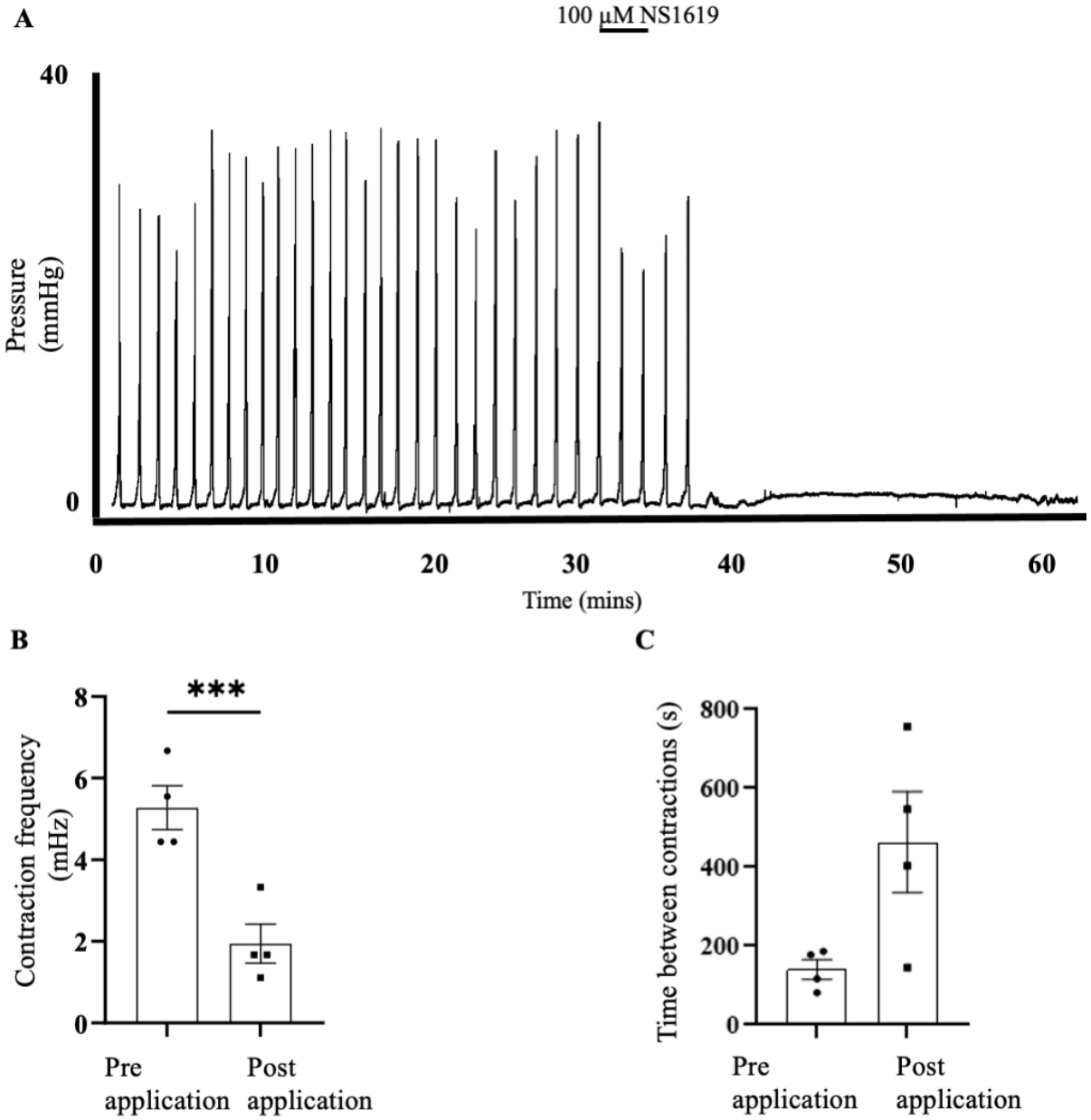


Figure 30. Effect of the BK_{Ca} opener on colonic contractions.

(A) Trace showing the effect of 100 µM NS 1619 on peristaltic colonic contractions. Addition of the drug is indicated by the black bar. (B) Average frequency of colonic contractions before and after the administration of 100 µM NS 1619. Contraction frequency before and after treatment with NS 1619. (C) Average time between contractions before and after the treatment with NS 1619. Data are shown as mean ±SEM.

Similarly, application of SKA 31 significantly decreased spontaneous colonic migrating motor complexes from an average of 5.97±0.53 mHz to 2.5±0.58 mHz (p=0.02, paired t test, N=4). Additionally, the time between colonic contractions increased, although not significantly following treatment with SKA 31 (123±12.68 s vs 438.5±134.2 s, p=0.1025, paired t test, N=4) (Figure 31).

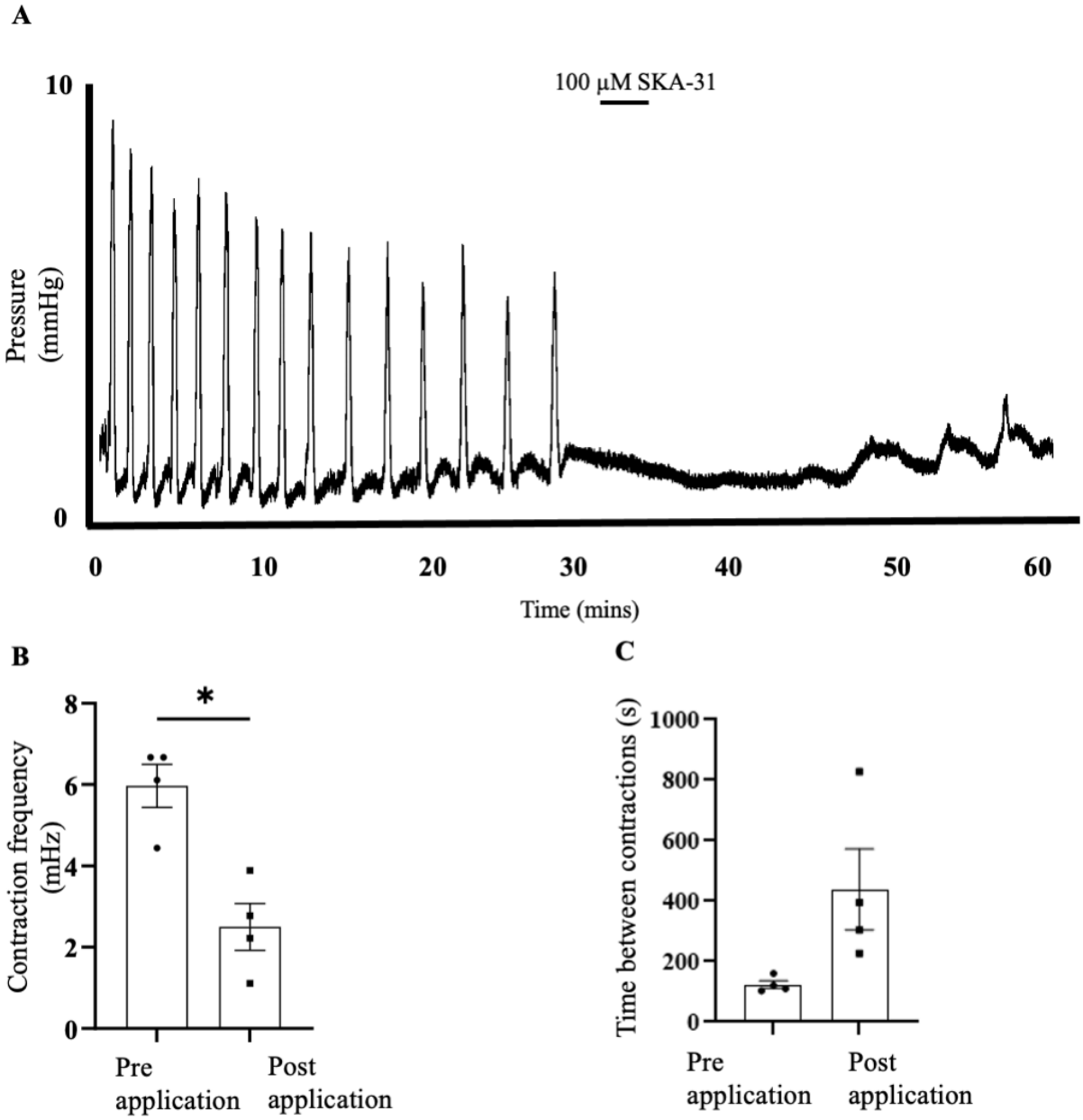


Figure 31. Effect of the IK_{Ca}/SK_{Ca} opener on colonic contractions.

Trace showing the effect of 100 µM SKA 31 on peristaltic colonic contractions. Addition of the drug is indicated by the black bar. (B) Average frequency of colonic contractions before and after the administration of 100 µM SKA 31 (p=0.02, paired t test, N=4). (C) Average time between contractions before and after the treatment with SKA 31. Data are shown as mean ±SEM.

Both SKA 31 and NS 1619 inhibited colonic contractions reducing the frequency of contractions. The large effects observed confirmed the efficacy of the concentrations used, suggesting we are engaging the target channel.

3.15 Summary of results

- Treatment with the BK_{Ca} channel opener NS 1619 had no inhibitory effect on LSN discharge to ATP.
- Similarly, the IK_{Ca}/SK_{Ca} opener and SKA 31 had no effect on the colonic afferent response to ATP, bradykinin and colonic ramp distension.
- By contrast pre-treatment with the K_V7 channel opener retigabine, significantly inhibited colonic afferent responses to ATP, bradykinin, and ramp distension. Additionally, retigabine significantly lowered spontaneous baseline activity.
- Both SKA 31 and NS 1619 significantly decreased colonic contractility indicating the drugs and concentrations used for these studies were sufficient for engaging calcium activated potassium channels.

3.16 Discussion

In the present study, I investigated the effect of potassium channel modulation on the colonic LSN activity in response to chemical (ATP and bradykinin) and mechanical (ramp distension) stimuli. The findings from these studies informing on the therapeutic potential of targeting K_{Ca} channels for the treatment of abdominal pain in gastrointestinal disease. Which, based on the data presented are unlikely to be effective visceral analgesics. Pre-treatment with the BK_{Ca} channel opener, NS 1619 was unable to suppress colonic afferent activation to ATP. Similarly, SKA 31, the combined IK_{Ca}/SK_{Ca} opener had no inhibitory effect on the LSN response to bradykinin, ATP and ramp distension. In contrast, pre-treatment with retigabine, the K_V7 channel opener had a marked inhibitory effect on colonic nerve activity in the same assays, demonstrating the utility of K_V7 openers as treatments for visceral pain.

3.16.1 Study design and algogenic stimuli selection

The goal of this study was to investigate the effect of potassium channel openers as an approach that would target nociceptors directly and work irrespective of the noxious stimulus driving pain in disease states. We utilized ATP and bradykinin as prototypic algogenic stimuli and colonic ramp distension as a relevant noxious stimulus. ATP is an important extracellular signalling molecule that plays a crucial role in nociception and chronic pain. Injection of ATP has been shown to activate nociceptors in humans whereas P2X3 deficient mice have reduced pain behaviours to ATP (Cockayne et al., 2000; Hilliges et al., 2002). Bradykinin which preferentially binds to the B2 receptor has been shown to stimulate visceral afferents and sensitize nociceptors (Longhurst & Dittman, 1987). Additionally, injection of bradykinin evokes pain in humans (Manning et al., 1991). Colonic ramp distensions (>40 mmHg) evoke visceral pain behaviours in vivo in mice cause pain in humans (Ness & Gebhart, 1988). Additionally, previous work in the lab demonstrated the ability of ATP and bradykinin to stimulate human and mouse colonic afferents (Hockley et al., 2016; McGuire et al., 2018; Peiris et al., 2017). Therefore, we determined that ATP, bradykinin, and ramp distension would be appropriate stimuli to use in nerve recording studies.

Administration of ATP resulted in a robust increase in colonic afferent output as previously demonstrated (Hockley et al., 2016). Repeat administration of ATP resulted in consistent response profiles and comparable changes in peak nerve activity thereby allowing investigation of K_{Ca} modulation in a repeat ATP application protocol. Similarly, application of bradykinin

resulted in a robust increase in LSN nerve activity as previously demonstrated (Peiris et al., 2017). Repeat application of bradykinin resulted in waning excitatory effects in subsequent applications, a phenomenon known as tachyphylaxis (Mizumura et al., 2009). The responses stabilized following the 2nd application of bradykinin, therefore allowing observation of the effects of the K^+ openers on the 3rd and 4th applications. Slow ramp distension increased nerve activity in a pressure dependant manner. Responses to repeat distension resulted in reduced nerve activity in subsequent responses with the responses stabilizing by the 3rd distension thereby allowing evaluation of K^+ channel modulation on the LSN response to ramp distension from the 4th distension.

3.16.2 K_{Ca} channel modulation on the colonic nerve response to ATP, bradykinin and ramp distension

K_{Ca} channels are involved in the repolarisation and hyperpolarization of neuronal membranes and therefore are important regulators of neuronal excitability. Several studies have examined the contribution of K_{Ca} channels on neuronal excitability and nociception to assess the therapeutic potential of targeting these ion channels. For example, one study showed that the cytokines IL-6 and TNF-α inhibited afferent mechanosensitivity in colonic afferents in remission mice through upregulation of BK_{Ca} channels, an effect that was blocked by the BK_{Ca} blocker, iberiotoxin, thereby demonstrating the protective role of the BK_{Ca} channels in GI mechanosensitivity (Campaniello et al., 2017). However, the direct effect of K_{Ca} channel modulation on colonic afferent signalling from the GI tract has not been fully explored.

In the present study, we started by examining the expression of K_{Ca} channels in gut projecting neurons using an online database containing whole transcriptome expression within single DRG neurons projecting to the colon. Data from this study shows that BK_{Ca} (*Kcnma1*) and $SK_{Ca}2.1$(*Kcnn1*) channels are present in DRG neurons projecting to the colon. However, despite their expression on colonic afferents, present data suggests that BK_{Ca} channel activation has no inhibitory effect on the colonic nerve response to ATP. The BK_{Ca} opener, NS 1619 did not alter the overall response profile, peak firing and duration of the response to ATP. Additionally, NS 1619 had no effect on spontaneous baseline activity.

Similarly, SK_{Ca}/IK_{Ca} channel activation had no effect on the response to ATP and bradykinin and colonic ramp distension. SKA 31 did not alter the nerve's response to ATP, bradykinin,

and ramp distension. However, SKA 31 significantly reduced spontaneous baseline activity. Given the inhibitory effects of SKA 31 were m only observed on baseline activity, we concluded that K_{Ca} openers would not be an effective strategy for inhibiting neuronal activity.

3.16.3 K_V7 channel modulation on colonic nerve response to ATP, bradykinin and ramp distension

K_V7 channels, which enable the generation of the M-current, a subthreshold current that stabilizes the membrane potential and controls neuronal excitability are expressed in DRG neurons projecting to the colon and have previously been shown to inhibit neuronal activation in response to bradykinin and luminal distension (Blackburn-Munro et al., 2005; Peiris et al., 2017). Therefore, retigabine, a K_V7 channel opener that opens neuronal $K_V7.2$-7.5 voltage-activated K^+ channels resulting in reduced neuronal excitability was employed as a positive control for these studies. In contrast to the K_{Ca} openers, administration of retigabine had a marked inhibitory effect LSN response to ATP, bradykinin, and ramp distension. Additionally, retigabine inhibited spontaneous baseline activity. These results in agreement with previous studies that demonstrated that retigabine can inhibit the LSN response to bradykinin and colonic ramp distension (Peiris et al., 2017).

3.16.4 K_{Ca} channel modulation on colonic peristaltic activity

Lastly, we examined the effect of K_{Ca} openers used in this study on colonic peristaltic activity to confirm the drugs were acting at their respective channels in the gut. Activation of K_{Ca} channels results in hyperpolarization of smooth muscle cells in the GI tract resulting in the relaxation of the smooth muscle (dela Pena et al., 2009). As anticipated, administration of K_{Ca} openers (NS 1619 and SKA 31) inhibited spontaneous colonic contractions. These results suggesting the drugs and concentrations used in these experiments were sufficient to engage the target ion channels. Additionally, this data suggests K_{Ca} channels can be evaluated for their potential to treat motility disturbances.

3.16.5 Considerations

The absence of an inhibitory effect from BK_{Ca} and the IK_{Ca}/SK_{Ca} channel activation on nerve activity was surprising given the expression of the channels in colon projecting neurons. We considered the possibility that these channels were tonically active in our preparations prior to administration of the drugs therefore their activity could not be enhanced further. However, by using the K_{Ca} openers to alter colonic contractility, we demonstrated that the drugs and concentrations used for these experiments were effective in engaging K_{Ca} channels in the system. Another possibility is that intracellular calcium levels were insufficient to stimulate K_{Ca} channels. However, this is very unlikely given the known role for Ca^{2+} in mediating the effects of algogenic mediators including bradykinin and ATP used in these studies.

Another possible explanation for the lack of an inhibitory response from the BK_{Ca} opener and the modest response with the IK_{Ca}/SK_{Ca} opener could be the stimuli used for these studies was too strong to be overcome by the K_{Ca} channel activation. If this was the case, we would expect to see an inhibitory response at reduced concentrations of either ATP or bradykinin. However, both the BK_{Ca} opener and the SK_{Ca}/IK_{Ca} had no effect on the response to ATP and bradykinin including the sustained response which occurs at reduced concentrations. While SKA 31 had a modest effect on baseline activity, NS 1619 had no effect spontaneous baseline effect. Additionally, the SK_{Ca}/IK_{Ca} opener did not alter the response to colonic ramp distension including at lower distension pressures.

The utilization of retigabine as a positive control in these studies demonstrates the utility of modulating K^+ channel function on nerve activity. Additionally, we were able to rule out methodological problems as a cause for the failure of the K_{Ca} openers to reduce colonic nerve activity as retigabine had a marked inhibitory effect on colonic afferent output in the same system. The differences in K^+ conductance between K_{Ca} and K_V7 channels could contribute to the observed differences in the channels' ability to alter neuronal firing. K_V7 channels are activated during the depolarization phase of the action potential whereas K_{Ca} channels are activated during the repolarization and afterhyperpolarization phase of the action potential. Therefore, targeting channels involved in the depolarization phase could be more effective in preventing action potential firing than targeting channels involved in the hyperpolarization that occurs after action potential firing.

Lastly, since K_{Ca} channels are widely expressed in multiple cell types throughout the GI tract and are involved in many physiological processes including regulation of immune cells, targeting K_{Ca} channels may present with undesired GI related side effects. For example, IK_{Ca} channels modulate many calcium dependent cellular processes in immune cells and blockade of these channels has been demonstrated to be beneficial in IBD (Di et al., 2010). Therefore, while K_{Ca} channels may have a beneficial effect in regulating motility, the involvement of these channels in the immune system may limit the therapeutic potential of K_{Ca} channel activators.

3.17 Future studies

Future work to confirm the expression of K_{Ca} channels on neurons projecting to the GI tract would be essential in validating the utility of K_{Ca} modulation for altering colonic afferent output. Immunohistochemistry and PCR could be utilized to confirm expression of K_{Ca} channels in sensory neurons. Additionally, future studies could evaluate differences in expression between the KCNQ and K_{Ca} channels to determine if the expression patterns account for the observed differences on their effect on neuronal excitability. Secondly, given that expression patterns of BK_{Ca} channels can be altered by colitis, studies to evaluate changes in expression of K_{Ca} channels in colitis vs healthy tissue would be key in establishing any potential role of K_{Ca} channel in pain in GI pathologies (Campaniello et al., 2017). Additionally, K_{Ca} modulation could be evaluated in disease models or behavioral studies to evaluate the contribution of these ion channels in pain in whole organisms. K_{Ca} channels are widely expressed in non-neuronal cells, therefore may indirectly contribute to pain whole organisms.

3.18 Conclusions

In conclusion, we show that targeting K_{Ca} channels has no effect on nerve in altering colonic afferent output in response to ATP, bradykinin, and colonic ramp distension. Therefore, K_{Ca} channels would not be viable target for treating pain in gastrointestinal disorders. However, since K_{Ca} channels can inhibit colonic motility, they could present as viable therapeutics for treating GI motility disorders. On the other hand, retigabine effectively inhibited neuronal discharge in response to bradykinin, and colonic ramp distension providing evidence for the utility of KCNQ openers but not K_{Ca} openers for the treatment of visceral pain.

4 Angiotensin II on colonic afferent activity

4.1 The renin angiotensin system

Angiotensin II (Ang II) is the main effector molecule of the renin-angiotensin system (RAS), which plays a central role in regulating renal and cardiovascular physiology (Fyhrquist & Saijonmaa, 2008). Ang II is an octapeptide hormone (Asp-Arg-Val-Tyr-Ille-His-Pro-Phe in humans) that exerts its effects by binding two GPCRs, angiotensin type 1 (AT_1) and angiotensin type 2 (AT_2) (de Gasparo et al., 2000). In the traditional or classical view of the RAS, angiotensinogen produced by the liver is converted into the decapeptide angiotensin I (Ang I) by renin, a proteolytic enzyme that is produced by the juxtaglomerular apparatus. Ang I is physiologically inactive but acts as the precursor for Ang II. Ang I is converted to Ang II by angiotensin converting enzyme (ACE), a zinc carboxypeptidase that is found primarily in the vascular endothelium of the lungs and kidneys (Atlas, 2007; Mehta & Griendling, 2007).

The complexity of the RAS has increased considerably in the past two decades with multiple new receptors and angiotensin peptides identified. While Ang II is still considered to be the main effector molecule of the RAS, the actions of other angiotensin peptides including Ang III, Ang IV, and Ang [1-7] have been demonstrated (Atlas, 2007). Ang III is formed by aminopeptidase A which removes a single amino acid from the N-terminus of Ang II and has similar biological activities to Ang II. Ang IV results from further conversion of Ang III by aminopeptidase B or N and plays a role in blood flow regulation, learning, memory, and neuronal development through binding the angiotensin type 4 receptor (AT_4) (Chai et al., 2004; Haulica et al., 2005). Ang II can also be broken down to Ang [1-7] by ACE 2, a homolog of ACE, and Ang [1-7] exerts its effects such as vasodilation and antiproliferation via the GPCR, Mas (Varagic et al., 2008). Additionally, ACE 2 can cleave Ang I to Ang [1-9] which binds to the AT_2 receptor and has beneficial effects in cardiovascular diseases (Figure 32) (Ocaranza et al., 2020).

4.2 Tissue production of Ang II

In addition to the classical RAS, RAS independent-angiotensin-generating systems have been demonstrated in various organs including the brain, kidneys, heart, liver, pancreas, reproductive organs, and the gastrointestinal tract (Carey & Siragy, 2003). Enzymes including cathepsin D (CTSD) produced by lysosomes, cathepsin G (CTSG) released by activated neutrophils, and tonin have been shown to cleave angiotensinogen to form Ang I (Nehme et al., 2019). Ang II can be formed through Ang I cleavage by cathepsin G[216] and chymase, a

serine protease that is synthesized in mast cell granules and released in response to inflammatory stimuli (Miyazaki & Takai, 2006). Alternatively, Ang II can be formed by the direct cleavage of angiotensinogen by cathepsin G, tonin and kallikreins produced by the pancreas (Maruta & Arakawa, 1983; Nehme et al., 2019). Additionally, an alternative Ang II precursor-Ang 1-12 has been identified that allows the production of Ang II independent from Ang I production by renin. Ang 1-12 can be cleaved by ACE to generate both Ang I and Ang II (Nehme et al., 2019). Another study also showed that chymase can cleave Ang 1-12 to Ang II in addition to Ang I in both humans and rodents (Ahmad et al., 2019).

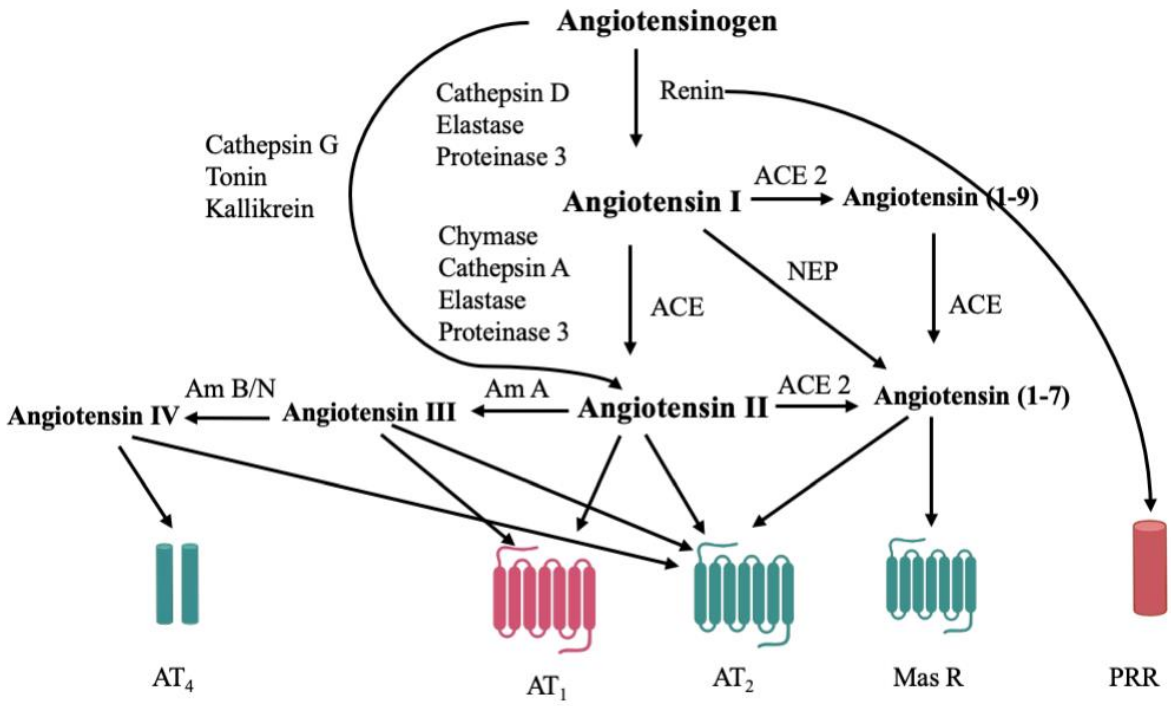


Figure 32. Schematic representation of the renin angiotensin system.

In the classic RAS, angiotensinogen is cleaved by renin to generate Ang I. ACE cleaves Ang I to generate Ang II, the major bioactive of the system. Ang II binds two GPCRs, AT_1 and AT_2. Ang II can be further broken down by ACE 2 and aminopeptidase A to generate Ang (1-7) and Ang III, respectively. Enzymes shown on the left can generate Ang II independent of the classic renin ACE-pathway.

Components of the RAS have been classified into two complementary pathways: the classical and alternative pathways. The classical and alternative RAS pathways mediate opposing effects on inflammation, fibrosis, and cell proliferation. The balance of signaling from these two pathways determines the overall homeostatic effect of the RAS. Components of the proinflammatory pathway include ACE, Ang II, renin, prorenin, chymase, and neutral endopeptidase (NEP). The classical pathway induces vasoconstriction, salt and water retention, tissue inflammation, and fibrosis. In contrast, the alternative RAS components, ACE 2, and

Ang [1-7] acting via Mas receptor, have vasodilatory, antihypertensive, anti-fibrotic, and anti-inflammatory effects (Garg et al., 2015; Santos et al., 2019).

4.3 Ang II

Ang II has diverse physiological and pathophysiological effects including vasoconstriction, blood pressure regulation, inflammation, and hypertension. The target tissues for Ang II include the adrenals, kidneys, brain, vascular smooth muscle, and the sympathetic nervous system (de Gasparo et al., 2000). Angiotensin receptors AT_1 and AT_2 mediate opposing effects of inflammation, fibrosis, and cell proliferation. Both AT_1 and AT_2 bind Ang II with nearly identical affinity. While many of the pathophysiological effects of Ang II are mediated by the AT_1 receptor, less is known about the AT_2 receptor. Most species, including humans, possess a single AT_1 receptor gene whereas rodents harbor two AT_1 receptor subtypes: AT_{1A} and AT_{1B} which are encoded by different genes *Agtr1a* and *Agtr1b* (de Gasparo et al., 2000). AT_{1A} and AT_{1B} are homologous and exhibit a 91% and 96% similarity on nucleic acid and amino acid levels, respectively. The AT_{1A} and AT_{1B} receptors are indistinguishable pharmacologically and functionally and exhibit similar ligand binding and signal transduction properties (Mehta & Griendling, 2007). However, AT_{1A} and AT_{1B} differ in their tissue distribution and transcriptional regulation (de Gasparo et al., 2000). AT_{1A} is dominantly expressed in most organs and is believed to be the closest homologue to the human AT_1 receptor (Forrester et al., 2018). The human AT_1 and rat AT_{1A} receptor nucleic acid and amino acid sequences share an 86% and 94% homology, respectively (Chung et al., 1999).

4.4 The RAS in pathological conditions

While the classical RAS has been mainly studied for its role in blood pressure regulation and body electrolyte balance, components of the RAS also plays key roles in cellular growth, proliferation, differentiation, migration, apoptosis, extracellular matrix remodeling, and inflammation (Mehta & Griendling, 2007; Nehme et al., 2019). Therefore, alterations in expression of RAS components are involved in multiple diseases including atherosclerosis, arthritis, pancreatitis, cardiac hypertrophy, renal fibrosis, and type 2 diabetes (Nehme et al., 2019; Ranjbar et al., 2019). Consequently, drugs that interfere with the RAS including angiotensin receptor blockers (ARBs) and angiotensin converting enzyme inhibitors (ACEIs) have proven to be beneficial in treating conditions that are associated with overactivity of the RAS. For example, eight ARBs: losartan, valsartan, irbesartan, candesartan, telmisartan,

eprosartan, olmesartan and azilsartan are currently marketed as commercial drugs to treat hypertension (Takezako et al., 2015).

4.5 The RAS and inflammation

Blockade of the RAS with ACE inhibitors and ARBs can diminish the expression of inflammatory mediators and is effective in treating proinflammatory diseases such as atherosclerosis, rheumatoid arthritis (RA) and colitis (Ranjbar et al., 2019). Ang II stimulates the production of inflammatory mediators including the reactive oxygen species (ROS), the proinflammatory transcription factor nuclear factor κB, chemokines including monocyte chemoattractant protein-1 (MCP-1), interleukin-8, cytokines including TNF-α, interleukin-1 (IL-1) and IL-6 and matrix metalloproteinases 1 and 9 (Dandona et al., 2007). Additionally, Ang II, through AT_1, is involved in all the key stages of inflammation such as increasing vascular permeability by stimulating the synthesis of prostaglandins and vascular endothelial growth factor (VEGF), leukocyte recruitment and activation through the release of several mediators including selectins, integrins, cellular adhesion molecules, cytokines and chemokines, and vascular repair processes through mediators of cell growth and fibrosis (Suzuki et al., 2003).

4.6 Components the RAS in the GI tract

Components of the RAS are widely distributed in the GI tract. Studies using RT-PCR and immunohistochemistry showed the presence of renin in the surface epithelium, lamina propria mesenchymal cells, microvascular walls, and muscular mucosa of the colon. AT_1 was detected on the surface of epithelial cells and in crypt bases, lamina propria macrophages, myofibroblasts, and mucosal vessel walls. ACE was weakly expressed in parts of the surface epithelium and more predominantly in the mesenteric microvascular walls, lamina propria, and submucosal mesenchymal cells. ACE 2 was localized to the mesenteric microvascular endothelium in the colon (Garg et al., 2012). Additional immunohistochemical studies confirmed the presence of ACE, ACE 2, Ang II, Ang (1-7), AT_1, and Mas receptors across the small and large intestines predominantly in the enterocytes (Garg et al., 2020).

4.7 The RAS in IBD

Several studies that have examined the role of the RAS components in IBD suggest that it plays a role in the pathogenesis of colitis. Components of the RAS have been shown to be upregulated in IBD patients and mice colitis models. For example, Ang II was found to be elevated in colonic biopsies obtained by endoscopy from patients with Crohn's compared with healthy controls (Jaszewski et al., 1990). Secondly, overexpression of components of the RAS has been shown to exacerbate disease in mice colitis models. For example, RenTgMK mice that overexpress active renin from the liver developed more severe colitis compared with the PBS-treated controls (Shi et al., 2016). Thirdly, targeting components of the RAS in colitis models has been shown to ameliorate disease. For example, treating RenTgMK mice with aliskiren, a renin inhibitor ameliorated colitis (TNBS) and inhibiting AT_1 receptor signaling in TBNS colitis with losartan, an AT_1 blocker ameliorated colitis (Shi et al., 2016). Additionally, deleting the AT_{1A} receptor ameliorated symptoms of colitis including diarrhea, rectal bleeding, and weight loss compared to WT controls. TNF-α expression was also significantly reduced in AT_{1A} deficient mice compared to controls (Katada et al., 2008). Similarly, in human biopsies, pro-inflammatory chemokines and cytokines including TNF-a, IL-17F, IL-6, IL-23, CCL2, IL-1b were suppressed in patients with IBD who were on ARB therapy compared to patients not receiving ARB therapy (Shi et al., 2016). Additionally, disease activity scores were lower, and the need for hospitalization/surgery was reduced in patients who were treated with RAS blockers (Garg et al., 2020).

RNA sequencing data generated in our group has also shown upregulation of components of the RAS in mucosal biopsy samples from patients with IBD. Angiotensinogen expression was upregulated in the mucosal biopsies of UC and CD patients compared with controls ($p<0.0001$, one-way ANOVA with Dunnett's multiple comparisons test). Cathepsin B, which cleaves the zymogen prorenin to renin was also upregulated in UC and CD patients compared with controls ($p<0.0001$, one-way ANOVA with Dunnett's multiple comparisons test). Similarly, ACE which converts Ang I to Ang II was upregulated in both UC and CD ($p<0.0001$, one-way ANOVA with Dunnett's multiple comparisons test). Renin expression on the other hand was comparable between IBS and IBD patients ($p=0.18$, one-way ANOVA with Dunnett's multiple comparisons test) (Figure 33).

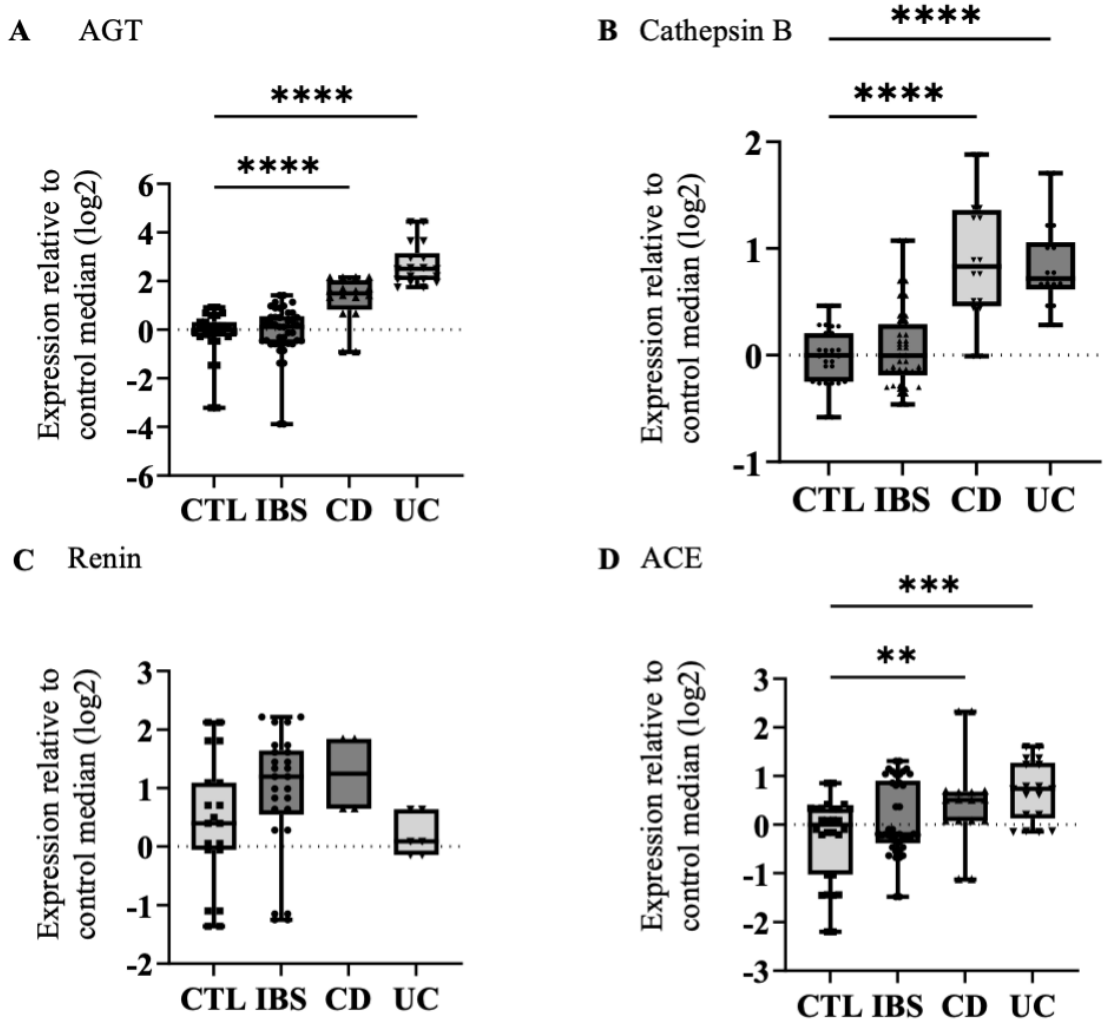


Figure 33. Components of the RAS in colonic biopsies of IBD patients.

Compared with controls (CTL), (A) angiotensinogen, (B) Cathepsin B and (D) ACE 2 were upregulated in colonic biopsies of UC and CD patients whereas renin expression was comparable between controls and IBD patients. Data are shown as Log2 fold change median expression in control samples. ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$).*

Taken together, these studies suggest that components of the RAS likely play a role in the pathogenesis of IBD and targeting components of the RAS could be an effective therapeutic strategy for the management of IBD. Moreover, targeting the RAS is an attractive option as it offers inexpensive and safe therapies that are already in widespread use.

4.8 The RAS on pain

The role of the RAS in nociception is not clearly understood with studies having demonstrated both nociceptive and antinociceptive effects of RAS blocking drugs (Bali et al., 2014).
For example, RAS blockers have demonstrated analgesic effects and have been shown to be beneficial in various models of neuropathic and nociceptive pain. Components of the RAS play a critical role in the activation of various processes of inflammation including accumulation of neutrophils, differentiation of dendritic cells, and production of inflammatory cytokines. Therefore, the ability of RAS blockers to attenuate angiotensin-mediated amplification of the inflammatory milieu has been considered the major mechanism for decreased pain perception (Bali et al., 2014). Clinical studies have demonstrated a pathophysiological role of the RAS in migraines and ACE inhibitors (such as enalapril and lisinopril) and AT_1 receptor blockers (such as telmisartan) have been shown to be effective in preventing attacks of migraine (Bali et al., 2014; Tronvik et al., 2006).

On the other hand, activation of the RAS has been shown to have anti-nociceptive effects in various pain models. For example, spontaneous hypersensitive rats exhibit decreased pain sensitivity which has been ascribed to over activation of the RAS (Irvine & White, 1997). Similarly, hypertensive patients have been reported to have reduced pain sensitivity which is antagonized by the different RAS inhibitors suggesting the anti-nociceptive actions of endogenous angiotensin peptides (Ghione et al., 1988; Guasti et al., 2002). Use of ACE inhibitors to reduce hypertension decreases pain thresholds independent of the effects on blood pressure in both rats and humans (Guasti et al., 1998; Irvine et al., 1995). ACEs are involved in the degradation of kinins like bradykinin and substance P, whose role in nociception is well documented (Erdos, 1975; Mauborgne et al., 1991). Therefore, the pain-inducing actions of ACE inhibitors have been attributed to the decrease in the breakdown of bradykinin and substance P (Irvine et al., 1995).

4.9 Ang II receptors and signaling pathways

4.9.1 AT_1 receptor

The gene coding AT_1 (*AGTR1*) is located on chromosome 3 in humans with AT_1 being composed of 359 amino acids with a molecular mass of 41 kDa (Mehta & Griendling, 2007). The AT_1 receptor is expressed in various tissues including vascular smooth muscle, endothelium, brain, heart, gastrointestinal tract, kidney adrenal gland, and adipose tissue

(Forrester et al., 2018). In the human colon, AT_1 is localized in vessel walls, myofibroblasts and macrophages in lamina propria, crypt bases and surface epithelium (Hirasawa et al., 2002). AT_1 activation is increased by acute exposure to Ang II, however, chronic exposure to Ang II downregulates the receptor (Mehta & Griendling, 2007).

Studies have shown that AT_1 can form homodimers and AT_1-GPCR heterodimers, which can alter ligand binding and receptor function. For example, AT_1 forms heterodimers with AT_2 and the Mas receptor, which inhibits the signalling pathways and functions of the AT_1 receptor (AbdAlla et al., 2001; Kostenis et al., 2005). AT_1 also forms a heterodimer with the bradykinin B2 receptor (AbdAlla et al., 2000). Both AT_1 dimers have been shown to play important roles in the development of pathological conditions such as atherosclerosis and preeclampsia (Takezako et al., 2017).

4.9.2 AT_1 signaling

AT_1 receptors are coupled to multiple signal transduction processes that lead to diverse biological actions such as smooth muscle contraction, neuronal activation, neurosecretion, ion transport, cell growth, and proliferation (de Gasparo et al., 2000). For example, AT_1 receptors couple to a range of intracellular signaling molecules including G proteins: $G\alpha_{q/11}$, $G\alpha_{12/13,}$ and G_i which activate downstream effectors including phospholipase C (PLC), phospholipase A_2 (PLA2), phospholipase D (PLD), adenylyl cyclase and ion channels such as L-type and T-Type voltage-sensitive calcium channels (de Gasparo et al., 2000; Mehta & Griendling, 2007). $G\alpha_{q/11}$ mediated signaling is the primary transduction mechanism initiated by Ang II in its major physiological target tissues. Ang II binding to $G\alpha_{q/11}$-coupled AT_1 results in dissociation of $G\alpha$ from $G\beta\gamma$ and activation of PLC. This results in the hydrolysis of phosphatidylinositol-4,5 biphosphate (PIP_2) and formation of inositol trisphosphate (IP_3) and diacylglycerol (DAG) which are involved in Ca^{2+} mobilization from the sarcoplasmic reticulum and stimulation of protein kinase C (PKC), respectively (Toyz & Berry, 2002). In addition to PLC, Ang II induced AT_1 activation via $G\alpha_{q/11}$ stimulates phospholipases A2, and D. The cascade induced by phospholipase D and A2 leads to the formation of fatty acids such as prostaglandins and leukotrienes (Chung et al., 1999). AT_1 signal transduction via G_i and $G_{12/13}$ leads to inhibition of adenyl cyclase, regulation of L-and T-type Ca^{2+} channels and activation of PLD, Rho GTPases or Rho kinase (Toth et al., 2018).

In addition to activating the G-protein-dependent pathways, Ang II also signals via non-G-protein-dependent pathways through activation of various intracellular protein kinases including receptor and non-receptor tyrosine kinases. Ang II also activates serine/threonine kinases including mitogen-activated protein kinase (MAPK) family kinases and PKC which are implicated in cell growth and hypertrophy (Forrester et al., 2018). Ang II also activates NAD(P)H oxidases and results in the generation of ROS which are involved in inflammation (Dandona et al., 2007). Additionally, the AT_1 receptor also couples to intracellular signaling that extends to the nucleases which regulate transcription and expression of proteins that control cell growth responses and cell proliferation in several Ang II target tissues (de Gasparo et al., 2000).

Agonist activated AT_1 also associates with β-arrestins to initiate signaling pathways independent of G protein signaling. β-arrestins, were originally characterized as terminators of G-protein signalling, however, studies have shown that β-arrestin recruitment results in extracellular signal-regulated kinases ERK1 and ERK2 (ERK1/2) activation, independent of G proteins (Kim et al., 2005). Several AT_1 ligands have been demonstrated to preferentially activate G protein independent signaling that is mediated by β-arrestins. Ligands that preferentially activate one pathway are known as biased agonists. AT_1 biased agonists that have been shown to selectively activate the β-arrestin mediated pathway include the Ang II analog SII Ang, TRV120023 and TRV027. The β -arrestin biased AT_1 agonists have exhibited beneficial cardiovascular effects (Takezako et al., 2017).

4.9.3 AT_1 receptor desensitization

In most GPCRs, agonist induced activation is transient and rapidly turned off by receptor desensitization (Kelly et al., 2008). Correspondingly, the effect of Ang II on its target tissues is transient and the tissue is desensitized to further agonism (Sasamura et al., 1994). Receptor desensitization of AT_1 can occur through a process that involves phosphorylation of the C terminal tail of the AT_1 by G protein-coupled receptor kinases (GRKs) followed by recruitment of β-arrestin that impairs further G protein coupling (Wang et al., 2018).

4.9.4 Ang II independent activation of AT_1

AT_1 was identified as the first mechanosensitive GPCR following the observation of AT_1 activation by mechanical stress through an Ang II independent mechanism in cardiomyocytes (Zou et al., 2004). AT_1 is also activated by agonistic autoantibodies. As such, agonist independent AT_1 activation in response to mechanical stress and AT_1-directed autoantibodies has been demonstrated and is implicated in the development of pathologic conditions including cardiac hypertrophy and hypertension and preeclampsia (Takezako et al., 2017; Yasuda et al., 2008). Mechanical activation of AT_1 is believed to involve various mechanisms including $G_{q/11}$ coupled PLC activation resulting in production of IP_3 and transient receptor potential canonical (TRPC) channel opening (Schnitzler et al., 2011). Membrane stretch is also thought to allosterically stabilize a distinct β-arrestin biased AT_1 conformation triggering ligand-independent β-arrestin mediated signaling (Wang et al., 2018). Several ARBs harbor variable inverse agonist activity for AT_1. Inverse agonists are compounds that stabilize the inactive conformation of receptors and reduce the constitutive activity of the receptor or the agonist-independent receptor activity (Yasuda et al., 2008). Thereby, AT_1 inverse agonists such as candesartan can inhibit ligand-independent activation of AT_1 to provide therapeutic effects for such disease states (Takezako et al., 2017).

4.9.5 AT_2 receptor

AT_2, which consists of 363 amino acids and has a mass of 41kDa, is only 34% identical to AT_1. In humans, AT_2 is encoded by the gene *AGTR2* which is located in the X chromosome resulting in sex differences in its expression and function (Juillerat-Jeanneret, 2020). AT_2 is highly expressed in fetal tissue but receptor expression declines rapidly after birth suggesting that AT_2 may play an important role in fetal development (Mehta & Griendling, 2007). In adults, AT_2 is expressed in the pancreas, heart, kidney, ovary, brain, and vasculature (Toyz & Berry, 2002). In the human colon, AT_2 receptors are localized in parts of mesenchymal cells, crypt cells and surface epithelium (Hirasawa et al., 2002).

Although the AT_2 receptor has basic structural features commonly shared by GPCRs, its signaling mechanisms are less clearly defined compared with the AT_1 receptor. The AT_2 receptor does not demonstrate any of the classical features of G-protein signaling such as modulation of Ca^{2+} or cyclic AMP (Porrello et al., 2009). A few studies have shown that AT_2

couples through G_i in some cell types (Hayashida et al., 1996). However, there is limited data and discrepancies in findings on AT_2 G-protein coupled signaling mechanisms.

The AT_2 receptor is thought to play a tissue-protective role and is upregulated in pathological conditions. AT_2 has an affinity for Ang II, Ang III, and Ang (1-7) and is believed to mediate effects counteracting AT_1 and analogous to those of the Mas receptor. The effects via AT_2 are known to antagonize AT_1 mediated effects by inhibiting its signaling pathways via activation of tyrosine or serine/threonine phosphatases (Hammer et al., 2017; Mehta & Griendling, 2007). Effects mediated by AT_2 include apoptosis, tissue hypertrophy, and vasodilation. Thus, AT_2 agonists have beneficial effects on cardiovascular and fibrosis diseases (Dasgupta & Zhang, 2011). AT_2 receptors are antagonized by tetrahydro-imidazopyridines such as PD123319 (Toyz & Berry, 2002).

4.10 Ang II and pain

Ang II and its receptors are expressed in various parts of the nervous system that are involved in pain and several studies have demonstrated the role of Ang II in nociception.
Ang II has been shown to participate in either nociceptive or antinociceptive transmission in different areas of the brain. For example, the injection of Ang II into the caudal ventrolateral medulla (CVLM), an area which is important in modulation of pain, elicited hyperalgesia that was attenuated by local administration of the AT_1 antagonist, losartan (Marques-Lopes et al., 2009). In contrast, injection of Ang II into the periaqueductal gray matter (PAG) which plays key roles in descending pathways that control nociceptive inputs into the spinal cord, induced hypoalgesia that was inhibited by both AT_1 and AT_2 antagonists (Pelegrini-Da-Silva et al., 2005). Additionally, intrathecal administration of Ang II induced nociceptive behavioral responses through activation of p38 MAPK signalling mediated through AT_1 (Nemoto et al., 2013).

Systemic administration of losartan has been shown to have analgesic effects by inhibiting NFκB and the production of proinflammatory cytokines and chemokines including IL-1β, TNF-α, and MCP in DRGs in a neuropathic pain model (Kim et al., 2019). Similarly, analgesic effects of AT_2 receptor antagonists have been reported in rodent neuropathic pain models and early trials evaluating the therapeutic potential of the highly selective AT_2 antagonist EMA401 in patients with postherpetic neuralgia showed promising results (Rice et al., 2014). However,

phase 2b studies evaluating the safety and efficacy of EMA401 were terminated prematurely due to side effects observed in cynomolgus monkeys, therefore efficacy of EMA401 could not be confirmed (Rice et al., 2021).

4.11 Ang II mechanism of action in neurons

AT_1 receptor mediated signaling pathways in neurons are similar to those observed in other Ang II target cells (de Gasparo et al., 2000). AT_1 receptor activation in neurons cocultured from the rat hypothalamus and brain stem results in activation of PLC with subsequent formation of DAG and IP_3 which leads to an increase of intracellular Ca^{2+} and activation of PKC and calcium/calmodulin kinase II. The kinases open Ca^{2+} and close K^+ channels resulting in neuronal excitation (de Gasparo et al., 2000; Sumners & Gelband, 1998). On the other hand, activation of the AT_2 receptor has been shown to decrease neuronal excitability in cultured rat hypothalamus and brain stem neurons by decreasing the activity of T-type Ca^{2+} channels and stimulating a delayed rectifier K^+ current (I_K) and a transient K^+ current (I_A) (de Gasparo et al., 2000; Sumners & Gelband, 1998).

4.12 Expression of angiotensin receptors in colon innervating nerves

We examined the expression of angiotensin receptors in sensory neurons projecting to the colon in a database from a study conducted in our group. Data from this study shows *Agtr1a* is restricted to the mNonPeptidergic (mNP) population of neurons which is associated with nociceptors, whereas *Agtr1b* is expressed across all the seven population. By contrast *Agtr2* is poorly expressed across all the seven populations (Hockley et al., 2018). The Expression of angiotensin receptors among the 7 neuronal populations is shown in Figure 34 below.

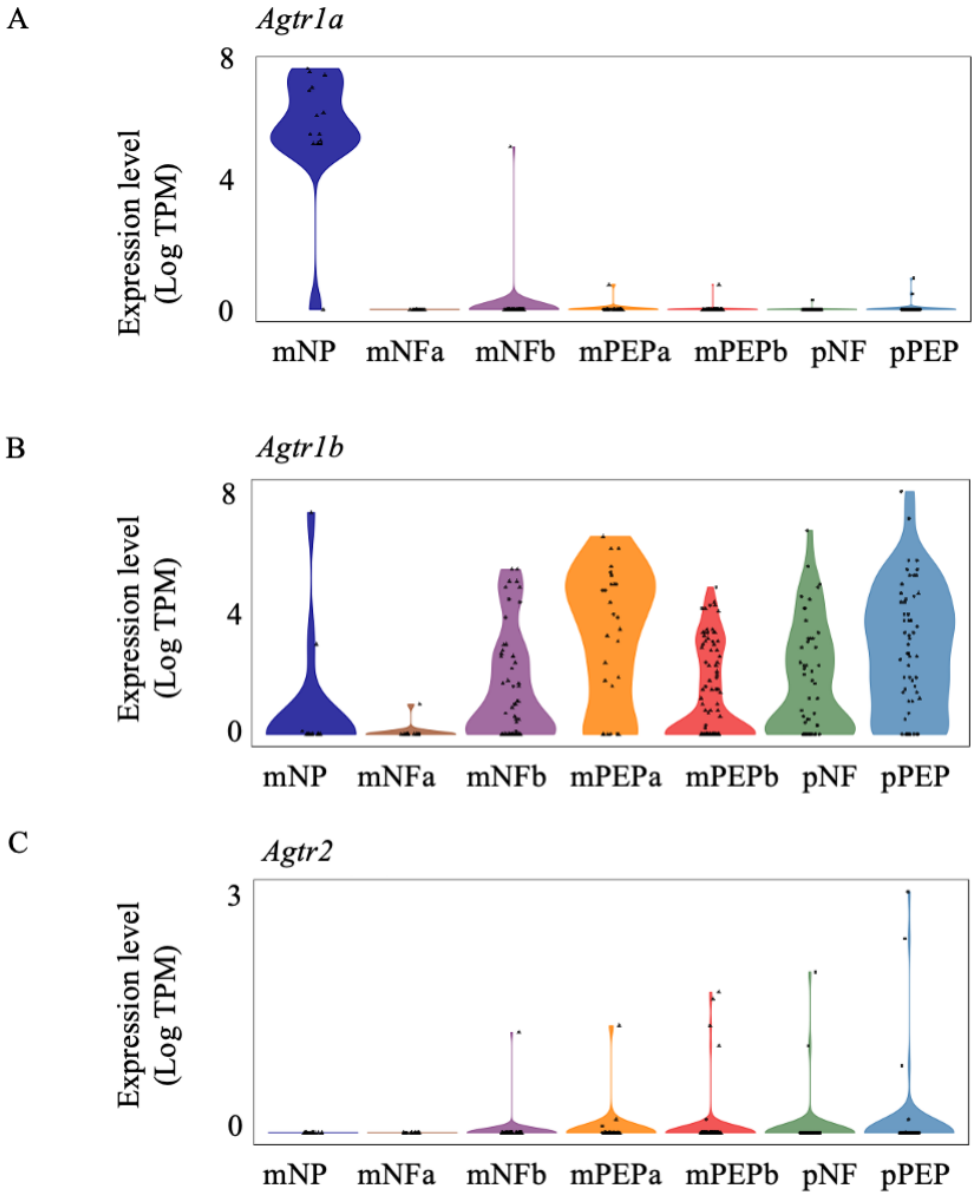


Figure 34. Expression of angiotensin receptors in mouse colonic DRG neurons.

(A) Agtr1a, (B) Agtr1b and (C)Agtr2 expression within colonic sensory neurons. Each black dot represents data from a single colonic sensory neuron isolated from either thoracolumbar (triangle) or lumbosacral (circle) DRG. The expression profile of each gene is demonstrated in each of the subtypes (mNeuroFilament-a (mNFa), mNeuroFilament-b (mNFb), mNonPeptidergic (mNP), mPeptidergic-a (mPEPa), mPeptidergic-b (mPEPb), pNeuroFilament (pNF) and pPeptidergic (pPEP)). Expression values are expressed in Transcript-Per-Million (Log [TPM]).

Additionally, we examined the co-expression of *Agtr1a and Agtr1b* with the Nav1.8 gene, *Scn10a* (Figure 35). Nav1.8 is exclusively expressed in nociceptors, therefore a strong co-expression of *Agtr1a* and *Scn10a* demonstrates *Agtr1a* receptors are expressed in nociceptive neurons and could thereby play a role in nociceptive signalling from the GI tract (Hockley et al., 2018; Lewin et al., 2004).

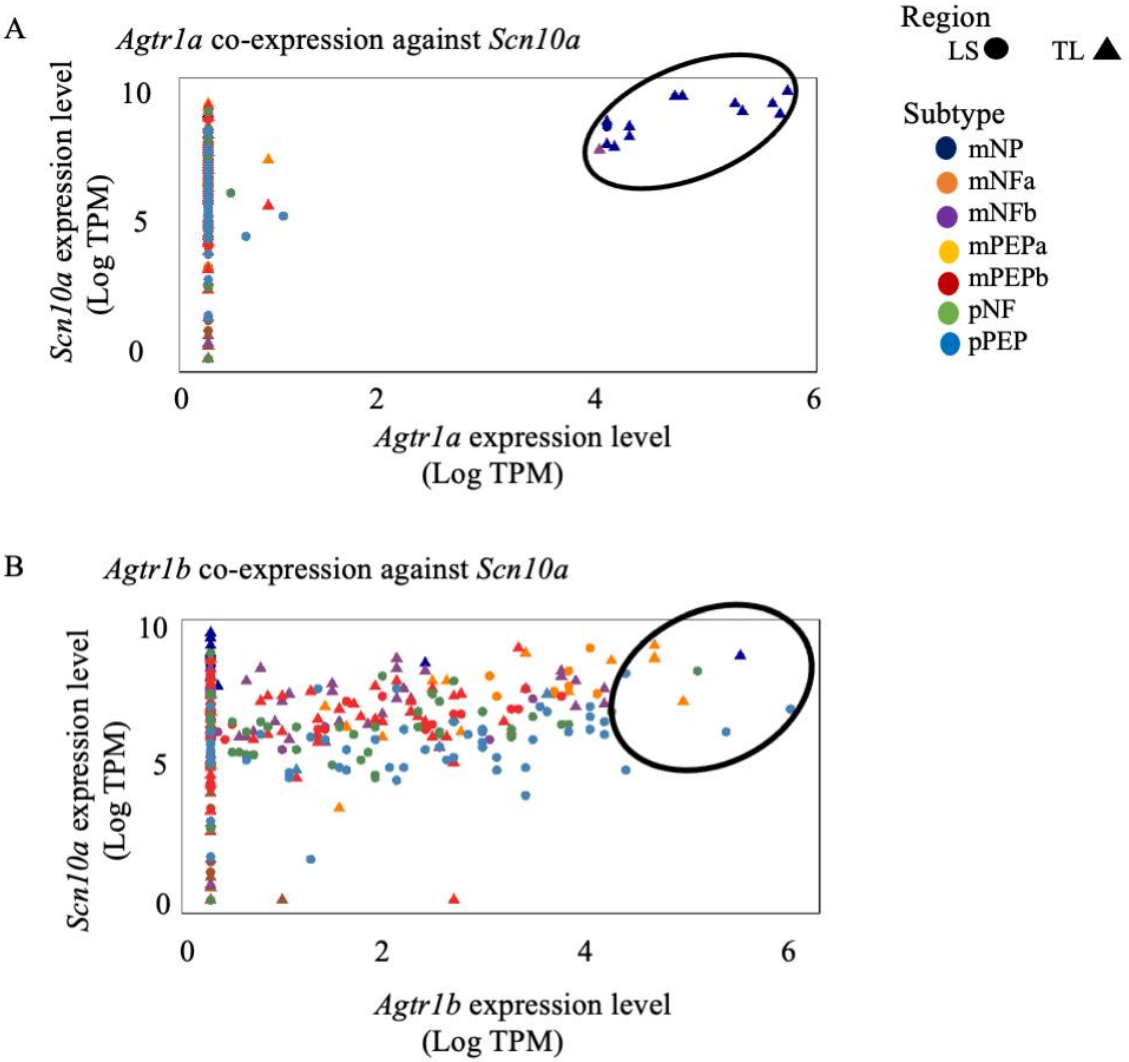


Figure 35. Co-expression of angiotensin receptors with Nav1.8.

Co-expression Agtr1a (A) with Scn10a and (B) Agtr1b with Scn10a. Each black dot represents data from a single colonic sensory neuron isolated from either thoracolumbar (triangle) or lumbosacral (circle) DRG. Expression values are expressed in Transcript-Per-Million (Log [TPM]). The subpopulation of neurons that highly co-express the angiotensin receptors (Agtr1a and Agtr1b) and Scn10a is highlighted by the black oval.

4.13 AT_1 and TRPC3

Activation of TRPC3 by DAG has been demonstrated to mediate downstream signalling effects of AT_1 in cardiomyocytes (Onohara et al., 2006). Additionally, studies performed in the lab have demonstrated that TRPC3 is involved in the response to Ang II in DRG neurons. TRPCs (TRPC1-7) are Ca^{2+} permeable, non-selective cation channels that typically serve signalling transduction pathways downstream of GPCRs. The TRPC3, 6 and 7 subfamily of the TRPC channels can be directly activated by DAG which is generated in response to receptor phospholipase pathways (Tiapko & Groschner, 2018). Activation of this subfamily of TRPCs by DAG results in cation influx that depolarizes the membrane potential (Chen et al., 2020). We evaluated co-expression of *Agtr1a* and *Agtr1b* with TRPC3 in DRG neurons projecting to the colon and data from this study shows TRPC3 is strongly co-expressed with *Agtr1a* in sensory neurons innervating the colon (Figure 36) (Hockley et al., 2018).

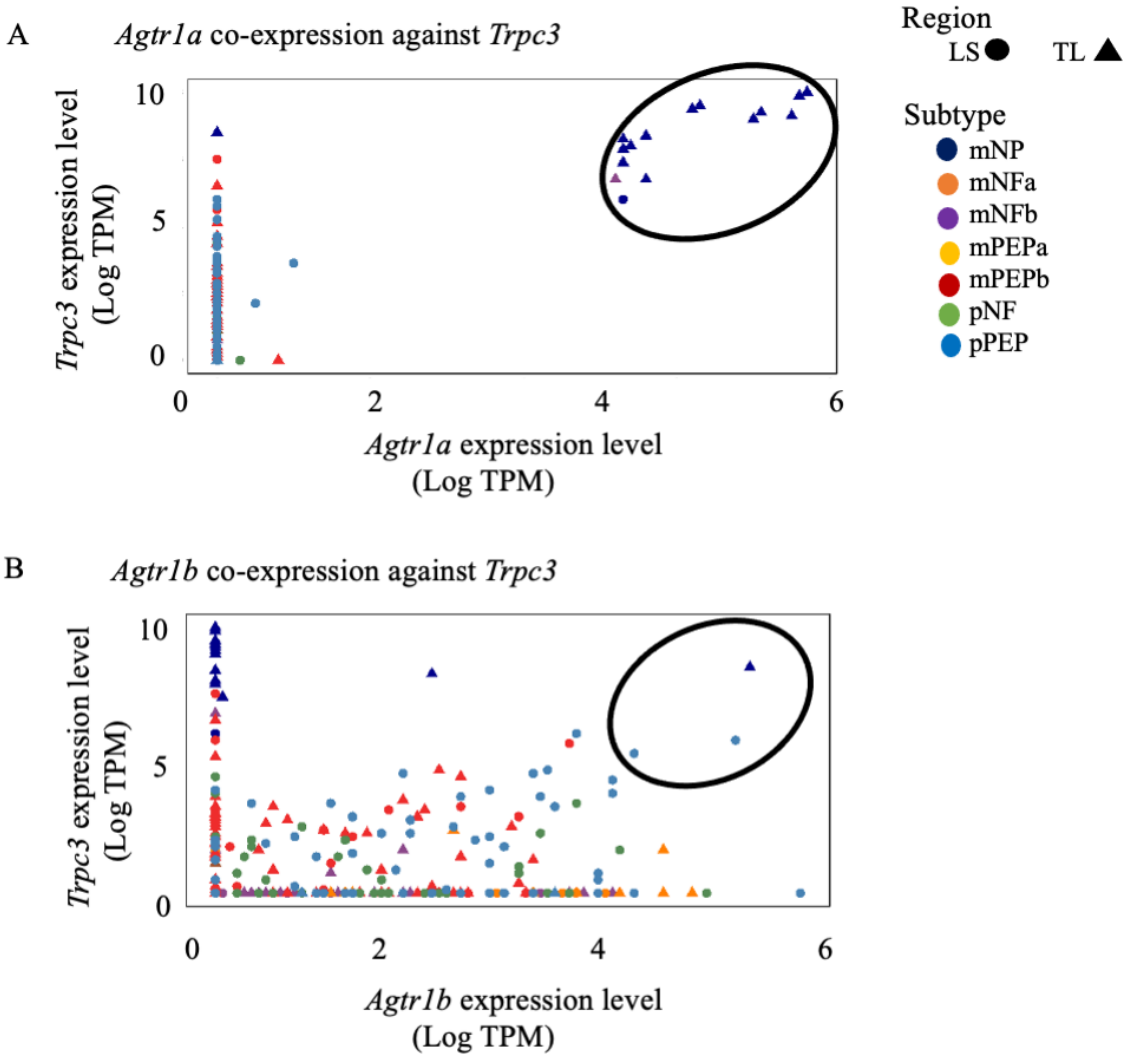


Figure 36. Co-expression angiotensin receptors with TRPC3

Co-expression of (A) Agtr1a against TRPC3. (B) Co-expression of Agtr1b against TRPC3. Each black dot represents data from a single colonic sensory neuron isolated from either thoracolumbar (triangle) or lumbosacral (circle) DRG. Expression values are expressed in Transcript-Per-Million (Log [TPM]). The subpopulation of neurons that highly co-express the TRPC3 and the angiotensin receptors is highlighted by the black oval.

4.14 Chapter aims

Ang II is implicated in inflammation and pain signalling in several pathological conditions. Studies have demonstrated the role of Ang II on the development of inflammation in IBD and the progression of the disease. However, the role of Ang II on pain transmission in IBD patients remains to be elucidated. Therefore, the goal of this study was to investigate the potential role of Ang II on pain signaling in the GI tract by examining its effects on mouse colonic nerves. Given that Ang II is elevated in IBD tissue, and the angiotensin receptors are present in sensory neurons innervating the gut, we hypothesized that Ang II stimulates colonic nerves and plays a role in nociceptive signaling from the GI tract by activating the angiotensin receptors expressed on the lumbar splanchnic nerve (LSN).

This chapter aims to:

1. Investigate the effects of Ang II on LSN activity.
2. Evaluate the effect of angiotensin receptor blockers on the LSN response to Ang II.
3. Determine the signaling mechanism of Ang II on the LSN.
4. Investigate the effect of Ang II on the LSN response to colonic ramp distension
5. Investigate the effect of AT_1 blockers on the LSN response to colonic ramp distension

4.15 Experimental protocols

4.15.1 Whole nerve LSN recordings

Electrophysiological recordings were used to evaluate the effects of Ang II on the LSN. The tissue was set up as described in the methods chapter. Once spontaneous nerve activity stabilized and baseline activity recorded, 20 ml of Ang II was administered to the colonic preparation by bath application via an inline heater at 7 ml/minute and changes in nerve activity in response to Ang II relative to the baseline were observed. In experiments with the antagonists, the antagonists were administered in 50 ml Krebs buffer prior to and during the administration of Ang II (Figure 37). In experiments with Ca^{2+} free Krebs, tissue bath perfusion was switched from supplemented carbogenated Krebs buffer to Ca^{2+} free Krebs (containing 2.5 mM Mg^{2+} and 0.1 mM EGTA) prior to treatment with Ang II.

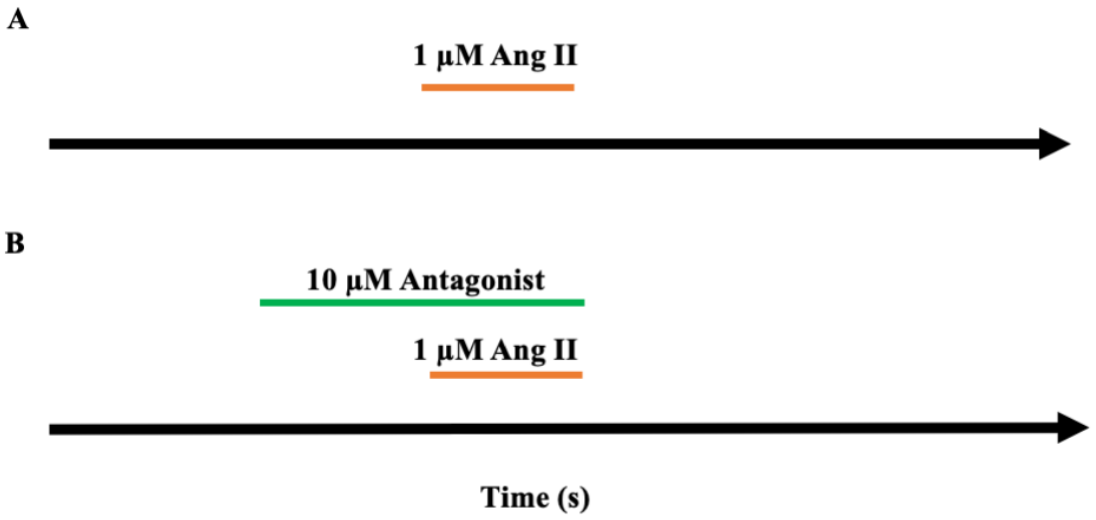


Figure 37. Schematic of the protocol used to evaluate the effect of Ang II on LSN activity.

(A) shows the protocol used to evaluate the effects of Ang II on whole nerve preparations. (B) shows the protocol used to evaluate the effects of angiotensin receptor blockers on the LSN response to Ang II.

To evaluate the effect of Ang II on the LSN response to ramp distension, five ramp distensions were performed with 20 ml of 1 µM Ang II administered between distension 3 and distension 4 (Figure 38). The AT_1 blockers valsartan, losartan and EMD 66684 were administered during distension 4 to evaluate the effects of the ARBs on the LSN response to mechanical stimulation. Control experiments were conducted in which the vehicle (0.1% DMSO) was administered prior to and during distension 4 (Figure 38).

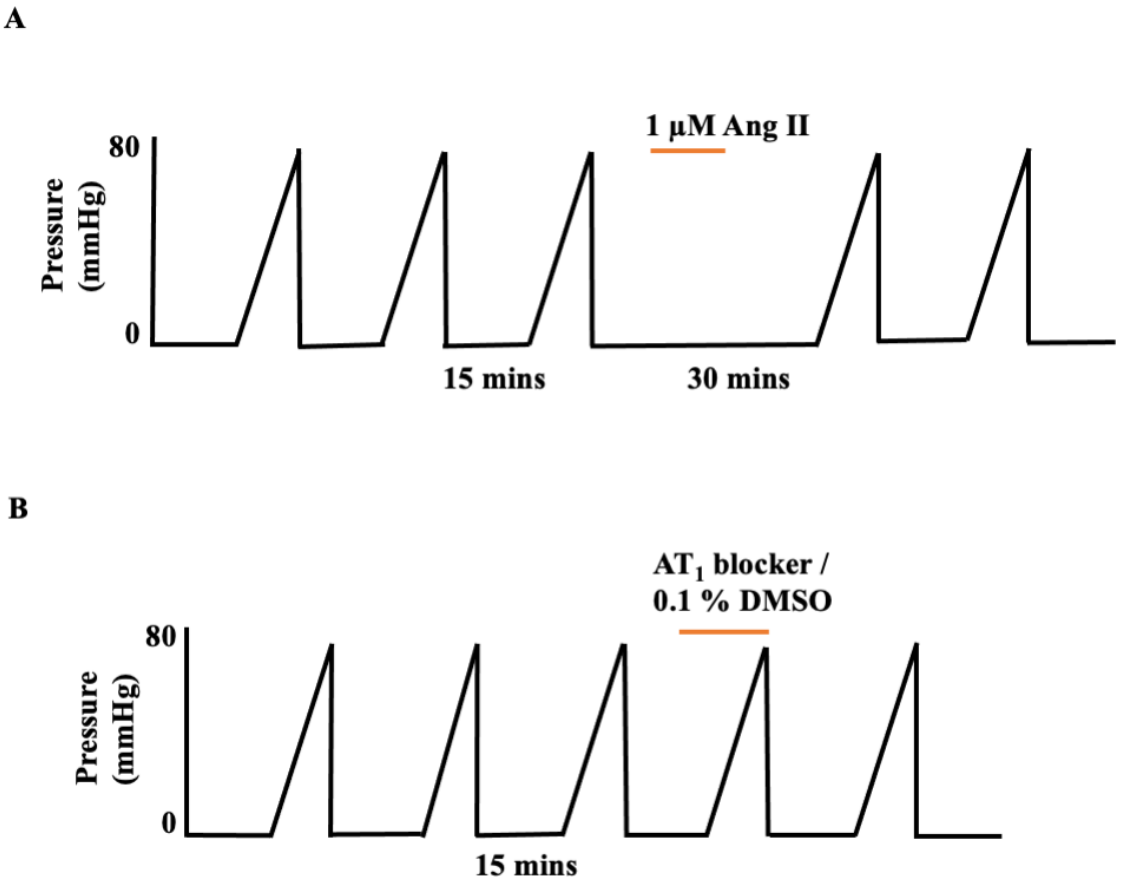


Figure 38. Schematic of protocols used to evaluate the effect of Ang II and AT receptor blockers on the LSN response to colonic ramp distension.

(A) Protocol used to evaluate the effect of Ang II on the LSN response to ramp distension. (B) Protocol used to evaluate the effect of AT_1 blockers on the LSN response to colonic ramp distension.

4.15.2 Few fibre studies

To classify the population of nerve fibres stimulated by Ang II, nerve activity was recorded from teased fibres of the LSN. Once nerve activity stabilized, two ramp distensions were performed followed by separate treatments of 1 µM Ang II, 1 µM bradykinin and 0.5 µM capsaicin (Figure 39).

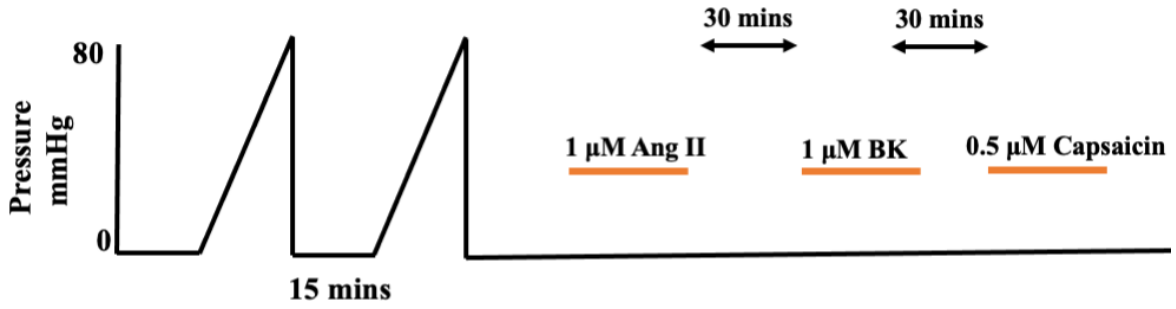


Figure 39. Schematic of the protocol used to evaluate Ang II on few fibres of the LSN.

Two ramp distensions 15 minutes apart were performed followed by LSN treatments with Ang II, bradykinin and capsaicin given 30 minutes apart.

4.15.3 Data analysis

The effect of the drug treatment was determined by subtracting the averaged baseline activity recorded 6 minutes before application of the drug from the nerve discharge over time following treatment with the drug. All statistical analysis were performed using GraphPad Prism 9.

4.16 Drugs

In the current study, the AT_1 blockers losartan, valsartan and EMD 66684 were utilized to block the effects of Ang II on colonic afferents. Losartan, which competitively binds to AT_1 was the first ARB to be developed and has a high affinity for AT_1 (Ki ~16 nM for AT_1 vs >100 µM for AT_2) and is commonly used to treat hypertension (Chung et al., 1999; Macari et al., 1993). Valsartan displays noncompetitive inhibition for the AT_1 receptor and is commonly used to treat high blood pressure, heart failure and diabetic kidney disease. EMD 66684 is a highly potent and very selective non-peptide AT_1 receptor antagonist. The selective AT_2 antagonist, PD123319, was used to block AT_2 to evaluate the effects of blocking the AT_2 receptor on colonic nerve activity in response to Ang II. PD123319 has a high affinity for AT_2 and is 10000 times more selective for AT_2 than AT_1 receptors (Macari et al., 1993).

The structures and IC_{50} values of the angiotensin receptor antagonists used in these studies is shown in Table 3 below.

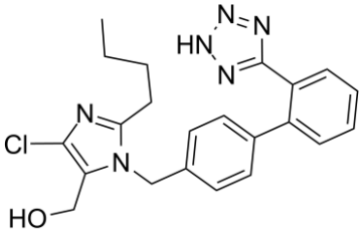
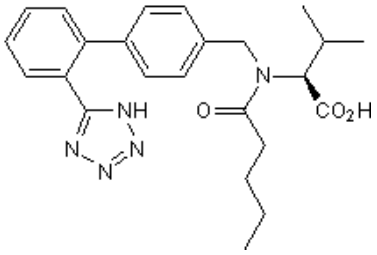
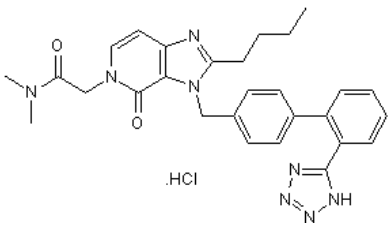
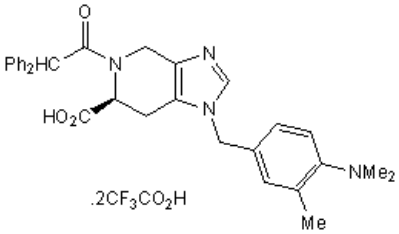
Antagonist	Target	Structure	IC_{50} (nM)
Losartan	AT_1		19 (rat adrenal cortex, (Chiu et al., 1989))
Valsartan	AT_1		20 (arterial smooth muscle cells, (Sironi et al., 2001))
EMD 66684	AT_1		0.7 (rat adrenal cortical membrane, (Mederski et al., 1994))
PD123319	AT_2		34 (rat adrenal tissue (Blankley et al., 1991))

Table 3. Angiotensin receptor blockers used in the present study.

4.17 Results

4.17.1 Angiotensin II on LSN activity.

Administration of Ang II resulted in a marked increase in LSN activity that subsided over time with ongoing nerve activity returning to baseline 20 minutes after drug administration. Increasing concentrations of Ang II (0.01, 0.1,1, and 3 μM) administered in separate preparations elicited a concentration dependent increase in afferent activity. Time to peak, determined as the time taken from the drug entering the bath to activity reaching its peak decreased with increasing concentrations of Ang II (e.g., time to peak: 0.01 μM Ang II, 525±37.75 s; 0.1 μM Ang II, 430±28.08 s; 1 μM Ang II, 350±24.08 s; 3 μM Ang II, 265.7±25.71 s) (Figure 40).

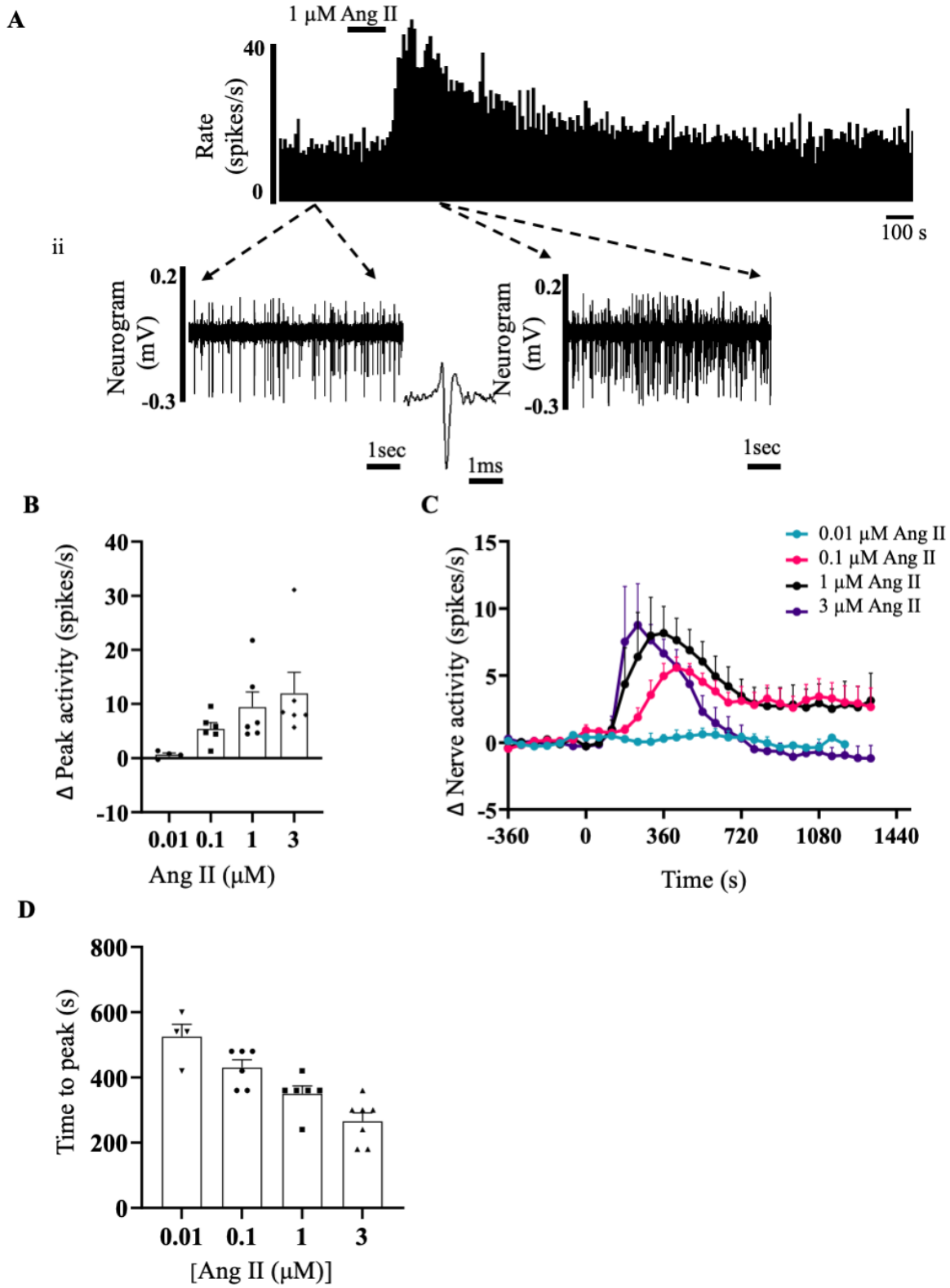


Figure 40. The effect of increasing concentrations of Ang II on LSN activity.

(A) Example rate histogram of the LSN response to 1 µM Ang II. Administration of Ang II is indicated by the black bar. (ii) Expanded trace of activity at baseline vs peak in response to Ang II. (B) Average change in peak activity in response to increasing concentrations (0.01, 0.1,1, and 3 µM) of Ang II. (C) Average response profiles to increasing concentrations of Ang

II. (D) Time taken from Ang II entering the bath to reach peak nerve activity. (N=4-6). Data are shown as mean ±SEM.

We elected to use 1 µM Ang II for subsequent studies evaluating the effect of ARBs on the LSN response to Ang II. Initial studies began by examining the LSN response to repeat application of 1 µM Ang II. The first application resulted in a robust increase in nerve activity (9.71±2.89 spikes/s, *N*=4), however, the 2nd application had no effect on LSN activity (0.78±1.09 spikes/s, *N*=4 animals) (Figure 41). Hence, experiments with the blockers were conducted separately.

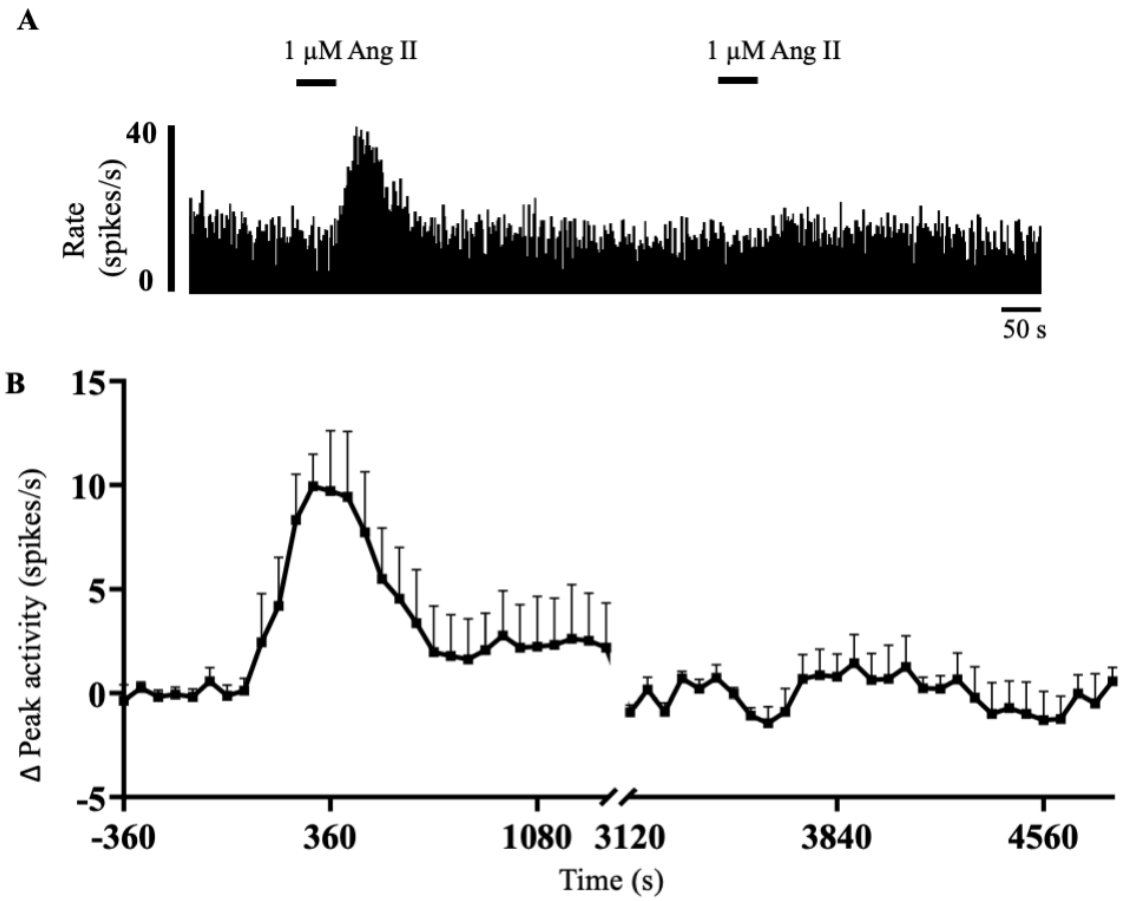


Figure 41. Repeat administration of Ang II on LSN activity.

(A) Example rate histogram of the colonic nerve response to repeat administration of 1 µM Ang II given 30 minutes apart. (B) Average response profile of LSN response to repeat administration of Ang II (N=4). Data are shown as mean ±SEM.

4.17.2 Angiotensin receptor blockers on the LSN nerve response to Ang II

Administration of 1 µM Ang II led to a robust increase in LSN activity with peak firing increasing by 9.43±2.79 spikes/s (*N*=6). The response to Ang II was abolished by pretreatment with AT_1 receptor blockers losartan, valsartan and EMD 66684 but not the AT_2 blocker PD123319 (Figure 42). Pretreatment with EMD 66684 (10 µM) resulted in an average change of 1.46±0.76 spikes/s (*p*=0.02, one-way ANOVA with Dunnett's multiple comparisons test, *N*=6), pretreatment with losartan (10 µM) resulted in an average change of -1.74±0.35 spikes/s (*p*=0.001, one-way ANOVA with Dunnett's multiple comparisons test, *N*=6) and valsartan (10 µM) resulted in an average change of 0.56±0.48 spikes/s (*p*=0.009, one-way ANOVA with Dunnett's multiple comparisons test, *N*=6) at peak activity. In contrast, Ang II administered in the presence of the AT_2 antagonist PD123319 (10 µM) resulted in an increase in nerve activity by 10.96±2.97 spikes/s (*p*=0.94, one-way ANOVA with Dunnett's multiple comparisons test, *N*=6) (Figure 43). Administration of the blockers had no effect on spontaneous baseline firing.

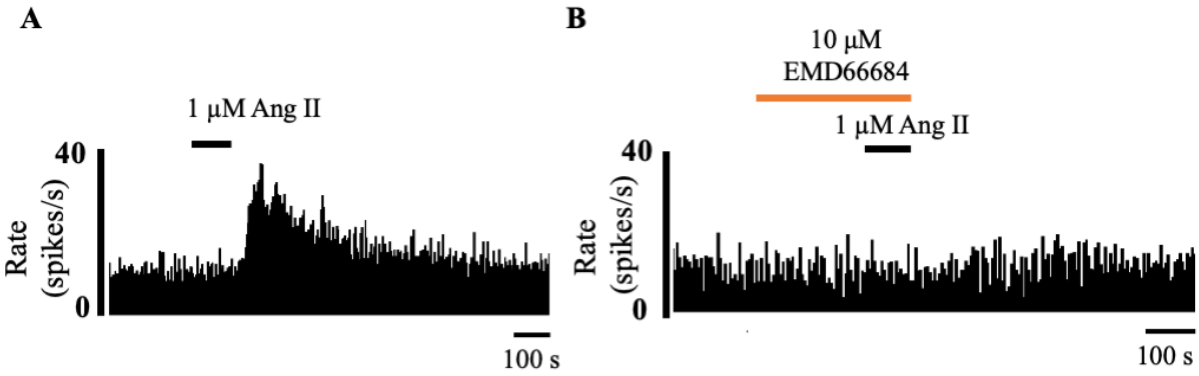


Figure 42. The effect of the AT1 antagonist, EMD 66684 on the LSN response to Ang II.

(A) Example rate histogram of the LSN response to 1 µM Ang II. (B) Example rate histogram of the LSN response to 1 µM Ang II administered following pre-treatment with EMD 66684, in a separate experiment. Application of Ang II is indicated by the black bar and EMD 66684 is indicated by the orange bar.

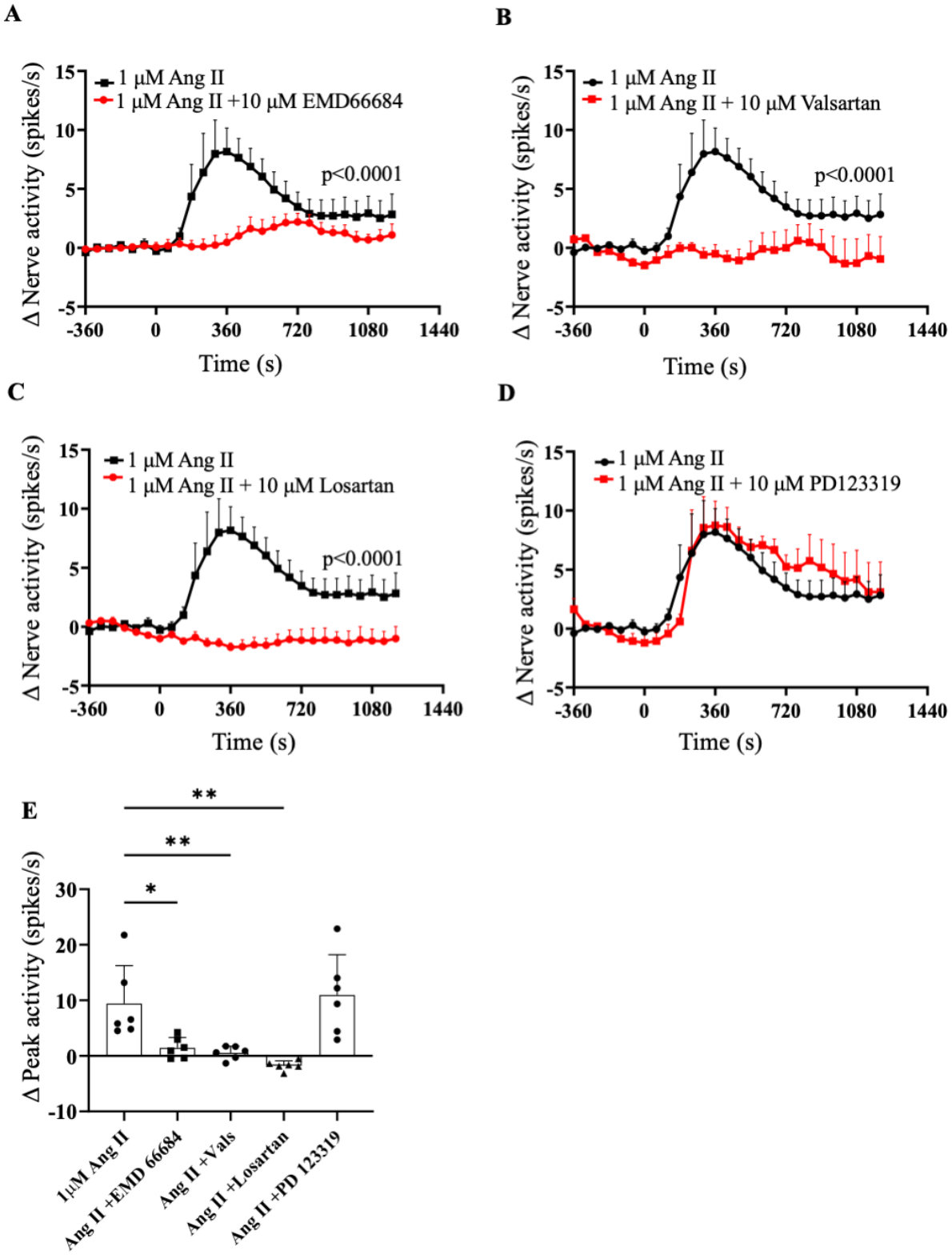


Figure 43. Angiotensin receptor blockers on the LSN response to Ang II.

Response profiles of LSN activity in response to Ang II with or without the AT_1 blockers: (A) EMD66684, (B) valsartan and (C) losartan and the AT_2 blocker: (D) PD 123319. (E) Mean peak LSN activity in response to Ang II following treatment with AT_1 and AT_2 blockers ($p < 0.05$, ** $p < 0.01$, one-way ANOVA with Dunnett's multiple comparisons test, N=6). Data are shown as mean ±SEM.*

4.17.3 AT_2 agonist on LSN activity

The absence of an inhibitory effect of the AT_2 antagonist on the response to Ang II indicated that blocking this receptor was ineffective in blocking the effects of Ang II. Therefore, we evaluated effect the AT_2 agonist CGP42112, on LSN activity to investigate any effect of AT_2 activation on the LSN. CGP42112 is a high affinity AT_2 agonist with Ki value of 0.24 nM. Administration of 1 µM CGP42112 did not alter colonic afferent activity (Figure 44). Combined, these results suggest that Ang II stimulates LSN activity via the AT_1 receptor.

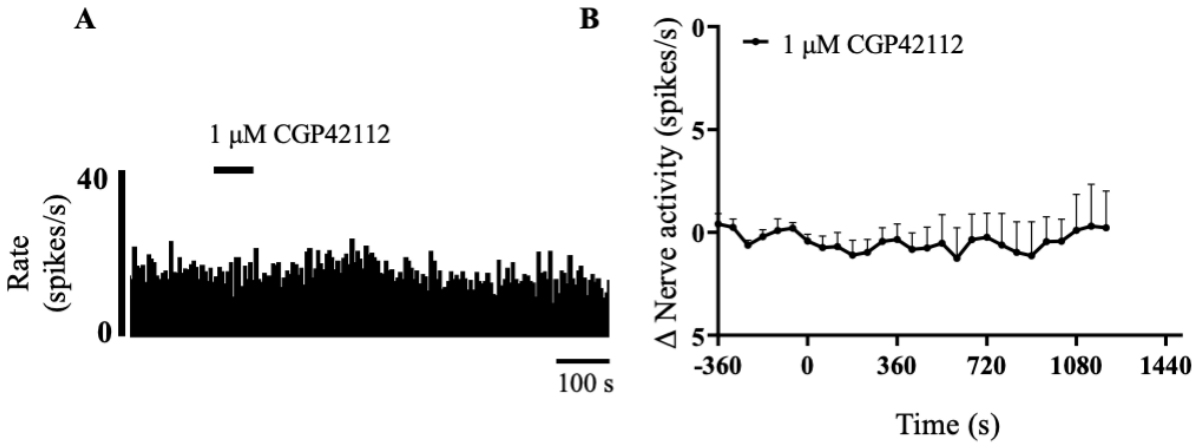


Figure 44. The effect of an AT_2 agonist (CGP42112) on LSN activity.

(A) Example rate histogram showing that the administration of 1 µM CGP42112 has no effect on LSN activity. (B) Average response profile of LSN activity following treatment with CGP 42112 (N=6). Data are shown as mean ±SEM.

4.17.4 Ang II on female mice

Next, we evaluated the effect of Ang II on female mice to assess any contribution of sex differences on the LSN response to Ang II. Spontaneous baseline activity was comparable between male and female mice (p=0.10, unpaired t test, N=4-6). Administration of Ang II in female mice resulted in nerve activity increasing by 5.09 ± 0.86 spikes/s at peak firing. The change in peak firing was comparable between the sexes (p=0.25, unpaired t test, N=4-6). However, statistical analysis using a two-way ANOVA indicate that the overall response to Ang II was reduced in female mice compared to males (p<0.0001, N=4-6) (Figure 45). These results suggest the response to Ang II may be reduced in female mice compared with male mice.

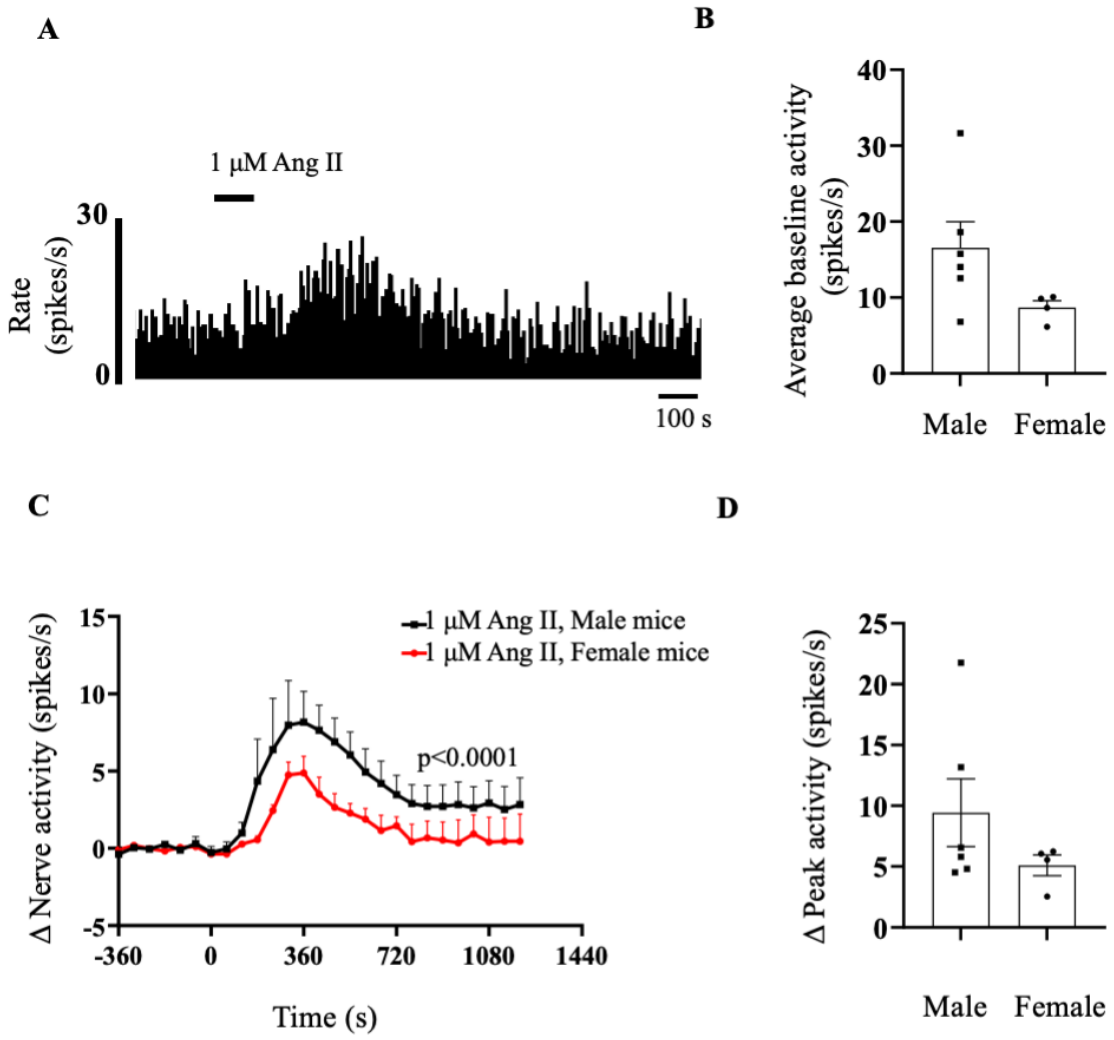


Figure 45. The effect of Ang II on female mice.

(A)Example rate histogram of the LSN response to 1 µM Ang II in female mice. (B) Bar graph showing average baseline activity 6 minutes prior to treatment with Ang II in male and female mice (p=0.10, unpaired t test, N=4-6). (C) Response profile showing the average colonic afferent response to Ang II in male vs female mice (p<0.0001, *two-way ANOVA with Bonferroni's multiple comparisons, N=4-6). (B) Bar graph showing the average change in peak activity in response to Ang II is comparable between male and female mice (p=0.25, unpaired t test, N=4-6). Data are shown as mean ±SEM.*

4.17.5 LSN response to Ang II in Ca^{2+} free Krebs

Next, we sought to determine the mechanism of action by which Ang II stimulates colonic afferents. We started by evaluating the effect of removing extracellular Ca^{2+} on the LSN response to Ang II. Nerve activity was allowed to stabilize for 30 minutes before Ang II was administered. Nerve activity significantly increased when perfusion was switched to Ca^{2+} free Krebs with the average baseline activity increasing by 43.36±10.54 spikes/s (p<0.05, paired t test, N=5). Administration of Ang II resulted in an increase in nerve activity that subsided in

magnitude over time. Peak nerve activity in response to Ang II increased by 20.44±5.34 spikes/s (*N*=5) (Figure 46). These results indicate the LSN response to Ang II does not depend on Ca^{2+} mediated mechanisms.

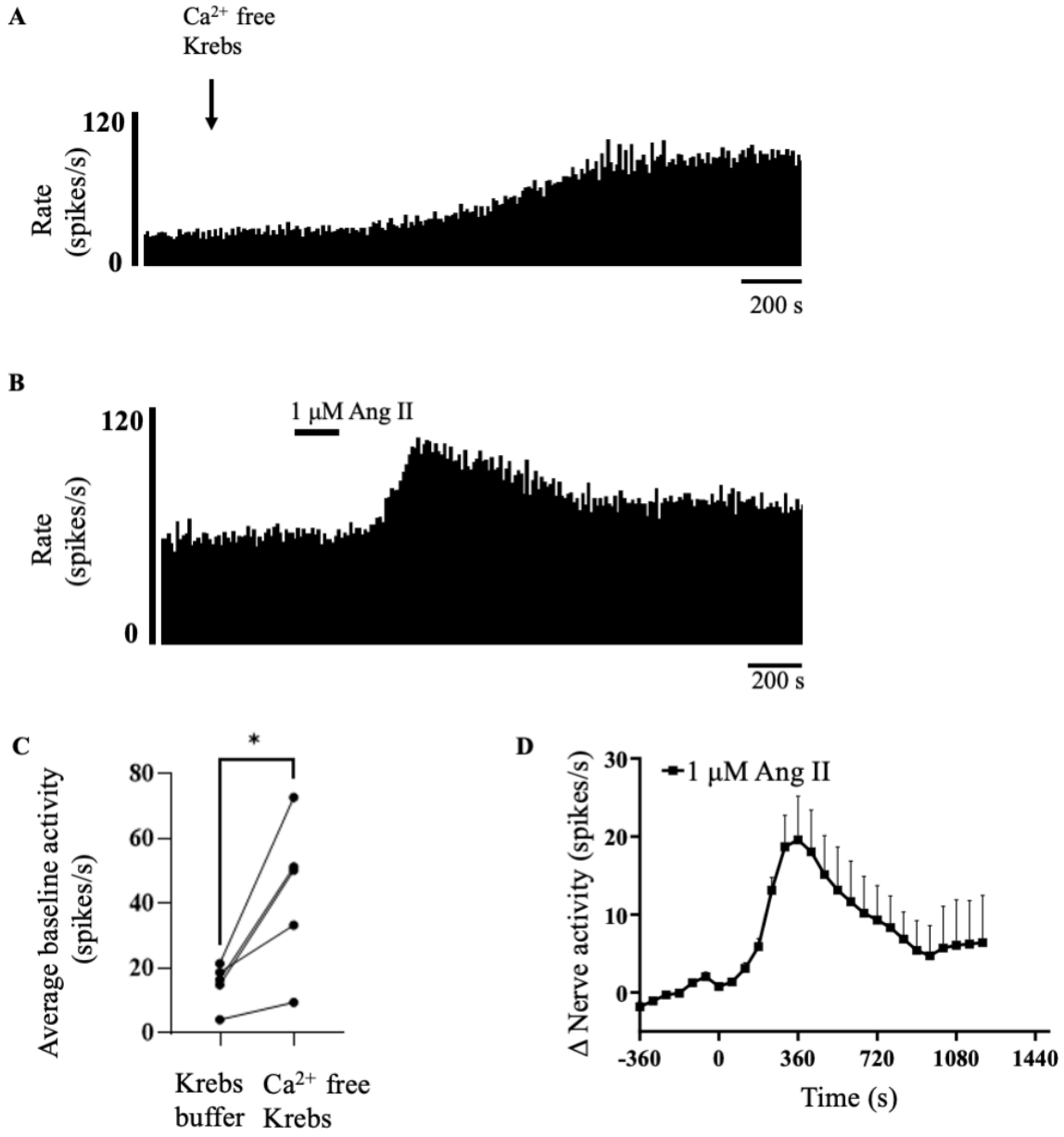


Figure 46. The effect on the LSN in Ca^{2+} free Krebs buffer.

***(A)** Example rate histogram of LSN activity in Ca^{2+} free Krebs buffer. (B) Example rate histogram of the colonic nerve response to 1 µM Ang II administered in Ca^{2+} free Krebs. (B) Average spontaneous nerve activity before and after switching to Ca^{2+} free Krebs (p=0.026, paired t test, N=5). (D) Average response profile of colonic afferent activity in response to Ang II in Ca^{2+} free Krebs. Data are shown as mean ±SEM.*

4.17.6 LSN response to Ang II in a gut free prep

Next, we examined the effect of Ang II on the isolated LSN by removing the colon and associated tissues from the preparation to measure nerve activity. To do this, the colonic preparation was set up as described in the methods chapter and once spontaneous baseline activity stabilized and was recorded for 20 minutes, the colon and surrounding tissues were carefully cut from the preparation.

Nerve activity was allowed to stabilize before administration of Ang II. Spontaneous nerve activity decreased following removal of the colon and surrounding tissues from the preparation (10.6±2.3 spikes/s vs 3.9±1.9 spikes/, p=0.21, paired t test, N=3). Administration of Ang II resulted in an increase in nerve activity (6.88±1.42 spikes/s, N=6) at peak activity that subsided over time to return to baseline activity (Figure 47). The findings from this study demonstrating that the effects of Ang II occur in part through the activation of vascular and mesenteric afferents and is not dependent on the activation of AT_1 receptors at non-neuronal sites within the gut.

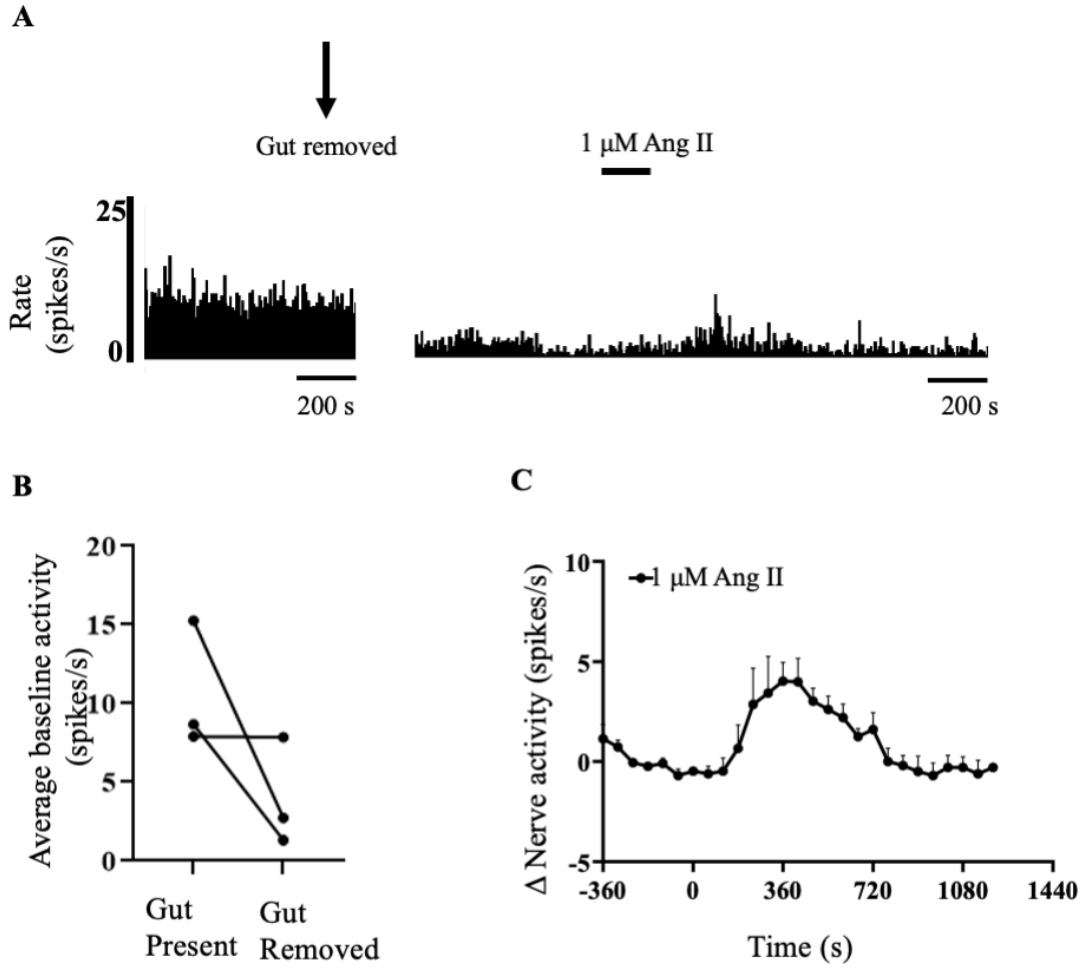


Figure 47. LSN response to Ang II in the gut free preparation.

(A)Example rate histogram of LSN activity prior to and after removal of the colon. (B) Average baseline activity before and after removal of the colon and associated tissues from the preparation (p=0.21, paired t test, N=3). (C) Response profile showing the average colonic afferent response to Ang II following removal of the colon (N=6). Data are shown as mean ±SEM.

4.17.7 The mechanism of action of Ang II on the LSN

Next, we aimed to elucidate the signalling mechanism that resulted in the increase in nerve activity following administration of Ang II. To do this, we targeted TRPCR3 which has been shown to mediate the DRG neuronal response to Ang II. In these studies, we hypothesized that blocking TRPC3 would inhibit the increase in neuronal activity of the LSN in response to Ang II.

Pre-treatment with 30 μM Pyr3, a selective TRPC3 antagonist that inhibits TRPC3 mediated Ca^{2+} influx (IC_{50}= 0.7 μM), did not alter the LSN response to Ang II compared with controls.

Nerve activity increased by 8.38±1.43 spikes/s following administration of Ang II in the presence of Pyr3 (Figure 48). These results suggest the colonic afferent response to Ang II is mediated by other mechanisms and blocking TRPC3 is not sufficient to block this response. The mechanism of action of Ang II on colonic afferents therefore remains to be elucidated.

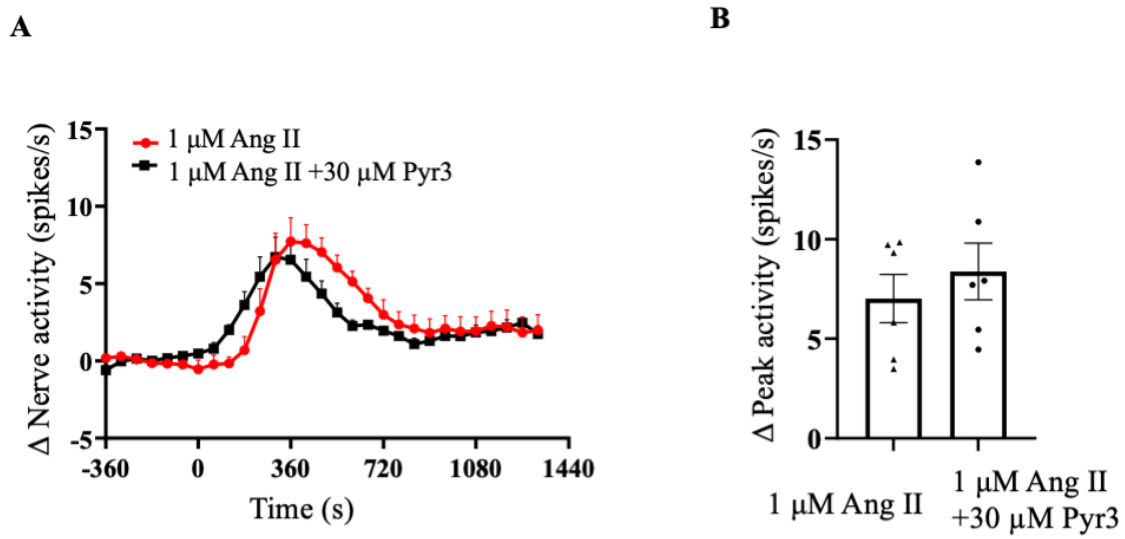


Figure 48. Effect of the TRPC3 blocker, Pyr3 on the LSN response to Ang II.

(A) Average response profile of colonic afferent activity in response to Ang II in the presence or absence of the TRPC3 blocker, Pyr3 (p=0.15, two-way ANOVA with Bonferroni's multiple comparisons test, N=6). (B) Bar graph showing the average change in peak activity in response to Ang II in the presence or absence with Pyr3 (p=0.48, unpaired t test, N=6). Data are shown as mean ±SEM.

4.17.8 Ang II on the LSN response to mechanical stimulation

Next, we sought to determine if Ang II would sensitize the LSN response to ramp distension. In control experiments, LSN responses to distension 3 and 4 were comparable, therefore, in these studies, Ang II was administered between distension 3 and 4. Distension 4 was performed after LSN activity had returned to baseline activity following treatment with Ang II and the LSN response to distension 4 was compared with the response to distension 3.

Increasing luminal pressure resulted in an increase in LSN discharge (for example, peak firing frequency in response to distension 1 increased by an average of 29.47±2.69 spikes/s, *N*=8). Application of Ang II between distension 3 and 4 resulted in a rapid increase in nerve discharge that subsided over time to return to baseline activity. LSN discharge increased by 21.90±7.72 spikes/s (*N*=8) at peak activity in response to Ang II. Peak activity in response to distension 4

was comparable with the peak response to distension 3 (p=0.7061, paired t test, N=8). Similarly, the overall response to distension 4 following treatment with 1 µM Ang II was unaltered compared with distension 3 (p=0.18, two-way ANOVA with Bonferroni's multiple comparisons test, N=8) (Figure 49). These results indicate there is no prolonged sensitization on the nerve response to ramp distension following treatment with Ang II.

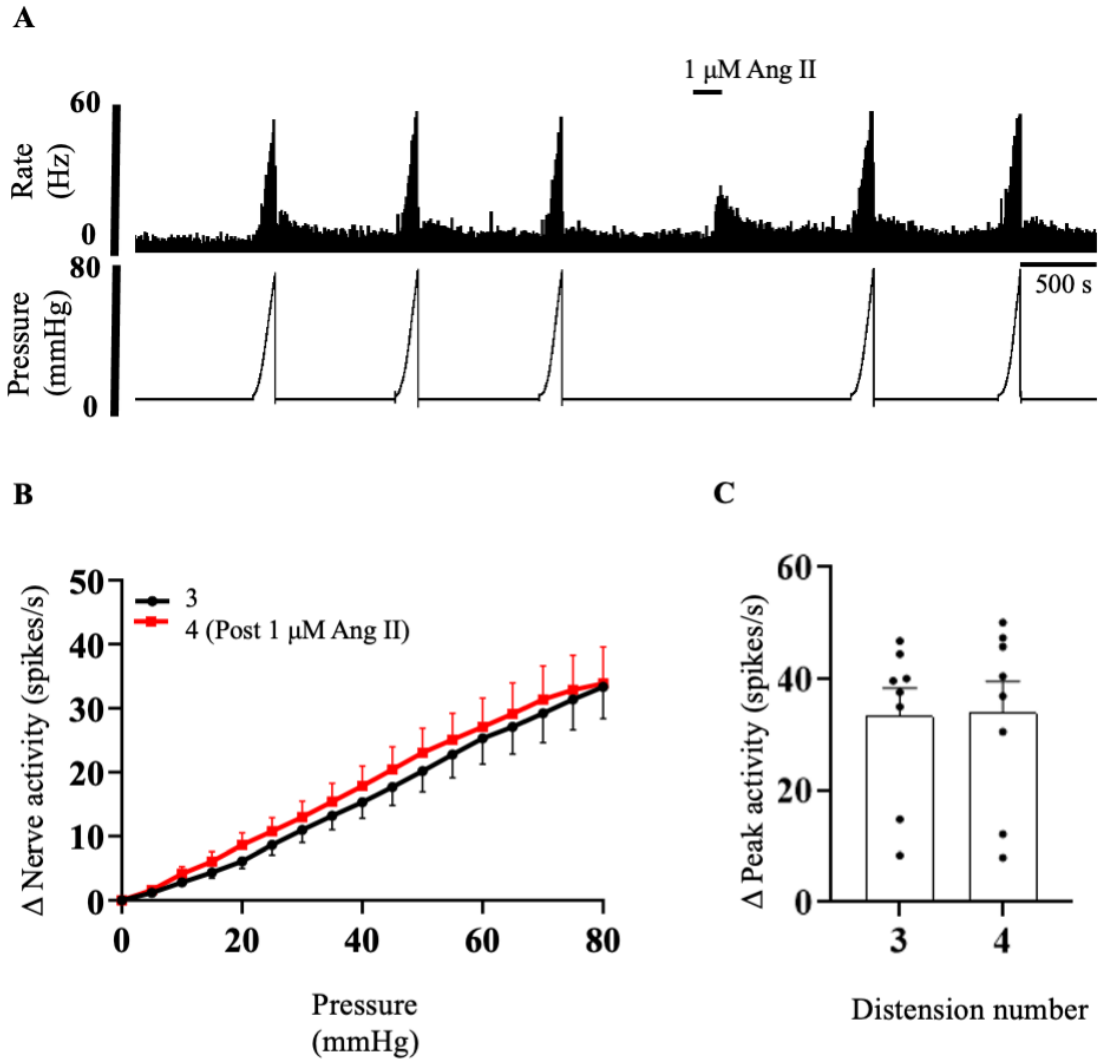


Figure 49. Effect Ang II on the LSN response to ramp distension.

(A) Example rate histogram of LSN activity in response to sequential (x5) slow ramp distensions (0-80 mmHg) with 1 µM Ang II administered between distension 3 and 4. (ii) Accompanying intraluminal pressure trace. (B) The response profile of LSN activity to distension 4 given after treatment with 1 µM Ang II (p=0.10, two-way ANOVA with Bonferroni's multiple comparisons test, N=8). (C) Peak firing in response to distension 3 and 4 with Ang II given before distension 4 (p=0.70, paired t test, N=8). Data are shown as mean ±SEM.

4.17.9 Few fibre experiments

Since there was no evidence of prolonged sensitization of the LSN by treatments with Ang II, additional studies were conducted to determine if Ang II stimulated mechanosensitive nerve fibers and to assess the sensitivity of these fibres to the algogenic mediators, capsaicin, and bradykinin. Electrophysiological recordings were made from teased fibres of the LSN and responses of these few fibres to ramp distension, Ang II, bradykinin, and capsaicin were examined. Figure 50 below is an example of a single unit responding to ramp distension, Ang II, bradykinin, and capsaicin.

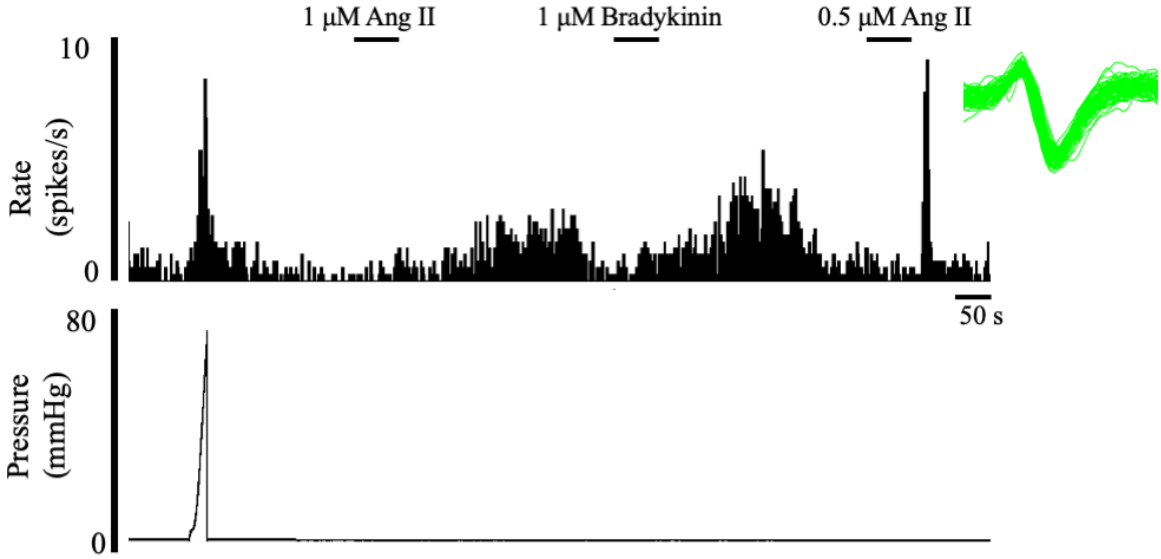


Figure 50. Single unit response to ramp distension, Ang II, bradykinin, and capsaicin.

Rate histogram of a single unit responding to ramp distension, Ang II, bradykinin and capsaicin and the accompanying pressure trace and overlay of action potential waveforms.

Using a combination of the waveform analysis function on spike 2 and manual discrimination of the action potential waveforms, I identified between 9-16 units per recording (*N*=3). In total 34 distinct fibres were identified across all 3 experiments. Of the 34 fibres, 7 responded to Ang II, bradykinin capsaicin and mechanical stimulation.

Table 4 summaries the proportion of ramp distension, bradykinin and capsaicin sensitive fibres that responded to Ang II. Combined, this data suggests Ang II stimulates nociceptors based on their response bradykinin, capsaicin and mechanical stimuli.

+Ang II +Mechanosensitive	**+Ang II +Bradykinin**	**+Ang II +Capsaicin**	**+Ang II +Bradykinin +Capsaicin +Mechanosensitive**
$\frac{14}{34}$	$\frac{10}{34}$	$\frac{18}{34}$	$\frac{7}{34}$

Table 4. Classification of Ang II Sensitive fibres.

4.17.10 AT_1 inverse agonists on LSN mechanosensitivity

Finally, we evaluated the potential contribution of AT_1 on the LSN response to mechanical stimulation. Mechanical stretch has been shown to activate ligand independent signaling of AT_1. ARBs with inverse agonist activity have been shown to inhibit the agonist independent mechanical stress induced by AT_1 receptor activation. Losartan and valsartan have been demonstrated to exhibit inverse agonist activity with valsartan displaying stronger inverse agonist than losartan (Miura et al., 2008; Takezako et al., 2015). Therefore, in the present study, we evaluated the effect of losartan, valsartan and EMD 66684 on the LSN response to mechanosensitivity.

In control experiments, the vehicle, DMSO, was given prior to and during distension 4. Repeated colonic ramp distension resulted in an increase in nerve activity, with the average peak change decreasing across the protocol. The responses stabilized by distension 3. Administration of DMSO had no effect on the LSN nerve response to colonic ramp distension (Figure 51).

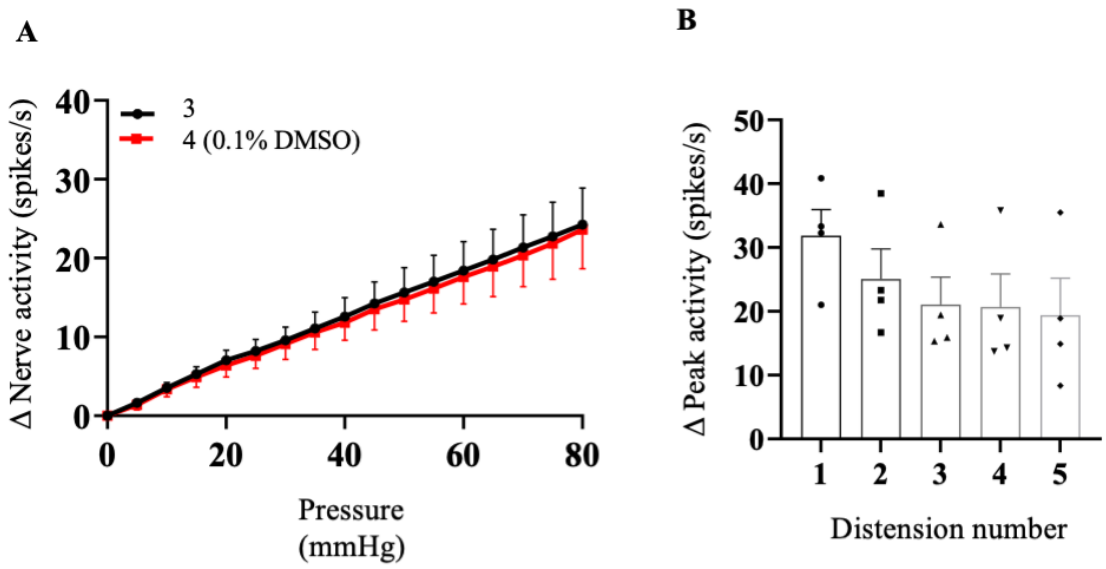


Figure 51. LSN response to ramp distension with DMSO treatments.

(A) The average change in nerve activity in response to distension 3 and distension 4 with 0.1% DMSO administered prior to and during distension 4 (p=0.51, two-way ANOVA with Bonferroni's multiple comparisons test, N=5). (B) The average peak firing in response to sequential (x5) slow ramp distension with DMSO administered prior to and during distension (N=5). Data are shown as mean ±SEM.

Initial experiments evaluated the effect of losartan on the LSN response to ramp distension. Losartan was administered prior to and during distension 4 and the response to distension 4 following treatment with losartan was compared with the response to distension 3. There was a decrease in the peak response to distension 4 compared with the response to distension 3 (31.19±5.58 spikes/s vs 26.46±4.7 spikes/s, p=0.03, paired t test, N=6). Similarly, the overall response was altered following treatment with losartan (p=0.015, two-way ANOVA with Bonferroni's multiple comparisons test, N=6) (Figure 52). Additional analysis was conducted to compare the losartan treated experiments with time matched DMSO controls. The responses following DMSO, or losartan were normalized to the response to D3 in each of the experiments and the % change in the response during D4 plotted. Compared with DMSO time matched controls, treatment with losartan significantly reduced the LSN response to colonic ramp distension (normalized overall response: p<0.0001, two-way ANOVA with Bonferroni's multiple comparisons test, N=6).

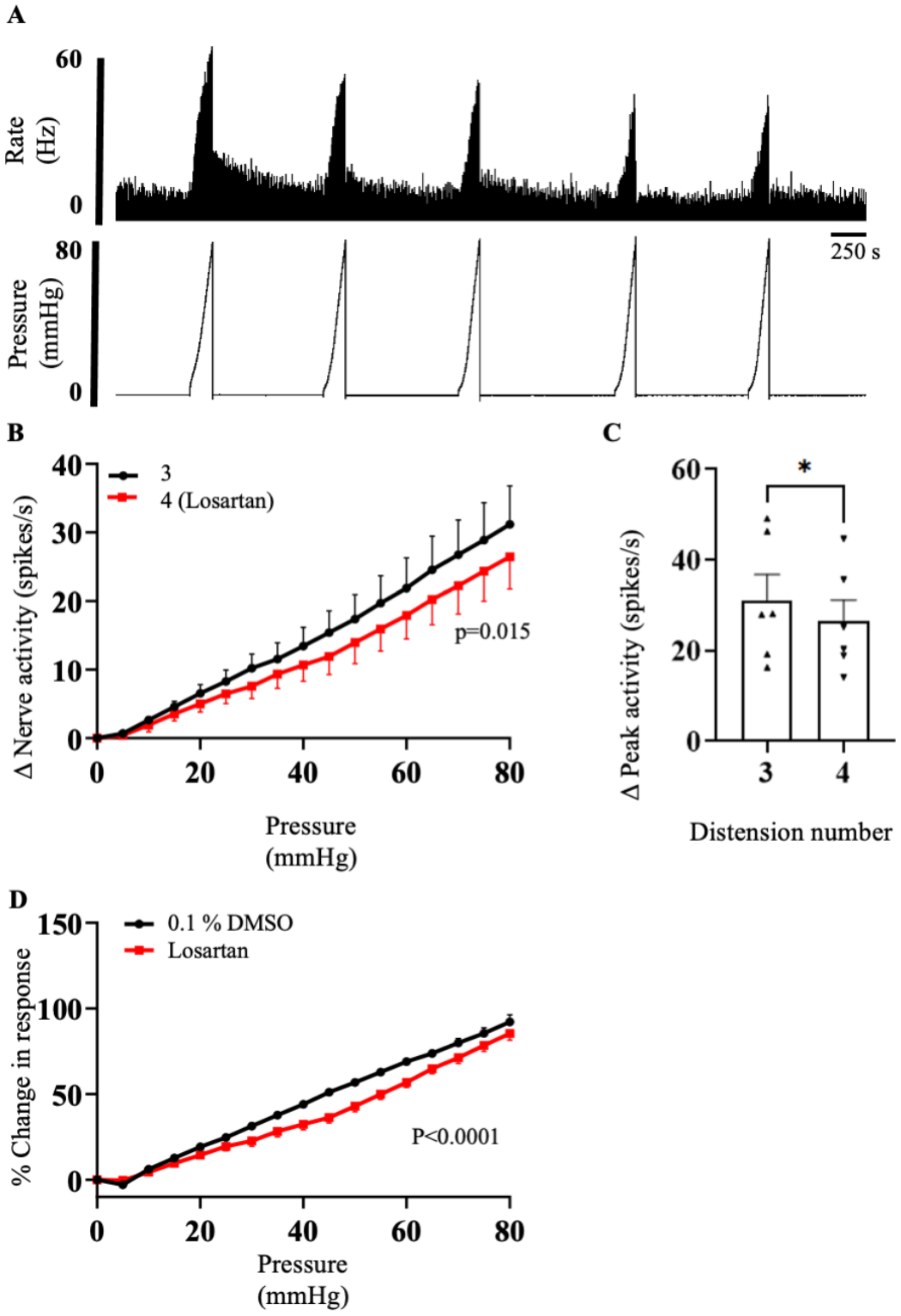


Figure 52. LSN response to ramp distension with losartan treatments.

A) Example rate histogram of LSN activity in response to repeat (x5) slow ramp distensions (0-80 mmHg) with 10 µM losartan administered prior to and during distension 4 and the Accompanying intraluminal pressure trace. (B) The response profile of LSN activity following treatment with 10 µM losartan. (C) Peak firing in response to distension 3 and 4 with 10 µM losartan given prior to and during distension 4. (D) The normalized overall response profiles to ramp distension with losartan and DMSO treatments. Data are shown as mean ±SEM.

Next, we examined the effects of EMD 66684 on the colonic afferent response to distension. Unlike losartan, EMD 66684 is an antagonist that competitively binds to AT_1 in the presence of Ang II. Therefore, we hypothesized that administration of EMD 66684 would have no effect on AT_1 contribution to LSN mechanosensitivity. Administration of 10 μM EMD 66684 had no effect on the colonic afferent response to ramp distension. The response to distension 4 following treatment with EMD 66684 was comparable to the response to distension 3 (p=0.66, two-way ANOVA, N=5) (Figure 53). These results suggesting that the inhibitory effect is only present with inverse agonists.

Therefore, we performed additional experiments with valsartan which has stronger inverse agonist properties compared with losartan, to determine if the results observed with losartan were real. We hypothesized that if the effect observed with losartan was real, valsartan would have a larger inhibitory effect on the nerve response to ramp distension. Valsartan was administered before and during distension 4 and the response to distension 4 compared with the response to distension 3. Disappointingly, valsartan had no inhibitory effect on the LSN response to colonic ramp distension (p=0.12, two-way ANOVA, N=3) (Figure 53). Therefore, the potential contribution of AT_1 to LSN mechanosensitivity remains unclear.

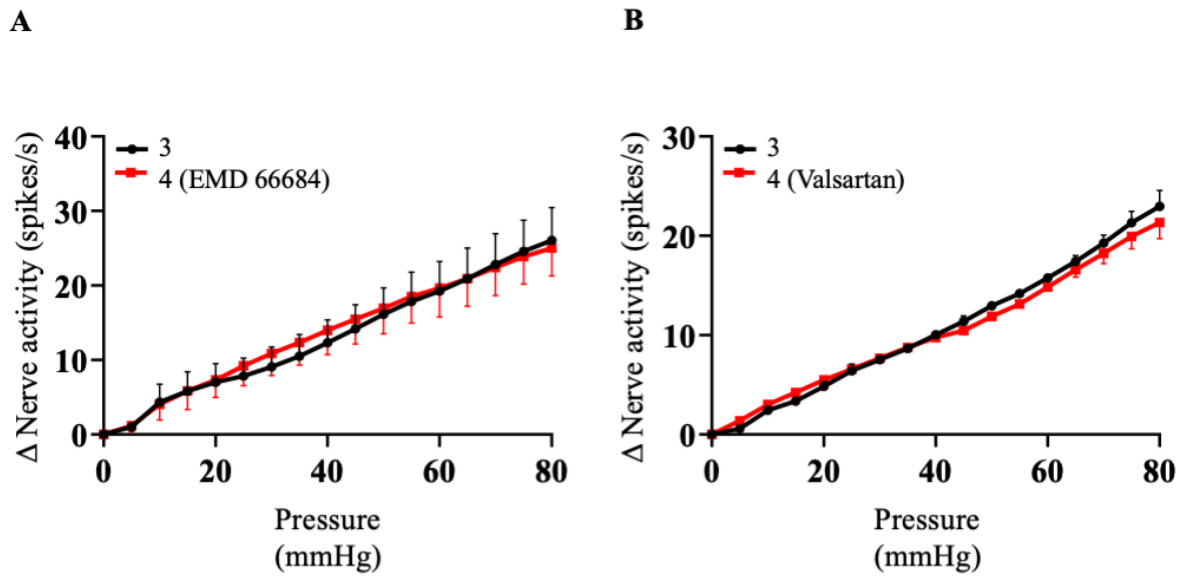


Figure 53. LSN response to ramp distension with EMD 66684 and valsartan treatments.

(A)The response profile of LSN activity in response to distension 3 and 4 with 10 μM EMD 6684 given prior to and during distension 4 (p=0.66, two-way ANOVA with Bonferroni's multiple comparisons test, N=6). (B) Response profile to LSN response to distension 3 and 4 with 10 μM valsartan administered prior to and during D4 (p=0.12, two-way ANOVA with Bonferroni's multiple comparisons test, N=6). Data are shown as mean ±SEM.

4.18 Summary of results

- Ang II stimulates LSN activity via AT_1, an effect that can be blocked by AT_1 blockers.
- The overall LSN response to Ang II is reduced in female mice.
- Removal of extracellular Ca^{2+} does not inhibit the LSN response to Ang II.
- Removal of the colon and associated tissues does not alter the LSN response to Ang II.
- Blocking TRPC3 had no inhibitory effects on the LSN response to Ang II. The signaling mechanism of Ang II on the LSN requires further investigation.
- There is no evidence of prolonged sensitization on the LSN response to ramp distension following treatment with Ang II.
- Ang II stimulates nociceptive fibres, classified by their response to mechanical stimulation, bradykinin, and capsaicin.
- The contribution of AT_1 to the LSN response to mechanical stimulation requires further investigation.

4.19 Discussion

The development of visceral hypersensitivity and nociceptor activation in response to mediators released from the inflamed bowel is thought to be the principal cause of pain for IBD patients. The mediators underpinning these events are not fully understood and consequently we investigated the potential contribution of Ang II, an octapeptide hormone upregulated in the bowel of IBD patients, to visceral nociception based on the marked expression of the AT_1 receptor on colonic nociceptors. Our findings revealed a potent excitatory effect of Ang II on visceral nociceptors indicating a likely contribution of Ang II to the production of abdominal pain in IBD.

4.19.1 Ang II on lumbar splanchnic nerve activity

Data from this chapter shows that Ang II stimulates the LSN in a concentration dependent manner. The Ang II mediated increase in afferent output was inhibited by AT_1 blockers losartan, valsartan and EMD 66684 but not by the AT_2 receptor blocker PD123319. Additionally, administration of the AT_2 agonist had no effect on colonic afferent activity. Taken together, these results indicate that Ang II stimulates colonic afferent firing via AT_1.
These observations are consistent with the expression profiles of angiotensin receptors in colonic sensory neurons. AT_{1A} and AT_{1B} are expressed across the various populations of sensory neurons whereas the AT_2 receptor is poorly expressed across all the neuronal populations (Hockley et al., 2018). Therefore, it is not unexpected that the AT_2 agonist had no effect on the LSN and treating the nerve with the AT_2 antagonist has no inhibitory effect on the LSN response to Ang II. In addition, our data shows a marked desensitization of the response to Ang II, consistent with the known internalization of the AT_1 receptor following activation (Mehta & Griendling, 2007).

Additionally, we evaluated whether sex differences would alter the response to Ang II in female mice compared with males. Males and females have been shown to have marked differences in basic biological processes such as pain processing and lack of pre-clinical research on female subjects likely results in poorer treatment outcomes for women (Beery, 2018). Additionally, since there are significant differences in the ways in which male and female animals experience diseases and react to drugs, deleterious effects of some drugs have been recorded on women (Lee, 2018). Therefore, it is important to evaluate the role sex differences in animal studies. While spontaneous baseline firing was comparable between male and female mice, present

data suggests the response to Ang II is reduced in female mice compared with males. However, additional experiments are needed to validate these observations.

4.19.2 The mechanism of action of Ang II in colonic afferents

AT_1 is widely expressed in the bowel. It is found in the muscularis of the colon in rats, the submucosal plexus of guinea pig distal colon and enteroendocrine L cells of the epithelium in mice (Hosoda et al., 2000; Pais et al., 2016; Sechi et al., 1993). In the human colon, AT_1 is expressed in vessel walls, myofibroblasts and macrophages in the lamina propria, crypt bases, and surface epithelium (Hirasawa et al., 2002). Therefore, administration of Ang II likely activates AT_1 receptors present in the GI tract to activate signaling pathways that induce physiological processes such as smooth muscle contraction and hormone and neurotransmitter release. In agreement with this, one study showed that Ang II induces smooth muscle contractions in rat and human via AT_1 receptors (Ewert et al., 2006). Another study showed that Ang II stimulated the release of gut hormones (glucagon-like peptide -1 and peptide Y) in mouse colonic L cells (Pais et al., 2016).

Therefore, in the present study, we sought to determine whether Ang II acted directly on the LSN or indirectly through release of mediators by cell types expressing the AT_1 receptor in the GI tract including the enteric nervous system. Firstly, all the ex vivo preparations in this study were treated with nifedipine, which blocks voltage dependent L type calcium channels to inhibit Ca^{2+} entry and block smooth muscle contraction. Thereby, we eliminated any contribution of smooth muscle contraction induced by Ang II on the LSN response to Ang II.

Secondly, Ang II was administered in the absence of extracellular Ca^{2+} to block neurotransmitter release and cross talk with the enteric nervous system. Neurochemicals and neurotransmitters released by the enteric nervous system have the potential to activate extrinsic afferents in a process that depends on calcium signaling. Ca^{2+} influx following the opening of Ca^{2+} channels in response to depolarization of the presynaptic nerve terminal triggers synaptic vesicle exocytosis, thereby releasing neurotransmitters contained in the vesicles and initiating synaptic transmission (Meir et al., 1999; Sudhof, 2012). Removal of extracellular Ca^{2+} attenuates this process and would inhibit any indirect activation of the LSN by neurochemicals released in the enteric nervous system.

Removal of extracellular Ca^{2+} significantly increased the activity and excitability of the LSN nerve prior to application of Ang II. This could be due to inhibition of ion channels that regulate neuronal excitability such as calcium activated potassium channels (K_{Ca}) whose activation depends on Ca^{2+} influx into sensory neurons during an action potential. K_{Ca} channels play a key role in the repolarization and hyperpolarization of the membrane, therefore, absence of Ca^{2+} could potentially inhibit these ion channels and increase neuronal excitability (Simons, 1988). However, our work on K_{Ca} channel modulation indicates there is not significant tonic K_{Ca} activity on the LSN nerve as K_{Ca} blockers had no effect on LSN baseline firing (Data shown in the appendix). Alternatively, the observed increase in spontaneous firing following the removal of extracellular Ca^{2+} could be due to the blockade of inhibitory peptides such as somatostatin (SST) that can suppress afferent excitability. Ca^{2+} plays a key role in release of gut hormones including SST which has been shown to play an inhibitory role in the control of visceral sensitivity through activation of the SST_2 receptor subtype (Rong et al., 2007). While removal of Ca^{2+} from the preparation increased baseline firing, the response to Ang II was unaltered by removal of Ca^{2+} suggesting Ca^{2+} dependent mechanisms such as neurotransmitter or hormone release are not involved in the response to Ang II.

Lastly, we isolated the LSN by removing the colon and surrounding tissue from the preparation to exclude any indirect effects of Ang II on the LSN response. Though removing the colon significantly reduced LSN firing, a response to Ang II was still observed, indicating that Ang II can act directly on nerve endings located in the mesentery and vascular supply to the colon.

4.19.3 The Ang II signalling pathway

The mechanism by which Ang II increases colonic afferent output remains to be elucidated. A major pathway of coupling for AT_1 is through $G_{q\alpha}$ which activates phospholipase C-β, leading to generation of IP_3 which mobilizes intracellular Ca^{2+} and DAG. In the present study, TRPC3, a non-selective cation channel that is activated by DAG was targeted to evaluate the effects of blocking this receptor on the colonic afferent response to Ang II. Activation of TRPC3 is thought to depolarize membrane potential and increase firing frequency. Therefore, if Ang II induced colonic afferent firing through TRPC3 activation, blocking this channel would inhibit Ang II induced neuronal discharge.

However, blocking TRPC3 did not alter the LSN response to Ang II suggesting the observed increase in colonic output following administration of Ang II does not depend on the activation of the ion channel. Additionally, the pronounced response to Ang II in the absence of extracellular Ca^{2+} suggests the response to Ang II is not dependent on Ca^{2+} permeable channels such as TRP channels. Additional studies are warranted to elucidate the signaling mechanism of Ang II in colonic afferents. One such study could evaluate the contribution of KCNQ channels to Ang II induced neuronal excitability. G_q coupled receptors including AT_1 have been demonstrated to inhibit KCNQ channels underlying the M current through PIP_2 depletion resulting in increased neuronal excitability (Adams et al., 1982).

4.19.4 Ang II on the LSN response to ramp distension

The development of chronic pain during remission from inflammatory disease is thought to be mediated by changes in the sensitivity of sensory nerves to stimuli such as distension of the bowel. Mechanosensitive visceral afferent fibers can be sensitized by inflammatory mediator release, thereby contributing to the altered sensations arising from the viscera (Gebhart, 2000). Therefore, identification of mediators that sensitize afferents is key in developing visceral analgesics. In the present study, we evaluated the ability of Ang II to mediate long-term changes in the sensitivity of sensory nerves to stimuli such as distension of the bowel. Data from this study suggests there is no evidence that Ang II sensitizes colonic afferents to ramp distension.

Next, we sought to classify mechanical and chemical (bradykinin and capsaicin) sensitivity of Ang II responsive fibres. Electrophysiological recordings of fibres teased from the LSN were conducted and waveform analysis used to identify single units with distinct amplitude and waveforms and their sensitivity to mechanical stimulation, Ang II, bradykinin and capsaicin examined. Present data suggests Ang II stimulates nociceptive fibres determined by their sensitivity to mechanical stimulation, capsaicin, and bradykinin. Although there is no evidence of prolonged sensitization after treatment with Ang II, persistent Ang II may increase excitability of mechanosensitive visceral afferents thus contributing to pain induced by distension of the gut.

Lastly, we evaluated the potential contribution of AT_1 to LSN mechanosensitivity. Ang II independent stimulation of the AT_1 has been implicated in pathological conditions such as

hypertension, preeclampsia or cardiac overload conditions which can be attenuated by actions of inverse agonists such as candesartan (Takezako et al., 2015). Ligand-independent signaling by AT_1 can be activated by mechanical stretch and autoantibodies as well as receptor mutations. AT_1 inverse agonists have been demonstrated to inhibit the effects of mechanical stimulation of the AT_1.Valsartan and losartan have inverse agonist properties, therefore, were employed to evaluate Ang II independent activation of AT_1. EMD 66684 on the other hand served as a control for these studies. Antagonists such as EMD 66684 inhibit responses in competition with agonists but are not able to reduce the constitutive activity of the receptor or the agonist independent activity of the receptor.

Treatment with losartan resulted in a reduced colonic afferent response to ramp distension. However, these results were not reproducible with valsartan, which is a stronger inverse agonist compared to losartan. Therefore, additional studies to validate the effect losartan on LSN mechanosensitivity will be essential in establishing the contribution of AT_1 to LSN mechanosensitivity. As expected, EMD 66684 had no effects on the colonic nerve response to ramp distension.

4.20 Future work

Future work to determine the mechanism of action of Ang II on colonic afferents would be key in establishing the potential role of Ang II on pain signaling from the GI tract. The contribution of Ang II on pain signaling could be evaluated in colitis models or along with other inflammatory mediators to establish the nociceptive role of Ang II in IBD patients. Additionally, quantifying levels of Ang II in IBD patients and examining the effect of Ang II in human visceral afferents would be key in establishing the potential pronociceptive role of Ang II in IBD patients. Given that AT_1 receptor blockers are already in use to treat conditions such as hypertension and have been shown to have beneficial effects on IBD symptoms, it would be interesting to evaluate pain scores of IBD patients on ARBs. Additionally, since angiotensinogen is the precursor for other angiotensin peptides, future work could quantify levels of these peptides in IBD patients and evaluate their effect on colonic afferents.

Additional studies are also necessary to evaluate how sex differences impact the LSN response to Ang II and validate observations presented here. Further evaluating of the contribution of AT_1 on LSN mechanosensitivity is necessary. Given that many ion channels including GPCRs confer LSN mechanosensitivity, it may be necessary to block several channels to see an effect,

therefore, future experiments could target AT_1 along with other ion channels and receptors that play a role in mechanosensitivity.

4.21 Conclusions

In conclusion, present data shows that Ang II directly activates colonic afferents including high threshold mechanosensitive fibers through AT_1, indicating the potential role of Ang II in pain signaling from the GI tract.

5 MMP1 on sensory signalling from the GI tract

5.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of Zn^{2+} dependent endopeptidases whose main physiological role is to degrade proteins in the extracellular matrix (ECM) (Ravi et al., 2007). The ECM constitutes of fibrous proteins such as collagen and elastin, glycoproteins and proteoglycans. It functions to support adhesion of cells and harbors chemokines, cytokines and growth factors. By cleaving the ECM, MMPs change ECM structure and liberate biologically active growth factors, chemokines and cytokines from their membrane anchored proforms, all of which influence cellular behavior (Klein & Bischoff, 2011; Mott & Werb, 2004). In addition to cleaving components of the ECM, MMPs also proteolytically activate or degrade a variety of nonmatrix substances including chemokines, cytokines, growth factors, and junctional proteins (Ravi et al., 2007). In physiological conditions, MMPs are secreted at very low levels and are involved in many biological processes such as remodeling of the ECM, cell proliferation, migration and differentiation, wound healing, embryogenesis, immunity and inflammation (Cui et al., 2017).

There are 28 enzymes classified as MMPs in vertebrates, with 24 genes found in humans (Bassiouni et al., 2021). Members of the MMP family contain four distinct functional domains: a propeptide of about 80 amino acids that regulates MMP activity by interacting with Zn^{2+}, a catalytic domain of about 170 amino acids that is responsible for the proteolytic activity, a hemopexin like domain of about 200 amino acids that is involved in substrate recognition and a flexible hinge region of variable length that links the catalytic domain and the hemopexin-like C domain (Figure 54) (Cui et al., 2017; Nissinen & Kahari, 2014).

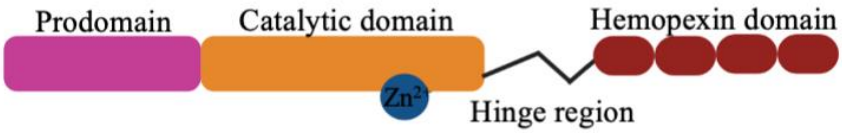


Figure 54. Schematic of the basic structure of MMPs.

A typical MMP consists of a propetide, a catalytic domain, a linker peptide and hemopexin domain.

MMPs are grouped into various subclasses according to their structure, substrate specificity and function and are shown in Table 5 below.

Class	**MMPs**
Collagenases	MMP1, MMP8, MMP13, MMP18
Gelatinases	MMP2 and MMP9
Stromelysins	MMP3, MMP7, MMP10, and MMP11
Elastase	MMP12
Membrane Types	MMP14, MMP15, MMP16, MMP17, MMP24 and MMP25
Other	MMP19, MMP20, MMP23, MMP26, MMP27 and MMP28

Table 5. Classification of MMPs.

MMPs are secreted as inactive zymogens by multiple tissues and cell types including myofibroblasts, T cells, macrophages, monocytes, neutrophils, and epithelial cells (Maronek et al., 2021). These zymogens are processed by other proteolytic enzymes such as serine proteases and other MMPs to generate the active forms (Verma & Hansch, 2007). The majority of MMPs are secreted outside the cell, with only a few anchored to the cell surface via a transmembrane domain (Sternlicht & Werb, 2001).

Production, activation, and activity of MMPs is tightly regulated to prevent excessive tissue degradation. MMPs are regulated at several levels from transcription, translation and activation as precursor zymogens. The expression of MMPs can be influenced by hormones, growth factors and cytokines. For example, inflammatory cytokines including TNF-α and growth factors including epidermal growth factor (EGF) upregulate the expression of MMPs at the transcriptional level (Caley et al., 2015). Once activated, the activity of MMPs is regulated by protease inhibitors including the family of tissue inhibitors of metalloproteinases (TIMPs) which competitively and reversibly inhibit the activity of all MMPs (Nissinen & Kahari, 2014; O'Sullivan et al., 2015).

Dysregulated expression of MMPs has been implicated in several pathological conditions such as arthritis, atherosclerosis, myocardial infarction, cancer, and IBD (Ravi et al., 2007). MMP expression and activity are upregulated in most types of human cancer and this correlates with increased tumor stage and shortened survival (Egeblad & Werb, 2002). MMP expression and activity are also typically upregulated in tissue injury and inflammatory diseases processes where they have been demonstrated to play key roles in both pro and anti-inflammatory pathways (Nissinen & Kahari, 2014). MMPs serve a proinflammatory role by regulating the

recruitment and activity of chemokines and cytokines to the site of inflammation in the tissue. For example, MMP13 cleaves and activates TNF-α, a potent proinflammatory cytokine that plays a key role in the pathogenesis of IBD (Vandenbroucke et al., 2013). In contrast, MMPs present anti-inflammatory effects by cleaving chemokines. For example, MMP2 cleaves monocyte chemotactic protein 3 (MCP-3) generating a truncated form of the chemokine that can still bind to the receptor and act as an antagonist thus inhibiting inflammation (McQuibban et al., 2000).

5.2 MMPs and IBD

In inflamed IBD tissue, MMPs are produced in excess and the activity of TIMPs is insufficient to block the MMPs, thereby, MMPs play a key role in IBD-related mucosal degradation. Several MMPs including MMP1, MMP2, MMP3, MMP8, MMP9, and MMP12 are upregulated in IBD patients as well as in animal models of colitis (Ravi et al., 2007). Genetic studies have found several single nucleotide polymorphisms (SNPs) in MMP3 and MMP10 that are associated with increased risk of IBD whereas SNPs in the MMP8 gene were associated lowered risk for IBD (de Bruyn et al., 2016). Other studies have shown that MMP levels directly correlate with disease activity and MMP inhibitors marimastat and barimastat have been shown to ameliorate colitis in rodents (Di Sebastiano et al., 2001; Sykes et al., 1999). MMP3 and MMP12 have been shown to degrade anti-TNF-α agents, infliximab, adalimumab and etanercept contributing to the non-responsiveness to these biologic agents observed in IBD patients (Biancheri et al., 2015).

Given the pathological role of MMPs in conditions such as cancer and IBD, the therapeutic potential of MMP inhibitors (MMPIs) has been evaluated in clinical trials. However, the results have historically been disappointing as severe side effects have been associated with their use due to non-selectivity of the inhibitors. For example, early clinical studies with MMPIs were associated with musculoskeletal syndrome (MSS) which was caused by inhibition of several MMPs including MMP1, MMP2 and MMP9 (Fields, 2019; Peterson, 2006). Andecaliximab (ADX), a monoclonal antibody that inhibits MMP9 was evaluated in clinical trials in patients with moderate to severe UC and CD, however, trials were terminated during phase 2/3 as ADX showed no efficacy in UC and CD patients (de Bruyn & Ferrante, 2018).

5.3 MMP1

MMP1 (collagenase-1or interstitial collagenase) degrades collagen and gelatin. MMP1 also cleaves MMP9 into its active form (Cui et al., 2017). MMP1 has a gene locus on chromosome 11 in humans, but mice lack a clear ortholog of human MMP1 (Klein & Bischoff, 2011). Two genes *Mcol-A* and *MCol-B* located on chromosome 9 in mice encode MMP1a and MMP1b, respectively and are considered to be the murine counterparts of MMP1 (Balbin et al., 2001). *Mcol-A* and *MCol-B* are 82% identical to each other and 74% identical to human MMP1. *Mcol-A* however is functionally closer to MMP1 than *Mcol-B* (Balbin et al., 2001).

MMP1 can be found in two distinct forms: a major unglycosylated form of ~57 kDa and a minor glycosylated form of 61 kDa (Pardo & Selman, 2005). MMP1 is expressed in various cell types including macrophages, endothelial and epithelial cells (Brinckerhoff et al., 2000). Like other MMPs, MMP1 is expressed at low levels under physiological conditions but is upregulated in inflammatory and autoimmune conditions and some cancers (Cui et al., 2017). For example, MMP1 has been demonstrated in various advanced cancers including colorectal cancer and bladder cancer, with a significant negative correlation between expression of MMP1 and survival (Arakaki et al., 2009; Brinckerhoff et al., 2000). The expression of MMP1 is transcriptionally regulated by growth factors, hormones, and cytokines. Cytokines that induce MMP1 include EGF, TNF-α, interferon beta and gamma, platelet-derived growth factor and interleukin (IL)-1, -4, -5.-6, -8 and -10 (Pardo & Selman, 2005). MMP1 activity can be inhibited by TIMP-1 that acts by forming a 1:1 complex with the activated catalytic Zn^{2+} in the MMP1 (Pardo & Selman, 2005).

5.3.1 MMP1 in IBD

MMP1 is overexpressed in IBD tissue. MMP1 transcripts are present in colonic epithelial cells and MMP1 levels are increased in cells from inflamed IBD mucosa (Pedersen et al., 2009). MMP1 expression levels strongly corelate with severity of disease in UC whereas, in CD, MMP1 is upregulated even in the non-inflamed patients with CD (Stumpf et al., 2005; von Lampe et al., 2000; Wang & Mao, 2007). A broad-spectrum inhibitor ilomastat, was shown to protect rats from TNBS induced colitis through inhibition of MMP1 (Wang & Wang, 2008).

RNA sequencing data generated in our group has also shown upregulation of MMP1 in mucosal biopsy samples from patients with IBD. MMP1 expression was upregulated in the

mucosal biopsies of UC and CD patients compared with controls (p<0.0001, one-way ANOVA with Dunnett's multiple comparisons test) (*Figure 55*).

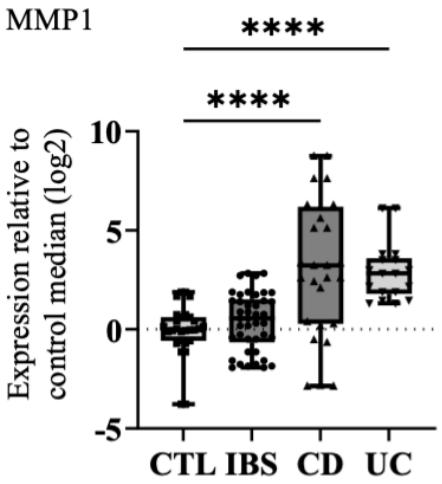


Figure 55. MMP1 expression in IBD patients.

*MMP1 expression in colonic biopsies of IBS and IBD (UC and CD) patients compared with controls. (*** p < 0.001, **** p < 0.001). Data are shown as Log2 fold change median expression in control samples.*

5.4 Protease activated receptors (PARs)

In an unexpected discovery, one study found that MMP1 can directly activate protease activated receptor-1 (PAR_1) to generate Ca^{2+} dependent signals in cancer cells (Boire et al., 2005). Since then, several others MMPs including MMP1a, MMP2, MMP3, MMP8, MMP9 and MMP13 have been shown to cleave and activate PAR_1 (Heuberger & Schuepbach, 2019). PAR_1 was the first of four members of the PAR family to be discovered and was characterized as the thrombin receptor in platelets (Kahn et al., 1999). To date, four protease activated receptors: (PAR_1, PAR_2, PAR_3, and PAR_4) have been identified. The four PARs (PAR_1, PAR_2, PAR_3, and PAR_4) are encoded by the genes *F2R, F2RL1, F2RL2, and F2RL3*, respectively (Heuberger & Schuepbach, 2019). PARs are predominantly expressed in vascular, immune and epithelial cells, astrocytes and neurons and are important mediators of hemostasis, thrombosis and inflammation (Soh et al., 2010). PARs are a class of GPCRs that are irreversibly activated after proteolytic cleavage of their N-terminal extracellular domain by proteases. The resulting N-terminal end becomes a tethered ligand that binds the receptor to induce an intracellular signal (Figure 56) (Ossovskaya & Bunnett, 2004).

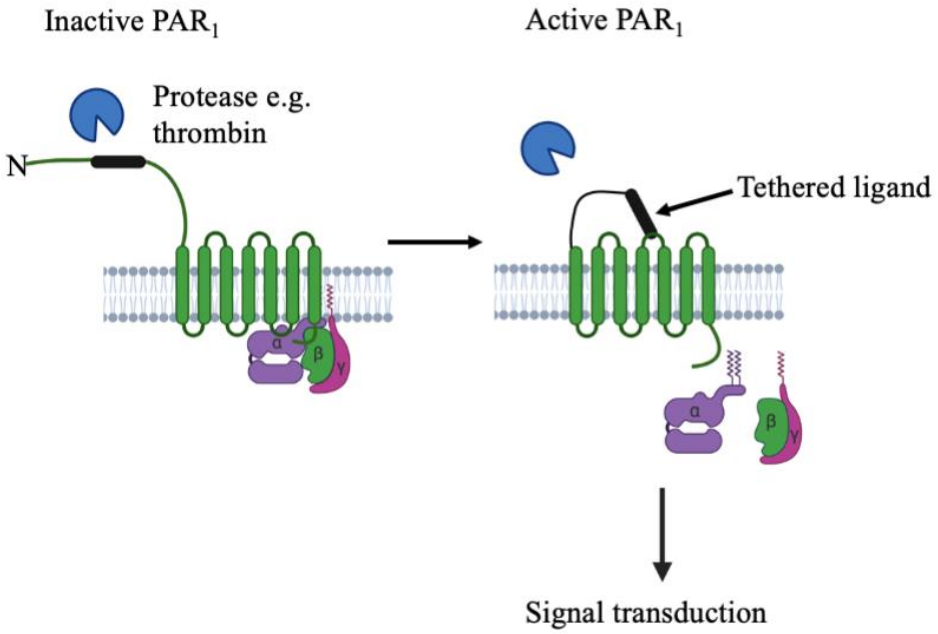


Figure 56. Mechanism of activation of PARs.

Proteases such as thrombin cleave the N-terminal extracellular domain of PARs, exposing a new N terminal domain that binds and activates the receptor, inducing an intracellular signal.

The discovery of PARs highlighted the potential role of proteases as signaling molecules, with the ability to regulate cell functions by cleaving receptors that are activated upon proteolysis. Different proteases can cleave the same PAR at divergent sites and can either activate distinct signals resulting in unique pathophysiological outcomes or disarm the receptor by removing or destroying the tethered ligand domains (Zhao et al., 2015). For example, PAR_1 can be activated by thrombin and trypsin and deactivated by cathepsin G or elastase (Cenac, 2013). Proteases are present at high levels in the GI tract, and therefore play key roles in regulating pathological processes such as inflammation and intestinal barrier permeability via PARs.

5.4.1 PARs and pain

Proteases have been suggested to play a role in the pathogenesis of visceral hypersensitivity via PARs. Activation of PAR_1 and PAR_4 has been demonstrated to have antinociceptive effects, whereas activation of PAR_2 induces pronociceptive effects in rodents (Ceuleers et al., 2016). For example, intracolonic infusion of sub inflammatory doses of PAR_2 agonists was demonstrated to activate spinal afferent neurons and produced delayed rectal hyperalgesia in

rats (Coelho et al., 2002). Additionally, intracolonic administration of IBS tissue supernatants was demonstrated to induce visceral hypersensitivity and allodynia via PAR_2 activation in mice, illustrating a key role for PAR_2 and PAR_2 activating proteases in visceral pain (Cenac et al., 2007). Tryptase which is released by mast cells has been demonstrated to be a key contributor in neuronal signalling of hypersensitivity via PAR_2 stimulation (Reed et al., 2003).

In contrast sub inflammatory doses of PAR_1 agonists provoked analgesia when injected into the colon or peritoneal cavity (Vergnolle, 2005). Similar results were obtained when PAR_1 agonists were injected into the rat paw with the PAR_1 agonists attenuating the nociceptive response to noxious mechanical or thermal stimuli (Asfaha et al., 2002). On the other hand, another study showed that supernatants from colonic biopsies of patients with IBS can induce calcium influx in human sensory neurons via PAR_1 activation. (Desormeaux et al., 2018). Additionally, PAR_1 is expressed on guinea pig myenteric neurons where the receptor is largely co-expressed with substance P, a neuropeptide whose role in promoting pain is well established (Corvera et al., 1999).

5.5 PAR_1

PAR_1 is expressed by a variety of cell types including platelets, endothelial cells, fibroblasts, smooth muscle cells, mast cells, neurons, and astrocytes (Dery et al., 1998). PAR_1 mediates most of the known proinflammatory actions of thrombin such as platelet aggregation, vasodilation, vasoconstriction and increased vascular permeability (Chin et al., 2003). In addition to thrombin, PAR_1 is activated by several other proteases including trypsin, cathepsin G, plasmin, and MMPs (MMP1, -2 and -13). MMP1 cleaves PAR_1 at a noncanonical site thereby generating a distinct tethered ligand that results in a signaling pattern differing from that of thrombin (Austin et al., 2013). PAR_1 activation induces $[Ca^{2+}]_I$ increase in multiple cells types including platelets, breast cancer cells and astrocytes from the rat brain (Boire et al., 2005; Ubl et al., 2000). MMP1 cleaves PAR_1 to give a robust Ca^{2+} response (Boire et al., 2005).

5.5.1 PAR_1 signalling

Like all GPCRs, PAR_1 couples with several α subunits including $Gq_{11\alpha}$, $G_{12/13\alpha}$ and $G_{i\alpha}$ thereby activating multiple mechanisms of signal transduction. The principal mechanism of activation is through G_q proteins resulting in activation of phospholipase C, PIP_2 hydrolysis and formation

of IP_3 and DAG leading to Ca^{2+} mobilization and activation of PKC (Dery et al., 1998). Activation of PAR_1 by the tethered ligand is intrinsically irreversible. PAR_1 activity is therefore regulated and limited by internalization and proteolytic desensitization of the receptor.

5.5.2 PAR_1 and MMP1 in IBD

The role of PAR_1 on the pathogenesis of IBD has been demonstrated. PAR_1 is upregulated in biopsies from CD and UC together with PAR_1 activating proteases: MMP1 and thrombin whose expression levels correlate with the severity of inflammation (Saeed et al., 2017; Vergnolle et al., 2004). Activation of PAR_1 has been shown to exacerbate chronic inflammation in mouse models of IBD whereas blockage of PAR_1 decreases the severity of IBD, and PAR_1 deficiency improved clinical signs of inflammation (Vergnolle, 2005).

5.6 Expression of PAR_1 on DRG neurons projecting to the colon

We examined the expression of *F2r*, the gene encoding the PAR_1 receptor in sensory neurons projecting to the colon in the RNA sequencing data base generated by our group. Data from this study suggests *F2r* is widely expressed across populations of neurons innervating the colon (Figure 57). Additionally, we examined the co-expression of *F2r* with *Scn10a*, the gene encoding $Na_V1.8$ and found a significant correlation between the genes, indicating *F2r* is present in nociceptive neurons (Figure 57) (Hockley et al., 2018).

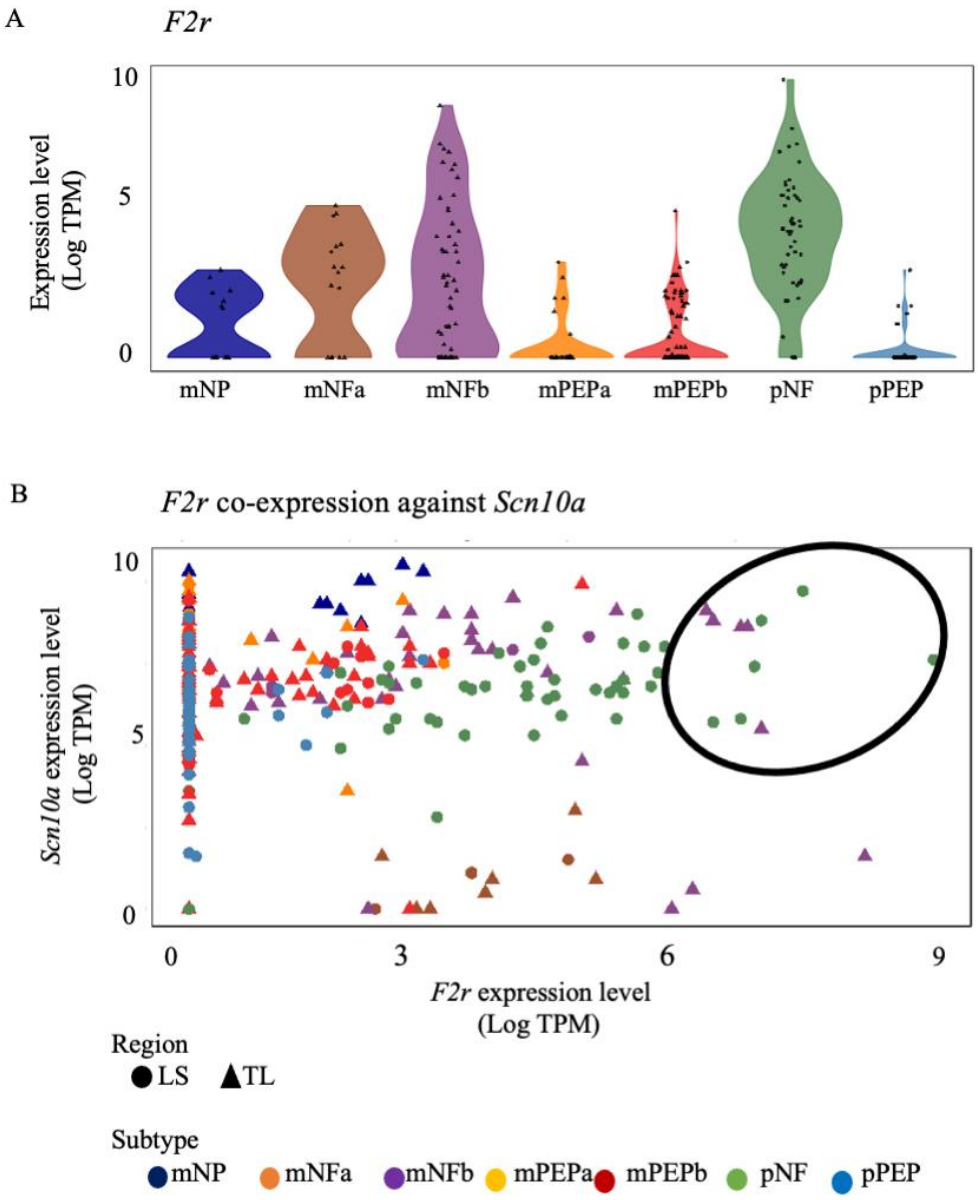


Figure 57. PAR$_1$ (F2r) in mouse colonic sensory neurons.

(A) F2r is expressed in DRG neurons projecting to the colon. The expression profile of F2r is demonstrated in each of the subtypes (mNeuroFilament-a (mNFa), mNeuroFilament-b (mNFb), mNonPeptidergic (mNP), mPeptidergic-a (mPEPa), mPeptidergic-b (mPEPb), pNeuroFilament (pNF) and pPeptidergic (pPEP)). Each black dot represents data from a single colonic sensory neuron isolated from either thoracolumbar (triangle) or lumbosacral (circle) DRG. Expression values are expressed in Transcript-Per-Million (Log [TPM]. (B) shows strong co-expression of with Scn10a. The subpopulation of neurons that highly co-express F2r and Scn10a is highlighted by the black oval.

5.7 PAR_1 in the GI tract

PAR_1 is widely distributed throughout the GI tract and is expressed by a variety of cell types including epithelial cells, enteric neurons, enterocytes, fibroblasts, smooth muscles, and immune cells (Ceuleers et al., 2016). In the GI tract, PAR_1 is involved in many physiological processes including ion secretion, permeability, inflammation, smooth muscle contraction, and prostaglandin release (Vergnolle, 2005).

5.7.1 PAR_1 and permeability

PAR_1 activation has been shown to lead to leakage in intestinal barrier functions increasing the passage of fluids across the gut mucosa (Vergnolle, 2005). Increased intestinal permeability on PAR_1 has been demonstrated to be mediated by direct activation of the receptor on enterocytes resulting in apoptosis (Chin et al., 2003). PAR_1 induced colitis has been shown to be partly due to an increase in intestinal barrier permeability and direct activation of B and T lymphocytes (Vergnolle et al., 2004). Therefore, while the principal aim of this study is to evaluate the effect of PAR_1 activation on sensory nerves, I conducted additional experiments to examine effects of PAR_1 activation on intestinal permeability to demonstrate a functional effect of PAR_1 activation on the colon.

Increased intestinal permeability is a characteristic of chronic inflammatory diseases of the intestine such as IBD and has been demonstrated to induce visceral hypersensitivity in animal models (Ait-Belgnaoui et al., 2005). Intestinal barrier integrity is essential in maintaining tissue homeostasis. The intestinal epithelium forms a barrier to prevent the absorption of potentially harmful compounds and organisms including bacteria and bacterially synthesized products (Greenwood-Van Meerveld et al., 2017). Under pathological conditions, increases in epithelial permeability allows luminal antigens, toxins and microbial fermentation to translocate the epithelial barrier (Greenwood-Van Meerveld et al., 2017). These products can activate and sensitize afferent nerve endings leading to visceral afferent sensitization.

5.8 Study Aims

Expression studies from our group confirmed the increased expression of MMP1 in IBD tissue. Given that PAR_1 is expressed on primary afferent neurons and sensory neurons in the gut, work done in this chapter aimed to evaluate the role of PAR_1 activation by MMP1 on pain signaling as a potential therapeutic target for treating pain in IBD patients.

This chapter aims to:

1. Examine the effect MMP1 on DRG neurons.
2. Determine whether MMP1 and TRAP-6 can activate PAR_1 to induce Ca^{2+} signals in DRG neurons.
3. Examine the effect of MMP1 and PAR_1 activation by TRAP-6 on colonic afferents.
4. Demonstrate the effect of PAR_1 activation on intestinal permeability.

5.9 Experimental protocols

5.9.1 Calcium imaging experiments

Calcium imaging was used to evaluate the effects of MMP1 on DRG neurons. Control experiments were conducted in which ECS was administered followed by 1 µM capsaicin and 50 mM KCl. Capsaicin was used to classify the responding cells as TRPV1 positive or negative to determine the effect of MMP1 on nociceptors. The MMP broad spectrum inhibitor marimastat was used to block the response to MMP1. The selective PAR_1 agonist, TRAP-6 was used to demonstrate PAR_1 activation and the selective PAR_1 antagonist, SCH 79797 was used determine whether the observed response to MMP1 was due to PAR_1 activation.

The MMP1 catalytic domain (Enzo Life Sciences) was thawed and prepared in ECS ~30 minutes before commencement of the protocol. For MMP1 and TRAP-6 treatments, images were captured for 10 seconds before 30 seconds of drug application, followed by a 20 second wash with ECS. For KCl and capsaicin treatments, baseline images were captured for 10 seconds followed by 10 seconds of KCl/capsaicin application and a 20 second wash with ECS. In control experiments, ECS was applied for 30 seconds. Cells were allowed to recover for 4 minutes between drug applications (Figure 58). For studies with the MMP inhibitor and the PAR_1 antagonist, the cells were pre-treated with 10 µM SCH 79797 or 10 µM marimastat prior to treatment with MMP1 or TRAP-6.

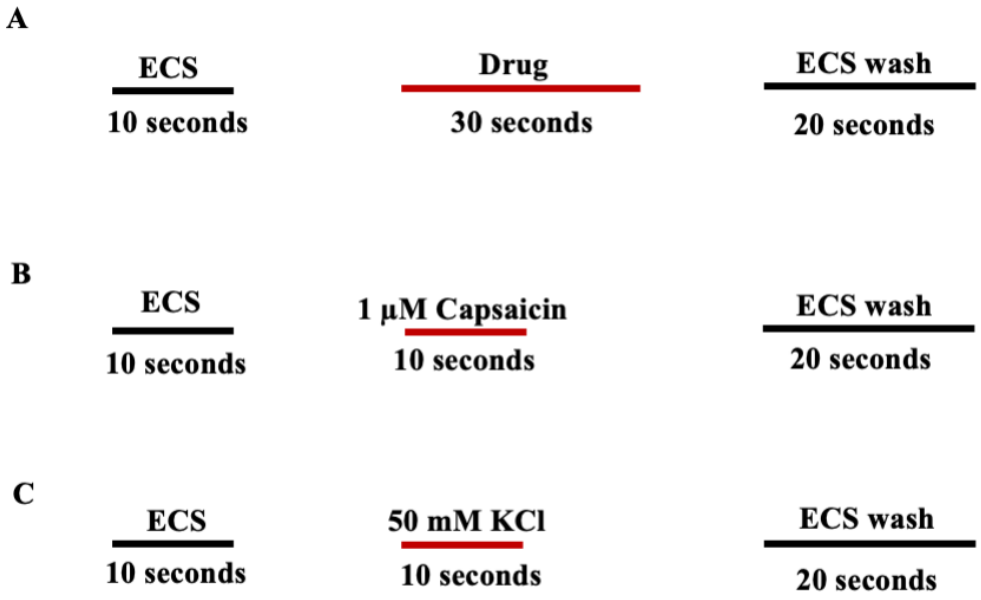


Figure 58. Schematic showing the protocols used during for calcium imaging experiments.

(A) Illustration of protocol used for treatment with MMP1 or TRAP-6. (B) Illustration of protocol used for capsaicin treatments. (C) Illustration of the protocol used for KCl treatments.

5.9.2 Ex vivo electrophysiology

To evaluate the effect of MMP1 on the LSN, the tissue was set up as described in the methods chapter. Once nerve activity stabilized, MMP1 was administered in supplemented Krebs solution via an inline heater at 7 ml/minute. Changes in LSN activity were observed.
In ramp distension experiments, TRAP-6 (10 µM) or the vehicle (0.1% DMSO) was administered after distension 2 and the effects of TRAP-6 on the LSN mechanosensitivity observed on distension 3. Changes in afferent activity was determined for every 5 mmHg increase in pressure and data normalized and presented as a percentage change in response to distension 2. Experiments evaluating the effect of TRAP-6 on the LSN response to ramp distension were conducted by Mr Rohit Gupta who is a PhD student in the Bulmer lab.

5.9.3 Ussing Chamber experiments

Intestinal tissue was fitted into the Ussing chambers as described in the methods section and tissues were clamped at zero potential. Changes in current were recorded on LabChart 8. Two voltage injections (20 mV) were given 5 minutes apart prior to addition of the drug to determine the resistance of the tissue. 10 µM TRAP-6 or 0.1 % DMSO was administered on the mucosal side of the tissue and an additional two voltage injections were given 10 and 20 minutes after drug administration.

5.10 Results

5.10.1 Control experiments

In control experiments, application of ECS resulted in Ca^{2+} influx in a small percentage (3.92±0.71%, *N*=5) of neurons. Application of capsaicin evoked a rapid Ca^{2+} influx in 37.34±6.12% (*N*=7) of neurons. Evaluation of the size of neurons demonstrates that capsaicin responsive cells are significantly smaller in size compared with non-capsaicin responders (Figure 59). This is consistent with published literature. TRPV1 expression is restricted to small and medium diameter neurons thereby, capsaicin responsive are smaller in size compared with the non-responders (Caterina et al., 1997).

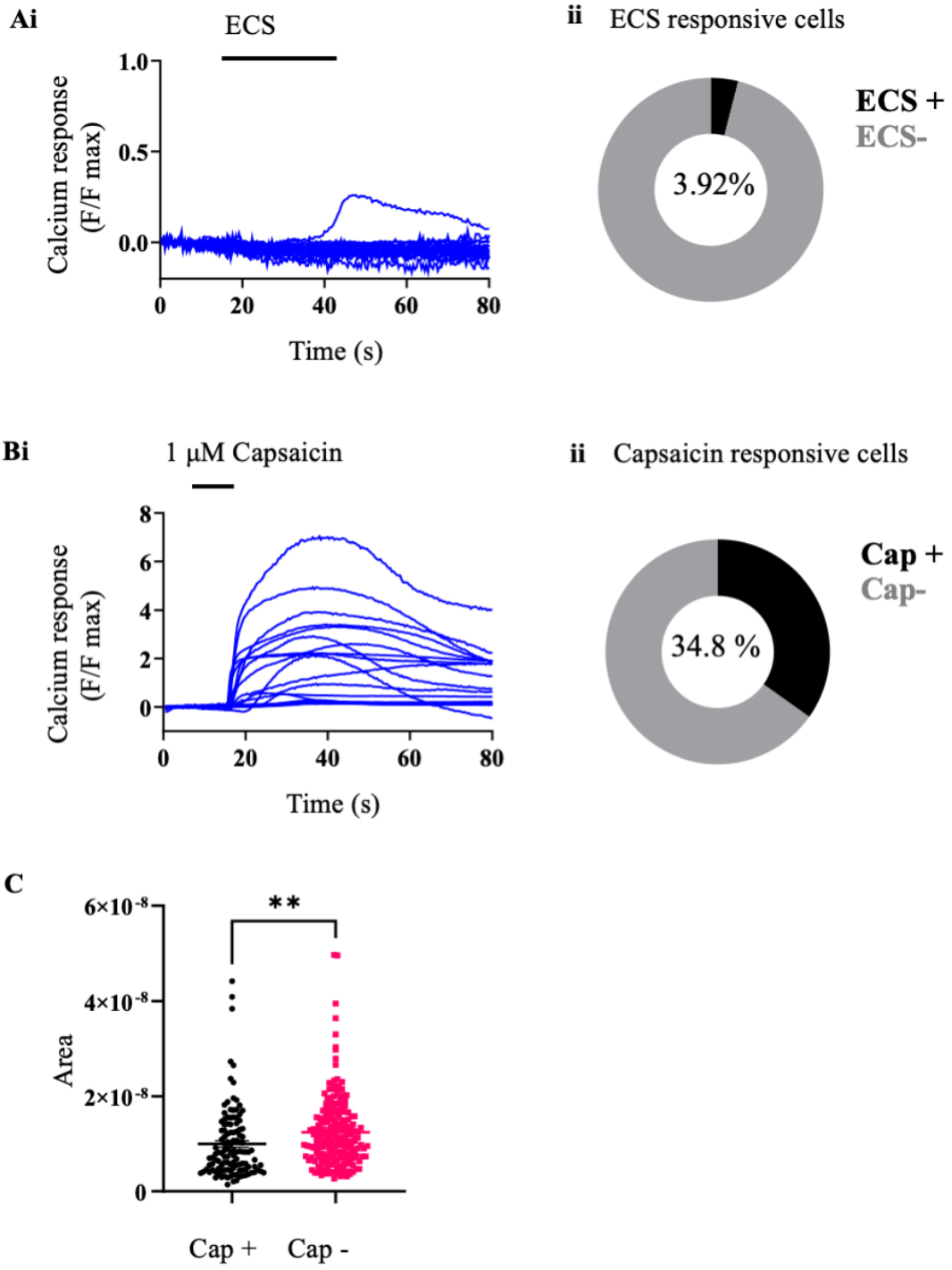


Figure 59. DRG neuron response to ECS, capsaicin and KCl in control experiments.

*(A) Representative response profiles of neurons responding to administration of ECS (n=1 dish containing 20 cells). (Ai)The proportion of neurons that responded to ECS application. (Bi) Representative response profiles of neurons responding to administration of 1 µM capsaicin (n=1 dish containing 20 cells). (Bii) The proportion of DRG neurons that responded to 1 µM capsaicin (n=7 dishes from N=7 independent cultures). (C) Comparison of the size of capsaicin responders vs non responders (** $p < 0.01$, Student's test; n=7 dishes from N=7 independent cultures).*

5.10.2 Effects of increasing concentrations of MMP1 on DRG neurons.

Next, I evaluated effects of increasing concentrations of MMP1 to determine an optimal concentration for use in subsequent studies. Application of increasing concentrations (1, 3 and 10 nM) of MMP1 resulted in a dose dependent Ca^{2+} influx in DRG neurons. The % of neurons that responded to MMP1 increased with increasing concentrations of MMP1 with 10.49±1.25, 14.84±3.04 and 17.03±2.64% of cells responding to 1, 3 and 10 nM MMP1, respectively. However, there was no significant difference in the peak response to 1, 3 and 10 nM MMP1 (p=0.46, one-way ANOVA with Tukey's multiple comparisons test). Evaluation of the size of the neurons that responded to 10 nM MMP1 showed that MMP- responders were significantly smaller in size compared with non-responders (p<0.0001, unpaired t test) (Figure 60).

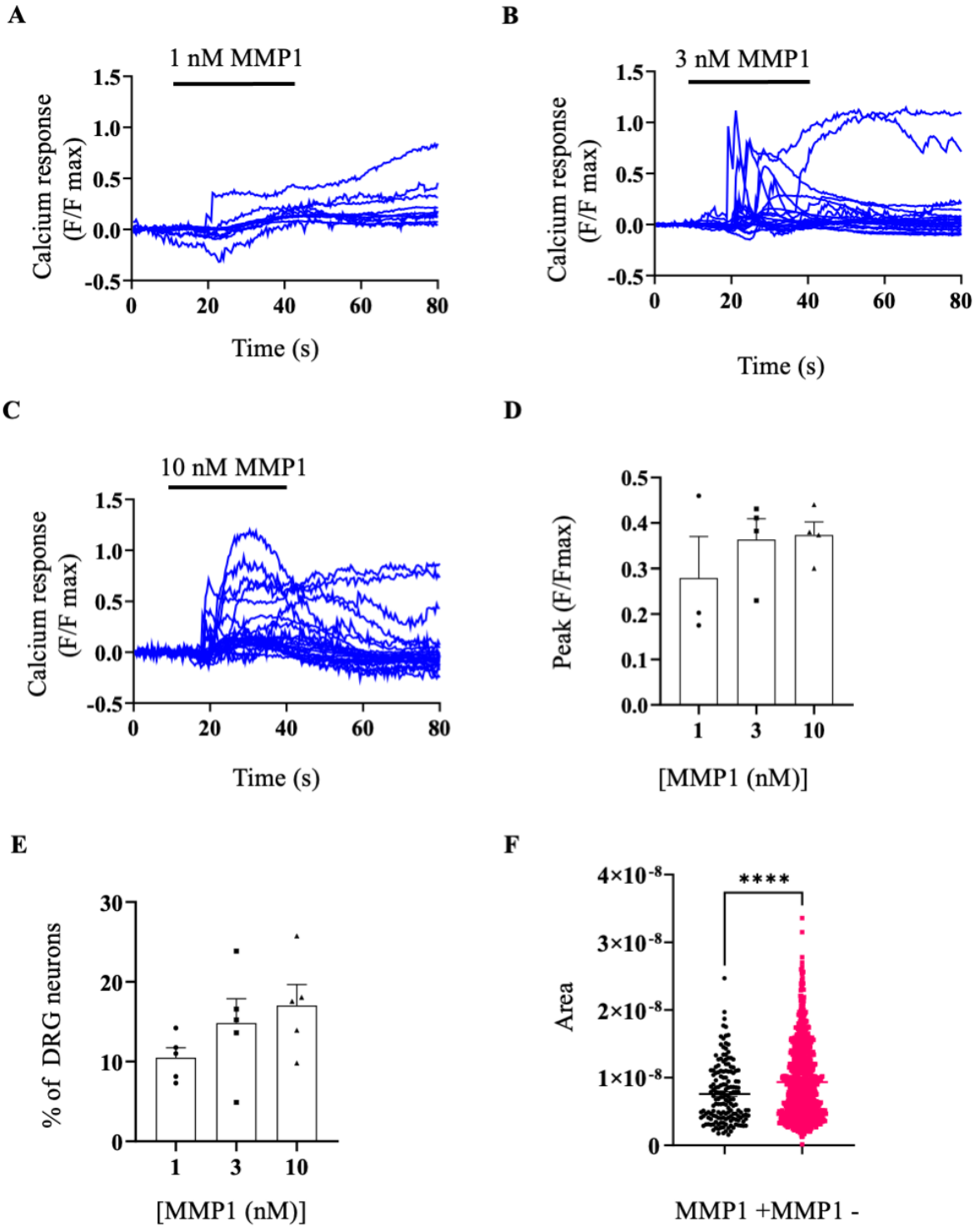


Figure 60. Effect of increasing concentrations of MMP1 on DRG neurons.

Representative response profiles showing Ca^{2+} influx in DRG neurons following treatment with (A) 1, (B) 3 and (C) 10 nM MMP1 for 30 seconds. (D) The average peak response of DRG neurons to increasing concentrations of MMP1. (E) The proportion of DRG neurons responding to MMP1 increased with increasing concentrations of MMP1. (F) Comparison of the size of MMP1 responsive vs the non-responders ($p < 0.0001$, n=5, N=5).

5.10.3 10 nM MMP1 on DRG neurons.

We elected to use 10 nM MMP1 for subsequent studies. Application of 10 nM MMP1 increased $[Ca^{2+}]i$ in 13±2.3% (*n*=6, *N*=6) of all neurons. The response to MMP1 was attenuated by pre-treatment of the neurons with marimastat (*p*=0.018, one-way ANOVA with Dunnett's multiple comparisons test, *n*=5, *N*=5). Similarly, the response to MMP1 was attenuated by SCH 79797 (*p*=0.024, one-way ANOVA with Dunnett's multiple comparisons test, *n*=5, *N*=5) (Figure 61).

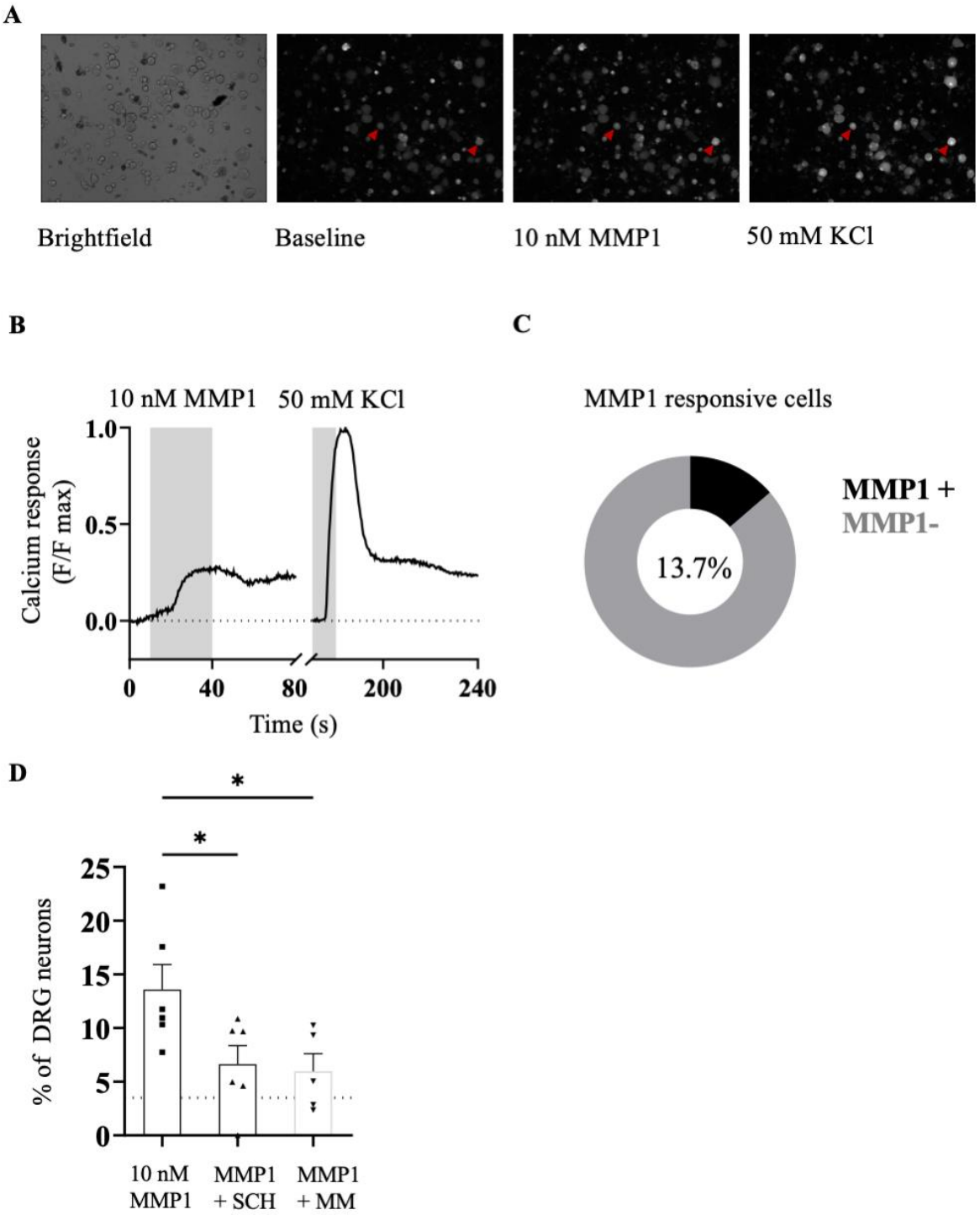


Figure 61. Effect of the PAR$_1$ inhibitor (SCH 79797) and the MMP inhibitor (marimastat) on the DRG neuron response to MMP1.

(A) Representative images showing (i) typical brightfield image of the cells, (ii) image captured before addition of MMP1, (iii) image captured during the response to MMP1 and (iv) image captured during the response to 50 mM KCl. (B) Response profile of a single DRG neuron responding to 10 nM MMP1 followed by 50 mM KCl. (C) The proportion of neurons that responded to 10 nM MMP1. (D) Bar graph showing proportion of DRG neurons responding to 10 nM MMP1 following pretreatment with 10 µM SCH 79797.

5.10.4 MMP1 co-sensitivity with capsaicin

The stimulation of nociceptors by MMP1 was confirmed by response to subsequent application of the TRPV1 agonist capsaicin. Application of capsaicin following treatment with MMP1 resulted in a rapid increase in Ca^{2+} influx in 30.86±3.25% of DRG neurons. The proportion of neurons that responded to capsaicin following treatment with MMP1 was comparable with the proportion of neurons responding in control experiments (p=0.39, unpaired t test, N=6-7). Similarly, the peak response to capsaicin was comparable between control experiments in which cells were treated with ECS and the MMP1 treated cells. 68.34±5.58% of MMP responsive cells also responded to capsaicin indicating MMP1 stimulates a proportion of nociceptive neurons (Figure 62).

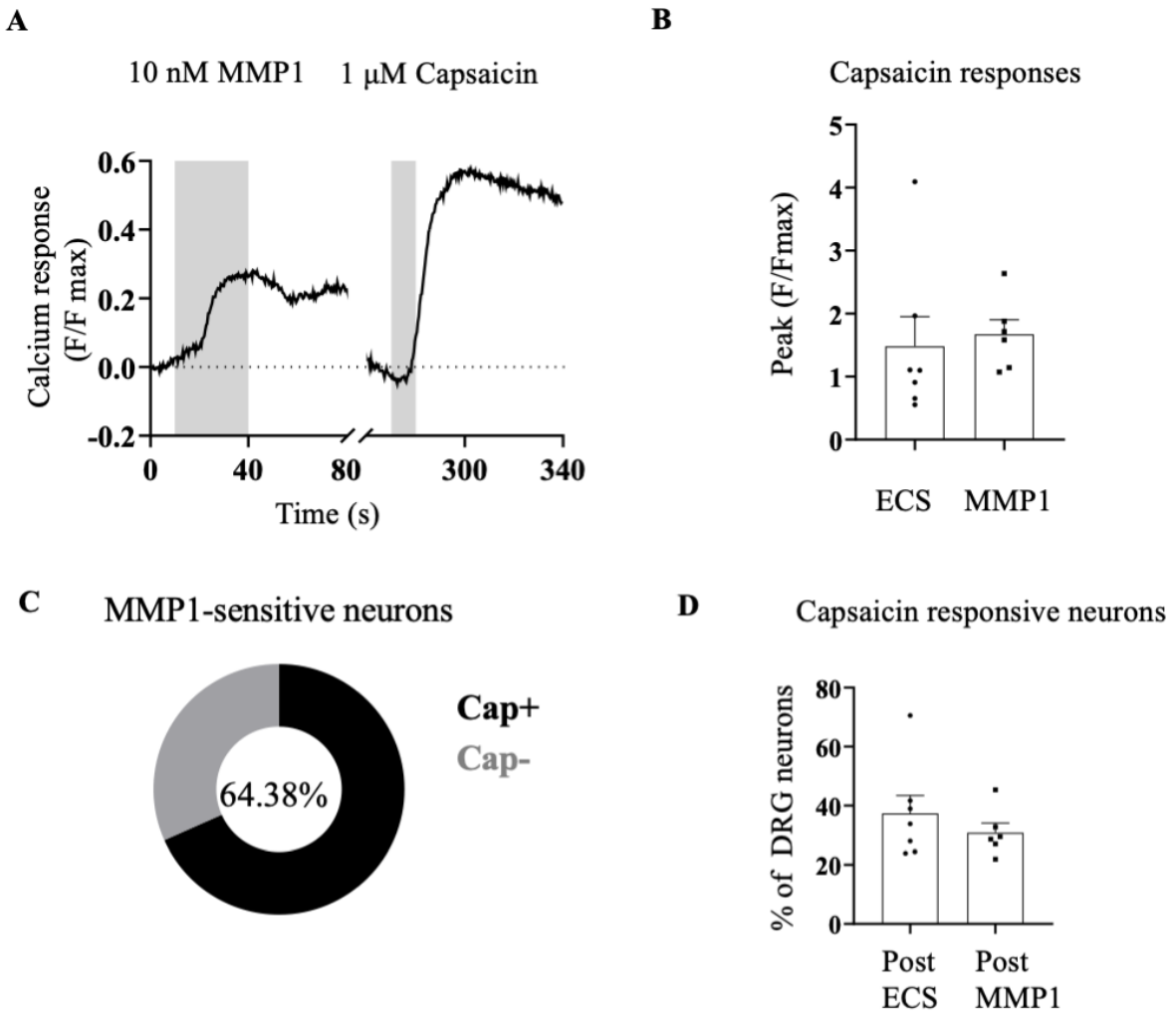


Figure 62. MMP1 co-sensitivity with capsaicin.

(A) Response profile of a single DRG neuron responding to 10 nM MMP1 followed by 1 µM capsaicin. (B) The proportion of neurons responsive to capsaicin in cells treated with the vehicle, ECS or the cells treated with 10 nM MMP1 (p=0.39, unpaired t test, N=6-7). (C) The proportion of MMP1 responsive neurons that also respond to capsaicin. (D) The peak response to capsaicin averaged per dish between ECS treated and MMP1 treated cells (p=0.73, unpaired t test, N=6=7).

5.10.5 TRAP-6 on DRG neurons

Lastly, we conducted experiments to evaluate the DRG neuronal response to PAR_1 activation using TRAP-6, a selective PAR_1 peptide agonist. Application of TRAP-6 increased Ca^{2+} influx in 22.70±2.26% (*n*=3, *N*=3) of all DRG neurons. The proportion of neurons responding to TRAP-6 was reduced by preincubation of the cells with the selective PAR_1 antagonist SCH 79797 (*p*=0.039, unpaired t test). The proportion of neurons responding to capsaicin following treatment with TRAP-6 was comparable with the proportion of capsaicin responders following treatment with the vehicle, ECS. Consistent with the MMP1 data, 58.16±9.126% of TRAP-6 responsive neurons were sensitive to capsaicin suggesting PAR_1 is expressed in a population of nociceptive neurons (Figure 63).

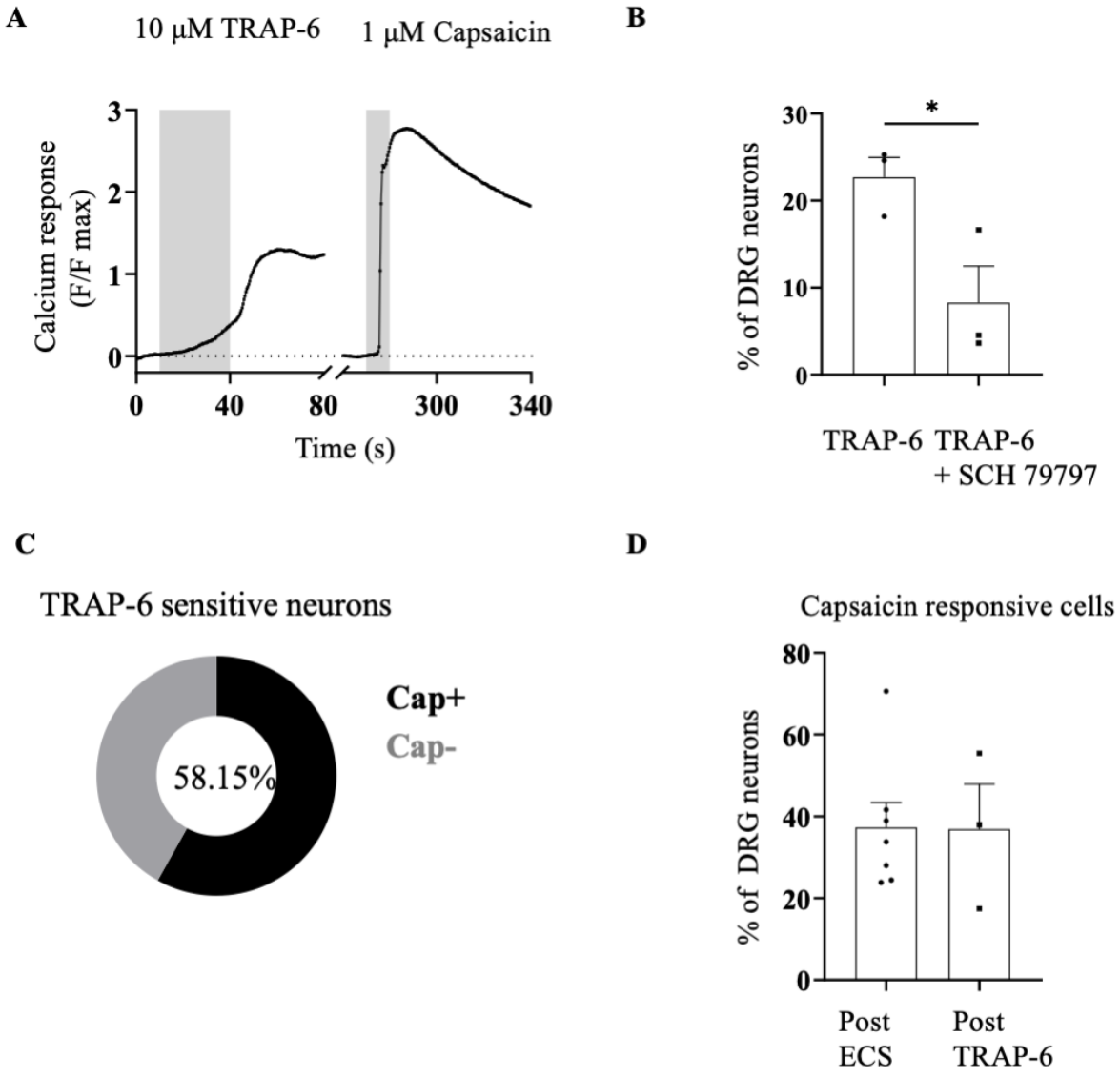


Figure 63. Effect of TRAP-6 on DRG neurons.

(A) Response profile of a single DRG neuron responding treatment with 10 µM TRAP-6. (B) The % of DRG neurons responsive to TRAP-6 in the presence or absence of the PAR_1 antagonist, SCH 79797 (p=0.039, unpaired t test, n=3, N=3). (C) The proportion of TRAP-6 responsive neurons that also responded to capsaicin compared. (D) The proportion of neurons responding to capsaicin in cells treated with the vehicle, ECS or the cells treated with TRAP-6 (p=0.48, unpaired t test, N).

5.10.6 MMP1 on colonic afferents

Following calcium imaging studies which demonstrated that MMP1 stimulates DRG neurons via PAR_1, we evaluated the effect of administration of MMP1 on colonic afferents.
Given that PAR_1 receptors are expressed on colon projecting DRG neurons, we hypothesized that application of MMP1 or TRAP-6 would stimulate colonic afferent firing. Serosal perfusion of increasing concentrations of MMP1 (10, 30 nM) had no effect on colonic afferent activity (Figure 64). Due to time constraints, we were not able to test higher concentrations or different protocols, therefore additional studies to evaluate the MMP1 on colonic afferents are necessary.

Additionally, we evaluated the potential of TRAP-6 to sensitize the LSN response to ramp distension. Administration of 10 µM TRAP-6 administered between distension 2 and 3 had TRAP-6 had no direct effect on the LSN or the response to ramp distension (p=0.80, two-way ANOVA with Bonferroni's multiple comparisons test, N=3 for TRAP-6 and N=8 vehicle treatment) (Figure 64).

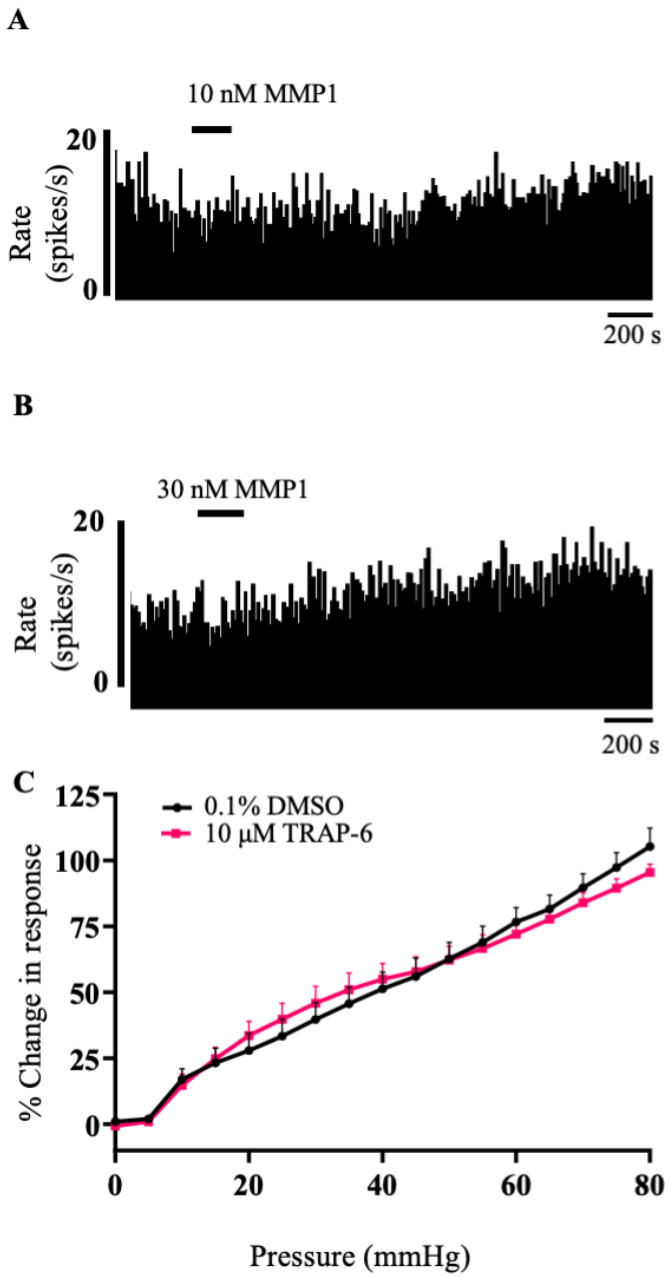


Figure 64. MMP1 and TRAP-6 on LSN activity.

***(A)** Example rate histogram of the colonic nerve response to 10 nM MMP1. (B) Example rate histogram of the colonic nerve response to 30 nM MMP1. Application of MMP1 is indicated by the black bar. (C) The response profile of LSN activity to distension 3 is unaltered following treatment with 10 µM TRAP-6 (p=two-way ANOVA with Bonferroni's multiple comparisons test, N=3 for TRAP-6 and N=8 vehicle treatment).*

5.10.7 PAR_1 activation on permeability

Since we did not observe an effect of PAR_1 activation on LSN activity, additional experiments were conducted to demonstrate the effect of PAR_1 activation by TRAP-6 on intestinal tissue. PAR_1 activation has been demonstrated to increase intestinal permeability (Chin et al., 2003). Therefore, we used the Ussing chamber system to measure changes in permeability of mouse intestinal tissue in response to the concentration tested on the LSN colonic preparation (10 µM). A reduction in TEER values would indicate a reduction in membrane integrity, therefore changes in TEER values across different time points were evaluated in these experiments. Administration of 10 µM TRAP-6 on the mucosal membrane did not have a significant overall effect on TEER values compared with DMSO treated controls (p=0.09, two-way ANOVA with Bonferroni's multiple comparisons test, N=3-5). The average TEER value was reduced at t=30 mins (p=0.04, two-way ANOVA with Bonferroni's multiple comparisons test, N=3-5) however, the subsequent responses were unaltered. (Figure 65). Combined, the results suggest TRAP-6 may alter intestinal permeability, however additional experiments are needed to verify these observations.

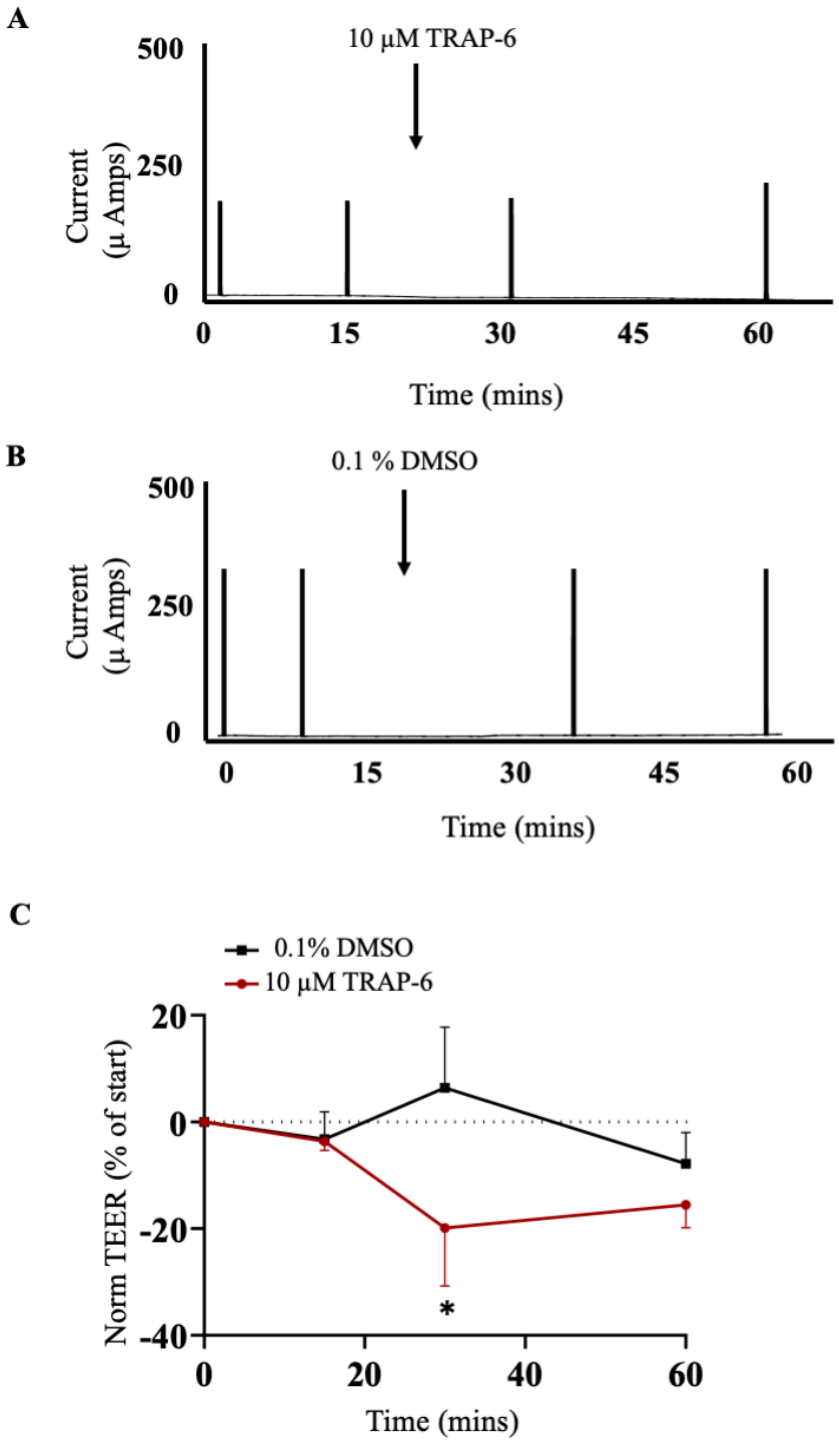


Figure 65. Effect of TRAP-6 on intestinal tissue permeability.

(A) Raw trace showing changes in current following voltage injections at different time points before and after TRAP-6 administration and (B) DMSO. Changes in TEER measurements before and after the addition of TRAP-6 and DMSO at t=20 minutes in intestinal tissue.

5.11 Summary of findings

- Application of MMP1 results in an increase in Ca^{2+} influx in DRG neurons via activation of PAR_1.
- The effect of MMP1 is attenuated by pretreatment with marimastat, the broad-spectrum MMP inhibitor and the PAR_1 antagonist SCH 79797.
- MMP1 activates a proportion of nociceptors classified by their sensitivity to capsaicin.
- TRAP-6 results in Ca^{2+} influx in DRG neurons including nociceptors.
- Administration of MMP1 and TRAP-6 had no observable direct effect on LSN nerve activity.
- Additional studies are needed to examine the effect of TRAP-6 on intestinal permeability.

5.12 Discussion

MMP1 is a member of the zinc-dependent endopeptidases matrix metalloproteinase family and has been shown to be upregulated in IBD tissue along with several other MMPs. The role of MMPs in the pathogenesis of inflammatory diseases such as rheumatoid arthritis and osteoarthritis has been well documented (Nissinen & Kahari, 2014). MMPs have been implicated in the development of IBD and treatment with the broad-spectrum MMP inhibitors batimastat and marimastat has been shown to ameliorate TNBS-induced colitis in rats (Di Sebastiano et al., 2001; Sykes et al., 1999). MMP1 cleaves PAR_1 generating a tethered ligand that subsequently binds and activates the receptor leading to Ca^{2+} mobilization (Boire et al., 2005). PAR_1 activation has been implicated in the development of IBD. PAR_1 is overexpressed in the colon of IBD patients and deletion or blockade of PAR_1 activation has been shown to decrease disease severity in murine models of IBD (Vergnolle et al., 2004). Behavioural studies have demonstrated an analgesic role for PAR_1 in animal models. However, there is no evidence that such analgesic effect is due to a direct activation on primary afferents and further studies are warranted to determine the effects of PAR on sensory neurons (Vergnolle, 2004). In contrast IBS supernatants have been shown to induce calcium influx in human DRG neurons and induce nerve activation via PAR_1, demonstrating a potential role PAR_1 in symptoms associated with IBS (Desormeaux et al., 2018).

PAR_1 is expressed in rodent and human DRG neurons and PAR_1 agonists including thrombin have been shown to stimulate Ca^{2+} mobilization in DRG neurons through PAR_1 activation (de Garavilla et al., 2001; Desormeaux et al., 2018). Although MMP1 is known to cleave PAR_1, the effect of MMP1-PAR_1 signaling on DRG neurons has not been demonstrated. Therefore, in this study, I examined the effects of MMP1 on DRG neurons to evaluate the potential role of PAR_1 cleavage by MMP1 on pain signaling. The data presented demonstrates that MMP1 can stimulate DRG neurons including nociceptors via PAR_1 activation. Following this observation, I evaluated the effect of administration of MMP1 and TRAP-6 on colonic afferents. Surprisingly, the serosal application of both TRAP-6 and MMP1 had no observable direct effect on colonic afferents. Lastly, I evaluated the effect of PAR_1 activation on intestinal tissue permeability using TRAP-6 to confirm its response using the concentration tested in our studies. Administration of TRAP-6 resulted in decreased TEER values indicating reduced tissue permeability.

5.12.1 MMP1 signaling on sensory neurons

PAR_1 is expressed in 10-15% of DRG neurons in adult mice (Vellani et al., 2010). Consistent with these data, 15% of DRG neurons were responsive to the PAR_1 agonist TRAP-6 and 13% of neurons responded to MMP1. Marimastat, an MMP inhibitor, and the PAR_1 antagonist SCH 79797 significantly reduced the proportion of neurons responding to MMP1. The percentage of neurons responding to TRAP-6 was also reduced by pre-treatment with SCH 79797. Further evaluation of the MMP1 responsive neurons showed that MMP1 stimulates nociceptive neurons, thereby could play a role in pain signaling. MMP1 responsive neurons were significantly smaller in size compared with non-responders and 68% of the MMP1 responders were also responsive to capsaicin. Nociceptors are associated with neurons which have small cell bodies and TRPV1, which functions as a receptor for capsaicin and noxious stimuli is expressed in small and medium size DRG (O'Neill et al., 2012). Therefore, capsaicin was used to classify neurons as nociceptors and in agreement with published literature, capsaicin responsive neurons were significantly smaller in size compared with the non-responsive neurons. While studies have demonstrated the ability of PAR_1 agonists to sensitize TRPV1 in DRG neurons, present data shows no evidence of TRPV1 sensitization by either MMP1 or TRAP-6 (Vellani et al., 2010). The average peak response and percentage of neurons responding to capsaicin was comparable between ECS and MMP1 or TRAP-6 treated cells.

5.12.2 MMP1 and TRAP-6 on colonic afferents

Given that MMP1 and TRAP-6 induced Ca^{2+} signaling in DRG neurons and that PAR_1 is expressed in DRG neurons projecting the colon, we hypothesized that administration of MMP1 or TRAP-6 would stimulate the LSN, resulting in increased neuronal output. PAR_1 activation has previously been demonstrated to increase nerve discharge. For example, one study showed that mucosal biopsy supernatants from IBS patients evoked nerve discharge in human submucous neurons, an effect that was attenuated by the PAR_1 antagonist SCH 79797 (Buhner et al., 2018). In another study, trypsin, a nonselective PAR agonist, and thrombin, a PAR_1 and PAR_3 agonist, induced action potential discharge via PAR_1 activation in vagal afferent C fibres demonstrating a direct neuronal effect of PAR_1 activation (Kwong et al., 2010).

Surprisingly, despite *F2r* expression on DRG neurons projecting to the colon, application of MMP1 or TRAP-6 had no observable direct effects on LSN nerve activity. Additionally, we

did not observe any sensitization to the LSN response to ramp distension. More extensive studies of the effects of MMP1 and TRAP-6 on colonic afferents could be conducted to assess the effect of PAR_1 activation on colonic afferents.

5.12.3 TRAP-6 on intestinal tissue permeability

Since we observed no effect of PAR_1 activation on colonic afferents, we used the Ussing chamber to demonstrate a functional effect of PAR_1 activation by TRAP-6 in colonic tissue. Given that PAR_1 activation has been demonstrated to increase permeability and is upregulated in biopsies from IBD patients, PAR_1 induced increased permeability has been implicated in the pathogenesis of IBD (Vergnolle et al., 2004). Additionally, alterations in intestinal permeability have been implicated in the development of visceral hypersensitivity in IBS patients, therefore PAR_1 activation may contribute to visceral hypersensitivity and pain through altering colonic permeability (Zhou et al., 2009). Disappointingly, administration of TRAP-6 did not have a significant effect of colonic permeability in the present study. Given the n numbers are low., additional experiments are essential in establishing the effect of PAR_1 activation on intestinal permeability to demonstrate a functional effect of PAR_1 activation in colonic tissue.

5.13 Future work

Several studies are required to consolidate the findings presented here to determine the potential contribution of MMP1-PAR_1 signalling on visceral pain. Additional calcium imaging studies should be conducted to elucidate the mechanism of action of MMP1 and TRAP-6 on sensory neurons. Furthermore, evaluation of the effect of PAR_1 activation on colonic afferents is key to establishing the potential role of MMP1-PAR_1 signalling on the development of visceral pain. Different protocols can be utilized to evaluate direct and indirect effects of MMP1 on LSN activity. The effect of MMP1 could be evaluated in colitis disease models or in the presence of other MMPs or inflammatory mediators to evaluate the contribution of MMP1 on pain in disease vs healthy tissue. Behavioural studies could be conducted to determine whether PAR_1 activation by MMP1 contributes to hyperalgesia and if these effects can be attenuated by PAR_1 -MMP1 inhibition.

The present study highlights the potential of MMPs to play a role in pain signalling and opens way for additional studies that will evaluate the effect of other MMPs on sensory signalling. Several other MMPs including MMP2, -9 and 13 can cleave PAR_1, therefore, future work can expand on present findings and evaluate the effects of these MMPs on PAR_1 in sensory neurons. Different MMPs cleave different sites along the PAR_1 receptor, raising the possibility that different MMPs induce different signalling pathways, therefore may be capable of producing different effects. Additionally, MMP2, -3, -8 and 13 were also upregulated in the mucosal biopsy samples of IBD patients compared with controls. In Figure 66, below MMP2, -3, and 8 are upregulated in both UC and CD compared with controls, whereby MMP13 is upregulated CD. Thereby, MMPs may be an important target for treating visceral pain.

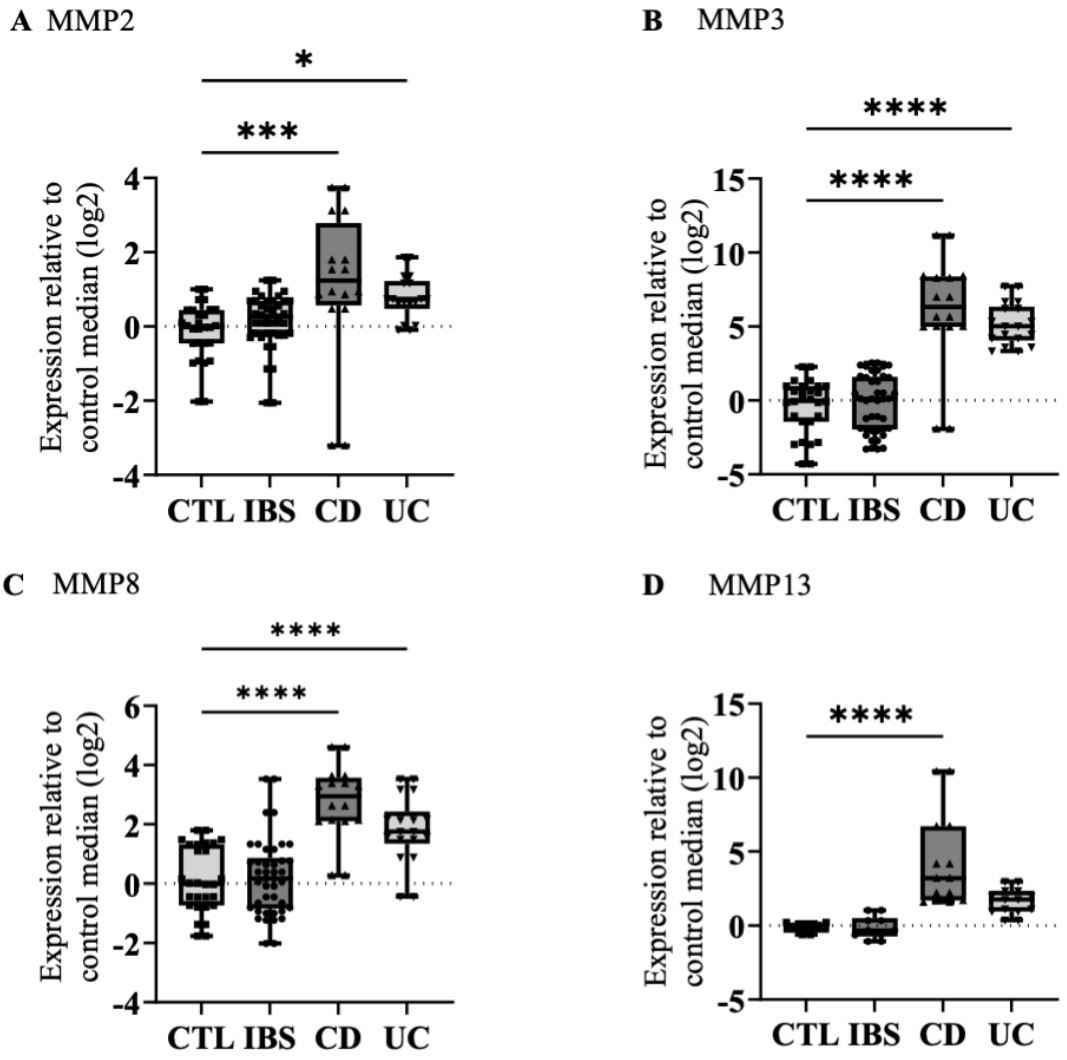


Figure 66. MMPs are upregulated in mucosal biopsies of IBD patients compared with controls.

MMP2, -3 and 8 are upregulated in IBD patients compared with controls whereases MMP13 is only upregulated in CD ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$). Data are shown as Log2 fold change median expression in control samples.*

5.14 Conclusions

In summary, data from this chapter demonstrates that MMP1 can stimulate nociceptors via PAR_1 receptors, thereby providing a mechanism by which MMPs may contribute to abdominal pain in IBD patients. The effects of MMP1 on colonic afferents should be investigated in more detail to establish the role of MMP-PAR1 signalling on visceral pain.

6 Discussion

6.1 Inflammatory mediators excite and sensitise afferents and points of intervention

Visceral pain represents a significant clinical problem in IBS and IBD patients contributing to life-long morbidity in these conditions. Inflammatory mediators including bradykinin, ATP and inflammatory molecules released following tissue damage and inflammation activate and sensitize nociceptors resulting in increased pain perception (Bueno & Fioramonti, 2002). The identification of such mediators is key in developing analgesics to treat pain in IBD patients. Additionally, receptors and ion channels present on sensory neurons are important targets for developing new therapeutics for treating pain. Work done towards this thesis evaluated the potential of K_{Ca} channel modulation on sensory nerve signalling from the GI tract and examined the effects of inflammatory mediators (Ang II and MMP1) that are upregulated in IBD tissue on neuronal excitability and afferent activity to determine their therapeutic potential in treating pain in GI diseases.

6.2 K_{Ca} channels as therapeutic targets.

Potassium channels are important regulators of neuronal excitability and K^+ channel deficiencies are linked with overexcitability of neuronal membranes. In contrast, activation of K^+ channels provide anti-excitatory effects therefore, pharmacological enhancement of K^+ serves as an attractive strategy for management of pain (Du & Gamper, 2013; Humphries & Dart, 2015). The present study evaluated the therapeutic potential of K_{Ca} channel activation on colonic afferent activity in response to noxious mechanical and chemical stimuli. Surprisingly, despite K_{Ca} channels expression in sensory neurons innervating the colon, activation of BK_{Ca} channels demonstrated no inhibitory effect on LSN activity to ATP. Similarly, the combined IK_{Ca}/SK_{Ca} channel opener only had no effect on the LSN response to ATP and bradykinin and ramp distension. In contrast, K_V7 channel activation significantly inhibited colonic afferent activation in response to ATP, bradykinin and colonic ramp distension indicating K_V7 but not K_{Ca} openers may have a therapeutic potential for treatment of abdominal pain. In separate experiments, the K_{Ca} openers attenuated peristaltic contractions demonstrating the utility of K_{Ca} openers in treating GI motility disorders.

Following on from this, we next sought to understand the utility of targeting mediators whose expression is elevated in IBD tissue and cognate receptor expression can be found in colonic nociceptors. Consistent with this approach we looked at the effects of Ang II on colonic afferent activity and the effect of MMP1 on DRG neurons.

6.3 Ang II on colonic afferent activity and therapeutic potential

Angiotensinogen, the precursor for Ang II is upregulated in mucosal biopsies of IBD patients and the AT_1 receptor is expressed in colonic sensory neurons. Given the expression of angiotensin receptors in sensory neurons innervating the colon, we evaluated the pronociceptive potential of Ang II on colonic afferents. In keeping with this receptor expression, we observed a robust activation of the lumbar splanchnic nerve through AT_1 receptor activation. Single unit data confirmed that the majority of these fibres are nociceptors as evidenced by their sensitivity to ramp distension, capsaicin and bradykinin.

We also examined the ability of Ang II to sensitise the LSN nerve response to colonic ramp distension to evaluate its potential role in the development of chronic pain and found no evidence of prolonged colonic afferent sensitization following treatment with Ang II. Lastly, we evaluated the contribution of AT_1 on LSN mechanosensitivity. AT_1 was the first GPCR demonstrated to be mechanosensitive, with mechanical activation of the receptor playing key roles in the development of cardiac hypertrophy. AT_1 inverse agonists can inhibit mechanical activation of AT_1 receptor (Zou et al., 2004). Treatment with losartan decreased LSN activity in response to distension. However, the results could not be reproduced with valsartan which has stronger inverse properties that losartan. Therefore, additional studies are required to examine the inhibitory effect of blocking AT_1 in the LSN response to ramp distension.

The signalling mechanism of Ang II on colonic afferents remains to be elucidated. Future studies to confirm elevated Ang II on IBD patients would be crucial in establishing the contribution of Ang II to pain in IBD patients.

Overall, present data suggests that Ang II may play a role in pain signalling in the GI tract. Targeting AT_1 using ARBs to treat GI pain would be an attractive therapeutic intervention as AT_1 blockers are widely available and used in the clinic to treat high blood pressure.

6.4 MMP1 on sensory signalling and therapeutic potential

MMP1 is upregulated in IBD patients and has been shown to cleave and activate PAR_1 (Boire et al., 2005). PAR_1 is expressed on sensory neurons innervating the GI tract, therefore, we evaluated the ability of MMP1 to activate PAR_1 on sensory neurons and likely contribute to pain. In the present studies, MMP1 induced Ca^{2+} signalling in DRG neurons including a subset of neurons identified as nociceptors due to their co-sensitivity with capsaicin. The response to MMP1 was attenuated by the broad spectrum MMP1 inhibitor marimastat and the PAR_1 inhibitor SCH 79797. These results suggest MMP1 cleaves PAR_1 in DRG neurons to induce calcium signalling. However, administration of TRAP-6 and MMP1 had no effect colonic nerve activity suggesting the modulation of PAR_1 may be more subtle by comparison with other mediators such as Ang II which induce a robust afferent activity. Lastly, we examined the effects of TRAP-6 on colonic permeability to demonstrate a functional effect of PAR_1 activation. As previously demonstrated, PAR_1 activation increased tissue permeability indicating the concentration of TRAP-6 used in these studies was sufficient for engaging the receptor in the system (Chin et al., 2003).

In conclusion data from this study suggests a potential role for MMP1-PAR_1 signalling on pain therefore could be a viable therapeutic target for treating pain symptoms. However additional studies are needed to determine the mechanism of action of MMP1 on sensory neurons and the effects of PAR_1 activation on visceral nociception.

6.5 Conclusions

Activation and sensitization of visceral afferents by inflammatory mediators released in the GI tract contributes to visceral hypersensitivity and development of chronic pain. Understanding the role of inflammatory mediators and mechanisms of sensory signalling from the GI tract is paramount in developing new analgesics to treat visceral pain. Work from this thesis highlights the utility of targeting KCNQ channels and the AT_1 receptor with respective openers and blockers to treat visceral pain. Work with MMP1 opens up an exciting opportunity for this family of proteases to modulate colonic afferent activity although further work is warranted to establish the role of MMP1 on visceral nociception. Data on the K_{Ca} channels suggests targeting this class of ion channels demonstrates no therapeutic potential in treating visceral pain as no inhibitory effects were observed with either the combined IK_{Ca}/SK_{Ca} opener SKA 31 or the BK_{Ca} opener NS1619.

Additionally, this thesis, in part, highlights the potential role of proteases in development of visceral pain in IBD patients. Here, we demonstrate that Ang II can stimulate visceral nociceptors thus could contribute pain in IBD patients. Ang II can be produced directly from angiotensinogen by proteases such as cathepsin G, tonin, trypsin and tissue kallikrein. Additionally, chymase, cathepsin G, tonin, trypsin and kallikrein generate Ang II from Ang I (Belova, 2000). Therefore, upregulation of such proteases in IBD tissue could be contributing to Ang II generation. Here we also demonstrate that MMP1 cleaves and activates PAR_1 in sensory neurons including nociceptors, suggesting a potential role for MMP1- PAR_1 activation in development of visceral pain. Thereby affirming the importance of proteases on pain signalling from the GI tract.

The findings from this thesis are summarised in Figure 67 below.

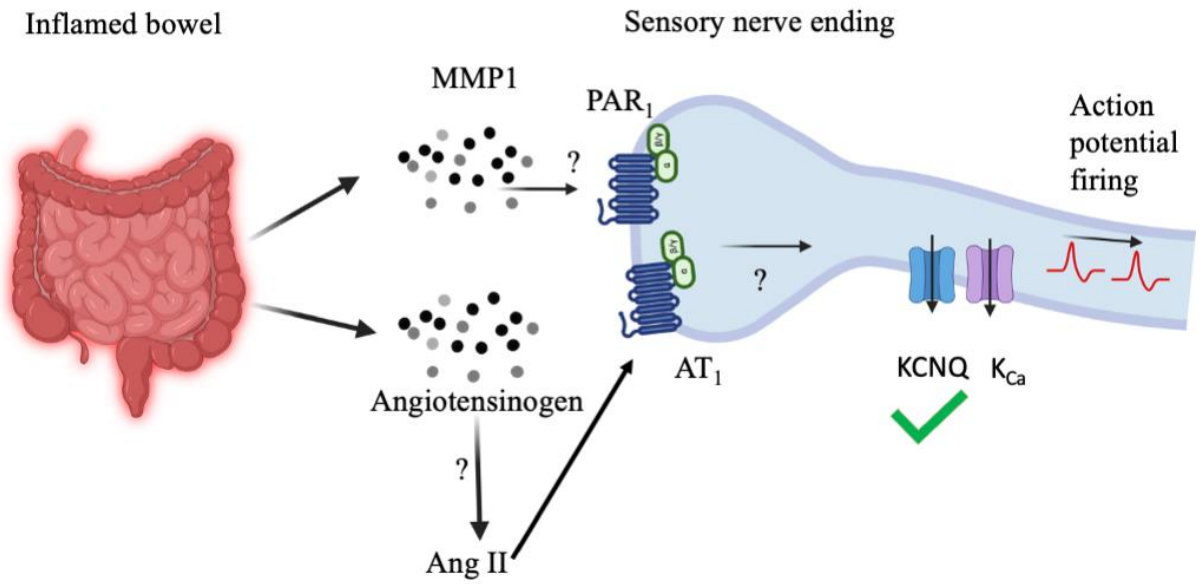


Figure 67. Thesis summary.

MMP1 is highly expressed in IBD tissue and can induce Ca^{2+} mobilization in DRG neurons via PAR_1. The effects of MMP-PAR_1 signalling on colonic afferents remains to be elucidated. On the other hand, Ang II, generated by proteolytic cleavage of angiotensinogen induces action potential firing in colonic afferents including nociceptive fibres demonstrating a potential role for Ang II on visceral pain via AT_1 activation. KCNQ channel activation attenuated action potential discharge in response to noxious chemical and mechanical stimuli whereas only modest effects were observed following K_{Ca} channel activation.

In conclusion, targeting ion channels and receptors involved in nociception is an important strategy for developing novel analgesics with minimum side effects and low abuse potential. Present studies demonstrate a potential role for AT_1 and KCNQ channels on pain signaling whereas additional work is needed to evaluate the role of MMP1 on pain signaling. Therefore, future studies should evaluate the therapeutic potential of targeting AT_1 and KCNQ receptors on pain. Additional work is needed to determine the mechanisms by which the inflammatory mediators Ang II and MMP1 may contribute to pain in order to validate them as targets for treatment of visceral pain.

7 Appendices

7.1 K_{Ca} channel blockers on spontaneous baseline firing

Removal of extracellular calcium from the colonic preparation resulted in a marked increase in nerve discharge that could be attributed to closure of K_{Ca} channels. This would suggest that K_{Ca} channels, whose activity depends on extracellular Ca^{2+} were tonically active in the system. Therefore, we treated the LSN with the SK_{Ca} and IK_{Ca} blockers apamin and TRAM-34, respectively, to evaluate any excitatory effects of inhibiting these channels. However, both the SK_{Ca} blocker, apamin and the IK_{Ca} blocker, TRAM-34 had no effect on spontaneous baseline firing compared with the time matched DMSO controls (TRAM-34: p=0.28, two-way ANOVA with Dunnett's multiple, apamin: p=0.44, two-way ANOVA with Dunnett's multiple, N=4-6) (Figure 68). These results indicating that there is no tonic K_{Ca} activity in the system and other mechanisms were responsible for the observed increase in nerve following removal of extracellular calcium.

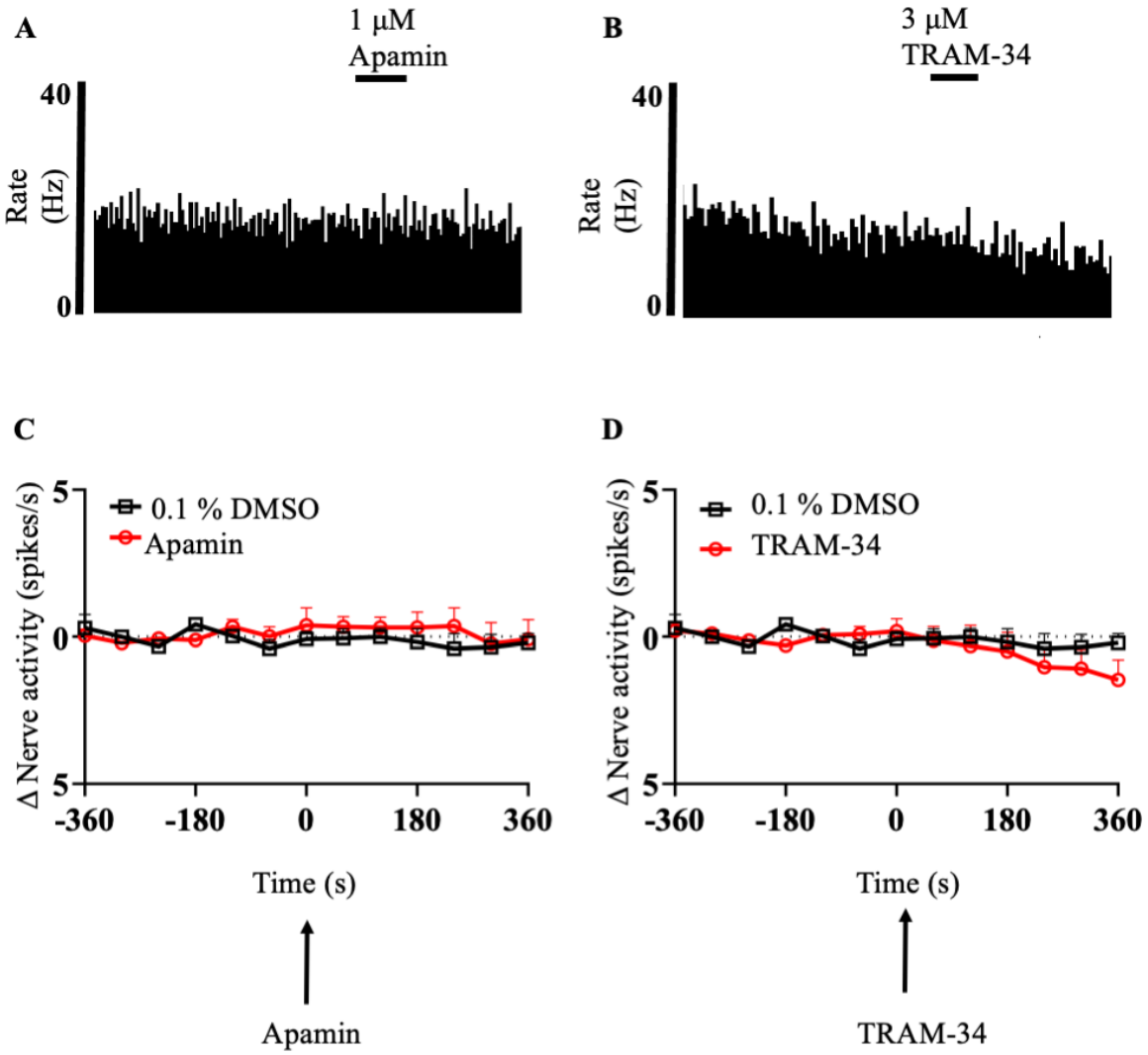


Figure 68. The effect of SK_{Ca} and IK_{Ca} blockers on spontaneous firing

(A) Example rate histogram of spontaneous baseline firing before and treatment with (A) apamin. (B) Example rate histogram of spontaneous baseline firing before and treatment with TRAM-34. Average response profiles illustrating the effects of (C) apamin and (D) TRAM-34 on LSN spontaneous activity in comparison with the time matched DMSO controls. Addition of apamin, TRAM-34, or DMSO is at T=0. N=5-6 animals. Data are shown as mean ±SEM.

7.2 K_{Ca} openers on baseline activity

Additional analysis was conducted to evaluate the effect of the K_{Ca} openers on baseline activity across the different treatment groups (ATP, bradykinin and ramp distension).

SKA 31 had no inhibitory effect on baseline activity in ATP treated and ramp distension experiments whereas retigabine had no inhibitory effect on baseline activity in ATP treated experiments. The inconsistencies in these observations could be attributed to differences in magnitude of responses in male C57B6 (bradykinin treated and ramp distension experiments) mice compared with female CD1 mice (ATP treated) Figure 69.

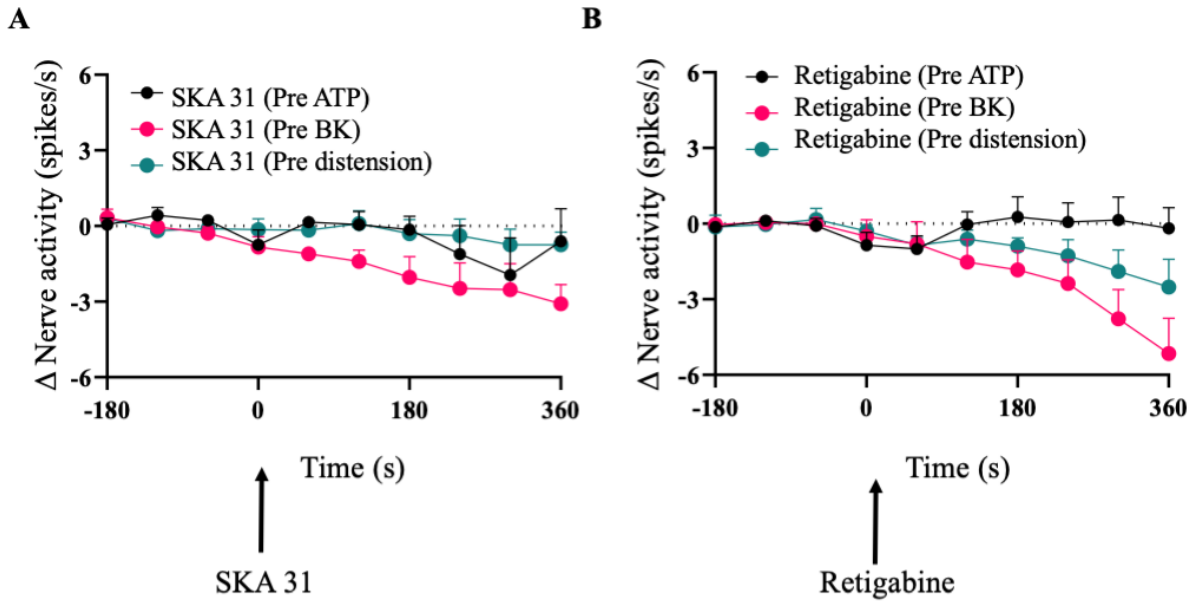


Figure 69. Potassium openers on baseline activity

Average response profiles illustrating the effects of (A) SKA 31 and (B) retigabine on LSN spontaneous activity across three treatment groups (ATP, bradykinin, and ramp distension). Addition of SKA 31 or retigabine is at T=0. N=5-6 animals. Data are shown as mean ±SEM.

7.3 TRAP 6 on colonic permeability

Lastly, although there were no significant effects observed with TRAP-6 on intestinal permeability, preliminary experiments with TEER measurements taken every 60 seconds suggested the TRAP 6 induced changes in current and hence TEER values. However, these results were not observed with protocol used in the reported studies. Therefore, future studies could utilise different protocols to evaluate the effect of TRAP 6 on intestinal permeability.

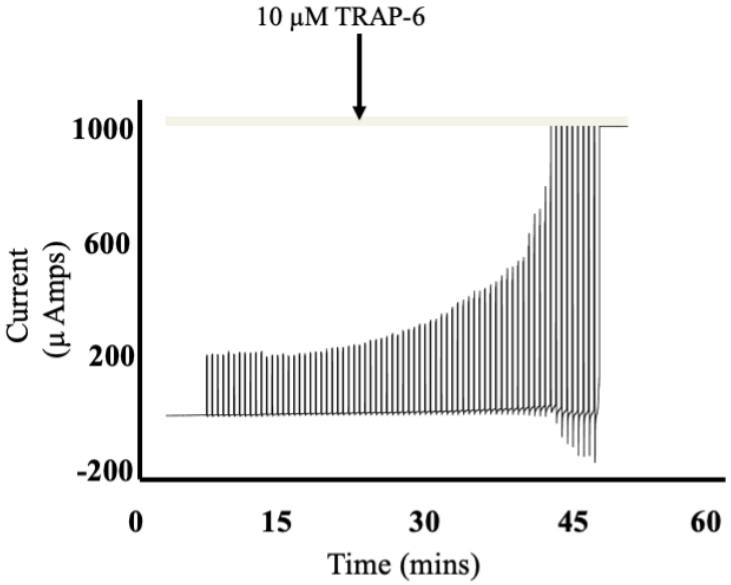


Figure 70. TRAP-6 on intestinal permeability

Raw trace showing changes in current following voltage injections at different time points before and after TRAP-6 administration.

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