MECHANO AND CHEMOSENSORY FUNCTION OF COLONIC PRIMARY AFFERENT FIBRES IN SPLANCHNIC AND PELVIC PATHWAYS

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Stuart Matthew Brierley
B.Sc (Hons, First Class)

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If the doors of perception were cleansed,

everything would appear to man as it is....

INFINITE

-William Blake-

Publications arising from thesis

i) Peer Reviewed journal articles

1. **Brierley S.M,** Jones III R.C.W, Gebhart G.F & L.A Blackshaw (2004). *Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice.* Gastroenterology, 127(1), 166-178. *(Impact factor:* 12.80)

2. *Page A.J, ***Brierley S.M,** Martin C.M, Martinez-Salgado C, Wemmie J.A, Brennan T.J, Symonds E, Omari T, Lewin G.R, Welsh M.J & L.A Blackshaw (2004). *The ion channel ASIC1 contributes to visceral but not cutaneous mechanoreceptor function.* Gastroenterology, 127, 1739–1747 ***(* Equal first authorship)*** *(Impact factor:* 12.80)

3. *Page A.J, ***Brierley S.M,** Martin C.M, Price M.P, Wemmie J.A, Symonds E, Butler R, & L.A Blackshaw (2005). *Different contributions of ASIC channels 1a, 2 and 3 in gastrointestinal mechanosensory function.* Gut, 54(10) 1408-1415 ***(* Equal first authorship).*** *(Impact factor:* 5.9)

4. **Brierley S.M,** Jones III R.C.W, Xu L, Robinson D.R, Hicks G.A, Gebhart G.F & L.A Blackshaw (2005). *Differential chemosensory function and receptor expression of splanchnic and pelvic colonic afferents in mice.* The Journal of Physiology, 567(1) 267-281.*(Impact factor:* 4.4)

5. **Brierley S.M,** Jones III R.C.W, Xu L, Gebhart G.F & L.A Blackshaw (2005). *Activation of splanchnic and pelvic colonic afferents by bradykinin in mice.* Neurogastroenterology & Motility, 17(6) 854-862. *(Impact factor:* 2.5).

ii) Book Chapters

1. **Brierley S.M,** Coldwell J.R, Cooper N.J, Howarth G.S and Blackshaw L.A. (2002). *Colonic spinal primary afferents: anatomical, histochemical, mechanosensory and chemosensory subtypes and effects of inflammation.* Falk Symposium, 130, 14, 120-125

2. **Brierley S.M,** and Blackshaw L.A. *The neurobiology of visceral nociceptors.* Pasricha, Willis Gebhart: Chronic abdominal and visceral pain: theory and practise. *In press*

iii) Conference proceedings:

1. **Brierley S.M**, Price M.P, Wemmie J.A, Welsh M.J and Blackshaw L.A. (2005). *Varying contributions of ASIC1, 2 & 3 to colonic mechanonociceptor function.* Visceral Pain Satellite of the World Congress on Pain Adelaide. *13*

2. Hughes P, **Brierley S.M,** Cooper N.J, Young R.L & L.A Blackshaw (2005). *Localisation of ASIC3 mRNA in colonic afferents in the mouse.* Visceral Pain Satellite of the World Congress on Pain Adelaide. *21*

3. **Brierley S.M**, Jones R.C.W III, Xu L, Gebhart G.F and Blackshaw L.A (2005). *Bradykinin is a major stimulus for splanchnic but not pelvic colonic serosal afferents.* Neurogastroenterology and Motility. In press.

4. Hughes P, **Brierley S.M,** Cooper N.J, Young R.L & L.A Blackshaw (2005). *Localisation of ASIC3 mRNA in colonic afferents in the mouse.* Neurogastroenterology and Motility. In press.

5. **Brierley S.M**, Price M.P, Wemmie J.A, Welsh M.J and Blackshaw L.A. (2005). *ASIC1, 2 & 3 contribute differently to serosal & mesenteric mechanoreceptor function.* Gastroenterology, 128; 4 Suppl *2;* A-124.

6. **Brierley S.M**, Price M.P, Wemmie J.A, Welsh M.J and Blackshaw L.A. (2003). *Role of ASIC1, 2 & 3 ion channels in colonic splanchnic afferent mechanotransduction.* Neurogastroenterology and Motility. 15, 65-66.

7. Jones C, **Brierley S.M**, Blackshaw L.A and Gebhart G.F (2003). *Mechanosensitive properties of pelvic afferents in the mouse.* Society for Neuroscience.

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9. **Brierley S.M** and Blackshaw L.A (2003). *Mouse colonic primary afferents in the lumbar splanchnic nerve: Mechanosensory and pharmacological subtypes.* Proceedings of the Australian Neuroscience Society. Vol. 14; 37

10. Blackshaw L.A, **Brierley S.M,** Coldwell J.R and Cooper N.J (2002). *Colonic spinal primary afferents: anatomical, histochemical, mechanosensory and chemosensory subtypes and effects of inflammation.* Falk Symposium, 130, P39.

11. **Brierley S.M,** Cooper N.J and Blackshaw L.A. (2002). *Pelvic Colonic Primary Afferents: Mechanosensory and Pharmacological Subtypes.* Gastroenterology, 122(4) Suppl.1: A527.

12. **Brierley S.M,** Cooper N.J, Page A.J and Blackshaw L.A. (2001). *A comparative study of P_{2x} receptors on vagal and spinal primary afferents in the three species.* Neurogastroenterology and Motility, 13(4), 379.

13. **Brierley S.M,** Cooper N.J, Lynn P.A. and Blackshaw L.A. (2001). *Pelvic vs Splanchnic colonic primary afferents.* Gastroenterology, 120(5), A178.

Abbreviations

α,β-meATP; α,β-methylene adenosine 5'-triphosphate

AMPA; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AM; A-fiber mechanonociceptors

ASIC; Acid Sensing Ion Channels

C; carboxyl terminus

CNS; central nervous system

CRD; colorectal distension

CT; Cycle threshold

DEG/ENaC; Degenerin/Epithelial Na^+ Channel

ΔΔCT; (ΔCT [ASICx -/-] - ΔCT [ASICx +/+])

ΔCT; (Cycle threshold (CT) of ASICx transcript - Cycle threshold (CT) of β-actin)

DRG; dorsal root ganglia

D-hair; down hair mechanoreceptors

ECD; extracellular domain

ES cells; embryonic stem cells

GalR; galanin receptor

GABA; γ-Amino butyric acid

IC_{50}; concentration at which 50% inhibition of response is observed

IMG; inferior mesenteric ganglion

IGLEs; intraganglionic laminar endings

IMAs; intramuscular arrays

iGluR; ionotropic glutamate receptors

IBS; irritable bowel syndrome

IB_4; isolectin-B_4

LSN; lumbar splanchnic nerve

mGlur; metabotropic glutamate receptor

MPG; major pelvic ganglion

N; amino terminus

-/- ; null mutant

neo; PGK-neo cassette

NMDA; *N*-methyl-Daspartate

PN; sacral pelvic nerve

PCR; polymerase chain reaction

PARs; proteinase-activated receptors

PPADS; pyridoxyl 5-phosphate 6-azophenyl-2',4'-disulfonic acid

RA; rapidly adapting mechanoreceptor

rIGLEs; rectal intraganglionic laminar endings

RT; reverse transcription

5-HT; serotonin

SA; slowly adapting mechanoreceptor

SST; Somatostatin receptors

spikes / sec; spikes per second

TM; transmembrane domain

TLOSRs; transient lower oesophageal relaxations

TRP; transient receptor potential

TRPV1; transient receptor potential vanilloid receptor 1

ANOVA; analysis of variance

NaV; voltage-gated Na+ channels

+/+; wild-type

Summary

Background: A direct comparison of the mechano and chemosensory properties of lumbar splanchnic (LSN) and sacral pelvic (PN) spinal afferents innervating the colon is lacking. In addition, the mechanotransduction mechanisms involved in transmitting mechanosensory information from the colon are unknown.

Aims: 1) To determine the range of mechanosensory information encoded by LSN and PN afferents innervating the distal colon, 2) determine whether these pathways differ in their chemosensitivity and 3) directly compare the roles of the Acid Sensing Ion channels (ASIC) 1, 2 and 3 in LSN afferent mechanotransduction.

Methods: Novel *in vitro* preparations of mouse colon with attached LSN or PN were developed. Mechanosensitive primary afferents were distinguished based on their response to 3 distinct mechanical stimuli: probing (70 mg–4 g), circular stretch (1–5g), and mucosal stroking (10–1000 mg). Serosal afferent fibres from both pathways were tested with α,β-meATP (1mM), bradykinin (1μM) and capsaicin (3μM). In separate experiments the mechanosensitivity of serosal and mesenteric LSN afferents was examined in ASIC1a, 2 and 3 wild-type (+/+) and null mutant (-/-) mice.

Results: Five different classes of afferent were identified in the LSN and PN. Three of these classes (serosal, muscular, and mucosal) were conserved between both pathways but their respective proportions, receptive field distributions, and response properties differed greatly. In general PN afferents responded to lower stimulation intensities, displayed greater response magnitudes, and adapted less completely to mechanical stimulation. In addition, each pathway contained a specialized class of afferent fibre; mesenteric (LSN) and muscular/mucosal (PN). The majority of chemosensory afferents responding to α,β-meATP, bradykinin and capsaicin were found in the LSN.

Disrupting ASIC1a increased the mechanosensitivity of both serosal and mesenteric LSN afferents, whilst disrupting ASIC2 increased the mechanosensitivity of only serosal afferents. By contrast disrupting ASIC3 markedly reduced the mechanosensitivity of both afferent classes. Benzamil concentration-dependently inhibited serosal mechanosensitivity in +/+ mice, which was unaltered in ASIC1a +/+ but significantly reduced in ASIC2 and 3 -/-.

Conclusions: Splanchnic and pelvic pathways contain distinct populations of mechanosensitive afferents. These afferents are capable of detecting an array of mechanical and chemical stimuli and are individually tuned to detect the type, magnitude, and duration of the stimulus. ASIC1a, 2 and 3 have contrasting roles in the mechanotransduction of LSN colonic afferents, in both molecular and pharmacological terms, which holds promise for therapeutic targeting.

INTRODUCTION

Overview

From a clinical point of view, Irritable Bowel Syndrome (IBS) is the most common disorder diagnosed by gastroenterologists[1]. Enhanced colonic mechanosensation and abdominal pain are hallmarks of this functional bowel disease. Since its original description by Ritchie in 1973[2], increased perception of mechanical distension of the distal colon/rectum has become one of the best characterised clinical manifestation of IBS, along with altered bowel function. This finding exists generally in the relative absence of overt colon pathology, suggesting maladaptive changes in the function of colonic mechanosensory pathways. Recent evidence indicates that peripheral mechanosensation plays an important role in the aetiology of this disease[3-7]. Many studies have focused on sensation from the colon in an effort to understand how these changes may occur, however the precise nature of the sensory information encoded has remained elusive. This is because sensory information from the colon is conducted via two pathways: the lumbar splanchnic nerves (LSN) and sacral pelvic nerves (PN) to the spinal cord. To date interpretation of the roles of these pathways in colonic sensation has been limited by a failure to directly compare the roles of these two innervations of the colon using the same techniques in the same species. Understanding the precise nature of the sensory information encoded by each pathway is fundamental in the physiological mechanisms responsible for colonic sensation and potentially the pathophysiological mechanisms which underlie the visceral hypersensitivity observed in IBS. There are a number of gaps in our knowledge concerning the types of afferent present within these pathways and the functional properties they display and therefore the types of signals which are sent to the spinal cord. Similarly, there are gaps concerning whether or not afferents are chemosensitive and the comparative chemosensitivity of LSN and PN pathways, in particular the predominant pathway responsible for the signalling of chemical events. Understanding differences or similarities between the two pathways would be an enormous advance in designing new therapies for IBS. Although recent evidence from behavioural and functional imaging studies of IBS patients suggest changes

occur at the level of the primary afferent neuron and/or spinal cord but not in higher cortical centres[5, 7] thereby supporting the notion that peripheral mechanosensation plays an important role in the aetiology of of IBS, as yet no potential therapy has targeted the molecules responsible for transducing mechanical signals from the colon. Perhaps this is because the identity of mechanotransduction mechanisms within the viscera is as yet unknown; however, knowledge of mechanosensation in other systems provides important clues to their identity. The Degenerin/Epithelial Na^+ Channel (DEG/ENaC) cation channels are attractive candidates to serve as transducers of mechanical stimuli[8], in particular the Acid Sensing Ion Channels (ASICs)[9, 10]. This thesis documents the development of two novel *in vitro* electrophysiological techniques for recording from the LSN and PN nerves innervating the mouse colon, allowing a direct comparison of the mechanosensitive and chemosensitive properties of afferents from these two pathways. In addition, this thesis also identifies the molecules which contribute to the mechanotransduction of mechanical stimuli from the colon via LSN colonic afferents.

Clinical significance: Irritable bowel Syndrome

Diagnosis and prevalence

IBS is defined by the Rome II criteria as a functional bowel disorder in which there is the presence of continuous or recurrent abdominal pain or discomfort that is relieved with defecation, or associated with a change in frequency or consistency of stool for at least 3 months in a 12 months period[11]. The severity of these symptoms can range from mild to severe[12]. Traditionally it has been thought that these changes occur in the absence of any overt pathology and as such cannot be explained by structural or biochemical abnormalities[1, 12], however recent studies suggest a low grade mucosal inflammation may be involved at least in a subgroup of IBS patients[4, 13-16]. IBS is a common disease afflicting approximately 10% of the population, however the precise prevalence can range from the 2.9% up to 20% depending on the criteria used[1, 17-20]. The most common age group effected

by IBS are between 18 and 34 years of age[17] with a female/male ratio ranging from 1.1 to 2.6 and an overall prevalence ranging from 14% to 24% in women and 5% to 19% in men[19, 21, 22].

The impact of IBS on the individual and on the community as a whole is considerable. IBS patients report reduced quality of life[19, 23], with extraintestinal symptoms including; anxiety, depression, lethargy, urinary frequency, fibromyalgia, backache, headache, poor sleep and dyspareunia[19]. It is estimated that only 10%–50% of patients with IBS seek medical care and those who do have more symptoms including severe pain and psychological symptoms[1, 19]. IBS patients report a 3 times greater absenteeism from work due to their symptoms. Estimates of the total cost of IBS in the USA through direct costs of health care use and indirect costs of absenteeism from work is $25 billion per annum[1].

Sub-classification of IBS and Aetiology

IBS can be sub-classified into 3 groups based on altered bowel habit; constipation predominant, diarrhea-predominant and alternating, with a similar prevalence of each subclass[16, 19, 24-26]. The gender ratio in these subgroups is similar except in constipation-predominant IBS, which is more common in women[1, 27]. It has recently been suggested that these sub-classes of IBS represent a heterogeneous population of patients who are classified together despite widely differing symptoms which is suggestive of differing underlying pathologies[14, 16]. As such another subclass has now been added, post-infectious IBS[14]. However, despite this heterogeneous population of patients, enhanced colonic mechanosensation is a hallmark of all subtypes of IBS and as such increased perception of mechanical distension of the distal colon/rectum has become one of the best characterized clinical manifestation of IBS[1, 2, 28]. The extent of this enhanced colonic sensation is considerable, as a colorectal distending volume of approximately 60 ml evokes pain in less than 10% of normal subjects compared with over 50% of IBS patients. Therefore there is leftward shift in the

psychophysical function of IBS patients suggesting the presence of hyperalgesia in IBS[2] (*Figure 1*). There is a general agreement that this visceral hypersensitivity and hyperalgesia correlates well with the overall severity of the disease[1, 4], which is significant as pain is the symptom that affects quality of life the most[23].

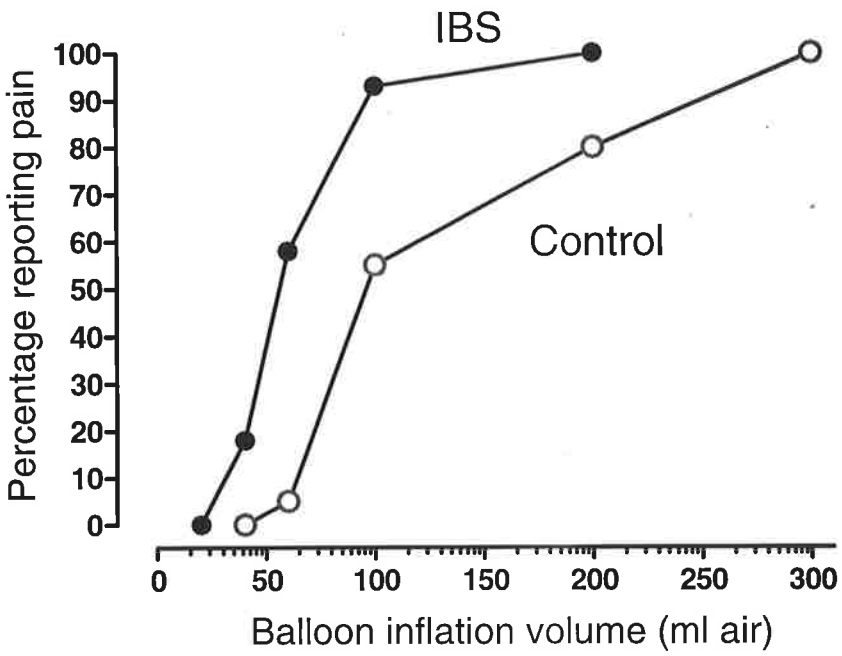


***Figure 1**. Percentage of normal controls and IBS patients reporting pain from colorectal distension.* Patients with IBS (close circles) report pain at lower volumes of balloon distension than do controls (open circles). The stimulus–response curve is shifted to the left in IBS suggesting visceral hypersensitivity in these patients. Figure adapted from Ritchie (1973); Gut 6:105–112.

There are various mechanisms which are thought to be involved in the visceral hypersensitivity experienced by IBS patients; however there is no clear consensus, which may reflect the heterogeneity of the disease. The hypothesized mechanisms contributing to visceral hypersensitivity include 1) sensitization of the extrinsic sensory afferent endings within the gut wall[3, 29], 2) hyperexcitability of dorsal horn neurons[3, 29] and 3) modulation of pain control systems in response to information signalled by the gut[3, 29-31]. However, recent evidence from behavioural and functional imaging studies of patients with IBS suggest that changes may occur at the level of the

primary afferent neuron and/or spinal cord but not in higher cortical centers[5, 7], suggesting peripheral mechanosensation plays an important role in the aetiology of this disease. In particular there is evidence suggesting that hypersensitivity of LSN afferents causes hyperalgesia in IBS patients[5, 32]. Consistent with the role of peripheral mechanosensation, subsets of IBS patients have increased numbers of inflammatory cells in the colonic mucosa[33], whilst activated mast cells have been found in close proximity to colonic nerves which correlate with abdominal pain in IBS patients[4]. This recent evidence suggests that activation or sensitization of extrinsic sensory endings within the gut wall may play a key role in IBS. Recent hypotheses support this notion by suggesting a low-grade inflammatory response at the level of the gut wall could be involved[4, 14]. In contrast post-infectious IBS patients appear to have a clearer aetiology as they describe an acute onset of symptoms (as classified by Rome II criteria) after a gastroenteritis episode yet previously had entirely normal bowel habit[14, 34]. For patients who had experienced gastroenteritis the relative risk of developing IBS within the following year is approximately 11 times greater[14]. These patients typically have the diarrhoea-predominant IBS and account for 6–17% of the IBS population[16]. A role for stress in the pathophysiology of IBS has also been suggested whilst psychological and environmental stressors have been associated with onset and symptom exacerbation in IBS, possibly via activation of sensitized immune cells within the gut wall[19, 20, 34-40].

Treatments

Despite the differing symptoms and aetiologies of IBS and the fact that enhanced colonic mechanosensation and hyperalgesia is a hallmark of all subtypes of IBS patients, most treatments to date are directed at altering bowel habit[16, 41], despite pain affecting quality of life the most[23]. This strategy has to a large extent been unsuccessful[19, 41, 42]. Such concerns are highlighted by the first drug to be approved by the Food and Drug Administration in the USA as a specific therapy for IBS. Alosetron is a potent selective 5-HT_3 receptor antagonist and is effective in normalizing bowel

frequency and reducing pain[43], however, reports of significant side effects including severe constipation, faecal impaction and ischemic colitis resulted in an initially withdrawal from the market[41, 44]. Altering serotonergic function has been a popular target for IBS therapies with the 5-HT_4 agonist, tegaserod, used for the treatment for constipation-predominant IBS by increasing gut transit[45]. More recent experimental therapies have targeted modulation of tachykininergic, opioid, cannabinoid and other serotonergic receptor function[41, 45]. However questions remain concerning their specific target of action due to a lack of basic studies, and their success in clinical trials due to the potential of unwanted central and peripheral actions. Non-pharmacological alternatives to IBS therapies are also emerging. Probiotics have been identified as being useful in alleviating symptoms in IBS as a decreased incidence of symptoms correlates with normalization of anti-inflammatory mediators, suggesting an immune-modulating role for probiotics[46, 47].

Targeting visceral afferents in the treatment of visceral hyperalgesia in IBS patients is a valid rationale for a multitude of reasons. First, visceral afferent endings are located within the wall of the gut and generate sensory signals that are transmitted from the gut via their associated afferent axons to the CNS. Although much of the information sent in response to intraluminal nutrients or normal gastrointestinal motility is rarely perceived, the main conscious sensations that are perceived from the gut are discomfort and pain. This is because visceral sensory afferents can serve a nociceptive function as they are polymodal. These afferents can detect and transduce mechanical, chemical and even thermal stimuli, via their different afferent classes and the multitude of membrane receptors and ion channels that can modulate their sensitivity[42, 48-54]. Therefore, there is the opportunity of targeting the periphery, avoiding potentially deleterious CNS effects[54]. Secondly, these same visceral afferents can modulate gastrointestinal motor and secretory activity, via local and central reflexes. Although these help to enable the gastrointestinal tract to maintain normal digestive function, in disease states they may be disturbed, altering motility leading to diarrhea or

constipation[42, 48-54]. Thus targeting of afferent endings may develop strategies that can attenuate the increased visceral sensation and therefore control the visceral hyperalgesia experienced by IBS patients. The ultimate therapy would control the visceral hyperalgesia and the abnormal reflex activity that is the basis of altered motility[54].

Sensory innervation of the gastrointestinal tract

The afferent innervation of the gastrointestinal tract mediates sensations from the gut and initiates reflex control of digestive function. The afferent fibres innervating the gastrointestinal tract follow two main anatomical branches, the vagal pathway and the spinal pathway. Vagal afferents have axons which project directly into the brainstem to the nucleus tractus solitarius while their cell bodies are located in the nodose ganglia. Vagal afferents are important in the sensory innervation of the upper gastrointestinal tract in particular the oesophagus and stomach and small intestine[51,48, 55]. However, the vagal innervation decreases down the length of the gastrointestinal tract and is particularly sparse in the distal colon[55, 56]. As such vagal fibres are associated with sensation in the upper gut such as fullness, bloating, and nausea and can induce vomiting via autonomic reflex control of digestive function. In contrast, pain evoked from the upper gut is though to be mediated via spinal nerves[57].

Spinal afferent endings are distributed throughout the entire gastrointestinal tract and the central projections of these afferent neurons enter the spinal cord, make synaptic contacts in prevertebral ganglia and the spinal dorsal horn with their cell bodies located within the dorsal root ganglia (DRG). Spinal afferents can be subdivided further into splanchnic and pelvic afferents. The splanchnic nerves terminate in the thoracolumbar spinal cord, whilst the paired pelvic nerves terminate in the lumbosacral spinal cord[48, 55]. Pelvic afferents are limited to the colon, the distal colon and rectum in particular[58]. Spinal afferents are associated with graded sensations of fullness,

bloating, discomfort and pain from the stomach and bloating, urgency, stool and pain from the colon, and are also thought to mediate discomfort and pain from the small bowel. However, in the rectum and distal colon, spinal afferents from the gut also give rise to sensations, from fullness to urgency and discomfort, in addition to pain evoked by more intense stimulation[48, 58, 59].

In terms of the afferent innervation pertinent to this project, the sensory information from the distal colon/rectum travels to the central nervous system through spinal afferents via two distinct spinal anatomical pathways: the lumbar splanchnic nerves (LSN), which terminate in the thoracolumbar spinal cord, and the paired sacral pelvic nerves (PN), which terminate in the lumbosacral spinal cord (*Figure 2*), within more than one level (laminae I, II, V and X) of the dorsal horn[29, 60, 61]. These spinal afferents arborise synapsing onto second order neurons in the dorsal horn of the spinal cord which contributes to the diffuse nature of visceral pain[29]. The second order neuronal pathways then ascend centrally via pathways including the spinothalamic, spinohypothalamic, spinosolitary, spinoreticular and spinoparabrachial tracts to the brainstem and thalamus with noxious signals relayed to sensory and limbic cortical sites[3, 29]. The majority of these dorsal horn second order neurons also have convergent inputs from visceral and somatic sensory neurons which may be responsible for viscero-somatic referred pain[29, 60, 61]. The excitability of dorsal horn neurons can be altered by a number of transmitters, in particular activation of N-methyld-aspartate (NMDA) receptors by glutamate and neurokinin 1 (NK-1) receptors by substance P increases neuronal excitability, whilst activation of γ-Amino butyric acid (GABA) receptors and opiate receptors decreases excitability[29, 62, 63]. Both thoracolumbar and lumbosacral dorsal horn neurons respond to colonic distension with short latency-abrupt responses and short latency-sustained responses[60, 64]. However, comparative studies of thoracolumbar and lumbosacral dorsal horn neurons suggest that acute colorectal pain is largely processed in the lumbosacral spinal cord whilst the thoracolumbar regions are recruited during pathophysiological conditions[60, 64-67].

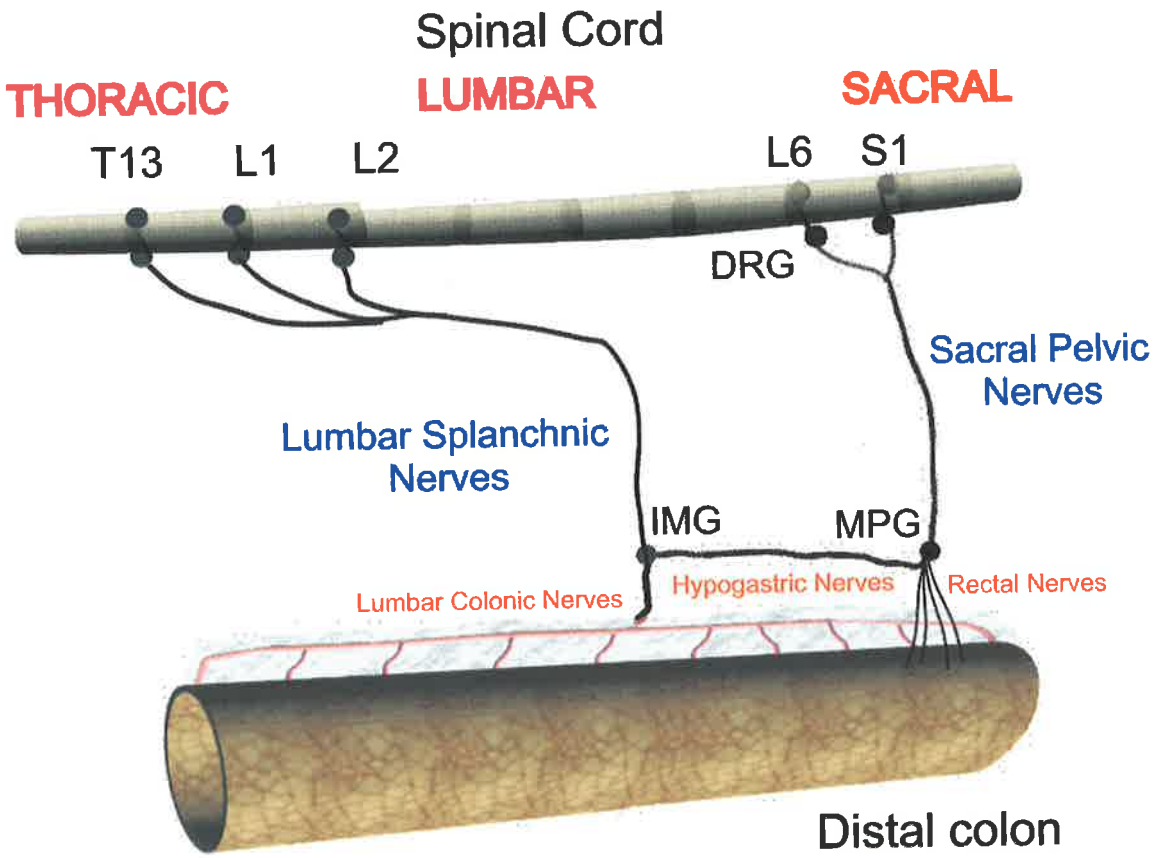


Figure 2. *Extrinsic spinal innervation of the colon. The sensory information from the distal colon/rectum travels to the central nervous system through spinal afferents via two distinct anatomical pathways: the lumbar splanchnic nerves (LSN), which terminate in the thoracolumbar spinal cord, and the paired pelvic nerves (PN), which terminate in the lumbosacral spinal cord,*

The thoracolumbar afferents, with receptive fields in the colonic wall, travel via the lumbar colonic nerves via the insertions into the colonic wall and into the mesenteric attachment of the colon, where they juxtapose with blood vessels supplying the colon. These afferent fibres then pass through the IMG and pass into the intermesenteric nerves containing the LSN.

The lumbosacral afferents travel though an entirely separate anatomical pathway via the rectal nerves which pass through the MPG and into the paired PN to the lumbosacral spinal cord. Afferents in each pathway can also travel through the hypogastric nerve and innervate the colon traveling in different directions via the MPG and IMG.

IMG; inferior mesenteric ganglion, MPG; major pelvic ganglion, DRG; dorsal root ganglia

The thoracolumbar afferents, with receptive fields in the colonic wall, travel via the lumbar colonic nerves via the insertions into the colonic wall and into the mesenteric attachment of the colon, where they juxtapose with blood vessels supplying the colon. These afferent fibres then pass through the inferior mesenteric ganglion, where collaterals can form peptidergic synapses with noradrenergic neurons to alter reflex activity[68], and pass into the intermesenteric nerves containing the LSN[69-74]. The lumbosacral afferents travel though an entirely separate anatomical pathway via the paired pelvic nerves which pass through the major pelvic ganglion to the lumbosacral spinal cord[73-76]. Afferents in each pathway can also travel through the hypogastric nerve and innervate the colon traveling in different directions via the major pelvic ganglia and inferior mesenteric ganglia[77].

In the rat the afferent innervation of the descending colon and rectum originates in the thoracolumbar DRG, at the anatomical levels of T_{13}-L_2, and the lumbosacral DRG, at the anatomical levels of L_6-S_2[60]. The LSN innervation of the colon in rat is comprised of approximately 1500 afferent fibres and 1250 efferent fibres[73], compared with the pelvic innervation of the colon comprising approximately 1600 afferent fibres and 3200 efferent fibres[78]. Other reports indicate significantly more colonic afferents are present in the T_{13}-L_2 ganglia than in the L_6-S_2 ganglia[79].

Previous studies in rat have reported a range in size of retrogradely labelled colonic primary afferents between 12–30μm in diameter[80], with the mean cell diameters of thoracolumbar and lumbosacral cells being approximately equal (28μm). These cells are therefore classified as small- to medium-sized reinforcing the suggestion that the majority of retrogradely labelled colonic neurons are Aδ or C fibre afferents (see below). In the mouse a similar afferent distribution to the rat is observed in thoracolumbar DRG, although the distribution is slightly wider at the anatomical levels of T_8-L_1, and the lumbosacral DRG, at the anatomical levels of L_6-S_1[81]. There are no studies to date comparing the relative proportion of afferent and efferent fibres from either the thoracolumbar or

lumbosacral innervation of the mouse colon/rectum. However, a comparison of the two pathways reveals a greater preponderance of retrogradely labelled afferent cells per mm^2 of DRG cell bodies within the thoracolumbar DRG[81]. The majority (92%) of retrogradely labelled cells in mouse thoracolumbar and lumbosacral DRG have diameters of 11–30 μm, suggesting that these cells can also be classified as small- to medium-sized, and therefore possibly functionally as Aδ or C fibre afferents.

Anatomical identification of visceral afferent endings

Unlike the cutaneous afferent innervation, where Aβ fibres, Aδ fibres innervate specific anatomical structures like Merkel cells, Ruffini, Hair Lanceolates and Pacinian or Meissner Corpuscles, the dogma associated with vast majority of visceral sensory endings are that the peripheral arborizations of small myelinated and unmyelinated afferent fibres terminate as free nerve endings without any clear anatomic specialization[53]. Despite this it is clear from numerous studies utilizing neuronal tracing techniques that these peripheral terminals of vagal and spinal afferents can be localized within the different layers gastrointestinal tract, giving an indication to their physiological characteristics and functional roles. Three types of specialized endings have been identified in the gut wall, intraganglionic laminar endings (IGLEs), intramuscular arrays (IMAs), and mucosal afferents (*Figure 3*)[48, 58, 59, 82, 83].

Intraganglionic laminar endings (IGLES)

Vagal Intraganglionic laminar endings (IGLEs) are special terminal structures that are located within the myenteric plexus throughout the gastrointestinal tract of a variety of species including rats, mice and guinea-pigs[55, 84-89]. IGLEs, traced from nodose ganglia are distributed throughout the entire gastrointestinal tract, with greatest densities in the stomach in particular the corpus (6.3 IGLEs/mm^2) the antrum (3.8 IGLEs/mm^2) and the forestomach (2.8 IGLEs/mm^2). The

density of IGLEs in other regions of the gastrointestinal tract is highest in the proximal duodenum (3.3 IGLES/mm^2) with very few IGLES in the distal colon (0.2 IGLES/mm^2)[84, 85,89, 90]. IGLEs are typically characterized as single axons entering a myenteric ganglion that have ramifying endings on the surfaces of the ganglia that are flattened, highly arborizing "leaf-like" processes[58, 84-89, 91]. In many cases a single axon gives rise to several IGLEs of various sizes in different ganglia[86, 87].These endings been hypothesized to detect mechanical shearing forces between the orthogonal muscle layers[58, 92]. It has recently been demonstrated, using a combination of rapid anterograde tracing and *in vitro* electrophysiology, that IGLEs are specialized transduction sites of mechanosensitive vagal afferent neurons in the guinea-pig oesophagus and stomach[86-88]. These studies show that morphologically identified IGLEs in the oesophagus and stomach correspond with the receptive fields or "hot spots" of stretch-sensitive afferents[86-88].

Spinal IGLEs with special terminal structures that are located within the myenteric plexus in the guinea-pig rectum have been classified as rectal IGLEs (rIGLEs). These rIGLES specifically innervate the rectum but not the distal colon probably via the pelvic nerve from the lumbosacral DRG[93, 94]. These rIGLES share characteristics of vagal IGLEs in the upper gastrointestinal tract in that they display branched, flattened, lamellar endings. However, rIGLEs are approximately 10 fold smaller (~630μm^2) than vagal IGLEs innervating the guinea-pig oesophagus (6900 μm^2) and stomach (6100μm^2) with fewer leaflets and less extensive branching patterns[86, 87, 93, 95, 96]. Using a combination of anterograde tracing and electrophysiological techniques it has been demonstrated that rIGLEs are functionally similar to vagal IGLEs in the upper gut as the are mechanotransduction sites which are sensitive to both distension and muscle contraction[86, 87, 93-95]. These morphologically identified rIGLEs in the rectum correspond with the receptive fields or "hot spots" of low threshold, slowly adapting, stretch-sensitive mechanoreceptors[93-95].

Intramuscular Arrays (IMAs)

Vagal Intramuscular Arrays (IMAs) are special terminal structures that have a parent axon that branches several times before terminating within the circular and longitudinal muscle layers. The size of the arrays can vary from several hundred microns to several millimetres in length[97]. In the muscle layers, the individual terminals run for several millimetres, creating a distinct pattern of parallel elements, which are commonly associated with interstitial cells of Cajal[56, 85, 87, 89, 98]. In contrast to IGLEs, IMAs have a distinctly different distribution, and are concentrated in the lower oesophageal sphincter, forestomach, and pyloric sphincter. The highest concentration of IMAs is in the forestomach (17.3 IMAs/mm^2) with fewer in the corpus (2.9 IMAs/mm^2) and antrum (0.6 IMAs/mm^2)[90]. IMAs have been suggested to be in-series tension receptors that serve as stretch or length detectors, that possibly respond to both passive stretch and active contraction of the muscle[58, 85, 90]. However, recent studies in the guinea-pig oeseophagus and stomach provide no evidence to support the notion that IMAs function as length receptors[87]. Spinal IMAs have also been located within the guinea-pig colon however as yet they do not have a known functional correlate[93].

Mucosal endings

Vagal afferent fibres have been shown to have nerve endings within the mucosa of the gastrointestinal tract. These fibres pass through the muscle layers and submucosa and have multiply branching axons within the lamina propria of both villi and crypts[58]. These mucosal fibres have been located within rat duodenum and jejunum[58, 99]. The evidence for mucosal afferents arises mainly from electrophysiological studies (see below).

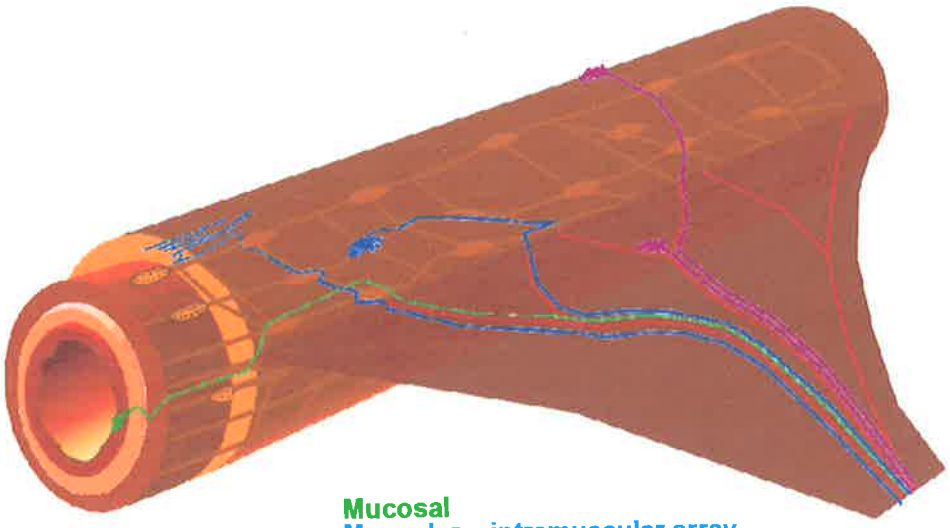


Figure 3 *Several different classes of mechanoreceptor within the gastrointestinal tract.*

Mucosal afferents have been extensively documented throughout the upper gastrointestinal tract in addition to the distal colon and perianal mucosa. Mucosal are silent at rest; respond to fine stroking of the luminal surface with rapidly adapting responses and are unresponsive to distension

Intramuscular Arrays (IMAs) have been documented anatomically and are special terminal structures that have a parent axon that branches several times before terminating within the circular and longitudinal muscle layers. IMAs have been suggested to be in-series tension receptors that serve as stretch or length detectors, that possibly respond to both passive stretch and active contraction of the muscle

Intraganglionic laminar endings (IGLEs) have been documented anatomically and are typically characterized as single axons entering a myenteric ganglion that have ramifying endings on the surfaces of the ganglia that are flattened, highly arborizing "leaf-like" processes. IGLES have been recently shown to be the specialized transduction sites of mechanosensitive tension sensitive vagal afferent neurons and tension sensitive rectal mechanoreceptors (rIGLES).

Mesenteric and serosal afferents have endings that are located close to or on blood vessels or branching points of capillaries supplying the serosa. They are classified by their response to probing or distortion of the colon but not to low intensity circular stretch or fine mucosal stroking. However these afferents can be activated briefly by intense stretch or distortion of the mesenteric attachment and high intensity colonic distension. (Figure adapted from Blackshaw, 2004)

Classification of Afferent Fibres

Much of the terminology used in the classification of visceral afferents has been translated from that used in the study of cutaneous sensation. These physiological classifications are based on afferent conduction velocities, which in turn, relates to axon diameter and the degree of myelination and their responsiveness to mechanical and thermal stimuli[100]. Cutaneous afferents can be subdivided into three classes based on conduction velocity alone; Aβ fibres, Aδ fibres and C-fibres. Each of these classes has subclasses of afferents based on mechanosensory responses. Large diameter myelinated Aβ fibres can be sub-classified into rapidly adapting mechanorecptors, which respond exclusively to movement of the skin but not to static indentation, and slowly adapting mechanoreceptors, which respond to both[100]. Aδ fibers have thin axons and a thin myelination and can be subclassified into either low-threshold D-hair mechanoreceptors which have relatively large receptive fields or nociceptive neurons with high-thresholds (AM) mechanoreceptors[100, 101]. Small diameter unmyelinated C-fibres can be subclassified into C-mechanonociceptors that have high mechanical thresholds and respond to mechanical but not thermal stimuli. Polymodal C-fibres that respond to mechanical and thermal stimuli are termed C-mechanoheat receptors. These C-fibres are designed to transmit exclusively noxious response in responses to noxious stimuli[100, 101]. Thus cutaneous afferents have highly specific functions and as such different classes of sensory neurons carry information for distinct sensory modalities. By contrast, studies of visceral afferents throughout the gastrointestinal tract have demonstrated that conduction velocities are limited to either small diameter unmyelinated C-fibres or thinly myelinated Aδ fibers[53, 68, 74, 75, 102-105]. However, visceral afferent fibres differ considerably in their basic physiological properties as they can signal normal functional events in addition to signalling pain in noxious environments[75]. In contrast to cutaneous afferents, visceral afferents lack a standardized nomenclature of afferent subclasses. As such visceral afferents have been classified based on 1) the layer of gut containing their receptive field, 2) on the type of mechanical stimuli that they are responsive to or 3) their general response properties.

However, the location of the endings of their receptive field is crucial in determining their mechanical sensitivity and responsiveness to varying mechanical stimuli.

Functional classes of visceral afferents

Combinations of *in vivo* and *in vitro* electrophysiological techniques have led to the identification and classification of three distinct patterns of afferent endings distributed within the wall of the gastrointestinal tract (*Figure 3*). Recent *in vitro* preparations have allowed manipulation of isolated afferent receptive fields resulting in a more controlled application of mechanical and chemical stimuli. Vagal and spinal afferents can be loosely divided into four classes; Distension/tension sensitive, mucosal, serosal/mesenteric and silent nociceptors (*Figure 3*) [48, 53, 59, 74, 82, 83].

Distension/tension sensitive afferents

Afferents within the wall of the gastrointestinal tract that broadly respond to distension or stretch of a region of gut have been extensively characterised[48, 53, 58, 82]. However, to add complexity these afferents have been described by a variety of names including; distension sensitive, tension sensitive, stretch sensitive, muscular afferents, tonic, phasic, low threshold, high threshold and wide dynamic range fibres to name but a few. Recent reviews indicate differences in the signals generated by these afferents in vagal and spinal pathways[48, 53, 58, 82]. For example in the upper gastrointestinal tract tension receptors have low resting activity and have low thresholds of activation. These afferents are responsive to both distension and contraction of the gut with a slowly adapting, linear relationship to wall tension and reach maximal responses within the physiological range of distension[48, 53, 58, 82]. By contrast, spinal afferents have higher thresholds of activation and encode within both physiological and noxious levels of stimulation[48, 53, 58, 82]. Due to these different

response profiles it has been suggested that vagal afferents are involved in physiological regulation, such as triggering reflexes controlling gastrointestinal function and signalling satiety and fullness, while spinal afferents are responsible for mediating pain[48, 53, 58, 82]. Because few vagal fibres innervate the distal colon and because the thoracolumbar and lumbosacral spinal pathways have been demonstrated as the important pathways in signalling colonic distension, vagal afferents henceforth will not be discussed in detail. However, it should be noted that recent *in vitro* studies utilizing single fibre recordings in the mouse, ferret and guinea-pig have identified tension-sensitive vagal afferents that have functional properties that are comparable to those recorded *in vivo*[53, 86, 87, 106, 107]. These tension-sensitive afferents respond in a graded manner to circular tension with slowly adapting responses[86, 87, 106, 107] and are insensitive to fine mucosal stimulation[106]. The receptive fields of these stretch-sensitive afferents in the guinea-pig oesophagus and stomach correspond with morphologically identified IGLEs demonstrating that IGLEs are the specialized transduction sites of mechanosensitive tension sensitive vagal afferent neurons[86-88].

Distension of the colon has been the primary stimulus used to study LSN and PN afferents in a multitude of species. As such afferents that respond to colonic stretch, applied either directly *in vitro* or indirectly using colorectal distension *in vivo*, have been identified and characterized[75, 104, 105, 108-115] (*Figure 4*). Distension sensitive colonic afferents recorded from the LSN of the cat generally display spontaneous activity and have be classified into four categories ranging between tonic and phasic discharge response patterns to distension[105]. Two percent were classified as Type I units, which displayed rapidly adapting responses, 10% were Type II units that displayed adapting responses, whilst 31% were Type III units displaying transient responses that adapted to steady state. The remaining 48% were Type IV units responding with steady state discharges. Eighty five percent of Type I-III units had activation thresholds below 25mm Hg, whilst 45% of Type IV units had activation thresholds above 25mm Hg[105]. These afferents also respond to contraction of the colon, whilst 43% of units could be activated by a discrete probing stimulus with mechanoreceptive sites

identified near to or on the arteries of the colonic wall[105]. Overall these results indicate heterogeneous populations of distension-sensitive afferents from the LSN suggesting that distension may not be the adequate stimulus for all LSN afferents.

In contrast to LSN afferents in the cat, PN distension sensitive colonic afferents generally display little to no spontaneous activity and have low thresholds to intraluminal pressure[75, 76, 102]. Moreover, PN afferents can be classified into either rapidly adapting phasic (47%) responses or tonic (53%) responses; most phasic afferents are classed as Aδ-fibres while most tonic afferents classed C-fibres. Afferents with tonic responses respond linearly to increasing intraluminal pressure throughout the innocuous and noxious levels of distension. These slowly adapting responses correspond best with Type III or IV units in the cat LSN, whilst rapidly adapting fibres correspond best with Type II LSN fibres. There also appears to be a greater response of distension-sensitive PN afferents at high intracolonic pressures relative to LSN afferents in the cat[75].

Recordings from the PN in rat colon show similar findings to those seen in the PN of cats. Distension sensitive PN afferents can be classified into dynamic responses which displayed slow adaptation (45%) or tonic non-adapting (55%) responses[102]. These phasic afferents were only transiently excited during filling or emptying of the colon, whereas tonic afferents discharged throughout the distension stimulus. However, these afferents could also be sub-classified as low threshold, responding to 10mg Hg of less (77%), or high threshold, responding to greater than 28 mm Hg (23%).

More recently, various *in vitro* preparations have been developed to allow greater accessibility of receptive fields utilizing a combination of stretch, stoking and probing stimuli to classify all afferent subtypes, not just distension or stretch sensitive afferents. In rat distal colon

muscular afferents have been identified which respond with an excitation that adapts during the stimulus[112, 113]. In addition to responding to circular stretch these afferents also responded to blunt probing of their receptive field but not to low intensity mucosal stroking[112, 113]. However, these afferents only account for 5-19% of the LSN innervation and are optimally activated by maintained circular stretch. Similarly, in the guinea-pig colon there are few stretch-sensitive mechanoreceptors in the distal colon, however in the rectum there is a high density of stretch-sensitive mechanoreceptors that display low thresholds and slowly adapting response to maintained distension[93, 94]. The receptive fields of these low threshold, slowly adapting, stretch-sensitive mechanoreceptors correspond with morphologically identified IGLEs in the rectum termed rIGLES[93-95]. These specialized rectal mechanoreceptors also bear many similarities to vagal IGLE mechanoreceptors in the upper gut described above[93, 94]. These data indicate that muscular afferents, as a whole, are responsive to small changes in intraluminal pressure, respond to colonic stretch or distension with a linear relationship to wall tension, and are likely to encode these stimuli well into the noxious range. However, differences may exist between the LSN and PN innervation.

Mucosal afferents

Mucosal afferents in the vagal pathway have been extensively documented throughout the upper gastrointestinal tract including the oesophagus, stomach, antrum, duodenum and small intestine[116-123]. These afferents are silent at rest; respond to fine stroking of the luminal surface with rapidly adapting responses and are unresponsive to distension[116-123]. Moreover, they are polymodal as they are also chemosensitive to a range of chemical and osmotic stimuli including serotonin (5-HT), bradykinin, purines, cholecystokinin and prostaglandins[48,116-119, 121-123]. Recent *in vitro* studies have highlighted the relative importance of these afferents in terms of their exact location, proportions, and their modality. In the ferret oesophagus mucosal afferents account for 47% of the afferents recorded. These afferents have small receptive receptive field sizes (1-3mm^2), which are

randomly distributed across the width of the oesophagus, with 36% of mucosal receptors classified as C-fibres[106]. They respond in a graded manner to the application of mucosal stroking with von Frey hairs (10-1000mg)[106]. Similarly in the mouse gastro-oesophageal region, mucosal afferents account for 41% of afferents recorded. These afferents also have small distinct receptive fields (<0.5 mm) and respond in a graded manner to the application of low intensity mucosal stroking[107]. When comparing the two species mouse mucosal receptors elicit significantly greater responses to mucosal stroking than ferret mucosal receptors[107]. Mucosal afferents in the mouse and ferret are polymodal and can elicit responses to α,β-meATP, capsaicin, HCl, 5-HT, bradykinin prostaglandin E2 and bile[106, 107]. This *in vitro* approach has also led to the discovery of tension/mucosal afferents in the ferret oesophagus, which respond to both circular stretch and fine mucosal stroking and therefore display the properties of both tension and mucosal afferents[106]. They account for 16% of vagal afferents recorded in the oesphagus and have been suggested to have a specialised role in detection of rapidly moving boli[48]. However, the major role of gastric and intestinal vagal mucosal receptors is thought to be in the generation of sensations such as satiety, nausea and vomiting, with a minor role in direct generation of reflex responses[48].

Mucosal afferents in the lower gastrointestinal tract have not been studied as extensively as those in the upper gut; however spinal mucosal afferents have been characterized in the distal colon, anal canal and perianal mucosa. In the distal colon mucosal afferents have been identified using a rat *in vitro* preparation recording from the LSN. These colonic mucosal afferents have similar properties to vagal mucosal afferents[112], responding to fine tactile mechanical stimulation of the mucosa with a 10 mg von Frey hair and do not respond to circumferential stretch of the colon. Mucosal afferents account for 24% of the colonic afferents recorded from the LSN and are also polymodal as they are responsive to a variety of chemical stimuli including 5-HT, NaCl, HCl, bile and capsaicin[112, 113].

Afferents with similar properties to mucosal afferents have also been identified from the PN *in vivo* with receptive fields in the anal canal of the cat[75, 114] and perianal mucosa of the rat[110]. In the anal canal of the cat these afferents respond to a proximodistal shearing stimuli within the lumen, have discrete receptive fields and were usually not activated by distension. The afferents also had significantly faster conduction velocities than colonic afferents[75]. Similarly, eighteen afferents documented in the perianal mucosa of the rat responded with a burst of firing to stroking of the mucosa or by rotation or movement of the experimental balloon within the colon. These afferents also had discrete receptive fields with the majority unresponsive to colorectal distension[110]. Unlike the distension sensitive afferents (which were C-fibres) the majority of these perianal mucosal afferents were classified as Aδ fibres[102]. As mucosal afferents are sensitive to mechanical deformation of the mucosa, they may respond to particulate material within the lumen which can refine the quality of perceived stimuli and alter reflexes controlling motility[124-126].

Serosal/mesenteric afferents

Spinal afferent fibres with endings within the serosa and mesenteric attachment of the colon have been reported in the cat and rat[103, 105, 109, 112, 113]. These afferents have endings that are located close to or on blood vessels or branching points of capillaries supplying the serosa. They may have up to seven punctate receptive fields[75, 103, 105, 108-110, 112, 127]. Studies from rat and cat colon, recording from the LSN, show that punctate mechanical stimulation of the mesentery or stretch of the mesenteric attachment elicits afferent firing[103, 105]. These afferents are also capable of responding to distension with a rapidly adapting response, particularly at noxious levels of distension[105] and are polymodal as the majority respond to chemical stimuli including 5-HT, NaCl, HCl, bile, bradykinin and capsaicin[112, 113, 128]. Recent *in vitro* studies in rat colon have demonstrated that serosal/mesenteric afferents account for between 50-80% of the afferents recorded from the LSN[112, 113]. These afferents are classified by their response to probing or distortion of the colon but not to circular stretch or fine

mucosal stroking and have small (2-4 mm^2) punctate receptive fields[109, 112, 113]. Serosal afferents also display a greater sensitivity to mechanical stimulation on the serosal surface compared with the mucosal surface[112]. It is clear that mesenteric and serosal afferents can be activated briefly by intense stretch or distortion of the mesenteric attachment and colonic distension in addition to stimuli applied to their receptive fields[103, 105, 112]. It is possible that these afferents could relate to the high threshold (or phasic) mechanoreceptors that have been described previously *in vivo* as they have low resting activity and respond only to noxious levels of distension. As such they are likely to be considered mechanonociceptors.

Silent nociceptors

Silent nociceptors have been mainly studied in somatic tissues, where some have been characterized as chemonociceptors, and as a consequence have been implicated in the transmission of painful stimuli[53, 100]. Large populations of afferents in the viscera that are silent at rest and are insensitive to innocuous and noxious colorectal distension[53, 74, 75, 105] have been suggested to be "silent nociceptors". The term "silent nociceptors" has been used to describe these silent afferents as they subsequently generate spontaneous activity and mechanosensitivity during and after inflammation or chemical application[48, 53, 74]. For example recordings from the rat PN reveal colonic afferents that are unresponsive to colorectal distension up to 100mg Hg. However, after 30 minutes treatment with acetic acid, these afferents developed spontaneous activity and began to respond to distension as low as 10mm Hg[74]. However, it has been suggested that many of the afferents described previously as silent nociceptors, due to their insensitivity to distension, may in fact be another class of physiologically activated afferent for which colonic distension is not an adequate stimulus, and these afferents are then sensitized by inflammation or chemical application[53, 112]. Indeed colonic mucosal afferents described from the rat LSN share many features with afferents that have been called silent nociceptors. These mucosal afferents (described above) are normally

insensitive to distension, show no resting activity, respond to chemical stimuli and may develop spontaneous firing after exposure to chemical stimuli during a study[112]. However, this same study, which used variety of mechanical stimuli (circular stretch, fine mucosal stroking and probing) to identify subclasses of colonic afferents, showed that 6% of the afferents recorded, initially had no mechanoreceptive fields, were not spontaneously active and could not be classed as muscular, serosal or mucosal afferents. However, these afferents were recruited during application of chemicals (NaCl, HCl, Bile and capsaicin) during the investigation of another mechanically sensitive unit and retesting mechanical responsiveness revealed that these afferents became responsive to mechanical probing[112]. Clearly, further investigation is required in the classification and existence of visceral silent nociceptors.

Overall these studies suggest important differences in the nature and sensitivity of mechanosensory afferents within the gut and in particular between the LSN and PN pathways. Such a finding that would imply unique sensory signalling functions for these two nerve supplies. A meaningful functional comparison of these data described above from these two pathways is prevented, however, by the diversity of species and experimental techniques that have been used to characterize one or the other of these pathways individually. To date no study to date of has compared the various classes of mechanosensitive afferent in the pelvic and LSN pathways using the same technique in the same species.

***Figure 4**. Colonic afferents recorded previously from the LSN and PN*

A) Responsiveness of LSN colonic afferents.

i) Distension sensitive colonic afferents recorded from the LSN of the cat in vivo have be classified into four categories ranging between tonic and phasic discharge responses patterns to distension. Type I units (2%) displayed rapidly adapting responses, Type II units (10%) displayed adapting responses, whilst Type III units (31%) display transient responses that adapt to steady state. The remaining 48% were Type IV units responding with steady state discharges. Eighty five percent of Type I-III units had activation thresholds below 25mm Hg, whilst 45% of Type IV units had activation thresholds above 25mm Hg. Overall these results indicate heterogeneous populations of distension-sensitive afferents from the LSN and that distension may not be the adequate stimulus for all LSN afferents. (Figure adapted from Blumberg et al., 1983[105]).

ii) Recording from the rat LSN in vitro 3 distinct classes of afferent can be classified in response to 3 distinct stimuli. Serosal afferents are activated only by probing the tissue and are insensitive to colonic stretch or fine mucosal stroking. Muscular afferents are activated by probing and by a maintained circular stretch. Mucosal afferents respond to probing and to fine mucosal stroking of their receptive field with a von Frey hair but is insensitive to colonic stretch. (Figure adapted from Hicks et al., 2002[113]).

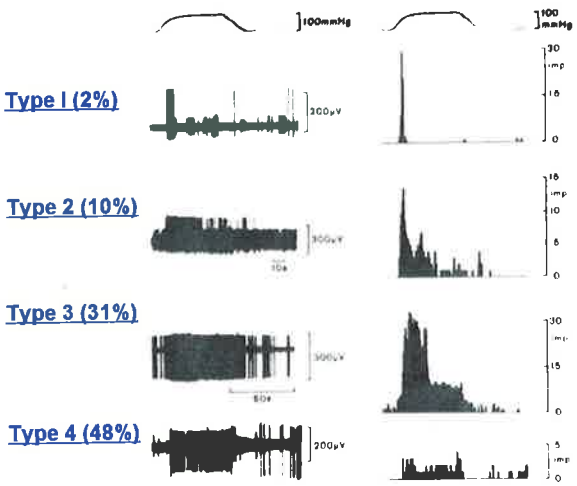
B) Responsiveness of PN colonic afferents.

i) In contrast to LSN afferents in the cat, PN distension sensitive colonic afferents can be classified into either rapidly adapting phasic (47%) responses or tonic (53%) responses. These slowly adapting responses correspond best with Type III or IV units in the cat LSN, whilst rapidly adapting fibres correspond best with Type II LSN fibres. (Figure adapted from Janig & Koltzenburg, 1991[75]).

ii) Distension sensitive afferents from the rat PN can also be classified into dynamic responses which displayed slow adaptation (45%) or tonic non-adapting (55%) responses[98]. However, these afferents can also be sub-classified as low threshold, responding to 10mg Hg or less (77%), or high threshold, responding to greater than 28 mm Hg (23%). (Figure adapted from (Sengupta & Gebhart, 1994[102]).

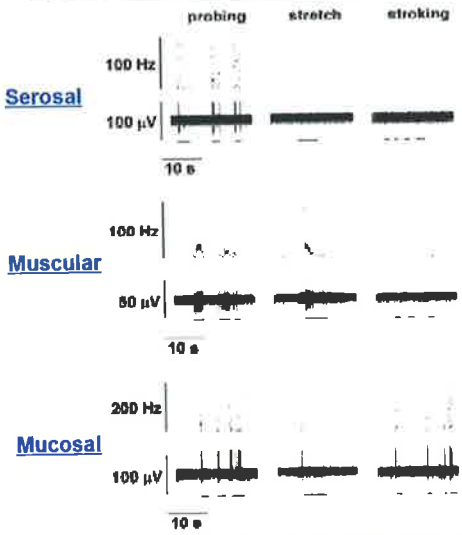
A) Responsiveness of LSN colonic afferents

i) Cat distension sensitive afferents



(Blumberg *et al.*, Pflugers Arch, 1983, *398:* 33-40)

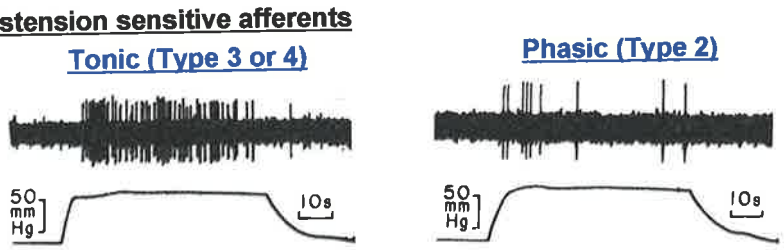
ii) Rat all afferent classes



(Lynn & Blackshaw, J Physiol, 1999, *518:* 271-282)
(Hicks *et al.*, J Physiol, 2002, *544:* 861-869)

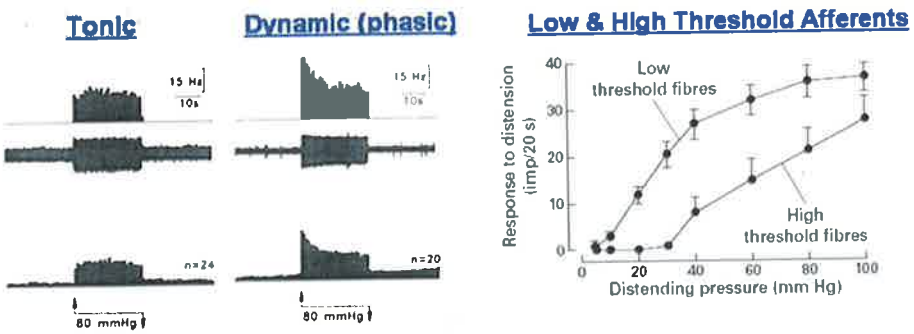
B) Responsiveness of PN colonic afferents

i) Cat distension sensitive afferents



(Janig & Koltzenburg, J Neurophysiol, 1991, *65:* 1067-1077)

ii) Rat distension sensitive afferents



(Sengupta & Gebhart, J Neurophysiol, 1994, *71:* 2046-2060)

Chemosensory targets

A multitude of factors, receptors and ion channels have been shown to be involved in altering afferent firing within the gastrointestinal tract. These targets are numerous and can be classified into either having excitatory or inhibitory effects on afferent discharge and have been the subject of a multitude of recent reviews[42, 48, 54, 82, 129-134]. However, there are three important issues to consider when determining the most relevant targets to be investigated in this study. Firstly, what is the most problematic symptom associated with IBS? Secondly, what is the main aetiological basis of the disease and thirdly, which receptors are altered. The P2X purinoceptor, $P2X_3$, the bradykinin B receptor, and the Transient Receptor Potential Vanilloid receptor 1 (TRPV1) (formerly known as VR1) are prime candidates for study as they meet all of the three criteria for investigation. Firstly, pain is the symptom that affects quality of life of IBS patients the most[23], and $P2X_3$, B and TRPV1 receptors have all been strongly implicated in the transmission of pain in various systems[105, 128, 135-147]. Secondly, visceral hypersensitivity is a hallmark of all IBS subtypes, and all three receptors are reported to alter mechanosensitivity either directly or indirectly[147-153]. Thirdly, $P2X_3$, B and TRPV1 receptors have all been shown to be increased in patients with inflammatory bowel disease[154-157]. The Acid sensing ion channels (ASICs) also fulfill these criteria and will be discussed later.

$P2X_3$ receptors

$P2X_3$ is a member of the P2X purinoceptor family of ATP-gated ion channels, of which there are seven cloned subunits $P2X_{1-7}$, that can assemble to form homomeric and heteromeric receptors, which are activated by extracellular ATP[139, 140, 158]. Studies in rat, monkey and mouse have localized the expression of $P2X_3$ receptors on small diameter primary afferent neurons (*C*-fibres) in DRG, usually with the lectin Isolectin-B_4 (IB_4)[81, 159-167], whilst functionally, $P2X_3$ mediated currents have been detected in these same neurons[139, 140, 164, 168-172]. As such $P2X_3$ receptors have been strongly implicated in nociception and pain[135-140], although it should be noted that the majority of these

studies have been performed in levels of DRG which are devoid of colonic innervation[60, 81]. These data suggest that in DRG $P2X_3$ may play a role in the processing of nociceptive information through either homomeric $P2X_3$ channels or $P2X_{2/3}$ heteromultimeric channels expressed either separately or together on individual neurons[139, 140, 166, 169, 173].

Activation of $P2X_3$ receptors by ATP or the selective agonist α,β-methylene adenosine 5'-triphosphate (α,β-meATP) evokes excitation of gastrointestinal afferents in the oesophagus, stomach jejunum, and colon[88, 107, 165, 174]. The proportion of afferents that evoke a response to these agonists varies between different species and different regions of the gastrointestinal tract. In rat jejunum α,β-meATP activates 100% of afferent bundles tested[174] whilst 89% of vagal tension receptors were activated in the guinea-pig oesophagus[88]. By contrast, in the mouse oesophagus only 30% of mucosal and 43% of tension receptors respond to α,β-meATP[107], while no afferents in the ferret oesophagus elicit a response[175]. Lower down the gastrointestinal tract, 65% of pelvic distension sensitive colonic afferents in the rat respond to ATP or α,β-meATP[165].

The role $P2X_3$ plays in sensory signalling is evident in $P2X_3$ -/- mice. These mice exhibit marked urinary bladder hyporeflexia, have reduced pain-related behaviour in response to injection of ATP and formalin and are unable to code the intensity of non-noxious "warming' stimuli"[151, 152]. These results lead to the suggestion that the $P2X_3$ receptors are involved in mechanosensation. This mechanism is proposed to occur via ATP acting as the molecular messenger that is released from the epithelial cells in response to distension, and $P2X_3$ receptors detect ATP and trigger the neuronal pathway signalling bladder fullness[151, 152, 176]. A similar mechanism has been proposed in the colon whereby ATP present in the colon is released by colorectal distension and that responses of pelvic distension sensitive afferents are inhibited by P2X receptor antagonists[165]. This mechanism appears to have an enhanced role in mechanosensory transduction during inflammation as augmented

distension-evoked sensory nerve responses are observed after application of ATP and α,β-meATP[177]. There is also a role for $P2X_3$ receptors on gastrointestinal afferents in the ferret oesophagus during inflammation as α,β-meATP is able to sensitize vagal afferents to mechanical stimuli[175]. Notably, from a clinical gastroenterology point of view, expression of $P2X_3$ is increased in colonic nerve fibers of patients with inflammatory bowel disease[154].

Bradykinin receptors

Bradykinin is one of the best established chemical stimuli and most relevant to tissue injury and pain. The direct effects of bradykinin are mediated via two G protein-coupled receptors, B_1 which is highly inducible in states of inflammation or injury[178] and B_2 which is constitutively expressed[178, 179]. Evidence suggests a role for B_2 receptors in acute inflammatory events, such as oedema and inflammatory pain, whereas B_1 receptors appear to be involved in chronic inflammatory responses, including certain forms of persistent visceral hyperalgesia[180]. This suggests B_2 receptors are a good target in normal conditions while B_1 receptors may be a good target in studies of altered afferent function in colonic inflammation. Bradykinin has been shown to be an important mediator in signalling of pain and irritation in skin, muscle, joints, vasculature, and all visceral organs[105, 128, 142-147, 181]. Bradykinin excites 55% of skin C-fibres in rat[145], 71% of joint afferents in cat[143], and 100% of cardiac afferents in cat[182]. Almost 100% of guinea-pig airway vagal afferents respond to bradykinin, with the exception of fast-conducting fibres with cell bodies in the nodose ganglion, which were unresponsive[183]. In the gastrointestinal tract bradykinin powerfully activates all afferents tested via B_2 receptors in an *in vitro* rat jejunum preparation[147, 184]. Some of these effects are also mediated via bradykinin-induced release of prostaglandins[147, 184]. In the cat colon bradykinin evokes a response in 67% of cat colonic LSN afferents recorded *in vivo*[128], while a study of 9 pelvic distension-sensitive colonic afferents in rats *in vivo* showed that 7 (77%) responded to bradykinin[102]. Administration of B_2 receptor antagonists can attenuate disease severity in dextran sulphate sodium-induced colitis in

rats[185]. Interestingly, B_1 but not B_2 receptor protein is significantly increased in the intestines of both active ulcerative colitis and Crohn's disease patients compared with controls, although the cell type on which they are expressed is not known[157].

Recently it has been demonstrated that bradykinin activation of afferent fibres may have numerous downstream effects, including the production of 12-lipoxygenase metabolites of arachidonic acid which activate vanilloid (TRPV) receptors intracellularly[145], which are involved directly in mechanical, thermal and pH sensitivity[136, 186]. A similar mechanism is responsible for bradykinin activation of the mechano- and thermo-sensitive channel TRPA1[187]. There is also direct evidence in some models that bradykinin induces increased responsiveness of afferents to other stimuli, in particular mechanical sensitization in studies of articular afferents[148, 149].

TRPV1

TRPV1 belongs to the transient receptor potential (TRP) channel family and is activated by heat, protons and vanilloid ligands such as capsaicin, the pungent ingredient in chillies[135-137, 188-195]. Studies have localized the expression of TRPV1 receptors on small diameter primary afferent neurons (C-fibres) in DRG, usually with $P2X_3$ and IB_4, whilst functionally, TRPV1 mediated currents have been detected in these same neurons. As such TRPV1 receptors have been strongly implicated in nociception and pain[135-140], including thermal nociception, inflammatory hyperalgesia and allodynia[138] and neuropathic pain[196]. As is the case with $P2X_3$ receptors, it should be noted that the majority of these studies have been performed in levels of DRG which are devoid of colonic innervation[60, 81]. Recent reports using immunohistochemistry demonstrate that 69% of rat DRG neurons innervating the urinary bladder expressed TRPV1, in contrast to only 32% of DRG neurons innervating the skin[197], suggesitng differences in TRPV1 expression between cutaneous and visceral afferents.

Capsaicin is a TRPV1-selective agonist that can activate sensory afferents. It's action is generally regarded as involving two phases: an initial excitation leading to transmitter release, followed by desensitization and damage after prolonged or repeated exposure[135, 137, 190, 198, 199]. In the gastrointestinal tract, capsaicin evokes a powerful excitation of discharge in all classes of vagal and spinal afferents, however the relative proportion varies between location and species[106, 150, 200]. Early reports in the cat found that the majority of vagal and spinal afferents were activated by capsaicin[201]. Similarly, in the mouse, 80% of isolated retrogradely labelled colonic lumbosacral DRG cells responded to capsaicin with an inward current[202]. By contrast, in rat isolated retrogradely labelled cells capsaicin evoked responses in 42% of nodose ganglion cells[203], and 46% of colonic lumbosacral DRG cells[204]. From the LSN capsaicin activated 29% of rat colonic afferents, including 17% of mucosal afferents, 40% of serosal afferents but no muscular afferents[112]. In the rat stomach capsaicin activated 32% of spinal afferents[109], whilst in the ferret gastro-oesophageal region capsaicin activated approximately 30% of vagal afferents[106, 200], including 20% of mucosal and tension mucosal afferents and 60% of tension sensitive afferents[106, 200]. One notable finding is the ability of capsaicin to cause mechanical desensitization *in vitro* in gastroesophageal[200] and jejunal[150] preparations. However, in the gastroesophageal preparation, mechanical desensitization was also observed in capsaicin unresponsive afferents in addition to capsaicin-responsive afferents. The desensitization and subsequent degeneration of primary afferents by capsaicin is thought to follow from uncontrolled cation influx into afferent endings, resulting in depolarization block and subsequent osmotic damage[199]. More recently a study of mechanosensitivity in TRPV1 +/+ and -/- mice showed more directly that mechanotransduction was reduced in the -/- compared to the +/+. Additionally the TRPV1 antagonist capsazepine was effective in reducing mechanical responses in the +/+, an effect that was totally lost in TRPV1 -/- mice[150]. From a clinical perspective, expression of TRPV1 is increased in colonic nerve fibers of patients with inflammatory bowel disease[155], and in patients with

rectal hypersensitivity[205], while administration of TRPV1 antagonists can attenuate disease severity in dextran sulphate sodium-induced colitis in mice[206].

There are a number of other identified targets, which evoke either excitatory or inhibitory effects on gastrointestinal afferents, to potentially consider when comparing the functional receptors expressed by LSN and PN afferents. Some of these are detailed below but are outside the scope of this study due to the time constraints associated with a three year project.

Other excitatory targets

Serotonin receptors

Altering serotonergic function has been a popular target for IBS therapies mainly for altering bowel habit[41, 45]. However, 5-HT can also have a direct effect on exciting gastrointestinal afferents. 5-HT activates a proportion of vagal afferent endings in ferret stomach and small intestine and mesenteric afferents innervating the rat jejunum exclusively via 5-HT_3 receptors[123, 207]. Similarly 5-HT activates a proportion of mucosal, tension and tension/mucosal vagal gastro-oesophegeal afferents in the ferret or mouse[106, 107]. In rat colon 56% of LSN colonic afferents respond to 5-HT via both 5-HT_3 and non-5-HT_3 receptors, which correlates with the percentage of thoracolumbar DRG cell bodies retrogradely labeled from the colon that display 5-HT_3 receptors[113]. In the rat 5-HT_1, 5-HT_2 and 5-HT_3 receptor subtypes have been demonstrated to modulate responses to noxious colorectal distension[208].

Proteinase-activated receptors (PARs)

PARs are G protein-coupled receptors, consisting of four receptors PAR1-4[209]. These receptors are activated by serine proteases such as mast cell tryptase, thrombin and trypsin, and as such are likely to be important when mast cells degranulate following inflammation[210, 211]. This is

highlighted by recent studies suggesting an important role for PAR1 in the pathogenesis of experimental colitis[54, 210, 211], whilst PAR-2 agonists have been show to evoke discharge of rat jejunal mesenteric afferents[212, 213]. Interestingly PAR-2 can sensitize TRPV1 to induce hyperalgesia.[214]

Ionotropic Glutamate receptors

Glutamate is a major transmitter in the CNS and can act via ionotropic NMDA receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA]/kainite receptors. Peripheral ionotropic GluR receptors (iGluR) have been suggested to be involved in normal visceral pain transmission, via activation of endogenous glutamate. This has been proposed as NMDA receptor antagonists have been shown reduce the responses to mechanical stimuli in LSN and pelvic afferents from the rat colon whilst decreasing the visceromotor response to colorectal distension[215, 216]. NMDA antagonists also reduced the response of vagal afferent fibers innervating the rat stomach[217]. Similarly, AMPA/kainate receptor antagonists also reduced the response of vagal afferent fibers innervating the rat stomach[217].

Prostaglandins

Prostaglandins are synthesized endogenously from the precursor arachidonic acid by cyclooxygenase in a variety of cell types and are important mediators of pain[48, 54, 130, 131]. EP1 receptors appear to play a major role in the rapid excitation of small intestinal afferents whilst EP2 receptors appear to cause longer term activation and sensitisation[218]. Jejunal afferent responses to bradykinin are also dependent on the presence of prostaglandins in particular PGE_2[147, 184]. Patients with inflammatory bowel disease have markedly elevated level of prostaglandins[219].

Voltage-gated Na+ channels (Nav)

Na*v channels* can be classified into two broad classes; Tetrodotoxin (TTX)-sensitive (TTX-S) and resistant (TTX-R) on the basis of their sensitivity to the Na^+ channel blocker TTX. The TTX-R channels are of particular interest because colonic inflammation induces increased neuronal excitability of mouse thoracolumbar DRG colonic afferents via a Na*v*1.8 Na^+ current[220].

Inhibitory targets

γ-Amino butyric acid (GABA) receptors

GABA is another transmitter that has a major role in the CNS, which modulates its effect via three classes of receptors $GABA_A$, $GABA_B$, and $GABA_C$. The inhibitory G-protein-coupled $GABA_B$ receptor is particularly important in the upper gastrointestinal tract as it is expressed on gastric vagal afferent neurons. $GABA_B$ receptor agonists also inhibit afferent mechanosensitivity and reduce transient lower oesophageal relaxations (TLOSRs)[129, 221-224]. This has lead to interest in these receptors as therapeutics for gastro-oesophageal reflux disease by reducing the TLOSRs which are the major cause of this disease.

Metabotrophic glutamate receptors (mGluR)

The actions of glutamate are also mediated via metabotropic glutamate receptors (mGluR). These receptors can be divided into eight molecular subtypes that can be classified into three pharmacological and functional groups: group I (mGlu1 and 5 receptors) are excitatory, whereas group II (mGlu2 and 3 receptors) and group III (mGlu4, 6, 7 and 8 receptors) are inhibitory to neuronal function[48]. Glutamate in the presence of kynurenate (to block iGluRs) can concentration-dependently inhibit vagal afferent mechanosensitivity in the mouse and ferret. This inhibition can be mimicked by selective group II and III mGluR agonists[225]. Conversely, group III mGluR antagonists can increase mechanosensitivity to intense stimuli[225]. Therefore there appears to be a delicate

balancing act in the way in which glutamate can act via mGluR and iGluR to regulate of afferent mechanosensitivity[225].

Galanin receptors

Galanin is a transmitter that is found throughout the central and enteric nervous systems. Three G protein–coupled receptors (GalR1-3) mediate the effects of galanin. Galanin itself causes inhibition of mechanosensitivity in 80% of mouse and 58% of ferret gastro-oesophageal afferents respectively. However it does cause potentiated responses in 12% of afferents[226]. The inhibitory effects are likely to be mediated via GalR1 and 3 receptors whilst the potentiation is mediated via GalR2 receptors[226].

Somatostatin receptors

Somatostatin receptors (SST1–5) have been detected throughout the rat gastrointestinal tract[227], whilst clinical studies show that a somatostatin analogue decreases colorectal pain in patients with IBS[228]. Interestingly octerotide, a SST2 receptor agonist, significantly inhibited the mechanosensitivity of spinal, but not vagal, rat jejunal afferents, suggesting a possible involvement in the transmission of nociceptive information[229].

It is clear from the literature that although there has been considerable investigation into the responses of afferents to a variety of endogenous and exogenous, excitatory and inhibitory mediators (described above) including agonists of $P2X_3$, B and TRPV1 receptors[48, 54, 102, 112, 128, 165, 177, 230], in terms of colonic signalling there are a number of gaps in our knowledge. This is in regards to which is the predominant pathway through which these signals are transmitted, the types of afferent fibres activated and which receptors underlie the pharmacology of its action. This is relevant firstly to understanding the sensory physiology of the colon, as sensitization of mechanoreceptors and perhaps chemoreceptors may give rise to the abdominal pain and discomfort experienced by

IBS patients[231] and secondly to future targeting of pharmacotherapies towards particular types of sensation and regions of the gut. In addition it is also important to identify the mechanotransduction mechanisms by which afferents signal mechanical stimuli.

Molecular basis of mechanotransduction

An organism's perception of mechanical sensation is vital in determining how they respond to their environment. However, understanding the molecular basis of mechanotransduction is currently a major challenge. External mechanosensation is responsible for the senses of touch, hearing, proprioception and several aspects of somatic pain, whilst the importance of visceral sensation has been discussed at length in the previous sections of this introduction. Knowledge is increasing about the molecular identity and function of the proteins that transduce mechanical stimuli into electrical signals at the body surface; however mechanotransduction within the viscera is particularly poorly understood. The number of candidate molecules that serve as mechanotransducers is increasing with the discovery of novel molecules and improved understanding of established molecules. At the start of this project the clear candidates in touch sensation were the degenerin/epithelial sodium channel (DEG/ENaC) family, which in mammals are comprised mainly of the Acid Sensing Ion Channels (ASICs)[232-240]. At present it appears that in addition to the ASIC channels the TRP family are mechanosensory candidates, in particular TRPV1, TRPV4, TRPA1 and TRPC1[235, 236, 239-258]. Apart from TRPV1 these TRP channels will not be specifically addressed in this thesis in terms of their involvement in mechanosensation from the colon, however their roles are currently being determined by this investigator.

The key components in the process of mechanotransduction are speed and sensitivity. First, mechanotransduction needs to be fast and therefore mechanical forces need be focused directly to transduction channels themselves, without intervening second messengers[236]. Secondly, sensitivity

requires that the maximal amount of stimulus energy be directed to the transduction channel[236]. Therefore, mechanical forces need to be directed to specific ion channels, which can open rapidly and amplify the signal by allowing entry of large numbers of ions[236]. The preferred hypothesis of how this occurs is via linking or tethering of the channels to the extracellular matrix and/or the intracellular cytoskeleton[233, 236]. Movement of this complex structure changes tension in all elements of the system, and the transduction channel responds by changing its open probability, therefore increasing or decreasing the mechanosensory signal *(Figure 5)*[236]. As mentioned above candidates to fulfill the role of the transduction channels are the DEG/ENaC channel family.

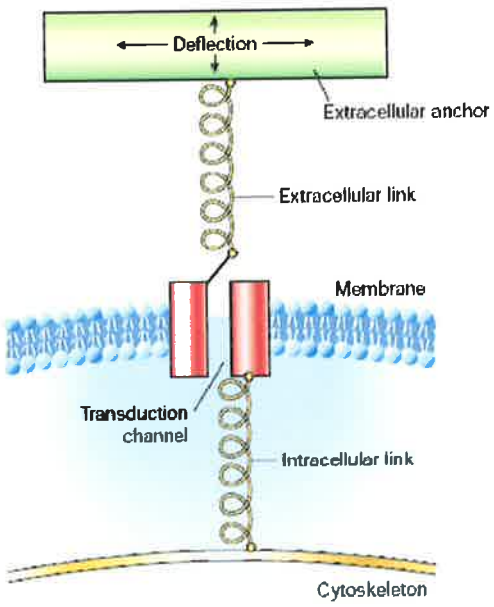


Figure 5: *Basic features of mechanosensory transduction. Transduction channels are present within the membrane which is anchored by both intracellular and extracellular anchors. These anchors are attached to the the cytoskeleton and possibly to an extracellular structure to which forces are applied. Displacement between intracellular and extracellular structures causes tension in the system through which the the open probability of the channel is increased therefore increasing the mechanosensory signal. (Figure adapted from Gillespie and Walker, 2001).*

The degenerin/epithelial sodium channel (DEG/ENaC) family

To be considered a molecular component of mechanotransduction a given protein must be located within the mechanoreceptor, particularly at the site at which mechanical stimuli is detected[236]. The speed which is required for the transduction of mechanical forces suggests that this signal is generated directly to transduction channels[236]. There is an abundance of indirect evidence suggesting that the DEG/ENaC superfamily of ion channels *(Figure 6)* may form the molecular components of receptors that detect mechanical stimuli. Genetic and localization studies in a variety

of species have been critical in elucidating their roles in mechanosensation, as these channels are located within a wide variety of mechanoreceptors in a multitude of species and disruption of these channels results in alterations in the detection of the mechanical environment[233, 236-238, 259].

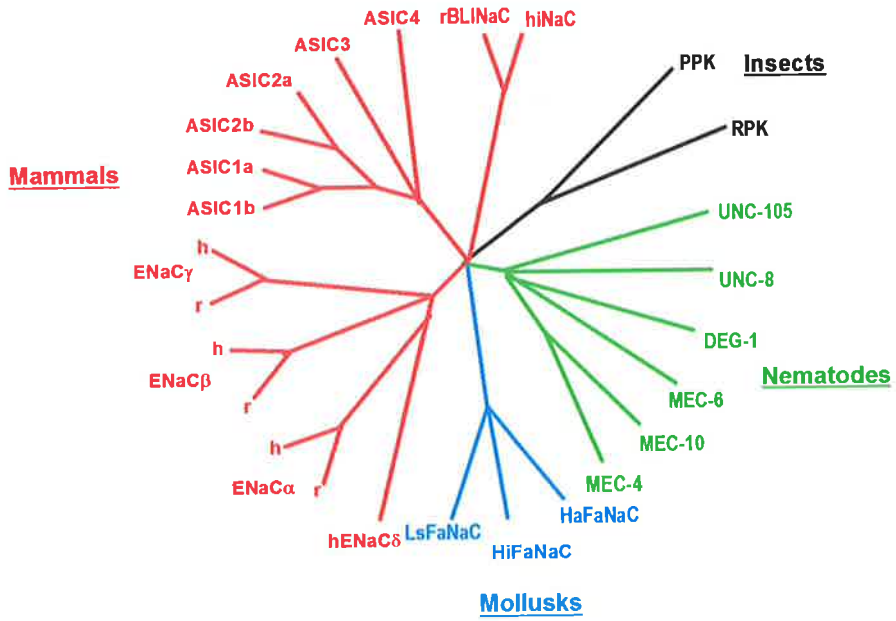


Figure 6: *Phylogentic tree of DEG/ENac channel family showing the organization into subfamilies of related sequences. Figure adapted from Kellenberger and Schild 2002.*

First, in the invertebrate *Caenorhabditis elegans* the degenerins have been implicated in mechanotransduction as mutations result in major deficits in body touch perception[233, 234, 236-238, 240, 260, 261]. Specifically, mutations disrupting the DEG/ENaC proteins *MEC-4* and *MEC-10* impair responses to touch[260-264], whilst *MEC-6* is a part of the degenerin channel complex that may mediate mechanotransduction in touch cells[265]. Moreover, *UNC-8* has been implicated in proprioception[266] whilst *UNC-105* is implicated in the detection of muscle stretch[267, 268]. Importantly this corresponds with the expression of a multitude of *MEC* genes in the touch receptor neuron responsible for gentle touch in *Caenorhabditis elegans*[269].

Second, in *Drosophila melanogaster* the DEG/ENaC protein *Pickpocket* has been localized to the peripheral dendrites of a subset of mechanosensory neurons at the exterior surface[270, 271] and disruption of this protein enhanced locomotion[272].

Third, in the rat, β and γ ENaC proteins have been localized in specialized mechanosensory structures in the skin and aortic arch[273, 274]. Specifically βENaC and γENaC subunits are expressed in medium to large lumbar DRG neurons, Merkel cell–neurite complexes, Meissner-like corpuscles, and small lamellated corpuscles, the specialized mechanosensory structures of the skin[275]. RT-PCR analysis has revealed expression of β and γ ENaC proteins in rat nodose ganglion whilst the γ ENaC subunit has been shown to be localized to the site of mechanoreception in baroreceptor nerve terminals innervating the aortic arch and carotid sinus, where they are proposed to form mechanically gated channels involved in the sensing of blood pressure[276, 277]. ENaCs are also expressed in epithelia of the lumen within the kidney, colon, and lung which form channels that constitutively reabsorb sodium but are not gated directly by pH or mechanical stimuli[278, 279].

Acid sensing ion channels (ASICs)

The Acid sensing ion channels (ASICs) are probably the most important branch of the DEG/ENaC family, at least in terms of mammalian mechanotransduction, and have been extensively localized in a multitude of species. There are four different genes that encode six polypeptides that have been identified for these channels: ASIC1a[280] and ASIC1b[281] (which differ in the first 172 amino acids), ASIC2a[282-284] and ASIC2b[285] (which differ in the first 236 amino acids), ASIC3 [280, 286, 287], and ASIC4[288-290]. Generally, ASIC1, 2 and 3 have been localized within the central nervous system, peripheral mechanosensory structures and sensory ganglia, although the specific location is species dependent[9, 10, 280, 281, 291-293]. In the rat ASIC1, 2 and 3 have been located in rat DRG using RT-PCR analysis, *in situ* hybridization and immunohistochemistry. Specifically, ASIC1 localizes to the plasma

membrane of small, medium, and large diameter cells, whereas ASIC2 and ASIC3 are preferentially expressed in medium to large cells[292]. Within the cells themselves, ASIC1, 2 and 3 are present mainly on the plasma membrane of the soma and cellular processes[292]. *In situ* hybridization has revealed that in DRG the highest level of ASIC1a mRNA expression is in small neurons[280, 294], whereas ASIC1b is present in 20–25% of small and large diameter neurons[281]. ASIC2a and ASIC2b and ASIC3 mRNA are detected in small-diameter neurons[281, 295, 296]. Overall these results imply a significant expression and localization of ASIC1, 2 and 3 in nociceptive fibres. The relevance of this is highlighted as, ASIC1, 2 and 3 expression is significantly upregulated in L_4-L_5 DRG during inflammatory conditions[296]. Ligation studies have indicated that ASIC subunits are transported from DRG cell bodies to sensory nerve terminals in the periphery[291]. In contrast to ASIC1, 2 and 3 in the rat, ASIC4 mRNA is present in very low levels in DRG if at all and was actually cloned from the pituitary gland[288-290, 293]. Because of the low or lack of presence of ASIC4 in DRG and the observations that ASIC1, 2 and 3 but not ASIC4 are activated by protons and only ASIC1, 2 and 3 form functional heteromultimers ASIC4 has not been considered as important. However, ASIC4 is present in brain, the inner ear and pancreas[288, 290]. Similarly ASIC1, 2 and 3 have a widespread distribution outside of DRG. *In situ* hybridization and immunohistochemistry studies have localized ASIC1, ASIC2 and ASIC2 showing a widespread distribution in the brain including the olfactory bulb, cerebral cortex, hippocampus, basolateral amydgaloid nuclei, subthalamic nuclei and cerebellum[237, 280, 282, 285, 291-293, 295, 297].

In the mouse, ASIC2 and 3 are located at the sites of sensation as they are present in mechanosensory lanceolate nerve endings surrounding the hair shaft and other cutaneous sensory structures[9, 10, 291, 298]. Similarly, ASIC1, 2 and 3 are located within the DRG where the cell bodies of their mechanoreceptors are located. Specifically, ASIC1, 2 and 3 have been located in DRG using RT-PCR or northern blot analysis[9, 10, 281, 285, 298-300] where they have been shown to form functional

heteromultimeric channels *(Figure 7)*[298, 301]. The location of ASICs corresponds directly with the deficits observed in cutaneous mechanosensation in mice with disruptions to the *ASIC2* and *ASIC3* genes respectively which taken together indicate they play specific, distinct and diverse roles in cutaneous mechanotransduction. As observed in the rat ASICs are not only located within DRG and have also been located within various regions of the brain. Specifically ASIC1 is located within the glomerulus of the olfactory bulb, whisker barrel cortex, cingulate cortex, striatum, nucleus accumbens, amygdala, and cerebellar cortex[281, 299, 302, 303]. ASIC2 is also located within the Purkinje, granule cell layers of cerebellum, in dentate gyrus and regions CA1–CA4 of hippocampus, and in the olfactory bulb[281, 282, 285].

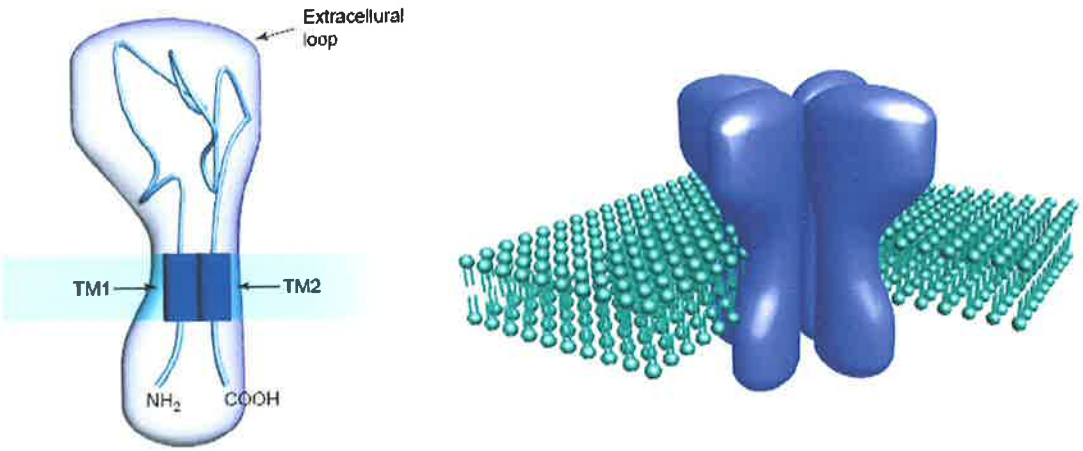


Figure 7: *Structure of the Acid Sensing Ion Channels (ASICs). A single subunit is composed of two transmembrane domains (TM1 and 2), a large extracellular loop, which is responsible for gating, and C and N intracellular termini facing the intracellular space. At least four of these individual sub-units are assembled in the membrane to form a functional unit. In native mouse DRG neurons ASIC1, 2 and 3 subunits combine to form functional heteromultimers. (Figure adapted from Krishtal 2003).*

Insights from electrophysiological studies using null mutant mice

A more direct implication in mechanotransduction has come from studies of five classes of cutaneous mechanoreceptors in mutant mice lacking individual *ASIC* family members. Data from mice with disruptions to the respective *ASIC* genes indicate they play specific, distinct and diverse roles in cutaneous mechanotransduction[9, 10]. There are five classes of cutaneous mechanoreceptors in the mouse; rapidly adapting (RA) and slowly adapting (SA) mechanoreceptors; down hair (D-hair) receptors; A-fiber mechanonociceptors (AM), and high threshold C-fibers. Mice with a disruption of the *ASIC2* gene displayed reduced responses of RA and SA mechanoreceptors, thus these mice showed a reduced sensitivity in mechanosensitive neurons detecting light touch, but not in other populations[10]. Mice with a disrupted *ASIC3* gene have reduced responsiveness of AM nociceptors, whereas RA mechanoreceptors in contrast showed increased mechanosensitivity, thus these mice displayed a reduced sensitivity of mechanoreceptors detecting a noxious pinch but an increased sensitivity to light touch[9]. In addition, these ASIC3 -/- mice also displayed a reduced response to mechanically-induced pain[294]. In contrast, mice with a disruption of *ASIC1a* gene displayed no change in the function of any class of the five classes of cutaneous mechanoreceptor[304]. These data identify the ASIC2 and ASIC3 subunits as essential for the normal function of cutaneous mechanoreceptors, but no role for ASIC1a. Moreover, the demonstration of both positive and negative effects of ASIC2 and 3 mutations suggests that ASIC subunits form components of a mechanosensory complex. Further indications of functional heteromultimeric complexes are clear from -/- mice, patch clamp and expression studies that conclude ASIC1, 2 and 3 coexist in the same sensory neurons[233, 298, 301]. Whether these channels also contribute to colonic mechanosensation is unknown and will be addressed in this study.

The hypotheses to be tested in the present study were:

1) There are differences in the mechanosensitive properties of LSN and PN afferents innervating the distal colon/rectum.

2) There are differences in the responsiveness of LSN and PN colonic afferents to chemical stimuli.

3) ASIC1a, 2 and 3 contribute to the mechanotransduction of LSN mesenteric and serosal colonic afferents and the loss of *ASIC1a, 2 or 3* produces alterations in afferent mechanosensitivity.

4) The amiloride analogue benzamil causes a dose-dependent inhibition of LSN colonic afferent mechanosensitivity and the loss of *ASIC1a, 2 or 3* produces alterations in the efficacy of benzamil.

5) ASIC1, 2 and 3 mRNA transcripts are expressed in thoracolumbar DRG and the loss of *ASIC1a, 2 or 3* via gene deletion does not result in compensatory changes in the expression of the other ASICs

As such the aims of the present study were to:

1) Address the lack of fundamental knowledge of afferent signalling from the distal colon/rectum by using an *in vitro* electrophysiological approach to directly compare the mechanosensitive afferents found in the LSN and PN;

2) Address the lack of knowledge concerning the comparative chemosensitivity of LSN and PN colonic afferents from the distal colon by using an *in vitro* electrophysiological approach.

3) Identify and compare the roles of ASIC1a, 2 and 3 in the mechanotransduction of LSN mesenteric and serosal colonic afferents from the distal colon by using an *in vitro* electrophysiological approach.

4) Investigate the possible mechanisms by which disrupting *ASIC1a, 2 or 3* produces different alterations in LSN colonic afferent mechanosensitivity by using the amiloride analogue Benzamil.

5) To investigate the relative expression of ASIC1, 2 and 3 in thoracolumbar DRG, and determine whether there are compensatory changes in ASIC transcript expression when one of the other *ASIC* genes is disrupted using Quantitative RT-PCR.

CHAPTER 1

SPLANCHNIC AND PELVIC MECHANOSENSORY AFFERENTS SIGNAL DIFFERENT QUALITIES OF COLONIC STIMULI IN MICE

Summary

Background & Aims: Mechanosensory information from the colon is conducted via the LSN and PN to the spinal cord. The precise nature of mechanosensory information encoded by each pathway has remained elusive. This study characterized and directly compares the properties of mechanosensitive primary afferents from these two pathways in the mouse. *Methods:* Using a novel *in vitro* mouse colon preparation, mechanosensitive primary afferents were recorded from the LSN and PN and distinguished based on their response to receptive field stimulation with three distinct mechanical stimuli: probing (70mg-4g), circular stretch (1-5g), and mucosal stroking (10-1000mg). *Results:* Five different classes of afferent were recorded from the LSN and PN. Three of these classes of afferent (serosal, muscular and mucosal) were conserved between both pathways, however their respective proportions, receptive field distributions and response properties differed greatly. In general, these three classes of afferent recorded from the PN responded to lower stimulation intensities, displayed greater response magnitudes and adapted less completely to mechanical stimulation compared with their counterparts in the LSN. In addition, the LSN and PN each contain a specialized class of afferent (mesenteric and muscular/mucosal, respectively) which is unique to their respective pathway. *Conclusions:* The LSN and pelvic pathways contain distinct populations of mechanosensitive afferents. These afferents are capable of detecting an array of mechanical stimuli and are individually tuned to detect the type, magnitude, and duration of the stimulus. The result of these differences between pathways in terms of sensations from the colon is probably that the pelvic pathway signals maintained distension and the passage of material over the mucosal epithelium, and the LSN pathway signals transient events such as the onset of rapid distension, contraction or torsion on the mesentery. This knowledge contributes to our understanding of the role these two pathways play in conveying mechanical information from the mouse colon.

Introduction

Irritable bowel syndrome is the most common disorder diagnosed by gastroenterologists[1]. Enhanced colonic mechanosensation is the hallmark of this functional bowel disease. Since its original description by Ritchie in 1973[2], increased perception of mechanical distension of the distal colon/rectum has become one of the best characterized clinical manifestation of IBS, along with altered bowel function. This finding exists in the relative absence of overt colon pathology, suggesting maladaptive changes in the function of colonic mechanosensory pathways. Indeed, evidence from behavioral and functional imaging studies of patients with IBS suggest that these changes occur at the level of the primary afferent neuron and/or spinal cord, but not in higher cortical centers[5, 7], supporting the notion that peripheral mechanosensation plays an important role in the aetiology of this disease.

Sensory information from the distal colon/rectum travels to the central nervous system through two distinct anatomical pathways: LSN, which terminate in the thoracolumbar spinal cord, and the paired pelvic nerves (PN), which terminate in the lumbosacral spinal cord. Mechanosensitive afferents have been identified in both of these nerve supplies using a combination of *in vivo* and *in vitro* electrophysiological techniques in the cat[75, 105, 108] and rat[109-113]. These studies suggest important differences in the nature and sensitivity of mechanosensory afferents in the LSN and PN, a finding that would imply unique sensory signalling functions for these two nerve supplies. A meaningful functional comparison of the two pathways is prevented, however, by the diversity of species and experimental techniques that have been used to characterize one or the other of these pathways individually and, to date, no study to date of has compared the various classes of mechanosensitive afferent in the pelvic and LSN pathways using the same technique in the same species. The present study had two purposes: 1) to address the lack of fundamental knowledge of afferent signalling from the distal colon by using an *in vitro* electrophysiological approach to directly

compare the mechanosensitive afferents found in the LSN and PN; and 2) to document the mechanosensory properties of colonic afferents in +/+ C57BL/6 mice, a mouse strain commonly used to generate a growing library of transgenic mice, some of which have proven invaluable for elucidating mechanotransduction mechanisms in the skin[9, 10].

These data indicate that mechanosensitive afferents exist in both nerves that respond to mechanical stimulation of the colonic mucosa, muscle layer, serosa, and/or mesentery, but that the LSN and pelvic pathways exhibit major differences in the proportion of each class, the location of their receptive fields, and their sensitivity to mechanical stimulation. This study provides definitive evidence that LSN and PN are distinct, not only anatomically, but also functionally and likely serve unique roles in the detection of mechanical stimuli in the distal colon.

Materials and Methods

All experiments were performed in accordance with the guidelines of the Animal Ethics Committees of the Institute for Medical and Veterinary Science and the University of Adelaide, Adelaide, Australia, and the Institutional Animal Care and Use Committee of The University of Iowa, Iowa City, Iowa, USA.

In vitro mouse colonic primary afferent preparations

A novel *in vitro* preparation was developed for these studies (*Figure 1.1*). Male and female mice (C57BL/6) 20-30g were killed via CO_2 inhalation and cervical dislocation. The colon (5-6cm) and mesentery (containing the lumbar colonic nerves) were removed intact along with either the attached neurovascular bundle containing the inferior mesenteric ganglion and LSN or in separate preparations with the major pelvic ganglion and PN. The tissue was transferred to ice cold Krebs' solution and, following further dissection, the distal colon was opened longitudinally along the anti-

mesenteric border to orientate lumbar colonic insertions to lie along the edge of the open preparation. The tissue was pinned flat, mucosal side up, in a specialized organ bath consisting of two adjacent compartments machined from clear acrylic (Danz Instrument Service, Adelaide, South Australia), the floors of which were lined with Sylgard® (Dow Corning Corp., Midland, MI, USA). The PN or neurovascular bundle containing the LSN were extended from the tissue compartment into the recording compartment where they were laid onto a mirror. A movable wall with a small "mouse hole" (to allow passage of the nerves) was lowered into position and the recording chamber filled with paraffin oil. The colonic compartment was superfused with a modified Krebs' solution (in mM: 117.9 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4(H_2O)_7$, 2.5 $CaCl_2$, 11.1 d-glucose, 2 sodium butyrate, and 20 sodium acetate), bubbled with carbogen (95% O_2 / 5% CO_2) at a temperature of 34°C. All preparations contained the L-type calcium channel antagonist nifedipine (1 μM) to suppress smooth muscle activity and the prostaglandin synthesis inhibitor indomethacin (3 μM) to suppress potential inhibitory actions of endogenous prostaglandins[112]. Under a dissecting microscope, the LSN were dissected away from the neurovascular bundle and the nerve sheath surrounding the LSN or PN peeled gently back exposing the nerve trunk. Using fine forceps, the nerve trunk was teased apart into 6-10 bundles which were individually placed onto a platinum recording electrode. A platinum reference electrode rested on the mirror in a small pool of Krebs' solution adjacent to the recording electrode.

Characterisation of LSN and pelvic afferent properties

Receptive fields were identified by systematically stroking the mucosal surface or the mesenteric attachment with a brush of sufficient stiffness to activate all types of mechanosensitive afferent. Once identified, receptive fields were tested with three distinct mechanical stimuli to enable classification: focal compression of the receptive field via a perpendicular probing stimulus with calibrated von Frey hairs (70, 160, 400, 1000mg, 2g and 4g force; each force applied 3 times for a

period of 3 sec), mucosal stroking with calibrated von Frey hairs (10, 200, 500, and 1000mg force; each force applied 10 times) and circular stretch (1-5g, in 1g increments; each weight applied for a period of 1 min, with a 1 min interval between each application). Stretch was applied using a claw made from bent dissection pins attached to the tissue adjacent to the afferent receptive field and connected to a cantilever system via thread[106, 107]. Weights were applied to the opposite side of the cantilever system to initiate graded colonic stretch. Only circular, and not longitudinal, stretch was tested in this study. Unlike the human, there were no observable structural differences of the muscle layers when comparing the distal colon and rectum in the mouse. There were no observable differences in the amount of tissue stretch when the same load was applied to either the distal colon or rectum suggesting that the compliance of the muscle in the two regions was similar. Although differenes in compliance between the two regions cannot be totally excluded any such differences may have been reduced by the addition of nifedipine in the current study. Moreover, a recent study in guinea-pig rectum indicates that studying the afferent firing evoked by von Frey hair compression allows a more accurate comparison of afferent properties when smooth muscle tone is altered [95]. Colonic afferents were characterized using the classification system previously applied in the rat colon[109, 112, 113].

Data recording and analysis

Electrical signals generated by nerve fibers placed on the platinum recording electrode were fed into a differential amplifier, filtered, sampled at a rate of 20 kHz using a 1401 interface (Cambridge Electronic Design, Cambridge, UK) and stored on a PC. The amplified signal was also used for online audio monitoring. Action potentials were analysed off-line using the Spike 2 wavemark function and discriminated as single units on the basis of distinguishable waveform, amplitude and duration. A maximum of two active units on each recorded strand was allowed so as to avoid errors in discrimination. Data are expressed as mean ± SEM. n = the number of individual

afferents. Adaptation profiles to probing were calculated as the mean number of spikes per 100 msec bin over the entire 3 sec of a 1g probing stimulus. Adaptation profiles to stretch were calculated as the mean number of spikes per 10 sec bin over the entire 60 sec of a 3g circular stretch. Curve slopes were derived from regression analyses performed on stimulus-response functions and adaptation data to compare the gain and rates, respectively, of afferent responses. The limited number of stimuli used at lower stimulus intensities prevented meaningful extrapolation of afferent thresholds to von Frey probing, mucosal stroking, and circular stretch. Extrapolation of threshold values for stimulation based on a linear regression analysis of stimulus-response functions was also not performed because of the non-linear nature of the data within this stimulus range and nonsensical (i.e. negative) x-intercepts that were obtained from this analysis. Therefore, to compare the sensitivity of LSN and pelvic afferents to von Frey probing, the percentage of afferents that responded to each probe force was calculated based on the total number of fibers tested in each afferent class ("percent responding"). Data were analysed using Prism 4 software (GraphPad Software, San Diego, CA, USA), and when appropriate, were analysed using a two-way analysis of variance (ANOVA) or repeated measures, two-way ANOVA to determine significant differences between curves. Differences were considered significant at a level of $P < 0.05$.

Results

Basic mechanosensory properties

Four classes of LSN afferent fiber could be distinguished from one another by their responses to mechanical stimuli (*Figure 1.2A*). These four fiber classes were termed mesenteric, serosal, muscular and mucosal. All four classes were responsive to focal compression of their receptive fields via a perpendicular probing stimulus. However, it was their location and response, or lack thereof, to the other types of mechanical stimuli (i.e. fine mucosal stroking and circular stretch) that clearly determined their class. Mesenteric afferents had receptive fields that were located on the

mesenteric attachment directly adjacent to the colon and responded in a graded manner to probing of their receptive fields with calibrated von Frey hairs (*Figure 1.2A(i)*). Due to their location on the mesenteric attachment, these afferents were not tested with circular stretch or mucosal stroking. Serosal afferents had receptive fields located in the colonic wall that were reproducibly activated only by focal compression of their receptive fields via a perpendicular probing stimulus and did not respond to colonic stretch or fine mucosal stroking with a 10mg von Frey hair (*Figure 1.2A(ii)*). Muscular afferents had receptive fields in the colonic wall that were optimally activated by focal compression of their receptive fields via a perpendicular probing and maintained circular stretch ≥ 2mm, but were not responsive to fine mucosal stroking (*Figure 1.2A(iii)*). In response to circular stretch, muscular afferents responded with an excitation that adapted completely during the stimulus (waning to pre-stimulus levels within 10-20 sec). Both serosal and muscular afferents responded to mucosal stroking at higher stimulus intensities (500-1000mg; data not shown), which distort the underlying muscle and serosal tissue layers. However, mucosal receptors were most sensitive to stroking of their receptive fields with a brush or a 10 mg von Frey hair (*Figure 1.2A(iv)*). Their exact thresholds to mucosal stroking could not be determined because smaller hairs would not penetrate the surface tension of the superfusate. These afferents responded in a graded manner to an ascending series of stroking stimuli (10-1000mg; data shown for 10mg), but not to circular stretch.

All classes of LSN afferent had small (≤ 0.5mm) punctate receptive fields from which responses could be most readily evoked. The vast majority of LSN afferents possessed a single mechanosensitive receptive field; only a small minority (15%) of mesenteric afferents possessed more than one receptive field, a property previously described for LSN mesenteric afferents[103, 109, 127, 305, 306]. Overall, the majority of mesenteric, serosal and muscular afferents and all mucosal afferents recorded from the LSN were silent at rest. However, a minority of mesenteric (27%), serosal (37%), and muscular (40%) afferents in this pathway did exhibit low rates of spontaneous activity (0.38 ±

0.12 spikes/sec, 0.48 ± 0.09 spikes/sec, 0.68 ± 0.36 spikes/sec, respectively) that were not significantly different among fiber classes ($P > 0.05$). The discharge rate of spontaneously active LSN afferents observed were similar to those previously reported[105, 112].

Pelvic afferents, similar to LSN afferents, could be categorized into four different classes; three were also found in the LSN (serosal, muscular, and mucosal) and one was unique to the PN (muscular/mucosal). All PN afferents responded in a graded manner to probing of their receptive field but could be distinguished from one another by their sensitivity to fine mucosal stroking and circumferential stretch. Unique to the PN were afferents that responded to both fine stroking and stretch and this class was therefore termed muscular/mucosal (*Figure 1.2B(i)*). Serosal, muscular, and mucosal afferents in the PN possessed the same general response properties as their respective counterparts in the LSN: serosal afferents responded only to focal compression of their receptive fields via a perpendicular probing stimulus (*Figure 1.2B(ii)*), muscular afferents responded to stretch but not fine stroking (*Figure 1.2B(iii)*) and mucosal afferents responded to fine stroking but not stretch (*Figure 1.2B(iv)*). Notably, mesenteric afferents, often recorded from the LSN nerve, were never found in the pelvic nerve. No pelvic afferents exhibited spontaneous activity, a trait similar to that previously reported for mechanosensitive pelvic afferents *in vivo*[75, 114], and possessed single receptive fields that were, in general, punctate like those in the found in the LSN, although no formal analysis of their exact dimensions was performed.

Topographical and population distribution of afferent subtypes

Of the fibers recorded from the LSN, 50% had receptive fields in the mesenteric attachment (*Figure 1.3A and 1.3B*). These were scattered along the mesentery, primarily aboral to the point where the LSN and inferior mesenteric artery branch out to form the lumbar colonic nerves and lumbar colonic arteries. Commonly, mesenteric afferent receptive fields were located on or near

these primary lumbar colonic blood vessels. Serosal afferents were the second most abundant class of afferent fiber (36%; *Figure 1.3A and 1.3B*). Their receptive fields were concentrated near the mesenteric attachment throughout the entire length of the colon except for the rectum and anal canal. Muscular and mucosal afferents were relatively scarce, consisting of only 10% and 4%, respectively, of the afferents recorded from the LSN (*Figure 1.3A and 1.3B*). Muscular afferents were clustered in the lower regions of the distal colon, whilst mucosal afferents were located more proximally. In general, the distribution of LSN afferent receptive fields located within the colonic wall (i.e. serosal, muscular and mucosal) were concentrated near the mesenteric attachment and none were found more than 180 degrees around the colon from the mesenteric attachment (*Figure 1.3A*), a pattern described previously in a similar *in vitro* preparation of the LSN innervation of the rat colon[112, 113].

Compared to the LSN, the afferent population of the PN had a very different representation and receptive field distribution (*Figures 1.3C and 1.3D*). Receptive fields of pelvic afferents were distributed throughout the distal three centimetres of the colon, including the rectum and anal canal, a region devoid of LSN receptive fields in this study. Consequently, the majority of pelvic receptive fields were situated distal to those of LSN afferents. Also, pelvic afferent receptive fields were found throughout the circular axis of the colon, and unlike those found in the LSN, were not clustered near the mesenteric attachment (*Figure 1.3C*). No obvious differences were noted in the distribution of receptive fields among the four classes of PN afferents. Like the LSN, serosal afferents were the most abundant class found in the PN (33%), but considerably more afferents responsive to fine mucosal stroking and circular stretch were found in the PN than the LSN, with mucosal, muscular, and muscular/mucosal afferents together comprising over two-thirds (23%, 21%, and 23%, respectively) of all mechanosensitive pelvic afferents (*Figure 1.3D*). As stated above, no mesenteric afferents were found in the PN.

Dynamic responsiveness to graded probing

All LSN afferents displayed graded responses to focal compression of their receptive fields via an ascending series of perpendicular probing stimuli with similar stimulus-response functions and adaptation profiles despite major differences in their receptive field location (*Figure 1.4A and 1.4B*). No significant differences were detected in either the magnitude or slope of stimulus-response functions (*Figure 1.4A*) or adaptation curves (*Figure 1.4B*) among the four classes of LSN afferents ($P > 0.05$ for all analyses). To compare the sensitivity of LSN afferents to perpendicular probing, the percentage of afferents in each class that were activated by the range of probe forces used in this study were plotted (*Figure 1.4C*). All mucosal LSN afferents were activated by the lowest probing force (70mg). Significantly fewer serosal (21%; 4/19), muscular (20%; 1/5), and mesenteric (34%; 9/26) LSN afferents were activated by this force ($P < 0.05$; *Figure 1.4C*). The percentage of these afferent classes activated by ascending probing stimuli increased similarly until 100% were activated by 2g and 4g ($P > 0.05$; *Figure 1.4C*). Splanchnic afferents in the mesentery and serosa, despite originating from different tissue types, were notably similar in their responsiveness, adaptation profiles, and activation thresholds to probing (*Figure 1.4A-C*).

Pelvic serosal, muscular, mucosal, and muscular/mucosal afferents all demonstrated graded responses to perpendicular probing that adapted throughout the stimulus (*Figure 1.4D and 1.4E*). No significant differences were detected among the four pelvic afferent classes, in terms of either the magnitude or slope of the probing stimulus-response functions ($P > 0.05$ for all analyses). However, among pelvic afferents, the adaptation profile of mucosal afferents were significantly shallower than those of serosal or muscular afferents ($P < 0.05$). All muscular/mucosal afferents were activated by the lowest probing stimulus (70mg), while significantly fewer serosal, muscular and mucosal afferents (28%; 5/18, 42%; 5/12, and 62%; 8/13, respectively) were activated by this probe intensity

($P < 0.05$; *Figure 1.4F*). Probing with a 1g von Frey hair activated 100% of all four classes of pelvic afferents.

These data described above on the dynamic mechanosensory properties of mouse LSN and pelvic afferents confirm that responses to focal compression of receptive fields via von Frey probing discriminate poorly among afferent classes compared to stimuli directed at specific tissue layers (fine mucosal stroking and circular stretch).

Comparison of dynamic mechanosensory properties between LSN and PN afferents

The most prevalent afferent classes encountered in both the LSN and PN were serosal and muscular afferents. A comparison of the dynamic properties of these afferents revealed that they differed considerably between the two pathways. Despite the fact that serosal afferents from both nerves displayed graded responses to focal compression of increasing probing stimuli, LSN afferents were significantly less responsive to stimulation than pelvic afferents ($P < 0.001$, *Figure 1.5A*). Splanchnic and pelvic serosal afferents also displayed significantly different adaptation profiles to probing ($P < 0.001$, *Figure 1.5B*). Splanchnic serosal afferents adapted more completely to background activity levels than those in the pelvic pathway, taking 0.3 sec compared to 1.4 sec to adapt to 50% of initial response, respectively. Furthermore, LSN afferents adapted to 25% of initial response in 1.1 sec, whilst pelvic afferents adapted only to 32% of initial response by the end of the 3 sec stimulus. Although similar proportions of LSN (21%; 4/19) and pelvic (28%; 5/18) serosal afferents responded to the lowest probing stimulus, pelvic afferents had a significantly higher percent responding curve, with 100% activated by a 1g probe compared with only 70% of LSN serosal afferents ($P < 0.05$; compare *Figures 1.4C and 1.4F*). Therefore, LSN serosal afferents required higher intensity probing stimuli to be activated, evoked smaller responses to a given probing stimulus and adapted more completely to probing than pelvic serosal afferents.

A comparison of the functional properties of muscular afferents in the LSN and PN revealed differences comparable to those observed between serosal afferents in the two pathways (*Figure 1.5C-F*). Splanchnic muscular afferents were less responsive to focal compression with von Frey probing than pelvic muscular afferents, with significantly lower stimulus-response functions ($P < 0.001$, Figure 5C) and percent responding curves, with 25% of LSN and 42% of pelvic afferents responding to a 70mg probe and 75% of LSN and 100% of pelvic muscular afferents activated by a 1g probe ($P < 0.05$, compare *Figures 1.4C and 1.4F*). In contrast to serosal afferents, there were no significant differences in the slopes of the adaptation profiles to probing stimuli when comparing LSN and pelvic muscular afferents ($P > 0.05$). Overall, these data show that pelvic muscular afferents evoked larger responses to focal compression via probing stimuli and were activated by lower probing stimulus intensities than LSN muscular afferents. The functional differences between LSN and pelvic muscular afferents were also manifested in their response to stretch. Pelvic muscular afferents were more responsive to circular stretch than LSN afferents, with significantly higher stretch stimulus-response functions ($P < 0.01$, *Figure 1.5E*) and stretch adaptation profiles (*Figure 1.5F*). Splanchnic muscular afferents adapted completely to background levels within 20 sec of the onset of the stretch stimulus (*Figure 1.5F and Figure 1.2A(iii)*), whilst most pelvic muscular afferents fired throughout the entire duration of the 1 min stretch (*Figure 1.5F and Figure 1.2B(iii)*). Thus, in response to stretch, pelvic muscular afferents evoked larger responses and adapted less completely than LSN muscular afferents.

Dynamic properties of PN muscular/mucosal afferents

The pelvic nerve contained afferent fibers with properties not found in those recorded from the LSN. These fibers possessed properties of both muscular and mucosal afferents in that they were excited by circular stretch and fine mucosal stroking and were therefore termed muscular/mucosal afferents. *Figure 1.6A* depicts mucosal stroking stimulus-response functions for

all four afferent classes found in the PN. Muscular/mucosal afferents were similar to mucosal afferents in their response to stroking. Specifically, only mucosal and muscular/mucosal afferents generated responses to receptive field stroking with the lowest intensity filament (10mg) and these two classes of afferent had stroking stimulus-response functions significantly different from other PN afferents ($P < 0.001$, *Figure 1.6A*). Muscular/mucosal afferent responses to circular stretch were more complex than their responses to stroking. Seven of 13 muscular/mucosal afferents exhibited stretch stimulus-response functions that were similar to those of muscular afferents while the remaining six muscular/mucosal afferents responded much more robustly, with significantly higher stimulus-response functions compared to muscular afferents ($P < 0.001$, *Figure 1.6B*). Consequently, the former subclass of muscular/mucosal afferents were termed low-responders and the latter subclass termed high-responders. This distinction was manifest as a significantly elevated response to stretch during both the initial dynamic phase, immediately following the application of stretch, and the sustained tonic phase ($P < 0.001$, *Figure 1.6C*).

Discussion

The present study defines the mechanosensory properties of spinal afferents from the mouse colon that are found in the LSN and PN. This study has identified five different classes of afferent fiber, each capable of detecting different types of mechanical stimuli. Three of these fiber classes (serosal, muscular, and mucosal) were conserved between both pathways but displayed significantly different functional properties. Each pathway also contained a unique class of afferent fiber, mesenteric afferents found in the LSN and muscular/mucosal afferents in the PN. Consequently, these data indicate that both nerves are critical in relaying mechanosensory information from the colon but that each contains afferent fibers individually tuned to detect distinct types of mechanical stimuli and respond with differing sensitivities. These findings demonstrate the great detail in which mechanical events in the colonic environment are signalled to the central

nervous system. They lead us to consider that the rich diversity in mechanosensory properties of colonic afferents may be comparable with those innervating the skin[101]. Moreover, this investigation provides the first information on colonic afferents in mice, establishing a basis for studies of the role that specific gene products play in colon sensory function.

Differences in the topographical distribution of LSN and PN afferents

In general, the distribution of LSN and PN afferent receptive fields was very different. Receptive fields from the LSN were located either on the mesentery or within the colonic wall but concentrated near the mesenteric attachment and none were found more than 180 degrees around the colon from the mesenteric attachment. In contrast, the receptive fields of PN afferents were distributed throughout the distal three centimetres of the colon, including the rectum and anal canal, a region devoid of LSN receptive fields in this study. Consequently, the majority of PN receptive fields were situated distal to those of LSN afferents. Also, PN afferent receptive fields were found throughout the circular axis of the colon, and unlike those found in the LSN, were not clustered near the mesenteric attachment. These differences may reflect the respective roles in which the LSN and PN pathway are involved in the signalling of colonic events. For example 85% of LSN afferents innervate the mesentery and serosa and as such this distinctive distribution may represent the most efficient layout of receptive fields that were designed to detect events on or close to the mesenteric attachment. By contrast, the vast majority of PN afferents were equipped to detect either events in the lumen or colorectal stretch and as such receptive fields located throughout the circular axis of the colon may represent the most efficient layout for the detection of events within the colonic wall.

Function of afferent classes shared by LSN and pelvic pathways

Based on their functional properties, serosal, muscular, and mucosal afferents found in the LSN and pelvic pathways likely signal very different mechanical events. Serosal afferents were the

most abundant population, accounting for approximately one third of all fibers found in this study. They were activated optimally by a perpendicular probing stimulus applied directly to their receptive fields. Serosal afferents have been previously described in the LSN of the rat[112, 113] and the cat[105, 108], this study shows a similar distribution of LSN afferents in approximately equal proportions as those found previously in the LSN of the rat using an *in vitro* preparation[112, 113]. However, the present study is the first to use graded stimuli to establish stimulus-response relationships and are, therefore, unable to qualitatively compare functional differences with previous reports. Serosal afferents have not been encountered in prior studies of mechanosensitive PN afferents in rat[110] or cat[75] using *in vivo* single-fiber recording techniques, probably because afferents were identified in these studies based on their response to distension without direct access to the location of their receptive fields. Indeed, these studies estimated that colon distension activated just 5%[110] and 16%[75] of all pelvic nerve afferents, making it likely that serosal afferents in these species were overlooked by the employed mechanical stimulus. Pelvic serosal afferents have also been identified in preliminary studies recording from the sacral hypogastric nerves innervating the colon of the rat displaying similar properties to those characterised here[307]. Serosal afferents have probably been encountered before in the PN of the rat however either an adequate stimulus was not applied or were classified as not responding to colorectal distension (CRD) or urinary bladder distension[110]. Alternatively serosal afferents could correspond with high threshold fibres (for response to CRD >30mm Hg) which are traditionally thought to be nociceptive[110]. Interestingly, the proportions of PN serosal afferents in the present study are comparable to the percentage of high threshold afferents in response to CRD. Moreover, it is of note that the proportion of high threshold afferents is less when tested with fluid colorectal distension compared with balloon CRD[110, 111]. Indeed 43% of all distension sensitive afferents from the LSN had receptive fields which are located in the mesentery or in the colonic wall close to the mesentery[105]. It is possible that over certain pressures (>30 mm Hg) CRD with a balloon induces a focal compression equivalent to a probing stimulus directly to the serosal receptive field

located within the colonic wall. However the precise function of serosal afferents remains unclear. It is equally conceivable that neither pelvic nor LSN serosal afferents are activated by physiological levels of distension or contraction *in situ*. In this case they may signal transient, sharp pain at the onset of spasm or distension due to rapid transit of contents or experimental balloon inflation, during which acute intense mechanical stimulation might be achieved. Although each pathway contributes equally in serosal afferent signalling from the colon they send very different signals to the spinal cord. This can be deduced from a number of functional differences between PN and LSN afferents. Pelvic afferents were clustered more distally, exhibited less adaptation, and responded across a wider stimulus range than LSN afferents. Consequently, PN serosal afferents would be expected to generate a more intense and sustained afferent barrage in response to these acute mechanical events when they occur in more distal portions of the descending colon and rectum than serosal afferents in the LSN would generate in response to this type of event.

Muscular afferents accounted for 21% and 10% of all PN and LSN afferents, respectively, recorded in this study. For the LSN, the proportion of muscular afferents found in this study is similar to previous reports of muscular afferents in the LSN of rat using an *in vitro* technique[112, 113], however it is much lower than the percentage of distension-sensitive afferents (presumably equivalent to muscular afferents) among all mechanosensitive afferents reported in the LSN of the cat *in vivo*[105]. It is possible that the proportion of stretch-sensitive afferents was underestimated in the *in vitro* studies, including the current one, because only circular, and not longitudinal, stretch was used. Alternatively, the LSN innervation of the cat may contain many more stretch-sensitive afferents than the rat or mouse. Because this study is the first report of muscular afferents in the PN using an *in vitro* technique in any species, it is difficult to compare with previous *in vivo* studies of muscular afferents in the rat[110] and cat[75] that used only colon distension to identify mechanosensitive fibers. However, stretch-sensitive afferents constitute over 50% of the total mechanosensitive PN afferent population

in this study (muscular and muscular/mucosal afferents combined) and is similar to the predominance of distension-sensitive afferents among afferents responding to mechanical stimulation of the colon and anal canal described in both rat[110] and cat[75].

Muscular afferents in the mouse LSN and PN were located primarily in the distal colon and activated by low intensity circular stretch and direct probing of their receptive fields. However, they differed in three critical aspects. First, LSN muscular afferents were less likely to respond to probing at lower stimulus intensities (< 1g) than PN muscular afferents (compare *Figures 1.4C* and *1.4F*). Second, PN muscular afferents showed greater responses to both probing and stretch (*Figures 1.5C* and *1.5E*) and did not adapt completely during stimulus application (*Figures 1.5D and 1.5F*) compared to LSN muscular afferents. Third, stretch-sensitive PN afferents (including muscular/mucosal afferents - see below), greatly outnumber stretch-sensitive LSN afferents (compare *Figures 1.3B* and *1.3D*). Taken together, these differences indicate that the PN is better equipped to respond to stretch of the colonic wall and contains muscular afferents that respond more robustly to tonic stretch than those found in the LSN. Also, their sustained response during maintained stretch suggests that PN muscular afferents are more likely to signal tonic changes in the calibre or wall tension of the distal colon, such as during the presence of stool or gas. In contrast, the higher stimulus intensities required to activate and more completely-adapting responses of LSN muscular afferents, which occur in much lower proportions, would be better tuned to signal the onset of higher intensity mechanical events, such as muscular contraction or passage of material, which are of a more acute nature. This notion is supported by a study in rats demonstrating that the PN alone is sufficient to maintain behavioural responses to sustained colorectal distension[60] and a discussion of human data proposing that LSN afferents are better able to convey phasic rather than tonic changes in colonic pressure[2]. However, stretch-sensitive afferents in both pathways encode beyond the physiological range of distension and may therefore transmit nociceptive information.

Although the greater mechanosensitivity of PN muscular afferents relative to LSN muscular afferents has been previously described in the cat[76, 104], it was not until the current study that direct comparisons between the two pathways in the same setting have been performed to clearly delineate the different sensitivity of muscular afferents in the PN and LSN.

Afferents that respond to colonic stretch, applied either directly *in vitro* or indirectly using colon distension *in vivo*, have been identified and extensively characterized previously[75, 104, 105, 108-115]. Muscular afferents, as a whole, are responsive to small changes in intraluminal pressure, respond to colonic stretch or distension with a linear relationship to wall tension, and encode these stimuli well into the noxious range. Additionally, two basic types of distension-evoked afferent responses have been described: phasic and tonic[75, 105, 108, 110, 111, 115]. The roles of these two types of afferent likely differ as phasic afferents are only transiently excited during stimulus onset and offset, whereas tonic afferents discharge throughout the stimulus duration[75]. Comparisons of these results with those previously published are complicated by the heterogeneity of species, techniques, and mechanical stimuli used in these studies. However, the stretch-sensitive muscular afferents reported here closely resemble muscular afferents described in the LSN of the rat colon *in vitro*[109, 112, 113] and the distension-sensitive, low threshold afferents described *in vivo* in the LSN of the cat[105] and PN of the cat[105, 108, 115] and rat[110, 111]. All muscular and muscular/mucosal afferents recorded in this study responded to the lowest intensity stretch (1g), encoded stimuli well into the noxious, nonphysiological range, and exhibited exclusively tonic-type responses.

Mucosal afferents accounted for 23% of the PN and 4% of the LSN innervation in the present study. They exhibited different distributions, with PN mucosal afferents localised in the most distal region of the colon whilst the few LSN mucosal afferents were found more proximally. Although the rarity of LSN mucosal afferents makes it difficult for direct comparison with those in the PN, both

display adapting responses to low threshold mucosal stroking or probing (compare *Figures 1.4B* and *1.4E*) and an insensitivity to circular stretch (compare *Figures 1.2A(iv)* and *1.2B(iv)*). These data suggest that the signalling of fine mechanical stimulation of the colonic mucosa occurs predominantly via the PN pathway as nearly half of the PN afferent population (including muscular/mucosal afferents) were capable of detecting fine mucosal stroking (*Figure 1.3D*). Colonic mucosal afferents have been characterized *in vitro* from the LSN[112] and colonic hypogastric nerves of the rat[308], and in the PN *in vivo* with receptive fields in the anal canal of the cat[75, 114] and perianal mucosa of the rat[110]. However, the current study is the first to fully characterize the mechanical response properties of colorectal mucosal afferents in the LSN and PN of the mouse and demonstrate a widespread distribution of mucosal afferents throughout the distal colon and not restricted solely to the anal mucosa. In fact, it is seems surprising to find so many afferents sensitive to fine mucosal stimulation in such a large region of the distal colon and rectum considering the vagueness of colonic sensations. Therefore the only logical conclusion is that these and muscular/mucosal afferents (see below) must be placed either to provide fine mucosal input to reflexes controlling motility[124-126] and/or to refine the quality of perceived stimuli. Whichever is the case, mechanical signals from the colonic mucosa are carried predominantly by the PN and may contribute to the large proportion of distension-insensitive afferents reported previously[75, 110].

Mesenteric afferents specific to the LSN pathway

Mesenteric afferents are the largest fiber population recorded from the colon, accounting for 50% of the LSN innervation, and were specific to this pathway. Mesenteric afferents displayed similar probing response properties to LSN serosal afferents. Consistent with other studies, mesenteric afferents in the present study were located close to or on blood vessels or branching points of capillaries supplying the serosa and occasionally displayed multiple receptive fields, similar to mesenteric afferents previously reported in other species[75, 103, 105, 108-110, 112, 127]. Although not

routinely tested in the present study, it is clear that mesenteric afferents can be activated briefly by intense mesenteric stretch and colonic distension in addition to stimuli applied to their receptive fields[103, 105, 112]. It has been proposed that these afferents may detect twisting and torsion of the colon and pulsatile changes in blood pressure in mesenteric blood vessels, possibly critical during plasma extravasation resulting from colonic inflammation[74, 309]. The preponderance of mesenteric and serosal afferents in the LSN that required higher intensity mechanical stimulation for activation supports its role as a higher intensity threshold pathway than the PN.

Muscular/mucosal afferents specific to the PN pathway

Muscular/mucosal afferents accounted for 23% of all PN afferents, yet they have not been reported previously in the colon. Similar afferents have, however, been reported in the vagus nerve supply to the ferret esophagus known as tension/mucosal receptors[106]. The present study showed that PN muscular/mucosal afferents were clustered in the distal colon (*Figure 1.3C*). They displayed graded responses to mucosal stroking that were similar to PN mucosal afferents (*Figure 1.6A*) and adapted only partially to circular stretch (*Figure 1.6C*). Pelvic muscular/mucosal afferents could be divided into two distinct populations, high-responders and low-responders, based on their stretch stimulus-response functions (*Figure 1.6B*): low-responding muscular/mucosal afferents were comparable to muscular afferents while the high-responding population was the most sensitive of all afferents to circular stretch, generating larger responses during both the initial phase and throughout the entire duration of stretch (*Figure 1.6C*). The purpose of these two distinct subpopulations of muscular/mucosal afferents that are unique to the PN pathway is unknown. In addition, the anatomical structure of the receptive endings of these afferents that confers upon them the ability to detect both fine mucosal stimuli within the lumen and stretch of the colon wall is unknown, however it has been suggested that their endings might have a receptive field interposed in the muscularis mucosa and play a specialized role in the detection of rapidly moving boluses[48, 106]. Alternatively,

muscular/mucosal afferents may have two separate endings, one in the mucosa and another in the muscle layers of the colon that are spatially indistinguishable with the technique used here. Whichever may be true, PN muscular/mucosal afferents are situated to detect both fine mucosal stroking and circular stretch, thereby enabling them to relay information on a diversity of mechanical events from the distal colon to the spinal cord.

Advantages and disadvantages of in vitro preparations

The relatively recent advent of *in vitro* preparations has allowed a greater understanding of the functional biology of visceral afferents. These *In vitro* preparations, such as the ones developed in the current study, have many advantages over the previously described *in vivo* preparations. Firstly, *in vitro* preparations allow greater accessibility of afferent receptive fields resulting in a more discrete and highly reprodubile application of both mechanical and chemical stimuli. In the current study this has allowed accuarate mapping of receptive field distributions and the precise application of three types of mechanical stimuli (stroking, stretch and focal compression via probing) which would not have been possible in more conventional *in vivo* models. This is important as it has allowed the classification of individual afferents into five separate subclasses and as such a greater understanding of the functional properties of these afferents and how they may contribute to colonic signalling. The highly reprodubile application of chemical stimuli has also allowed the identifcation of a new class of chemosensitive afferent in Chapter 2. Secondly, *in vitro* preparations allow the investigation of afferents in a controlled environment, allowing afferent function to be determined in isolation from the CNS which may complicate analysis via subsequent motor outputs and functional behaviours. Thirdly, recent *in vitro* preparations have also allowed the structure and function of individual afferents to be determined[86, 87, 93-95]. Conversely *in vitro* preparations also have some disadvantages over *in vivo* preparations. Firstly, the vascular supply is interrupted (which is the source of many inflammatory mediators and their precursors), whilst the tissue is constantly

superfused with fresh Krebs solution, which would wash away any locally released mediators. Secondly, the colon is opened longitudinally and placed in an artifical environment. As such it remains unclear how the application of various stimuli, such as circumferential stretch, correlates functionally with an *in vivo* distension stimulus. Finally as these preparations were studied in isolation it remains unclear how afferent signalling is interpreted and processed within the CNS and the subsequent motor outputs and functional behaviours which are generated.

Functional significance

Until now, the full complement of afferent fibers involved in detecting mechanical stimuli and the role they potentially play in the physiology and pathophysiology of the colon were unknown. The present study permits, for the first time, a direct comparison of the functional properties of mechanosensitive afferents from the LSN and PN spinal innervation of the mouse colon. From these data it can be concluded that these two pathways are equipped to detect multiple types of mechanical stimuli and are comprised of three classes of mechanosensitive afferent common to both pathways but possessing distinct functional properties and a class of afferent unique to each pathway. Afferents from both pathways are individually tuned to detect the type of stimulus and the magnitude and duration of stimulus intensity. Overall, the PN pathway contains a greater number of afferents tuned to detect circular stretch, likely the primary mechanical stimulus generated by colorectal distension in natural or experimental situations. They are, as a whole, more sensitive and responsive to mechanical stimulation than the LSN pathway. Therefore, these data indicate that the PN and LSN pathways constitute high- and low-gain pathways, respectively, for the detection of mechanical stimuli in the distal colon. The sheer scale of mechanosensory information detected from the colon evident from this study is staggering and surely contributes to intrinsic and extrinsic reflexes, as well as conscious innocuous and noxious perceptions from the colon. This study provides a detailed understanding of colonic mechanotransduction that is crucial to assessing the

function of colon sensory afferents under normal and diseased conditions. Moreover, the current study provides the basis for experiments using transgenic mice on the very nature of mechanotransduction, an area in which pharmacological tools are limited and where genetic approaches have proven invaluable[9, 10]. Such studies will be useful in revealing promising therapeutic molecular targets for the treatment of functional bowel disorders.

Figure 1.1: Diagram of the novel in vitro electrophysiological preparation developed for these studies. Mouse distal colon/rectum with attached LSN or PN nerves was dissected and placed in a specialized organ bath. The distal colon/rectum was opened longitudinally along the anti-mesenteric border to orientate lumbar colonic insertions to lie along the edge of the open preparation. The tissue was pinned flat, mucosal side up, in a specialized organ bath consisting of two adjacent compartments machined from clear acrylic the floors of which were lined with Sylgard. The PN or neurovascular bundle containing the LSN were extended from the tissue compartment into the recording compartment where they were laid onto a mirror. A movable wall with a small "mouse hole" (to allow passage of the nerves) was lowered into position and the recording chamber filled with paraffin oil. The nerve sheaths were dissected free and single units recorded. Receptive fields were identified by systematically stroking the mucosal surface or the mesenteric attachment with a brush of sufficient stiffness to activate all types of mechanosensitive afferent. Once identified, receptive fields were tested with three distinct mechanical stimuli to enable classification: probing with calibrated von Frey hairs (70, 160, 400, 1000mg, 2g and 4g force; each force applied 3 times for a period of 3 sec), mucosal stroking with calibrated von Frey hairs (10, 200, 500, and 1000mg force; each force applied 10 times) and circular stretch (1-5g, in 1g increments; each weight applied for a period of 1 min, with a 1 min interval between each application). Stretch was applied using a claw made from bent dissection pins attached to the tissue adjacent to the afferent receptive field and connected to a cantilever system via thread. Weights were applied to the opposite side of the cantilever system to initiate graded colonic stretch.

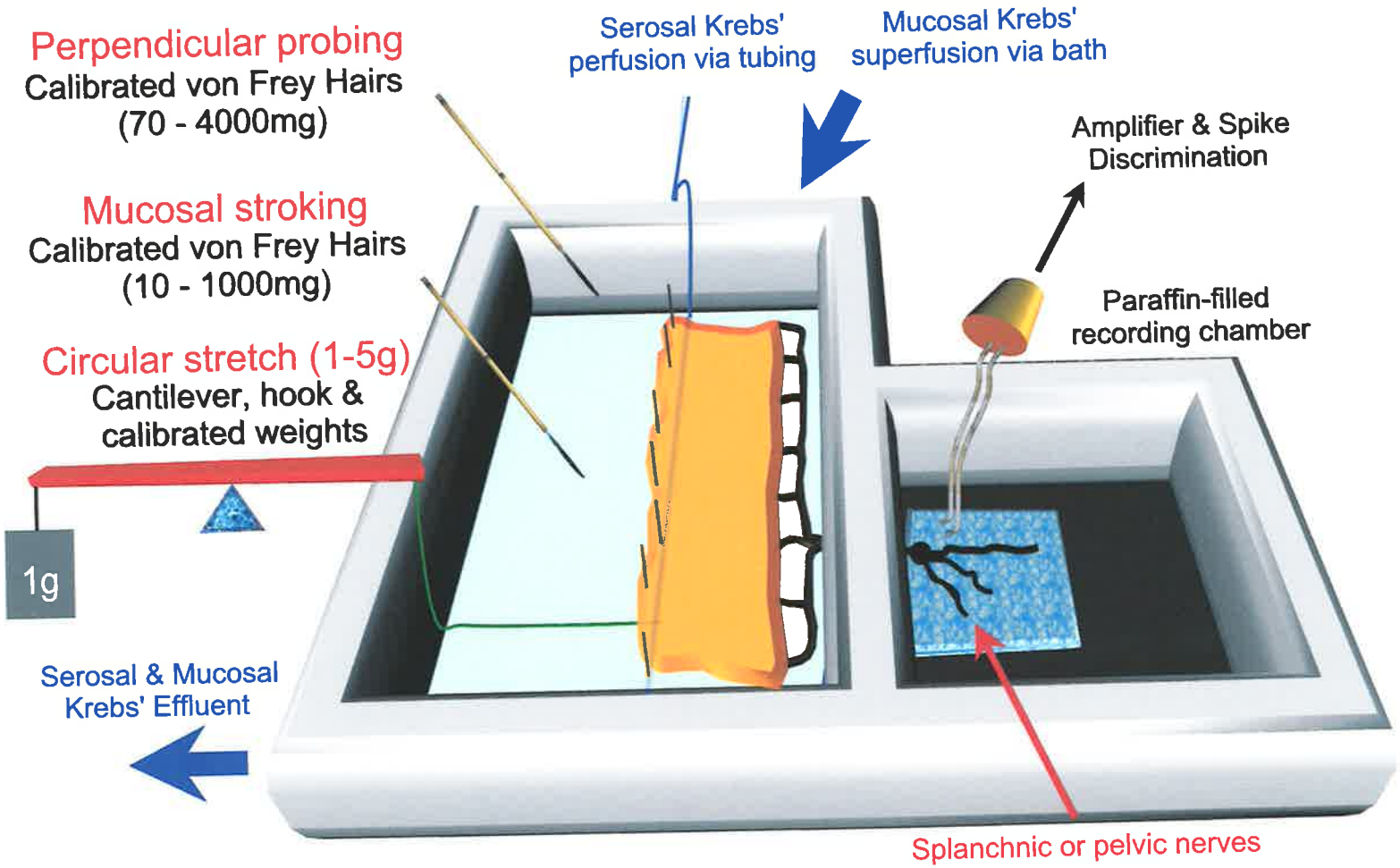

Perpendicular probing
Calibrated von Frey Hairs
(70 - 4000mg)
Mucosal stroking
Calibrated von Frey Hairs
(10 - 1000mg)
Circular stretch (1-5g)
Cantilever, hook &
calibrated weights
1g
Serosal & Mucosal
Krebs' Effluent
Serosal Krebs'
perfusion via tubing
Mucosal Krebs'
superfusion via bath
Amplifier & Spike
Discrimination
Paraffin-filled
recording chamber
Splanchnic or pelvic nerves

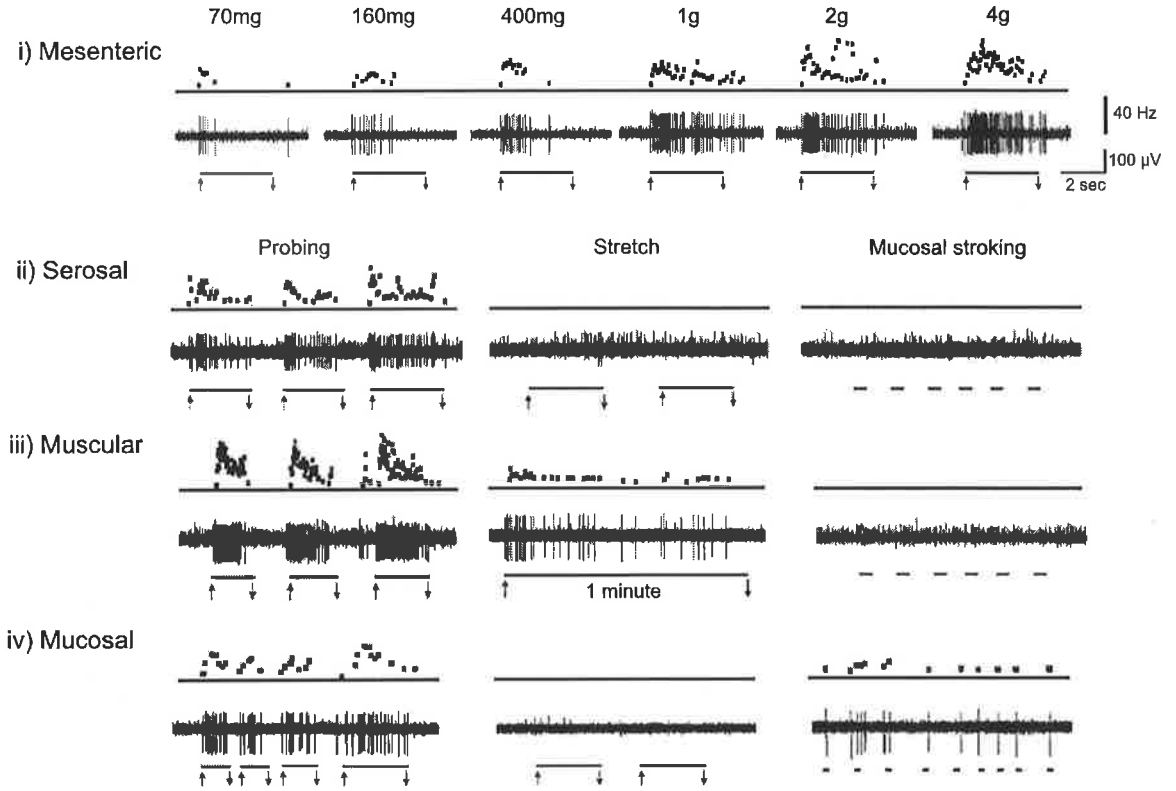
Figure 1.2: A) Four types of LSN afferent classified on the basis of their receptive field location and response to mechanical stimuli. **i)** Mesenteric afferents responded in a graded manner to focal compression via an ascending series of probing stimuli (70mg-4g). **ii)** Serosal afferents were activated only by focal compression of their receptive fields via a perpendicular probing stimulus. **iii)** Muscular afferents were activated by focal compression of their receptive fields via a perpendicular probing stimulus and maintained circular stretch but did not respond to fine mucosal stroking (10mg). **iv)** Mucosal afferents were activated by focal compression of their receptive fields via a perpendicular probing stimulus and fine mucosal stroking but did not respond to circular stretch.

B) Four types of PN afferent classified on the basis of their responses to mechanical stimuli. **i)** Muscular/mucosal afferents were activated by probing, stretch, and fine mucosal stroking (10mg). **ii)** Serosal afferents were activated by focal compression via probing of their receptive field and did not respond to maintained circular stretch or fine mucosal stroking. **iii)** Muscular afferents were activated by focal compression via probing and circular stretch but did not respond to fine mucosal stroking. **iv)** Mucosal afferents were activated by focal compression via probing and fine mucosal stroking but not stretch.

Upper traces show instantaneous firing frequency and lower traces show raw electrophysiological data. Horizontal bars indicate application of stimulus. Scale bars apply throughout except where indicated.

Data from the PN in all figures are provided by Dr Carter Jones, University of Iowa, USA. SMB designed and developed the LSN preparation and was involved in the subsequent development of the PN preparation with CJ. SMB was responsible for designing all of the mechanical stimulation protocols and analytical procedures for both pathways.

A) Splanchnic



B) Pelvic

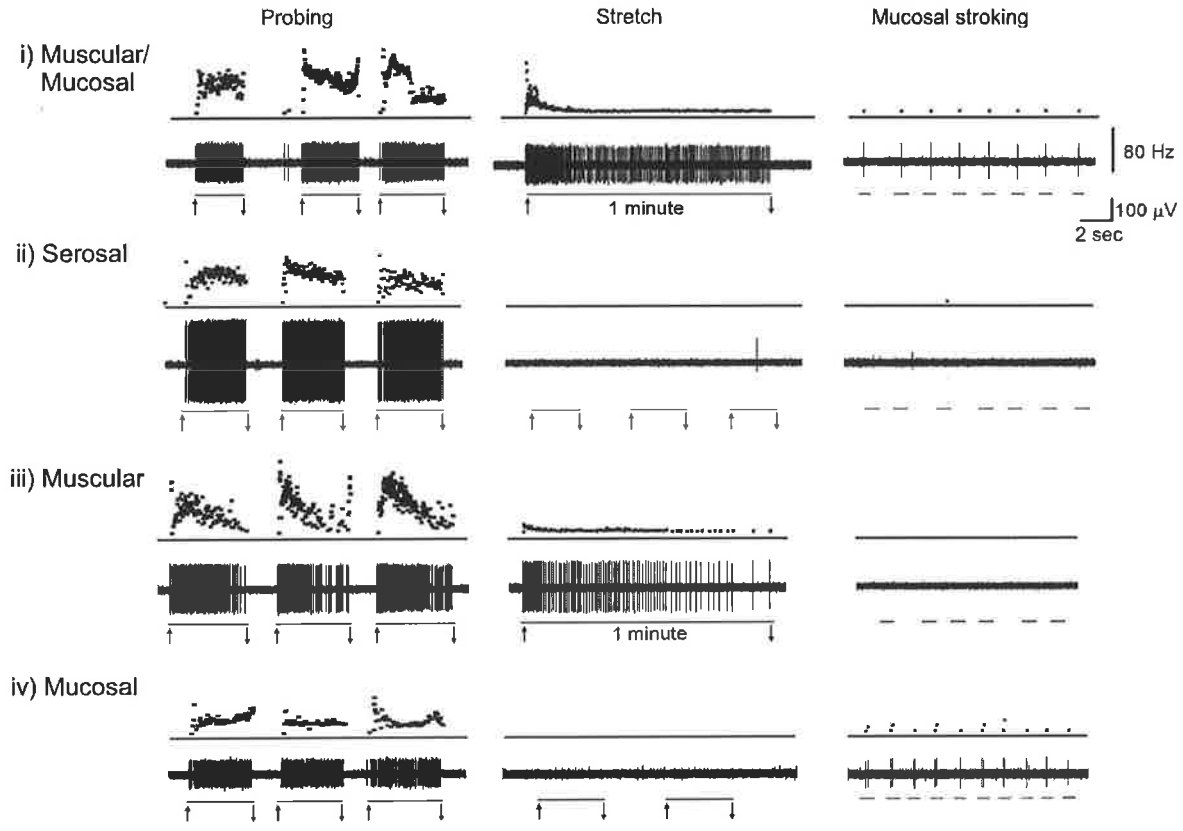


Figure 1.3: Distribution and proportions of afferent classes recorded from the LSN and PN pathways.

A) Splanchnic receptive fields were concentrated on or near the mesenteric attachment and were scattered down the entire length of the colon except for the rectum and anal canal. **B)** The majority of LSN afferents encountered were mesenteric (striped segment) and serosal afferents (grey segment), with the remaining few comprised of muscular (black segment) and mucosal afferents (white segment). **C)** Pelvic afferent receptive fields were scattered across the entire width of the colon and were generally clustered in the lower region of distal colon and rectum. No receptive fields were found on the mesentery. **D)** The largest population of PN afferents encountered were serosal (grey segment), with similar proportions of muscular (black segment), mucosal (white segment) and muscular/mucosal (chequered segment) afferents.

IMG: inferior mesenteric ganglion; LSN: lumbar splanchnic nerve; PN: pelvic nerve; MPG: major pelvic ganglion.

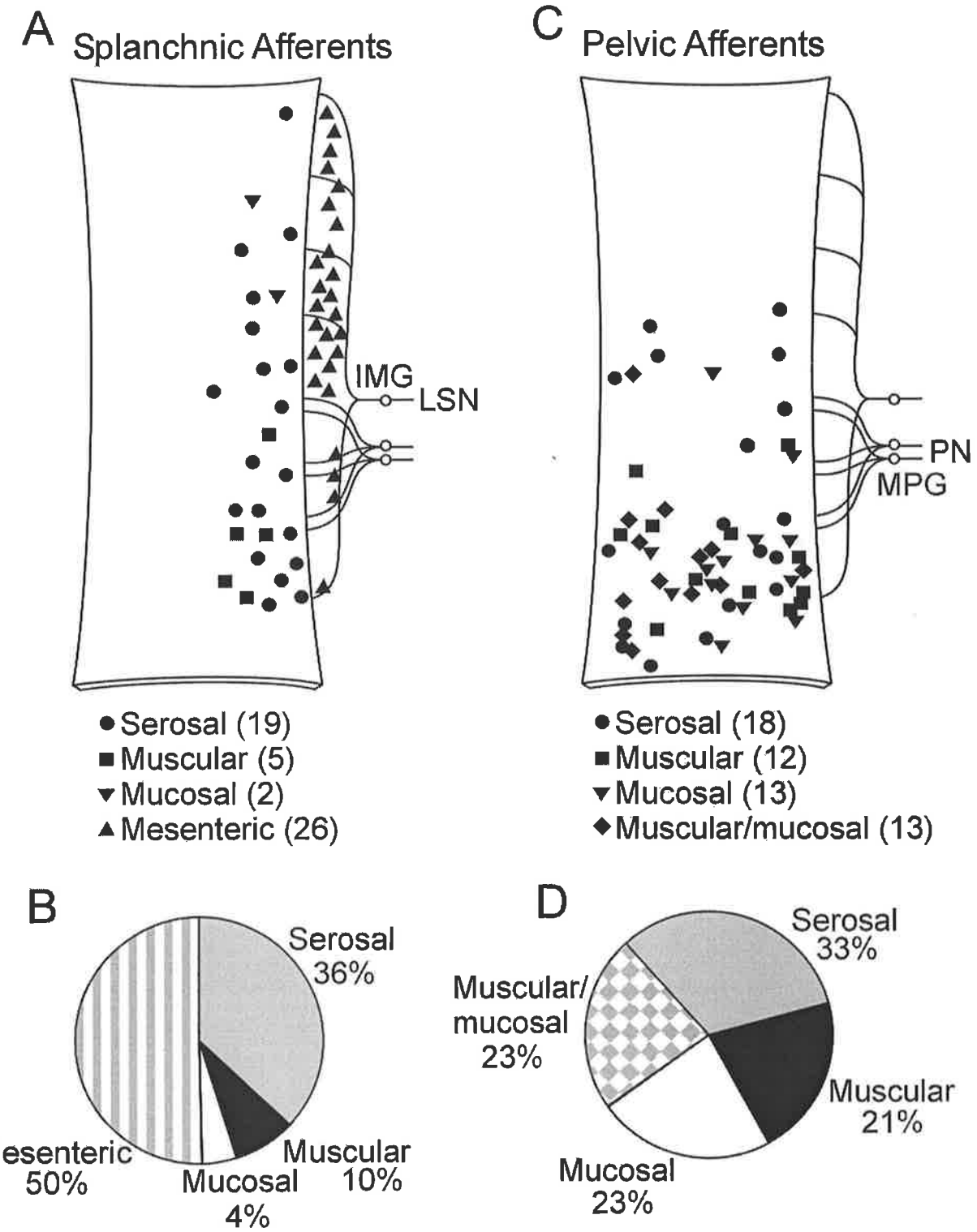

A
Splanchnic Afferents
IMG
LSN
Serosal (19)
Muscular (5)
Mucosal (2)
Mesenteric (26)
C
Pelvic Afferents
PN
MPG
Serosal (18)
Muscular (12)
Mucosal (13)
Muscular/mucosal (13)
B
Serosal 36%
Muscular 10%
Mucosal 4%
Mesenteric 50%
D
Serosal 33%
Muscular 21%
Mucosal 23%
Muscular/ mucosal 23%

Figure 1.4: Mechanosensitivity, adaptation, and activation characteristics of LSN and PN afferents to graded stimulation with von Frey hairs.

A and D) Stimulus-response functions of LSN and PN afferents to focal compression via probing stimuli. All afferent subtypes from both pathways displayed graded responses to increasing probing stimuli (70mg-4g). Grouped responses to probing of LSN and PN afferents were significantly different ($P < 0.05$). However, within each pathway, probing responses of individual afferent classes did not differ significantly nor were the slopes of stimulus-response functions significantly different ($P > 0.05$ for all analyses). **B and E)** Adaptation profiles of LSN and PN afferent responses during a 3 sec application of a 1g probe. The adaptation curves of all LSN afferents displayed similar slopes (B; $P > 0.05$). Among PN afferents, the adaptation profile of mucosal afferents was significantly shallower than those of serosal or muscular afferents (E; $P < 0.05$). **C and F)** von Frey hair force required to activate LSN and PN afferents. Significantly fewer serosal and muscular afferents in the LSN were activated by probe intensities ≤ 1g compared to similar afferents in the PN ($P < 0.05$). **C)** Mucosal afferents were significantly more sensitive to probing than all other LSN afferent classes, with 100% recruited by the lowest probe intensity tested ($P < 0.05$). A probing force of 2g was required to activate all LSN afferents. F) In contrast to LSN afferents, muscular/mucosal afferents were the most sensitive to probing among all PN afferents, with a significantly different percent responding curve compared to the other PN afferent classes. In addition, more mucosal afferents were activated by probing intensities ≤ 1g than were serosal afferents ($P < 0.05$). A probing force of 1g was required to activate 100% of PN afferents.

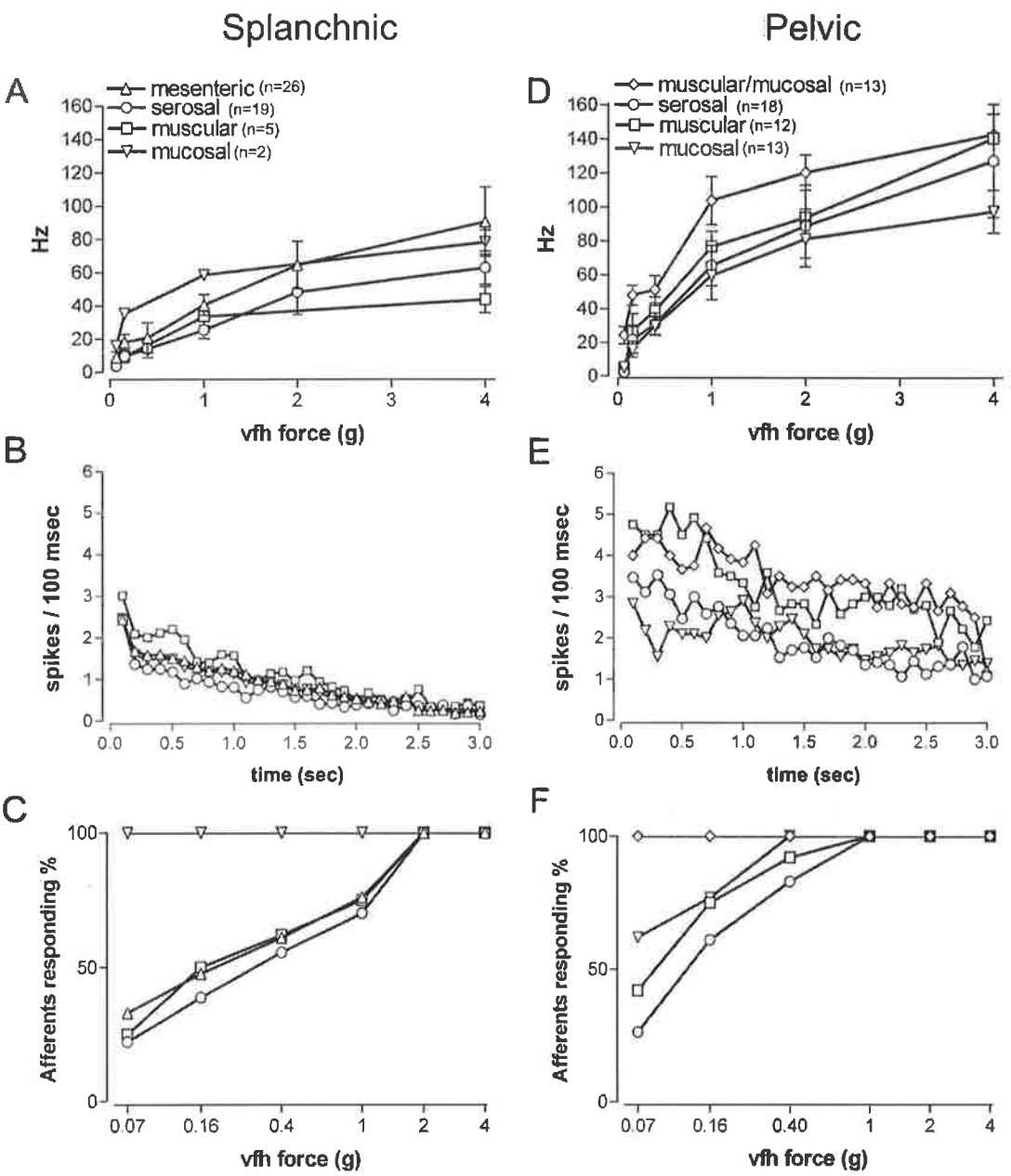

Splanchnic
Pelvic
A
mesenteric (n=26)
serosal (n=19)
muscular (n=5)
mucosal (n=2)
Hz
vfh force (g)
D
muscular/mucosal (n=13)
serosal (n=18)
muscular (n=12)
mucosal (n=13)
Hz
vfh force (g)
B
spikes / 100 msec
time (sec)
E
spikes / 100 msec
time (sec)
C
Afferents responding %
vfh force (g)
F
Afferents responding %
vfh force (g)

Figure 1.5: Comparison of serosal and muscular afferents between LSN and PN pathways.

A) Stimulus-response functions of LSN (n=19) and PN (n=18) serosal afferents to probing. Serosal afferents from both pathways displayed graded responses to focal compression of increasing probing stimuli (70mg-4g). However, PN serosal afferents were significantly more sensitive to probing, displaying larger stimulus-response functions ($P < 0.001$; 2-way ANOVA) and steeper slopes than LSN serosal afferents ($P < 0.001$; PN slope 29.63 ± 4.31 vs. LSN slope 15.02 ± 1.88). **B)** Adaptation profiles of serosal afferents recorded from the PN (n=18) and LSN (n=19) to a 3 sec, 1g probing stimulus. The PN response was significantly larger throughout ($P < 0.001$, n=18 vs. n=19) and fitted a linear regression, whereas LSN data were non-linear. However, LSN serosal afferents demonstrated significantly faster adaptation over the first 0.5 sec of the response ($P < 0.01$) and more complete adaptation than PN serosal afferents. **C)** Stimulus-response functions of muscular afferents in the LSN (n=5) and PN (n=12) to graded probing stimuli. Muscular afferents from both pathways displayed graded responses to increasing probing stimuli (70mg-4g). However, PN muscular afferents are more sensitive to probing, displaying significantly larger stimulus-response functions ($P < 0.001$) with steeper slopes than LSN muscular afferents ($P < 0.001$; PN slope 30.89 ± 5.5 vs. LSN slope 9.01 ± 3.3). **D)** Adaptation profiles of LSN (n=5) and PN (n=12) muscular afferents to a 3 sec, 1g probing stimulus. Pelvic muscular afferents responded significantly higher throughout the stimulus than LSN muscular afferents ($P < 0.001$), however there was no difference in the slopes of the two curves ($P > 0.05$). **E)** Circular stretch stimulus-response functions of LSN (n=5) and PN (n=12) muscular afferents. All PN and LSN muscular afferents responded to the full range of stretch stimuli (1-5g). However, PN muscular afferents were significantly more responsive to stretch, displaying higher stimulus-response functions ($P < 0.001$) with steeper slopes than those of LSN muscular afferents ($P < 0.001$, PN slope 0.30 ± 0.03 vs. LSN slope 0.11 ± 0.02). **F)** Adaptation profiles of LSN and PN muscular afferents to a 1 min, 3g stretch. Pelvic muscular afferents displayed significantly more spikes per 10 sec bin across the entire stimulus period than LSN afferents ($P < 0.001$). Both LSN and PN muscular afferents adapted over the first 20 sec of the stimulus. Whilst PN afferents continued to discharge over the remainder of the stimulus, LSN afferents returned to their spontaneous level of firing 20 sec after the stimulus onset. Thus, muscular afferents in the LSN adapted more completely to circular stretch than those in the PN. Note that PN afferents showed no spontaneous activity.

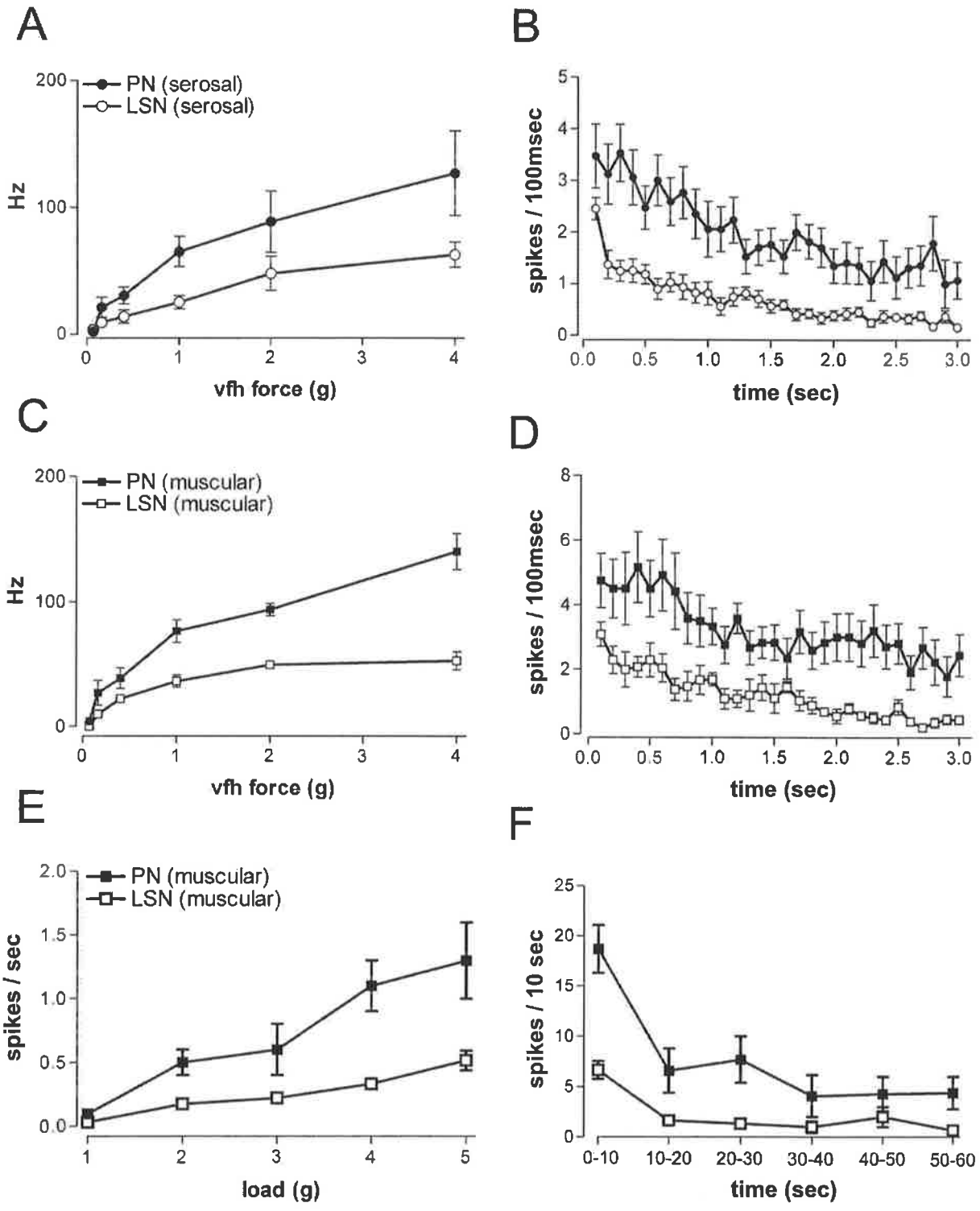
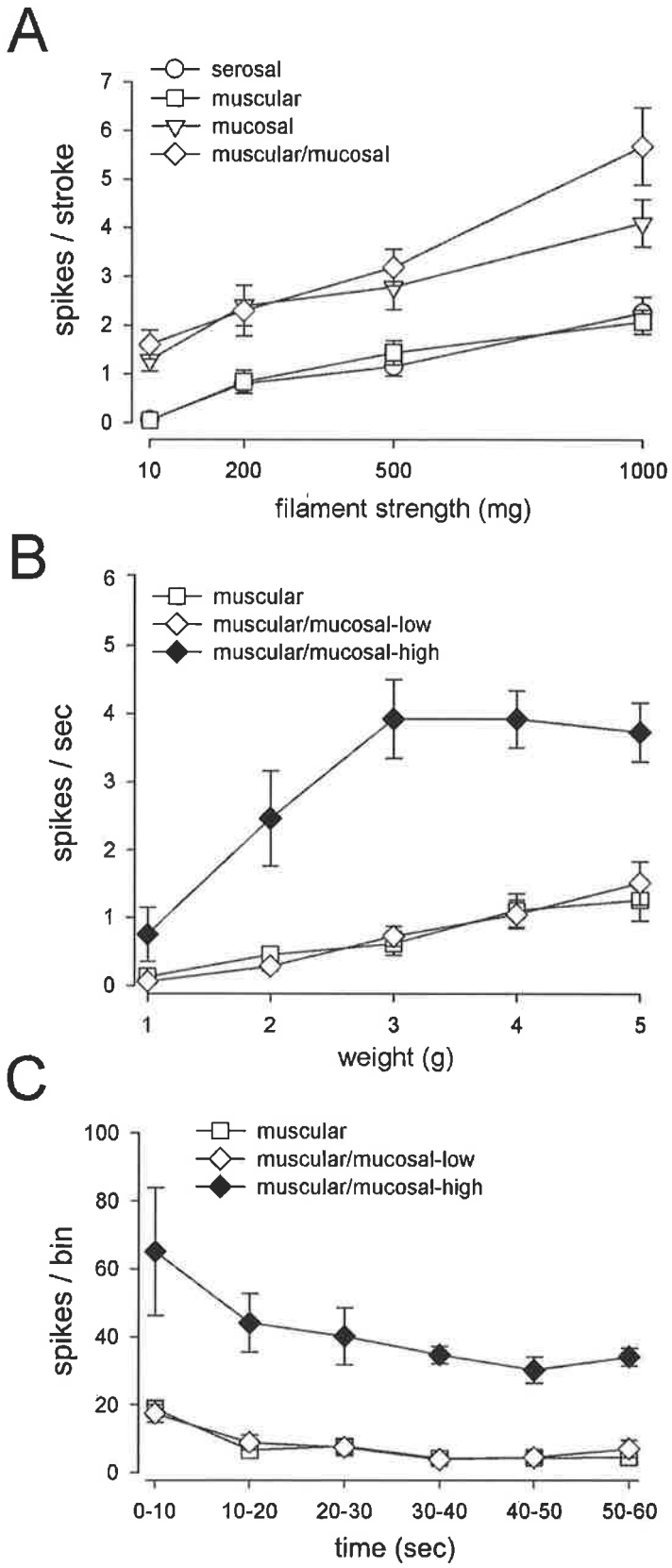

A
PN (serosal)
LSN (serosal)
Hz
vfh force (g)
B
spikes / 100msec
time (sec)
C
PN (muscular)
LSN (muscular)
Hz
vfh force (g)
D
spikes / 100msec
time (sec)
E
PN (muscular)
LSN (muscular)
spikes / sec
load (g)
F
spikes / 10 sec
0-10
10-20
20-30
30-40
40-50
50-60
time (sec)

Figure 1.6: Comparison of dynamic properties of PN afferent classes

A) Mucosal stroking stimulus-response functions of PN afferents. All four classes demonstrated graded responses to mucosal stroking (10-1000mg). Notably, mucosal (n=13) and muscular/mucosal (n=13) afferents were the only classes to respond to the 10mg filament and exhibited stimulus-response functions to mucosal stroking that were significantly higher than those of PN serosal (n=18) and muscular (n=12) afferents ($P < 0.001$). **B)** Circular stretch stimulus-response functions of muscular and muscular/mucosal PN afferents. All muscular and muscular/mucosal afferents responded to the full range of stretch stimuli (1-5g). Two subclasses of muscular/mucosal afferents could be distinguished based on their responses to stretch: a high-responding population, with responses significantly different than muscular afferents ($P < 0.001$, n = 6) and a low-responding population, with responses similar to those of muscular afferents ($P > 0.05$, n = 7). **C)** Adaptation profiles of muscular and muscular/mucosal PN afferents to a 1 min, 3g stretch. High-responder muscular/mucosal afferents displayed significantly more spikes per 10 sec bin across the entire 1 min stimulus period compared to both low-responder muscular/mucosal and muscular afferents ($P < 0.001$). No significant difference in the rate of adaptation, defined as the slope of the adaptation curve, was observed among PN muscular and muscular/mucosal afferents ($P > 0.05$).


A
serosal
muscular
mucosal
muscular/mucosal
spikes / stroke
0
1
2
3
4
5
6
7
10
200
500
1000
filament strength (mg)
B
muscular
muscular/mucosal-low
muscular/mucosal-high
spikes / sec
0
1
2
3
4
5
6
1
2
3
4
5
weight (g)
C
muscular
muscular/mucosal-low
muscular/mucosal-high
spikes / bin
0
20
40
60
80
100
0-10
10-20
20-30
30-40
40-50
50-60
time (sec)

CHAPTER 2

DIFFERENTIAL CHEMOSENSORY FUNCTION OF SPLANCHNIC AND PELVIC COLONIC AFFERENTS IN MICE

Summary

Background & Aims: Lumbar splanchnic and pelvic nerves convey different mechanosensory information from the colon to the spinal cord. This study determined whether these pathways also differ in their chemosensitivity. *Methods:* Using an *in vitro* mouse colon preparation primary afferents were tested with selective P2X, bradykinin and TRPV1 receptor ligands, α,β-meATP, bradykinin and capsaicin respectively. *Results:* Forty percent of LSN afferents responded to the P2X receptor agonist, α,β-methyleneATP (α,β-meATP; 1 mM), an effect that was concentration-dependent and reversed by the P2X antagonist PPADS (100 μM). Significantly fewer PN afferents (7%, $P<0.05$) responded to α,β-meATP. Sixty five percent (17/31) of LSN afferents elicited a powerful response to bradykinin, an effect that was concentration-dependent (EC_{50} 0.16μM) and reversed by the selective B_2 receptor antagonist HOE-140 (10nM). The proportion of LSN afferents responding to bradykinin was significantly greater than that of PN afferents (65% vs. 11%, $P < 0.001$) and their responses were significantly larger. Bradykinin-responsive LSN serosal afferents displayed a modest but significant mechanical sensitization after responding to bradykinin, an effect which was not observed in bradykinin-responsive PN serosal afferents. The TRPV1 receptor agonist, capsaicin (3 μM), also excited more LSN than PN afferents (61% versus 47%). Capsaicin-responsive LSN serosal afferents displayed marked mechanical desensitization after responding to capsaicin, an effect which was not observed in capsaicin-responsive PN serosal afferents. Mechanically-insensitive afferents were recruited by α,β-meATP (1mM), bradykinin (1 μM) and capsaicin (3 μM), and these remained mechanically-insensitive and were almost exclusive to the LSN. *Conclusions:* Colonic LSN and PN pathways differ in their chemosensitivity to known noxious stimuli. As these pathways relay information that may relate to symptoms in functional gastrointestinal disease these results may have implications for the efficacy of therapies targeting receptor modulation.

Introduction

One of the most common pain syndromes seen in the clinic is IBS[1]. Enhanced visceral sensation and abdominal pain are hallmarks of this disease[1, 2, 28] and identification of drugs that can normalize symptom occurrence has been considered critical in the search for effective IBS therapies[310]. Pain is the symptom that affects quality of life the most,[23] therefore it is a logical approach to investigate receptors as potential drug targets that have been strongly implicated in pain in other systems. Thus the $P2X_3$, TRPV1 (formerly known as VR1) and bradykinin receptors are prime candidates for study.

$P2X_3$ is a member of the P2X purinoceptor family of ATP-gated ion channels[139, 140] while TRPV1 belongs to the transient receptor potential (TRP) channel family and is activated by heat, protons and vanilloid ligands such as capsaicin, the pungent ingredient in chilies[135, 137]. Both of these receptors are predominantly expressed in small diameter primary afferent neurons (*C*-fibers)[136, 138, 160, 162, 163, 166]. Both $P2X_3$ and TRPV1 have been strongly implicated in nociception and pain[135-140], although these studies have been performed in levels of DRG which are devoid of colonic innervation[60, 81]. Although it is unknown whether the expression of these receptors is altered in IBS, the expression of both $P2X_3$ and TRPV1 is increased in colonic nerve fibers of patients with inflammatory bowel disease[154, 155, 311].

Bradykinin is one of the best established chemical stimuli and most physiologically relevant to tissue injury and pain. Bradykinin has been shown to be an important mediator in signalling of pain and irritation in skin, muscle, joints, vasculature, and all visceral organs[105, 128, 142-147, 181]. Recently it has been demonstrated that bradykinin activation of afferent fibres may have numerous downstream effects, including the production of 12-lipoxygenase metabolites of arachidonic acid which activate vanilloid (TRPV) receptors[145], which are involved directly in mechanical, thermal and

pH sensitivity[136, 186]. There is also direct evidence in some models that bradykinin induces increased responsiveness of afferents to other stimuli[148, 149].

Activation of $P2X_3$, TRPV1 and bradykinin receptors, by the selective agonists α,β-methylene adenosine 5'-triphosphate (α,β-meATP), capsaicin and bradykinin respectively, will excite gastrointestinal afferents[88, 102, 107, 128, 165, 174, 177, 230], including colonic afferents[102, 112, 128, 165, 177, 230]. However, the extrinsic spinal innervation of the colon makes the issue more complex as sensory information from the distal colon and rectum travels to the central nervous system through two distinct anatomical pathways: LSN, which terminate in the thoracolumbar spinal cord, and the PN, which terminate in the lumbosacral spinal cord. Mechanosensitive afferents have been identified in both of these nerve supplies[75, 105, 108, 110, 112, 113, 312]. Recent evidence (detailed in Chapter 1) suggests that LSN and PN afferents carry markedly different types of information, indicating that these pathways are distinct, both anatomically and functionally, and likely to serve unique roles in the detection of mechanical stimuli in the distal colon[312]. Despite considerable investigation into the responses of afferents from either pathway to various chemical stimuli, including these agonists[102, 112, 128, 165, 177, 230,48, 54, 102,128, 313], there are a number of gaps in our knowledge about which type of afferent fibres they activate, which is the predominant pathway and which receptors underlie the pharmacology of its action. This is relevant firstly to understanding the sensory physiology of the colon, as sensitization of mechanoreceptors and perhaps chemoreceptors may give rise to the abdominal pain and discomfort experienced by IBS patients[231] and secondly to future targeting of pharmacotherapies towards particular types of sensation and regions of the gut. To establish these facts it is important to study α,β-meATP, capsaicin and bradykinin actions on LSN and PN afferents in the same species and importantly in the same preparation. Thus the present study had the purpose of addressing the lack of knowledge concerning the comparative chemosensitivity of LSN and PN colonic afferents from the distal colon by using an *in vitro* electrophysiological approach.

These data indicate major differences in the chemosensitivity of afferents in the LSN and PN pathways, because by far the majority of chemosensory afferents responding to α,β-meATP, capsaicin and bradykinin are found in the LSN pathway.

Material and Methods

All electrophysiological experiments were performed in accordance with the guidelines of the Animal Ethics Committees of the Institute for Medical and Veterinary Science and the University of Adelaide, Adelaide, Australia, and the Institutional Animal Care and Use Committee of The University of Iowa, Iowa City, Iowa, USA.

In vitro mouse colonic primary afferent preparations

Dissections were carried out in male and female mice (C57BL/6; 20-30g) according to protocols described in detail in Chapter 1[312]. Briefly, mice were killed by CO_2 inhalation and cervical dislocation, the colon (5-6cm) and mesentery (containing the lumbar colonic nerves) removed intact with either the attached neurovascular bundle containing the inferior mesenteric ganglion and LSN or in separate preparations with the major pelvic ganglion and PN. The tissue was transferred to ice cold Krebs solution and, following further dissection, the distal colon was opened longitudinally along the anti-mesenteric border to orientate lumbar colonic insertions to lie along the edge of the open preparation. The tissue was pinned flat, mucosal side up, in a specialized organ bath consisting of two adjacent compartments machined from clear acrylic (Danz Instrument Service, Adelaide, South Australia), the floors of which were lined with Sylgard® (Dow Corning Corp., Midland, MI, USA). The PN or neurovascular bundle containing the LSN were extended from the tissue compartment into the recording compartment where they were laid onto a mirror. A movable wall with a small "mouse hole" (to allow passage of the nerves) was lowered into position and the recording chamber filled with paraffin oil. The colonic compartment was superfused with a modified Krebs' solution (in mM: 117.9

NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4(H_2O)_7$, 2.5 $CaCl_2$, and 11.1 d-glucose), bubbled with carbogen (95% O_2, 5% CO_2) at a temperature of 34°C. All preparations contained the L-type calcium channel antagonist nifedipine (1 μM) to suppress smooth muscle activity and the prostaglandin synthesis inhibitor indomethacin (3 μM) to suppress potential inhibitory actions of endogenous prostaglandins[112, 312]. Under a dissecting microscope, the LSN were dissected away from the neurovascular bundle and the nerve sheath surrounding the LSN or PN peeled gently back to expose the nerve trunk. Using fine forceps, the nerve trunk was teased apart into 6-10 bundles that were placed individually onto a platinum recording electrode. A platinum reference electrode rested on the mirror in a small pool of Krebs solution adjacent to the recording electrode.

Characterization of LSN and PN serosal afferents

Colonic afferents were characterized using the classification system previously applied in mouse and rat colon[112, 113, 312]. Receptive fields were identified by systematically stroking the mucosal surface with a brush of sufficient stiffness to activate all types of mechanosensitive afferent. The present study focused on serosal afferents in testing and comparing afferent chemosensitivity as they are by far the most conserved afferent class shared between the LSN and PN pathways Chapter 1 and [312]. Serosal afferents were classified in both pathways by their graded response to focal compression of their receptive field via perpendicular probing with calibrated von Frey hairs (70, 160, 400, 1000 and 2000mg; each force applied 3 times for a period of 3 s) and their insensitivity to circular stretch (1-5g) and fine mucosal stroking (10mg)[312].

Drug addition to receptive fields

Chemosensitivity of LSN and PN serosal afferents to the P2X-purinoceptor agonist α,β-meATP (1 mM), the bradykinin receptor agonist bradykinin (1 μM) and the TRPV1 receptor agonist capsaicin (3 μM) were determined after mechanical thresholds and stimulus response functions had

been established. These concentrations were chosen as they elicit powerful responses in similar preparations[107, 112,308]. A small metal ring was placed over the receptive field of interest, residual Krebs aspirated, and chemical stimuli (α,β-meATP, bradykinin or capsaicin) applied to the mucosal surface for 2 min before being aspirated from the ring. This route of administration has been previously shown to activate serosal afferents reproducibly[112, 113]. Drugs were administered in this order to avoid damage or desensitization. In all experiments, the mechanical sensitivity of receptive fields to probing (2000mg von Frey hair) was re-determined between each drug application to determine possible alterations in mechanical sensitivity and ensure continued viability of the unit under investigation. A probing stimulus of 2000mg was chosen because it was reproducibly effective in activating all LSN and PN serosal afferents. Responses to chemicals were counted when a maintained > 25% increase in discharge above basal levels occurred[113].

In separate LSN experiments, α,β-meATP sequential concentration (0.1μM – 1mM) response relationship experiments were performed. Full recovery from α,β-meATP application was allowed before proceeding to the next higher concentration. In separate experiments pyridoxyl 5-phosphate 6-azophenyl-2',4'-disulfonic acid (PPADS) was administered to the bath 15 minutes prior to a second α,β-meATP (1 mM) application and focal compression of the receptive field repeated with a 2000mg von Frey hair to determine the role of P2X receptors in the effect of the α,β-meATP and in LSN mechanotransduction. PPADS at a concentration of 100 μM was chosen as it has been previously shown to block the effects of α,β-meATP on rat colonic pelvic distension-sensitive afferents[165, 177].

In separate LSN experiments bradykinin sequential concentration (0.1nM – 1μM) response relationship experiments were performed. Full recovery from bradykinin application was allowed before proceeding to the next higher concentration. In separate LSN experiments after bradykinin

responsive afferents were found the B_2 receptor antagonist HOE-140 (10 nM) [184] was subsequently administered to the bath for 20 minutes prior to a second bradykinin (1 μM) application[184]. Only one concentration of capsaicin (3 μM) was used to avoid desensitization of responses.

Electrophysiological data recording and analysis

Electrical signals generated by nerve fibers placed on the platinum recording electrode were fed into a differential amplifier, filtered, sampled (20 kHz) using a 1401 interface (Cambridge Electronic Design, Cambridge, UK) and stored on a PC for off-line analysis. The amplified signal was also used for online audio monitoring. Action potentials were analysed off-line using the Spike 2 wavemark function and discriminated as single units on the basis of distinguishable waveform, amplitude and duration. Data are expressed as mean ± SEM. *n* indicates the number of individual afferents. Adaptation profiles to probing were calculated as the mean number of spikes per 100 ms bin over the entire 3 s of a 1g probing stimulus. Data were analysed using Prism 4.03 software (GraphPad Software, San Diego, CA, USA), and where appropriate, were analysed using a two-way analysis of variance (ANOVA) with Bonferroni post hoc tests (to determine significant differences between curves) or paired and unpaired *t*-tests. Linear regression analysis was used to compare slopes of adaptation profiles. Fisher's exact tests were used to compare significance differences between stimulus thresholds, responders and non-responders to chemical stimuli. Chemosensory responses were quantified in terms of their latency (calculated by the mean time delay between bradykinin application and onset of afferent response), area (calculated by the mean number of action potentials generated per response), amplitude (calculated by the mean spike discharge frequency evoked during the response) and duration (calculated by the time between onset of response and afferent firing returning to baseline levels). Responses to α,β-meATP , bradykinin or capsaicin application were counted when a maintained > 25% increase in discharge above basal levels occurred. EC_{50} values were calculated by a curve fit function in GraphPad Prism software

version 4.03 to the range of concentrations used. Differences were considered significant at a level of $P < 0.05$.

Drugs

Stock solutions of all drugs were kept frozen and diluted to their final concentration in Krebs solution. α,β-meATP, bradykinin, capsaicin, and HOE-140 and PPADS were obtained from Sigma-Aldrich.

Results

Mechanosensory properties of LSN and sacral PN afferents

Serosal afferents were recorded from both the LSN and PN pathways that had receptive fields located on the colonic wall. As shown previously in Chapter 1 these were activated reproducibly by focal compression via perpendicular probing of their receptive field with calibrated von Frey hairs and did not respond to colonic stretch (1-5g) or stroking of the colonic mucosa with a 10mg von Frey hair (*Figure 2.1A,B*). Fibres recorded in this part of the investigation showed dynamic properties that differed considerably between the two pathways, exactly as those encountered in Chapter 1's investigation. Despite the fact that serosal afferents from both pathways displayed graded responses to increasing probing stimuli, PN afferents were significantly more sensitive to probing than LSN afferents, displaying significantly greater stimulus response functions (*Figure 2.1C*). In addition PN serosal afferents displayed a significantly greater magnitude of response to probing with 400, 1000 and 2000mg von Frey hairs (*Figure 2.1C*). PN and LSN serosal afferents also displayed significantly different adaptation profiles to probing with a 1000mg von Frey hair (*Figure 2.1D*). PN serosal afferents displayed a more maintained response to probing than their LSN counterparts, indicated by significantly shallower linear regression slopes (*Figure 2.1D*). Pelvic serosal afferents also displayed significantly lower stimulus thresholds to probing as almost twice as

many PN afferents responded to lower probing stimuli (70, 160 and 400mg), while 100% of PN serosal afferents responded to a 1000mg probing stimulus compared with only 89% being activated in the LSN pathway (*Figure 2.1E).* Therefore, consistent with Chapter 1[35], PN serosal afferents required lower intensity probing stimuli to be activated, evoked larger responses to probing stimuli, and displayed a more maintained response to probing than LSN serosal afferents.

Chemosensory properties of LSN and PN afferents

LSN responses to α,β-methyleneATP

Ten of the 31 (32%) LSN serosal mechanoreceptive afferents tested responded to α,β-meATP (1 mM) (*Figure 2..2A and 2.3Ai*). They displayed a rapid excitation of discharge, after a short latency (5.3 ± 1.6 s; Figure *2.4A*), that was normally short in duration (6.6 ± 3.3 s; *Figure 2.4B*) before discharge returned to basal levels (*Figure 2.3Ai*). In all cases (10/10), the response was reproducible on a second application of α,β-meATP (1 mM) three minutes later. Splanchnic serosal afferents responded to α,β-meATP in a concentration-dependent manner (EC_{50}= 21.2 μM, *Figure 2.5A*) and this response was blocked by the non-selective P2X-receptor antagonist PPADS (100 μM,[165, 177] for 15 minutes, $P < 0.001$, *t*-test, *Figure 2.5B*). No mechanical sensitization or desensitization was observed after α,β-meATP as probing with a 2000mg von Frey hair after the removal of α,β-meATP gave the same response to probing before and after exposure (*Figure 2.6A*). Afferents responding to α,β-meATP and those not responding were similarly unaffected in their mechanosensitivity ($P > 0.05$, *t*-test, compare *Figure 2.6Aand D*). In separate experiments PPADS (100 μM) did not alter the mechanosensitivity of LSN afferents ($n = 6$, $P > 0.05$, *t*-test).

Recruitment of mechanically insensitive LSN afferents by α,β-methyleneATP

On seven occasions during testing of a mechanically sensitive serosal afferent, another separate mechanically-insensitive chemosensitive unit was recruited (*Figure 2.7A*). Mechanically-

insensitive afferents were also recruited following bradykinin and capsaicin addition (see later), giving a total population of 43 LSN afferents in this investigation. Therefore, in total 17 of 43 (40%; *Figure 2.2A*) LSN afferents responded to α,β-meATP (1 mM). Recruited afferents displayed reproducible responses to α,β-meATP with similar latencies (4.7 ± 2.7 s) to mechanically sensitive serosal afferents; however they displayed significantly longer durations of response (37.3 ± 9.8 s; $P < 0.01$, *t*-test). Recruited afferents did not subsequently become mechanosensitive.

PN responses to α,β-methyleneATP

In contrast to LSN afferents, fewer (1 of 15; 7%) PN serosal mechanoreceptive afferents responded to α,β-meATP (1 mM) (*Figure 2.2B, 3Bi*). Furthermore, no PN afferents were recruited during the addition of α,β-meATP. As seen in the LSN pathway mechanosensitivity was unaltered in afferents that did not respond to α,β-meATP ($P > 0.05$, *t*-test, *Figure 2.6D*). Due to the low number of PN afferents responding to α,β-meATP no statistical comparison could be performed between pathways *(Figure 2.6A)*. However, it is clear that far more LSN serosal afferents are responsive to α,β-meATP than PN serosal afferents ($P < 0.05$, Fisher's exact test).

LSN responses to bradykinin

Seventeen of the 31 (55%, *Figure 2.2C*) LSN serosal mechanoreceptive fields tested responded to bradykinin (1 μM). After a short latency (4.4 ± 0.8 s; *Figure 2.3A*) these afferents displayed a rapid and maintained excitation of discharge that had a mean duration of 89.6 ± 9.28 s (*Figure 2.3B*). Bradykinin elicited a mean spike discharge response of 13.1 ± 2.3 Hz and a mean number of spikes per response of 217.6 ± 44.8 *(Figure 2.3C)*. Splanchnic serosal afferents responded to bradykinin in a concentration-dependent manner (EC_{50}= 0.16 μM, *Figure 2.5C*). In separate experiments 20 minutes prior incubation with the B_2 receptor antagonist HOE-140 (10 nM, [165, 177, 184] significantly attenuated the response evoked by bradykinin, resulting in an almost complete

abolition of the bradykinin evoked response (1 μM; $P < 0.0001$, *t*-test, *Figure 2.5Dand E*). Mechanical sensitization was observed after bradykinin as probing with a 2000mg von Frey hair after the removal of bradykinin elicited a modest but significant increase in response (*Figure 2.6B*). By contrast, LSN afferents that did not respond to bradykinin did not display altered mechanosensitivity ($P > 0.05$, *t*-test, *Figure 2.6E*).

Recruitment of mechanically insensitive LSN afferents by bradykinin

On ten occasions (*Figure 2.2C)* during testing of a mechanically sensitive serosal afferent, a separate mechanically-insensitive chemosensitive unit was recruited (*Figure 2.7B*). Five of the seven mechanically-insensitive afferents recruited by α,β-meATP also responded to bradykinin (3 μM). In addition to these, five other mechanically insensitive units were recruited by bradykinin (1 μM) that were insensitive to α,β-meATP. Therefore, in total 27 of 41 (66%; *Figure 2.2C*) LSN afferents responded to bradykinin (1 μM). Recruited afferents displayed responses to bradykinin with similar latencies (6.5 ± 1.5 s), duration of response (100 ± 9.6 s), mean spike discharge (12.2 ± 1.8 Hz) and number of spikes per response (217.9 ± 46.15 s) to mechanically sensitive serosal afferents ($P > 0.05$, t-test, n = 17 serosal vs n=10 recruited, for all). Recruited afferents did not subsequently become mechanosensitive.

PN responses to bradykinin

Only 2 of 19 (11%) PN mechanosensitive serosal afferents responded to bradykinin (1 μM) significantly fewer than in the LSN pathway ($P < 0.001$, Fisher's exact test; *Figure 2.2D*). After a short latency (9 ± 7 s; *Figure 2.4A*) PN afferents displayed a maintained excitation of discharge that had a mean duration of 77 ± 22 s (*Figure 2.4B*). In contrast to the LSN pathway, no mechanically insensitive PN afferents were recruited during the addition of bradykinin *(Figure 2.2D)*. A comparison of the pattern of response to bradykinin revealed significant differences between the two pathways.

Splanchnic serosal afferents elicited significantly more spikes per response than PN serosal afferents in response to bradykinin 1 μM *(LSN:* 217.6 ± 44.8 vs *PN:* 52 ± 10; $P < 0.05$; *Figure 2.4C).* In addition, bradykinin-responsive PN serosal afferents did not display mechanical sensitization ($P > 0.05$, *t*-test, *Figure 2.6B*). As seen in the LSN pathway mechanosensitivity was unaltered in afferents that did not respond to bradykinin ($P > 0.05$, *t*-test, *Figure 2.6E*).

LSN responses to capsaicin

Sixteen of 31 (52%) LSN serosal mechanoreceptive afferents responded to capsaicin (3 μM) (*Figure 2.2E; 3Aiii*). They displayed a powerful excitation of discharge after a short latency (4.3 ± 1.1s; *Figure 2.4A*). The average duration of response was 35.7 ± 8.3 s before discharge returned to basal levels (*Figure 2.4B; 3Aiii*). Capsaicin-responsive LSN serosal afferents displayed pronounced mechanical desensitization when re-probed with a 2000mg von Frey Hair after exposure to capsaicin ($P < 0.05$, *t*-test, *Figure 2.6C*). By contrast, capsaicin-unresponsive LSN serosal afferents did not show mechanical desensitization ($P < 0.05$, *t*-test, *Figure 2.6F*). None of the chemicals were re-tested after capsaicin desensitization

Recruitment of mechanically-insensitive LSN afferents by capsaicin

Five of the seven mechanically insensitive afferents recruited by α,β-meATP responding to bradykinin also responded to capsaicin (3 μM, *Figure 2.2*). In addition to these, two other mechanically-insensitive units that were insensitive to α,β-meATP but recruited by bradykinin also responded capsaicin (3 μM). An additional three mechanically-insensitive units that were insensitive to α,β-meATP (but were not tested with bradykinin) were recruited by capsaicin (3 μM). Therefore, in total 26 of 43 (61%) LSN afferents responded to capsaicin (3 μM; *Figure 2.2E*). Recruited units displayed similar latencies (2.7 ± 1.3 s) and duration of responses (50.3 ± 7.8 s; $P > 0.05$, *t*-test) to

mechanically sensitive, capsaicin-responsive serosal afferents. Recruited afferents did not subsequently become mechanosensitive.

PN responses to capsaicin

In contrast to the LSN afferent responses, fewer (7 of 16; 44%) PN serosal mechanoreceptive afferents responded to capsaicin (3 μM) (*Figure 2.2F*). They displayed a powerful excitation of discharge lasting 87 ± 26 s after a latency of 16 ± 4 s (*Figure 2.3A and B*). A comparison of the pattern of responses to capsaicin revealed significant differences between the two pathways. PN afferents displayed significantly longer latencies of response ($P < 0.01$, *t*-test, *Figure 2.3A*) and significantly longer durations of response ($P < 0.05$, *t*-test, *Figure 2.3B*). Only one mechanically-insensitive PN afferent was recruited during the addition of capsaicin (3 μM), giving a total eight of 17 (47%) PN afferents that were responsive to capsaicin (3 μM; Figure *2.2F*). In contrast to their LSN counterparts, capsaicin-responsive PN serosal afferents did not display mechanical desensitization (*Figure 2.6C*). None of the chemical were re-tested after capsaicin desensitization

Discussion

This study has six major findings relating to transmission of sensory information from the colon. First, these results have revealed that LSN and PN colonic afferents transmit different patterns of information to the central nervous system, not only in terms of signalling mechanical events but also in terms of signalling chemical activation. Second, α,β-meATP in the colon is a selective stimulus for LSN afferents, however there was no evidence for a role of the P2X receptor in mechanical transduction, in contrast to studies of afferents innervating different targets in other species. Third, bradykinin is a selective stimulus for LSN afferents, and activation of bradykinin B_2 receptors by bradykinin causes mechanical sensitization in LSN but not PN afferents. Fourth,

capsaicin activates both LSN and PN afferents, however, it activates a higher proportion of LSN afferents and activation of TRPV1 receptors by capsaicin subsequently attenuates responsiveness to mechanical stimuli. Fifth, from their markedly different response profiles to capsaicin, these data suggest that LSN and PN afferents may differ in their mechanisms of downstream signalling via TRPV1. Sixth, this study has identified a population of afferents in both pathways that are recruited by chemical stimuli and remain mechanically insensitive, and therefore serve a novel role.

The results of this study show that that activation of $P2X_3$, B_2 and TRPV1 receptors is more likely to be signalled via the thoracolumbar LSN pathway than the lumbosacral PN pathway. This in turn means $P2X_3$, B_2 and TRPV1 receptor agonists activate afferents with higher mechanical thresholds and lower mechanical responsiveness that project to the thoracolumbar spinal cord. Pelvic afferents that project to the lumbosacral cord on the other hand are more infrequently activated by these agonists and their responses were smaller, in the case of bradykinin, but larger in the case of capsaicin. This contrasts to their mechanical responsiveness which is greater and occurs at lower thresholds than in LSN afferents. A picture thus emerges of the tuning of chemosensitivity in colonic afferents according to their mechanical responsiveness and central destination.

Differences between LSN and PN pathways

The findings concerning the mechanosensitivity of colonic serosal afferents corroborate the data obtained in Chapter 1[312]. These data have shown that PN serosal afferents differ from LSN afferents in several ways: they are more distally located, adapt more slowly to mechanical stimuli, and respond across a wider stimulus range. The present study focused specifically on one class of afferents, the serosal afferents. This class was chosen because they are the most abundant in both the LSN and PN, and may thus provide a meaningful comparison of chemosensitivity between pathways; plus they are likely linked to signalling of pain. Based on the findings in Chapter 1, it is

proposed that serosal afferents signal transient, sharp pain at the onset of spasm or distension. Distension of this nature would result from rapid transit of contents or experimental balloon inflation, during which acute intense mechanical stimulation might be achieved. Consequently, PN serosal afferents would provide a signal from more distal regions of the colon and rectum than LSN serosal afferents, and generate a more intense and sustained afferent barrage in response to acute mechanical events. An extra dimension to this profile of PN and LSN serosal afferents shows that LSN afferents are more likely to express functional $P2X_3$, bradykinin and TRPV1 receptors than PN afferents. In the whole animal these different profiles of receptor expression would mean that the distal colon and rectal region is less chemosensitive to bradykinin and vanilloid or purinergic stimuli than more proximal regions of the mid- to distal colon. It would also mean that pathways activated in the lumbosacral spinal cord are less likely to receive colorectal chemosensory input than those in the thoracolumbar cord. Because $P2X_3$ and TRPV1 receptors are likely to be continually activated endogenously (see below), this adds further complexity to the stream of afferent information reaching the spinal cord from the colon and rectum.

The differences between the LSN and PN pathways are further highlighted by the correlation of chemosensitivity of colonic afferents with receptor expression on their cell bodies[314]. In total 40% of LSN afferents were responsive to α,β-meATP with 36% of thoracolumbar colonic sensory neurons expressing $P2X_3$-LI. In comparison, only 7% of PN afferents responded to α,β-meATP with only 19% of lumbosacral colonic sensory neurons expressing $P2X_3$-LI [314]. A similar disparity between the two pathways is observed with capsaicin responsiveness and TRPV1-LI. In total 61% of LSN afferents responded to capsaicin compared with only 47% of PN afferents. Correspondingly 82% of thoracolumbar colonic sensory neurons expressed TRPV1-LI, and 50% of lumbosacral colonic sensory neurons expressed TRPV1-LI[314]. Although these data from electrophysiological and anatomical studies differ slightly in the exact proportions of neurons expressing each type of receptor,

they agree on the general trend of greater receptor expression in the LSN pathway[314]. The small differences between these findings could be due to a number of technical factors, but also a basic physiological feature of this approach - the fact that retrograde tracing of colonic afferent pathways does not discriminate between functional classes of primary afferents innervating the colonic wall (muscular, mucosal or serosal [312], whereas this electrophysiological analysis was restricted to serosal afferents. This was because serosal afferents constitute the only major population of afferents common to both pathways, and therefore comparable directly between the two. Thus, other functional classes of afferents may have made slightly different contributions to the total of retrogradely labeled populations that were TRPV1 and $P2X_3$-LI. Nonetheless it appears that activation of $P2X_3$, B_2 and TRPV1 receptors is more likely to be signalled via the thoracolumbar LSN pathway than the lumbosacral PN pathway. Therefore electrophysiological observations of serosal afferents are broadly representative of the whole population of afferents in each pathway.

Mechanically-insensitive chemosensitive afferents

A particularly novel finding in this study was the existence of a reasonably large population (approximately a quarter) of mechanically-insensitive afferents restricted almost exclusively to the LSN. These were recruited by addition of α,β-meATP and/or bradykinin and/or capsaicin, when added to a ring surrounding a serosal mechanoreceptive field, otherwise they would have been overlooked entirely. In total, 14 mechanically-insensitive chemosensitive afferents were observed in the LSN pathway compared with only one in the PN pathway. Mechanically-insensitive afferents showed similar overall distribution to mechanically sensitive afferents. Importantly, they appear to be distinct from the previously described "silent nociceptors" in skin[53, 315] and "chemospecific afferents" in colon[51, 112] because they remained insensitive to mechanical stimuli after they had been recruited by chemical stimuli[149, 182, 316-318]; they provide evidence for a novel class of truly chemospecific colonic afferents that are primarily confined to the LSN pathway. Thus these mechanically-insensitive

recruited afferents do not therefore appear to belong to the category of "silent afferents" which are proposed to play a role in inflammatory pain[53]. It will be interesting to determine how the proportion of mechanically-insensitive afferents alters in chronic inflammation studies. The existence of mechanically-insensitive afferents in healthy animals that are responsive to α,β-meATP, bradykinin and capsaicin suggests the presence of a highly tuned early warning system to alert the CNS about injury to the colon without the complication of having to signal mechanical events simultaneously. This would result in an unambiguous signal about the chemical environment which may be interpreted in a specific way and give rise to specific sensory and motor outcomes, perhaps in a comparable way to that by which the carotid body signals to the brain stem[319]. Although this study found only one mechanically-insensitive afferent (recruited by capsaicin) in the PN, this does not mean they are definitely absent, and may merely reflect the rarity of α,β-meATP and bradykinin responsiveness and the possibility remains they may be recruited by other mediators.

TRPV1 signalling and mechanosensitivity

Capsaicin evoked a powerful excitation of discharge in both LSN and PN afferents, comparable to that observed in similar gastrointestinal preparations[150, 200]. An interesting observation from the present study was the ability of capsaicin to cause marked mechanical desensitization. Notably this occurred only in LSN afferents that gave a response to capsaicin, whereas capsaicin-responsive PN afferents and capsaicin-unresponsive afferents in both pathways displayed no mechanical desensitization. Mechanical desensitization after capsaicin has been observed previously *in vitro* using gastro-oesophageal[200] and jejunal[150] preparations. However, in the gastroesophageal preparation mechanical desensitization was also observed in capsaicin-unresponsive afferents in addition to capsaicin-responsive afferents, although higher concentrations of capsaicin (1mM) were used[200]. Desensitization and subsequent degeneration of primary afferents by capsaicin is thought to follow from uncontrolled cation influx into afferent endings, resulting in

depolarization block and subsequent osmotic damage[199]. Based on this mechanism one would expect that stronger activation by capsaicin would result in greater desensitization. However, this study found that PN serosal afferents showed far less desensitization than LSN afferents, even though they showed significantly more prolonged capsaicin responses than LSN afferents. This suggests there are distinct mechanisms linking activation with desensitization, that these mechanisms are specific to the LSN pathway, and that they are independent of the degree of activation by capsaicin. Pelvic afferents also differed from LSN afferents in their latencies of response to capsaicin, even though their endings were distributed similarly. Although the differences in duration and latency of response could possibly be explained by microanatomical factors that may have influenced the rate of diffusion of the stimulus to the ending, there remains the possibility that result from differences in downstream signal transduction following activation of TRPV1. It was not possible to determine other aspects of the pharmacology of TRPV1 activation in each pathway because of the inherent problem of desensitization associated with this receptor, but these findings do suggest there may be different coupling of TRPV1 to mechanisms of initiation or termination of neuronal excitation in the two pathways.

Bradykinin signalling and mechanosensitivity

Bradykinin evoked a powerful excitation of discharge in LSN afferents which as virtually abolished by the B_2 receptor antagonist HOE 140, indicating a simple pharmacology. This is as one might expect, considering the prevalence of the B_2 receptor in other types of sensory neurons, and its involvement in the signalling of pain from numerous sources[320-322]. The other major class of neuronal bradykinin receptor, the B_1 receptor, is highly inducible in states of inflammation or injury [178], suggesting a good target of interest in studies of altered afferent function in colonic inflammation. The proportion of LSN afferents responding to bradykinin was approximately two-thirds. These proportions compare with 55% of skin C-fibres in rat [145], 71% of joint afferents in cat [143], and 100% of

cardiac afferents in cat[182]. Almost 100% of guinea-pig airway vagal afferents responded to bradykinin, with the exception of fast-conducting fibres with cell bodies in the nodose ganglion, which were unresponsive[183]. A similar proportion (67%) of cat colonic LSN afferents recorded *in vivo* were bradykinin responsive as in the present study[128]. A study of 9 pelvic distension-sensitive colonic afferents in rats *in vivo* showed that 7 (78%) of them responded to bradykinin[102]. In contrast, only two pelvic serosal afferents (11%) were responsive to bradykinin in the current study. The difference in species and the different classes of afferent studied may account for the large difference in proportions in the two studies. Alternatively some responses to bradykinin *in vivo* could have been secondary to contractile responses, as observed in a study of vagal afferents[122]. This possibility was minimised in this and another study of LSN bradykinin responsiveness by the use of blockers of smooth muscle contractility[128]. A question posed by these results is why do some colonic afferents respond to bradykinin and others do not? In this study bradykinin responsiveness does not appear to be connected with mechanical responsiveness, size and location of receptive fields, spontaneous discharge, or sensitivity to other chemical stimuli. The only predictive factor was the pathway of origin of the fibres, suggesting that expression of the B_2 receptor is determined developmentally as a particular feature of thoracolumbar as opposed to lumbosacral colonic afferents. Studies in mice and rats have clearly shown these populations to be anatomically quite distinct, with very few colonic afferents having cell bodies in the intermediate DRG (L2-L5)[60, 81, 323].

In LSN afferents the response to bradykinin was associated with subsequent mechanical sensitisation, so that the response to a focal compression of the receptive field with a 2000mg von Frey hair was potentiated by nearly 50%. Interestingly this phenomenon was not observable in PN afferents, although the number of responsive afferents may have been too small to reveal it. Another difference between the pathways was the size of response to bradykinin, which was nearly five times larger in LSN afferents. Thus mechanical sensitisation and response amplitude may be connected.

Mechanical sensitisation after bradykinin was previously observed in studies of articular afferents[149], but no subpopulations of sensitised and non-sensitised afferents were apparent. The mechanism of bradykinin-induced mechanical sensitisation is likely to involve an interaction with mechanosensitive ion channels, which underlie mechanotransduction in sensory neurons. Recent evidence indicates that bradykinin receptors are linked to mechanosensitive ion channels via membrane lipid metabolism which increases opening of the channel. The main candidates so far are the transient receptor potential channels TRPV1 and TRPA1 (see above)[145, 187, 324]. TRPV1 could be particularly important in LSN afferents as capsaicin-sensitive LSN afferents in the current study displayed pronounced mechanical desensitsation after responding to capsaicin (see above). Similarly, TRPA1 could be particularly important in LSN afferents as bradykinin-sensitive LSN afferents in the current study displayed pronounced mechanical sensitisation. Bradykinin has been shown to activate TRPA1, and this activation is coupled to activation of B_2 receptors, via a phospholipase C pathway[187]. As TRPA1 is a candidate mechanotransduction channel, at least in vertebrate hair cells[257], this could identify a new target for colonic mechanotransduction in the LSN pathway. The answer to this question is currently under investigation by this investigator in a separate series experiments to this thesis.

Bradykinin induced activation of other TRP channels may also occur as many TRP channels are linked via G-protein coupled receptor signalling[242]. In reference to the targets of this particular study there are contrasting reports to a possible interaction between TPRV1 and Bradykinin receptors. Bradykinin signalling via protein kinases can sensitise TRPV1 responses to capsaicin and low pH[242, 325, 326]. In addition TRPV1 has been shown to be required for bradykinin induced thermal hyperalgesia[325], with such interactions occurring via second messenger systems as inhibition of protein kinase C, phospholipase C or phospholipase A2 can markedly decrease the nociception caused by bradykinin, but not that of capsaicin[327]. A direct activation of TRPV1 by bradykinin has

also been suggested based on TRPV1s involvement in the bradykinin-induced excitation of vagal airway afferent nerve terminals. This was based on the observation that every bradykinin-responsive C-fiber tested was also responsive to capsaicin and that prior addition of the TRPV1 antagonist capsazepine or TRPV1 blocker ruthenium red both reduced bradykinin evoked firing by at least 75% [328]. However, inhibitors of lipoxygenase and cyclooxygenase enzymes also blocked the bradykinin evoked firing by a similar amount suggesting that lipoxygenase products were responsible for the bradykinin B_2 receptor-induced activation of TRPV1 in the peripheral terminals of afferent C-fibers. In fact direct activation of TRPV1 by bradykinin has not been clearly demonstrated, and appears unlikely based on multiple observations that bradykinin responses in TRPV1 -/- mice are not significantly altered [150,329].

The likelihood that TRPV1 activation is responsible for the colonic afferents responses to bradykinin application in the present study is unlikely for a multitude of reasons. First, bradykinin causes mechanical sensitisation whilst capsaicin causes mechanical desensitization in LSN serosal afferents. Second, the bradykinin evoked response can be almost completely blocked by a B_2 receptor antagonist. Third, not all bradykinin responsive afferents were also responsive to capsaicin, particularly in the PN pathway where only 11% of afferents responded to bradykinin but almost half responded to capsaicin. Fourth, there are considerable differences in afferent responses profiles evoked in response to bradykinin and capsaicin, suggestive of difference mechanisms of activation. Thus any interaction between TRP channels and bradykinin in the current study may be limited to the mechanical sensitization observed in response to bradykinin and the possible involvement of TRPA1.

Purinergic signalling and mechanosensitivity

α,β-meATP evoked a reproducible and concentration-dependent excitation of LSN serosal afferents that was reversed by the P2X antagonist PPADS and was comparable to that observed in similar gastrointestinal preparations[88, 107, 165]. The latency and duration of response to α,β-meATP in the current study is similar to those observed previously in the mouse stomach and esophagus using an almost identical *in vitro* electrophysiological preparation[107]. However, the latency of response to α,β-meATP in the current study is significantly shorter (5.3 ± 1.6 s) than observed in the guinea-pig esophagus [88] (18.5 ± 3 s) and rat colon [165] (13.7 ± 0.85 s), which may relate to the relative thickness of these preparations and therefore drug accessibility. Chemical or mechanical sensitization or desensitization was not observed following α,β-meATP treatment in either LSN or PN afferents, unlike the findings with capsaicin and bradykinin, indicating a difference in the coupling of P2X receptors and TRPV1 and B_2 receptors with mechanisms influencing long-term excitability.

Endogenous activation of $P2X_3$, TRPV1 and B_2 receptors?

Two of the important current questions concerning primary afferent purinergic, vanilloid and bradykinin receptors are whether they are endogenously activated, and whether endogenous activation contributes to mechanical transduction. The mechanical desensitization observed after capsaicin in LSN afferents is not inconsistent with a role for TRPV1 in mechanotransduction. A recent study of mechanosensitivity in TRPV1 +/+ and -/- mice showed more directly that mechanotransduction was reduced in the -/- compared to the +/+, and the TRPV1 antagonist capsazepine was effective in reducing mechanical responses in the +/+[150]. The presence and release of TRPV1 ligands by mechanical and other stimuli is a complex issue, because there are numerous candidates, including heat, low pH and endogenous vanilloids. Temperature changes in the colon are unlikely to be sufficient to activate TRPV1 in health, but in diseased states such as IBS, where there is increased exposure to mediators like 5-HT[330], TRPV1 can become sensitive to

temperatures in the physiological range[202]. Changes in pH are established consequences of both inflammation and luminal metabolism of colonic flora, and are therefore likely contributors to endogenous activation of TRPV1, although these are outside the scope of this study. Endogenous vanilloids are present in the colon, particularly in disease states[331-334], and are likely to be the subject of continued investigation in the context of post-inflammatory changes in colonic afferent fibre sensitivity. Notably TRPV1 receptors have been shown to be upregulated in colonic nerve fibers of patients with inflammatory bowel disease[155] while administration of TRPV1 antagonists can attenuate disease severity in dextran sulphate sodium-induced colitis in mice[206].

Although there were potent effects of exogenous bradykinin in these *in vitro* preparations it is difficult to observe endogenous activation in this setting. This is firstly because the vascular supply is interrupted (which is a major source of bradykinin and its precursors), and secondly because the tissue is constantly superfused with fresh Krebs solution, which would wash away any locally released mediators. Therefore it remains to be determined if endogenous bradykinin is able to activate colonic afferents. The threshold concentrations observed were in the same range as the concentrations detected in inflamed or ischemic tissues ($>10^{-8}$M [335]), which makes endogenous activation likely. A study of cardiac afferents showed that a bradykinin receptor antagonist was able to block their activation by ischaemia[321], whilst administration of B_2 receptor antagonists can attenuate disease severity in dextran sulphate sodium-induced colitis in rats[185]. Notably, B_1 but not B_2 receptor protein is significantly increased in the intestines of both active ulcerative colitis and Crohn's disease patients compared with controls[157]. Joint afferents, on the other hand, were unaffected by antagonists[336], suggesting endogenous bradykinin actions may be tissue-specific. As detailed above, recent evidence indicates that bradykinin receptors are linked to mechanosensitive ion channels via membrane lipid metabolism which increases opening of the channel. The main candidates so far are the transient receptor potential channels TRPA1 and TRPV1[145, 187, 324]. This

may explain the mechanical sensitization observed in bradykinin responsive LSN afferents in the current study and could suggest differences in the mechanotransduction mechanisms between LSN and PN afferents.

In contrast to the results obtained with bradykinin and capsaicin, no change in mechanosensitivity after treatment with α,β-meATP or the P2X antagonist PPADS, was observed which is evidence against a direct role for P2X receptors in mechanotransduction. Other recent studies suggests, however, that ATP is present in the colon and is released by distension[165, 177] and that responses to distension are inhibited by P2X receptor antagonists. Other studies of urinary bladder afferents in $P2X_3$ +/+ and -/- mice agree that these receptors play an important direct role in mechanotransduction[151, 152]. How, therefore, can the difference between these results be explained? The most plausible explanation relates to the different stimuli applied to activate these endings and the location of the afferent nerve endings themselves. Receptive fields of afferents in previous studies were sensitive to distension of their host organ, placing them in close proximity to sources of endogenous ATP, released in response to the distension stimulus [165]. In the present study, however, endings were located on the outermost layer of the colon, which is further from the epithelium and myenteric neurons, both of which are major potential sources of endogenous ligand. There are other possible explanations, but ultimately the present data would argue that the role of ATP in mechanotransduction may be restricted to specific sub-populations of visceral afferents. It is also possible that the purinergic system may play a greater role in disease in the afferents observed in this study, as ATP is released in inflammatory conditions from a number of cell types, and notably, $P2X_3$ receptors have been shown to be upregulated in colonic nerve fibers of patients with inflammatory bowel disease[154, 155]. In addition to ATP numerous other mediators may be released from the myenteric or submucosal plexus which may lead to the release of other transmitters that may act on colonic afferents. However, this is unlikely to influence the results of the current study as

the receptive fields of these afferents are located on the outermost layer of the colon close to the mesenteric attachment. Moreover, recent studies using similar preparations to those employed here have demonstrated that mechanosensory and chemosensory function remain intact in the presence of Ca^{2+}-free Krebs solution to block fast exocytotic synaptic transmitter release, suggesting a direct mechanism of mechanosensory and chemosensory afferent function[95, 109].

In conclusion this study has made a direct comparison of the chemosensitivity of LSN and PN colonic afferents which terminate in thoracolumbar and lumbosacral DRG respectively. This approach has demonstrated that LSN and PN pathways from the colon display vastly different mechanosensitivity, chemosensitivity and functional receptor expression. The results of this study show that that activation of $P2X_3$, B_2 and TRPV1 receptors is more likely to be signalled via the thoracolumbar LSN pathway than the lumbosacral PN pathway and in some cases does so in the absence of any mechanical sensitivity. This study also demonstrates contrasting roles for B_2 and TRPV1 receptors in the mechanical sensitivity of LSN afferents after B_2 and TRPV1 receptor activation. As LSN and PN pathways relay information that may relate to symptoms in functional gastrointestinal disease, these results may have implications in the efficacy of therapies targeting receptor modulation.

Figure 2.1: Mechanosensory properties of LSN and PN serosal afferents

A) LSN and **B)** PN serosal afferents were activated only by probing of their receptive fields and were insensitive to circular stretch (1-5g) and fine mucosal stroking (10mg), note the difference in magnitude of response to a 2000mg von Frey hair. **C)** Stimulus-response functions of LSN and PN serosal afferents to increasing probing stimuli (70-2000mg). Pelvic serosal afferents were significantly more sensitive to probing displaying greater stimulus response functions ($P < 0.001$, two-way ANOVA, LSN $n = 31$ vs PN $n = 21$) * indicates $P < 0.01$ Bonferroni posthoc test, data from 400 mg stimulus; ** indicates $P < 0.001$, Bonferroni posthoc test, data from 1000 and 2000 mg stimulus). **D)** Pelvic serosal afferents displayed a more maintained adaptation response profile ($P < 0.001$, two-way ANOVA, LSN $n = 31$ vs PN $n = 21$) displaying a significantly shallower slope ($P < 0.001$; linear regression; PN; -0.23 ± 0.03 vs. LSN; -0.52 ± 0.03). **E)** Pelvic serosal afferents display lower stimulus thresholds as almost twice the amount of PN serosal afferents respond to lower probing stimuli (70, 160 and 400mg; $P < 0.001$, Fisher's-exact test).

Data from the PN in all figures are provided by Dr Carter Jones and Lingjing Xu, University of Iowa, USA. SMB designed and developed the LSN preparation and was involved in the subsequent development of the PN preparation with CJ. SMB was responsible for designing all of the mechanical stimulation protocols and analytical procedures for both pathways. SMB also designed the chemosensitivity protocols, determined the chemosensory targets and performed all of the analysis for both pathways.

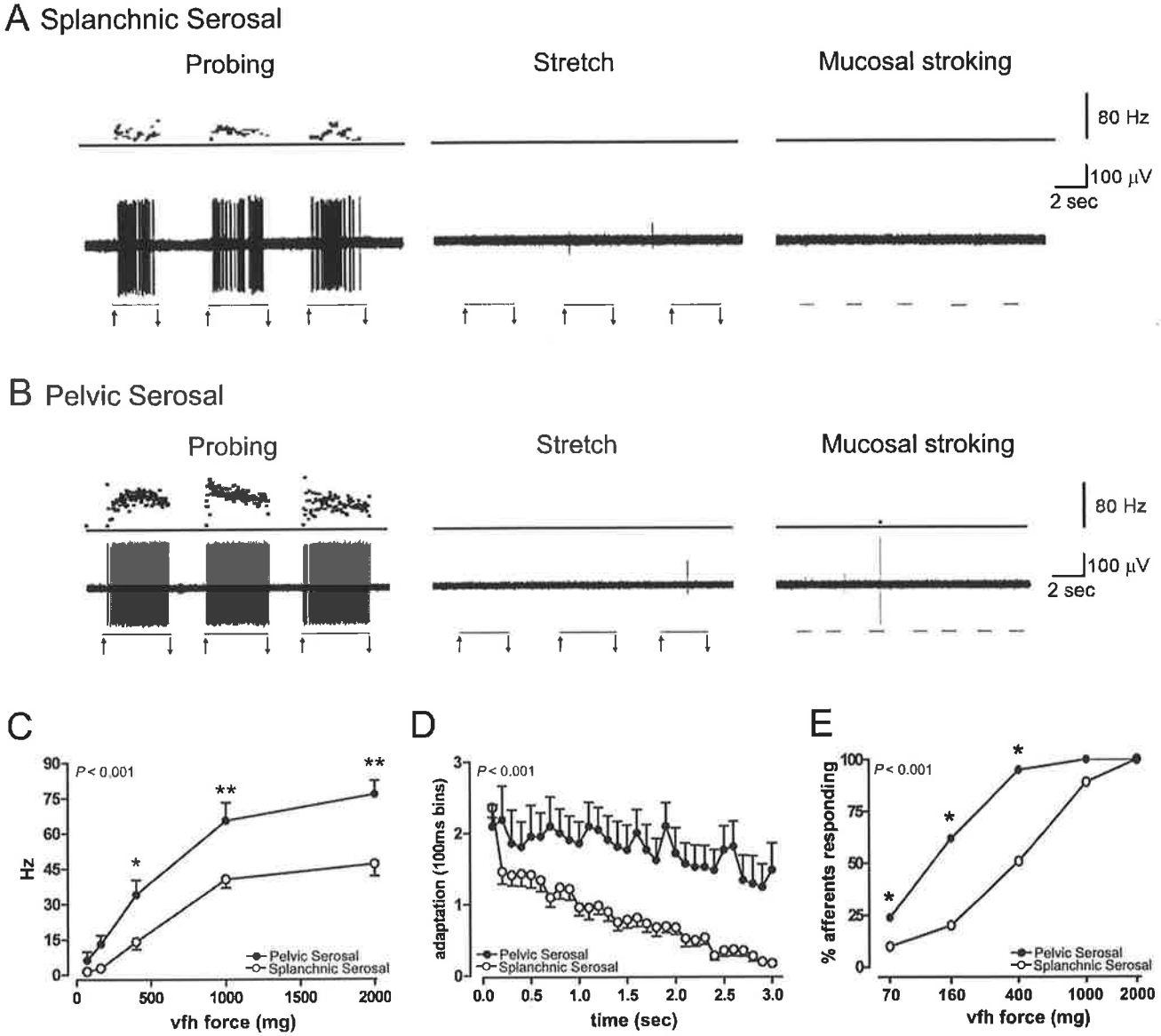

A Splanchnic Serosal
Probing
Stretch
Mucosal stroking
80 Hz
100 μV
2 sec
B Pelvic Serosal
Probing
Stretch
Mucosal stroking
80 Hz
100 μV
2 sec
C
P< 0.001
Hz
vfh force (mg)
Pelvic Serosal
Splanchnic Serosal
D
P< 0.001
adaptation (100ms bins)
time (sec)
Pelvic Serosal
Splanchnic Serosal
E
P< 0.001
% afferents responding
vfh force (mg)
Pelvic Serosal
Splanchnic Serosal

Figure 2.2: Proportions and distributions of LSN and PN afferents responding to α,β-meATP and capsaicin

(A) 40% (17 of 43) of LSN afferents responded to α,β-meATP (1 mM). Ten of these were mechanically sensitive serosal afferents, 7 others were recruited additionally during these tests and were not possible to activate mechanically. ***(B)*** By contrast only 7% (1 of 15) of PN serosal afferents responded to α,β-meATP (1 mM). In contrast to LSN serosal afferents no PN afferents were recruited during the addition of α,β-meATP. ***(C)*** 66% (27 of 41) of LSN afferents responded to bradykinin (1 μM). Seventeen of these afferents were mechanosensitive serosal afferents, whilst 10 were mechanically-insensitive afferents recruited during the addition of bradykinin into a ring surrounding a mechanically sensitive afferent being tested. ***(D)*** By contrast, only 11% (2 of 19) of PN serosal afferents responded to bradykinin (1 μM). In contrast to LSN serosal afferents no mechanically insensitive PN afferents were recruited during the addition bradykinin (1 μM). ***(E)*** In total 26 of 43 (61%) LSN afferents responded to capsaicin (3 μM). 51% (16 of 31) of these were LSN serosal afferents that responded to capsaicin (3 μM) whilst five of the chemosensitive mechanically insensitive units recruited by α,β-meATP also responded to capsaicin (3 μM). An additional five units which were insensitive to α,β-meATP and were recruited by bradykinin (1 μM) also responded to capsaicin (3 μM) ***(F)***. In contrast, only 47% (8 of 17) of PN serosal afferents responded to capsaicin (3 μM). Seven of these were serosal afferents and only one additional mechanically-insensitive afferent was recruited by capsaicin. Unlike the LSN pathway, only one PN afferent was recruited during capsaicin addition. IMG: inferior mesenteric ganglion; LSN: lumbar splanchnic nerve; PN: pelvic nerve; MPG: major pelvic ganglion.

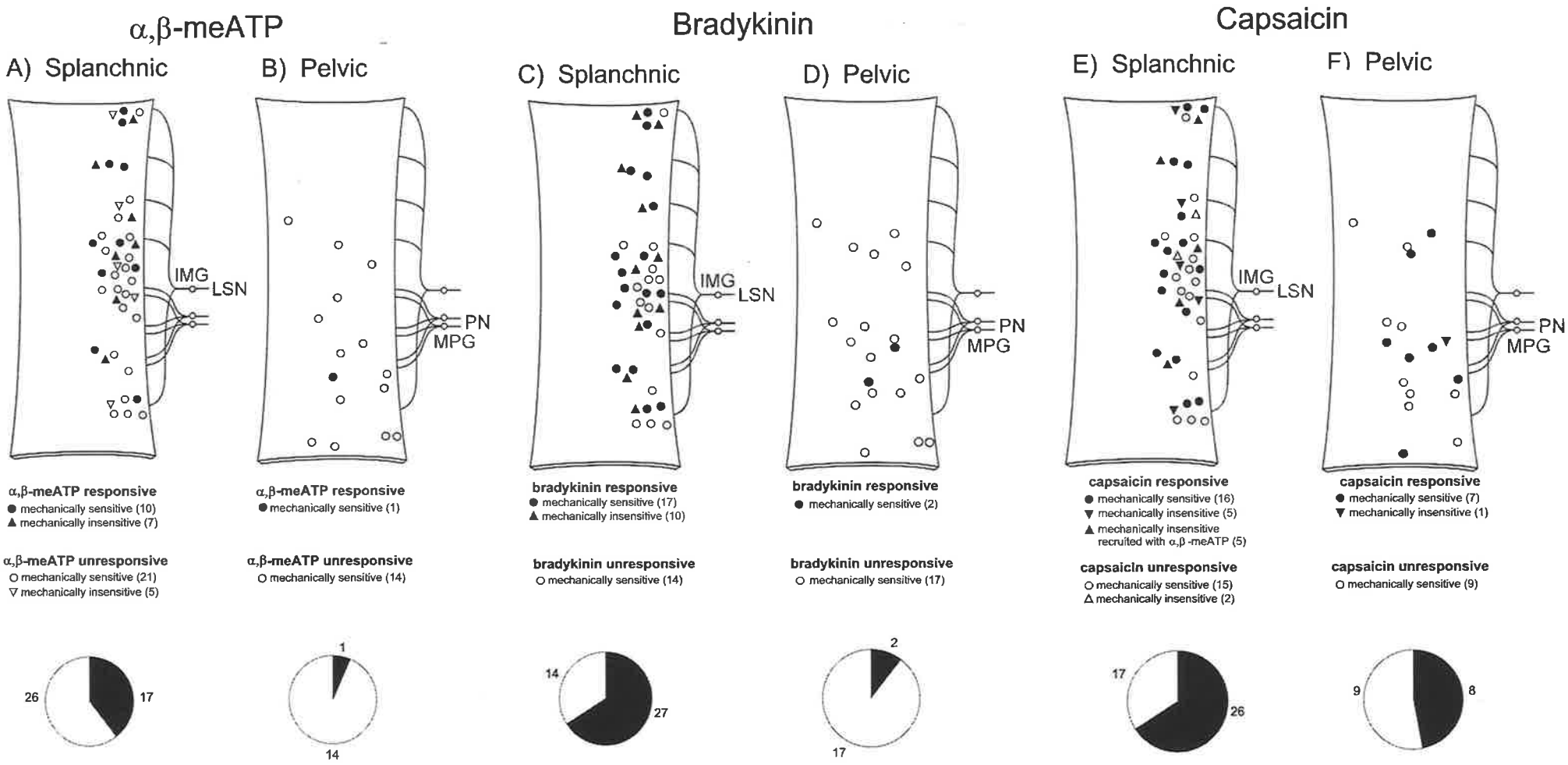
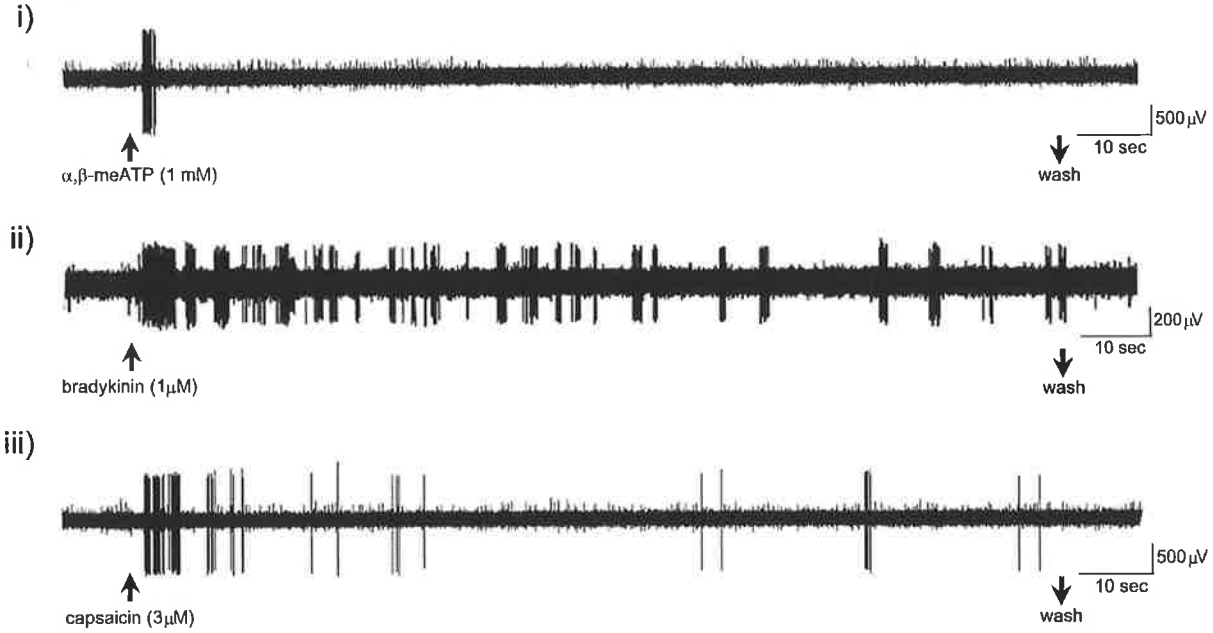

α,β-meATP
Bradykinin
Capsaicin
A) Splanchnic
B) Pelvic
C) Splanchnic
D) Pelvic
E) Splanchnic
F) Pelvic
IMG
LSN
PN
MPG
α,β-meATP responsive
● mechanically sensitive (10)
▲ mechanically insensitive (7)
α,β-meATP unresponsive
○ mechanically sensitive (21)
▽ mechanically insensitive (5)
26
17
α,β-meATP responsive
● mechanically sensitive (1)
α,β-meATP unresponsive
○ mechanically sensitive (14)
1
14
bradykinin responsive
● mechanically sensitive (17)
▲ mechanically insensitive (10)
bradykinin unresponsive
○ mechanically sensitive (14)
14
27
bradykinin responsive
● mechanically sensitive (2)
bradykinin unresponsive
○ mechanically sensitive (17)
2
17
capsaicin responsive
● mechanically sensitive (16)
▼ mechanically insensitive (5)
▲ mechanically insensitive recruited with α,β -meATP (5)
capsaicln unresponsive
○ mechanically sensitive (15)
△ mechanically insensitive (2)
17
26
capsaicin responsive
● mechanically sensitive (7)
▼ mechanically insensitive (1)
capsaicin unresponsive
○ mechanically sensitive (9)
9
8

Figure 2.3: LSN and PN afferent responses to α,β-meATP, bradykinin and capsaicin

(A) Example of LSN serosal afferents responding to ***i)*** α,β-meATP (1 mM) ***ii)*** bradykinin (1 μM) and to ***iii)*** capsaicin (3 μM). ***(B) i)*** All but one PN serosal afferents were unresponsive to α,β-meATP (1 mM) ***ii)*** Example of one of the few PN afferents that was responsive to bradykinin application ***iii)*** Example of a PN afferent responding to capsaicin. Note the differences in latency and duration of response compared with LSN *(Aiii). Upper panels show spike rate histograms (1 sec bin width) of single unit activity. Lower panels show raw electrophysiological data.*

A) Splanchnic



B) Pelvic

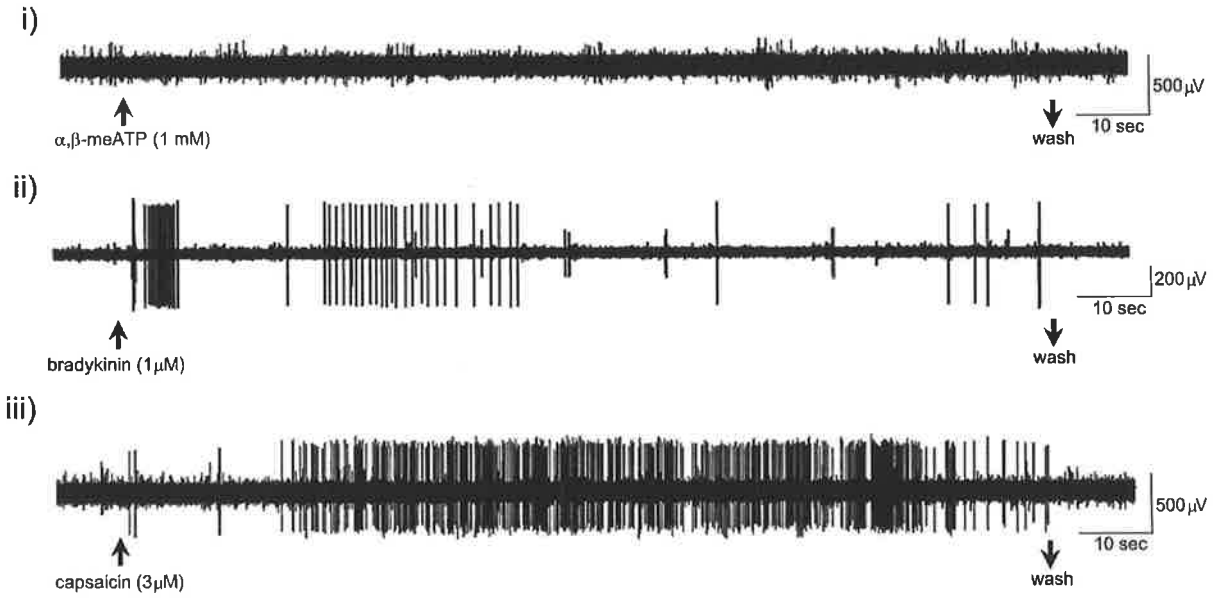


Figure 2.4: ***(A)*** Latency of response of LSN and PN afferents that responded to α,β-meATP (1 mM), bradykinin (1 μM) and capsaicin (3 μM). PN afferents took a significantly longer time to respond to capsaicin compared with LSN afferents (*, $P < 0.05$, *t*-test, PN $n=7$ vs LSN $n=16$). ***(B)*** Duration of response of LSN and PN afferents that responded to α,β-meATP and capsaicin. Pelvic afferents displayed significantly longer durations of response to capsaicin (*, $P < 0.05$, PN $n=7$ vs LSN $n=16$, *t*-test). ***(C)*** Bradykinin (1 μM) elicited significantly more spikes per response in bradykinin responsive LSN serosal afferents than PN serosal afferents ($P < 0.05$, t-test).

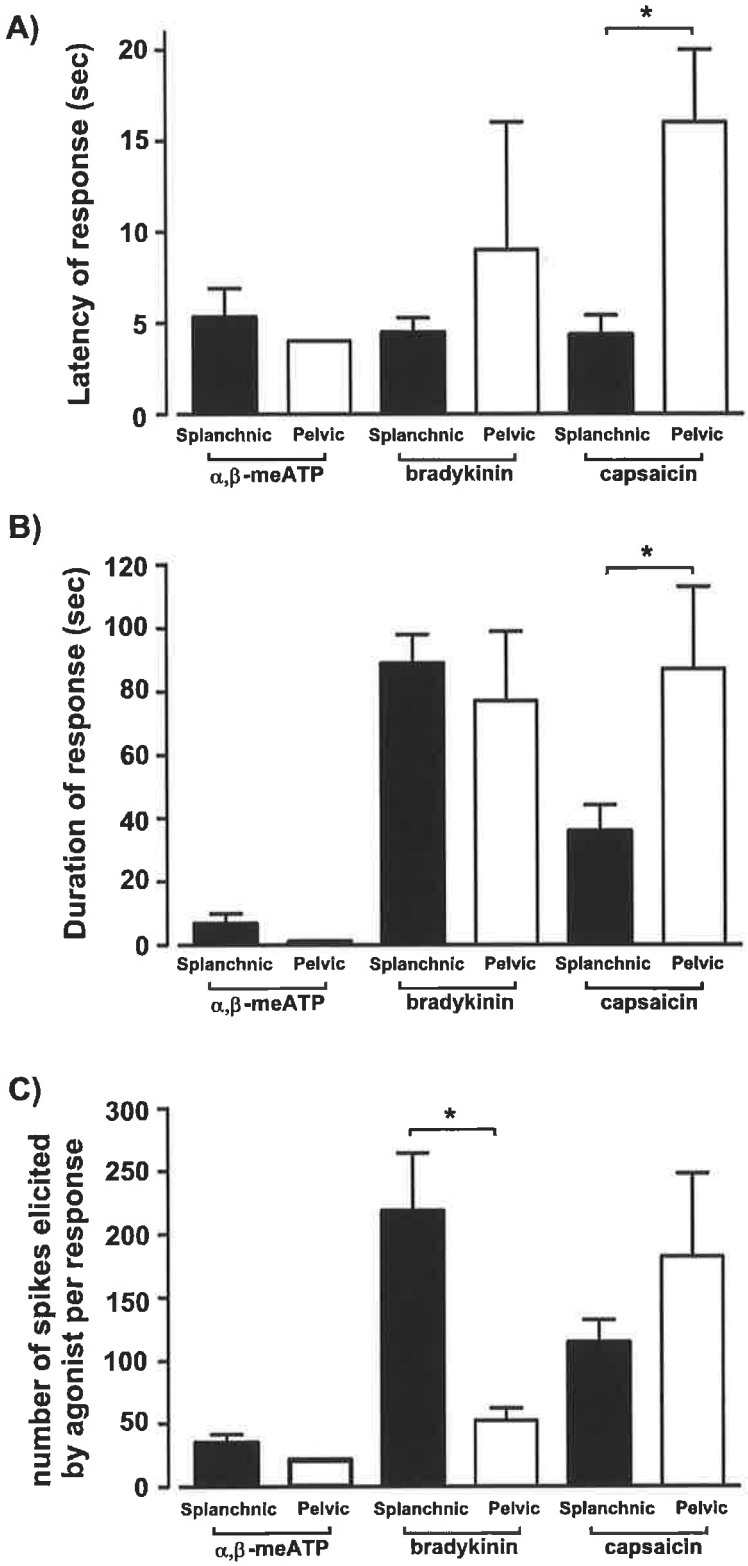

A)
Latency of response (sec)
20
15
10
5
0
*
Splanchnic
Pelvic
Splanchnic
Pelvic
Splanchnic
Pelvic
α,β-meATP
bradykinin
capsaicin
B)
Duration of response (sec)
120
100
80
60
40
20
0
*
Splanchnic
Pelvic
Splanchnic
Pelvic
Splanchnic
Pelvic
α,β-meATP
bradykinin
capsaicin
C)
number of spikes elicited by agonist per response
300
250
200
150
100
50
0
*
Splanchnic
Pelvic
Splanchnic
Pelvic
Splanchnic
Pelvic
α,β-meATP
bradykinin
capsaicin

Figure 2.5: ***(A)*** α,β-meATP caused a concentration-dependent excitation of LSN serosal afferents (EC_{50}: 21.2 μM, *dashed line indicates sigmoidal dose response curve, n=6*). ***(B)*** The LSN serosal afferent response to α,β-meATP (1 mM) was blocked by the non-selective P2X receptor antagonist PPADS (100 μM; *n=6). Note only one PN afferent responded to α,β-meATP.* ***(C)*** Bradykinin caused a concentration-dependent excitation of LSN serosal afferents (EC_{50}: 0.16 μM, *dashed line indicates sigmoidal dose response curve, n=6*). ***(D)*** The LSN serosal afferent response to bradykinin (1 μM) was blocked by the B_2 receptor antagonist HOE-140 (10nM; *n=4).* ***(E) i)*** Response of a LSN serosal afferent to bradykinin (1 μM) and ***ii)*** lack of response to bradykinin (1 μM) in the presence of HOE-140 (10nM, applied for 20 mins). *Upper traces show spike rate and lower traces show raw electrophysiological data. Scale bars apply throughout. Note only two PN serosal afferents responded to bradykinin.*

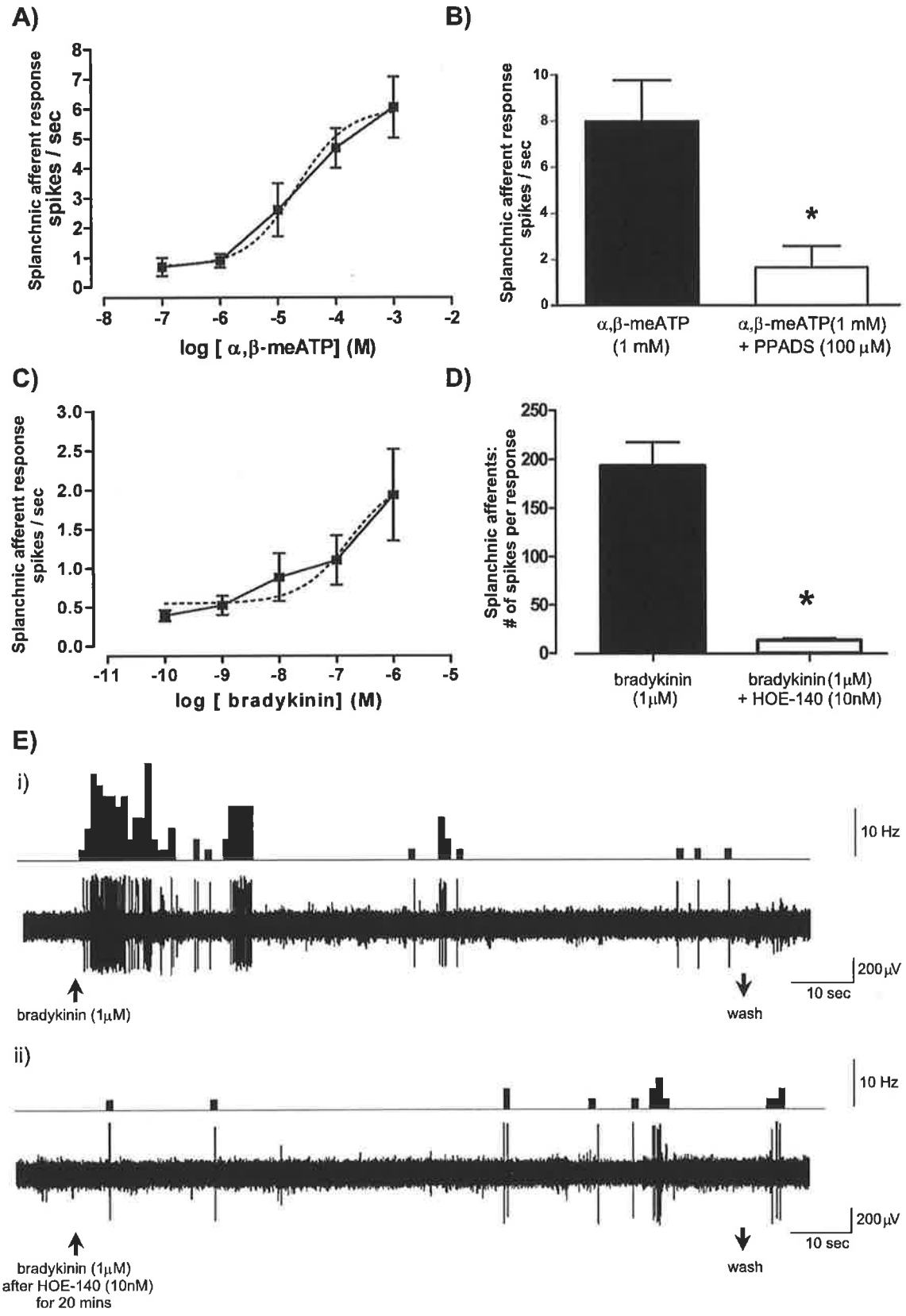

A)
Splanchnic afferent response spikes / sec
log [α,β-meATP] (M)
B)
Splanchnic afferent response spikes / sec
α,β-meATP (1 mM)
α,β-meATP(1 mM) + PPADS (100 μM)
C)
Splanchnic afferent response spikes / sec
log [bradykinin] (M)
D)
Splanchnic afferents: # of spikes per response
bradykinin (1μM)
bradykinin (1μM) + HOE-140 (10nM)
E)
i)
10 Hz
200μV
10 sec
bradykinin (1μM)
wash
ii)
10 Hz
200μV
10 sec
bradykinin (1μM) after HOE-140 (10nM) for 20 mins
wash

Figure 2.6: Altered mechanosensitivity of LSN serosal afferents after responding to bradykinin and capsaicin (A) Splanchnic afferents did not display altered mechanosensitivity after responding to α,β-meATP. (LSN *n=10*; *Note only one PN afferent responded to α,β-meATP*). ***(D)*** Similarly, LSN afferents that did not respond to α,β-meATP also did not display altered mechanosensitivity. ***(B)*** Splanchnic afferents displayed a modest but significant mechanical sensitization after responding to bradykinin (1 μM; ($P < 0.05$, t-test). ***(E)*** In contrast, LSN afferents that were unresponsive to bradykinin did not display altered mechanosensitivity ($P > 0.05$, t-test). ***(B&E)*** Pelvic afferents did not display altered mechanosensitivity in either bradykinin responsive or unresponsive populations ($P > 0.05$, t-test). ***(C)*** Capsaicin-responsive LSN afferents displayed significant mechanical desensitization after responding to capsaicin (3 μM; $P < 0.01$, *t*-test *n=16*). ***(C)*** This is in direct contrast to capsaicin-responsive PN afferents which did not display altered mechanosensitivity ($P > 0.05$, *t*-test, *n=7*). ***(C&F)*** Pelvic afferents did not display altered mechanosensitivity in capsaicin responsive or unresponsive populations ($P > 0.05$, t-test). *Afferents were mechanically tested with a 2000 mg von Frey hair before and after drug addition* ***A&D)*** *α,β-meATP and* ***B&E)*** *bradykinin* ***C&F)*** *capsaicin.*

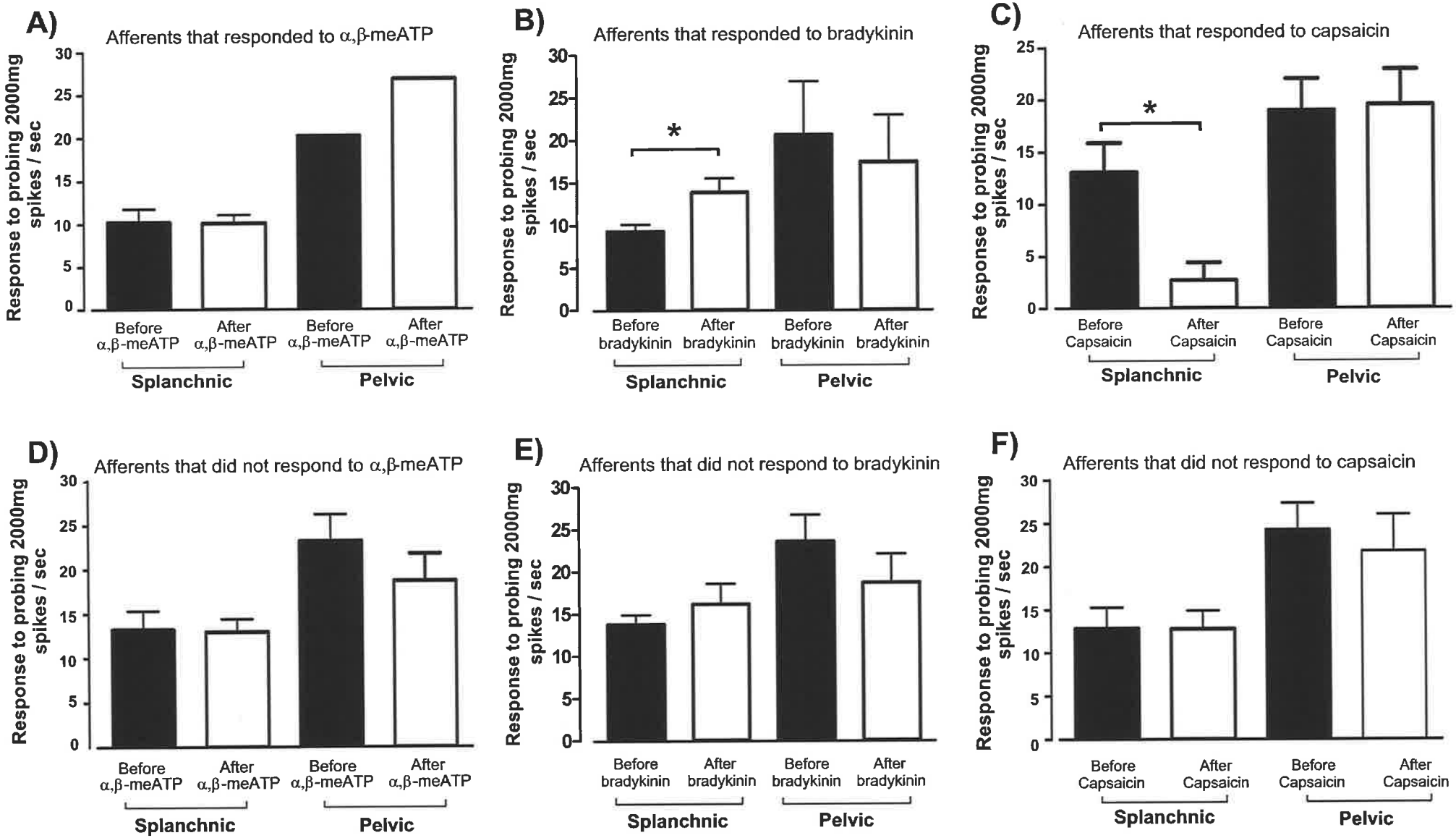
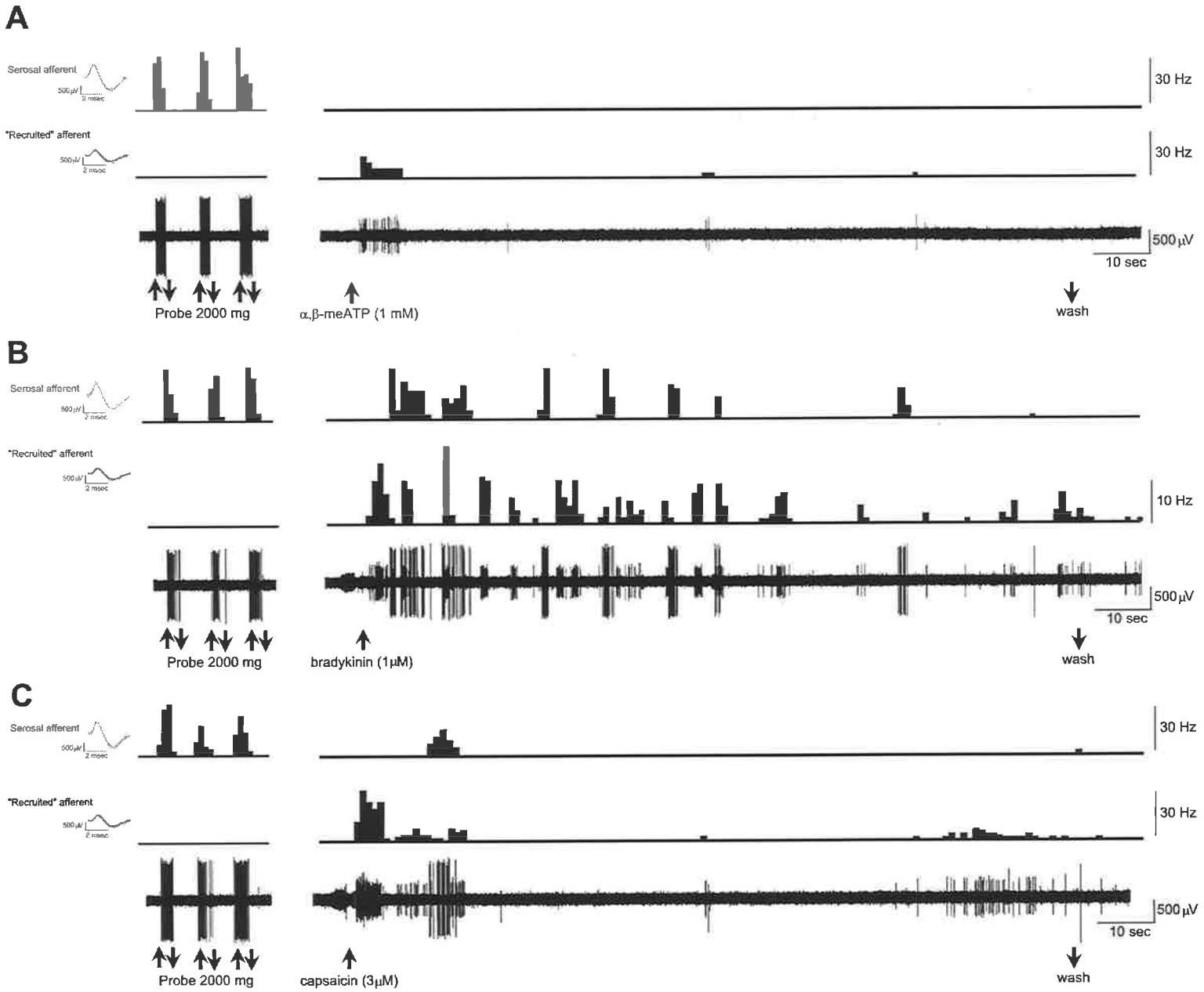

A)
Afferents that responded to α,β-meATP
Response to probing 2000mg spikes / sec
Before α,β-meATP
After α,β-meATP
Before α,β-meATP
After α,β-meATP
Splanchnic
Pelvic
B)
Afferents that responded to bradykinin
Response to probing 2000mg spikes / sec
*
Before bradykinin
After bradykinin
Before bradykinin
After bradykinin
Splanchnic
Pelvic
C)
Afferents that responded to capsaicin
Response to probing 2000mg spikes / sec
*
Before Capsaicin
After Capsaicin
Before Capsaicin
After Capsaicin
Splanchnic
Pelvic
D)
Afferents that did not respond to α,β-meATP
Response to probing 2000mg spikes / sec
Before α,β-meATP
After α,β-meATP
Before α,β-meATP
After α,β-meATP
Splanchnic
Pelvic
E)
Afferents that did not respond to bradykinin
Response to probing 2000mg spikes / sec
Before bradykinin
After bradykinin
Before bradykinin
After bradykinin
Splanchnic
Pelvic
F)
Afferents that did not respond to capsaicin
Response to probing 2000mg spikes / sec
Before Capsaicin
After Capsaicin
Before Capsaicin
After Capsaicin
Splanchnic
Pelvic

Figure 2.7: Recruitment of mechanically insensitive LSN afferents by α,β-meATP bradykinin and capsaicin. ***(A)*** A mechanically sensitive LSN (large amplitude) afferent that was unresponsive to α,β-meATP. However, the addition of α,β-meATP recruited a mechanically insensitive (small amplitude) afferent. ***(B)*** A mechanically sensitive LSN (large amplitude) afferent that was responsive to bradykinin. The addition of bradykinin also recruited a mechanically insensitive (small amplitude) afferent. Mechanically insensitive afferents recruited by bradykinin were limited to the LSN pathway. ***(C)*** Both the mechanically sensitive (large amplitude) and mechanically insensitive (small amplitude) afferent responded to capsaicin. *Insets*: the average spike shape of the LSN serosal afferent and the recruited mechanically insensitive afferent. *Upper traces* show spike rate and *lower traces* show raw electrophysiological data. Scale bars apply throughout.


A
Serosal afferent
500 µV
2 msec
"Recruited" afferent
500 µV
2 msec
30 Hz
30 Hz
500 µV
10 sec
Probe 2000 mg
α,β-meATP (1 mM)
wash
B
Serosal afferent
500 µV
2 msec
"Recruited" afferent
500 µV
2 msec
10 Hz
500 µV
10 sec
Probe 2000 mg
bradykinin (1µM)
wash
C
Serosal afferent
500 µV
2 msec
"Recruited" afferent
500 µV
2 msec
30 Hz
30 Hz
500 µV
10 sec
Probe 2000 mg
capsaicin (3µM)
wash

CHAPTER 3

THE ACID SENSING ION CHANNELS ASIC1a, 2 AND 3 CONTRIBUTE DIFFERENTLY TO COLONIC SEROSAL AND MESENTERIC MECHANORECEPTOR FUNCTION

Summary

Background & Aims: Mechanosensitive ion channels are essential for the detection of noxious and non-noxious stimuli. Deletion of the Acid Sensing Ion Channels (ASIC) 2 or ASIC3 alters mechanosensory function in cutaneous afferents; however the roles of ASIC1, 2 and 3 in colonic mechanotransduction are not yet reported. Therefore this study directly compares the roles of ASIC1, 2 and 3 in the mechanotransduction of high threshold colonic primary afferents. *Methods:* An *in vitro* preparation of mouse colon with attached LSN nerves was used to examine the mechanosensitivity of serosal and mesenteric afferents to a range of probing forces. ASIC1a, 2 and 3 +/+ and -/- mice were used and in each genotype recordings were compared. *Results*: A total of 112 mesenteric and 139 serosal afferents were recorded. Disrupting ASIC1a significantly increased the sensitivity of both serosal ($P < 0.01$) and mesenteric ($P < 0.001$) afferents; however adaptation was only altered in mesenteric afferents. Disrupting ASIC2 significantly increased the sensitivity of serosal ($P < 0.01$) afferents but had no effect on mesenteric mechanosensitivity. Disrupting ASIC2 did not alter either mesenteric or serosal adaptation. Disrupting ASIC3 significantly decreased the sensitivity of both serosal ($P < 0.001$) and mesenteric ($P < 0.01$) afferents; however adaptation was altered only in mesenteric afferents. *Conclusions:* This study identifies contrasting roles for ASIC1a, 2 and 3 in mechanosensory function. It also shows differences in their contribution to serosal and mesenteric afferents. Thus, all 3 ASICs contribute to serosal mechanosensitivity, but only ASIC1a and 3 contribute to mesenteric mechanosensitivity and adaptation. Only ASIC3 makes a positive contribution to mechanosensitivity. These findings indicate diversity in mechanisms of mechanotransduction which holds promise for therapeutic targeting.

It is clear from the literature that the defining feature in all IBS subgroups is colonic mechanical hypersensitivity[5, 337] and although peripheral mechanosensation plays an important role in the etiology of this disease[3-7], as yet no potential therapy has targeted the molecules responsible for transducing mechanical signals from the colon. Perhaps this is because the identity of mechanotransduction mechanisms within the viscera is as yet unknown; however, knowledge of mechanosensation in other systems provides important clues. The Degenerin/Epithelial Na^+ Channel (DEG/ENaC) cation channels are attractive candidates to serve as transducers of mechanical stimuli[8]. In mammals the DEG/ENaC family comprises α, β and γ ENaC and the acid sensing ion channels ASIC1 (or BNaC2), ASIC2 (or BNC1 or MDEG), ASIC3 (or DRASIC) and ASIC4 (or SPASIC). Various lines of evidence suggest that DEG/ENaC ion channels may involved in the mechanotransduction process. First, DEG/ENaC proteins have been localized in specialized mechanosensory structures in many species. A multitude of *MEC* genes are expressed in the touch receptor neuron responsible for gentle touch in *Caenorhabditis elegans*[269]. The *Drosophila melanogaster* gene *Pickpocket* is localized in multiple dendritic mechanosensory neurons in the embryo[270]. In the rat, β and γ ENaC proteins have been localized in specialized mechanosensory structures in the skin and aortic arch[273, 274]. ASICs have a widespread expression in the CNS of zebrafish[338]. Importantly, ASICs have also been localized within mammalian peripheral mechanosensory structures and sensory ganglia[9, 10, 280, 281, 291-293]. Specifically in rat and mouse, ASIC2 and 3 are present in mechanosensory lanceolate nerve endings surrounding the hair shaft and other cutaneous sensory structures[9, 10, 291, 298]. ASIC1, 2 and 3 have been located in rat and mouse DRG (L4-L5)[9, 10, 280, 291-295], and rat and mouse brain[281, 292-294, 299, 302, 339]. Nerve ligation studies have indicated that ASIC subunits are transported from DRG cell bodies to sensory nerve terminals in the periphery[291].

A more direct implication of a mechanotransduction role for these channels has come from studies utilising gene ablation whereby a lack of DEG/ENaC channels results in major deficits in body touch perception and mechanosensory function[233, 234, 236, 237, 240, 260, 261]. Specifically, in the invertebrate *Caenorhabditis elegans* mutations disrupting the DEG/ENaC proteins MEC-4 and MEC-10 impair responses to touch[260-264], whilst MEC-6 is a part of the degenerin channel complex that may mediate mechanotransduction in touch cells[265]. Moreover, UNC-8 has been implicated in proprioception[266] whilst UNC-105 is involved in the detection of muscle stretch[267, 268]. As such ASICs, by their close relationships to the above invertebrate channels, have been implicated in mechanotransduction. This has been strengthened by more direct methods using mice lacking individual ASIC family members. Data from mice with disruptions to the *ASIC2* and *ASIC3* genes respectively indicate they play specific, distinct and diverse roles in cutaneous mechanotransduction[9, 10]. There are five classes of cutaneous mechanoreceptors in the mouse; rapidly adapting (RA) and slowly adapting (SA) mechanoreceptors; down hair (D-hair) receptors; A-fiber mechanonociceptors (AM), and high threshold C-fibers. Mice with a disruption of the *ASIC2* gene displayed reduced responses of RA and SA mechanoreceptors, thus these mice showed a reduced sensitivity in mechanosensitive neurons detecting light touch, but not in other populations[10]. Mice with a disrupted *ASIC3* gene have reduced responsiveness of AM nociceptors, whereas RA mechanoreceptors in contrast showed increased mechanosensitivity, thus these mice displayed a reduced sensitivity of mechanoreceptors detecting a noxious pinch but an increased sensitivity to light touch[9]. In addition, these ASIC3 -/- mice also displayed a reduced response to mechanically-induced pain[294]. In contrast, mice with a disruption of *ASIC1a* gene displayed no change in the function of any class of the five classes of cutaneous mechanoreceptor[304]. These data identify the ASIC2 and ASIC3 subunits as essential for the normal function of cutaneous mechanoreceptors, but no role for ASIC1a. Moreover, the demonstration of both positive and negative effects of ASIC2 and 3 mutations suggests that ASIC subunits form components of a mechanosensory complex.

Thus the present study had the purpose of addressing the lack of knowledge concerning the comparative roles of ASIC1, 2 and 3 in the mechanotransduction of LSN mesenteric and serosal colonic afferents from the distal colon by using an *in vitro* electrophysiological approach. This approach allowed this study to determine not only if the ASIC1, 2 and 3 subunits were involved in mechanosensory function but also if the role of ASIC1a, 2 and 3 differed between subsets of mechanosensory afferents. The results of this study identify contrasting roles for ASIC1a, 2 and 3 in colonic mechanosensory function. It shows differences in the respective ASIC subunits contribution to serosal and mesenteric afferent function. These results show that all three ASIC subunits contribute to serosal mechanosensitivity, but only ASIC1a and 3 contribute to mesenteric mechanosensitivity and adaptation of response. Only ASIC3 makes a positive contribution to mechanosensitivity.

Materials and Methods

All electrophysiological experiments were performed in accordance with the guidelines of the Animal Ethics Committees of the Institute for Medical and Veterinary Science and the University of Adelaide, Adelaide, Australia.

Generation of ASIC1a -/- mice

Mice with disrupted a *ASIC1a* gene were generated at the University of Iowa, Iowa City, USA by homologous recombination in embryonic stem (ES) cells as previously described[299]. Briefly, in the targeted allele, a PGK-neo cassette replaces the first exon of the *ASIC1a* gene and approximately 400 bp of upstream sequence (*Figure 3.1A*)[299]. The *ASIC1* gene gives rise to two proteins (ASIC1a and 1b) through alternative splicing. Disruption of *ASIC1* using the currently available strategy leads to loss of transcript only for ASIC1a, but expression of both ASIC1a and 1b proteins is successfully abolished[299, 304]. This knockout is therefore referred to henceforth specifically

as ASIC1a -/-. Heterozygote pairs of mice were mated to generate animals for the current investigation. Animals were genotyped by GENSA (IMVS, Adelaide). Briefly, individual mice were tail tipped, DNA extracted via a phenol-chlorophorm procedure and Polymerase Chain Reactions (PCR) performed with primers specific for the wild type and mutant alleles of ASIC1. Gel electrophoresis was performed to distinguish between null mutant (-/-), heterozygote (+/-) and wild-type (+/+) mice. Homozygous ASIC1a +/+ or -/- littermates were blindly selected for experiments after genotyping and results were encoded until statistical analysis. Littermates were otherwise indistinguishable by body weight, general behavior or gross anatomical features of the gut or elsewhere.

Generation of ASIC2 -/- mice

Mice with disrupted *ASIC2* genes were generated at the University of Iowa, Iowa City, USA by homologous recombination in (ES) cells as previously described[10]. Briefly, in the targeted allele, a PGK-neo cassette replaces the region encoding M2 and exon 3' to this of the *ASIC2* gene (*Figure 3.1B*)[10]. Heterozygote pairs of mice were mated to generate animals for the current investigation. Animals were genotyped by GENSA (IMVS, Adelaide) as described for ASIC1 above. Homozygous ASIC2 +/+ or -/- littermates were blindly selected for experiments after genotyping and results were encoded until statistical analysis. Littermates were otherwise indistinguishable by body weight, general behavior or gross anatomical features of the gut or elsewhere.

Generation of ASIC3 -/- mice

Mice with disrupted *ASIC3* genes were generated at the University of Iowa, Iowa City, USA by homologous recombination in ES cells as previously described[9]. Briefly, the region of the gene containing the first exon, including the start ATG codon, was replaced with the PGK-neo cassette to this of the *ASIC3* gene (*Figure 3.1C*)[9]. Gene-targeting experiments were done essentially as previously described[340]. A 4.5 kb Not1/Xho1 fragment, located 5' of exon 1, and a 3.5 kb Asc1/Kpn1

fragment, located 3' of exon 1, were subcloned into the targeting vector, P1339 LoxPIGK-Neo, on either side of the neomycin resistance cassette sequence. The linearized targeting vector was introduced into ES cells by electroporation. Two different ES cell lines carrying a targeted ASIC3 allele were injected into C57BL/6 blastocysts to generate chimeras[9]. Heterozygote pairs of mice were mated to generate animals for the current investigation. Animals were genotyped by GENSA (IMVS, Adelaide) as described for ASIC1 above. Homozygous ASIC3 +/+ or -/- littermates were blindly selected for experiments after genotyping and results were encoded until statistical analysis. Littermates were otherwise indistinguishable by body weight, general behavior or gross anatomical features of the gut or elsewhere.

In vitro mouse colonic primary afferent preparation

Dissections were carried out in ASIC1a, 2 or 3 +/+ and -/- male and female mice (20-30g) according to protocols described in detail in Chapters 1 and 2.

Characterization of LSN mesenteric and serosal afferents

Lumbar splanchnic colonic afferents were characterized as described in Chapters 1 and 2. Receptive fields were identified by systematically stroking the mucosal surface with a brush of sufficient stiffness to activate all types of mechanosensitive afferent. The present study focused on mesenteric and serosal afferents as these afferent classes represent 86% of the LSN innervation of the colon (see Chapter 1). Mesenteric afferents were located on the mesenteric attachment and were tested with focal compression of their receptive fields via perpendicular probing with calibrated von Frey hairs (70, 160, 400, 1000, 2000 and 4000mg; each force applied 5 times for a period of 3 s). Serosal afferents were classified by their graded response to focal compression of their receptive fields via perpendicular probing with calibrated von Frey hairs (70, 160, 400, 1000, 2000 and

4000mg; each force applied 5 times for a period of 3 s) and their insensitivity to circular stretch (1-5g) and fine mucosal stroking (10mg)[312].

Electrophysiological data recording and analysis

Electrical signals generated by nerve fibers placed on the platinum recording electrode were fed into a differential amplifier, filtered, sampled (20 kHz) using a 1401 interface (Cambridge Electronic Design, Cambridge, UK) and stored on a PC for off-line analysis. The amplified signal was also used for online audio monitoring. Action potentials were analysed off-line using the Spike 2 wavemark function and discriminated as single units on the basis of distinguishable waveform, amplitude and duration. Data are expressed as mean ± SEM. *n* indicates the number of individual afferents; N indicates the number of mice. Stimulus-response functions were constructed by assessing the peak number of action potentials/sec (maximum instantaneous frequency), mean frequency, spike rate and spikes per second of the response to probing with calibrated von Frey hairs (70, 160, 1000, 2000 and 4000 mg) for 3 sec each. Adaptation profiles during the probing response were calculated as the mean number of spikes per 200 ms bin over the entire 3 s of a 1g probing stimulus. These measures reflect changes in the initial dynamic phase (instantaneous frequency) and the overall response (mean frequency, spike rate and spikes/sec). Data were analysed using Prism 4 software (GraphPad Software, San Diego, CA, USA), and where appropriate, were analysed using a two-way analysis of variance (ANOVA; to determine significant differences between curves) with Bonferroni post hoc tests (to determine differences at individual stimulus strengths). Linear regression analysis was used to compare slopes of adaptation profiles. Fisher's exact tests were used to compare significance differences between stimulus thresholds between ASIC1a, 2 or 3 +/+ and -/- mice. Differences were considered significant at a level of $P < 0.05$.

Results

Effects of ASIC1a deletion on colonic mesenteric afferent function

ASIC1a +/+ mesenteric afferents displayed graded responses to an ascending series of probing stimuli (70-4000mg) when expressed as either maximum instantaneous frequency *(Figure 3.2A),* mean frequency *(Figure 3.2B),* spike rate *(Figure 3.2C) or* spikes per second *(Figure 3.2D).* Deletion of *ASIC1a* significantly increased the sensitivity in colonic mesenteric afferents, as indicated by significantly increased stimulus response functions *($P < 0.0001$, Figure 3.2A, B, C and D).* Bonferroni post hoc tests also indicated significant increases in ASIC1a -/- responses at 2000 and 4000mg probing stimuli (max IF * $P < 0.05$, *Figure 3.2A*), 1000 and 4000mg probing stimuli (mean frequency * $P < 0.05$, *Figure 3.2B*), and at a 1000mg probing stimulus (spikes/sec, * $P < 0.05$, *Figure 3.2D*).

A small but significant alteration in the adaptation profile of mesenteric afferents was evident (*Figure 3.3A and B*). ASIC1a -/- colonic mesenteric mechanoreceptors displayed altered adaptation when expressed as raw data, indicated by a significantly steeper adaptation slope than in ASIC1a +/+ ($P < 0.0001$, linear regression, *Figure 3.3A*), which represents a more rapid adaptation overall. The adaptation profile also was altered significantly ($P < 0.001$; 2-way ANOVA, *Figure 3.3A*). Bonferroni post hoc test indicates significant increases in -/- mice responses at indicated time points (* $P < 0.05$, *Figure 3.3A*). When the data were normalized to allow for the increased responses in ASIC1a -/- mice, a significantly steeper adaptation slope in ASIC1a -/- was observed ($P < 0.001$, linear regression, *Figure 3.3B*) indicating a more rapid adaptation overall. The adaptation profile also was altered significantly ($P = 0.004$; 2-way ANOVA, *Figure 3.3B*). Overall these data indicate that disrupting *ASIC1a* causes a linear adaptation profile of mesenteric afferent responses to probing resulting in a more rapid adaptation response than in ASIC1a +/+ afferents.

To compare the sensitivity of mesenteric afferents to probing in ASIC1a +/+ vs. -/- the percentage of mesenteric afferents that were activated by the range of probe forces used in this study were plotted. Overall, no significant changes in threshold were observed between ASIC1a +/+ and -/- mesenteric afferents *(Figure 3.4A)*. Mesenteric afferents in ASIC1a +/+ and -/- mice displayed similar receptive field distributions, indicating no major anatomical abnormalities in -/- mice *(Figure 3.5)*.

Effects of ASIC1a deletion on colonic serosal afferent function

ASIC1a +/+ serosal afferents displayed graded responses to an ascending series of probing stimuli (70-4000mg) when expressed as either maximum instantaneous frequency *(Figure 3.6A)*, mean frequency *(Figure 3.6B)*, spike rate *(Figure 3.6C) or* spikes per second *(Figure 3.6D)*. Deletion of *ASIC1a* significantly increased the sensitivity in colonic serosal afferents, as indicated by significantly increased stimulus response functions *(Figure 3.6A, B, C and D)*. Bonferroni post hoc tests also indicated significant increases in ASIC1a -/- mice responses at a 4000mg probing stimulus (max IF * $P < 0.05$, *Figure 3.6A*, and spikes/sec, * $P < 0.05$, *Figure 3.6D*).

In contrast to mesenteric afferents, no significant alteration in the adaptation profile of serosal afferents was evident when ASIC1a -/- colonic serosal mechanoreceptor adaptation responses where expressed as raw data ($P > 0.05$, linear regression and 2-way ANOVA, *Figure 3.7A*) or normalized data ($P > 0.05$, linear regression and 2-way ANOVA, *Figure 3.7B*).

To compare the sensitivity of serosal afferents to probing in ASIC1a +/+ vs. -/- the percentage of serosal afferents that were activated by the range of probe forces used in this study were plotted. Overall, the thresholds required to activate serosal afferents observed between ASIC1a +/+ and -/- serosal afferents were almost identical *(Figure 3.4B)*. As observed in the mesenteric

population, serosal afferents in ASIC1a +/+ and -/- mice displayed similar receptive field distributions, indicating no major anatomical abnormalities in -/- mice *(Figure 3.5)*.

Effects of ASIC2 deletion on colonic mesenteric afferent function

ASIC2 +/+ mesenteric afferents displayed graded responses to an ascending series of probing stimuli (70-4000mg) when expressed as either maximum instantaneous frequency *(Figure 3.8A)*, mean frequency *(Figure 3.8B)*, spike rate *(Figure 3.8C) or* spikes per second *(Figure 3.8D)*. Deletion of *ASIC2* had no effect on the sensitivity in colonic mesenteric afferents, as indicated by almost identical stimulus response functions *($P > 0.05$, Figure 3.8A, B, C and D)*.

Similarly no alteration in the adaptation profile of mesenteric afferents was evident ($P > 0.05$, linear regression, and 2-way ANOVA, *Figure 3.9A and B*). ASIC2 -/- colonic mesenteric mechanoreceptors displayed almost identical adaptation profiles when expressed as raw and normalized data ($P > 0.05$, 2-way ANOVA) and as indicated by a similar adaptation slopes compared with ASIC2 +/+ mice ($P > 0.05$, linear regression, *Figure 3.9A*),

In addition, no significant changes in threshold were observed between ASIC2 +/+ and -/- mesenteric afferents *(Figure 3.10A)*, whilst mesenteric afferents in ASIC2 +/+ and -/- mice displayed similar receptive field distributions, indicating no major anatomical abnormalities in -/- mice *(Figure 3.11)*. Overall these data indicate that *ASIC2* makes little if any contribution to mesenteric afferent mechanoreceptor function.

Effects of ASIC2 deletion on colonic serosal afferent function

ASIC2 +/+ serosal afferents displayed graded responses to an ascending series of probing stimuli (70-4000mg) when expressed as either maximum instantaneous frequency *(Figure 3.12A),* mean frequency *(Figure 3.12B),* spike rate *(Figure 3.12C) or* spikes per second *(Figure 3.12D).* In contrast to mesenteric afferents, deletion of *ASIC2* significantly increased the sensitivity in colonic serosal afferents, as indicated by significantly increased stimulus response functions *(Figure 3.12A, B, C and D).* Bonferroni post hoc tests also indicated significant increases in ASIC2-/- mice responses at a 4000mg probing stimulus (all parameters * $P < 0.05$, *Figure 3.12A,B,C and D*).

Although the adaptation profile was altered ($P < 0.01$; 2-way ANOVA) in ASIC2-/- mice when expressed as raw data, serosal mechanoreceptors displayed a similar adaptation slope to ASIC2 +/+ mice ($P > 0.05$, linear regression), Moreover, when these data were normalized to account for the increased response in ASIC2-/-, no significant alteration in the adaptation profile or the slope of serosal afferents was evident in ASIC2 -/- ($P > 0.05$, linear regression and 2-way ANOVA, *Figure 3.13A*). Overall, these results indicate little if any contribution of ASIC2 to the adaptation of serosal afferent mechanoreceptors. Similarly, the thresholds required to activate serosal afferents observed between ASIC2 +/+ and -/- serosal afferents were almost identical *(Figure 3.10B).* As observed in the mesenteric population, serosal afferents in ASIC2 +/+ and -/- mice displayed similar receptive field distributions, indicating no major anatomical abnormalities in -/- mice *(Figure 3.11).*

Effects of ASIC3 deletion on colonic mesenteric afferent function

ASIC3 +/+ mesenteric afferents displayed graded responses to an ascending series of probing stimuli (70-4000mg) when expressed as either maximum instantaneous frequency *(Figure 3.14A),* mean frequency *(Figure 3.14B),* spike rate *(Figure 3.14C) or* spikes per second *(Figure 3.14D).* Deletion of *ASIC3* significantly decreased the sensitivity in colonic mesenteric afferents, as

indicated by significantly shallower stimulus response functions *(Figure 3.14A, B, C and D).* Bonferroni post hoc tests also indicated significant decreases in ASIC3-/- mice responses at 1000, 2000 and 4000mg probing stimuli (max IF, * $P < 0.05$, *Figure 3.14*A) and at 2000 and 4000mg probing stimuli (mean frequency, spike rate and spikes/sec, * $P < 0.05$, *Figure 3.14B,CandD*).

No alteration in the adaptation profile or slope of mesenteric afferents was evident ($P > 0.05$, linear regression, and 2-way ANOVA, *Figure 3.15AandB*) when expressed as raw data. However, when these data were normalized to allow for the decreased response in ASIC3-/-, a small but significant alteration in the adaptation profile of mesenteric afferents was evident ($P < 0.05$, linear regression, *Figure 3.15A and B*). ASIC3 -/- colonic mesenteric mechanoreceptors displayed altered adaptation profiles as indicated by a significantly shallower adaptation slope than in ASIC3 +/+ mice ($P < 0.05$, linear regression, *Figure 3.15A*), indicating a more rapid adaptation overall. The adaptation profile was also altered significantly ($P < 0.001$; 2-way ANOVA, *Figure 3.15A*). Overall these data indicate that disrupting *ASIC3* causes a linear adaptation profile of mesenteric afferents resulting in a more rapid adaptation response than in ASIC3+/+ afferents.

To compare the sensitivity of mesenteric afferents to probing in ASIC3 +/+ vs. -/- the percentage of mesenteric afferents that were activated by the range of probe forces used in this study were plotted Overall, no significant changes in threshold were observed between ASIC3 +/+ and -/- mesenteric afferents *(Figure 3.16A).* Mesenteric afferents in ASIC3 +/+ and -/- mice displayed similar receptive field distributions, indicating no major anatomical abnormalities in -/- mice *(Figure 3.17).*

Effects of ASIC3 deletion on colonic serosal afferent function

ASIC3 +/+ serosal afferents displayed graded responses to an ascending series of probing stimuli (70-4000mg) when expressed as either maximum instantaneous frequency *(Figure 3.18A),* mean frequency *(Figure 3.18B),* spike rate *(Figure 3.18C) or* spikes per second *(Figure 3.18D).* Deletion of *ASIC3* significantly decreased the sensitivity in colonic serosal afferents, as indicated by significantly shallower stimulus response functions *(Figure 3.18A, B, C and D).* Bonferroni post hoc tests also indicated significant decreases in ASIC3 -/- responses at 2000 and 4000mg probing stimuli (max IF * $P < 0.05$, *Figure 3.18A),* at a 2000mg probing stimulus (spike rate, * $P < 0.05$, *Figure 6c)* and at 2000 and 4000mg probing stimuli (spikes/sec, * $P < 0.05$, *Figure 3.18D*). In contrast to mesenteric afferents, no significant alteration in the adaptation profile of serosal afferents was evident when ASIC3 -/- colonic serosal mechanoreceptor adaptation responses were expressed as raw data ($P > 0.05$, linear regression and 2-way ANOVA, *Figure 3.19A*) or normalized data ($P > 0.05$, linear regression and 2-way ANOVA, *Figure 3.19B*).

To compare the sensitivity of serosal afferents to probing in ASIC3 +/+ vs. -/- the percentage of serosal afferents that were activated by the range of probe forces used in this study were plotted. Overall, the thresholds required to activate serosal afferents observed were not significantly different between ASIC3 +/+ and -/- serosal afferents *($P > 0.05$,* Fisher's exact test, *Figure 3.16B).* As observed in the mesenteric population, serosal afferents in ASIC3 +/+ and -/- mice displayed similar receptive field distributions, indicating no major anatomical abnormalities in -/- mice *(Figure 3.17).*

Discussion

This study demonstrates widespread changes in the sensitivity of LSN mesenteric and serosal afferents with the disruption of ASIC1a, 2 or 3, indicating that they are all required for normal colonic mechanotransduction. Disrupting *ASIC1a* significantly increased the sensitivity of both

serosal and mesenteric afferents; whilst only altering the adaptation response profile of mesenteric afferents. Disrupting *ASIC2* significantly increased the sensitivity of serosal afferents only and had no effect on mesenteric afferent mechanosensitivity. Disrupting *ASIC2* however did not alter either mesenteric or serosal afferent adaptation response profile. In contrast, disrupting ASIC3 significantly decreased the sensitivity of both serosal and mesenteric afferents; whilst altering mesenteric afferent adaptation response profiles. Thus ASIC3 appears to make a direct and positive contribution to mechanotransduction in both classes of colonic afferent. It is worthy of note that fibres respond in a graded manner regardless of genotype. However, responsiveness of colonic afferents to weak stimuli is relatively unchanged in -/- yet their responses to higher stimulus intensities are markedly altered, suggesting a greater contribution of ASIC subunits as noxious intensities are reached. Based on the results of this study overall it is unlikely that ASIC subunits function simply as individual mechanically gated cation channels, and in some cases they may in fact dampen the mechanotransduction process. This would be the case for ASIC1a in both mesenteric and serosal afferents and for ASIC2 in serosal afferents because in both instances mechanosensitivity was increased in the -/- animals. This negative modulatory or "dampening" role is probably a result of existence of all three ASIC subtypes as heteromultimers[298, 301], in which each member contributes directly or indirectly to mechanotransduction. ASIC1a and ASIC2 clearly appear to make little if any direct contribution to mechanotransduction of LSN colonic afferents, because without them mechanosensitivity is universally increased in mesenteric and serosal LSN colonic afferents, suggesting that the heteromultimeric mechanotransducer becomes more efficient. However, the role of ASIC2 is intriguing by its capacity to influence mechanosensitivity negatively in colonic serosal afferents and positively in gastro-oesophegeal and cutaneous afferents (see below for discussion). More evidence for ASIC subunits forming functional heteromultimers arises from studies of recombinant and native channels[233, 298, 301]. These showed that low pH-induced currents in cells expressing ASIC1, 2 or 3 individually showed quite different characteristics to the current in native

sensory neurons. On the other hand, the low pH-induced current in cells co-expressing all three ASIC subunits was identical to the native response, indicating some interaction between them must occur. Thus, the emerging picture from these positive and negative effects of ASIC mutation is of a complex interplay between individual members of the heteromultimeric complex, which explains the positive and negative effects of deletion of each member.

Effects of disrupting ASIC1a, 2 and 3 in colonic afferents relative to vagal gastro-oesophageal and cutaneous afferents

A comparison of results from the present study with those of previous investigations on cutaneous afferents and concurrent studies on gastro-oesophageal afferents reveals contrasting contributions of the ASIC subunits in the colon compared with gastro-oesophageal and cutaneous mechanotransduction (*Table 3.1*). Such a comparison is possible because these studies have consistently used a direct approach of measuring mechanosensitivity in single primary afferent fibers with identified receptive fields in the colon, gastro-oesophageal region or skin[9, 10, 304]. The results of the current study show that disrupting *ASIC1a* results in a marked increase in mesenteric and serosal colonic afferent mechanosensitivity. There are two types of vagal afferent which innervate the gastro-oesophaegeal region in the mouse; mucosal receptors and tension receptors[107]. As observed in the colon disrupting *ASIC1a* results in a marked increase in both types (mucosal and tension) of vagal gastro-oesophegeal afferent. However, ASIC1a influenced mechanosensation slightly differently in the colon versus the upper gut. Although mechanosensitivity in both regions was enhanced by loss of ASIC1a, its absence had different effects on adaptation. In rapidly-adapting mesenteric colonic afferents, the loss of ASIC1a led to changes in adaptation profile, resulting in these afferents displaying a more linear adaptation profile compared with ASIC1a +/+ mesenteric afferents, whereas adaptation was unaffected in slowly-adapting gastro-oesophageal afferents. In

contrast to the viscera deletion of *ASIC1a* had no effect on the mechanosensitivity or adaptation of any of the 5 classes of cutaneous afferent[304].

The results of the current study show that *ASIC2* disruption led to a potent increase in the mechanosensitivity of colonic LSN serosal afferents but no change in mesenteric colonic afferents. Disruption of *ASIC2* also increased the mechanosensitivity of gastro-oesophageal mucosal receptors, but in direct contrast reduced the mechanosensitivity gastro-oesophageal tension receptors and two out of five cutaneous mechanoreceptor types, and had no effect on the other three[10]. Therefore taken together these results suggest the contribution of ASIC2 is specific to individual fibre types and regions of gut, and is notably opposite in the colon and skin.

The predominant effect of *ASIC3* disruption in the colon was a reduced sensitivity of both types of afferent. Similarly, *ASIC3* disruption reduced the sensitivity of gastro-oesophageal tension afferents and one class of cutaneous afferent. However, in the skin *ASIC3* disruption did result in an increased sensitivity of RA mechanoreceptors[9]. As observed in ASIC1a -/- mice disruption of *ASIC3* influenced the adaptation of response differently in the colon versus the upper gut. Although mechanosensitivity in both mesenteric colonic afferents and gastro-oesophageal tension receptors was reduced by the loss of ASIC3, its absence had different effects on adaptation. In rapidly-adapting mesenteric colonic afferents, the loss of ASIC3 led to changes in adaptation profile, resulting in these afferents displaying a more linear adaptation profile compared with ASIC3 +/+ mesenteric afferents, whereas adaptation was unaffected in slowly-adapting gastro-oesophageal afferents. However, such a widespread positive influence on mechanosensitivity suggests ASIC3 is an important potential target for modulating mechanosensory function in viscera, particularly in colonic high-threshold afferents. Taken together, these data relating to changes in ASIC1a, 2 and 3

-/- mice indicate there is a high degree of tissue- and pathway-specificity to the contributions of ASIC subunits to mechanotransduction.

How does disrupting ASIC1a, 2 or 3 cause intra- and inter- pathway variations between the mechanosensitivity of colonic, vagal gastro-oesophageal and cutaneous afferents?

It is clear from these studies that there is a high degree of tissue- and pathway-specificity to the contributions of ASIC subunits to mechanotransduction. Firstly, these differences may reflect structural and/or functional divergence between mechanoreceptors in these tissues. It is clear from the results in Chapter 1 and from numerous studies[53, 86-88, 93, 94, 106, 107, 112, 306, 341-347] and reviews[58, 74] that different classes of afferent from the same nerve supply innervating the same organ terminate in endings within different layers of the gut. The same also applies for different classes of cutaneous afferents[100, 101]. This divergence is also highlighted by the relatively non-adapting nature of gastro-oesophageal vagal tension receptors relative to colonic LSN afferents. Slowly- or non- adapting afferents are suggested to signal maintained events such as filling or increases in smooth muscle tone, whereas rapidly adapting afferents are suggested to signal rapid events such as spasm and resultant torsion on the mesentery[48, 74, 312]. Therefore tissue-specific differences in mechanoreceptor structure and force transduction could be responsible for the contrasting results. However, there are several other potential explanations for these contrasting observations and the notion that mechanoreceptors may differ in molecular composition and function between tissues is further emphasized by the comparison of colonic, gastro-oesophageal and cutaneous mechanosensation. ASIC1a, ASIC2 and ASIC3 subunits heteromultimerize in cultured DRG neurons to mediate acid-evoked currents[298, 301]. The first direct indication of ASICs role as a mechanosensor resulted from disrupting the closely related *ASIC2* and *ASIC3* genes which had a significant impact on cutaneous mechanosensation; therefore one might anticipate that deleting ASIC1a would also have some effect, however, this was not the case. It is possible that the differences observed between

mesenteric and serosal colonic afferents in the current study when disrupting the *ASIC* genes could relate to the absolute number or the relative proportions of ASIC subunits contributing to the mechanosensory complex in each afferent type. Alternatively, the associated intracellular and extracellular proteins might confer distinct properties on mechanoreceptors in the two environments. Similar explanations could also account for the differences observed between mucosal and tension gastro-oesophageal receptors and the differences between the 5 types of cutaneous afferents, and ultimately for the differences observed between colonic, gastro-oesophageal and cutaneous afferent function.

In studies such as the present one when gene disruption is used, interpretation is complicated because when one of the channels is lost from the heteromultimeric complex, the others may compensate either by changes in expression levels or by reassembly into different configurations. Previous studies have investigated the up- or downregulation of other subunits in previous quantitative studies of ASIC expression, and showed that ASIC 1 and 2 levels are unchanged in ASIC3 -/-, and ASIC 1 and 3 levels are unchanged in ASIC2 -/-[9, 10, 298]. The possibility remains that there may be changes specific to thoracolumbar DRG which will be investigated in Chapter 5. However, the possibility remains that there may be changes in subunit configuration other than just the subtraction of the deleted channel and that by ASICs may reassemble into different configurations. These scenarios will be addressed in Chapters 4 and 5.

Two recent studies have called into question the relevance of ASIC2 and 3 in mechanotransduction. However, their evidence was based either on studies of the responses of cell bodies to mechanical stimuli[348], on limited analyses of cutaneous sensory fibers[349], and on an indirect measure of visceral mechanosensation[349]. Firstly, in the study on mechanically gated conductances in the membrane of acutely isolated cultured DRG neurons there was no evidence of

any role in mechanosensory function for ASIC2 and ASIC3[350], which is in direct contrast to electrophysiological recordings of cutaneous afferents[9, 10]. Although some heterogeneity in the kinetics of evoked currents was observed, a clear correspondence with identified mechanoreceptor spike responses to natural stimuli found *in vivo* was not apparent[100]. This discrepancy could also be explained as in isolated cells there is a lack of tissue-specific factors and connections to the extra and intracellular matrices which are potential mechanisms by which a channel responds to a tactile stimulus[233]. Indeed various integral membrane proteins, such as stomatin have recently been implicated in the mechanotransduction process[262, 274, 351-353], and stomatin itself can bind to and alter the gating of ASICs. This may be a one of the reasons responsible for the inability to demonstrate a mechanosensory response of ASICs in isolated DRG cells[350]. Secondly, a study on the role of ASIC2 in viscera used colonic stretch-induced peptide release as an indirect measure of mechanosensation[349], and found no change in ASIC2 -/-. However, as detailed in Chapters 1 and 2 there are a total of eight classes of mechanosensitive afferent innervating the colon from the LSN and PN and that their respective chemosensitivities to known noxious stimuli are different. The current study detailed a role for ASIC2 in serosal but not mesenteric LSN colonic afferents. Stretch-sensitive (muscular) afferents from the LSN were not tested in the current study due to their low abundance and PN afferents were not part of this study. These results taken together suggest that stretch-sensitive colonic afferents may be similar to mesenteric colonic afferents in their apparent lack of requirement for ASIC2.

Although the negative results described above and the different approaches taken make it difficult to construct a true picture of the role that ASICs play at peripheral endings, given the identical approaches taken to sub-classify colonic, gastro-oesophageal and cutaneous primary afferent endings a picture is emerging at least at this level. This role is highlighted by the observation that disrupting either the *ASIC1a, 2 or 3* genes alters digestive activity in the whole animal[304, 354] and

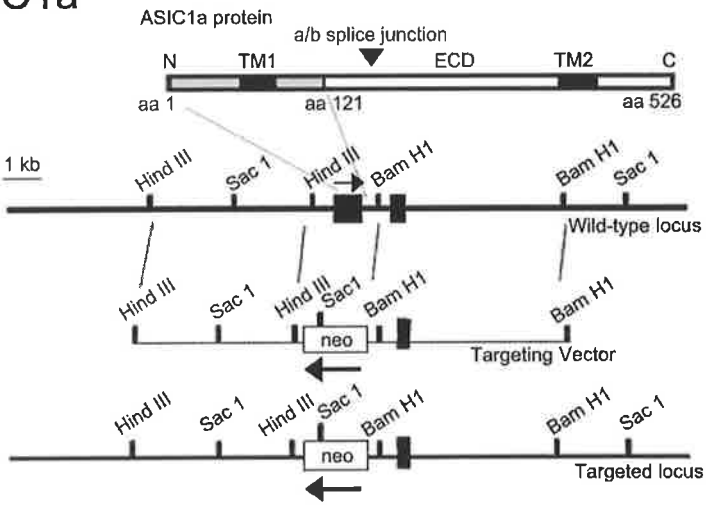
the behavioural responses to somatic stimuli[9, 10, 304] in a predictable manner based on the changes observed in visceral and cutaneous mechanosensory function, demonstrating the physiological relevance of the alterations in mechanosensory function.

In conclusion this study has made a direct comparison of the roles of ASIC1a, 2 and 3 in the mechanosensory function of LSN afferents. These results identified contrasting roles for ASIC1a, 2 and 3 in colonic mechanosensory function and shows differences in the respective ASIC subunits contributing to serosal and mesenteric afferent function. All three ASIC subuints contribute to serosal mechanosensitivity, but only ASIC1a and 3 contribute to mesenteric mechanosensitivity and adaptation of response. Only ASIC3 makes a positive contribution to colonic mechanosensitivity. The roles of ASIC1a, 2 and 3 in colonic mechanosensory function differs from their role in vagal gastro-oesophageal and cutaneous mechanoreceptor function. Findings of both positive and negative effects of *ASIC* mutations on mechanosensitivity suggest a complexity in the way they contribute to mechanotransduction.

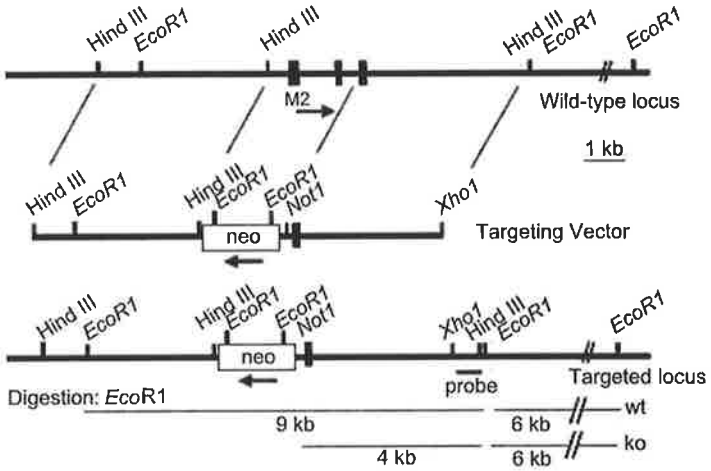
Figure 3.1: Generation of ASIC1a, 2 and 3 Knockout Mice

A) ASIC1a +/+ and -/- mice were generated at the University of Iowa, Iowa, USA and acquired via collaboration with Associate Professor John Wemmie and Professor Michael Welsh. Briefly, ASIC1a -/- mice were generated by homologous recombination in embryonic stem cells using a standard approach previously reported[299]. In the knockout allele, PGK neo cassette replaces the first exon of the *ASIC1a* gene and approximately 400 bp of upstream sequence. The top bar illustrates the anticipated topology of the ASIC protein (N, amino terminus; C, carboxyl terminus; TM, transmembrane domain; ECD, extracellular domain; stippled region is coded by targeted exon; arrowhead, splice junction). Also shown are +/+ genomic locus, targeting vector, and targeted locus. Figure adapted from[299]. ***B)*** ASIC2 +/+ and -/- mice were acquired via collaboration with Dr Margaret Price and Professor Michael Welsh, University of Iowa, Iowa, USA). Briefly, ASIC2 -/- mice were generated by homologous recombination in embryonic stem cells using a standard approach similar to that previously reported[10]. In the knockout allele, a PGK-neo cassette replaces the region encoding M2 and exon 3' to this of the *ASIC2* gene. Simplified restriction map of mouse ASIC2 locus and structure of the targeting vector. The region of the gene containing the first exon, including the start ATG codon, was replaced with the PGK-neo cassette (neo). Exons are shown as black rectangles. Arrows denote the direction of transcription. (N, amino terminus; C, carboxyl terminus; TM, transmembrane domain; ECD, extracellular domain; stippled region is coded by targeted exon; arrowhead, splice junction). Also shown are +/+ genomic locus, targeting vector, and targeted locus. Figure adapted from[10]. ***C)*** ASIC3 +/+ and -/- mice were acquired via collaboration with Dr Margaret Price and Professor Michael Welsh, University of Iowa, Iowa, USA. Briefly, ASIC3 -/- mice were generated by homologous recombination in embryonic stem cells using a standard approach previously reported[10]. In the knockout allele, the region of the gene containing the first exon, including the start ATG codon, was replaced with the PGK-neo cassette (neo). Figure adapted from[9].

A) ASIC1a



B) ASIC2



C) ASIC3

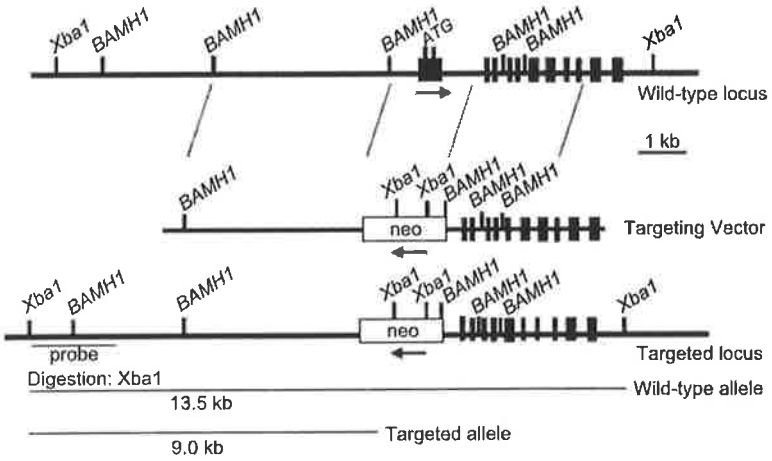


Figure 3.2: Effects of disrupting ASIC1a on LSN mesenteric afferent mechanosensitivity

A) Increased sensitivity of colonic mesenteric mechanoreceptors with responses expressed as maximum instantaneous frequency (Max IF). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC1a (+/+) (•, n=10, N=8) and (-/-) mice (□, n=17, N=11), showing a significant increase in stimulus-response function in ASIC1a -/- mice ($P < 0.0001$, two-way ANOVA). Bonferroni post hoc test indicates significant increases in ASIC1a-/- mice responses at 2000 and 4000mg stimuli (* $P < 0.05$).

B) Increased sensitivity of colonic mesenteric mechanoreceptors with responses expressed as mean frequency (Mean Freq). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC1a (+/+) (•, n=10, N=8) and (-/-) mice (□, n=17, N=11), showing a significant increase in stimulus-response function in ASIC1a -/- mice ($P < 0.0001$, two-way ANOVA). Bonferroni post hoc test indicates significant increases in -/- mice responses at 1000 and 4000mg stimuli (* $P < 0.05$).

C) Increased sensitivity of colonic mesenteric mechanoreceptors with responses expressed as spike rate (rate). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC1a (+/+)(•, n=10, N=8) and (-/-) mice (□, n=17, N=11) showing a significant increase in stimulus-response function in ASIC1a -/- mice (P = 0.001, two-way ANOVA).

D) Increased sensitivity of colonic mesenteric mechanoreceptors with responses expressed as spikes per second (spikes / sec). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC1a (+/+)(•, n=10, N=8) and (-/-) mice (□, n=17, N=11) showing a significant increase in stimulus-response function in ASIC1a -/- mice (P < 0.001, two-way ANOVA). Bonferroni post hoc test indicates significant increases in -/- mice responses at a 1000mg stimulus (* P < 0.05).

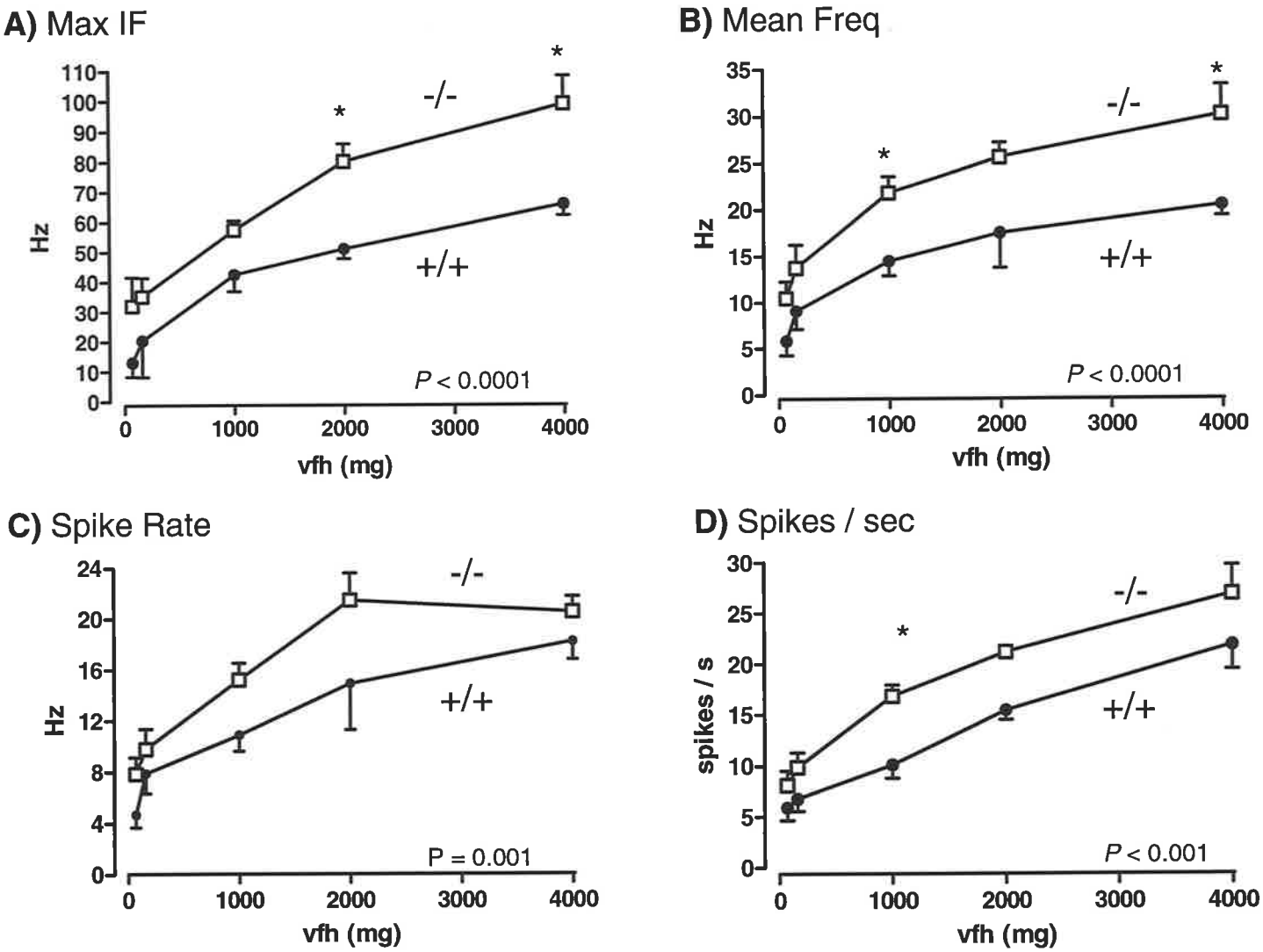
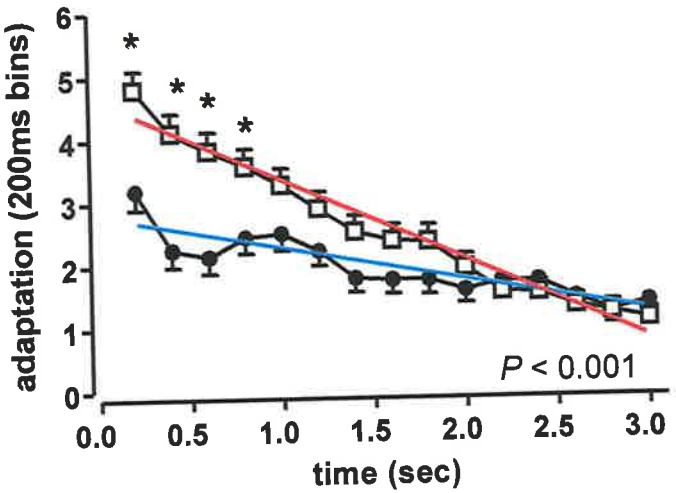

A) Max IF
Hz
vfh (mg)
-/-
+/+
*
*
P < 0.0001
B) Mean Freq
Hz
vfh (mg)
-/-
+/+
*
*
P < 0.0001
C) Spike Rate
Hz
vfh (mg)
-/-
+/+
P = 0.001
D) Spikes / sec
spikes / s
vfh (mg)
-/-
+/+
*
P < 0.001

Figure 3.3: Effects of disrupting ASIC1a on LSN mesenteric afferent adaptation

A) Adaptation profiles of colonic mesenteric mechanoreceptors from ASIC1a (+/+) (•, n=10, N=8) and (-/-) mice (□, n=17, N=11) expressed as raw data. ASIC1a -/- colonic mesenteric mechanoreceptors displayed altered adaptation profiles as indicated by a significantly steeper adaptation slope than in ASIC1a +/+ mice ($P < 0.0001$, linear regression), indicating a more rapid adaptation overall. The adaptation profile also was altered significantly ($P < 0.001$; 2-way ANOVA). Bonferroni post hoc test indicates significant increases in -/- mice responses at indicated time points (* $P < 0.05$).

B) Adaptation profiles of colonic mesenteric mechanoreceptors from ASIC1a (+/+)(•, n=10, N=8) and (-/-) mice (□, n=17, N=11) expressed as percentage of maximum response (This normalized the data and allowed direct comparison between the adaptation slopes and allowed for the increased responses in ASIC1a -/- mice). ASIC1a -/- displayed a significantly adaptation steeper slope than in ASIC1a +/+ mice ($P < 0.001$, linear regression), indicating a more rapid adaptation overall. The adaptation profile also was altered significantly ($P < 0.001$; 2-way ANOVA).

A) Adaptation (mean data)



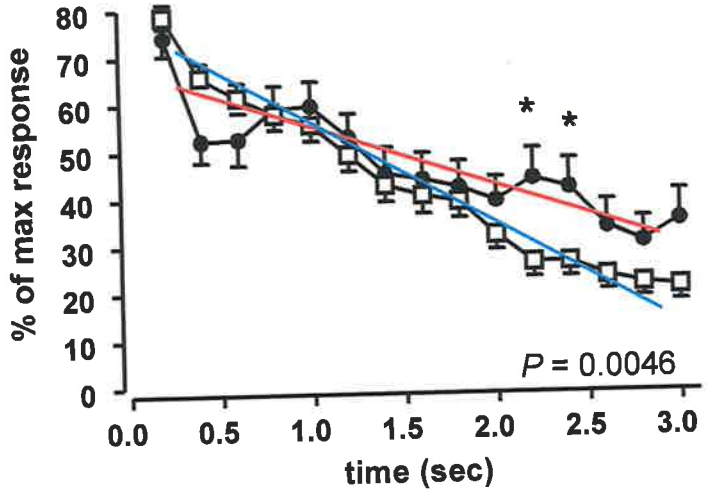
* *P*< 0.001 (post-hoc test)

ASIC1a -/- slope: -1.273 ± 0.06294

ASIC1a +/+ slope: -0.5179 ± 0.07272

Slopes are significantly different *P*< 0.001

B) Adaptation (% of max response)



* *P*< 0.05 (post-hoc test)

ASIC1a -/- slope: -19.90 ± 1.069

ASIC1a +/+ slope: -11.63 ± 1.606

Slopes are significantly different *P*< 0.001

Figure 3.4: A) Effects of disrupting ASIC1a on the percentage of LSN mesenteric afferents responding to different intensities of mechanical stimuli

Disrupting ASIC1a did not significantly alter the percentage of mesenteric afferents responding to 70, 160, 1000, 2000 or 4000mg perpendicular probing via calibrated von Frey hairs. ASIC1a (+/+) (•, n=10, N=8) and (-/-) mice (□, n=17, N=11).

B) Effects of disrupting ASIC1a on the percentage of LSN serosal afferents responding to different intensities of mechanical stimuli

Disrupting ASIC1a did not significantly alter the percentage of serosal afferents responding to 70, 160, 1000, 2000 or 4000mg perpendicular probing via calibrated von Frey hairs. ASIC1a (+/+) (•, n=17, N=12) and (-/-) mice (□, n=16, N=11).

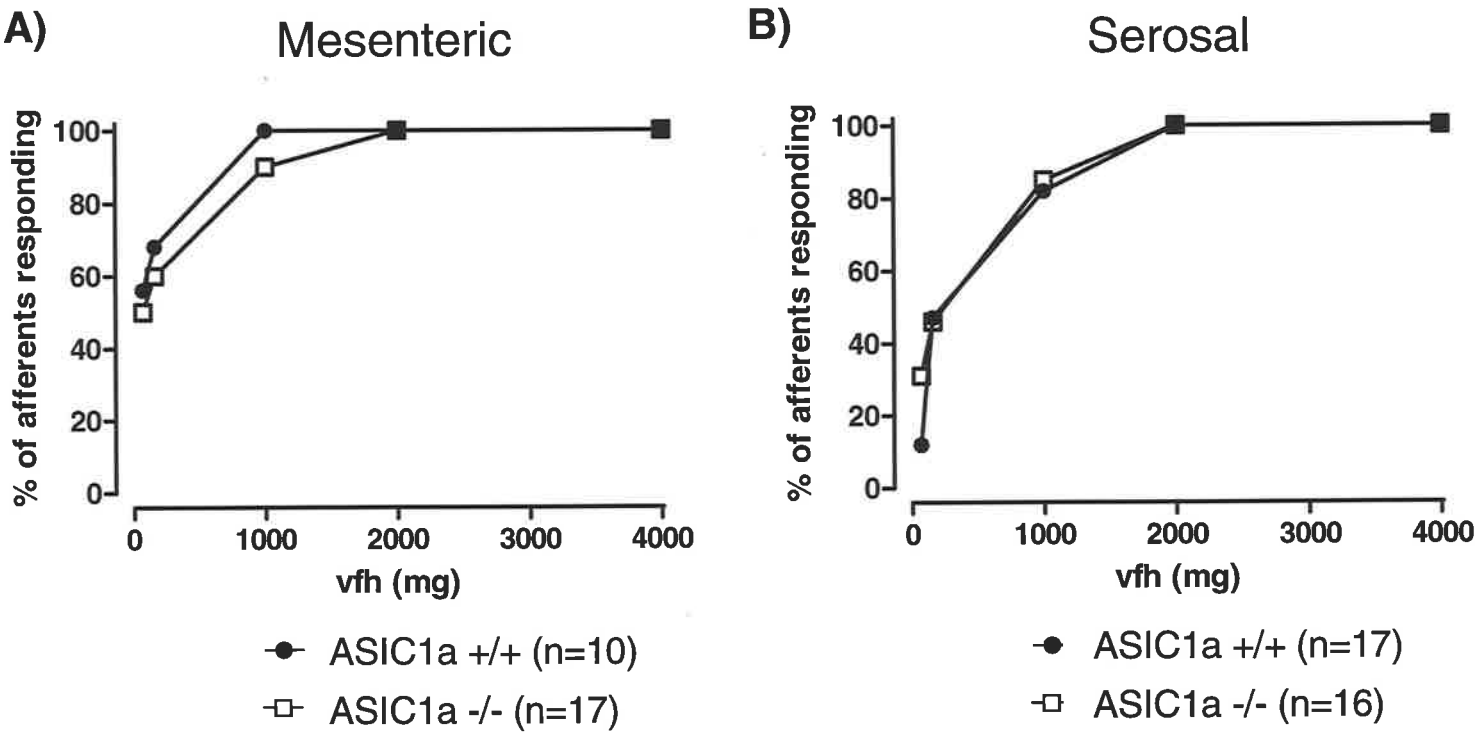

A)
Mesenteric
% of afferents responding
100
80
60
40
20
0
0
1000
2000
3000
4000
vfh (mg)
ASIC1a +/+ (n=10)
ASIC1a -/- (n=17)
B)
Serosal
% of afferents responding
100
80
60
40
20
0
0
1000
2000
3000
4000
vfh (mg)
ASIC1a +/+ (n=17)
ASIC1a -/- (n=16)

Figure 3.5: Receptive field distributions of mesenteric and serosal LSN afferents in ASIC1a +/+ and -/- mice.

Figure demonstrates that mesenteric and serosal afferents in ASIC1a +/+ and -/- mice displayed similar receptive field distributions.

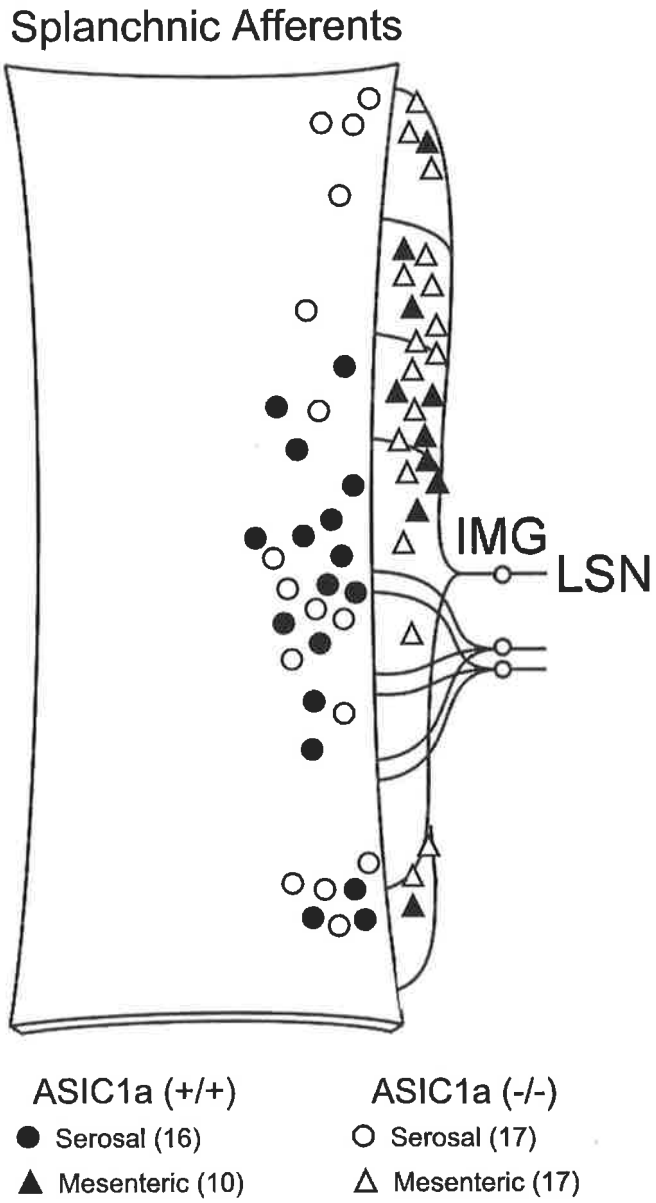

Splanchnic Afferents
IMG
LSN
ASIC1a (+/+)
Serosal (16)
Mesenteric (10)
ASIC1a (-/-)
Serosal (17)
Mesenteric (17)

Figure 3.6: Effects of disrupting ASIC1a on LSN serosal afferent mechanosensitivity

A) Increased sensitivity of colonic serosal mechanoreceptors with responses expressed as maximum instantaneous frequency (Max IF). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC1a (+/+) (•, n=17, N=12) and (-/-) mice (□, n=16, N=11), showing a significant increase in stimulus-response function in ASIC1a -/- mice ($P < 0.001$, two-way ANOVA). Bonferroni post hoc test indicates significant increases in -/- mice responses at a 4000mg stimulus (* $P < 0.05$).

B) Increased sensitivity of colonic serosal mechanoreceptors with responses expressed as mean frequency (Mean Freq). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC1a (+/+) (•, n=17, N=12) and (-/-) mice (□, n=16, N=11), showing a significant increase in stimulus-response function in ASIC1a -/- mice ($P < 0.05$, two-way ANOVA).

C) The sensitivity of colonic mesenteric mechanoreceptors when expressed as spike rate (rate) was unaltered in ASIC1a -/-. Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC1a (+/+) (•, n=17, N=12) and (-/-) mice (□, n=16, N=11).

D) Increased sensitivity of colonic mesenteric mechanoreceptors with responses expressed as spikes per second (spikes / sec). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC1a (+/+) (•, n=17, N=12) and (-/-) mice (□, n=16, N=11), showing a significant increase in stimulus-response function in ASIC1a -/- mice ($P < 0.01$, two-way ANOVA). Bonferroni post hoc test indicates significant increases in -/- mice responses at a 4000mg stimulus (* $P < 0.05$).

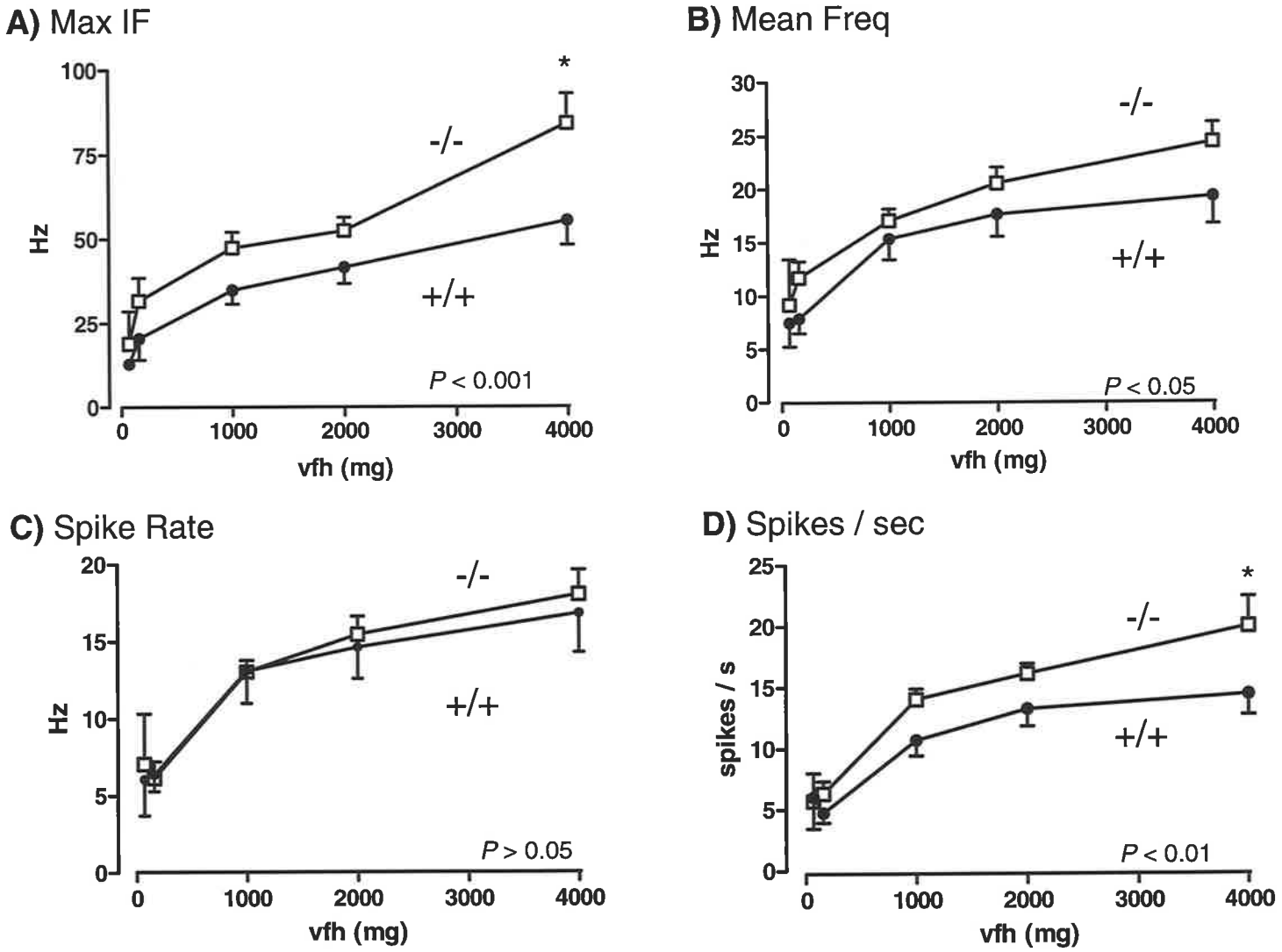
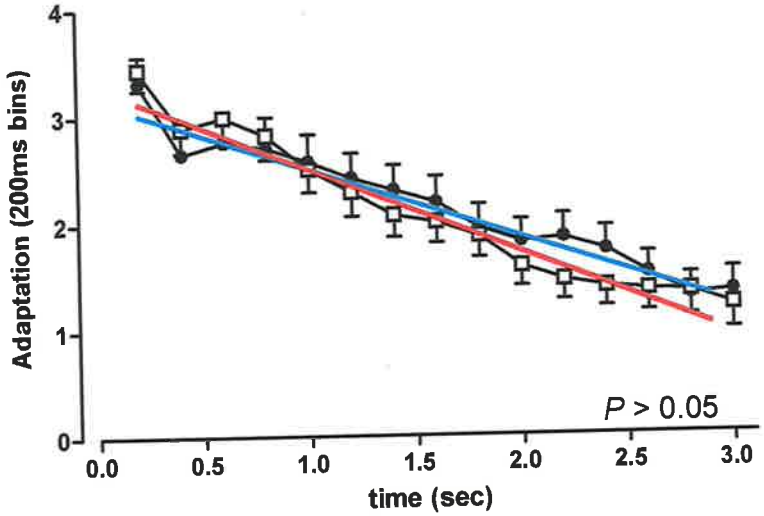

A) Max IF
100
75
50
25
0
Hz
*
-/-
+/+
P < 0.001
0
1000
2000
3000
4000
vfh (mg)
B) Mean Freq
30
25
20
15
10
5
0
Hz
-/-
+/+
P < 0.05
0
1000
2000
3000
4000
vfh (mg)
C) Spike Rate
20
15
10
5
0
Hz
-/-
+/+
P > 0.05
0
1000
2000
3000
4000
vfh (mg)
D) Spikes / sec
25
20
15
10
5
0
spikes / s
*
-/-
+/+
P < 0.01
0
1000
2000
3000
4000
vfh (mg)

Figure 3.7: Effects of disrupting ASIC1a on LSN serosal afferent adaptation

A) Adaptation profiles of colonic serosal mechanoreceptors from ASIC1a (+/+) (•, n=17, N=12) and (-/-) mice (□, n=16, N=11), as raw data. ASIC1a -/- colonic mesenteric mechanoreceptors displayed similar adaptation profiles to ASIC1a +/+ mice ($P > 0.05$, linear regression). The adaptation profile was also unaltered ($P > 0.05$; 2-way ANOVA).

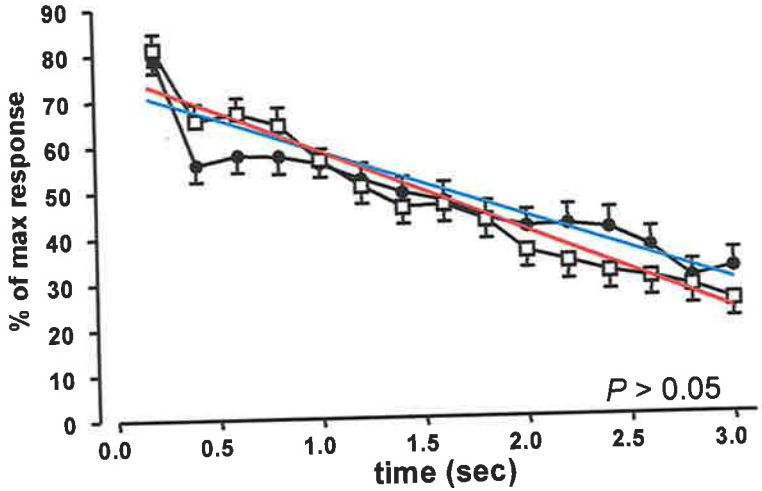
B) Adaptation profiles of colonic serosal mechanoreceptors from ASIC1a (+/+)(•, n=17, N=12) and (-/-) mice (□, n=16, N=11), expressed as percentage of maximum response. ASIC1a -/- colonic serosal mechanoreceptors displayed similar adaptation profiles to ASIC1a +/+ mice ($P > 0.05$, linear regression). The adaptation profile was also unaltered ($P > 0.05$; 2-way ANOVA).

A) Adaptation (mean data)



ASIC1a -/- slope : -0.7783 ± 0.4650
ASIC1a +/+ slope : -0.6405 ± 0.3582
Slopes are not significantly different $P > 0.05$

B) Adaptation (% of max response)



ASIC1a -/- slope : -0.7783 ± 0.4650
ASIC1a +/+ slope: -0.6405 ± 0.3582
Slopes are not significantly different $P > 0.05$

Figure 3.8: Effects of disrupting ASIC2 on LSN mesenteric afferent mechanosensitivity

The sensitivity of colonic mesenteric mechanoreceptors was unaltered in ASIC2-/- mice when expressed as A) maximum instantaneous frequency, B) mean frequency, C) spike rate (rate) and as D) spikes per second (spikes / sec). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC2 (+/+) (•, n=20, N=14) and (-/-) mice (□, n=26, N=15).

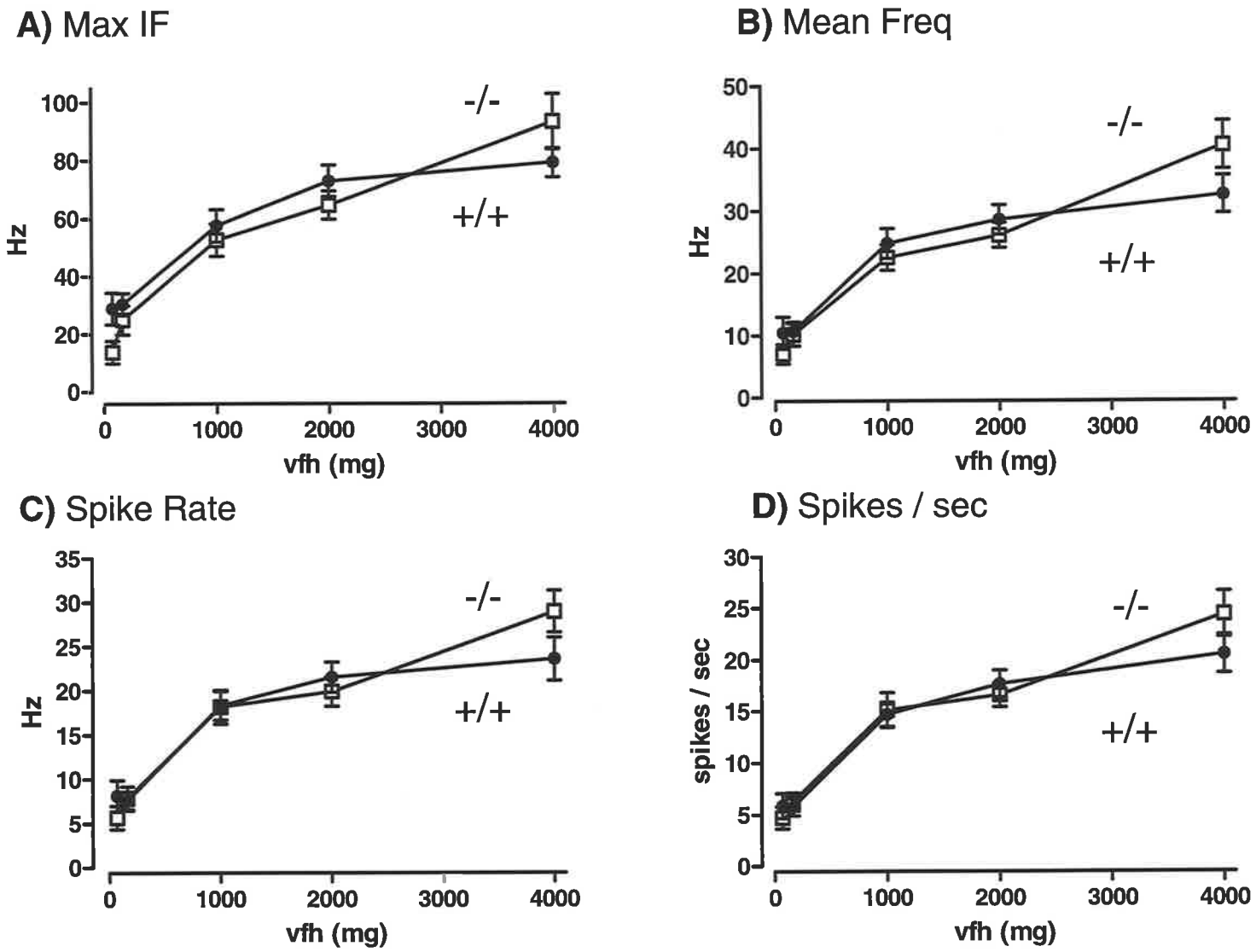
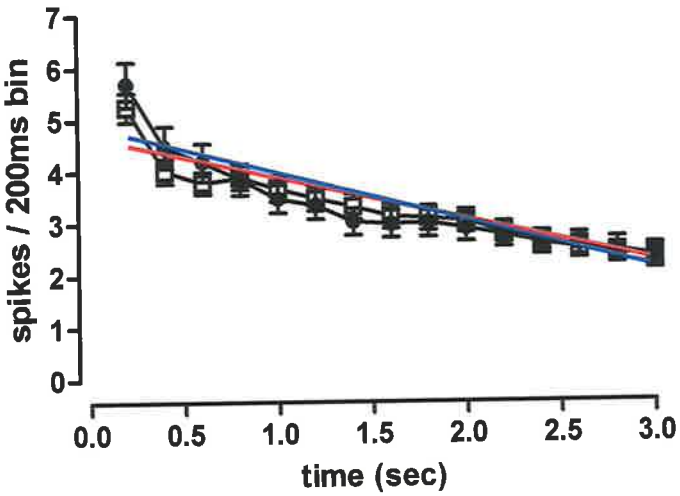

A) Max IF
Hz
vfh (mg)
-/-
+/+
B) Mean Freq
Hz
vfh (mg)
-/-
+/+
C) Spike Rate
Hz
vfh (mg)
-/-
+/+
D) Spikes / sec
spikes / sec
vfh (mg)
-/-
+/+

Figure 3.9: Effects of disrupting ASIC2 on LSN mesenteric afferent adaptation

A) Adaptation profiles of colonic serosal mechanoreceptors from ASIC2 (+/+) (•, n=20, N=14) and (-/-) mice (□, n=26, N=15), as raw data. ASIC2 -/- colonic mesenteric mechanoreceptors displayed similar adaptation slopes to ASIC2 +/+ mice ($P > 0.05$, linear regression). The adaptation profile was also unaltered ($P > 0.05$; 2-way ANOVA).

B) Adaptation profiles of colonic serosal mechanoreceptors from ASIC2 (+/+) (•, n=20, N=14) and (-/-) mice (□, n=26, N=15), expressed as percentage of maximum response. ASIC2 -/- colonic serosal mechanoreceptors displayed similar adaptation slopes to ASIC2 +/+ mice ($P > 0.05$, linear regression). The adaptation profile was also unaltered ($P > 0.05$; 2-way ANOVA).

A) Adaptation (mean data)

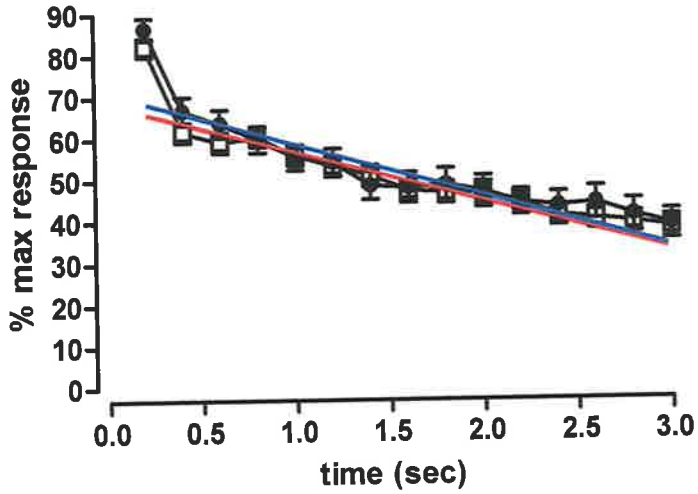


ASIC2 -/- slope : -0.8078 ± 0.08169

ASIC2 +/+ slope :-0.9262 ± 0.1188

Slopes are not significantly different $P > 0.05$

B) Adaptation (% of max response)



ASIC2 -/- slope : -11.87 ± 1.419

ASIC2 +/+ slope: -12.53 ± 1.709

Slopes are not significantly different $P > 0.05$

Figure 3.10: A) Effects of disrupting ASIC2 on the percentage of LSN mesenteric afferents responding to different intensities of mechanical stimuli

Disrupting ASIC2 did not significantly alter the percentage of mesenteric afferents responding to 70, 160, 1000, 2000 or 4000mg perpendicular probing via calibrated von Frey hairs. (+/+) (•, n=20, N=14) and (-/-) mice (□, n=26, N=15).

B) Effects of disrupting ASIC2 on the percentage of LSN serosal afferents responding to different intensities of mechanical stimuli

Disrupting ASIC2 did not significantly alter the percentage of serosal afferents responding to 70, 160, 1000, 2000 or 4000mg perpendicular probing via calibrated von Frey hairs. ASIC2 (+/+) (• , n=27, N=19) and (-/-) mice (□ , n=34, N=21).

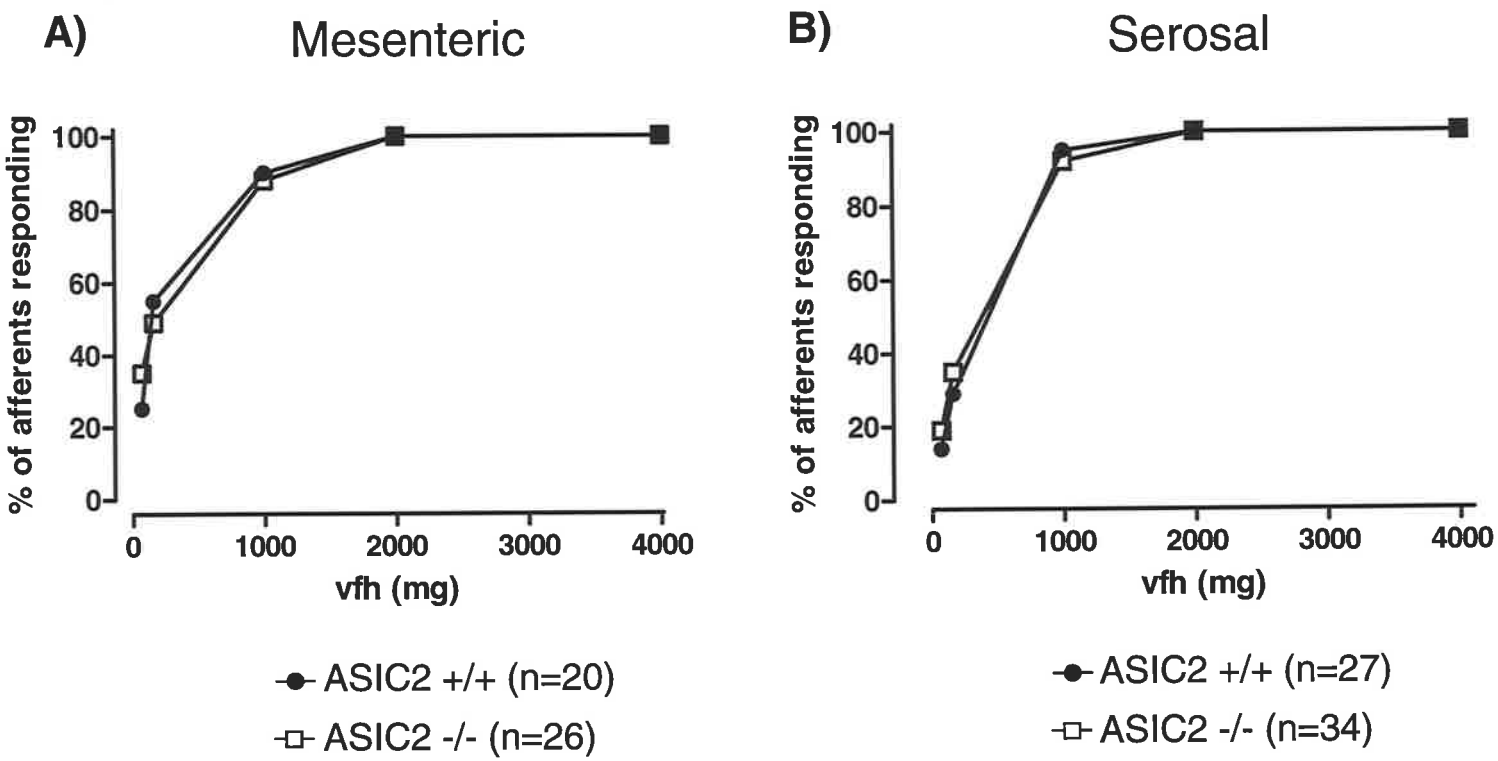

A)
Mesenteric
% of afferents responding
100
80
60
40
20
0
0
1000
2000
3000
4000
vfh (mg)
ASIC2 +/+ (n=20)
ASIC2 -/- (n=26)
B)
Serosal
% of afferents responding
100
80
60
40
20
0
0
1000
2000
3000
4000
vfh (mg)
ASIC2 +/+ (n=27)
ASIC2 -/- (n=34)

Figure 3.11: Receptive field distributions of mesenteric and serosal LSN afferents in ASIC2 +/+ and -/- mice.

Figure demonstrates that mesenteric and serosal afferents in ASIC2 +/+ and -/- mice displayed similar receptive field distributions.

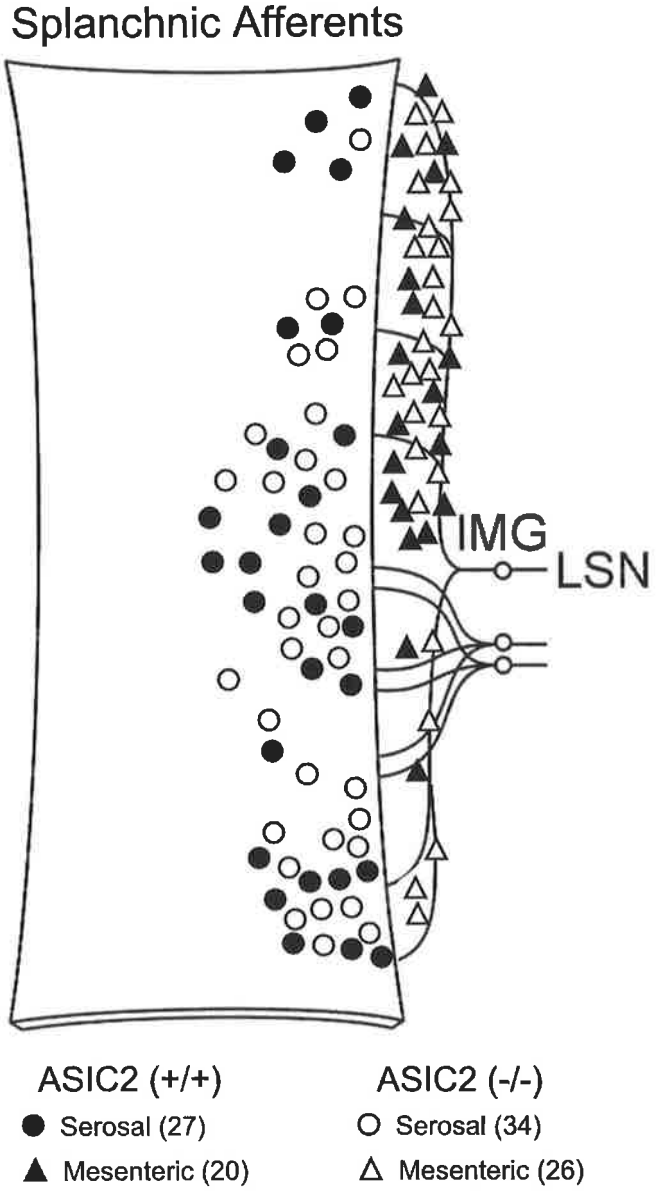

Splanchnic Afferents
IMG
LSN
ASIC2 (+/+)
Serosal (27)
Mesenteric (20)
ASIC2 (-/-)
Serosal (34)
Mesenteric (26)

Figure 3.12: Effects of disrupting ASIC2 on LSN serosal afferent mechanosensitivity

A) Increased sensitivity of colonic serosal mechanoreceptors with responses expressed as maximum instantaneous frequency (Max IF). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC2 (+/+) (•, n=27, N=19) and (-/-) mice (□, n=34, N=21), showing a significant increase in stimulus-response function in ASIC2 -/- mice ($P < 0.001$, two-way ANOVA). Bonferroni post hoc test indicates significant increases in -/- mice responses at a 4000mg stimulus (* $P < 0.05$).

B) Increased sensitivity of colonic serosal mechanoreceptors with responses expressed as mean frequency (Mean Freq). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC2 (+/+) (•, n=27, N=19) and (-/-) mice (□, n=34, N=21), showing a significant increase in stimulus-response function in ASIC2 -/- mice ($P < 0.001$, two-way ANOVA). Bonferroni post hoc test indicates significant increases in -/- mice responses at 2000 and 4000mg stimuli (* $P < 0.05$).

C) Increased sensitivity of colonic serosal mechanoreceptors with responses expressed as spike rate (rate). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC2 (+/+) (•, n=27, N=19) and (-/-) mice (□, n=34, N=21), showing a significant increase in stimulus-response function in ASIC2 -/- mice ($P < 0.001$, two-way ANOVA). Bonferroni post hoc test indicates significant increases in -/- mice responses at 2000 and 4000mg stimuli (* $P < 0.05$).

D) Increased sensitivity of colonic mesenteric mechanoreceptors with responses expressed as spikes per second (spikes / sec). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC2 (+/+) (•, n=27, N=19) and (-/-) mice (□, n=34, N=21), showing a significant increase in stimulus-response function in ASIC2 -/- mice ($P < 0.001$, two-way ANOVA). Bonferroni post hoc test indicates significant increases in -/- mice responses at 2000 and 4000mg stimulus (* $P < 0.05$).

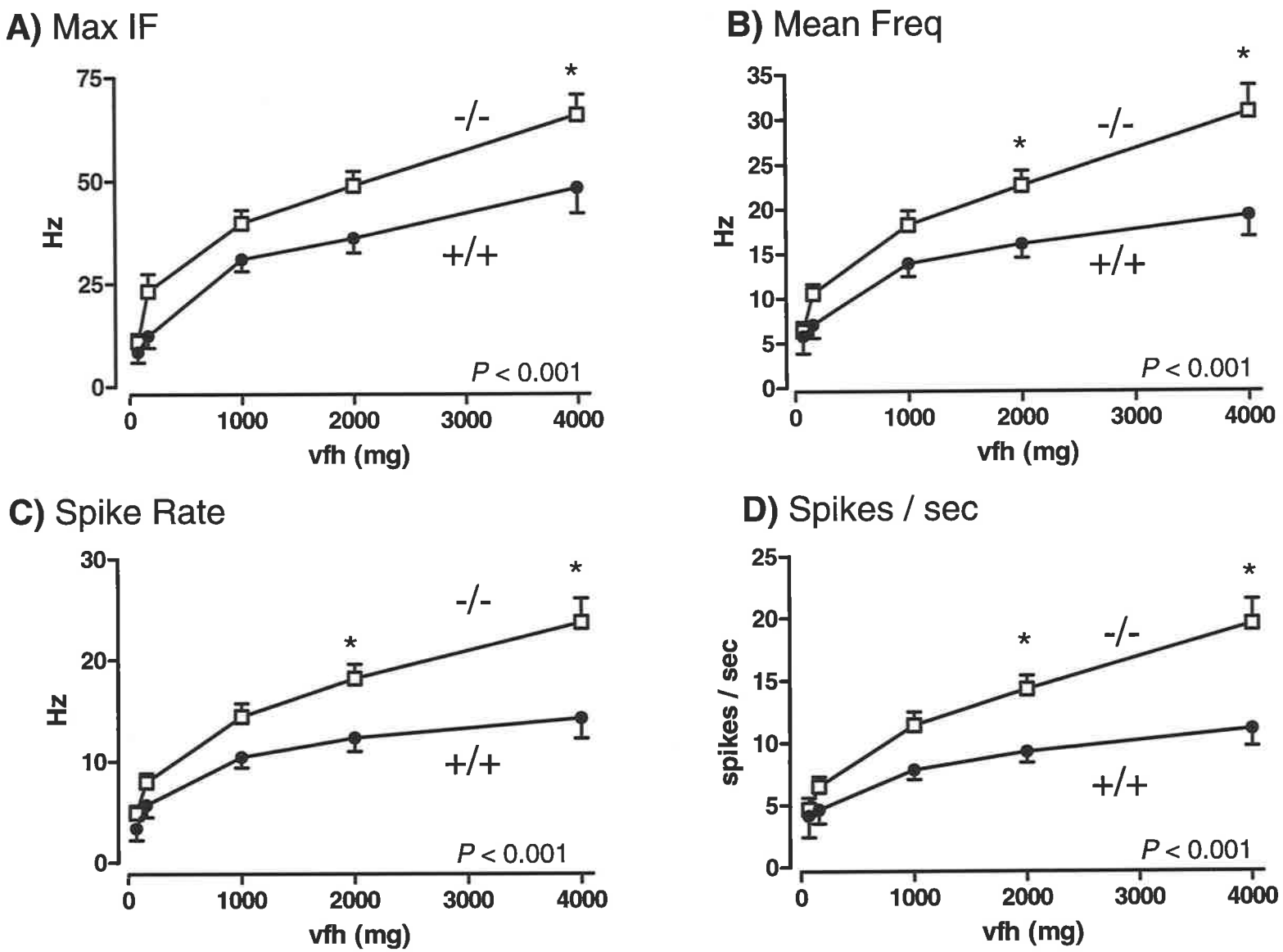
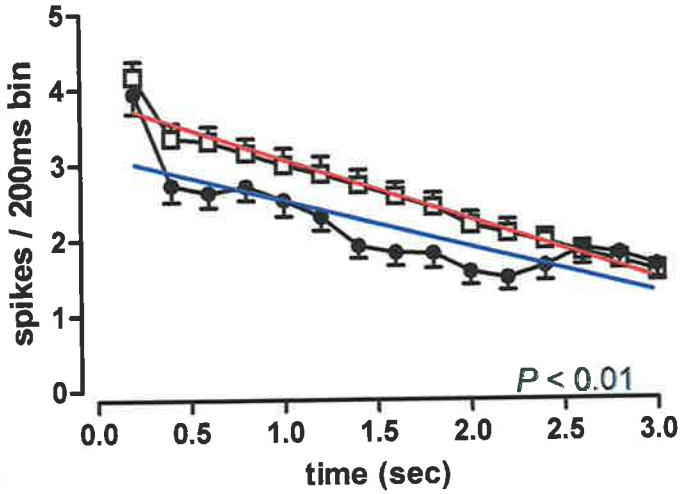

A) Max IF
75
50
25
0
Hz
-/-
+/+
*
P < 0.001
0
1000
2000
3000
4000
vfh (mg)
B) Mean Freq
35
30
25
20
15
10
5
0
Hz
*
-/-
+/+
P < 0.001
vfh (mg)
C) Spike Rate
30
20
10
0
Hz
*
-/-
+/+
P < 0.001
vfh (mg)
D) Spikes / sec
25
20
15
10
5
0
spikes / sec
*
-/-
+/+
P < 0.001
vfh (mg)

Figure 3.13: Effects of disrupting ASIC2 on LSN serosal afferent adaptation

A) Adaptation profiles of colonic serosal mechanoreceptors from ASIC2 (+/+) (•, n=27, N=19) and (-/-) mice (□, n=34, N=21), as raw data. Although the adaptation profile was altered *(P* > 0.05; 2-way ANOVA) in ASIC2-/- mice, serosal mechanoreceptors displayed a similar adaptation slope to ASIC2 +/+ mice (*P* > 0.05, linear regression), indicating no significant change in adaptation.

B) Adaptation profiles of colonic serosal mechanoreceptors from ASIC2 (+/+) (•, n=27, N=19) and (-/-) mice (□, n=34, N=21) expressed as percentage of maximum response. ASIC2 -/- colonic serosal mechanoreceptors displayed a similar adaptation slope to ASIC2 +/+ mice (P > 0.05, linear regression). The adaptation profile was also unaltered (P > 0.05; 2-way ANOVA). Overall there was no significant change in adaptation in ASIC2-/-.

A) Adaptation (mean data)

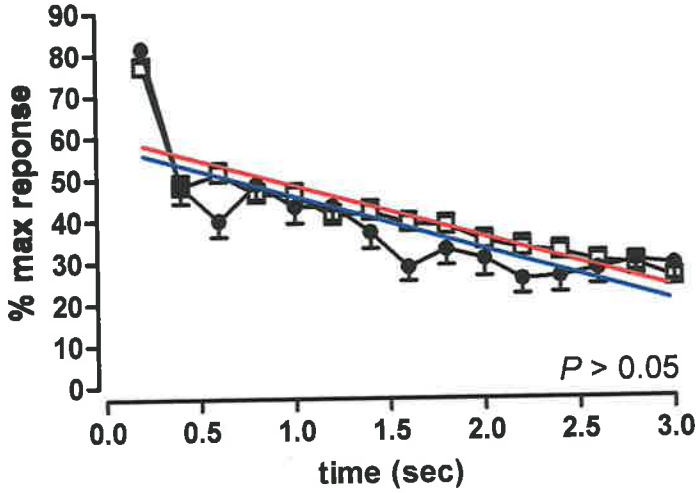


ASIC2 -/- slope : -0.7983 ± 0.04524

ASIC2 +/+ slope: -0.6159 ± 0.1111

Slopes are not significantly different $P > 0.05$

B) Adaptation (% of max response)



ASIC2 -/- slope : -12.39 ± 1.844

ASIC2 +/+ slope: -12.71 ± 2.729

Slopes are not significantly different $P > 0.05$

Figure 3.14: Effects of disrupting ASIC3 on LSN mesenteric afferent mechanosensitivity

A) Decreased sensitivity of colonic mesenteric mechanoreceptors with responses expressed as maximum instantaneous frequency (Max IF). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC3 (+/+) (•, n=23, N=14) and (-/-) mice (□, n=26, N=15), showing a significant decrease in stimulus-response function in ASIC3 -/- mice ($P < 0.001$, two-way ANOVA). Bonferroni post hoc test indicates significant decreases in -/- mice responses at 1000, 2000 and 4000mg stimuli (* $P < 0.05$).

B) Decreased sensitivity of colonic mesenteric mechanoreceptors with responses expressed as mean frequency (Mean Freq). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC3 (+/+) (•, n=23, N=14) and (-/-) mice (□, n=26, N=15), showing a significant decrease in stimulus-response function in ASIC3 -/- mice ($P < 0.001$, two-way ANOVA). Bonferroni post hoc test indicates significant decreases in -/- mice responses at 2000 and 4000mg stimuli (* $P < 0.05$).

C) Decreased sensitivity of colonic mesenteric mechanoreceptors with responses expressed as spike rate (rate). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC3 (+/+)(•, n=23, N=14) and (-/-) mice (□, n=26, N=15), showing a significant decrease in stimulus-response function in ASIC3 -/- mice ($P < 0.001$, two-way ANOVA). Bonferroni post hoc test indicates significant decreases in -/- mice responses at 2000 and 4000mg stimuli (* $P < 0.05$).

D) Decreased sensitivity of colonic mesenteric mechanoreceptors with responses expressed as spikes per second (spikes / sec). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC3 (+/+) (•, n=23, N=14) and (-/-) mice (□, n=26, N=15) showing a significant decrease in stimulus-response function in ASIC3 -/- mice ($P < 0.001$, two-way ANOVA). Bonferroni post hoc test indicates significant decreases in -/- mice responses at 2000 and 4000mg stimuli (* $P < 0.05$).

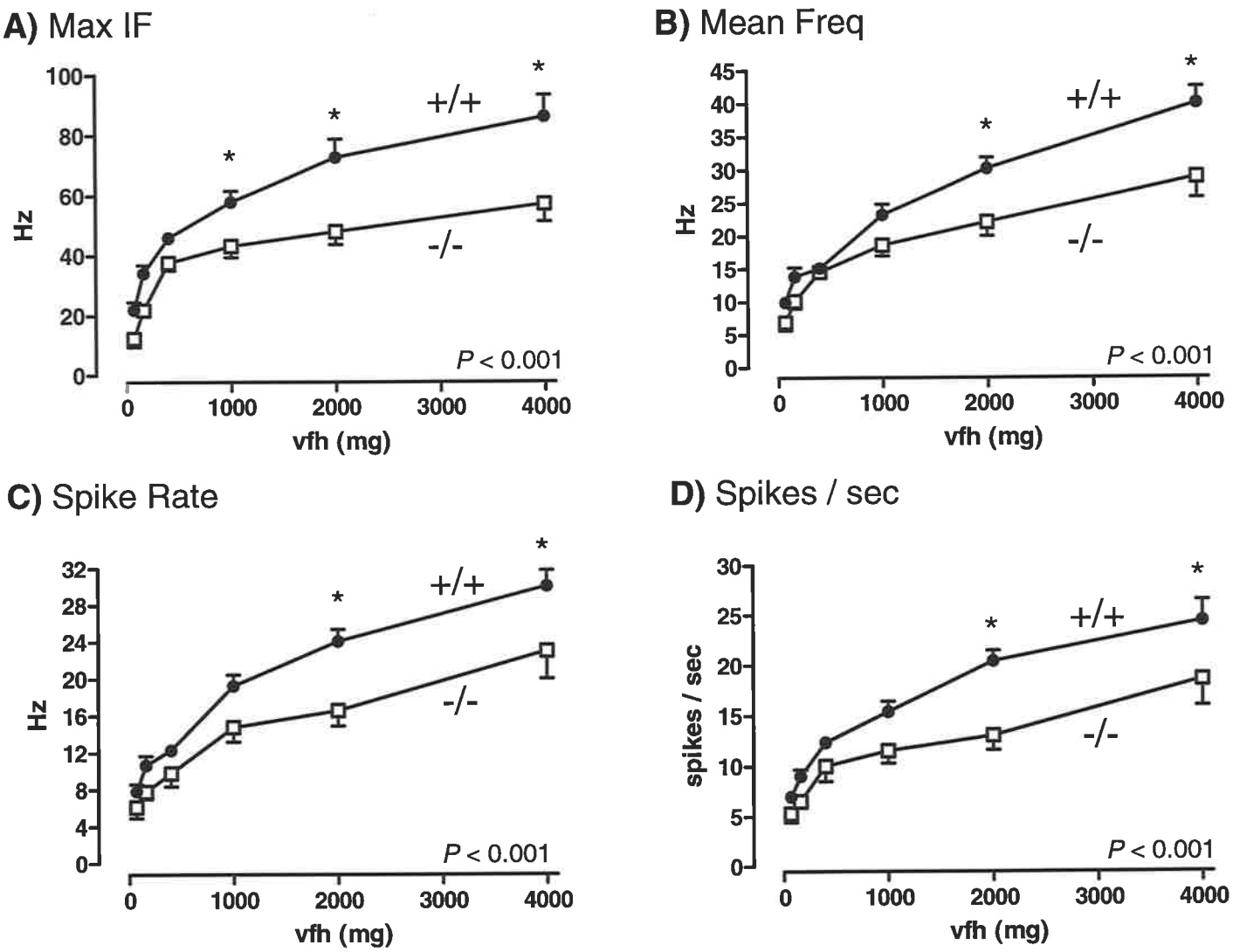
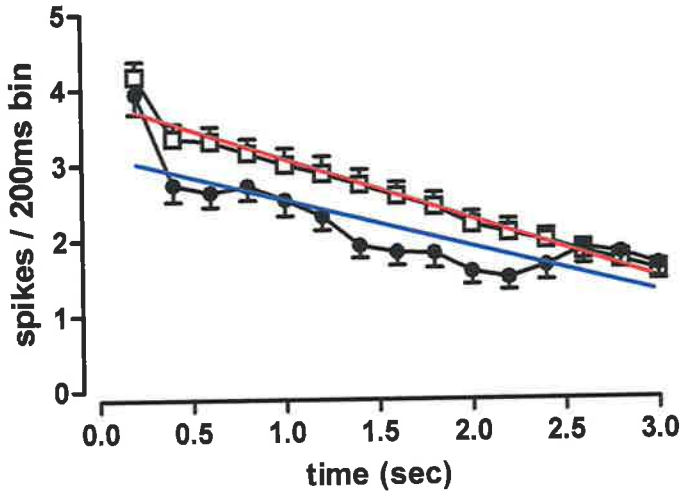

A) Max IF
Hz
vfh (mg)
+/+
-/-
P < 0.001
B) Mean Freq
Hz
vfh (mg)
+/+
-/-
P < 0.001
C) Spike Rate
Hz
vfh (mg)
+/+
-/-
P < 0.001
D) Spikes / sec
spikes / sec
vfh (mg)
+/+
-/-
P < 0.001

Figure 3.15: Effects of disrupting ASIC3 on LSN mesenteric afferent adaptation

A) Adaptation profiles of colonic mesenteric mechanoreceptors from ASIC3 (+/+)(•, n=23, N=14) and (-/-) mice (□, n=26, N=15), as raw data. ASIC3 -/- colonic mesenteric mechanoreceptors displayed a similar adaptation slope to ASIC3 +/+ mice ($P > 0.05$, linear regression). However, the adaptation profile was also altered ($P < 0.05$; 2-way ANOVA).

B) Adaptation profiles of colonic mesenteric mechanoreceptors from ASIC3 (+/+) (•, n=23, N=14) and (-/-) mice (□, n=26, N=15), expressed as percentage of maximum response. ASIC3 -/- colonic mesenteric mechanoreceptors displayed a significantly steeper adaptation slope to ASIC3 +/+ mice ($P < 0.05$, linear regression). The adaptation profile was also altered ($P < 0.05$; 2-way ANOVA). Overall these data indicate a quicker adaptation of ASIC3 -/- mesenteric afferents.

A) Adaptation (mean data)

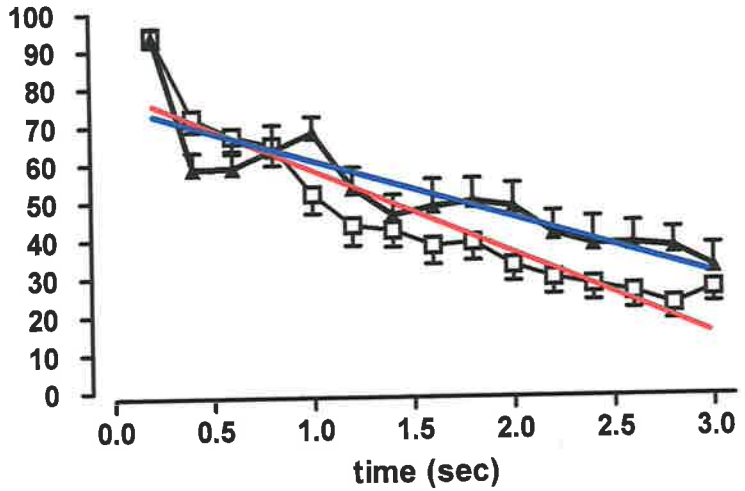


ASIC3 -/- slope : -0.8246 ± 0.09956

ASIC3 +/+ slope: -0.8763 ± 0.08211

Slopes are not significantly different $P > 0.05$

B) Adaptation (% of max response)



ASIC3 -/- slope : -21.79 ± 2.377

ASIC3 +/+ slope: -15.19 ± 2.368

Slopes are significantly different * $P< 0.05$

Figure 3.16: A) Effects of disrupting ASIC3 on the percentage of LSN mesenteric afferents responding to different intensities of mechanical stimuli

Disrupting ASIC3 did not alter the percentage of mesenteric afferents responding to 70, 160, 1000, 2000 or 4000mg perpendicular probing via calibrated von Frey hairs. ASIC3 (+/+) (•, n=23, N=14) and (-/-) mice (□, n=26, N=15).

B) Effects of disrupting ASIC3 on the percentage of LSN serosal afferents responding to different intensities of mechanical stimuli

Disrupting ASIC3 did not significantly alter the percentage of serosal afferents responding to 70, 160, 1000, 2000 or 4000mg perpendicular probing via calibrated von Frey hairs. ASIC3 (+/+) (•, n=26, N=18) and (-/-) mice (□, n=20, N=14).

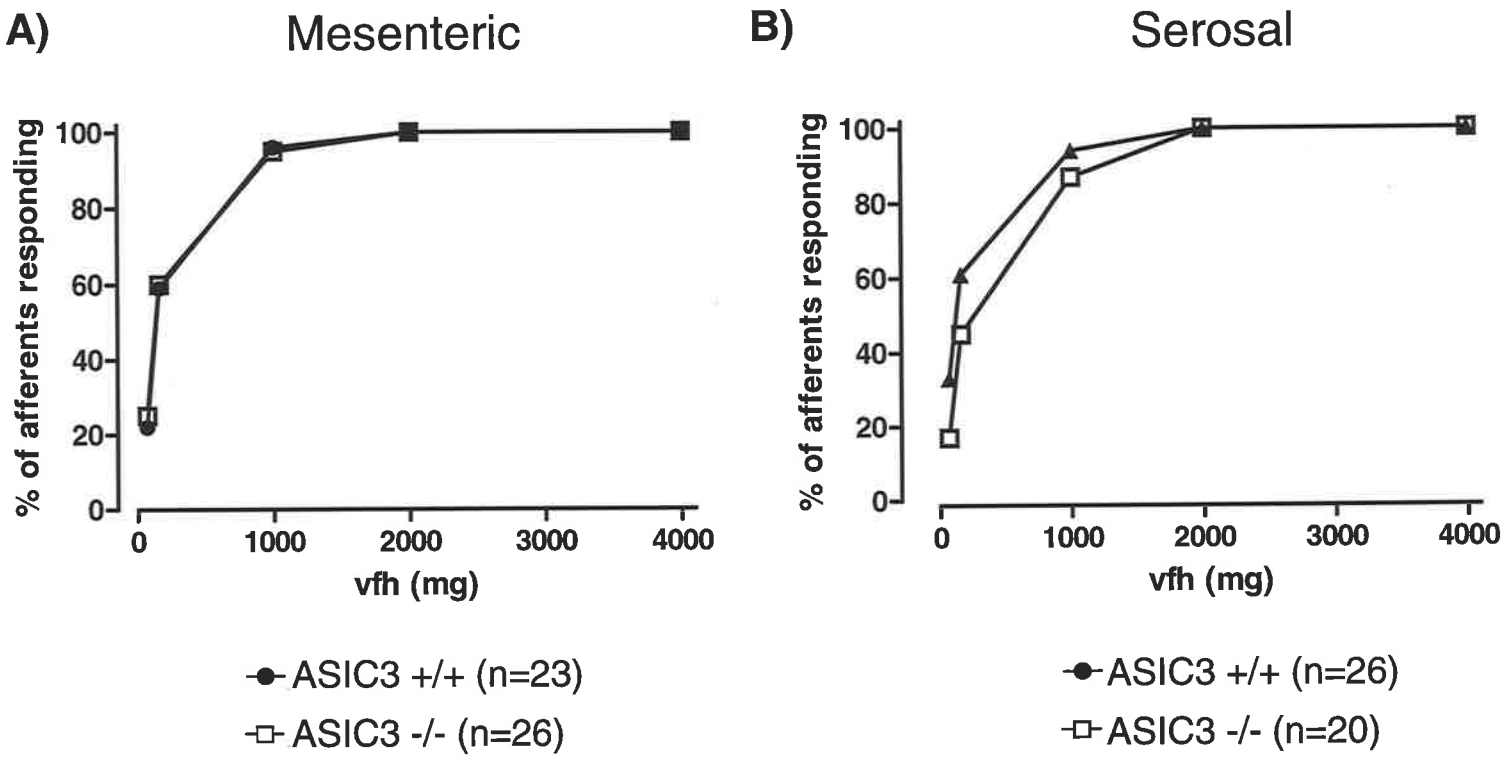

A)
Mesenteric
% of afferents responding
100
80
60
40
20
0
0
1000
2000
3000
4000
vfh (mg)
ASIC3 +/+ (n=23)
ASIC3 -/- (n=26)
B)
Serosal
% of afferents responding
100
80
60
40
20
0
0
1000
2000
3000
4000
vfh (mg)
ASIC3 +/+ (n=26)
ASIC3 -/- (n=20)

Figure 3.17: Receptive field distributions of mesenteric and serosal LSN afferents in ASIC3 +/+ and -/- mice.

Figure demonstrates that mesenteric and serosal afferents in ASIC3 +/+ and -/- mice displayed similar receptive field distributions.

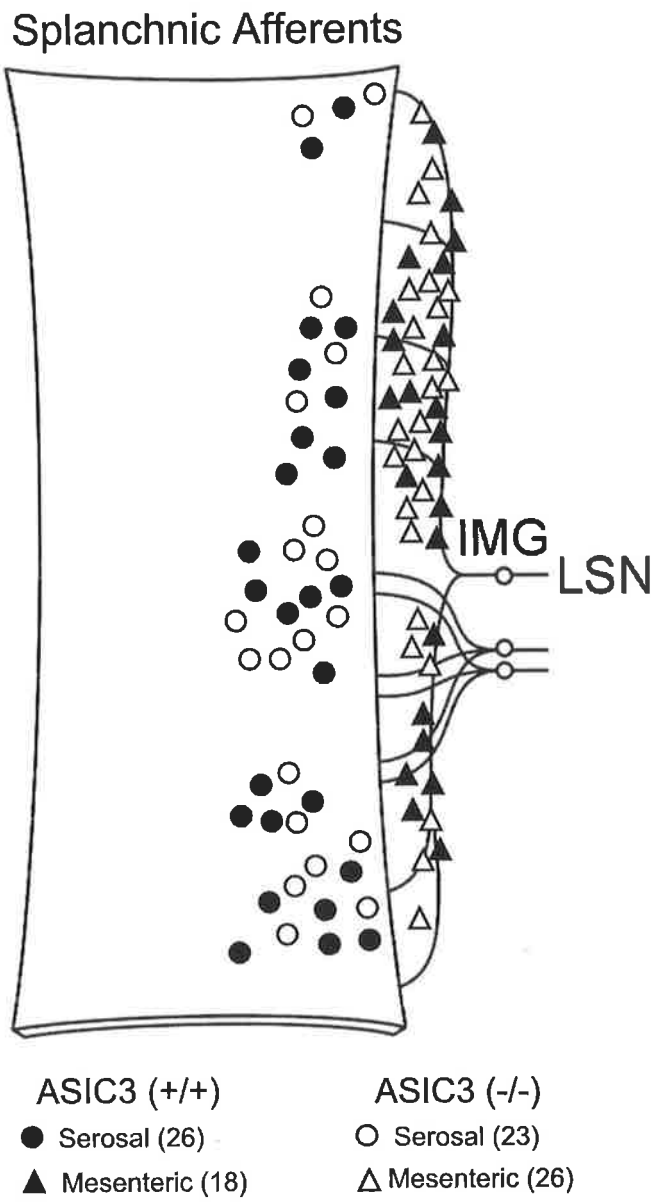

Splanchnic Afferents
IMG
LSN
ASIC3 (+/+)
Serosal (26)
Mesenteric (18)
ASIC3 (-/-)
Serosal (23)
Mesenteric (26)

Figure 3.18: Effects of disrupting ASIC3 on LSN serosal afferent mechanosensitivity

A) Decreased sensitivity of colonic serosal mechanoreceptors with responses expressed as maximum instantaneous frequency (Max IF). Stimulus-response functions of colonic serosal mechanoreceptors from ASIC3 (+/+) (•, n=26, N=18) and (-/-) mice (□, n=20, N=14), showing a significant decrease in stimulus-response function in ASIC3 -/- mice ($P < 0.001$, two-way ANOVA). Bonferroni post hoc test indicates significant decreases in -/- mice responses at 2000 and 4000mg stimuli (* $P < 0.05$).

B) Decreased sensitivity of colonic serosal mechanoreceptors with responses expressed as mean frequency (Mean Freq). Stimulus-response functions of colonic serosal mechanoreceptors from ASIC3 (+/+) (•, n=26, N=18) and (-/-) mice (□, n=20, N=14), showing a significant decrease in stimulus-response function in ASIC3 -/- mice ($P < 0.01$, two-way ANOVA).

C) Decreased sensitivity of colonic serosal mechanoreceptors with responses expressed as spike rate (rate). Stimulus-response functions of colonic mesenteric mechanoreceptors from ASIC3 (+/+) (•, n=26, N=18) and (-/-) mice (□, n=20, N=14), showing a significant decrease in stimulus-response function in ASIC3 -/- mice ($P < 0.001$, two-way ANOVA). Bonferroni post hoc test indicates significant decreases in -/- mice responses at a 2000 mg stimuli (* $P < 0.05$).

D) Decreased sensitivity of colonic serosal mechanoreceptors with responses expressed as spikes per second (spikes / sec). Stimulus-response functions of colonic serosal mechanoreceptors from ASIC3 (+/+)(•, n=26, N=18) and (-/-) mice (□, n=20, N=14), showing a significant decrease in stimulus-response function in ASIC3 -/- mice ($P < 0.001$, two-way ANOVA). Bonferroni post hoc test indicates significant decreases in -/- mice responses at 2000 and 4000mg stimulus (* $P < 0.05$).

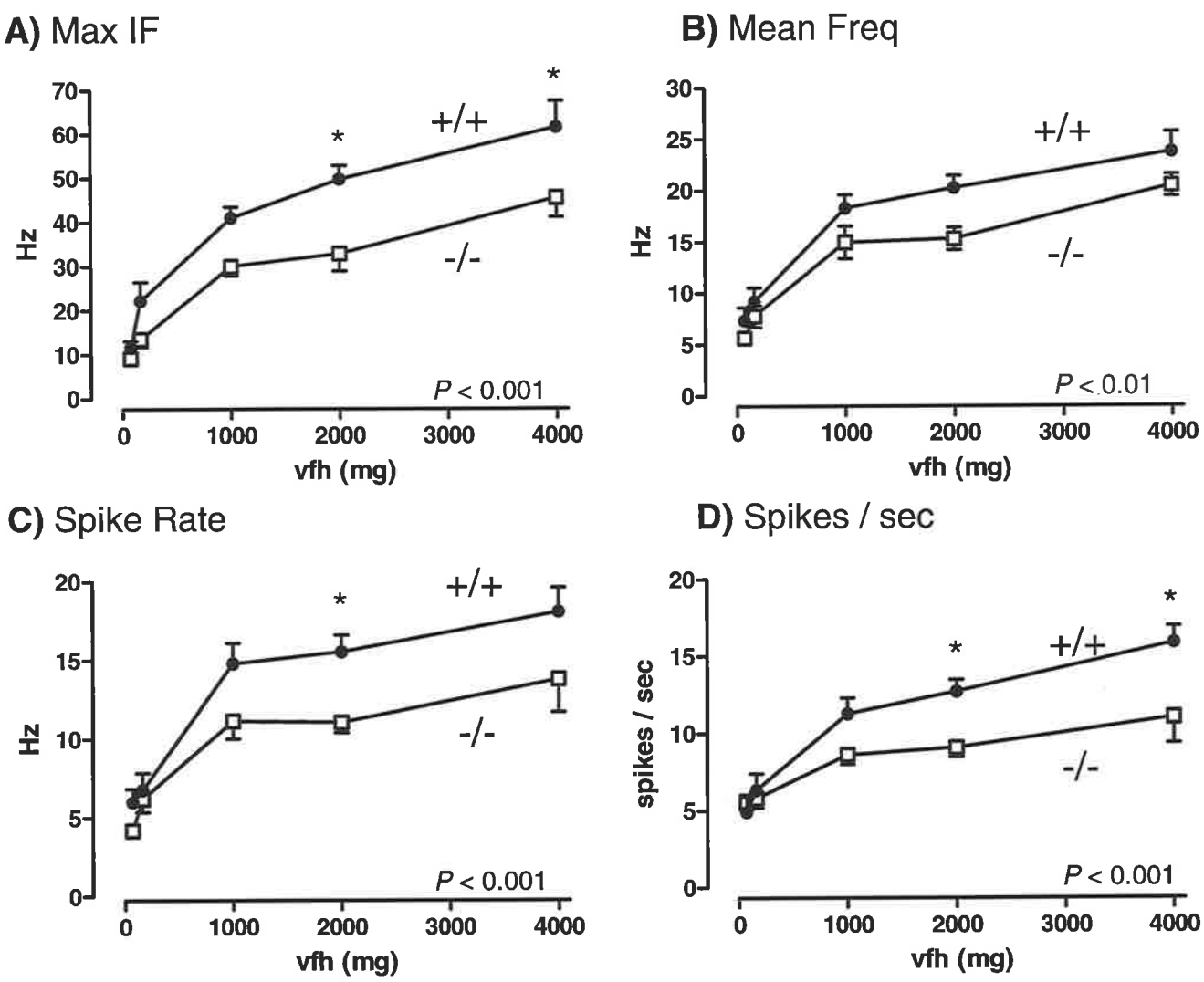
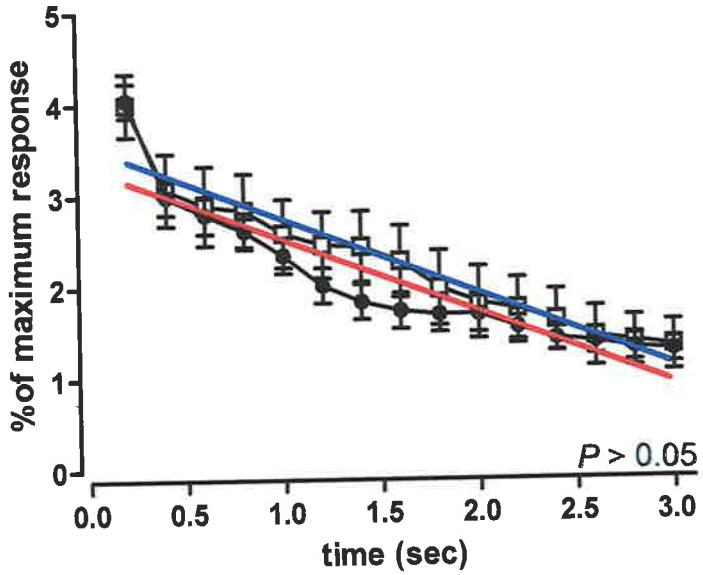

A) Max IF
Hz
70
60
50
40
30
20
10
0
+/+
-/-
*
*
P < 0.001
0
1000
2000
3000
4000
vfh (mg)
B) Mean Freq
Hz
30
25
20
15
10
5
0
+/+
-/-
P < 0.01
0
1000
2000
3000
4000
vfh (mg)
C) Spike Rate
Hz
20
15
10
5
0
+/+
-/-
*
P < 0.001
0
1000
2000
3000
4000
vfh (mg)
D) Spikes / sec
spikes / sec
20
15
10
5
0
+/+
-/-
*
*
P < 0.001
0
1000
2000
3000
4000
vfh (mg)

Figure 3.19: Effects of disrupting ASIC3 on LSN serosal afferent adaptation

A) Adaptation profiles of colonic serosal mechanoreceptors from ASIC3 (+/+) (•, n=26, N=18) and (-/-) mice (□, n=20, N=14), as raw data. ASIC3 -/- colonic mesenteric mechanoreceptors displayed a similar adaptation slope to ASIC3 +/+ mice ($P > 0.05$, linear regression). The adaptation profile was also unaltered ($P > 0.05$; 2-way ANOVA).

B) Adaptation profiles of colonic serosal mechanoreceptors from ASIC3 (+/+) (•, n=26, N=18) and (-/-) mice (□, n=20, N=14), expressed as percentage of maximum response. ASIC3 -/- colonic serosal mechanoreceptors displayed a similar adaptation slope to ASIC3 +/+ mice ($P > 0.05$, linear regression). The adaptation profile was also unaltered ($P > 0.05$; 2-way ANOVA).

A) Adaptation (mean data)

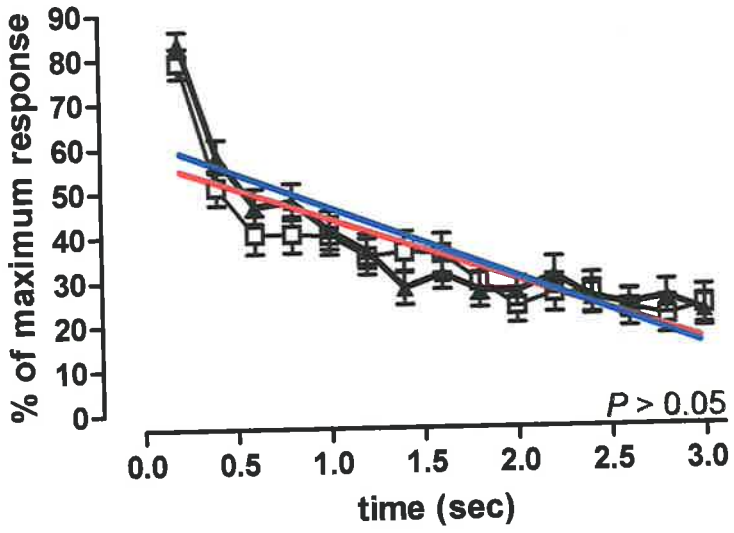


ASIC3 -/- slope : -0.8007 ± 0.0615

ASIC3 +/+ slope: -0.7933 ± 0.09894

Slopes are not significantly different $P > 0.05$

B) Adaptation (% of max response)



ASIC3 -/- slope : -13.92 ± 2.451

ASIC3 +/+ slope: -15.74 ± 2.712

Slopes are not significantly different $P > 0.05$

Table 3.1: Summary of changes in the mechanosensitivity of LSN colonic afferents, vagal gastro-oesophageal afferents and spinal cutaneous afferents in ASIC1a, 2 and 3 null mutant mice.

Disruption of *ASIC1a* increases the mechanosensitivity of all classes of visceral (colonic and gastro-oesophageal) afferent but does not influence cutaneous mechanoreceptor function. Disruption of *ASIC2* increases the mechanosensitivity of serosal colonic afferents and gastro-oesophageal mucosal afferents. In direct contrast disruption of *ASIC2* decreases the mechanosensitivity gastro-oesophageal tension afferents and cutaneous RA and SA afferents. Disruption of *ASIC3* decreases the mechanosensitivity of mesenteric and serosal colonic afferents, gastro-oesophageal mucosal afferents and cutaneous AM afferents. In contrast, disruption of *ASIC3* increased the mechanosensitivity of cutaneous RA afferents. Overall these data suggest that different mechanisms underlie mechanosensory function in the colon, gastro-oesophageal region and skin. Data summarized from this Chapter and [9, 10, 304].

Abbreviations: Rapidly adapting (RA), slowly adapting (SA) mechanoreceptors; down hair (D-hair) receptors; A-fiber mechanonociceptors (AM), and high threshold C-fibers (C-fibres).

Tissue	Colon		Gastro-esophageal		Cutaneous				
Afferent	Mesenteric	Serosal	Tension	Mucosal	RA	SA	D-hair	AM	C-fibres
ASIC1 (-/-)	↑	↑	↑	↑	↔	↔	↔	↔	↔
ASIC2 (-/-)	↔	↑	↓	↑	↓	↓	↔	↔	↔
ASIC3 (-/-)	↓	↓	↔	↓	↑	↔	↔	↓	↔

CHAPTER 4

THE ACID SENSING ION CHANNELS ASIC1a, 2 and 3 CONTRIBUTE DIFFERENTLY TO THE PHARMACOLOGY OF BENZAMIL ON COLONIC SEROSAL MECHANORECEPTORS

Summary

Background and Aims: The Acid sensing ion channels ASIC1a, 2 and 3 all contribute to the mechanosensory function of LSN serosal colonic mechanoreceptors and are all blocked by amiloride and it's analogues. This study directly compares the contributions of ASIC1a, 2 and 3 to the pharmacological effects of the potent amiloride analogue benzamil. *Methods:* An *in vitro* preparation of mouse colon with attached LSN nerves was used to examine the mechanosensitivity of serosal afferents. ASIC1a, 2 and 3 +/+ and -/- mice were used and in each genotype recordings were compared in the presence and absence of Benzamil (1-300μM). *Results*: Benzamil caused a dose-dependent inhibition of mechanical stimulus response functions in serosal afferent mechanoreceptors in ASIC1a, 2 and 3 +/+ mice. Disrupting *ASIC1a* significantly increased the sensitivity of serosal afferents ($P < 0.01$); however, the loss of *ASIC1a* had no significant effect on the inhibitory effect of benzamil on serosal afferents. Disrupting *ASIC2* significantly increased the sensitivity of serosal afferents ($P < 0.01$) whilst significantly reducing the effect of benzamil. Disrupting *ASIC3* significantly decreased the sensitivity of serosal afferents and significantly reduced the effect of benzamil ($P < 0.01$). Intriguingly, in *ASIC3* -/- afferents benzamil appeared to potentiate mechanosensory responses at low concentrations of benzamil. This was not seen in ASIC3+/+ afferents. *Conclusions:* This study identifies differences in the effects of benzamil in LSN colonic serosal afferents with disruptions to *ASIC1a, 2* and *3.* These results possibly suggest differences to the contributions of ASIC1a, 2 and 3 in the pharmacology of benzamil. ASIC2 and 3 appear to play a crucial role in the pharmacology of benzamil on colonic afferents.

An organism's ability to perceive mechanical sensation is vital in determining how it responds to their environment. Internal mechanosensation is responsible for information reaching the brain from the viscera encoding sensation and pain. It is also critical in the autonomic reflex control of digestive, cardiovascular, respiratory and endocrine function. In the viscera for example, distension or contraction of a region of gut may evoke sensations ranging from a pleasant fullness to bloating, cramping and severe pain, particularly in pathophysiological states. Mechanical stimuli also trigger motor patterns as colorectal distension may trigger peristalsis and defecation[355]. Thus alterations in the signal sent to higher centers by the viscera may have significant consequences for the organism. The results of Chapter 3 indicate that disrupting *ASIC1a, 2 or 3* produces different alterations in colonic afferent mechanosensitivity. In summary, disrupting *ASIC1a* significantly increased the sensitivity of both serosal and mesenteric afferents. Disrupting *ASIC2* significantly increased the sensitivity in only serosal afferents and had no effect on mesenteric afferent mechanosensitivity. In contrast, disrupting ASIC3 significantly decreased the sensitivity of both serosal and mesenteric afferents. Thus ASIC3 appears to make a direct and positive contribution to mechanotransduction in both classes of colonic afferent. It is possible that these changes may reflect a multitude of alterations at the level of the mechanotransduction complex as ASIC1a, ASIC2 and ASIC3 subunits heteromultimerise in cultured DRG neurons[298, 301]. Thus these changes in mechanosensitivity may relate to changes in subunit configuration other than just the subtraction of the deleted channel and that ASIC subunits may reassemble into different configurations.

Although it is useful to know how each ASIC subunit contributes to mechanotransduction, it may be of greater importance to determine which of them, if any, makes the more attractable pharmacological target. Thus it may be possible to interfere with mechanotransduction specifically in distinct classes of afferents. Sensitivity to amiloride has been a valuable marker of currents

generated by DEG/ENaC channels from vertebrates, insects, mollusks, and nematodes[259, 356-359]. A particular characteristic that is shared by all DEG/ENaC cation channels, including ASIC1, 2 and 3 is the ability of extracellular amiloride to inhibit their current, evoked either by pH[238, 276, 278, 279, 360-363] or possibly by mechanical stimuli[364]. Amiloride has been previously used to investigate pH evoked currents in ASIC2 -/- and ASIC3 -/- large-diameter sensory neurons[10, 301] and thus may also be a useful tool in dissecting out which ASIC subunits are the most important as pharmacological targets in altering colonic mechanosensitivity.

The mechanism of amiloride's action in blocking the DEG/ENaC cation channel current has been controversial. For example, early data indicated that amiloride blocks current by occluding the ion-conducting pore itself[365], whereas other evidence suggests that amiloride binds to a predicted extracellular domain of ENaC[366, 367]. A more recent report indicates that amiloride can have two distinct effects on the ASIC2 channel and suggest that there may be two different sites of interaction. One site appears to lie within the channel pore and when amiloride interacts with this site it blocks the channel. The other site appears to be different from the blocking site and is located in the extracellular domain. When amiloride interacts with this site it can cause a conformational change that activates the channel[368]. Amiloride also has many analogues such as Benzamil which has a 10 times greater affinity of channel block[356, 369]. Notably, benzamil has been shown to almost completely block the mechanically-activated currents in somatosensory neurons using patch clamp experiments on rat lumbar DRG neurons in culture[364].

The present study had the purpose of addressing the question which ASIC subunits are responsible for the inhibitory actions of benzamil on primary afferents. The results of this study indicate differences in the contribution of ASIC1a, 2 and 3 to the pharmacology of benzamil in LSN

colonic serosal afferents and therefore which ASIC subunits are the major targets for amiloride analogues.

Material and Methods

All electrophysiological experiments were performed in accordance with the guidelines of the Animal Ethics Committees of the Institute for Medical and Veterinary Science and the University of Adelaide, Adelaide, Australia.

Generation of ASIC1a, 2 and 3 -/- mice

Mice with disrupted *ASIC1a, 2 and 3* genes were generated at the University of Iowa, Iowa City, USA by homologous recombination in embryonic stem cells as described in Chapter 3.

In vitro mouse colonic primary afferent preparation

Dissection, were carried out in ASIC1a, 2 or 3 +/+ and -/- male and female mice (20-30g) according to protocols for the LSN as described in detail in Chapter 1 and 2.

Characterization of LSN serosal afferents

Lumbar splanchnic colonic afferents were characterized as described in Chapters 1, 2 and 3. Receptive fields were identified by systematically stroking the mucosal surface with a brush of sufficient stiffness to activate all types of mechanosensitive afferent. The present study focused serosal afferents as the mechanosensitivity this afferent class was altered in all three *ASIC* (ASIC1a, 2 and 3) -/- mice (see Chapter 3). Serosal afferents were classified by their graded response to focal compression of their receptive fields via perpendicular probing with calibrated von Frey hairs (70, 160, 400, 1000, 2000 and 4000mg; each force applied 5 times for a period of 3 s) and their insensitivity to circular stretch (1-5g) and fine mucosal stroking (10mg)[312].

Electrophysiological data recording and analysis

Electrical signals generated by nerve fibers placed on the platinum recording electrode were fed into a differential amplifier, filtered, sampled (20 kHz) using a 1401 interface (Cambridge Electronic Design, Cambridge, UK) and stored on a PC for off-line analysis. The amplified signal was also used for online audio monitoring. Action potentials were analysed off-line using the Spike 2 wavemark function and discriminated as single units on the basis of distinguishable waveform, amplitude and duration. Data are expressed as mean ± SEM. *n* indicates the number of individual afferents; N indicates the number of mice. Data were analysed using Prism 4 software (GraphPad Software, San Diego, CA, USA), and where appropriate, were analysed using a two-way analysis of variance (ANOVA) with Bonferroni post hoc tests (to determine significant differences between curves) to compare significance differences between ASIC1a, 2 and 3 +/+ and -/- mice. Differences were considered significant at a level of $P < 0.05$.

Drug addition to receptive fields

The amiloride analogue Benzamil was tested on ASIC1a, 2 and 3 +/+ and -/- LSN serosal afferents to examine potential deficits in ASIC1a, 2 and 3 -/- serosal afferents and therefore determine the role of ASIC1a, 2 and 3 in the inhibition caused by benzamil. Once identified and stimulus-response functions generated sequential concentration-response curves were constructed for benzamil (1- 300μM). A small metal ring was placed over the serosal receptive field of interest, residual Krebs aspirated, and benzamil (1 μM) applied to the mucosal surface for 10 min before re-determining the mechanical sensitivity of the receptive field to focal compression via probing (2000mg von Frey hair). A probing stimulus of 2000mg was chosen because it was reproducibly effective in activating all LSN serosal afferents. Benzamil was then aspirated from the ring and the next concentration applied. This protocol was repeated for all Benzamil concentrations (1, 10, 30,

100 and 300 μM) and after the final concentration (300 μM) a 15 minute wash out period with normal Krebs was allowed before re-determining mechanical sensitivity.

Drugs

Stock solutions of benzamil were kept frozen and diluted to their final concentration (1, 10, 30, 100 and 300 μM) in Krebs solution. Benzamil was obtained from Sigma-Aldrich, Sydney Australia.

Results

Effects of ASIC1a deletion on colonic serosal afferent function

As shown in Chapter 3, ASIC1a +/+ serosal afferents displayed graded responses to an ascending series of probing stimuli (70-4000mg) when expressed as either maximum instantaneous frequency *(Figure 3.6A),* mean frequency *(Figure 3.6B),* spike rate *(Figure 3.6C) or* spikes per second *(Figure 3.6D).* Deletion of *ASIC1a* significantly increased the sensitivity in colonic serosal afferents, as indicated by significantly increased stimulus response functions *(Figure 3.6A, B, C and D).* Bonferroni post hoc tests also indicated significant increases in ASIC1a -/- mice responses at a 4000mg probing stimulus (max IF * $P < 0.05$, *Figure 3.6A*, and spikes/sec, * $P < 0.05$, *Figure 3.6D*).

Effects of ASIC1a deletion on the inhibition of colonic serosal afferents evoked by Benzamil

Benzamil caused a dose-dependent inhibition of ASIC1a +/+ serosal afferent mechanoreceptor function with significant inhibition observed at 100 and 300μM when expressed as either maximum instantaneous frequency *($P < 0.05$; Figure 4.1A),* mean frequency *(Figure 4.1B),* spike rate *(Figure 4.1C) or* spikes per second *(Figure 4.1D).* At a concentration of 100μM benzamil reduced the response of serosal afferents to a 2000mg perpendicular probing stimulus by

approximately 60%. Benzamil at a concentration of 300μM reduced the control response by approximately 85%. This effect was significantly reversed by a 15 minute Krebs washout.

Deletion of *ASIC1a* had no significant effect on the ability of benzamil to attenuate the response of serosal afferents to a 2000mg perpendicular probing stimulus expressed as either maximum instantaneous frequency *(P > 0.05; Figure 4.1A),* mean frequency *(P > 0.05; Figure 4.1B),* spike rate *(P > 0.05; Figure 4.1C) or* spikes per second *(P > 0.05; Figure 4.1D).* The concentration at which 50% inhibition (IC_{50}) of response was achieved was similar between ASIC1a +/+ and -/- when expressed as either maximum instantaneous frequency (+/+:60.1 μM; vs. -/-: 58.4μM), mean frequency (+/+:62.5 μM; vs. -/-: 65.7μM), spike rate (+/+:67.1 μM; vs. -/-: 63.7μM) *or* spikes per second (+/+:73.0 μM; vs. -/-: 63.1μM).

Effects of ASIC2 deletion on colonic serosal afferent function

As shown in Chapter 3, ASIC2 +/+ serosal afferents displayed graded responses to an ascending series of probing stimuli (70-4000mg) when expressed as either maximum instantaneous frequency *(Figure 3.12A),* mean frequency *(Figure 3.12B),* spike rate *(Figure 3.12C) or* spikes per second *(Figure 3.12D).* Deletion of *ASIC2* significantly increased the sensitivity in colonic serosal afferents, as indicated by significantly increased stimulus response functions *(Figure 3.12A, B, C and D).* Bonferroni post hoc tests also indicated significant increases in ASIC2-/- mice responses at a 4000mg probing stimulus (all parameters * $P < 0.05$, *Figure 3.12A, B, C and D*).

Effects of ASIC2 deletion on the inhibition of colonic serosal afferents evoked by Benzamil

Benzamil caused a dose-dependent inhibition of ASIC2 +/+ serosal afferent mechanoreceptor function with significant inhibition of mechanoreceptor function observed at 30, 100

and 300μM when expressed as either maximum instantaneous frequency *(Figure 4.2A),* mean frequency *(Figure 4.2B),* spike rate *(Figure 4.2C) or* spikes per second *(Figure 4.2D).* At a concentration of 30μM benzamil reduced the response of serosal afferents to a 2000mg perpendicular probing stimulus by approximately 55% of the control response. Benzamil at a concentration of 100μM caused a 70% reduction, whilst 300μM Benzamil caused a 95% reduction of the control response. This effect was significantly reversed by a 15 minute Krebs washout. Deletion of *ASIC2* significantly reduced the ability of benzamil to attenuate the response of serosal afferents to a 2000mg perpendicular probing stimulus ($P < 0.01$; two-way ANOVA, *Figure 4.2A, B, C and D).* Bonferroni post hoc tests also indicated significantly greater probing responses in ASIC2-/- mice at 10μM (** $P < 0.01$), 30μM, and 100μM benzamil (* $P < 0.05$), when expressed as spike rate *(Figure 4.2C*), or spikes per second *(Figure 4.2D*). Significantly greater probing responses in ASIC2 -/- mice were observed at 30μM, and 100μM benzamil when expressed as either maximum instantaneous frequency *(** $P < 0.01$; Figure 4.2A*), or mean frequency *(* $P < 0.05$; Figure 4.2B*).* The IC_{50} of benzamil was different between ASIC2 +/+ and -/- when expressed as either maximum instantaneous frequency (+/+:33.3 μM; vs. -/-: 153.8μM), mean frequency (+/+:35.2 μM; vs. -/-: 215.3μM), spike rate (+/+:41.9 μM; vs. -/-: 168.8μM) *or* spikes per second (+/+:23.2 μM; vs. -/-: 100.1μM).

Effects of ASIC3 deletion on colonic serosal afferent function

As shown in Chapter 3, ASIC3 +/+ serosal afferents displayed graded responses to an ascending series of probing stimuli (70-4000mg) when expressed as either maximum instantaneous frequency *(Figure 3.18A),* mean frequency *(Figure 3.18B),* spike rate *(Figure 3.18C) or* spikes per second *(Figure 3.18D).* Deletion of *ASIC3* significantly decreased the sensitivity in colonic serosal afferents, as indicated by significantly shallower stimulus response functions *(Figure 3.18A, B, C and*

D). Bonferroni post hoc tests also indicated significant decreases in ASIC3-/- responses at 2000 and 4000mg probing stimuli (max IF * $P < 0.05$, *Figure 3.18A)*, at a 2000mg probing stimulus (spike rate, * $P < 0.05$, *Figure 6c)* and at 2000 and 4000mg probing stimuli (spikes/sec, * $P < 0.05$, *Figure 3.18D*).

Effects of ASIC3 deletion on the inhibition of colonic serosal afferents evoked by Benzamil

Benzamil caused a dose-dependent inhibition of ASIC3 +/+ serosal afferent mechanoreceptor function with significant inhibition of mechanoreceptor function observed at 100 and 300μM when expressed as either maximum instantaneous frequency *(Figure 4.3A),* mean frequency *(Figure 4.3B),* spike rate *(Figure 4.3C) or* spikes per second *(Figure 4.3D).* At a concentration of 100μM benzamil reduced the response of serosal afferents to a 2000mg perpendicular probing stimulus by approximately 70% of the control response. Benzamil at a concentration of 300μM caused an 85% reduction of the control response. This effect was significantly reversed by a 15 minute Krebs washout. Deletion of *ASIC3* significantly reduced the ability of benzamil to attenuate the response of serosal afferents to a 2000mg perpendicular probing stimulus ($P < 0.01$; two-way ANOVA, *Figure 4.3A, B, C and D).* Bonferroni post hoc tests also indicated significantly greater probing responses in ASIC3-/- mice at 1, 10μM and 30μM (* $P < 0.05$) and 100μM benzamil (** $P < 0.01$), when expressed as spikes per second *(Figure 4.2D).* Significantly greater probing responses in ASIC3-/- mice were observed at 30μM, and 100μM benzamil when expressed as spike rate *(* $P < 0.05$; *Figure 4.2C).* Significantly greater probing responses in ASIC3-/- mice were observed at 100μM benzamil when expressed as maximum instantaneous frequency *(** $P < 0.01$; *Figure 4.2A)* or mean frequency (* $P < 0.05$; *Figure 4.2B).* Not only were these results different but the responses appear to be potentiated compared with control responses *(Figure 4.2A, B, C and D).* The IC_{50} of benzamil was different between ASIC3 +/+ and -/-

when expressed as either maximum instantaneous frequency (+/+:67.8 μM; vs. -/-: 143.2μM), mean frequency (+/+:67.2 μM; vs. -/-: 205.1μM), spike rate (+/+:54.6 μM; vs. -/-: 173.0μM) *or* spikes per second (+/+:51.5 μM; vs. -/-: 127.5μM).

Discussion

This study demonstrates clear differences in the effects of benzamil on the mechanosensitivity of LSN serosal afferents with disruptions to ASIC1a, 2 or 3. As expected benzamil caused a dose-dependent inhibition of ASIC1a, 2 and 3 +/+ serosal afferent mechanoreceptor function. However, benzamil caused varied effects in the various *ASIC* -/- mice. Although disrupting *ASIC1a* significantly increased the sensitivity of serosal afferents, the loss of *ASIC1a* had no effect on the ability of benzamil to attenuate the response of serosal afferents. Disrupting *ASIC2* also significantly increased the sensitivity of serosal afferents; however in this case the ability of benzamil to attenuate the response of serosal afferents was markedly reduced. Disrupting *ASIC3* significantly decreased the sensitivity of serosal afferents to mechanical stimuli and also reduced the ability of benzamil to attenuate the response of serosal afferents. Intriguingly, disrupting *ASIC3* actually appeared to result in a potentiated mechanosensory response at low concentrations of benzamil when compared with ASIC3+/+. These differences in the effects of benzamil indicate differences in the contributions of ASIC1a, 2 and 3 to the pharmacology of benzamil.

The observation of varied effects of Benzamil and the various alterations in serosal afferent mechanosensitivity in the current study is suggestive of a multitude of effects happening at the level of the heteromultimeric complex. Studies of acid evoked currents in isolated DRG from ASIC1a, 2 and 3 -/- mice have gone some way to clarifying what happens when a ASIC channel is deleted and it is likely that the loss of one subunit can change the subunit composition and perhaps kinetic

properties of the channel complex[298]. As discussed in Chapter 3, the issue of explaining the varied effects caused by the disruption of ASIC1, 2 or 3 on the mechanosensory response of afferents is complex. Similarly, the issue of explaining the varied results obtained with benzamil is equally if not more complex as one must take into account the changes responsible for the alterations in mechanosensitivity in addition to the changes which must occur to explain the differences evoked by benzamil. The simplest explanation of the changes in mechanosensitivity and the results of benzamil is that they are a direct result from the specific loss of the properties of the individual *ASIC* protein that has been disrupted via gene deletion. Therefore, if similar numbers of ASIC1, 2 and 3 are required to assemble heteromultimers, based on data that co-expression of ASIC1, 2 and 3 best represents the properties of native DRG neurons, at least in response to pH-evoked currents[298], then deletion of ASIC1, for example, would cause varied effects. This is because not only would the ability of benzamil to bind to the ASIC1 protein be lost, but the respective effects of benzamil on the ASIC2 and ASIC3 subunits to the complex would be amplified. To add further complexity there is a possibility of changes in subunit configuration other than just the subtraction of the deleted ASIC channel. It is possible that the remaining ASICs subunits may reassemble into different structural configurations of the heteromultimeric complex. As shown previously there are two binding sites for amiloride and its analogues such as benzamil, one site which appears to lie within the channel pore and another site which is located in the extracellular domain[368]. Altering the structural configuration of the heteromultimeric complex may therefore occlude or open the site at which benzamil can bind, preventing or enhancing the ability of benzamil to bind to the respective sites. Such a scenario may explain why the ability of benzamil to inhibit mechanoreceptor firing was unaltered in ASIC1a -/- mice, but was reduced in ASIC2 and 3 -/- mice. However, the scenario is further complicated as amiloride can have two distinct effects on the ASIC2 channel through interaction at two different binding sites. When amiloride interacts with the site within the channel pore it blocks the channel, whilst interaction with the site at the extracellular domain can stimulate the channel[368]. Therefore, in

ASIC3-/- serosal afferents, for example, where the properties of ASIC3 are lost and the properties of ASIC2 may be amplified a conformation change in the heteromultimeric complex may facilitate binding to the extracellular site potentially explaining the resultant increase in mechanoreceptor firing caused at lower concentration of benzamil when compared with ASIC3 +/+. However, the scenario is further complicated as not only does amiloride and benzamil block ASIC1a[280, 281], ASIC1b[26], ASIC2a[10, 284] and ASIC3[10, 284, 301], but also all other DEG/ENaC channels[8, 259, 356-358, 370-372]. Therefore it is not only the ASIC channels which contribute to the effect of benzamil. Amiloride also has different affinities for the various DEG/ENaC channels. For example, ENaC channels are the most sensitive channels to amiloride, with a concentration of 100 nM causing a 50% inhibition of H^+-gated currents[278] . ASIC1a and ASIC1b have IC_{50s} of approximately 10 and 20 μM respectively whilst ASIC2 has an IC_{50} of approximately 30 μM[280, 281, 295, 297, 373-375]. ASIC3 is the least sensitive channel to amiloride with an IC_{50} of 60–100 μM[280, 281, 287, 295, 297, 360, 373-375]. The complexities of these issues are highlighted by previous studies that have used amiloride in ASIC3 -/- mice. These studies show that a loss of ASIC3 in DRG cells almost doubles the inhibition of transient H^+-gated currents evoked by 10 μM amiloride. Therefore deleting an ASIC channel actually increased the sensitivity to amiloride rather than reducing it. This increased sensitivity to amiloride is consistent with the loss of the relatively amiloride-resistant ASIC3 subunit, and consistent with ASIC subunits forming heteromultimeric channels[301].

Future studies, combining the techniques used in this study with patch-clamp techniques on identified colonic neurons[202] may ultimately resolve the issues highlighted in this thesis. Such a scenario would allow intracellular recordings to be performed from retrogradely labelled LSN afferents located within the thoracolumbar DRG whose axons are still attached to afferent endings within the colonic wall. This would allow mechanical stimulation of the various classes of afferents within the colonic wall using the approach documented in this thesis, whilst allowing analysis of

mechanotransduction and pharmacological mechanisms at the cellular level. The availability of highly selective blockers for individual ASIC subtypes will also help to address these issues, although some candidates are already emerging. The first identified potent and specific peptide blocker of ASIC1a channels was psalmotoxin 1 (PcTX1) which was isolated from the venom of the South American tarantula *Psalmopoeus cambridgei*[376, 377]. Although, PcTX1 is specific for ASIC1a, can distinguish between ASIC1a and ASIC1b and can distinguish between ASIC1a, ASIC2 and ASIC3; PcTX1 is however unable to block ASIC1a as soon as this subunit is associated with either ASIC2a or ASIC3[376, 377]. Thus PcTX1 would not be able to answer the questions posed by this study where heteromultimeric ASIC complexes are almost certainly present. Even more recently an ASIC3 selective compound APETx2 was discovered from the sea anemone *Anthopleura elegantissima*[378, 379]. This may be more promising as APETx2 inhibits homomeric ASIC3 channels and ASIC3 containing heteromeric channels both in heterologous expression systems and in primary cultures of rat sensory neurons[378, 379].

In conclusion, this study has identified differences in the effects of benzamil in LSN colonic serosal afferents with disruptions to *ASIC1a, 2* and *3* possibly suggesting differences in the contributions of ASIC1a, 2 and 3 in the pharmacology of benzamil. ASIC2 and ASIC3 appear to be particularly significant in the pharmacology of benzamil on serosal afferents. These findings indicate diversity in mechanisms of mechanotransduction in both molecular and pharmacological terms which holds promise for therapeutic targeting.

Figure 4.1: Effects of disrupting ASIC1a on the ability of benzamil to inhibit probing induced mechanoreceptor firing

Benzamil at concentrations of 100 and 300 μM caused a dose-dependant inhibition of serosal mechanoreceptor firing evoked by a 2000mg von Frey Hair probing stimulus when expressed as maximum instantaneous frequency ***(A)***, mean frequency ***(B)***, spike rate ***(C)*** *or* spikes per second ***(D)***. Disrupting ASIC1a did not significantly alter the ability of benzamil to inhibit serosal mechanoreceptor firing. ASIC1 (+/+) (•, n=8, N=6) and (-/-) mice (□, n=14, N=11) for all parameters ***(A, B, C, D)***.

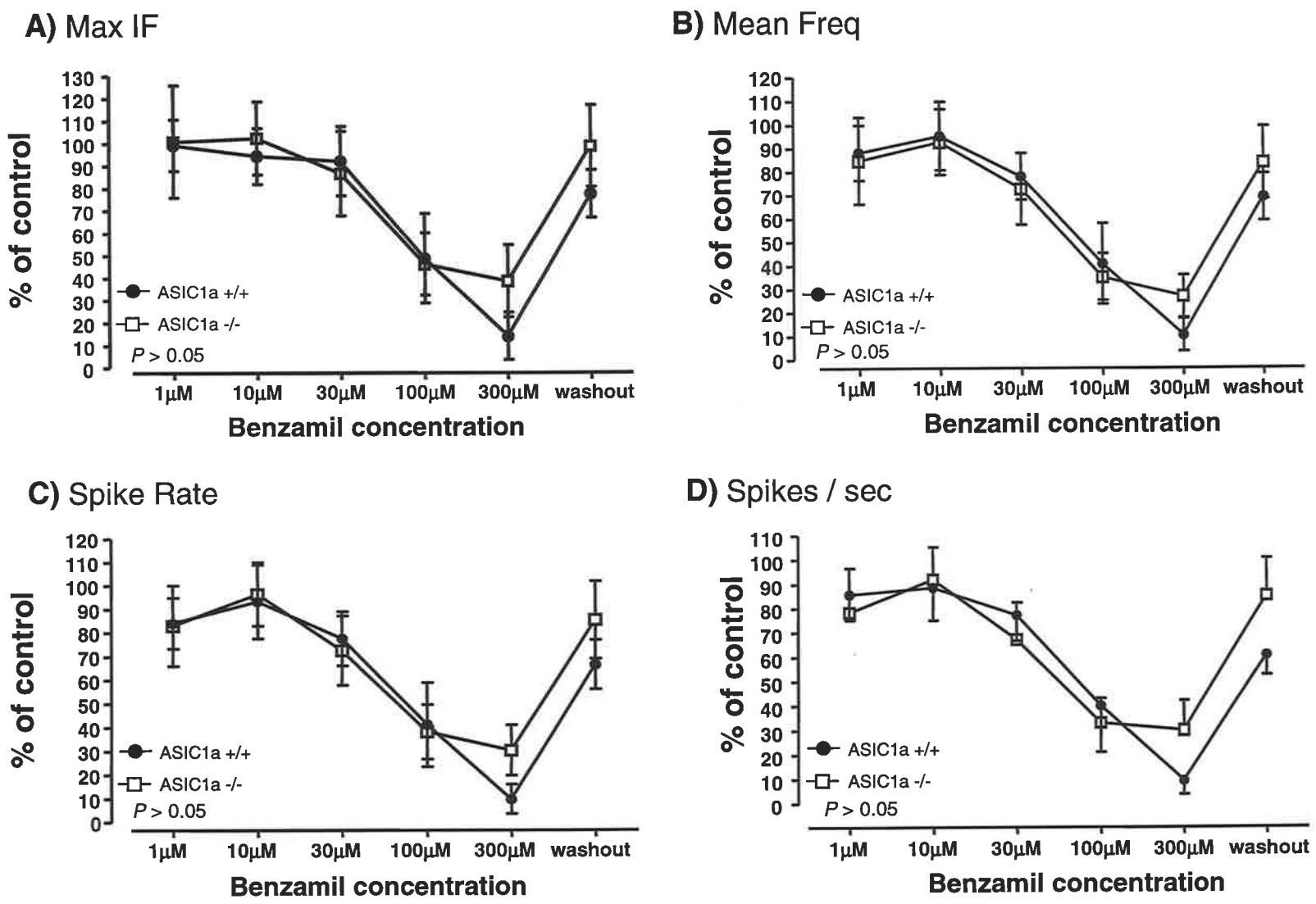

A) Max IF
% of control
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ASIC1a +/+
ASIC1a -/-
P > 0.05
1μM
10μM
30μM
100μM
300μM
washout
Benzamil concentration
B) Mean Freq
% of control
120
110
100
90
80
70
60
50
40
30
20
10
0
ASIC1a +/+
ASIC1a -/-
P > 0.05
1μM
10μM
30μM
100μM
300μM
washout
Benzamil concentration
C) Spike Rate
% of control
120
110
100
90
80
70
60
50
40
30
20
10
0
ASIC1a +/+
ASIC1a -/-
P > 0.05
1μM
10μM
30μM
100μM
300μM
washout
Benzamil concentration
D) Spikes / sec
% of control
110
100
90
80
70
60
50
40
30
20
10
0
ASIC1a +/+
ASIC1a -/-
P > 0.05
1μM
10μM
30μM
100μM
300μM
washout
Benzamil concentration

Figure 4.2: Effects of disrupting ASIC2 on the ability of benzamil to inhibit probing induced mechanoreceptor firing

Benzamil at concentrations of 30, 100 and 300 μM caused a dose-dependant inhibition of serosal mechanoreceptor firing evoked by a 2000mg von Frey Hair probing stimulus when expressed as maximum instantaneous frequency ***(A)***, mean frequency ***(B)***, spike rate ***(C)*** *or* spikes per second ***(D)***.

Deletion of *ASIC2* significantly reduced the ability of benzamil to attenuate the response of serosal afferents to a 2000mg perpendicular probing stimulus ($P < 0.01$; two-way ANOVA, ***(A, B, C and D)***. Bonferroni post hoc tests also indicated significantly greater probing responses in ASIC2-/- mice at 30μM, and 100μM benzamil when expressed as either maximum instantaneous frequency (** $P < 0.01$; ***(A)***, or mean frequency (* $P < 0.05$; ***(B)***. Significantly greater probing responses in ASIC2-/- mice were observed at 10μM (** $P < 0.01$), 30μM, and 100μM benzamil (* $P < 0.05$), when expressed as spike rate ***(C)***, or spikes per second ***(D)***. ASIC2 (+/+) (•, n=11, N=9) and (-/-) mice (□, n=11, N=9).

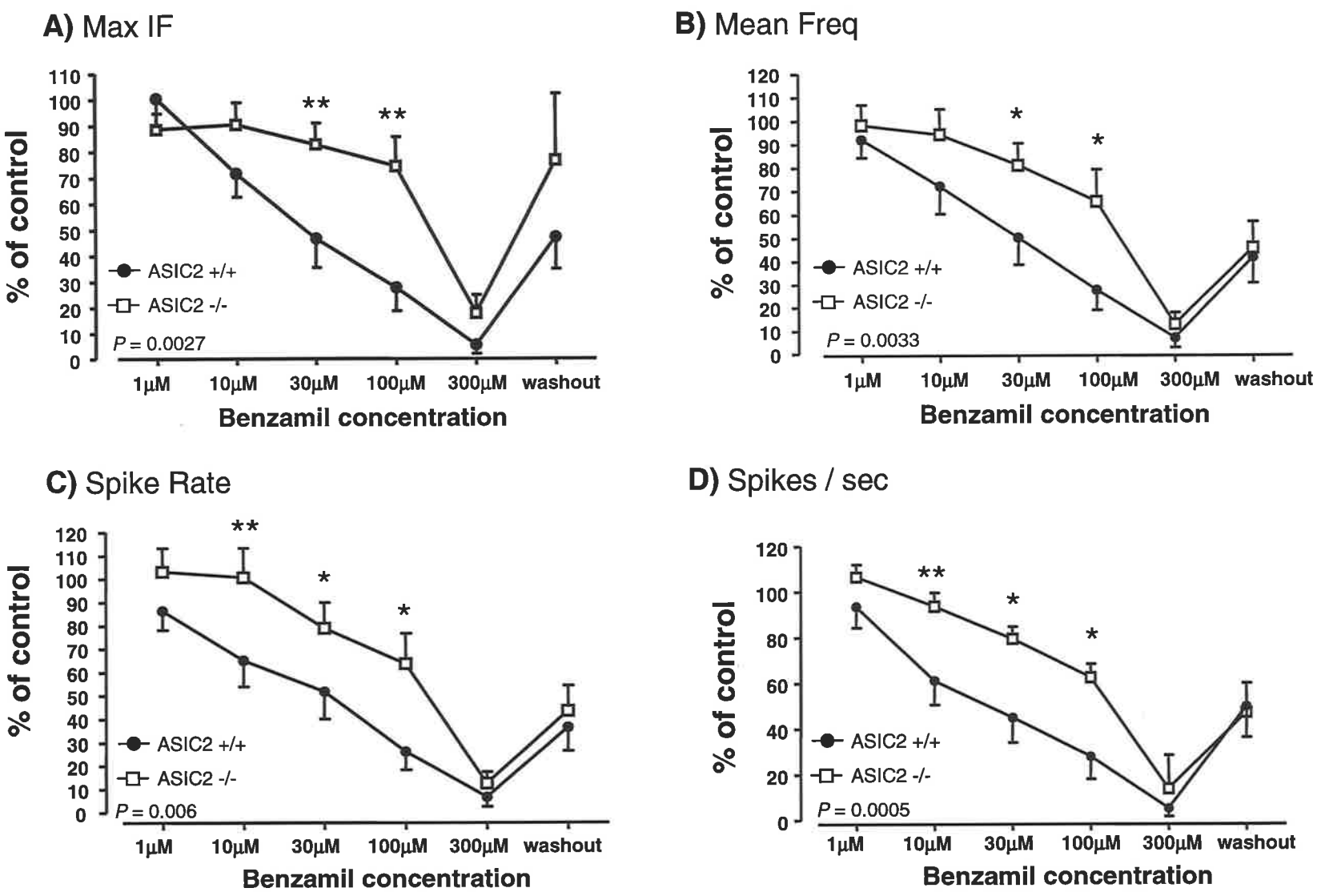

A) Max IF
% of control
110
100
90
80
70
60
50
40
30
20
10
0
**
**
ASIC2 +/+
ASIC2 -/-
P = 0.0027
1μM
10μM
30μM
100μM
300μM
washout
Benzamil concentration
B) Mean Freq
% of control
120
110
100
90
80
70
60
50
40
30
20
10
0
*
*
ASIC2 +/+
ASIC2 -/-
P = 0.0033
1μM
10μM
30μM
100μM
300μM
washout
Benzamil concentration
C) Spike Rate
% of control
120
110
100
90
80
70
60
50
40
30
20
10
0
**
*
*
ASIC2 +/+
ASIC2 -/-
P = 0.006
1μM
10μM
30μM
100μM
300μM
washout
Benzamil concentration
D) Spikes / sec
% of control
120
100
80
60
40
20
0
**
*
*
ASIC2 +/+
ASIC2 -/-
P = 0.0005
1μM
10μM
30μM
100μM
300μM
washout
Benzamil concentration

Figure 4.3: Effects of disrupting ASIC3 on benzamils ability to inhibit mechanoreceptor firing

Benzamil at concentrations of 30, 100 and 300 μM caused a dose-dependant inhibition of serosal mechanoreceptor firing evoked by a 2000mg von Frey Hair probing stimulus when expressed as maximum instantaneous frequency ***(A)***, mean frequency ***(B)***, spike rate ***(C)*** *or* spikes per second ***(D)***.

Disrupting ASIC3 significantly alter benzamils ability to inhibit serosal mechanoreceptor firing. Deletion of *ASIC3* significantly reduced the ability of benzamil to attenuate the response of serosal afferents to a 2000mg perpendicular probing stimulus ($P < 0.01$; two-way ANOVA, ***(A, B, C and D)***. Bonferroni post hoc tests also indicated significantly greater probing responses in ASIC3-/- mice at 100μM benzamil when expressed as maximum instantaneous frequency (** $P < 0.01$; ***(A)***. Significantly greater probing responses in ASIC3-/- mice were observed at or mean frequency (* $P < 0.05$; *Figure 4.2B*). Moreover, significantly greater probing responses in ASIC3-/- mice were observed at 30μM, and 100μM benzamil when expressed as spike rate (* $P < 0.05$; ***(C)***, whilst significantly greater probing responses in ASIC3-/- mice were observed at 1, 10μM and 30μM (* $P < 0.05$) and 100μM benzamil (** $P < 0.01$), when expressed as spikes per second ***(D)***. Not only were these results different but the responses appear to be potentiated compared with control responses ***(A, B, C and D)***. ASIC3 (+/+) (•, n=11, N=9) and (-/-) mice (□, n=16, N=13).

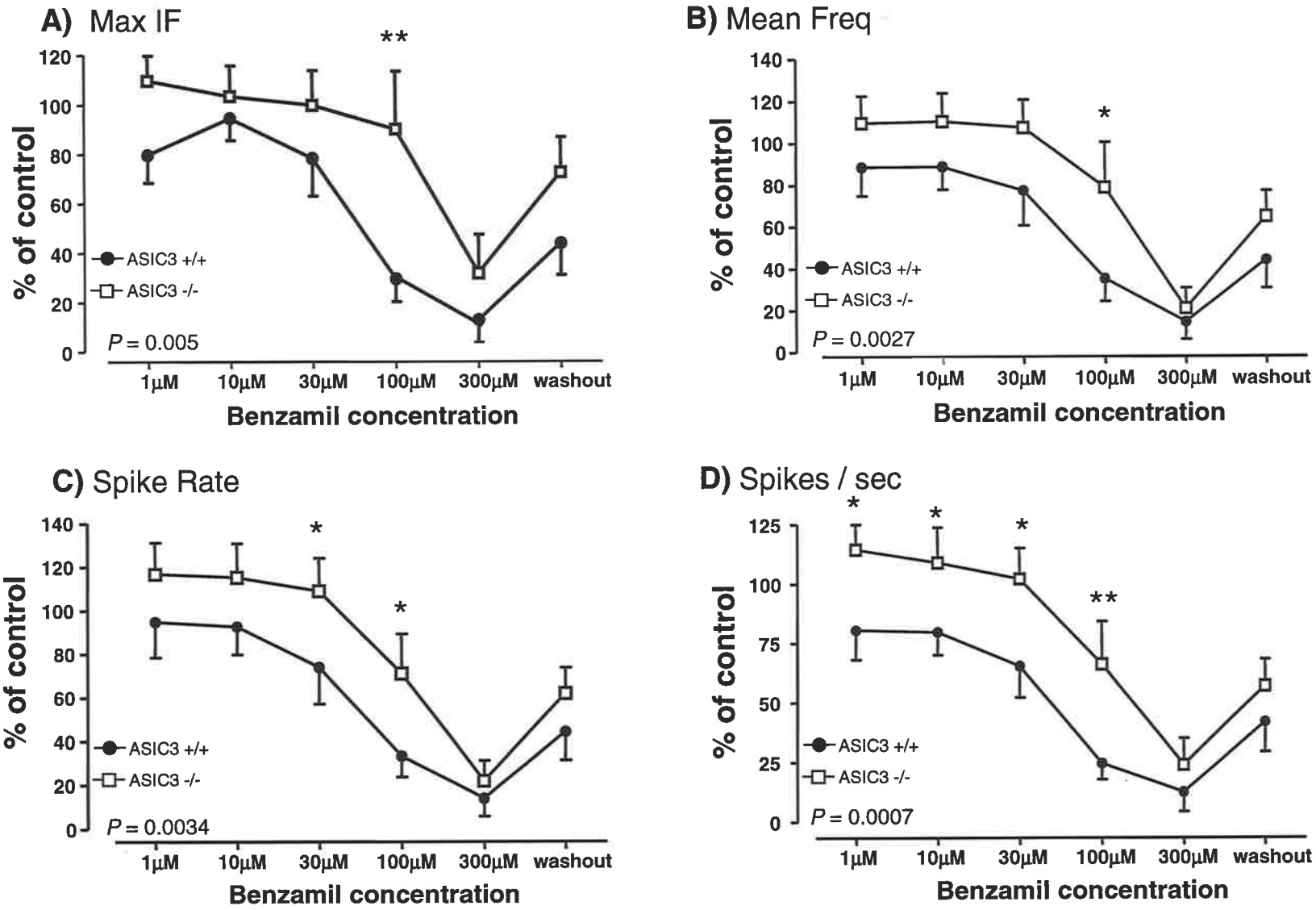

A) Max IF
**
120
100
80
60
40
20
0
% of control
ASIC3 +/+
ASIC3 -/-
P = 0.005
1µM
10µM
30µM
100µM
300µM
washout
Benzamil concentration
B) Mean Freq
140
120
100
80
60
40
20
0
*
% of control
ASIC3 +/+
ASIC3 -/-
P = 0.0027
1µM
10µM
30µM
100µM
300µM
washout
Benzamil concentration
C) Spike Rate
140
120
100
80
60
40
20
0
* *
% of control
ASIC3 +/+
ASIC3 -/-
P = 0.0034
1µM
10µM
30µM
100µM
300µM
washout
Benzamil concentration
D) Spikes / sec
125
100
75
50
25
0
* * * **
% of control
ASIC3 +/+
ASIC3 -/-
P = 0.0007
1µM
10µM
30µM
100µM
300µM
washout
Benzamil concentration

Table 4.1: Summary of the effects of disrupting ASIC 1a, 2 or 3 on the mechanosensitivity of afferents and the efficacy of benzamil.

ASIC1, 2 & 3 Summary of Results

		Mechano-sensitivity	Adaptation	von Frey threshold	Benzamil
ASIC1 (-/-)	Mesenteric	↑	Altered	No change	Not tested
	Serosal	↑	No change	No change	No change
ASIC2 (-/-)	Mesenteric	No change	No change	No change	Not tested
	Serosal	↑	No change	No change	↓ efficacy
ASIC3 (-/-)	Mesenteric	↓	Altered	No change	Not tested
	Serosal	↓	No change	No change	↓ efficacy

CHAPTER 5

EXPRESSION OF ASIC1, 2 AND 3 TRANSCRIPTS IN THORACOLUMBAR DORSAL ROOT GANGLIA

Summary

Background & Aims: ASIC1a, 2 and 3 all contribute to the mechanosensory function of LSN colonic mechanoreceptors. However, studies utilizing -/- mice are complicated by the possibility of compensatory changes in expression levels of other transcripts. Although previous studies have indicated that levels of other ASIC transcripts remain unchanged in *ASIC* -/- the possibility remains that there may be changes specific to thoracolumbar DRG. *Methods:* Thoracolumbar (T10-L1) DRG were removed bilaterally from 5 animals from each genotype, ASIC1a, 2 and 3 +/+ and -/- and RNA extracted. Standard reverse transcription (RT) and polymerase chain reaction (PCR) was performed to determine the expression of ASIC1, 2 and 3 transcripts in ASIC1a, 2 and 3 +/+ and -/- thoracolumbar DRG respectively. Quantitative RT-PCR was also used to determine the relative expression of ASIC1, 2 and 3 transcripts in ASIC1a, 2 and 3 +/+ thoracolumbar DRG and whether there were any significant compensatory changes in ASIC1a, 2 and 3 -/- thoracolumbar DRG. *Results*: RT-PCR and gel electrophoresis of the amplified products generated by primers specific for ASIC1, 2 and 3 revealed intense single bands, corresponding to the predicted sizes of ASIC1, 2 and 3 transcripts in *ASIC1a, 2* and *3* +/+ mice. In contrast, deletion of *ASIC1a, 2* or *3* resulted in loss of the corresponding transcript in all cases. Quantitative RT-PCR revealed in all ASIC +/+ thoracolumbar DRG that ASIC2 had the highest relative transcript expression, followed by ASIC1 and ASIC3. Quantitative RT-PCR revealed in all ASIC -/- thoracolumbar DRG that deletion of either ASIC1a, 2 or 3 did not significantly alter the mRNA levels of the other respective ASIC transcripts. Preliminary data using laser capture microdissection demonstrates expression of ASIC1, 2 and 3 mRNA specifically in retrogradely labeled colonic cells *Conclusions:* This study identifies the presence of ASIC1, 2 and 3 transcripts in thoracolumbar DRG, which correspond with the innervation of LSN colonic afferents. There are no compensatory changes in ASIC transcript expression when one of the other *ASIC* genes is disrupted. The changes observed in colonic afferent mechanosensitivity utilizing electrophysiological studies are likely the direct result of the specific loss of the individual *ASIC* gene.

To be considered a molecular component of mechanotransduction a given protein must be located within the mechanoreceptor, specifically at the site at which mechanical stimuli is detected[236]. The speed at which transduction of mechanical forces occurs suggests that this signal is generated directly by transduction channels, without intervening second messengers systems[236]. The cation channels in the DEG/ENaC family are proposed to fulfill this function. There indirect evidence suggesting that the DEG/ENaC superfamily of ion channels may form the molecular components of receptors that detect mechanical stimuli. As detailed previously in Chapter 3 genetic and localization studies in a variety of species have been critical in elucidating their roles in mechanosensation, as these channels are located within a wide variety of mechanoreceptors in a multitude of species and disruption of these channels results in alterations in the detection of the mechanical environment[233, 236-238, 259].

ASICs are probably the most important branch of the DEG/ENaC family, at least in terms of mammalian mechanotransduction, and have been extensively localized in the rat and mouse. Generally, ASIC1, 2 and 3 have been localized within the peripheral mechanosensory structures and sensory ganglia, although the specific location is species dependent[9, 10, 280, 281, 291-293]. In the rat ASIC1, 2 and 3 have been located in rat DRG using RT-PCR analysis using a combination of techniques. Specifically, ASIC1 has been localized to the plasma membrane of small, medium, and large diameter cells, whereas ASIC2 and ASIC3 were preferentially expressed in medium to large cells[292]. Within the cells themselves, ASIC1, 2 and 3 are present mainly on the plasma membrane of the soma and cellular processes[292]. *In situ* hybridization has revealed that in DRG the highest level of ASIC1a mRNA expression is in small neurons[280, 294], whereas ASIC1b is present in 20–25% of small and large diameter neurons[281]. ASIC2a and ASIC2b and ASIC3 mRNA are detected in small-diameter neurons[281, 295, 296]. Thus these results indicate a significant expression and localization of

ASIC1, 2 and 3 in nociceptive fibres. Nerve ligation studies indicate that ASIC subunits are transported from DRG cell bodies to sensory nerve terminals in the periphery[291].

In the mouse, ASIC2 and 3 are located at the sites of sensation as they are present in mechanosensory lanceolate nerve endings surrounding the hair shaft and other cutaneous sensory structures[9, 10, 291, 298]. Similarly, ASIC1, 2 and 3 are located within the DRG where the cell bodies of their mechanoreceptors are located. Specifically, ASIC1, 2 and 3 have been located in DRG using RT-PCR or northern blot analysis[9, 10, 281, 285, 298-300]. Overall the location of ASICs corresponds directly with the deficits observed in cutaneous mechanosensation in mice with disruptions to the *ASIC2* and *ASIC3* genes respectively which taken together indicate they play specific, distinct and diverse roles in cutaneous mechanotransduction.

As detailed in Chapters 3 and 4, ASIC1a, 2 and 3 all contribute to the mechanosensory function of LSN colonic mechanoreceptors. However, ASICs have not been specifically located within the DRG regions innervating the colon, the thoracolumbar DRG (representing LSN pathway) and lumbosacral spinal cord (representing sacral PN pathway)[81], nor in colonic afferents within DRG. Studies utilizing -/- mice are complicated by the possibility of compensatory changes in expression levels of other transcripts. Although previous studies have indicated that levels of other ASIC transcripts remain unchanged in ASIC1a, 2 and 3 -/- mice[9, 10, 299] the possibility remains that there may be changes specific to thoracolumbar DRG.

Thus the present study had the purpose of addressing the lack of knowledge concerning the expression of ASIC1, 2 and 3 in thoracolumbar DRG, and determining whether there are compensatory changes in ASIC transcript expression when one of the other *ASIC* genes is disrupted. Quantitative RT-PCR will be used as it is currently the most precise technique for determining

transcript expression[380-391]. The results of this study identify the presence of ASIC1, 2 and 3 transcripts in thoracolumbar DRG, which correspond with the innervation of LSN colonic afferents. There are no compensatory changes in ASIC transcript expression when one of the other *ASIC* genes is disrupted. The changes observed in colonic afferent mechanosensitivity utilizing electrophysiological studies must be a direct result from the specific loss of the individual *ASIC* gene.

Materials and Methods

All experiments were performed in accordance with the guidelines of the Animal Ethics Committees of the Institute for Medical and Veterinary Science and the University of Adelaide, Adelaide, Australia,

Dorsal root ganglia dissection

Five male or female mice (20-30g) from each genotype (ASIC1a, 2 and 3 +/+ and -/-) were killed via CO_2 inhalation and cervical dislocation. Thoracolumbar (T10-L1) DRG were removed bilaterally and the level of each noted and recorded, using the costae fluctuantes as an anatomical guide as detailed previously[81]. Dissections were carried out in a dissection chamber under sterile condition in ice-cold sterile saline. DRG from each individual genotype were pooled and stored in RNAlater® (Qiagen, Australia) at -20°C for subsequent RNA extraction.

RNA extraction

DRG from each genotype were transferred to a glass "pestle and mortar" homogenizer, excess RNAlater® removed with a sterile Pasteur pipette and snap frozen with liquid nitrogen and crushed with a sterile glass pestle. A total volume of 1.0ml of TRIzol® reagent (Invitrogen, Australia) was added, the ganglia homogenized and the contents transferred to Qiashredders® (Qiagen, Australia) and centrifuged (12,000 rpm) at room temperature for 2 minutes for further

homogenization. The Qiashredder columns were removed and the tubes containing the homogenate capped and the samples vortexed and left to stand for 15 minutes at room temperature, promoting dissociation of nucleoprotein complexes. Subsequently 0.2ml of chloroform was added, the samples vortexed for 1 minute, and left to stand for 15 minutes at room temperature. The samples were cold centrifuged (4°C at 12,000 rpm) for 15 minutes and the upper aqueous phase containing the RNA transferred to a new sterile eppendorf tube, 0.5ml of isopropanol added, vortexed and cold centrifuged for 10 minutes, allowing RNA precipitation. The upper aqueous phase was discarded, and 1ml of 75% ethanol added to the RNA pellet. Following a 1 minute vortex and a 5 minute cold centrifugation the supernatant was removed, the pellet re-suspended in RNAse free water and heated to 60°c for 2 minutes. Template RNA for each genotype was dispensed into 5µl aliquots and stored at -80°C. RNA quantification was determined by measuring the absorbance at 260 nm (*A*260) using a spectrophotometer (Biorad) RNA quality was estimated by the A260 and A280 nm ratio.

Determination of ASIC expression using RT-PCR

Reverse transcription (RT) and polymerase chain reaction (PCR) was performed using a Qiagen® one-step RT-PCR kit, with primers used as indicated in *Table 5.1A*. The primers used were previously published[9, 10] and tested for specificity using NCBI's BLAST software. RT-PCR master-mix was prepared according to the manufacture's specifications as follows: for each reaction 25µL RT-PCR Master Mix, 10µL RNAse free water, 10µL of 5x QIAGEN OneStep RT-PCR Buffer, 2µL dNTP Mix (containing 10 mM of each dNTP), 2µL QIAGEN OneStep RT-PCR Enzyme Mix and), 1µL of RNAse inhibitor (Ambion).

RT–PCR reactions were performed using an alpha unit block for PTC DNA engine systems (MJ Research) attached to a PTC-200 Peltier thermal cycler (MJ Research). The following

amplification program was applied: Reverse Transcription: 50°C for 30mins, Initial PCR activation: 95°C for 15 mins, PCR cycles: denaturing: 94°C for 1 min, annealing: 47°C for 1 min and extension: 72°C for 1 min repeated for 40 cycles followed by a final extension step of 72°C for 10 min.

For each reaction 25μL of Master Mix was added to 0.2ml clear thin walled PCR tubes (Axygen) with 10μL (3μM) forward primer (for a final concentration of 0.6 μM), 10μL (3μM) backward primer (for a final concentration of 0.6μM) and 5μL of 50 ng template RNA. Control PCRs were performed by substituting RNA template with 5μL distilled RNA-free water. Amplified products (5μL PCR product/2μL loading buffer) were resolved by 1.5-3% agarose gel electrophoresis and visualised via ethidium bromide staining.

Determination of relative ASIC expression using Quantitative RT-PCR

Reverse transcription (RT) and polymerase chain reaction (PCR) was performed using a Qiagen QuantiTect® SYBR® Green RT-PCR one-step RT-PCR kit, with primers used as indicated in *Table 5.1B.* All primers were designed with Primer 3.0 software (Applied Biosystems) and tested for specificity using NCBI's BLAST software. All product lengths were restricted between 100-150bp, to allow the highest efficiency of reaction when using SYBR® Green, as specified in the Qiagen QuantiTect® SYBR® Green handbook.

Real-time PCR master-mix was prepared according to the manufactures specifications as follows: for each reaction 24.5μL of 2x QuantiTect SYBR Green RT-PCR Master Mix, (containing, HotStarTaq® DNA Polymerase, QuantiTect SYBR Green RT-PCR Buffer, dNTP Mix including dUTP, SYBR® Green I (fluoresces in the presence of double stranded DNA), ROX (passive reference dye), 5 mM MgCl2) and 0.5μL of QuantiTect RT Mix, (containing Omniscript® Reverse Transcriptase and

Sensiscript® Reverse Transcriptase). For each reaction 25μL of the total Master Mix was added to MJ White PCR tubes (MJ Research) with 10μL (3μM) forward primer, 10μL (3μM) backward primer and 5μL of 50ng template RNA. Control PCRs were performed by substituting RNA template with 5μL distilled RNAse-free water.

RT–PCR reactions were performed using a Chromo4 (MJ Research) real-time instrument (designed to detect SYBR® green fluorescence and therefore amplified cDNA products) attached to a PTC-200 Peltier thermal cycler (MJ Research) and analyzed using Opticon Monitor software (MJ Research). The following amplification program was applied: Reverse Transcription: 50°C for 30mins; Initial PCR activation: 95°C for 15 mins; PCR cycles; denaturing: 94°C for 15 sec, annealing: 47°C for 30 sec and extension: 72°C for 30 sec repeated for 44 cycles followed by a final extension step of 72°C for 10 min. Subsequently, in all but one experiment (to allow gel electrophoresis to be performed) a melting curve program was performed to verify the specificity and identity of the RT-PCR products.

Each assay was run in triplicate in separate experiments. The comparative Ct method was used to quantify the abundance of target transcripts in -/- RNA relative to +/+ RNA, as previously described (PE Applied Biosystems User Bulletin 2 and [380-390]). Standard curves for ASIC1, 2, 3 and β-actin demonstrated that the efficiencies of targets (ie ASICs) and reference (β-actin) are approximately equal. The size of amplified products (5μL PCR product / 2μL loading buffer) was confirmed by 1.5% agarose gel electrophoresis and visualised via ethidium bromide staining.

The read-out for quantitative RT-PCR using the opticon monitor software was given as the number of PCR cycles or the "cycle threshold" (CT) needed to achieve a certain level of fluorescence. In all experiments the CT was fixed in the exponential/linear part phase of the PCR

fluorescence curve. During the initial PCR cycles (0-15), the fluorescence signal emitted by SYBR®-Green bound to the specific PCR product was normally in such a low intensity that it failed to register above a background level *(Figure 5.1)*. In all experiments β–actin was the first product to register a signal above background after approximately 20 PCR cycles. Comparing the CT was an effective way of determining the relative expression of transcripts as the CT was proportional to the logarithm of initial amount of target in a sample, the relative concentration of one target with respect to another was reflected in the difference in cycle number (ΔCt) necessary to achieve the same level of fluorescence[380].

To obtain relative expression values for ASIC transcripts the CT for each ASIC transcript was compared to the internal reference β–actin using the equation ΔCT (Cycle threshold (CT) of ASICx transcript - Cycle threshold (CT) of β-actin). Briefly, the greater the ΔCT value the less of the target transcript present. To determine the potential relative expression change of these transcripts in -/- the ΔΔCT was calculated using the following formula ΔΔCT=ΔCT [ASICx -/-] - ΔCT [ASICx +/+]) and the relative fold change calculated using the formula $2^{-\Delta\Delta CT}$ *(Table 5.2)* [380-390].

Verification of relative expression of data in thoracolumbar DRG and nodose ganglia

Melt curve analysis performed at the end of two of the quantitative PCRs revealed no primer dimers when performing quantitative RT-PCR on thoracolumbar DRG suggesting that only a single specific product was present and contributing to the overall fluorescent signal detected from SYBR® green *(Figure 5.2a)*. A lack of primer dimers was also observed in nodose ganglia *(Figure 5.2b)*.

To confirm the validity of the comparative CT method titration curves were obtained by taking template RNA of known concentration from thoracolumbar DRG and nodose ganglia and using them to create a five log dilution series (50, 25, 12.5, 6.25 and 3.125ng RNA). The linearity of

the plots shown in *Figure 5.3a and b* indicated equal amplification of the assay over a range of input RNA concentrations for β-actin, ASIC1, ASIC2 and ASIC3. Further validation was indicated by calculating ΔCT (Cycle threshold (CT) of ASICx transcript - Cycle threshold (CT) of β-actin) from the titration curves for thoracolumbar DRG and nodose ganglia *(Figure 5.4a and b).* A slope of less than 0.1 indicated that the efficiencies of target (ie ASICs) and the reference (ie. β-actin) were approximately equal and therefore relative comparative analysis using the comparative CT method was appropriate.

Results

ASIC1, 2 and 3 are expressed in thoracolumbar DRG

ASIC1, 2 and 3 have been identified in mouse DRG previously[9, 10, 298, 304], however they have not been specifically located in thoracolumbar DRG, the spinal region corresponding to the LSN innervation of the colon[81]. Using RT-PCR and gel electrophoresis of the amplified products generated by primers specific for ASIC1, 2 and 3, UV analysis of the gel revealed intense single bands, corresponding to the predicted sizes of ASIC1, 2 and 3 transcripts. These transcripts were detected in ASIC1a, 2 and 3 +/+ mice *(Figure 5.5).* Correspondingly, in ASIC1a -/-, ASIC1 was absent whilst ASIC2 and 3 transcripts were detected, in ASIC2 -/-, ASIC2 was absent whilst ASIC1 and 3 transcripts were detected and in ASIC3 -/-, ASIC3 was absent whilst ASIC1 and 2 transcripts were detected. Thus deletion of *ASIC1a, 2* or *3* resulted in loss of the corresponding transcript in all cases. Where the RNA template was substituted with distilled water (no), no evidence of amplified product was seen in +/+ or -/-, and expression of the housekeeping gene β-actin was observed in all cases.

Relative expression of ASIC1, 2 and 3 in thoracolumbar DRG using quantitative RT-PCR

To determine the possibility that disrupting one ASIC gene might alter expression of other ASICs quantitative RT-PCR was used to compare transcript levels of ASIC1, 2 and 3 genes. Using quantitative RT-PCR and gel electrophoresis of the amplified products generated by primers specific for ASIC1, 2 and 3 (*Table 5.2*), UV analysis of the gel revealed single bands, corresponding to the predicted sizes of ASIC1, 2 and 3 transcripts in ASIC1a, 2 and 3 +/+ mice *(Figure 5.6)*. This is important as it is the first step in verifying the relative expression data obtained from SYBR® green fluorescence intensity.

ASIC1, 2 and 3 were all detected in thoracolumbar DRG of ASIC1a, 2 and 3 +/+ mice. ASIC2 and 3 transcripts were not detectable in ASIC2 and ASIC3 -/- mice respectively. Some ASIC1 transcript was detected in ASIC1a -/- mice as a pan-ASIC1 primer was used for quantification. The ASIC1 detected was presumably that of the other splice variant ASIC1b. Where the RNA template was substituted with distilled RNA-free water (no), no evidence of amplified product was seen in +/+ or -/-, and expression of the housekeeping gene β-actin was observed in all cases.

Relative expression of ASIC1, 2, 3 and β-actin in thoracolumbar DRG in +/+ and -/- mice

The mean ± SD of the ΔCT of the ASIC transcripts in ASIC1a, 2 and 3 +/+ and -/- thoracolumbar DRG are shown in *Table 5.2*. Quantitative RT-PCR revealed in all ASIC +/+ thoracolumbar DRG that ASIC2 had the highest relative transcript expression, followed by ASIC1 and ASIC3. Relative expression data indicate that ASIC2 is the predominant transcript in ASIC1a, 2 and 3 +/+ thoracolumbar DRG, followed by ASIC1 and ASIC3 *(Figure 5.7A)*. Specifically there was a 11.36 ± 1.38 fold greater abundance of ASIC2 than ASIC1, a 22.84 ± 3.65 fold greater abundance of ASIC2 than ASIC3. There was a 3.91± 2.26 fold greater abundance of ASIC1 than ASIC3 *(Figure 5.7A)*.

Quantitative RT-PCR revealed that disruption of the *ASIC1* gene did not significantly alter the mRNA levels of either ASIC2 or ASIC3 in thoracolumbar DRG. Similarly, disruption of *ASIC2* and *ASIC3* did not result in significant compensatory changes in ASIC1 and 3 and ASIC1 and 2 transcript expression in the respective thoracolumbar DRG. In ASIC1a -/- mice, some ASIC1 was detected as a pan ASIC1 primer was used for quantification *(Figure 5.7B)*. As described earlier the ASIC1 expression detected was most probably that of the other splice variant ASIC1b as shown previously[304].

Relative expression of ASIC1, 2, 3 and β-actin in thoracolumbar DRG and nodose ganglia

As there are significant differences in the mechanosensitivity of both LSN colonic afferents and gastro-oesophageal vagal afferents when *ASIC* genes are deleted (see Chapter 3) separate quantitative RT-PCR experiments were performed to compare ASIC transcript expression between these two pathways of innervation. Such results may help to explain the contrasting effects of *ASIC* disruption in the mechanosensitivity of afferents between the two. In ASIC+/+ mice ASIC2 was the predominant transcript present in both thoracolumbar DRG and nodose ganglia followed by ASIC1 and ASIC3. Despite this general pattern of expression the relative abundance varied. In ASIC+/+ there was approximately a 2.5 fold (2.55 ± 0.62) greater amount of ASIC1 transcript in thoracolumbar DRG than nodose ganglia. Similarly, there was approximately a 2.8 (2.85 ± 0.35) fold greater amount of ASIC2 transcript in thoracolumbar DRG than nodose ganglia relative to β-actin and a 9.3 (9.35 ± 2.75) fold greater amount of ASIC3 transcript in thoracolumbar DRG than in nodose ganglia *(Figure 5.8)*.

Discussion

This study demonstrates five key findings with relation to ASIC transcript expression. One, RT-PCR analysis revealed there was widespread expression of ASIC1, 2 and 3 transcripts in thoracolumbar DRG, which corresponds with the LSN innervation of the colon. Two, these transcripts were not detectable in the corresponding ASIC1a, 2 or 3 -/- mice. Three, quantitative RT-PCR revealed that ASIC2 has the highest relative transcript expression in thoracolumbar DRG, followed by ASIC1 and ASIC3. Fourth, quantitative RT-PCR revealed in all ASIC -/- thoracolumbar DRG that deletion of either ASIC1a, 2 or 3 does not significantly alter the mRNA levels of the other respective ASIC transcripts. Five, quantitative RT-PCR revealed that ASIC1, 2 and 3 transcript expression was greater in thoracolumbar DRG than nodose ganglion.

Thus this study identifies the presence of ASIC1, 2 and 3 transcripts in thoracolumbar DRG and deletion of any *ASIC* gene does not result in compensatory changes in the expression of other ASIC transcripts. Therefore the changes observed in colonic afferent mechanosensitivity in ASIC knockouts are likely a direct result from the specific loss of the individual *ASIC* gene rather than functional compensatory mechanisms. The differences in ASIC expression between thoracolumbar DRG and nodose ganglia could explain the differences in mechanosensitivity observed between the two pathways.

The presence of ASIC1, 2 and 3 transcripts in thoracolumbar DRG is consistent with the expression of these transcripts in other levels of DRG[9, 10, 294, 298, 351]. However, this is the first documentation of the relative expression of these transcript demonstratting that ASIC2 is the predominant transcript in thoracolumbar DRG. In studies such as the present using gene disruption interpretation is complicated because when one gene is deleted the others may compensate by changes in expression levels. Previous studies from the same colony of ASIC2 and 3 -/- mice have

investigated the up- or down regulation of other ASIC transcripts using quantitative RT-PCR on RNA isolated from all levels of DRG[9, 10]. These studies showed that ASIC 1 and 2 levels are unchanged in ASIC3 -/-, and ASIC1 and 3 levels are unchanged in ASIC2 -/-[9]. The present study has shown using similar quantitative RT-PCR techniques but on RNA isolated specifically from thoracolumbar DRG shows that ASIC2 and 3 levels are unchanged in ASIC1a -/-, ASIC 1 and 3 levels are unchanged in ASIC2 -/- and that ASIC1 and 2 levels are unchanged in ASIC3 -/-. These results have a significant bearing on the interpretation of the results of the changes in mechanosensitivity of afferents recorded with electrophysiological preparations described in Chapters 3 and 4. It can now be concluded that the changes observed in colonic afferent mechanosensitivity are not due to compensatory changes in ASIC transcript expression "replacing" the deleted *ASIC* gene. The simplest explanation of changes in mechanosensitivity would conclude that they must be a direct result from the specific loss of the properties of the individual *ASIC* protein that has been disrupted via gene deletion. Although compensatory changes in expression levels can now be ruled out the issue is more complicated as ASIC1, 2 and 3 form functional heteromultimers in DRG[298, 301]. Therefore, if similar numbers of ASIC1, 2 and 3 are required to assemble heteromultimers, based on previous data that co-expression of ASIC1, 2 and 3 best represents the properties of native DRG neurons, at least in response to pH-evoked currents[298], then deletion of ASIC1a, for example, would cause varied effects. This is because not only would the properties of the ASIC1a subunit be lost, but the respective properties of the ASIC2 and ASIC3 subunits to the complex would be amplified.

To add further complexity there is a possibility of changes in subunit configuration other than just the subtraction of the deleted channel. It is possible that the remaining ASICs subunits may reassemble into different structural configurations of the heteromultermeric complex. Such a scenario would alter the functionality of the heteromultermeric complex, potentially making it more or less efficient at transducing mechanical stimuli. This may explain the respective gain and loss of

function in colonic, gastro-oesophageal and cutaneous afferents when various *ASIC* genes are deleted.

The issue is further complicated by the possibility of substitution of the deleted channel for another. Based on this studies quantitative RT-PCR results this scenario would not occur via up regulation of expression but instead may occur by the formation of fewer heteromultimeric complexes overall. What is apparent from these studies is that disruption of any one of the *ASIC* genes fails to abolish any one sensory modality completely; and instead, the loss of the deleted subunits results in modified sensory transduction suggesting that ASIC subunits may have both overlapping functions and some functional redundancy[298]. Such an observation would be consistent for colonic, gastro-oesophageal and cutaneous mechanosensation.

The complexity of the role of ASICs in mechanotransduction is amplified when contrasting results between different mechanoreceptors within the same pathway and between mechanoreceptors in different pathways. For example, in colonic afferents disrupting *ASIC2* resulted in an increase in serosal afferent mechanosensitivity but no change in mesenteric afferents. It is possible that the differences observed between these afferent classes could relate to the absolute number or the relative proportions of ASIC subunits contributing to mesenteric and serosal afferents mechanosensory complex. This would suggest that the heteromultimeric complexes within serosal afferent mechanoreceptors contain similar proportions of ASIC1, 2 and 3 as disrupting any one of these results in significant changes in mechanosensitivity. Conversely, the heteromultimeric complexes within mesenteric afferent mechanoreceptors may contain a higher proportion of ASIC1 and 3 as disrupting ASIC2 did not affect mechanosensitivity. It could be argued that such a conclusion may seem inappropriate given that the quantitative RT-PCR data indicate ASIC2 is by far the most expressed transcript in thoracolumbar DRG. However, these data represent the global

ASIC transcript expression within thoracolumbar DRG but does not necessarily represent specific proportions within colonic cells or for that matter between mesenteric and serosal populations. Differences in the absolute number or the relative proportions of ASIC subunits within the heteromultermeric complex could also account for 1) the differences observed between mucosal and tension gastro-oesophageal receptors, 2) the differences between the 5 types of cutaneous afferents, and 3) for the differences observed between colonic, gastro-oesophageal and cutaneous afferent function. Alternatively, the associated intracellular and extracellular proteins might confer distinct properties on mechanoreceptors within their different environments[233, 351]. Recent data indicate that the integral membrane protein, stomatin, (which is found in lipid/protein-rich microdomains) binds to ASIC1a, 2a, and 3 subunits and can differentially alter the function of specific ASIC subunits[351].

Limitations of this study

It could be argued that RT-PCR analysis from whole thoracolumbar DRG does not necessarily indicate that ASIC transcripts are present specifically within colonic cells. This is a possibility as only a small percentage (between 5 and 10%) of all thoracolumbar cells are of colonic origin[81]. However, a subsequent PhD study within the laboratory has utilized *in situ* hybridization to specifically locate ASIC1, 2 and 3 mRNA in retrogradely labeled cells within thoracolumbar DRG traced from the colon[392]. These studies have shown that ASIC1, 2 and 3 are all expressed in a large proportion of colonic neurons[392]. Similarly, this investigator (S.M.B) using techniques unavailable at the time of the other studies present within this thesis has detected ASIC1, 2 and 3 transcripts within isolated colonic cells[392]. Specifically, using retrograde tracing methods and laser capture microdissection, similar to that used previously[393], to isolate traced colonic cells within thoracolumbar DRG, RT-PCR analysis has revealed intense bands corresponding to ASIC1, 2 and 3 *(see Figure 5.9).*

It cannot be discounted that the relative expression studies between ASIC1a, 2 and 3 +/+ and -/- in whole thoracolumbar DRG could have masked specific differences contained within colonic neurons. Similarly the relative expression between thoracolumbar DRG and nodose ganglia represents the global ASIC transcript differences between thoracolumbar DRG and nodose ganglia but does not necessarily represent specific differences between colonic and gastro-oesophageal populations. Studies which are currently underway within the laboratory using laser capture microdissection (which was unavailable during the current study) to isolate colonic or gastro-oesopheageal retrogradely labeled cells and quantitative RT-PCR may resolve these discrepancies between the two pathways. Indeed differences in the relative expression of transcript of various channels and receptors between visceral afferents (LSN vs PN vs vagal) and non-visceral afferents could potentially lead to promising therapeutic targets for various disease states. The power of such techniques is demonstrated by Shin *et al.,* (2003)[393] who have used DNA microarrays and subtracted cDNA libraries to isolate genes that are specifically expressed by one type of mouse mechanoreceptor to show that the T-type calcium channel Ca(v)3.2 is exclusively expressed in the DRG by D-hair receptors.

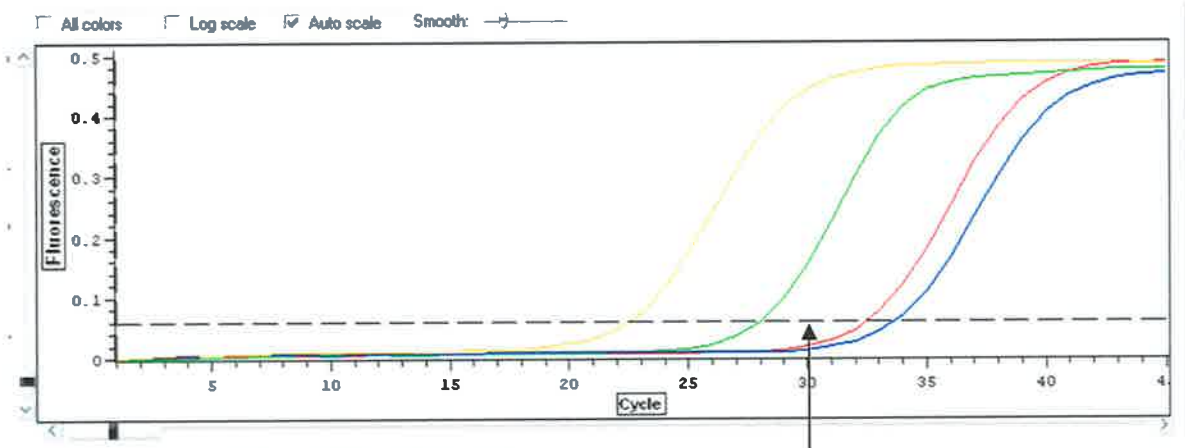
In the absence of quantifiable protein expression data the functional outcomes of the mRNA expression data of this study are discussed on the premise that mRNA expression relates directly to the functional protein expression of the ASIC subunits contributing to the afferent mechanosensory complexes. Although, it has been previously demonstrated in mouse DRG that mRNA expression of ASIC1, 2 and 3 results in the expression of ASIC1, 2 and 3 proteins respectively[9, 10, 304], it is possible that changes in the functional protein expression of the individual ASIC subunits may occur with or without changes in overall gene expression. Similarly, although this study did not detect statistically significant changes in the transcript levels of the remaining ASIC subunits when one is disrupted it

cannot be excluded that secondary changes in the proteins levels of the remaining ASIC subunits occurs.

In conclusion this study identifies the presence of ASIC1, 2 and 3 transcripts in thoracolumbar DRG. Deletion of any *ASIC* gene does not result in compensatory changes in other ASIC transcripts; therefore the changes observed in colonic afferent mechanosensitivity are likely a direct result from the specific loss of the individual *ASIC* gene. The differences in ASIC expression between thoracolumbar DRG and nodose ganglia could explain the differences in mechanosensitivity observed between the two pathways.

Figure 5.1: **Example of quantitative PCR transcript curves and setting of the cycle threshold.**

In all experiments the CT was fixed in the exponential/linear part phase of the PCR fluorescence curve. During the initial PCR cycles (0-15), the fluorescence signal emitted by SYBR-Green bound to the specific PCR product was normally in such a low intensity that it failed to register above a background level. In all experiments β–actin was the first product to register a signal above background after approximately 20 PCR cycles



β-actin CT: 22.13

ASIC2 CT: 27.95

ASIC1 CT: 32.21

ASIC3 CT: 33.87

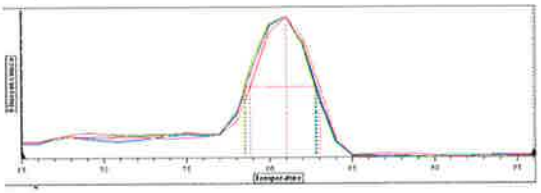
Cycle threshold (CT)

Figure 5.2: **Melt curve analysis for Quantitative RT-PCR in ASIC1a, 2 and 3 +/+ and -/- mice.**

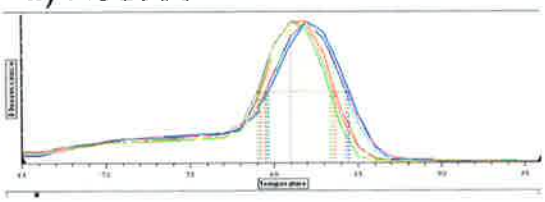
Melt curve analysis revealed a lack of primer dimers when performing quantitative RT-PCR on **i)** thoracolumbar DRG when detecting transcripts **A)** ASIC1, **B)** ASIC2, **C)** ASIC3 and **D)** β-actin suggesting that only a single specific product was present and contributing to the overall fluorescent signal detected from SYBR® green. Similarly melt curve analysis also revealed a lack of primer dimers when performing quantitative RT-PCR on **ii)** nodose ganglia when detecting transcripts **A)** ASIC1, **B)** ASIC2, **C)** ASIC3 and **D)** β-actin.

A) ASIC1 product

i) DRG

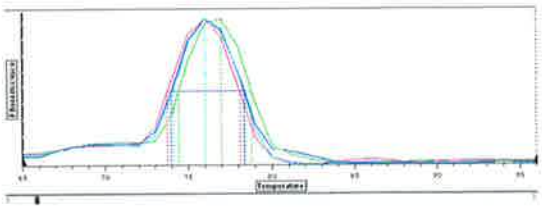


ii) Nodose

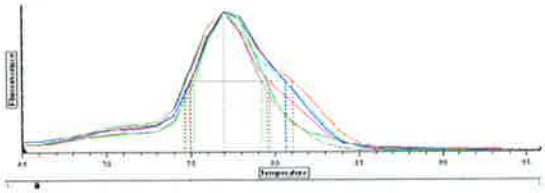


B) ASIC2 product

i) DRG

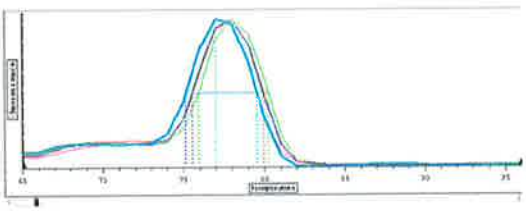


ii) Nodose

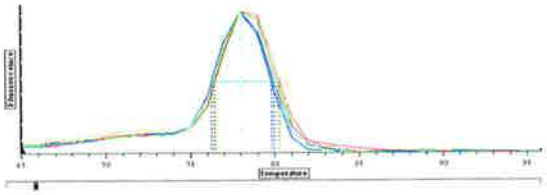


C) ASIC3 product

i) DRG

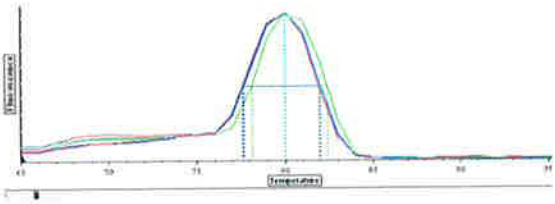


ii) Nodose



D) β-actin product

i) DRG



ii) Nodose

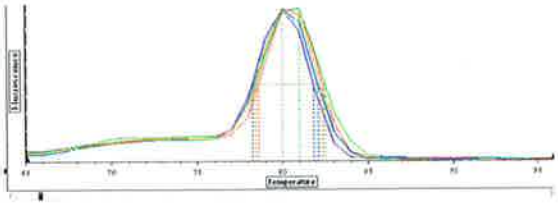
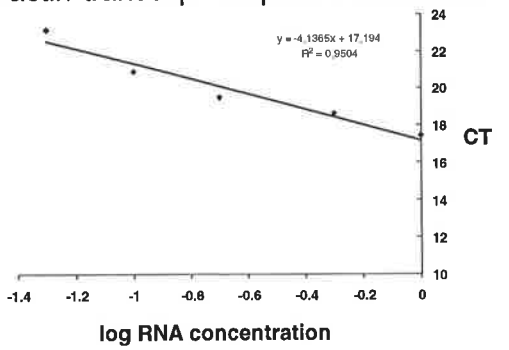


Figure 5.3: **Template dilution standards for each of the transcripts detected using Quantitative RT-PCR.**

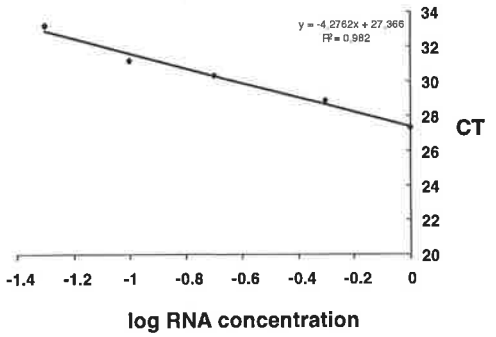
Titration curves were obtained by taking template RNA of known concentration from ***A)*** thoracolumbar DRG or ***B)*** nodose ganglia and using these to create a five log dilution series (50, 25, 12.5, 6.25 and 3.125ng RNA). The linearity of the plots shows the equal amplification of the assay over a range of input RNA concentrations for **(i)** β-actin, **(ii)** ASIC1, **(iii)** ASIC2 and **(iv)** ASIC3. CT refers to the cycle threshold.

A) DRG

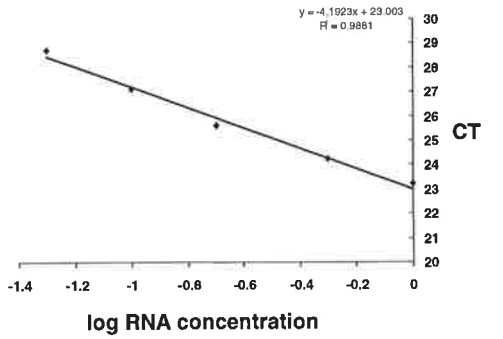
i) β-actin transcript template RNA dilution



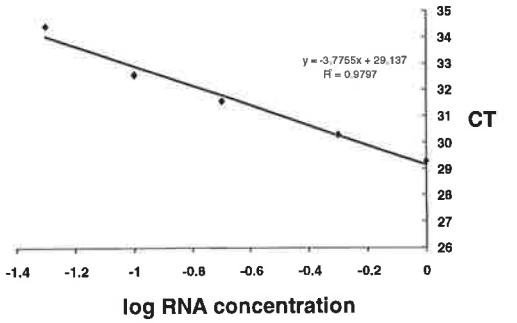
ii) ASIC1 transcript template RNA dilution



iii) ASIC2 transcript template RNA dilution

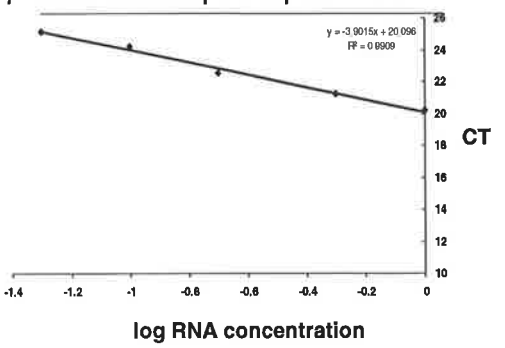


iv) ASIC3 transcript template RNA dilution

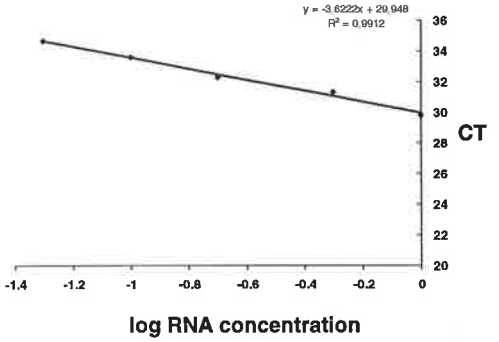


B) Nodose

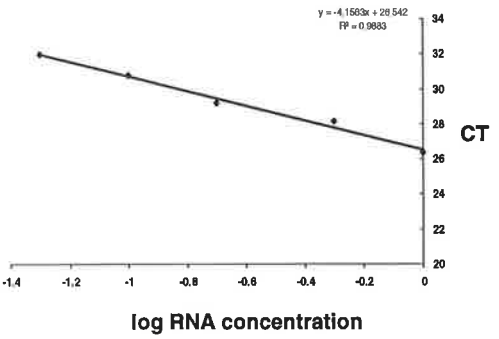
i) β-actin transcript template RNA dilution



ii) ASIC1 transcript template RNA dilution



iii) ASIC2 transcript template RNA dilution



iv) ASIC3 transcript template RNA dilution

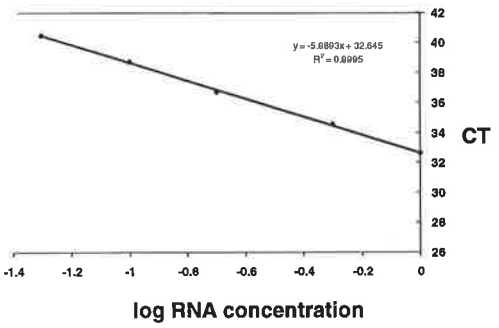
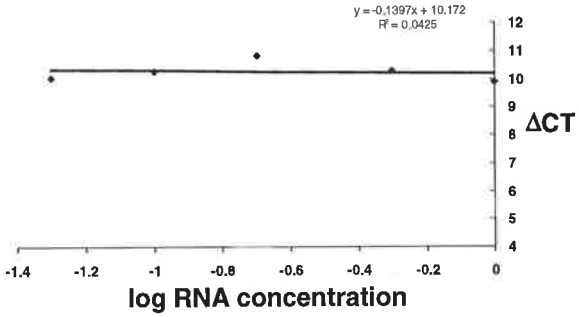


Figure 5.4: **Validation of the use of the comparative CT method for each of the ASIC transcripts detected using Quantitative RT-PCR.**

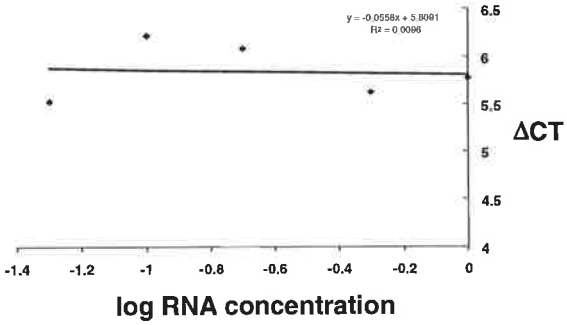
Graphical representation of the ΔCT (Cycle threshold (CT) of ASICx transcript - Cycle threshold (CT) of β-actin) from the titration curves obtained from ***A)*** thoracolumbar DRG and ***B)*** nodose ganglia in *Figure 5.3*. A slope of less than 0.1 indicates that the efficiencies of target (ie ASICs) and the reference (ie. β-actin) are approximately equal and therefore relative comparative analysis using the comparative CT method is appropriate.

A) DRG

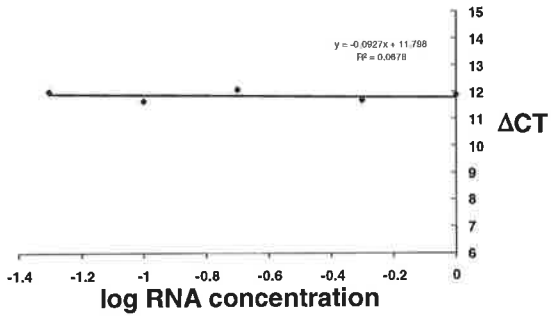
i) ASIC1 transcript vs. β-actin



ii) ASIC2 transcript vs. β-actin

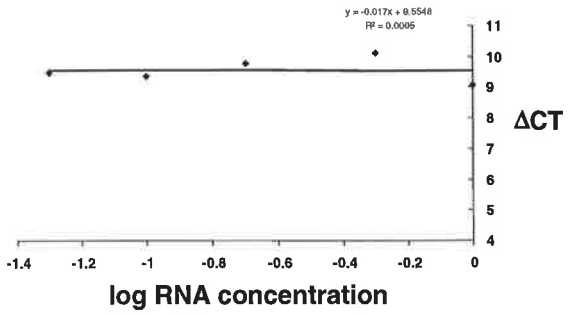


iii) ASIC3 transcript vs. β-actin

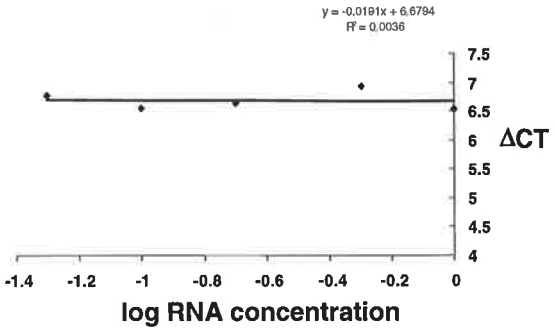


B) Nodose

i) ASIC1 transcript vs. β-actin



ii) ASIC2 transcript vs. β-actin



iii) ASIC3 transcript vs. β-actin

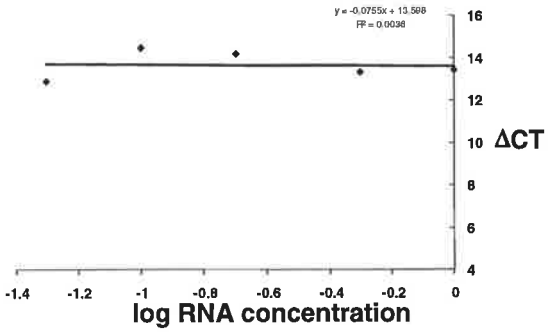


Figure 5.5: **RT-PCR of thoracolumbar DRG in ASIC1a, 2 and 3 +/+ and -/- mice.**

ASIC 1a, 2 and 3 were all detected in thoracolumbar DRG of +/+ mice, but were not detectable in -/- mice of each gene. Where the RNA template was substituted with RNA free distilled water (no), no evidence of the amplified product was seen in +/+ or -/-. Expression of the housekeeping gene β-actin was observed in both +/+ and -/- mice.

DRG

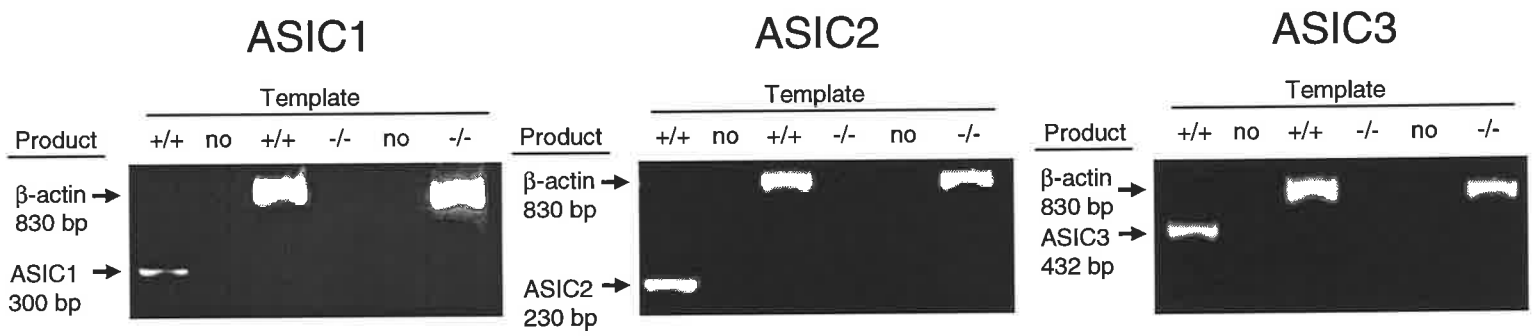

ASIC1
Template
Product
+/+ no +/+ -/- no -/-
β-actin
830 bp
ASIC1
300 bp
ASIC2
Template
Product
+/+ no +/+ -/- no -/-
β-actin
830 bp
ASIC2
230 bp
ASIC3
Template
Product
+/+ no +/+ -/- no -/-
β-actin
830 bp
ASIC3
432 bp

Figure 5.6: **Confirmation of ASIC transcript product size amplified during Quantitative RT-PCR of thoracolumbar DRG in ASIC1a, 2 and 3 +/+ and -/- mice.**

ASIC1, 2 and 3 were all detected in thoracolumbar DRG of ASIC1, 2 and 3 +/+ mice. ASIC2 and 3 transcripts were not detectable in ASIC2 and ASIC3 -/- mice respectively. Some ASIC1 transcript was detected in ASIC1a-/- mice as a pan-ASIC1 primer was used for quantification. The ASIC1 detected is presumably that of the other splice variant ASIC1b as shown previously[304]. Where the RNA template was substituted with distilled water (no), no evidence of amplified product was seen in +/+ or -/-, and expression of the housekeeping gene β-actin was observed in both +/+ and -/- mice.

DRG

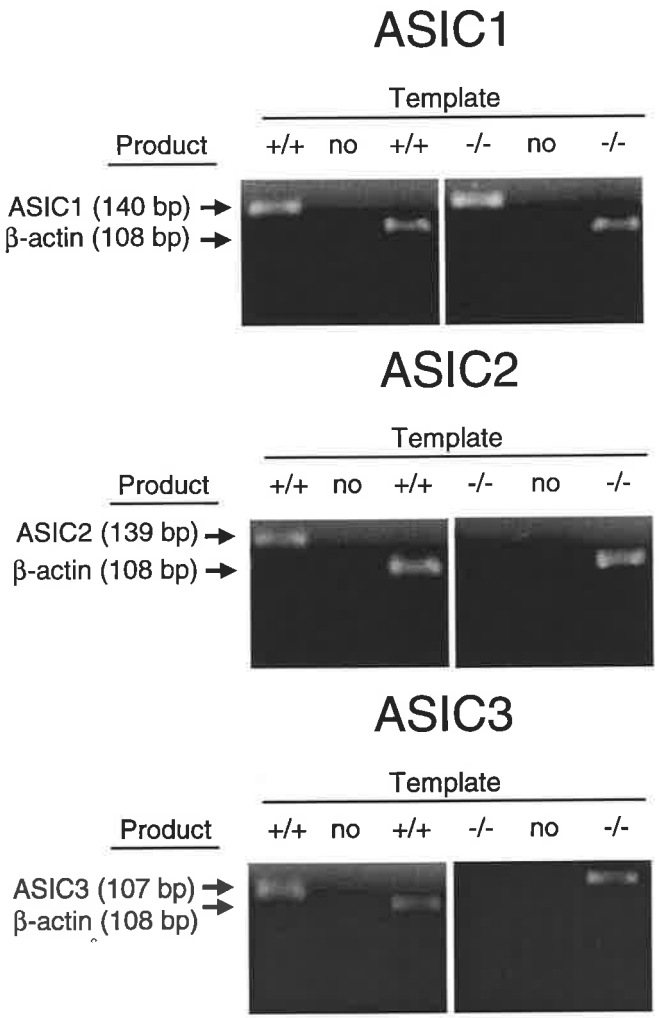
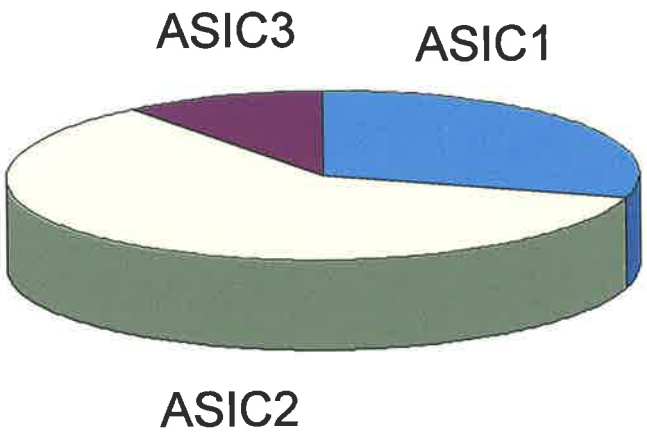

ASIC1
Template
Product
+/+ no +/+ -/- no -/-
ASIC1 (140 bp)
β-actin (108 bp)
ASIC2
Template
Product
+/+ no +/+ -/- no -/-
ASIC2 (139 bp)
β-actin (108 bp)
ASIC3
Template
Product
+/+ no +/+ -/- no -/-
ASIC3 (107 bp)
β-actin (108 bp)

Figure 5.7: Graphical representation of relative expression analysis of ASIC transcripts in Table 2

A) Relative expression of ASIC1, 2 and 3 transcripts in ASIC+/+ thoracolumbar DRG. Quantitative RT-PCR revealed that ASIC2 is the predominant transcript followed by ASIC1 and ASIC3. Specifically there is an 11.36 ± 1.38 fold greater abundance of ASIC2 than ASIC1, a 22.84 ± 3.65 fold greater abundance of ASIC2 than ASIC3. ASIC1 has a 3.91± 2.26 fold greater abundance of ASIC3.

B) Quantitative RT-PCR revealed that there were no significant compensatory changes in ASIC expression in ASIC1a, 2 or 3 -/- thoracolumbar DRG except for the transcript in the respective -/- mice ($P > 0.05$, Mann-Whitney test). Some ASIC1 was detected in ASIC1a-/- mice as a pan-ASIC1 primer was used for quantification, but ASIC1 expression was significantly decreased in ASIC1a -/- ($P = 0.0057$, Mann-Whitney test). The ASIC1 detected is presumably that of the other splice variant ASIC1b as shown by [304].

A)



B)

Quantitative PCR of ASIC expression in null mutant thoracolumbar DRG (mean ±SD)

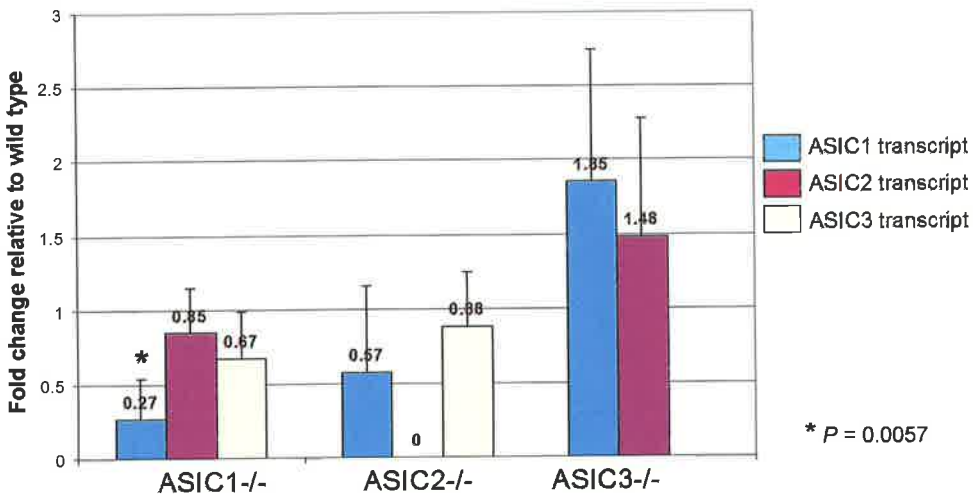


Figure 5.8: **Relative expression of ASIC transcripts in thoracolumbar DRG compared with nodose ganglia using Quantitative RT-PCR.**

Graphical representation of data presented in Tables 5.2 showing the relative expression of ASIC1, 2 and 3 transcripts in thoracolumbar DRG compared with nodose ganglia.

The relative expression values were calculated using ΔΔCT (ΔCT [DRG] - ΔCT [nodose]) of the ASIC transcripts in ASIC1a, 2, and 3 +/+ and -/-. The fold difference in transcript expression in DRG relative to nodose is calculated using the formula $2^{-\Delta\Delta CT}$.

In ASIC+/+ mice ASIC2 is the predominant transcript present in both thoracolumbar DRG and nodose ganglia followed by ASIC1 and ASIC3 transcripts. Despite this general pattern of expression the relative abundance varied considerably. In ASIC+/+ there is approximately a 2.5 fold greater amount of ASIC1, 2.8 fold greater amount of ASIC2 and a 9.3 fold greater amount of ASIC3 transcript in thoracolumbar DRG than in nodose ganglia.

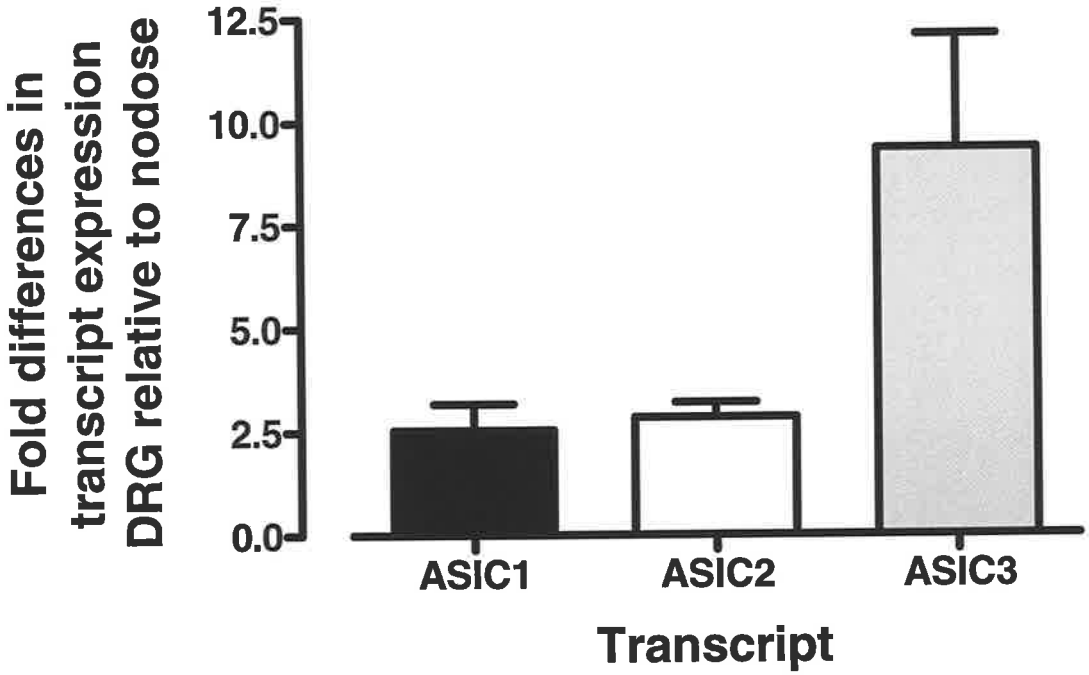

Fold differences in transcript expression DRG relative to nodose
12.5
10.0
7.5
5.0
2.5
0.0
ASIC1
ASIC2
ASIC3
Transcript

Figure 5.9: **Additional results using techniques unavailable at the time of the other studies to confirm presence of ASIC1, 2 and 3 in LSN colonic cells**

This investigator has detected ASIC1, 2 and 3 transcripts specifically within isolated colonic cells using retrograde tracing methods and laser capture microdissection (a technique which was not available during other RT-PCR and quantitative RT-PCR studies), to isolate traced colonic cells within +/+ thoracolumbar DRG. RT-PCR analysis has revealed intense bands corresponding to ASIC1, 2 and 3, confirming the presence of ASIC1, 2 and 3 transcripts are specifically located within colonic cells within the thoracolumbar DRG.

RT-PCR from retrograde labeled laser capture microdissection colonic cells from thoracolumbar DRG (T10-L1)

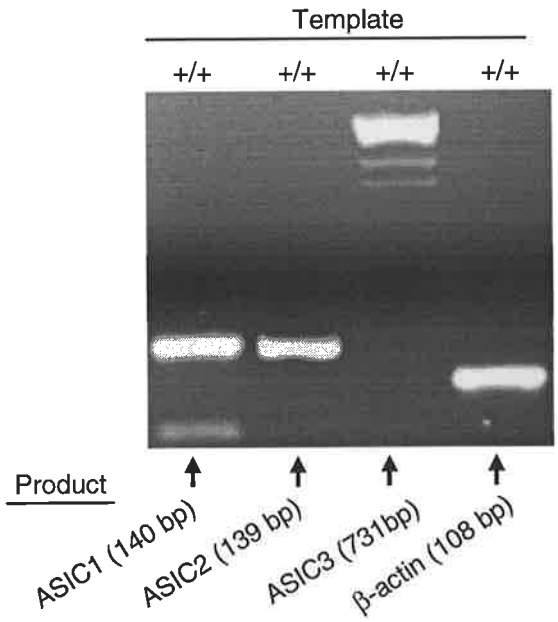


Table 5.1

A) Nucleotide primer sequences used for RT-PCR identification of ASIC transcripts and ***B)*** Nucleotide primer sequences used for relative quantification of ASIC transcripts using quantitative RT-PCR. Primer sequences in both ***A)*** and ***B)*** were designed to detect both ASIC1 splice variants (ASIC1a and ASIC1b), both ASIC2 splice variants (ASIC2a and ASIC2b) and ASIC3.

Table 1A: Nucleotide primer sequences used for RT-PCR identification of ASIC transcripts

GENE	FORWARD PRIMER (5' – 3')	REVERSE PRIMER (5' – 3')	PRODUCT LENGTH (bp)
ASIC1	CAGCTAGAGATATTGCAGGACAAG	CCACACAGGCAAGTACTTATCTTG	300
ASIC2	TCGAGAACATTCTTGTTCTGGAT	GTTCTCATCATGGCTCCCTTCCTC	230
ASIC3	TGAGAGCCACCAGCTTACC	GGCAGATACTCCTCCTGCT	432
β-actin	ATCATGTTTGAGACCTTCAACAC	TCTGCGCAAGTTAGGTTTTGTC	830

Table 1B: Nucleotide primer sequences used for relative quantification of ASIC transcripts

GENE	FORWARD PRIMER (5' – 3')	REVERSE PRIMER (5' – 3')	PRODUCT LENGTH (bp)
ASIC1	CAGATGGCTGATGAAAAGCA	AAGTGGCACGAGAGAAGCAT	140
ASIC2	TGACATTGGTGGTCAAATGG	ATCATGGCTCCCTTCCTCTT	139
ASIC3	AGGGAGAAGTCCCAAAGCAT	GACACTCCATTCCCAGGAGA	107
β-actin	GACCTCTATGCCAACACAGT	GATCTTCATGGTGCTAGGAG	108

Table 5.2: **Relative expression analysis of ASIC1, 2 and 3 transcripts from thoracolumbar DRG in ASIC1a, 2 and 3 +/+ and -/- mice using Quantitative RT-PCR.**

Upper panels indicate the mean ± SD of the ΔCT (Cycle threshold (CT) of ASICx transcript - Cycle threshold (CT) of β-actin) of the ASIC transcripts in ASIC1a, 2 and 3 +/+ and -/- thoracolumbar DRG.

Lower panels indicate the mean ± SD of the ΔΔCT (ΔCT [ASICx -/-] - ΔCT [ASICx +/+]) of the ASIC transcripts in ASIC1a, 2 and 3 +/+ and -/- thoracolumbar DRG. The relative fold change in ASICx -/- relative to ASICx +/+ is made using the formula $2^{-\Delta\Delta CT}$.

Quantitative RT-PCR revealed that there were no significant compensatory changes in ASIC expression in thoracolumbar DRG except for the transcript in the respective -/- mice. Some ASIC1 was detected in ASIC1a-/- mice as a pan-ASIC1 primer was used for quantification[10, 304]. The ASIC1 detected is presumably that of the other splice variant ASIC1b as previously shown[304].

Q-PCR relative expression analysis: DRG

	ΔCT ASIC1- β-actin	ΔCT ASIC2- β-actin	ΔCT ASIC3- β-actin
ASIC1 WT	8.30±0.61	4.52±0.43	8.505±0.118
ASIC2 WT	7.49±0.79	4.03±0.47	8.665±0.204
ASIC3WT	7.85±0.16	4.65±0.48	10.983±0.809
ASIC1 KO	10.15±0.87	4.7±0.71	9.07±0.40
ASIC2 KO	8.3±0.71	N/A	8.845±0.441
ASIC3 KO	7.8±0.16	4.6±0.48	N/A

Transcript	ΔΔCT (ΔCT ASIC1KO- ΔCT ASIC1WT)	ASIC1KO relative fold change to ASIC1WT	ΔΔCT (ΔCT ASIC2KO- ΔCT ASIC2WT)	ASIC2KO relative fold change to ASIC2WT	ΔΔCT (ΔCT ASIC3KO- ΔCT ASIC3WT)	ASIC3KO relative fold change to ASIC3WT
ASIC1	1.84±1.60	*0.27x (0.133x-0.58x) * $P = 0.0057$	0.81±1.06	0.57x (0.27x-1.12x)	-0.89±0.61	1.85x (1.21x-2.83x)
ASIC2	0.22±0.84	0.85x (0.47x-1.53x)	-	-	-0.57±1.00	1.48x (0.74x-2.97x)
ASIC3	0.56±0.41	0.67x (0.51x-0.91x)	0.18±0.48	0.88x (0.63x-1.24x)	-	-

Mean fold change / StDev Fold change

Relative fold change KO vs WT calculated using $2^{-\Delta\Delta CT}$ with ΔΔCT + s and ΔΔCT – s, where s = the standard deviation of the ΔΔCT value.

DISCUSSION

This thesis documents the mechanosensory and chemosensory properties of spinal afferents from the mouse colon. Five different classes of afferent fibre innervate the colon from the LSN and PN innervation. Each pathway possesses a distinct class of afferent fibre, mesenteric afferents in the LSN and muscular/mucosal afferents in the PN. Each pathway also contains three classes of conserved afferents; serosal, muscular, and mucosal. However, these classes display different functional properties, suggesting that each pathway has a specific role in the signalling of colonic events. Moreover, this study demonstrates that activation of $P2X_3$, B_2 and TRPV1 receptors is more likely to be signalled via the thoracolumbar LSN pathway than the lumbosacral PN pathway in mice. This is because α,β-meATP and bradykinin activate significantly higher proportions of LSN afferents. Although capsaicin activates both LSN and PN afferents, it activates a higher proportion of LSN afferents whilst activation of TRPV1 receptors by capsaicin subsequently attenuates LSN responsiveness to mechanical stimuli. This thesis also identifies a population of afferents which are almost exclusive to the LSN that are recruited by chemical stimuli; remain mechanically insensitive and are likely to serve a novel role during colonic injury or inflammation.

This thesis also identifies several key findings concerning the influence of the Acid Sensing Ion Channels on LSN colonic mechanosensory function. This study employed various molecular techniques identifying several key features of ASIC mRNA expression. Importantly, ASIC1, 2 and 3 mRNA is present in thoracolumbar DRG and specifically within isolated identified colonic neurons. Quantitatively ASIC2 has the highest relative transcript expression in thoracolumbar DRG, followed by ASIC1 and ASIC3. Critically, deletion of either *ASIC1a, 2* or *3* does not alter the mRNA expression of the other ASIC subunits suggesting that compensatory changes of the other ASIC transcripts do not occur. Moreover, these results indicate greater expression of ASIC1, 2 and 3 mRNA in thoracolumbar DRG than in nodose ganglia, which may account for some of the observed

differences in mechanosensitivity between the two pathways when the respective *ASIC* genes are disrupted.

Functional significance of LSN and PN afferents

The results of this thesis suggest that LSN and PN pathways serve unique roles in the detection and signalling of events in the colon and rectum. Although each pathway contains afferents that can detect various mechanical stimuli, their different functional properties indicate they are likely to signal very different mechanical events. For example, almost half of the afferents in the PN pathway are tuned to detect circular stretch and mucosal events, whilst the LSN pathway is solely responsible for detecting events in the mesenteric attachment. These differences also extend to the regions in which afferents from either pathway are located. For example, LSN afferent receptive fields are located through out the distal colon but not in the rectum. By contrast, PN afferent receptive fields are located in the rectum and the more aboral regions of the distal colon. Therefore, these data indicate that the LSN pathway constitutes a low gain pathway, for the detection of mechanical stimuli in the distal colon whilst the PN pathway constitutes a high gain pathway in the more aboral region of the distal colon and the rectum. Overall, these results indicate that the amount and variety of mechanosensory information signalled from the colon is far larger than previously considered. As the PN innervation is particularly adept at detecting events within the lumen and muscle contraction or relaxation in the rectum, it has recently been suggested that these afferents serve similar functions to vagal afferents in the upper gut in the perception of conscious non-noxious sensations[58]. Although it should be noted that PN afferents also have the capacity mediate noxious sensations. In contrast, the LSN innervation contains both low and high threshold afferents suggesting they generate diverse sensations. Both of these pathways are likely to contribute to reflexes altering colonic motility via the prevertebral ganglia or at higher centres via lumbar sympathetic and sacral parasympathetic outflow[68, 126, 394-398].

Signalling of intraluminal events by LSN and PN afferents

Mucosal afferents have been previously characterized in the distal colon *in vitro* from the LSN[102] and hypogastric nerves of the rat[308]. Mucosal afferents have also been recorded from the PN *in vivo* with receptive fields in the anal canal of the cat[75, 114] and perianal mucosa of the rat[110]. The majority of these anal and perianal mucosal afferents have faster conduction velocities (Aδ fibres)[102] than distension sensitive afferents (C-fibres) and as such have been proposed to play a role in the conscious perception of anal continence[399]. As mucosal afferents are sensitive to mechanical deformation of the mucosa, they may respond to particulate material within the lumen which can refine the quality of perceived stimuli and alter reflexes (i.e. ano-colonic reflex) controlling motility[124-126].

The current study shows that both pathways contain afferents with rapidly adapting responses to low intensity mucosal stroking and insensitivity to circular stretch. These afferents are consistent with previous reports of mucosal afferents throughout the gastrointestinal tract, suggesting that these afferents may respond to particulate material within the colonic lumen. In the present study these afferents accounted for nearly 50% of the PN afferent population (mucosal and muscular/mucosal afferents) but only 4% of the LSN innervation (mucosal afferents). Moreover, these afferents exhibited different distributions, with PN mucosal and muscular/mucosal afferents localised in the rectum and most aboral regions of the distal colon whilst the few LSN mucosal afferents were found more orally in the distal colon. Although the responsiveness of individual afferents to mucosal stroking is similar between the two pathways these results suggest that fine mechanical stimulation of the colonic mucosa is signalled predominantly via the PN pathway to the lumbosacral spinal cord. In particular this signal occurs when mechanical stimulation of the colonic mucosa occurs in the distal colon/rectum. The high proportion of PN mucosal afferents recorded in

this study may in fact constitute the large proportion of distension-insensitive PN afferents reported previously *in vivo*[75, 110].

Signalling of colonic stretch by LSN and PN afferents

Afferents that respond to colonic stretch, applied either directly *in vitro* or indirectly using colorectal distension *in vivo*, have been identified and extensively characterized previously[75, 104, 105, 108-115]. These afferents, as a whole, are responsive to small changes in intraluminal pressure, respond to colonic stretch or distension with a linear relationship to wall tension, and encode these stimuli well into the noxious range. Additionally, two broad types of distension-evoked afferent responses have been described: phasic and tonic[75, 105, 108, 110, 111, 115]. The roles of these two types of afferent are likely to differ as phasic afferents are only transiently excited during stimulus onset and offset, whereas tonic afferents discharge throughout the stimulus duration[75].

In the current study muscular afferents in the mouse LSN and PN were activated by low intensity circular stretch and focal compression of their receptive fields. These afferents closely resemble muscular afferents described in the LSN of the rat colon *in vitro*[109, 112, 113] and the distension-sensitive, low threshold afferents described *in vivo* in the LSN of the cat[105] and PN of the cat[105, 108, 115] and rat[110, 111]. The proportion of muscular LSN afferents (10%) found in the current study is similar to previous reports of muscular afferents in the LSN of rat using an *in vitro* technique[112, 113]. Stretch-sensitive PN afferents constitute over 50% of the total mechanosensitive PN afferent population in this study (muscular and muscular/mucosal afferents combined). This is a similar proportion to the distension-sensitive afferents responding to mechanical stimulation of the colon and anal canal described in both rat[110] and cat[75].

Although stretch-sensitive afferents were recorded from both pathways in the present study they differ in five critical aspects. First, stretch-sensitive PN afferents (including muscular/mucosal afferents), greatly outnumber stretch-sensitive LSN afferents (~50% vs. 10% respectively). Second, PN afferent receptive fields, in particular those of muscular/mucosal afferents are located in the rectum. Third, LSN muscular afferents are less likely to respond to focal compression of their receptive field at lower stimulus intensities (< 1g), suggesting they have higher thresholds. Fourth, PN muscular and muscular/mucosal afferents have greater responses to both probing and stretch. Finally, PN muscular and muscular/mucosal afferents display a more maintained response to stretch compared with LSN muscular afferents.

Overall, these findings would indicate that the PN is better equipped to respond to stretch particular of the distal colon and rectal wall. Moreover, these PN afferents are activated at lower stimulation intensities and respond more robustly to tonic stretch than those found in the LSN. The sustained response of PN afferents during maintained stretch suggests that they are more likely to signal tonic changes in the calibre or wall tension of the distal colon and rectum, such as during the presence of stool or gas. In contrast LSN muscular afferents, with their higher stimulus intensities and their more completely-adapting responses, would be better tuned to signal the onset of higher intensity mechanical events, such as muscular contraction or passage of material, which are of a more acute nature. Moreover, due to their different receptive field distributions, signalling of muscular contraction or passage of material in the more oral region of the distal colon is likely to be signalled via LSN muscular afferents. By contrast, presence of stool or gas in the rectum and most aboral regions of the distal colon are transmitted via the PN muscular and muscular/mucosal afferents.

These conclusions perhaps explain why behavioural responses to sustained colorectal distension are lost after sectioning the PN[60] and why LSN afferents are proposed to be better at

conveying phasic rather than tonic changes in colonic pressure in humans[2]. These findings also concur with recent *in vitro* studies in the guinea-pig indicating few stretch-sensitive mechanoreceptors in the distal colon, but a high density in the rectum[93-95]. These rectal afferents also show a greater responsiveness to stretch compared with the colonic afferents[93-95]. These rectal stretch-sensitive mechanoreceptors display low thresholds and slowly adapting responses to maintained distension and also respond to contraction[93, 94]. The receptive fields of these low threshold, slowly adapting, stretch-sensitive mechanoreceptors correspond with morphologically identified IGLEs in the rectum termed rIGLES[93-95]. Taken together this evidence indicates that colorectal distension/stretch is predominantly signalled via afferents in the PN to the lumbosacral spinal cord. Moreover, the signal generated by the two pathways is different, with the PN transmitting a more intense and maintained response to the lumbosacral spinal cord at the same stimulation intensities. Afferents in both pathways can probably signal throughout the physiological and noxious stimulus ranges and as such are likely to give rise to a numerous conscious sensations from fullness to discomfort and pain at high stimulation intensities by either distension or contraction of the distal colon and rectum. It is also clear that these afferents are also polymodal contributing to chemosensation[48, 74, 105, 128, 400].

Signalling of serosal and mesenteric events by LSN and PN afferents

Serosal afferents have been previously described in the LSN of the rat[112, 113] and the cat[105, 108]. However, they have not been encountered in prior studies of mechanosensitive PN afferents in rat[110] or cat[75] using *in vivo* single-fibre recording techniques. However, it is possible that serosal afferents may correspond with high threshold fibres (responding to CRD of greater than 30mm Hg) which are traditionally thought to be nociceptive[110]. In the present study serosal afferents are the most abundant population, accounting for approximately one third of all fibers found in this study. In both pathways these afferents were activated optimally by focal compression of their receptive field

via a perpendicular probing stimulus. Although each pathway contributes equally in serosal afferent signalling they differ in several features. First, PN serosal afferents are generally clustered more distally, particularly in the rectum. Second, PN serosal afferents responded across a wider stimulus range than LSN afferents and finally, PN serosal afferents displayed a more intense and maintained response to focal compression than LSN afferents. Consequently, these pathways send very different signals to the spinal cord. In particular, PN serosal afferents in more aboral regions of the distal colon and rectum would respond at lower stimulation intensities and generate a more intense and sustained afferent barrage in response to acute mechanical events. It is possible that serosal afferents in each pathway may signal transient, sharp pain at the onset of contraction or distension due to rapid transit of contents or experimental balloon inflation, during which acute intense mechanical stimulation might be achieved. It is also clear that these afferents are polymodal and LSN serosal afferents in particular contribute to chemosensation (see Chapter 2)[112, 113].

Mesenteric afferents have been previously described located close to or on blood vessels or branching points of capillaries supplying the serosa and occasionally displayed multiple receptive fields[75, 103, 105, 108-110, 112, 127]. These afferents can be activated briefly by intense mesenteric stretch and colonic distension in addition to stimuli applied directly to their receptive fields[103, 105, 112]. It has been proposed that these afferents may detect twisting and torsion of the colon and pulsatile changes in blood pressure in mesenteric blood vessels, which may be critical during plasma extravasation resulting from colonic inflammation[74, 309]. In the current study mesenteric afferents were the largest fibre population recorded from the colon, accounting for 50% of the LSN innervation, and were specific to this pathway. It is perhaps surprising that such a large population of afferents exist for the signalling of relatively rare events such as twisting and torsion, therefore it is possible that these afferents have an as yet unidentifed physiological role.

Comparative role of LSN and PN colonic afferents in the signalling of mechanosensory events

To best illustrate the likely role of each afferent population it is perhaps best to consider the scenario of passage of material through the lower bowel in various circumstances.

Fine mechanical stimulation of the colonic mucosa is likely to occur during events such as pellet or stool passage (*Figure D1a*). These events will activate mucosal afferents in the LSN pathway and mucosal and muscular/mucosal afferents in the PN pathway. However, the contribution of each pathway in signalling these events will be different due to the relative abundance of these afferents between the two pathways and their receptive field locations. Based on receptive field distributions, pellet or stool passage in the more oral region of the distal colon is signalled via LSN mucosal afferents. However, as these afferents are rare (constituting only 4% of the LSN innervation) the signal sent to the thoracolumbar spinal cord is small. As the pellet or stool passes into the most aboral regions of the distal colon deformation of the mucosa will be signalled equally by mucosal and muscular/mucosal afferents via the PN. These afferents in total represent 50% of the PN innervation and therefore the signal sent to the lumbosacral spinal cord is large. Deformation of the mucosa in the rectum by pellets or stool will be signalled exclusively by the mucosal and muscular/mucosal afferents via the PN resulting in the transmission of a large signal to the lumbosacral spinal cord. Based on previous studies this signal leads to conscious perception of anal continence[399].

Contraction or distension at low stimulation intensities is likely to activate muscular afferents in the LSN pathway and both muscular and muscular/mucosal afferents in the PN pathway (*Figure D1b*). However, each pathway will contribute differently in signalling these events due to the disparity in afferents numbers, their receptive field locations and general response properties. Events in the

more aboral regions of the distal colon will be signalled by both the LSN and PN pathway. LSN muscular afferents are rare constituting 10% of the innervation, and not all will be activated at this intensity, therefore the signal sent to the thoracolumbar spinal cord is small and is therefore unlikely to reach consciousness. Moreover, based on their more completely-adapting responses to maintained stretch LSN muscular afferents would be better tuned at signalling the onset of contraction or distension. In contrast, most of the PN muscular and muscular/mucosal afferents will be activated (which represent 50% of the PN innervation). Not only are more afferents activated in the PN but they also generate a more intense afferent barrage, particularly the high-responding muscular/mucosal afferents, when compared with LSN muscular afferents. Therefore the signal sent to the lumbosacral spinal cord is large and would allow perception of colonic filling or contraction (*Figure D1b*). Contraction or distension of the rectum will be signalled exclusively by the PN pathway via muscular and muscular/mucosal afferents resulting in a large signal sent to the lumbosacral spinal cord, allowing perception of rectal continence (*Figure D1b*). As the stimulus intensity of contraction or distension increases more LSN muscular afferents are recruited and their afferent discharge increases, however the relative signal sent to the thoracolumbar spinal cord is still relatively small. The predominant signal is still being sent to the lumbosacral spinal cord via the PN as more muscular and muscular/mucosal afferents are recruited with greater afferent discharges than LSN afferents. In particular the high-responding PN muscular/mucosal afferents will have reached firing saturation (*Figure D1b*).

As the stimulation intensity increases during passage of large faeces or strong contraction during mass movement in the distal colon (*Figure D1c*), all LSN muscular afferents will be recruited sending an increased afferent discharge to the thoracolumbar spinal cord leading to perception of discomfort and possibly pain. Some of the lower threshold serosal and mesenteric afferents may also be recruited. However, the predominant signal from the distal colon is still transmitted via the PN

pathway to lumbosacral spinal cord as all muscular and muscular/mucosal afferents will be recruited firing more intensely than previously and a large proportion of serosal afferents will also be activated leading to the perception of bloating or discomfort and possibly pain. In the rectum passage of large faeces or strong contraction will be transmitted exclusively by the PN pathway via muscular, muscular/mucosal and serosal afferents, leading to the perception of urgency.

At high or noxious stimulation intensities during events such as spasm, twisting or intususseption the signal generated by both pathways is considerable as all afferent classes will be activated *(Figure D1d)*. In particular the signal generated by the LSN in the distal colon is considerably greater than at lower stimulation intensities as all mesenteric and serosal afferents will be activated which constitute 85% of the LSN innervation. Similarly, in the distal colon PN muscular, low-responding muscular/mucosal and serosal afferents will be firing more intensely than at lower stimulation intensities. The considerable afferent barrages sent to both the thoracolumbar and lumbosacral spinal cord via the LSN and PN respectively are likely to be perceived as intense pain and extreme discomfort. In the rectum these events are also likely be perceived as intense pain and extreme discomfort via signalling of muscular and muscular/mucosal and serosal afferents exclusively by the PN pathway.

Differences in functional receptor expression between the LSN and PN pathways

The current study indicates that activation of $P2X_3$, B_2 and TRPV1 receptors is more likely to be signalled via the thoracolumbar LSN pathway. In the whole animal these different profiles of receptor expression would mean that the rectum and more aboral regions of the distal colon are less chemosensitive to bradykinin and vanilloid or purinergic stimuli than more oral regions of the distal colon. Activation of B_2 or TRPV1 receptors may also result in alterations in the mechanosensitivity of LSN afferents in the distal colon. A particularly novel finding of this study is the existence of a

reasonably large population (approximately a quarter) of mechanically-insensitive afferents. These afferents were almost exclusively found in the LSN and displayed similar distributions to mechanically-sensitive LSN afferents. However these afferents, after being recruited by chemical stimuli, remain insensitive to mechanical stimuli and therefore appear to be different from the previously described "silent nociceptors" in skin and "chemospecific afferents" in colon[51, 53, 112, 315,149, 182, 316-318]. The identification of this afferent population suggests the presence of a highly tuned early warning system to alert the CNS about injury to the colon without the complication of signalling mechanical events simultaneously. This would result in an unambiguous signal being transmitted to the thoacolumber spinal cord concerning the chemical environment which may be interpreted in a specific way.

Is there endogenous activation of $P2X_3$, B_2 and TRPV1 receptors?

An important question concerning primary afferents is whether purinergic, vanilloid and bradykinin receptors are activated endogenously. Endogenous chemosensory activation of P2X receptors may occur via two processes. First, ATP exists within cells in millimolar concentrations; therefore, any significant cellular damage is likely to increase local release. Secondly, ATP can be released from endothelial cells subjected to shear stress and in response to distension[165, 177, 401]. It is also possible that the purinergic system may play a greater role in disease as ATP is released in inflammatory conditions from a number of cell types[165, 177]. In particular ATP has an enhanced role in mechanosensory transduction in the inflamed rat colorectum[177]. The underlying mechanisms appears to involve increased distension-evoked release of ATP as well as an increase in the number of DRG neurons supplying the colorectum expressing $P2X_3$ receptors[177]. Notably, $P2X_3$ receptors have been shown to be upregulated in colonic nerve fibers of patients with inflammatory bowel disease[154, 155].

Figure D1. Role of LSN and PN colonic afferents in the signalling of mechanosensory events

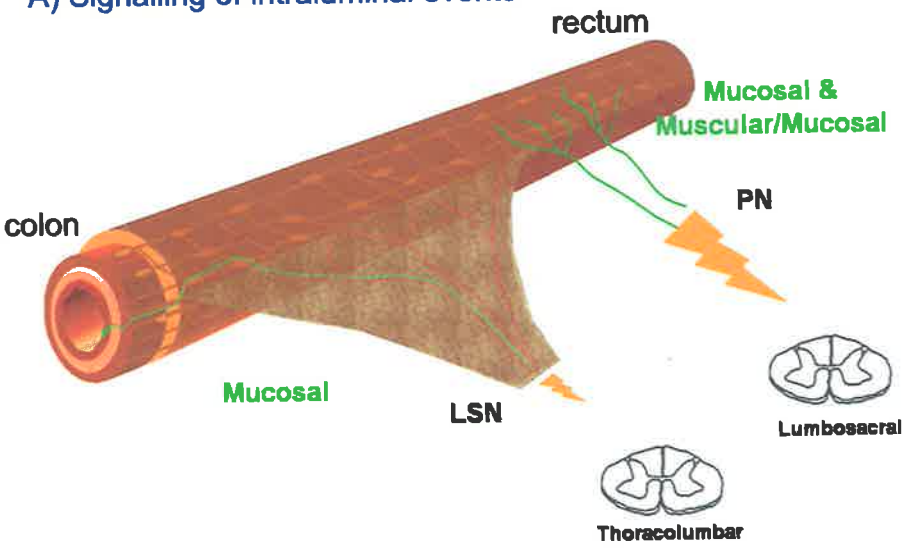
A) Signalling of intraluminal events such as pellet or stool passage
Mechanical stimulation of the colonic mucosa in the more oral regions of the distal colon is signalled via LSN mucosal afferents. However, these afferents are rare constituting 4% of the LSN innervation therefore the signal sent to the thoracolumbar spinal cord is small, and is unlikely to reach consciousness. By contrast, mechanical stimulation of the colonic mucosa in the rectum and most aboral regions of the distal colon are transmitted via the PN. These afferents represent 50% of the PN innervation (mucosal and muscular/mucosal) therefore the signal sent to the lumbosacral spinal cord is large, leading to the conscious perception of continence. Thus although the responsiveness of individual afferents to mucosal stroking is similar between the two pathways mucosal events are signalled predominantly via the PN pathway to lumbosacral spinal cord.

B) Signalling of contraction or distension at low stimulation intensities
Signalling of low threshold events in the more oral region of the distal colon is signalled via LSN muscular afferents. However, these afferents are rare constituting 10% of the LSN innervation therefore the signal sent to the thoracolumbar spinal cord is small and is unlikely to reach consciousness. By contrast, low threshold events in the rectum and most aboral regions of the distal colon are transmitted via the PN. These afferents represent 50% of the PN innervation (muscular and muscular/mucosal) therefore the signal sent to the lumbosacral spinal cord is large. Moreover, the signal generated by the two pathways is different, with the PN transmitting a more intense and maintained response to the lumbosacral spinal cord at the same stimulation intensities. Therefore the signal sent to the lumbosacral spinal cord is likely to lead to the perception of colonic filling or contraction

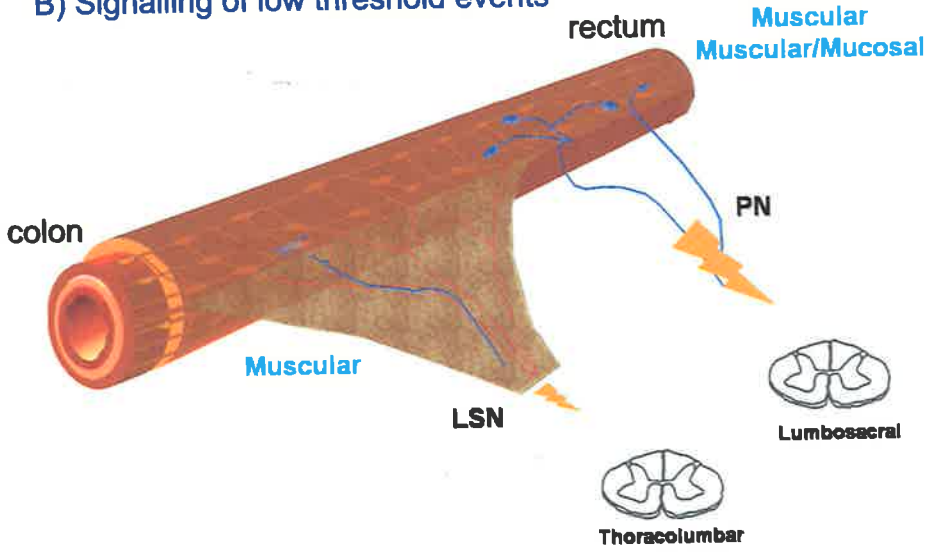
C) Signalling of passage of large faeces or strong contraction during mass movement
All LSN muscular afferents will be recruited sending an increase in afferent discharge to the thoracolumbar spinal cord. Some of the lower threshold serosal and mesenteric afferents will also be recruited, perhaps leading to the perception of discomfort or pain. However, the predominant signal is still transmitted via the PN pathway to lumbosacral spinal cord as all muscular and muscular/mucosal afferents will be recruited firing more intensely than previously, with a large proportion of serosal afferents also activated. This is likely to be perceived as bloating, discomfort or pain and in the rectum a perception or urgency.

D) Signalling of high intensity noxious events such as spasm, twisting or intususseption
At these stimulation intensities the signal generated by both pathways is considerable as all afferent classes will be activated. The signal generated by the LSN is considerably greater than at lower stimulation intensities as all mesenteric and serosal afferents will be activated which constitute 85% of the LSN innervation leading to the perception of intense pain and extreme discomfort. However, due to the pathways different receptive field distributions high threshold/noxious events in oral region of the distal colon are signalled via LSN, whilst events in the rectum and most aboral regions of the distal colon are transmitted via the PN.

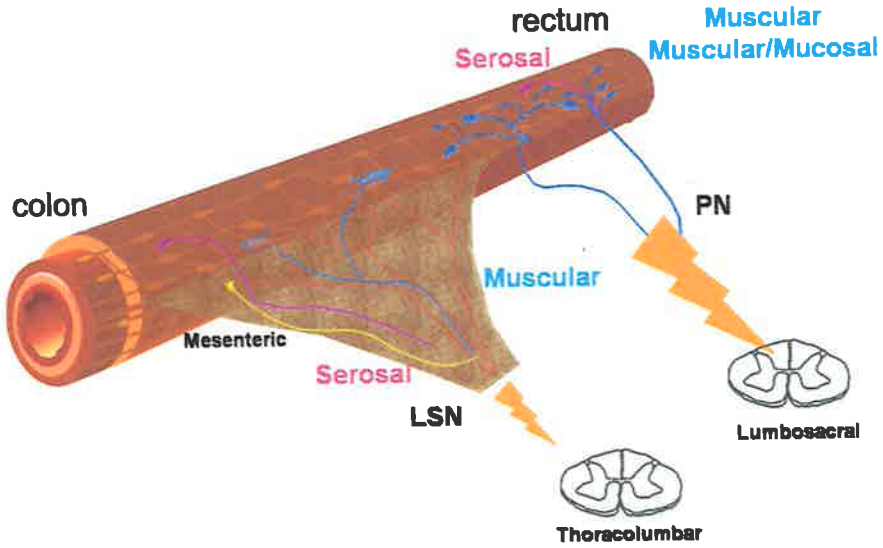
A) Signalling of intraluminal events



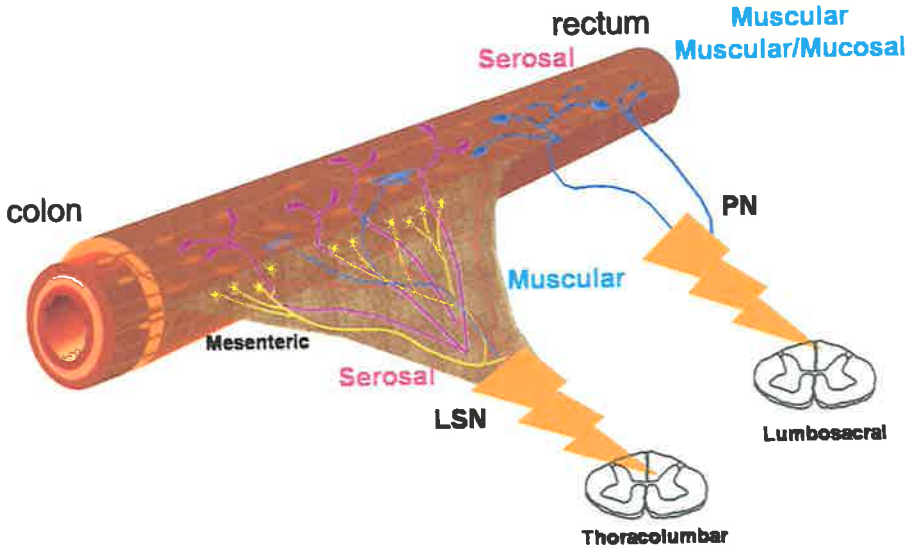
B) Signalling of low threshold events



C) Signalling of medium threshold events



D) Signalling of high threshold/noxious events



In the case of bradykinin B_2 receptors, the threshold concentrations observed in the current study are similar to concentrations detected in inflamed or ischemic tissues[335], suggesting that endogenous activation in these situations is likely. This is in keeping with previous studies which indicate bradykinin receptor antagonists are able to block cardiac afferent activation by ischaemia[321], whilst administration of B_2 receptor antagonists can attenuate disease severity in dextran sulphate sodium-induced colitis in rats[185]. Notably, B_1 but not B_2 receptor protein is significantly increased in the intestines of both active ulcerative colitis and Crohn's disease patients compared with controls[157].

The presence and release of TRPV1 ligands by mechanical and other stimuli is a more complex issue, because there are numerous candidates, including heat, low pH and endogenous vanilloids. Although temperature changes in the colon are unlikely to be sufficient to activate TRPV1 in normal conditions, in diseased states such as IBS, where there is increased exposure to mediators like 5-HT[330], TRPV1 can become sensitive to temperatures in the physiological range[202]. Both inflammation and luminal metabolism of colonic flora results in changes in pH, whilst endogenous vanilloids are present in the colon, particularly in disease states[331-334]. These factors are therefore all likely contributors to endogenous activation of TRPV1. Notably, TRPV1 receptors have been shown to be upregulated in colonic nerve fibers of patients with inflammatory bowel disease[155] while administration of TRPV1 antagonists can attenuate disease severity in dextran sulphate sodium-induced colitis in mice[206].

Overall, the results of the current study suggest initial signalling of injury or inflammation via $P2X_3$, B_2 or TRPV1 receptors in the more oral regions of the distal colon occurs predominantly via the LSN. It is also clear that signalling via TRPV1 receptors in the more aboral regions of the distal colon and the rectum can be signalled to the lumbosacral spinal cord via the PN. However, the

predominant chemosensory signal is transmitted to the thoracolumbar spinal cord via the LSN, leading to the perception of pain.

ASIC1a, 2 and 3 contribute to LSN afferent mechanotransduction

This study has also identified contrasting roles for ASIC1a, 2 and 3 in LSN colonic mechanosensory function. However, these respective ASIC subunits contribute differently to serosal and mesenteric afferent function. Although all three ASIC proteins contribute to serosal mechanosensitivity only ASIC1a and 3 contribute to mesenteric mechanosensitivity and adaptation of response. In particular only ASIC3 makes a positive contribution to LSN colonic mechanosensitivity. The discovery of both positive and negative effects of *ASIC* mutations on afferent mechanosensitivity suggests a complex interaction in the way that each ASIC subunit contributes to mechanotransduction. This complexity is highlighted by the results obtained with the ASIC blocker benzamil. Benzamil concentration-dependently inhibited serosal mechanosensitivity in +/+ mice. Although this effect was unaltered in ASIC1a +/+ it was significantly reduced in ASIC2 and 3 -/- mice. As deletion of any *ASIC* gene does not result in compensatory changes in other ASIC transcripts the alterations in colonic afferent mechanosensitivity and pharmacology are likely a direct result from the specific loss of the individual ASIC subunit and complex interactions within the heteromultimeric complex. However, a comparison of these results with studies on cutaneous and gastro-oesophageal afferents suggests a high degree of tissue- and pathway-specificity in the way ASIC subunits contribute to mechanotransduction.

Respective contributions of ASIC1a, 2 and 3 to the mechanotransduction of LSN afferents

This study demonstrates *ASIC1a*, *2* and *3* are all required for normal colonic mechanotransduction as disruptions of these genes results in widespread changes in the sensitivity of LSN mesenteric and serosal afferents. Specifically, disrupting *ASIC1a* significantly increases the

sensitivity of both serosal and mesenteric afferents; whilst only altering the adaptation response profile of mesenteric afferents. Disrupting *ASIC2* significantly increases the sensitivity of serosal afferents and has no effect on mesenteric afferent mechanosensitivity. Disrupting *ASIC2* however does not alter either mesenteric or serosal afferent adaptation. In contrast, disrupting ASIC3 significantly decreases the sensitivity of both serosal and mesenteric afferents and alters mesenteric afferent adaptation. Therfore ASIC3 appears to make a direct and positive contribution to mechanotransduction in both classes of LSN colonic afferent.

Determining the respective contributions of the individual ASIC subunits is difficult as studies of recombinant and native channels indicate ASIC1, 2 and 3 combine to form functional heteromultimers[233, 298, 301]. This is because low pH-induced currents in cells co-expressing all three ASIC subunits are identical to the native DRG response[298]. By contrast, cells expressing only the individual ASIC1, 2 or 3 subunits display markedly different characteristics to the low-pH currents in native DRG neurons, even if the three currents are mathematically summed. Moreover, deletion of any one ASIC subunit does not abolish the low pH-induced currents, but alters the currents in a manner consistent with heteromultimerization of the two remaining subunits. Therefore interaction between ASIC1, 2 and 3 subunits, in relatively equal proportions, must occur within a heteromultimeric complex. Although, it is not clear exactly how many subunits must come together to form the complex, various studies indicate that between 4 and 9 subunits are required to arrange around a central pore to form a functional complex[375].

The positive and negative effects of *ASIC* mutation in the current study also suggest a complex interplay between the individual subunits within a heteromultimeric complex. This is because the results cannot be simply explained by loss of the individual ASIC subunit, via gene deletion, and in some cases ASIC subunits may in fact dampen the mechanotransduction process.

Overall this suggests ASIC subunits are unlikely function simply as individual mechanically gated channels. As such this negative modulatory or dampening role is best explained by the of existence of all three ASIC subunits as heteromultimers[298, 301], in which each member contributes either directly or indirectly to mechanotransduction. This would be the case for ASIC1a in both mesenteric and serosal afferents and for ASIC2 in serosal afferents because in both instances mechanosensitivity was increased in -/- mice. Therefore, ASIC1a and ASIC2 clearly appear to make little if any direct contribution to the mechanotransduction of LSN colonic afferents, because without them mechanosensitivity is universally increased in mesenteric and serosal LSN colonic afferents, suggesting that the heteromultimeric mechanotransducer becomes more efficient. However, the role of ASIC2 is intriguing by its capacity to influence mechanosensitivity negatively in colonic serosal afferents whilst having no apparent role in mesenteric afferents. In contrast, ASIC3 appears to make a direct contribution to mechanotransduction as mechanosensitivity is universally decreased in ASIC3 -/- mesenteric and serosal LSN colonic afferents, suggesting that the heteromultimeric complex becomes less efficient.

When gene disruption is used, interpretation can be complicated by compensatory changes in mRNA expression levels. The results of the current study demonstrate that deletion of any individual *ASIC* gene does not result in compensatory changes in the expression of other ASIC transcripts. This is important in terms of interpreting the alterations in mechanosensory function when ASIC subunits are deleted. It can now be concluded that the changes observed in colonic afferent mechanosensitivity are not due to compensatory changes in ASIC transcript expression simply "replacing" the deleted *ASIC* gene. However, the question remains how can the differences in mechanosensory function be explained between the different ASIC -/- mice? Studies of acid evoked currents in isolated DRG from ASIC1, 2 and 3 -/- mice have gone some way to clarifying this process and it appears likely that loss of one subunit can change the subunit composition and perhaps kinetic

properties of the channel complex[298]. Therefore there are three potential scenarios which may occur at the level of the heteromultimeric complex that could explain the alterations in mechanosensory function after ASIC deletion.

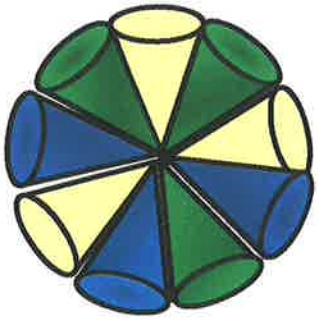
First, the simplest explanation of changes in mechanosensitivity would conclude that they must be a direct result from the specific loss of the properties of the individual ASIC subunit that has been disrupted via gene deletion. However, the deletion of *ASIC1*, *2* or *3* would cause varied effects due to differences at the level of the heteromultimeric complex. This is due to the loss of the properties of the deleted ASIC (e.g. ASIC1) subunit, but also the possible amplification of the respective contributions of the remaining ASIC subunits (e.g. ASIC2 and 3) in the complex *(Figure D2)*. Second, if the same total number of subunits are required to form the heteromultimeric complex then there is the possibility of substitution of the deleted ASIC (e.g. ASIC1) subunit for the other remaining ASIC (e.g. ASIC2 and 3) subunits. Although this would not occur via up-regulation of mRNA levels of the remaining ASIC subunits, this process could occur via the formation of fewer heteromultimeric complexes overall *(Figure D2)*. Third, it is also possible that the remaining ASIC subunits reassemble into different structural configurations of the heteromultimeric complex. Overall any of these scenarios would alter the functionality of the heteromultimeric complex, potentially making it more or less efficient at transducing mechanical stimuli *(Figure D2)*. This may explain the respective gain and loss of function in colonic afferents when various *ASIC* genes are deleted. However, what is apparent from these studies is that disruption of any one of the *ASIC* genes fails to abolish any one sensory modality completely; and instead, the loss of the deleted subunits results in modified sensory transduction suggesting that ASIC subunits may have both overlapping functions and some functional redundancy[298]. The observation of varied effects of Benzamil in the current study also suggests several sites of action within the heteromultimeric complex that could be explained by the various scenarios proposed above.

Recent data also indicate that the tethering of the ASICs is critical in their function. The integral membrane protein, stomatin, (which is found in lipid/protein-rich microdomains) binds to ASIC1a, 2, and 3 subunits and can alter each of their functions, with strikingly different functional effects between subunits. Notably, stomatin has the most prominent effect on ASIC3, potently reducing acid-evoked currents[351]. The question of how these acid-evoked currents relate to the differences observed with mechanical stimuli remains to be elucidated. However, this system is similar to a suggested a model of mechanotransduction in *Caenorhabditis elegans* whereby *MEC-2* (which shares a 65% identity and an 85% similarity to stomatin) functions to link *MEC-4* and *MEC-10* (related to ASICs) channels to the intracellular cytoskeleton and the extracellular matrix. Deformation of this system by mechanical stimuli is then thought to open the channel complex[362, 402]. As benzamil did not completely abolish afferent responses to mechanical stimuli in the current study it seems likely that ASIC and DEG/ENaC channels are not the only mechanotransduction mechanism. Recent evidence would also indicate that various members of the TRP family may be involved[188, 242, 245, 258, 403, 404].

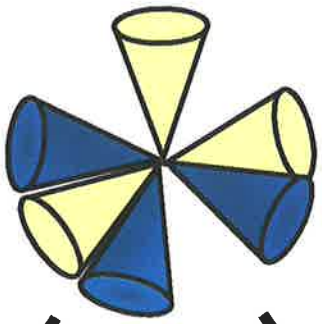
Figure D2) How does disruption of ASIC1, 2 & 3 alter mechanosensitivity?

ASIC1, 2 and 3 subunits assemble to form functional heteromultimeric complexes in DRG neurons. When one of the ASIC subunits is lost via gene deletion various changes in the complexes may occur. Loss of ASIC subunits (e.g. ASIC1) via gene deletion will result in the loss of the properties of that particular subunit. This will also result in a greater contribution of the remaining subunits (e.g. ASIC2 and 3). This may occur via two scenarios. 1) Substitution of the disrupted ASIC subunits (e.g. ASIC1) for other ASIC subunits (e.g. ASIC2 and 3). As quantitative PCR indicates there is no significant up regulation of these other subunits, this would mean that there are fewer heteromultimeric complexes overall. 2) Alternatively, it is possible that the remaining ASIC subunits may reassemble into different structural configurations of the heteromultimeric complex. Either of these two scenarios would alter the functionality of the heteromultimeric complex, potentially making it more or less efficient at transducing mechanical stimuli. This may explain the respective gain of function in ASIC1a and 2 -/- colonic afferents and the loss of function in ASIC3-/-.

"Normal" ASIC heteromultimeric complex

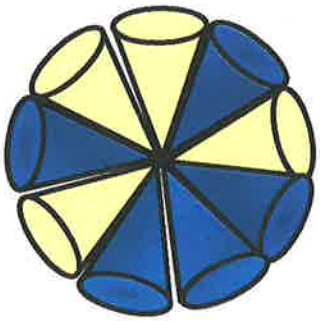


Loss of ASIC subunit via gene disruption

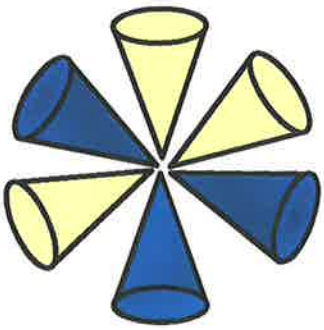


- Loss of the properties of the individual ASIC subunit

- Greater contribution of the other ASIC subunits to the complex



Substitution of other ASIC subunits for disrupted ASIC gene (less complexes overall)



Overall conformational change in the ASIC heteromultimeric complex (less subunits per complex)

Altered efficiency of the complex and therefore altered mechanosensory response to mechanical stimuli

Differences in the contribution of ASIC subunits to the mechanosensory function between colonic afferent subtypes

Comparing the results of LSN mesenteric and serosal colonic afferents amplifies the complexity of the role of ASICs in mechanotransduction. For example, LSN colonic afferents with disruptions to *ASIC2* display increased serosal afferent mechanosensitivity but no change in mesenteric afferent mechanosensitivity. There are several different possibilities that could explain these differences. First, the total number of ASIC subunits contributing to either the mesenteric or serosal afferent mechanosensory complex could be different. In this scenario, for example, fewer ASIC subunits may be required in mesenteric afferents to form a function heteromultimeric complex. This scenario may be unlikely as significant differences in mesenteric afferent mechanosensory function are observed when *ASIC1* and *3* are disrupted. Alternatively, both serosal and mesenteric mechanosensory complexes may require the same number of ASIC subunits but the relative proportions of ASIC1, 2 and 3 subunits may differ. In this scenario the heteromultimeric complexes within serosal afferent mechanoreceptors would contain similar proportions of ASIC1, 2 and 3 as disrupting any one of these results in significant changes in mechanosensitivity. Conversely, the heteromultimeric complexes within mesenteric afferent mechanoreceptors may contain a higher proportion of ASIC1 and 3 subunits as disrupting ASIC2 does not affect mechanosensitivity *(Figure D3)*. As discussed above intracellular and extracellular proteins, such as stomatin, might confer distinct properties on mechanoreceptors within their different environments[233, 351]. These scenarios, either individually or together, would alter the functionality of the heteromultimeric complex, potentially making it more or less efficient at transducing mechanical stimuli. This may explain the respective gain of function observed in mesenteric and serosal afferents when *ASIC1a* is deleted and serosal afferents when *ASIC2* is deleted. Similarly, this may explain the loss of function in mesenteric and serosal afferents when *ASIC3* is deleted and the apparent lack of a functional role of *ASIC2* in mesenteric afferents.

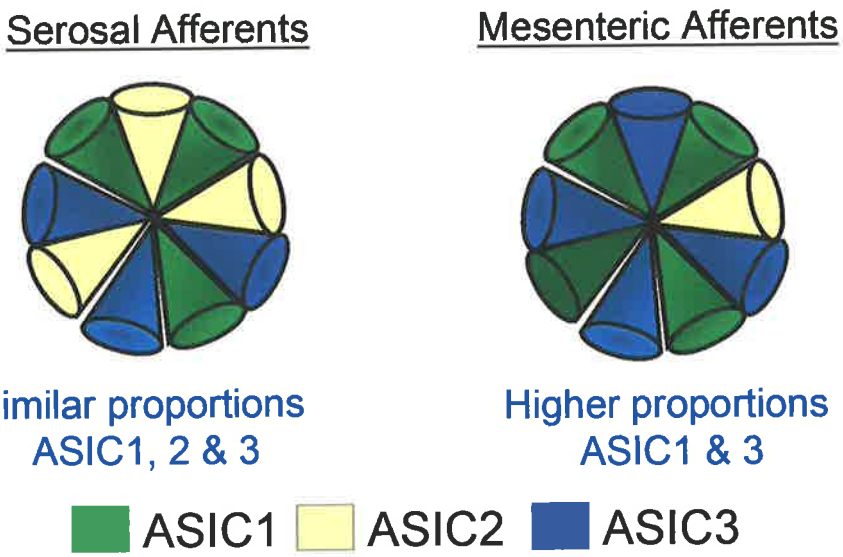


Figure D3) Why does disrupting different ASIC subunits cause varied effects in different LSN afferents?

The differences observed between mesenteric and serosal afferents, particularly in ASIC2 -/- mice, may result from differences in the heteromultimeric complexes between the two afferent classes. For example serosal afferents may have similar proportions of ASIC1, 2 and 3 contributing to the complex as disrupting any one of these genes results in alterations in serosal afferent function. By contrast, mesenteric afferents may have higher proportions of ASIC1 and 3 and a lower proportion of ASIC2 contributing to the complex as deleting ASIC2 has no effect on mesenteric afferent function.

ASIC1, 2 and 3 contribute differently to colonic, gastro-oesophageal and cutaneous afferent mechanosensory function

It is abundantly clear that afferents recorded in the colon, gastro-oesophageal region and skin signal vastly different mechanical events. The colonic and gastro-oesophageal afferents recorded in this study are likely to signal noxious and physiological events respectively, whilst the cutaneous afferents that were altered by disrupting *ASIC2* and *3* detect either light touch or noxious

pinch[9, 10]. Therefore it is of little surprise that a comparison of colonic, gastro-oesophageal and cutaneous data indicates a high degree of tissue- and pathway-specificity to the contribution of ASIC subunits to mechanotransduction. For example, disrupting *ASIC1a* results in a marked increase in both types of colonic and gastro-oesophageal afferents but has no effect on any of the 5 classes of cutaneous afferent[304]. This would suggest that ASIC1 influences visceral but not cutaneous mechanoreceptor function, suggesting that different mechanisms underlie mechanosensory function in gut and skin. This is highlighted by the contribution of ASIC2, as its role is specific to individual afferent classes, different regions of gut, and is notably opposite in the colon and skin. ASIC3 by contrast has a universal positive influence on mechanosensitivity particularly in colonic high-threshold afferents suggesting that it is an important potential target for modulating LSN colonic mechanosensory function. As such it seems apparent that particular mechanoreceptors in different tissues may differ in their molecular composition. Such widespread differences are highlighted by the comparative mRNA expression of ASIC3 transcripts. In thoracolumbar DRG there is almost a 10 fold greater expression of ASIC3 mRNA than in nodose ganglia, suggesting a greater importance of ASIC3 in DRG. This may explain why in ASIC3 -/- mice both mesenteric and serosal colonic afferents display functional deficits compared with only tension receptors in the gastro-oesophageal region. As discussed earlier a variety of scenarios at the level of the heteromultimeric complex could occur to alter mechanosensory function. As such this may account for the differences observed between mucosal and tension gastro-oesophageal receptors and the differences between the 5 types of cutaneous afferents, and ultimately for the differences observed between colonic, gastro-oesophageal and cutaneous afferent function.

Functional significance of ASICs in mechanosensation

The relative importance of ASICs to the mechanotransduction process is evident from the behaviour changes in the whole animal. These changes occur in a predictable manner based on the

alterations in visceral and cutaneous mechanosensory function and as such demonstrate the physiological relevance of the alterations in mechanosensory function. In particular, ASIC1 -/- mice display decreased gastric emptying which correlate with increases in gastro-oesophageal mechanoreceptor function. ASIC2 -/- mice display reduced faecal output which is associated with an increase in colonic serosal afferent sensitivity, presumably due to altered feedback control of colonic motility. Finally, ASIC3 -/- mice display reduced somatic responses to acid and noxious heat which corresponds with reduced sensitivity of myelinated (AM) mechanonociceptors[9].

From a clinical perspective a reduction in mechanoreceptor signalling would be beneficial in a therapeutic treatment for IBS, as these patients display visceral hypersensitivity. From this study ASIC3 in particular may be a good target, with an ASIC3 channel blocker a potentially beneficial therapeutic treatment option for reducing colonic signalling. Alternatively, an opener of ASIC1 or 2 channels may be beneficial in reducing colonic mechanosensitivity. However, it will be important to determine if the increases in mechanosensitivity of colonic afferents in ASIC1a and 2 -/- mice translates to increases in visceromotor response to experimental balloon distension of the colon. Similarly, it will be important to determine if the decrease in mechanosensitivity of colonic afferents in ASIC3 -/- mice translates to a decrease in visceromotor responses. In addition, determining whether ASICs contribute to visceral hypersensitivity during and after inflammatory events will have an important bearing in translating these findings to functional bowel disorders such as IBS.

A number of other diseases involving the gastrointestinal system may benefit from altering mechanoreceptor signalling. A reduction in mechanoreceptor signalling would be a beneficial therapeutic treatment option for gastro-oesophageal reflux disease, where reduced signalling from proximal gastric mechanoreceptors leads to reduced triggering of lower oesophageal sphincter relaxations which underlie reflux events[129]. Similarly, a number of clinical situations involving bowel

obstruction or blockage of ducts may lead to intense pain[53]. By contrast, in obesity, increasing the response of gastric vagal mechanoreceptors may be a non-surgical option to increase satiety signals and reduced food intake[405]. Therefore either positive or negative modulation of the mechanotransduction process in visceral mechanoreceptors is a highly desirable option, depending on the clinical problem[354].

CONCLUSIONS

This study has identified five different classes of afferent fibre. Each pathway contains a unique class, mesenteric and muscular/mucosal afferents in the LSN and PN respectively. Three classes (serosal, muscular, and mucosal) are conserved between both pathways; however, they display different proportions, response thresholds and magnitudes, receptive field distributions and adaptation profiles. Consequently, these data indicate that both nerves are critical in relaying mechanosensory information from the colon but that each contains afferent fibres individually tuned to detect distinct types of mechanical stimuli and respond with differing sensitivities. The result of these differences indicates the PN pathway signals maintained distension and the passage of material over the mucosal epithelium, while the LSN pathway signals transient events such as the onset of rapid distension, contraction or torsion of the mesentery. Furthermore these pathways differ in their signalling of chemical activation. The results of this study show that activation of $P2X_3$, B_2 and TRPV1 receptors is more likely to be signalled via the thoracolumbar LSN pathway than the lumbosacral PN pathway. The functional implications of these results mean that $P2X_3$, B_2 and TRPV1 receptor agonists activate afferents with higher mechanical thresholds and lower mechanical responsiveness that project to the thoracolumbar spinal cord. Overall this means that LSN colonic afferents are continually in a state of readiness to signal inflammation or injury due to their constitutive functional expression of $P2X_3$, B_2 or TRPV1 receptors. The identification of afferents that are solely recruited by chemical stimuli is intriguing and suggests an unambiguous signal concerning the chemical environment that is sent primarily to the thoracolumbar spinal cord during injury to the colon. This may be interpreted in a specific way and allow a specific sensory and motor responses. It is clear that LSN and PN pathways have vastly differing roles in the signalling of both mechanical and chemosensory information. As such they should be considered separately when designing therapies targeting receptor modulation in functional bowel disorders.

This study has also identified the molecules that influence colonic mechanosensation and identifies contrasting roles for ASIC1a, 2 and 3 in colonic mechanosensory function. ASIC1, 2 and 3 transcripts are present in thoracolumbar DRG and disrupting any one of these genes alters mechanosensitivity. ASIC1a, 2 and 3 subunits contribute to serosal mechanosensitivity, but only ASIC1a and 3 contribute to mesenteric mechanosensitivity. However, only ASIC3 makes a positive contribution to colonic mechanosensitivity, suggesting ASIC1a and 2 provide a negative modulatory or dampening role. These positive and negative effects of ASIC mutation suggest a complex interplay between the individual subunits within a heteromultimeric complex in which each member contributes either directly or indirectly to mechanotransduction.

ASIC1a, 2 and 3 subunits also display a high degree of tissue- and pathway-specificity in their contribution to mechanotransduction in the viscera and skin and their disruption can result in the heteromultimeric complex becoming either more or less efficient. The physiological relevance of these alterations in mechanosensory function is highlighted by the observations that *ASIC* gene disruption alters functional behavioural responses in a predictable manner based on the changes observed in mechanosensory function. These findings indicate diversity in mechanisms of mechanotransduction in both molecular and pharmacological terms which holds promise for targeting them therapeutically.

Appendices

Publication arising from this thesis

Chapter 1

Brierley S.M, Jones III R. C W, Gebhart G. F & L.A Blackshaw (2004). *Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice.* Gastroenterology, 127(1), 166-178. *(Impact factor:* 12.80)

Chapter 2

Brierley S.M, Jones III R.C.W, Xu L, Robinson D.R, Hicks G.A, Gebhart G.F & L.A Blackshaw (2005). *Differential chemosensory function and receptor expression of splanchnic and pelvic colonic afferents in mice.* The Journal of Physiology, 567(1), 267–281 *(Impact factor:* 4.4)

Brierley S.M, Jones III R.C.W, Xu L, Gebhart G.F & L.A Blackshaw (2004). *Activation of splanchnic and pelvic colonic afferents by bradykinin in mice.* Neurogastroenterology & Motility, 17(6) 854-862. *(Impact factor:* 2.5).

Chapter 3

*Page A.J, ***Brierley S.M,** Martin C.M, Martinez-Salgado C, Wemmie J.A, Brennan T.J, Symonds E, Omari T, Lewin G.R, Welsh M.J & L.A Blackshaw (2004). *The ion channel ASIC1 contributes to visceral but not cutaneous mechanoreceptor function.* Gastroenterology, 127, 1739–1747 ***(* Equal first authorship)*** *(Impact factor:* 12.80)

*Page A.J, ***Brierley S.M,** Martin C.M, Price M.P, Wemmie J.A, Symonds E, Butler R, & L.A Blackshaw (200). *Different contributions of ASIC channels 1a, 2 and 3 in gastrointestinal mechanosensory function.* Gut, 54(10) 1408-1415 ***(* Equal first authorship).*** *(Impact factor:* 5.9)

Brierley, SM., Jones III R C W., Gebhart G F and Blackshaw, L A. (2004) Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice.
Gastroenterology, v. 127(1), pp. 166–178, July 2004

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Gut, v. 54 (10), pp. 1408-1415, October 2005

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