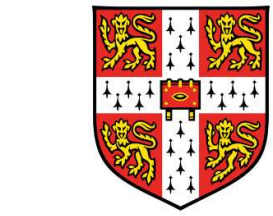
Regulation of Visceral Nociception by GPR35



UNIVERSITY OF CAMBRIDGE

Dissertation Submitted for the Degree of Doctor of Philosophy

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I. Preface

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. All work presented was carried out at the Department of Pharmacology, University of Cambridge between October 2018, and September 2022.

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

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

II. Abstract

Regulation of Visceral Nociception by GPR35

Rohit Gupta

Abdominal pain and discomfort are common symptoms of Inflammatory bowel disease (IBD) and diagnostic criteria for irritable bowel syndrome (IBS) that significantly impair quality of life. Pain in IBD is thought to be mediated by the activation of pain-sensing nerves (nociceptors) that innervate the bowel by mechanical modalities, such as the distention of visceral organs or by mediators released in response to gut inflammation. Pain management in these conditions is challenging due to the side effects associated with commonly used analgesics, and so a significant unmet clinical need exists for the development of new visceral analgesics. GPR35 is a G- protein-coupled receptor (GPCR) which preferentially signals through the activation of $G_{\alpha i/o}$ subunits. GPR35 is designated as an “orphan” GPCR due to the ambiguity of its cognate ligand. However, a few synthetic (e.g., zaprinast and cromolyn) and endogenous agonists (e.g., kynurenic acid) have been identified, which have facilitated research into its function (O’Dowdl et al., 1998; Divorty et al., 2015). In recent years, many of these agonists have been shown to be anti-nociceptive in experimental studies of pain signalling. These effects are abolished in GPR35 -/- mice, thereby providing target validation for the analgesic potential of GPR35 agonists (Ohshiro et al., 2008; Cosi et al., 2011; Alexander et al., 2015). In this thesis, I investigated the role of the GPR35 receptor in the regulation of visceral nociception. Our *in-silico* analysis of previously published transcriptomic data reveals significant co-expression of GPR35 with noxious transducer TRPA1 in the nociceptive neuronal population of colonic DRG neurons. We showed that stimulation of TRPA1 vigorously excites colonic afferents, induces afferent mechanosensitivity and releases neuropeptide Substance-P (SP) from the

colonic tissues, which exerts excitatory effects on colonic afferents. Application of the GPR35 receptor agonists cromolyn or zaprinast attenuates TRPA1-induced afferent excitation, relieves mechanosensitivity, and inhibits the release of SP from colonic tissues, thereby restricting the afferent excitation and colonic contractility induced by SP. GPR35 agonists also inhibited the excitatory action of the disease-relevant mediator PGE2. Finally, the involvement of GPR35 as a molecular determinant of cromolyn or zaprinast action was confirmed by repeated experiments in GPR35-/- animal tissues. These findings suggest that GPR35 represents a high-value target for the development of visceral analgesics.

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IV. Abbreviations

5-HT	5-hydroxytryptamine
5-HTR	5-hydroxytryptamine receptors
AC	adenylyl cyclase
ADP	adenosine-5'-diphosphate
ANOVA	Analysis of variance
AP	action potential
ASIC	Acid-sensing ion channel
ATP	Adenosine triphosphate
BK	Bradykinin
cAMP	cyclic adenosine-5'-monophosphate
Cap	capsaicin
$Ca_V s$	voltage-gated calcium channel
CD	Crohn's disease
CFA	complete Freud's adjuvant
CG	celiac ganglion
CGRP	calcitonin gene-related peptide
CNS	central nervous system
COX	cyclooxygenase
COX-2	cyclooxygenase-2
CRD	colorectal distension
CV	conduction velocity
CXCL1	chemokine ligand 1
DEG/ENaC	degerin/epithelial Na^+channel
DH	dorsal horn
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglia
DSS	Dextran sulphate sodium
EC_{50}	Half-maximal effective concentration
ELISA	Enzyme-linked immunosorbent assay
ENaC	Epithelial sodium channel
ENS	Enteric nervous system
ERK	extracellular signal-regulated kinase

GI	Gastrointestinal
K_{ir}	Inwardly rectifying potassium channel
GPCR	G-protein coupled receptors
GPR35	*GPR35*
HRP	Horseradish peroxidase
I.P.	Intraperitoneal
IB4	Isolectin B4
IBD	Inflammatory bowel disease
IBS	irritable bowel syndrome
IBS-C	Constipation predominant IBS
IBS-D	Diarrhoea predominant IBS
IC_{50}	Half-maximal inhibitory concentration
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IMG	Inferior mesenteric ganglia
IS	Inflammatory soup
KO	Knock-out
LS	Lumbosacral
LSN	Lumbar splanchnic nerve
MMP-1	Matrix metalloproteinase-1
MPO	Myeloperoxidase *5*
mRNA	Messenger ribonucleic acid
$Na_V s$	voltage-gated sodium channel
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG	nodose ganglia
NGF	Nerve growth factor
NPY	Neuropeptide Y
NSAIDs	Non-steroidal anti-inflammatory drugs
P2X	ionotropic P2X purinoceptor
$P2_X3$	P2X purinoceptor 3
P2Y	metabotropic P2Y purinoceptor
PAR2	Proteinase-activated receptor 2
PBS	Phosphate-buffered saline

PG	Pelvic ganglion
PGE2	Prostaglandin E2
Piezo2	Piezo-type mechanosensitive ion channel component 2
PKA	protein kinase A
PKC	protein kinase C
PLC	Phospholipase C
PN	Pelvic nerve
PNS	Peripheral nervous system
qPCR	Quantitative polymerase chain reaction
SD	Standard deviation
SEM	Standard error mean
siRNA	Small interfering Ribonucleic Acid
SMG	Superior mesenteric ganglion
SNRI	Serotonin-noradrenaline reuptake Inhibitor
SP	substance P
SSRI	selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressants
TG	Trigeminal ganglion
TGHβ1	Transforming growth factor beta 1
TL	Thoracolumbar
TNBS	2,4,6-trinitrobenzene sulfonic acid *6*
TNFα	Tumour necrosis factor alpha
TrkA	High-affinity nerve growth factor receptor
TRP	transient receptor potential channel
TRPA1	Transient receptor potential cation channel, subfamily A, member 1
TRPV1	Transient receptor potential cation channel subfamily V member 1
TTX-R	Tetrodotoxin resistant
TTX-S	tetrodotoxin-resistant
UC	Ulcerative colitis
VMR	Visceromotor response

Poster Presentations

Some results contained within this thesis have been presented at both national and international scientific meetings.

- Rohit Gupta, Alastair Brown, Rie Suzuki, David C Bulmer. **Cromolyn inhibits TRPA1 channel mediated visceral nociceptor sensitisation**.
  Digestive Disease week-2022
  21-24 May
  San Diego, CA.

- Rohit Gupta, Alastair Brown, Rie Suzuki, David C Bulmer. Critical role of **Substance P in TRPA1 channel mediated stimulation of colonic nociceptors.**
  Digestive Disease week-2022
  21-24 May
  San Diego, CA.

- Rohit Gupta, Rie Suzuki, Alastair J.H. Brown, Nicole Kaneider-Kaser, David Bulmer. **Evidence of GPR35 receptor mediated inhibition of TRPA1 stimulated colonic afferent activation.**
  IASP-world congress on pain,
  4-8 August-2020,
  Amsterdam, UK

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Investigation into regulation of colonic afferent signalling by GPR35

1. Introduction

1.1. Visceral pain

Chronic abdominal pain arising from the gastrointestinal tract of patients suffering from irritable bowel syndrome (IBS) or inflammatory bowel disease (IBD) is a significant global healthcare problem, affecting millions of people worldwide (Bielefeldt et al., 2009). Abdominal pain is typically evoked from the hollow organs of the gut such as the stomach or intestines in response to mechanical stimuli such as sustained contraction, stretching or tension in the gut wall, although the presence of ischaemia, injury or inflammation can also cause pain (Miranda et al., 2018). By contrast pain is not commonly experienced from the solid organs of the gut such as the liver or spleen, a phenomenon attributed to their lack of sensory innervation (Cervero and Laird 1999). Extensive tissue injury and damage to solid visceral organs can therefore go unnoticed leading to delayed detection typically with more advanced disease, for example pancreatic cancer (Greenwald et al. 1987) or liver cirrhosis (Marotta et al. 1900). In addition, painful stimuli, such as burning, crushing, and cutting, evoke little pain when applied to most visceral structures underscoring the fundamental differences in the processing of pain from visceral compared with cutaneous structures (Ness et al., 1990, Cervero and Laird 1999). The characteristics of these pains are often different. For example, visceral pain is typically dull, diffuse, poorly localised, and referred to overlying structures (Ness et al., 1990, and Cervero, 1994) by comparison with the immediate, sharp, localised nature of somatic pain which is typically accompanied by

a motor reflex designed to move the affected body part away from harmful stimuli. On the contrary, gastrointestinal (GI) visceral pain can lack a rapid withdrawal response, evoking instead quiescent grounding behaviour (Cervero and Laird 2004) which is accompanied by autonomic reflexes such as vomiting and more robust emotional and psychological components than cutaneous pain (Cueva et al. 2007).

1.2. Burden of pain in gastrointestinal (GI) disease

1.2.1. Inflammatory bowel diseases (IBD)

Inflammatory bowel disease (IBD), comprising of Crohn's disease (CD) and ulcerative colitis (UC), is a group of colletids with distinct morbidity, affecting over 6.8 million people worldwide (Alatab et al., 2020). Clinically IBD presents as recurrent chronic inflammation of the GI-tract associated with purulent stool, colonic oedema, bowel obstruction and abdominal pain. Abdominal pain is a common and recurring symptom with up to 70% of patients experiencing pain during the initial onset or exacerbations of IBD (Zeitz et al., 2016; Perler et al., 2019). According to the National report of UK IBD audit 3rd round inpatient experience questionnaire responses-2012, "*IBD patients frequently experienced pain during their stay in hospital. Eighty-five percent (85%) of adult respondents reported some pain, with 52% indicating that pain was usually severe and 42% moderate"* (https://www.rcplondon.ac.uk/file/1082/download). Pain during IBD flare may arise through multifactorial aetiologies including inflammation, surgical complications (abscesses, intestinal fistula, intestinal fibrostenosis or strictures) (Morrison et al., 2013; Srinath et al., 2012; Rieder and Fiocchi, 2008), that can be exacerbated by disrupted gut-microbiota (Khan et al., 2019) and concurrent psycho-social pathologies (Odes et al., 2017). Pain in UC is often episodic varying in intensity and duration; painful spells may occur over a few seconds or whole days,

while CD patients typically experience more persistent pain (Perler et al., 2019; Coates et al., 2013). Chronic pain during active IBD is an indication of disease severity and is primarily governed by inflammatory burden (Peyrin-Biroulet et al., 2016). Furthemore, a subset (~30%) of IBD patients in clinical remission or quiescent states still report significant pain, indicative of a longer-term change in pain processing (Jonefjäll et al., 2015; Gracie and Ford, 2014). The cause of pain in this subset of patients is poorly understood. However, multiple plausible etiologies have been proposed, especially the development of visceral hypersensitivity in response to long term sensitisation of nociceptors, a specialised subset of sensory neurons responsible for pain generation, within the gut by inflammatory mediators has gained much support (Gold and Gebhart et al., 2010; Gebhart and Feng, 2013; Feng and Guo, 2020). In addition, subsequent sensitisation of central pain processing pathways in response to prolonged nociceptor stimulation or psychological factors contributes to chronic pain by amplifying pain experienced by IBD patients (Regueiro et al., 2017).

Pain in IBD negatively impacts on a patient's quality of life (QOL) and may have a high financial burden (Baumgart et al., 2012; Zeitz et al., 2016; Perler et al., 2019). Moreover, IBD patients suffering from abdominal pain typically visit emergency health departments more frequently (Ding et al., 2021). With pain during disease flare necessitating hospitalisation at some point for 20% to 47% of IBD patients (Agrawal et al., 2020; Lewin and Velayos, 2020). Although care must be taken with the management of severe pain in IBD patients which may be due to obstruction, stricture or transmural inflammation necessitating surgical intervention (Rieder et al., 2013 and 2017).

1.2.2. Irritable bowel syndrome (IBS)

Irritable bowel syndrome (IBS) is the most prevalent functional gastrointestinal disorder (FGIDs), commonly affecting up to 15% of the western population (Oka et al., 2020). The diagnostic criteria for IBS include abdominal pain and discomfort, altered bowel habits, bloating and disordered defecation. However, international best practice guidelines promote positive diagnosis using the Rome criteria-III and IV (Thompson et al., 1999; Oka et al., 2020). A distinct predominance symptom pattern exists in IBS patients based on which they are categorised as constipation prevalence (IBS-C), diarrhoea prevalence (IBS-D), mixed IBS (IBS-M) showing both constipation and diarrhoea symptoms or alternating IBS (IBS-A) where bowel habits of patients vary over time (Longstreth et al., 2006). The symptoms such as diarrhoea, constipation and abdominal pain impose distress, lead to absenteeism, and account for an increased burden of medical costs on the patient, altogether deteriorating the quality of life (QOL) (Talley et al., 1995; Thompson et al.,1999). IBS is not accompanied by any marked histological or biochemical alteration detectable with traditional diagnostic tools, (Thompson et al., 1999). However, a growing body of evidence suggests an imbalance of mucosal immunity with signs of microscopic inflammation such as increased accumulation of lamina propria mononuclear cells, immunoglobulin A plasma cells, increased rectal mucosal enteroendocrine cells and T lymphocytes is found in post infectious (PI)-IBS (Gwee *et al.* 2003; Spiller et al., 2000). Additionally, increased proteolytic activity and mast cell accumulation in proximity of colonic nerves (Cenac *et al.* 2007; Barbara *et al.* 2004) and impaired mucosal barrier function in subpopulations of IBS patients has been observed (Piche *et al.,* 2009; Zhou *et al.* 2009). A prevailing hallmark clinical feature of IBS is colorectal hypersensitivity to barostat balloon distension (Mertz et al., 1995; Barbara et al., 2011). Visceral hypersensitivity (VH)

refers to an increased perception of stimuli arising from the viscera. Specific terms that are commonly used to describe hypersensitivity: *are allodynia* and *hyperalgesia* (Farzaei et al., 2016). The perception of pain in response to stimuli that usually are not perceived as painful is referred to as allodynia. In comparison, an increase in pain perception to stimuli usually perceived as painful is referred to as hyperalgesia (Farzaei et al., 2016). Visceral hypersensitivity is also accountable for the generation of heightened visceral stimuli in IBD, perceived as pain. For example, enhanced visceral hypersensitivity in IBD, following transient inflammation in the gut, is evident in clinical studies, where patients with moderate to active colitis show decreased sensation thresholds and reduced maximal tolerable volume in response to anorectal balloon distention in comparison to healthy volunteers (Farthing and Lennard-jones, 1978; Rao et al., 1987).

1.3. Pain management in IBD and IBS

1.3.1. Medication reducing inflammation to achieve pain relief

Pain management in IBD patients is focused on remission of inflammation (Jostins et al., 2012) with support from commonly prescribed analgesics. For many patients pain relief accompanies disease remission however as mentioned previously, a significant minority of patients continue to experience pain and discomfort largely because of visceral hypersensitivity during remission, highlighting a need for new analgesic strategies. Furthermore, a recently published retrospective study of analgesic use in newly diagnosed IBD patients, approximately a fifth to a quarter of patients were prescribed NSAIDs, half opioids and nearly a third neuromodulators/ antidepressants to mitigate pain symptoms (Hunter et al., 2021). However, many patients reported

limited effectiveness and drug associated adverse effects with these treatment regimens. For example, frequent and long-term (≥15 times/month) use of NSAIDs can precipitate injury to the gut mucosa (Wolfe et al., 1999) and is associated with increased incidence of CD and UC (Ananthakrishnan et al., 2012) and early clinical relapse of quiescent IBD (Takeuchi et al., 2006), a risk more associated with active CD than UC patients (Bonner et al., 2001). These effects are greater for dual inhibition of COX1 and COX2 enzymes by non-selective NSAIDs (Takeuchi et al., 2006) with selective cyclooxygenase-2 (COX-2) inhibitors (including celecoxib, rofecoxib and etoricoxib)) showing improved safety profiles compared with conventional NSAIDs in clinically inactive IBD patients (Bonner, 2001; Mahadevan et al., 2002; Sandborn et al., 2006; El Miedany et al., 2006; Biancone et al., 2004). However, conflicting data suggesting a low incidence of disease relapse (Bonner, 2001) and exacerbation of IBD is also reported (Wilcox and Mattia, 2005; Gornet et al., 2002). Frequent use of NSAIDs (≥ 5 times/month) is associated with active disease at treatment follow-up, indicating a lack of both treatment efficacy and disease remission in IBD patients (Long et al., 2016).

1.3.2. Medication improving colonic motility to achieve pain relief

In IBS, abnormal colonic motility or altered colonic transit is a pathophysiological mechanism behind pain symptoms and diarrhoea, particularly in IBS-C and IBS-D patients. Antispasmodics have been used to treat altered colonic motility. For example, hyoscyamine a non-selective muscarinic receptor inhibitor, functions by relaxing the intestinal smooth muscle to relieve spasms and abdominal cramps and have been proven effective in managing abdominal pain (Ford et al., 2008). A similar beneficial effect of another anticholinergic drug Mebeverine have been reported in Cochrane report signifying improvement of pain scores in IBS patients (Ruepert et al., 2011).

However, antispasmodics are known to reduce gastric motility; therefore, aftereffects such as constipation and bowel obstruction should be monitored during therapy. Additional side effects such as dry mouth, dizziness and blurred vision also limit their clinical utility. An herbal remedy implementing peppermint oil improved motility issues in IBS by inhibiting smooth muscle contractility and calcium influx in the gastrointestinal tract (Amato et al., 2014; Hawthorn et al., 1988), whereas menthol which is an active ingredient of peppermint oil additionally offers analgesic properties by activating transient receptor potential cation channel subfamily-M member 8 (TRPM8) which exerts antinociceptive effects on visceral afferents (Liu et al., 2013). However, the use of peppermint has its own side effects including gastroesophageal reflux, xerostomia, and belching. The antispasmodic offers some beneficial effects in IBS patients' but the conclusive evidence for their usefulness in IBD patients remains elusive (Makharia et al., 2011). Moreover, the meta-analysis of clinical trials studying the effectiveness of antispasmodics does not offer a conclusive viewpoint on the benefits of these therapy (Ruepert et al., 2011).

Opioids are commonly prescribed for the management of chronic pain following surgery in IBD patients, and there has been a surge in the use of opioid medication among CD and UC patients (Burr et al., 2018; Barnes, 2020), despite the risk of overdose, depression, anxiety, and abuse liability (Burr et al., 2018; and Cohen-Mekelburg et al., 2018). Observational studies on small cohorts of IBD patients report improvement in disease activity index (DAI) scores and QOL (relieving abdominal pain, cramping and diarrhoea) following opioid treatment but this does not translate to larger clinical trials (Kienzl et al., 2020).

Opioid receptor agonists such as loperamide and diphenoxylate (μ-receptor agonist) treatment have improved stool consistency, pain, and reduction in urgency and stool frequency in IBS-D patients (Hovdenak, 1987; Efskind et al., 1996). Similarly, Eluxadoline is a novel κ- and μ-opioid receptor agonist, and δ-opioid receptor antagonist has also been shown to improve diarrhoea and pain (Dove et al., 2013; Lembo et al., 2016). The common concerns over opioid agonists are nausea and headache, while the use of eluxadoline should be avoided in patients with pancreatitis, severe hepatic impairment, or severe constipation.

Finally, tricyclic antidepressants (TCAs; amitriptyline), selective serotonin reuptake inhibitors (SSRIs; paroxetine and citalopram) and serotonin-norepinephrine reuptake inhibitors (SNRIs; duloxetine and venlafaxine) have only been shown to be effective at relieving psychological comorbidities (anxiety and depression) as opposed to pain in IBD patients. However, antidepressants have shown improvement in QOL, anxiety, depression, (Brennan et al., 2009 and Kaplan et al., 2014), visceral hypersensitivity (Thoua et al., 2009) and pain symptoms (Ford et al., 2014) in IBS and functional pain disorders.

1.3.3. Medication improving constipation to relieve pain

Constipation is another pernicious symptom of IBS that leads to pain. Constipation developed either due to altered colonic motility or increased fluid absorption from the colonic lumen, causing stool hardening and reduced gastrointestinal transit. The drugs that improve intestinal fluid secretion or increase colonic transit would relieve constipation and pain. Lubiprostone is a prostaglandin derivative with intestinal secretagogues action. It softens the stool by facilitating the passive movement of Na^+

and H_2O into the lumen following chloride channel activation and secretion in the intestinal enterocyte's apical membrane and improve gastrointestinal transit. The drug is approved for chronic constipation (Camilleri et al., 2006).

Similarly, linaclotide, a peripherally restricted guanylate cyclase-C (GC-C) receptor agonist, causes secretion of chloride and bicarbonate into the intestinal lumen *via* the cystic fibrosis transmembrane regulator (CFTR), resulting in sodium and water secretion into the lumen (Busby et al., 2010). It has also been shown to inhibit sensory neuron activity by increasing cGMP levels leading to relief pain and has therefore been approved for the treatment of IBS-C patients (Chey et al., 2012; Rao et al., 2012; Rao et al., 2014). Another drug Plecanatide is a peptide analogue of uroguanylin, an endogenous GC-C agonist that is also efficacious in treating chronic idiopathic constipation in IBS-C patients by relieving severe pain (Miner et al., 2017; Brenner et al., 2018).

Agonist at $5HT_4$ receptor such as Tegaserod have demonstrated efficacy in patients with IBS-C and improved overall symptoms including constipation, pain, bloating and discomfort (Layer et al., 2007) whereas Mosapride, another agonist at $5HT_4$ receptor, was effective in IBS-C patient during pilot study but study investigating larger cohorts for longer duration have shown no significant improvement compared to placebo (Mansour et al., 2012). However, these drugs have associated side effects such as diarrhea, cramping, and rarely observed cardiac problems (Loughlin et al., 2010).

1.3.4. Medication reducing diarrhoea to achieve pain relief

Serotonin (5-HT) influences colonic motility and plays a constitutive role in visceral pain (Hicks et al., 2002). Patients with IBS-D have shown increased postprandial 5-HT release, while patients with IBS-C display reduced postprandial 5-HT levels (Atkinson et al., 2006). 5-HT3 antagonist such as Alosetron (Andresen et al., 2008), Ramosetron

(Fukudo et al., 2016) has shown improvement in symptoms of IBS-D patients, including stool frequency, bloating, abdominal pain and diarrhoea; consequently, they have been approved for use in IBS-D patients.

Antibiotics such as rifaximin and ciprofloxacin are also prescribed particularly in infectious diarrhoea. Rifaximin in non-constipated IBS patients provides adequate relief in global IBS symptoms and bloating moreover, with repeat therapy it significantly reduces urgency, bloating, abdominal pain, and stool consistency (Pimentel et al., 2000 and 2011). Rifaximin however accelerate colonic transit that may be deleterious for patients with IBS-D (Acosta et al., 2016).

Finally, lifestyle modification, probiotics, and dietary interventions including restricted use of provocative substances, such as alcohol and high sugar content diets have their own advantage and appear to reduce pain in remissive IBD patients; however, this approach needs further investigation (Norton et al., 2017). Considering the lack of success for pain management in IBD, and slender improvement in IBS, despite diverse empirical treatment approaches, it is evident that efficient and effective treatments are still an unmet clinical need.

Our understanding of the neural circuitry of the GI tract, physiological process of sensory transduction, the basis of nociception in specific spinal pathways and the pathophysiology that contributes to symptom presentation in functional bowel disorders may provide a basis for future rational drug design to address this unmet need.

1.4. Sensory innervation of the GI tract

The GI tract is innervated by intrinsic neurons of the enteric nervous system (ENS), motor neurons of the autonomic nervous system (sympathetic and parasympathetic), and primary afferent neurons which project to the central nervous system (CNS).

1.4.1. Enteric nervous system

The ENS comprises of two plexi, the myenteric (Auerbach) plexus and the submucosal (Meissner) plexus. The myenteric plexus is situated between the longitudinal and circular smooth muscle layers and regulates gut motility such as peristalsis and other motor reflex intrinsic to the bowel. The submucosal (Meissner) plexus is a network of neurons between the smooth muscle layer and mucosa which control epithelial cell functions, gastrointestinal blood flow, and secretion according to the lumen environment (Uesaka et al., 2016). The three main classes of enteric neurons, comprise of motor neurons, intrinsic primary afferent neurons (IPANs, or sensory neurons), and interneurons which act co-ordinately to execute reflex gut function (Lakhan and Kirchgessner, 2010; Vermeulen et al., 2014).

1.4.2. Extrinsic innervations

The extrinsic primary afferents innervating the GI tract provide sensory connection with the Central Nervous System (CNS), facilitating gut reflexes and sensations such as pain, satiety, and fullness. The extrinsic sensory afferents take cognisance of physical and chemical changes occurring within the lumen and the gut wall and convey the signals of noxious and non-noxious events to the CNS. The three main pathways involved in the transduction of sensory information include spinal afferents projecting to the thoracolumbar (TL) spinal cord *via* the splanchnic nerves, spinal afferents

projecting to the lumbosacral (LS) spinal cord *via* the pelvic nerve and vagal afferents which project to the brainstem. While the GI tract is extensively innervated, the sensation of pain is primarily transduced by afferents from the TL and LS pathways, as shown by its absence following bilateral sympathectomy (Ray & Neill, 1947).

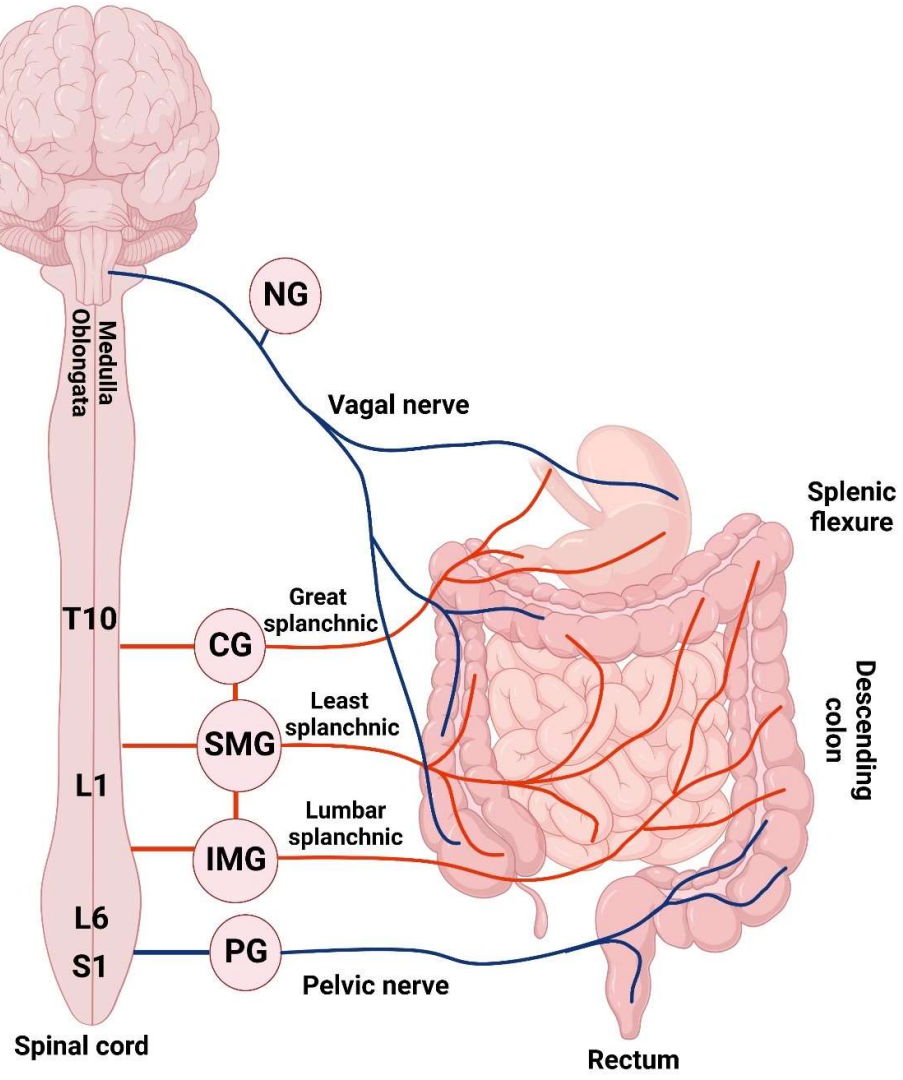


Figure 1: Spinal afferent innervation of the colon. The mammalian gut is innervated by three main afferent pathways such as vagal, thoracolumbar, and lumbosacral. Vagal afferent fibres originate from cell bodies located in the nodose ganglia (NG); project centrally to the brainstem and distributed peripherally throughout the GI tract. The vagal afferent fibres densely innervate proximal GI regions including stomach,

oesophagus, and small intestine. Thoracolumbar afferent fibres originate from DRG neurones (T7-L1) and project centrally to the dorsal horn and peripherally to the upper and lower GI tract. Thoracolumbar afferents pass through the celiac ganglia (CG), superior mesenteric ganglia (SMG) and inferior mesenteric ganglia (IMG). Finally, Lumbosacral afferents originate in DRG neurones (L6 and S1) project centrally to the dorsal horn of the spinal cord and peripherally innervate the distal colon and rectum by passing through the pelvic ganglia (PG).

Spinal thoracolumbar afferents innervate the gut from the oesophagus to the stomach, small intestine, and proximal colon *via* the great splanchnic and lesser splanchnic nerves. The transverse colon is innervated by spinal fibres of the lumbar splanchnic nerves (LSN), while the distal colon receives dual innervation from the lumbar splanchnic nerve and sacral pelvic nerves (PN). The latter principally innervate the rectum. The splanchnic nerve cell bodies are located within the thoracolumbar dorsal root ganglia (DRG) with the majority arising from T10-L1 spinal levels, whilst pelvic afferents have cell bodies within lumbosacral DRG, the majority of which coming from L6-S1 spinal levels (Grundy and Brierley, 2018). Spinal afferents project peripherally from DRG *via* sympathetic nerves which transverse pre-vertebral ganglia (celiac, superior, and inferior mesenteric ganglion) to reach the gut wall; and centrally to terminate within laminae I, II, V and X of the dorsal horn making synaptic contacts with 2nd-order neurons (Sadeghi et al., 2018, Cervero & Connell, 1984a, 1984b, Morgan et al., 1981). Finally, visceral sensory input is relayed to higher brain centres *via* ascending pathways which include spinoreticular, spinohypothalamic, spinomesencephalic and spinothalamic pathways.

Parallel, to these vagal afferent neurons whose cell bodies are in jugular/nodose ganglia (NG) project centrally to the brainstem and are peripherally distributed throughout the GI tract, with a dense innervation observed in the stomach, oesophagus, and small intestine. Vagal innervation of the colon is sparse and is not seen to extend beyond the transverse colon (Berthoud et al.,1991 and 1997). Vagal afferents mainly regulate food intake and autonomic reflexes such as gastric emptying and enzyme secretion (e.g., gastric/pancreatic secretion). It orchestrates behavioural aspects of food intake, such as the perception of hunger, fullness, satisfaction, and bloating (Wang et al., 2020), contributes to the initiation of motor reflexes (Grundy et al., 2002; Page et al., 2002) and triggers aversive responses such as nausea and emesis, in response to potentially noxious stimuli, although the vagus does not mediate pain behaviours (Lamb et al., 2003).

Apart from the anatomical segregation of the spinal afferents into broad groupings such as "pelvic afferents" and "splanchnic afferents", electrophysiological and tracing techniques have been used to further classify and characterise murine spinal afferents based on perceived location within the various layers of the GI tract (Brookes et al., 2013; Brierley and Linden, 2014; Spencer et al., 2014; Humenick et al., 2015; Hibberd et al., 2016), and functional response to different mechanical stimuli such as probing, circular stretch and mucosal stroking using calibrated von Frey hairs (vFh) (Page et al., 2004; Brierley et al., 2004, 2008 and 2009; Hughes et al., 2009b; Brookes et al., 2013; Castro et al., 2013 and Brierley and Linden et al., 2014) as muscular (respond to low intensity circumferential stretch); mucosal (respond to light vFh stroking of the mucosa), mesenteric (respond to focal compression of the mesentery) and serosal (respond to focal compression of the colon wall, but not mucosal stroking or low intensity circular stretch). Finally, a subset of "silent afferents" also exist, which, under

normal physiological conditions, are mechanically insensitive but can be activated by inflammatory stimuli. These mechanically insensitive afferents (MIA) can be further divided into those which subsequently develop mechanosensitivity in response to inflammatory mediators and those which do not (Brookes et al., 2013; Feng and Gebhart, 2011). These afferents therefore involved in increasing pain signalling from the periphery in response to distension or contraction of the gut in the presence of inflammation and as such are likely to be pathologically important (Bueno and Fioramonti, 2002; Knowles and Aziz, 2009; Takahashi et al., 2021).

Serosal and mesenteric afferents encode much higher intensities of circumferential stretch and balloon distension (~50mmHg) (Brierley et al., 2008; Hughes et al., 2009) and have previously been grouped together as 'vascular' afferents (Brookes, 2013). These vascular afferents, apart from being involved in the transduction of high-intensity mechanical stress, also respond to a diverse range of inflammatory mediators such as ATP, bradykinin, glutamate, nerve growth factor, histamine, prostaglandins, and 5-HT (Brunsden and Grundy, 1999; Maubach and Grundy, 1999; Blackshaw and Gebhart, 2002; Hicks et al., 2002; Rong et al., 2002; Grundy, 2004; Brierley et al., 2005; Sipe et al., 2008; Hockley et al., 2016), and are also sensitive to cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin (IL)-2, IL-6, IL-1β (Brierley et al., 2005b; Hughes et al., 2013; Campaniello et al., 2016). The vascular afferents comprise the major class of nociceptor in the colon and are most likely involved in abdominal pain *via* neuro-immune interactions during disease, and their activation has been specifically linked with inflammatory pain (Brookes, 2013; Brierley, 2004; Hughes, 2009; Brierley, 2008).

Muscular afferents are relatively rare in the splanchnic pathway but prevalent in pelvic pathways innervating the distal colon and rectum (Feng and Gebhart et al., 2011; Brierley et al., 2004; Hughes et al., 2009b). Muscular afferents respond to low-intensity stretch and to relatively small changes in intraluminal pressure. However, they can encode stimuli into the noxious range (>30mmHg) and therefore these afferents are thought to play a role in nociception (Brierley et al., 2004).

Finally, mucosal afferents constitute the most sparse afferent population in LSN as studied in mouse (4%) and rat (23%) colon (Brierley et al., 2004; Lynn and Blackshaw et al., 1999). The mucosal mechanosensitive afferents are primarily located in or below the mucosal epithelium. These afferents exhibit extremely dynamic firing to discrete probing of the mucosal receptive spot but are insensitive to circular stretch or luminal distention, and therefore they are unlikely to be involved in nociceptive signalling. The sensitivity profile of these afferents indicates their role in monitoring the dynamic events in the gut, such as mechanical positioning, motility control by providing fine mucosal input, and fine-tuning of perceived stimuli (Bahr et al., 1986a and 1896b). Most mucosal mechanosensitive afferents are also sensitive to the hyperosmolar solution, acid, bile, 5-HT, ATP, and capsaicin (Brierley et al., 2004; Lynn and Blackshaw, 1999; Hicks et al., 2002). However, their characterisation in the presence of other chemical mediators and during inflammation has not been extensively studied (Brierley et al., 2004; Hughes et al., 2009).

Class of Afferents	Mechanical stimulus		
	Stroke	**Probe**	**Stretch**
Mucosal	Yes	Yes	No
Muscular mucosal	Yes	Yes	Yes-low threshold
Muscular	No	Yes	Yes-low threshold
Serosal	No	Yes	Yes-high threshold
Mesenteric	No	Yes	Yes-high threshold
Mechanically insensitive (MIA) or Silent afferents	No (in naïve conditions)	No (in naïve conditions)	No (in naïve conditions)

Table 1: Summarising the firing response profiles of the functional classes of afferents innervating the murine colon and rectum to distinct mechanical stimulus (modified from Brierley et al., 2018).

The spinal afferent classification established in mice is comparable in humans. Studies on the resected human appendix, colon and rectum tissues have identified afferents displaying similar functional characteristics to muscular, serosal and mesenteric afferent groups (Hockley et al., 2016; Yu et al., 2016; McGuire et al., 2018). Similarly, comparable to the findings in the rodent tissues, human afferents are also sensitive to diverse algogenic and inflammatory mediators. For example, direct application of the inflammatory soup (bradykinin, 5-hydroxytryptamine (5-HT), histamine, and PGE2) elicited robust transient increase in the afferent activity (Peiris et al., 2011). Subsequently, several studies have shown activation of human afferents following the application of individual mediators such as histamine and PGE2 (McGuire et al., 2018), bradykinin (Peiris et al., 2011; McGuire et al., 2018 and Yu et al., 2016), and, finally, 5-HT (McGuire et al., 2018 and Yu et al., 2016). Additionally, agonist at purinergic receptor ATP (McGuire et al., 2018), TRPV1 agonist capsaicin (Jiang et al., 2011;

Peiris et al., 2011; Ng et al., 2016; Yu et al., 2016) and TRPA1 agonist, AITC (Yu et al., 2016) have also shown to excite human afferents.

The murine spinal afferents that innervate the colorectum are further distinguished based on sensitivity to colorectal distention in tube preparation (Malin et al., 2009; Hughes et al., 2009b; Hibberd et al., 2016). This has led to the grouping of colorectal afferents traveling with lumbar splanchnic nerves, into those which response at low pressure thresholds <20mmHg of colorectal distension whose high-frequency firing saturates at these low pressures; afferents which respond at pressure thresholds <20mmHg but continue to respond at moderate frequency across a wide-dynamic range of pressures and afferents which respond at low-frequency with a high-pressure threshold to activation >40mmHg.

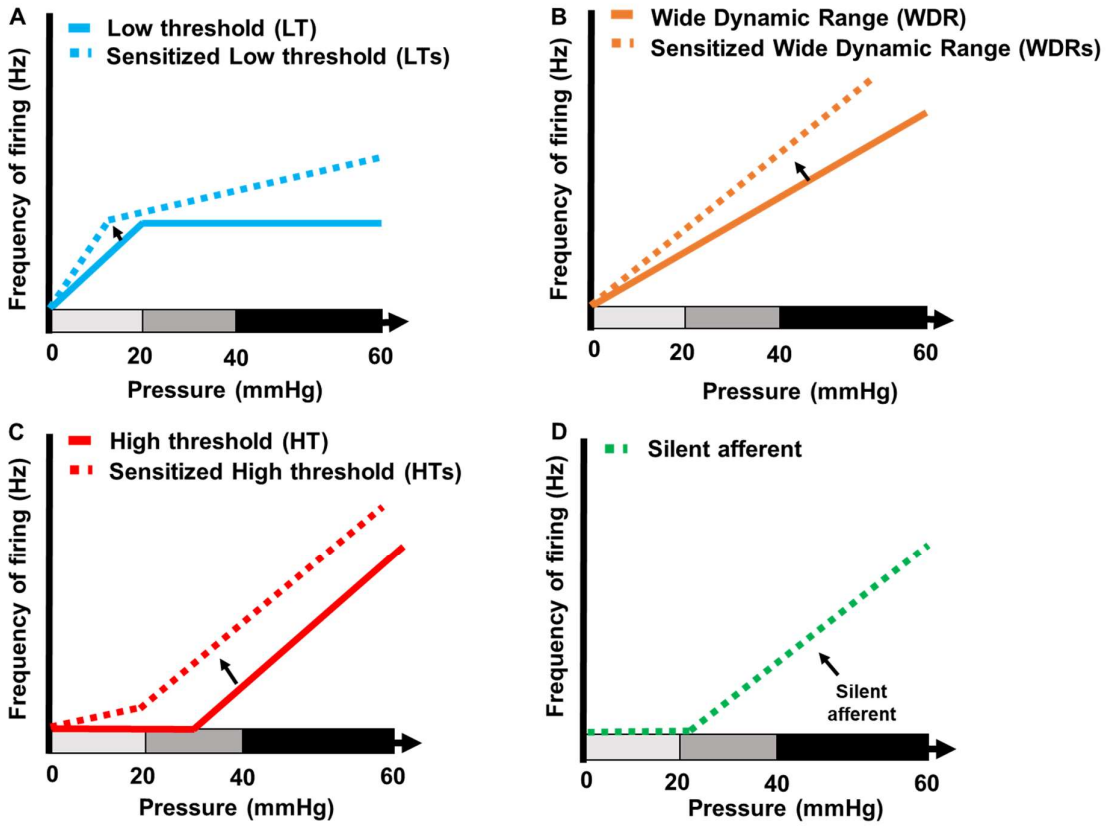


Figure 2: Distention response profiles of high, moderate, low-frequency and mechanically insensitive afferents (silent nociceptors) during normal and sensitised states. The murine colorectal afferents are functionally classified as A. Mucosal afferents (respond to low-threshold mechanical stimuli), B. Muscular afferents (respond to wide dynamic range (WDR) mechanical stimuli), and C. Serosal afferents (respond to high threshold mechanical stimuli), D. A population of mechano-insensitive "silent afferents" has also been identified which only responds when sensitised and activated by inflammatory and immune mediators (modified from Hibberd et al., 2016).

Distension-sensitive afferents with low thresholds and wide dynamic ranges are comparable to the functional classifications of "muscular" and "muscular/mucosal" afferent groups described in the classification developed by Brierley et al. (2004) using probing and stretch of the colorectum opened into a flat sheet preparation. While low-frequency, high threshold afferents are consistent with afferents originally described as serosal and later classified as vascular afferents which are only activated by probing or excessive tissue stretch. These high threshold afferents responding at noxious distention of the colorectum also displays sensitivity to a range of algogenic compound such as bradykinin or ATP, leading to their classification as putative polymodal nociceptors (Blackshaw et al., 2007; Bulmer and Grundy, 2011). A comparable classification also exists for pelvic afferents innervating the colorectum however the activation thresholds for these afferent following distension in a tubular preparation are markedly lower for example serosal afferent thresholds described by Malin et al. (2009) or Hibberd et al. (2016) are as low as 10 cm H_2O (compared with >40mmHg reported by Hughes et al., 2009b).

The Distension and contraction are powerful activators of pain pathways that can be monitored behaviourally by pseudaffective and visceromotor reflexes (Ness and Gebhart, 1990). Colorectal distention of the bowel is a relevant, reliable, and most widely used method of producing abdominal pain in humans and animals (Ness and Gebhart, 1990). The Lumbosacral afferents primarily mediate the visceromotor response to colorectal distension in normal mice, as severing pelvic pathways abolishes the response, but lesioning splanchnic pathways has little effect (Kyloh et al., 2011). Lumbosacral dorsal rhizotomy abolishes visceromotor reflexes, but inflammation reinstates a thoracolumbar contribution (Traub, 2000) which indicates that thoracolumbar vascular afferents can contribute to pain symptoms after inflammatory challenge. Furthermore, the threshold of mechanical stimulus required to elicit pain in humans during colorectal distension is comparable to the threshold required to activate putative polymodal nociceptors in visceral afferent recordings from the human appendix and colorectal afferents in rodent studies (Kuiken et al., 2005; Peiris et al., 2011). For example, in humans, distension to pressures of between 15 to 40mmHg evoked pain from the colorectum and small intestine evokes graded pain which is abolished caudal from the sigmoid colon following bilateral sympathectomy from T7 to L3 (Ray & Neill, 1947). By contrast, pain evoked by rectal distension (within 16cm of the sphincter) is unaffected by T7-L3 sympathectomy but abolished by pelvic nerve rhizotomy highlighting the dual innervation of the GI tract (splanchnic versus pelvic nerves) with pain from the rectum being transmitted *via* pelvic nerves and pain from the remaining small and large intestine transmitted *via* splanchnic afferent nerve fibres (Ray & Neill, 1947).

Most recently single cell RNA sequencing has been utilised to characterise colonic sensory neurons innervating the mouse colorectum based on discrete patterns of gene expression leading to the identification of seven distinct classes of colonic neurons, four of whom comprise of neurons terminating in DRG at TL and LS levels, one at TL levels only and two within LS levels only. This study has provided a comprehensive data of transcript expression in colonic neurons that has increased our understanding of the potential diversity of function within colonic sensory neurons (Hockley et al., 2019).

1.5. Stimulus transduction in visceral afferents

Pain is generated following the activation of nociceptive afferent terminals in response to noxious physiological stimulus (e.g., chemical, mechanical, heat). In the viscera, this occurs *via* unmyelinated C-type nerve fibre, with conduction velocity (CVs) <2.0 m/s (Fang et al., 2005) that are polymodal, responding to noxious mechanical stimuli and algogenic mediators such as bradykinin, while others may be silent afferents as discussed above.

The peripheral terminal of the nociceptive neurons is equipped with a rich repertoire of specific ion channels which serve as pain transducers, for example the ATP-gated P2X3 receptor, the classical heat/capsaicin-sensitive ion channel TRPV1 and the cold/redox-sensitive TRPA1 channel. These pain transducers transduce a variety of chemical or physical stimuli (algogens) leading to the opening of the channel pore and conductance of cations thereby promoting neuronal depolarisation, known as a generator potential, which if sufficient in magnitude can trigger action potential firing in nociceptors.

1.6. Mechanisms modulating excitability of visceral afferent endings

1.6.1 Mechanosensation and Mechanotransduction

Transduction of mechanical stimuli is the primary means of conveying conscious sensation arising from the gut such as fulness and pain in response to bowel distention (Brookes et al., 2013). The encoding of noxious stimuli occurs at the peripheral nerve terminal of a visceral afferent which innervates different layers of the gut wall (mucosa, muscle, and serosa/vascular) and shows distinct characteristic responses to mechanical stimuli of differing stimulus strength. The mechanisms by which afferent excitability is altered in response to mechanical stimuli can be broadly classified into direct or indirect activation. Direct transduction occurs at the nerve terminal itself. It is mediated by mechanically sensitive ion channels expressed at the afferent endings. In contrast, the Indirect, or 'chemical,' transduction occurs outside the afferent terminal, typically in a non-neuronal cell, releasing mediators to excite the afferent terminal. The mechanosensitive ion channels acting as direct transducers of mechanical stimulus are discussed below in detail, while the process of indirect transduction is described later in the thesis.

1.6.2. Mechanosensitive ion channels (Direct transducers of mechanical modalities)

The Mechanosensory circuits built into the GI luminal wall allow the integration of spatial and temporal mechanical stimuli and transduce them into coordinated physiological responses (e.g., peristalsis reflex). In contrast, connections to the extrinsic mechanosensory circuits are crucial for brain-gut communication (e.g., sense of fullness). The mechanosensitive ion channels integral to these circuits transduces

extreme mechanical modalities such as bowel distention, compression, and occlusion into GI pain. These pore-forming mechanosensitive structures are activated upon relative displacement of their structural proteins resulting in the opening of channel gates within a fraction of milliseconds (< 5ms), instigating an influx of cations and leading to depolarisation of afferent endings (Brierley et al., 2010). The mechanosensitive ion channels being widely investigated are ASICs, TRPV4, TRPA1 and recently studied Piezo2 (Coste et al., 2010; Page et al., 2005, 2007; Brierley et al., 2008, 2009 and 2010; Prato et al., 2017).

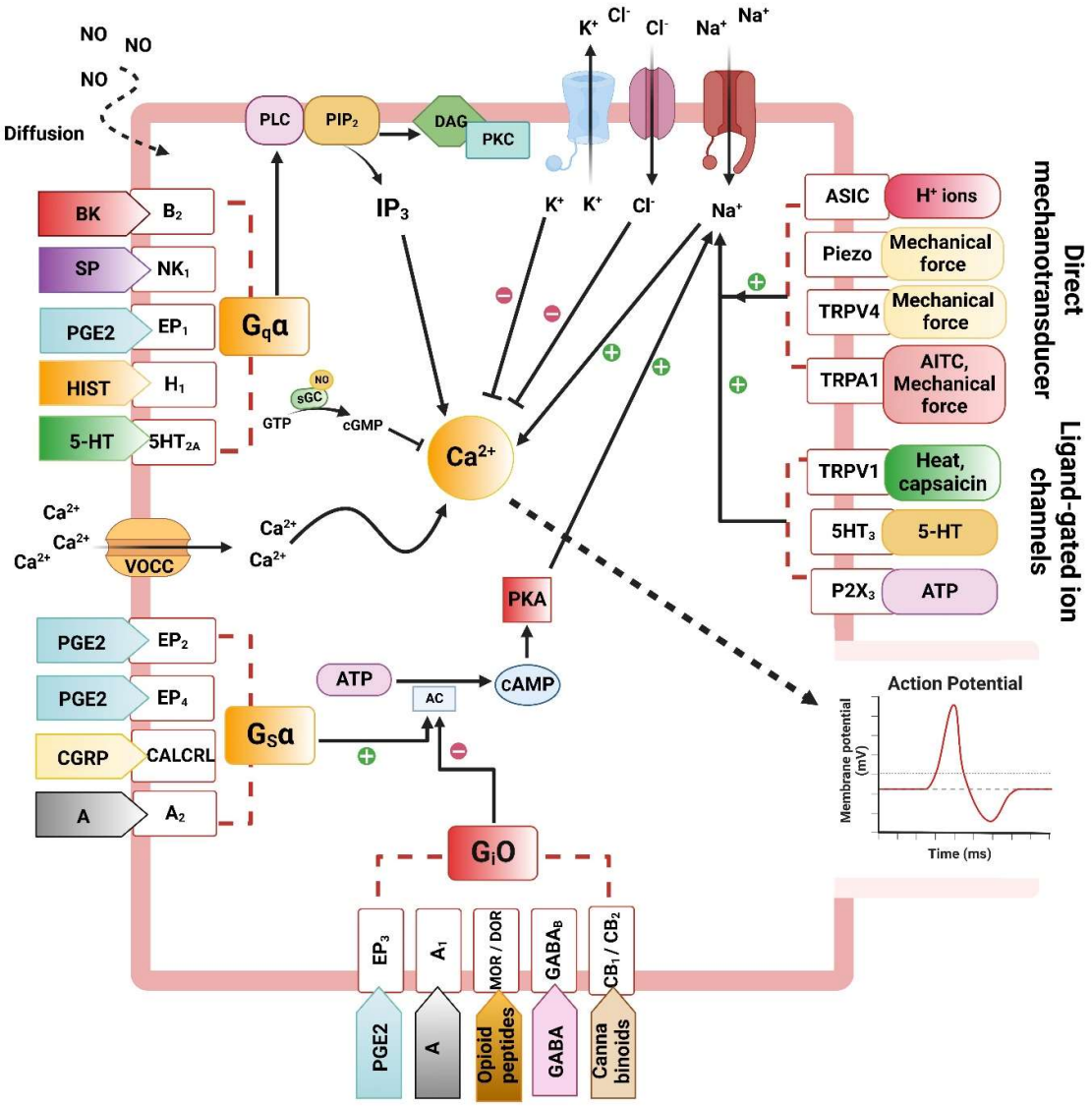


Figure 3: Mechanisms regulating visceral afferent excitation in gastrointestinal tract. Mechanical modalities (stretch, distention) are directly transduced through mechanically-gated channels (e.g., TRPV4, TRPA1, ASIC2, Piezo), leading to depolarisation of the afferent ending. Inflammatory mediators bound on their respective cognate G-protein coupled receptor (GPCR) to activate downstream intracellular signalling pathways such as excitatory G_q and G_S pathway leading to both depolarisation and sensitisation of the afferent ending, potentially through the post-translational modification of Voltage-gated ion channels (Na_V, K_V) and/or non-selective

ion channels (P2X3, TRPV1, $5HT_3$). The activation of the inhibitory G_io pathway by opioids, GABA, and Cannabinoids inhibits the adenyl cyclase enzyme activity to reduce cAMP generation and subsequently limits the activation of PKA and VGSC functioning to reduce afferent excitation. The Nitrous oxide diffuses across the membrane to the site of inflammation and, in the presence of soluble guanylate cyclase enzyme, converts GTP to cGMP, which has an inhibitory effect on afferent excitation. 5-HT: 5-hydroxytryptamine; $5\text{-}HT_{2A}$: 5-hydroxytryptamine type 2A receptor; $5\text{-}HT_3$: 5-hydroxytryptamine type 3 receptor; A: Adenosine; A_1: adenosine type 1 receptor; A_2: adenosine type 2 receptor; AC: adenylyl cyclase; ASIC: acid-sensing ion channels; ATP: adenosine triphosphate; B_1: bradykinin receptor type B1; B_2: bradykinin receptor type B2; BK: bradykinin; CALCRL: calcitonin receptor-like receptor; cAMP: cyclic adenosine monophosphate; CB_1: cannabinoid type 1 receptors; CB_2: cannabinoid type 2 receptors; cGMP: cyclic guanosine monophosphate; CGRP: calcitonin gene-related peptide; Cl^-: chloride ion; DAG: diacylglycerol; EP: prostaglandin E2 receptor; EP_1: prostaglandin E2 receptor type 1; EP_2: prostaglandin E2 receptor type 2; EP_3: prostaglandin E2 receptor type 3; EP_4: prostaglandin E2 receptor type 4; GABA: γ-aminobutyric acid; $GABA_B$: γ-aminobutyric acid type B receptor; H_1: histamine; IP_3: inositol triphosphate; K^+: potassium ion; KOR: κ-opioid receptors; MOR: μ-opioid receptors; Na^+: sodium ion; Na_v: voltage-activated Na^+ channels; NE: norepinephrine; NGF: nerve growth factor; NK_1: neurokinin type 1 receptor; NO: nitric oxide; $P2X_3$: purino receptor; PG: prostaglandins; PGE_1: prostaglandin E_1; PGE_2: prostaglandin E_2; PI3: phosphoinositide 3-kinase; PKA: protein kinase A; PKC: protein kinase C; PLC: phospholipase C; sGC: soluble guanylyl cyclase; SP: substance P.

1.6.3. Acid-sensing ion channels (ASICs)

Acid-sensing ion channels (ASICs), formed by ASIC proteins, are members of the degenerin/epithelial Na^+ channel protein superfamily and were first identified to form mechanoreceptors in *Caenorhabditis elegans* (Hanukoglu et al., 2016; Chalfie et al., 1989). Five genes for ASICs have been identified (ASICs1–5) that encode five subunits, each with distinct biophysical properties (Hanukoglu et al., 2016). ASICs are voltage-insensitive cationic channels that are directly activated following a drop in extracellular pH (Grunder and Pusch, 2015). However, apart from their pH sensing role, the ASICs 1-3 have been shown to exert a putative role in cutaneous and visceral mechanosensation (Chen et al., 2013, Omerbasic et al., 2015). Their physiological involvement in mechanosensation is further corroborated by the expression in mechanosensitive colonic sensory neurons and observed mechanosensory deficits in mutant null mice (Page et al., 2005).

ASIC1 is localized in 30 % of the thoracolumbar afferents and DRG innervating the colon (Hughes et al., 2007, Page et al., 2005). ASIC1a gene is markedly associated with the visceral mechanosensory function of mice (Page et al., 2004). Ablation of ASIC1a has increased sensitivity to mechanical stimuli in both vagal gastro-oesophageal mechanoreceptors and spinal splanchnic afferents (Page et al., 2004; 2005). Intriguingly, no change in Asic1a gene deletion was observed in cutaneous mechanoreceptors. This suggests that ASIC1 exerts an inhibitory influence on visceral mechanosensitivity but does not affect cutaneous mechanoreceptor function.

Expression of ASIC2 was observed in almost 50% of the splanchnic DRG neurones and vagal gastro-oesophageal neurones (Hughes et al., 2007). However, deletion of ASIC2 differentially affects the mechanosensitivity in distinct subsets of colonic

afferents. Thus, conflicting effects were observed with deletion of ASIC2, where serosal splanchnic afferents showed increased mechanosensitivity and vagal intraganglionic laminar endings (IGLEs) showed reduced mechanosensitivity. In contrast, the mesenteric splanchnic afferents were unaffected (Page et al., 2005). Moreover, genetic deletion of ASIC2 has been shown to reduce the sensitivity of low threshold rapidly adapting mechanoreceptors (Price et al., 2000).

A marked expression of ASIC3 is observed in most afferent classes innervating the upper and the lower gut (Hughes et al., 2007). ASIC3 null mice show consistent mechanosensory deficits in the recordings from mesenteric and serosal splanchnic afferents and pelvic muscular/mucosal afferents (Bielefeldt et al., 2008; Page et al., 2005) without affecting gastroesophageal mucosal receptors (Page et al., 2005). Similarly, ASIC3 knock-out mice were significantly less sensitive to phasic colon distension (15-60 mmHg) in CRD experiments. Additionally, chemical inflammatory mediators failed to enhance stretch-evoked afferent fibre responses in ASIC3 knock-out mice as observed in control mice (Page et al., 2005). This explains the contribution of ASIC3 in visceral pain behaviour with possible involvement in peripheral sensitization during inflammatory insult. The role of ASIC3 in peripheral sensitization is further endorsed by 2-guanidine-4-methylquinazoline, an endogenously produced inflammatory mediator induced activation of ASIC3 at pH 7.4 and modulation of pH responses of ASIC1 and 2 (Alijevic et al., 2012). From the above studies contribution of ASICs in visceral and somatic mechanotransduction is very well defined. However, its intrinsic ability to respond to direct mechanical force is questioned in recent literature but supports the role of ASICs as a mechanosensor in specific local environments (Barth et al., 2019).

1.6.4. Transient receptor potential (TRP) ion channels (Transducer of noxious stimuli)

Transient receptor potential (TRP) ion channels superfamily incorporates more than 30 non-selective cation ions channels, which are broadly divided into six subfamilies (TRPC, TRPV, TRPM, TRPA, TRPP and TRPML) based on their homological sequence (Clapham et al., 2003; Startek et al., 2019). TRP ion channels are archetypal sensors that commonly feature six transmembrane domains, a pore-forming channel gating region between the fifth and sixth transmembrane domains, and intracellular N and C termini. The transmembrane and extracellular pore domains are sensitive to environmental cues and directly sense endogenous and exogenous chemical, mechanical, and thermal stimuli, for example, temperature and pH (TRPV1), mechanical stimuli (TRPV4), Inflammatory mediators and reactive oxygen species (TRPA1), and direct stimulation by natural products, such as capsaicin (TRPV1), allyl isothiocyanate (AITC) from horseradish (TRPA1), and menthol from mint (TRPM8) (Story et al., 2003; Kobayashi et al., 2005). The importance of the TRP channels is widely explored and described in pathological conditions of pain, itch, cough, and neurogenic inflammation (Basbaum et al., 2009; Bautista et al., 2006; Grace et al., 2014b). TRP channels are also implicated in visceral mechanosensation of which TRPV4 and TRPA1 are attributed as direct transducer of mechanical stimuli.

1.6.4.1. Transient receptor potential channel, vanilloid-4 (TRPV4)

TRPV4 is a mammalian homologue of the OSM-9 gene from *Caenorhabditis elegans*, and in line with its function, TRPV4 is also sensitive to membrane stretch or hypotonic conditions (Alessandri-Haber et al., 2003). TRPV4 expression is found in DRG, with comparatively high expression observed in free nerve endings and cutaneous Aδ- and

C-fiber terminals (Brierley et al., 2008, Alessandri-Haber et al., 2003, Suzuki et al., 2003), indicative of its extended role beyond osmosensation such as mechanosensation and pain (Alessandri-Haber et al., 2003, Grant et al., 2007, Brierley et al., 2008). Studies using TRPV4 null mice and TRP antagonists have shown to reduce mechanosensitivity in colonic serosal and mesenteric afferents, while no effects were observed on subtypes of vagal and pelvic afferents (Brierley et al., 2008). Similarly, inhibitory effects on mechanosensitivity were also observed during *in vivo* CRD experiments using both TRPV4 null mice and following small interfering RNA (siRNA) knock-down of TRPV4 mice (Brierley et al., 2008; Cenac et al., 2007). TRPV4 is stimulated by inflammatory markers, including proteases enzyme and endogenous inflammatory lipid mediators, anandamide, and AA metabolites (e.g., 5',6'-EET) (Watanabe et al., 2003, Grant et al., 2007). Moreover, TRPV4 is also vital in chronic inflammatory conditions, such as colitis, through transcriptional regulation and the release of cytokines, SP, and CGRP (Brierley et al., 2008; D'Aldebert et al., 2011).

1.6.4.2. Transient receptor potential ankyrin-1 (TRPA1)

TRPA1 is a calcium (Ca^{2+}) permeable channel expressed in nociceptors and plays a critical role in mechanotransduction by splanchnic, and pelvic afferents innervating the colon and distention-induced visceral pain (Brierley et al., 2009; Kondo et al., 2009). Specifically, TRPA1 contributes to mechanotransduction in splanchnic mesentery and serosal afferents whereas it prominently modulates mechanosensitivity in pelvic serosal, mesenteric and mucosal afferents but did not affect pelvic muscular afferents responsive to stretch (Brierley et al., 2009). TRPA1 agonists (mustard oil, AITC and TCA) augment mechanical hypersensitivity and induce visceral hyperalgesia in mice colonic afferents (Brierley et al., 2009; Cattaruzza et al., 2010). TRPA1 -/- mice and

rats treated with TRPA1 antisense oligonucleotides to knock down TRPA1 expression show a reduced VMR to CRD (Brierley et al., 2009; Yang et al., 2008).

1.7. Putative mechanotransducer

1.7.1. PIEZOs

Piezos are large transmembrane proteins conserved among various species of which Piezo1 and Piezo2 represents mechanically activated ion channels (Coste et al., 2010). In mammals, Piezos are broadly expressed in a wide range of mechanosensitive cells with Piezo1 expression observed in colon, kidney, lung, and skin (Coste et al., 2010, Satoh et al., 2006). However, its relatively low expression in DRG suggest that Piezo1 may not be crucial for mechanotransduction (Coste et al, 2010). In contrast studies using heterologous expression systems and reconstituted lipid bilayers have confirmed mechanical force as a direct regulator of channel opening (Syeda et al., 2016). Furthermore, chemical induced activation of Piezo1 further add complexity to its gating properties, which is yet to be elucidated (Syeda et al., 2015). Piezo2, a related mammalian homolog, is strongly expressed in DRG neurons suggesting a potential role in somatosensory mechanotransduction (Coste et al., 2010; Ranade et al., 2014). Genetically ablated Piezo2 mice lack normal touch sensation and proprioception and similarly in humans inactivating variants in Piezo2 is associated with prominent mechanosensory deficiencies (Chesler et al., 2016). Intriguingly, Piezo2 is also expressed in colonic sensory neurones and may be relevant for colonic mechanotransduction (Hockley et al., 2019), a role which is not yet evaluated.

1.8. Indirect mechanotransducer

In addition to the direct transducers of colorectal mechanotransduction, the channels that are indirectly modulated by mechanical forces, e.g., TRPV1 and P2X3, are equally crucial for GI-related visceral pain.

1.8.1. Transient Receptor Potential Vanilloid 1 (TRPV1)

TRPV1 channel is a heat sensor activated at noxious heat (43^{o}C) or capsaicin (Tominaga et al., 1998, Caterina et al., 1997). TRPV1 is expressed by 80% of thoracolumbar and 50-60% of lumbosacral colonic DRG neurones (Christianson et al., 2006; Robinson et al., 2004). TRPV1 knock out mice has shown reduced mechanosensitivity in the recordings from distention-sensitive afferents (Rong et al., 2004, Jones et al., 2005, Bielefeldt et al., 2008), while the somatic mechanosensory function was unaffected (Caterina et al., 2000). Additionally, TRPV1 knock out mice poorly respond to noxious heat and lack the ability to release neuropeptides illustrating the critical involvement of TRPV1 in nociception and neurogenic inflammation (Caterina et al., 2000). The TRPV1 is not directly regulated by mechanical force. However, it may indirectly influence the excitability of mechanosensitive afferents upon activation by several inflammatory mediators such as proteases, serotonin, histamine, tachykinins, and bradykinins to augment afferent sensitisation (Veldhuis et al., 2015).

1.9. Indirect/ stimulus-induced mediator release alter afferent excitability

Indirect, or ‘chemical,’ transduction occur when mechanotransduction occurs outside the afferent terminal, typically in a non-neuronal cell, which in turn release mediators to excite the afferent terminal. Examples, of this would be the release of 5-HT or ATP from enterochromaffin cells or enterocytes respectively in response to stretch of the

gut lumen, which subsequently stimulate colonic afferents through the activation of respective 5-HT_3 and $P2X_{2/3}$ receptors. It has been shown that the mechanical stress or distention of the hollow visceral organs, trigger the release of ATP from the epithelial lining resulting into the stimulation of visceral nociceptors (Burnstock, 1999). Further evidence to support the purinergic contribution was obtained from a rat pelvic sensory nerve-colorectal preparation where distension of the colorectum led to pressure-dependent increase in release of ATP from mucosal epithelial cells and evoked pelvic nerve excitation. This excitation was mimicked by application of ATP and α, β-methyl-ATP and attenuated by the selective $P2X_3$ and $P2X_{2/3}$ antagonist (Wynn et al., 2003). In addition, release of ATP by colorectal distension is greatly enhanced during colitis suggesting a more prominent role for this pathway during inflammatory pain (Wynn et al., 2004). The excitability of visceral afferent nerves is enhanced in IBS, and ATP has been reported to cause sensitization of afferent nerves to mechanical or chemical stimuli. For example, chronic functional visceral hyperalgesia induced in a rat model for IBS, is associated with potentiation of ATP-evoked responses and an enhanced expression of $P2X_3$ receptors in colon-specific sensory neurons and consistent with this role, mechanical hypersensitivity to colitis does not develop in mice lacking P2X receptor suggesting a key role for this pathway in the development of visceral hypersensitivity in disease states (Xu et al., 2008; Shinoda et al., 2010). ATP has also been shown to interact with several mediators of inflammation including 5-hydroxytryptamine (5-HT), bradykinin, prostaglandins, and substance P (SP) which are known to activate colonic afferents (Wynn and Burnstock, 2006). In addition, TRPV1 channels are activated and sensitised by ATP that is released during distension, augmenting peripheral sensitisation, and increasing pain signalling (Lakshmi and Joshi, 2005).

1.10. Inflammatory mediators modulate afferent excitability and sensitises nociceptors

Visceral nociceptors are sensitive to a wide range of noxious mediators released as a consequence of tissue damage or inflammation, such as 5-HT, histamine, bradykinin, substance P, ATP, adenosine, and prostaglandins (PGE2) in addition to cytokines and chemokines (e.g., TNFalpha, IL-1β and IL-6) (Grundy, 2004; Baral et al., 2019). A reflection of inflammatory mediator-induced colonic afferent sensitisation is shown by Jones, in which the application of inflammatory soup (bradykinin, adenosine triphosphate (ATP), histamine, prostaglandin E2, and noradrenaline) to receptive fields of the colon caused nociceptor hyperexcitability (Jones et al., 2005). Similarly, intracolonic application of inflammatory soup increases resting afferent activity and the magnitude of firing in the rat (Su & Gebhart, 1998). Moreover, the studies have implemented the application of inflammatory soup to investigate mechanically insensitive afferents which are otherwise ‘silent’ but acquire mechanosensitivity following inflammatory challenges (Feng & Gebhart, 2011).

A compelling body of evidence over the sensitising role of mediators at visceral nociceptors has been derived from the studies using resected diseased human tissues. The supernatants derived from inflamed and normal human bowel tissues facilitated the study to understand the impact of human-derived inflammatory milieu on visceral afferent modulation. For example, visceral hyperalgesia is evoked by supernatants derived from colonic mucosal biopsies of IBS patients (Cenac et al., 2007). Moreover, it has been shown that the supernatants from IBS biopsies, but not from controls, induce visceral hypersensitivity or are capable of increasing nerve discharge in animals (Barbara et al., 2007; Cenac et al., 2007) Additionally,

supernatants generated from the biopsies of a post-infectious patient with IBS (PI-IBS) stimulate colonic afferent activity and evoke mechanosensitivity despite clinical remission of the gastroenteritis (Balemans et al., 2017). The disease supernatants induced visceral hypersensitivity and increased afferent discharge were significantly attenuated by histamine H_1 receptor blockade and PAR2 antagonist, implicating the role of mediators and suggesting that persistent changes in afferent signalling contribute to the underlying pathophysiology of IBS (Cenac et al., 2007; Barbara et al., 2007). In line to these observations, treatment with mast cell stabilizer ketotifen or the histamine H_1 receptor antagonist significantly attenuated abdominal pain scores in IBS patients with abrupt hypersensitivity while having no effect on normosensitive IBS patients (Klooker et al., 2010; Wouters et al., 2016). In addition to tissue supernatant, faecal supernatants from IBS-D patients, when instilled into the colorectum of mice, induce colonic hypersensitivity (Annaházi et al., 2009). A recent study investigated the correlation between supernatant-mediated nociceptor activation and the average daily pain score, where a significant correlation was found between biopsy supernatant-mediated colonic afferent activation and patient pain severity scores. However, no correlation was observed between biopsy-mediated colonic afferent activity and symptom scores for bowel habit and psychological factors. This data indicates that biopsy supernatant-mediated nociceptor activation provides mechanistically relevant insight into the generation of pain symptomology in patients independent of disease activity (Cibert-Goton et al., 2021). Collectively these findings provide robust evidence for a pro-nociceptive environment in the bowel of IBS patients.

1.11 Voltage-Gated Channels regulate afferent excitability and sensitises nociceptors

1.11.1 Voltage gated sodium ion channels

Voltage-gated ion channels (VGSCs) are important determinants of sensory neuron excitability: they are essential for the initial transduction of sensory stimuli, the electrogenesis of the action potential, and neurotransmitter release from sensory neuron terminals. Among the nine ($Na_V1.1$-$Na_V1.9$) α-subunit isoforms of VGSCs, the $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ are expressed primarily on nociceptive afferents. The biophysical characteristics of these VGSCs channels, as well as their unique expression patterns within subtypes of sensory neurons, define their functional role in pain signaling. Changes in the expression of VGSCs, as well as post-translational modifications, contribute to the sensitization of sensory neurons in chronic pain states. Mutations in peripherally expressed sodium channels $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ are associated with congenital insensitivity to pain, primary erythromelalgia, paroxysmal episodic pain syndrome and painful neuropathy (Faber et al., 2012; Huang et al., 2013; Huang et al., 2014). The expression of Na_V channels is significantly altered in disease condition or during tissue injury as such spinal cord injury or severing of axon is associated with down-regulation of tetrodotoxin-resistant (TTX-R) sodium channels $Na_V1.8$ and $Na_V1.9$ (Benn et al., 2001; Decosterd et al., 2002). In addition, inflammatory mediators increase expression of $Na_V1.7$ and $Na_V1.8$ (Black et al., 2004).

The TTX-resistant $Na_V1.9$ isoform is expressed primarily by small diameter neurones of the peripheral nervous system, and its unique biophysical characteristics produce a persistent sodium current. Also, the activation and inactivation threshold for $Na_V1.9$ is

close to resting membrane potential, which allows it to depolarise the membrane following subthreshold excitation regulating neuronal excitability and nerve terminal sensitivity (Coste et al., 2004). The $Na_V1.9$ channel is also implicated in inflammatory pain, and several inflammatory mediators, such as ATP, Bradykinin; PGE2, TNF-α potentiate the $Na_V1.9$ current and increase neuronal excitability (Hockley et al., 2014; Maingret et al., 2008; Baker, 2005; Rush & Waxman, 2004).

The regulation of sensory neurone excitability mediated by $Na_V1.9$ currents significantly impacts behaviour pain phenotypes in rodent models. For example, visceral hyperalgesia elicited by intraplantar administration of prostaglandin E2, bradykinin, interleukin-1β, capsaicin, and purinergic agonist was reduced or absent in $Na_V1.9$ -/- mice (Amaya et al., 2006). Similarly, complete Freud adjuvant (CFA) induced thermal hypersensitivity was absent in $Na_V1.9$ -/- mice (Amaya et al., 2006). Moreover, intracolonic instillation of inflammatory insult significantly increases response to phasic CRD in wild-type animals; however, the responses remained unchanged in $Na_V1.9$ -/- animals (Martinez & Melgar, 2008).

In a post-infectious inflammatory model, isolated DRG neurones from wild-type mice exhibited marked hyperexcitability, which was absent in $Na_V1.8$ -/- mice but unchanged in $Na_V1.9$ -/- animals, suggesting a more prominent involvement of $Na_V1.8$, over $Na_V1.9$ channels in post-infection neuronal excitation (Hillsley et al., 2006).

1.11.2. Voltage gated potassium ion channels

In addition to the VGSCs discussed above, the voltage-gated potassium channels (K_V7) that mediate the M current also regulate excitability in peripheral sensory nociceptors and central pain pathways. Similar to the $Na_V1.9$, the activation threshold

for the K_V7 channel is below the threshold for action potential initiation and near resting membrane potential. Therefore, K_V7 channels significantly influence the resting membrane potential and contribute to a membrane-potential clamping effect that stabilizes neuronal excitability and restricts repetitive firing. However, mediators of visceral hypersensitivity in IBS, PAR-2, have been shown to inhibit the M current *via* phosphoinositide phospholipase (PLC) activation (Linley et al., 2008). Additionally, inflammatory mediators such as bradykinin have shown to attenuate M current by Gq/11-coupled mediated action to evoke membrane depolarisation and increased neuronal excitability (Du et al., 2014), while treatment with the K_V7 opener retigabine almost completely abolished visceral afferent firing evoked by the bradykinin (Peiris et al., 2017). Retigabine also attenuated responses elicited by noxious bowel distension (0–80 mmHg) in a concentration-dependent manner, whereas the K_V7 blocker XE991 potentiated such responses (Peiris et al., 2017). Retigabine, when injected into the peritoneal cavity, reduces visceral pain behaviours in mice following intracolonic application of capsaicin (Hirano et al., 2007).

Apart from the ion channels associated with the transduction of mechanical stimuli, the contribution of other known regulators of neuronal excitability, including T-type calcium channels, $Na_V1.1$ and HCN2, have been studied but need further investigation (Osteen et al., 2016; Hirano et al., 2007).

The following literature explains the pivotal contribution of voltage-gated ion channels in setting the tone for action potential generation and nociceptor activation. The literature also describes that function of these voltage-gated ion channels is greatly influenced by the inflammatory insult explaining their crucial involvement in the exacerbation of disease symptoms in IBD and IBS, especially pain. Therefore,

compound modulating the activity of these channels could lead to an effective analgesic.

1.12. Role of neurogenic inflammation in visceral afferent sensitisation

The manifestation of the inflammatory process with moderate to severe injury or exposure to corrosive chemicals are redness, swelling, heat and pain in the affected area. The redness is due to dilatation of blood vessels; swelling or wheel formation is due to the exudation of fluid from the vasculature (driven by increase vascular permeability); heat is due to increase blood flow in injured area and finally pain due to irritation of afferent terminus surrounding the area of injury. These are the cardinal response observed on skin demonstrating early phases of acute inflammation and the phenomenon is described as “The Triple response” (Bruce, 1913). A similar inflammatory environment defined as “neurogenic inflammation” augment around the nociceptive colonic afferents during the disease’s conditions to heighten pain responses in IBD. The nociceptive afferents serve dual functions, i.e., Activation of nociceptors by noxious stimuli not only sends action potentials to the central nervous system but can also change the response properties of the afferents themselves by the local release of neurogenic peptides such as SP and CGRP from Aδ and C-fiber axons (Richardson and Vasko, 2002). The local release of these neurogenic peptides is described to have extensive role in colorectal hypersensitivity such that a CGRP receptor antagonist reduced chronic colorectal hypersensitivity in rat (Bourdu et al., 2005). Correspondingly, the content of SP is increased in the inflamed colon of patients with ulcerative colitis (Goldin et al., 1989) and in animal models of intestinal inflammation (Castagliuolo et al., 1997; Sturiale et al., 1999). There are, however,

relatively few studies that have examined the direct evidence for contribution of these neuropeptides in colonic mechanosensitivity.

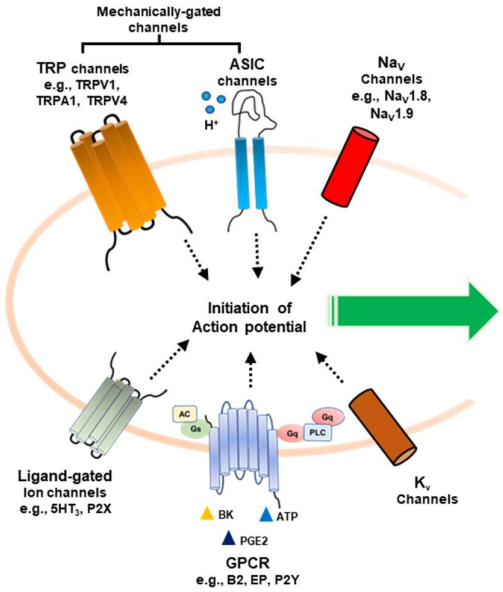
The excitation of colonic afferents can trigger neurogenic inflammation in the gut which is characterized by arteriolar vasodilation and extravasation of plasma proteins, neutrophils and immune cells thereby reinforces the likelihood of extensive physiological crosstalk between afferents and immune cells.

1.13. Neuroimmune interaction augments sensitisation of visceral afferents.

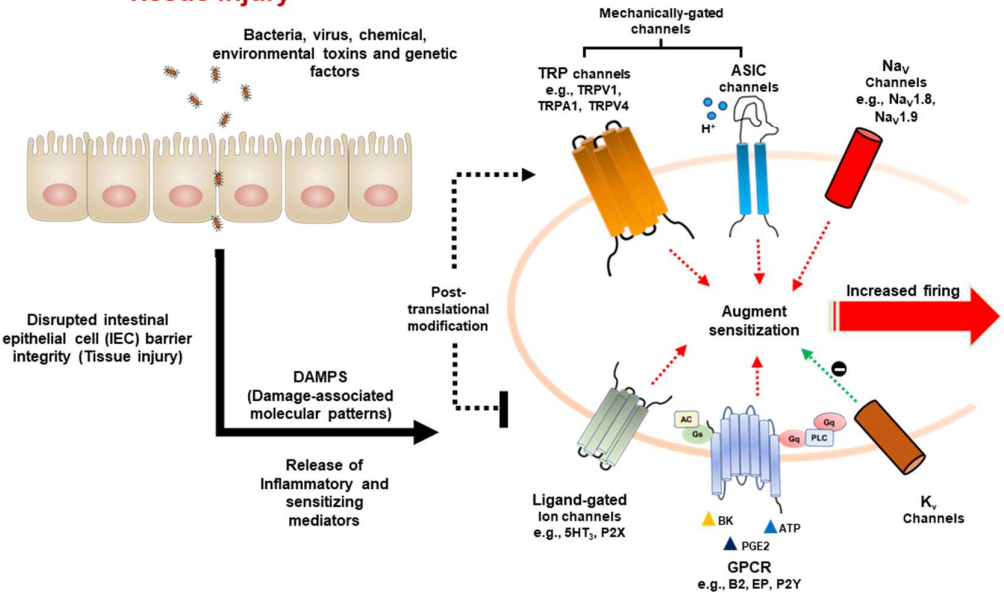
The immune system has a critical role in the sensitisation of nociceptor neurons. Immune cells release cytokines, lipids and growth factors that act on peripheral nociceptors and central nervous system (CNS) neurons to sensitise pain signalling pathways (Baral et al., 2019). In turn, nociceptors actively release neuropeptides from their peripheral nerve terminals that modulate the activity of innate and adaptive immune cells (Cook et al., 2018). This bi-directional molecular dialogue between nociceptor neurons and the immune system is a fundamental aspect of inflammation (acute and chronic) and commonly constitute visceral hypersensitivity driven pain (Baral et al., 2019; Cook et al., 2018). The pain tends to reduce with the resolution of the tissue immune response in IBD patients, highlighting the immune system's importance in neuronal sensitisation. However, a significant population of patients in the IBD group under remission and those from the IBS group (including post-inflammatory or post-infective IBS) reflects pain symptoms. A better understanding of these interactions could produce approaches to treat chronic pain in inflammatory diseases.

Normal low-grade chemical & mechanical stimuli

E.g., Distention/fluid flow



Tissue Injury



Autoimmune / Inflammatory Diseases

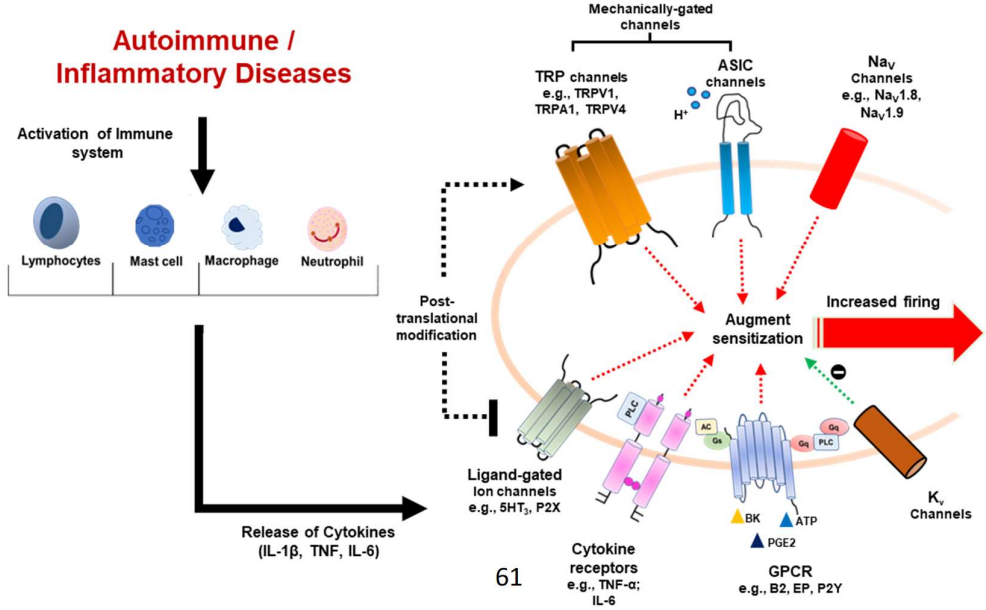


Figure 4: Neuroimmune interactions at peripheral nerve terminals augment sensitisation and induces pain. Impairment of intestinal barrier integrity permits translocation of commensal bacteria into the lumen, subsequently activating the immune system. The resulting cascade releases prominent inflammatory mediators, which act on their respective receptors to induce phosphorylation and activation of membrane-embedded voltage-sensitive-Na^+ channels ($Na_V1.8$, $Na_V1.9$ and $Na_V1.7$) and inhibition of voltage-gated K^+ channels that contributes to increased synaptic plasticity and membrane excitability. Consequently, this allows non-selective cation channels, such as TRPV1, TRPV4 and TRPA1 to trigger action potential firing more readily.

During IBD, impairment of intestinal barrier integrity, induced by a combination of genetic (antimicrobial peptides, autophagy, chemokines) and environmental factors (microorganisms, diet, infection, smoking, appendectomy, stress, excessive use of NSAIDs and antibiotics), allows translocation of commensal bacteria into the lumen. This infiltration instigates an aberrant and excessive cytokine response, inducing acute mucosal inflammation. Failure to resolve this early inflammation in a genetically susceptible host promotes uncontrolled activation of the mucosal immune system, including macrophages, T cells, and innate lymphoid cells. The activated immune cells interact with antigens and microbial products of the commensal microbiota to develop chronic inflammation of the GIT. Additionally, mucosal injuries caused by infiltrating pathogens promote endogenous host-derived molecules, known as damage-associated molecular patterns (DAMPs), which can further activate the immune system, augment inflammation, and contribute to the development of inflammatory disease (Gong et al., 2020). Pro-inflammatory and sensitising mediators, such as cytokines (e.g., TNFα, IL-6, IL-1β), prostaglandins, bradykinin, bioactive lipids

(sphingosine-1-phosphate), chemokines, neuropeptides (CGRP and substance-P), protons and ATP, is also released following inflammation and tissue injury. These mediators act on their cognate receptors at the GPCR-TRP channel axis, stimulating nociceptors to evoke action potential firing and augment neuronal sensitisation to other stimuli (Santos et al., 1998; Raithel et al., 2001; Lapointe et al., 2015 and Balemans et al., 2017). This cascade of events includes multiple intracellular mechanisms, including phosphorylation of ligand-gated ion channels (e.g., TRPV1 and TRPA1) or voltage-gated ion channels (e.g., Na_V and K_V) changes in gene expression. Given the shared interaction between nociceptor neurons and the immune system in VH induced colonic nociception, it is essential to understand the influence of individual immune components involved in this neuro-immune signalling.

1.13.1. Mast cell

There is burgeoning research interest for investigating the interplay of the mast cell-nerve axis in gut-visceral nociception. Mast cells are the major player of the immune system playing a pivotal role in maintaining gut homeostasis, but during tissue injury or inflammation, they also augment hypersensitivity-like conditions in the colon (Stead et al., 1989; Wang et al., 2014). Mast cell degranulation by multivalent antigen releases allergic mediators such as histamine, serotonin, serine proteases enzymes, prostaglandins (*e.g.*, PGE2, PGD2), leukotrienes (*e.g.,* LTC4, LTD4) and platelet-activating factor (PAF) (Buhner and Schemann, 2012; Vermeulen et al., 2014). Moreover, the literature also describes that mast cell degranulation upregulates pro-inflammatory cytokine (*e.g.,* TNFα, IL-6) levels in IBD patients (Holtmann et al., 2016). The plethora of inflammatory mediators and pro-inflammatory cytokines sensitise primary afferent neurons, augment VH, and induce pain during active IBD conditions. The influence of mast cell degranulation mediated VH and pain during inflammatory

conditions have been validated in an animal model of IBD. The experimental DSS-model of colitis enrichment of mast cells in the proximity of afferent fibres, which were more reactive to compound 48/80 induced degranulation, have been reported (Coldwell et al., 2007). Similarly, in TNBS induced colitis model, VH was attenuated with mast cell stabiliser ketotifen and was abolished in mast cell-deficient rats (Ohashi et al., 2008). In humans, mast cells are found in the closed association with nerves of GI mucosa (Stead et al., 1989). Also, activation of mast cells in the proximity of the colonic nerve has been correlated with abdominal pain in IBS (Barbara et al., 2004). Mast cell mediators released from the colonic mucosal biopsies from IBS patients have been shown to excite nociceptive visceral afferents in rats, confirming mast cells' involvement in VH-induced pain (Barbara et al., 2007; Hughes et al., 2009). Finally, despite the poor correlation of mast cell activation in biopsies from IBS patients, the mast cell stabiliser ketotifen decreases VH and improves intestinal symptoms (Klooker et al., 2010).

1.13.2. Nerve Growth Factor (NGF)

The sensitisation of nociceptors by inflammatory mediators is further instantiated by the pivotal role of the Nerve Growth factor (NGF). NGF expression and its receptors are concomitantly increased in CD and UC patients (di Mola et al., 2000). During inflammation, NGF is produced by mast cells and macrophages. This Neurotrophin directly sensitise the nociceptors by binding to the TRKA receptor. NGF binding to TRKA receptor also upregulates bradykinin receptor expression, and expression of TRPV1 and ASICs ion channels (Mantyh et al., 2011). NGF also produces sensitisation of voltage-dependent Na^+ ion channels; for example, NGF drives $Na_V1.8$ sensitisation, and NGF fails to produce thermal hyperalgesia in $Na_V1.8$-deficient mice (Kerr et al., 2001). NGF promotes the release of histamine, serotonin (5-HT) and

protons from the mast cell and indirectly augment afferent sensitisation. Finally, NGF upregulates the synthesis of nociceptive markers such as CGRP, Substance-P (SP) and Brain-derived neurotrophic factor (BDNF), which have already been described to sensitise first and second order neurons in the spinal cord.

1.13.3. Leukotriene-(LTB4)

Another sensitising mediator produced as a downstream product of the arachidonic metabolism pathway is Leukotrienes. Increased tissue synthesis of the leukotriene B4 (LTB4) is reported in the colonic mucosa and rectal dialysates of patients with active inflammatory bowel disease, contributing to the neutrophil influx and colonic injury (Jupp et al., 2007). LTB4 at low doses drives PKC-dependent TRPV1 sensitisation and augment thermal hyperalgesia (Zinn et al., 2017).

1.13.4. Enteroendocrine cells

ECCs are enteroendocrine cells that reside in the luminal epithelium. ECC's increased expression and overactivity are reported in patients with IBD (Lakhan and Kirchgessner, 2010). ECC consist of peptides such as serotonin (5-HT), cholecystokinin and secretin, which influences the neuromuscular junctions and further regulates colonic motility and secretions. The 5-HT is a significant player of ECC and has been reported to stimulate vagal and afferent nerves, provoking emesis, and pain (Bueno et al., 2007; Anand et al., 2007). The 5-HT influence and excites the colonic visceral afferent endings through its respective 5-HT_3 and 5-HT_4 receptors and augments visceral hypersensitivity (Greenwood-van Meerveld et al., 2006). TRPA1 is a highly enriched sensory receptor transcript observed in EC cells (Nozawa Katsura et al., 2009). Additionally, allyl isothiocyanate (AITC), a TRPA1 agonist and reactive chemical agent from wasabi, consistently activated the EC cells and released 5-HT (Nozawa Katsura et al., 2009; Bellono et al., 2017). This activity profile of TRPA1

agonist corroborates the aetiology of GI inflammation. The preclinical and clinical evidence suggests the beneficial role of 5-HT_3 antagonist (alosetron & cilansetron) and 5-HT_4 partial agonist (Tegaserod) in attenuation of hypersensitivity and abdominal pain in IBS (Coffin et al., 2003).

1.13.5. Macrophages & neutrophils

In addition to the above-described essential promoters of VH, macrophages are also amongst the prominent contributors. Upon activation during inflammation, the macrophage initiates phagocytosis and provides host immunity. However, they also release prostaglandins (PGE2) and cytokines such as IL-1β, IL-6, IL-8 and TNF-α, which may act directly on the receptors located on afferent endings and sensitise the neurons or they can precipitate sensitivity for different algogenic mediators like bradykinin, substance-p, histamine, and ATP (Chen et al., 2020; Barry et al., 2019). Finally, the neutrophils mediated release of eicosanoids such as 15S di-HETE and modulation of adenyl cyclase activity in afferent neurons during mucosal injury and inflammation are also crucial for augmenting VH. Consequently, this cascade of events augments permanent sensitisation of primary afferents and triggers persistent pain even in response to the low-grade stimulus. (Dennis and Norris, 2015).

1.13.6. Cytokines sensitising nociceptors

Cytokines are attributed as critical drivers and regulators of inflammation (Gebhart, 1999; Barbara et al., 2002; Gotteland et al., 1999; Imamura et al., 2015; Torossian et al., 2017). Moreover, cytokines can directly stimulate and sensitise nociceptors to amplify visceral pain stimuli (Baral et al., 2019; Vermeulen et al., 2014; Cook et al., 2018). A recent study from our lab confirmed that cytokine receptors are expressed on colonic sensory neurons, predominantly in a neuronal subset enriched with key nociceptive ion channels, such as TRPA1 and TRPV1, implicating a role of cytokines

in visceral pain processing (Hockley et al., 2019). However, the underlying mechanisms by which cytokines interact with visceral nociceptors remains obscure. Enhanced VH in IBD, following transient inflammation in the gut, is further evident in clinical studies, where patients with moderate to active colitis show decreased sensation thresholds and reduced maximal tolerable volume in response to anorectal balloon distention in comparison to healthy volunteers (Farthing and Lennard-jones, 1978; Rao et al., 1987). Moreover, supernatants derived from the inflamed human tissues of CD and UC patients were reported to increase colonic nerve activity in mice (Hockley et al., 2014). Additionally, cytokine quantification in tissue supernatants of IBD patients reveals significantly increased levels of TNFα, IL-6 and IL-1β (Hockley et al., 2014).

1.13.6.1. Interleukin-1β (IL-1β)

IL-1β is pivotal in hyper inflammation, and it increases the susceptibility of sensory neurons to noxious thermal stimuli (Obreja et al., 2002). IL-1β-induced hyperalgesia occurs through upregulation of nociceptive mediators, such as NGF, prostaglandins, IL-6, substance-p, CGRP and MMP9 (Ren and Torres, 2009). Furthermore, IL-1β increases TRPV1 expression in cultured rat DRG neurons and promote thermal hyperalgesia (Ebbinghaus et al., 2012). The downstream signalling pathway of IL-1β-induced mechanical and thermal hyperalgesia is linked to p38 mitogen-activated protein kinase (MAPK)-mediated phosphorylation of $Na_V1.8$ (Binshtok et al., 2008). Moreover, an approach to attenuate pain symptoms in rheumatoid arthritis (RA) and gout using Anakinra, a recombinant humanised IL-1 receptor antagonist, has proven effective (Ruscitti et al., 2019; Janssen et al., 2019). Anakinra is used clinically for treatment in RA and other inflammatory conditions (O'Dell, 2004), but treatment

exacerbates CD (Carter et al., 2004), creating uncertainty around the therapeutic utility of IL-1β inhibitors for IBD-induced pain.

1.13.6.2. Tumor necrosis factor-α (TNF-α)

Apart from IL-1β, the cardinal cytokine TNFα is also involved in the inflammatory cascade and simultaneously plays a crucial role in developing hyperalgesia-like conditions. TNFα directly mediates nociceptive effects through TNFR1 and TNFR2 receptors (Constantin et al., 2008; Cunha et al., 2005), and can also indirectly sensitise neurons through interaction with non-selective cation channels, such as TRPV1 and TRPA1 (Constantin et al., 2008; Fernandes et al., 2011). A recent investigation from our lab revealed that TNFα sensitises sensory neurons and colonic afferents to the TRPV1 agonist capsaicin. The sensitisation action of TNFα is dependent on TNFR1 expression, and p38 MAPK activity as the sensitisation response was abolished following p38 MAPK inhibition and was absent in tissue from TNFR1 knockout mice (Barker et al., 2022). TNFα also alters neuronal excitability by interacting with $Na_V1.8$ and $Na_V1.9$ (Gudes et al., 2015; Ibeakanma and Vanner, 2010). Besides influencing ion channel function, TNFα can indirectly augment neuronal sensitisation by increasing prostaglandin production (Nicol et al., 1997). Knockdown of TNFR1 or COX-2 inhibitors, despite adverse effects and toxicity concerns, successfully abolished TNFα mediated hyperalgesia (Cunha et al., 2005; Nicol et al., 1997). Treatment of UC and CD patients with anti-TNFα drugs has become the most suitable choice for clinicians; however, this therapy is still primarily intended to achieve clinical remission of inflammation. Sequestration of TNFα by monoclonal antibodies, such as infliximab, adalimumab, and golimumab, significantly improved Crohn's Disease Activity Index (CDAI) scores in both CD and UC patients (Levin et al., 2016). Recently, it was reported that anti-TNFα therapy reduced visceral hypersensitivity (sense of fullness)

in patients following standardised nutrient challenges and improved psychological well-being (Gray et al., 2018). However, despite the successful outcomes of anti-TNFα agents in clinical practice and improved remission rates in IBD patients, only 30% of patients can be successfully treated with anti-TNFα drugs. Moreover, loss of primary (13-40%) and secondary (23-46%) treatment response to anti-TNF treatment over time is a primary concern for clinicians (Ding et al., 2016). Additionally, long-term use of anti-TNFαs is associated with potential health complications, such as a compromised immune system and increased risk of opportunistic infections, as well as induction of new-onset CD (Levin et al., 2016). Immediate and delayed hypersensitivity reactions are also observed in 30-40% of patients, limiting their clinical utility (Steenholdt et al., 2012).

1.13.6.3. Interleukin-6 (IL-6)

IL-6 is the most comprehensively explored cytokine in inflammatory diseases such as RA, IBD, IBS and cancer-associated inflammation. IL-6 is increased in the serum and plasma of IBD patients and correlates with disease severity (Jonefjall et al., 2016; Lochhead et al., 2016; Zhu et al., 2017; Nikolaus et al., 2018). In preclinical rodent models of colitis, IL-6 mRNA is significantly increased (Wang et al., 2010), and elevated serum IL-6 in UC patients can induce visceral hypersensitivity (Jonefjall et al., 2016). IL-6 also induces visceral hypersensitivity and altered colonic motility by modulation of enteric neurons in animal models of IBD (Buckley et al., 2014). IL-6 can also sensitise nociceptive pathways (De Jongh et al., 2003). IL-6 and Soluble-IL-6 (sIL-6) receptors induce sensitisation of rat skin nociceptors (Obreja et al., 2002b; Obreja et al., 2005). In rat skin models, IL-6 induces sensitisation of nociceptors as observed by heat-evoked CGRP release *ex vivo* (Opree and Kress, 2000). Genetic ablation of IL-6 reduces mechanical hyperalgesia compared to wild-type mice following

subcutaneous administration of carrageenan (Xu et al., 1997). IL-6 also upregulates TRPV1 receptors in DRG neurons and increases pain perception in a JAK/PI3K-dependent manner (Fang et al., 2015).

1.14. G-protein-coupled receptors (GPCRs)

GPCRs are a large family of cell surface receptors comprising the most diverse proteins (G-protein) involved in signal transduction across the biological membrane. GPCRs are divided into families based on structural similarities. The class A (rhodopsin-like) family is the largest group, followed by the class B (secretin) family. However, other families exist, including class C and the frizzled and adhesion classes (Fredriksson et al., 2003). GPCRs are chemically tractable for the most part making them attractive therapeutic targets with more than 45% of currently available pharmaceutical drugs working against GPCRs (Bleicher *et al.*, 2003; Hopkins and Groom, 2002). They represent the largest class of the 'druggable genome' representing approximately 19% of the currently available drug targets (Hopkins and Groom, 2002; Rask-Andersen et al., 2014; Lu and Zhang, 2019). While GPCRs are the most exploited therapeutic target for drug design and discovery, a subset of largely uncharacterised and poorly understood GPCRs mainly belong to the rhodopsin-like receptors family exist (Alavi et al., 2018). This subset of GPCRs is designated as "Orphan" GPCRs and comprised of more than 150 orphan GPCRs without a recognised endogenous ligand. Looking at the successful therapeutic history of GPCRs, deorphanizing these receptor subsets and understanding their role in human physiology could provide promising targets for future therapies (Civelli et al., 2006; Levoye et al., 2006; Shore and Reggio, 2015).

GPCRs regulating physiological and pathological pain are widely expressed in pain pathways. Nociceptors are equipped with GPCRs which in tandem with ion channels regulate the detection of noxious stimuli at peripheral terminals and transmission of pain at central terminals. GPCRs enable sensory nerves to detect and respond to distinct molecular mediators such as endogenous biogenic amines (serotonin and histamine), peptides (kinins, tachykinins, opioids), purines and nucleotides (adenosine, ATP), lipids (prostaglandins), steroids (bile acids), and enzymes like proteases (serine and cysteine). It is important to note that GPCRs can stimulate or inhibit pain transmission, and the expression of GPCRs is highly regulated with tissue injury, inflammation, and disease, significantly alter the expression and functional dynamics of GPCR signalling.

1.14.1. GPCR signalling pathways

All GPCRs comprise seven α-helical transmembrane domains, an extracellular N terminus with three extracellular loops, and an intracellular C terminus with three intracellular loops. These seven transmembrane helices of the GPCR serve as key signal transduction interfaces. They recognise a large diversity of hormones and neurotransmitters, link the extracellular stimuli with diverse intracellular responses (Li et al., 2002). The G-proteins associated with GPCRs are heterotrimeric and consist of three subunits: α, β, and γ. Based on the G protein-coupling preference, GPCRs can be broadly classified into four major categories: $G_{\alpha s}$-, $G_{\alpha i/o}$-, $G_{\alpha q/11}$-, and $G_{\alpha 12/13}$-coupled receptors (Hur and Kim, 2002; Neves et al., 2002). Typically, pronociceptive GPCRs such as bradykinin receptors (B_2), purinergic receptors ($P2Y_{1\&2}$), prostaglandin E2 (PGE2) receptors (EP1 and EP4), and protease-activated receptor 2 (PAR2), are primarily coupled to excitatory $G_{\alpha q}$ and $G_{\alpha s}$ signalling pathways, which can induce

nociceptor excitability and lower thresholds for activation. Ligand binding to a GPCR induces conformational change and promote the interaction between GPCR and heterotrimeric protein subunits. This interaction triggers the exchange of guanosine 5′-diphosphate (GDP) for guanosine 5′-triphosphate (GTP) on the Gα subunit and the $G_{\beta\gamma}$ dimer couples to GPCR, providing stability to receptor–Gα interface (Li et al., 2002). The subsequent cascade results in the dissociation of the G protein from the receptor and later the Gα subunit from the $G_{\beta\gamma}$ subunits. The $G_{\beta\gamma}$ subunits can activate a diverse array of effectors such as enzymes and ion channels (Neves et al., 2002; Sadja et al., 2003). The GTP-bound Gα subunits activate a series of subunit specific signalling cascades. For example, $G_{\alpha s}$ activates membrane-associated adenylyl cyclase (AC) enzyme and catalyses the synthesis of the second messenger cAMP from ATP molecules. The increased cAMP concentration acts as a second messenger resulting in the activation of cAMP-dependent protein kinase A (PKA). The PKA mediate diverse and complex cellular functions by phosphorylating ion channels and transcription factors, typically mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK). By contrast $G_{q/11}$ coupled GPCRs activate phospholipase C (PLC), particularly the PLCβ isoform, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). DAG and IP3 subsequently activate protein kinase C (PKC) and cause a transient increase of intracellular Ca^{2+} respectively (Li et al., 2002). Protein kinase activation phosphorylates and sensitises ligand-gated and voltage-gated ion channels. Ion fluxes depolarise nociceptors and trigger action potentials leading to acute nociception. GPCRs coupled via $G_{i/o}$ proteins inhibit adenyl cyclase enzyme activity, as opposed to “stimulatory” $G_{\alpha s}$ proteins. As previously described, Gs-coupled receptors (e.g., PGE2-receptor) generally increase the excitability of DRG neurons;

thus, contributing to inflammatory hypersensitivity. In principle, concurrent activation of G_i-coupled receptors counteracts this effect by decreasing cAMP levels.

1.14.2. Targets of $G_{i/o}$ GPCR inducing analgesic effects

1.14.2.1. Voltage-gated calcium (Ca^{2+}) channels

Voltage-gated calcium (Ca^{2+}) channels (Cavs) are vital transducers of membrane potential changes into intracellular Ca^{2+} transients. In neurons, Ca^{2+} influx through these channels modulates the presynaptic release of neurotransmitters (Simms and Zampone et al., 2014). There are five subtypes of Ca_Vs: The L-type (Ca_V1), N-type ($Ca_V2.2$), P/Q-type ($Ca_V2.1$), R-type ($Ca_V2.3$), and T-type (Ca_V3). The N-type and P/Q-type are the most critical Cavs for synaptic transmission. The N-type of Ca^{2+} channels is inhibited by Gβγ subunits of $G_{i/o}$ coupled GPCR (Currie et al., 2010). Inhibition of N-type VGCCs in the spinal cord produces profound analgesia (Wang et al., 2000; Huang et al., 2015). Additionally, inhibition of low-voltage activated Ca^{2+} channel (T-type) in DRG neurons and the peripheral nerve termini by G_i-coupled $GABA_B$ receptor agonist baclofen have been reported to alter the response to mechanical stimuli (Rose et al., 2013).

1.14.2.2. GIRK (Kir3.x) channels

The G protein-coupled inward-rectifying potassium channels (GIRK; Kir3.x) are characterised by the property of inward-rectification, which is described as allowing large inward currents and smaller outward currents. Kir3 channels are ubiquitously expressed in the CNS and regulate neuronal excitability (Gao et al., 2007). Kir3.x subfamily (Kir3.1-Kir3.4) mRNA expression in rat and human DRG neurons has been detected (Gao et al., 2007). Additionally, Unbiased RNA sequencing and single-cell

RNA sequencing of mouse DRG neurons revealed low levels of GIRK channel expression with a significant proportion of GIRK2 expression (Thakur et al., 2014). GIRK channels are activated by receptors that couple to $G\alpha_{i/o}$ (Wickman and Clapham et al., 1995). The $G_{\beta\gamma}$ subunits liberated from G protein heterotrimers bind directly to GIRK channels to enhance channel activity and hyperpolarise the second-order neurons (Peleg et al., 2002, Sadja et al., 2003). The μ-opioid receptor agonist, DAMGO, have restored peripheral analgesia in nociceptor-specific GIRK2 expressed mouse in vivo (Nockemann et al., 2013). Moreover, the $GABA_B$ and SST receptors have been shown to stimulate GIRK channels in rat DRG neurons indicating the analgesic potential of $G_{i/o}$ signalling (Gao et al., 2007, Gorham et al., 2014).

Apart from the above-mentioned targets the $G_{i/o}$ GPCR also inhibits the functions of Transient receptor potential melastatin 3 (TRPM3) channels. TRPM3 channels are expressed in a subset of sensory neurons is vital for noxious heat perception. The expression of TRPM3 is also reported in DRG. Temperatures near 40°C activate this channel, and it can also be stimulated by chemical agonists such as pregnenolone sulphate. The $G_{\beta\gamma}$ subunit of the $G_{i/o}$ GPCR interacts with TRPM3 and attenuates its functions (Vriens et al., 2011). Additionally, activation of μ-opioid, $GABA_B$, and SST receptors inhibited Ca^{2+}signals evoked by pregnenolone sulphate in DRG neurons (Badheka et al., 2017).

Finally, phosphoinositide 3-kinase-γ (PI3Kγ) and mitogen-activated protein kinases (MAPK) are also downstream targets of $G_{i/o}$ GPCRs. These kinases are implicated in the sensitisation of nociceptive neurons in the presence of inflammatory mediators (Cesare et al., 1999; Aley et al., 2001).

1.15. GPCR -TRP channel axis: A point of convergence for noxious stimuli in GI tract

GPCRs and TRP channels are crucial regulators of gastrointestinal function and the integral role of TRP channels in colonic nociception and visceral hypersensitivity is well defined (Veldhuis et al., 2015). TRP channels are widely expressed on the extrinsic sensory neurons innervating the colon and upregulated during colitis (Chen et al., 2020). For example, increased density of TRPV1-positive fibres innervating the colonic mucosa in IBD, and IBS patients has been reported and positively correlated to visceral pain scores (Akbar et al., 2008; Hughes et al., 2009). During colitis, sensitisation of TRPV1, TRPV4, and TRPA1 by inflammatory mediators acting at GPCRs drives colonic afferents' hyperexcitability through the activation of GPCR signalling cascades (e.g., cAMP-dependent protein kinase A [PKA], PKC) and the synthesis of lipid second messengers (PIP2) that phosphorylate TRPs to increase cell-surface expression and interactions with adaptor proteins. The multiple signals that emanate from GPCRs converge on a relatively small number of TRP channels, influencing their activation threshold or augmenting sensitisation. For example, activated PKC and PKA phosphorylate the membrane or cytoplasmic residues of TRPV1 and alter its gating properties to facilitate its opening (Cheng and Ji, 2008). Additionally, NGF and bradykinin- mediate potentiation and sensitisation of TRPV1 and TRPA1 (Chuang et al., 2001) channel and cAMP–PKA and PKC signalling in TRPV4 ion channel contribute to mechanical hyperalgesia induced by carrageenan or by an inflammatory soup containing bradykinin, substance P, PGE2, serotonin and histamine (Alessandri-Haber et al., 2006). In primary sensory neurons, this TRP-mediated cation influx produces generator potentials which activate voltage-gated Na^+ channels resulting to generate action potentials that relay nociceptive signalling. For

example, modulation of TRPV1, TRPV4, and TRPA1 signalling contributes to the nociceptive actions of GPCRs activated by proteases (PAR2), bradykinin (B_1R, B_2R), prostaglandins (EP, IP receptors), ATP ($P2Y_{1,2}$), histamine (H_1R), and serotonin ($5HT_4$) (Veldhuis et al., 2015). TRP channels also stimulate cation-sensitive signalling pathways (e.g., Ca^{2+} stimulated protein kinase C activity) and transcriptional changes to promote the expression and release of pro-nociceptive or pro-inflammatory peptides, such as SP and CGRP, from peptidergic neurons. The release of these neuropeptides from peripheral nociceptive endings of nociceptors promotes arteriolar vasodilation, plasma extravasation, and granulocyte infiltration in postcapillary venules, a condition defined as neurogenic inflammation. Compared to voltage-gated ion channels (Ca^{2+}, Na^+ and K^+), TRP channels exhibit slower kinetics and possess high activation thresholds, whereby ion conductance occurs in response to substantial changes in voltage, temperature, exogenous ligands, and intracellular lipids. These functional dynamics of TRP channels allow them to respond only upon exposure to noxious stimuli, thereby generating critical sensory feedback to reflect pain. Unsurprisingly TRP channels have been identified as therapeutic targets for pain and inflammation. The compounds targeting the TRP channels within peripheral cells are currently providing the promise of being an effective analgesic in the clinic (Brederson et al., 2013).

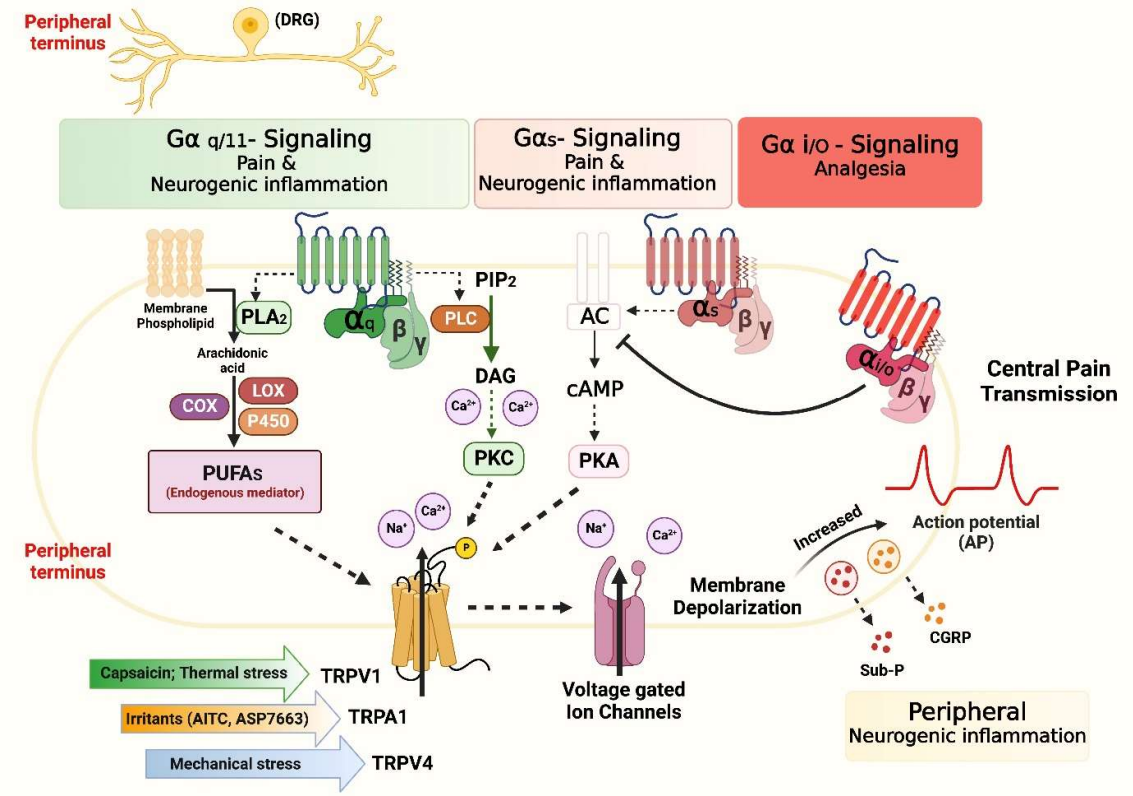


Figure 5. The GPCR-TRP channel axis in pain signalling. The GPCRs activate multiple pathways that converge on TRP channels. $G\alpha_q$ protein signaling (green) stimulates PLA_2 which converts membrane Phospholipids (PIP_2) to produce arachidonic acid (AA) and downstream polyunsaturated fatty acid (PUFA) acting as endogenous TRP channel ligands via LOX, cytochrome P450, and COX activity. Additionally, activation of PLC metabolizes PIP_2 to produce second messenger such as DAG, IP3, and stimulate intracellular Ca^{2+} release, increasing PKC kinase activity and TRP channel phosphorylation. Gαs protein signaling (light brown) stimulates adenylate cyclase (AC) activity; increases cAMP production and PKA activity to promote TRP channel phosphorylation and sensitization. Together, these pathways increase the likelihood of TRP channels responding to endogenous stimuli. Conversely, $G\alpha_{i/o}$ signaling (Red) promotes analgesia through inhibition of AC-dependent cAMP formation. TRP channel activity stimulates voltage-gated ion flux, membrane depolarization and downstream action potentials (AP) for central

transmission of pain. Stimulated primary afferent terminals also release peptides in the periphery (e.g., SP, CGRP) to mediate neurogenic inflammation.

1.15.1. Strategic approaches for treatment of pain

As discussed above, mediator-induced afferent sensitisation is a well-established phenomenon that drives the visceral pain experienced by IBD patients (Grover et al., 2009). Moreover, inflammatory mediators' long-term mechanistic changes in the sensory nerve terminal likely cause visceral hypersensitivity-induced pain in IBS patients (Lembo et al., 1994). These data collectively provide evidence supporting a role of continued peripheral nociceptor input as a critical driver in chronic visceral pain and simultaneously supports the prominent role of inflammatory mediators in the stimulation of visceral nociceptors and augmentation of VH. The TRP-channels, voltage gated ion channels, inflammatory mediators and their cognate GPCRs are central to this inflammation-driven visceral hypersensitivity-induced pain. Therefore, they reflect the most obvious target for pain relief. However, some TRP channel antagonists have disappointed in clinical trials owing to their unacceptable on-target adverse effects. For example, first-generation TRPV1 antagonists were withdrawn from clinical trials, including AMG517, because of febrile reactions due to marked hyperthermia (Gavva et al., 2008) and others, like MK2295, because of burn injuries (R Eid et al., 2011) whereas antagonists that progressed into phase-II efficacy trials failed to demonstrate analgesic activity, such as a terminated trial of AZD1386 for osteoarthritic pain (Miller et al., 2014). Similarly, a highly potent TRPA1 antagonist, ODM-108, developed by Orion Pharma to treat neuropathic pain, failed due to complex pharmacodynamic properties (ClinicalTrials.gov Identifier: NCT02432664).

Peripheral sensitization in patients with organic and functional visceral pain syndromes has significant implications for clinical treatment. Therefore, it may be therapeutically beneficial to either "switch off" nociceptors or inhibit mediator-induced neuronal sensitisation when treating visceral pain in IBS/IBS.

1.15.2. Targeting peripheral sensitization for pain relief

The therapeutic approaches attenuating sensitisation by targeting the pain-sensing nerves, so-called nociceptors, are widely being evaluated. Clinical evidence to support the utility of this approach concerning visceral pain is growing and has achieved modest success. For example, a peripherally restricted guanylate cyclase-C (GC-C) receptor agonist, linaclotide, has reduced constipation-induced pain in IBS patients (Casto et al., 2013). Linaclotide potently binds to and activates the GC-C receptor expressed on the luminal surface of intestinal epithelial cells to stimulate production of the intracellular secondary messenger cyclic guanosine-3′,5′-monophosphate (cGMP) which regulates intestinal fluid and electrolyte secretion into the lumen (Busby et al., 2010), a mechanism well appreciated for reducing the visceral pain elicited by noxious mechanical activation of colonic afferents (Feng et al., 2013; Castro et al., 2013; Silos-Santiago et al., 2013). Moreover, with consistent and reproducible efficacy against pain end-points across several clinical trials, linaclotide has been approved for the treatment of IBS-C patients (Chey et al., 2012; Rao et al., 2012; Rao et al., 2014). Linaclotide significantly improved pain scores in sensitized animal tissue aligned with the inhibitory phenomenon observed with kappa opioid receptor agonist asimadoline (Hughes et al., 2014), the gamma-aminobutyric B (GABA) receptor agonists baclofen and α-conotoxin Vc1.1 (Castro et al., 2017), and activation of the oxytocin receptor by selenoether oxytocin analogues (de Araujo et al., 2014). Moreover, eluxadoline, a μ/κ-

opioid receptor agonist and δ-opioid receptor antagonist, has effectively attenuated pain in clinical trials focusing on IBS patients with predominant diarrhoea (IBS-D) (Dove et al., 2013). The biologic approach, which focuses on blocking mediators involved in the sensitisation of nociceptors, has proven effective in attenuating pain in various inflammatory diseases. The efficiency of this approach has been demonstrated in RA and UC by blocking the hyperalgesic potential of IL-1β (Obreja et al., 2002). These data collectively provide and evidence supporting the strategy that blocking the actions of inflammatory mediators on nociceptive afferents or inhibiting peripheral sensitisation could lead to a better treatment of chronic visceral pain.

1.15.3 Blocking nerve conduction for pain relief

An approach, blocking the activation of pain-sensing nerves thereby reducing transduction and transmission of noxious stimuli from the gut to the central nervous has also been effective in attenuation of visceral pain for example, rectal administration of local anaesthetic has proven effective in reducing spontaneous and stimulus-evoked visceral pain (e.g., visceral hypersensitivity to intrarectal balloon distension) associated with functional GI disorders (Verne et al., 2003 and 2005). Although, complete blockade of the nerve functions could not be a practical long-term solution, but selective agents attenuating the functions of nociceptors could offer several advantages over the empirical pain relief treatment.

1.15.4 Targeting GPCRs in the treatment of pain

GPCRs expressed by primary sensory neurons and second-order spinal neurons can stimulate and inhibit pain transmission. Our growing understanding of the structure, functions, and complex signalling pathways of these dynamic signalling proteins

provides insights into how GPCRs control pain and has revealed new opportunities for therapy.

1.15.4.1. Targeting biased agonism paradigm of GPCRs

The ability of GPCRs to differentially activate downstream signalling pathways by stabilisation of distinct receptor conformational states in response to the binding of two distinct agonists on the same GPCR explains the phenomenon of biased agonism. Biased agonists are expected to have different functional and physiological consequences from conventional balanced agonists, given that they activate only a select portion of a receptor's signalling cascade while inhibiting others. The biased agonism is observed in PAR2 receptor signalling. The activation of PAR2 by trypsin activates canonical pathways and induces coupling to $G_{\alpha q}$ and β-ARRs, stimulates endocytosis and augments sustained sensitisation of colonic sensory nerves during IBS (Jimenez-Vargas et al., 2018; DeFea et al., 2000). However, activation of PAR2 by cathepsin S does not lead to receptor endocytosis (Jimenez-Vargas et al., 2018). The biased agonism is also a characteristic phenomenon that reflects in MOPr signalling. The endogenous enkephalins are balanced agonists at MOPr, activating both G protein- and β-arrestin-mediated pathways, whereas morphine is biased toward G protein-mediated signalling (Bohn et al., 2004). Moreover, β-arrestin-mediated pathways control peripheral side effects of morphine, such as constipation, respiratory depression, and nausea, while the antinociceptive effects are mediated through G-protein signalling as validated in β-arrestin-2 knockout mice (Bohn et al., 1999). Considering the biased agonism phenomenon of MOPr, biased agonists oliceridine that evoke MOPr signalling by G proteins but not β-ARR2 have been developed. They have shown promising results in preclinical (Altarifi et al., 2017) and early phase clinical trials in humans (Soergel et al., 2014). However, in comparison to morphine, abuse

liability and other risks outweigh the utility of oliceridine, consequently declining FDA approval. Therefore, the transition of this promising approach to novel therapies is being questioned, and their clinical utility is currently uncertain (Singla et al., 2019).

1.15.4.2. Targeting GPCR dimerisation theory

The concept of GPCR dimerisation is controversial, and preferentially, contentions exist around the dimerization of opioid receptors (OPrs) (Geppetti et al., 2015; Gomes et al., 2000). Despite the controversy, bifunctional ligands "Eluxadoline" (MOPr agonist and DOPr antagonist) have been developed and shown to attenuate pain in IBS-D patients without evidence for abuse potential in phase II and III trials (Dove et al., 2013).

1.15.4.3 Focusing on the tissue-specific distinct physiological role of GPCRs

GPCRs can produce different sensory outputs in different tissues such that GPCRs like mas-related G-protein receptors (Mrgprs) and GPCR 5 (TGR5), a receptor for secondary bile acids that mediate itch in the skin, can also induce irritant sensation and visceral hypersensitivity in the colon (Castro et al., 2019). Moreover, Mrgpra3, Mrgprc11 and TGR5 are expressed in DRG neurons innervating the mouse colon and have been implicated in afferent nerve sensitisation associated with IBS pain (Bautzaova et al., 2018; Catsro et al., 2019). The downstream signalling of these receptors is mediated through TRPA1 channels, which makes this an efficient target for the novel treatment of pain in IBD/IBS. Therefore, the drugs developed for combating itch can be repurposed to treat pain in IBS/IBD.

1.15.4.4. Inhibiting endosomal signaling in GPCRs

More recently, the ability of GPCRs to signal through their direct interactions with β-arrestin has also emerged (Galandrin and Bouvier, 2006). The GPCR signalling at the plasma membrane is often transient. The effectors, β-arrestin, sterically interdict the GPCR association with G proteins and desensitise signalling at the plasma membrane. Moreover, the interaction with β-arrestin further promotes the transfer of ligand-bound receptors from the cell surface to early endosomes *via* dynamin- and clathrin-dependent endocytosis (Goodman et al., 1996). Endosomal signalling in GPCR generates sustained intracellular signals *via* secondary messengers (e.g., cAMP and GRKs). This continual and sustained endosomal signal sensitises ion channels (TRPV1) and drives chronic visceral pain (Amadesi et al., 2004). The Endosomal signalling is a characteristic feature of protease-activated receptor-2 (Jimenez-Vargas Nestor N et al., 2018), the NK_1-Receptor (Jensen et al., 2017), the μ-opioid receptor (MOPr) and δ-opioid receptor (DOPr) (Jimenez-Vargas Nestor N et al., 2020). The role of endosomal signalling in PAR2 induce sensitisation of colonic nociceptors and hyperalgesia is well defined. (Jimenez-Vargas Nestor N., et al., 2018, Cenac et al., 2007). However, the reports explaining the crucial involvement of endosomal signalling mediated effects of NK_1-R and the μ-opioid receptors on GI-related pain are obscure. A recently performed study with a modified drug delivery approach targeting the NK_1-receptor endosomal signalling has shown a positive outcome. A lipid-anchored neurokinin 1 receptor antagonist prolongs pain relief in a preclinical model by multiple mechanisms, including an increased local concentration of NK_1-antagonist at membranes, a prolonged decrease in NK_1R endocytosis, and persistent inhibition of signalling from endosomes suggesting this approach may have clinical potential to treat pain (Mai et al., 2021).

1.15.4.5. Targeting $G_{i/o}$ GPCRs for pain relief

It is evident from the above studies that GPCRs have been the focus of drug discovery and development, and different approaches have been implemented around GPCRs for developing an efficient treatment for pain relief in GI diseases, particularly around IBD and IBS. However, the efficacious treatment is still an unmet clinical need. The $G_{i/o}$ coupled GPCRs possess the potential to alter the excitability and expression of vital ionotropic proteins (TRP channels), controlling short- and long-term changes in the synaptic activity of nociceptors. This ability of $G_{i/o}$ coupled GPCRs are shown in different studies, for example, systemic administration of Cannabinoid CB1 receptor ($G_{i/o}$ coupled GPCR) agonist WIN55,212-2 suppresses thermal and mechanical hyperalgesia induced by intradermal injection of capsaicin, an agonist of nociceptive TRPV1 receptors (Patwardhan et al., 2006). Similarly, activation of $GABA_B$ receptors in DRG neurons by baclofen have shown to inhibit TRPV1 sensitisation without affecting the normal functioning of the capsaicin on TRPV1. This effect was independent of $G_{\beta\gamma}$ signalling and was related to protein-protein interaction between $GABA_B$ and TRPV1 due to their close juxtaposition (Hanack et al., 2015). This raises the possibilities where such interaction between closely associated $G_{i/o}$ GPCRs and the components of pain circuitry could be crucial in pain signalling and should be explored for the purpose of therapeutic intervention. Similarly, another $G_{i/o}$ GPCR, SST1-4 receptors are expressed in DRG neurons (L1-L5), of which significant proportions of SST2a receptors are co-expressed in TRPV1 positive neurons (Carlton et al., 2003). SST has shown anti-inflammatory and antinociceptive effects locally and systemically (Corsi et al., 1997). Intraplantar injection of SST reduced mechanical allodynia in a rat inflammatory pain model (Helyes et al., 2001). The Intraplantar Injection of SST receptor agonist, octreotide has inhibited formalin-induced nociceptive

behaviours and attenuated C-fibers to bradykinin-induced excitation and heat sensitisation (Carlton et al., 2001; Carlton et al., 2003). Intraplantar injection of octreotide reduces capsaicin-induced pain behaviours in rats, suggesting that SST may inhibit TRPV1 channels expressed on primary afferent nerves (Carlton et al., 2004). Additionally, SCR007, a selective non-peptide SSTR2 agonist, significantly increases the nociceptive threshold (Ji et al., 2006).

A neuropeptide, Galanin, has also been implicated for its inhibitory influence on the excitability of colonic sensory afferents (Taylor et al., 2020). Galanin execute its downstream action by binding to its three G protein-coupled receptors such as GalR1, GalR2, and GalR3 which are expressed by putative nociceptors originating from the LSN in the distal colon (Taylor et al., 2020). The GalR1 and GalR3 are mostly $G_{i/o}$-coupled receptors and functions by inhibiting adenyl cyclase activity and inwardly rectifying K^+ (GIRK) channels, resulting in neuronal hyperpolarization. $G_{\alpha i/o}$-subunits and β_γ -subunit heterodimer G-proteins mediate these actions. By contrast, GalR2 is predominantly Gq-coupled, leading to the release of intracellular Ca^{2+} and the stimulation of protein kinase C (PKC), events associated with neuronal excitation (Freimann et al., 2015). GalR1 KO mice showed increased hyperalgesia after hind-paw tissue injury and inflammation (Malkmus et al., 2005), whereas GalR1-agonist has been shown to suppress colorectal mechanosensitivity and hypersensitivity to inflammatory insult (Taylor et al., 2020). The following study further strengthens our hypothesis that $G_{i/o}$ coupled GPCRs can inhibit neuronal excitability, which can be used to modulate colonic afferent excitability as a measure to treat visceral pain.

Recently, a novel approach using chemogenetically engineered mutated GPCR defined as “Designer receptors exclusively activated by designer drugs (DREADDs)”

have been used to study the potential of $G_{i/o}$ signalling in attenuation of visceral pain. This G_i-coupled designer receptor does not respond to endogenous ligands but can be activated by synthetic ligands. G_i-coupled DREADD in small diameter nociceptors has increased thermal and mechanical threshold when stimulated by a known chemical activator (Iyer et al., 2016).

The role of GPCRs in visceral pain is complex, and our knowledge in this area is still growing. Additionally, a significant proportion of GPCRs predominantly expressed in the colon are still in the orphan stage, and their functional roles are still elusive. The expression pattern of such GPCRs and their interaction with principal regulators of nociception such as TRP channels is still under evaluation. However, looking at the previous history and treatment potential of $G_{i/o}$ GPCRs in pain, we have focused our investigation on one such $G_{i/o}$ coupled orphan GPCR, the GPR35 receptor, for its therapeutic intervention in GI pain.

1.16. GPR35 is highly co-expressed with TRPA1 ion channel in mouse DRG

1.16.1. *In silico* analysis of RNA-Sequencing database.

To begin we performed an *in-silico* analysis of RNA-sequencing data previously generated and published by Hockley et al., 2019. The study identified seven neuronal subtypes of colonic sensory neurons by transcriptomic profiling and unsupervised clustering of thoracolumbar (TL) and lumbosacral (LS) DRG neurons from mice (Hockley et al. 2019). Expression profiles by colonic neuronal subtype can be found at https://hockley.shinyapps.io/ColonicRNAseq/. I identified significant expression of GPR35, TRPA1 and TRPV1 in the ‘mPEPb’ subtype of neurons, which represents

peptidergic nociceptors *(**see figure 6.A***). The GPR35 transcripts are broadly expressed across all colonic afferent populations, while TRPA1 and TRPV1 is more selectively expressed in peptidergic afferent populations (***see figure 6.B***). I have also found significant genetic co-expression between GPR35 receptors and TRPA1 or TRPV1 (***see figure 6.C***), which may allude to the involvement of GPR35 receptor in processing or modulating visceral nociception. This forms the rationale for investigating the interplay between GPR35 receptor and TRPA1 channel functioning.

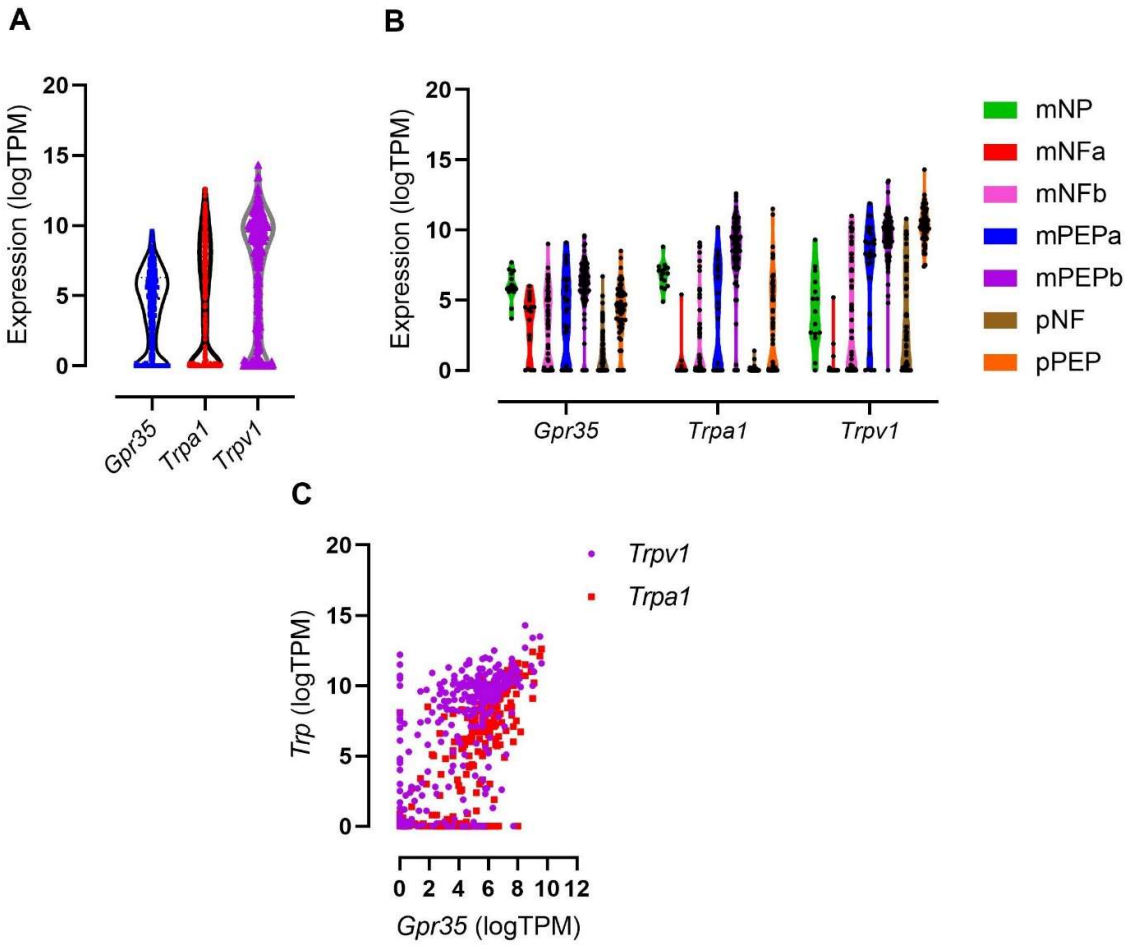


Figure 6: Single-cell RNA-seq of colonic sensory neurons in mouse. **A**. GPR35, TRPA1 and TRPV1 transcripts were found to be expressed in colonic sensory neurons. **B.** While the GPR35 transcripts are broadly expressed across all colonic afferent populations, the TRPA1 and TRPV1 is more selectively expressed in

peptidergic afferent populations. **C.** High degree of co-expression between GPR35 and TRPA1 or TRPV1 (nociceptive marker) is evident. Expression values are presented in Transcript-Per-Million (Log [TPM]). Neuronal subsets are abbreviated as follows; mNFa, mNeuroFilament-a; mNP, mNonPeptidergic; mPEPa, mPeptidergic-a, and mPEPb, mPeptidergic-b where prefix 'm' represents 'mixed' subtypes (neurons isolated from both TL and LS regions of DRG) and prefix 'p' represents neurons exclusively isolated from LS region of DRG. The data is previously published by Hockley, J.R.F, et al., 2019.

1.17. Principal Aim:

GPR35 is highly expressed in TRPV1 and TRPA1-positive colonic nociceptors. Based on the reported Gi/o coupling of GPR35 and the observed analgesic activity of its surrogate ligands in behavioural models of pain discussed briefly in Chapter -2, we hypothesise that GPR35 receptor activation would reduce colonic nociceptor firing, particularly in response to TRP channel activation.

1.18. Research objectives

To investigate this further we sought to:

1. Investigate the effect of GPR35 agonists (zaprinast and cromolyn) on colonic afferent response to algogenic stimuli (bradykinin and the TRPA1 receptor agonist ASP7663).

2. Investigate the effect of GPR35 agonists on (zaprinast and cromolyn) mechanosensitivity and its sensitisation by algogenic stimuli (TRPA1 receptor agonist ASP7663 and PGE2).

3. Investigate the mechanism of action by which GPR35 agonists modulate colonic afferent activity.

4. Repeat studies in tissue from GPR35-/- mice to confirm that the effects of zaprinast and cromolyn are specifically mediated through GPR35.

Chapter-2

Investigating the effect of GPR35 agonist on TRPA1 induced colonic afferent activation.

2.1. GPR35 receptor

GPR35 is a 7-transmembrane domain, GPCR, that transmits function *via* interaction with $G_{\alpha i/o}$, $G\alpha_{13}$, and β-arrestin (Milligan et al., 2011; Mackenzie et al., 2011; Divorty et al., 2015; Shore and Reggio, 2015). Since the discovery of GPR35 in 1998, it has been designated as an "orphan" receptor of the GPCR family due to a lack of clarity on its endogenous cognate ligand (O'Dowdl et al., 1998; Divorty et al., 2015). However, Kynurenic acid (KYNA) and lysophosphatidic acid have been reported to be endogenous ligands of GPR35 (Wang et al., 2006; Oka et al., 2010). Additionally, synthetic agonist such as pamoic acid, cromolyn sodium and zaprinast have demonstrated agonist activity at GRP35 receptors.

2.2. GPR35 receptor expressions

GPR35 is highly expressed in the gastrointestinal tract, stomach, small intestine, and colon of humans and mice, along with the spleen, pancreas, and immune cells (Wang et al., 2006; Yang et al., 2010). In comparison, relatively lower expression of GPR35 were reported in heart, lung, and skeletal muscle (Horikawa et al.,2000; Taniguchi et al., 2006; Min et al., 2010). Literature also reports high expression of GPR35 in the

discrete regions of the CNS and peripheral nervous system, spinal cord, and dorsal root ganglia (DRG) of the mouse (Wang et al., 2006; Taniguchi et al., 2006; Ohshiro et al., 2008; Cosi et al., 2011; Berlinguer-Palmini et al., 2013). Additionally, GPR35 receptors are expressed in TRPV1 positive small to medium diameter DRG sensory neurons, indicative of a role in processing noxious sensory information (Ohshiro et al., 2008; Cosi et al., 2011). In humans, apart from GPR35 (also designated as GPR35a) with 309 amino acids, 7-transmembrane domain polypeptide, an alternatively spliced protein GPR35b, which is identical in sequence apart from containing a 31 amino acid N-terminal extension has also been reported (Marti-Solano et al., 2020a; Marti-Solano et al., 2020b). However, the research has broadly focused on human GPR35 with a short (GPR35a) isoform. The regulation and functional information on the similarities or dissimilarities between these spliced variants are limited. However, concerning our study, which focuses on the GI area and the colonic afferents, recent literature demonstrates the presence of similar expression patterns for both the GPR35 isoforms in the colon and nerves (Marti-Solano et al., 2020). Moreover, both the isoforms of GPR35 respond similarly to GPR35 agonists as shown in *in vitro* pharmacological studies (Guo et al., 2008; Zhao et al., 2010; Mackenzie et al., 2014; Marti-Solano et al., 2020).

2.3. GPR35 endogenous ligands

The endogenous physiological roles and transducer signalling pathways of GPR35 are still poorly defined. However, Kynurenic acid (KYNA) and lysophosphatidic acid have been reported as endogenous ligands of GPR35 (Wang et al., 2006; Oka et al., 2010). Kynurenic acid is an endogenous tryptophan metabolite produced *via* the kynurenine pathway. Kynurenic acid is a competitive antagonist of the N-methyl-D-aspartate

(NMDA) receptor (Kessler et al., 1989; Hilmas et al., 2001), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainite receptors (Patel et al., 2001; Prescott et al., 2006; Mok et al., 2009). Kynurenic acid exhibits neuroprotective effects, which are attributed to its antagonism of ionotropic excitatory amino acid receptors (Kemp et al., 1988; Kessler et al., 1989). Kynurenic acid is also described as an agonist of GPR35. However, kynurenic acid displays species selectivity over its agonist action on the GPR35 receptor in humans, rats, and mice with differences in EC_{50} values (***see table -1***) (Wang et al., 2006; Zhao et al., 2010; Jenkins et al., 2012; Southern et al., 2013). The Literature also shows that kynurenic acid is substantially more potent against the rat ortholog than the human (Jenkins et al., 2010), creating uncertainty over its endogenous regulation of the GPR35 receptor. Additionally, the selective action of kynurenic acid through GPR35 agonism has been questioned, considering its multiple actions on glutamate receptors (NMDA and AMPA). To answer the following, specific glutamatergic receptor antagonist studies have been performed, however, recently reported agonistic action of AMPA and kainite receptor antagonists 6,7-dinitro- 2,3-quinoxalinedione (DNQX) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) for GPR35 receptor has further complicated the story (Southern et al., 2013).

2.4. Synthetic agonists and antagonists for GPR35

The synthetic agonists such as pamoic acid, and zaprinast have demonstrated agonist activity at GRP35 receptors (Jenkins et al., 2010; Yang et al., 2010; Taniguchi et al., 2006). Compared to zaprinast, pamoate shows high potency towards the human GPR35 receptor but exhibits a partial agonistic activity as determined by the BRET-based GPR35-βarrestin-2 interaction assay. Also, it is described to have very little

agonistic activity toward rat GPR35 receptor (Jenkins et al., 2010). However, these findings contrast with studies where pamoate has elicited an agonist effect in mouse GPR35 orthologue (Zhao et al., 2010). The anti-asthma and anti-allergic agents cromolyn disodium (Jenkins et al., 2010; Yang et al., 2010) and nedocromil sodium (Yang et al., 2010) were recently shown to act as moderately potent agonists of GPR35. After which several mast cell stabilisers have been screened using a PathHunter human GPR35a–β-arrestin-2 interaction and complementation assay of which lodoxamide, and bufrolin have been identified as an equipotent agonist of human and rat GPR35 (MacKenzie et al., 2014). However, their effects at mouse ortholog have not been investigated. Moreover, a functional link between GPR35 agonism and mast cell stabilisation remains to be demonstrated. Amongst these exogenous GPR35 agonists, zaprinast has emerged as a reference standard agonist for probing the functions of the GPR35 receptor (Yang et al., 2010; Jenkins et al., 2010; Zhao et al., 2010; Divorty et al., 2015). The relative potency of zaprinast for the human, rat, and mouse GPR35 receptors is described as rat > mouse > human. (Jenkins et al., 2012; Milligan et al., 2011 and Zhao et al., 2010). Zaprinast is also a potent inhibitor of phosphodiesterase (PDEs) (Lugnier et al., 1986) and is mainly reported as an inhibitor of subclasses of cGMP-specific PDEs including PDE5 and PDE6 (Beavo, 1995; Loughney et al., 1998). Antagonists of GPR35 receptor, CID-2745687 and ML-145, are also available but are specific to human. These are utilised as valuable tools for human-specific GPR35 exploration due to the lack of cross reactivity at the rat/mouse receptor (Zhao et al., 2010; Heynen-Genel et al., 2010; Jenkins et al., 2012).

GPR55 agonist Compounds	pEC50 values [-log(M)]			Additional Pharmacology	Reference
	Human	Rat	Mice		
Kynurenic acid	39 – 217 (μM)	7 – 66 (μM)	11 (μM)	AMPA, NMDA and Kainate glutamate receptor antagonist	Milligan et al., 2017
Zaprinast	5.30 ± 0.03	7.02 ± 0.05	6.01 ± 0.06	PDE-inhibitor	Jenkins et al., 2012
Cromolyn sodium	4.78 ± 0.10	5.30 ± 0.03	4.24 ± 0.06	Mast cell stabilizer	Jenkins et al., 2012
Pamoate	7.28 ± 0.07	> 3	79 (nM)		Zhao et al., 2010 Jenkins et al., 2012
Lodoxamide	1.61 ± 0.42 (nM)	12.5 ± 0.6 (nM)	NA	Mast cell stabilizer	MacKenzie et al., 2014
Bufrolin	2.93 ± 0.71 (nM)	9.9 ± 0.4 (nM)	NA	Mast cell stabilizer	MacKenzie et al., 2014
GPR35 antagonist compounds	**pIC50 values [-log(M)]**				
	Human	**Rat**	**Mice**		
CID-2745687	6.70 ± 0.09	Not-active	Not-active		Jenkins et al., 2012
ML-145	10 (μM)	Not-active	Not-active		Jenkins et al., 2012

Table 2. Potency values for agonist ligands at human, rat, and mouse GPR35. The values are determined by BRET-based GPR35-β-arrestin-2 interaction assays and PathHunter protein complementation assay respectively.

2.5. Role of GPR35 in GI diseases and pain

The GPR35 receptor has emerged as a putative target for IBD from human genome wide association studies where it was identified as a potential risk locus in IBD patients (Imielinski et al., 2009). Additionally, single-nucleotide polymorphism (SNP), rs3749171 in GPR35, that results in a threonine to a methionine substitution at the transmembrane helix may impact activation of GPR35 and has been associated with both UC and primary sclerosing cholangitis (PSC) (Imielinski et al., 2009; Jostins et al., 2013 and Ellinghaus et al., 2013). This association is of interest given that one of the comorbidities of IBD is PSC. It is estimated that 60-80% of PSC patients of northern Europe suffer from IBD (Karlsen et al., 2010). Additionally, the results of another GWAS and ImmunoChip single-nucleotide polymorphism screening have reported close association between GPR35 and Crohn's disease (Yang et al., 2015). In animals, activation of GPR35 receptors protected the mice against the development of colitis in the DSS-induced mouse model *via* upregulation of fibronectin and integrin α5 expression that promotes mucosal repair (Tsukahara et al., 2017). Consistent with this observation, GPR35-/- mice develop more severe colitis (Farooq et al., 2018), and this is attributed to a significant reduction in intestinal epithelial integrity due to a reduction in the expression of several proteins critical for maintaining tight junction integrity. These include Zonula occluden-1, E-cadherin, and Claudin-1. In addition, the expression of many proinflammatory cytokines such as IL-1β, CXCL1, CXCL2, CCL2, HMGB1, TGFβ1, TGFβ3 and proteases such as MMP1/9/12 are significantly elevated in the colonic mucosa of GPR35-/- mice during colitis (Farooq et al., 2018).

2.6. GPR35 in nociception

GPR35 is a potential target for pain treatment (Ohshiro et al., 2008; Cosi et al., 2011 and Alexander et al., 2015). GPR35 receptors are highly expressed in small to medium diameter DRG sensory neurons and this has led to the proposal for a role of GPR35 in the processing of noxious sensory information (Ohshiro et al., 2008; Cosi et al., 2011). This is further supported by evidence showing significant co-expression of GPR35 receptors with transient receptor potential vanilloid 1 (TRPV1) channel which is responsible for the transduction of noxious heat and acid (Ohshiro et al., 2008; Cosi et al., 2011). The literature surrounding GPR35 suggests an inhibitory function of GPR35 on synaptic transmission by nociceptive neurones in Ret conditional knock-out mice. Ret is a glial-derived neurotrophic factor family ligand receptor and signalling *via* Ret impacts the development of sensory neurons. Ret also regulates many ion channels and receptors including $Na_V1.8$, $Na_V1.9$, P2X3, TRPA1, ASIC2a, MrgD and delta opioid receptor (Franck et al., 2011). In a conditional knockout mouse, Ret deleted in small- and medium-size sensory neurons, showed a significant reduction in GPR35 expression with cold hyperalgesia and hypersensitivity to mechanical stimuli (Franck et al., 2011). This suggests that elevated pain responses in these mice could be a consequence of reduced GPR35 expression further suggesting a regulatory role of GPR35 in nociception.

GPR35 agonist's kynurenine (kynurenic acid precursor) and zaprinast pre-treatment are reported to attenuate chemical and mechanically induced nociception in animal models (Ohshiro et al., 2008; Cosi et al., 2011 and Alexander et al., 2015). In the acetic acid-induced writhing test, activation of GPR35 with zaprinast (5 mg/kg s.c.)

significantly decreased the number of the writhing response by more than 50%. Similarly, activation of GPR35 through elevation of kynurenic acid levels with L-kynurenine (300 mg/kg s.c.) administration drastically decreased the number of writhes in treated mice compared to vehicle control. Additionally, in isolated, cultured glial cells, zaprinast and kynurenic acid have reduced cAMP levels following pre-stimulation with forskolin, exemplifying $G_{\alpha i/o}$- receptor activation. The zaprinast and kynurenic acid are assumed to share a common mechanism site as maximal doses of these two agents had no additive effect on analgesia (Cosi et al., 2011). Additionally, *In vivo* studies employing the formalin test investigated zaprinast in acute and facilitated visceral pain. Pre-treatment with zaprinast (10 to 100 mg) by intrathecal injection, followed by subcutaneous injection of formalin (50µl, 5%) into the planar surface of the hind paw significantly reduced flinches during phase 1 and phase 2 in the formalin test suggesting activity on both acute and the facilitated pain (Yoon et al., 2005). The stimulation of GPR35 heterologously expressed in rat sympathetic neurons by zaprinast inhibits N-type calcium channels, suggesting a potential role for GPR35 in regulating neuronal excitability and transmitter release (Guo et al., 2008). Moreover, GPR35 agonism has also been demonstrated to reduce excitatory postsynaptic currents at the neurons of the rat hippocampus by zaprinast and kynurenic acid treatment (Berlinguer-Palmini et al., 2013). Pamoic acid, a newly identified GPR35 agonist, is also reported with antinociceptive actions in the acetic acid-induced abdominal constriction pain model (Zhao et al., 2010).

Cromolyn is described as GPR35 receptor agonist (Yang et al., 2010; Jenkins et al., 2010). Cromolyn has shown analgesic effects by alleviating visceral hypersensitivity in preclinical and clinical studies (Daryani et al., 2009; Stefanini et al., 1992 and 1995;

Carroll et al., 2013). It also suppresses the synaptic excitation provoked by inflammatory mediators (Wang et al., 2014; Zhang et al., 2018).

Cromolyn is also a clinically established therapy for treating asthma, through prevention of the degranulation of mast cells. In humans, mast cells are found in the close association with nerves of GI mucosa (Stead et al., 1989). Activation of mast cells in the proximity of the colonic nerve has been correlated with abdominal pain in IBS (Barbara et al., 2004). Mast cell degranulation releases a plethora of inflammatory mediators and proinflammatory cytokines, which sensitises primary afferent neurons, augment VH and induces pain during active IBS and IBD conditions (Santos et al., 1998; Raithel et al., 2001; Lapointe et al., 2015 and Balemans et al., 2017).

However, the precise action of cromolyn i.e., agonism of GPR35 receptor Vs mast cell stabilisation responsible for its analgesic effects remains elusive. Cromolyn reduces colorectal distention induced abdominal pain behaviours in stress-sensitive Wistar Kyoto rats, and this has been shown to be independent of its mast cell stabilising activity (Carroll et al., 2013).

2.7. Transient receptor potential ankyrin-1 subtype (TRPA1)

TRPA1 is a transducer of noxious stimuli in the colon and will be a focus for this thesis (Chung et al., 2011; Brierley et al., 2009). TRPA1 responds to mechanosensation and chemical irritants such as mustard oil (allyl isothiocyanate, AITC), cinnamaldehyde, allicin, acrolein, and noxious cold (< 17 °C) (Bandell et al., 2004; Jordt et al., 2004; Brookes et al., 2013; Bautista et al., 2006; Kojima et al., 2014; Meseguer et al., 2014). These TRPA1 agonists have been found to stimulate colonic afferents and provoke visceral nociception in mice (Brierley et al., 2009; Kondo et al., 2009; Mueller-

Tribbensee et al., 2015; Lennertz et al., 2012). The topical application of TRPA1 causes acute pain and the development of neurogenic inflammation through release of neuropeptide, purines, and other transmitters (Bautista et al., 2006). Primarily these irritant compounds activate TRPA1 through covalent modification of reactive cysteine residues in the ankyrin repeat domains (Macpherson et al., 2007). In addition to environmental irritants, a few endogenous TRPA1 agonists such as 4-hydroxy-2-nonenal (4-HNE) and 4-oxononenal (4-ONE) have also been identified. These endogenous ligands are oxidative products of membrane phospholipids produced during tissue injury and inflammation (Trevisani et al., 2007; Graepel et al., 2011). Additionally, a cyclopentane PGD2 metabolite, 15-deoxy-prostaglandin J2 (15d-PGJ2) (Cruz-Orengo et al., 2008), and nitrooleic acid (Taylor-Clark et al., 2009) were also found to activate TRPA1.

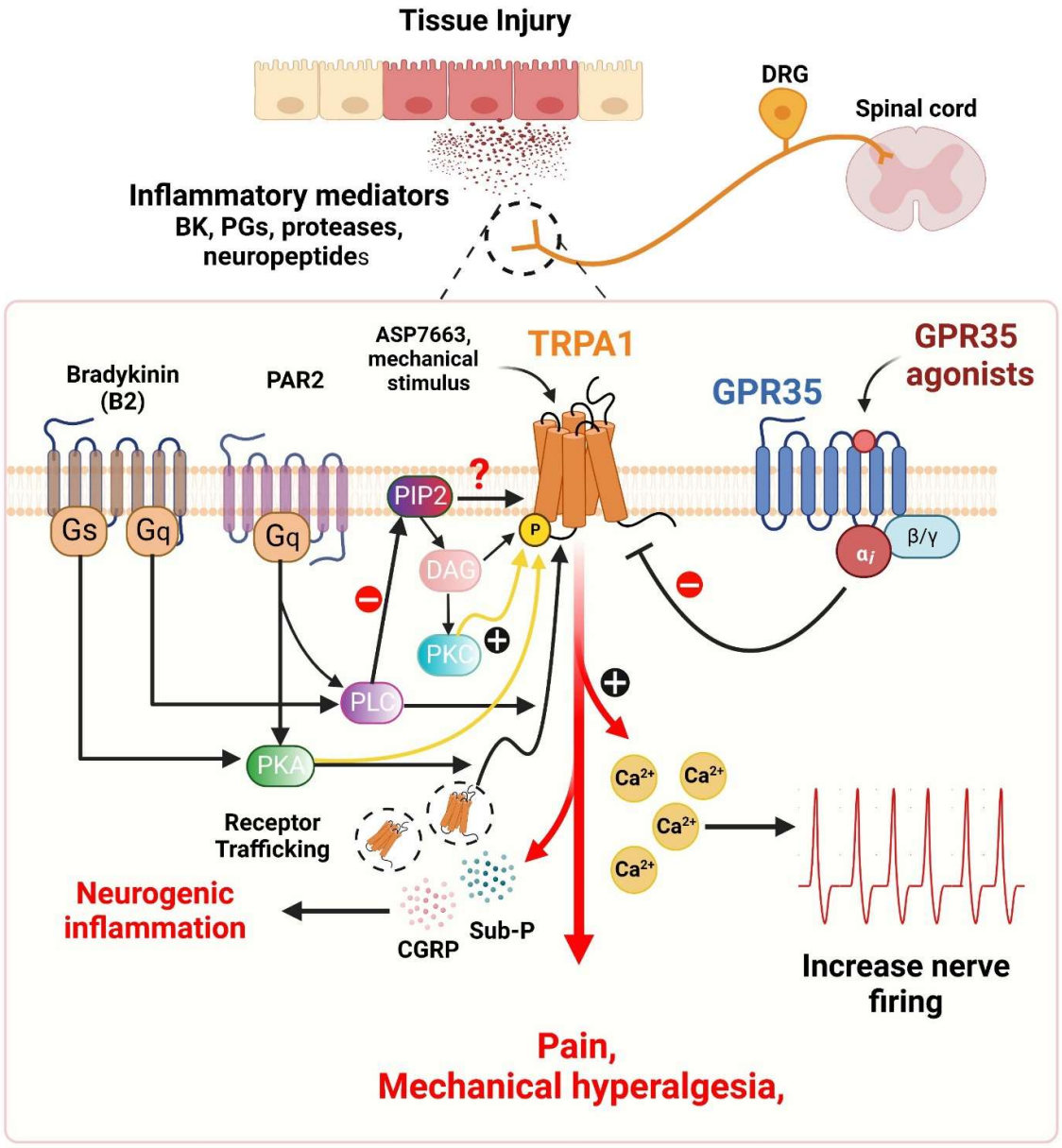


Figure 7: Schematic illustration of TRPA1 activation. TRPA1 is expressed in a subset of mouse colonic DRG and in different layers of the mouse colon. TRPA1 can be directly activated by cold temperatures (< 17^{o}C), pungent natural compounds such as mustard oil, Allyl isothiocyanate (AITC) cinnamaldehyde and acrolein or by noxious mechanical stimulus. Indirect activation of TRPA1 may involve modulation by G protein-coupled receptors such as bradykinin and PAR2 and by inflammatory

mediators released during tissue injury. TRPA1 activation elicits acute nociceptive behaviour in mice, neurogenic inflammation and marked hypersensitivity to thermal and mechanical stimuli.

2.7.1. TRPA1 expression

TRPA1 is highly expressed in the Nodose ganglion (NG) and DRG neurons, specifically innervating mouse intestines, as identified by *in-situ* hybridisation (Brierley et al., 2009). TRPA1 is expressed alone or co-expressed in a subset of TRPV1-expressing extrinsic primary sensory afferents and lumbosacral DRG neurons innervating the colon (Kobayashi et al., 2005; Malin et al., 2011). While TRPV1 is expressed by 80% of thoracolumbar and 50-60% of lumbosacral colonic DRG neurones (Christianson et al., 2006; Robinson et al., 2004), approximately 50% of these colonic afferents express TRPA1 (Brierley et al., 2009; Yang et al., 2008). In addition to extrinsic neurons, TRPA1 mRNA has been detected in mouse intestinal specimens (Penuelas et al., 2007), particularly localised in muscularis externa and mucosal layers (Poole et al., 2011). Furthermore, TRPA1 is highly expressed in human and rat EC cells (Nozawa et al., 2009). Immunoreactivity for TRPA1 has been identified in a subset of mouse colonic DRG neurons containing sensory neuropeptide SP and CGRP (Cattaruzza et al., 2010). It is also found in peripheral nerve endings in different layers of mouse colon frequently colocalised with CGRP (Brierley et al., 2009).

2.7.2. Role of TRPA1 in GI diseases

TRPA1 has emerged as an effector of visceral hypersensitivity induced pain in IBD and IBS (Blackshaw, 2014; Cseko et al., 2019; Balemans et al., 2019). TRPA1

transcript expression is highly upregulated during chronic/active and remitted CD/UC disease conditions (Malin et al., 2011; Kun et al., 2014; Li et al., 2016; Cseko et al., 2019), and similarly, increased TRPA1 protein expression is found in the surface epithelium, submucosal and muscle layers of animal colons during colitis (Cseko et al., 2019). TRPA1 activation exacerbates Dextran sulphate sodium (DSS) induced colitis in mice, while TRPA1-/- mice are protected from colonic inflammation (Utsumi et al., 2018). TRPA1 has also been reported to be responsible for hyperalgesia evoked by pro-inflammatory mediators such as TNF-α (Brierley et al., 2009), bradykinin (Bautista et al., 2006) and prostaglandin-E2 (Dall'Acqua et al., 2014). For example, bradykinin, a proalgesic and pro-inflammatory mediator, indirectly activates TRPA1 through Gq coupled bradykinin receptor 2 (B_2) mediated potentiation of TRPV1 (Bandell et al.,2004; Bautista et al., 2006). Moreover, in a model of bradykinin-induced colonic hypersensitivity, wildtype mice showed an increase in mechanosensitivity after bradykinin application, whereas TRPA1 -/- mice showed no change in mechanosensitivity after bradykinin application, corroborating the crucial role of TRPA1 in sensitisation and mechanical hypersensitivity (Brierley et al., 2009).

The above studies firmly implicate the antinociceptive effects of GPR35 agonists such as kynurenic acid, zaprinast, pamoic acid and cromolyn. However, the downstream mechanism of the GPR35 receptor responsible for the antinociceptive effect remains unclear. It is evident that TRPA1 is a noxious transducer involved in visceral hypersensitivity. Moreover, our *in-silico* analysis of RNA-seq database revealed that GPR35 is highly co-expressed with TRPA1-channels in the DRGs. Based on the observed analgesic activity in behavioural pain models, we hypothesised that GPR35 agonists may inhibit the activation of TRPA1 channel positive visceral nociceptors and

bring benefit on pain regulation and processing. This chapter is therefore aimed to investigate the inhibitory potential of the GPR35 receptor on TRPA1 induced colonic afferent activation.

2.8. Research objectives:

To investigate the effect of GPR35 receptor stimulation on colonic afferent activity I will:

- Determine the activation of colonic afferents by the TRPA1 agonist (ASP7663), confirm this effect is TRPA1 mediated and examine the reproducibility of these response.

- Investigate the effect of GPR35 agonist (zaprinast and cromolyn) pre-treatment on the colonic afferent response to the TRPA1 receptor activation.

- Confirm whether responses to zaprinast and cromolyn are mediated through the activation of GPR35 receptors using tissue from GPR35 -/- mice.

2.9. Materials and methods:

2.9.1. Animals

Experiments were performed using male CD-1, C57BL/6 and GPR35-/- mice (10-14 weeks of age) obtained from suppliers such as Charles River or Envigo (Cambridge UK). Mice were conventionally housed in temperature-controlled rooms (21°C) with a 12-h light/dark cycle and provided with nesting material, a red plastic shelter and

access to food and water *ad libitum*. GPR35 knockout (GPR35-/-) C57BL/6N-Gpr35tm1b(EUCOMM)Hmgu/WtsiH mice were rederived by the MRC Harwell, Oxfordshire, UK. We received 6 female and 7 male Gpr35-tm1b: Homozygous mice. The mice were intercrossed in the animal house unit and developed into a GPR35-/- colony. The animals were genotyped using external genotyping services (TransnetYX, USA).

2.9.2. In Vitro mouse colonic splanchnic afferent preparations

Adult male CD-1; C57BL/6, and GPR35-/- mice were euthanised using a CO_2 chamber (Vet-Tech solutions; flow rate of CO_2 0.8-1.0 L/min) followed by cervical dislocation in accordance with schedule-1 of the UK Animal (Scientific Procedures) Act 1986. The abdominal cavity was exposed by laparotomy, and internal organs such as pancreas, liver, kidney, and spleen were carefully removed under a stereomicroscope. The large intestine running from the anus to the caecum was identified. The bladder and the reproductive organs were carefully transacted at their junctions to the urethra. The pubic symphysis and the right and left acetabular joints were cut to remove the iliac bone. The Iliac bone was carefully resected to expose the distal colorectum. The distal colon from the ascending/descending inflexion point to the anus, including the rectum, with associated lumbar splanchnic nerves, was removed. Simultaneously, the skeletal muscle and neurovascular bundles (including the aorta) were cut ventrally from the spinal column whilst retaining the colon's inferior mesenteric artery and lumbar splanchnic innervation. During experiments, faecal materials from the colon were gently flushed out with Krebs solution to achieve unrestricted luminal infusion. The lumbar colonic nerve was traced into the aortic neurovascular bundle at the point of the iliac bifurcation, and the inferior and superior mesenteric ganglia were identified.

The extraneous skeletal muscle and connective tissues were removed from the neurovascular bundle using superfine forceps, allowing unrestricted access to the intermesenteric nerve. The epineurium (nerve sheath) was carefully peeled back from the nerve using fine forceps, and suction electrode recordings were made from one of the two intermesenteric nerves between these two ganglia, rostral to the inferior mesenteric ganglion.

2.9.3. Electrophysiological Recordings

The distal colon with associated lumbar splanchnic nerve attachment tissues was cannulated, luminally perfused (100µl/min) against a 2-3mmHg end pressure and serosally superfused (7ml/min; 32-34°C) with carbogenated Krebs buffer (in mM: 124 NaCl, 4.8 KCl, 1.3 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4.7H_2O$, 11.1 glucose, and 25 $NaHCO_3$) supplemented with nifedipine (10µM) and atropine (10µM) to block smooth muscle contraction.

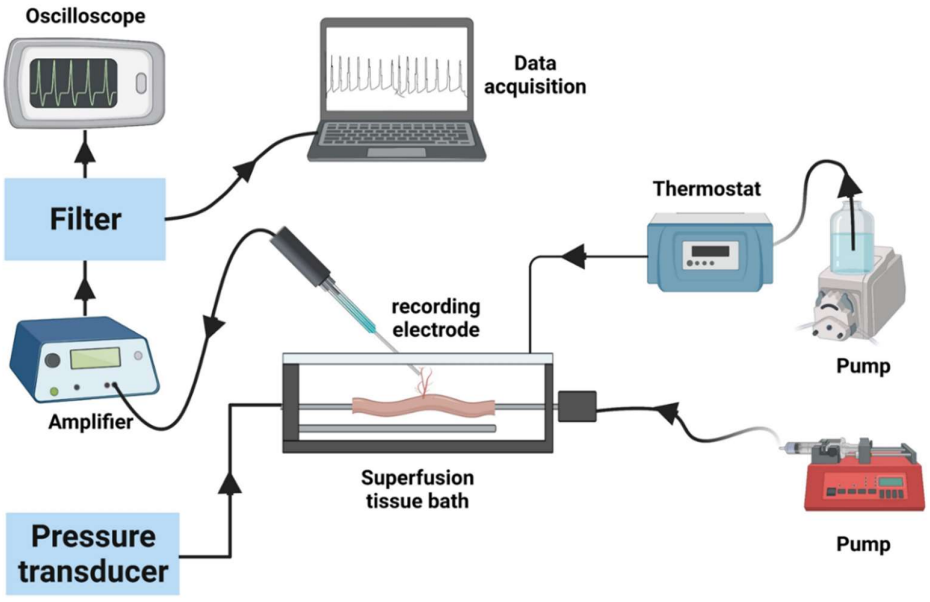


Figure 8: Illustration for electrophysiology method and instrumental setup.

A borosilicate glass suction electrode was used to recorded multi-unit activity from lumbar splanchnic nerve bundles (rostral to the inferior mesenteric ganglia). Signals were amplified, bandpass filtered (gain 5K; 100-1300 Hz; Neurolog, Digitimer Ltd, UK), and digitally filtered for 50Hz noise (Humbug, Quest Scientific, Canada). Analogue signals were digitised at 20 kHz (micro1401; Cambridge Electronic Design, UK) and displayed on a PC using Spike 2 software (Cambridge Electronic Design, UK). Spike firing was determined using a threshold counting at a level set at twice the background noise (typically 50-100μV). Recordings were stabilised over a 45 min period prior to experimental protocols after which basal ongoing nerve activity was recorded for 15 minutes prior to the start of experimental protocols.

2.9.4. Drugs and chemicals

The Drugs were purchased from Tocris bioscience or from Sigma Aldrich (UK). All compounds were diluted to working concentrations in a buffer on the day of experimentation.

Compound	Vehicle	Concentration Applied	Supplier	Application
ASP7663	DMSO	10μM, 30μM and 100μM	Tocris	TRPA1 agonist
Cromolyn sodium	H_2O	1μM, 10μM and 100μM	Tocris	GPR35 agonist
Zaprinast	DMSO	10μM and 100μM	Tocris	GPR35 agonist
AM0902	DMSO	1μM	Tocris	TRPA1 antagonist
IBMX	DMSO	50μM	Tocris	PAN-PDE inhibitor
Sildenafil	DMSO	1μM	Tocris	PDE-inhibitor
Compound 48-80	H_2O	50μg/ml	Tocris	Mast cell degranulator

Atropine	Ethanol	10μM	Sigma Aldrich	Cholinergic receptor antagonist
Nifedipine	DMSO	10μM	Sigma Aldrich	Ca^{2+} channel blocker

2.10. Experimental protocols

In preliminary studies, we determine the reproducibility of ASP7663 response by repeat application of ASP7663 bath superfusion (100μM, 20ml) following the interval of 40 minutes in CD-1 mice (***see figure 9.1***). We also determined the dose-response curve for ASP7663 using concentrations ranging from 10μM, 30μM and 100μM bath superfusion of 20ml in a separate set of experiments (***see figure 9.2***). While repeating the experiments in C57BL/6-WT and GPR35-/- mice, we first evaluated the response reproducibility of the ASP7663 (100μM, 20ml) application (***see figure 9.5***) and compared it with the vehicle (0.1% DMSO) treatment (data not shown), followed with which we examined the inhibitory effects of zaprinast (100μM, 50ml) and cromolyn (100μM, 100ml) pre-treatment on the colonic afferent response to ASP7663 (100μM, 20ml) given in C57B6-WT and GPR35-/- mice tissues (***see figure 9.6 and 9.7***).

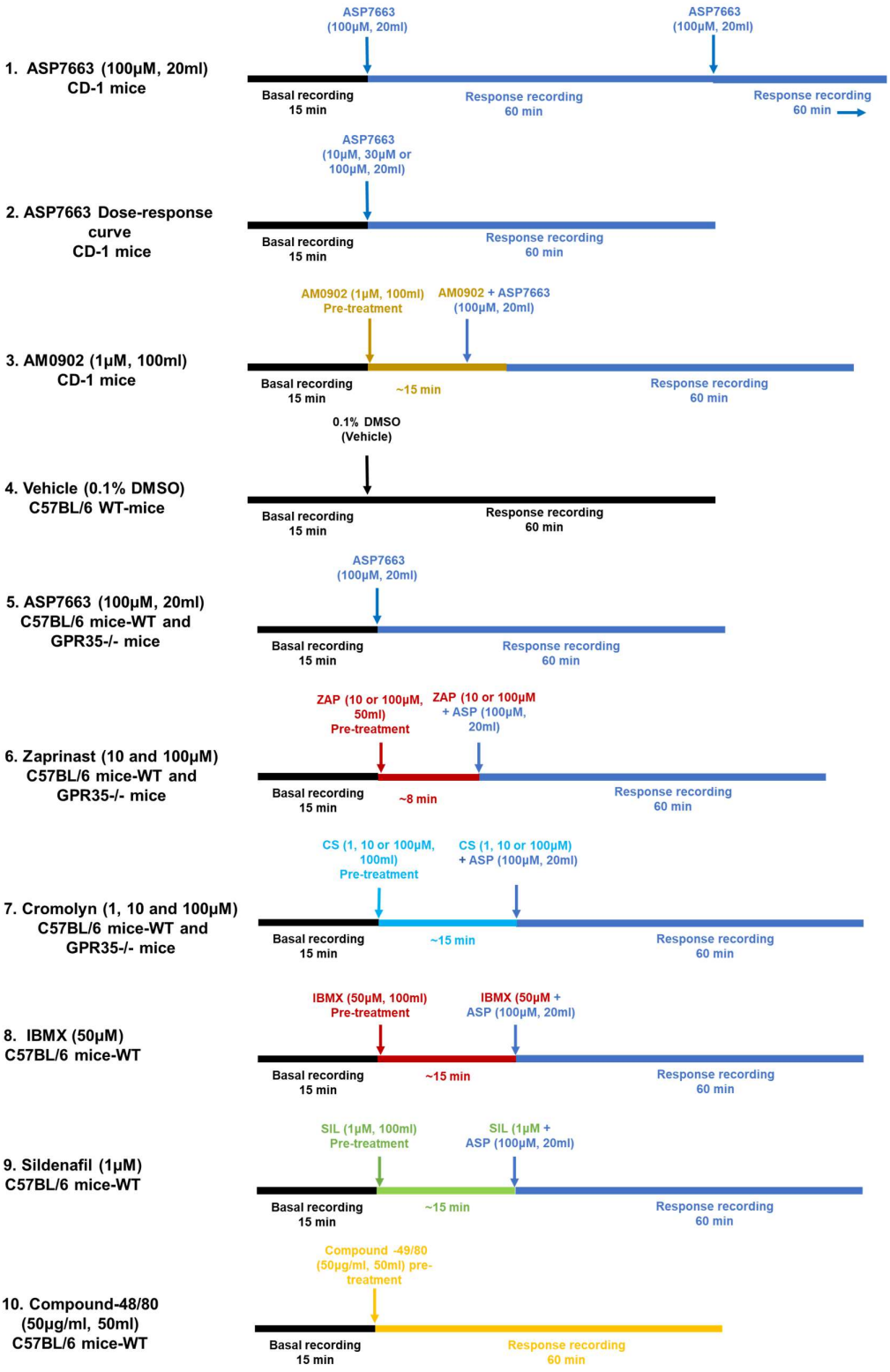


Figure 9. Experimental protocols for ex-vivo electrophysiology recordings

2.11. Data analysis

2.11.1 *Ex vivo* LSN recordings: Changes in LSN nerve activity was determined by subtracting baseline nerve discharge (mean nerve discharge calculated from the ongoing activity 6 minutes prior to drug application) from ongoing nerve activity measured at 1min intervals from the start of bath perfusion with ASP7663 and displayed as the mean change in nerve activity over time. Mean peak change in nerve activity following ASP7663 application was calculated using the peak increase in nerve discharge observed within 20min of ASP7663 application from individual experiments. Changes in peak nerve activity following application of ASP7663 were compared between vehicle, cromolyn and zaprinast pre-treatments using an unpaired Student's t-test or one-way ANOVA followed by Dunnett's or Tukey's multiple comparisons as appropriate. Changes in afferent nerve discharge following application of ASP7663 were compared over time between vehicle, cromolyn and zaprinast pre-treated tissues using a two-way ANOVA followed by Dunnett's multiple comparisons between the groups.

2.12. Results

2.12.1 TRPA1 selective agonist ASP7663 vigorously stimulate colonic afferents

TRPA1 agonist ASP7663 has been described as a novel and selective TRPA1 receptor agonist that evokes visceral pain (Ko et al., 2019; Kojima et al., 2014). While determining the reproducibility of afferent responses to ASP7663 we found that bath perfusion with ASP7663 (100μM, 20ml) produced a robust increase in nerve discharge (e.g., 27.6 ± 6.3 spikes/s increase in nerve discharge *n=3*) which was markedly reduced in magnitude and duration (e.g., 6.9 ± 0.1 spikes/s increase in nerve discharge *n=3*, $p < 0.05$, ***see figure- 10.C***) to repeat application, indicating that a repeat challenge protocol would not be feasible with ASP7663. Next, we examined the colonic afferent response to different concentrations of ASP7663 (one concentration tested per experiment), observing a minimal increase in nerve discharge following bath perfusion with 10μM, 20ml of ASP7663 (e.g., 0.5 ± 0.06 spikes/s increase in nerve discharge *n=5; P>0.05*), a significant increase in firing at 30μM ASP7663 (e.g., 8.9 ± 0.4 spikes/s increase in nerve discharge *n=5; P<0.001* as analysed by one-way ANOVA) and a marked increase in activity at 100μM ASP7663 (e.g., 29.4 ± 7.0 spikes/s increase in nerve discharge *n=5; P<0.001* as analysed by one-way ANOVA; ***see figure 10.E***).

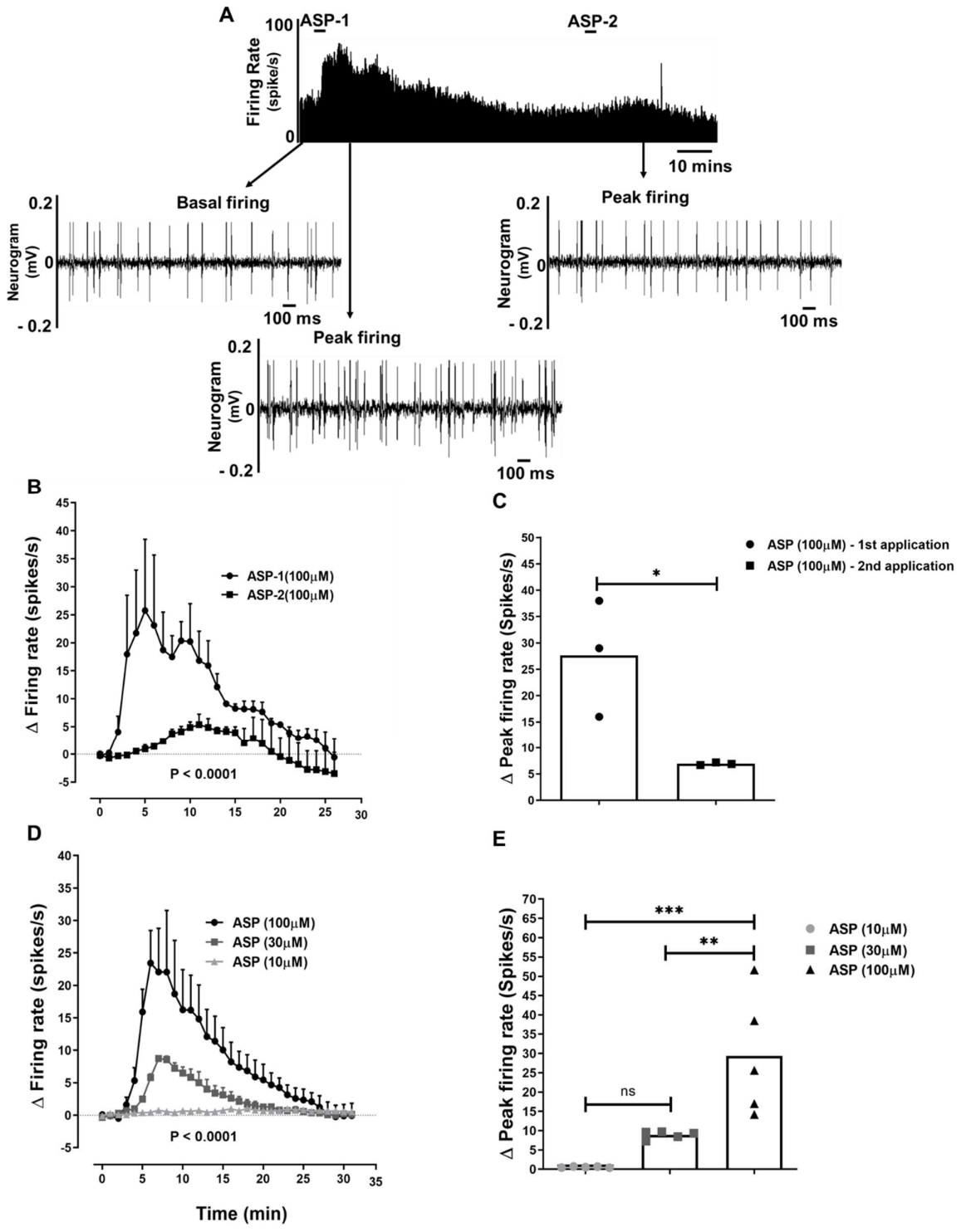


Figure 10: Preliminary screening and dose-dependent effect of ASP7663 on lumbar splanchnic nerve (LSN) preparation. A: Raw trace illustrating examples of the change in rate histogram of ongoing nerve discharge and exerts of neurogram

illustrating the effect of repeated ASP7663 applications on LSN activity. **B**: Line graph showing the change in nerve discharge over time to repeated application of ASP7663 (100μM, 20ml). **C**: Bar chart showing the mean ± sem change in peak nerve discharge following repeated application of ASP7663 (100μM); *n=3*. **D**: Line graph showing the change in nerve discharge over time following the application of ASP7663 at (10, 30 and 100μM). The statistical analysis was performed using Two-way ANOVA where, the significance is defined as **** $P < 0.0001$. **E**: Bar chart showing the mean ± sem change in peak nerve discharge to the application of ASP7663 (10, 30 and 100μM) in separate experiments, *n=5*. The statistical analysis was performed using unpaired student-t test and 1-way ANOVA as applicable where, the significance is defined as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, respectively.

Following on from this study, we next confirm that colonic afferent response to ASP7663 (30μM) was selectively mediated through the activation of TRPA1 receptors, for which we applied pre-treatment of selective TRPA1 antagonist AM0902 (1μM, 100ml), which prevented any increase in colonic afferent activity (e.g., 0.72 ± 0.47 spikes/s; *n=6; P<0.0001;* ***see figure 11.D***) following ASP7663 (30μM, 20ml) bath superfusion, confirming the selectivity of ASP7663 for TRPA1 receptor.

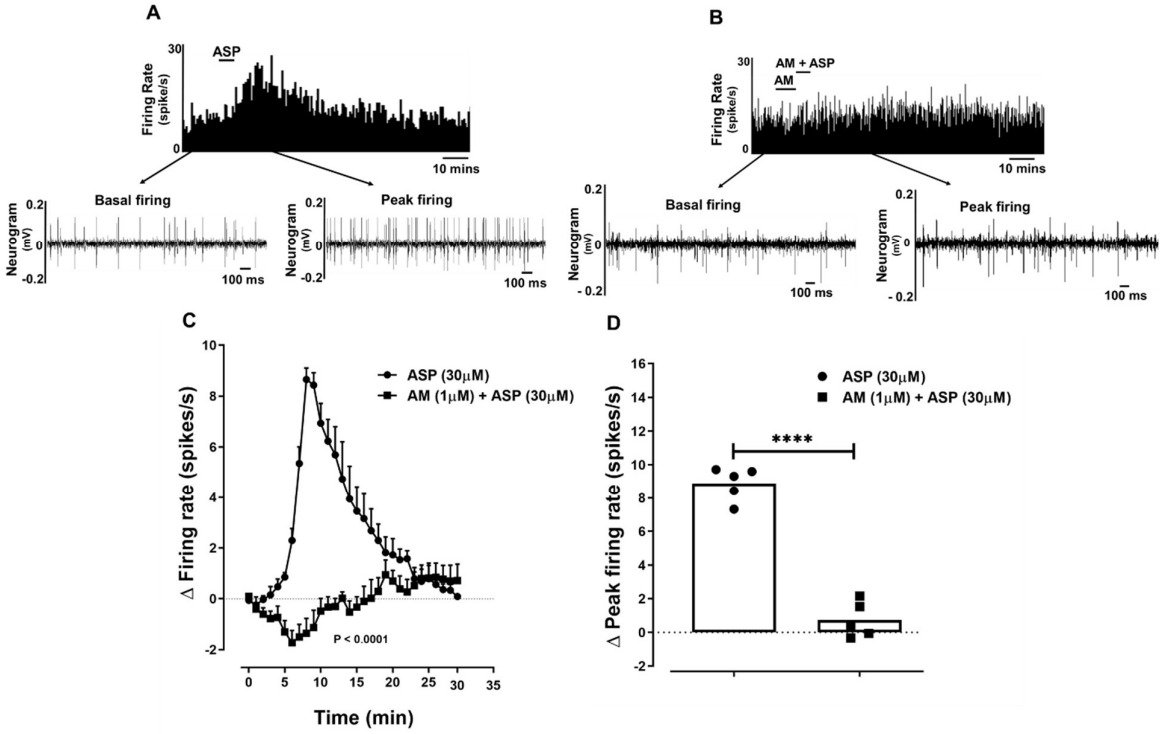


Figure 11. Effect of pre-treatment of AM0902 (AM; 1μM; TRPA1 antagonist) on ASP7663 (TRPA1 selective agonist) response in LSN preparation isolated from CD-1 mice. A: Example of a rate histogram and neurogram trace illustrating the colonic afferent response to ASP7663 (30μM). **B:** Example of a rate histogram with neurogram trace illustrating the colonic afferent response to ASP7663 (30μM) in presence of AM0902 (1μM). **C:** Line graph showing the change in colonic afferent activity over time following administration of ASP7663 alone or in presence of AM0902. The statistical analysis was performed using Two-way ANOVA where, the significance is defined as **** *P < 0.0001*. **D**: Bar chart showing the mean ± sem change in peak firing rates to ASP7663 alone and in presence of AM0902; *n=5*. The statistical analysis was performed using unpaired student-t test where, the significance is defined as **** *P < 0.00001.*

2.12.2 GPR35 agonist zaprinast and cromolyn sodium (CS) inhibits TRPA1 induced colonic afferent response

Given that GPR35 is extensively co-expressed with TRPA1 in colon projecting neurons isolated from mouse DRG (Hockley et al., 2019) we hypothesized that GPR35 agonists could be expected to have an inhibitory effect on TRPA1 mediated colonic nociception. To explore this possibility, we examined the effects of GPR35 agonists cromolyn and zaprinast on TRPA1 mediated colonic afferent stimulation.

To begin we determined the reproducibility of afferent responses to ASP7663 (100μM) in C57B6 mice in a new set of experiments. The initial bath perfusion with ASP7663 (100μM, 20ml) produced a robust increase in nerve discharge (e.g., 13.13 ± 2.8 spikes/sec, *n=6;* ***see figure 12.A***) from the basal firing. However, the response to ASP7663 (100μM) was comparably less in magnitude to the response obtained in CD-1 mice, (e.g., 29.4 ± 7.0 spikes/s increase in nerve discharge *n=5; P<0.001;* ***see figure 12.A***) although the duration of response was similar.

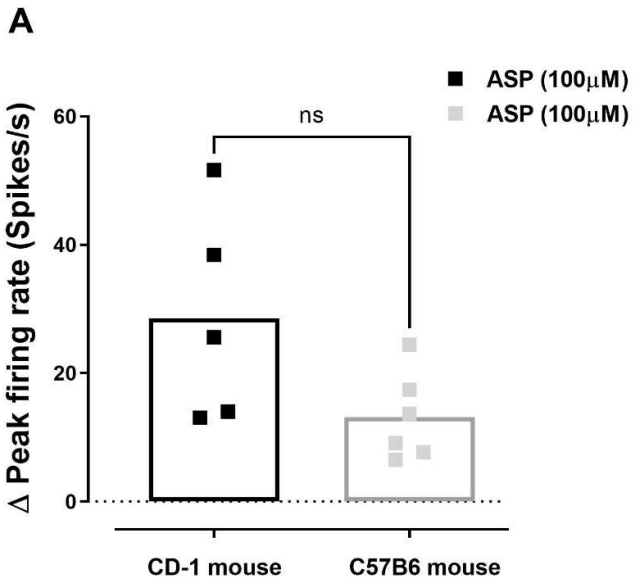


Figure 12. Response to ASP7663 application in LSN preparation isolated from CD-1 mice and C57B6-WT mice. A. Bar chart showing the mean ± sem change in peak firing rates to ASP7663 (100μM) in CD-1 *(n=5)* and C57B6 *(n=6)* mice. The statistical analysis was performed using unpaired student-t test where, no significance difference was observed.

We next investigated the effect of increasing concentration of zaprinast (10μM and 100μM) and cromolyn (1μM, 10μM, and 100μM) pre-treatment on the colonic afferent response to ASP7663 (100μM). Zaprinast (100μM, 50ml) pre-treatment significantly attenuated the colonic afferent response of ASP7663 (100μM) (e.g., 3.3 ± 1.4 spikes/sec, *n=5, **P< 0.01;* ***see figure 13.E***). Although pre-treatment with a lower concentration of zaprinast did not inhibit ASP7663-induced response to colonic afferents (e.g., 12.3 ± 1.5 spikes/sec, *n=6*). Similarly, cromolyn treatment produced a concentration-dependent reduction in the response to ASP7663 (100μM). With a moderate reduction in afferent response to ASP7663 (100μM) following cromolyn (1μM,

100ml) pre-treatment (e.g., 9.8 ± 1.7 spikes/sec, *n=5*), and a significant reduction was observed following pre-treatment with Cromolyn (CS) (10μM, 100ml) (e.g., 4.0 1.3 spikes/sec, *n=5, P<0.05*) and CS (100μM, 100ml) (e.g., 4.7 1.8 spikes/sec, *n=5, P<0.05;* ***see figure 13.E***).

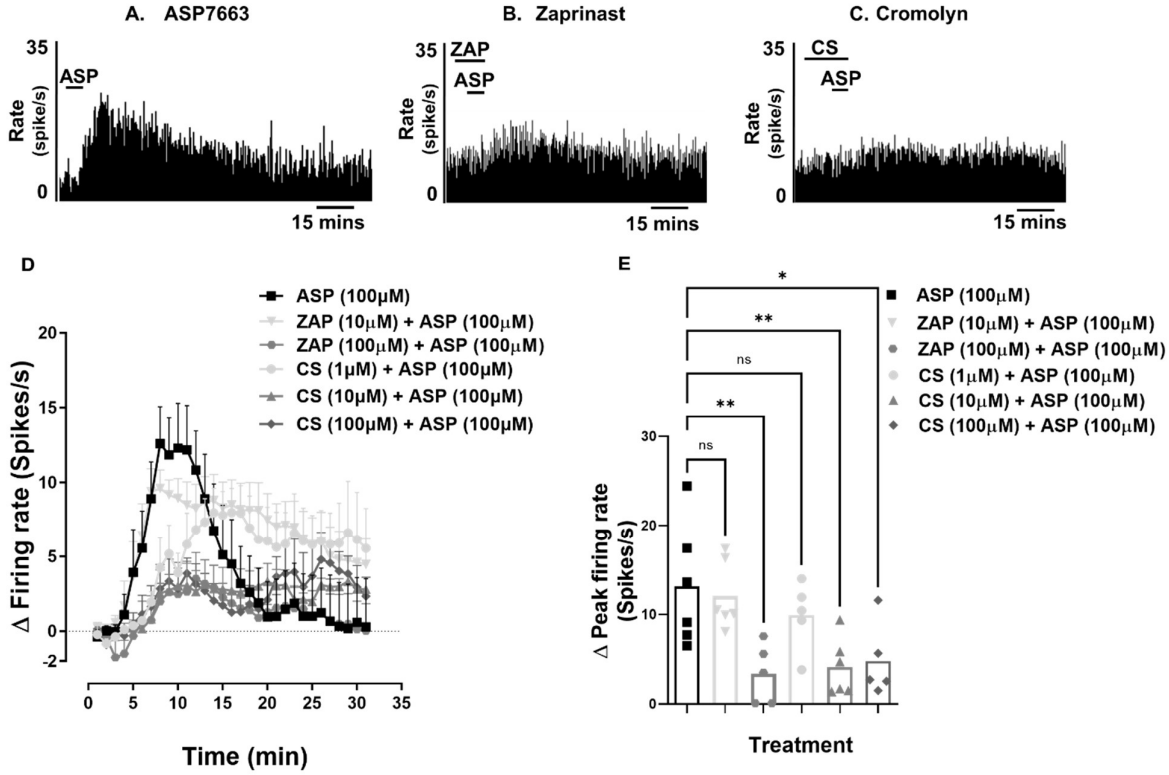


Figure 13. Effect of GPR35 agonist pre-treatment on ASP7663 (TRPA1 selective agonist) response in LSN preparation isolated from C57B6-WT mice. A. Example of a rate histogram with neurogram trace illustrating the colonic afferent response to ASP7663 (100μM) alone and following pre-treatment with **(B)** ZAP (100μM) and **(C)** CS (100μM). **D:** Line graphs showing the change in colonic afferent activity over time following the application of ASP7663 (100μM; *n=6*) alone and following pre-treatment with ZAP (10μM and 100μM) and CS (1, 10 and 100μM). **E.** Bar charts showing the

mean ± sem peak change in afferent firing following the application of ASP7663 (100μM) alone and following pre-treatment with zaprinast 10μM, *n=6* and 100μM, *n=5* along with CS 1μM, *n=5*; 10μM, *n=6* and 100μM, *n=5* respectively. The statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparison test where, the significance is defined as, * *P < 0.05* and ***P <0.01.*

2.12.3. Investigation of zaprinast and cromolyn in GPR35-/- mice

We further examined the effects of zaprinast (100μM, 50ml) and cromolyn (100μM, 100ml) pre-treatment on ASP7663 (100μM, 20ml) responses in the tissue isolated from GPR35-/- mice. The experimenter was blinded to treatment and experimental protocols randomised with support from colleagues within the lab.

2.12.4. Zaprinast pre-treatment failed to attenuate TRPA1-induced afferent activation in GPR35-/- tissue

In tissue isolated from GPR35-/- mice application of ASP7663 (100μM, 20ml) by bath superfusion produced a robust increase in the afferent response (e.g., 10.7 ± 1.0 spikes/sec; *n=5;* ***see figure- 14.B*****)** whereas zaprinast (100μM, 50ml) pre-treatment failed to inhibit the response to ASP7663 (100μM) in tissue from GPR35-/- mice (e.g., 15.46 ± 3.7 spikes/s, *n=5,* ***see figure 14.B***), indicating that the inhibitory effect of zaprinast on TRPA1 activity was abolished in GPR35-/- mice tissues.

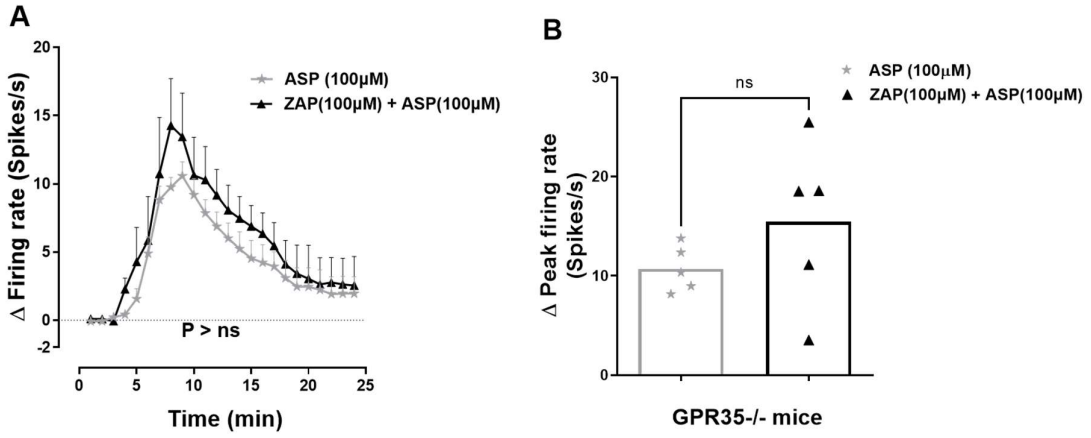


Figure 14: Response of ASP7663 (100μM; TRPA1 agonist) alone and following the pre-treatment of Zaprinast (100μM) on LSN tissue preparation isolated from GPR35-/- mice. A: Line graph showing the change in colonic afferent activity over time following administration of ASP7663 alone and following pre-treatment of zaprinast (100μM) in LSN tissue preparation isolated from GPR35-/- mice. The statistical analysis was performed using Two-way ANOVA where, no significance difference was observed. **B**: Bar chart showing the mean ± sem change in peak firing rates to ASP7663 alone and following pre-treatment of zaprinast (100μM) in GPR35-/- mice (*n=5*). The statistical analysis was performed using unpaired student-t test where, no significance difference was observed.

2.12.5. Cromolyn pre-treatment failed to attenuate TRPA1-induced afferent activation in GPR35-/- tissue

Similarly, in a separate study, cromolyn (100μM, 100ml) pre-treatment also failed to inhibit the response to ASP7663 (100μM, 20ml) in tissue from GPR35-/- mice (e.g., 8.4 ± 1.3 spikes/s, *n=5;* ***see figure-15.D***) when compared to the response elicited by ASP7663 (100μM, 20ml) alone in GPR35-/- mice tissues (e.g., 9.4 ± 1.0; *n=5;* ***see***

figure- 15.D). The results of the following experiments on GPR35-/- mice tissues support the role of GPR35-receptor as a molecular determinant of direct afferent inhibition produced by zaprinast and cromolyn.

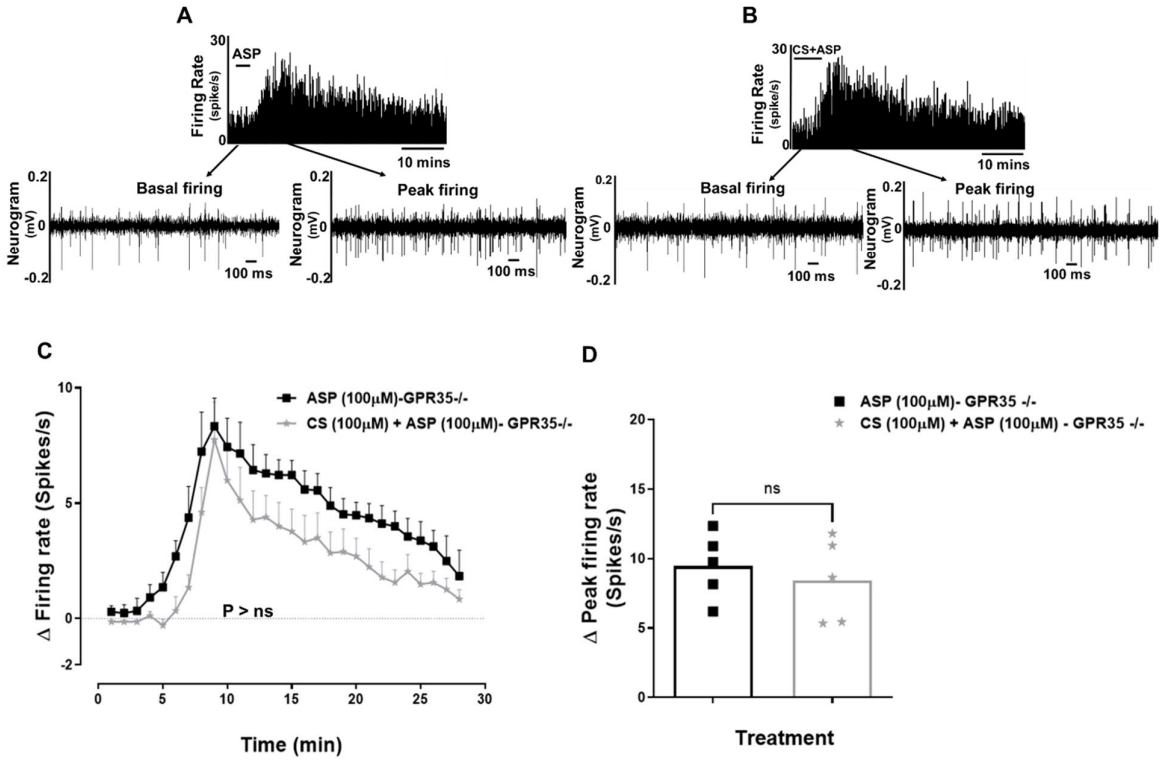


Figure 15. Effect of pre-treatment of Cromolyn sodium (CS; GPR35 selective agonist) on ASP7663 (TRPA1 selective agonist) response in LSN preparation isolated from GPR35-/- mice. A & B: Example of a rate histogram with neurogram trace illustrating the colonic afferent response to ASP7663 (100μM) alone and following pre-treatment with CS (100μM). **C:** Line graphs showing the change in colonic afferent activity over time following the application of ASP7663 (100μM) alone and following pre-treatment with CS (100μM); (P > ns at all points, two-way ANOVA). **D.** Bar charts showing the mean ± sem peak change in afferent firing following the application of ASP7663 (100μM) alone and following pre-treatment with CS (100μM); *n=5* respectively. The statistical analysis was performed using unpaired student-t test where, no significance difference was observed.

2.12.6. Adjuvant studies for CS and zaprinast to confirm their off-target studies

2.12.6.1. Zaprinast as phosphodiesterase (PDEs) PDE5/6 inhibitor

Zaprinast is an inhibitor of c-GMP specific phosphodiesterase (PDEs) PDE5 and PDE6 inhibitor (Beavo, 1995; Loughney et al., 1998). To better understanding the contribution of PDE5 inhibition, we examined the effects of pre-treatment with sildenafil a PDE inhibitor that lacks agonism at GPR35 (Berlinguer-Palmini et al., 2013) on the colonic afferent response to TRPA1 agonist ASP7663. Additionally, studies with PAN PDE-inhibitor compound IBMX were also conducted.

The response to ASP7663 (100μM) was significantly increased following sildenafil (1μM) (e.g., AUC: 237.6 ± 18.9; *n=5; p< 0.01*) or IBMX (50μM) pre-treatment (e.g., AUC: 219.0 ± 21.0; *n=7; p< 0.05*) as compared to ASP7663 (100μM) alone (e.g., AUC: 121.1 ± 23.9; *n=6;* ***see figure 16.B***). These results demonstrating that inhibition of PDE5/6 activity or PAN PDE inhibition promotes TRPA1 mediated colonic afferent activation. The above findings providing an explanation for the increased area under curve observed with lower concentration of zaprinast (10μM) pre-treatment (e.g., AUC: 190.7 ± 15.83; *n=6;* ***see figure 16.B***) as compared to ASP7663 (100μM) alone which may also be due to the non-specific phosphodiesterase enzyme inhibition by zaprinast (Taniguchi et al., 2006).

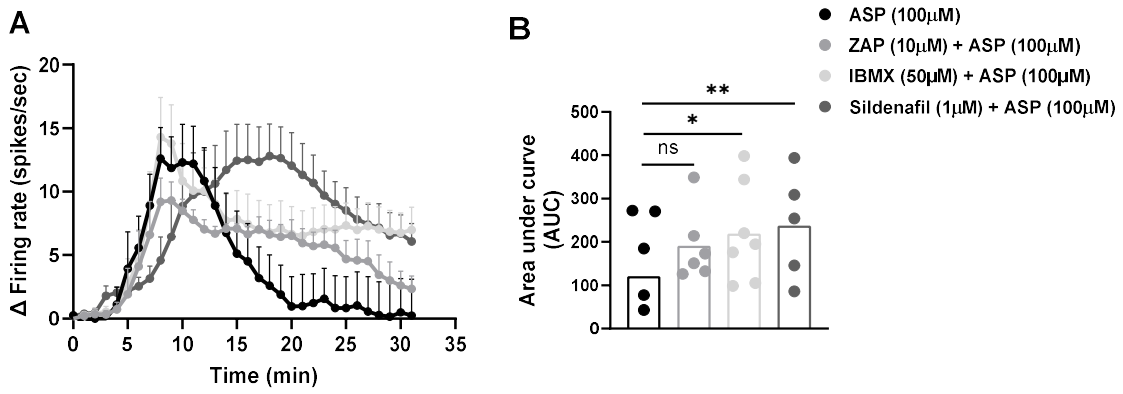


Figure 16: PDE-inhibitors increases ASP7663 response duration. A: Line graph showing the change in colonic afferent activity over time following administration of ASP7663 alone and following pre-treatment of zaprinast (10μM); IBMX (50μM) and sildenafil (1μM) in LSN tissue preparation isolated from C57B6-WT mice. B: Bar charts showing the Area under curve obtained following the application of ASP7663 (100μM; *n=6*) alone and zaprinast (10μM; *n=5*); IBMX (50μM; *n=7*) and sildenafil (1μM; *n=5*) in LSN tissue preparation isolated from C57B6-WT mice. The statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparison test where, the significance is defined as, ** P < 0.05* and ***P <0.01.*

2.12.6.2. Compound 48/80 (mast cell degranulator) does not excite colonic afferents

Cromolyn is a mast cell stabiliser, an action which may contribute to its inhibitory action on TRPA1 induced afferent activation. To study this further, we investigated the effects of mast cell degranulator, compound 48/80, on colonic visceral afferents. Application of compound 48/80 (50μg/ml, 50ml) by bath superfusion failed to increase colonic afferent nerve activity compared to basal nerve discharge suggesting that mast cells are unlikely to influence colonic afferent activity in our experiments and as such the

mast cell stabilising activity of CS is unlikely to be responsible for the inhibitory effect on TRPA1 induced afferent activation.

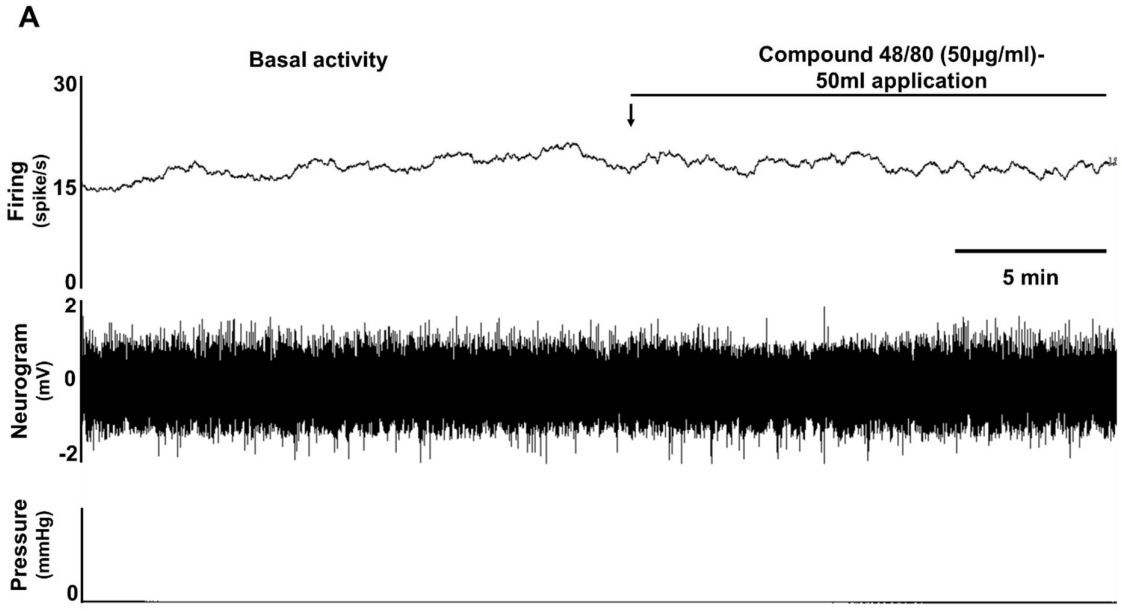


Figure 17: Compound 48/80 does not affect colonic afferent discharge in LSN preparation isolated from C57B6 mice. A. Example of a rate histogram with neurogram trace illustrating the colonic afferent response to compound 48/80 (50µg/ml; 50ml; *n=5*) application.

Finally, although the magnitude of the response obtained following the bath superfusion of ASP7663 (100µM) in GPR35-/- mice tissues was comparable (e.g., 10.72 ± 1.0 spikes/s, *n=5*) in with tissue from wild type C57B6/J mice (e.g., 13.13 ± 2.8 spikes/s, *n=6*).

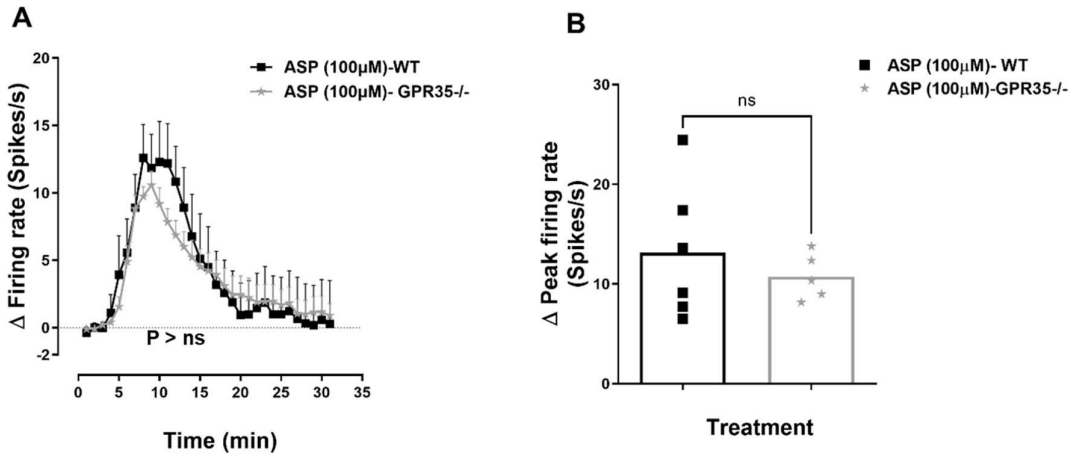


Figure 18: Comparative response of ASP7663 (100μM; TRPA1 agonist) pre-treatment on LSN tissue preparation isolated from C57B6-WT and GPR35-/- mice. A: Line graph showing the change in colonic afferent activity over time following administration of ASP7663 in LSN tissue preparation isolated from C57B6-WT and GPR35-/- mice; (P > ns at all points, two-way ANOVA).. **B**: Bar chart showing the mean ± sem change in peak firing rates to ASP7663 in C57B6-WT (*n=6*) and GPR35-/- mice (*n=5*). The statistical analysis was performed using unpaired student-t test where, no significance difference was observed.

A significant reduction of basal afferent activity could be observed in tissue from GPR35-/- mice (e.g., 7.1 ± 0.9 spikes/sec; *n=15*; *P< 0.0001*) compared with tissue from wild type C57B6 mice (e.g., 18.9 ± 2.2 spikes/sec; *n=17,* ***see figure 19***).

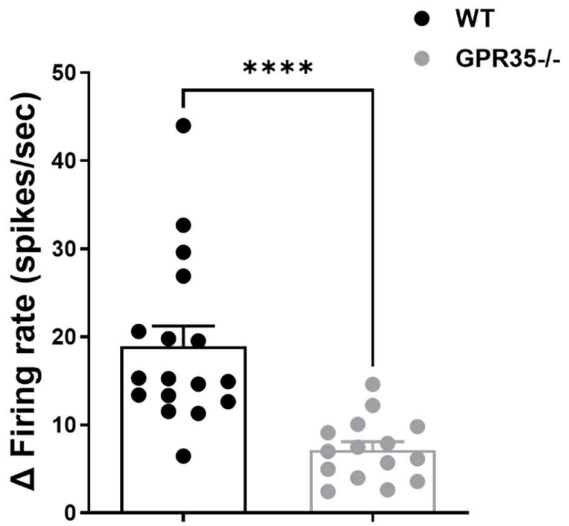


Figure 19: Basal activity obtained in the LSN tissue preparation isolated from C57B6-WT and GPR35-/- mice. Bar chart showing the (mean ± sem) average firing rate (10min) for LSN tissue preparation isolated from C57B6-WT (*n=17*) and GPR35-/- mice (*n=15*). The statistical analysis was performed using unpaired student-t test where, statistical significance was defined a ****P < 0.0001.

2.13. Discussion

I have shown that the GRP35 agonists cromolyn and zaprinast, attenuate colonic afferent excitation elicited by the TRPA1 selective agonist ASP7663. These effects are lost in tissue from GPR35-/- mice indicating that they are mediated through GPR35 receptor activation.

The orphan receptor GPR35 is widely expressed in sensory neurones that project to the bowel (Hockley et al., 2018) consistent with previous reports of GPR35 expression in rodent DRGs (Taniguchi et al., 2006; Ohshiro et al., 2008). The *in-silico* analysis of RNA-sequencing data previously generated and published by our group revealed significant coexpression of GPR35 with TRPA1 in colonic DRGs (Hockley et al., 2019). Which, based on the coupling of GPR35 to $G_{i/o}$ alpha G protein subunits, would suggest the GPR35 agonists will have an inhibitory effect on afferent signalling elicited by TRPA1 activation (Taniguchi et al., 2006; Wang et al., 2006; Guo et al, 2008).

To test this, I utilised the compound ASP7663 as a TRPA1 receptor agonist, which we demonstrated increased colonic afferent activity. The contribution of TRPA1 channels to the effects of ASP7663 being confirmed by AM0902 pre-treatment which abolished the response of ASP7663 on colonic afferents. Following which we demonstrated the inhibitory effect of GPR35 agonist cromolyn and zaprinast on TRPA1 induced colonic afferent activation. It is important to remember that cromolyn is also a mast cell stabilizer, and mast cell mediators have been shown to excite nociceptive visceral afferents (Barbara et al., 2007; Hughes et al., 2009) and are enriched with TRPA1 ion channels (Prasad et al., 2008). It is possible that the mast cell stabilizing activity of cromolyn could be responsible for its inhibitory action on TRPA1 instead of neuronal GPR35 activation. To test the influence of this plausible mechanism we tested

compound 48/80 (50µg/ml) to degranulate mast cells present releasing any preformed mediators such as histamine, 5-HT, Leukotrienes, and prostaglandins (Schemann et al., 2012; Wang et al., 2014). However, we do not observe any increase in colonic afferent activity following the application of compound 48/80 suggesting that in our experiments mast cell are unlikely to contribute to TRPA1-induced colonic afferent activation and that cromolyn most likely attenuate TRPA1-induced colonic excitation by its agonistic action at neuronally expressed GPR35 receptors. We further confirmed a role for GPR35 receptors showing that the inhibitory responses to cromolyn were lost in tissue from GPR35-/- mice.

We also demonstrated the inhibitory effect of GPR35 receptors by investigating the GRP35 agonist zaprinast (PDE5/6 inhibitor). Zaprinast has been used as a reference agonist for GPR35 in several studies (Jenkins et al., 2010, 2011; Taniguchi et al., 2006; Milligan et al., 2011) and previously reported to attenuate chemical and mechanically induced nociception in animal models (Ohshiro et al., 2008; Cosi et al., 2011; Alexander et al., 2015). Moreover, zaprinast agonism of GPR35 elicits inhibition of N-type calcium channels; and effect that was blocked by pertussis toxin pre-treatment demonstrating an involvement of $G_{i/o}$ proteins (Guo et al., 2008). We found that zaprinast pre-treatment significantly attenuated the ASP7663 (100µM) response on colonic afferents. Intriguingly, at lower concentration of zaprinast (10µM) pre-treatment failed to attenuate ASP7663-induced afferent activation and instead zaprinast increased the area under curve for TRPA1 response. Our data suggests the loss of inhibitory effects at lower concentrations of zaprinast was related to inhibition of phosphodiesterase (PDE) enzymes (Taniguchi et al., 2006). PDE inhibition increases intracellular cAMP levels in comparison to cGMP (Endres et al., 1990). cAMP has been shown to activate abdominal visceral afferents (Guo and Longhurst, 2000) consistent

with this we found pre-treatment with the selective PDE-5/6 inhibitor, sildenafil citrate or the PAN-PDE inhibitor IBMX significantly increased the AUC of the colonic afferent response to ASP7663 indicating that that inhibition of PDE5/6 activity by zaprinast limited its inhibitory effect on TRPA1 mediated colonic afferent activation at lower concentrations of zaprinast.

TRPA1 is transducer of colonic nociception which our studies demonstrate can be inhibited by GPR35 agonist cromolyn (10μM and 100μM) and zaprinast (100μM) highlighting their utility as potential treatments for visceral pain from the gut.

Chapter-3

Effect of GRP35 agonists on colonic afferent mechanosensitivity

3.1. Role of TRPA1 in mechanotransduction and mechanosensitisation

TRPA1 is expressed on colonic afferents where it acts as a mechanotransducer and modulates the mechanosensitivity of colonic afferents to algogenic mediators such as bradykinin and by doing so contributes to visceral allodynia and hyperalgesia in disease states. For example, bradykinin enhances colonic afferent response to mechanical stimulus in wild type mice but fails to evoke a mechanical hypersensitivity in TRPA1 knock-out mice (Brierley et al., 2009). In addition, TRPA1 -/- mice display attenuated pain behaviours to cutaneous injection of bradykinin (Kwan et al., 2006) and reduced visceral pain behaviours measured by abdominal contractions referred to as the visceromotor response or VMR to colorectal distension (up to 90 mmHg) along with a significant reduction in colonic CGRP release following noxious distention (Mueller-Tribbensee et al., 2015). Several studies emphasise that pharmacological blockade of the TRPA1 ion channel effectively attenuates inflammation and visceral hyperalgesia in an animal model of colitis (Engel et al., 2011; Mitrovic et al.,2010) in addition to other nociceptive stimuli (Petrus et al., 2007; Kondo et al., 2009; Mueller-Tribbensee et al., 2015; Lennertz et al., 2012). For example, TRPA1 inhibitors reduces colitis induced by TNBS or DSS (Engel et al., 2011). Additionally, reducing TRPA1 expression by antisense oligodeoxynucleotide significantly attenuates colonic hypersensitivity induced by TNBS-mediated colitis in mice (Yang et al., 2008). Moreover, intracolonic AITC-induced afferent signalling to the spinal cord with increased c-Fos expression was significantly inhibited by TRPA1 channel blocker HC-

030031, implicating protective role in gastrointestinal pain conditions (Mitrovic et al., 2010). Therefore, given the involvement of TRPA1 in mechanotransduction and mechanosensitisation along with data from the previous chapter where GPR35 agonists attenuate TRPA1 mediated activation of colonic afferents, we sought to investigate the inhibitory activity of GPR35 agonists on direct and TRPA1 sensitised colonic mechanosensitivity.

3.2. Aim

The aim of studies in this chapter was to study the effect of GPR35 agonists on colonic afferent response to ramp distension of the colorectum and TRPA1 agonist mediated sensitisation of colonic afferent mechanosensitivity.

3.3. Research objectives

1. Investigate the effect of GPR35 agonists (zaprinast and cromolyn) on colonic afferent response to TRPA1-induced colonic afferent mechanosensitivity

2. Investigate the effect of GPR35 agonists (zaprinast and cromolyn) on colonic afferent response to TRPA1-induced colonic afferent mechanosensitivity to ramp distention in tissue from GPR35+/+ & -/- mice.

3.4. Methods

Electrophysiological recordings were made from lumbar splanchnic nerve bundles innervating mouse (C57B6-WT and GPR35-/-) colon that had been cannulated and mounted in a tissue bath as described in the methods section of Chapter-2.

Additionally, for the ramp distensions paradigm, the luminal outflow cannula was blocked, and a subsequent increase in pressure was observed until the desired maximum pressure was reached (e.g., 80 mmHg). It took approximately 70 to100 seconds to reach 80mmHg. The afferent activity was recorded with the subsequent rise in pressure.

3.5. Experimental protocols

A repeat of five ramp dissentions were applied following an interval of 10 min. From the five successive dissentions, the response to distention-2 and 3 was the most similar, suggesting pre-treatment would be best given between these distensions. For determining the involvement of TRPA1 in mechanotransduction, antagonist AM0902 (3μM, 100ml) pre-treatment was applied immediately after distention-2, and its inhibitory effect was observed on distention-3. Additionally, the inhibitory effects of the GPR35 agonist such as ZAP (100μM, 50ml) and cromolyn (100μM, 100ml) on the mechanotransduction was also evaluated following their pre-treatment immediately after distention-2 (***see figure 20***)

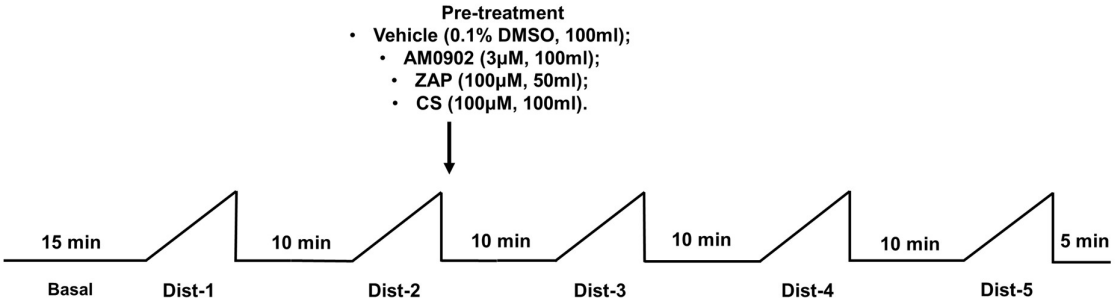


Figure 20. Experimental protocols for evaluating effects of TRPA1-antagonist and GPR35-agonist on colonic mechanotransduction during ramp distention paradigm.

For evaluating TRPA1-induced mechanosensitivity bath superfusion of ASP7663 (100μM,20ml), pre-treatment was applied after distention 2, and its sensitising effect was observed on distention-3. Similarly, pre-treatment of GPR35 agonist cromolyn (1μM, 10μM, and 100μM; 100ml) and zaprinast (10μM, and 100μM; 50ml) were also applied immediately after distention-2 followed by the application of ASP7663 (20ml) with subsequent distention stimulus-3 (***see figure 21.1 and 21.3***). The pre-treatment with AM0902, cromolyn and zaprinast was continued after the distention-3 till the afferent activity reached the basal level. Similar experimental protocols were repeated for ASP7663 (100μM, 100ml) alone and following pre-treatment with cromolyn (100μM, 100ml) in the tissues obtained from GPR35-/- mouse. The details of the experimental protocols are described in **figure 21**.

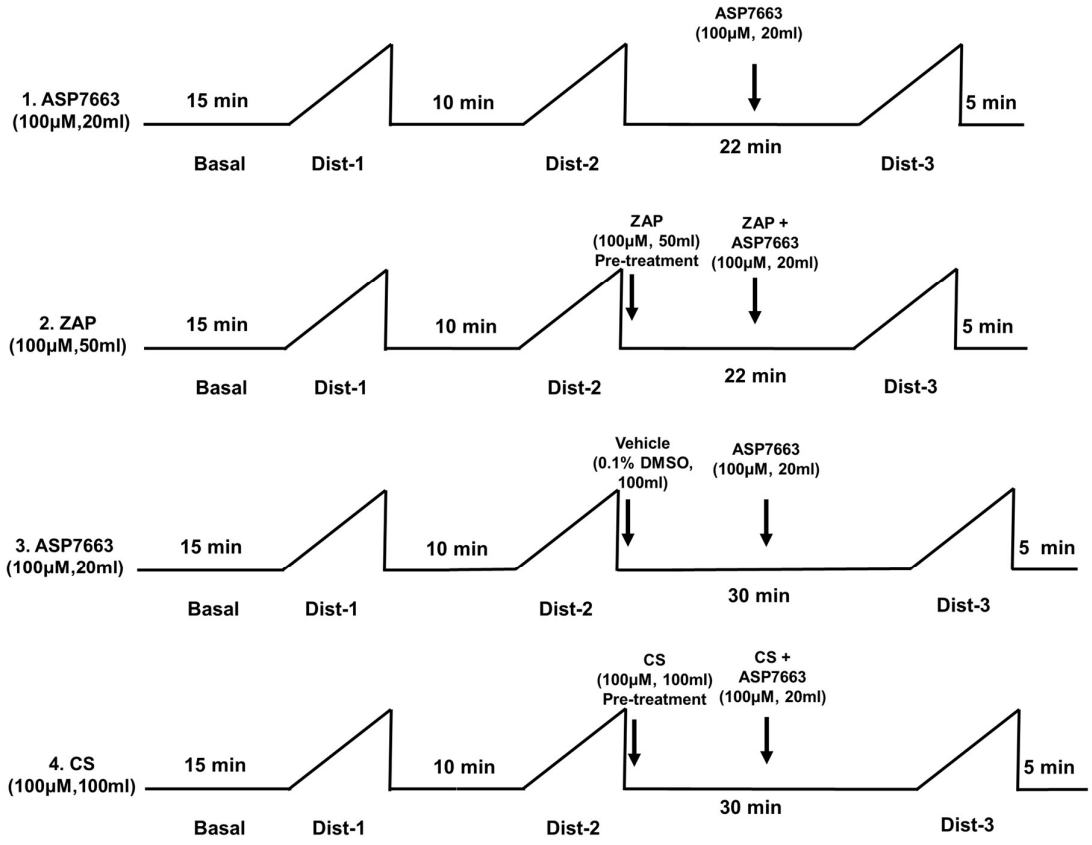


Figure 21. Experimental protocols for determining the effects of TRPA1-agonist and GPR35-agonist on colonic mechanosensitivity during ramp distention paradigm.

3.6. Data analysis

3.6.1 *Ex vivo* LSN recordings during ramp distention: The afferent activity was calculated using a spike-2 software pressure script which determines the spike frequency against pressure. The spike frequency was calculated for every 5mmHg rise in pressure starting from 0 to 80mmHg for each ramp distention. Changes in neuronal firing rates were calculated by subtracting baseline firing (mean nerve discharge calculated from the ongoing activity 60s prior to individual ramp distention) from increases in nerve activity following ramp distension or drug treatment. The data obtained was normalised and expressed as percentage change in afferent response, plotted against increasing pressure stimulus (0-80mmHg with every 5mmHg rise in pressure) to visualize as ramp profiles. For profiles, the statistical analysis was performed using 2-way ANOVA followed by Dunnett's, Šídák's or Bonferroni post-test analysis for multiple comparisons as applicable. Additionally, the change in peak effect obtained at 80mmHg pressure (mean ± sem) is expressed as percent change in peak response or change in peak firing rate (spike/s) represented as bar chart for which the statistical analysis was performed using unpaired student T-test or by ordinary one-way ANOVA followed by Dunnett's Multiple comparisons as applicable.

3.7. Results:

3.7.1 Distention stimuli evoke robust excitation of LSN

Preliminary studies were performed to determine the reproducibility of afferent response to repeated ramp distension of the colorectum. A progressive reduction in afferent response to colorectal distension was observed with each distension which was greatest between the first and second distension (e.g., 44.2 ± 5.05 spikes/s at 80mmHg**; *see figure* 22.C** vs 35.0 ± 5.66 spikes/s, *n=6*), and very modest between the second and third distention (33.41 ± 6.30 spikes/s, *n=6***; *see figure* 22.C**). Consequently, subsequent experiments were performed by applying test agents between the second and third distension.

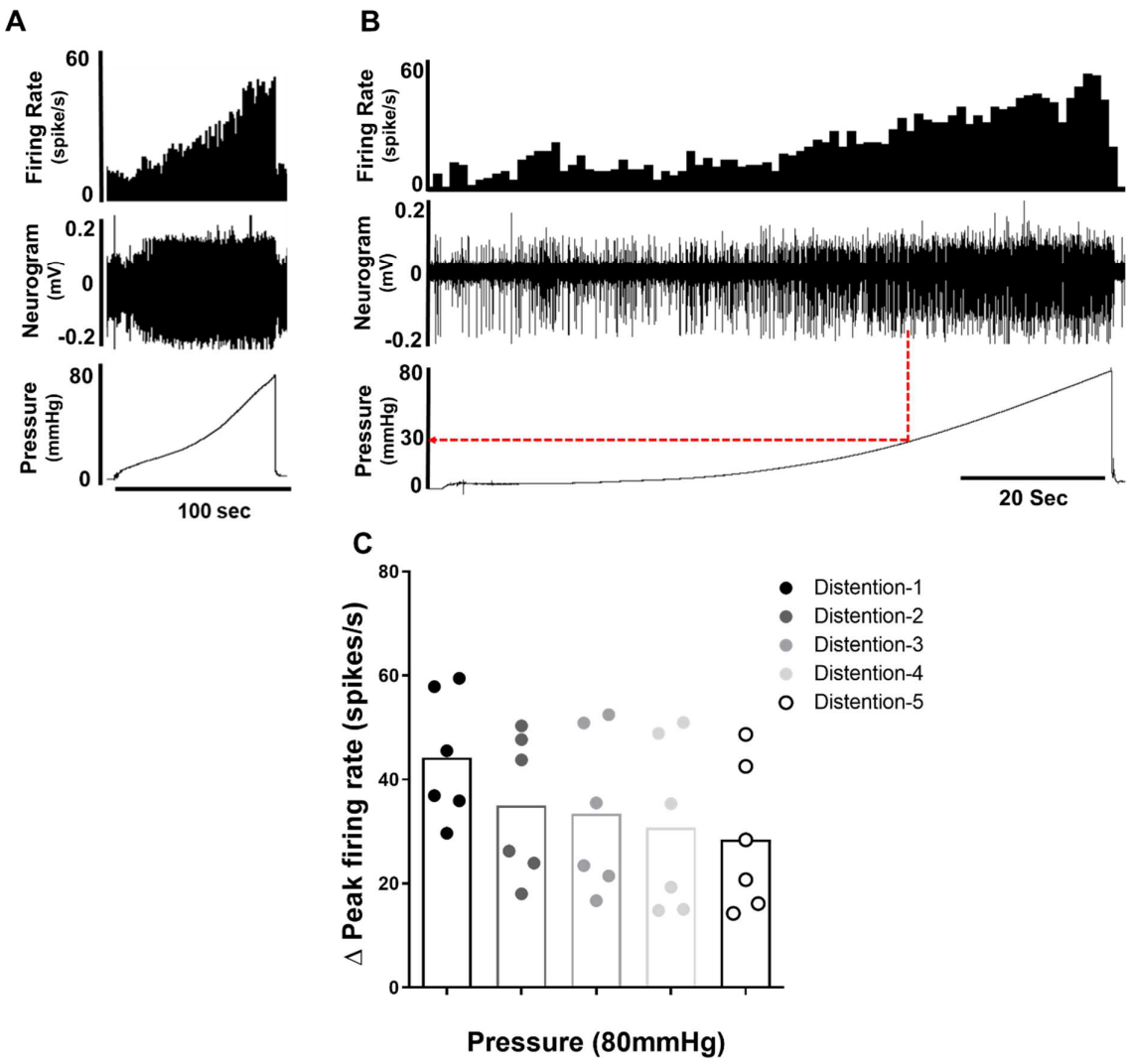


Figure 22: Colonic splanchnic nerve responses to 0-80mmHg ramp distension. **A.** Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg; 100sec; 10 min intervals) in C57B6/J mice. **B:** An expanded trace illustrating the marked increase in afferent activity above 30mmHg of pressure stimulus. **C:** Bar graph showing average change in nerve activity for five individual ramp distention's at 80mmHg.

3.7.2 Compliance for distention -2 and 3

To avoid the influence of uncontrolled variables affecting colonic nerve activity in our experiments, such as colon tissue length and tissue elasticity difference between preparations, we also investigated the pressure and volume compliance between the ramp distention-2 and distention-3. For both the distention, maximum pressure of 80mmHg was achieved in a similar time frame (e.g., 110.7 sec ± 8.7 sec vs 111.0 sec ± 5.4 sec; ***see figure 23.A***). Similarly, the cumulative volume of fluid infused within the colonic tissues to reach 80mmHg pressure was also similar (e.g., 332.2µl ± 26.2µl vs 333.2µl ± 16.2µl; ***see figure 23.B***), where no significant difference was observed statistically using Two-way ANOVA.

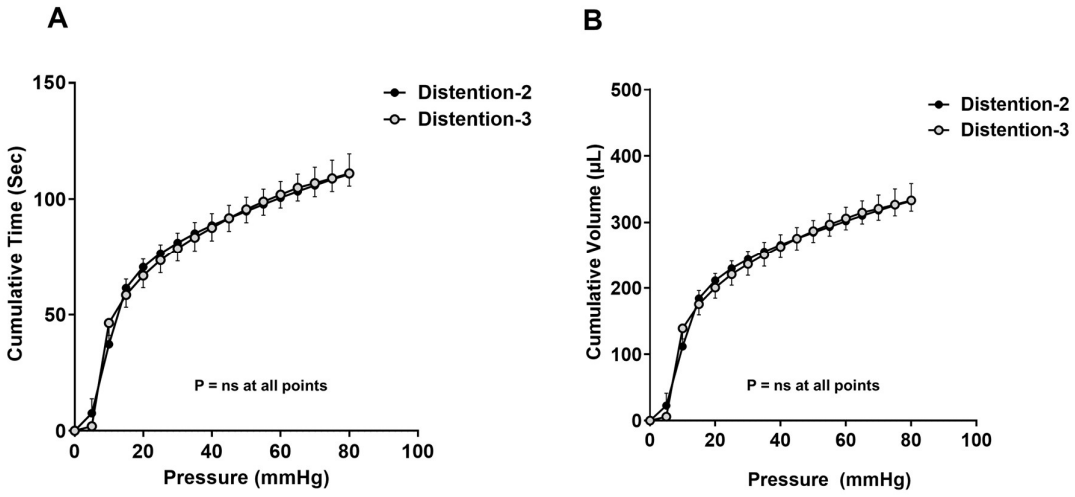


Figure 23: Compliance for ramp distention. **A**: cumulative time (sec) is plotted against increasing pressure (0-80mmHg, with every 5mmHg rise in pressure) for ramp distention -2 and 3 **B**. Cumulative volume (µl) is plotted against increasing pressure (0-80mmHg, with every 5mmHg rise in pressure) for ramp distention -2 and 3. Compliance was equivalent between sequential ramp distensions within protocols and

no differences between treatment groups were observed ($p > 0.999$, two-way ANOVA, *n=6*).

3.7.3 TRPA1 selective antagonist AM0902 inhibits distention induced mechanosensitivity

To begin we first determined the contribution of TRPA1 channels to colonic mechanosensitivity by examining the colonic afferent response to ramp distension following pre-treatment with the selective TRPA1 antagonist-AM0902 (3μM) observing a significant reduction in the afferent response to the third ramp distension applied during AM0902 (3μM) pre-treatment (e.g., 63.0% ± 4.5%, *n=6*, ** *P < 0.01*; **see figure 24.C**) by comparison to the vehicle (0.1% DMSO) treatment (e.g., 94.0% ± 4.0%, *n=6*). We also determined the compliance for the pre-AM0902 and post-AM0902 (3μM, 100ml) treated distention and found that the cumulative time (e.g., 118.1 ± 13.5 sec vs 116.6 ± 13.1 sec; *n=6;* ***see figure 24.D***) and cumulative volume (e.g., 192.4 ± 22.9μl vs 192.4 ± 22.4μl; *n=6;* ***see figure 24.E***) was comparable.

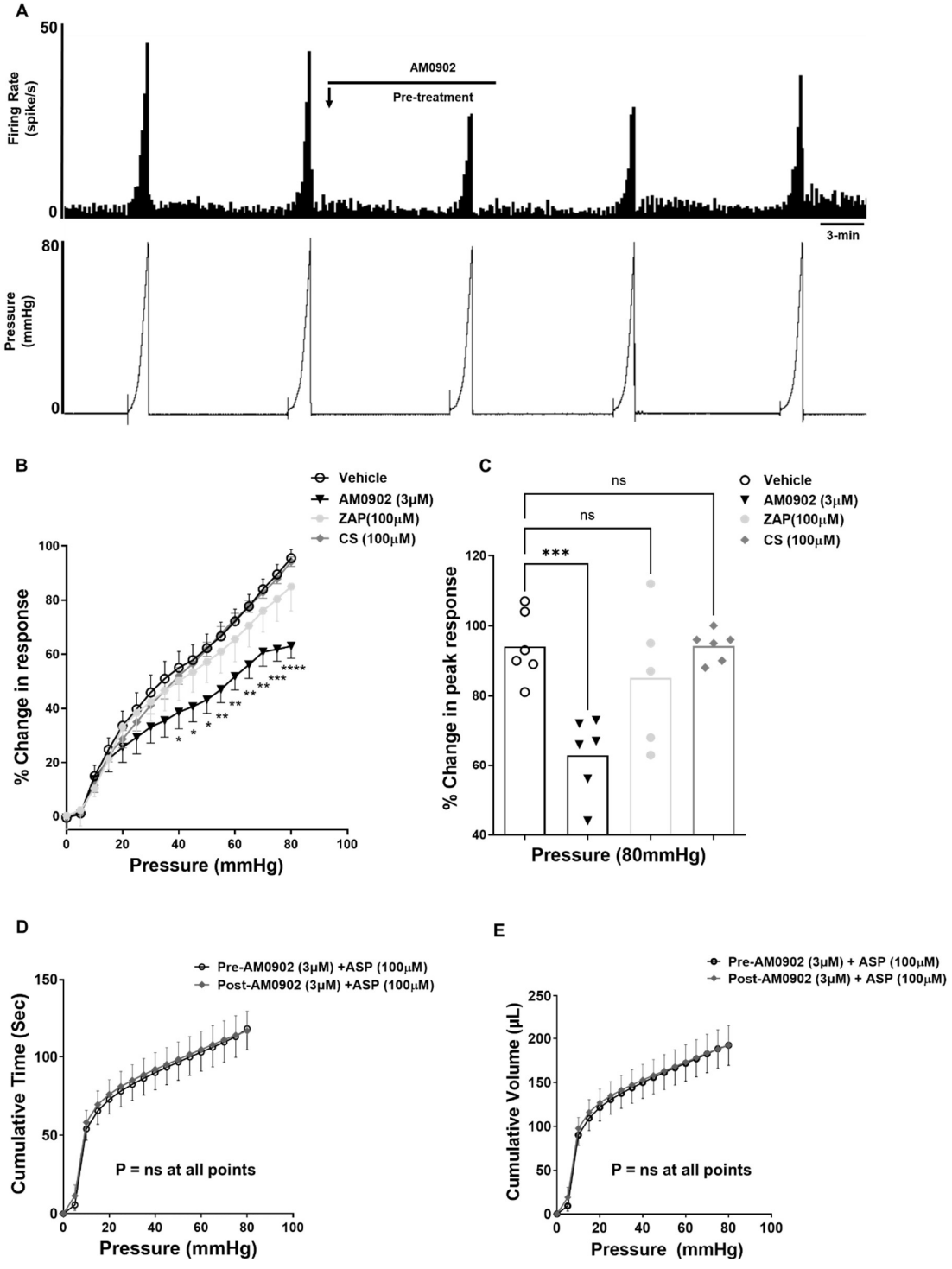


Figure 24: TRPA1 antagonist attenuates colonic mechanosensitivity. **A.** Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg; 10min intervals) in C57B6/J mice alone and pre-treated with TRPA1 selective

antagonist AM0902 (3μM) and GPR35 receptor agonist cromolyn (CS) and zaprinast (ZAP). **B**. Line graph showing effect of AM0902, cromolyn and zaprinast on colonic splanchnic nerve response to 0-80mmHg ramp distention. The statistical analysis was performed using 2-Way ANOVA followed by Dunnett's multiple comparisons test where, the significance is defined as, * P < 0.05; ** P < 0.01; *** P< 0.001 and **** P < 0.0001, respectively. **C**. The bar graph shows the peak effect of AM0902, cromolyn and zaprinast on colonic splanchnic nerve response at 80mmHg of distention. The statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's Multiple comparison test where, the significance is defined as, *** P < 0.001, respectively. **D**. Cumulative time (sec) and **E**. Cumulative volume (μl) is plotted against increasing pressure (0-80mmHg, with every 5mmHg rise in pressure) for pre-AM0902 and post-AM0902 distention's (P > 0.99; two-way ANOVA, *n=6*).

Moreover, we observed a marked inhibitory effect of AM0902 in the range above 20mmHg up to 80mmHg where mean percent change in peak response calculated as a proportion to the total pre-treatment response was significantly reduced following the application of AM0902 (3μM, 100ml) pre-treatment (e.g., 43.6% ± 5.2%, *n=6*, * *P < 0.05*) as compared to vehicle treated tissue (e.g., 66.8% ± 6.3%, *n=6*; ***see figure 25***) whereas minimal inhibitory effect of AM0902 was observed on colonic afferents with low activation threshold (i.e., below 20mmHg).

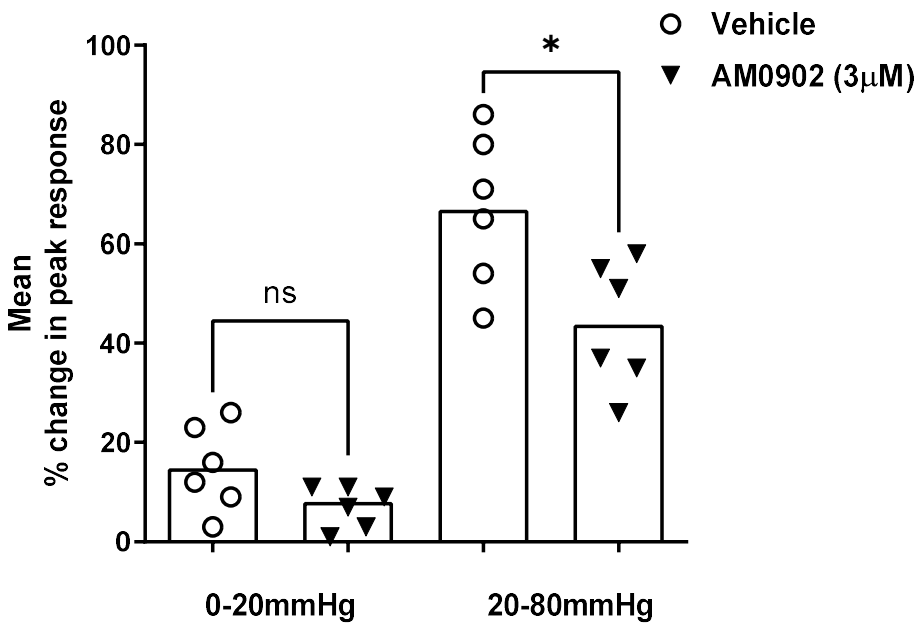


Figure 25: TRPA1 preferentially sensitizes high threshold colonic afferents to ramp distention. A. The bar charts showing the mean ± sem percent change in peak response calculated as a proportion to the total pre-treatment response between (0-20mmHg) and (20-80mmHg) following the application of vehicle and AM0902 (3μM, 100ml) pre-treatment. The statistical analysis was performed using unpaired student-t test where is *P < 0.05.

By contrast pre-treatment with cromolyn (100μM; 100ml) or zaprinast (100μM; 50ml) had no significant effect on colonic afferent discharge to ramp distension (e.g., **vehicle**: 94.0% ± 4.0% *n=6* vs **CS**: 94.1% ± 1.7% *n=6;* **zaprinast** 85.0% ± 8.9%; *n=6*). Demonstrating that although TRPA1 channels contribute to colonic afferent mechanosensitivity acute application of GPR35 agonists such as cromolyn and zaprinast has no effect on colonic afferent mechanosensitivity despite attenuating the colonic afferent response to TRPA1 agonists (***see figure 24.C***).

3.7.4. TRPA1 activation evoke mechanical hypersensitivity in colonic afferent

TRPA1 has also been implicated in the augmentation of mechanical hypersensitivity in the colon (Mueller-Tribbensee et al., 2015; Lennertz et al., 2012). Therefore, our next goal was to investigate the effects of TRPA1 activation on the sensitisation of distension response and the impact of GPR35 agonists on this sensitisation. In a new set of an experiment, we first applied the TRPA1 agonist ASP7663 (100μM; 20ml) prior to, and during a third distention. Application of ASP7663 produced increase in afferent discharge and sensitised the colonic afferent response to a subsequent distension (applied once the direct response to ASP7663 had returned to baseline) with significant change in peak afferent firing at 80mmHg (e.g., 13.2 ± 2.4 spikes/s, *n= 5*, * *P < 0.001;* ***see figure 26.C***) in comparison to peak afferent activity determined from the distention response prior to the application of ASP7663. (10.1 ± 2.3 spikes/s; *n=5;* ***see figure 26.C***). We also looked for the distention compliance for pre-ASP7663 and post-ASP7663 (100μM, 100ml) treatment and found that the cumulative time (e.g., 73.39 ± 4.8 sec vs 72.4 ± 4.5 sec; *n=5;* ***see figure 26.D***) and cumulative volume (e.g., 122.3 ± 8.6μl vs 120.7 ± 7.5μl; *n=5;* ***see figure 26.E***) was comparable. Moreover, we observed significant sensitising effects of ASP7663 (100μM, 100ml) pre-treatment on colonic afferents at higher pressure ranging from 20mmHg to 80mmHg (e.g., 87.6% ± 7.6%; *n=5*; * P < 0.05*;* ***see figure 26.F***) as compared to control distention (68.0% ± 2.5%; *n=5*) with minimal or no effects at lower pressure range (0-20mmHg) suggesting that TRPA1 may have prominent sensitizing effect on high threshold afferent fibres in the colorectum that are functionally important for visceral nociception.

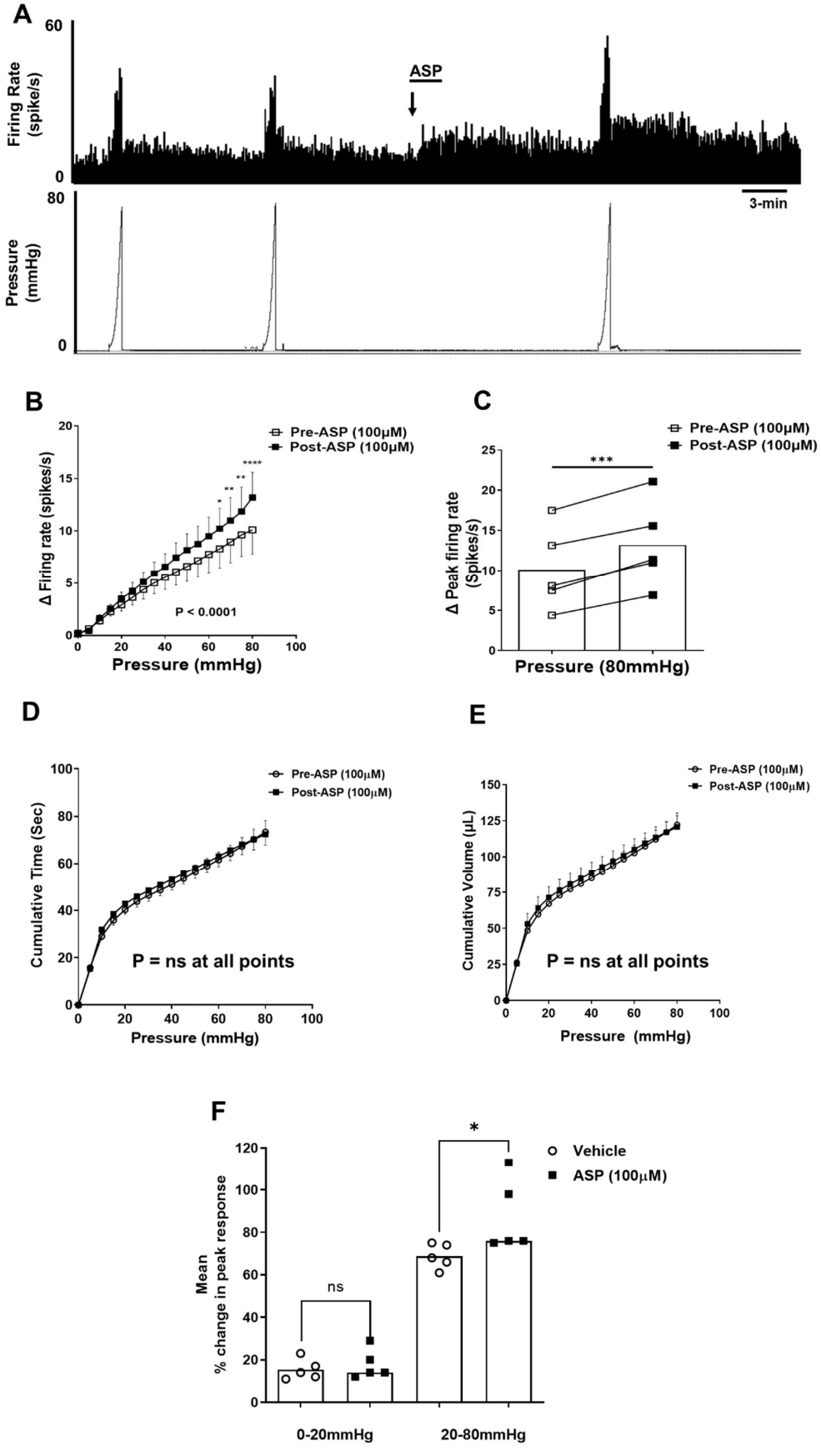

A
Firing Rate (spike/s)
60
0
ASP
3-min
Pressure (mmHg)
80
0
B
Pre-ASP (100μM)
Post-ASP (100μM)
Δ Firing rate (spikes/s)
P < 0.0001
Pressure (mmHg)
C
Pre-ASP (100μM)
Post-ASP (100μM)
***
Δ Peak firing rate (Spikes/s)
Pressure (80mmHg)
D
Pre-ASP (100μM)
Post-ASP (100μM)
Cumulative Time (Sec)
P = ns at all points
Pressure (mmHg)
E
Pre-ASP (100μM)
Post-ASP (100μM)
Cumulative Volume (μL)
P = ns at all points
Pressure (mmHg)
F
*
Vehicle
ASP (100μM)
Mean % change in peak response
ns
0-20mmHg
20-80mmHg

Figure 26: TRPA1 agonist sensitizes colonic afferents to ramp distention. A. Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg; 100sec; 10 min intervals) in C57B6/J mice alone and pre-treated with TRPA1 selective agonist ASP7663 (100μM) **B**. Line graph showing the change in colonic afferent activity following the application of ASP7663 on colonic splanchnic nerve response to 0-80mmHg ramp distention. The statistical analysis was performed using Two-way RM ANOVA followed by Šídák's multiple comparisons test where, the significance is defined as, * P< 0.05; ** P< 0.01 and **** P< 0.0001 respectively. **C**. The bar charts showing the mean ± sem peak change in afferent firing following the application of ASP7663 on colonic splanchnic nerve response at 80mmHg of distention. The statistical analysis was performed using paired student-t test where, the significance is defined as, *** P < 0.001, respectively. **D**. Cumulative time (sec) and **E**. Cumulative volume (μl) is plotted against increasing pressure (0-80mmHg, with every 5mmHg rise in pressure) for pre-ASP7663 and post-ASP7663 distention's (P > 0.99; two-way ANOVA, *n=5*). **F**. The bar charts showing the mean ± sem percent change in peak response calculated as a proportion to the total pre-treatment response between (0-20mmHg) and (20-80mmHg) following the application of vehicle and ASP7663 (100μM, 100ml) pre-treatment. The statistical analysis was performed using unpaired student-t test where is *P < 0.05.

3.7.5. Zaprinast attenuates TRPA1 induced colonic mechanosensitivity

Having established that TRPA1 agonist application sensitises response to mechanical stimuli we next aim to determine the effects of zaprinast pre-treatment on ASP7663 induced sensitization of colonic afferents to ramp distention. Pre-treatment of zaprinast

(100μM, 50ml) significantly attenuated the ASP7663 induced mechanosensitivity to colonic ramp distention (e.g., 89.40% ± 5.6%, *n=5*, **** P < 0.0001) as compared to ASP7663 *per se* treatment (e.g., 137.0% ± 7.7%; *n=5,* ***see figure 27.C****)*. However, response following pre-treatment with zaprinast (10μM, 50ml) was comparable to the ASP7663 (100μM) pre-treated group (125.2% ± 4.7%; *n=5*). We also found that the compliance of the distention's including cumulative time (e.g., 70 ± 5.2 sec vs 65.4 ± 2.4 sec; *n=5;* ***see figure 27.D***) and cumulative volume (e.g., 116.7 ± 8.7μl vs 109.1 ± 4.1μl; *n=5;* ***see figure 27.E***) was comparable between the pre-zaprinast and post-zaprinast (100μM, 50ml) treatment.

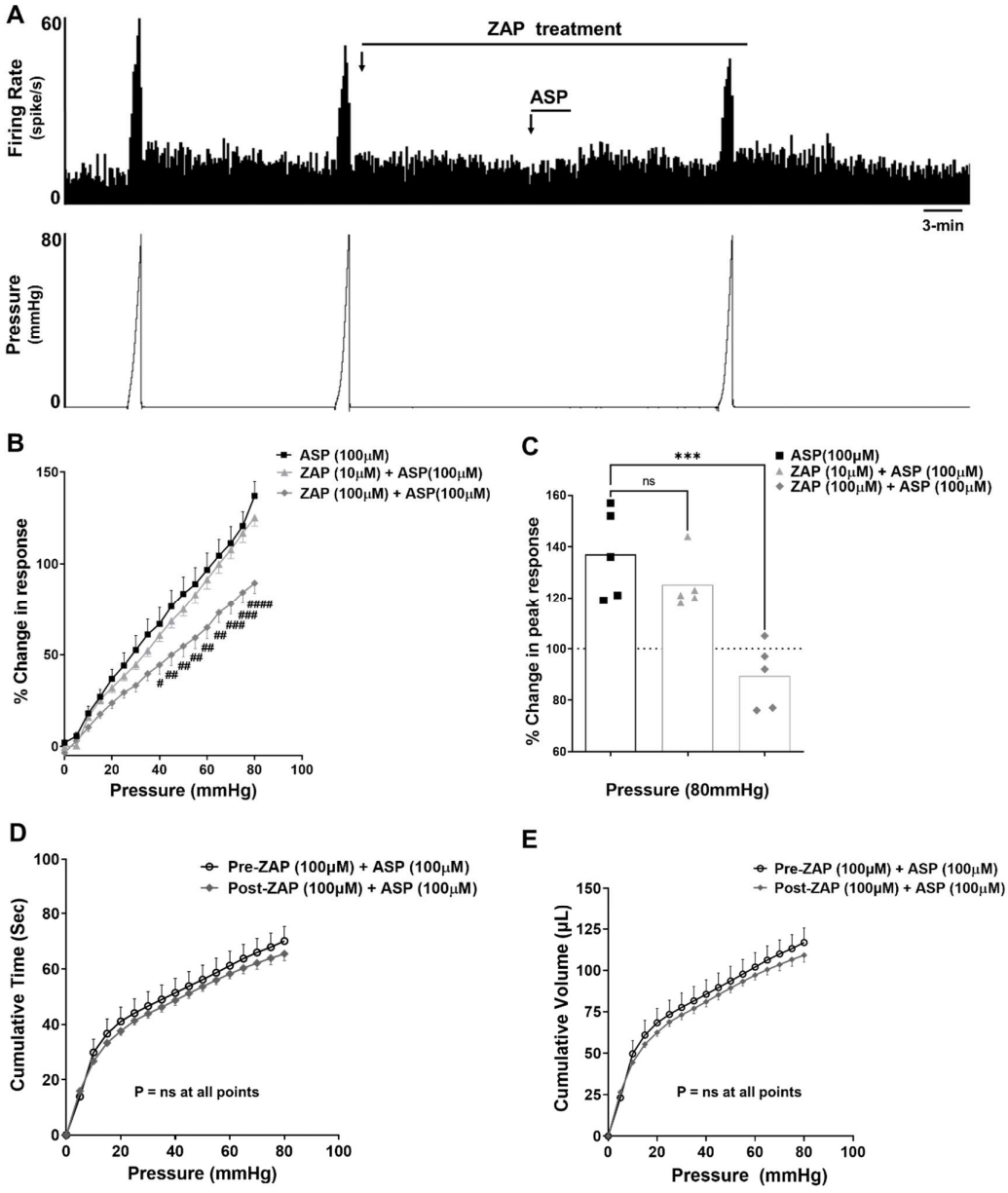


Figure 27: Zaprinast attenuates TRPA1 induced colonic afferents sensitization to ramp distention. A. Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg) in C57B6/J mice pre-treated with TRPA1 selective agonist ASP7663 (100μM) in presence of GPR35 agonist zaprinast (100μM). **B**. Line graph showing sensitising effect of ASP7663 on colonic afferents to ramp distension (0-80mmHg) and inhibitory effects of different concentration of zaprinast (10μM, 100μM) on ASP7663 induced mechanosensitivity of colonic afferents to ramp

distension (0-80mmHg). The statistical analysis was performed using 2-Way ANOVA followed by Tukey's multiple comparisons test where, the significance is defined as, # P < 0.05; ## P < 0.01; ### P < 0.001 and #### P < 0.0001 as compared to ASP7663 (100μM) respectively. **C**. Bar charts showing the sensitizing effect of ASP7663 on colonic afferents to ramp distension (0-80mmHg) along with inhibitory effects of different concentration of zaprinast (10μM and 100μM) on ASP7663 induced mechanosensitivity of colonic afferents to noxious ramp distension at peak pressure of 80mmHg. The dotted line represents peak effects of vehicle group. The statistical analysis was performed using One-Way ANOVA followed by Dunnett's multiple comparisons test where, the significance is defined as, *** P < 0.001 as compared to ASP7663 (100μM) respectively. **D**. Cumulative time (sec) and **E**. Cumulative volume (μl) is plotted against increasing pressure (0-80mmHg, with every 5mmHg rise in pressure) for pre-zaprinast and post-zaprinast distention's (P > 0.99; two-way ANOVA, *n=5*).

3.7.6 Cromolyn attenuates TRPA1 induced colonic mechanosensitivity

In a separate set of experiments conducted in blinded manner we found that application of ASP7663 (100μM, 20ml) sensitizes the colonic afferents and increases the splanchnic nerve afferent firing with subsequent increase in pressure from 0-80mmHg with peak effect observed at 80mmHg in vehicle treated group (e.g., 118.4% ± 5.6%, *n= 5*, ** P < 0.05 ;* ***see figure 28.C***). The mechanosensitisation observed following application of ASP7663 was attenuated by cromolyn (100μM, 100ml) pre-treatment (e.g.., 90.4% ± 4.5%, *n=5*, ***P<0.01* at 100μM*;* ***see figure 28.C***). However, pre-treatment with lower concentration of cromolyn (1μM and 10μM) produced a comparable response to ASP7663 (e.g., 118.6% ± 4.4%, *n=5* at 1μM; 112.4% ±

6.0%, *n=5* at 10μM) suggesting that lower concentration of cromolyn pre-treatment was not effective in reducing ASP7663 induced mechanosensitivity in colonic afferents. We also investigated to the see the compliance for pre-cromolyn and post-cromolyn (100μM, 100ml) treated distention such as cumulative time (e.g., 81.4 ± 2.9 sec vs 85.1 ± 1.5 sec; *n=5;* ***see figure 28.D***) and cumulative volume (e.g., 135.7 ± 4.8μl vs 141.9 ± 2.6μl; *n=5;* ***see figure 28.E***) where no significant difference was observed.

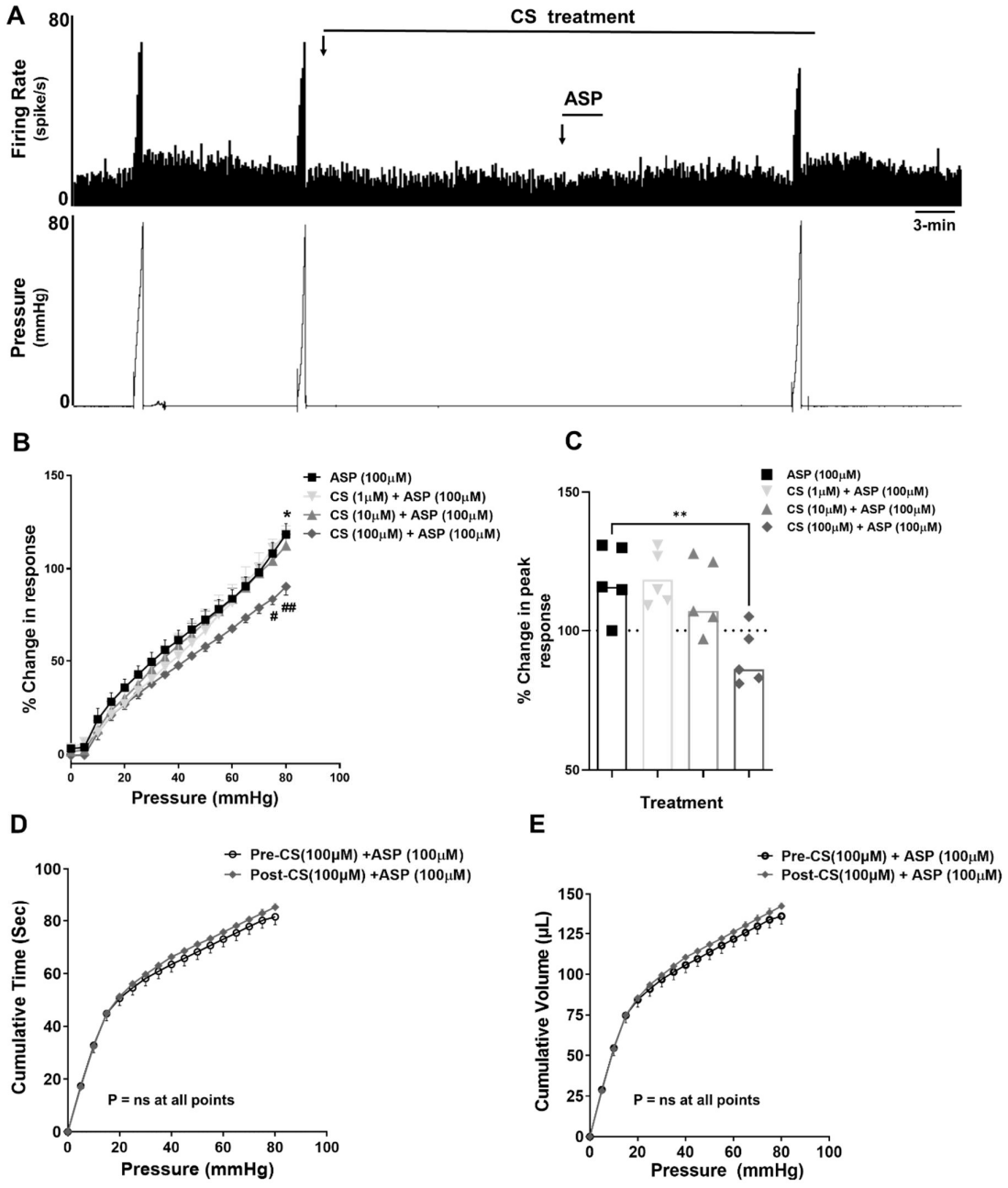


Figure 28: Cromolyn attenuates TRPA1 induced colonic afferents sensitization to ramp distention. A. Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg) in C57B6/J mice pre-treated with TRPA1 selective agonist ASP7663 (100μM) in presence of GPR35 agonist cromolyn (100μM). **B**. Line graph showing sensitising effect of ASP7663 on colonic afferents to ramp distension (0-80mmHg) and inhibitory effects of different concentration of cromolyn

(1μM, 10μM and 100μM) on ASP7663 induced mechanosensitivity of colonic afferents to ramp distension (0-80mmHg). The statistical analysis was performed using 2-Way ANOVA followed by Tukey's multiple comparisons test where, the significance is defined as, # $P < 0.05$; and ## $P < 0.01$ as compared to ASP7663 (100μM) respectively. **C**. Bar charts showing the sensitizing effect of ASP7663 on colonic afferents to ramp distension (0-80mmHg) along with inhibitory effects of different concentration of cromolyn (1μM, 10μM and 100μM) on ASP7663 induced mechanosensitivity of colonic afferents to noxious ramp distension at peak pressure of 80mmHg. The dotted line represents peak effects of vehicle group. The statistical analysis was performed using One-Way ANOVA followed by Dunnett's multiple comparisons test where, the significance is defined as, ** $P < 0.01$, as compared to ASP7663 (100μM) respectively. **D**. Cumulative time (sec) and **E**. Cumulative volume (μl) is plotted against increasing pressure (0-80mmHg, with every 5mmHg rise in pressure) for pre-cromolyn and post-cromolyn distention's ($P > 0.99$; two-way ANOVA, *n=5*).

Intriguingly, both the GPR35 agonist has shown significant inhibitory effects at higher pressure point (above 60mmHg - 80mmHg) further reinforcing our assumptions that TRPA1 prominently sensitises high threshold afferents and that GPR35 agonists are capable of inhibiting TRPA1 induced afferent sensitisation of these high threshold afferent fibres thereby showing potential to treat colonic nociception.

In the above studies, we observed a potential inhibitory effect of GPR35 agonist zaprinast and cromolyn on ASP7663 induced afferent activation and

mechanosensitisation. Similar observations have been reported for cromolyn, where it reduced colorectal distention induced abdominal pain behaviours in stress-sensitive Wistar Kyoto rats (Carroll et al., 2013). To further confirm that inhibitory effect exerted by cromolyn pre-treatment on TRPA1 induced colonic mechanosensitivity was selectively mediated by its potential to activate GPR35 receptor we reinvestigated the effect of cromolyn on TRPA1 mediated mechanosensitisation in colonic afferent preparation isolated from GPR35-/- animals.

3.7.7. Mechanosensitivity in GPR35-/- mice.

We initiated our study by investigating changes in the electrophysiological conductance of colonic afferents in GPR35-/- mice using *ex-vivo* mouse colonic whole nerve splanchnic afferent preparation during ramp distention studies. Intriguingly, the GPR35-/- mice showed significant mechanical deficit in comparison to the C57B6-WT mice (***see figure* 29**).

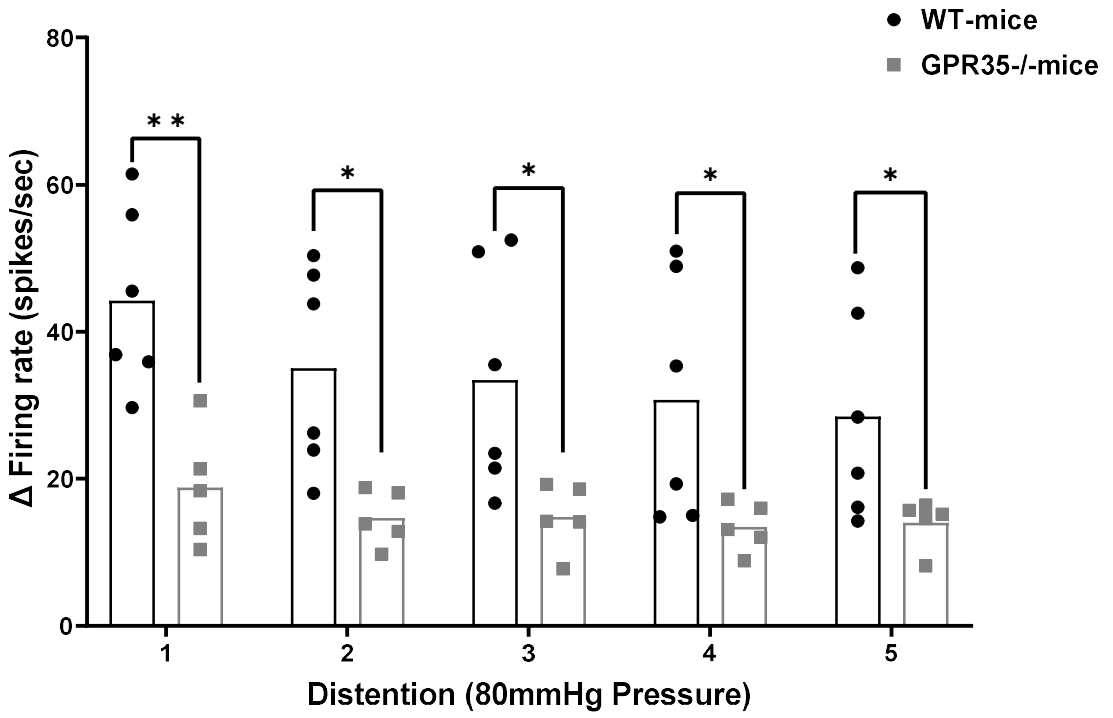


Figure 29: Comparison of colonic afferent response to ramp distention between C57B6-WT and GPR35-/- mice. Bar graph showing average change in nerve activity for five individual ramp distention's at 80mmHg for C57B6-WT and GPR35-/- mice. The statistical analysis was performed using multiple unpaired t-test where significance was defined as *P < 0.05; ** P <0.01 respectively.

However, similar to C57B6-WT mice, we observed a robust increase in colonic afferent discharge with a notably steep rise in the afferent activity above 40 mmHg, which gradually increases with increased pressure up to 80mmHg. The firing rates drop suddenly following pressure release and gradually return to baseline levels. The average peak firing rate of 18.7 ± 3.5 spikes/s at 80mmHg was observed during the first distention. However, with each subsequent colorectal distension the peak firing rate was declined (distention's 1> 2 > 3 > 4 > 5; ***see figure 29***). The electrophysiological responses over five ramp distensions of the colorectum were

determined. The peak firing rate was comparable between the second (14.6 ± 1.6 spikes/s, *n=5*) and third distention (14.7 ± 2.0 spikes/s, *n=5;* ***see figure 29***). The distention 2nd and the 3rd were used as the bracket to evaluate the sensitising effect of ASP7663 (100μM) on colonic afferents during ramp distention. While investigating the effect of ASP7663, we found that application of ASP7663 (100μM) sensitises the colonic afferents and increases the splanchnic nerve afferent firing with subsequent increase in pressure from 0-80mmHg with peak effect observed at 80mmHg (e.g., 22.7 ± 2.4 spikes/s, *n=5*, **P < 0.05*) in comparison to controlled distention before application of ASP7663 (e.g., 18.3 ± 2.3 spikes/s, *n=5*; ***see figure 30.B***).

Additionally, the percentage change in the peak response obtained following the application of ASP7663 (100μM) during ramp distention was comparable in wild type C57B6 mice and GPR35-/- mice implicating that although the genotypes show significant mechanical deficits, they are functionally responding to noxious chemical stimuli particularly ASP7663 (***see figure* 30.C**).

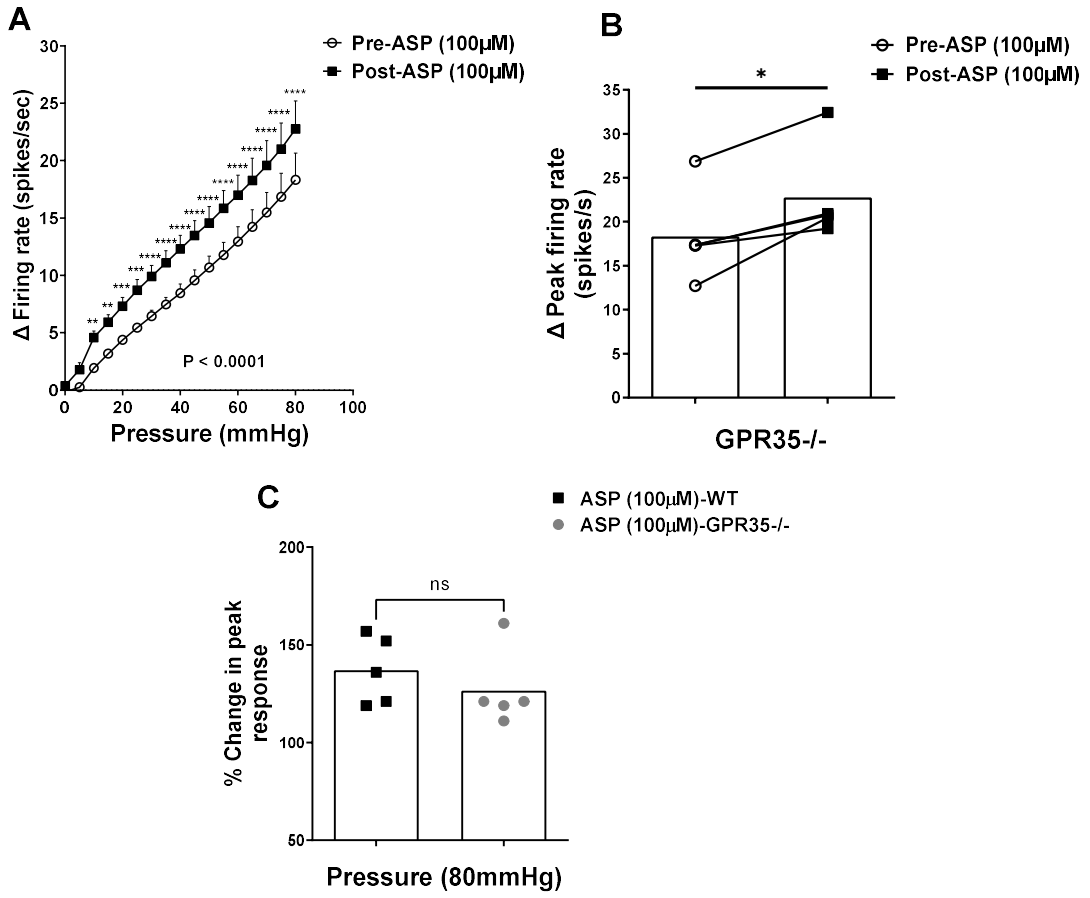


Figure 30: ASP7663 (TRPA1 agonist) induces mechanosensitisation of colonic afferents to ramp distention in GPR35-/- mice. A. Line graph showing the change in colonic afferent activity following the application of ASP7663 on colonic splanchnic nerve response to 0-80mmHg ramp distention in GPR35-/- mice colonic tissues. The statistical analysis was performed using Two-way RM ANOVA followed by Šídák's multiple comparisons test where, the significance is defined as, ** P< 0.01, *** P< 0.001 and ****P< 0.0001 respectively. **B**. The bar charts showing the mean ± sem peak change in afferent firing following the application of ASP7663 on colonic splanchnic nerve response at 80mmHg of distention in GPR35-/- mice colonic tissues. The statistical analysis was performed using paired student-t test where, the significance is defined as, * P < 0.5, respectively. **C**. Bar charts comparing percent change in peak

response (mean ± sem) following the application of ASP7663 (100μM; *n=5*) in the mice tissues obtained from GPR35-/- and C57B6-WT mice (*n=5*). The statistical analysis was performed using one way ANOVA followed by Dunnett's post hoc comparisons where, significance is defined as **P <0.01 and *** *P < 0.001* respectively.

Finally, the involvement of GPR35-receptor as a molecular determinant of direct inhibition of colonic afferent mechanosensitivity produced by cromolyn (100μM) pre-treatment in ramp distention induced by ASP7663 (100μM) was confirmed by repeat studies in tissue from GPR35-/- mice. We found the peak afferent response produced by ASP7663 following the cromolyn (100μM) pre-treatment was comparable to peak response produced by ASP7663 alone (e.g., 114.6% ± 7.1%; *n=5* vs 118.4% ± 5.6%, *n=5*, ** *P < 0.01;* ***see figure 31.C***). Also, the compliance such as cumulative time (e.g., 50.9 ± 6.9 sec vs 51.8 ± 5.8 sec; *n=5;* ***see figure 31.D***) and cumulative volume (e.g., 84.8 ± 11.5μl vs 86.4 ± 9.7μl; *n=5;* ***see figure 31.E***) was comparable between the pre-cromolyn and post-cromolyn (100μM, 100ml) treated distention. The following results implicate that the inhibitory effects of cromolyn on TRPA1 mediated colonic afferent mechanosensitisation are driven through the stimulation of GPR35 receptors.

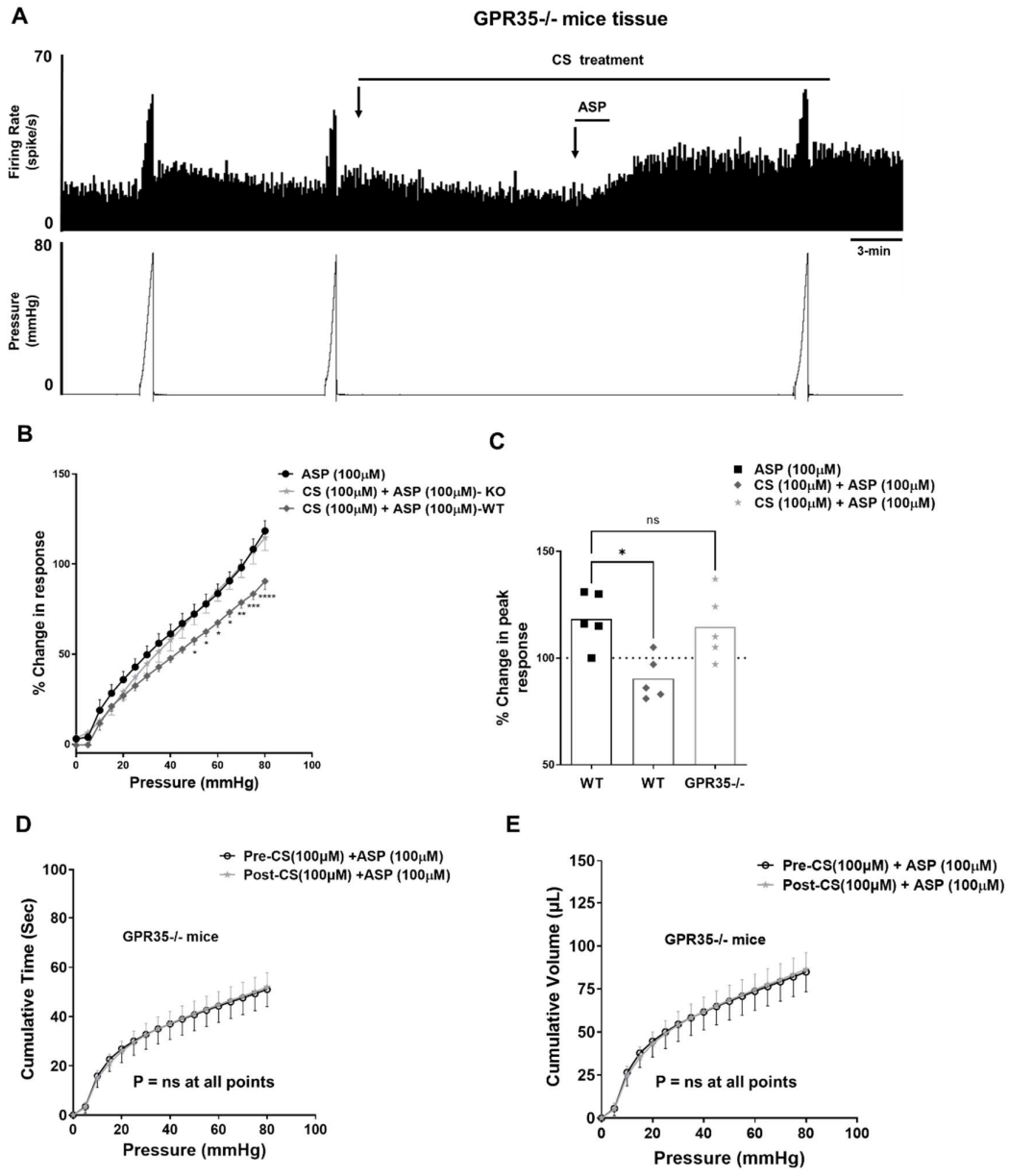


Figure 31. Cromolyn failed to inhibit TRPA1 induced mechanosensitisation of colonic afferents to ramp distention in GPR35-/- mice. A. Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg) in GPR35-/- mice alone and pre-treated with GPR35 agonist CS (100μM) following the application of selective TRPA1 agonist ASP7663 (100μM). **B.** Line graph showing percent change in peak response (mean ± sem) following the application of ASP7663 (100μM; *n=5*) alone and following pre-treatment with CS (100μM) at 0-80mmHg of pressure. The

statistical analysis was performed using Two-way ANOVA followed by Bonferroni's multiple comparison test where, statistical significance is defined as *P<0.05; **P<0.01; ***P<0.001 and ****P< 0.0001 as compared to ASP7663 (100μM) respectively. **C**. Bar charts showing percent change in peak response (mean ± sem) following the application of ASP7663 (100μM; *n=5*) alone and following pre-treatment with CS (100μM) 80mmHg of pressure. The dotted line represents peak effects of vehicle group. The statistical analysis was performed using ordinary One-way ANOVA followed by Šídák's multiple comparisons test where, significance is defined as *P< 0.05 as compared to ASP7663 (100μM). **D**. Cumulative time (sec) and **E**. Cumulative volume (μl) is plotted against increasing pressure (0-80mmHg, with every 5mmHg rise in pressure) for pre-cromolyn and post-cromolyn distention's (P > 0.99; two-way ANOVA, *n=5*) in GPR35-/- mice tissues.

3.8. Discussion

The result from our study demonstrates the inhibitory effect of GPR35 agonists zaprinast or cromolyn on TRPA1-induced mechanosensitisation of colonic afferents in mice. Moreover, we confirmed that cromolyn attenuates TRPA1-induced mechanosensitivity *via* GPR35 receptors using tissue from GPR35-/- mice.

Pain arising from mechanosensitive visceral afferents following distension or contraction of the bowel is a common problem in IBS and IBD patients (Brookes et al., 2013). In humans, distension of the colorectum evokes pain with thresholds to the perception of pain being typically reported between 30-40mmHg. A similar threshold is required to activate “high threshold” lumbar splanchnic afferents following luminal distension of mouse colon (Kanazawa et al., 2008; Brierley et al., 2008; Hughes et al., 2009). This highlights the translational utility of mouse colorectal afferent response to luminal distension as a reliable and reproducible model for the preclinical investigation of visceral analgesics (Hockley et al., 2014).

Consistent with this, we observed an increase in LSN activity following luminal distension which was significantly reduced across the noxious pressure range (40-80mmHg), by the TRPA1 inhibitor AM0902 confirming the explicit role of TRPA1 in activation of high-threshold afferent fibres functionally important for visceral nociception. Similarly, TRPA1 activation by the selective agonist ASP7663 sensitised colonic afferents to mechanical stimuli at higher distending pressures in our studies. Following on from the studies in the previous chapter we evaluated the inhibitory effects of GPR35 agonist cromolyn and zaprinast on colonic afferent responses to

ramp distention observing no significant influence on afferent mechanosensitivity. However, both cromolyn and zaprinast did inhibit TRPA1 agonist augmented mechanosensitivity in colonic afferents which was reversed in tissue from GPR35-/- mice. These findings and the marked inhibitory effects of cromolyn and zaprinast on TRPA1 agonist induced colonic afferents excitation suggesting that the effects of GPR35 agonist occur on agonist but not mechanically mediated activation of TRPA1 channels.

Previous work has found that GPR35 activation by zaprinast in sympathetic neurons inhibited N-type calcium channel activity responsible for controlling cellular excitability by inhibiting the release of excitatory transmitters (Guo et al., 2008). This effect of zaprinast was PTX-sensitive, suggesting GPR35 coupling to the endogenous $G_{i/o}$ protein (Guo et al., 2008). Moreover, cromolyn has been shown to directly influence nociceptive signalling by inhibiting capsaicin-induced activity on sensory nerve endings (Dixon et al., 1980). Furthermore, cromolyn also inhibits neurogenic inflammation (Yamawaki et al., 1997) and release of SP in human skin (Crossman et al., 1993).

Agonist activation of TRPA1 has been reported to release SP and CGRP from isolated mouse colon (Engel et al., 2011; Cattaruzza et al., 2010) or cultured mouse DRG neurons innervating the colon (Nakamura et al., 2012). This led us to consider that cromolyn and zaprinast may also inhibit tachykinin release in response to TRPA1 activation and this tachykinin release may be responsible for the colonic afferent excitation and sensitisation of mechanical responses following TRPA1 agonist activation. The afferent activation following mechanical activation of TRPA1 by contrast is not mediated by SP release and hence is not inhibited by cromolyn or zaprinast.

However, the effects of SP on colonic afferents are not very well elucidated and so, our goals were subsequently to confirm SP release from colonic afferents following TRPA1 activation, the contribution of SP to colonic afferent activity, and determine the effects of cromolyn and zaprinast on TRPA1 mediated SP release.

Chapter-4

Role of GPR35 in attenuation of TRPA1 induced neuropeptide release

4.1. Neurogenic inflammation

The concept of “neurogenic inflammation” has evolved from seminal experiments by Goltz in 1874 and by Bayliss in 1901, showing that electrically stimulating dorsal roots induces vasodilation in the skin, independent of that produced by the immune system. Later it was established that the neurogenic inflammation is mediated by neuropeptides released from the peripheral terminals of sensory afferents such as substance P (SP) and calcitonin gene-related peptide (CGRP) (Chiu et al., 2012; Edvinsson et al., 1987). Neurogenic inflammation has been implicated in the pathophysiology of conditions such as asthma, psoriasis, colitis and contributes to the generation of pain symptomology (Geraghty et al., 2021; Engel et al., 2011). Moreover, neurogenic inflammation is broadly responsible for the pain component in migraine and bone fracture (Wei et al., 2009). Inhibition of neurogenic inflammation with nerve blocking agents such as Botox (Botulinum neurotoxin A) or anti-CGRP antibodies significantly reduces inflammatory pain, and headache (Chiu et al., 2012). Neurogenic inflammation is not always harmful and is speculated to exert a protective effect by causing vasodilation and enhanced plasma extravasation which facilitated immune response to infection and dilutes noxious stimuli for example preventing mucosal damage by acid in the gastric mucosa (Holzer & Holzer-Petsche, 1997). However, the neuropeptide functions in the colon may contribute to chronic inflammation (Holzer, 1998).

In the GI tract, TRP channel activation for example TRPV1, TRPA1, and TRPV4, can evoke neurogenic inflammation by releasing neuropeptides such as SP and CGRP, from sensory nerve fibres innervating the bowel (Engel et al., 2011). Similar response can also be elicited by the activation of G protein coupled receptors expressed on sensory nerves which the activation of protease-activated receptor 2 (PAR2) being a focus of much research due to the increase in proteolytic activity found in many gastrointestinal diseases leading to the development of neurogenic inflammation through the release of SP and CGRP (Julius & Basbaum, 2001; Dai et al., 2007).

CGRP is largely responsible for the vasodilation of blood vessels whereas SP also increases capillary permeability leading to plasma extravasation and oedema (Edvinsson et al., 1987; Saria, 1984). In addition, a broad range of neuropeptides, including Neurokinins A and B, Vasoactive intestinal peptide (VIP), neuropeptide (NPY), gastrin-releasing peptide (GRP) and Adrenomedullin can also be found in sensory nerve terminals and may contribute to neurogenic inflammation.

4.2. Role of TRPA1 in neurogenic inflammation

TRPA1 mediated inflammatory pain and hyperalgesia is partly driven by neuropeptide (SP and CGRP) release and subsequent neurogenic inflammation (Engel et al., 2011; Cattaruzza et al., 2010). Retrograde labelling studies have confirmed TRPA1 expression in peptidergic (CGRP positive) DRG neurons innervating gastrointestinal regions (Brierley et al., 2009; Dai et al., 2007; Engel et al., 2011; Cattaruzza et al., 2010; Kondo et al., 2009). Furthermore, release of SP and CGRP from isolated mouse colon (Engel et al., 2011; Cattaruzza et al., 2010) or cultured mouse DRG neurons innervating the colon (Nakamura et al., 2012) during visceral pain and inflammatory conditions have also been reported. The release of SP from the peripheral endings of primary afferents activates the tachykinin NK_1 receptor on endothelial cells of

postcapillary venules to cause extravasation of plasma proteins and inflammatory oedema, and it has also been reported to activate immune cells (Chiu et al., 2012; Saria, 1984; Baral et al., 2019), with TRPA1 activation aggravating colitis (Utsumi et al., 2018). For example, lipopolysaccharide (LPS), a constituent of bacterial cell wall has shown to sensitize TRPA1 receptor channel during experimental colitis which initiates and maintaining colitis through SP release (Macpherson et al., 2007), and TRPA1-/- mice are protected from colonic inflammation (Utsumi et al., 2018). Blocking this process for example through treatment with GPR35 agonists would therefore alleviate inflammation and inflammation-induced sensitisation and pain.

4.3. Downstream mechanism of Substance-P in Neurogenic inflammation

Substance P (SP) is an undecapeptide belonging to the tachykinin family, which also includes neurokinin A and neurokinin B. It binds to neurokine receptors (NKs) in the order of NK1R >NK2R >NK3R (Gerard et al., 1991; Mantyh et al., 1997). SP binding to NK_1 receptor activate phospholipase-C and components of the second messenger system such as IP3 and DAG, which subsequently increases cytosolic Ca^{2+} levels and activates the protein kinase-C enzyme. NK1R signalling can also activate adenylyl cyclase, which causes the generation of cAMP, and the latter activates protein kinase-A (Suvas et a., 2017) by a process called endosomal signalling (Jensen et al., 2017). The following process generates sustained intracellular signals, sensitises TRP-channels and drives chronic visceral pain (Amadesi et al., 2004). Together activation of these downstream cellular components transduces the NK1 mediated SP effects, which includes the other cellular signalling pathways such as ERK1/2, p38 MAPK and NFkB regulating proliferative, antiapoptotic and immune regulatory processes (Fiebich

et al., 2000; Hong et al., 2020). The desensitisation process of SP involves internalisation of the SP-NK_1R complex in the endosome, where endosomal acidification dissociates SP from the NK_1R complex, followed by degradation of SP by endothelin-converting enzyme-1, a membrane metalloendopeptidase named as neprilysin (Skidgel et al., 1984; Erdos & Skidgel, 1989). Within the immune system NK_1 receptors are expressed on dendritic cells, monocytes, eosinophils, neutrophils, mast cells, natural killer cells, and T cells, indicative of the immune regulatory role of SP (Suvas et a., 2017). SP also promotes the activation of mast cells and induces the release of proinflammatory cytokines and chemokines to reinforce the process of neurogenic inflammation (Suvas et al., 2017; Baral et al., 2019).

4.4. Role of TRPV1 neurogenic inflammation

Like TRPA1, TRPV1 is expressed by the peptidergic subset of sensory fibres. In mice, 59% of TRPV1-immunoreactive DRG neurons projecting to the colon colocalise with CGRP, and 70% of the CGRP-positive neurons express TRPV1 (Tan et al., 2008). In conformity to this expression pattern, activation of TRPV1 and TRPA1 has shown increased release of CGRP from the distal colon segment compared to the proximal colon. (Engel et al., 2011).

The above literature describes TRPV1 and TRPA1 as crucial correlates of the neurogenic inflammation process in which the neuropeptides such as SP and CGRP play a vital role.

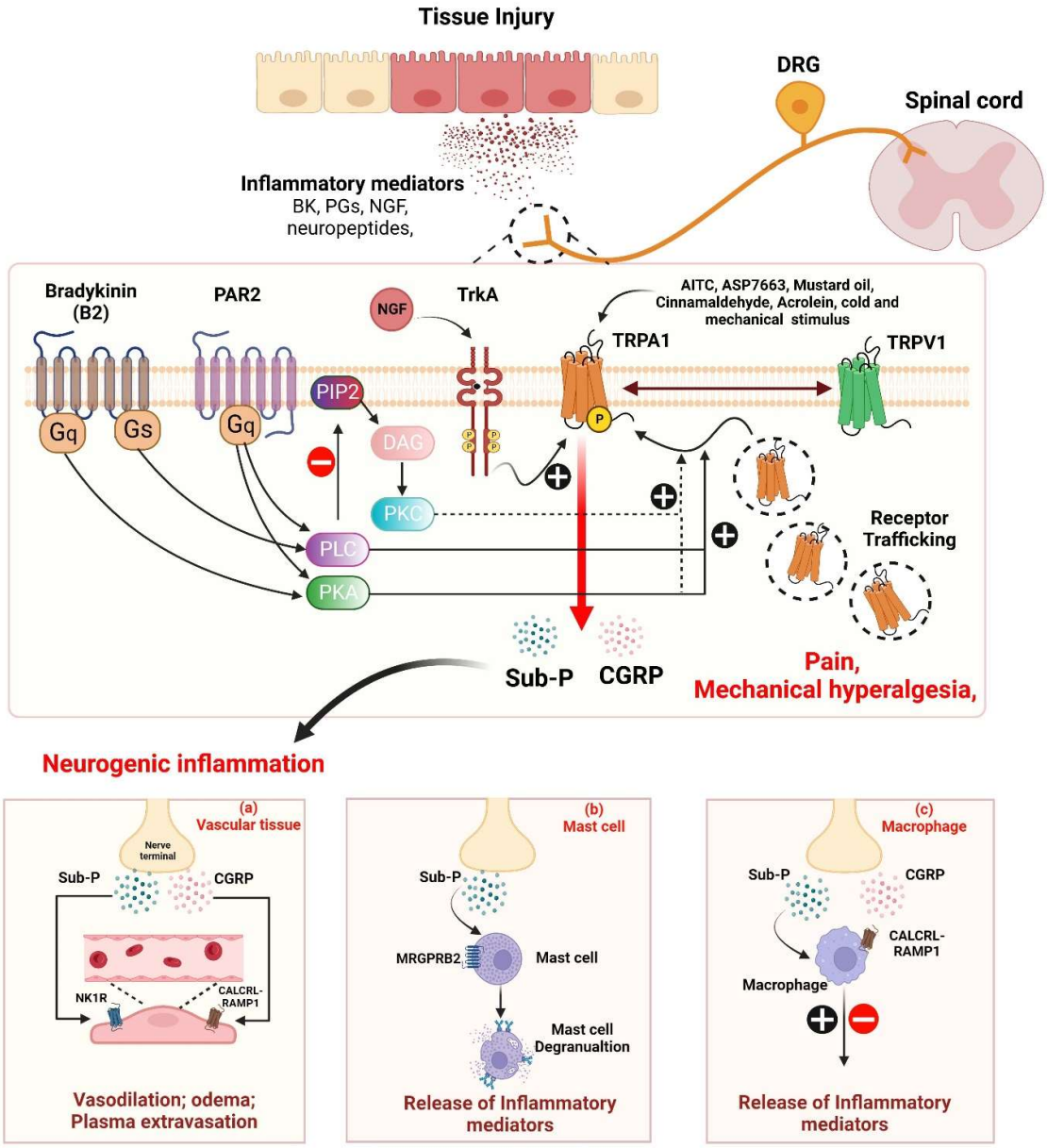


Figure 32: The overview of TRPA1 channels involved in nociception, inflammation, and neurogenic inflammation in lower gastrointestinal region. The TRPA1 channels are mainly expressed on enterochromaffin cell (EC), extrinsic primary afferents and on the DRGs projecting to the colon and intestine. TRPA1 channels directly detect various stimuli in the GI lumen and act as secondary transducers for GPCR. Upon activation, TRP channels transduce the sensory signal (primarily nociception) to the central nervous system and lead to autonomic reflex

responses. This mechanism could be enhanced by inflammatory mediators such as bradykinin, proteases and are responsible for the augmentation of visceral hypersensitivity during pathological conditions. **(a)** Meanwhile, the activation of TRPA1 channels triggers neurogenic inflammation with neuropeptides, such as CGRP and SP release from peripheral nerve terminals that act on the vasculature to drive neurogenic inflammation. Substance P activates NK1R on endothelial cells to promote vascular permeability and oedema. CGRP acts *via* the calcitonin receptor- like receptor (CALCRL)– receptor activity- modifying protein 1 (RAMP1) receptor complex on vascular and lymphatic smooth muscle cells to promote relaxation and acts on endothelial cells to release nitric oxide, leading to vasodilation. **(b)** Moreover, Substance P activates MAS- related G protein- coupled receptor member B2 (MRGPRB2) on mast cells, leading to mast cell degranulation and pro- inflammatory mediator release. **(c)** Additionally, nociceptor releases substance P, which can interact with immune cells such as monocytes and macrophages *via* ERK–p38 mitogen-activated protein kinase (MAPK)-mediated NF-κB activation to drive expression of pro-inflammatory cytokines, thus promoting or restraining the initiation or process of inflammation. Moreover, CGRP acts on CALCRL–RAMP1 to drive cAMP–protein kinase A (PKA)-dependent modulation of CREB to upregulate IL-10 and inducible cAMP early repressor (ICER)-dependent transcriptional repression of pro-inflammatory cytokines. Therefore, based on the immunomodulatory effects, TRP channels play a role in nociception, neurogenic inflammation and in the immune pathogenesis of Inflammatory disease in GIT such as IBD or IBS. GPCR, G-protein coupled receptors; SP, substance P; CGRP, calcitonin-gene-related peptide; MCP-1, macrophage chemoattractant protein-1.

4.5. Aim:

I have shown that TRPA1 activation stimulates colonic afferents and sensitises response to colorectal distension. These effects are attenuated by pre-treatment with GPR35 agonists. TRPA1 (and TRPV1) are reported to promote neuropeptide release, (SP and CGRP) and so we sought to understand a) the contribution of SP and CGRP to TRPA1 (and TRPV1) evoked colonic afferent activation and mechanosensitisation, and b) the effect of GPR35 agonist pre-treatment on these responses.

4.6. Research objectives:

To investigate the effect of GPR35 receptor stimulation on TRPA1 and TRPV1-induced SP release and response, we will:

- Determine the effects of selective NK1-antagonist aprepitant on TRPA1 and TRPV1- induced afferent excitation.
- Examine the effect of SP on colonic afferent activity and mechanosensitivity.
- Investigate the role of SP and CGRP in the intestinal contraction mediated by TRPA1 and TRPV1 agonists.
- Investigate the release of SP from colonic tissue in response to TRPA1 agonist application.
- Investigate the effect of the GPR35 agonist cromolyn on TRPA1 mediated tissue contractility and SP release assay.
- Confirm responses are mediated through the activation of GPR35 receptors using tissue from GPR35 -/- mice.

4.7. Experimental methods and protocols

4.7.1 Ex-vivo electrophysiology (effect of drug treatment on colonic afferents)

The electrophysiological recording was made from the mice (C57B6-WT and GPR35-/-) colon tissue following the described procedure in the methods section of chapter-2 and 3. At the start of the experiments, the recordings were allowed to stabilise over a 45 min period, after which basal activity of the afferent was recorded for 15 minutes prior to experimental protocols. The effect of drug treatment on colonic afferent for ASP7663 (100μM, 20ml), capsaicin (500ng, 20ml) and 20 ml application of SP [10μM, 30μM; 50μM and SP-50μM + peptidase inhibitors (100ml-pre-treatment)] in C57B6-WT colon tissues were determined alone or following the pre-treatment with aprepitant (10μM, 100ml) in a separate set of experiments (***See figure 33***) . The inhibitory effect of JYL1421 (TRPV1 antagonist) was also determined on direct action elicited by capsaicin (500ng, 20ml) application (***See figure 33.3***). We also investigated the inhibitory effects of CGRP antagonist BIBN (3μM) on ASP7663-induced colonic afferent excitation (***See figure 33.9***). The afferent firings were recorded and analysed.

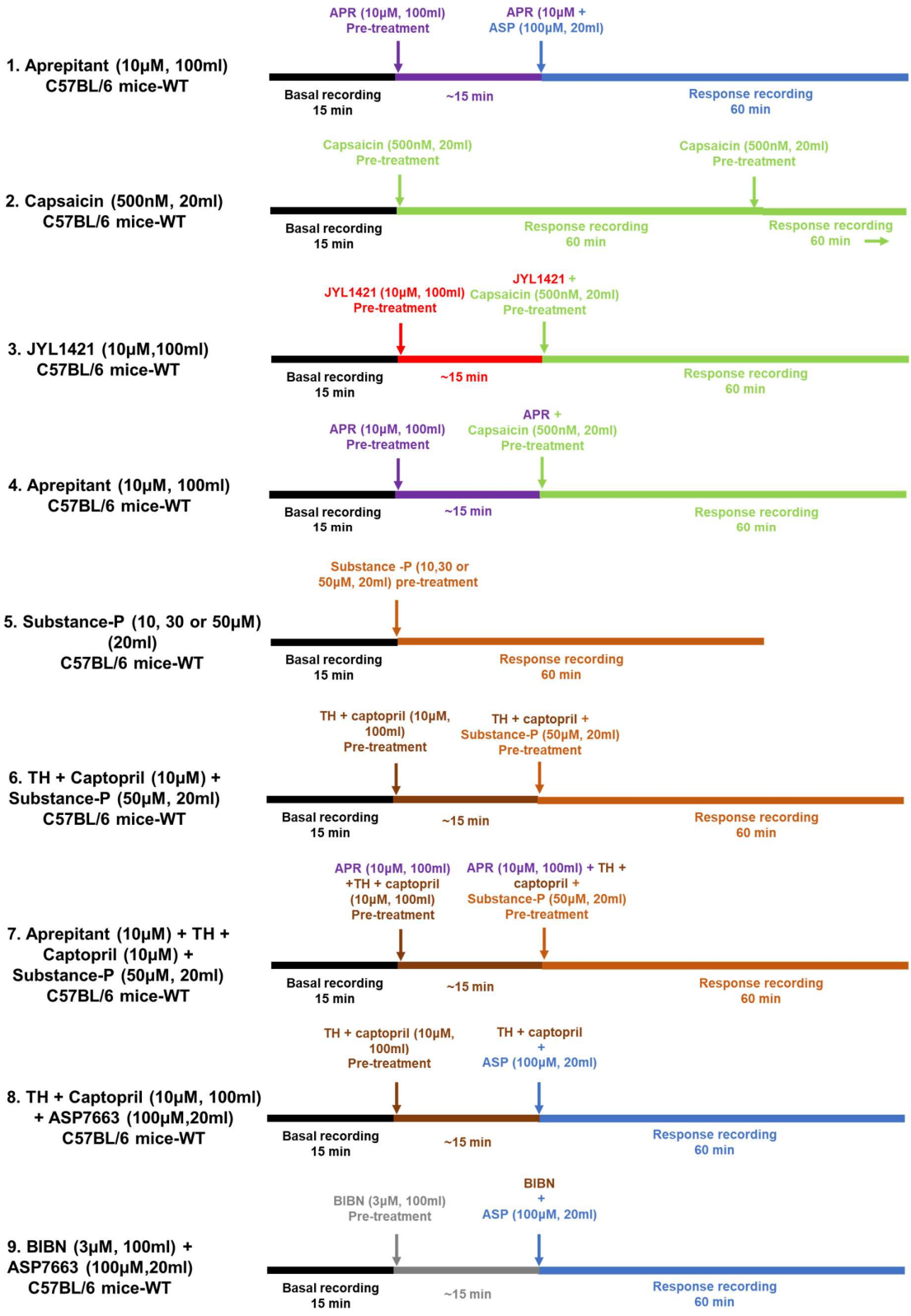


Figure 33. Experimental protocols for ex-vivo electrophysiology recordings.

4.7.2 Data analysis

4.7.3 *Ex vivo* LSN recordings: Changes in LSN nerve activity were determined by subtracting baseline nerve discharge (mean nerve discharge calculated from the ongoing activity 6 minutes prior to drug application) from ongoing nerve activity measured at 1min intervals from the start of bath perfusion with ASP7663, capsaicin and SP. The response obtained was displayed as the mean change in nerve activity over time. Mean peak change in nerve activity following ASP7663, capsaicin and SP application was calculated using the peak increase in nerve discharge observed within 20min of ASP7663, capsaicin and SP application from their respective experiments. Changes in peak nerve activity following application of ASP7663; capsaicin and SP were compared between aprepitant pre-treatments using an unpaired Student's t-test or one-way ANOVA followed by Tukey's Multiple comparisons. Changes in afferent nerve discharge following application of ASP7663, capsaicin and SP were compared over time between aprepitant pre-treated tissues using a two-way ANOVA followed by Dunnett's or Šídák's multiple comparisons between the groups.

4.7.4 Ex-vivo electrophysiology (ramp distention)

For the ramp distensions paradigm, the experiments were conducted according to the described procedure in chapter 3. The effect of selective NK_1-antagonist aprepitant (10μM, 100ml) pre-treatment was evaluated on mechanotransduction for which aprepitant (10μM, 100ml) pre-treatment was applied immediately after the distention-2, and its inhibitory effect was observed on distention-3 (***see figure 34.1***). In another set of experiments, we also determined the inhibitory effects of aprepitant on ASP7663

induced mechanosensitivity for which aprepitant (10μM, 100ml) pre-treatment was applied followed by the bath superfusion of ASP7663 (100μM, 20ml) in the presence of aprepitant. As soon as the ASP7663 direct response returned to basal activity (20 min), the distention stimulus was applied to the colon tissue. The response at every 5mmHg increase in pressure (0-80mmHg) was determined and compared with the previously generated ASP7663 (100μM) group (***see figure 34.2***). Similarly, the effects of SP (50μM) in the presence of peptidase inhibitors (thiorphan + captopril) were determined on colonic mechanosensitivity in separate experiments and compared with the vehicle (Kreb's solution) added with peptidase inhibitors (thiorphan (10μM) + captopril (10μM) (***see figure 34.3***).

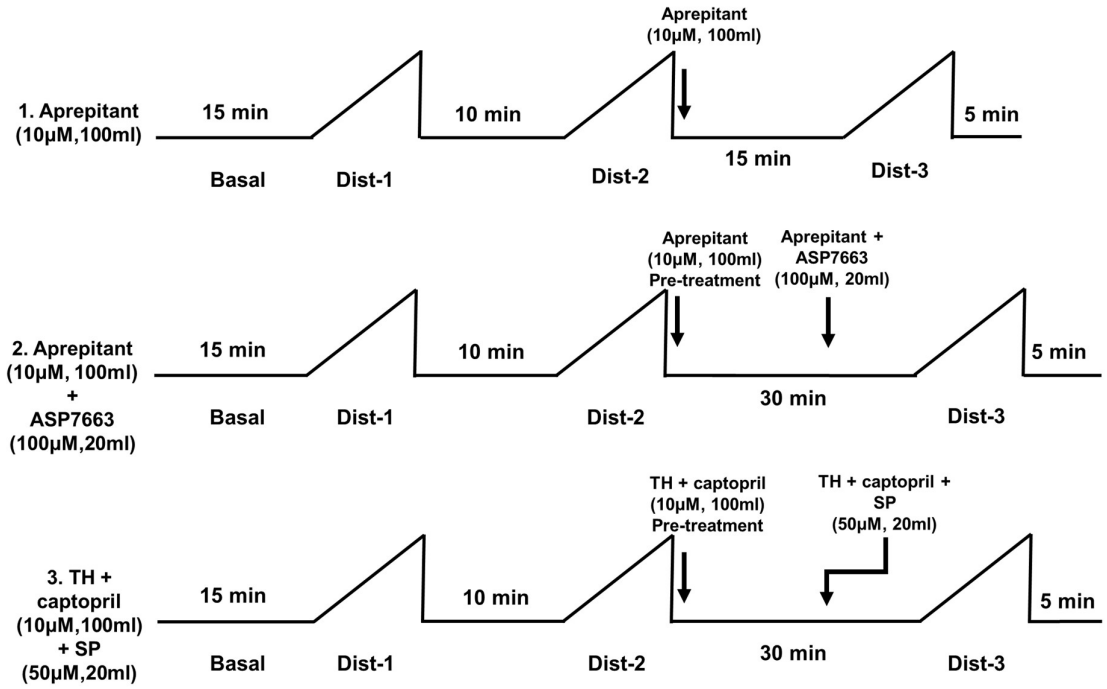


Figure 34. Experimental protocols for evaluating effects of NK_1-agonist and antagonist on colonic mechanosensitivity during ramp distention paradigm.

4.7.4.1 Data analysis (ramp distention experiments)

The afferent activity was calculated using a spike-2 software pressure script which determines the spike frequency against pressure. The spike frequency was calculated for every 5mmHg rise in pressure starting from 0 to 80mmHg for each ramp distention. The baseline nerve discharge (mean nerve discharge calculated from the ongoing activity 1 minute prior to individual ramp distention) was subtracted from the afferent activity for individual ramp distention. The data obtained were normalised and expressed as a percentage change in afferent responses. For normalisation, the response to distention-2 and distention-3 were normalised with the peak effect (at 80mmHg) of distention-2. The data is expressed as a percentage change in response to increasing pressure stimulus (0-80mmHg with every 5mmHg rise in pressure) as a profile. For profiles, the statistical analysis was performed using 2-way ANOVA followed by Dunnett's, Šídák's or Bonferroni's post-test analysis for multiple comparisons. Additionally, the change in peak afferent activity obtained at 80mmHg pressure (mean ± sem) is expressed as the bar chart for which the statistical analysis was performed using paired student T-test or by ordinary one-way ANOVA followed by Tukey's Multiple comparisons as applicable.

4.8. Tissue contractility assay

The distal colon segment (~ 2cm) was removed from euthanised C57B6-WT and GPR35-/-mice, cleared of the content, and placed in ice-cold aerated Krebs-Ringer solution until use. A silk thread was attached at the upper and lower ends of the colonic segment, which was mounted vertically in the tissue chamber (volume 10ml) filled with Krebs-Ringer solution (37°C, aerated with 5% CO_2/ 95% O_2) contained within a heated

water bath (37°C). The lower thread was attached to a fixed hook within the tissue chamber, and the upper thread was attached to an isotonic force transducer to measure contractile activity. The contraction response generated by the colon tissue was converted into voltage (V). The signals were amplified, digitised, and displayed on chart recorder software (LabChart). Tissue was allowed to equilibrate for 60 minutes (washed every 15 minutes with fresh buffer) before experimental protocols.

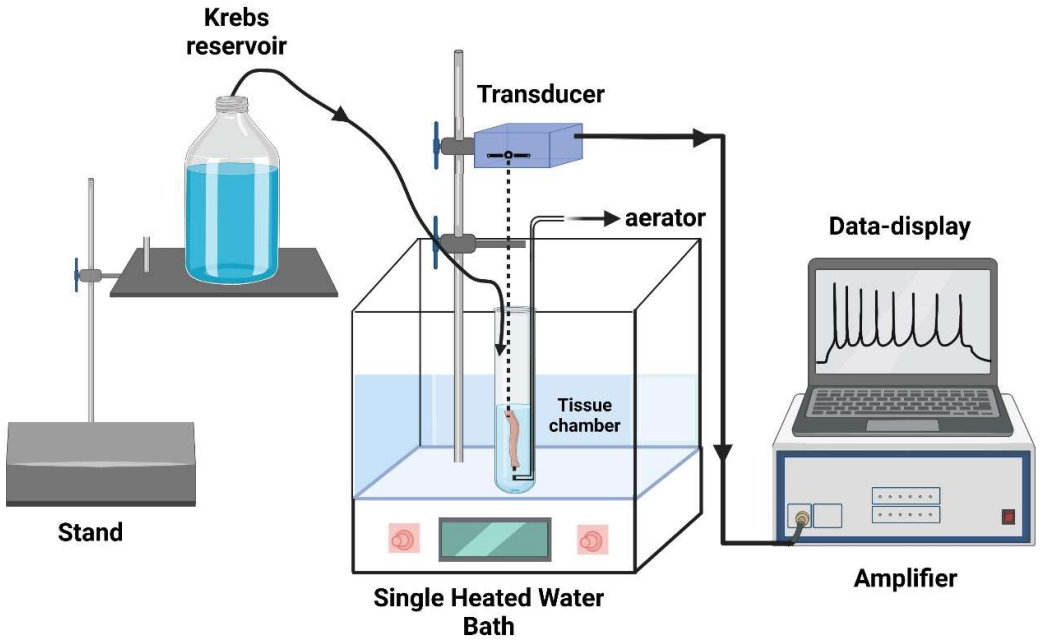


Figure 35: **Illustration of lab station setup used for tissue contractility assay.**

4.8.1 Tissue contractility assay protocol: Tissue was first challenged with acetylcholine (100μM) to confirm contractile response and washed. Subsequently, tissues were challenged with TRPA1 agonist ASP7663 (100μM) and capsaicin (1μM) alone in the separate experiments and the tissue contraction response as a change in the voltage (V) was recorded. Each tissue was challenged only once with either

ASP7663 or capsaicin looking over their extreme desensitisation effects on colonic afferents. For evaluating the inhibitory effects of selective TRPA1-antagonist, NK_1-antagonist and GPR35-agonist, the tissues were incubated with AM0902 (3μM), aprepitant (10μM), and cromolyn (100μM) for 10min prior to application of ASP7663 (100μM) or capsaicin (1μM) respectively in separate set of experiments. The inhibitory effect of cromolyn (100μM) was also evaluated in the tissues obtained from GPR35-/- mice. At the end of each experiment, the tissues were washed with fresh buffer, and acetylcholine (100μM) was reapplied to confirm the presence of contractile activity in the tissues.

4.8.2 Data analysis (Tissue contractility assay):

For tissue contractility assay responses were normalised to the initial acetylcholine response (100%), and the magnitude of contractile effects elicited by ASP7663 statistically compared between ASP7663 alone or tissues pre-incubated with AM0902 and aprepitant. For assessment of the cromolyn effect, the magnitude of contractile response elicited by ASP7663 was statistically compared between ASP7663 alone or cromolyn pre-incubated tissue obtained from C57B6-WT and GPR35-/- mice. Similarly, the response from the capsaicin-treated group was compared with the separate group pre-treated with aprepitant. Statistical analysis was carried out using GraphPad Prism 9.0 software with significance set at $P<0.05$. Data are expressed as an individual or mean ± SEM values.

4.9 Chemiluminescent immunoassay (CLIA)

The descending colon tissue from the C57B6-WT and GPR35-/- mouse was removed in one piece, cleared of the faecal material, and placed in a wet dish with oxygenated Krebs solution. The tissue segment was washed 3-times before the experiment to remove any adhering tissue debris or blood that may interfere with the experiment. The tissue segments were weighted prior to the release assay. The SP-release experiment started by transferring the distal colon segment (~2 cm) in a test tube filled with oxygenated and filtered (Whatman filter paper-150mm) Krebs solution placed on a heated water bath maintained at 37°C and was equilibrated for 15 min. The enkephalinase inhibitor thiorphan (10μM) and peptidase inhibitor captopril (10μM) were added during experiments to prevent the breakdown of SP. The tissue was separately incubated with ASP7663 (100μM) alone or following the pre-incubation with cromolyn (10μM; 100μM) and AM0902 (3μM) in an Eppendorf tube (2ml) with 500μl of Krebs solution. As soon as the colon preparation was removed from the Eppendorf tube, the remaining incubation fluid was aliquoted, centrifuged (5000-rpm) for 5 minutes and used immediately to measure SP content using a commercially available CLIA-kit (Cloud-Clone Corp, catalogue no. CCA393Mu, Houston, USA) following the manufacturer's instructions. Briefly, a competitive inhibition reaction is launched between the biotin labelled-SP and unlabelled-SP using standard and the tissue samples (50μl) in a microplate-well pre-coated with an antibody specific to SP followed by immediate addition of detection reagent-A (50μl). The kit was incubated for 1 hour at 37°C. After incubation, the wells were washed with wash buffer (3-times), and the remaining fluid was aspirated. Further, detection reagent-B was added (100μl) to each well and allowed to incubate for 30 minutes at 37°C. The wells were washed (5-times) with washed buffer, and the remaining fluid was aspirated. A mixture of substrate

solution (100µl) was added to each well, and the microplate was incubated for 10min at 37°C to generate glow light emission kinetics. Upon plate development, the intensity of the emitted light was determined by the CLARIOstar microplate reader (BMG-Labtech-430-0202). The intensity of the emitted light is inversely proportional to the SP level in the sample. Finally, the SP levels were calculated, normalised for the tissue weight, and represented as a fold increase in SP release. The values are expressed as (mean ± sem), and the statistical analysis was performed using ordinary one-way ANOVA followed by Tukey's Multiple comparison test, where the significance is defined as * P< 0.05 and ** *P < 0.01.*

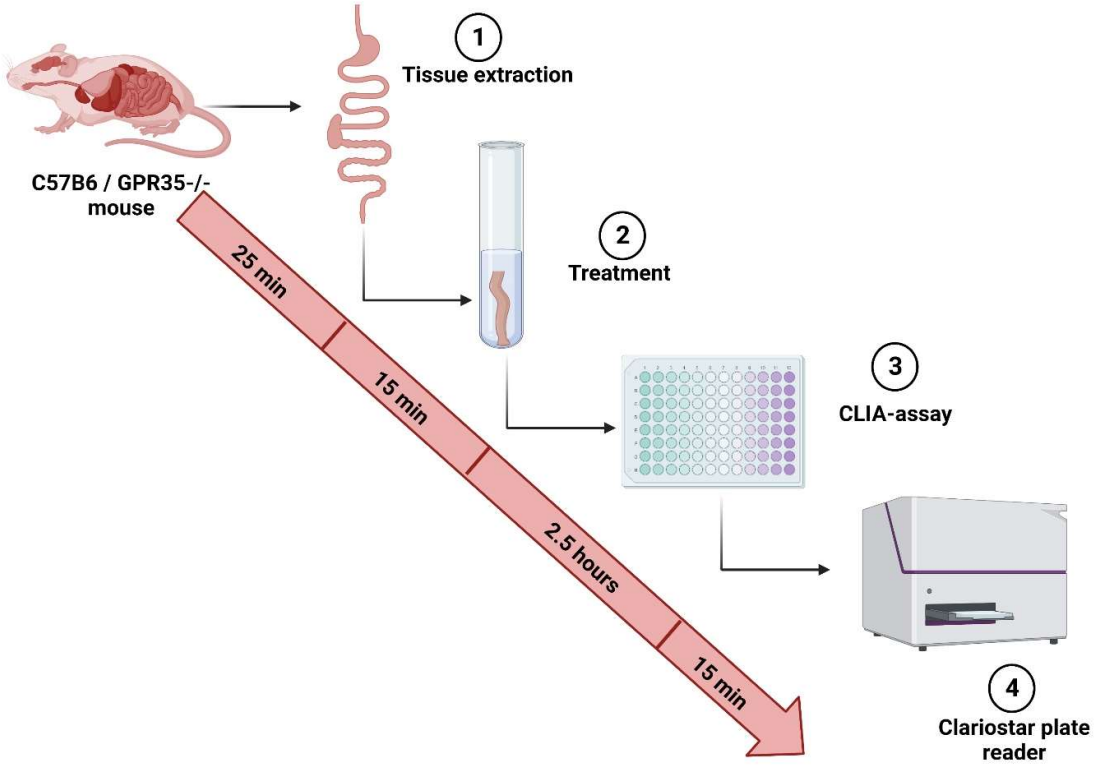


Figure 36: Timelines for CLIA-assay.

4.10. Drugs and chemicals

The Drugs were purchased from Tocris bioscience or from Sigma Aldrich (UK). All compounds were diluted to working concentrations in a buffer on the day of experimentation.

Compound	Vehicle	Concentration applied	Supplier	Application
Substance-P	H_2O	10μM, 30μM and 50μM	Sigma	Neuropeptide (NK_1- agonist)
Thiorphan	Ethanol	10μM	Sigma	Enkephalinase inhibitor
Captopril	H_2O	10μM	Tocris	Peptidase inhibitor
AM0902	DMSO	1μM, and 3μM	Tocris	TRPA1 antagonist
Aprepitant	DMSO	10μM		NK1 antagonist
BIBN	DMSO	3μM	Tocris	CGPR receptor antagonist
Capsaicin	Ethanol	500nM	Sigma Aldrich	TRPV1 agonist
JYL1421	DMSO	10μM	Tocris	TRPV1 antagonist
Acetylcholine	H_2O	100μM	Sigma Aldrich	Cholinergic receptor agonist
Atropine	Ethanol	10μM	Sigma Aldrich	Cholinergic receptor antagonist
Nifedipine	DMSO	10μM	Sigma Aldrich	Ca^{2+} channel blocker

4.11. Results

4.11.1 NK_1 receptor antagonist attenuates TRPA1-induced colonic afferent excitation

Bath superfusion with ASP7663 (100μM) produced a robust increase in afferent discharge (12.05 ± 2.8, spikes/s; *n=5;* ***see figure 37.D***) which was significantly inhibited by aprepitant (10μM) pre-treatment (e.g., 4.4 ± 1.6 spikes/s; *n=5; P < 0.05;* ***see figure 37.D****)* indicating that the afferent response to TRPA1 agonist stimulation is mediated in part through the release of SP.

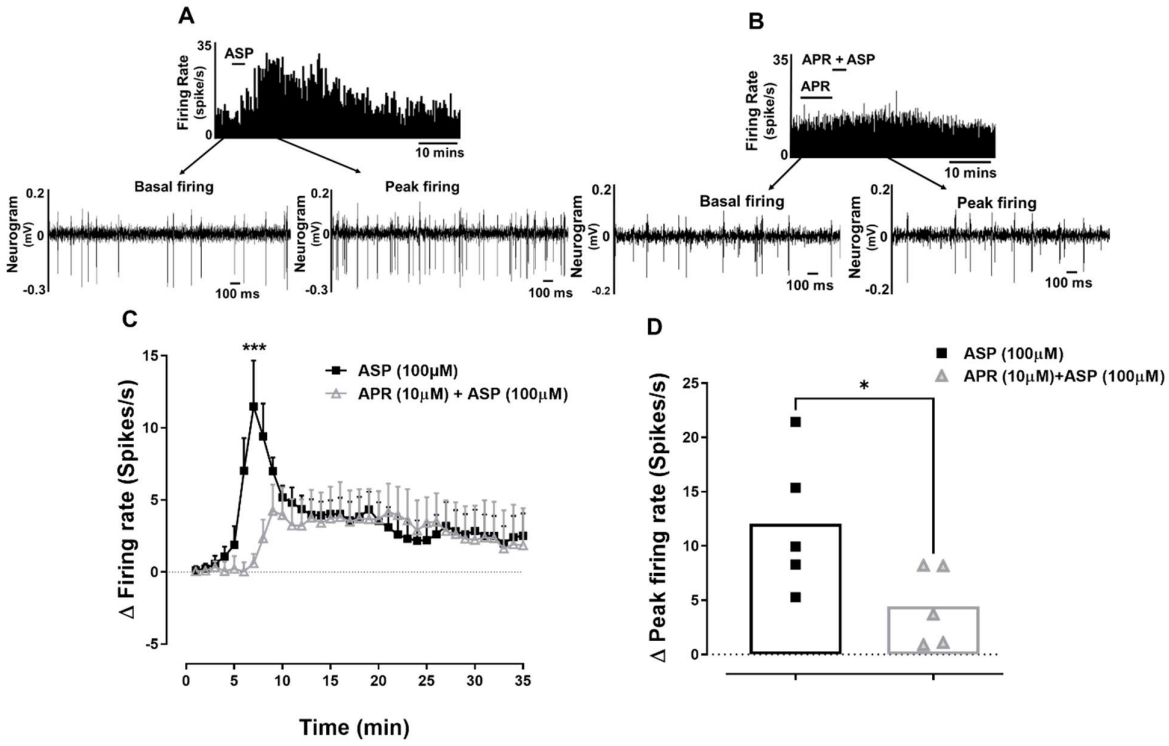


Figure 37: Aprepitant (APR; selective NK1 antagonist) pre-treatment attenuates ASP7663 (TRPA1 selective agonist) response in LSN preparation. A & B: Example of a rate histogram with neurogram trace illustrating the colonic afferent response to ASP7663 (100μM) alone and following pre-treatment with Aprepitant (10μM). **C**: Line graphs showing the change in colonic afferent activity over time

following the application of ASP7663 (100μM) alone and following pre-treatment with Aprepitant (10μM) where *** P < 0.001 analyzed by Two-way ANOVA followed by Šídák's multiple comparisons test. **D.** Bar charts showing the mean ± sem peak change in afferent firing following the application of ASP7663 (100μM) alone and following pre-treatment with aprepitant (10μM); *n=5.* The statistical analysis was performed using unpaired student-t test where, the significance is defined as, * *P < 0.05*.

4.11.2 Capsaicin (TRPV1 agonist) excites colonic afferents and increases afferent activity

Bath perfusion with capsaicin (500nM) produced a robust increase in nerve discharge (e.g., 38.4 ± 5.9 spikes/s increase in nerve discharge *n=5;* ***see figure 38.C***) followed with cessation of afferent firing after attaining the peak response. Ongoing nerve activity returned to levels comparable with those seen prior to capsaicin treatment after an interval of around 30-40 min however, no appreciable colonic afferent response was observed to a second application of capsaicin (e.g., 5.4 ± 1.1 spikes/s increase in nerve discharge *n=3*, *p* < 0.01, ***see figure 38.C***) indicating that the use of a repeat challenge protocol would not be possible with capsaicin.

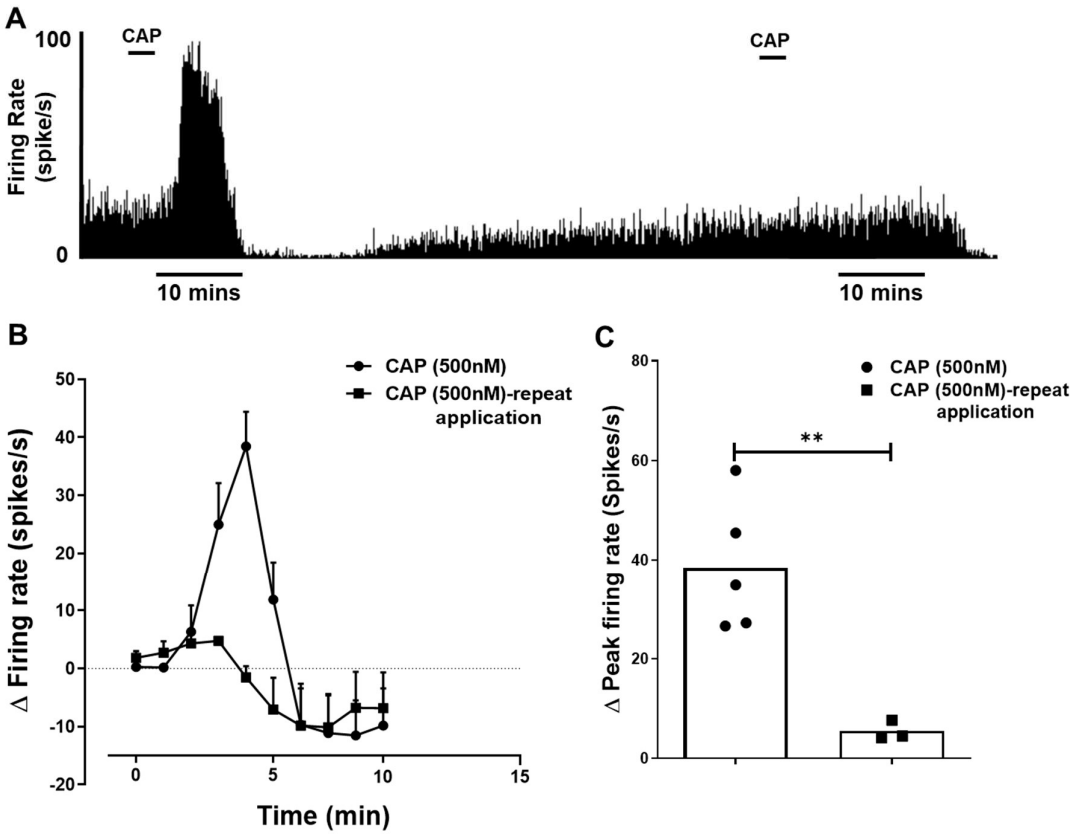


Figure 38: Capsaicin produces robust colonic afferents excitation but shows excessive tachyphylaxis. A: Raw trace illustrating examples of the change in rate histogram of ongoing nerve discharge and exerts of neurogram illustrating the effect of repeated capsaicin (500nM) applications on LSN activity. **B**: Line graph showing the change in nerve discharge over time to first and repeated application of capsaicin (500nM). **C**: Bar chart showing the mean ± sem change in peak nerve discharge following first (*n=6*) and repeated application of capsaicin (500nM); *n=3*. The statistical analysis was performed using unpaired student-t test and where, the significance is defined as ** $P < 0.01$.

Following on from this study we next sought to confirm that colonic afferent response to capsaicin was selectively mediated through the activation of TRPV1 receptors using

the selective TRPV1 antagonist compound-JYL1421 (10μM, 100ml), which prevented the increase in colonic afferent activity to capsaicin (e.g., 4.5 ± 1.3 spikes/s; *n=5; P<0.001;* ***see figure 39.B***).

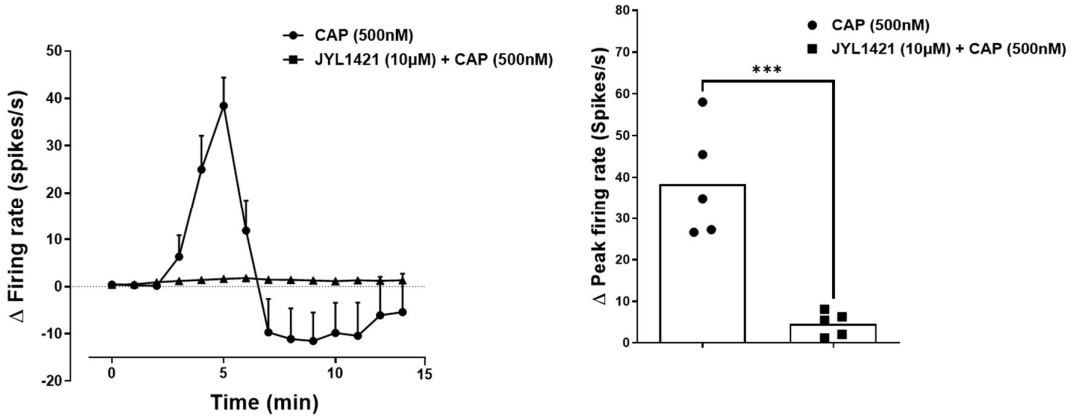


Figure 39: JYL1421 (selective TRPV1 antagonist) abolish response to capsaicin. **A**: Line graph showing the change in nerve discharge over time by application of capsaicin (500nM) alone or by following JYL1421 (3μM) pretreatment. **B**: Bar chart showing the mean ± sem change in peak nerve discharge by application of capsaicin (500nM) alone (n=5) or by following the application of JYL1421 (3μM, *n=5*) pretreatment. The statistical analysis was performed using unpaired student-t test and where, the significance is defined as *** *P < 0.001*.

However, pre-treatment with aprepitant (10μM) does not inhibit the colonic afferent response to capsaicin (e.g., 37.9 ± 11.2 spikes/s; *n=5;* ***see figure 40.D***). Indicating that unlike the colonic afferent response to TRPA1 agonist stimulation the response to capsaicin is not mediated through the release of SP in this preparation.

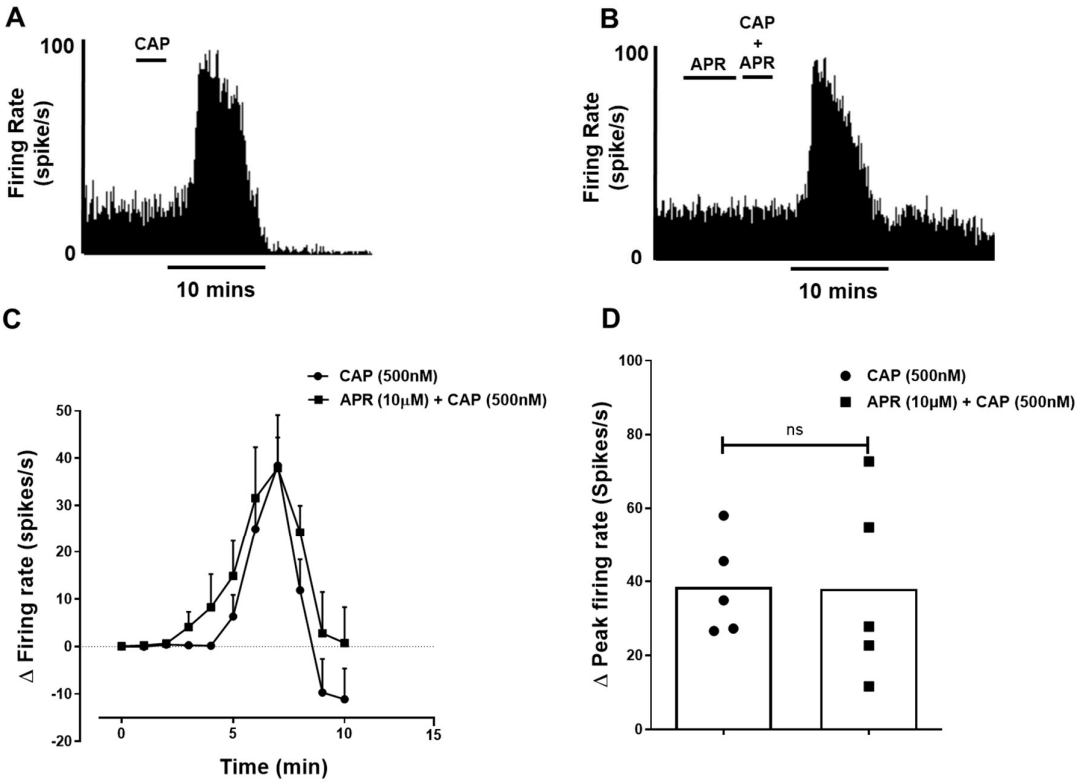


Figure 40: NK_1-antagonist failed to inhibit capsaicin induced colonic afferent excitation. A: Raw trace illustrating examples of the change in rate histogram of ongoing nerve discharge and exerts of neurogram illustrating the effect of capsaicin (500nM) applications alone or (**B**) following aprepitant (10μM) pretreatment on LSN activity. **C**: Line graph showing the change in nerve discharge over time after application of capsaicin (500nM) alone or following aprepitant (10μM) pretreatment on LSN activity. **D**: Bar chart showing the mean ± sem change in peak nerve discharge after application of capsaicin (500nM, *n=5*) alone or following aprepitant (10μM, *n=5*) pretreatment on LSN activity. The statistical analysis was performed using unpaired student-t test where, no significant deference was observed.

4.11.3. Substance-P (SP) excites colonic afferents

To confirm a contribution of SP to colonic afferents activation we examined the response to increasing concentrations of Substance-p (10μM, 30μM and 50μM; one concentration tested per experiment) observing only a modest increase in afferent activity. However, the response to SP was significantly increased following co-application with enkephalinase and peptidase inhibitors (e.g., SP (50μM) elicited an increase in afferent discharge of 4.7 ± 1.3 spikes/s; *n=5* compared with 11.4 ± 1.8 spikes/s, *n=5, P<0.01 in the absence vs presence of peptidase inhibition;* ***see figure 41.B***). This response was attenuated by pre-treatment with aprepitant (e.g., 3.3 ± 0.9 spikes/s, *n=6,* P<0.001).

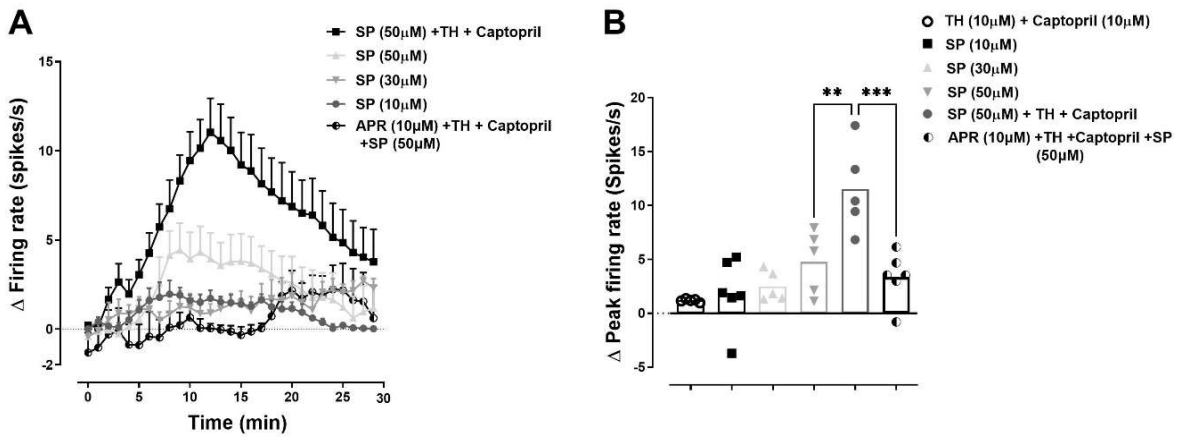


Figure 41: Substance P excites colonic afferent. A: Line graphs showing the change in colonic afferent activity over time following the application of increasing concentrations of Substance-p (10μM, 30μM and 50μM) alone and the response of SP (50μM) in presence of peptidase inhibitors [captopril (10μM) + thiorphan (10μM)] and selective NK_1-antagonist aprepitant (10μM). **B.** Bar charts showing the mean ± sem peak change in afferent firing following the application of increasing concentrations of

Substance-p (10μM, 30μM and 50μM) alone and the response of SP (50μM) in presence of peptidase inhibitors [captopril (10μM) + thiorphan (10μM)] and selective NK_1-antagonist aprepitant (10μM). The statistical analysis was performed using ordinary one-way ANOVA followed by Tukey's Multiple comparison test where, the significance is defined as, ** *P < 0.01* and *** *P < 0.001.*

4.11.4 Peptidase inhibitors increases duration of action for TRPA1 agonist

Following on from this we speculated that peptidase inhibitor pre-treatment would also enhance the activity of ASP7663. However, the magnitude of the ASP7663 response obtained in presence of peptidase inhibitors (e.g., 13.03 ± 1.8, *n=7*) was comparable to ASP7663 treatment alone group (e.g., 13.13 ± 2.8, *n=6;* ***see figure 42.B***) but , the duration of the colonic afferent response to ASP7663 was substantially greater, as shown by calculating the area under curve (AUC) following the application of ASP7663 (100μM, 20ml) alone (e.g., AUC: 178 ± 40.2; *n=6;* ***see figure 42.C)*** and in presence of peptidase inhibitors (e.g., AUC: 211.3 ± 43.9; *N=7*; ***see figure 42.C***). The following results further provides evidence supporting the contribution from SP to the colonic afferent response to TRPA1 agonist stimulation. We do not observe any substantial effects of thiorphan and captopril on its own in colonic afferents.

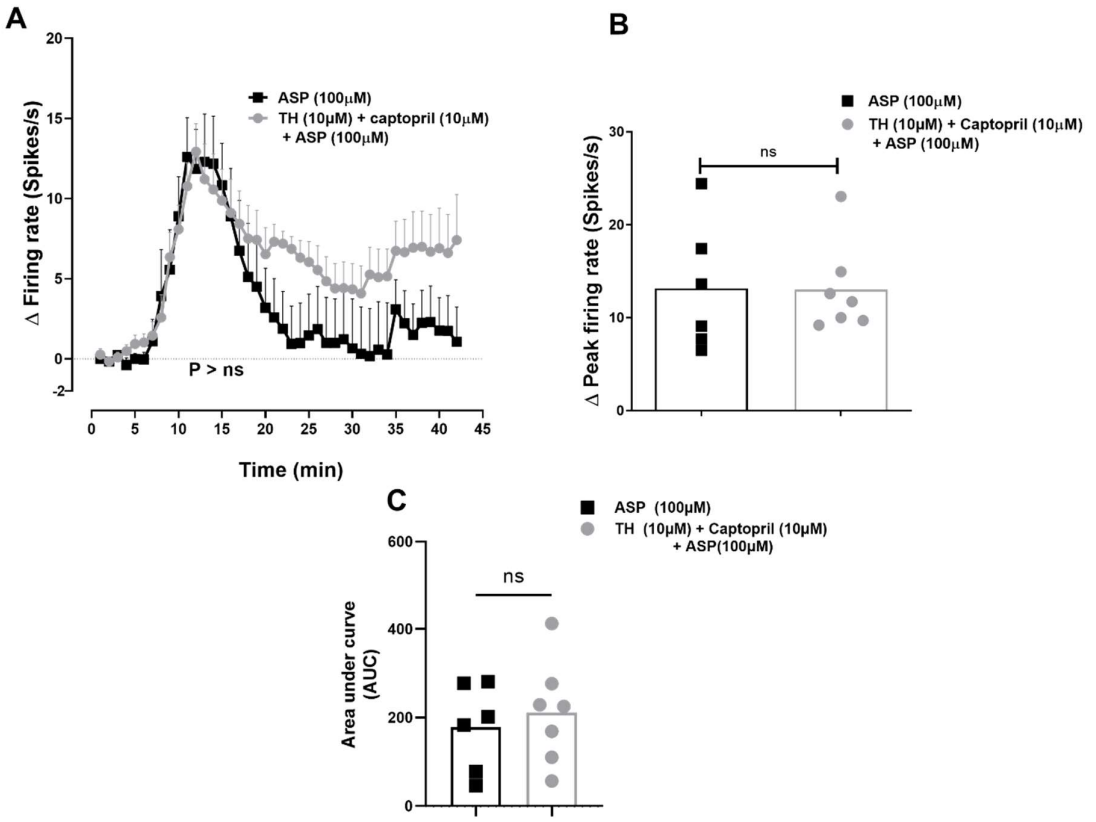


Figure 42: Peptidase inhibitor pre-treatment increases ASP7663 (TRPA1 selective agonist) response duration in LSN preparation. A: Line graphs showing the change in colonic afferent activity over time following the application of ASP7663 (100μM) alone and following pre-treatment with peptidase inhibitors [captopril (10μM) and Thiorphan (10μM)]. The statistical analysis was performed using Two-way ANOVA where, no significant difference was observed. **B.** Bar charts showing the mean ± sem peak change in afferent firing following the following pre-treatment with peptidase inhibitors [captopril (10μM) and Thiorphan (10μM]; *n=7.* **C**. Bar charts showing the area under curve (AUC) obtained following the application of ASP7663 (100μM; *n=6*) alone and in presence of peptidase inhibitors thiorphan (10μM) and captopril (10μM). The statistical analysis was performed using unpaired student-t test where, no significant difference was observed for the graph B and C.

4.11.5 CGRP is relatively less involved in TRPA1 induced colonic afferent excitation as compared to SP

Upon confirming the crucial involvement of SP, we next investigated the role of CGRP, to TRPA1 activation of colonic afferents. However, the response to application of ASP7663 (100μM) in presence of selective CGRP receptor antagonist, BIBN (3μM) was not significantly different to ASP7663 alone (10.16 ± 3.1 spikes/s vs 13.13 ± 2.8 spikes/s, *n=6;* ***see figure 43.B***). Simultaneously, the AUC for ASP7663 pre-treatment alone and in presence of BIBN (3μM) shows a non-significant reduction (e.g., 178 ± 40.2 vs 103.5 ± 32, ***See figure 43.C***) suggesting that CGRP release have limited contribution to the change in nerve activity elicited by TRPA1 agonist stimulation.

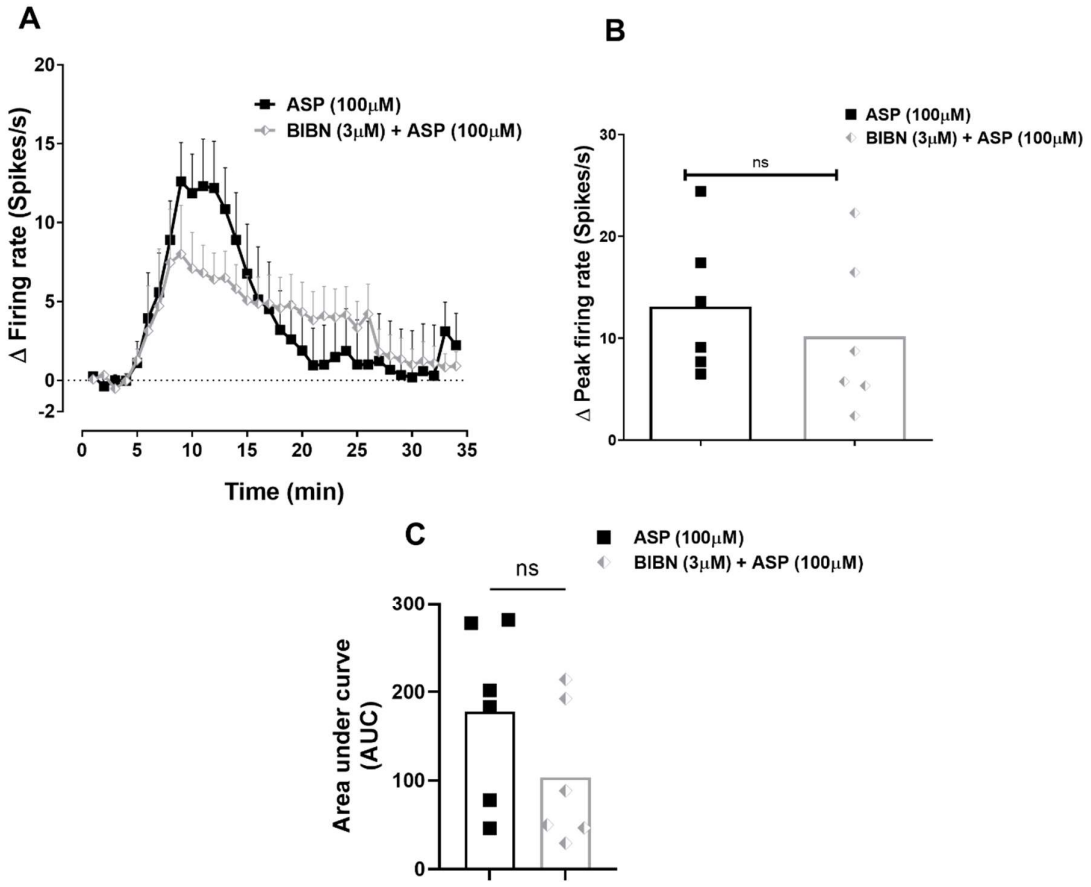


Figure 43: CGRP antagonist moderately reduces TRPA1 induced colonic afferent excitation. A: Line graphs showing the change in colonic afferent activity over time following the application of ASP7663 (100μM) alone and following pre-treatment with BIBN (3μM). The statistical analysis was performed using Two-way ANOVA where, no significant difference was observed.. **B.** Bar charts showing the mean ± sem peak change in afferent firing following the application of ASP7663 (100μM*; n=6*) alone and following the pre-treatment with BIBN (3μM, *n=6*). **C**. Bar charts showing the area under curve (AUC) obtained following the application of ASP7663 (100μM; *n=6*) alone and in presence of CGRP receptor antagonist BIBN (3μM, *n=6*). The statistical analysis was performed using unpaired student-t test where, no significant difference was observed for graph B and C.

4.11.6. Substance-P increase colonic afferents sensitivity to ramp distention

We next sought to determine the contribution of SP to the sensitisation of colonic afferents to luminal distension following pre-treatment with the TRPA1 agonist ASP7663. To begin we first examined the effect of SP (50μM) given in the presence of peptidase inhibitors sensitizes colonic afferent response to ramp distention observing a significant increase in response 130.8% ± 3.0%, *N=5*, P< 0.0001 compared with 95.5% ± 3.1%, *N=8* in vehicle treated tissue*; **see figure 44.C***.

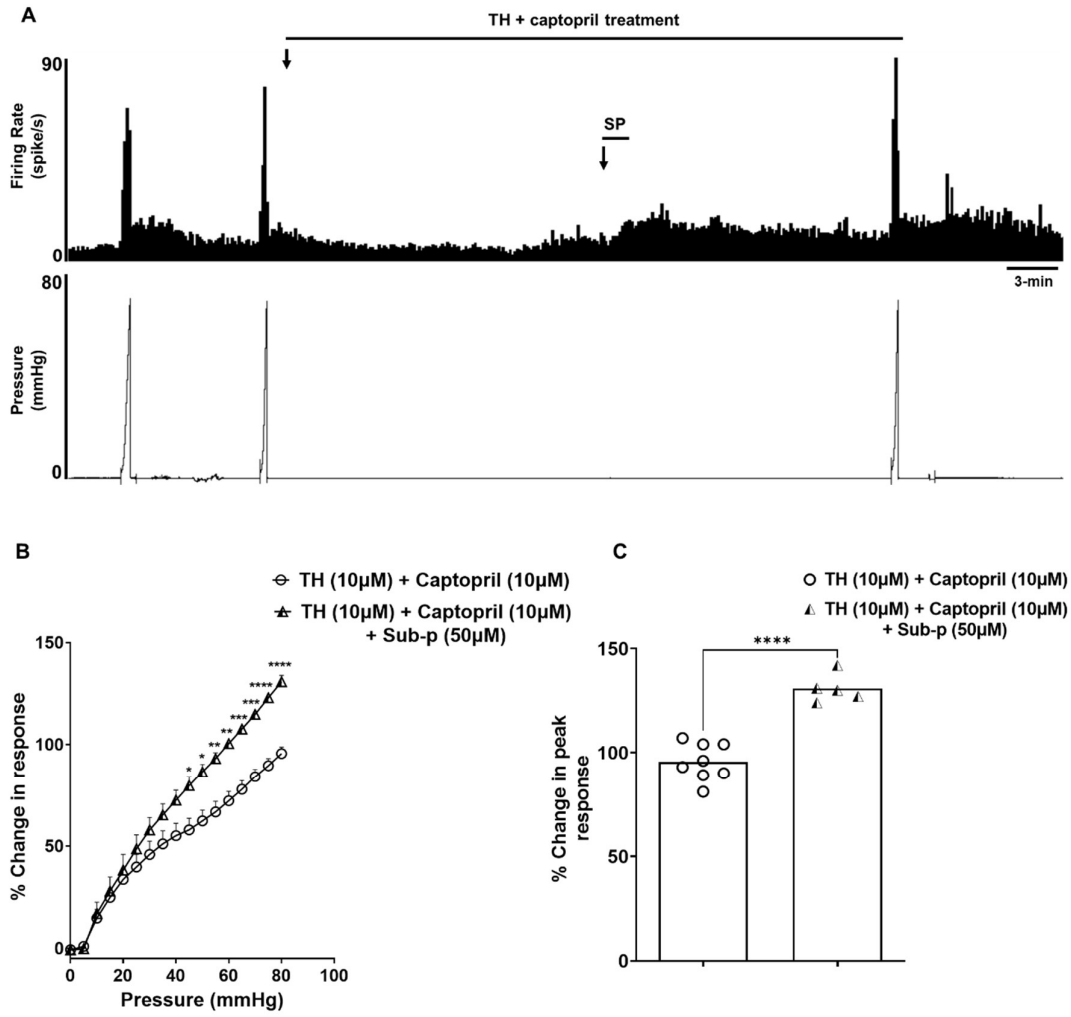


Figure 44: Substance P sensitizes colonic afferents to ramp distention. **A.** Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg; 100sec; 10 min intervals) in C57B6/J mice alone and pre-treated with SP (50μM, 20ml) in presence of peptidase inhibitors [captopril (10μM) and Thiorphan (10μM)]. **B.** Line graph showing effect of Substance P in presence of peptidase inhibitors [captopril (10μM) and Thiorphan (10μM)] on colonic splanchnic nerve response to 0-80mmHg ramp distention. The statistical analysis was performed using 2-Way ANOVA followed by Bonferroni's multiple comparisons test where, the significance is defined as, * $P < 0.05$, ** $P < 0.01$, ***$P< 0.001$ and **** $P < 0.0001$,

respectively. **C**. The bar graph shows the peak effect of Substance P in presence of peptidase inhibitors [captopril (10μM) and Thiorphan (10μM)] on colonic splanchnic nerve response to 0-80mmHg ramp distention. The statistical analysis was performed using unpaired student-t test where, significant difference was defined as **** P < 0.0001, respectively.

Next we examined the effect of NK_1 antagonist pre-treatment with aprepitant (10μM) on the colonic afferent in ramp distention alone and during the sensitisation of the colonic afferent response to ramp distension following ASP7663 (100μM). We found that pre-treatment of aprepitant (10μM) has no effect on distention induced afferent activation (e.g., 24.4 ± 3.7 spikes/s vs 24.7 ± 4.5 spikes/s, *n=7,* ***see figure 45.C***).

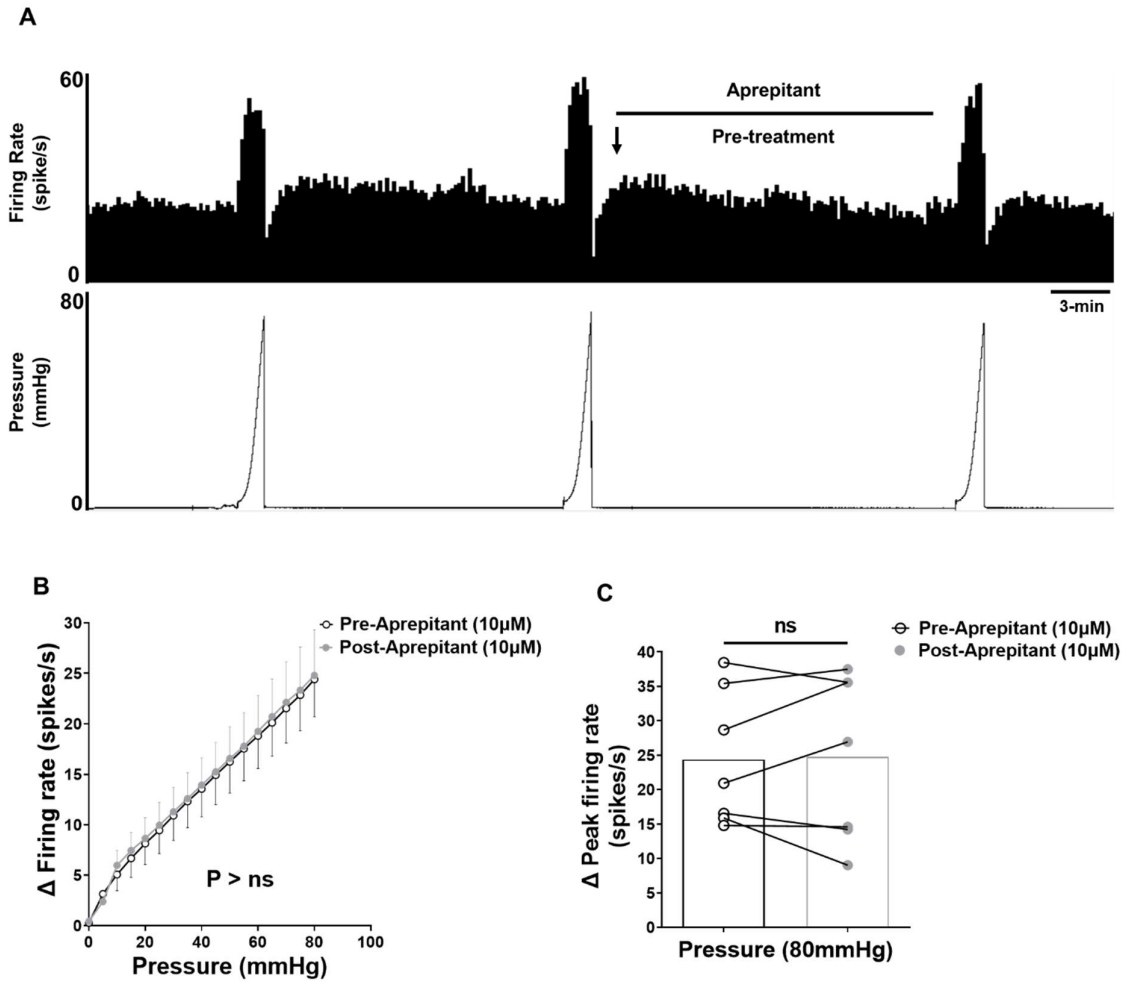


Figure 45: Aprepitant does not influence mechanotransduction in colonic afferents. A. Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg; 100sec; 10 min intervals) in C57B6/J mice alone and pre-treated with aprepitant (10μM, 100ml). **B.** Line graph showing the change in colonic afferent activity following the application of aprepitant (10μM, 100ml) on colonic splanchnic nerve response to 0-80mmHg ramp distention. The statistical analysis was performed using Two-way RM ANOVA where no significant difference was observed. **C**. The bar charts showing the mean ± sem peak change in afferent firing following the application of aprepitant (10μM, 100ml) on colonic splanchnic nerve response at 80mmHg of distention. The statistical analysis was performed using paired student-t test where no significant difference was observed.

However, pre-treatment with aprepitant abolished ASP7663 (100μM) induced mechanosensitisation (e.g., 98.8% ± 3.3%; *n=7*; ****P<0.001*) as compared to ASP7663 treatment alone (e.g., 132.0 ± 6.4%, *N=7;* ***see figure 46.C***). Additionally, we also checked for the compliance including cumulative time (e.g., 45.8 ± 2.7 sec vs 44.3 ± 1.7 sec; *n=5;* ***see figure 46.D***) and cumulative volume (e.g., 76.3 ± 4.5μl vs 73.9 ± 2.9μl; *n=5;* ***see figure 46.E***) between the pre-aprepitant and post-aprepitant (10μM, 100ml) treated distention and found no significant difference. The above result implicates that the TRPA1 agonist induced SP release contributes to the sensitisation of colonic afferents to luminal distension following TRPA1 activation.

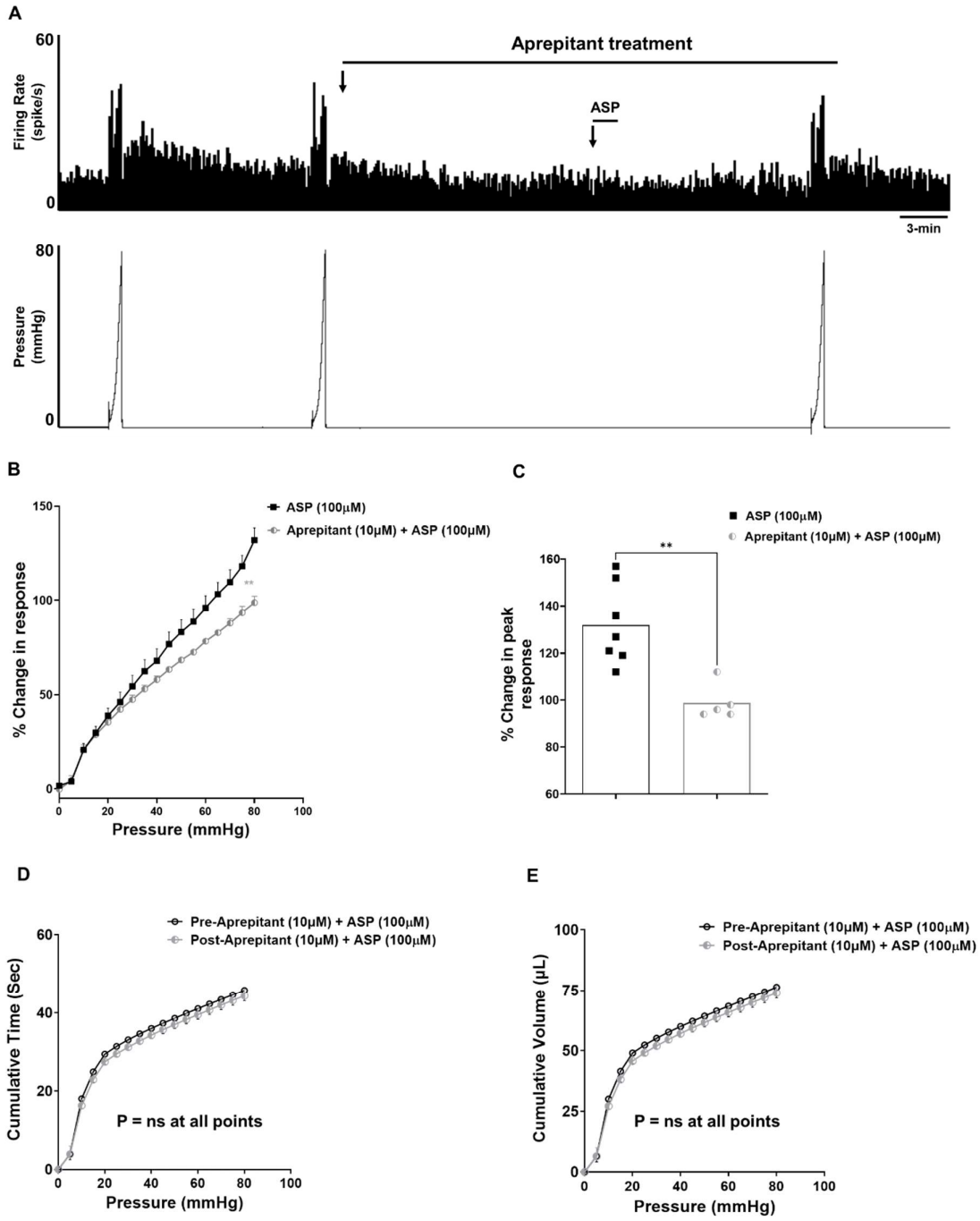


Figure 46: Aprepitant attenuates ASP7663 induced mechanosensitisation in colonic afferents to ramp distention. **A.** Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg; 100sec; 10 min intervals) in C57B6/J mice alone and pre-treated with ASP (100μM, 20ml) in presence of aprepitant. **B**. Line graph showing effect of ASP7663 (100μM) alone and following pre-

treatment with Aprepitant (10μM) on colonic splanchnic nerve response to 0-80mmHg ramp distention. The statistical analysis was performed using 2-Way ANOVA followed by Tukey's multiple comparison test where, significance is defined as ** P < 0.01 respectively. **C**. The bar graph shows the peak effect of ASP7663 (100μM) alone and following pre-treatment with Aprepitant (10μM) on colonic splanchnic nerve response to 0-80mmHg ramp distention. The statistical analysis was performed using unpaired student t-test where, significance is defined as ** P < 0.01 respectively. **D**. Cumulative time (sec) and **E**. Cumulative volume (μl) is plotted against increasing pressure (0-80mmHg, with every 5mmHg rise in pressure) for pre-aprepitant and post-aprepitant distention's (P > 0.99; two-way ANOVA, *n=5*).

4.11.7. TRPA1 and SP induced colonic contractility

Substance P contributes to the regulation of gut motility following its release from neurons within the enteric nervous. Additionally, stimulation of extrinsic sensory nerves innervating the bowel, typically through the application of TRP channel agonists has been shown to induce colonic contraction through the release of SP (Yang et al., 2019). For example, the TRPA1 agonist AITC mediates contraction of mouse proximal and distal colon, and this effect is attenuated by tetrodotoxin pre-treatment but not muscarinic antagonists suggesting involvement of neurogenic process. The downstream neurotransmitter of this TRPA1 induced colonic contraction is SP with atropine-insensitive contractions in the porcine ileum being abolished by the neurokinin-1 (NK_1) receptor antagonists (Schmidt & Hoist, 2002). The regulation of gut motility by SP is disease relevant with decreased SP levels being found in patients

with chronic severe constipation (Goldin et al., 1989) and low SP levels reported in the right transverse colon of the paediatric patient suffering from slow-transit constipation (King et al., 2010) or rectal biopsies from patients with slow transit constipation (Tzavella et al., 1996).

We therefore next sought to exploit the measurement of gut contraction as a means of investigating the effect of the GPR35 agonist cromolyn on TRPA1 agonist mediated SP release from sensory nerves. Consistent with the literature we found that bath application of ASP7663 (100μM) elicited a robust contraction of colonic tissue which was abolished following pre-treatment with either the TRPA1 antagonist AM0902 (3μM) or the NK_1 antagonist aprepitant (10μM). For example, ASP7663 evoked contractions were 58.06 ± 6.30 % (N=7; P<0.0001) of respective ACh responses compared with -4.8 ± 4.0%, in tissues pre-treated with AM0902; *n=5*, p<0.0001 and -0.5 ± 5.7 in aprepitant pre-treated tissues; *n=8*, p < 0.0001*;* ***see figure 47.B***. Contractile response could still be elicited in response to ACh (100μM) following treatment with AM0902 or aprepitant and these compounds does not elicit any effect on gut contractility when given alone (data not shown).

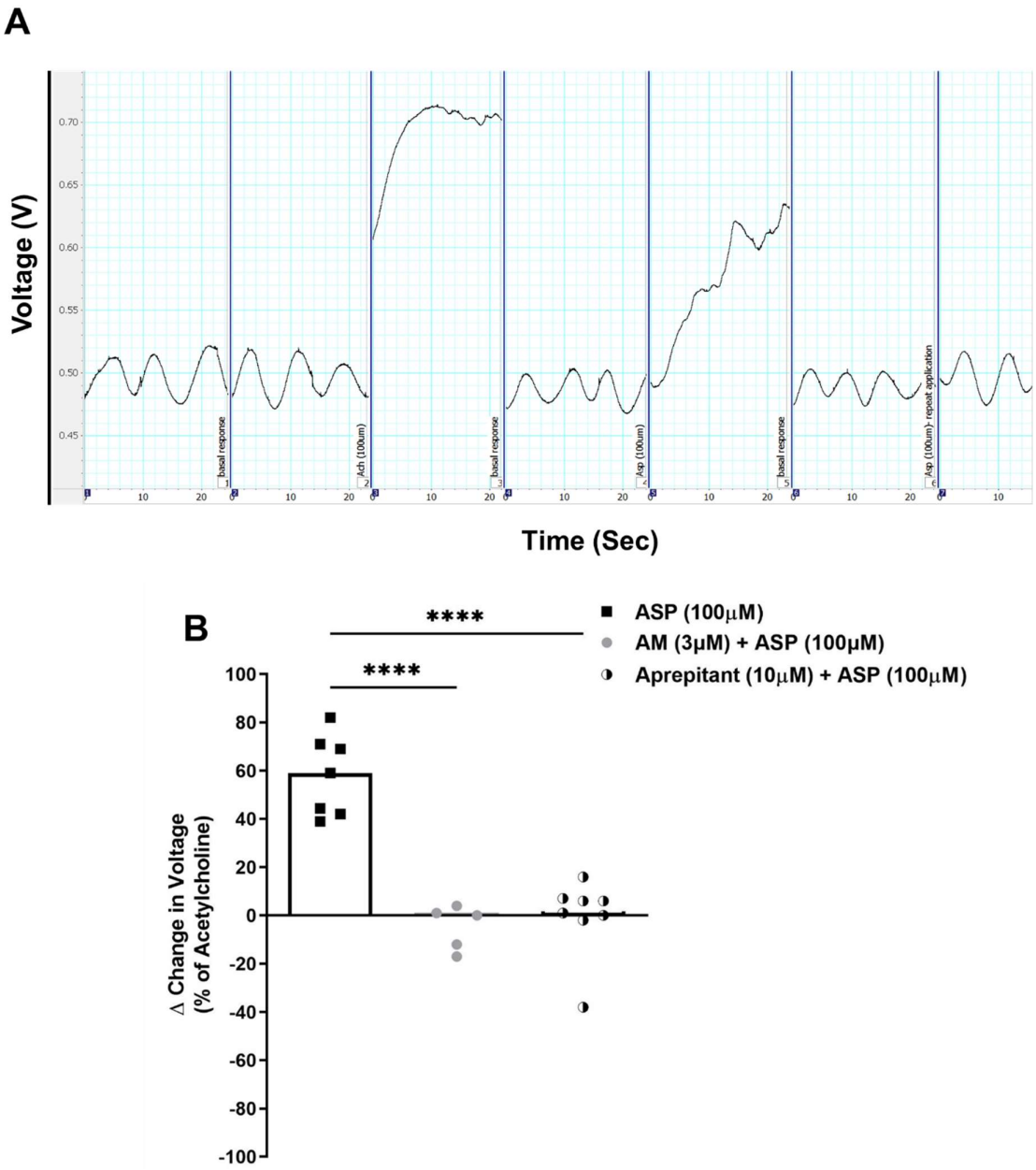


Figure 47: TRPA1-agonist (ASP7663, 100μM) induced transient contraction of mouse distal colon was abolished by pre-treatment with TRPA1-antagonist (AM0902, 3μM) and selective NK1-antagonist (aprepitant, 10μM). A: Raw data showing change in voltage (V) following application of acetylcholine (100μg/ml, 5μl) and ASP7663 (100μM, 20μl) repeat application. **B**: Data plots and bar graphs showing respective isotonic contractions of mouse distal colon tissue, elicited by ASP7663 alone (100μM; *n*=7) alone or following pre-treatment with AM0902 (3μM, *n*=*5*) and

Aprepitant (10μM; *n=8*). Responses are normalised to preceding application of acetylcholine (100μM). Bar graphs represent mean values. The Data is expressed as (mean ± sem) and the statistical comparisons were performed using an unpaired Student t-test **** denoting P<0.0001.

Furthermore we found that pre-treatment with cromolyn (100μM) significantly attenuated the contractile response to ASP7663 (100μM) in wild type C57B6 mice (e.g., 15.86 ± 3.3%; *n=7*; p<0.0001*;* ***see figure 48.B***) but not in the colonic tissue isolated from GPR35-/- mouse (e.g., 49.33 ± 2.7%; *n=6*) where the response was comparable to the contractile response produced by ASP7663 (100μM) alone (e.g., 58.06 ± 6.30%, *n=7*).

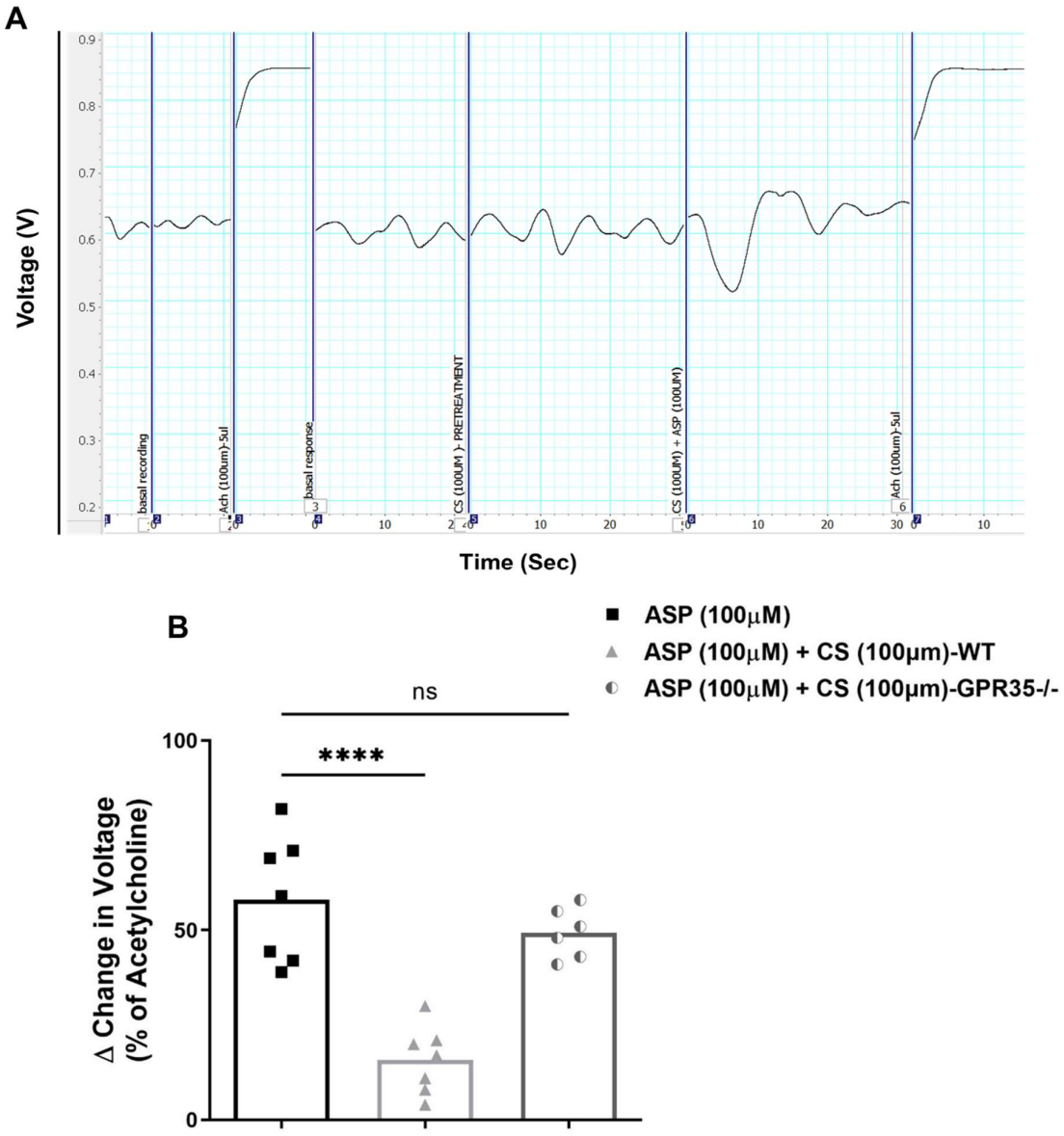


Figure 48: TRPA1-agonist (ASP7663, 100μM) induced transient contraction of mouse distal colon was abolished by pre-treatment with CS (100μM) in C57B6-WT mouse, but CS failed to antagonise contraction effects in colonic tissues obtained from GPR35-/- mouse. A: Raw data showing change in voltage (V) following application of acetylcholine (100μg/ml, 5μl) and ASP7663 (100μM, 20μl) in presence of CS (100μM).**B:** Data plots and bar graphs showing respective isotonic contractions of mouse distal colon tissue, elicited by ASP7663 alone (100μM; *n*=7)

alone or following pre-treatment with CS (100μM, *n=7*) in C57B6-WT mouse and CS (100μM; *n=6*) pre-treatment in GPR35-/- mouse. Responses are normalised to the preceding application of acetylcholine (100μM). Bar graphs represent mean values. The Data is expressed as (mean ± sem), and the statistical comparisons were performed using an unpaired Student t-test ****, denoting P<0.0001.

4.11.8. GPR35 receptor activation by CS attenuates SP release in colonic tissue of mice

Finally, having confirmed that agonist activation of TRPA1 receptor stimulate colonic afferents, augment mechanosensitisation and induces colonic contractility in an NK_1 dependent manner, and that these effects could be blocked by GPR35 agonists, we next sought to generate direct evidence for TRPA1 agonist mediated colonic SP release and its inhibition by the GPR35 agonist cromolyn through GPR35 receptors. Consistent with our functional data we found that incubation of the colonic tissue for 10 min in ASP7663 (100μM) induces a significant increase in the release of SP (e.g., 11.31 ± 3.84; *n=10; P < 0.05*) which was significantly attenuated by 10min pre-incubation with AM0902 (3μM) prior to the application of ASP7663 (100μM) (e.g.,1.39 ± 0.48; *n=8; p< 0.05;* ***see figure 49***). Furthermore pre-treatment with either cromolyn (10μM) or cromolyn (100μM) substantially inhibited ASP7663 mediated SP release from colonic tissues (e.g., 0.85 ± 0.33; *n=6* and 0.56 ± 0.13; *n=7; p< 0.05;* ***see figure 49***) a response lost in colonic tissue segments from GPR35-/- mice (e.g., 14.08 ± 3.7; *n=7*; *P< 0.01;* ***see figure 49***) confirming responses to cromolyn were mediated *via* the GPR35 receptor. ASP7663 mediated SP release was comparable between tissue from wild type and GPR35 -/- mice.

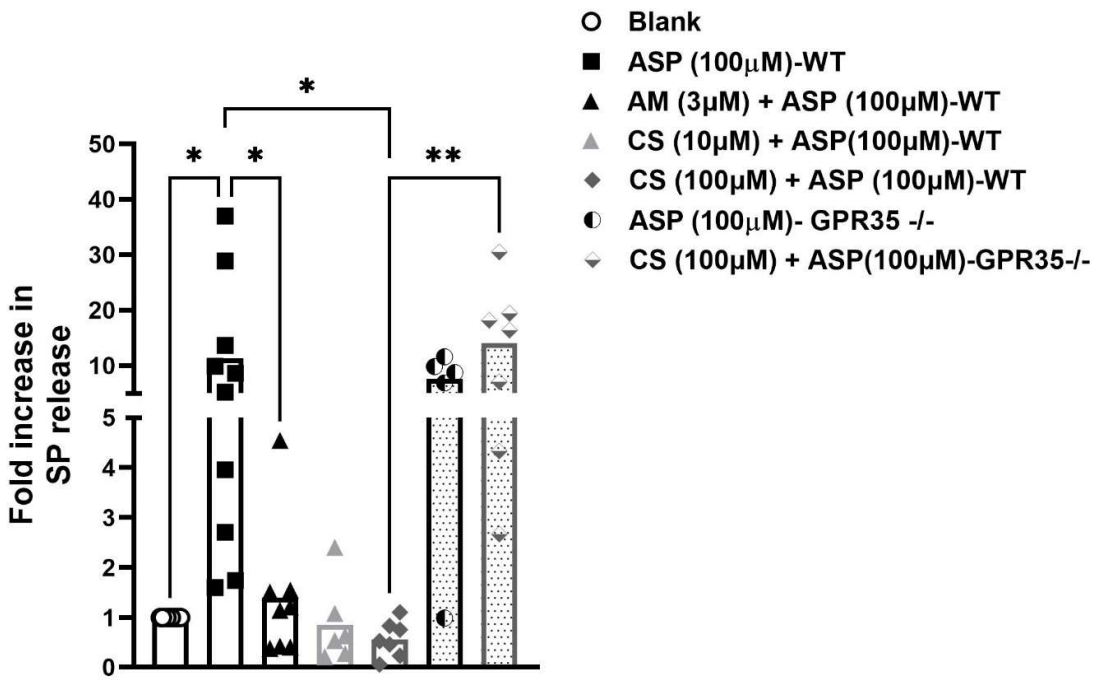


Figure 49: TRPA1 agonist ASP7663 induced SP release is attenuated by CS pre-treatment in C57B6-WT mice, but not in GPR35-/- mice distal colon tissues. The release of SP was determined using chemiluminescent immunoassay kit (CLIA). The data plots and bar graph showing fold change increase in SP release from distal colon tissues after application of ASP7663 (100μM) alone or following pre-treatment of CS (10, 100μM) in C57B6-WT mice and GPR35-/- mice. The values are expressed as (mean ± sem) and the statistical analysis was performed using ordinary one-way ANOVA followed by Tukey's Multiple comparison test where, the significance is defined as, * P< 0.05, and ** *P < 0.01.*

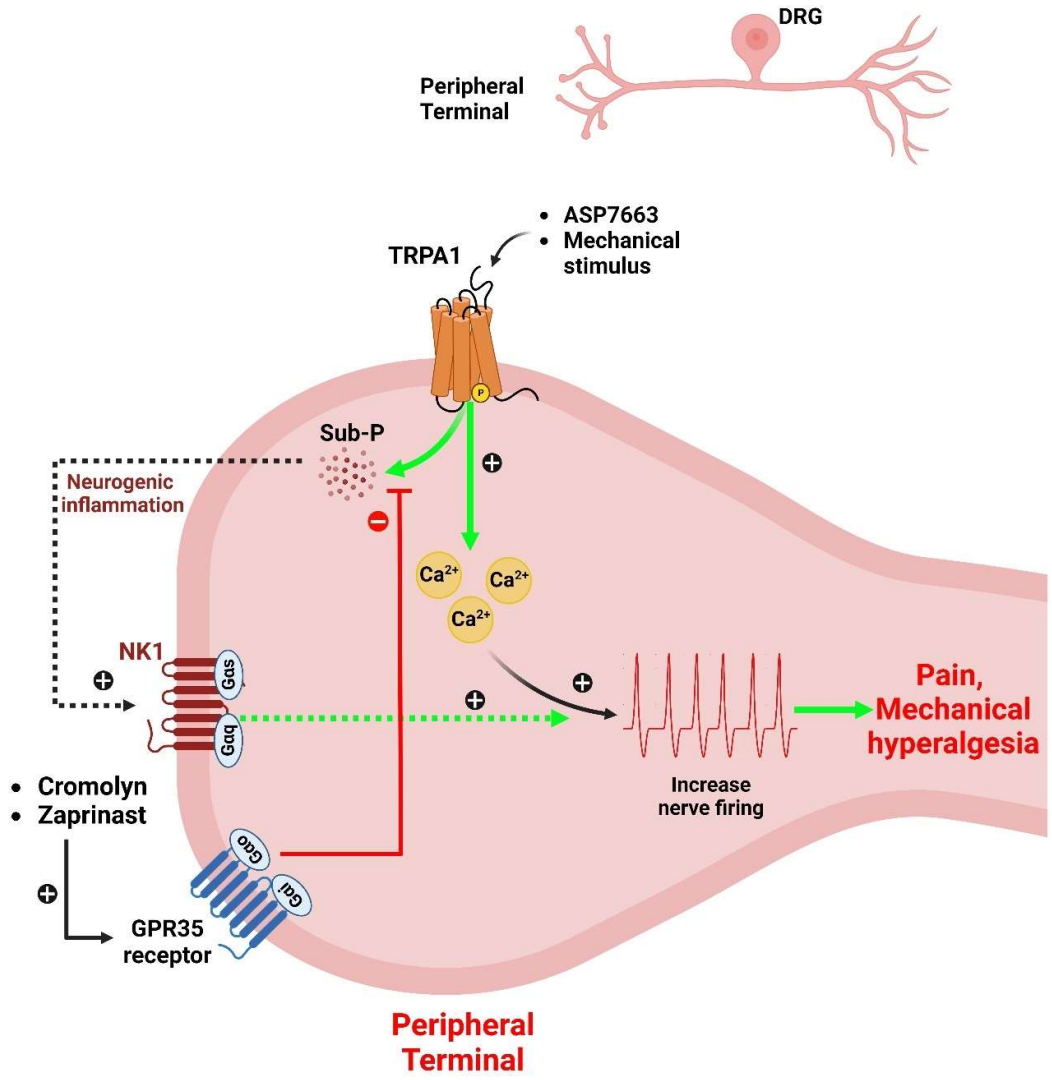


Figure 50. GPR35 activation inhibit TRPA1-induced SP release and restrict its subsequent excitatory effects on colonic afferents. TRPA1 activation induces influx of Ca^{2+} ions resulting into excitation and sensitisation of the colonic afferents to noxious mechanical stimuli. Activation of TRPA1 also induces the release of SP, which exerts excitatory and sensitising effects on colonic afferents. SP also increases colonic contractility and augments neurogenic inflammation, indirectly contributing to increase afferent firing.

4.12. Discussion

Our results collectively show that the GPR35 agonist cromolyn attenuates TRPA1-induced colonic contraction and the SP release from the distal colon tissues by activating the GPR35 receptors. These findings providing an explanation for how cromolyn inhibits TRPA1-induced activation of colonic afferents and sensitisation of colonic mechanosensitivity. In support of this we also show that SP can stimulate colonic afferents and sensitise their response to noxious bowel distension. This effect is NK_1 receptor dependent.

TRPA1 channels can evoke neurogenic inflammation by releasing neuropeptides such as SP and CGRP exacerbating pain, inflammation, and gut motility in colitis (Meseguer et al., 2014; Engel et al., 2011). Although SP is a well-studied neuropeptide, surprisingly, the effect of SP on colonic afferent activity and its contribution to TRP-channel mediated visceral nociceptor activation have not been extensively studied. We hypothesise that the release of SP from sensory nerve endings may contribute to TRPA1 channel-mediated colonic afferent activation and sensitisation. Investigating further, we found that SP in the presence of peptidase inhibitors can stimulate colonic afferents and consistent with this NK_1 antagonist pre-treatment significantly attenuates TRPA1 induced colonic afferent excitation and peptidase inhibitor pre-treatment significantly enhances TRPA1 induced colonic afferent excitation suggesting a crucial involvement of SP in TRPA1 mediated responses.

Interestingly, the afferent response to capsaicin was unchanged in the presence of aprepitant, however it may be the significant inhibition of colonic afferent activity seen

following application of capsaicin and putative response to TRPV1 mediated SP release.

Apart from the SP, CGRP may also be released following the activation of TRPA1 on colonic afferents. Although CGRP is a vital contributor to neurogenic inflammation (Engel et al., 2011) we found that pre-treatment with selective CGRP receptor antagonist BIBN had only a modest effect on the ASP7663 response profile and did not alter the peak response, suggesting limited contribution of CGRP in TRPA1 mediated stimulation of colonic afferents.

SP produced mechanical allodynia in wild-type mice (Yamashita et al., 1993; Sahbaie et al., 2009). while a mouse with deleted pre-protachykinin A gene (*ppt-A* −/−) which codes for SP, displayed reduced mechanical allodynia and heat hyperalgesia in post-incisional pain. Similarly, NK_1 receptor antagonist has also been shown to attenuate mechanical allodynia (Sahbaie et al., 2009). Although it is widely accepted that SP signalling facilitates nociceptive sensitisation and widely influences mechanosensitivity, a study investigating the direct action of SP on colonic afferent mechanosensitivity is missing from the literature. To bridge the gap and understand SP's contribution to colonic mechanosensitivity, we investigated its effects in the ramp distention paradigm. Pre-treatment of SP in the presence of peptidase inhibitors thiorphan and captopril stimulates colonic afferent and sensitises the colonic afferent response to distention. These effects are NK_1 mediated although NK receptors are not directly involved in the afferent response to gut distension.

Consistent with this observation we also found that TRPA1-induced contraction was attenuated by NK_1 antagonist aprepitant pre-treatment and significantly inhibited by

cromolyn pre-treatment in tissue from WT but not GPR35-/- mice confirming that cromolyn attenuates TRPA1-induced colonic contraction by its GPR35 agonist effect. These data and the observations from the previous chapters of this thesis indicating that GPR35 agonists inhibit agonist but not mechanical activation of TRPA1 channels, and this effect is due to an inhibitory effect on SP release downstream to TRPA1 activation. With our SP release assay confirming that colonic TRPA1 agonist mediated SP release is inhibited by cromolyn in a manner dependent on the expression of GPR35.

Chapter-5

Role of GPR35 in attenuation of colonic afferent response to the prototypic inflammatory and algogenic mediator

5.1. Introduction

While assessing the antinociceptive role of GPR35 in visceral afferent function, it is useful to consider the pro-nociceptive stimuli which may be present in the disease condition. Such “disease relevant” stimuli will likely participate in the pathogenesis of visceral pain associated with IBD or IBS. In these conditions, tissue damage, inflammation or altered function will lead to the release of multiple mediators that act to sensitise afferent terminals (Grundy, 2004; Su and Gebhart, 1998). The mediators may include algogenic mediators such as bradykinin, ATP, PGE2, and 5-HT, which are released by damaged or stressed cells, as well as the immune and vascular systems. The continuous exposure to these mediators modulates the response properties of sensory nerve fibres to subsequent exogenous stimuli such as bowel distension leading to prolonged nerve stimulation due to a reduced threshold for activation (Grundy, 2004). Therefore, it is essential to investigate the effect of GRP35 agonist on the modulation of colonic afferent function by such inflammatory or algogenic mediators as such we sought to examine the effect of the GPR35 agonists on the afferent response to PGE2 or bradykinin.

Prostaglandin-E2 (PGE2) is an autacoid derived from arachidonic acid by the enzymatic actions of cyclooxygenase 1 (COX1) and COX2 enzymes. The COX-pathway generate five bioactive prostanoids such as prostaglandin E2 (PGE2), PGI2, PGD2, PGF2α and thromboxane. Amongst these prostaglandin-E2 (PGE2) is the most studied being abundantly produced in inflamed tissues and contributes to the

generation of inflammatory hyperalgesia (Ferreira, 1972; Grundy, 2004). For example, prostaglandin-E2 infusion and I.M injections are painful in humans and lead to hyperalgesia (Ferreira, 1972). PGE2 can directly excite nociceptors and potentiate the sensitising effects of other algogenic mediators such as ATP, bradykinin, and capsaicin (Moriyama et al., 2005; Vanegas and Schaible, 2001; Wang et al., 2007; Zhang et al., 2008). These effects are mediated *via* EP1-EP4 receptor subtypes with the different subtypes contributing to nociception *via* their different downstream signalling cascades. For example, PGE2 sensitises TRPV1 responses in cultured sensory neurons by activating adenylyl cyclase and PKA-mediated TRPV1 phosphorylation, *via* EP2 receptors (Kwong and Lee, 2002) and EP4 receptors (St-Jacques and Ma, 2014). In addition, PGE2 also enhances TRPV1 channel activity through EP1 receptors *via* a PKC-dependent pathway (Moriyama et al., 2005). Recently PGE2 produced by colonic mast cells from IBS patients have shown to augment VH to mechanical distention in mice (Grabauskas et al., 2020).

5.2. Aim:

To investigate the effects of GPR35 agonists on the colonic afferent response to the prototypic inflammatory and algogenic mediators we will:

- Investigate the effect of GPR35 agonists (zaprinast and cromolyn) on colonic afferent response to PGE2-induced sensitisation of colonic afferent response to distension.
- Investigate the GPR35 agonists on the colonic afferent response to bradykinin

5.3. Experimental protocols

The mice (C57B6-WT and CD-1) colon tissue was prepared, and the electrophysiological recording was made following the previously described procedure in method section. The excitatory effect of repeated bradykinin (1μM, 20ml) bath superfusion was determined on the colonic preparation isolated from CD-1 mice following an interval of 1-hour. The inhibitory effects of cromolyn (50μM, 50ml) and NK_1-antagonist, aprepitant (10μM, 100ml) pre-treatment was also determined on bradykinin (1μM, 20ml) bath application using isolated colonic preparation from C57B6-WT mice.

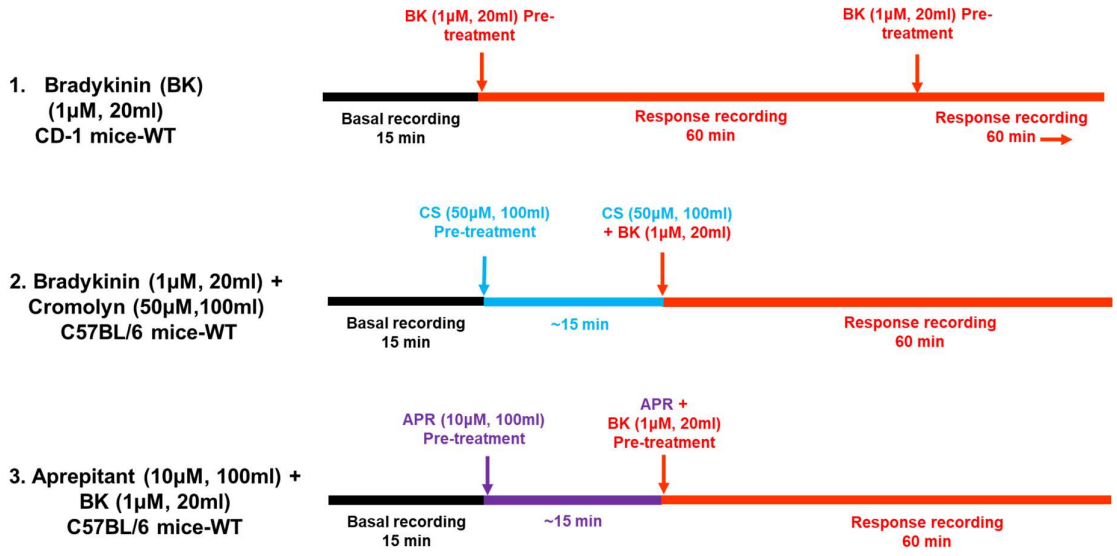


Figure. 51 Experimental protocols for ex-vivo electrophysiology recording evaluating effects of bradykinin, aprepitant and cromolyn pre-treatment on mice colonic afferents.

For the ramp distensions paradigm, the experiments were conducted on isolated colonic preparation from C57B6-WT mice by following the previously described procedure in the chapter-3. For determining the effect of PGE2 on colonic

mechanosensitivity, PGE2 (10μM, 100ml) was superfused immediately after distention-2, and its effect on mechanosensitivity was observed on distention-3 (***see figure 52.1***). Similarly, the effect of PAN-PDE inhibitor IBMX (50μM, 100ml) pre-treatment on distention-induced mechanosensitivity was also evaluated (**see figure 52.2**). For evaluating inhibitory effects of GPR35 agonist zaprinast and cromolyn on PGE2-induced mechanosensitivity, both zaprinast (100μM), and cromolyn (100μM) pre-treatment (20ml) was applied immediately after distention 2, followed with bath superfusion of PGE2 (10μM, 100ml) in the presence of zaprinast (100μM) and cromolyn (100μM) in separate experiments. The inhibitory effects of cromolyn and zaprinast pre-treatment was observed and calculated from distention-3 (***see figure 52.3 and 52.4***).

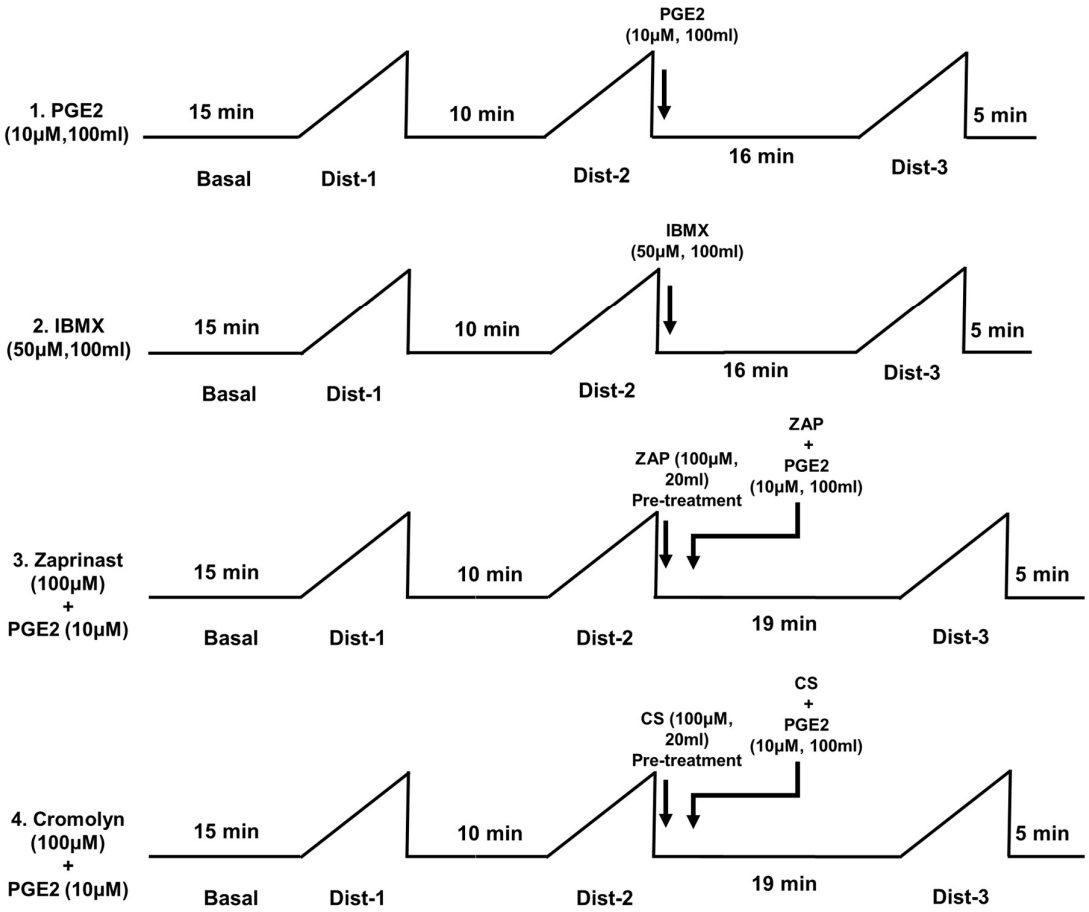


Figure 52. Experimental protocol for determining effects of PGE2, IBMX and GPR35 agonist on colonic mechanosensitivity during ramp distention paradigm.

5.4. Data analysis

5.4.1. *Ex vivo* LSN recordings: Changes in LSN nerve activity was determined by subtracting baseline nerve discharge (mean nerve discharge calculated from the ongoing activity 6 minutes prior to drug application) from ongoing nerve activity measured at 1min intervals from the start of bath perfusion with bradykinin and displayed as the mean change in nerve activity over time. Mean peak change in nerve activity following bradykinin application was calculated using the peak increase in nerve discharge observed within 20min of bradykinin application from individual experiments. Changes in peak nerve activity following application of bradykinin were compared between vehicle, CS and aprepitant pre-treatments using an unpaired Student's t-test.

5.4.2. *Ex vivo* LSN recordings during ramp distention: The afferent activity was calculated using a pressure script for every 5mmHg rise in pressure starting from 0 to 80mmHg for each ramp distention. The baseline nerve discharge (mean nerve discharge calculated from the ongoing activity 1 minutes prior to individual ramp distention) was subtracted from the afferent activity calculated using a pressure script for individual ramp distention and the data obtained was normalised and expressed as percentage change in afferent response. For normalisation, the response to distention-2 and distention-3 were normalised with the peak effect (at 80mmHg) of distention-2. The data is expressed as a percentage change in response to increasing pressure stimulus (0-80mmHg with every 5mmHg rise in pressure) as profile. For profiles, the statistical analysis was performed using 2-way ANOVA followed by Šídák's, Tukey's or Bonferroni post-test analysis for multiple comparisons as applicable. Additionally,

the average peak effect obtained at 80mmHg pressure (mean ± sem) is expressed as the bar chart for which the statistical analysis was performed using unpaired student T-test, ordinary one-way ANOVA or Two-way ANOVA followed by Šídák's or Tukey's post-test analysis for multiple comparisons as applicable.

5.5. Results

5.5.1. PGE2 sensitisation of colonic afferents to ramp distention

To begin we investigated the effect of PGE2 on colonic afferent response to ramp distention. Bath superfusion of PGE2 (10μM, 100ml) sensitises colonic afferents to ramp distention with significant change in peak afferent firing at 80mmHg (e.g., 29.5 ± 5 spikes/s, *n=5*, ** P < 0.01;* ***see figure 53.C***) in comparison to the change in peak afferent activity determined from the distention response prior to the application of PGE2. (23.9 ± 5 spikes/s; *n=5;* ***see figure 53.C***). Moreover, we also checked for the compliance between the pre-PGE2 and post-PGE2 treated distention's and observed no significant difference for the cumulative time (e.g., 53.4 ± 2.8 sec vs 51.7 ± 2.1 sec; *n=5;* ***see figure 53.D***) and cumulative volume (e.g., 89.1 ± 4.7μl vs 86.2 ± 3.5μl; *n=5;* ***see figure 53.E***) respectively.

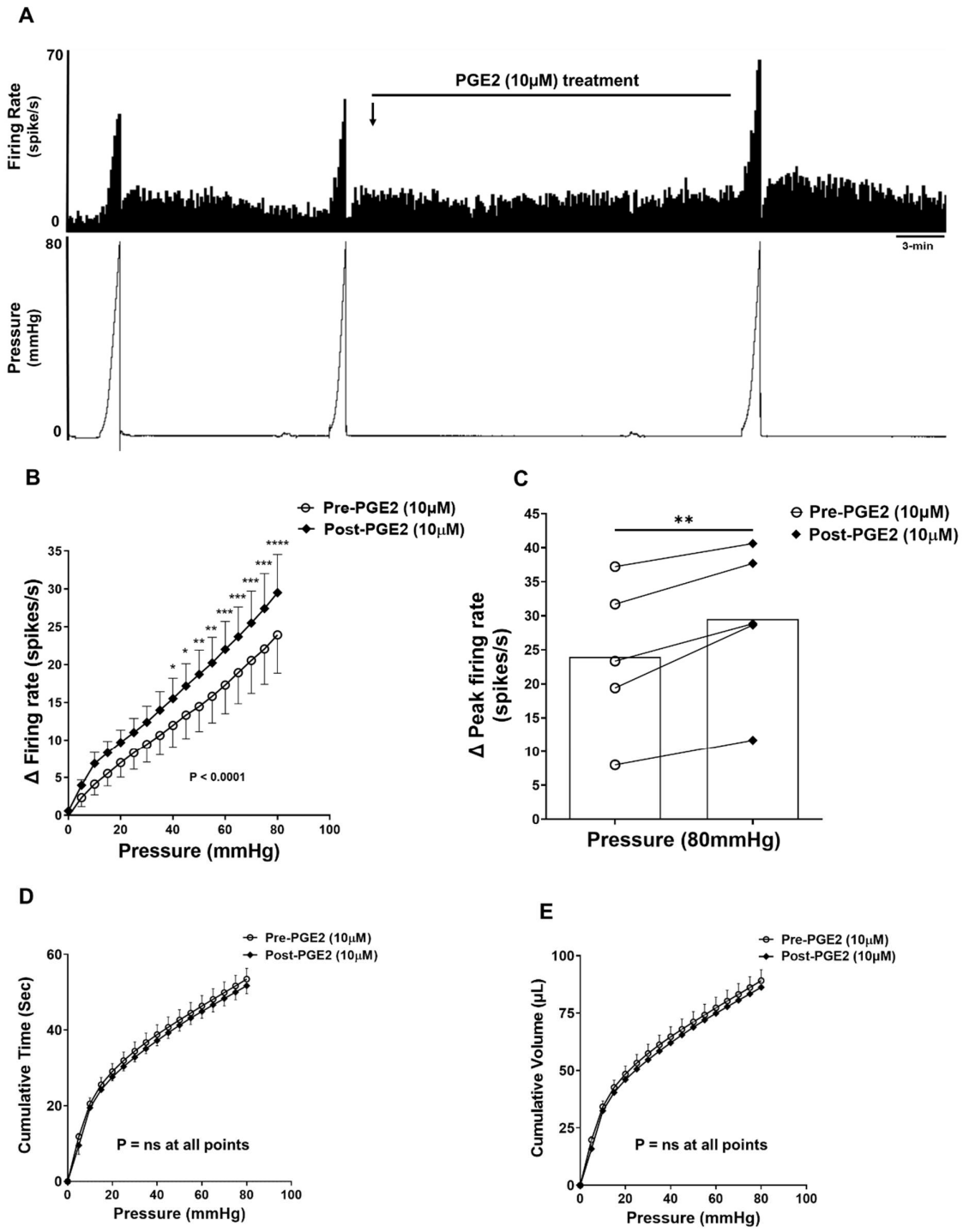


Figure 53. PGE2 induces mechanosensitisation of colonic afferents to ramp distention. A. Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg; 100sec; 10 min intervals) in C57B6/J mice alone and pre-treated with PGE2 (10μM, 100ml). **B**. Line graph showing the change in colonic

afferent activity following the application of PGE2 (10μM, 100ml) on colonic splanchnic nerve response to 0-80mmHg ramp distention. The statistical analysis was performed using Two-way RM ANOVA followed by Šídák's multiple comparisons test where, the significance is defined as, * P< 0.05, **P< 0.01, *** P< 0.001 and **** P< 0.0001 respectively. **C**. The bar charts showing the mean ± sem peak change in afferent firing following the application of PGE2 (10μM, 100ml) on colonic splanchnic nerve response at 80mmHg of distention. The statistical analysis was performed using paired student-t test where, the significance is defined as, ** P < 0.01, respectively. **D**. Cumulative time (sec) and **E**. Cumulative volume (μl) is plotted against increasing pressure (0-80mmHg, with every 5mmHg rise in pressure) for pre-PGE2 and post-PGE2 distention's (P > 0.99; two-way ANOVA, *n=5*).

Next we determined the inhibitory effect of zaprinast and cromolyn on PGE2 (10μM) induced mechanosensitisation of colonic afferents to ramp distention. Interestingly a robust sensitisation of colonic afferent response to luminal distension was still observed to PGE2 in the presence of zaprinast (e.g., 121.0% ± 7.0%; *n=5* Vs 129.2% ± 7.6%, *n= 5;* ***see figure 54.C***).

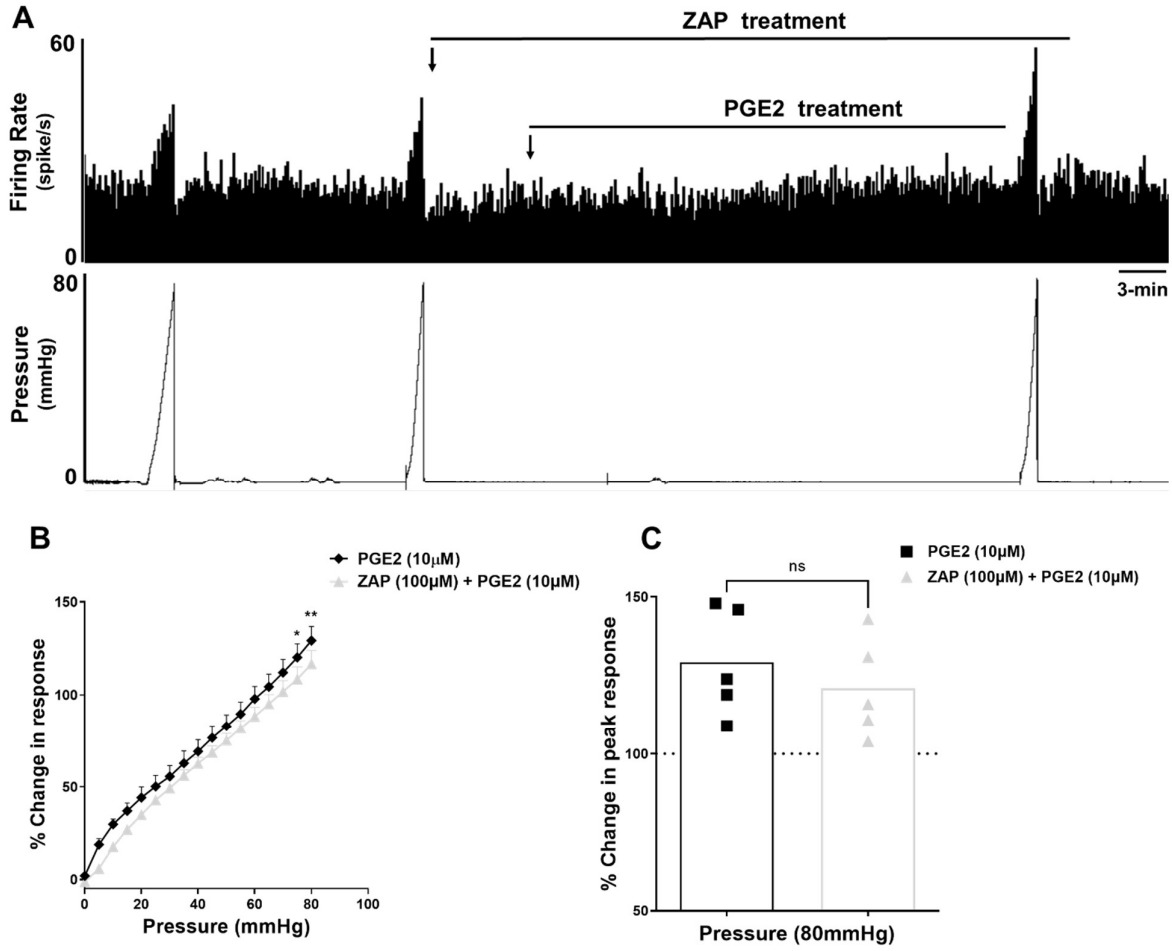


Figure 54: Zaprinast failed to attenuate PGE2 induced mechanosensitisation of colonic afferents to ramp distention. A. Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg) in C57B6/J mice alone and pre-treated with PGE2 (10μM, 100ml) in presence of zaprinast (100μM). **B.** Line graph showing percent change in peak response (mean ± sem) following the application of vehicle; PGE2 (10μM; *n=6*) alone and following pre-treatment with zaprinast (10μM; *n=5*) at 0-80mmHg of pressure. The statistical analysis was performed using Two-way ANOVA followed by Šídák's multiple comparisons test where, statistical significance is defined as *P<0.05, **P< 0.01 respectively. **C**. Bar charts showing percent change in peak response (mean ± sem) following the application of vehicle; PGE2 (100μM; *n=6*) alone and following pre-treatment with zaprinast (100μM; *n=5*), at 80mmHg of

pressure. The dotted line represents peak effects of vehicle group. The statistical analysis was performed using unpaired student t-test where no significant difference was observed.

However, a concentration dependent decrease in PGE2 induced sensitisation of colonic afferent mechanosensitivity was observed following cromolyn pre-treatment (117.4% ± 7.3%, *n=5* at 1μM; 111.6% ± 12.7%, *n=5* at 10μM and 103.0% ± 4.9%, *n=5*, *P<0.05* at 100μM; ***see figure 55.C***).

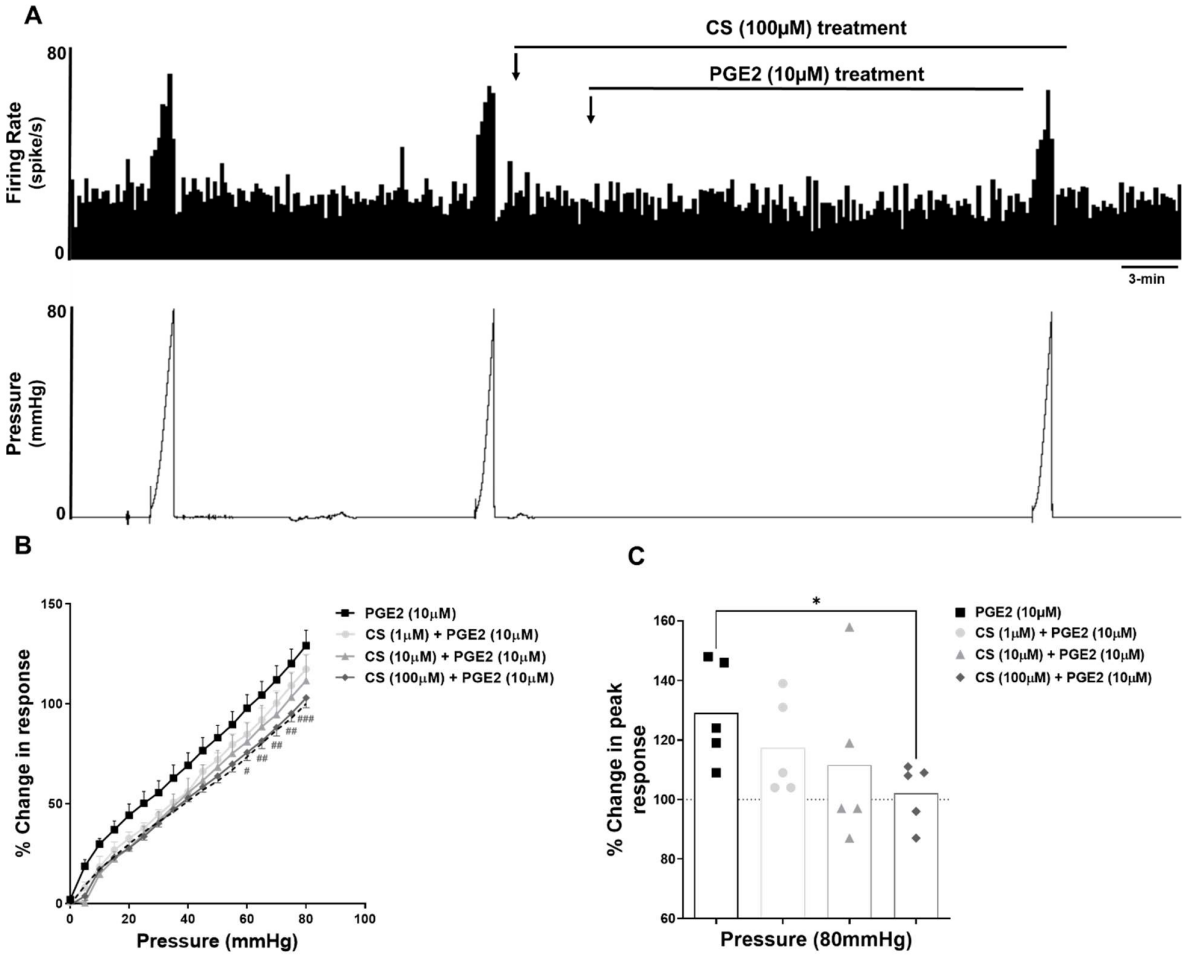


Figure 55: **Cromolyn inhibits PGE2 induced mechanosensitisation of colonic afferents to ramp distention. A.** Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg; 100sec; 10 min intervals) in C57B6/J mice alone and pre-treated with PGE2 (10μM, 100ml) in presence of CS (100μM). **B.** Line graph showing percent change in peak response (mean ± sem) following the application of vehicle; PGE2 (10μM; *n=6*) alone and following pre-treatment with CS [1μM (*n=5*), 10μM (*n=5*) and 100μM (*n=5*)] at 0-80mmHg of pressure. The statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparison test where, statistical significance is defined as *P<0.05, **P< 0.01 and ***P<0.001 respectively. **C**. Bar charts showing percent change in peak response (mean ± sem) following the application of vehicle; PGE2 (100μM; *n=6*) alone and following pre-treatment with CS [1μM (*n=5*), 10μM (*n=5*) and 100μM (*n=5*)] at 80mmHg of pressure. The statistical analysis was performed using One-way ANOVA followed by Šídák's multiple comparisons test multiple comparison test where, statistical significance is defined as *P<0.05.

5.5.2. Non-specific PDE-inhibitor, IBMX induced sensitization of colonic afferents to ramp distention

The activation of EP2 and EP4 receptors by PGE2 leads to an increase in cAMP and activation of PKA which in turn promotes mechanical hypersensitivity (Gold et al., 1998). These modulatory actions of PGE2 are mediated in part by effects on voltage-gated ion channels, including (a) enhancement of the TTX-resistant Na^+ in nociceptive neurons (Gold *et al.,* 1998), (b) suppression of the sustained (delayed rectifier) outward K^+ current (England *et al.,* 1996; Nicol *et al.,* 1999) and (c) enhancement of

the hyperpolarization-activated cation current (I_h) that is thought to facilitate repetitive firing. These actions of PGE2 could contribute to increased excitability in nociceptors. In addition to being a GPR35 agonist zaprinast is also a phosphodiesterase inhibitor. This action would enhance cAMP mediated sensitisation of colonic afferents by PGE2, and so to understand if this additional activity contributed to the lack of effect seen to zaprinast pre-treatment on PGE2 induced mechanosensitisation of colonic afferents we repeated studies in the presence of the phosphodiesterase inhibitor IBMX (50μM), which is known to raise intracellular cAMP levels (Kaneda et al., 1997). Consistent with this hypothesis pre-treatment with IBMX (50μM) sensitizes the colonic afferents to ramp distention with significant peak change in afferent firing at 80mmHg (e.g., 24.8 ± 2.5 spikes/s, *n= 5*, ** P < 0.01;* ***see figure 56.C***) in comparison to peak change in afferent activity determined from the distention response prior to the application of IBMX (21.2 ± 2 spikes/s; *n=5;* ***see figure 56.C***) suggesting that the PDE-inhibitory activity of zaprinast may functionally antagonise any inhibitory effect of zaprinast mediated by its GPR35 agonist activity on PGE2 induced mechanosensitisation.

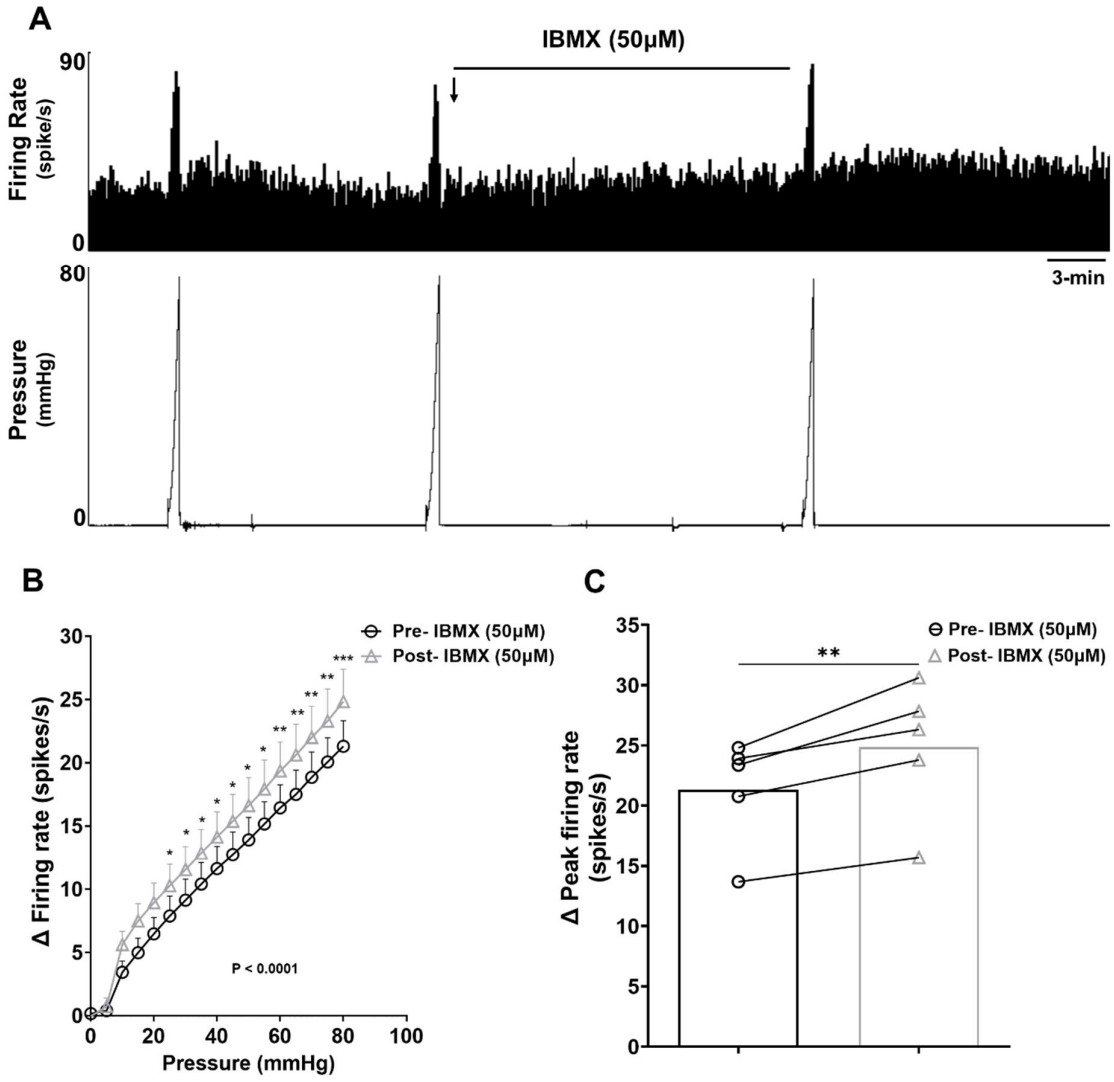


Figure 56: IBMX pre-treatment sensitizes colonic afferents to ramp distention (0-80mmHg). **A.** Example rate histogram and raw trace of LSN response to repeat ramp distension (0-80mmHg) in C57B6/J mice alone and pre-treated with IBMX (50μM, 50ml). **B**. Line graph showing the change in colonic afferent activity following the application of IBMX (50μM, 100ml) on colonic splanchnic nerve response to 0-80mmHg ramp distention. The statistical analysis was performed using Two-way RM ANOVA followed by Šídák's multiple comparisons test where, the significance is defined as, * P< 0.05, **P< 0.01 and *** P< 0.001 respectively. **C**. The bar charts

showing the mean ± sem peak change in afferent firing following the application of IBMX (50μM, 100ml) on colonic splanchnic nerve response at 80mmHg of distention. The statistical analysis was performed using paired student-t test where, the significance is defined as, ** $P < 0.01$, respectively.

5.5.3. Bradykinins evoke noxious afferent stimulation, which is unaffected by CS and aprepitant pre-treatment.

Finally, we also studied the effect of the GPR35 agonist cromolyn on bradykinin (BK) mediated colonic afferent activation. Application of bradykinin (1μM) by bath superfusion elicited a marked and sustained increase in afferent activity (e.g., 24.4 ± 9.4 spikes/s increase in nerve discharge *n=5;* ***see figure 57.B***) within 5 minutes of application. Responses were stable to repeat bradykinin application with a second bradykinin application eliciting a comparable increase in afferent activity (e.g., 22.2 ± 10.4 spikes/s increase in nerve discharge *n=5; P>0.05* as analysed by unpaired student t-test; ***see figure 57.B***). Pre-treatment with cromolyn had no effect on the magnitude of bradykinin evoked colonic afferent activation (e.g., 13.7 ± 2 spikes/s increase in nerve discharge *n=5* vs 13.2 ± 4.0 spikes/s increase in nerve discharge *n=5;* ***see figure 57.C***). Furthermore, pre-treatment with the selective NK_1 antagonist, aprepitant (10μM) also had no effect on the afferent response to bradykinin (1μM) demonstrating that the direct activation of colonic afferents by bradykinin was not dependent on SP release (***see figure 57.D***).

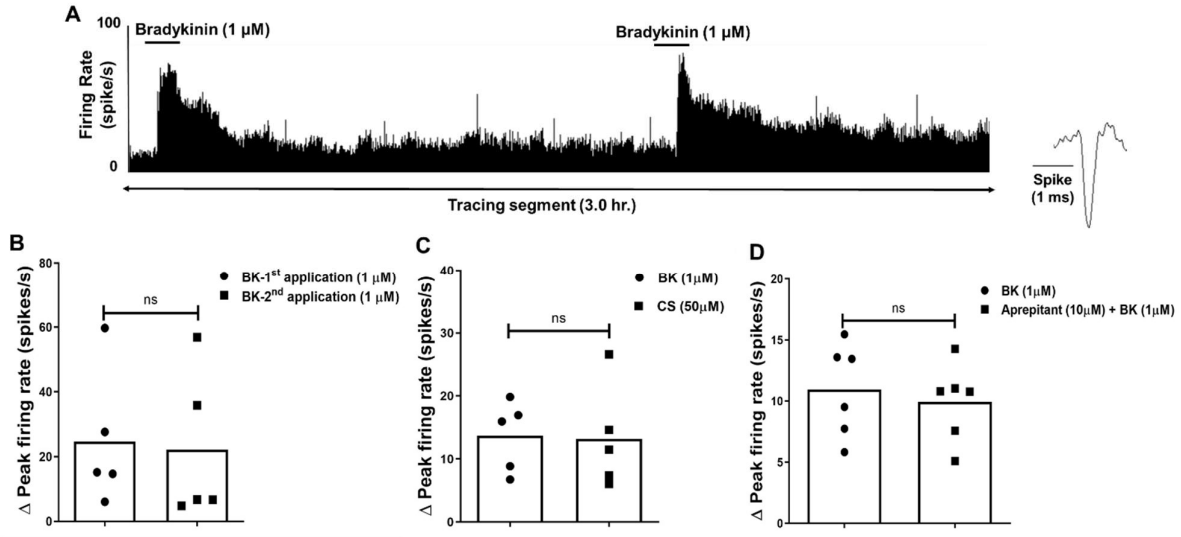


Figure 57: Effect of Bradykinin (BK; 1μM) on colonic afferent response in LSN preparation isolated from C57B6 mice. A. Example rate histogram with neurogram trace, illustrating the effect of repeat application of bradykinin (BK; 1μM) along with an example of single resolved action potential obtained from Lumbar splanchnic nerve preparation isolated from CD-1 mice. The effect of BK was recorded for 60 min from its application, further second response of BK was recorded after inter-response interval of 60 min. **B.** Bar charts showing the mean ± sem peak change in afferent firing following application of BK. **C.** Bar charts showing the mean ± sem peak change in afferent firing following application of BK (1μM) alone and following pre-treatment with CS (100μM) in LSN preparation isolated from C57B6 mice. **D.** Bar charts showing the mean ± sem peak change in afferent firing following application of BK (1μM) alone and following pre-treatment with Aprepitant (10μM) in LSN preparation isolated from C57B6 mice. The statistical analysis was performed using unpaired student-t test where, the significance is defined as, $* P < 0.05$.

5.6. Discussion

Data from our studies demonstrate that the GPR35 agonist cromolyn can inhibit PGE2-mediated sensitisation of colonic afferents but has no effect on the direct activation of colonic afferents to bradykinin. The GPR35 agonist zaprinast does not affect PGE2-mediated mechanosensitisation; however, further studies with the PDE inhibitor IBMX suggest that the PDE inhibitory activity of zaprinast acts to oppose any GPR35 mediated inhibitory effect of zaprinast.

Multiple mediators released during tissue damage and inflammation synergise to sensitise afferent endings. Their sensitising effects have been studied using artificial inflammatory soups (IS), typically consisting of mediators such as bradykinin, ATP, PGE2, histamine and 5-HT, which have been shown to induce robust afferent hypersensitivity to mechanical stimuli and direct stimulation of afferent fibres (Su & Gebhart, 1998; Jones, 2005; Feng & Gebhart, 2011 and Kiyatkin et al., 2013). In our study, we also observed that PGE2 sensitises the colonic afferents to mechanical stimulation; this was abolished by cromolyn (100μM) pre-treatment. These results were in line with recent findings that show intracolonic infusions of rats with IBS-D biopsy supernatants containing elevated PGE2 levels elicited hypersensitivity of response to colorectal distension measured using the visceromotor response, and this effect was prevented by cromolyn treatment (Grabauskas et al., 2020). This inhibitory effect of cromolyn was attributed to its mast cell stabilising activity; however, we found no effect of compound 48/80 on colonic activity in tissue from “normal” mice suggesting that mast cells do not contribute to the sensitising effects of PGE2 in our experiments or the inhibitory actions of cromolyn. Instead, we speculate that PGE2 sensitises colonic afferents by stimulating cAMP production and subsequent activation of PKA

through the activation of EP_2 and EP_4 receptors (Grabauskas et al., 2020). Evidence supports this from our experiments with a non-specific PDE inhibitor, IBMX, which sensitises colonic afferent response to ramp distention and will elevate cAMP levels. Following on from this, our data would be consistent with the GPR35 agonist activity of cromolyn, which is known to decrease cAMP production due to the inhibitory effect of the GPR35, which inhibits adenyl cyclase activity due to the coupling of GPR35 to inhibitory $G_{i/o}$ proteins. Furthermore, we speculate that the potential inhibitory effects of zaprinast-mediated activation of the GPR35 are not seen as the PDE-inhibitory activity of zaprinast acts in opposition to this $G_{i/o}$ coupled effects of GPR35 receptor activation and as such, zaprinast acts as a functional antagonist of its own GPR35 agonist activity.

Bradykinin elicits acute pain by activating nociceptors, followed by a longer-lasting sensitisation to thermal and mechanical stimuli (Dray & Perkins, 1993). These responses are attenuated in TRPA1-deficient mice, which exhibit pronounced deficits in bradykinin-evoked nociceptor excitation and pain hypersensitivity (Bautista et al., 2006). Given that we have previously shown that cromolyn attenuates TRPA1-induced colonic afferent activation, we sought to investigate the inhibitory effects of cromolyn on bradykinin-induced colonic excitation. However, cromolyn failed to inhibit colonic afferent response to bradykinin, suggesting that the mechanism by which bradykinin stimulates colonic afferents is not dependent on TRPA1 activation. Consistent with this possibility, the afferent response to bradykinin, unlike the afferent response to the TRPA1 agonist ASP7663, was not inhibited by aprepitant results, suggesting that SP is not involved in the activation of colonic afferents by bradykinin. Furthermore, the direct activation of colonic afferents by bradykinin is mediated *via* the bradykinin B2

receptor, which activates Gq-coupled G-proteins to stimulate PLC activity. Therefore, unlike PGE2, which can sensitise colonic afferents through the activation of adenyl cyclase and increase in cAMP production, it is not immediately apparent how the activation of $G_{i/o}$ G-proteins coupled to GPR35 receptors may inhibit the downstream signalling cascades activated by bradykinin.

6. Overall Summary and conclusion:

6.1 Summary

An unmet clinical need exists for novel visceral analgesics to effectively treat pain in IBD and IBS. The goals of this thesis were to investigate the potential utility of GPR35 agonists to treat pain in these conditions by studying the effect of GPR35 agonists on the activation of colonic afferent and sensitisation of response to luminal distension following TRPA1 activation and treatment with algogenic mediators PGE2 and bradykinin.

We chose to study GPR35 as investigation of the single-cell database of the mouse colonic neurone transcriptome revealed extensive co-expression of GPR35 with the transducers of noxious stimuli TRPA1 and TRPV1 (Hockley et al., 2019). Furthemore as GPR35 is reported to couple to $G_{i/o}$ G-protein subunits its effects on nociceptor signalling would be expected to be inhibitory (Imielinski et al., 2009; Jostins et al., 2013; Ellinghaus et al., 2013; Yang et al., 2015), a hypothesis supported by studies with GPR35 agonists kynurenine, zaprinast (Ohshiro et al., 2008; Cosi et al., 2011 and Alexander et al., 2015) and Cromolyn (Daryani et al., 2009; Stefanini et al., 1992 and 1995; Carroll et al., 2013; Wang et al., 2014; Zhang et al., 2018) that demonstrate inhibitory effects on chemical, mechanical and inflammatory mediator evoked pain.

Consistent with our hypothesis that GPR35 agonists would inhibit the activation of colonic nociceptors by TRPA1 channel activation demonstrated a concentration dependent effect of the GPR35 agonists cromolyn and zaprinast on the activation of colonic afferents by a TRPA1 agonist which was dependent on GPR35 receptor expression. Interestingly the inhibitory effect of GPR35 on TRPA1 activity appeared to

be modality specific with GPR35 agonists having no effect on the colonic afferent response to ramp distension in contrast to the inhibitory effect of TRPA1 inhibitors. However, GPR35 agonists did prevent TRPA1 agonist mediated sensitisation of colonic afferent response to ramp distention. A response again lost in tissue from GPR35 -/- mice.

Our investigations indicated that colonic afferent response to agonist activation of TRPA1 were mediated through the release of SP and subsequent NK1 receptor activation and suggested that the contribution of mechanically activated TRPA1 channels is not dependent on SP release and NK_1 receptor activation. Furthermore, our studies suggest that the inhibitory effects of GPR35 agonists on TRPA1 mediated colonic activation and sensitisation are driven through the inhibition of this SP release, which we were able to demonstrate through both functional studies looking at TRPA1 mediated colonic contractility which is known to be mediated *via* the release of SP and by directly measuring colonic SP release to TRPA1 activation by CLIA-assay.

Finally, we also demonstrated that the GPR35 agonist cromolyn can inhibit the sensitisation of colonic afferents to PGE2 a mediator implicated in mediating visceral pain in both IBD and IBS. There was insufficient time to investigate this mechanism action fully although data generated with zaprinast and the PDE inhibitor IBMX suggests that the inhibitory effects of cromolyn may be due to the inhibitory effects of GPR35 receptors on adenyl cyclase activity due to the coupling of GPR35 to $G_{i/o}$ G-proteins.

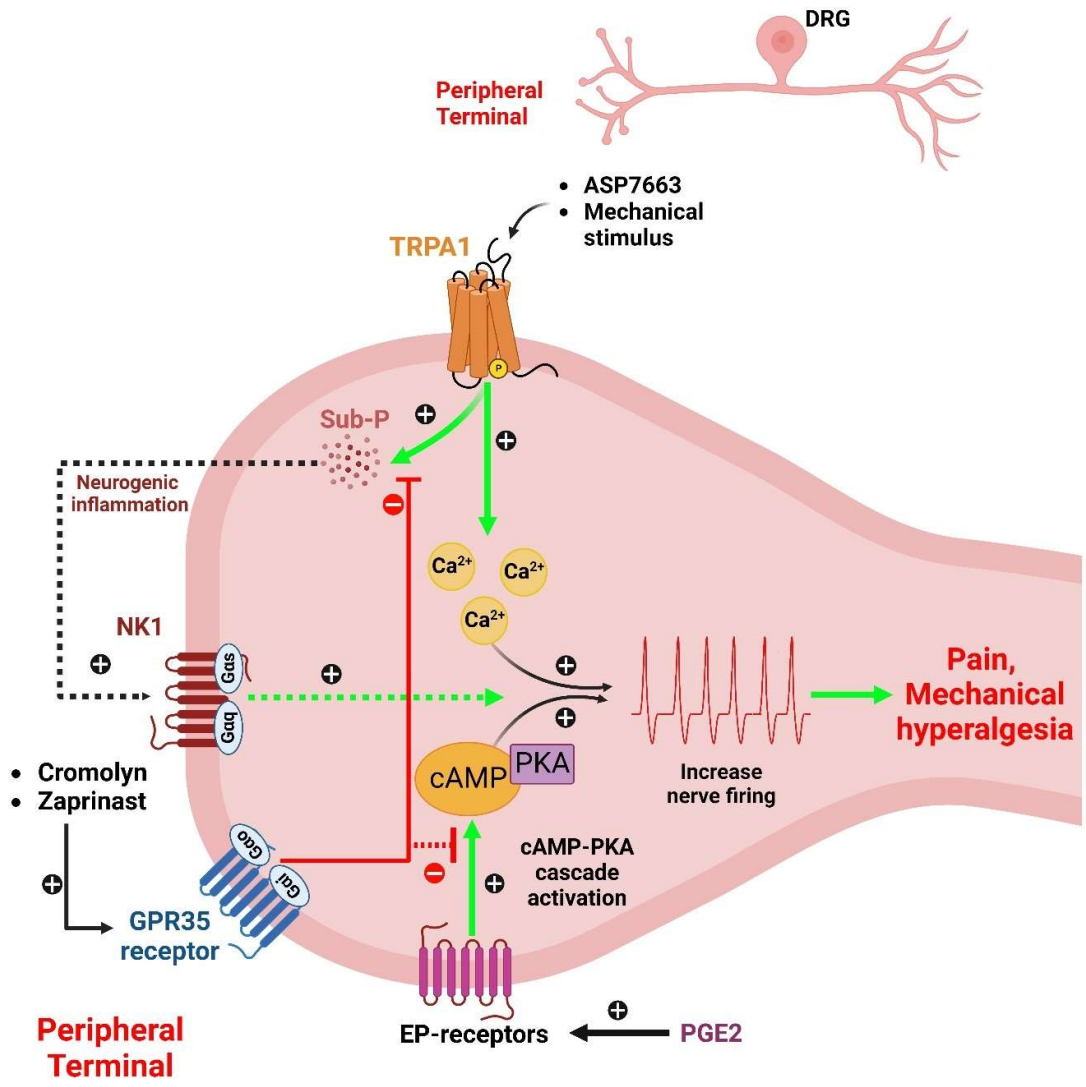


Figure 58: GPR35 activation attenuates TRPA1 and PGE2 induced afferent excitation. TRPA1 activation induced Ca^{2+} ion influx resulting into excitation and sensitisation of the colonic afferents to noxious mechanical stimuli. It also induces the release of SP, which exerts excitatory and sensitising effects on colonic afferents. SP also increases colonic contractility and augments neurogenic inflammation, indirectly contributing to increase afferent firing. A disease-relevant inflammatory mediator, PGE2 also sensitises colonic afferents by activating the cAMP-PKA cascade. Activation of GPR35 receptor by synthetic agonist CS and zaprinast attenuates TRPA1

induced excitatory and sensitising effects whereas, CS attenuates PGE2-induced colonic afferents mechanosensitisation.

6.2. Conclusion

Our results showed that GPR35 receptor agonists cromolyn and zaprinast, inhibit TRPA1-mediated excitation and sensitisation of colonic afferents. This is due to the inhibitory effects of GPR35 receptor activation on TRPA1-mediated SP release and provides a mechanism of action by which GPR35 agonists may inhibit abdominal pain in conditions such as IBD and IBS.

7. Future work:

7.1. Investigating the effects of GPR35 activation on CaV currents

Voltage-gated calcium (Ca^{2+}) channels (Cavs) facilitate the intracellular entry of external Ca^{2+} in response to depolarisation of membrane potential. These Ca^{2+} transients trigger neurotransmitter release which at the level of sensory nerve ending promotes neurogenic inflammation through the release of SP and CGRP. The N-type and P/Q-type are the most critical for this response and represent a mechanism by which $G_{i/o}$ coupled GPCRs inhibit neurogenic inflammation due their direct inhibition of N-type of Ca^{2+} channels *via* the $G_{\beta\gamma}$ subunits or by reducing PKA mediated increase in Cav channel conductance (Currie et al., 2010). Further investigation into the effects of GPR35 agonist activation with cromolyn on Ca_V-current using the patch clamp technique in mouse DRG neurons isolated from C57B6-WT and GPR35-/- mice would help provide further insight into the contribution of this mechanism of action to GPR35 signalling.

7.2. Investigating direct effects of GPR35 activation on TRPA1 channel functioning

TRPA1 selective antagonist, AM0902 significantly reduced distention-induced afferent excitation implicating a crucial involvement of TRPA1 in mechanotransduction. However, both cromolyn and zaprinast did not affect the colonic mechanosensitivity directly only inhibiting agonist induced TRPA1 responses. Further investigation into the effects of GPR35 agonists on TRPA1-channel function using Ca^{2+} imaging in mouse DRGs would help understand the stimulus dependent effects of GPR35 agonists on TRPA1 responses.

7.3. Determination of cAMP and cGMP levels

Findings from our study indicate that the PDE inhibition by zaprinast may attenuate its inhibitory action via GPR35 receptors and the inhibition of cAMP production by GPR35 agonists may be important in their inhibitory effects on the action of PGE2. Further experiments are therefore needed to study the effect of GRP35 agonists on cAMP production elicited by PGE2 in DRG neurones.

7.4. Bridging the GAP using Human tissues experiments

As previously discussed, the approaches that have allowed the recording of nerve discharge *in-vitro* from the resected human bowel tissue segments have been tremendously helpful in bridging the translational gap and inconsistencies of findings from animal models (Peiris et al., 2011, Ng et al., 2016; Hockley et al., 2017) to clinical trial. Therefore, where possible responses will be translated to recordings of visceral nociceptor activity in human bowel. Our main focused would be to first confirm the reproducibility of ASP7663 response in resected human bowel tissue and later confirm the inhibitory effects of zaprinast and cromolyn pre-treatment on TRPA1-induced afferent activation.

8. Appendices:

8. 1. Preliminary experiments in CD-1 mice

8.1.1. Zaprinast (GPR35 agonist) inhibits colonic afferent response to the TRPA1-agonist ASP7663.

The effect of pre-treatment with the GPR35 agonist (and PDEV inhibitor) zaprinast was examined on the colonic afferent response to 30μM and 100μM of ASP7663. Pre-treatment with zaprinast significantly attenuated the afferent response to 30μM (e.g., 2.5 ± 0.7 spikes/s increase in nerve discharge *n=5, P<0.0001 see figure 1.D*) and 100μM (e.g., 6.3 ± 4.0 spikes/s increase in nerve discharge *n=5, P<0.05*, *see figure 1.F*) of ASP7663.

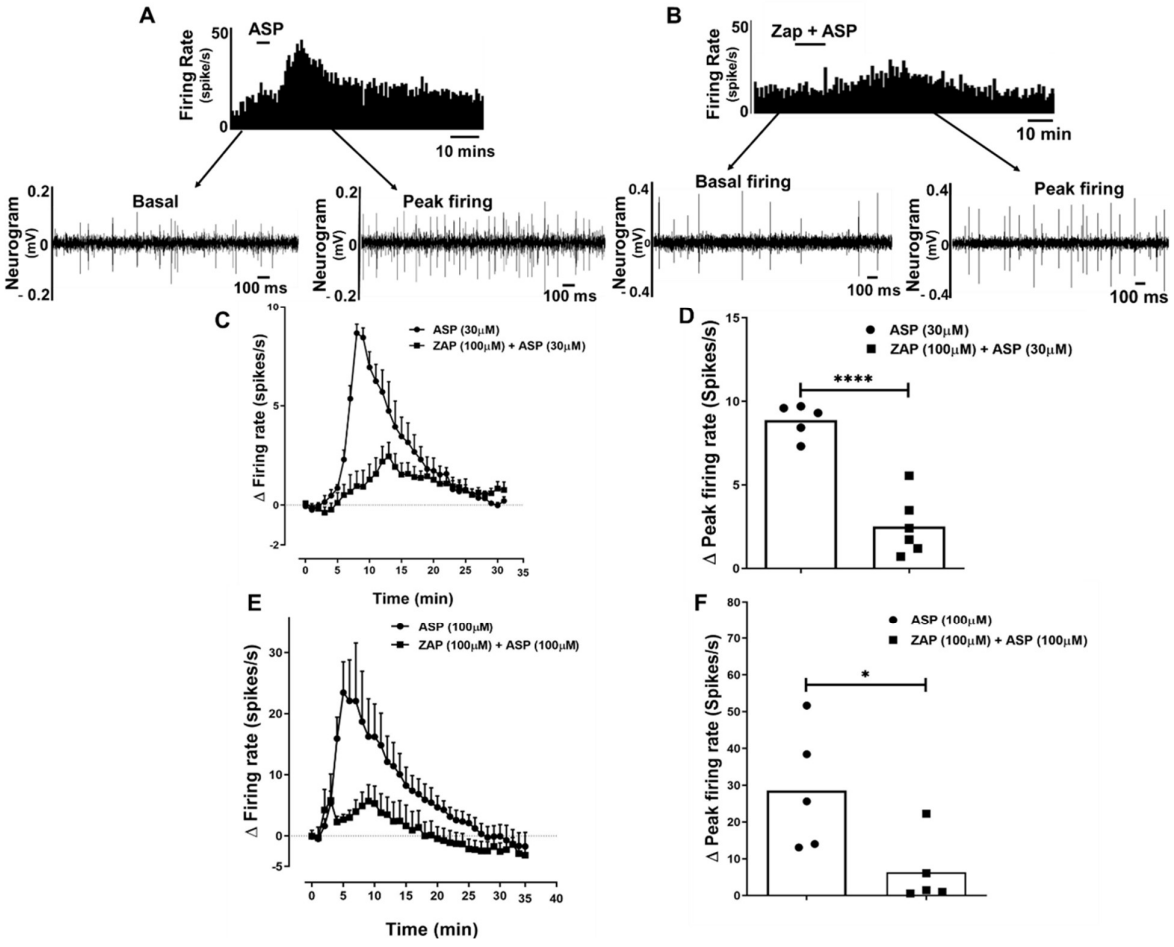


Figure 1. Effect of pre-treatment of Zaprinast (ZAP; GPR35 agonist;) on ASP7663 (TRPA1 selective agonist) response in LSN preparation isolated from CD-1 mice. **A & B**: Example of a rate histogram with neurogram trace illustrating the colonic afferent response to ASP7663 (100μM) alone and following pre-treatment with zaprinast (100μM). **C & E**: Line graphs showing the change in colonic afferent activity over time following the application of ASP7663 (30μM & 100μM) alone and following pre-treatment with zaprinast (100μM). **D & F**: Bar charts showing the mean ± sem peak change in afferent firing following the application of ASP7663 (30μM & 100μM; *n=5*) alone and following pre-treatment with zaprinast (100μM); *n=5* and *n=6* respectively. The statistical analysis was performed using unpaired student-t test where, the significance is defined as, **** *P<0.0001* and * *P < 0.05* respectively.

9. Research Impact statement

Student name	Rohit Gupta
USN	304332759
Department	Pharmacology
Supervisor	Dr David Bulmer
Thesis title	Regulation of Visceral Nociception by GPR35

Background

I am Rohit Gupta, PhD student in the Department of Pharmacology, University of Cambridge. Despite of the COVID challenges I have successfully completed experiments needed to achieve the overall objectives of my research project. The research data I have generated shows an antinociceptive effect of GPR35 receptor agonists in the viscera. To do this, I have learned ex-vivo electrophysiology techniques, immunohistochemistry, and tissue-bath assays. Moreover, I worked with sensory neurones (DRGs) in culture (Ca^{2+} imaging, CGRP and Substance-P release). I have successfully established a Tissue bath-assay and CLIA-essay for my project. My findings have been presented at the World Congress on Pain-2021, Amsterdam and in the meeting of the American Gastroenterology Association (DDW) 2022 in San Diego, CA.

Research Impact Statement:

The COVID-19 situation has been disruptive to my PhD mainly the year-2020 was very challenging for me, considering the pandemic lockdown. Starting the year 2020, I started to work with sensory neurones (DRGs) in culture (Ca^{2+} imaging and CGRP release), in addition to studies on GPR35-/- mice and human tissue (*via* a collaboration with Queen Mary University London).

However, at the Start of March 2020, in response to the instruction from my college Hughes Hall, to leave the UK in response to COVID-19, I returned to India on 17th March 2020. I got stuck in a pandemic at my native place in Nagpur, India and have

almost lost 10 months of my laboratory working time. When one considers that students usually need six months at the start of their PhD to learn techniques and six months at the end of their PhD to write up, I had lost significant productive lab time for my PhD.

The pandemic has also impacted the delivery of our transgenic mouse line being specifically bred at the MRC-Harwell for my project. This work was similarly put on hold due to lockdown. Furthermore, the third national lockdown and restrictions on working distance/occupancy limits have also delayed the opportunity for me to be trained in experimental techniques essential for the experiments outstanding for my PhD. For example, I have been unable to prosecute human tissue work at QMUL (Queen Merry University London) and set up tissue bath assays with collaborators (Sosesi Heptares, Ltd, UK).

Finally, after a sequential delay in the delivery of transgenic animals (**we received the ordered mice in December-2021 rather than the original date of July 2020 with almost a delay of more than one year**), we obtained the animals for our experimentation in December-2021. However, the breeding process took up to 3 months to develop colonies. I then started my experiments using transgenic mice from March-2023 and completed the experiments in the month of June-2022. Finally, as a result of hard work and continuous supervisor support and input, I completed an impactful thesis.

Future plans:

I would say that learning from Cambridge is once a lifetime opportunity for me, and I want to make effective use of this opportunity for shaping my carrier. I am interested in defining the downstream signalling pathway for the GPR35-receptor responsible for its antinociceptive potential. Therefore, as a future plan I want to validate and translate the research findings using human tissues for which experimental data will be generated from the recordings of visceral nociceptor activity in human bowel. We are in the process to establish an extended collaboration with Sosei Heptares and GSK, Stevenage, UK, to set up and validate cell-based assays for estimation of

cAMP and CGRP levels. We are also planning to establish a research lab, particularly for human tissue samples (tissue/supernatant) experiments.	
Student's signature *[by typing your name, you are providing your electronic signature]*	Rohit Gupta
Supervisor signature *[by typing your name, you are providing your electronic signature]*	Dr David Bulmer

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