The role of voltage-gated sodium channel 1.9 ($Na_v1.9$) in visceral afferent signalling

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To my parents . . .

I am here because you were there

Abstract

Abdominal pain places a large burden on society and its current management is sub-optimal due to the lack of visceral-specific analgesics. Voltage-gated sodium channels (Na_v) are crucial for action potential generation and $Na_v1.9$ has the lowest activation threshold of all these channels. In addition, $Na_v1.9$ expresses a unique, slow, persistent current and is peripherally expressed on small diameter dorsal root ganglion (DRG) neurons. Whereas, at the level of the DRG, the $Na_v1.9$ current is enhanced in response to a number of inflammatory mediators, no data exist for the role of $Na_v1.9$ in signalling at the nerve terminal. Behavioural studies in mice have established a role for $Na_v1.9$ in the response of somatic afferents to inflammatory mediators where it has a role in the immediate response of the afferents as well as in the development of hyperalgesia. No studies have investigated the role of $Na_v1.9$ in the response of visceral afferents to inflammatory and mechanical stimuli.

This thesis investigated the role of $Na_v1.9$ in the response of visceral afferents to a variety of chemical and mechanical stimuli. Electrophysiological recording of colonic and intestinal afferents from $Na_v1.9$ wild type and knockout mice were made using a bespoke recording chamber. The tissue was superfused with a number of chemical mediators and the change in peak afferent activity compared between wild type and knockout mice. For some experiments, human inflammatory supernatant was generated from inflamed and control appendices. The effect of this supernatant on visceral afferents and the role of $Na_v1.9$ in this response were studied. The response of visceral afferents to intraluminal distension was also studied in $Na_v1.9$ wild type and knockout mice.

The data presented in this thesis demonstrate a pivotal role for $Na_v1.9$ in the activation of visceral afferents by chemical and mechanical stimuli as well as a human inflammatory supernatant. The response of colonic and intestinal afferents

to bradykinin, capsaicin and intraluminal distension is significantly attenuated in $Na_V1.9$ knockout mice. Although the peak response of afferents to acetic acid was not significantly different between the genotypes, there were significant differences in the profile of the responses. Additionally, the data show that cyclooxygenase blockade enhances the effect of elimination of $Na_V1.9$ on the response of afferents to noxious stimuli. Finally, the activation of visceral afferents by human inflammatory supernatants was significantly decreased in $Na_V1.9$ knockout mice suggesting that blockade of this channel in man could provide analgesic effects that, due to its restricted peripheral distribution, would not be expected to be associated with the side effect profile of the current non-specific sodium channel blockers.

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List of abbreviations

-/-	Knockout
+/+	Wildtype
µ	micro
ANOVA	Analysis of variance
ASIC	Acid sensing ion channel
ATP	Adenosine-5'-triphosphate
B_1	Bradykinin receptor 1
B_2	Bradykinin receptor 2
bp	Base pair
$CaCl_2$	Calcium chloride
CFA	Complete Freund's adjuvant
cm	Centimetre
CNS	Central nervous system
CO_2	Carbon dioxide
COX	Cyclooxygenase
DAG	diacylglycerol
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion/ganglia
EDTA	Ethylenediaminetetraacetic acid
GDNF	Glial-derived neurotrophic factor
GI	Gastrointestinal
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
Hz	Hertz
IASP	International Association for the Study of Pain
IB4	Isolectin B4
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome

IGLE	Intraganglionic laminar ending
IL	Interleukin
IMA	Intramuscular array
IMG	Inferior mesenteric ganglion/ganglia
IMN	Intermesenteric nerve
IP3	inositol 1,4,5-trisphosphate
KCl	Potassium chloride
LSN	Lumbar splanchnic nerve
m	milli
M	Molar
$MgSO_4$	Magnesium sulphate
mRNA	Messenger ribonucleic acid
n	nano
NACC	National Association for Colitis and Crohn's Disease
NaCl	Sodium chloride
NaH_2PO_4	monosodium phosphate
$NaHCO_3$	Sodium bicarbonate
Na_v	Voltage-gated sodium channel
NG	Nodose ganglion/ganglia
NGF	Nerve growth factor
NSAID	Non-steroidal anti-inflammatory drug
O_2	Oxygen
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E_2
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PN	Pelvic nerve
PNS	Peripheral nervous system
RPM	Revolutions per minute

SDS	Sodium dodecyl sulfate
sem	Standard error of the mean
siRNA	Small interfering ribonucleic acid
SMG	Superior mesenteric ganglion/ganglia
TG	Trigeminal ganglion/ganglia
TNBS	Trinitrobenzene sulfonic acid
TNFα	Tumour necrosis factor alpha
TRPA	Transient receptor potential ankyrin
TRPV	Transient receptor potential vanilloid
TTX	Tetrodotoxin
TTX-r	Tetrodotoxin-resistant
TTX-s	Tetrodotoxin-sensitive

1

Introduction

1.1 Abdominal pain

1.1.1 Burden of abdominal pain

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage" (IASP 2011). It is clear from this definition that there are two elements to feeling pain: the actual stimulation of nerve endings and the psychological element that often influences how a person reacts to the stimulus. Acute abdominal pain is one of the three most common presentations to emergency departments, with 7 million presentations in the United States in 2007-8 (Bhuiya *et al.* 2010). There are three main causes of pain: inflammation of an organ, e.g. appendicitis or inflammatory bowel disease (IBD), occlusion of flow causing distension, e.g. ureteric stones or an obstructing tumour, or functional disorders, e.g. irritable bowel syndrome (IBS) or non-ulcer dyspepsia. Although the vast majority of episodes resolve either spontaneously or after medical management, the prevalence

of chronic abdominal pain is estimated at 25% (Halder and Locke 2009). Pain, both acute and chronic, places a large burden on health and social care systems with the cost of functional digestive diseases estimated at over $40 billion in 8 major industrialised countries (Fullerton 1998). Only 25% of this is in the form of direct healthcare costs, while the majority relates to indirect costs incurred from loss of productivity (Fullerton 1998).

1.1.2 Treatment of abdominal pain

In patients where the cause of abdominal pain is established, medical or surgical management of the underlying condition usually results in resolution of the pain, e.g. appendicectomy for appendicitis or antibiotics followed by cholecystectomy for cholecystitis. In addition to the medical treatment, patients are often prescribed analgesics that have differing modes of action, e.g. paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs), anti-spasmodics and opioids. Although a combination of these drugs usually provides adequate analgesia, this is complicated by their side effect profiles, e.g. nausea or constipation. In addition, surveys have shown that only 10-30% of patients seek medical advice for chronic pain (Fullerton 1998; Halder *et al.* 2002; Halder and Locke 2009) with the majority accepting chronic pain as part of daily life (Halder and Locke 2009).

A recent survey of IBD patients conducted by the National Association for Colitis and Crohn's Disease (NACC) revealed that 61% of patients experienced abdominal pain during remission, with three quarters ranking their pain as 5 or greater on a 1-10 scale (NACC 2008). Of the patients who approached a medical practitioner, 68% were prescribed analgesia (commonly paracetamol, NSAIDs, anti-spasmodics or tramadol), which provided a degree of relief in only 70% of patients (NACC 2008). The survey findings demonstrated that over 50% of those who approached a medical practitioner for management of their abdominal pain did not get adequate relief. This correlates with a large pan-European survey which revealed that 40% of people with chronic pain of various types had inadequate management of their pain (Breivik

et al. 2006). Inadequate pain control interferes with a person's ability to carry out activities of daily living including going to work, maintaining relationships and enjoying hobbies. In addition, patients may experience anxiety, fear, anger and depression (Becker *et al.* 1997). The consequent deterioration in their quality of life can prompt them to consider the extreme measure of suicide as their only coping strategy (Hitchcock *et al.* 1994). These surveys highlight the current poor pain management of patients who have both life-long inflammatory conditions and functional gastrointestinal (GI) diseases and illustrate the need for the development of visceral specific analgesics which will have more favourable side effect profiles.

1.2 Nociception in the gastrointestinal tract

Chronic abdominal pain is associated with distorted signalling of visceral afferents. Our understanding of this signalling is derived from experiments in animals and the following description relates mainly to experiments in rodents. While it is likely that the innervation is representative of that in man, there is a paucity of studies that have investigated the extrinsic innervation of the human GI tract.

1.2.1 Anatomy of visceral afferents

The GI tract is served by both intrinsic and extrinsic nerves. Anatomically, the extrinsic nerves are made up of vagal and spinal nerves with the spinal nerves further subdivided into splanchnic and pelvic populations. The vagus nerve innervates the GI tract down to the transverse colon (Berthoud and Neuhuber 2000) while spinal afferents innervate the whole of the GI tract (Christianson and Davies 2010) (Figure 1). The majority of extrinsic afferents are unmyelinated C fibres with a minority of thinly-myelinated A delta (Aδ) fibres (Prechtl and Powley 1990; Sengupta and Gebhart 1994; Robinson *et al.* 2004; Tan *et al.* 2009). These afferents have unencapsulated nerve endings in all layers of the gut wall with subspecialisation of function dependent on the location of the afferent terminal as well as the receptors and ion channels expressed on the terminal (Figure 2).

1.2.1.1 Vagal afferents

Vagal afferents have their cell bodies in nodose ganglia (NG) and terminate in the nucleus tractus solitaries. Vagal afferents have 3 distinct terminals that exist in specific locations within the gut wall: intramuscular arrays (IMAs) within longitudinal and circular muscle layers especially in the stomach (Berthoud and Powley 1992), intraganglionic laminar endings (IGLEs) which form basket-like structures surrounding myenteric ganglia (Berthoud *et al.* 1997; Zagorodnyuk and Brookes 2000) and mucosal endings in the lamina propria in close proximity to the mucosal

epithelium but not exposed directly to the contents of the lumen. The first two groups of terminals act as mechanoreceptors within the muscle layers while the mucosal afferents have a role in 'tasting' the lumen via a number of 'helper' cells, e.g. enteroendocrine cells (Berthoud and Patterson 1996). These mucosal afferents are most abundant in the proximal duodenum (Berthoud and Neuhuber 2000). Due to the proximity of IGLEs and myenteric ganglia, there has also been speculation that this might be the site of 'cross-talk' between the intrinsic and extrinsic systems (Grundy 2006).

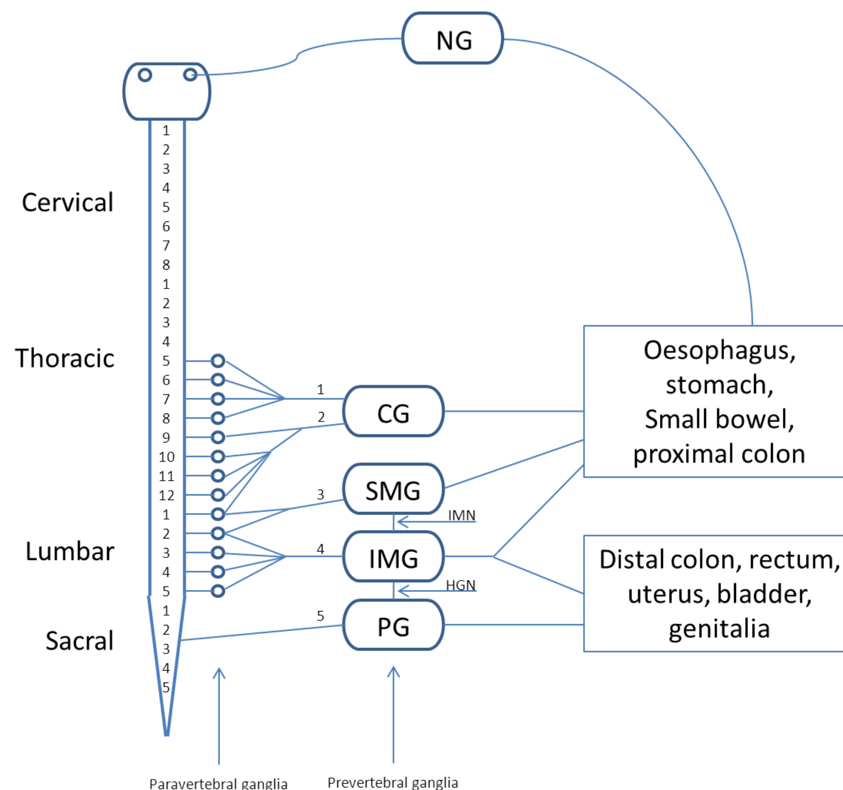


Figure 1 Visceral afferent pathways in rodents. From oesophagus to proximal colon, the GI tract is dually innervated by vagal and spinal afferents in contrast with the lower GI tract which is innervated by spinal afferents only. NG - nodose ganglion, CG - coeliac ganglion, SMG/IMG – superior/inferior mesenteric ganglion, PG – pelvic ganglion, IMN – intermesenteric nerve, HGN – hypogastric nerve. 1-4 greater, lesser, least and lumbar splanchnic nerves respectively, 5 pelvic nerve.

1.2.1.2 Spinal afferents

Spinal afferents have their cell bodies in dorsal root ganglia (DRG) and pass through para- and pre-vertebral ganglia to synapse in the dorsal horn, where they converge with somatic afferents. It has been established that fewer than 15% of DRG neurons are visceral in origin (Neuhuber *et al.* 1986; Perry and Lawson 1998; Peeters *et al.*

2006). In contrast to somatic afferents that have dense terminations, the visceral afferents terminate extensively and thinly in laminae I, V and X of the spinal cord, with terminations spread over at least five spinal segments and some crossing into contralateral lamina (Sugiura *et al.* 1989; Knowles and Aziz 2009). The convergence of visceral and somatic input explains the phenomenon of referred pain (Bielefeldt and Gebhart 2006) while the scattered distribution amongst numerous spinal segments may explain the poor localisation of visceral pain by patients (Sugiura *et al.* 1989).

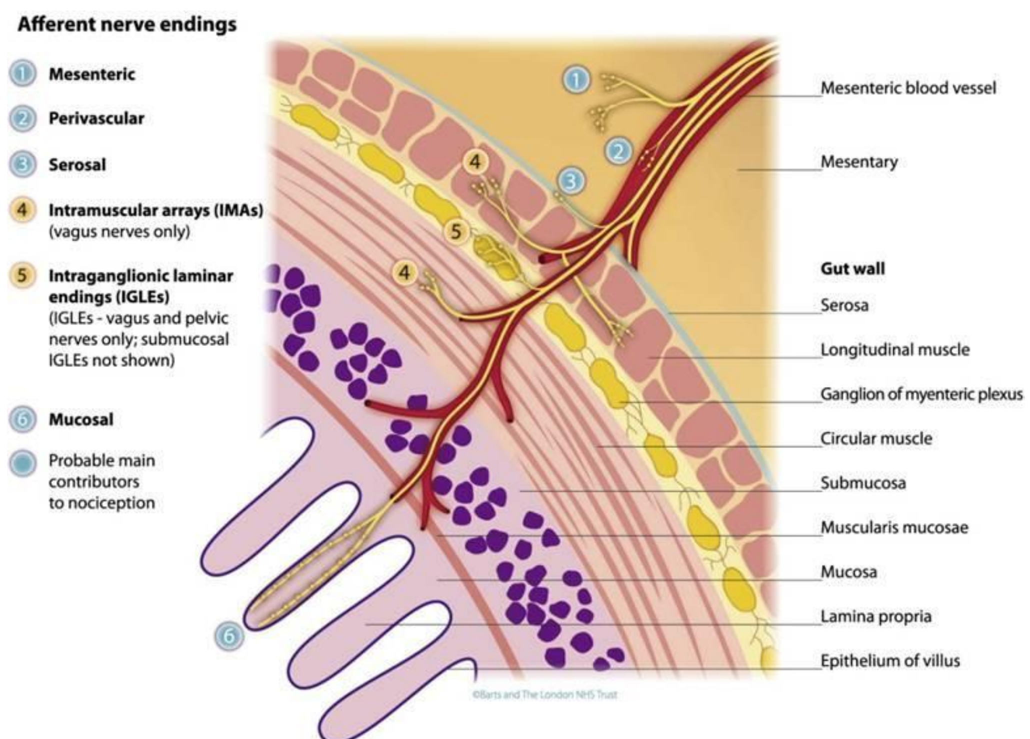


Figure 2 Nerve endings in the GI tract. From Knowles and Aziz 2009.

The second order neurons project to the brain through the spinomesencephalic, spinohypothalamic, spinorecticular and spinothalamic tracts that lie in the anterolateral quadrant of the spinal cord. The spinothalamic tract transmits conscious somatic and visceral sensation by its projections to the somatosensory cortex, anterior cingulate cortex and the insula while the other three tracts mainly trigger subconscious responses to the visceral and somatic input (Almeida *et al.* 2004; Anand *et al.* 2007). The transmission of nociception from spinal afferents can

be modulated by gating influences from converging viscerosomatic neurons. Visceral pain thresholds are increased by viscerosomatic inputs with transient inhibition of transmission demonstrated (Tattersall *et al.* 1986). This may explain the pain relief experienced by patients by the application of a hot water bottle to the abdominal wall.

A spinal visceral afferent consists of peripheral terminals in the gut wall, a peripheral nerve axon, a cell body in the DRG, central axons in the dorsal root and central axon terminals in the spinal cord. In rodents, spinal afferents have been shown to consist of five classes based on their response to mechanical stimuli: mesenteric, serosal, muscular, muscular/mucosal and mucosal (Brierley *et al.* 2004). Three of these classes (serosal, muscular and mucosal) are common to both lumbar splanchnic nerve (LSN) and pelvic nerve (PN) afferents but their relative prevalence differs between the two populations. Mesenteric afferents are only found in the LSN (where they accounted for 50% of all afferents detected) while muscular/mucosal afferents are only present on the PN (Brierley *et al.* 2004). In a study using fast blue to label afferents from the descending colon of the mouse, Robinson *et al* demonstrated labelling of DRG from T4 to S3 with two peaks around T8-L1 and L6/S1, which represent splanchnic and pelvic populations respectively (Robinson *et al.* 2004). These data are supported by other studies in the mouse and rat that have shown the greatest density of labelling of the LSN at T8-T12 and a narrow peak at L6/S1 or L6-S2 likely to be due to innervation from the PN (Nadelhaft and Booth 1984; Neuhuber *et al.* 1986; Payette *et al.* 1987). Labelling of gastric afferents has been demonstrated between T4 and L2 with a narrow peak at T9/10 (Ozaki and Gebhart 2001) while the peak labelling of jejunal afferents is at T12 (Tan *et al.* 2008). This overlap between gastric, jejunal and colonic input into second order neurons explains the poor localisation of visceral pain by patients.

1.2.2 Signalling of extrinsic visceral afferents

Most of the information transmitted from the viscera to the central nervous system (CNS) is unconsciously processed with the principle conscious sensation being pain. Visceral afferents can be classified either anatomically (mucosal/muscular/serosal/ mesenteric) or functionally (mechanical/chemical/thermal). Vagal mechanosensitive afferents (IMAs and IGLEs) are thought to code mainly within the physiological range with a minority that detect noxious gastric distension (Ozaki *et al.* 1999). Chemosensitive vagal afferents detect a wide range of luminal stimuli both physiological and noxious including acid, cholecystokinin, glucose and fatty acids (Berthoud and Neuhuber 2000) using 'helper cells' that present mediators to the afferent terminal.

Whereas vagal afferents encode mainly within the physiological range, splanchnic afferents encode well into the pathological range and are thought to be the main pathway for mediating pain perception (Berthoud *et al.* 2004). Recently, IGLEs have been reported in guinea pig rectum which, like the vagal IGLEs, are low threshold, slowly adapting receptors (Lynn *et al.* 2003). These receptors are not seen in splanchnic afferents. This suggests that the more focused distribution of vagal and pelvic afferents may correspond to areas of the GI tract where graded, innocuous sensations can be evoked by distension (Berthoud *et al.* 2004).

In *in vitro* recordings, the majority of mechanosensitive spinal afferents have been found to be silent at rest (Brierley *et al.* 2004). In addition, significant differences exist in the response of splanchnic and pelvic afferents to mechanical stimuli: splanchnic afferents are overall less responsive to mechanical stimulation than pelvic afferents and also adapt more completely to mechanical probing. Nearly half of pelvic afferents respond to circular stretch or mucosal stroking compared to less than 10% of splanchnic afferents (Brierley *et al.* 2004; Brierley *et al.* 2005a). Pelvic afferents also have lower activation thresholds to electrical stimulation (Feng and Gebhart 2011). These characteristics suggest that the pelvic pathway signals maintained distension and passage of material whereas the splanchnic pathway

signals transient events, e.g. rapid distension or torsion of the mesentery (Brierley *et al.* 2005b).

Mechanosensitivity is influenced by a wide range of mediators released as a consequence of inflammation, e.g. bradykinin or prostaglandins, which can reduce the activation threshold of afferents leading to hypersensitivity (Kirkup *et al.* 2001). Indeed, some mechanically insensitive afferents develop mechanosensitivity during inflammation which may persist after the acute insult (Gebhart 2000). A recent study has shown that over a quarter of the afferent population in the distal 3 cm of the mouse colon/rectum was mechanically insensitive and that approximately half of these acquired mechanical sensitivity after exposure to an inflammatory soup (Feng and Gebhart 2011). The study also demonstrated significant differences between pelvic and splanchnic afferents: a higher proportion of LSN (33%) than PN (23%) afferents was mechanically insensitive but a significantly lower proportion of LSN (23%) than PN (71%) gained mechanosensitivity after the inflammatory soup. In agreement with results from other *in vitro* studies, the responses of PN afferents were greater than LSN afferents (Feng and Gebhart 2011). An inflammatory soup has also been shown to reduce the threshold for, and increase the response to, distension of the stomach *in vivo* (Ozaki and Gebhart 2001). In addition, a role for spinal afferents in IBS-related hypersensitivity has been highlighted by a study assessing response to mechanical stimuli in mice subjected to trinitrobenzene sulfonic acid (TNBS) colitis (Hughes *et al.* 2009). Splanchnic afferents demonstrated increased responses to mechanical stimulation associated with a reduction in the activation threshold during the acute and recovery phases. In contrast, pelvic afferents only demonstrated these effects in the recovery phase with no change in their response during the acute inflammatory phase. The sensitisation of afferents was restricted to the high-threshold serosal and mesenteric afferents (Hughes *et al.* 2009). Reducing these afferents' threshold may be the major factor underlying hyperalgesia.

Spinal afferents also respond directly to application of a number of chemical mediators including capsaicin, adenosine-5'-triphosphate (ATP) and bradykinin (Brierley *et al.* 2005a; Brierley *et al.* 2005b; Song *et al.* 2009). More splanchnic than pelvic afferents respond to these mediators (ATP 40% vs. 7%; capsaicin: 61% vs. 47%; bradykinin 66% vs. 11%). Following exposure to capsaicin, splanchnic afferents demonstrate desensitisation to mechanical stimuli which is not seen in pelvic afferents (Brierley *et al.* 2005a) while exposure to bradykinin sensitised the mechanical response of splanchnic but not pelvic nerves (Brierley *et al.* 2005b). The differential response of afferents to chemical and mechanical stimuli can be explained by the differential expression of receptors and ion channels on their terminals. Visceral afferents express calcitonin gene-related peptide, Substance P and transient receptor potential vanilloid channel (TRPV) 1 and 4 in greater proportion than somatic afferents (Perry and Lawson 1998; Robinson *et al.* 2004; Brierley *et al.* 2008). In addition, DRG neurons express significantly more TRPV1, transient receptor potential ankyrin channel (TRPA) 1 and voltage-gated sodium channel 1.9 ($Na_v1.9$) than NG neurons (Peeters *et al.* 2006). More significantly, there is a difference in the expression of some receptors amongst spinal visceral afferents, with splanchnic afferents expressing significantly more TRPV1 and $P2X_3$ channels than pelvic afferents (Brierley *et al.* 2005a).

1.2.3 Mediators and channels involved in signalling

Transduction is the conversion of an extracellular stimulus into a generator potential. A generator potential is produced by the opening of ion channels in response to a depolarising stimulus and is terminated by time, voltage-gated inactivation of these channels and the opening of a voltage sensitive outward potassium conductance (Glazebrook *et al.* 2002). When the membrane potential exceeds a threshold, an action potential is initiated at the terminal and propagated along the axon. The arrival of an action potential at the central terminal results in the release of neurotransmitters which bind to receptors on the second order neurons, allowing propagation of the signal. There are 3 classes of receptors on afferent

terminals: ligand-gated ion channels, voltage-gated ion channels and G-protein coupled receptors (GPCRs) (Figure 3).

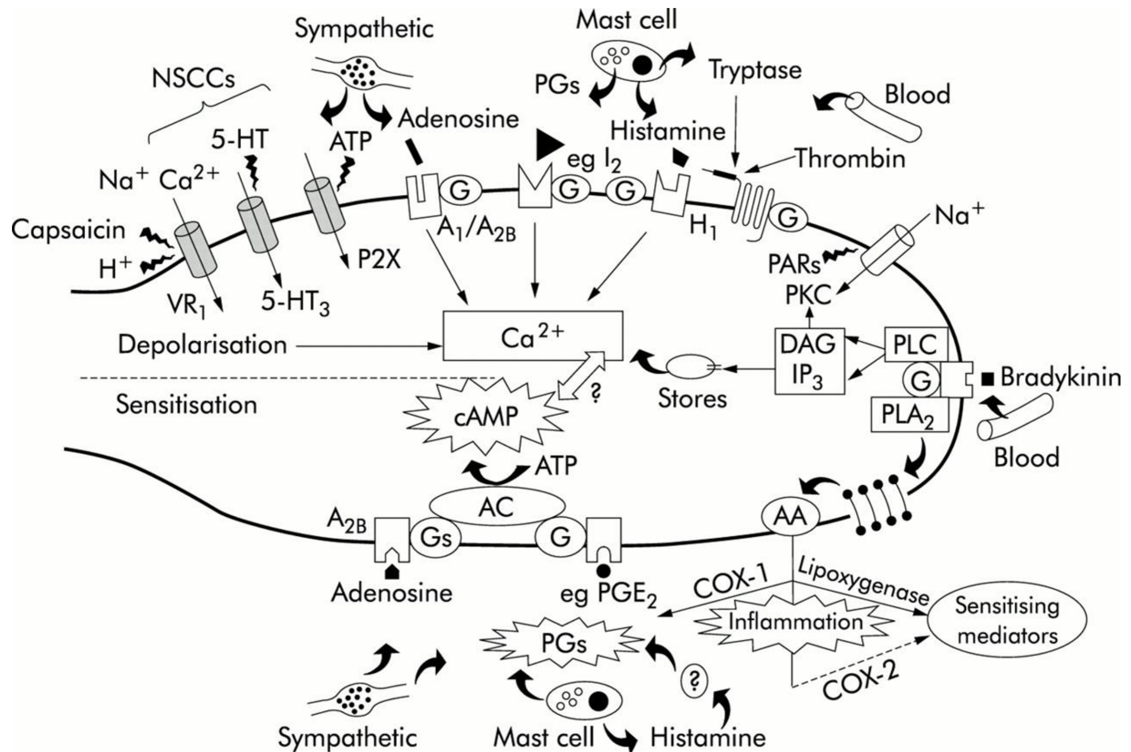


Figure 3 Receptors and mechanisms underlying activation and sensitisation of visceral sensory afferents. 5-HT - 5-hydroxytryptamine, ATP - Adenosine-5'-triphosphate, COX – cyclooxygenase, DAG – diacylglycerol, IP3 - inositol 1,4,5-triphosphate, PARs - protease activated receptors, PGs – Prostaglandins, PKC - protein kinase C, PLA2 - phospholipase A2, PLC - phospholipase C, VR_1 - Transient receptor potential V1. From Grundy 2002.

Inflammatory mediators are released by numerous cell types in response to tissue injury and produce their effects on visceral afferents via three processes: direct activation of an ion channel, sensitisation of the afferent through second messenger molecules and alteration of the phenotype of the afferent through changes in the expression of channels, receptors or other mediators (Kirkup *et al.* 2001). In addition, some receptors share second messenger pathways and hence can be modulated by feedback from different mediators (Wood 2000). The major ion channels and mediators involved in signalling are discussed further in the following sections.

1.2.3.1 Ligand-gated ion channels

The transient receptor potential (TRP) channel family consists of over 30 members grouped into six subfamilies (Wu *et al.* 2010). They have six transmembrane domains and assemble as homo- or hetero-tetramers to form cation channels that allow calcium into the cells when activated. A number of TRP channels have been implicated in nociception.

TRPV1 is the receptor for capsaicin, protons and temperatures over 43 ˚C (Caterina *et al.* 1997). It is preferentially located on small to medium diameter neurons (Caterina *et al.* 2000) and modulated by protein kinase C (PKC) (Premkumar and Ahern 2000; Vellani *et al.* 2001; Numazaki *et al.* 2002). TRPV1 expression is increased in colorectal tissue from patients with an acute exacerbation of their IBD (Yiangou *et al.* 2001b) or rectal hypersensitivity (Chan *et al.* 2003; Akbar *et al.* 2008) while TRPV1 -/- mice lack the afferent hypersensitivity associated with inflammation (Caterina *et al.* 2000; Davis *et al.* 2000; Jones *et al.* 2005). In addition, DRG of adult mice that were subjected to acetic acid infusion as neonates revealed significant increase in TRPV1 expression (Winston *et al.* 2007). *In vivo* studies of colorectal distension in rats have demonstrated an important role for TRPV1 in the mechanical hypersensitivity of sensitised visceral afferents (Ravnefjord *et al.* 2009; Vermeulen *et al.* 2013). In addition, a TRPV1 antagonist has been shown to reduce the thermal hyperalgesia expressed after injection of complete Freund's adjuvant (CFA) into the hind paw of rats (Joshi *et al.* 2009). These studies highlight a significant role for TRPV1 in the sensitisation of visceral and somatic afferents.

TRPV4 is an osmo-sensitive channel that responds to mechanical stimulation and temperatures between 27 and 34 ˚C (Wu *et al.* 2010). It is postulated to play a significant role in mechanical transduction in the gut. Colonic instillation of a TRPV4 agonist provokes allodynia and hyperalgesia to colonic distension in mice and this was reduced in mice injected with small interfering ribonucleic acid (siRNA) to TRPV4 (Cenac *et al.* 2008). In agreement with the above, knock out of TRPV4 causes an

increased threshold to mechanical stimulation and a reduction in the response to von Frey probing *in vitro* and balloon distension of the distal colon *in vivo* (Brierley *et al.* 2008). Tissue from patients suffering from an acute exacerbation of their IBD demonstrates increased labelling for TRPV4 (Brierley *et al.* 2008).

TRPA1 is the receptor for mustard and cinnamon as well as temperatures below 17 ˚C (Story *et al.* 2003; Wu *et al.* 2010) and is co-expressed with TRPV1 (Story *et al.* 2003; Kobayashi *et al.* 2005). In addition, TRPA1 has been shown to play an important role in the responses to balloon distension of the mouse colon and rat stomach (Brierley *et al.* 2009; Kondo *et al.* 2009). This has been corroborated by *in vitro* studies demonstrating a reduced response and increased threshold to mechanical stimulation in TRPA1 -/- mice (Brierley *et al.* 2009). TRPA1 agonists cause significantly greater sensitisation to mechanical stimuli after colonic inflammation compared to no inflammation. This effect was absent in TRPA1 -/- mice, highlighting a role for TRPA1 in mediating IBD associated hyperalgesia (Brierley *et al.* 2009).

ATP is released as a result of inflammation, hypoxia, cell acidosis or distension and binds to P2X (ligand-gated ion channels) and P2Y (GPCR) receptors to cause an inward flow of calcium ions (Bouvier *et al.* 1991). Of the seven P2X receptors, $P2X_3$ is selectively expressed on small diameter sensory neurons (Chen *et al.* 1995; Vulchanova *et al.* 1998) and $P2X_3$ -/- mice experience attenuated pain behaviours (Souslova *et al.* 2000). Colonic $P2X_3$ expression is increased in IBD in man (Yiangou *et al.* 2001a) and the release of ATP by colorectal distension has been shown to be increased in a rat model of colitis, demonstrating a prominent role for ATP in the response to inflammation (Wynn *et al.* 2004).

1.2.3.2 Voltage-gated ion channels

Nerve fibres contain numerous voltage-gated ion channels including potassium, calcium and sodium channels. Only those channels involved in membrane depolarisation are considered in this section.

Voltage-gated calcium channels comprise of an α1 channel-forming subunit associated with α2δ, β and γ subunits. Each α1 subunit comprises of four repeat domains of six transmembrane segments (Dolphin 2012). Activation of voltage-gated calcium channels alters the membrane potential and contributes to the excitability of sensory neurons. These channels also play a crucial role in neurotransmitter release from the presynaptic terminals in the dorsal horn.

Voltage-gated sodium channels are transmembrane receptors that are essential for the generation of action potentials. They confer excitability on neurons by opening in response to membrane depolarisation, thus allowing influx of sodium ions. Because of the direct relevance of these channels to the thesis, they are discussed separately in section 1.3 below.

1.2.3.3 G-protein coupled receptors

GPCRs are the largest family of cell surface mediators of signal transduction with approximately 800 human genes coding for functional GPCRs. They share a common structure comprising seven α-helical trans-membrane domains connected by extracellular and intracellular loops. They interact with G-proteins which then modulate the activity of a number of effectors, e.g. phospholipases and ion channels (Neer 1995). Only the most relevant substrates of GPCRs are discussed here.

A major aspect of the inflammatory response is the direct release of cytokines, e.g. Interleukin (IL) -1β, IL-8 and Tumour necrosis factor alpha (TNFα), and the

recruitment of leukocytes that causes further release of cytokines. Cytokines are low molecular weight regulatory proteins that commonly stimulate the release of other mediators and cause phosphorylation of ion channels, leading to sensitisation of afferents (Kidd and Urban 2001). The inflammatory response is normally resolved by the release of anti-inflammatory cytokines (e.g. IL-4, IL-6 and IL-10). An imbalance in the proportion of pro- and anti-inflammatory cytokines leads to the development of chronic inflammation and associated chronic pain. Studies of peritoneal fluid from patients suffering from acute appendicitis or IBD show an elevation in the concentrations of IL-1β, IL-6, IL-8 and TNFα amongst others (Rivera-Chavez *et al.* 2003; Dalal *et al.* 2005; Yamamoto *et al.* 2005) suggesting a role for these cytokines in the body's response to visceral inflammation.

Tissue damage leads to the activation of kallikreins which cleave kininogens to generate kinins. Kinins are a group of 9-11 amino acid peptides that include bradykinin and its active metabolite, des-Arg^9-bradykinin (Couture *et al.* 2001). Bradykinin is an initial mediator of inflammation, induces pain and is a potent vasodilator. Its actions are mediated via two GPCRs: bradykinin receptors 1 and 2 (B_1 and B_2). B_2 is constitutively expressed whereas B_1's expression is induced as a result of tissue damage (Couture *et al.* 2001). Both receptors signal through G_q and activate phospholipase C (PLC) which, in turn, cleaves Phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG activates PKC while IP3 leads to the opening of Ca^{2+} channels and consequent release of Ca^{2+} into the cytoplasm (Leeb-Lundberg *et al.* 2005). PKC modifies the function of other proteins, including transmembrane channels, e.g. $Na_v1.8$, $Na_v1.9$ and TRPV1 (Khasar *et al.* 1999; Premkumar and Ahern 2000; Vellani *et al.* 2001; Numazaki *et al.* 2002; Shin *et al.* 2002; Baker 2005). Loss of either bradykinin receptor (either in knockout mice or by using antagonists) results in reduced responses to painful stimuli (Rupniak *et al.* 1997; Pesquero *et al.* 2000).

Cyclooxygenase (COX) mediates the conversion of arachidonic acid into prostanoids. Analogous to the B_1 and B_2 receptors, COX-1 is constitutively expressed whereas COX-2 is induced by inflammation. Prostaglandins act as sensitisors by stimulating the production of Protein kinase A (PKA) and PKC, which mediate voltage-gated sodium channel and TRPV1 function, thus increasing sensory afferent excitability (Fitzgerald *et al.* 1999; Matsumoto *et al.* 2005). Prostaglandin E_2 (PGE_2) also increases the proportion of DRG neurons that respond to the application of bradykinin (Stucky *et al.* 1996) as well as the magnitude of response (Mense 1981; Brunsden and Grundy 1999). Addition of PGE_2 to a jejunal nerve preparation restores the bradykinin response that was partially blocked by a COX blocker, naproxen, suggesting that PGE_2 is required for the full response to bradykinin (Maubach and Grundy 1999).

1.2.4 Sensitisation

Persistent inflammation can cause increased expression of a channel or increased trafficking of the channel to the peripheral terminal (Ji *et al.* 2002). In addition, the release of pro-inflammatory agents at the site of injury may lead to peripheral sensitisation (Bueno *et al.* 2000) which is a form of stimulus-induced nociceptor plasticity characterised by increased spontaneous activity, an increased response to stimuli and a reduction in the threshold required to initiate an action potential. These characteristics may clinically manifest as spontaneous pain, hyperalgesia (exaggerated response to a stimulus) and allodynia (response to a previously innocuous stimulus). These phenomenon occur both in the somatic and visceral systems. Peripheral sensitisation can occur through 3 different mechanisms: a change in receptor sensitivity, an increase in the numbers of receptors or through the expression of new receptors (Kirkup *et al.* 2001). In principal, peripheral sensitisation is transient with the response properties of primary afferents returning to their normal state after resolution of the inflammatory episode (Mayer and Tillisch 2011).

A similar chain of events occurs centrally. The increased release of pre-synaptic neurotransmitters that occurs with peripheral sensitisation increases the activation of PKA and PKC. This leads to changes in CNS receptor kinetics, e.g. lowering of the activation threshold, that enhance their responsiveness (Knowles and Aziz 2009). As the majority of synaptic input is subthreshold (Woolf and King 1989), the lowering of the threshold allows conversion of these previously subthreshold inputs into action potentials. This is termed central sensitisation. In central sensitisation, the pain is not coupled to the presence, intensity or duration of a noxious stimulus and is due to a shift in the sensory system from high-threshold nociception to low-threshold pain hypersensitivity (Latremoliere and Woolf 2009). This corresponds to an enhancement in the functional status of the nociceptive pathways by an increase in membrane excitability, synaptic efficacy or reduced inhibition. Due to the convergence of numerous nerves in the CNS, central sensitisation often has effects on neurons adjacent to those that exhibit peripheral sensitisation. This leads to hyperalgesia in those neighbouring nerves, termed secondary hyperalgesia, and affects both visceral and somatic afferents (Figure 4).

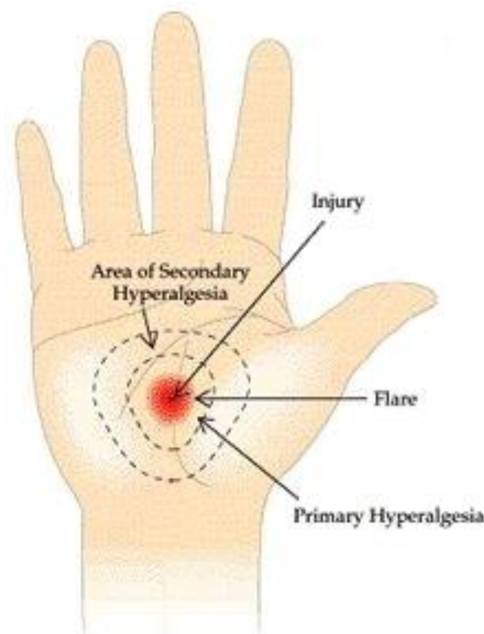


Figure 4 Illustration of an area of primary hyperalgesia surrounding a focus of injury. In addition, central sensitisation leads to development of a larger area of secondary hyperalgesia. Modified from Galer *et al* 2002.

1.3 Role of voltage-gated sodium channels in nociception

1.3.1 Introduction

Voltage-gated sodium channels (Na_v) are transmembrane proteins that are essential for the generation of action potentials. Each channel is coded for by 1700-2000 amino acids and formed by an α subunit (260 kDa) which acts as the core protein and auxiliary β subunits (33-36 kDa) which modify channel function (Catterall 2000). Each α subunit has 4 homologous domains each of which is made up of 6 transmembrane segments with a pore-forming loop between segments 5 and 6 (Figure 5). The short intracellular loop connecting domains III and IV serves as the inactivation gate for the channel (Catterall *et al.* 2005). The α subunit contains all the features of a functional ion channel including the voltage sensor, the activation and inactivation gates and the binding sites for tetrodotoxin and other blockers as well as phosphorylation sites (Cantrell and Catterall 2001). Four β subunits exist and they have differential distribution depending on the size of the neuron with β1 and β3 being the predominant subunits of large and small neurons respectively (Ho *et al.* 2012). The individual β subunits have been shown to have differing modulatory effects on Na_v function, for example β3 regulates $Na_v1.7$ activation while β1 regulates inactivation of the channel (Ho *et al.* 2012).

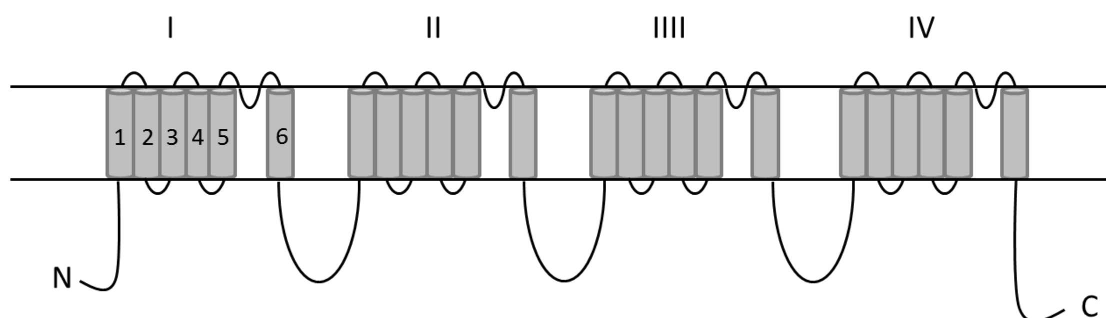


Figure 5 Structure of the α subunit of voltage-gated sodium channels. Each subunit is made of 4 homologous domains (I-IV) which are made of 6 trans-membrane segments (1-6). The pore is formed by loops between segments 5 and 6 of each domain.

There are 10 α subunits in total, with nine belonging to the same family (Figure 6). The tenth member (Na_x) is 50% identical to the other channels but is thought to be activated by changes in Na^+ concentration rather than the membrane potential (Hiyama *et al.* 2002; Catterall *et al.* 2005). From an evolutionary standpoint, Na_v1.1, Na_v1.2, Na_v1.3 and Na_v1.7 are thought to be closely related having genes located on human chromosome 2q23-24, while Na_v1.5, Na_v1.8 and Na_v1.9 form another subfamily located on 3p21-24 (Catterall *et al.* 2005).

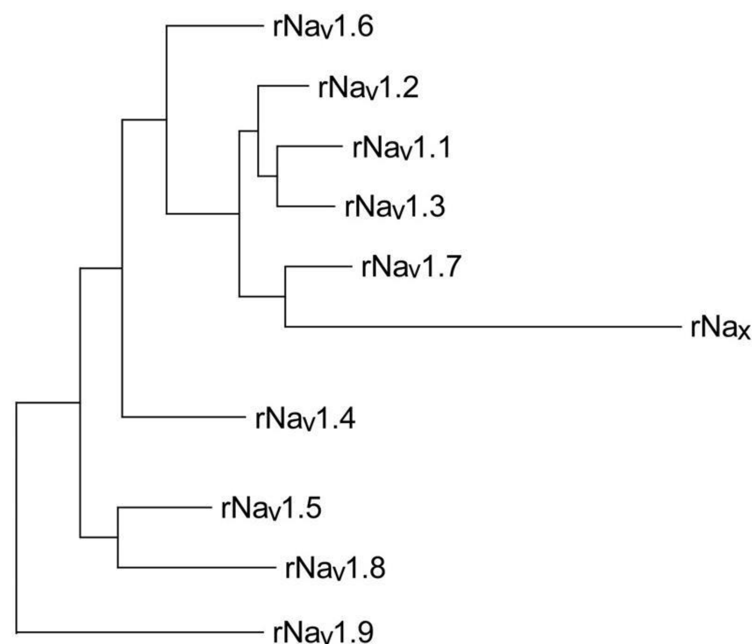


Figure 6 Phylogenetic tree of rat voltage-gated sodium channel α subunits. From Catterall *et al* 2005.

Collectively, Na_v channels are distributed throughout the body but individual channels have restricted distribution (Table 1 and figure 7). The channels are also categorised by their sensitivity to the puffer fish poison, tetrodotoxin (TTX) with most channels sensitive to nM concentrations of TTX while others (Na_v1.5, Na_v1.8 & Na_v1.9) are resistant to mM concentrations of TTX. Na_v1.7-1.9 are the dominant peripheral nervous system (PNS) channels with Na_v1.8 and Na_v1.9 expressing a TTX resistant (TTX-r) current while Na_v1.7 expresses a TTX sensitive current (TTX-s) (Catterall *et al.* 2005).

Channel	Predominant location	Sensitivity to tetrodotoxin	Chromosomal location
$Na_v1.1$	CNS/PNS	Sensitive	2q24
$Na_v1.2$	CNS	Sensitive	2q23-24
$Na_v1.3$	Embryonic CNS	Sensitive	2q24
$Na_v1.4$	Skeletal muscle	Sensitive	17q23-25
$Na_v1.5$	Heart	Resistant	3p21
$Na_v1.6$	CNS/PNS	Sensitive	12q13
$Na_v1.7$	PNS	Sensitive	2q24
$Na_v1.8$	PNS	Resistant	3p22-24
$Na_v1.9$	PNS	Resistant	3p21-24

Table 1 Distribution, chromosomal location and tetrodotoxin sensitivity of the voltage-gated sodium channels. PNS – peripheral nervous system, CNS – central nervous system. From Ogata and Ohishi 2002.

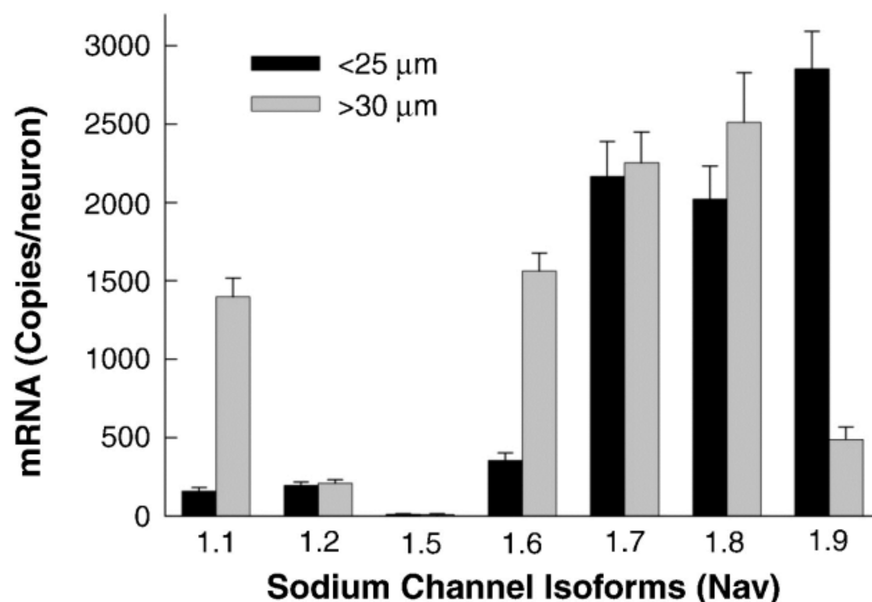


Figure 7 Expression of Nav transcripts in rat DRG neurons. Small (<25µm) and large (>30µm) diameter neurons were individually harvested with the mRNA in the cell lysates reverse transcribed and quantified using TaqMan real-time polymerase chain reaction. The data are the means and standard errors of 71 small and 90 large diameter neurons. mRNA messenger ribonucleic acid. From Ho and O’Leary 2011.

1.3.2 Role of Na_v in pain models

Because of their role in action potential generation, Na_vs contribute to the development of neuropathic and inflammatory pain. Neuropathic pain results from changes in the nerve structure, e.g. transection or avulsion of the nerve, whereas inflammatory pain develops as a result of changes in the environment surrounding the nerve, e.g. release of mediators during the inflammatory process. Thus, inflammatory pain may be somatic or visceral in origin whereas there is no established visceral model of neuropathic pain (although peripheral nerves are transected or ligated at surgery whenever the mesentery is divided).

1.3.2.1 Neuropathic pain models

A number of voltage-gated sodium channels have been shown to play a role in the development of neuropathic pain and subsequent hyperalgesia.

1.3.2.1.1 Na_v1.3

Na_v1.3, which is normally present at very low levels in adult DRG (Waxman *et al.* 1994), is significantly up-regulated following peripheral nerve injury in both rodents (Fjell *et al.* 1999; Waxman *et al.* 1999; Hains *et al.* 2003) and man (Black *et al.* 2008); and consequently its current becomes the dominant TTX-s current (Black *et al.* 1999). These changes are attenuated by application of nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF) which reverse the increase in Na_v1.3 expression and ameliorate behavioural responses (Boucher *et al.* 2000; Leffler *et al.* 2002). In addition, application of antisense oligodeoxynucleotides to Na_v1.3 decreases the mechanical and thermal hyperalgesia induced by the nerve injury implying a role for Na_v1.3 in the maintenance of the hyperalgesia (Hains *et al.* 2003).

1.3.2.1.2 Na_v1.7

Peripheral nerve injury causes down-regulation of Na_v1.7 transcript levels in rodents (Kim *et al.* 2002; Berta *et al.* 2008) whereas in man, immunoreactivity to Na_v1.7

increases in human neuromas (Kretschmer *et al.* 2002; Bird *et al.* 2007; Black *et al.* 2008). This contradiction may reflect inter-species differences in the contribution of $Na_v1.7$ to pain signalling as evidenced by the debilitating hereditary pain syndromes caused by gain-of-function mutations in $Na_v1.7$ in man (see section 1.3.2.2.1) or methodological issues, particularly the specificity of human antibodies.

1.3.2.1.3 $Na_v1.8$

$Na_v1.8$ expression is downregulated in injured neurons in rodent neuropathic pain models (Dib-Hajj *et al.* 1996; Dib-Hajj *et al.* 1999a; Decosterd *et al.* 2002; Lai *et al.* 2002; Zhang *et al.* 2004). However, in uninjured neighbouring neurons, the expression of $Na_v1.8$ is increased (Decosterd *et al.* 2002; Gold *et al.* 2003; Zhang *et al.* 2004). Data from studies using antisense or $Na_v1.8$ knockout mice show a reduction in mechanical and thermal hyperalgesia after elimination of $Na_v1.8$ (Akopian *et al.* 1999; Lai *et al.* 2002; Nassar *et al.* 2005). The hyperalgesia is also reduced by application of a small molecule blocker of $Na_v1.8$ (Jarvis *et al.* 2007). The expression of $Na_v1.8$ is also upregulated in painful neuromas in man (Coward *et al.* 2000; Black *et al.* 2008). The apparently paradoxical findings of decreased expression of $Na_v1.8$ in injured neurons and the decrease in hypersensitivity derived from blocking $Na_v1.8$ suggests that the role played by $Na_v1.8$ in neuropathic pain results from its redistribution to uninjured neurons.

1.3.2.1.4 $Na_v1.9$

In both rodent (Dib-Hajj *et al.* 1998; Sleeper *et al.* 2000; Decosterd *et al.* 2002; Priest *et al.* 2005; Amaya *et al.* 2006) and man (Coward *et al.* 2000; Black *et al.* 2008), studies have failed to show a role for $Na_v1.9$ in the development of neuropathic pain.

1.3.2.2 Inflammatory pain models

There is significant evidence in support of a role for voltage-gated sodium channels in the development of pain and hypersensitivity after inflammation in both the somatic and visceral systems.

1.3.2.2.1 $Na_v1.7$

In the rodent somatic system, $Na_v1.7$ expression is upregulated after injection of CFA (Gould *et al.* 2004), carrageenan (Black *et al.* 2004) or NGF (Gould *et al.* 2000). Removal of $Na_v1.7$ activity from nociceptors, e.g. by using conditional knock out or antisense, reduces the inflammatory pain responses as well as the ensuing hyperalgesia (Nassar *et al.* 2004; Yeomans *et al.* 2005). No studies have investigated a role for $Na_v1.7$ in the development of visceral inflammatory pain.

In man, $Na_v1.7$ gain of function mutations cause primary erythromelalgia and paroxysmal extreme pain disorder while loss of function mutations lead to congenital insensitivity to pain in individuals who have a reduced sense of smell but are otherwise normal (Dib-Hajj *et al.* 2007). There have been reports of individuals suffering from osteomyelitis, cellulitis and acute appendicitis without experiencing any pain (Cox *et al.* 2006; Goldberg *et al.* 2007). A recent clinical study showed that the administration of a $Na_v1.7$ blocker reduces pain in patients suffering from primary erythromelalgia (Goldberg *et al.* 2012). These studies provide compelling evidence of a role for $Na_v1.7$ in both somatic and visceral inflammatory pain in man.

1.3.2.2.2 $Na_v1.8$

In the rodent somatic system, $Na_v1.8$ expression and current are increased in response to a variety of inflammatory mediators including CFA, carrageenan and GDNF (Cummins *et al.* 2000; Black *et al.* 2004; Coggeshall *et al.* 2004). The expression of $Na_v1.8$ has also been shown to be increased in a TNBS model of colitis (King *et al.* 2009). Studies using $Na_v1.8$ -/- mice, antisense oligodeoxynucleotides or small

molecule blockers have demonstrated a role for $Na_v1.8$ in the response of visceral (Laird *et al.* 2002; Jarvis *et al.* 2007) and somatic (Akopian *et al.* 1999; Kerr *et al.* 2001; Abrahamsen *et al.* 2008; Joshi *et al.* 2009) afferents to inflammatory mediators and the consequent development of thermal and mechanical hyperalgesia.

In man, increased expression of $Na_v1.8$ has been observed in pulp from painful teeth (Renton *et al.* 2005). These studies highlight the role that $Na_v1.8$ plays in the early stages of the inflammatory response as well as in the development of hyperalgesia that is the hallmark of sensitisation.

1.3.2.2.3 $Na_v1.9$

$Na_v1.9$ expression and current are increased in response to a variety of inflammatory mediators suggesting that $Na_v1.9$ plays a role in inflammatory pain signalling. As it is the subject of this thesis, $Na_v1.9$ is fully discussed in section 1.4 below.

1.4 $Na_v1.9$

1.4.1 Identification

Dorsal root ganglia neurons express a sodium current which includes a strong TTX-r element (Rizzo *et al.* 1994) and $Na_v1.8$ was identified as the channel responsible for this current in 1996 (Akopian *et al.* 1996). However, patch clamp studies demonstrated an underlying heterogeneity in the TTX-r current which implied that more than one channel was responsible for the overall TTX-r current (Rush and Elliott 1997; Schild and Kunze 1997). Indeed, a TTX-r current was recorded in $Na_v1.8$ -/- mice (Cummins *et al.* 1999). $Na_v1.9$ was first cloned in rat in 1998 and expressed a TTX-r current with different characteristics to $Na_v1.8$ (Dib-Hajj *et al.* 1998; Tate *et al.* 1998). This was followed by the first reports in mouse and man one year later (Dib-Hajj *et al.* 1999b; Dib-Hajj *et al.* 1999c).

1.4.2 Structure

As with all sodium channels, the structure of $Na_v1.9$ consists of α and β subunits. The types of β subunits are yet to be established (Catterall *et al.* 2005) but are likely to be β2 and β3 as these are the predominant β subunits in small diameter afferents (Ho *et al.* 2012). In rat and mouse, $Na_v1.9$ is formed of 1765 residues (1792 residues in man) with a serine in domain 1 predicting resistance to TTX (Dib-Hajj *et al.* 2002). The rat α subunit exhibits only 42-53% similarity to other mammalian Na_vs (Dib-Hajj *et al.* 1998). Mouse and rat $Na_v1.9$ are 91% similar to each other and the human channel is 80% similar to both (Dib-Hajj *et al.* 1999b).

1.4.3 Biophysical characteristics

Recordings of $Na_v1.9$ current have been made from dorsal root and trigeminal ganglia (TG) soma and neurons as well as myenteric ganglia in mouse, rat, guinea pig and man. The current is described as slow, persistent and resistant to TTX, with activation thresholds ranging from -80 mV to -58 mV depending on the cell line and

protocol used (Tate *et al.* 1998; Cummins *et al.* 1999; Dib-Hajj *et al.* 1999b; Baker *et al.* 2003; Coste *et al.* 2004; Priest *et al.* 2005; Amaya *et al.* 2006; Li and Schild 2007; Padilla *et al.* 2007; Zheng *et al.* 2007; Maingret *et al.* 2008; Ostman *et al.* 2008; Qiao *et al.* 2009; Ho and O'Leary 2011). Thus, the $Na_v1.9$ activation threshold is more negative than that of the other Na_vs, particularly $Na_v1.8$ (Tate *et al.* 1998; Fjell *et al.* 1999; Baker *et al.* 2003; Coste *et al.* 2004; Zheng *et al.* 2007) with which it is closely expressed (Figure 8) (Tate *et al.* 1998; Amaya *et al.* 2000; Sleeper *et al.* 2000; Amaya *et al.* 2006). Because of its activation at potentials close to the resting membrane potential, $Na_v1.9$ is thought to contribute to setting the membrane potential (Cummins *et al.* 1999; Herzog *et al.* 2001; Baker *et al.* 2003), modulation of neuronal excitability close to the resting potential (Cummins *et al.* 1999) and enhancement of the response to inputs that are sub-threshold (Herzog *et al.* 2001; Rush and Waxman 2004).

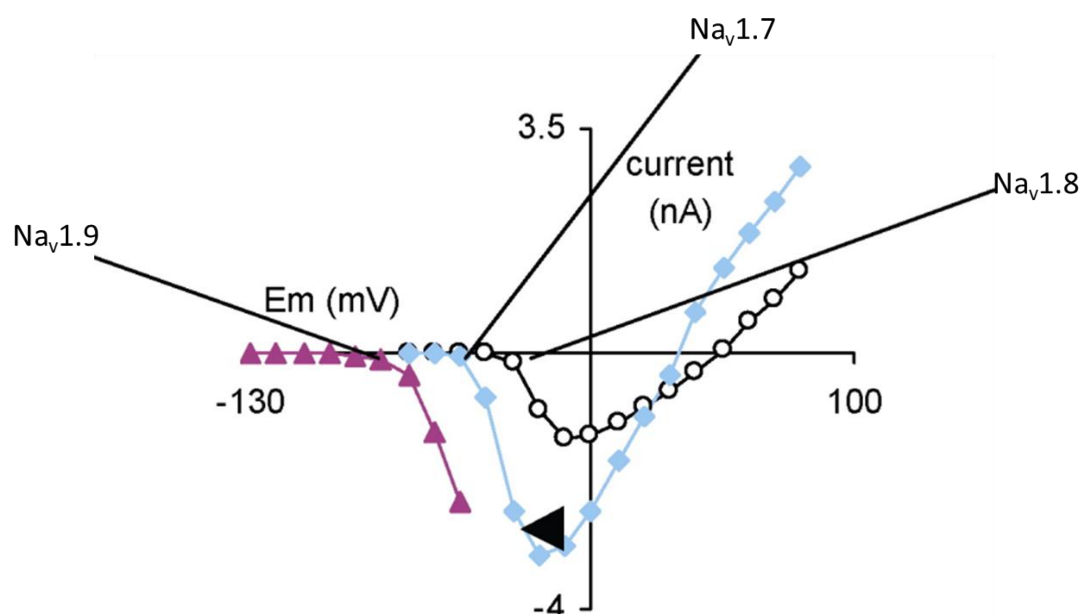


Figure 8 The voltage dependence of activation of $Na_v1.7$-1.9. $Na_v1.9$ activates at potentials more negative and closer to the resting membrane potential than $Na_v1.8$ and $Na_v1.7$. Modified from Momin and Wood 2008.

1.4.4 Cellular distribution

$Na_v1.9$ is preferentially distributed in small diameter neurons and has been shown to be present in 56-83% of fibres less than 30 µm in diameter (Dib-Hajj *et al.* 1998; Fjell *et al.* 2000; Fang *et al.* 2002). Although an early report found no $Na_v1.9$ labelling of myelinated fibres (Amaya *et al.* 2000), others, including from the same group, have shown $Na_v1.9$ in a small minority of Aδ and Aβ fibres (Fjell *et al.* 2000; Decosterd *et al.* 2002; Fang *et al.* 2002; Coggeshall *et al.* 2004; Fukuoka *et al.* 2008). A significant negative relationship has been described between $Na_v1.9$ staining intensity and DRG neuronal size and conduction velocity (Coward *et al.* 2000; Fang *et al.* 2002) as well as TG neuronal size (Wells *et al.* 2007). This indicates that $Na_v1.9$ is expressed most intensively on nociceptive C fibres.

1.4.4.1 Tissue distribution

$Na_v1.9$ has been described in a variety of tissues including rodent DRG and TG (Dib-Hajj *et al.* 1998; Tate *et al.* 1998; Amaya *et al.* 2000; Fang *et al.* 2002; Beyak *et al.* 2004; Amaya *et al.* 2006; Fang *et al.* 2006; Hillsley *et al.* 2006; Padilla *et al.* 2007; Fukuoka *et al.* 2008; Strickland *et al.* 2008; Fukuoka and Noguchi 2011; Ho and O'Leary 2011) as well as on nodes of Ranvier of small diameter fibres (Fjell *et al.* 2000). In addition, $Na_v1.9$ has been demonstrated in rodent cornea (Dib-Hajj *et al.* 1998; Fjell *et al.* 2000), dental pulpal afferents (Padilla *et al.* 2007), digital nerves (Coggeshall *et al.* 2004) and in the epidermis of the paws (Persson *et al.* 2010) and lips (Padilla *et al.* 2007). In the visceral system, $Na_v1.9$ has been demonstrated on bladder (Black *et al.* 2003) and colonic (Hillsley *et al.* 2006; King *et al.* 2009) afferents. In man, $Na_v1.9$ has been shown in DRG soma and neurons (Coward *et al.* 2000), TG neurons and dental pulpal afferents (Wells *et al.* 2007) as well as brachial nerves and painful neuromas (Black *et al.* 2008).

In the enteric nervous system, $Na_v1.9$ has been demonstrated in guinea pig (Rugiero *et al.* 2003), rat (Rugiero *et al.* 2003; Padilla *et al.* 2007) and mouse (Padilla *et al.* 2007) myenteric neurons and submucosal plexi and in the interplexus tracts running

between myenteric and submucosal ganglia (Padilla *et al.* 2007). $Na_v1.9$ was not seen in rat cerebellum, spinal cord, heart, kidney, liver or muscle (Dib-Hajj *et al.* 1998).

The above studies demonstrate that $Na_v1.9$ is distributed widely in both the somatic and visceral systems with no evidence of its presence in the CNS thus positioning it as an attractive analgesic target.

1.4.4.2 Co-localisation of $Na_v1.9$ with other markers

$Na_v1.9$ has been co-localised with a number of neuronal markers and other ion channels. Between 72% and 99% of Isolectin B4 (IB4) +ve and 27% and 56% of IB4 -ve fibres demonstrated staining for $Na_v1.9$ (Fjell *et al.* 1999; Fjell *et al.* 2000; Fukuoka *et al.* 2008) with the difference probably accounted for by the differing criteria used to define positive staining. IB4 is used in the somatic system as a marker of nociceptors and Fang *et al* (2006) concluded that intensity of IB4 staining was positively related to $Na_v1.9$ staining. $Na_v1.9$ has also been shown to co-localise with the bradykinin B_2 receptor (Amaya *et al.* 2006), TRPV1 (Amaya *et al.* 2000; Amaya *et al.* 2006; Padilla *et al.* 2007) and $P2X_3$ (Amaya *et al.* 2006). This is unsurprising as the presence of these receptors is a hallmark of nociceptors. This close co-expression allows for modulation of the function of these receptors by one another.

1.4.5 Effect of inflammatory mediators on $Na_v1.9$ current and expression

Various studies, mostly *in vitro*, have investigated the effect of inflammatory mediators on $Na_v1.9$ current. Guanosine triphosphate (GTP) and its analogue GTP-γ-S have been shown to enhance the $Na_v1.9$ current in both mouse and rat (Baker *et al.* 2003; Maingret *et al.* 2008) by acting through PKC (Baker 2005). ATP has been shown to increase $Na_v1.9$ current via PKC (Baker 2005). TNFα also increases the $Na_v1.9$ current in cultured rat DRG (Chen *et al.* 2011). Conflicting data have been reported with regards to the relationship between PGE_2 and $Na_v1.9$. A number of authors were unable to demonstrate an effect of PGE_2 on $Na_v1.9$ current (Baker 2005; Zheng

et al. 2007; Maingret *et al.* 2008). On the other hand, two studies demonstrated an increase in $Na_v1.9$ current after application of PGE_2 (Rush and Waxman 2004; Li and Schild 2007). Maingret *et al* (2008) failed to demonstrate an effect of bradykinin, PGE_2, histamine, noradrenalin or ATP on $Na_v1.9$ current when these mediators were added separately. However, an 'inflammatory soup' containing all the mediators caused an increase in the current and a decrease in the threshold for excitability. The discrepancy in these findings may be a reflection of the inherent difficulties in using an artificial system to replicate complex intracellular events.

These studies demonstrate that, at DRG level, the $Na_v1.9$ current is enhanced in response to a variety of inflammatory mediators with PKC as one mechanism for the action of these mediators. Investigators have also used rodent pain models to investigate the role of $Na_v1.9$ in the development of neuropathic and inflammatory pain.

1.4.6 Role of $Na_v1.9$ in pain models

1.4.6.1 Neuropathic pain models

A number of models are used to induce neuropathic pain in rodents. These include spinal nerve ligation (Kim and Chung 1992), spared nerve injury (ligation and axotomy of 2 of the 3 branches of the sciatic nerve) (Decosterd and Woolf 2000), and peripheral axotomy. These models induce spontaneous pain and hyperalgesia in the affected dermatomes.

Using these models, several authors have demonstrated decreased expression of $Na_v1.9$ with a concurrent reduction in its current after induction of the injury (Dib-Hajj *et al.* 1998; Dib-Hajj *et al.* 1999a; Boucher *et al.* 2000; Cummins *et al.* 2000; Sleeper *et al.* 2000; Decosterd *et al.* 2002; Berta *et al.* 2008). A model of neuropathic

pain involving leg lengthening leading to sural nerve elongation also demonstrated a reduction in $Na_v1.9$ messenger ribonucleic acid (mRNA) levels (Ohno *et al.* 2010). Behavioural studies have failed to demonstrate any differences between $Na_v1.9$ -/- and +/+ mice in the development of mechanical or thermal hyperalgesia that are induced by these models (Priest *et al.* 2005; Amaya *et al.* 2006). In agreement with rodent studies which have failed to demonstrate a role for $Na_v1.9$ in the development of neuropathic pain, there is no change in $Na_v1.9$ expression in painful neuromas in man (Coward *et al.* 2000; Black *et al.* 2008).

1.4.6.2 Inflammatory pain models

Inflammatory pain models have been used to investigate the role of $Na_v1.9$ in the development of acute somatic pain behaviour or hyperalgesia after induction of inflammation by a variety of mediators including carrageenan, CFA, formalin, PGE_2, bradykinin and capsaicin (Bannon 2001; Fehrenbacher *et al.* 2012). Models of visceral inflammatory pain include the induction of inflammation in the small or large bowel (Stadnyk *et al.* 1990; Scheiffele and Fuss 2002) as well as instillation of inflammatory mediators into the viscera, e.g. colon or bladder.

Using the somatic pain models, a number of authors have shown increased expression of $Na_v1.9$ after intraplantar injection of CFA or carrageenan (Tate *et al.* 1998; Amaya *et al.* 2006; Strickland *et al.* 2008; Lolignier *et al.* 2011). Behavioural studies have demonstrated that knockout of $Na_v1.9$ results in a significant attenuation of the pain behaviour, e.g. licking or flinching, as well as the thermal and mechanical hyperalgesia that develop after injection of CFA, carrageenan or formalin (Priest *et al.* 2005; Amaya *et al.* 2006; Lolignier *et al.* 2011). In addition, injection of bradykinin, capsaicin, PGE_2 and ATP also produced behavioural pain responses and mechanical and thermal hyperalgesia that were attenuated in $Na_v1.9$ -/- mice (Amaya *et al.* 2006). In man, $Na_v1.9$ expression has been shown to increase in inflamed dental pulp axons (Wells *et al.* 2007).

While studies investigating TNBS colitis and transient jejunitis did not show any effect of inflammation on Na_v1.9 expression or current (Beyak *et al.* 2004; Hillsley *et al.* 2006; King *et al.* 2009), using a pain model for IBS, Martinez and Melgar (2008) demonstrated that, during an acute inflammatory episode, Na_v1.9 -/- mice do not show the increased visceromotor reflex to colorectal distension experienced by Na_v1.9 +/+ mice. In addition, a study investigating bladder afferents demonstrated a reduction in afferent activation in response to PGE_2 in Na_v1.9 -/- mice (Ritter *et al.* 2009). In contrast, Leo *et al* (2010) found an increase in the number of abdominal writhes in response to acetic acid in Na_v1.9 -/- as compared to +/+ mice. The conflicting results from models of colonic inflammation may be accounted for by the variation in the inflammation caused or by the time period between induction of infection and testing, e.g. 5 hours for the Martinez study compared to up to 25 days for the Hillsley study.

In summary, data from both somatic and visceral pain models provide evidence for a role for Na_v1.9 in the response of afferents to inflammation.

1.4.7 Effect of knock-out of Na_v1.9 on other Na_vs

Priest *et al* (2005) reported marginal increases in Na_v1.1, 1.2, 1.3, 1.7 and 1.8 mRNA in Na_v1.9 -/- mice but Amaya *et al* (2006) showed no change in mRNA levels of Na_v1.1, 1.3, 1.5, 1.7 and 1.8. They also found no change in the TTX-s or Na_v1.8 currents. The differences in mRNA levels between the studies may be explained by the fact that Amaya used 3 DRG from 3 animals in each group (Na_v1.9 +/+ vs. -/-) whereas Priest only used one DRG from each of the 4 animals in each group. Overall, the data show that there is minimal, if any, increase in the expression of the other Na_vs in the Na_v1.9 -/- animals. In addition, as discussed above, Na_v1.9 transduces a unique persistent current that is activated at thresholds below those of the other Na_v channels. Thus, even a small increase in the expression of these other channels in Na_v1.9 -/- animals will not replace the current lost due to knock out of Na_v1.9.

1.4.8 Effect of knock-out of $Na_v1.9$ on general behaviour

$Na_v1.9$ -/- mice are indistinguishable from +/+ littermates in respect of moving, climbing and fertility. Amaya *et al* (2006) and Priest *et al* (2005) demonstrated that the response to Von Frey probing and exposure to heat and cold stimuli were not changed in $Na_v1.9$ -/ - mice while Ritter *et al* (2009) found no difference in bladder capacity or voided volumes. This indicates that there are no global deleterious effects to knocking-out $Na_v1.9$ (in contrast to global knockout of $Na_v1.7$ which is fatal soon after birth (Nassar *et al.* 2004)).

1.5 Summary

Abdominal pain places a large burden on society and its current management is sub-optimal due to the lack of visceral-specific analgesics. Voltage-gated sodium channels are crucial for action potential generation and $Na_v1.9$ has the lowest activation threshold of all these channels. In addition, $Na_v1.9$ expresses a unique, slow, persistent current and is peripherally expressed on small diameter DRG neurons. Whereas, at the level of the DRG, the $Na_v1.9$ current is enhanced in response to a number of inflammatory mediators, no data exist for the role of $Na_v1.9$ in signalling at the nerve terminal. Behavioural studies in mice have established a role for $Na_v1.9$ in the response of somatic afferents to inflammatory mediators where it has a role in the immediate response of the afferents as well as in the development of hyperalgesia. No studies have investigated the role of $Na_v1.9$ in the response of visceral afferents to inflammatory and mechanical stimuli.

1.6 Aim and hypotheses

1.6.1 Aim

The aim of this project was to investigate the role of $Na_v1.9$ in visceral afferent signalling. A number of individual inflammatory mediators as well as a human inflammatory supernatant were tested to investigate whether $Na_v1.9$ plays a role in the response of afferents to these mediators. In addition, luminal distension of segments of gastrointestinal tissue was used to investigate whether $Na_v1.9$ plays a role in the response of the afferents to mechanical stimulation.

1.6.2 Hypotheses

The following hypotheses were tested:

1. The visceral afferent response to a variety of individual inflammatory mediators is reduced in $Na_v1.9$ -/- mice.
2. The visceral afferent response to an inflammatory supernatant generated from inflamed human gastrointestinal tissue is reduced in $Na_v1.9$ -/- mice.
3. Prostaglandins play a role in the activation of visceral afferents in response to inflammatory stimulation via a $Na_v1.9$ mediated pathway.
4. $Na_v1.9$ plays a role in the response of visceral afferents to luminal distension.

2

Materials and Methods

2.1 Animals

Colonies of $Na_v1.9$ +/+ and $Na_v1.9$ -/- mice bred on a C57BL/6 background were re-derived from $Na_v1.9$ +/- mice in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. The $Na_v1.9$ -/- mice used in the generation of the heterozygous breeding pairs were generated as previously described (Ostman *et al.* 2008). Briefly, an RPCI-22 129S6/SvEvTac mouse BAC library was screened for $Na_v1.9$. Deoxyribonucleic acid (DNA) from $Na_v1.9$ BAC clones was prepared and subcloned into pBluescript (BS-SKII). The 5'-arm, containing exons 2 and 3, of the SCN11A gene was a 4.8 kb EcoRI–NsiI fragment. The 3' arm containing exons 6–10 was a 7.2 kb AccI–SmaI fragment. The two arms were inserted around a neomycin cassette. Hence, exons 4 and 5 of the SCN11A gene were replaced by the neomycin resistance cassette which deletes domain 1 in the S4 voltage sensor of the $Na_v1.9$ channel. Cells were selected with G418 and correctly targeted single-copy integrations were identified using Southern blots. Chimeras were crossed with C57BL/6 and germ line transmission tested using Southern blotting of genomic DNA from pinna biopsies. For Southern blots, DNA was digested with BamHIand probed with a 500 bp SacI-EcoRI fragment 5'-to 5'-arm. The mice were maintained in a

barrier facility with a 12 hour light/dark cycle and had *ad libitum* access to food and water. At the time of weaning, pinna biopsies were obtained for genotyping. In the initial experiments on intestinal nerves, C57BL/6 mice were used as controls (Charles Rivers, UK). Follow-up intestinal experiments used $Na_v1.9$ +/+. There was no difference in the response between C57BL/6 and $Na_v1.9$ +/+ so the two groups were analysed as one for this thesis.

2.1.1 Genotyping

Pinna biopsies were submerged in 500 µl of lysis buffer (0.1 M Tris-HCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.2% sodium dodecyl sulfate (SDS), 20 mM sodium chloride (NaCl), 50 µg proteinase K) for 6 hours at 50 ˚C. The mixture was then centrifuged at 5000 revolutions per minute (RPM) for 15 seconds and the supernatants transferred to clean tubes. The supernatant was then mixed 1:1 with isopropanol and centrifuged at 13000 RPM for 15 minutes at 4 ˚C. The supernatant was decanted, 500 µl of 70% ethanol added, and then centrifuged at 13000 RPM for 10 minutes at 4 ˚C. The supernatant was again decanted and the pellet allowed to dry overnight. On the next morning, 40 µl of sterile water was added and the concentration of DNA in each sample was determined using a Nanodrop spectrophotometer (Thermo Scientific, UK). Approximately 50-70 ng of DNA were mixed with HotStarTaq (Qiagen, UK) and purpose designed primers (common: 5’-ATGTGGCACTGGGCTTGAACTC-3’; wildtype: 5’-AACAGTCTTACGCTGTTCCGATG-3’; mutant: 5’-CTCGTCGTGACCCATGGCGAT-3’) and sterile water in a 1:5:1:1:2 ratio by volume (DNA: HotStarTaq: common primer: mutant/wild type primer: sterile water). The polymerase chain reaction (PCR) was started at 95 ˚C for 15 minutes followed by 35 cycles of: 95 ˚C for 1 minute, 57 ˚C for 1 minute and 72 ˚C for 2 minutes. Ten µl of PCR products were then mixed with 2 µl glycerol dye and loaded onto a 1% agarose gel containing 0.5% ethidium bromide. The electrophoresis reaction was run at 100 V for 25 minutes (VWR, UK) before the gel was transferred to a UV chamber (Syngene, UK) and images obtained using GeneSnap software (Syngene, UK). A band at 300

base pairs (bps) was visible for the wild-type gene and at 600 bp for the mutated gene.

2.2 Human Tissue

Human appendices were obtained from patients undergoing surgery at the Royal London Hospital (part of Barts Health NHS Trust). The patients underwent surgery either for cancer (normal appendices) or a clinical diagnosis of appendicitis (inflamed appendices). Patients were approached prior to surgery, the research project explained and an information leaflet provided. Patients who agreed to take part were asked to sign a consent form. The collection and use of human tissue was approved by the local NHS ethics committee (REC 10/H0703/71). After generation of the supernatants (described below), specimens were fixed in formalin and delivered to the NHS pathology laboratory to be subjected to routine histological examination. The histology report was subsequently checked to confirm whether the appendices were microscopically inflamed or not. Four of the six inflamed appendices were classified as suppurative while 2 were gangrenous (Carr 2000). The patients' white blood cell counts were also recorded.

2.2.1 Generation of human supernatants

The appendices were weighed and then incubated at 37 ˚C in carbogenated Krebs buffer – in mM: 124 NaCl, 4.8 potassium chloride (KCl), 1.3 monosodium phosphate (NaH_2PO_4), 2.5 calcium chloride ($CaCl_2$), 1.2 magnesium sulphate ($MgSO_4$), 11.1 glucose, and 25 sodium bicarbonate ($NaHCO_3$). The volume of buffer was 2.5 times the weight of tissue. After 25 minutes incubation, the appendix was removed and the buffer was spun at 2000 RPM for 10 minutes. The supernatant was then decanted and stored at -80 ˚C until use.

2.2.2 Measurement of cytokine concentration of human supernatants

The concentration of a selected number of cytokines in the human supernatants was measured using a Magpix reader (Luminex, UK). A five cytokine assay was chosen

(TNF-α, IL-1β, IL-6, IL-8 and GM-CSF; catalogue number LHC0003M, Invitrogen, UK). The system is based on ELISA principles. 50 µl of each supernatant were mixed with an equal volume of dilution buffer and then incubated with the magnetic beads for 2 hours. After 3 serial washes with wash buffer, the beads were incubated with a biotinylated antibody for 1 hour followed by further washes and then incubation with streptavidin-RPE for 30 minutes. Further washes were performed and then the bead fluorescence measured and compared with that of the standards (which were serially diluted 8 times). The supernatants were analysed in duplicates. The concentration of a particular cytokine in some samples was below the detection threshold and the software returned a reading of “<x.xxx”. In these situations, the value of x.xxx was taken as the concentration.

2.3 Electrophysiology

On the day of the experiment, the animal (male or female, 12-28 weeks) was sacrificed by exposure to rising carbon dioxide (CO_2) concentration followed by exsanguination. For intestinal nerve experiments, the intestine and mesentery were dissected from the ligament of Treitz to the terminal ileum. For colonic nerve experiments, the colon was dissected from the caecum down to anus including the aorta and associated nerve bundle. The intestine or colon was then placed in ice cold Krebs buffer (in mM: 124 NaCl, 4.8 KCl, 1.3 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 11.1 glucose, and 25 $NaHCO_3$). Preparations contained the prostaglandin synthesis inhibitor indomethacin (3 µM) to reduce the effect of prostaglandins on nerve activity and both the calcium channel antagonist nifedipine (10 µM) and muscarinic acetylcholine receptor antagonist atropine (10 µM) to suppress contractions due to smooth muscle activity which would interfere with the nerve recording. Following gross dissection of the segment of intestine or colon as described below, it was transferred to a bespoke recording chamber whose base was lined with sylgard (Dow Corning, USA) and which was filled with continuously carbogenated (95% O_2, 5% CO_2) Krebs buffer (7 ml/min; 32-34 ˚C). The ends of the segment of intestine or colon

were tied to cannula and the tissue luminally perfused at 0.1 ml/min. The dissected nerve fibre was then drawn into a borosilicate glass suction electrode (Harvard Apparatus, UK) to allow the acquisition of a multi-unit recording (Figure 9). Nerve activity was recorded on a Neurolog headstage (Neurolog, UK), amplified (gain 5k), filtered (band pass 100-2000 Hz) and acquired (20 kHz sampling rate) via a Micro 1401 MKII (Cambridge Electronic Design, UK) to a desktop computer running Spike 2 software (Cambridge Electronic Design). The threshold level for action potential counting was set to twice the background noise level.

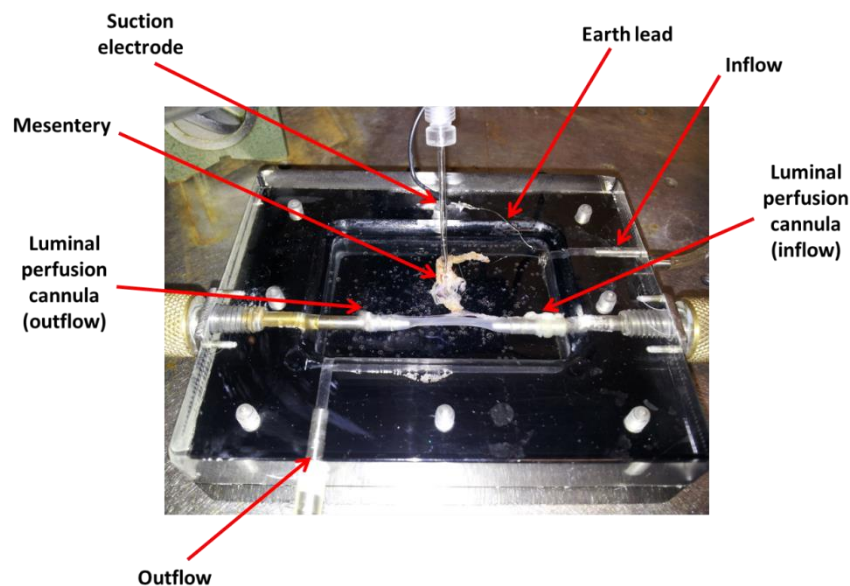


Figure 9 The recording chamber with a segment of colon tied to the perfusion cannula, its mesentery dissected and a nerve in the suction electrode.

2.3.1 Intestinal nerve dissection

For intestinal nerve recordings, a 3-4 cm segment of intestine, starting most distally, was dissected. If a second recording was made from the same animal, another segment of intestine was dissected, again starting distally. The mesentery was then pinned to the sylgard at right angle to the segment of intestine and the neurovascular bundle was dissected to allow isolation of the nerve bundle (Figure 10a).

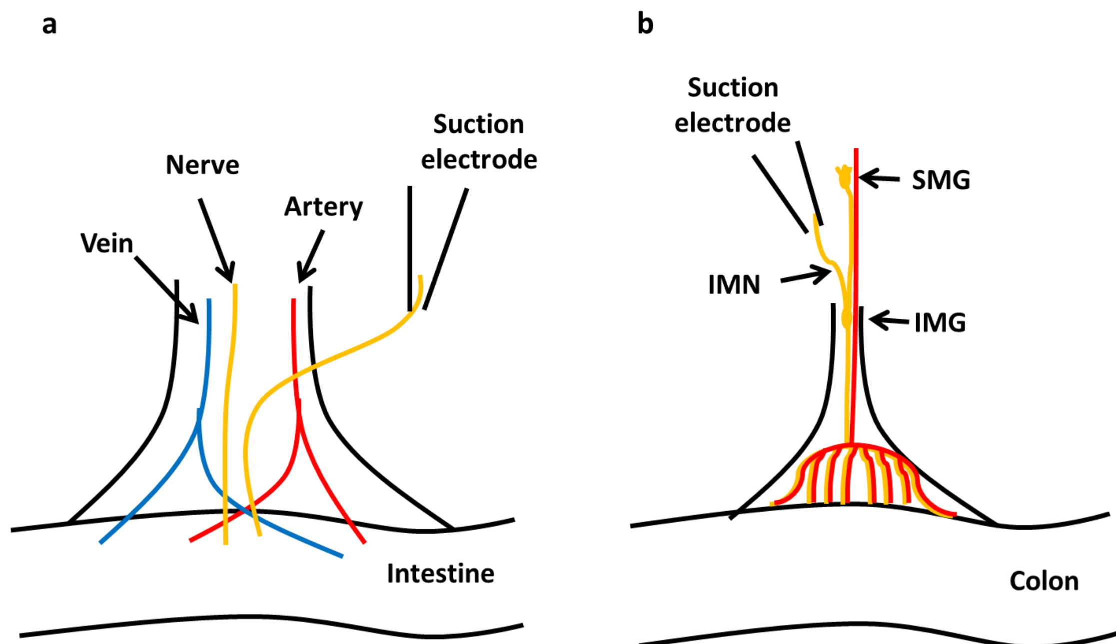


Figure 10 Schematic representation of the structures visible during the dissection of the (a) intestinal and (b) nerves. SMG: superior mesenteric ganglion, IMG: inferior mesenteric ganglion, IMN: inter-mesenteric nerve.

2.3.2 Colonic nerve dissection

For colonic nerve recordings, the segment of colon always included the anus. The neurovascular bundle was pinned flat at right angle to the colon and the lumbar splanchnic nerve was dissected between the inferior (IMG) and superior (SMG) mesenteric ganglia. The nerve naturally consists of 2 distinct and large bundles (intermesenteric nerves – IMN) and one of these was used for the recording (Figure 10b).

2.3.3 Experimental Protocols

A recording of baseline activity over approximately 30 minutes was made before the recording chamber was superfused with a 20 ml volume of the test mediator. Because of the low spontaneous activity of colonic nerves, ramp distension to 80 mmHg was undertaken at the beginning of the protocol to confirm the establishment of a recording. The specific protocol for each experiment is outlined in the relevant chapter.

2.4 Data analysis

A number of measures were used to analyse the data.

- Peak response: nerve activity was expressed as mean frequency over 60 seconds. Peak activity before and after application of the mediator was determined in spikes/second and the change in activity expressed as a mean ± standard error of the mean (sem).
- Response profile: nerve activity was expressed as mean frequency over 30 seconds. The activity was then plotted against time starting at the time the mediator entered the recording chamber.
- Duration of response: nerve activity was expressed as mean frequency over 60 seconds. Figure 11 highlights the different time points along the response curve. Three main measures were used: time to start of response (= B – A), time to peak response (= C – A) and the duration of response (= G – A). In addition, three secondary measurements were made: time for the response to lose 20% (= D – C), 50% (= E - C) and 80% (= F - C) of its peak response.

Comparisons were made between $Na_v1.9$ +/+ and -/- mice using student's t-test or two-way Analysis of variance (ANOVA) with bonferroni post hoc test as appropriate. Significance was set at $p<0.05$.

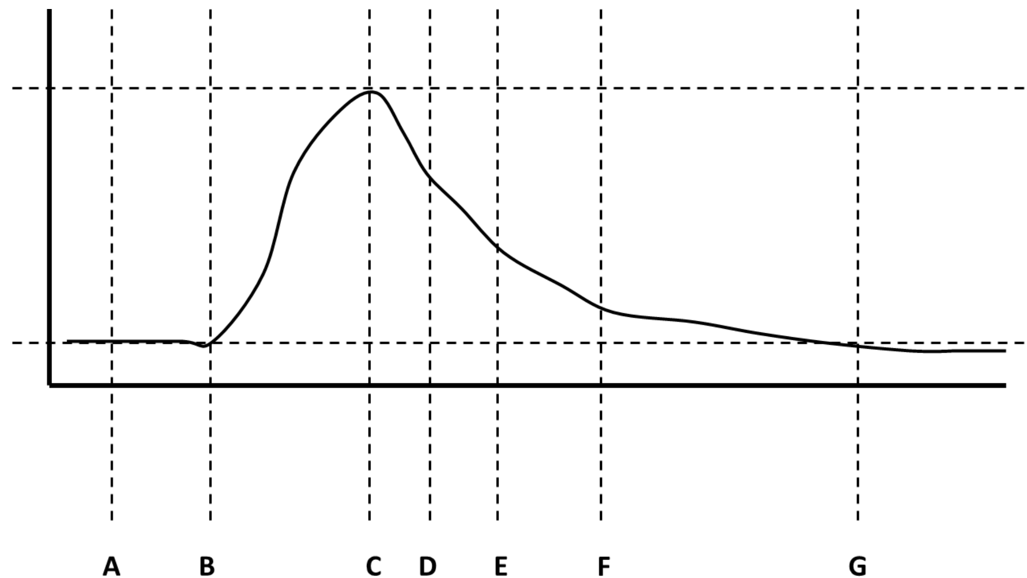


Figure 11 Schematic representation of the different time points along the response curve. A: time mediator entered the recording chamber, B: time when nerve activity increased above baseline, C: maximal firing rate after addition of the mediator, D: time when 20% of the response had been lost, E: time when 50% of the response had been lost, F: time when 80% of the response had been lost, G: time when nerve activity had returned to baseline.

2.5 Single unit discrimination

A consistent methodology was used to attempt discrimination of single units that constitute the multi-unit recordings. The analysis was performed using the "new wavemark" option in Spike 2. Templates were created when at least ten matching spikes were captured provided they were more frequent than one in 500 of the overall spike number. The spike width was set to a minimum of 32% of amplitude. Spikes were then matched to the template provided there was at least 75% points in common and there was no change in amplitude. The trigger thresholds were set to twice the background noise level. This analysis was applied to a subset of the colonic nerve recordings for five minutes before distension until 15 minutes after addition of 0.3µM bradykinin.

2.6 Chemicals

All chemicals were purchased from Sigma Aldrich. Stock concentrations of bradykinin (10 mM) were dissolved in distilled water. Stock concentrations of indomethacin (30 mM), nifedipine (100 mM) and capsaicin (10 mM) were dissolved in dimethyl sulfoxide (DMSO) and stock concentrations of atropine (100 mM) were dissolved in ethanol. Each aliquot was thawed immediately before use. Aliquots of human inflammatory supernatant were also thawed immediately before use.

3

The role of $Na_v1.9$ in the response of visceral afferents to chemical stimuli

3.1 Aims

The aims of the experiments discussed in this chapter are:

1. To establish the preparation as a way of studying the role of $Na_v1.9$ in afferent signalling
2. To investigate the role of $Na_v1.9$ in the response of visceral afferents to bradykinin, capsaicin and acetic acid when applied individually
3. To investigate the role of $Na_v1.9$ in the response of visceral afferents to a human inflammatory supernatant

3.2 Specific rationale

Bradykinin and capsaicin increase afferent activity and have been used in somatic studies to investigate the role of $Na_v1.9$ with attenuated responses demonstrated in $Na_v1.9$ -/- mice. The evidence for a role for $Na_v1.9$ in the response of visceral afferents to acetic acid is conflicting with a study in bladder afferents showing no role for $Na_v1.9$ whereas a pan-visceral afferent study concluded that loss of $Na_v1.9$ increased the response of the afferents to acetic acid. No studies have used a human inflammatory supernatant to investigate the role of $Na_v1.9$ in afferent signalling. The rationale for choosing these mediators is further discussed below.

3.2.1 Bradykinin signalling

Tissue damage leads to the activation of kallikreins which cleave kininogens to generate kinins. Kinins are a group of 9-11 amino acid peptides that include bradykinin and its active metabolite, des-Arg^9-bradykinin (Couture *et al.* 2001). Bradykinin is an initial mediator of inflammation, induces pain and is a potent vasodilator. Its actions are mediated via two members of the rhodopsin family of GPCRs: B_1 and B_2. Both receptors have 7 helix transmembrane domains but are only 36% identical at the amino acid level in human (Leeb-Lundberg *et al.* 2005). B_2 is constitutively expressed whereas B_1's expression is induced as a result of tissue damage (Couture *et al.* 2001). Both receptors are widely expressed including in vascular, smooth muscle, epithelial and neuronal cells and fibroblasts (Leeb-Lundberg *et al.* 2005). The receptors mainly signal through G_q and activate PLC that in turn cleaves PIP2 into DAG and IP3. DAG activates PKC while IP3 leads to the opening of Ca^{2+} channels and consequent release of Ca^{2+} into the cytoplasm (Leeb-Lundberg *et al.* 2005). PKC modifies the function of other proteins, including transmembrane channels, e.g. $Na_v1.8$, $Na_v1.9$ and TRPV1 (Khasar *et al.* 1999; Premkumar and Ahern 2000; Vellani *et al.* 2001; Numazaki *et al.* 2002; Shin *et al.* 2002; Baker 2005). Bradykinin also stimulates phospholipase A_2 and D activation through G-protein mediated mechanisms. Although both receptors couple to similar

transduction pathways, there are differences in their signalling patterns: stimulation of the B_2 receptor leads to rapid desensitization whereas B_1 is desensitized to only a small degree (Leeb-Lundberg *et al.* 2005). This, in addition to post translational modification, leads to B_2 signalling being transient while B_1 signalling is more sustained (Mathis *et al.* 1996). This is consistent with the fact that B_1 is induced by inflammation.

Bradykinin exerts its effects in two ways: by directly stimulating afferents and by enhancing the effects of other stimuli, e.g. mechanical stimulation. This has been shown in both the somatic and visceral systems in rodent and man (Whalley *et al.* 1987; Davis *et al.* 1996; Maubach and Grundy 1999; Brierley *et al.* 2005b). In addition, loss of the bradykinin receptors (either in knockout mice or by using antagonists) results in reduced somatic and visceral responses to inflammatory stimuli (Steranka *et al.* 1987; Heapy *et al.* 1993; Rupniak *et al.* 1997; Pesquero *et al.* 2000). Recently, there has been interest in the interplay between bradykinin and $Na_v1.9$ receptors. It is known that three quarters of $Na_v1.9$ expressing somatic afferents also express B_2 (Amaya *et al.* 2006). In addition, a somatic behavioural study has shown a significantly attenuated immediate response to intraplantar injection of bradykinin in $Na_v1.9$ -/- mice (Amaya *et al.* 2006). The study also showed reduced thermal and mechanical hypersensitivity after application of bradykinin in $Na_v1.9$ -/- mice. A parallel study in visceral afferents has not been conducted. Based on the above somatic study as well as patch clamp studies and extracellular multiunit recordings from visceral afferents in which bradykinin has been shown to activate neuronal cells, it is expected that the response of visceral afferents will be significantly reduced in $Na_v1.9$ -/- mice.

3.2.2 Transient receptor potential vanilloid-1 (TRPV1) signalling

TRPV1 is a cation channel that is the receptor for capsaicin, protons and temperatures over 43 ˚C amongst many others and was first cloned by Caterina and colleagues in 1997. In common with other members of the TRP channel family, it

consists of six transmembrane domains with a pore between segments 5 and 6. The channel assembles as a tetramer and upon opening, allows inflow of calcium ions into the cell (Clapham *et al.* 2005). TRPV1 is mostly intracellular and upon depolarisation, is trafficked to the membrane where it is activated by agonists, desensitised and then recycled to the intracellular compartment ready for another cycle (Szallasi *et al.* 2006). It is preferentially located on small to medium diameter neurons which are known to be nociceptors (Caterina *et al.* 2000). The function of TRPV1 is enhanced by a number of signalling pathways activated by bradykinin, prostaglandins, ATP and other inflammatory mediators (Premkumar and Ahern 2000; Vellani *et al.* 2001; Numazaki *et al.* 2002). In addition, acidic conditions lower the activation threshold of TRPV1 to other stimuli thus allowing the activation of the channel at normal body temperature (Tominaga *et al.* 1998). It can undergo desensitisation such that subsequent activation of the channel leads to reduced responses (Brierley *et al.* 2005a; Holzer 2008).

TRPV1 is involved in the pathophysiology of inflammatory conditions. Its expression is increased in colorectal tissue from patients with an acute exacerbation of their IBD (Yiangou *et al.* 2001b) or rectal hypersensitivity (Chan *et al.* 2003; Akbar *et al.* 2008) while TRPV1 -/- mice lack the afferent hypersensitivity associated with inflammation (Caterina *et al.* 2000; Davis *et al.* 2000; Jones *et al.* 2005). In addition, DRG of adult mice that were subjected to acetic acid infusion as neonates revealed a significant increase in TRPV1 expression (Winston *et al.* 2007). An *in vivo* study of colorectal distension in rats showed that repeated distension resulted in a significant increase in the visceromotor response which was blocked by application of a TRPV1 antagonist (Ravnefjord *et al.* 2009). In addition, a TRPV1 antagonist has been shown to reduce the thermal hyperalgesia expressed after injection of CFA into the hind paw of rats (Joshi *et al.* 2009). These studies highlight a significant role for TRPV1 in the sensitisation of both visceral and somatic afferents.

Over half of TRPV1 expressing neurons also express $Na_v1.9$ (Amaya *et al.* 2000; Amaya *et al.* 2006). In addition, a somatic behavioural study has shown a significantly attenuated immediate response to intraplantar injection of capsaicin in $Na_v1.9$ -/- mice (Amaya *et al.* 2006). The study also showed reduced mechanical hypersensitivity after capsaicin in $Na_v1.9$ -/- mice. A parallel study in visceral afferents is lacking. Based on the above somatic study as well as patch clamp studies and extracellular multiunit recordings from visceral afferents in which capsaicin has been shown to activate neuronal cells, it is expected that the response of visceral afferents will be significantly reduced in $Na_v1.9$ -/- mice.

3.2.3 Acetic acid as a disease model

Even though instillation of acetic acid has been used as a model for colonic inflammation for over 30 years (MacPherson and Pfeiffer 1978), its exact mechanism of action is not fully understood. Intraperitoneal acetic acid induces strong abdominal contractions that start within a few minutes of instillation and reach maximal frequency within 15 minutes (Martinez *et al.* 1999). It is possible that acetic acid exerts its effects by activation of membrane receptors; for example TRPV1 or acid sensing ion channel (ASIC) channels may be activated by the low pH. Indeed, there is evidence that antagonism of TRPV1 reduces the visceromotor response to colorectal distension caused by acetic acid infusion (Wiskur *et al.* 2010). The writhing test has been criticised because the intraperitoneal application of acetic acid causes activation of both somatic and visceral afferents and because the stimulus is never naturally encountered by the animal. Infusion of acetic acid into the bladder causes a reduction in maximal filling and voided volumes in association with an increase in frequency and amplitude of bladder contractions reflecting a sensitised system (Ritter *et al.* 2009).

Two recent studies have investigated the role of $Na_v1.9$ in the response of the viscera to acetic acid. An *in vivo* study of mouse bladder concluded that there was no role for $Na_v1.9$ in the pathogenesis of the effects of acetic acid (Ritter *et al.* 2009)

while a study investigating the number of abdominal contractions after intraperitoneal instillation of acetic acid showed a doubling in the number of writhes in $Na_v1.9$ -/- mice (Leo *et al.* 2010). The latter finding is not consistent with other studies which have shown an analgesic effect to knock-out of $Na_v1.9$. This contradiction and the differing results between the two acetic acid studies may be due to the different doses used (0.25% in the bladder study vs. 1% in the colonic study), to the relative contribution of $Na_v1.9$ to each system or to differences between mouse strains.

3.2.4 Role of an inflammatory soup

Many studies have examined the effect of inflammatory mediators on afferent signalling with most investigating each mediator in isolation. *In vivo*, inflammatory reactions lead to the release or recruitment of a large number of mediators. Hence, studying mediators in isolation, *in vitro*, is a poor model for the pathophysiological process occurring *in vivo*. A number of studies have shown that application of an 'inflammatory soup' consisting of a number of mediators leads to more robust activation of visceral afferents (Alessandri-Haber *et al.* 2006; Maingret *et al.* 2008). Three studies have created human tissue supernatants from colonic biopsy samples from patients diagnosed with IBS and studied their effect on nerve activity (Barbara *et al.* 2007; Cenac *et al.* 2007; Buhner *et al.* 2009). No group has used acutely inflamed full thickness intestinal tissue to create an inflammatory supernatant.

Appendicitis is one of the most common reasons for admission to a surgical ward with over 45,000 appendicectomies performed every year (DoH 2012). The diagnosis is usually made on clinical history and examination along with the results of routine biochemical investigations. In some instances, imaging studies are used to inform the decision making process. Three groups have attempted to analyse the inflammatory mediators released during appendicitis. At the beginning of the operation, the groups either aspirated peritoneal fluid (Dalal *et al.* 2005) or instilled 0.9% saline into the peritoneal cavity and then aspirated it 1 minute later (Rivera-Chavez *et al.* 2003;

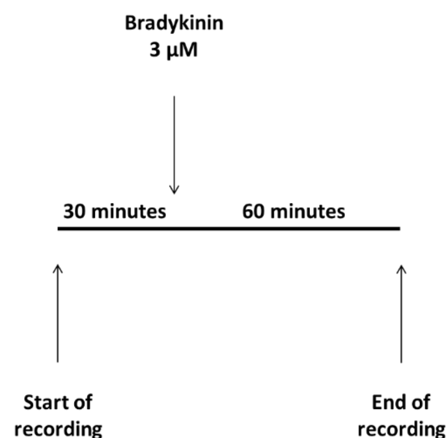
Yamamoto *et al.* 2005). The cytokine levels in the fluid were measured but were highly variable between the three studies with one group reporting detectable cytokines in only 50% of patients (Yamamoto *et al.* 2005). Indeed, a technique which relies on instillation of a saline solution into the peritoneal cavity and its aspiration after 60 seconds is unlikely to be representative of the cytokine environment in the inflamed appendix. No attempt was made to measure cytokine levels in the appendicular tissue itself which would be a more accurate reflection of the environment that the visceral afferents experience. Based on somatic studies using individual mediators and patch clamp studies using individual mediators as well as combinations of mediators, it is expected that the response of visceral afferents to a human inflammatory supernatant will be significantly reduced in $Na_V1.9$ -/- mice.

3.3 Experimental protocols

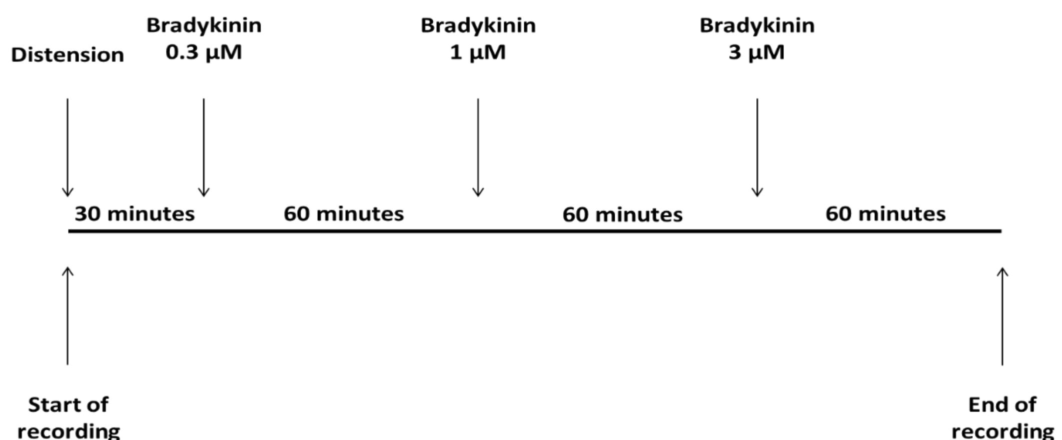
3.3.1 Bradykinin

The effect of bradykinin on nerve activity was investigated in both intestinal and colonic preparations. In intestinal preparations, a single 3 µM dose was used. In colonic preparations, consecutive applications of 0.3 µM, 1 µM and 3 µM were superfused 60 minutes apart. The nerve activity had returned to baseline before the next dose was applied.

3.3.1.1 Intestinal recordings



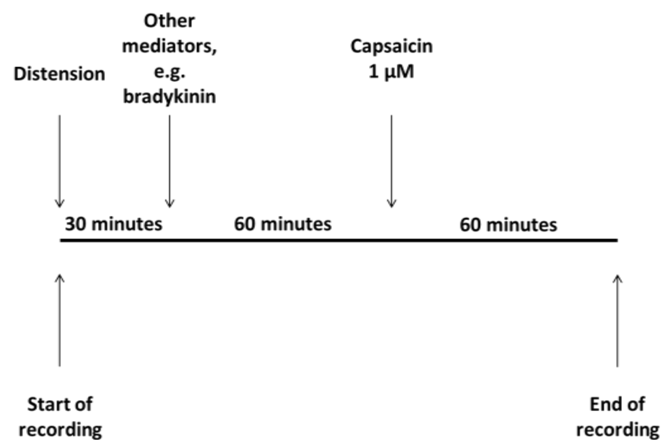
3.3.1.2 Colonic recordings



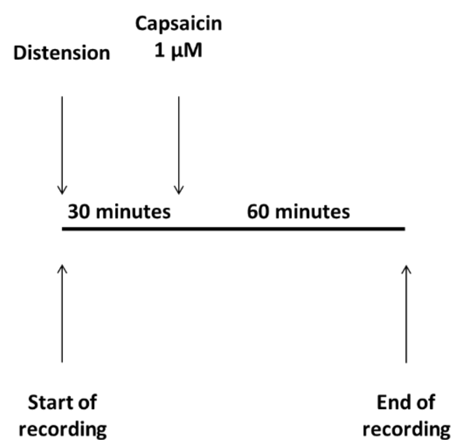
3.3.2 Capsaicin

The effect of 1 μM capsaicin was investigated in both intestinal and colonic preparations. In the initial intestinal preparations, the capsaicin was applied after other mediators. Further experiments were performed where capsaicin was the only mediator applied and there was no difference in the magnitude of response so the data was analysed as one group. In colonic preparations, capsaicin was the only mediator applied to the preparation.

3.3.1.1 Intestinal recordings

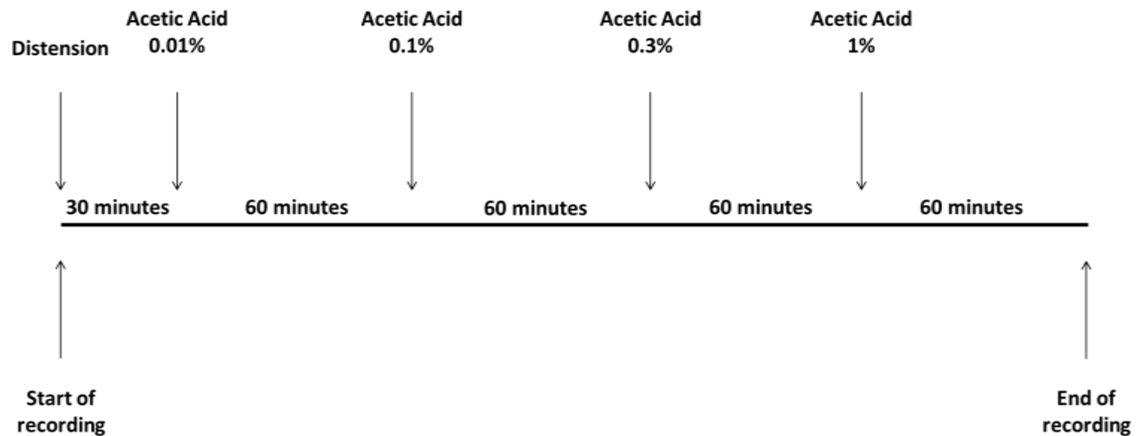


3.3.1.2 Colonic recordings



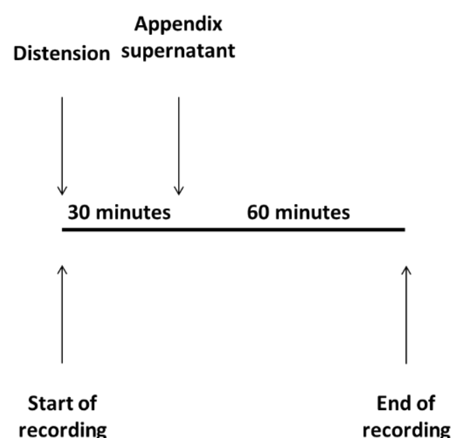
3.3.3 Acetic acid

The effect of acetic acid was investigated in colonic preparations only. Consecutive applications of 0.01%, 0.1%, 0.3% and 1% acetic acid were superfused 60 minutes apart. The nerve activity had returned to baseline before the next dose was applied.



3.3.4 Human inflammatory supernatant

The effect of human supernatants was investigated in colonic preparations only. A single dose of the supernatant was applied to each preparation. Control supernatants were tested in $Na_v1.9$ +/+ mice only whereas inflammatory supernatants were tested in both $Na_v1.9$ +/+ and -/- mice. In contrast to the other stimuli, the volume of supernatant superfused varied between 10 and 15 ml due to the natural variability in the size of the appendices.



3.4 Results

3.4.1 Baseline activity

Baseline firing was significantly lower in intestinal and colonic nerves from $Na_v1.9$ -/- compared to +/+ mice (intestinal: $Na_v1.9$ +/+ 40.4 ± 4.7 vs. $Na_v1.9$ -/- 16.3 ± 2.2 Hz; n=13; $p<0.001$. Colonic: $Na_v1.9$ +/+ 3.1 ± 0.6 vs. $Na_v1.9$ -/- 1.1 ± 0.2 Hz; n=26-34; $p<0.05$. Figures 12a and 12b).

In addition, the baseline firing of colonic nerves was significantly lower than intestinal nerves in both $Na_v1.9$ +/+ and -/- mice ($Na_v1.9$ +/+: intestinal 40.4 ± 4.7 vs. colonic 3.1 ± 0.6 Hz; n=13-34; $p<0.001$. $Na_v1.9$ -/-: intestinal 16.3 ± 2.2 vs. colonic 1.1 ± 0.2 Hz; n=13-26; $p<0.001$. Figure 12c).

Single unit analysis of a subset of the colonic recordings revealed a higher number of units per recording from $Na_v1.9$ +/+ compared to -/- mice ($Na_v1.9$ +/+ 7.2 (range 5-11) vs. $Na_v1.9$ -/- 2.8 (range 1-5) units; n=5-6). A similar proportion of these single units were active prior to stimulation across $Na_v1.9$ +/+ and -/- recordings ($Na_v1.9$ +/+ 16/36 (44.4%) vs. $Na_v1.9$ -/- 6/17 (35.3%) units; n=5-6).

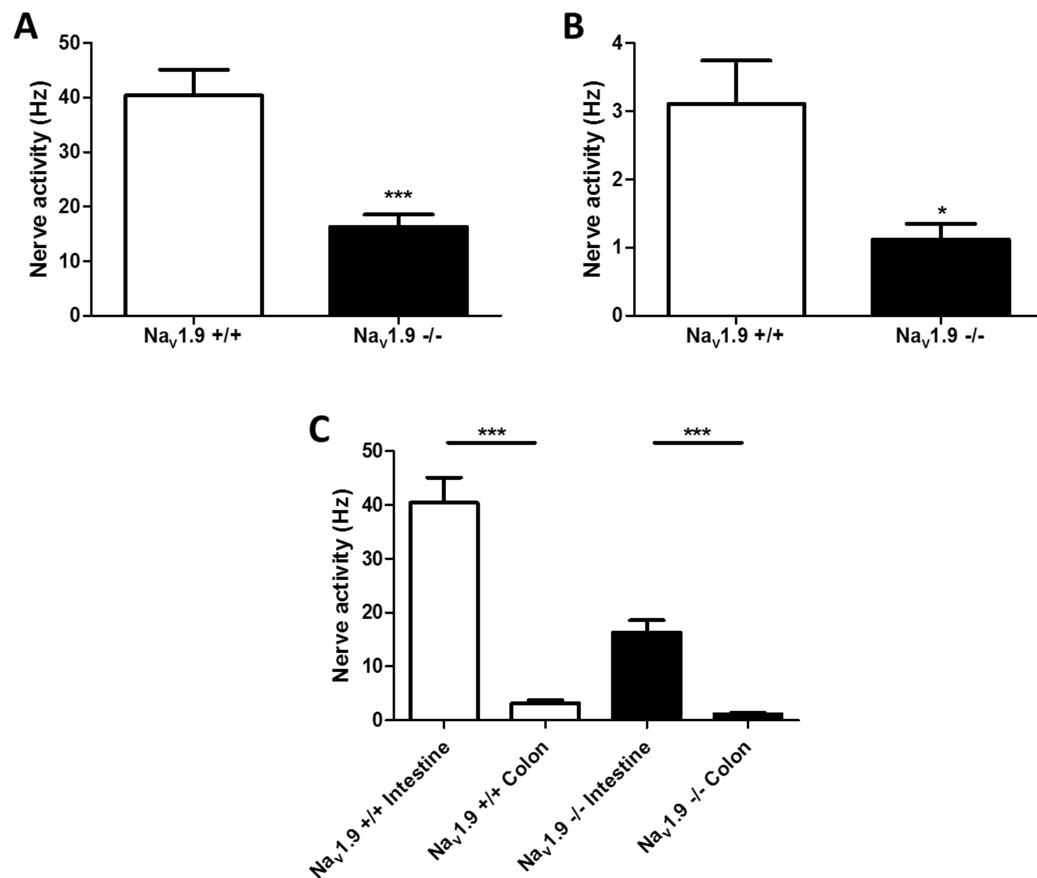


Figure 12 Baseline nerve activity in $Na_V1.9$ +/+ and -/- mice. (a) histogram comparing the baseline activity of intestinal nerves (b) histogram comparing the baseline activity of colonic nerves (c) histogram comparing the baseline activity of intestinal and colonic nerves. * $p < 0.05$ *** $p < 0.001$.

3.4.2 Response to bradykinin

Intestinal nerves were subjected to a single application of 3 µM bradykinin. The peak response of nerves from $Na_v1.9$ -/- mice was significantly reduced as compared to +/+ mice ($Na_v1.9$ +/+ 73.6 ± 7.3 vs. $Na_v1.9$ -/- 22.1 ± 4.5 Hz; n=5-6; p<0.001. Figure 13). Although there was a significant difference between the response profiles (two-way ANOVA p<0.001. Figure 13c), the difference in specific durations was not significant (Figure 14).

Colonic nerve preparations were subjected to consecutive applications of 0.3 µM, 1 µM and 3 µM bradykinin 60 minutes apart. The response was almost completely abolished in Nav1.9 -/- mice (0.3 µM: $Na_v1.9$ +/+ 7.8 ± 1.9 vs. $Na_v1.9$ -/- 0.6 ± 0.3 Hz; p<0.01. 1 µM: $Na_v1.9$ +/+ 6.4 ± 1.7 vs. $Na_v1.9$ -/- 0.4 ± 0.2 Hz; p<0.01. 3 µM: $Na_v1.9$ +/+ 7.8 ± 2.6 vs. $Na_v1.9$ -/- 0.4 ± 0.2 Hz; p<0.05; n=6. Figure 15).

Single unit analysis of the response of colonic afferents to 0.3µM bradykinin revealed that in $Na_v1.9$ +/+ recordings, the firing rate of 34 of the 36 (94.4%) units increased after application of 0.3µM bradykinin. In recordings from $Na_v1.9$ -/- mice, the firing rate of only three out of 17 (17.6%) units increased after 0.3µM bradykinin. The number of action potentials over time increased by 958% in $Na_v1.9$ +/+ mice compared to a 628% increase in $Na_v1.9$ -/- mice. Of the $Na_v1.9$ +/+ units which experienced an increase in firing in response to 0.3µM bradykinin, 14 had not responded to intra luminal distension and five were not active prior to application of the bradykinin. Neither of these phenomena were observed in $Na_v1.9$ -/- recordings.

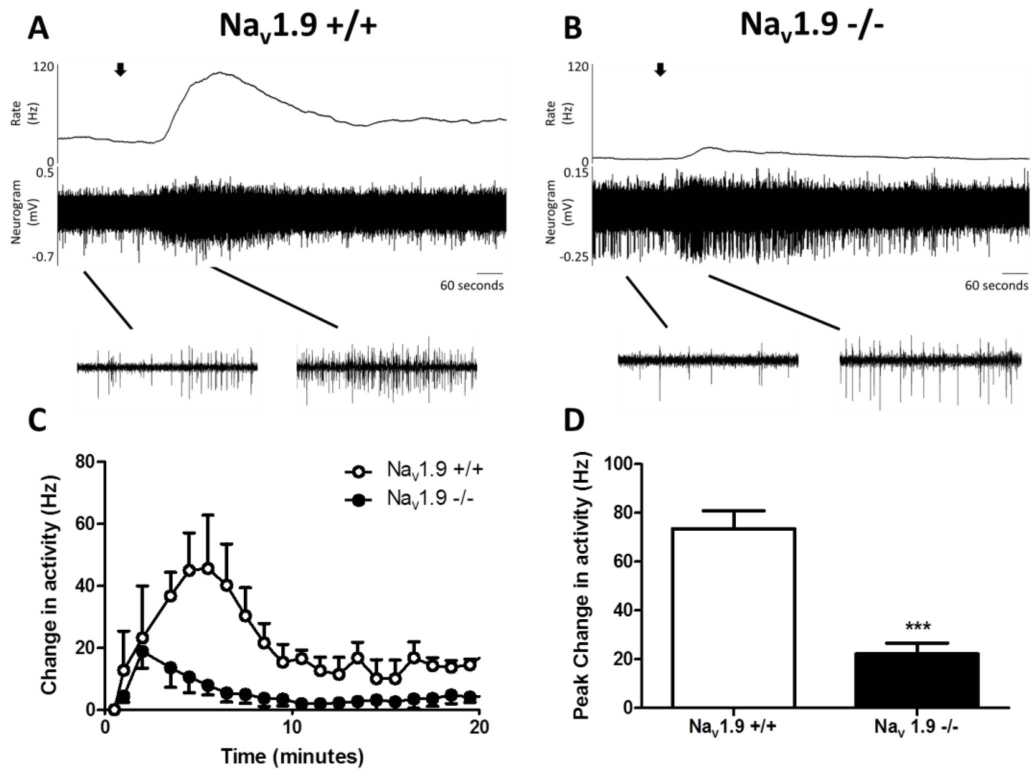


Figure 13 The effect of 3 µM bradykinin on intestinal nerve activity. Representative raw trace recordings and response rate (mean frequency over 60 seconds) from (a) $Na_v1.9$ +/+ and (b) $Na_v1.9$ -/- mice. The arrows indicate the addition of bradykinin. Below the raw trace, 1 second extracts are shown before and after the addition of bradykinin. (c) Average profile of response (two-way ANOVA $p<0.001$). (d) Histogram comparing the peak response. *** $p < 0.001$

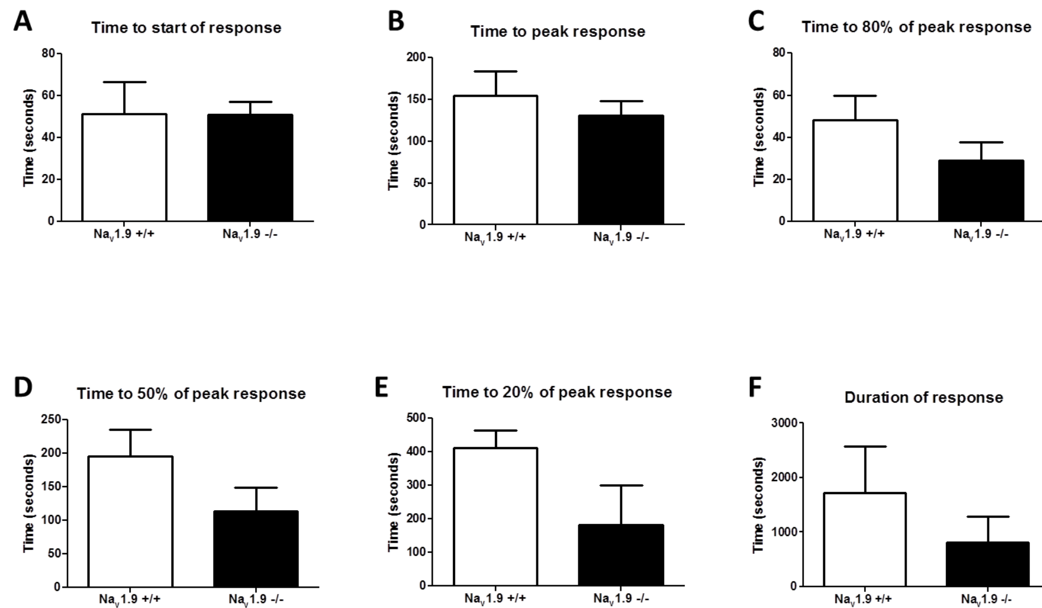


Figure 14 The duration of the intestinal response to 3 µM bradykinin. (a) time from bradykinin entering the bath to nerve activity rising above baseline (Na$_V$1.9 +/+ 51.5 ± 15.1 vs. Na$_V$1.9 -/- 51.0 ± 6.0 seconds; n=4-5; p=0.97). (b) time from bradykinin entering the bath to peak response (Na$_V$1.9 +/+ 154.5 ± 28.9 vs. Na$_V$1.9 -/- 130.8 ± 17.3 seconds; n=4-5; p=0.48). (c) time from peak response to 80% of the peak response (Na$_V$1.9 +/+ 48.3 ± 11.4 vs. Na$_V$1.9 -/- 29.2 ± 8.5 seconds; n=4-5; p=0.21). (d) time from peak response to 50% of the peak response (Na$_V$1.9 +/+ 194.7 ± 40.8 vs. Na$_V$1.9 -/- 113.2 ± 35.1 seconds; n=3-5; p=0.19). (e) time from peak response to 20% of the peak response (Na$_V$1.9 +/+ 412.0 ± 51.9 vs. Na$_V$1.9 -/- 182.3 ± 117.9 seconds; n=3; p=0.15). (f) time from bradykinin entering the bath to nerve activity returning to baseline (Na$_V$1.9 +/+ 1727.0 ± 845.0 vs. Na$_V$1.9 -/- 809.3 ± 483.1 seconds; n=2-3; p=0.38).

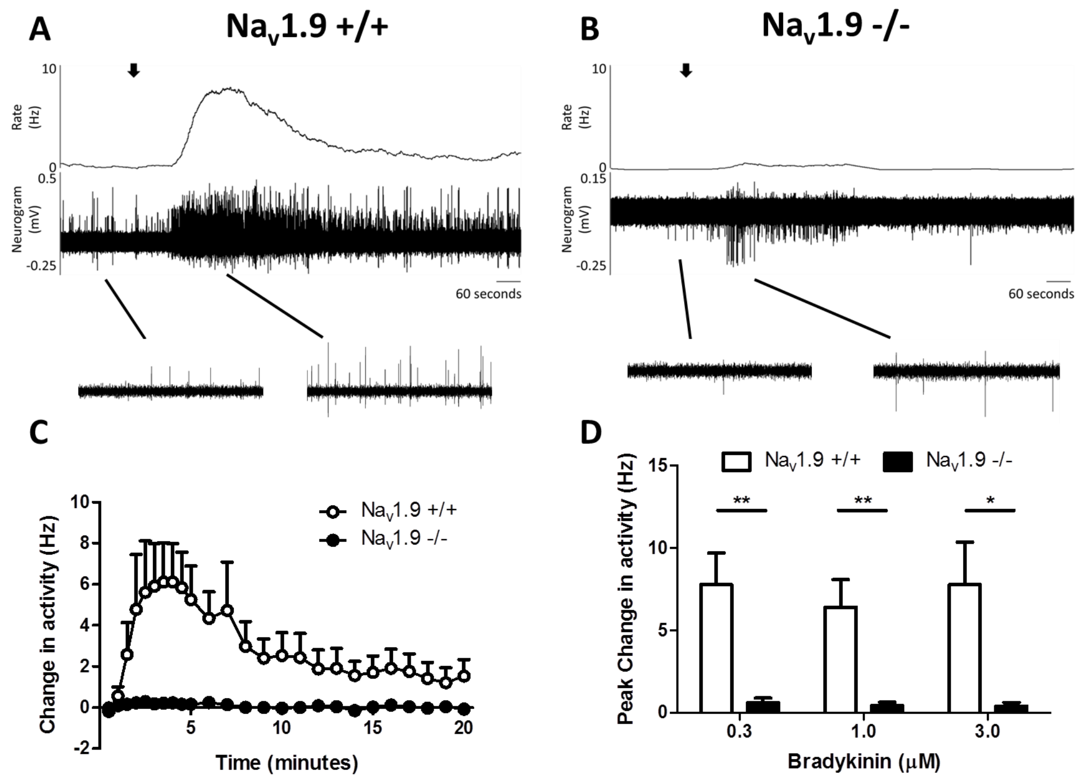


Figure 15 The effect of bradykinin on colonic nerve activity. Representative raw trace recordings and response rate (mean frequency over 60 seconds) from (a) $Na_V1.9$ +/+ and (b) $Na_V1.9$ -/- mice after addition of 3 μM bradykinin. The arrows indicate the addition of bradykinin. Below the raw trace, 2 second extracts are shown before and after the addition of bradykinin. (c) Average profile of response to 3 μM bradykinin (two-way ANOVA $p<0.001$). (d) Histogram comparing the peak response to three concentrations of bradykinin. * $p < 0.05$ ** $p < 0.01$

3.4.3 Response to capsaicin

A single application of 1 µM capsaicin was applied to both intestinal and colonic nerves. In intestinal recordings, the peak response (Na_v1.9 +/+ 39.0 ± 5.2 vs. Na_v1.9 -/- 17.8 ± 3.2 Hz; n=10-12; $p<0.01$. Figure 16) was followed (in 9/10 Na_v1.9 +/+ and 10/12 Na_v1.9 -/- recordings) by inhibition of the nerve activity (Na_v1.9 +/+ 28.5 ± 3.9 vs. Na_v1.9 -/- 14.7 ± 3.2 Hz; n=9-10; $p<0.01$). The difference between the response profiles was significant (two-way ANOVA $p<0.01$. Figure 16c). There was a trend for a longer time to reach peak response and a less steep decline in firing in the Na_v1.9 -/- mice but this was not significant (figure 17).

The peak response of colonic nerves was also significantly lower in Na_v1.9 -/- mice (Na_v1.9 +/+ 8.2 ± 1.1 vs. Na_v1.9 -/- 3.9 ± 0.9 Hz; n=7; $p<0.01$. Figure 18). Due to the low baseline firing of colonic nerves, there was no appreciable inhibition of firing after the initial response. The time taken to reach the peak response was significantly shorter in Na_v1.9 -/- mice (Na_v1.9 +/+ 179.1 ± 24.4 vs. Na_v1.9 -/- 103.6 ± 9.6 seconds; n=7; $p<0.05$. Figure 19b).

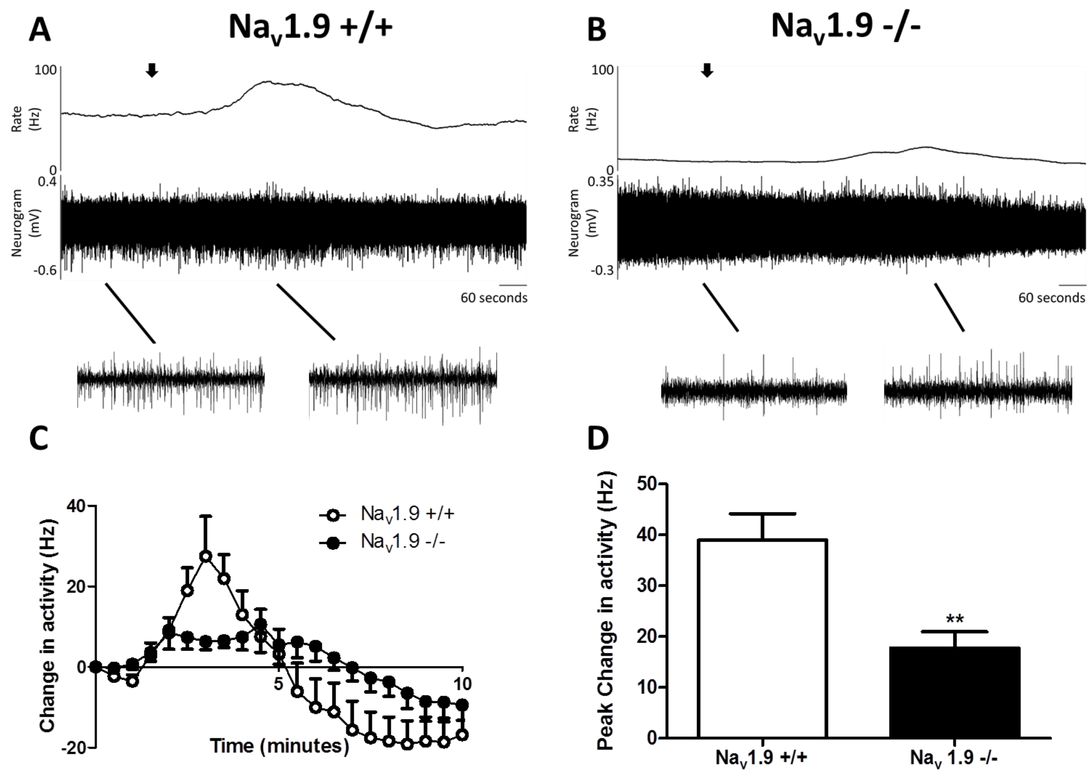


Figure 16 The effect of 1 μM capsaicin on intestinal nerve activity. Representative raw trace recordings and response rate (mean frequency over 60 seconds) from (a) Na$_v$1.9 +/+ and (b) Na$_v$1.9 -/- mice. The arrows indicate the addition of capsaicin. Below the raw trace, 1 second extracts are shown before and after the addition of capsaicin. (c) Average profile of response (two-way ANOVA $p<0.01$). (d) Histogram comparing the peak response ** $p < 0.01$

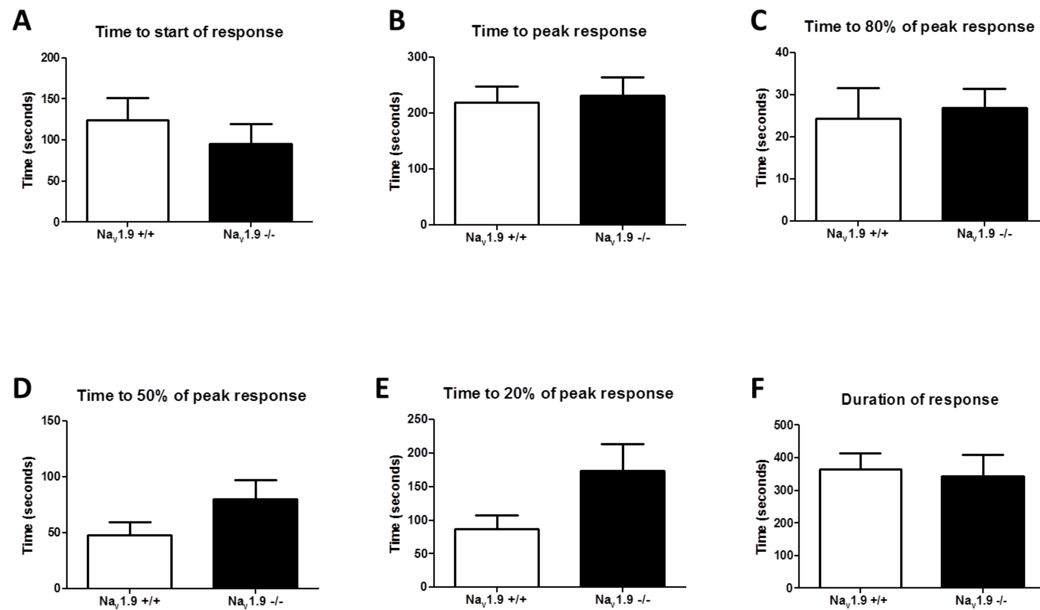


Figure 17 The duration of the intestinal response to 1 µM capsaicin. (a) time from capsaicin entering the bath to nerve activity rising above baseline (Na$_V$1.9 +/+ 124.4 ± 27.2 vs. Na$_V$1.9 -/- 95.6 ± 23.9 seconds; n=10-11; p=0.43). (b) time from capsaicin entering the bath to peak response (Na$_V$1.9 +/+ 218.8 ± 29.2 vs. Na$_V$1.9 -/- 231.2 ± 33.4 seconds; n=10-11; p=0.78). (c) time from peak response to 80% of the peak response (Na$_V$1.9 +/+ 24.5 ± 7.1 vs. Na$_V$1.9 -/- 27.0 ± 4.5 seconds; n=10-11; p=0.77). (d) time from peak response to 50% of the peak response (Na$_V$1.9 +/+ 48.3 ± 11.6 vs. Na$_V$1.9 -/- 80.3 ± 17.1 seconds; n=10-11; p=0.15). (e) time from peak response to 20% of the peak response (Na$_V$1.9 +/+ 87.7 ± 20.2 vs. Na$_V$1.9 -/- 174.2 ± 40.3 seconds; n=10-11; p=0.08). (f) time from capsaicin entering the bath to nerve activity returning to baseline (Na$_V$1.9 +/+ 365.1 ± 48.0 vs. Na$_V$1.9 -/- 343.8 ± 65.2 seconds; n=9-10; p=0.79).

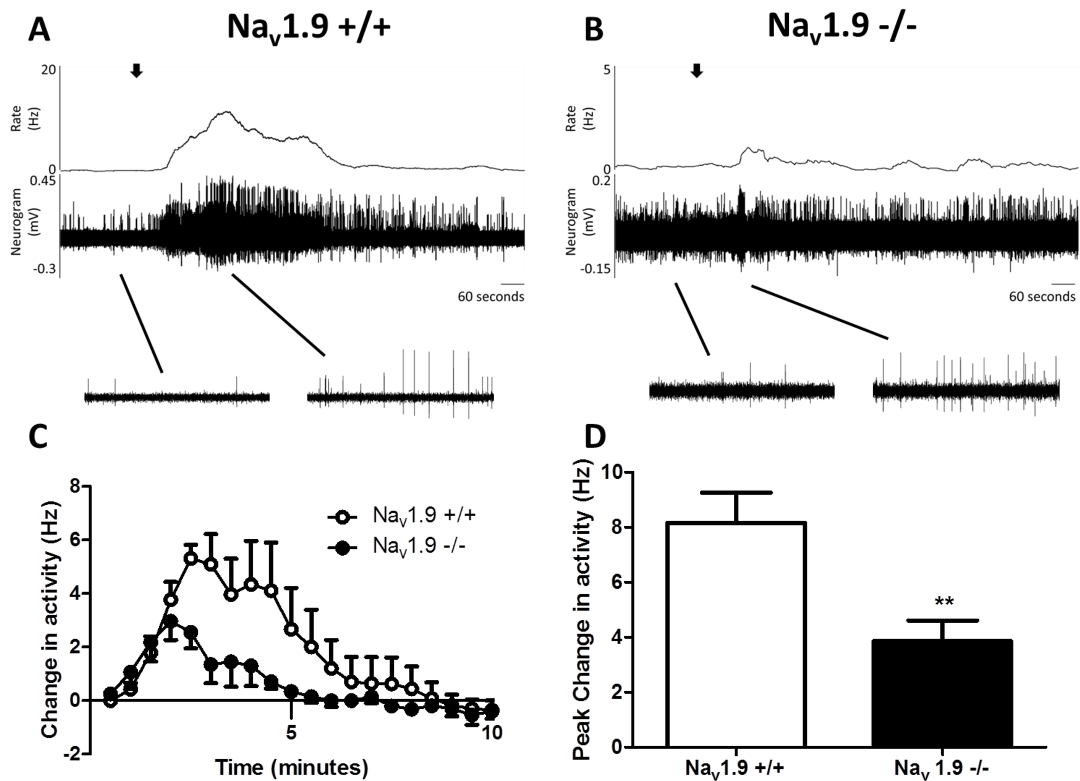


Figure 18 The effect of 1 µM capsaicin on colonic nerve activity. Representative raw trace recordings and response rate (mean frequency over 60 seconds) from (a) Na$_V$1.9 +/+ and (b) Na$_V$1.9 -/- mice. The arrows indicate the addition of capsaicin. Below the raw trace, 2 second extracts are shown before and after the addition of capsaicin. (c) Average profile of response (two-way ANOVA $p<0.001$). (d) Histogram comparing the peak response. ** $p < 0.01$

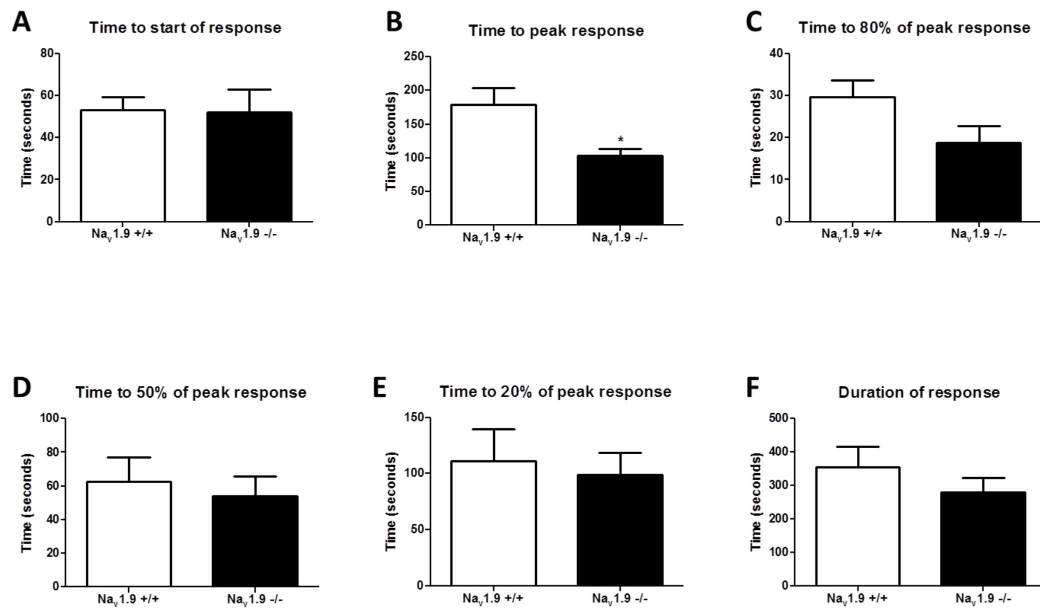


Figure 19 The duration of the colonic response to 1 µM capsaicin. (a) time from capsaicin entering the bath to nerve activity rising above baseline (Na_V1.9 +/+ 53.0 ± 6.4 vs. Na_V1.9 -/- 52.1 ± 10.7 seconds; n=7; p=0.95). (b) time from capsaicin entering the bath to peak response (Na_V1.9 +/+ 179.1 ± 24.4 vs. Na_V1.9 -/- 103.6 ± 9.6 seconds; n=7; p<0.05). (c) time from peak response to 80% of the peak response (Na_V1.9 +/+ 29.6 ± 4.0 vs. Na_V1.9 -/- 18.7 ± 4.0 seconds; n=7; p=0.08). (d) time from peak response to 50% of the peak response (Na_V1.9 +/+ 62.6 ± 14.4 vs. Na_V1.9 -/- 54.0 ± 12.0 seconds; n=7; p=0.66). (e) time from peak response to 20% of the peak response (Na_V1.9 +/+ 111.0 ± 28.6 vs. Na_V1.9 -/- 98.9 ± 19.9 seconds; n=7; p=0.73). (f) time from capsaicin entering the bath to nerve activity returning to baseline (Na_V1.9 +/+ 354.9 ± 60.5 vs. Na_V1.9 -/- 279.6 ± 42.2 seconds; n=7; p=0.33). * $p < 0.05$

3.4.4 Response to acetic acid

The response of colonic nerves to acetic acid was investigated by consecutive applications of 0.01%, 0.1%, 0.3% and 1% acetic acid. The response to 0.01% acetic acid was minimal in both groups ($Na_v1.9$ +/+ 0.4 ± 0.3 vs. $Na_v1.9$ -/- 0.0 ± 0.0 Hz; n=6; p=0.29. Figure 20d).

There was a trend for the peak response to 0.1% acetic acid to be lower in Nav1.9 -/- mice but this was not significant ($Na_v1.9$ +/+ 8.2 ± 2.9 vs. $Na_v1.9$ -/- 4.7 ± 2.2 Hz; n=6; p=0.35. Figure 20d). However, the overall response was significantly different between the two groups (two-way ANOVA p<0.01. Figure 20c). There were no significant differences in the sub-durations of the response to 0.1% acetic acid (Figure 21).

The response to 0.3% acetic acid was more complicated. All nerves exhibited a rise in activity in response to the acetic acid ($Na_v1.9$ +/+ 19.7 ± 6.3 vs. $Na_v1.9$ -/- 13.8 ± 3.4 Hz; n=6; p=0.43. Figure 20d). Four out of six nerves from $Na_v1.9$ -/- and five out of six nerves from $Na_v1.9$ +/+ mice exhibited a second smaller peak ($Na_v1.9$ +/+ 10.0 ± 3.8 vs. $Na_v1.9$ -/- 4.1 ± 2.0 Hz; n=4-5; p=0.24. Figure 22d). The time taken to reach this second peak was significantly longer in $Na_v1.9$ -/- mice ($Na_v1.9$ +/+ 562.6 ± 37.8 vs. $Na_v1.9$ -/- 727.3 ± 15.3 seconds; n=4-5; p<0.01. Figure 23d).

The response to 1% acetic acid was very short. Interestingly, in contrast to the lower doses tested, the peak response of $Na_v1.9$ -/- mice was higher than +/+ mice but this was not significant ($Na_v1.9$ +/+ 10.9 ± 3.3 vs. $Na_v1.9$ -/- 13.9 ± 4.6 Hz; n=6; p=0.60. Figure 20d). This was followed by total cessation of nerve activity in both groups. The response profiles of the four concentrations of acetic acid are shown in Figure 24. The profiles at 0.1% and 0.3% were significantly different between the groups (two-way ANOVA: 0.1% p<0.01, 0.3% p<0.001).

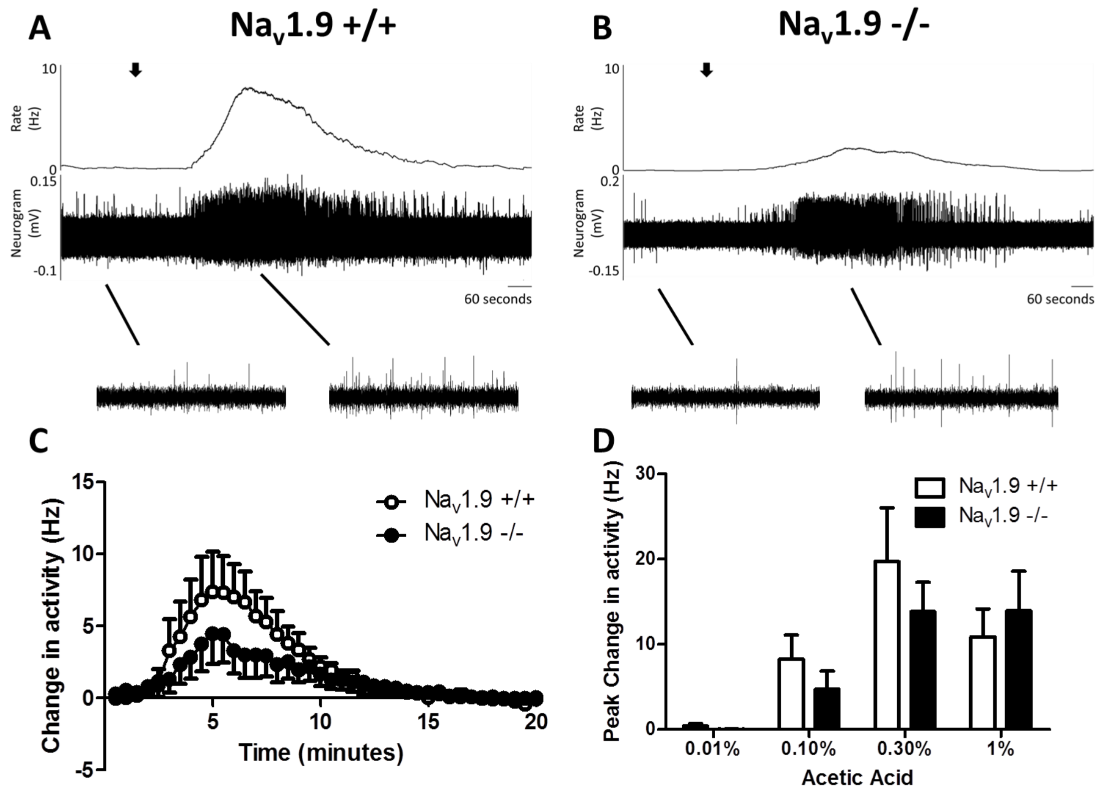


Figure 20 The effect of acetic acid on colonic nerve activity. Representative raw trace recordings and response rate (mean frequency over 60 seconds) from (a) Na$_V$1.9 +/+ and (b) Na$_V$1.9 -/- mice in response to 0.1% acetic acid. The arrows indicate the addition of acetic acid. Below the raw trace, 2 second extracts are shown before and after the addition of acetic acid. (c) Average profile of response to 0.1% acetic acid (two-way ANOVA $p<0.01$). (d) Histogram comparing the peak response to four concentrations of acetic acid.

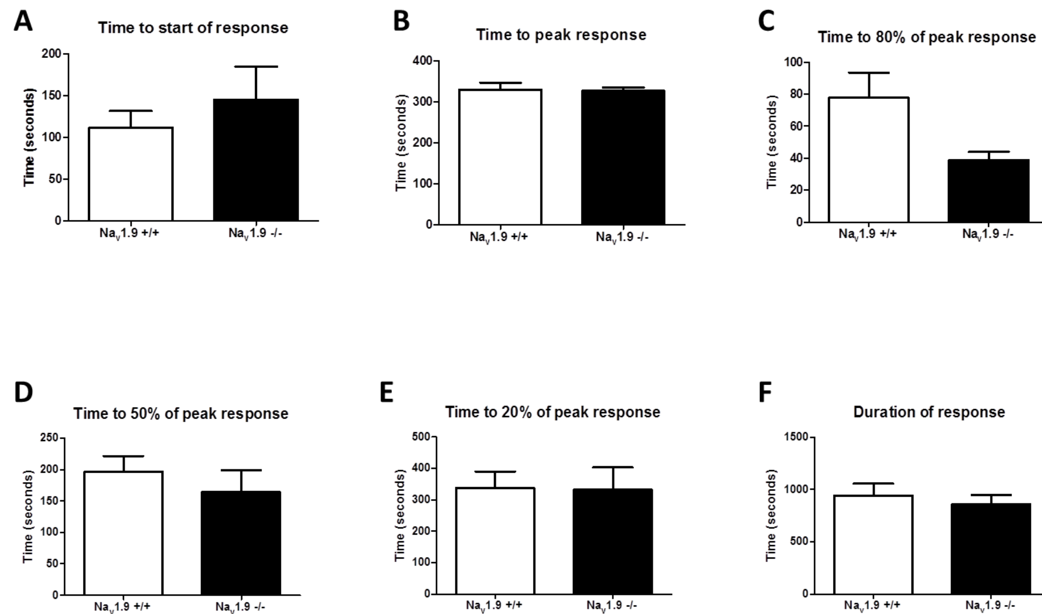


Figure 21 The duration of the colonic response to 0.1% acetic acid. (a) time from acetic acid entering the bath to nerve activity rising above baseline (Na$_V$1.9 +/+ 111.3 ± 20.2 vs. Na$_V$1.9 -/- 145.0 ± 39.4 seconds; n=5-6; p=0.44). (b) time from acetic acid entering the bath to peak response (Na$_V$1.9 +/+ 330.2 ± 16.7 vs. Na$_V$1.9 -/- 327.0 ± 8.0 seconds; n=5-6; p=0.88. (c) time from peak response to 80% of the peak response (Na$_V$1.9 +/+ 77.7 ± 15.8 vs. Na$_V$1.9 -/- 38.6 ± 5.2 seconds; n=5-6; p=0.06). (d) time from peak response to 50% of the peak response (Na$_V$1.9 +/+ 196.5 ± 25.3 vs. Na$_V$1.9 -/- 164.0 ± 35.0 seconds; n=5-6; p=0.46). (e) time from peak response to 20% of the peak response (Na$_V$1.9 +/+ 336.8 ± 53.4 vs. Na$_V$1.9 -/- 331.8 ± 71.49 seconds; n=5-6; p=0.96). (f) time from acetic acid entering the bath to nerve activity returning to baseline (Na$_V$1.9 +/+ 941.2 ± 113.5 vs. Na$_V$1.9 -/- 858.0 ± 89.1 seconds; n=5-6; p=0.59).

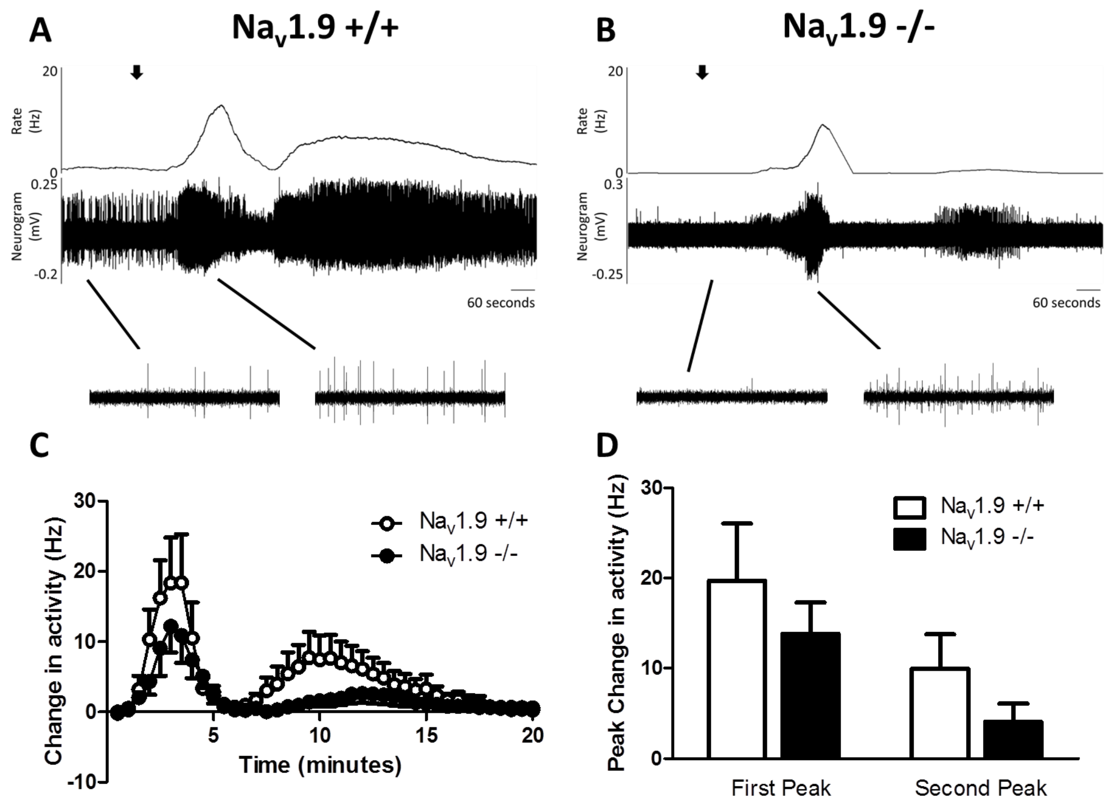


Figure 22 The effect of 0.3% acetic acid on colonic nerve activity. Representative raw trace recordings and response rate (mean frequency over 60 seconds) from (a) Na$_V$1.9 +/+ and (b) Na$_V$1.9 -/- mice in response to acetic acid. The arrows indicate the addition of acetic acid. Below the raw trace, 2 second extracts are shown before and after the addition of acetic acid. (c) Average profile of response to 0.3% acetic acid (two-way ANOVA $p<0.001$). (d) Histogram comparing the two peak responses.

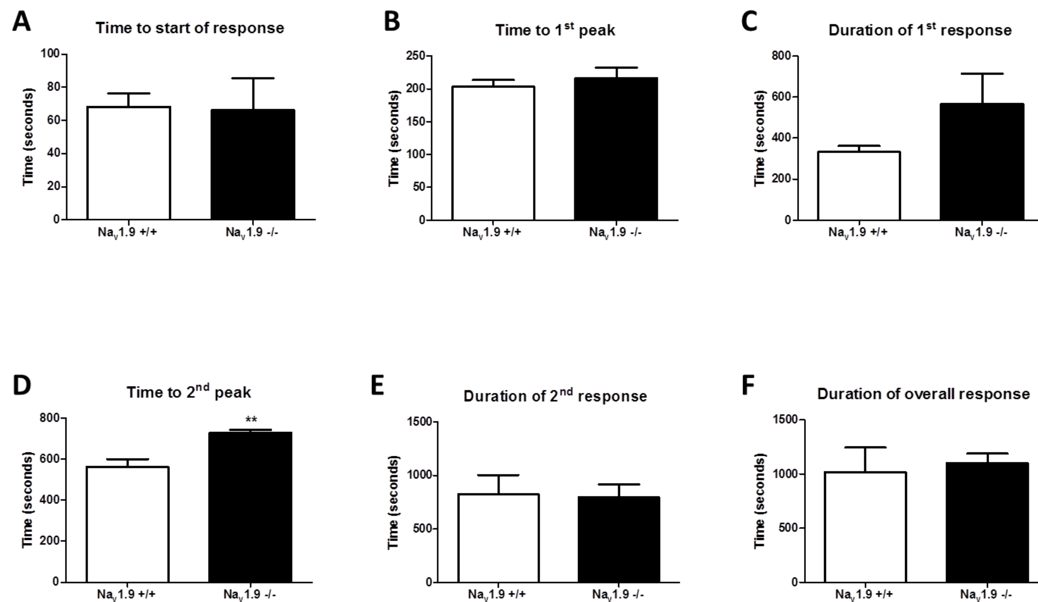


Figure 23 The duration of the colonic response to 0.3% acetic acid. (a) time from acetic acid entering the bath to nerve activity rising above baseline (Na$_v$1.9 +/+ 68.2 ± 8.2 vs. Na$_v$1.9 -/- 66.2 ± 19.3 seconds; n=6; p=0.93). (b) time from acetic acid entering the bath to 1st peak response (Na$_v$1.9 +/+ 203.3 ± 10.3 vs. Na$_v$1.9 -/- 216.0 ± 16.6 seconds; n=6; p=0.53). (c) duration of the 1st response (Na$_v$1.9 +/+ 331.7 ± 30.0 vs. Na$_v$1.9 -/- 567.8 ± 147.5 seconds; n=6; p=0.15). (d) time from acetic acid entering the bath to 2nd peak response (Na$_v$1.9 +/+ 562.6 ± 37.8 vs. Na$_v$1.9 -/- 727.3 ± 15.3 seconds; n=4-5; p<0.01). (e) duration of the 2nd response (Na$_v$1.9 +/+ 821.4 ± 182.2 vs. Na$_v$1.9 -/- 795.0 ± 119.3 seconds; n=4-5; p=0.91). (f) time from acetic acid entering the bath to nerve activity returning to baseline (Na$_v$1.9 +/+ 1016.0 ± 227.6 vs. Na$_v$1.9 -/- 1098.0 ± 89.0 seconds; n=6; p=0.75). ** p < 0.01

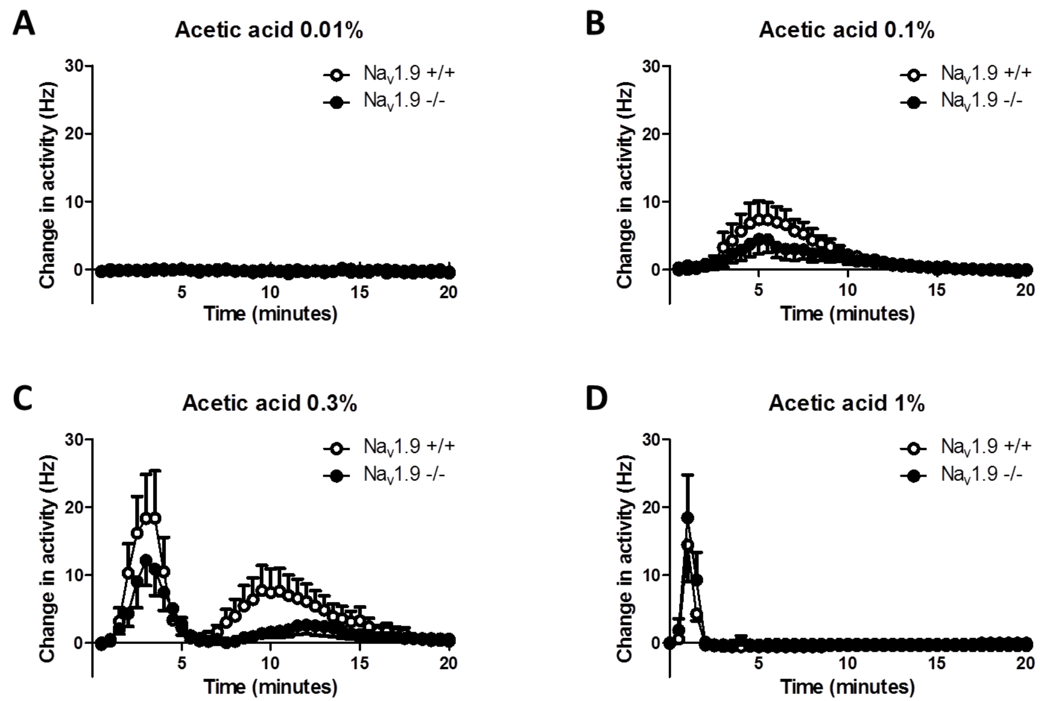


Figure 24 The effect of acetic acid on colonic nerve activity. Average profile of response to (a) 0.01% (two-way ANOVA $p=0.14$), (b) 0.1% (two-way ANOVA $p<0.01$), (c) 0.3% (two-way ANOVA $p<0.001$) and (d) 1% (two-way ANOVA $p=0.39$) acetic acid.

3.4.5 Response to human inflammatory supernatants

The response of human supernatant on nerve activity was tested in colonic preparations only. In $Na_v1.9$ +/+ mice, application of the control supernatant caused a small increase in nerve activity. This was significantly lower than that caused by the inflammatory supernatant (control supernatant 0.6 ± 0.1 vs. inflammatory supernatant 1.4 ± 0.1 Hz; n=5; $p<0.001$. Figure 25). Application of the inflammatory supernatant to colonic nerves from $Na_v1.9$ -/- mice caused an increase in nerve activity significantly lower than that in $Na_v1.9$ +/+ mice ($Na_v1.9$ +/+ 1.4 ± 0.1 vs. $Na_v1.9$ -/- 0.8 ± 0.1 Hz; n=3-5; $p<0.05$. Figure 26). The volume of inflammatory supernatant generated from the appendices of two patients was sufficient to allow multiple tests. Hence, each supernatant was tested on a nerve from $Na_v1.9$ +/+ and -/- mice. Although the number of tests (2) does not allow meaningful statistical comparison, the increase in nerve activity in response to application of the supernatant was higher in nerves from $Na_v1.9$ +/+ than $Na_v1.9$ -/- mice ($Na_v1.9$ +/+ 1.5 ± 0.4 vs. $Na_v1.9$ -/- 0.8 ± 0.3 Hz; n=2).

The profiles of the response to the inflammatory mediators were not significantly different between the two groups (two-way ANOVA p=0.05. Figure 26c). There was a trend towards a shorter response in Nav1.9 -/- mice but this was also not significant (Figure 27).

The cytokine content of inflammatory and control supernatants was assayed. There was a trend for higher concentrations of cytokines in the inflammatory supernatants but this did not reach significance (IL-1β: control 6.0 ± 2.9 vs. inflamed 20.9 ± 9.3 pg/ml; p=0.21. IL-6: control 248.9 ± 71.2 vs. inflamed 356.1 ± 157.6 pg/ml; p=0.59. GM-CSF: control 14.4 ± 4.8 vs. inflamed 28.2 ± 9.8 pg/ml; p=0.28. TNF-α: control 2.3 ± 0.5 vs. inflamed 3.0 ± 1.0 pg/ml; p=0.59. IL-8: control 178.7 ± 129.5 vs. inflamed 1197.0 ± 502.8 pg/ml; p=0.12; n=4-5. Figure 28).

Table xxxx shows patient demographics and white blood cell counts. The difference in ages between patients who donated normal appendices and those who donated inflamed appendices is a reflection of their disease aetiology as patients in the former group had their surgery for bowel cancer. The white blood cell count of the patients who had inflamed appendices trended higher than those who had normal appendices (normal 6.9 ± 1.0 vs. inflamed 11.4 ± 1.8 x10^3 cells/µl; n=5-6; p=0.06. Figure 29). When the white cell count of the patients who donated inflamed supernatants were analysed based on whether the supernatants were used on nerves from $Na_v1.9$ +/+ or -/- mice, there was no difference between the groups ($Na_v1.9$ +/+ 11.0 ± 2.1 vs. $Na_v1.9$ -/- 12.6 ± 0.9 x10^3 cells/µl; n=3-5; p=0.61. Figure 29). C-reactive protein levels were only available from some patients who had inflamed appendices (it is not consistently checked in suspected acute appendicitis and not before planned cancer surgery). Therefore, no comparison is possible between the normal and inflamed groups. In addition, no information on patient co-morbidities was collected at the time of collection of samples.

	Normal supernatant on $Na_v1.9$ +/+ nerves	Inflamed supernatant on $Na_v1.9$ +/+ nerves	Inflamed supernatant on $Na_v1.9$ -/- nerves
Gender M:F	2:3	3:2	1:2
Age (yrs)	64	32	43
White cell count (cells/µl)	6.9	11.0	12.6

Table 2 Age, gender and white cell count of patients who donated appendices.

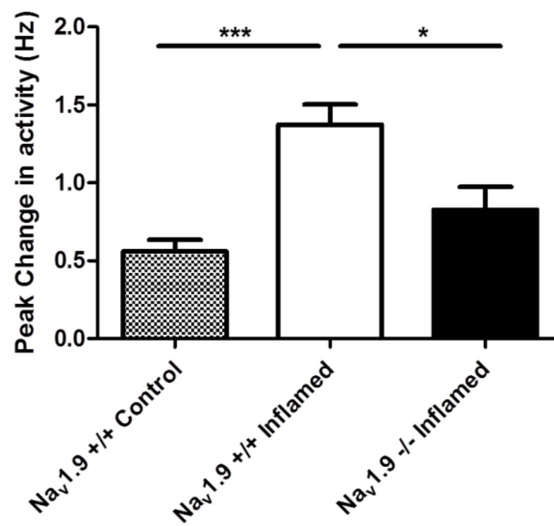


Figure 25 The effect of human supernatant on colonic nerve activity. * $p < 0.05$ *** $p < 0.001$

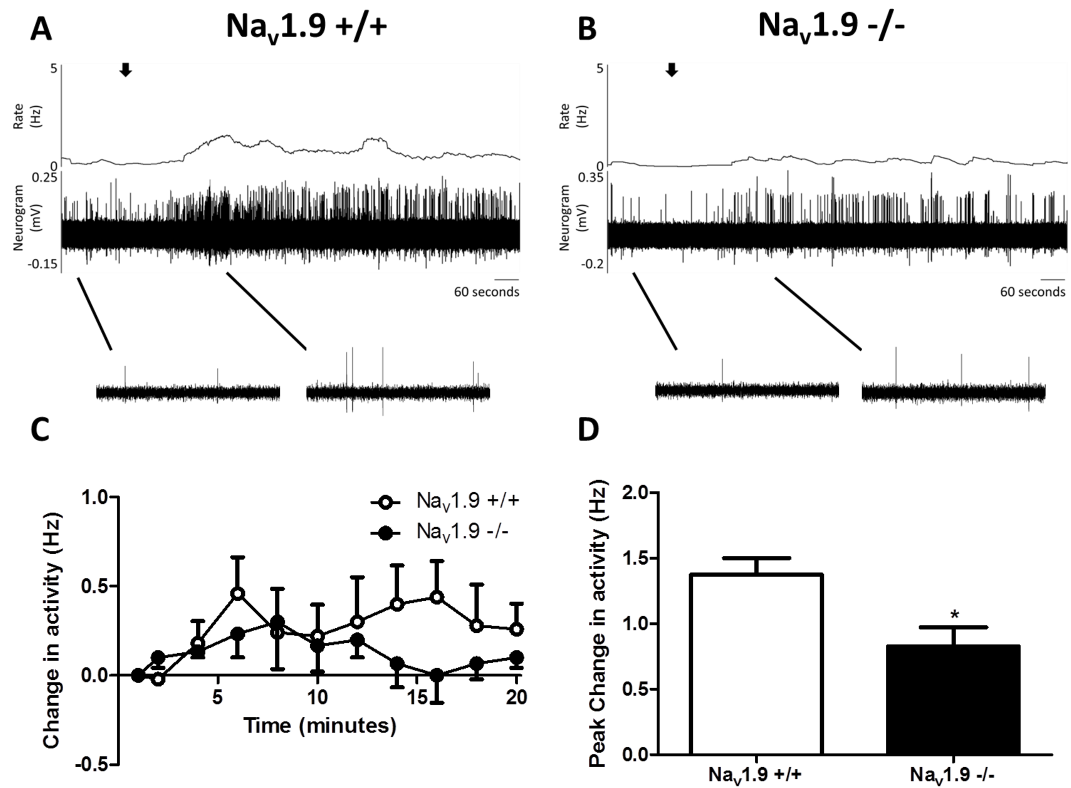


Figure 26 The effect of human supernatant on colonic nerve activity. Representative raw trace recordings and response rate (mean frequency over 60 seconds) from (a) Na$_V$1.9 +/+ and (b) Na$_V$1.9 -/- mice after addition of human supernatant. The arrows indicate the addition of supernatant. Below the raw trace, 2 second extracts are shown before and after the addition of supernatant. (c) Average profile of response (two-way ANOVA p=0.05). (d) Histogram comparing the peak response. * $p < 0.05$

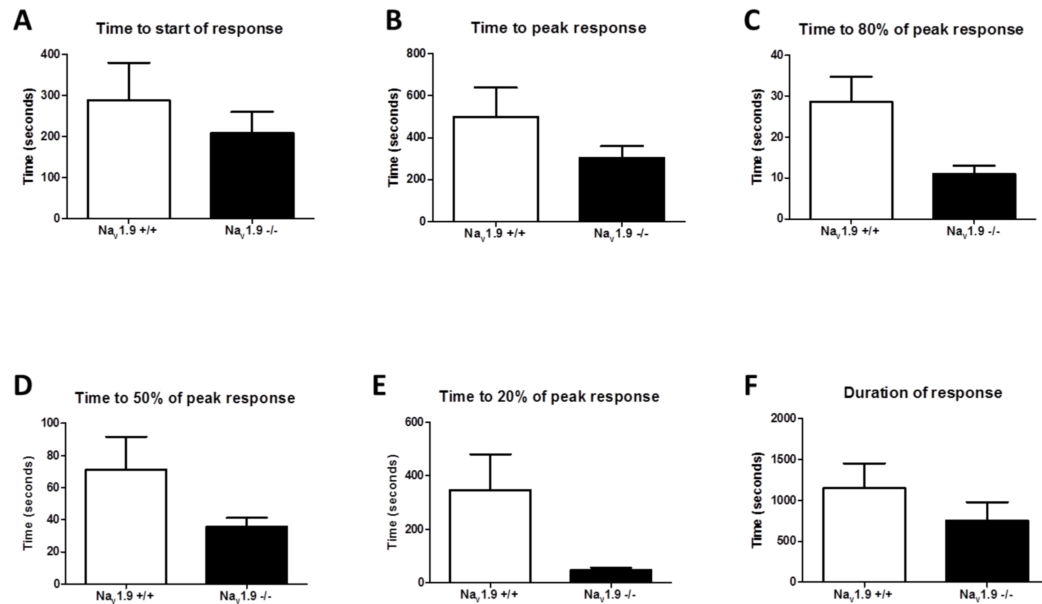


Figure 27 The duration of the colonic nerve response to human supernatant. (a) time from supernatant entering the bath to nerve activity rising above baseline ($Na_v1.9$ +/+ 288.2 ± 91.5 vs. $Na_v1.9$ -/- 208.3 ± 52.0 seconds; n=3-5; p=0.55). (b) time from supernatant entering the bath to peak response ($Na_v1.9$ +/+ 499.6 ± 139.2 vs. $Na_v1.9$ -/- 304.3 ± 55.5 seconds; n=3-5; p=0.34). (c) time from peak response to 80% of the peak response ($Na_v1.9$ +/+ 28.6 ± 6.1 vs. $Na_v1.9$ -/- 11.0 ± 2.0 seconds; n=3-5; p=0.08). (d) time from peak response to 50% of the peak response ($Na_v1.9$ +/+ 71.0 ± 20.6 vs. $Na_v1.9$ -/- 35.7 ± 5.5 seconds; n=3-5; p=0.25). (e) time from peak response to 20% of the peak response ($Na_v1.9$ +/+ 346.5 ± 133.2 vs. $Na_v1.9$ -/- 47.7 ± 8.4 seconds; n=3-4; p=0.12). (f) time from supernatant entering the bath to nerve activity returning to baseline ($Na_v1.9$ +/+ 1147.0 ± 305.8 vs. $Na_v1.9$ -/- 754.0 ± 224.2 seconds; n=3-4; p=0.38).

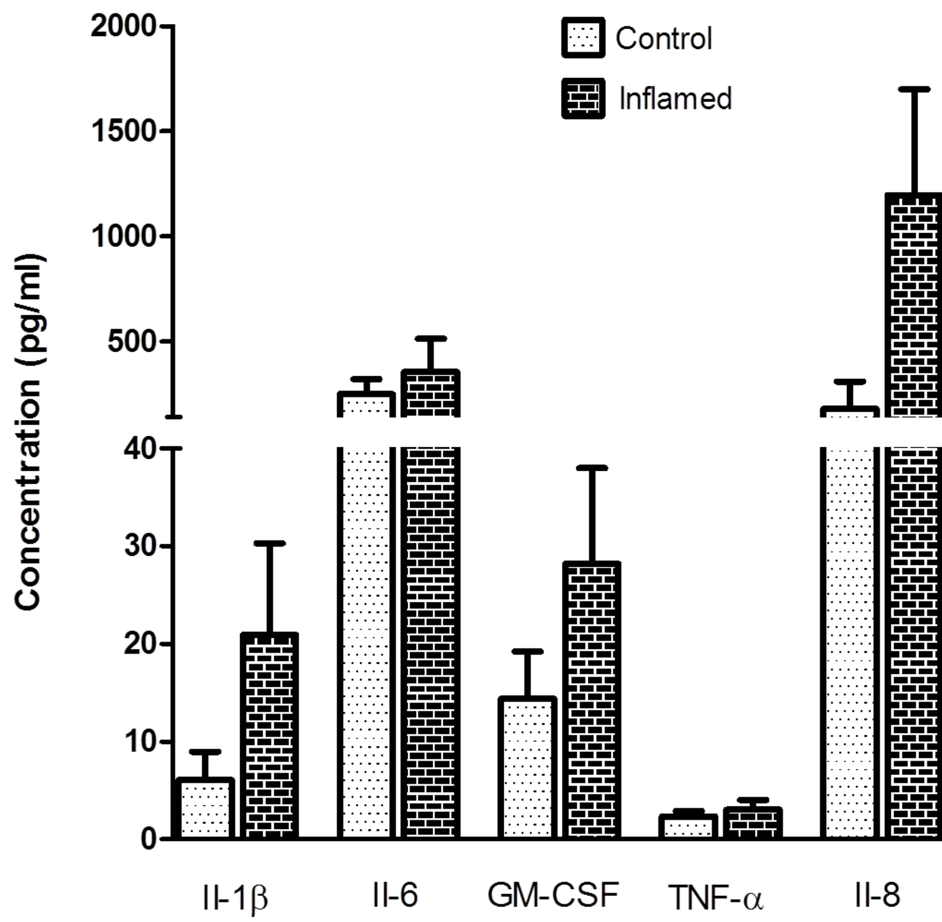


Figure 28 Cytokine content of inflammatory and control supernatants.

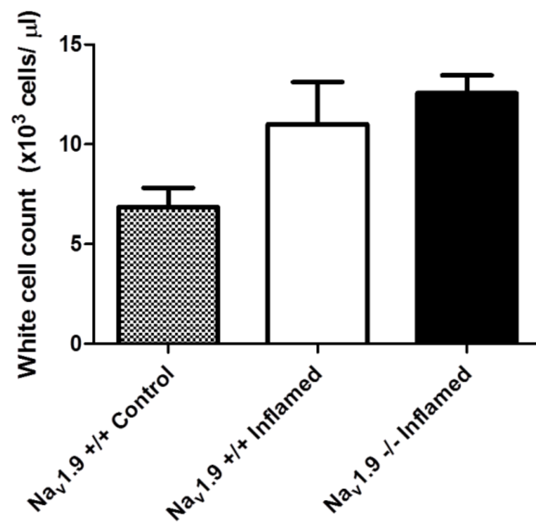


Figure 29 The white blood cell count of patients who provided appendix supernatants.

3.5 Specific discussion

3.5.1 The role of $Na_v1.9$ in the spontaneous activity of visceral afferents

The data generated demonstrate an important role for $Na_v1.9$ in the level of spontaneous visceral afferent activity. In both intestinal and colonic recordings, the level of spontaneous activity was approximately 60% lower in $Na_v1.9$ -/- compared to +/+ mice. In addition, the spontaneous activity of colonic afferents was less than 10% of the activity of intestinal afferents. This difference was seen across wild type and knockout mice. Off-line single unit analysis of a subset of the colonic afferent recordings revealed a greater number of units per recording of nerve activity in $Na_v1.9$ +/+ compared to -/- mice. The proportion of these units that were 'spontaneously' active was similar across both genotypes.

$Na_v1.9$ expresses a unique slow, persistent, tetrodotoxin resistant current (Dib-Hajj *et al.* 1998). It is known to become activated at a threshold which is close to the resting membrane potential and lower than those of other voltage gated sodium channels. Thus, $Na_v1.9$ is thought to contribute to setting the membrane potential (Herzog *et al.* 2001). Knock-out of $Na_v1.9$ leads to loss of this unique current which, when activated, 'nudges up' the membrane potential closer to the threshold necessary for action potential generation. Loss of $Na_v1.9$ therefore leads to an increase in the stimulus necessary for action potential generation. This is not always present in the 'resting' state which may explain the lower 'spontaneous' activity in -/- mice. In the single unit analysis of multi-unit colonic recordings, the number of units per recording was approximately 60% lower in $Na_v1.9$ -/- compared to $Na_v1.9$ +/+ mice. This corresponds to the drop in recoded overall activity. This suggests that it maybe the reduced number of units rather than less active units that contribute to the lower activity noted in recordings from $Na_v1.9$ -/- mice. Unfortunately, because of the low absolute number of 'spontaneously' active units in recordings from $Na_v1.9$ -/- mice, it was not possible to compare the activity of the single units between the genotypes.

A number of studies have reported that a majority of splanchnic afferents are silent at rest (Brierley *et al.* 2004; Brierley *et al.* 2005b; Feng and Gebhart 2011) while intestinal nerve recordings demonstrate significant spontaneous activity (Brunsden and Grundy 1999; Maubach and Grundy 1999). Data presented here confirm that the spontaneous activity of intestinal afferents is significantly higher than that of colonic afferents. There are a number of possible explanations. Firstly, it could be due to a difference in the number of fibres in the mesenteric as compared to the IMN nerve bundles from which the recordings are made. Secondly, mesenteric bundles contain vagal and spinal afferents while IMN bundles consist of only spinal afferents. Vagal afferents are activated at low threshold which may explain the higher 'spontaneous' activity of the mesenteric bundles. Alternatively, two scenarios may explain the difference between intestinal and colonic afferent: either a similar number of units are spontaneously active in both pathways but the splanchnic units have much lower spontaneous activity or secondly that significantly fewer units are spontaneously active in the splanchnic pathway. Unfortunately, the nature of extracellular, multi-unit recordings does not permit the investigation of these theories. Due to the high activity of intestinal afferents, off-line single unit analysis of the data does not provide meaningful data. Based on single unit data from other groups suggesting that the majority of splanchnic afferents are inherently silent at rest, it seems that the second scenario is the more likely explanation for the lower spontaneous activity demonstrated by my multi-unit recordings.

3.5.2 The role of $Na_v1.9$ in the activation of visceral afferents by bradykinin

The data generated demonstrate an important role for $Na_v1.9$ in the response of visceral afferents to bradykinin. The response of colonic afferents to bradykinin was reduced by 90% in $Na_v1.9$ -/- mice suggesting that $Na_v1.9$ plays a crucial role in the bradykinin signalling pathway in spinal afferents. The reduction in the response of intestinal afferents was 70% which, while lower than in colonic afferents, implies that $Na_v1.9$ plays a key role in bradykinin signalling in both vagal and spinal visceral

afferents. Single unit analysis of the response of colonic afferents to 0.3µM bradykinin revealed that a much higher proportion of $Na_v1.9$ +/+ units responded to the bradykinin as compared to $Na_v1.9$ -/- units. The increase in activity of these units was also greater in $Na_v1.9$ +/+ compared to $Na_v1.9$ -/- mice. In addition, a proportion of the $Na_v1.9$ +/+ units that responded to bradykinin did not respond to intra-luminal distension, a property not observed in $Na_v1.9$ -/- mice.

This data is consistent with that from a behavioural study in somatic afferents investigating the immediate paw licking response caused by intraplantar injection of bradykinin. Amaya *et al* investigated this response in $Na_v1.9$ +/+ and -/- mice. Injection of 300 ng bradykinin caused an immediate response in both groups of mice but the response in $Na_v1.9$ -/- mice was reduced by over 80% (Amaya *et al.* 2006). In addition, $Na_v1.9$ -/- mice also exhibited significantly reduced mechanical and thermal hypersensitivity. The data from the behavioural study are consistent with the findings reported here with regards to the magnitude of the effect of eliminating $Na_v1.9$ from spinal afferents.

Bradykinin mediates its effects though activation of PKC amongst other mechanisms (Leeb-Lundberg *et al.* 2005). PKC has been shown to upregulate the $Na_v1.9$ current (Baker 2005) providing a possible mechanism for bradykinin to enhance the $Na_v1.9$ current. In addition to $Na_v1.9$, other ion channels have been implicated in the bradykinin signalling pathway, e.g. TRPV1 and TRPA1 (Premkumar and Ahern 2000; Bandell *et al.* 2004). While this has not been investigated as part of this thesis, the substantial contribution by $Na_v1.9$ to the bradykinin signalling pathway demonstrated by these data implies that other contributors have relatively minor roles. Indeed, the response of splanchnic serosal afferents to bradykinin was not significantly different between TRPA1 +/+ and -/- mice (Brierley *et al.* 2009). Interestingly, the mechanical hypersensitivity to bradykinin was reduced in TRPA1 -/- mice. The Amaya study also demonstrated reduced mechanical hypersensitivity in $Na_v1.9$ -/- mice implying that, while the direct response to bradykinin is closely

related to $Na_v1.9$, its secondary sensitisation effects occur via modulation of a number of ion channels.

While there are no data quantifying expression of bradykinin receptors in visceral afferents, data from non retrogradely-labelled DRG demonstrate bradykinin labelling in 43 - 52% of the total cultured DRG (Segond von Banchet *et al.* 1996; Petersen *et al.* 1998). Brierley *et al* showed that 50 - 60% of mechanosensitive serosal lumbar splanchnic afferents responded to application of bradykinin (Brierley *et al.* 2005b; Brierley *et al.* 2009). In addition, a number of mechanically insensitive afferents also responded to bradykinin although it was not possible to calculate their proportion of the total mechanically insensitive population as they were found incidentally rather than being systematically searched for (Brierley *et al.* 2005b). A study which systematically investigated the whole lumbar splanchnic afferent population using electrical stimulation found that a third were mechanically insensitive (Feng and Gebhart 2011). Only 12% of these mechanically insensitive afferents responded to an inflammatory soup containing bradykinin, serotonin, histamine and PGE_2. The Brierley study only investigated serosal afferents but if the results are extrapolated to all afferent classes then it can be assumed that approximately half of lumbar splanchnic afferents are responsive to bradykinin. Amalgamating these results with those of Feng and Gebhart suggests that approximately 40% of all lumbar splanchnic afferents respond to bradykinin and so must express receptors for bradykinin. This figure is consistent with that found in the somatic system (Petersen *et al.* 1998).

No studies have explored the distribution of $Na_v1.9$ in visceral afferents. Data from the somatic system show that $Na_v1.9$ is expressed on 35 – 61% of L4-5 DRG and approximately 70% of L6 and S1 DRG (Amaya *et al.* 2000; Decosterd *et al.* 2002; Black *et al.* 2003; Fukuoka *et al.* 2008). The almost complete abolition of response to bradykinin in colonic afferents suggests that $Na_v1.9$ and B_2 are very closely co-expressed. The intestinal data relate to a mixed population of spinal and vagal

afferents. The decrease in response to bradykinin was significant but not as great in magnitude as the purely spinal population of the colonic nerve. This suggests that B_2 may be expressed in a wider population of nerves some of whom do not express $Na_v1.9$. A co-expression study in somatic afferents showed that 63% of B_2 expressing neurons co-express $Na_v1.9$ (Amaya *et al.* 2006). Thus, as an explanation for the findings presented here, I postulate that nearly all B_2 expressing spinal visceral afferents express $Na_v1.9$ with a significant majority of B_2 expressing vagal afferents co-expressing $Na_v1.9$.

3.5.3 The role of $Na_v1.9$ in the activation of visceral afferents by capsaicin

The data generated provide evidence for an important role for $Na_v1.9$ in the response of visceral afferents to capsaicin. The response of colonic and intestinal afferents to capsaicin was reduced by approximately 50% in $Na_v1.9$ -/- mice. In contrast to the response to bradykinin, the attenuation in response to capsaicin was consistent between intestinal and colonic afferents.

These data are consistent with that from a behavioural study in somatic afferents investigating the immediate paw licking response caused by intraplantar injection of capsaicin. Amaya *et al* investigated this response in $Na_v1.9$ +/+ and -/- mice. Injection of 2.5 μg capsaicin caused an immediate response in both groups of mice but the response in $Na_v1.9$ -/- mice was reduced by nearly 50% (Amaya *et al.* 2006). In addition, $Na_v1.9$ -/- mice also exhibited significantly reduced mechanical hypersensitivity. Data from this behavioural study are thus consistent with the findings reported here with regards to the magnitude of the effect of eliminating $Na_v1.9$.

Capsaicin is the agonist for TRPV1, a cation channel (Caterina *et al.* 1997). Binding of capsaicin to TRPV1 leads to inflow of calcium ions with consequent changes of the membrane potential. This may lead to a generator potential which can be a

precursor to an action potential. In addition, TRPV1 mediated neuropeptide release may exert GPCR mediated modulation of afferent activity (Luo *et al.* 2013). Studies of back labelled visceral DRG showed that approximately 82% of spinal, 50% of pelvic and 32% of vagal DRG express TRPV1 (Robinson *et al.* 2004; Brierley *et al.* 2005a; Tan *et al.* 2009). Due to the lack of studies investigating he expression of $Na_v1.9$ in visceral afferents, there are no data on the co-expression of $Na_v1.9$ and TRPV1. However, somatic co-expression studies reveal that approximately 55% of TRPV1 expressing neurons co-express $Na_v1.9$ (Amaya *et al.* 2000; Amaya *et al.* 2006). It is intriguing that despite evidence from back labelled visceral DRG showing significantly more TRPV1 positive neurons in spinal vs. vagal afferents, the reduction in response in colonic and intestinal afferents was similar in the current studies. Intestinal nerves contain a mixed population of vagal and spinal afferents. What is not known is the relative contribution of each population to the overall nerve bundle. There is some evidence that the density of $Na_v1.9$ positive afferents is significantly higher in spinal vs. vagal afferents (Peeters *et al.* 2006). It is tempting to speculate that as the proportion of afferents expressing TRPV1 and $Na_v1.9$ is lower in the vagal pathway, the percentage of afferents which express both receptors is similar to that in the spinal pathway. This is a potential explanation for the similar reduction in response of intestinal and colonic afferents to capsaicin in $Na_v1.9$ -/- mice.

While it is evident that activation of TRPV1 is sufficient to cause action potential generation independently of $Na_v1.9$, the data presented here clearly demonstrate a contribution by $Na_v1.9$ to visceral afferent excitability. Based on its low activation threshold and the voltage dependant nature of its activation, it is likely that $Na_v1.9$ acts as an amplifier of the generator potential caused by activation of TRPV1. The loss of this amplifying role in $Na_v1.9$ -/- mice leads to a reduced response of afferents to stimulation by capsaicin.

3.5.4 The role of $Na_v1.9$ in the activation of colonic afferents by acetic acid

The data generated demonstrate a complex relationship between $Na_v1.9$ and acetic acid activation of visceral afferents. Taken as peak responses, there was no significant difference between afferents from $Na_v1.9$ +/+ and -/- mice at all concentrations tested. However, this obscures nuances within the responses. The 0.01% did not activate afferents from either genotype. At the 0.1% concentration, the peak response was lower in $Na_v1.9$ -/- mice but this was not significant. The complete profile of the response was, however, strongly significant ($p<0.01$) resulting from the total number of action potentials activated by the acetic acid being significantly lower in $Na_v1.9$ -/- mice. The 0.3% dose elicited a unique response in both genotypes. The afferents increased their firing rate to reach a peak response within approximately 200 seconds before returning to baseline activity. In the majority of afferents, the discharge rate increased again to reach a second, smaller peak. Both peaks were lower in the $Na_v1.9$ -/- mice but the difference did not reach significance. The time from administration of acetic acid to the second peak was, however, significantly longer in $Na_v1.9$ -/- mice. In addition, the combined response profile was significantly different between the genotypes reflecting the lower number of action potentials generated in $Na_v1.9$ -/- mice. Interestingly, at the top dose of 1%, the peak response was higher in $Na_v1.9$ -/- mice but this was not significant. In addition, the peak was reached within 30 seconds followed by total cessation of nerve activity (spontaneously and in response to mechanical stimulation) for at least one hour.

The findings reported here are in contrast to those in the literature. A behavioural study conducted in $Na_v1.9$ +/+ and -/- mice demonstrated a significant increase in pain behaviour in $Na_v1.9$ -/- mice (Leo *et al.* 2010). The authors injected 1% acetic acid intraperitoneally and then counted the number of abdominal contractions between 5 and 20 minutes later. The authors counted twice the number of writhes in $Na_v1.9$ -/- compared to +/+ mice. This is counterintuitive, as loss of $Na_v1.9$ has been shown to have a protective effect on pain behaviour after application of

algogenic mediators. Intraperitoneal injection of acetic acid can be criticised as a non-natural inflammatory stimulus. In addition, it causes activation of visceral and somatic afferents which will both contribute to pain behaviour. Indeed, the authors state that a number of 'incorrectly' injected mice were excluded from their analysis. At the top dose in my study (1%), the response of visceral splanchnic afferents was not significantly higher in $Na_v1.9$ -/- as compared to +/+ mice. Although the concentration of acetic acid at addition to the recording chamber was 1%, there was inevitably some dilution as it mixed with the Krebs buffer in the chamber. Thus, it is likely that the afferents in the current study were not exposed to the full effect of 1% acetic acid. Nevertheless, even this reduced dose caused a great increase in afferent activation which was difficult to fully capture and lasted for less than 2 minutes followed by complete cessation of afferent activity. This is in contrast with the behavioural study in which the authors monitored pain behaviour 5 - 20 minutes after injection of the acetic acid.

A second behavioural study found no role for $Na_v1.9$ on the response of visceral afferents to acetic acid (Ritter *et al.* 2009). The authors infused the bladder with 0.25% acetic acid and recorded the maximal infused and voided volumes as well as bladder pressures in $Na_v1.9$ +/+ and -/- mice. The results of this study are in agreement with data presented here where there was no difference in the peak response of colonic splanchnic afferents between $Na_v1.9$ +/+ and -/- mice. The difference in the results between the two behavioural studies maybe due to the different concentrations used (as suggested by my data) or due to the relative contribution of $Na_v1.9$ to each signalling system ($Na_v1.9$ has been shown to be rarely expressed in non-inflamed bladder afferents (Black *et al.* 2003)).

The mechanism of action of acetic acid is not fully understood. It is possible that acetic acid exerts its effects by activating membrane receptors, for example TRPV1 or ASIC may be activated by the low pH. Indeed, there is evidence that antagonism

of TRPV1 reduces the visceromotor response to colorectal distension caused by acetic acid infusion (Wiskur *et al.* 2010). From the current data it appears that acetic acid acts on visceral afferents via more than one mechanism. At lower doses, the activation of afferents seems to be partially via a Na_v1.9 pathway as there was a non-significant decrease in the peak response in Na_v1.9 -/- mice. The 0.3% dose produced a biphasic response. The first peak was reached quickly and the afferent activity returned to baseline. The second phase of the response reached a peak which was lower than the first in mice from both genotypes. This peak was lower and took significantly longer in Na_v1.9 -/- mice. It is tempting to speculate that this second phase may be due to neuropeptide release by the afferents which activates second messenger pathways linked to Na_v1.9. The top dose of acetic acid caused a large and very brief increase in activity which was not significantly higher in Na_v1.9 -/- mice. That this top dose seems to have an opposite effect on the afferents as compared to the lower doses could be due to the fact that such a strong stimulus bypasses the threshold setting properties of Na_v1.9 and may make other Na_v channels more available and indeed activate other ion channels. In addition, acetic acid is an artificial stimulus that is not normally encountered by afferents and so its value as a disease model is questionable.

3.5.5 The role of Na_v1.9 in the activation of colonic afferents by human inflammatory supernatants

This study is the first to demonstrate activation of visceral afferents by human inflammatory supernatants from diseased tissue that is the cause of pain. The data generated provide evidence for an important role for Na_v1.9 in the response of visceral afferents to the inflammatory supernatant. The response of colonic afferents was reduced by nearly 50% in Na_v1.9 -/- mice. The data also demonstrate that visceral afferents can be activated by supernatant from non-inflamed tissue but that this activation is significantly lower than that by supernatant from inflamed tissue. This allows the use of the non-inflamed supernatant as a control. The use of the same supernatant on nerves from Na_v1.9 +/+ and Na_v1.9 -/- mice acted as an internal

control. While this was only possible in 2 supernatants, this difference in activation of $Na_v1.9$ -/- and $Na_v1.9$ +/+ nerves in both cases provides another layer of evidence supporting a role for $Na_v1.9$ in the response of the nerve to the inflammatory stimuli contained within the supernatants.

While no group has investigated the activation of visceral afferents by human 'overtly' inflammatory supernatants, two studies have been published that demonstrate activation of extrinsic and enteric visceral afferents by supernatants generated from biopsy samples of patients suffering from IBS (Barbara *et al.* 2007; Buhner *et al.* 2009). In addition, another group have demonstrated the development of mechanical hyperalgesia to colorectal distension in mice whose colon were instilled with supernatant created from biopsy samples of IBS patients (Cenac *et al.* 2007).

While there are a number of studies measuring serum cytokine levels in patients suffering from acute appendicitis, none measured the cytokine levels in the appendix itself. Three groups have measured the local release of inflammatory mediators by analysing peritoneal fluid, either by aspirating it at the beginning of the operation or by instilling 0.9% saline and then aspirating 1 minute later (Rivera-Chavez *et al.* 2003; Dalal *et al.* 2005; Yamamoto *et al.* 2005). This method is unlikely to result in a supernatant whose contents are a true reflection of the cytokine content of the inflamed appendix. The groups tested for a range of cytokine including IL-1β, IL-6, IL-8, GM-CSF and TNF-α. The cytokine levels measured by the groups were highly variable with a wide range for each cytokine, for example in one study the average IL-6 level was 3,910 pg/ml with the 25th and 75th percentiles stated as 869 and 17,500 respectively (Rivera-Chavez *et al.* 2003). The variability between groups was also high with average levels for IL-8 reported as 35 pg/ml in one study and 1,416 pg/ml in another (Rivera-Chavez *et al.* 2003; Dalal *et al.* 2005). Cytokines were not detected in every sample tested including in perforated or gangrenous appendicitis (Rivera-Chavez *et al.* 2003; Yamamoto *et al.* 2005). The cytokine levels detected in the current experiments reflected this variability. Although cytokine levels in

supernatant from inflamed tissue were higher than that from normal tissue, this did not reach significance. This is partly due to the inter-sample variability and small sample size but also due to the presence of moderate amount of some cytokines, e.g. IL-6, in the control tissue. In addition, the detection of very low levels of TNF-α in inflamed tissue was surprising considering its pro-inflammatory role. These findings may be related to the method used to produce the supernatant: during the 30 minute incubation of the appendix in buffer, further cytokines are being released in both control and inflamed tissue. This may explain the relatively high level of some cytokines in non-inflamed tissue. Variation in the disease process may also account for the variability between samples. Unlike laboratory animals and off the shelf mediators, human disease samples are not uniform. Patients do not present at a similar time in the disease process and their immune system responses to the inflammatory process are highly variable. This is one of the features that plague clinical trials of compounds that have been successful in the laboratory. One of the solutions would be to increase the sample size to account for the natural variability in human disease.

The white cell count of the patients who donated inflamed appendices were higher than those who donated normal appendices as would be expected based on their respective aetiologies (inflammatory process vs. cancer). The difference in ages is also a consequence of the disease aetiology.

The colonic afferents tested produced a robust response to application of the inflammatory supernatants. The magnitudes of the responses were smaller than those after application of single mediators, e.g. bradykinin or capsaicin. This may be accounted for by two factors. First, all studies involving single mediators had a 20 ml volume of mediator applied to the recording chamber and this was sufficient to replace the chamber volume. The size of the appendices limited the volume of supernatant available with a range of 10 - 15 ml used. Secondly, more concentrated

supernatants are likely to have stimulated the afferents to a greater degree. The calculation of supernatant volume = 2.5 x appendix weight was used as a compromise between concentration of cytokines and volume of supernatant. An alternative would have been to combine supernatants from more than one patient to produce a more concentrated 20 ml of supernatant for use in each experiment. This, however, would have broken the link between an individual patient and the afferent experiment.

Another finding of my study is the longer time period between addition of the supernatant and the beginning of the afferent response when compared to the single mediators. This may be a function of the smaller volume being diluted in the recording chamber but it may also be a reflection that most of the mediators in the supernatants signal via second messenger pathways to initiate action potentials. The significantly lower peak activation in $Na_v1.9$ -/- mice suggests that $Na_v1.9$ may have a role in the downstream signalling pathways of these mediators.

4

The role of prostaglandins in afferent signalling

4.1 Aims

The aims of the experiments discussed in this chapter are:

1. To investigate the role of $Na_v1.9$ in the response of visceral afferents to prostaglandin E_2
2. To investigate the role of prostaglandins in the spontaneous activity of visceral afferents in $Na_v1.9$ +/+ and -/- mice
3. To investigate the role of prostaglandins in the response of visceral afferents to bradykinin in $Na_v1.9$ +/+ and -/- mice

4.2 Specific rationale

Prostaglandins are known to increase afferent activity in rodents. However, patch clamp data are conflicting with regards to the role of PGE_2 on the $Na_v1.9$ current with some authors demonstrating enhancement of the current while others failing to demonstrate an effect. PGE_2 has also been shown to augment the response of visceral afferents to bradykinin. In view of the conflicting patch clamp data, I investigated whether this enhancement of bradykinin response occurs in the absence of $Na_v1.9$. A further discussion of the rationale for the experiments in this chapter is outlined below.

4.2.1 Cyclooxygenase pathway

Cyclooxygenase (COX) mediates the conversion of arachidonic acid into prostaglandin H_2 which is then converted to one of a number of prostanoids (Figure 30). COX exists as 2 isoforms termed COX-1 and COX-2. Analogous to the bradykinin B_1 and B_2 receptors, COX-1 is constitutively expressed whereas COX-2 is induced by inflammation (Ricciotti and FitzGerald 2011).

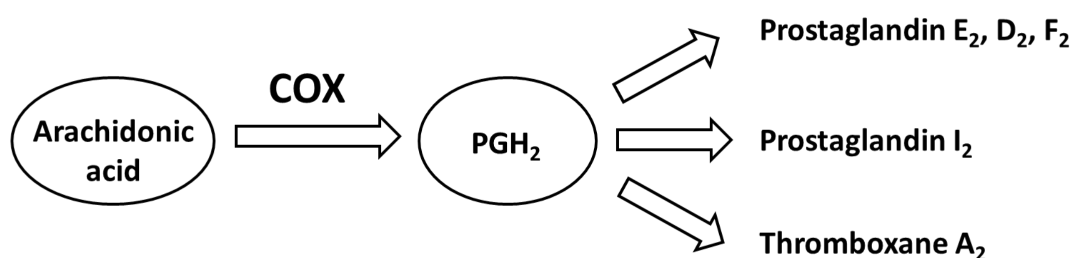


Figure 30 An outline of the cyclooxygenase (COX) pathway for metabolism of arachidonic acid. PGH_2: prostaglandin H_2.

NSAIDs are non-selective blockers of COX-1 and COX-2 with consequent inhibition of prostaglandin synthesis. This leads to a reduction in inflammation and also has

analgesic effects. In addition to their pro-inflammatory roles, prostaglandins also have a physiological role in the GI, renal and cardiovascular systems (Ricciotti and FitzGerald 2011). Consequently, one of the side effects of NSAIDs is a change in the balance between the protective and damaging influences on the GI mucosa leading to ulceration and risk of significant haemorrhage. Other effects of COX blockade include cardiac and renal failure. This range of side effects stimulated the development of COX-2 specific blockers. While these drugs had lower GI side effects, their cardiovascular side effect profile led to their withdrawal.

4.2.2 Role of prostaglandins in inflammation

Prostaglandins exert their effects by activating GPCRs which have been named EP, DP, IP and FP for prostaglandin E_2, D_2, I_2 and F_2 respectively (Woodward *et al.* 2011). Some of these receptors (e.g. EP and DP) have a number of subtypes which couple to different messenger pathways. The stimulation of PKA and PKC production mediates voltage-gated sodium channel and TRPV1 function thus increasing sensory afferent excitability (Fitzgerald *et al.* 1999; Matsumoto *et al.* 2005). PGE_2 is one of the most abundant prostaglandins and exhibits a variety of functions depending on the conditions: it is a mediator of many physiological functions e.g. immune responses and GI protection. During inflammation, it is significantly up regulated and plays a role in many of the constituents of the inflammatory response e.g. vasodilatation and pain (Ricciotti and FitzGerald 2011).

Evidence for a role for prostaglandins in afferent activity is available from studies in somatic and visceral afferents. In an *in vivo* experiment, systemic administration of PGE_2 significantly increased the activity of jejunal afferents (Haupt *et al.* 2000). In addition, application of a non-specific COX blocker, naproxen, reduces the intensity of spontaneous afferent activity (Maubach and Grundy 1999). Naproxen also blunts the response of visceral afferents to high dose bradykinin. This blunting of the response to bradykinin is reversed by the subsequent addition of PGE_2. The afferent response to low dose bradykinin was not affected by naproxen. This suggests that

although bradykinin can activate visceral afferents independently of the COX pathway, products of this pathway are necessary for the full effect of bradykinin to be sensed by the afferents (Maubach and Grundy 1999). PGE_2 also increases the number of DRG neurons that respond to bradykinin as well as the magnitude of the response in somatic and visceral afferents in the absence of COX blockade (Mense 1981; Stucky *et al.* 1996; Brunsden and Grundy 1999). In addition, PGE_2 sensitises the activity of TRPV1 on sensory neurons while blockade of COX attenuates the increase in visceral afferent activity due to application of histamine (Brunsden and Grundy 1999; Zhang *et al.* 2008).

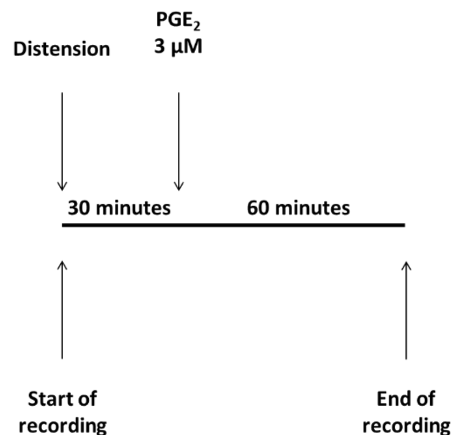
4.2.3 Interplay between PGE_2 and $Na_v1.9$

There is some evidence for a relationship between PGE_2 and $Na_v1.9$. Patch clamp studies have demonstrated an increase in $Na_v1.9$ current after application of PGE_2 (Rush and Waxman 2004; Li and Schild 2007) while multiunit recordings of bladder afferents show that the increase in activity caused by instillation of PGE_2 into the bladder is absent in $Na_v1.9$ -/- mice (Ritter *et al.* 2009). Two behavioural studies have also investigated the relationship between $Na_v1.9$ and PGE_2 albeit in somatic afferents. Injection of PGE_2 into the hind paw of mice produces mechanical allodynia to probing with von Frey hairs and thermal hyperalgesia during a hot plate test. In $Na_v1.9$ -/- mice, the mechanical allodynia was attenuated while the thermal hyperalgesia was absent (Priest *et al.* 2005; Amaya *et al.* 2006). A parallel study in visceral afferents is lacking. Based on these somatic studies as well as patch clamp studies and extracellular multiunit recordings from visceral afferents in which COX blockade has been shown to blunt the response of visceral afferents to bradykinin, it is expected that prostaglandins play a significant role in the response of visceral afferents to bradykinin in both $Na_v1.9$ -/- and +/+ mice. In addition, PGE_2 is expected to have a direct stimulatory effect on visceral afferents which is reduced in $Na_v1.9$ -/- mice.

4.3 Experimental protocols

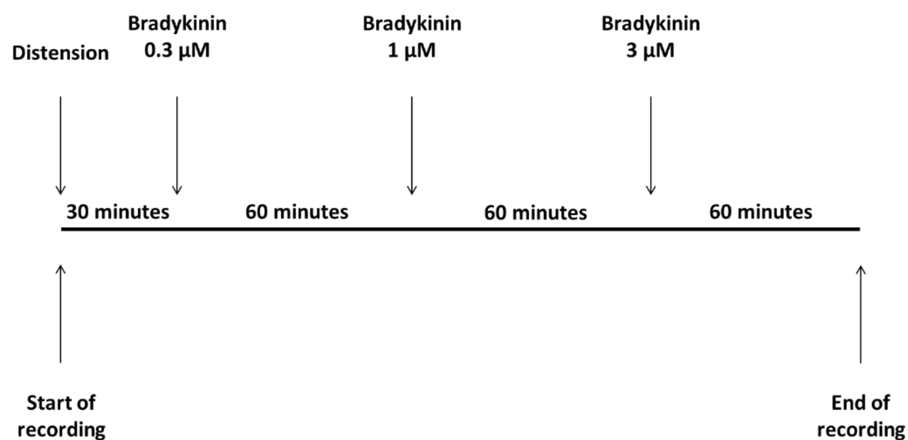
4.3.1 Prostaglandin E_2

The effect of PGE_2 on afferent activity was investigated in colonic preparations only. A single application of 3 µM PGE_2 was the only mediator applied to each preparation.



4.3.2 Bradykinin in the absence of indomethacin

The effect of bradykinin on afferent activity was investigated in indomethacin free colonic preparations. Consecutive applications of 0.3 µM, 1 µM and 3 µM were superfused 60 minutes apart. The nerve activity had returned to baseline before the next dose was applied.



4.4 Results

4.4.1 Response to prostaglandin E_2

The response of colonic nerves to PGE_2 was investigated by application of a single dose of 3 μM PGE_2 in the presence of indomethacin. The peak responses were significantly lower in $Na_v1.9$ -/- compared to +/+ mice ($Na_v1.9$ +/+ 4.1 ± 1.0 Hz vs. $Na_v1.9$ -/- 1.8 ± 0.3 Hz; n=6; $p<0.05$. Figure 31d). The overall responses were also significantly different (two-way ANOVA $p<0.001$. Figure 31c).

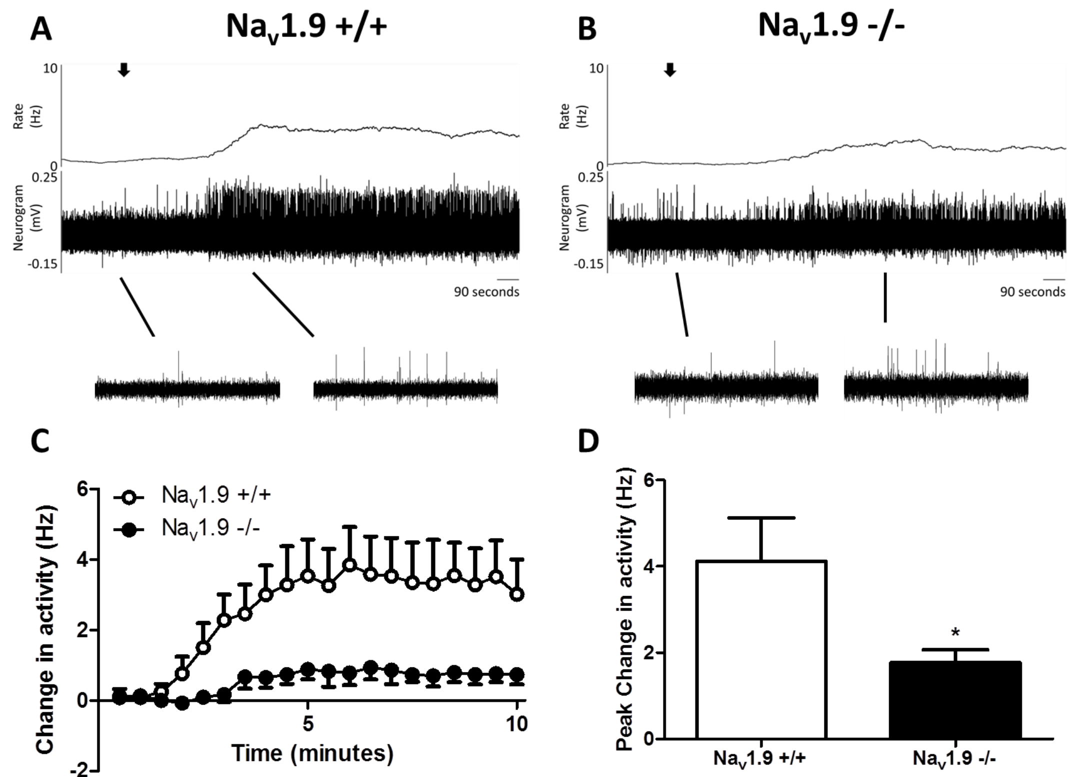


Figure 31 The effect of 3 µM PGE$_2$ on colonic nerve activity. Representative raw trace recordings and response rate (mean frequency over 60 seconds) from (a) Na$_V$1.9 +/+ and (b) Na$_V$1.9 -/- mice. The arrows indicate the addition of PGE$_2$. Below the raw trace, 2 second extracts are shown before and after the addition of PGE$_2$. (c) Average profile of response (two-way ANOVA $p<0.001$). (d) Histogram comparing the peak response. * $p < 0.05$

4.4.2 Role of prostaglandins in spontaneous afferent activity

In agreement with indomethacin-containing experiments, the baseline activity of $Na_v1.9$ -/- colonic nerves was significantly lower than +/+ mice in indomethacin-free experiments ($Na_v1.9$ +/+ 14.3 ± 4.2 vs. $Na_v1.9$ -/- 2.8 ± 0.6 Hz; n=7; $p<0.05$. Figure 32a). In addition, within each group, inclusion of indomethacin in the Krebs buffer significantly reduced baseline activity ($Na_v1.9$ +/+: indomethacin-free 14.3 ± 4.2 vs. indomethacin-containing 3.1 ± 0.6 Hz; n=7-34; $p<0.001$. $Na_v1.9$ -/-: indomethacin-free 2.8 ± 0.6 vs. indomethacin-containing 1.1 ± 0.2 Hz; n=7-26; $p<0.01$. Figure 32b).

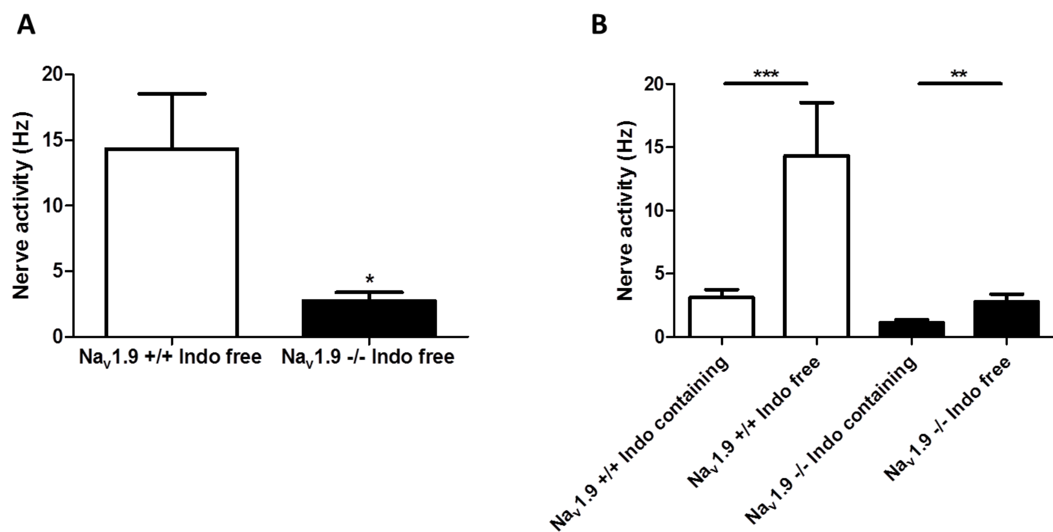


Figure 32 The role of indomethacin in baseline activity. (a) histogram comparing the baseline activity of colonic nerves in indomethacin-free preparations. (b) histogram comparing the baseline activity of colonic nerves in indomethacin-containing and indomethacin-free preparations. ** $p < 0.01$ *** $p < 0.001$

4.4.3 Role of prostaglandins in the response to bradykinin

In agreement with experiments containing indomethacin, the response to bradykinin was significantly greater in $Na_v1.9$ +/+ compared to -/- mice. However, in contrast with the indomethacin-containing experiments, the response was not eliminated in nerves from $Na_v1.9$ -/- mice (0.3 µM: $Na_v1.9$ +/+ 16.6 ± 4.1 vs. $Na_v1.9$ -/- 5.6 ± 1.7 Hz; $p<0.05$. 1 µM: $Na_v1.9$ +/+ 18.2 ± 4.0 vs. $Na_v1.9$ -/- 6.4 ± 1.8 Hz; $p=0.02$. 3 µM: $Na_v1.9$ +/+ 20.2 ± 5.2 vs. $Na_v1.9$ -/- 7.7 ± 1.3 Hz; $p<0.05$; n=7. Figure 33d). The overall response to 3 µM bradykinin was significantly different between $Na_v1.9$ +/+ and -/- mice in indomethacin-free experiments (two-way ANOVA $p<0.001$. Figure 33c). The duration of the response was not significantly different between the $Na_v1.9$ +/+ and -/- mice but the initial reduction in activity after the peak was steeper in -/- mice (Figure 34).

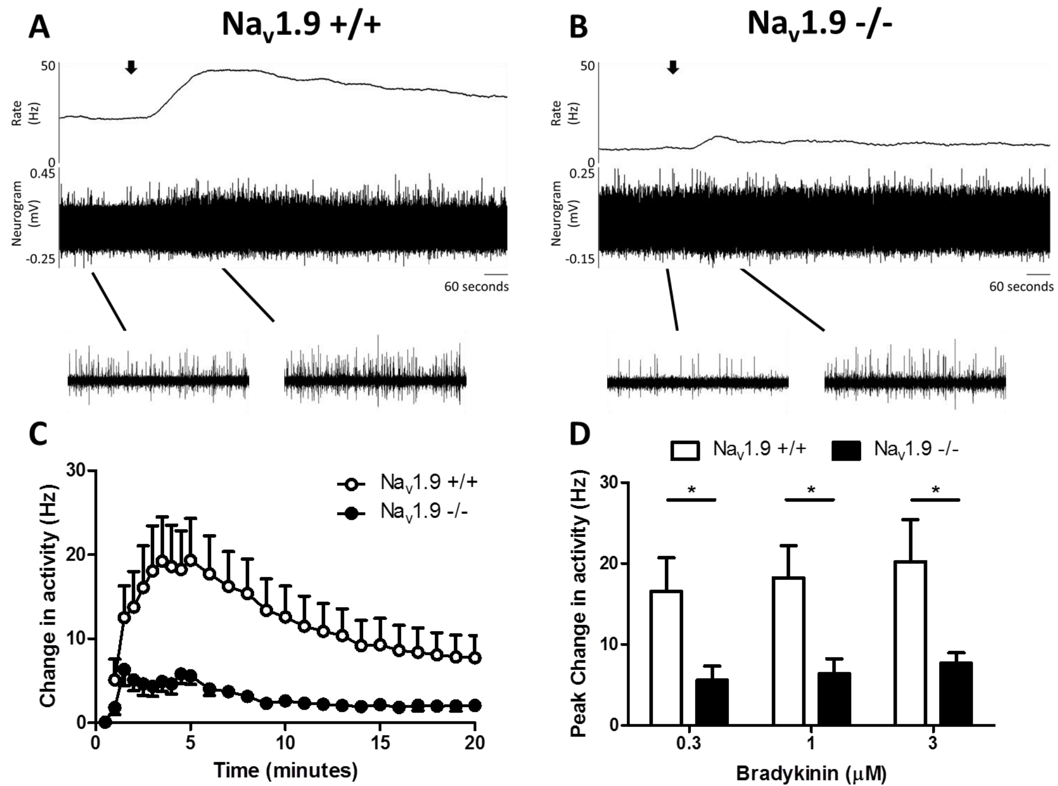


Figure 33 The effect of bradykinin on colonic nerve activity in indomethacin-free preparations. Representative raw trace recordings and response rate (mean frequency over 60 seconds) from (a) $Na_V1.9$ +/+ and (b) $Na_V1.9$ -/- mice after addition of 3 µM bradykinin. The arrows indicate the addition of bradykinin. Below the raw trace, 2 second extracts are shown before and after the addition of bradykinin. (c) Average profile of response to 3 µM bradykinin (two-way ANOVA $p<0.001$). (d) Histogram comparing the peak response to three concentrations of bradykinin. * $p < 0.05$

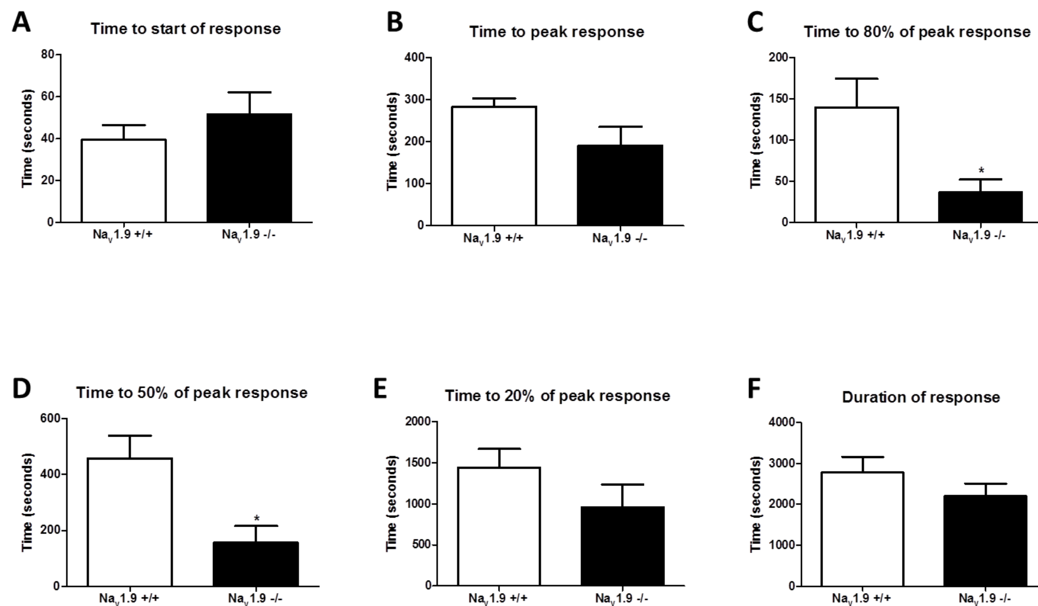


Figure 34 The duration of the colonic nerve response to 3 µM bradykinin in indomethacin-free preparations. (a) time from bradykinin entering the bath to nerve activity rising above baseline ($Na_V1.9$ +/+ 39.4 ± 6.9 vs. $Na_V1.9$ -/- 51.7 ± 10.4 seconds; n=7; p=0.35). (b) time from bradykinin entering the bath to peak response ($Na_V1.9$ +/+ 283.7 ± 19.4 vs. $Na_V1.9$ -/- 190.4 ± 45.7 seconds; n=7; p=0.08). (c) time from peak response to 80% of the peak response ($Na_V1.9$ +/+ 140.0 ± 34.5 vs. $Na_V1.9$ -/- 37.1 ± 15.6 seconds; n=7; p<0.05). (d) time from peak response to 50% of the peak response ($Na_V1.9$ +/+ 458.0 ± 81.6 vs. $Na_V1.9$ -/- 157.4 ± 59.6 seconds; n=7; p<0.05). (e) time from peak response to 20% of the peak response ($Na_V1.9$ +/+ 1443.0 ± 224.2 vs. $Na_V1.9$ -/- 961.0 ± 271.8 seconds; n=7; p=0.20). (f) time from bradykinin entering the bath to nerve activity returning to baseline ($Na_V1.9$ +/+ 2776.0 ± 387.8 vs. $Na_V1.9$ -/- 2200.0 ± 309.4 seconds; n=5-6; p=0.27). * $p < 0.05$

4.5 Specific discussion

4.5.1 The role of $Na_v1.9$ in the activation of colonic afferents by Prostaglandin E_2

The data generated demonstrate an important role for $Na_v1.9$ in the response of visceral afferents to PGE_2. The peak response of colonic afferents to PGE_2 was reduced by nearly 50% in $Na_v1.9$ -/- mice.

These data are consistent with findings from electrophysiological and behavioural studies. PGE_2 has been shown to increase $Na_v1.9$ current in patch clamp experiments (Rush and Waxman 2004; Li and Schild 2007). In addition, while two studies did not show an increase in afferent activity in response to PGE_2, (Brunsden and Grundy 1999; Maubach and Grundy 1999), a third study from the same group did (Haupt *et al.* 2000). One reason that may account for the difference between the three studies is that, in the two studies that did not find an effect of PGE_2 on afferent activity, the mesentery was detached from the jejunum while in the third study this did not occur. Two behavioural studies have investigated the role of $Na_v1.9$ in the response of somatic afferents to PGE_2. Injection of PGE_2 into the hind paw of mice produces mechanical allodynia to probing with von Frey hairs and thermal hyperalgesia during a hot plate test. In $Na_v1.9$ -/- mice, the mechanical allodynia was attenuated while the thermal hyperalgesia was absent (Priest *et al.* 2005; Amaya *et al.* 2006). In addition, extracellular recordings of bladder afferents show that the increase in activity caused by instillation of PGE_2 into the bladder is completely absent in $Na_v1.9$ -/- mice (Ritter *et al.* 2009).

Prostaglandin E_2 produces its effects by binding to GPCRs termed $EP_{1\text{-}4}$ (Woodward *et al.* 2011). The effect of PGE_2 on visceral afferent activity has been shown to be mainly mediated via EP_1 (Haupt *et al.* 2000). Although the signalling pathways of EP_1 are not fully understood, it is known that EP_1 couples to G_q which ultimately activates

PKC (Woodward *et al.* 2011). The $Na_v1.9$ current has been shown to be up regulated via a number of second messenger pathways including PKC (Baker 2005). It is thus likely that the modulation of $Na_v1.9$ current by PGE_2 occurs via this EP_1/PKC mechanism.

4.5.2 The role of prostaglandins in the spontaneous activity of colonic afferents

The data generated demonstrate that prostaglandins play a significant role in the spontaneous activity of colonic afferents. In the presence of indomethacin, a non-specific COX blocker, spontaneous colonic afferent activity was reduced by 60 - 80% in both $Na_v1.9$ +/+ and -/- mice.

These data are consistent with that of Maubach and Grundy (1999) who demonstrated a near 60% reduction in spontaneous activity in jejunal mesenteric afferents after application of naproxen, a non-specific COX blocker. The effect of COX blockade on spontaneous afferent activity in $Na_v1.9$ -/- has not been investigated before but patch clamp studies from both dorsal root and nodose ganglia have demonstrated an increase in $Na_v1.9$ current upon application of PGE_2 (Rush and Waxman 2004; Li and Schild 2007).

It is unclear whether the higher level of spontaneous activity in the absence of COX blockade is due to the physiological prostanoid tone of the tissue which would have been present *in vivo* or whether this is a result of the dissection and transfer of tissue to the recording chamber with the inevitable ensuing inflammatory reaction. To exclude the influence of prostaglandins on the $Na_v1.9$ current, indomethacin was added to the Krebs buffer for all preparations except where the role of COX blockade was under investigation.

4.5.3 The role of $Na_v1.9$ and prostaglandins in the activation of colonic afferents by bradykinin

The data generated demonstrate an important role for prostaglandins in the response of colonic afferents to bradykinin in both $Na_v1.9$ +/+ and -/- mice. In indomethacin containing preparations, the current experiments show a 90% reduction in the peak response to bradykinin in $Na_v1.9$ -/- mice. In the absence of COX blockade, the peak responses to bradykinin were reduced by approximately 60% in $Na_v1.9$ -/- mice.

Maubach and Grundy (1999) have demonstrated a reduction in the bradykinin response in the presence of naproxen, a non-specific COX blocker. The subsequent addition of PGE_2 restored the bradykinin response close to its pre naproxen levels. This effect was more evident at the higher concentrations of bradykinin (up to 10μM). A study on rat DRG has shown that, in the absence of COX blockade, the presence of PGE_2 significantly increases the proportion of DRG that are responsive to bradykinin (Stucky *et al.* 1996). Data from both studies suggest that PGE_2 causes a leftward shift of the stimulus-response curve and that the major part of the response at the higher concentrations was dependant on prostaglandins. This is confirmed by data presented here. The stimulus-response curve was flattened in the presence of COX blockade but not in indomethacin free experiments. This was even more evident in experiments in $Na_v1.9$ -/- mice where the bradykinin response is virtually abolished in the absence of prostaglandins but shows a clear stimulus-response pattern in the presence of prostaglandins.

Bradykinin exerts its effects partly through PKC which is known to enhance the $Na_v1.9$ current (Baker 2005; Leeb-Lundberg *et al.* 2005). In addition, one of the PGE_2 receptors, EP_1, couples to G_q which activates the production of PKC (Woodward *et al.* 2011). It is thus possible that the two pronged production of PKC causes an increase in the activation of visceral afferents in a synergistic way. An alternative is that PGE_2 acts to sensitise the afferents to stimulation by bradykinin. In a sense, the afferents

are primed by PGE_2 and further activation by a second mediator causes an enhanced response compared to single mediator activation. While experiments presented here did not explore which of the prostaglandins were responsible for the enhanced response to bradykinin, data generated by other laboratories and discussed above suggest that PGE_2 is a major candidate. It is interesting to note that while the effect of bradykinin on afferent activation is significantly reduced in $Na_v1.9$ -/-, its combination with prostaglandins is not completely blocked by knockout of $Na_v1.9$. This multi-pronged activation of afferents may also be conducted via other channels, e.g. TRPV1, which at high enough stimulation are able to change the membrane potential to overcome the lack of contribution of $Na_v1.9$.

5

The role of $Na_V1.9$ in the response of visceral afferents to distension

5.1 Aims

The aims of the experiments discussed in this chapter are:

1. To investigate the role of $Na_V1.9$ in the response of visceral afferents to luminal distension of the gastrointestinal tract
2. To investigate the role of prostaglandins in the response of visceral afferents to luminal distension in $Na_V1.9$ +/+ and -/- mice

5.2 Specific rationale

Due to the widespread expression of mechanosensitive channels on nociceptive afferents, it is probable that there is considerable co-expression between these channels and $Na_v1.9$. Loss of $Na_v1.9$ is likely to reduce the response of visceral afferents to mechanical stimulation due to its low activation threshold and contribution to resting membrane potential. Surprisingly, an *in vivo* study of $Na_v1.9$ -/- mice did not show a reduction in the visceromotor response to colorectal distension (Martinez and Melgar 2008). The experiments in this chapter investigated the effect that loss of $Na_v1.9$ has on the response of visceral afferents to luminal distension *in vitro*.

5.2.1 Classification of colonic mechanoreceptors

Colonic distension is a recognised cause of visceral pain. It has been known for a long time that people suffering from both IBS and IBD have a reduced threshold to feeling pain caused by distension as well as an increased response at each distension pressure (Ritchie 1973; Farthing and Lennard-jones 1978). In rodents, the inflation of a balloon inserted via the anus has been used to induce visceral pain for many years. Inflation of the balloon induces contraction of the abdominal muscles, known as the visceromotor response, which is correlated with the intensity of the stimulus (Ness and Gebhart 1988). This model of visceral pain, as well as the application of mechanical stimuli (e.g. von Frey hairs or circumferential stretching of the colon) during electrophysiological recordings, has enabled the characterisation of mechanosensitive visceral afferents.

In the rat, only 16% of pelvic afferents respond to colorectal distension. Two thirds of these fibres respond to an average pressure of 3 mmHg and were classified as low threshold mechanoreceptors while the rest respond to an average pressure of 33 mmHg and were classified as high threshold receptors (Sengupta and Gebhart 1994).

Similar ratios of low to high threshold afferents are found in the splanchnic nerves of the cat colon (2:1) and rat stomach (3:1) (Blumberg *et al.* 1983; Ozaki and Gebhart 2001). The response of the low threshold afferents is greater than the high threshold afferents at all distension pressures (Sengupta and Gebhart 1994; Ozaki and Gebhart 2001).

In addition to colorectal distension studies, investigation of the mechanoreceptors of the colon and rectum have been conducted using a flat sheet preparation (Lynn and Blackshaw 1999). This allows a number of stimuli to be applied to the colon (e.g. probing or circular stretch). Using this technique, five classes of mechanoreceptors have been identified in mice (Brierley *et al.* 2004). Three classes are common to the lumbar splanchnic and pelvic nerves. Each nerve also contains a 4th unique class of mechanoreceptors (Table 2).

Nerve	Class of afferent	Prevalence	Probing	Mucosal stroking	Circular stretch
LSN	Mucosal	4%	✓	✓	
	Muscular	10%	✓		✓
	Serosal	36%	✓		
	Mesenteric	50%	✓		
PN	Mucosal	23%	✓	✓	
	Muscular/Mucosal	23%	✓	✓	✓
	Muscular	21%	✓		✓
	Serosal	33%	✓		

Table 3 Classes of mouse mechanoreceptors and the stimuli that activate them. LSN lumbar splanchnic nerve, PN pelvic nerve

As can be seen in Table 2, the proportion of serosal afferents is consistent between the two groups. In the pelvic nerve, the other afferents are equally divided between the 3 classes of afferents whereas in the splanchnic nerve, mesenteric afferents account for half of the total. A significantly higher proportion of afferents are mucosal in the pelvic nerve possibly reflecting the necessary sensitivity of the lower part of the colon and of the rectum to the passage of faecal matter. Pelvic afferents are also more responsive to mechanical stimuli in that they respond at lower thresholds, to lower intensity stimuli and to a greater extent than splanchnic afferents (Brierley *et al.* 2004). A study which measured the visceromotor response to colorectal distension in mice using electromyogram recordings of the abdominal wall muscles concluded that the response is transmitted solely via the pelvic nerve (Kyloh *et al.* 2011). The results from that study concur with data showing that only 10% of splanchnic afferents respond to circular stretch (Brierley *et al.* 2004). These studies highlight the possible specialisation of afferents depending on where they terminate both in the gut and the spinal cord.

There is evidence from both rat and mouse that mechanoreceptors also respond to chemical mediators including bradykinin, ATP, capsaicin and an inflammatory soup containing bradykinin, PGE_2, serotonin and histamine (Sengupta and Gebhart 1994; Su and Gebhart 1998; Brierley *et al.* 2005a; Brierley *et al.* 2005b). The response of rodent splanchnic afferents to distension is sensitised by the application of bradykinin alone or as part of the inflammatory soup (Su and Gebhart 1998; Brierley *et al.* 2005b). On the other hand, application of capsaicin causes desensitisation of the afferents to distension (Brierley *et al.* 2005a). No sensitisation or desensitisation is seen in pelvic afferents. These results, along with data confirming the responsiveness of colonic afferents to heat, highlight the polymodal nature of visceral afferents.

5.2.2 Mechanical transduction channels

A number of channels have been implicated in the transduction of mechanical stimuli into action potentials. These include members of the transient receptor potential (TRP) and ASIC families (Brierley 2010). The effect of elimination of these channels on response to mechanical stimuli is considered in this section.

TRPV1 is expressed on 50% of pelvic and 82% of splanchnic afferents (Robinson *et al.* 2004; Brierley *et al.* 2005a). Deletion of TRPV1 reduces the response of pelvic muscular/mucosal afferents and leads to a decreased visceromotor response to colorectal distension (Jones *et al.* 2005). TRPV4 is expressed on around 60% of colonic afferents (Brierley *et al.* 2008). Knock-out of TRPV4 leads to decreased responses and increased thresholds of splanchnic serosal and mesenteric afferents as well as pelvic serosal afferents. This reduction in response translates into reduced abdominal electromyography in response to colorectal distension (Brierley *et al.* 2008). In addition, application of a TRPV4 agonist in wild-type mice sensitised splanchnic and pelvic responses to mechanical stimulation (Brierley *et al.* 2008). TRPA1 is also expressed on over half of colonic afferents (Brierley *et al.* 2009). Mirroring TRPV4, knock-out of TRPA1 leads to reduced responses and increased mechanical thresholds of splanchnic serosal and mesenteric afferents as well as pelvic serosal afferent which also translates into reduced abdominal electromyography in response to colorectal distension. In addition, application of a TRPA1 agonist in wild-type mice sensitised splanchnic and pelvic responses to mechanical stimulation which was more pronounced in mice suffering from TNBS colitis (Brierley *et al.* 2009). While each channel seems to have an independent and significant role in visceral mechanotransduction, there is substantial interplay between channels as is highlighted by the lack of the usual mechanical desensitisation after capsaicin in TRPA1 -/- mice (Brierley *et al.* 2009). This interaction is enabled by the close co-expression of these channels with 97% of TRPA1 expressing DRG neurons also expressing TRPV1 (Story *et al.* 2003).

Amongst the ASICs, ASIC3 seems to play a role similar to the TRP channels. It is the most abundant of the ASICs and is expressed on 73% of retrogradely labelled colonic afferents (Hughes *et al.* 2007). Deletion of ASIC3 leads to significantly reduced responses in splanchnic serosal and mesenteric afferents as well as pelvic muscular/mucosal afferents (Jones *et al.* 2005; Page *et al.* 2005). *In vivo*, knock-out of ASIC3 leads to a reduced visceromotor response to colorectal distension (Jones *et al.* 2005). In contrast to ASIC3, other members of the ASIC family seem to have a dampening effect on the transduction of mechanical stimuli in the gut: elimination of these channels tends to increase the sensitivity of afferents. ASIC1 is expressed on 30% of colonic afferents and its deletion increases the response of splanchnic mesenteric and serosal afferents to probing without altering their thresholds (Page *et al.* 2005; Hughes *et al.* 2007). ASIC2 is expressed on 47% of colonic afferents (Hughes *et al.* 2007). Its knock-out has variable effects depending on the class of afferents: the response of serosal afferents was increased whereas mesenteric afferents were unaffected. The thresholds of neither class of afferents were influenced by deletion of ASIC2 (Page *et al.* 2005). The contrasting effect of elimination of ASICs suggests that rather than functioning as individual mechanically gated ion channels, they operate together and with other mechanotransducers to exert their effects (Brierley 2010).

5.2.3 Colorectal distension and Na_V1.9

Because of the wide expression of TRPV1, TRPV4, TRPA1 and ASIC3, it is likely that there is considerable co-expression between these channels and Na_V1.9. Indeed, over half of TRPV1 expressing neurons also express Na_V1.9 (Amaya *et al.* 2000; Amaya *et al.* 2006; Padilla *et al.* 2007). Due to its low activation threshold and persistent, slow current, it is likely that loss of Na_V1.9 reduces the response of visceral afferents to mechanical stimulation. Surprisingly, an *in vivo* study of Na_V1.9 knock-out mice did not show a reduction in the visceromotor response to colorectal distension to 60 mmHg (Martinez and Melgar 2008). The authors used the pressure difference between the pressure generator used to inflate the balloon and the

pressure transducer as a surrogate measure for the visceromotor response and did not make electromyographic recordings. To accurately investigate a possible role for $Na_v1.9$ in the response of visceral afferents to distension of a segment of GI tract, I studied the electrophysiological output of the splanchnic nerves in $Na_v1.9$ wild type and knockout mice during distension to 60 (intestine) or 80 mmHg (colon).

5.3 Experimental protocols

The segment of intestine or colon was distended by occluding the outflow cannula. Due to the laxity of intestinal tissue, the maximum intraluminal pressure that could be consistently achieved was 60 mmHg whereas pressures of 80 mmHg were achieved in colonic tissue.

5.3.1 Colonic experiments

Because of the low spontaneous activity of colonic nerves, ramp distension to 80 mmHg was undertaken at the beginning of each recording to confirm the establishment of a recording.

5.3.2 Intestinal experiments

In a subset of the capsaicin experiments, ramp distension to 60 mmHg was undertaken at the beginning of the recording.

5.4 Results

5.4.1 Response to intraluminal distension

In intestinal nerves, there was a trend for a lower response in $Na_v1.9$ -/- mice (at 20 mmHg: $Na_v1.9$ +/+ 47.3 ± 15.9 vs. $Na_v1.9$ -/- 18.8 ± 5.7 Hz; n=5-6; p=0.15, at 40 mmHg: $Na_v1.9$ +/+ 68.3 ± 19.9 vs. $Na_v1.9$ -/- 27.0 ± 10.3 Hz; n=5-6; p=0.12 and 60 mmHg: $Na_v1.9$ +/+ 82.4 ± 28.0 vs. $Na_v1.9$ -/- 55.6 ± 19.0 Hz; n=5; p=0.45. Figure 35d). The overall response was significantly different between $Na_v1.9$ +/+ and -/- mice (two-way ANOVA; p<0.001. Figure 35c).

In colonic nerves, there was a significantly lower response in $Na_v1.9$ -/- mice (at 20 mmHg: $Na_v1.9$ +/+ 2.1 ± 0.6 vs. $Na_v1.9$ -/- 0.5 ± 0.2 Hz; n=17-26; p<0.05, at 40 mmHg: $Na_v1.9$ +/+ 3.4 ± 1.1 vs. $Na_v1.9$ -/- 0.8 ± 0.4 Hz; n=17-26; p=0.08, at 60 mmHg: $Na_v1.9$ +/+ 4.6 ± 0.9 vs. $Na_v1.9$ -/- 1.5 ± 0.4 Hz; n=17-26; p<0.05 and at 80 mmHg: $Na_v1.9$ +/+ 5.3 ± 0.9 Hz vs. $Na_v1.9$ -/- 1.8 ± 0.5 Hz; n=17-25; p<0.01. Figure 36d). The overall response was also significantly different between $Na_v1.9$ +/+ and -/- mice (two-way ANOVA; p<0.001. Figure 36c).

Single unit analysis of the response of colonic afferents to intraluminal distension revealed that a similar proportion of units from $Na_v1.9$ +/+ and -/- mice responded to distension ($Na_v1.9$ +/+ 24/36 (66.7%) vs. $Na_v1.9$ -/- 11/17 (64.7%); n=5-6). Of those distension sensitive units from $Na_v1.9$ -/- mice, the majority did not respond to application of 0.3µM bradykinin. This is contrast to the distension sensitive units from $Na_v1.9$ +/+ mice where the majority also responded to bradykinin ($Na_v1.9$ +/+ 2/24 (8.3%) vs. $Na_v1.9$ -/- 9/11 (81.8%); n=5-6).

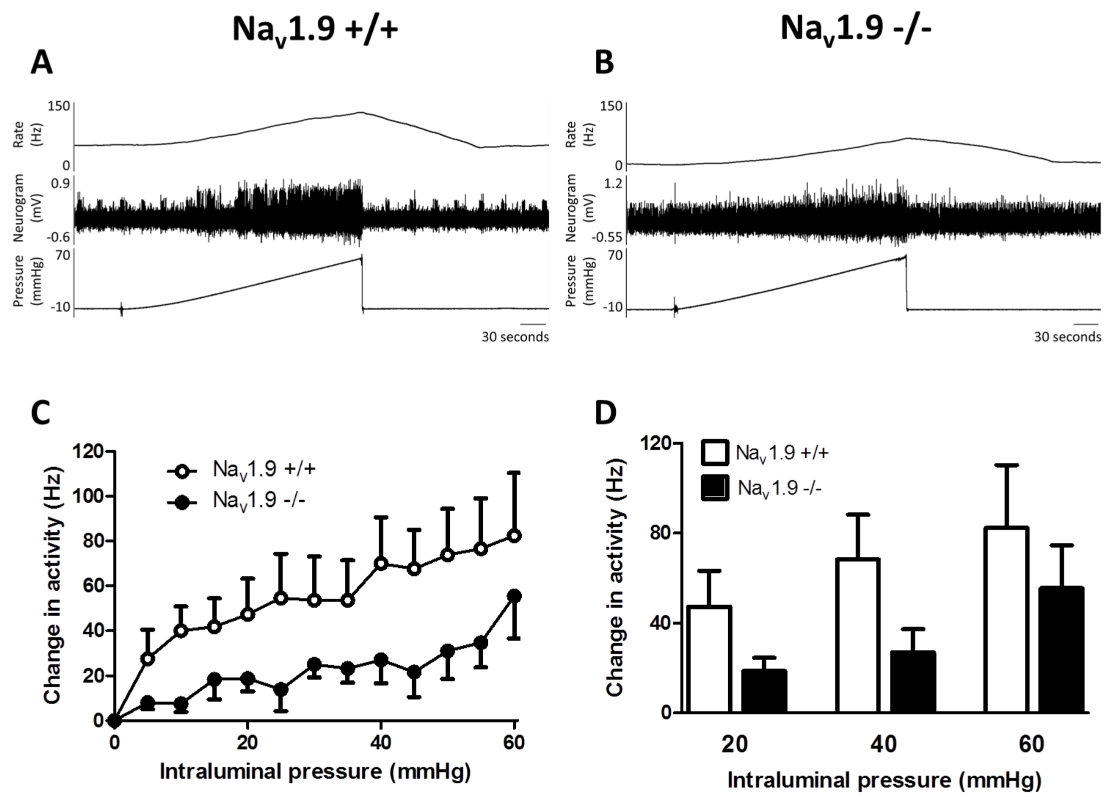


Figure 35 The effect of intraluminal distension to 60 mmHg on intestinal nerve activity. Representative raw trace recordings and response rate (mean frequency over 60 seconds) from (a) $Na_V1.9$ +/+ and (b) $Na_V1.9$ -/- mice. (c) Average profile of response (two-way ANOVA $p<0.001$). (d) Histogram comparing the responses at 20, 40 and 60 mmHg.

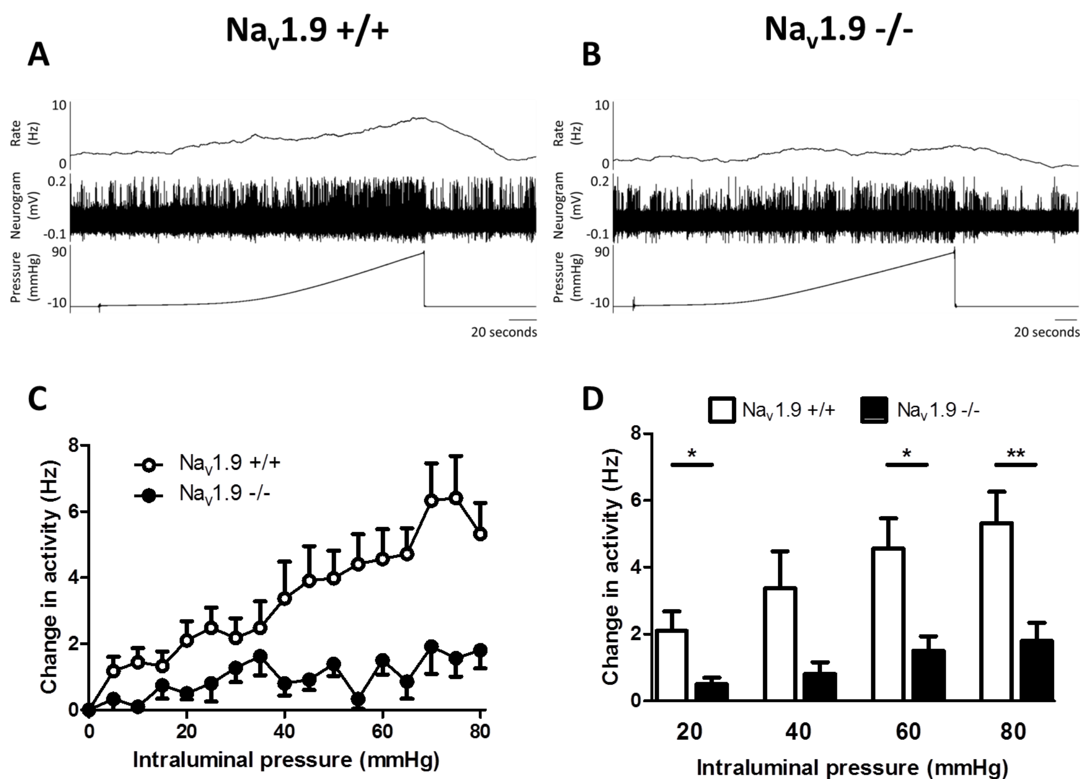


Figure 36 The effect of intraluminal distension to 80 mmHg on colonic nerve activity. Representative raw trace recordings and response rate (mean frequency over 60 seconds) from (a) $Na_v1.9$ +/+ and (b) $Na_v1.9$ -/- mice. (c) Average profile of response (two-way ANOVA $p<0.001$). (d) Histogram comparing the response at 20, 40, 60 and 80 mmHg. * $p < 0.05$ ** $p < 0.01$

5.4.2 Role of prostaglandins in the response to intraluminal distension

There was a trend for a lower response of $Na_v1.9$ -/- colonic nerves to intraluminal distension to 80 mmHg as compared to Nav1.9 +/+ in indomethacin-free experiments (at 80 mmHg $Na_v1.9$ +/+ 20.4 ± 4.2 vs. $Na_v1.9$ -/- 9.9 ± 3.3 Hz; n=7; p=0.07. Figure 37d). The overall responses were significantly different (two-way ANOVA p<0.001. Figure 37c). In addition, the distension responses were significantly higher in indomethacin-free compared to indomethacin-containing experiments (two-way ANOVA: $Na_v1.9$ +/+ p<0.001; $Na_v1.9$ -/- p<0.001. Figure 38).

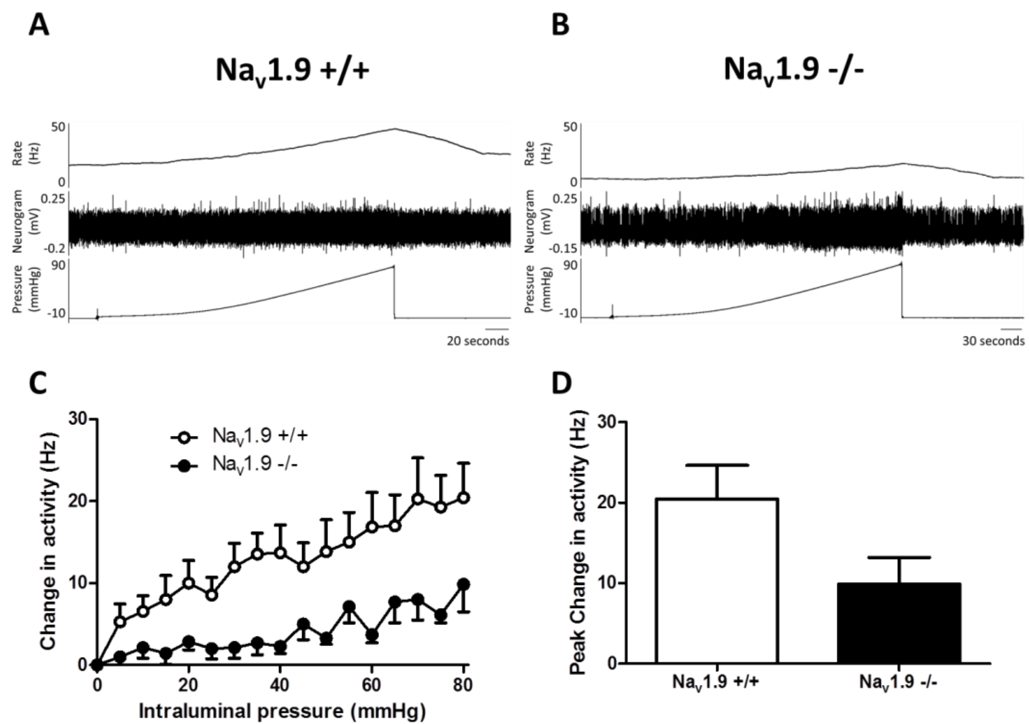


Figure 37 The effect of intraluminal distension to 80 mmHg on colonic nerve activity in indomethacin-free preparations. Representative raw trace recordings and response rate (mean frequency over 60 seconds) from (a) $Na_v1.9$ +/+ and (b) $Na_v1.9$ -/- mice. (c) Average profile of response (two-way ANOVA $p<0.001$). (d) Histogram comparing the response at 80 mmHg.

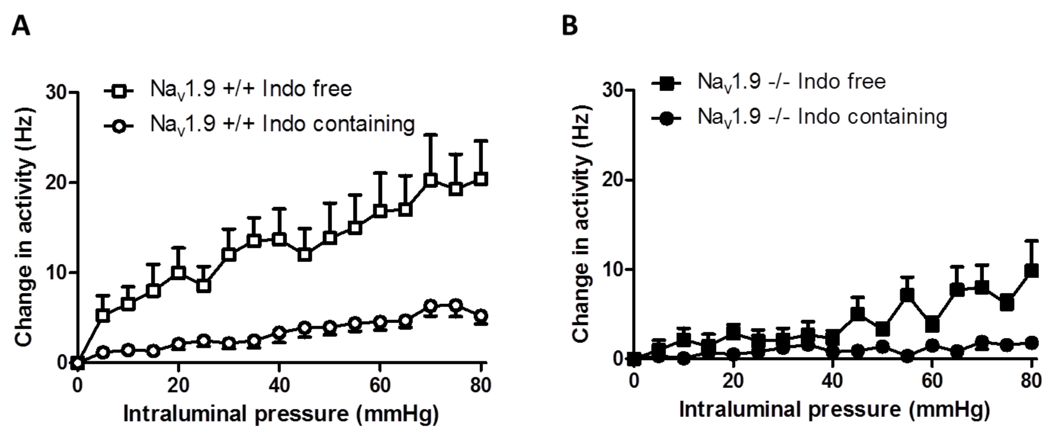


Figure 38 The role of indomethacin in the response of colonic nerves to intraluminal distension in (a) $Na_v1.9$ +/+ (two-way ANOVA $p<0.001$) and (b) $Na_v1.9$ -/- (two-way ANOVA $p<0.001$) mice.

5.5 Specific discussion

5.5.1 The role of $Na_v1.9$ in the activation of visceral afferents by distension

The data generated demonstrate an important role for $Na_v1.9$ in the response of visceral afferents to intraluminal distension. The overall response of splanchnic afferents was significantly attenuated in $Na_v1.9$ -/- compared to +/+ mice. It was approximately 75% lower at 20 and 40 mmHg and 65% lower at 60 and 80 mmHg. The overall response of intestinal afferents was also significantly attenuated in $Na_v1.9$ -/- compared to +/+ mice albeit with smaller differences between the genotype. It was approximately 60% lower at 20 and 40 mmHg and 30% lower at 60 mmHg. Single unit analysis of the response of colonic afferents to intraluminal distension revealed that a similar proportion of units from $Na_v1.9$ +/+ and -/- mice responded to distension. The vast majority of these distension sensitive units from $Na_v1.9$ +/+ mice also responded to application of bradykinin whereas only a small minority of units from $Na_v1.9$ -/- mice did so.

These findings are in contrast to those in the literature. A behavioural study conducted in $Na_v1.9$ +/+ and -/- mice found no difference in the visceromotor response to colorectal distension (Martinez and Melgar 2008). The authors inserted a 2 cm long balloon 5 mm from the anus, inflated it to 60 mmHg and measured the consequent visceromotor response. No difference was detected between $Na_v1.9$ -/- and +/+ mice. A possible explanation of this contradiction is provided by a study into the pathways activated by colorectal distension (Kyloh *et al.* 2011). The authors inserted an 8 mm balloon 4 - 7 mm from the anus, inflated it to 100 mmHg and obtained EMG recordings from the abdominal wall muscles. The authors then made sequential lesions in the splanchnic and pelvic afferent pathways and demonstrated that the whole of the colorectal distension response is transmitted via pelvic afferents. As my recordings are in splanchnic afferents, they are not expected to be a major contributor to the colorectal distension response measured by Martinez and Melgar (2008). It is thus possible that knockout of $Na_v1.9$ has different effects on the

two colonic afferent populations, splanchnic and pelvic: there is no change in the response of pelvic afferents whereas the response of splanchnic afferents is significantly reduced.

The main mechanotransduction channels in visceral afferents are TRPV1, TRPV4, TRPA1 and ASIC3. Studies of back labelled visceral DRG showed that approximately 82% of spinal, 50% of pelvic and 32% of vagal DRG express TRPV1 (Robinson *et al.* 2004; Brierley *et al.* 2005a; Tan *et al.* 2009). In addition, over half of colonic afferents express TRPV4, TRPA1 and ASIC3 (Hughes *et al.* 2007; Brierley *et al.* 2008; Cenac *et al.* 2008; Brierley *et al.* 2009). As these receptors are expressed on a large proportion of visceral afferents, it is likely that many afferents will express a number of these channels. In fact, data from back labelled rat stomach afferents showed that 80% of TRPA1 expressing NG and DRG also express TRPV1 (Kondo *et al.* 2009). Due to the lack of studies investigating he expression of $Na_v1.9$ in visceral afferents, there are no data on the co-expression of $Na_v1.9$ and these channels. Data from the somatic system reveal that approximately 55% of TRPV1 expressing neurons co-express $Na_v1.9$ (Amaya *et al.* 2000; Amaya *et al.* 2006). It is thus not unreasonable to postulate that there is close co-expression of $Na_v1.9$ and these mechanotransducers.

Intestinal nerves contain a mixed population of vagal and spinal afferents but the proportion of each population is not known. Vagal afferents are known to have lower mechanical thresholds in contrast to spinal and pelvic afferents (Knowles and Aziz 2009). The data presented here showed a significant attenuation of the mechanical responses across both vagal and spinal pathways. The nature of multi-unit recordings did not allow the investigation of the role of $Na_v1.9$ on separate populations of low and high threshold afferents.

While $Na_v1.9$ is not a transducer of mechanical stimuli, data presented here clearly demonstrate a role for $Na_v1.9$ in the mechanical responsiveness of visceral afferents. This is likely to be a result of the biophysical properties exhibited by $Na_v1.9$. Lack of

the $Na_v1.9$ current means that a stronger stimulus is needed to raise the membrane potential to the threshold required for action potential generation. Thus, $Na_v1.9$ acts as an amplifier of other stimuli.

5.5.2 The role of $Na_v1.9$ and prostaglandins in the activation of colonic afferents by distension

The data generated demonstrate an important role for prostaglandins in the response of colonic afferents to intraluminal distension in both $Na_v1.9$ +/+ and -/- mice. There is an approximately 75% attenuation of the response at 80 mmHg in the absence of indomethacin, a non-specific COX blocker, across both genotypes.

It is known that TRPV1 and TRPV4 currents are modulated via PKC (Vellani *et al.* 2001; Alessandri-Haber *et al.* 2006). In the absence of indomethacin, tissue prostaglandins bind to their GPCRs which lead to the formation of PKC amongst other messengers. Thus it is likely, that these endogenous prostaglandins sensitise the afferents to mechanical transduction via PKC. In a similar manner to the synergistic activation of afferents by bradykinin and prostaglandins, while the response of visceral afferent to intraluminal distension is significantly reduced in $Na_v1.9$ -/-, the combination of endogenous prostaglandins and distension is not completely blocked by knockout of $Na_v1.9$.

6

Discussion

The data presented here demonstrate an essential role for $Na_v1.9$ in the activation of visceral afferents by chemical and mechanical stimuli as well as a human inflammatory supernatant.

6.1 Methodological considerations

To investigate whether $Na_v1.9$ plays a role in the response of visceral afferents to various stimuli, a colony of global $Na_v1.9$ knockout mice was used which raises the possibility of compensation for this loss by other sodium channels. This is very unlikely. $Na_v1.9$ expresses a unique slow, persistent tetrodotoxin-resistant current. No other voltage-gated sodium channel is able to express this current. In addition, there is no appreciable change in the expression or current of the other sodium channels in $Na_v1.9$ -/- mice (Priest *et al.* 2005; Amaya *et al.* 2006). Nevertheless, an alternative would have been to use either a conditional knockout colony or siRNA to eliminate $Na_v1.9$ from the tissue of interest. Both of these techniques require specific technical skills which are not available in our laboratory.

Data were acquired using an extracellular multiunit recording protocol which allowed measurement of the overall response in all classes of afferents that were activated by each test stimulus. An alternative would have been to make single unit recordings as described by the Blackshaw laboratory (Lynn and Blackshaw 1999). An advantage of the Blackshaw technique is that it allows detailed information about the effect of a stimulus on a single neuronal unit to be investigated, e.g. if the overall response to a mediator is decreased then the technique would permit the investigation into whether all units responded at a lower level or whether some units responded at the 'normal' level while others did not respond at all. This technique takes considerably longer to acquire the recordings with consequent degradation of tissue. In addition, technical issues such as establishing an adequate seal around the brass rods and damage to the tissue by repeated probing are potential disadvantages of the technique. The main advantage of extracellular multiunit recordings is that they provide a more representative model for the afferent input to the spinal cord. An alternative to both techniques would have been to use patch clamp studies. As the aim of the study was to investigate the role of $Na_V1.9$ at the afferent terminal, the use of patch clamp studies would not have been appropriate.

Other groups have shown that PGE_2 increases the $Na_V1.9$ current (Rush and Waxman 2004; Li and Schild 2007). To prevent the response of visceral afferents to mediators, e.g. bradykinin or capsaicin, being 'influenced' by the presence of prostaglandins, indomethacin was added to the Krebs buffer in all experiments other than those investigating the role of prostaglandins. As can be seen from the results, prostaglandins act to sensitise the afferents to both chemical and mechanical stimuli. What is not clear is whether the prostaglandins present in preparations without indomethacin would have been present *in vivo* or whether they are a function of the inevitable degradation of tissue that occurs in the course of *in vitro* experiments. In a similar fashion, the effects of a number of mediators, e.g. bradykinin and PGE_2, have been shown to be partly associated with muscle contraction (Floyd *et al.* 1977; Haupt *et al.* 2000). To avoid the 'interference' that

both these and spontaneous muscle contractions would cause to the afferent discharge, atropine and nifedipine were added to the Krebs buffer in all experiments. The intraluminal pressure was continuously monitored and confirmed the lack of smooth muscle contraction throughout the experiments including after addition of the mediators of interest. In addition, no contractions were visible during the experiments.

6.2 Summary of results

$Na_v1.9$ has been shown to play an important part in visceral afferent signalling. The elimination of $Na_v1.9$ significantly reduces spontaneous afferent activity in both colon and intestine. This is probably because $Na_v1.9$ is activated at potentials close to the resting membrane potential. When this current is present, it raises the membrane potential closer to the action potential threshold thus allowing more afferents to generate action potentials. $Na_v1.9$ has not been thought to play a role in the response of visceral afferents to mechanical stimulation in the absence of tissue inflammation (Martinez and Melgar 2008). Data presented here demonstrate clear attenuation of the distension response in $Na_v1.9$ -/- mice. An explanation for the contradiction is the different populations of afferents studied as there is evidence that the colorectal distension response is mediated via the pelvic pathway whereas the current recordings were of the splanchnic pathway (Kyloh *et al.* 2011).

The response of colonic and intestinal afferents to bradykinin and capsaicin is significantly attenuated in $Na_v1.9$ -/- mice suggesting that $Na_v1.9$ plays a key role in their signalling pathways. This finding is consistent with data from the somatic system in which $Na_v1.9$ has been shown to have an important role in the primary response of afferents as well as the development of hypersensitivity to mechanical and thermal stimuli (Amaya *et al.* 2006). In addition, the response of colonic afferents to PGE_2 is also significantly attenuated in $Na_v1.9$ -/- mice which is consistent with data from the somatic and visceral systems (Priest *et al.* 2005;

Amaya *et al.* 2006; Ritter *et al.* 2009). The data also demonstrate that prostaglandins play important roles in afferent activity, both spontaneous, and in response to chemical and mechanical stimuli. This two-pronged stimulation of afferents is still significantly attenuated by loss of $Na_v1.9$, albeit to a lesser degree.

Acetic acid has been used for many years as a model for visceral pain. Recent data has been conflicting as to whether $Na_v1.9$ plays a role in the response of visceral afferents to stimulation by acetic acid (Ritter *et al.* 2009; Leo *et al.* 2010). Data presented here demonstrate a complex interaction between acetic acid and $Na_v1.9$. The peak change in response to various concentrations of acetic acid did not differ significantly between $Na_v1.9$ -/- and +/+ mice. In contrast, the total number of action potentials recorded was different at concentrations of 0.1% and 0.3%. At the 0.3% dose, most nerve bundles produced a twin peaked response suggesting two mechanisms are responsible for the effect of acetic acid. The time to the second peak was significantly lower in $Na_v1.9$ -/- mice indicating that the second response is conducted via a signalling pathway that involves $Na_v1.9$.

The activation of visceral afferents by human inflammatory supernatant is a new and exciting finding. I have demonstrated a clear difference in the level of activation of afferents between 'normal' and inflammatory human supernatants. In addition, the response of afferents to the inflammatory supernatants was significantly attenuated in $Na_v1.9$ -/- mice. This finding has great translational potential and should allow for the conduction of subsequent experiments to test human supernatants from a variety of inflammatory conditions.

6.3 $Na_v1.9$ in context

To date, investigation of the role of $Na_v1.9$ has focused on events at the DRG level or on behavioural studies using a knockout colony. This thesis is the first to explore the

role of $Na_v1.9$ at the afferent terminal. Data presented here confirm a significant role for $Na_v1.9$ in visceral afferent activity. $Na_v1.9$ has been shown to have a role in the setting the rate of spontaneous activity as well as the response of the afferents to a host of chemical mediators whether applied in isolation or as part of a more physiological mixture. In addition, $Na_v1.9$ has been shown to play a significant role in the response of the afferents to mechanical stimulation. The response to chemical stimuli was reduced by 50-90% in $Na_v1.9$ knock out mice while the response to intra luminal distension was reduced by 30-75% depending on region of gut and distension pressure. Only the peak response to acetic acid was not different between $Na_v1.9$ wild type and knock out mice. The fact that $Na_v1.9$ has a role in the response of afferents to a variety of stimuli indicates that it is at the heart of signal transduction of afferents. It is likely that $Na_v1.9$ is co-expressed with a number of receptors (e.g. B2) and ion channels (e.g. TRPV1). This allows the channel to impact a variety of signalling pathways. Indeed, it is likely that nearly all B2 expressing visceral afferents co-express $Na_v1.9$ while the majority of TRPV1, TRPV4 and TRPA1 expressing afferents co-express $Na_v1.9$. $Na_v1.9$ is also likely to have a role in second messenger pathways which would explain the difference in the response to the inflammatory human supernatant and the second response to 0.3% acetic. Evidence for the role of $Na_v1.9$ as an amplifier of other stimuli is seen in the afferent response to intra-luminal distension. $Na_v1.9$ is not a transducer of mechanical stimuli. But, as a result of its biophysical properties, the presence of $Na_v1.9$ on an afferent produces a significantly higher response to a given distension pressure. Therefore, the role of $Na_v1.9$ seems to be at the confluence of a variety of signalling pathways. This, along with its restricted distribution make it an ideal target for visceral specific analgesics.

6.4 Further work

The data presented demonstrate a significant role for $Na_v1.9$ in the response of visceral afferents to chemical and mechanical stimuli. Time and resource permitting, the results of further experiments would have complemented the dataset and

allowed a more comprehensive understanding of the magnitude of the role of $Na_v1.9$. Some these further experiments are outlined in this section.

The experiments performed as part of this thesis investigated either splanchnic afferents in isolation or as part of a bundle with vagal afferents. The data showed that the magnitude of effect of $Na_v1.9$ is different between the two pathways. Specific experiments investigating the vagal nerve in isolation as well as the pelvic nerve are necessary to enable a fuller understanding of the contribution of $Na_v1.9$ to signalling of the whole of the GI tract. In addition, single unit studies would add more detail to the contribution of $Na_v1.9$ to each afferent class.

The data presented here was acquired *in vitro*. While this is an appropriate model in the initial investigation of a target, it is necessary to perform *in vivo* and behavioural studies to understand the effect that manipulation of this channel has on the response of the subject to the stimuli being studied. Furthermore, experiments in animals that have been subjected to a disease model, e.g. TNBS colitis, would provide further data on the effect that elimination of this channel may have in man.

The obvious gap in our understanding of $Na_v1.9$ is its expression on visceral afferents. There is an urgent need for immunohistochemical studies of retrogradely labelled visceral afferents which will allow us to better understand the distribution of $Na_v1.9$ as well as its co-localisation with channels that are also involved in the afferent response to noxious stimuli. In addition to these retrograde labelling studies in rodents, experiments should be performed on human colonic tissue and associated nerves to provide an understanding of the potential translatability of the results from rodent to man.

An abstract at the recent meeting of the International Association for the Study of Pain highlighted a possible role for $Na_v1.9$ in erythromelalgia in man (Zhang *et al.* 2012). Two mutations of $Na_v1.9$ were found in patients who described ongoing 'burning', 'pricking' or 'stinging' pain. A search for $Na_v1.9$ loss of function mutations amongst patients who have congenital insensitivity to pain should also be instituted. Should these be found, they would provide more evidence of a role for $Na_v1.9$ in the pathogenesis of pain.

6.5 Summary

Data presented here highlight the hitherto under investigated role for $Na_v1.9$ in visceral afferent signalling. The magnitude of effect that $Na_v1.9$ has on the activation of afferents suggests that a number of signalling pathways converge on $Na_v1.9$ rather than $Na_v1.9$ being one of many pathways that these mediators utilise to exert their effects. $Na_v1.9$ has been shown to be involved in the response to stimuli which act via GPCRs and also through activation of ion channels. A thought provoking explanation is that $Na_v1.9$ acts as an amplifier of these disparate signalling pathways. This would necessitate a fewer number of each receptor to be expressed on a particular afferent thus allowing 'more room' for expression of a variety of receptors on a single afferent. Thus it is the presence of $Na_v1.9$ that allows afferents to be truly polymodal. $Na_v1.9$ seems to have more significant effects on splanchnic rather than vagal afferents. Whether this is due to differing expression patterns or the inherent characteristics of the different pathways is not clear. Thus, a $Na_v1.9$ blocker might be more effective on the splanchnic rather than the vagal afferents. Additionally, the data show that COX blockade enhances the effect of elimination of $Na_v1.9$ on the response of afferents to noxious stimuli. Notwithstanding the side effects of non-specific COX blockade, the data suggest that simultaneous blockade of $Na_v1.9$ and COX could provide for a significant reduction in the activation of afferents by chemical and mechanical stimuli.

7

Conclusion

Using consistent methodology, this thesis has documented an important role for $Na_V1.9$ in the response of visceral afferents to a range of chemical and mechanical stimuli. In addition, $Na_V1.9$ has been shown to play a role in the spontaneous activity of visceral afferents. Most importantly, the activation of visceral afferents by human inflammatory supernatants was significantly attenuated in $Na_V1.9$ -/- mice suggesting that blockade of this channel in man could provide analgesic effects that, due to its restricted peripheral distribution, would not be expected to be associated with the side effect profile of the current non-specific sodium channel blockers.

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