HUMAN VISCERAL AFFERENT RECORDINGS: A PRE-CLINICAL HUMAN MODEL OF VISCERAL PAIN

by

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Submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy

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ABSTRACT

Aim: We have recently developed electrophysiological recordings of human visceral afferent (HVA) activity in isolated gastrointestinal tissues. The aim of the this study was 1) test the mechano- and chemosensitivity of HVAs, 2) characterise subpopulations of HVAs based on their response to mechanical stimuli, 3) test the effect of drugs that have/are in clinical trials on the mechanosensitivity (von Frey hair (VFH) probing and appendix distension) of HVAs. **Methods**: All experiments were performed in accordance with UK human ethics regulations [NREC09/H0704/2]. Surgically resected human ileum, colon, and appendix were obtained from consenting patients undergoing bowel resection. Tissues were pinned in a tissue bath, or cannulated (appendix), and superfused with carbongenated Krebs buffer, at 6ml/min, 32-34°C. Mesenteric nerve bundles were carefully dissected and afferent activity was recorded using suction electrodes. Tissues were tested for mechanosensitivity (VFHs, stretching, mucosal stroking, distension) and chemosensitivity (bradykinin (BK), ATP (adenosine trisphosphate), PGE_2 (prostaglandin E_2), serotonin (aka 5-hydroxytryptamine (5-HT)), histamine, adenosine). The receptors involved in the activation of HVAs by BK, or ATP were also investigated. The response of HVAs to VFH probing or distension was tested before and after the application of tegaserod, STa endotoxin, or a transient receptor potential vanilloid 4 (TRPV4) agonist (GSK1016790A) or antagonist (HC067047). **Results and Conclusion**: HVAs were characterised as mesenteric, serosal, muscular, or muscular-mucosal. HVAs were chemosensitive to all mediators. Bradykinin B2 receptors are the most important receptors involved in the activation of HVAs by BK. P2Y receptors may play an important role in the activation of HVAs by ATP. Application of tegaserod, HC067047 or STa endotoxin reduced the HVA response to mechanical stimuli. HVA recordings are feasible and practical and are suitable for both basic scientific mechanistic studies, and could potentially be used as a pre-clinical model, in conjunction with animal experiments, to help predict the efficacy of novel compounds before clinical trials.

TABLE OF CONTENTS

FIGURES

TABLES

ABBREVIATIONS

4αPDD	4-α- phorbol 12, 13- idecanoate
5-HT	5-hydroxytryptamine
ADP	Adenosine diphosphate
AMPA	α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid
AMP	Adenosine monophosphate
ANS	Autonomic nervous system
AP	Action potential
ARD	Ankyrin rich domain
ASIC	Acid sensing ion channel
ATP	Adenosine trisphosphate
BC	Approximate bath concentration
BDNF	Brain derived neurotrophic factor
BK	Bradykinin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CD	Crohn's Disease
CFTR	Cystic fibrosis transmembrane conductance regulator
CGRP	Calcitonin gene related peptide
Cl^{-}	Chloride
CNS	Central nervous system
COX	Cyclooxygenase
CRD	Colorectal distension
DA	Day after
DO	Day of
δ	Delta
DRG	Dorsal root ganglion
DSS	Dextran sulphate sodium
EET	5, 6-epoxyeicosatrienoic acid
EC	Enterochromaffin cell
E. coli	Escherichia coli
ENS	Enteric nervous system
GDP	Guanosine diphosphate
GI	Gastrointestinal
GPCR	G-protein coupled receptor
GTP	Guanosine trisphosphate
HDU	High dependence unit
HPA	Hypothalamic-pituitary-adrenal
HT	High threshold
HVA	Human visceral afferent
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IFAN	Intestinofugal afferent neuron
IGLE	Intraganglionic laminar ending
IHC	Immunohistochemistry
IMA	Intramuscular array
IPAN	Intrinsic primary afferent neuron
IR	Immunoreactivity
k^{+}	Potassium
κ	Kappa

LT	Low threshold
MAPK	Mitogen-activated protein kinase
me5-HT	2-methyl-5- hydroxytryptamine
meATP	α, β methylene adenosine trisphosphate
MIA	Mechanical insensitive afferent
mV	Millivolts
µ	Mu
Na^+	Sodium
Na_V/VGSC	Voltage gated sodium channel
NECA	5'-N-ethylcarboxyamidoadenosine
NGF	Nerve growth factor
NK	Neurokinin
NMDA	N-methyl-D-aspartate
NSAID	Non-steroidal anti-inflammatory drugs
NTS	Nucleus tractus solitarius
PAG	Periaqueductal gray
PGE_2	Prostaglandin E_2
PKA	Protein kinase A
PKC	Protein kinase C
PLA	Phospholipase A
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
PRD	Proline rich domain
PVG	Prevertebral ganglion
RR	Ruthenium Red
TNBS	2, 4, 6-trinitrobenzenesulfonic acid
TRPA	Transient receptor potential ankyrin
TRPC	Transient receptor potential canonical
TRPM	Transient receptor potential melastatin
TRPML	Transient receptor potential mucolipin
TRPN	Transient receptor potential no mechanoreceptor potential C
TRP	Transient receptor potential
TRPP	Transient receptor potential polycystin
TRPV	Transient receptor potential vanilloid
TTX-R	Tetrodotoxin-resistant
UC	Ulcerative colitis
UDP	Uridine diphosphate
UTP	Uridine trisphosphate
VFH	Von Frey hair
VIP	Vasoactive intestinal polypeptide
VMR	Visceromotor response
WDR	Wide dynamic range

CHAPTER 1: GENERAL INTRODUCTION

1.1 ABDOMINAL PAIN IN GASTROINTESTINAL DISEASES

Abdominal pain is one of the commonest symptoms upon presentation to a gastroenterologist (Shaheen et al., 2006), and is a feature of many organic (e.g. inflammatory bowel disease (IBD)) and functional gastrointestinal disorders (FGID) (e.g. irritable bowel syndrome (IBS)). In many of these diseases pain is the cause of substantial morbidity, impacting negatively on quality of life indicators such as fatigue, sleep, anxiety and depression (Wang et al., 2012, Greenley et al., 2013, Walter et al., 2013). Many functional and organic bowel disorders have been outlined by specialist authorities as a significant encumbrance on the healthcare system (Spiller, 2007).

IBS is one such functional bowel disorder that is characterised by abdominal discomfort and pain, which is associated with altered bowel habits, and not accompanied by abnormal structural changes in the gut (Longstreth et al., 2006, Hughes et al., 2013). The diagnostic criteria for IBS as set out most recently in the Rome III criterion is defined by; abdominal pain or discomfort, which is associated with either, improvement with defecation, associated with a change in the frequency or appearance of stool, that is recurring at least 3 days per month in the last 3 months, with the onset of symptoms beginning at least 6 months prior to diagnosis. There are also a number of supportive symptoms which help classify IBS into its subtypes, which occur with similar prevalence, diarrhoea predominant IBS (IBS-D), constipation predominant IBS (IBS-C) or alternating/mixed IBS (IBS-A/IBS-M) and un-subtyped IBS (IBS-U) (Longstreth et al., 2006, Camilleri et al., 2012). Additionally, IBS which has developed after a bout of gastroenteritis, may be categorised as post infectious IBS (PI-IBS) (Ohman and Simren, 2013).

It is estimated that 10-20% of adults, predominantly women, have intestinal symptoms consistent with IBS (Saito et al., 2002, Gwee, 2005), leading to diminished quality of life as a

consequence of disruption to work and sleep (Wang et al., 2012), and exerting a significant healthcare and economic burden in society (Sandler et al., 2002). Despite the prevalence and impact of IBS, its disease pathophysiology is still not understood, but hypothesises include dysfunction of information processing between the brain and the gut, low grade inflammation of the bowel, changes in the gut microbiota, and enhanced stress signalling particularly in the context of negative early life events (Camilleri et al., 2012).

By contrast, IBD represents a group of chronic lifelong organic gastrointestinal diseases, the most common of which are Crohn's disease (CD) and ulcerative colitis (UC) (Yen and Pardi, 2012). IBD is characterised by relapses and remission of inflammation predominantly of the small and large intestine. The commonest symptoms on presentation are a triad of abdominal pain, blood in the faeces/altered bowel habit, usually diarrhoea, and weight loss (Sobczak et al, 2014). IBD has a major impact on the patient's quality of life and ability to work, and by extension is a considerable economic burden on society (Busch et al., 2014).

CD can affect any part of the GI tract but is usually found in the ileocaecal region leading to a predominance of patients reporting right sided abdominal pain on presentation to a physician. The inflammation in CD affects the full thickness of the bowel wall leading to swelling and thickening of the smooth muscle, fibrosis and structuring, and in some patients fistulising disease (Cassinotti et al., 2008, Fakhoury et al., 2014). Consistent with the presence of small bowel disease it is common for CD patients to suffer from nutritional deficiencies due to the impact of inflammation on the absorption of nutrients in the intestine (Fakhoury et al., 2014). Overall CD has a slightly greater prevalence in women than men, with the average age of onset being 20-30 years old (Cosnes et al., 2011). It is most prevalent in North America, and Northern Europe, where the highest reported incidence rates varies between 19.2-24.3/100,000 (Molodecky et al., 2012).

By contrast UC is restricted to the rectum and colon, and is characterised by inflammation and ulcerations which is restricted to the mucosa and submucosa layers of the gut (Sobczak et al., 2014). Despite this pain is still one of the primary symptoms reported by UC patients on presentation is normally found in the lower left flank of the abdomen. UC patients experience significant rectal bleeding, diarrhoea, and weight loss (Fakhoury et al., 2014). UC affects more men than women, with the average age of onset being 30-40 years old (Cosnes et al., 2011). The highest reported incidence of UC varies between 12.7-20.2/100,000 in Europe and North America, where it is most prevalent (Molodecky et al., 2012).

The causes of pain in IBD are not fully understood, but are thought to involve the activation and sensitisation of pain sensing nerves which innervate the gastrointestinal (GI) tract by inflammatory mediators (Hughes et al., 2013). The innervation of the gut is complex with both extrinsic nerves projecting from and to the brain and spinal cord as part of the afferent and efferent limbs of the autonomic nervous system (ANS) , and intrinsic neurones found within the enteric nervous system (ENS) innervating the GI tract. From a sensory perspective the extrinsic nerves are responsible for the transmission of both noxious (pain) and non-noxious (physiological) information about the gut to the central nervous system (CNS). Intrinsic nerves may also indirectly modulate the transmission of noxious information from the gut by regulating secretomotor functions, which can contribute to a noxious event. The functional anatomy of these systems is described below.

1.2 OVERVIEW OF THE EXTRINSIC INNERVATION OF THE GASTROINTESTINAL TRACT

1.2.1 Overview

The GI tract is a group of organs involved in the ingestion, digestion and subsequent absorption of food and the defecation of waste products (Knowles and Aziz, 2009). The gut is unique in that it is innervated by both intrinsic (via the ENS) and extrinsic nerves (via the ANS). Together these 2 nervous systems act in concert to control the subconscious physiological

functions of the gut, while harnessing the potential to consciously communicate noxious stimuli (Knowles and Aziz, 2009). Extrinsically the gut is innervated by both parasympathetic and sympathetic divisions of the ANS, which consists of both sensory and motor nerves. Although for clarity it is now common for the afferent sensory innervation to be described by the nerves through which they connect to the central nervous system. Hence parasympathetic afferent fibres are referred to as vagal or pelvic afferents with reference to their projection with respective vagus or pelvic nerves. Similarly sympathetic afferent fibres may be referred to as splanchnic afferents with reference to the splanchnic nerves which innervates much of the gut. However, it is more common for these to be called spinal afferents, particularly with reference to the target organ innervated, for within colonic spinal afferents or gastric spinal afferents. Additionally pelvic afferents may also be referred to as pelvic spinal afferents to reflect the projection of pelvic nerves to the spinal cord. For this thesis we have adopted a nomenclature that refers to pelvic afferents as pelvic spinal afferents and sympathetic afferents as splanchnic spinal afferents. The motor efferent innervation by the ANS is still referred to as parasympathetic or sympathetic and is provided by the sacral and vagal parasympathetic nerves and sympathetic prevertebral ganglia (PVG) nerves (Ratcliffe et al., 2011).

1.2.2 Vagal afferents

Vagal afferents predominantly transmit physiological information such as fullness, satiety and sphincter control to the CNS (Ramkumar and Schulze, 2005, Berthoud, 2008, Goyal and Chaudhury, 2008), but can, in some vagal fibres communicate pain signals (Cervero, 1994, Lennerz et al., 2007, Blackshaw, 2014). Over 90% of the vagal nerve fibres are sensory (Berthoud and Neuhuber, 2000). Vagal afferents innervating the intestine have their cell bodies in the inferior vagal ganglion in humans (nodose ganglia in murine species). Vagal afferents project to the nucleus tractus solitarius (NTS) and area postrema in the brainstem. From the NTS vagal afferent input is relayed via direct projections mainly to the parabrachial

nucleus, nucleus ambiguus, dorsal vagal motor nucleus and ventrolateral brain stem, although a smaller number of fibres also project directly to the hypothalamus, locus coeruleus, amygdala, insular cortex and peri-aquaductal grey (PAG). Projections to the parabrachial nucleus are further relayed to the PAG, hypothalamus, amygdala, insular cortex and other limbic structures and are thought to influence emotional and behavioural responses to information from the gut (Berthoud et al., 2004, Knowles and Aziz, 2009) (figure 1.01). The innervation of the gut by vagal afferent endings is densest in upper GI tract and diminished moving oral to anal. The distal colon is not typically considered to be significantly innervated by the vagus (Berthoud et al., 1991, Berthoud and Neuhuber, 2000, Blackshaw et al., 2007). At the level of the gut 3 distinct types of ending have been attributed to vagal afferents, mucosal endings, intramuscular arrays (IMA), and intraganglionic laminar endings (IGLE) (table 1.01). These are described in greater detail below.

1.2.3 Spinal afferents

Spinal visceral afferents are important in the transmission of noxious pathophysiological and physiological information from the gut. The spinal afferent innervation of the intestine is largely derived through splanchnic nerves although the final third of the distal colon receives an overlapping innervation by splanchnic and pelvic nerves with the rectum innervated by pelvic spinal afferents. Approximately 10-30% of fibres in these 2 nerves are sensory afferent fibres (Blackshaw and Grundy, 1989, Blackshaw and Gebhart, 2002). Splanchnic and pelvic afferents have their cell bodies in the dorsal root ganglion (DRG) and terminate in the dorsal horn of the spinal cord at the thoracolumbar and sacral levels, respectively. Within the spinal cord they synapse with second order neurons within lamina I, V and X of the dorsal horn, and activate projection neurons either directly or indirectly by interneuron relays which relay input to the CNS via spinoreticular, spinohypothalamic, spinomesencephalic and spinothalamic tracts (Grundy, 2002, Almeida et al., 2004). The relay of inputs from the spinothalamic tract to the somatosensory cortex, the anterior cingulate cortex together with the insula by the

thalamus, is important for the localisation and intensity of, and the emotional response to, pain, respectively (Almeida et al., 2004, Anand et al., 2007) (figure 1.01). In addition to terminating within the spinal cord splanchnic and pelvic afferents may also send collateral projections as they pass through prevertebral ganglia. These projections synapse on the cell bodies of preganglionic sympatheic efferents within the ganglia and thereby act to regulate the effect of sympathetic motor nerves innervating the GI tract (Green and Dockray, 1988, Holzer et al., 2001). Spinal afferents have endings in all layers of the gut wall (Grundy, 2002). Based on the location of receptive fields and their response characteristics to mechanical stimuli (probing, stretching and stroking) it is possible to characterise spinal afferents into 5 distinct subtypes termed mesenteric, serosal, muscular, muscular-mucosal, and mucosal afferents (table 1.02). These subtypes are described in more detail below.

Vagal: **NTS**
- Thalamus
- Locus Coeruleus
- Periaqueductal Gray
- Amygdala
- Cortex

Spinal: Thalamus
- Anterior Cingulate Cortex
- Insula
- Amygdala
- Hypothalamus
- Cortex

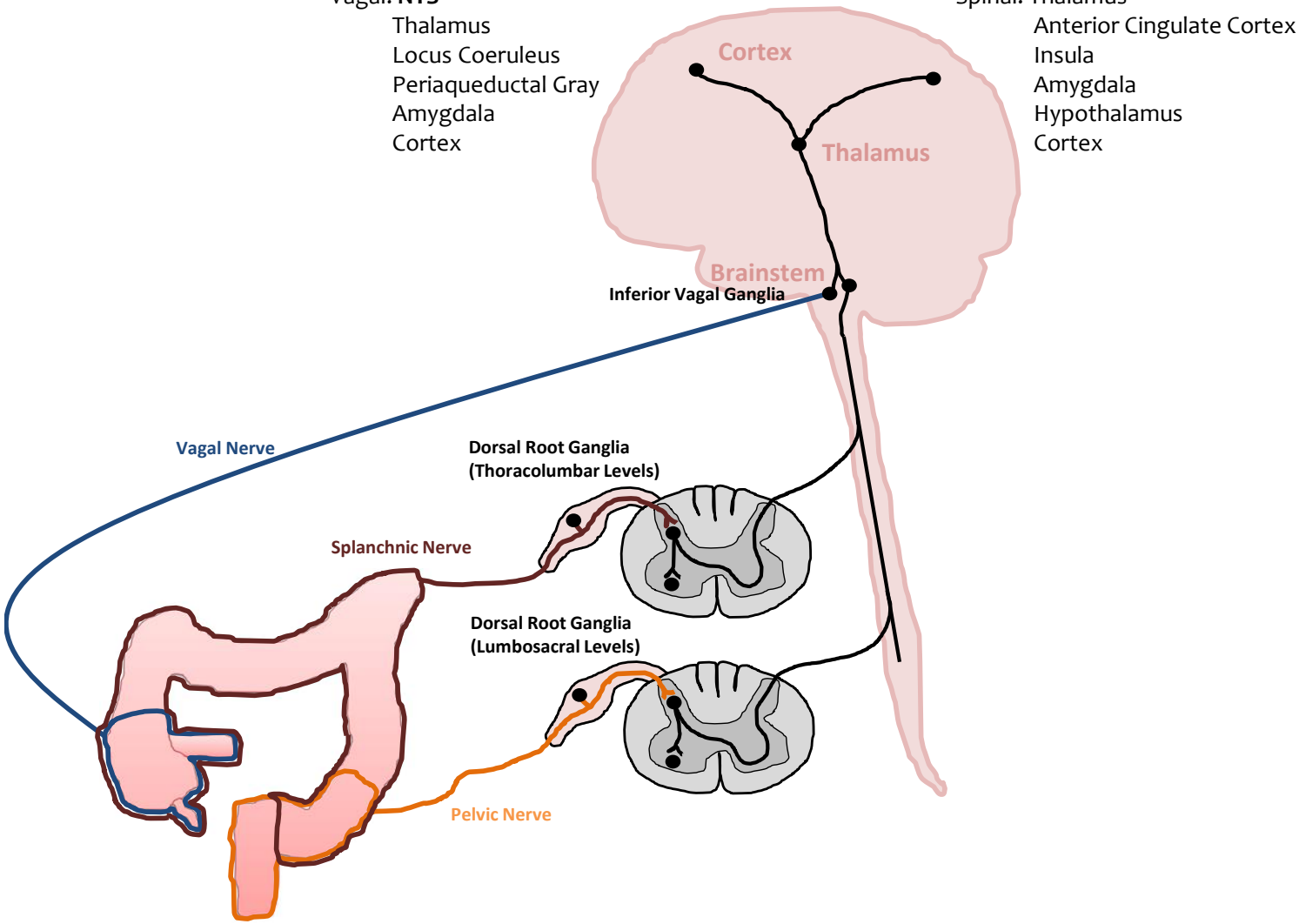


Figure 1.01: Extrinsic afferent innervation of the gut. Vagal afferents (blue) have their cell bodies in the inferior vagal ganglion in humans, and have denser innervation in the upper gastrointestinal tract and proximal colon. They mainly project to the nucleus tractus solitarius (NTS) in the brainstem, with a smaller number of fibres projecting to the hypothalamus, locus coeruleus, amygdala and peri-aquaductal grey, and the thalamus, where some projections are relayed to the cortex. Spinal visceral afferents innervate the GI tract through the splanchnic and pelvic nerves. Splanchnic (brown) and pelvic (orange) afferents have their cell bodies in the DRGs and terminate in the dorsal horn of the thoracolumbar and sacral spinal cord, respectively. Here they synapse with second order neurons, which ultimately project to the anterior cingulate cortex, the insula, amygdala, hypothalamus and thalamus and onto the somatosensory cortex.

Afferent Type	Cell Body	Central Projections	Peripheral Endings in the Gut
Vagal	Inferior Vagal Ganglion	Brainstem (NTS), PAG, Hypothalamus, LC, Amygdala,	Intramuscular arrays, Intra Ganglionic Laminar Endings, Mucosal
Splanchnic	Dorsal Root Ganglion	Thoracolumbar Spinal Cord	Mesenteric, Serosal, Muscular, Mucosal
Pelvic	Dorsal Root Ganglion	Sacral Spinal Cord	Serosal, Muscular, Muscular-mucosal, Mucosal

Table 1.01: Outlines the location of the cell body, and the central and peripheral projections for vagal, splanchnic and pelvic afferents.

1.2.4 Subtypes of vagal and spinal afferents innervating the gut

A number of methods have been employed to attempt to characterise extrinsic afferent neurons innervating the small and large intestine. These include but are not limited to receptor expression, neurotransmitters used, basal firing rate, conduction velocity, activation thresholds etc. (Brookes et al., 2013). For reference we will predominantly focus on the mucosal surface up, flat sheet characterisation method develop by Lynn and Blackshaw in rat (1999) and later refined in Brierley et al. This has been adopted by several groups in the field and is based on the characterisation of extrinsic afferents into subtypes using their response profile to probing of the gut wall and mesentery with calibrated von Frey hairs (VFH), stretching of the gut wall, and stroking of the mucosal surface. Mesenteric and serosal afferents respond to VFH probing of their receptive fields, and to high intensity stretch, but not to mucosal stroking, muscular afferents respond to low levels of stretch and VFH probing, but not to mucosal stroking, muscular-mucosal afferents respond to all 3 stimuli, and mucosal afferents only response to mucosal stroking. Multiple studies have used this type of afferent characterisation, although many are from the same group (Lynn and Blackshaw, 1999, Hicks et al., 2002, Brierley et al., 2004, Page et al., 2004, Page et al., 2005, Brierley et al., 2005b, Jones et al., 2005, Brierley et al., 2008, Brierley et al., 2009, Hughes et al., 2009a, Feng and Gebhart, 2011). A summary of the spinal subtypes described using this method can be found in figure 1.02, and table 1.02.

1.2.4.1 Vascular afferents

1.2.4.1.1 Splanchnic and pelvic

The term vascular afferents has been suggested to encompass both serosal and mesenteric afferents since they terminate predominantly on the vasculature and display similar response profiles to mechanical stimuli for example they respond to VFH probing of their receptive fields but not tissue stretching (up to 5g) or mucosal stroking (Song et al., 2009) (figure 1.02). More

recently it has been speculated that subgroups of vascular afferents may exist e.g. “silent” afferents (Brookes et al., 2013). The terms are used interchangeably here. The first “vascular” afferent was described in 1966, as a movement receptor, responding to light probing of areas surrounding the mesenteric artery. These afferents also responded to distortion of the mesentery and to balloon distension of the bowel to ~30 mm Hg (Bessou and Perl, 1966). Indeed, the first study to employ a myriad of mechanical stimuli to characterise splanchnic afferents in the mucosal surface up flat sheet preparation from the rat colon developed by Blackshaw, described serosal afferents as responsive to “firm blunt probing” of the mucosa, to circumferential stretch (0-10mm) but adapted rapidly to the stimulus, and to lighter than firm probing of the serosal surface, were considered serosal afferents However, the authors noted that a mucosa-up orientation of the tissue made serosal probing difficult and not always possible (Lynn and Blackshaw, 1999). Another study on splanchnic fibres innervating the flat sheet rat colon also identified serosal afferents, but these only responded to probing and not tissue stretch (0-8 mm) (Hicks et al., 2002). Additionally in this report, mesenteric afferents with receptive fields in the mesentery were also described (Hicks et al., 2002). In the mucosa up flat sheet preparation from the mouse colon, the splanchnic nerve also contains both serosal afferents and mesenteric afferents which are sensitive only to probing of their respective receptive fields i.e. serosa vs. mesentery (Brierley et al., 2004). The sensitivity of serosal units to tissue stretch appears to be linked to the intensity and nature of the applied stretch. For example responsiveness of serosal afferents to high intensity distension (>50mmHg) when kept as a tubular preparation and circumferential stretch (>5g) when applied by a claw attached to a cantilever system has recently been reported (Brierley et al., 2008, Brierley et al., 2009). Although stretching of the entire flat sheet by way of a clip attached the length of the tissue up to a force equivalent of ~17 grams (0.170 newtons over 34 seconds) has been reported to have no effect on serosal afferents (Feng et al., 2012b). In the studies from the Blackshaw lab using a claw and cantilever system to deliver a more focal stretch, the highest stretch used, 11g, was enough to activate 80% of serosal afferents (Hughes

et al., 2009a). It would seem likely that the gradual application of the stretching stimulus may account for these discrepancies, whereby slow application of stretch, even to potentially noxious levels, does not activate serosal afferents.

In both rat and mouse, these vascular afferents make up more than 80% of the afferent fibres in the splanchnic nerve reflecting a role for splanchnic spinal afferents in the detection of noxious stimuli in the colon (Hicks et al., 2002, Brierley et al., 2004). By contrast in the mouse colon, pelvic afferents do not contain mesenteric afferents, but only serosal afferents, which constitute only about a third of the afferent fibre population. (Brierley et al., 2004, Hughes et al., 2009a, Feng and Gebhart, 2011).

Serosal and mesenteric afferents (or vascular afferents) have been implicated as the principle pathway by which visceral pain is transduced and relayed to the CNS, due to their role as high threshold mechanoreceptors, and hence restricted response to only noxious levels of mechanical stimuli (Knowles and Aziz, 2009). Serosal and mesenteric afferents are also chemosensitive to a vast range of noxious or inflammatory mediators including capsaicin, 5-HT, BK, histamine, ATP etc. (Berthoud et al., 2001, Hicks et al., 2002, Brierley et al., 2005a).

1.2.4.2 "Silent" afferents

Another subtype of afferents termed either "silent" or "mechanically insensitive afferents" (MIAs) have been identified in a number of species. Their first description in visceral afferents was in cat urinary bladder (Habler et al., 1988, Habler et al., 1990). However, methodological issues in these studies such as restricted levels of pre-inflammation distension, to pressures which barely reached the activation threshold for high threshold mechanoreceptors, may be a contributing factor to their apparent mechanoinsensitivity in normal tissue (Cervero, 1994). "Silent" afferents have subsequently been reported in both pelvic and splanchnic afferent nerves (Lynn and Blackshaw, 1999, Feng and Gebhart, 2011). It has been report that up to 33% of splanchnic and 23% of pelvic afferents may be MIAs (Feng and Gebhart, 2011). However,

since electrically stimulating the axons peripherally may also stimulate sympathetic efferent fibres, there may have been an overestimation of the proportion of MIAs (Brookes et al., 2013).

"Silent" afferents are unresponsive to any mode of mechanical stimuli until sensitised by inflammatory mediators, such as BK, 5-HT, histamine, $PGE_{2,}$ and capsaicin (usually given as an inflammatory soup) after which they respond to probing stimuli, but not stretching of the tissue or stroking of the mucosa (Lynn and Blackshaw, 1999, Feng and Gebhart, 2011). Hence, the majority of "silent" afferents are likely to have vascular endings. "Silent" afferents are proposed to be functionally distinct to other visceral afferents, focused more on injury and inflammation (Cervero, 1994). However, whether they truly represent a functionally distinct subtype of afferent fibre is still unclear. For example, these fibres could represent a population of very high threshold vascular afferents that are not activated by the levels of noxious stimuli present in the viscera. Inflammation and subsequent sensitisation, acute and long term (changes in gene expression) may reduce their activation threshold and increasing their excitability. Hence their functionality may be similar to that of other high threshold vascular afferents (Cervero, 1994, Brookes et al., 2013).

1.2.4.3 Muscular afferents

1.2.4.3.1 Vagal

IMAs are located in the circular and longitudinal muscle of the gut wall and in the myenteric plexus and are more prevalent in the upper GI tract, especially in the fundus and pyloric sphincters (Berthoud and Neuhuber, 2000, Wang and Powley, 2000). IMAs consist of long axons tracking parallel to the respective muscle layer, with shorter perpendicular branches. IMAs may transmit information on muscle stretch and length but are unlikely to be important in nociception (Phillips and Powley, 2000, Powley and Phillips, 2002, Knowles and Aziz, 2009) (figure 1.02).

IGLEs terminate as numerous flattened endings that together with the connective tissue encapsulate the myenteric plexus (Nonidez, 1946). They have endings parallel to the muscle fibres in the wall of the intestine, with fine branching endings that extend into the myenteric plexus, allowing them to respond in-series to mechanical tension (Brookes et al., 2013) (figure 1.02). IGLEs are also present in the submucosal plexus in smaller numbers (Castelucci et al., 2003). IGLEs are low threshold slowly adapting mechanosensors, sensing physiological levels of sheering forces as the smooth muscle of the GI tract contracts (Neuhuber, 1987, Zagorodnyuk and Brookes, 2000, Lynn et al., 2003). IGLEs are found in the upper GI tract, the small intestine and to a lesser extent in the proximal colon (Berthoud et al., 1997, Fox et al., 2000). IGLEs can also sense chemical mediators such as ATP, the relevance of which is unknown (Page et al., 2002, Zagorodnyuk et al., 2003). There is no established role for IGLEs in nociception (Knowles and Aziz, 2009).

1.2.4.3.2 Splanchnic and pelvic

Afferents responsive to circumferential stretch, which adapted slowly, but were not responsive to mucosal stroking, were classified as muscular afferents (figure 1.02). These were initially described in the splanchnic innervation of the rat colon (Lynn and Blackshaw, 1999, Hicks et al., 2002), but have since been described in both the splanchnic and pelvic innervation of the mouse colon (Brierley et al., 2004). Muscular afferents constitute up to a fifth of splanchnic afferents, and up to a quarter of pelvic afferents. There is a dearth of evidence linking either splanchnic or pelvic muscular afferents to any nociceptor activity. Instead they are likely to signal physiological information about tension, length and contraction of the muscle (Knowles and Aziz, 2009).

1.2.4.3.2.1 rIGLEs

Pelvic afferents can have endings between the smooth muscle layers of the gut wall. These endings are similar structure, although smaller and less complex, to vagal IGLEs displaying

flattened laminar endings surrounding the myenteric plexus (Lynn et al., 2003). They have only been demonstrated in rectum, and are so called rectal IGLEs (rIGLE). rIGLEs are reported to be low threshold, slowly adapting tension receptors responding to both rectal distension and contraction (Lynn et al., 2003). It is likely that rIGLEs arise from pelvic afferents originating from sacral DRGs, and that they have physiological rather than nociceptive roles (Lynn et al., 2003).

1.2.4.4 Mucosal Afferents

1.2.4.4.1 Vagal

Vagal mucosal afferents were first reported in the cat in 1957 and responded to compression of the intestine but not to distension (Paintal, 1957). Subsequently they have been described in the oesophagus, stomach, or small intestine of the ferret (Page and Blackshaw, 1998), cat (Iggo, 1957), rat (Clarke and Davison, 1978) rabbit (Andrews and Andrews, 1971), mouse (Page et al., 2002) and sheep (Harding and Leek, 1972) where they respond to light stroking of the mucosa but not to circumferential stretch or distension. Vagal mucosal afferents also display a range of chemosensitivity, including responsiveness to 5-HT, the P2X receptor agonist α, β methylene ATP (meATP), cayenne pepper (transient receptor potential vanilloid 1 (TRPV1) agonist), mustard oil (transient receptor potential ankyrin 1 (TRPA1) agonist), organic and inorganic acids, H_20, casein hydrolysate etc. (Paintal, 1954, Clarke and Davison, 1978). Mediators such as cholecystokinin and peptide YY can activate vagal mucosal afferents to help regulate satiety (Smith et al., 1981, Blackshaw and Grundy, 1990, Abbott et al., 2005). Mucosal afferents compose up to 2/3s of the vagal afferent pathway (Page and Blackshaw, 1998, Berthoud et al., 2001). A number of vagal mucosal endings have been described, some spanning the length of the villi others terminating before they enter the villi (Powley and Phillips, 2002) (figure 1.02).

1.2.4.4.2 Splanchnic and Pelvic

Mucosal afferents also exist in the splanchnic and pelvic pathways of the spinal nerve in murine species (figure 1.02). These afferents are responsive to stroking of the mucosal surface with a 10mg VFH, and to probing but not to circumferential stretch (Lynn and Blackshaw, 1999, Hicks et al., 2002, Brierley et al., 2004, Feng and Gebhart, 2011). Mucosal afferents are relatively rare in mouse splanchnic pathways (1-4%), but constitute up to a quarter of pelvic afferents in mice (e.g. (Brierley et al., 2004, Feng and Gebhart, 2011). There is evidence for the reverse in rats, splanchnic pathways comprising 14-24% of afferent fibres, while 6% of pelvic afferents respond to stroking of the mucosa (Sengupta and Gebhart, 1994, Lynn and Blackshaw, 1999, Hicks et al., 2002). Spinal mucosal afferents respond to a variety of chemical mediators such as 5-HT and capsaicin (Lynn and Blackshaw, 1999, Hicks et al., 2002), suggesting a potential role in chemonociception (Knowles and Aziz, 2009).

1.2.4.5 Muscular-mucosal

1.2.4.5.1 Vagal tension-mucosal and pelvic muscular-mucosal afferents

Recordings from the vagal innervation the ferret oesophagus revealed a subtype of afferent fibre that was responsive to both stroking of the mucosa and to circumferential stretch (Page and Blackshaw, 1998). A similar subtype of afferent was described in pelvic afferents innervating the mouse colon. These were responsive to blunt probing of the mucosa, stretching of the colon wall, and to 10mg stroking of the mucosal surface and were termed muscular-mucosal afferents (Brierley et al., 2004) (figure 1.02, table 1.02). The location of these vagal tension-mucosal and pelvic muscular-mucosal afferent terminals is unclear. It has been suggested that the muscularis externa, and the lamina propria in the mucosa both contain muscular-mucosal terminals (Page and Blackshaw, 1998, Blackshaw and Gebhart, 2002, Brierley et al., 2004). However, it has also been speculated that endings at 1 site in the subepithelial plexus is enough to sense both modes of mechanical stimuli (Zagorodnyuk et al.,

2010, Brookes et al., 2013). Overall, tension-mucosal afferents phenotype has been suggested to contribute to approximately ~16% of vagal afferents innervating the oesophagus (Page and Blackshaw, 1998). Similarly, muscular-mucosal afferent make up about 1 quarter of the pelvic innervation of the mouse colon (Brierley et al., 2004, Hughes et al., 2009a, Feng and Gebhart, 2011). Although the function of these afferents is unclear, they have been proposed to play a role in sensing movement of material in the GI tract, considering their responsiveness to light mucosal stroking (Brookes et al., 2013).

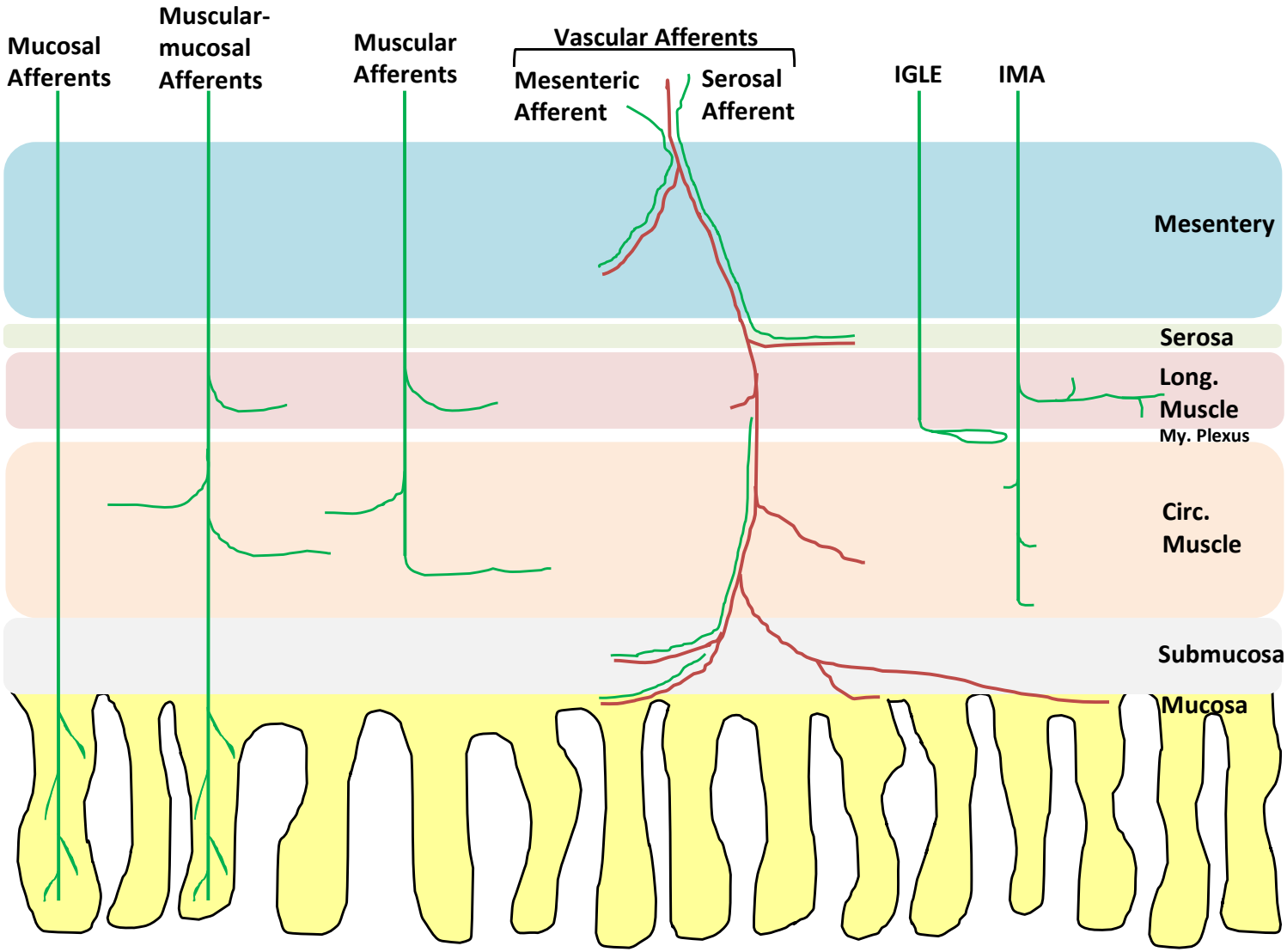

Mucosal Afferents
Muscular-mucosal Afferents
Muscular Afferents
Vascular Afferents
Mesenteric Afferent
Serosal Afferent
IGLE
IMA
Mesentery
Serosa
Long. Muscle
My. Plexus
Circ. Muscle
Submucosa
Mucosa

Figure 1.02: Spinal and vagal afferent terminals in the gut. Shows 5 subtypes of spinal afferent terminals in the gut; mesenteric, serosal, muscular, muscular-mucosal, and mucosal afferents, and 3 types of vagal afferent terminals, intra ganglionic laminar endings (IGLEs), Intra muscular arrays (IMAs), and mucosal afferents. Mesenteric and serosal afferents have terminals in the mesentery and serosa, respectively, both closely associated with blood vessels (red). Spinal muscular afferents have terminals in the longitudinal and circular muscle. Vagal IGLEs terminate as numerous flattened endings that together with the connective tissue encapsulate the myenteric plexus. They also have endings parallel to the muscle fibres in the wall of the intestine, with fine branching endings that extend into the myenteric plexus. Vagal IMAs have long axons that run parallel to the respective muscle layer, and have shorter perpendicular branches. The location of muscular mucosal afferent terminals is not fully understood but they are thought to have their terminals in either the muscularis externa, and the laminar propria, or the subepithelial plexus. Vagal and spinal mucosal afferents terminate in the mucosa of the gut.

Afferent Subtype	Von Frey Hair	Muscular Stretch	Mucosal Stroking
Mesenteric	✓	×*	×
Serosal	✓	×*	×
Muscular	✓	✓	×
Muscular-mucosal	✓	✓	✓
Mucosal	✓	×	✓

Table 1.02: Outlines each spinal afferent subtype and which mechanical stimuli they respond to. **(*)** Activated at very high levels of stretch

1.3 OVERVIEW OF THE ENTERIC NERVOUS SYSTEM

1.3.1 Overview

The ENS provides the postganglionic efferent innervation of the parasympathetic component of the ANS and a major final effector pathway for the action of postganglionic sympathetic efferent fibres, in addition to containing its own intrinsic populations of sensory and interneurons (Sasselli et al., 2012). The ENS is arranged as a complex network of glial cells and neurons, approximately 10^8 neurons, a number comparable to the spinal cord, that extend the entire length of the GI tract (Furness and Costa, 1979, Grundy and Schemann, 2007). It is organised into 2 plexuses, the myenteric plexus, located between the circular and longitudinal smooth muscle layers, and the submucosal plexus, between the circular muscle and the mucosa (Gershon, 2011). In humans, the submucosal plexus is divided into 3 layers, the outer, intermediate and inner plexus, the latter located just below the muscularis mucosae (Hoyle and Burnstock, 1989, Schemann and Neunlist, 2004).

The ENS consists of many different types of neurons including, intrinsic primary afferent neurons (IPAN), motor neurons, interneurons, vasomotor neurons, secretory neurons, rectospinal, and intestinofugal afferent neurons (IFAN) (Costa et al., 2000, Furness, 2000) (figure 1.03). The ENS, with limited contribution from the CNS, can regulate motility, via control of the smooth muscle, and mucosal secretion into the lumen of the GI tract (Goyal and Hirano, 1996, Costa et al., 2000, Furness, 2000, Grundy and Schemann, 2007). ENS neurons use a multitude of neurotransmitters including acetylcholine, nitric oxide, substance P, vasoactive intestinal polypeptide (VIP), ATP, dopamine, Neuropeptide Y, and 5-HT (Benarroch, 2007).

IPANs are contained in both plexuses and exhibit considerable branching, extending to the lamina propria of the mucosa, lying below the epithelial lining. Enteroendocrine cells such as enterochromaffin cells (EC) can sense mechanical stimuli such as distension of the gut wall, and mucosal deformation. In response they release 5-HT into the lamina propria, which subsequently can activate IPANs. Similarly, ECs can sense the chemical contents of the gut

lumen including nutrients such as glucose and toxins, and subsequently activate IPANs via a similar 5-HT dependent mechanism (Gershon, 2000, Gershon, 2003, Raybould et al., 2003). Hence mechanical and chemical stimuli indirectly activate IPANs (Gershon, 2005). IPANs transmit this information to ascending and descending interneurons, which synapse with the excitatory (oral side of IPAN) and inhibitory motor neurons (aboral side IPAN), that control the contraction and relaxation of the gut, respectively, through their interaction with the interstitial cells of Cajal, which regulate smooth muscle contractility (Costa et al., 2000, Furness, 2000, Benarroch, 2007). This coordinated oral contraction and aboral relaxation is the basis of peristaltic movement in the human GI tract (Schemann and Neunlist, 2004).

IPANs and enteroendocrine cells are involved in the detection of noxious stimuli in gut lumen (Furness, 2006). Toxins such as cholera or E. coli stimulate the release of 5-HT and/or peptides from EC cells or other enteroendocrine cells (Lundgren, 2002). These mediators activate IPANs, which in turn may alter motility and increase mucosal secretion through their interactions with interneurons and motor neurons, resulting in diarrhoea to promote expulsion of the toxins (Furness, 2006).

Secretory motor neurons are located in both the myenteric and the submucosal ganglia, where they are much more abundant, and project to the mucosa. Vasomotor neurons are restricted to the submucosa and project to the mucosa and to blood vessels in the local environment (Costa et al., 2000). They receive inputs from myenteric and submucosal IPANs, which regulate the secretory motor and vasomotor reflexes that control mucosal secretion and absorption as well as dilation of local blood vessels (Furness, 2000, Benarroch, 2007). These reflexes are influenced centrally by extrinsic sympathetic neurons, with which they synapse (Costa et al., 2000, Furness, 2000). IPANs themselves can act as secretory motor neurons via their mucosal terminations (Furness et al., 2004).

Interneurons project either orally (ascending) or anally (descending) forming chains as they link between different ENS neurons (Kunze and Furness, 1999). Different subtypes of interneurons can be characterised based on the specific groups of neurotransmitters they

possess. Interneurons can exert an inhibitory or excitatory effect upon neurons with which they synapse (Furness, 2000).

Another distinct type of intrinsic neuron has been identified in the rectum of rats (Doerffler-Melly and Neuhuber, 1988). These intrinsic neurons, termed rectospinal neurons, have their cell bodies within the myenteric plexus and project to the dorsal horn of the spinal cord. They are the only type of intrinsic neuron that directly project to the CNS. However, their distribution is restricted to the distal rectum, and to date they have only been identified in rats (Doerffler-Melly and Neuhuber, 1988, Neuhuber et al., 1993, Suckow and Caudle, 2008).

Similar to rectospinal neurones but far more numerous and widespread in their distribution IFANs also have cell bodies located in the myenteric plexus and project beyond the gut. However, unlike rectospinal neurones IFANs only project as far as the sympathetic PVG, where they synapse on postganglionic neurones and can help regulate autonomic function (Crowcroft et al., 1971, Szurszewski et al., 2002). In addition, IFANs are also reported to send projections within the gut that synapse with IPANs (Costa et al., 2000, Furness, 2000). IFANs are mechanoreceptors that respond to stretch not tension (Weems and Szurszewski, 1978). Paravertebral sympathetic post ganglionic neurones receive excitatory synaptic input from IFANs following activation by distension triggering a sympathetically mediated reduction in gut motility and secretion (Costa and Furness, 1984, Messenger and Furness, 1993, Miller and Szurszewski, 1997, Suckow and Caudle, 2008). This reflex is believed to control the inherent tendency of gastrointestinal smooth muscle to contract upon luminal filling. IFANs thereby facilitate the physiological stretching of the gut by preventing large increases intraluminal pressure (Szurszewski et al., 2002).

Enteric glia also play a vital role in enteric function. Enteric glia greatly out number neurons in the ENS and are located in the myenteric and submucosal plexi, where they surround axonal bundles (Wedel et al., 1999, Ruhl, 2005). Glial processes are in close contact not only with enteric neurones (which are often partially enveloped by flattened glial end feet), but also other cell types within the gut such epithelial cells, endothelial cells and smooth

muscle cells (Ruhl, 2005). Enteric glia contain many of the chemical precursors to various neurotransmitters, and express their receptors, which helps regulate neurotransmission (Ruhl, 2005, Benarroch, 2007). Glia may also influence blood flow, epithelial cell permeability and immunity in the GI tract (Ruhl, 2005).

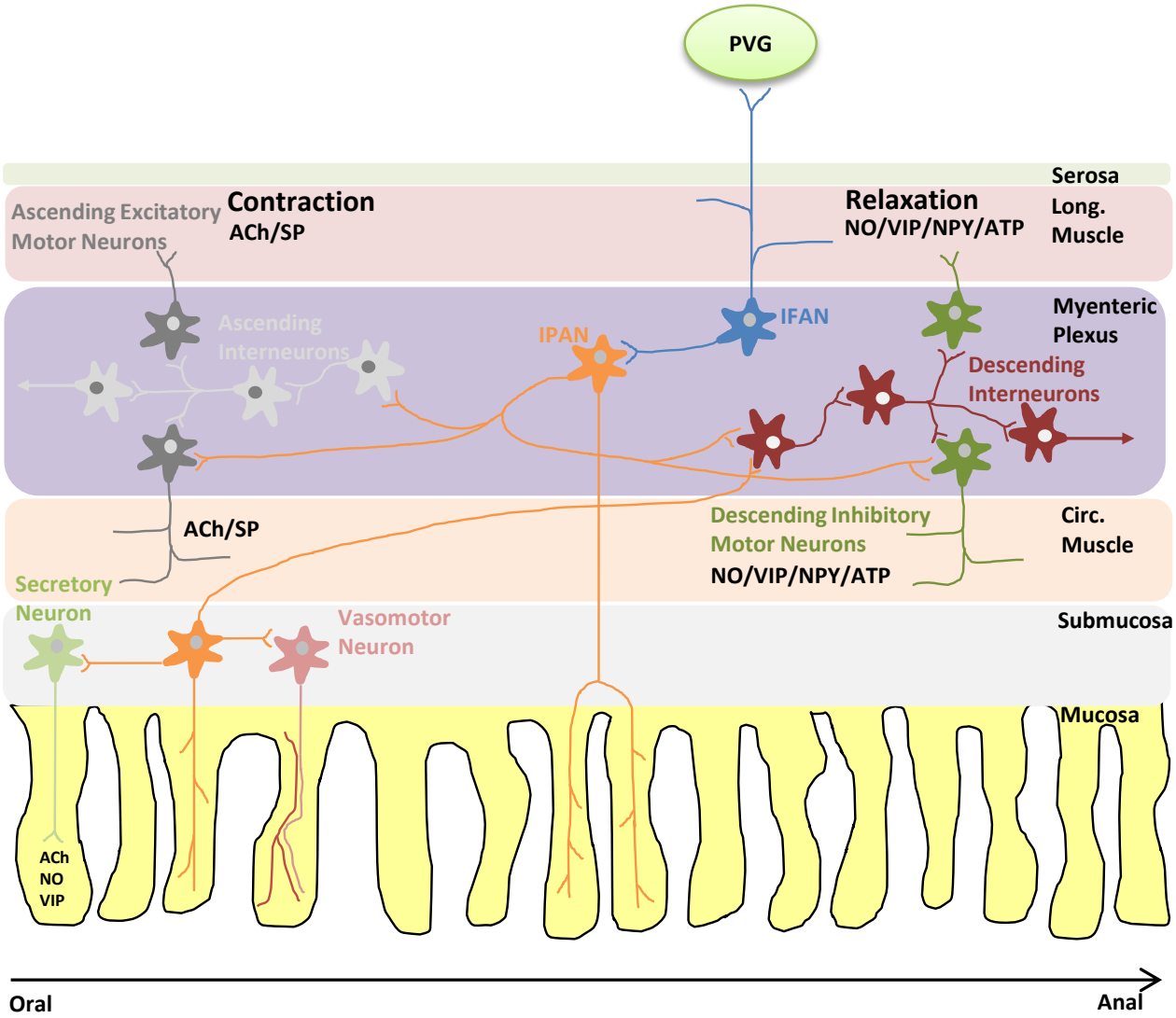

PVG
Serosa
Ascending Excitatory
Motor Neurons
Contraction
ACh/SP
Relaxation
NO/VIP/NPY/ATP
Long.
Muscle
Myenteric
Plexus
Ascending
Interneurons
IPAN
IFAN
Descending
Interneurons
ACh/SP
Descending Inhibitory
Motor Neurons
NO/VIP/NPY/ATP
Circ.
Muscle
Secretory
Neuron
Vasomotor
Neuron
Submucosa
Mucosa
ACh
NO
VIP
Oral
Anal

Figure 1.03: Overview of the enteric nervous system. Intrinsic primary afferent neurons (IPANs) (orange) have their cell bodies in the submucosal or myenteric plexus and project to the mucosa. Here they can detect both mechanical and chemical stimuli. IPANs have oral projections, which synapse with ascending interneurons (light grey) and ascending excitatory motor neurons (dark grey) that control the contractile peristaltic reflex. IPANs also have anal projections, which synapse with descending interneurons (red) and descending inhibitory motor neurons (dark green) and control the inhibitory reflex and resultant smooth muscle relaxation. Secretory motor (light green) and vasomotor neurons (pink) have projections to the mucosa. They receive inputs from myenteric and submucosal IPANs, which regulate the secretory motor and vasomotor reflexes that control mucosal secretion and absorption as well as dilation of local blood vessels. Intestinofugal afferent neurons (IFANs) (blue) cell bodies are located in the myenteric plexus. They have projections outside the gut wall to the sympathetic prevertebral ganglion (PVG). IFANs can sense stretch of the smooth muscle and send excitatory signals to the PVG, which in turn provide extrinsic input to the smooth muscle inhibiting motility and mucosal secretion, and controlling contractility of the smooth muscle in the wall of the gut. IFANs project to IPANs within the gut wall.

1.4 NERVE FUNCTION

1.4.1 Ionotropic and metabotropic signalling

Ion channels are porous transmembrane proteins that allow the passage of ions across the plasma membrane based on their reception of certain stimuli. Ion channels are selectively permeable to ions based on their size and charge. Typically ion channels are gated, whereby certain stimuli cause a conformation change in the channel structure, which can cause the ion channel to open or close (Purves, 2012). For example mechanically gated ion channels are regulated by mechanical stimuli such as stretch, ligand gated ion channels open and close in response to neurotransmitters, voltage gated ion channels are responsive to changes in the membrane potential and a number of "stimulus transducing" channels are gated by exogenous chemicals in the microenvironment. The opening of ion channels and subsequent flux of ions through the channel leads to fast changes in membrane potential and is the major pathway of stimulus transduction in sensory nerves There are also resting ion channels that are not gated and are generally open at rest, contributing the resting membrane potential (Kandel, 2012).

Metabotropic receptors are transmembrane proteins, whose intracellular domains are linked to effector proteins, which when activated initiate downstream signalling cascades. Thus metabotropic receptors modulate nerve excitability indirectly causing changes in the activity of other proteins and ultimately ion channel function. The most common metabotropic receptors are G-protein coupled receptors (GPCRs) which consist of 7 transmembrane spanning domains, and are linked intracellularly to GTP-proteins (G-proteins). Classically G-proteins have 3 subunits, α, β, and γ (Kandel, 2012). The α G protein subunit is separate from the β, and γ subunits. However, upon binding of a guanosine-5'-diphosphate (GDP) molecule, the α subunit binds to the β, and γ subunits to form an inactive G-protein trimer. When a metabotropic receptor is activated by its ligand, the subsequent conformational changes facilitates the replacement of GDP with Guanosine-5'-triphosphate (GTP) which in turn enables

the dissociation of the alpha subunit and its translocation into the cytoplasm which in turn leads either to 1) direct alterations of ion channel permeability or 2) activation of effector proteins e.g. adenylate cyclase, which in turn can stimulate second messenger systems e.g. cyclic adenosine mono phosphate (cAMP), causing downstream signalling cascades that can alter ion channel conductivity, neuronal metabolism and regulate gene transcription and protein expression (Kingsley, 2000).

There are different types of G-proteins, which are linked with distinct effector proteins and second messengers. G stimulatory proteins (G_s proteins) stimulate the activity of adenylate cyclase thereby increasing the production of cAMP, which in turns activates PKA, which can phosphorylate target proteins altering their function. In contrast, G inhibitory proteins ($G_{i/o}$ proteins) inhibit the activity of adenylate cyclase, resulting in a decreased production of cAMP and reduction in PKA activity (Purves, 2012). $G_{q/11}$ proteins utilise a different intracellular pathway, stimulating the activity of phospholipase C, which in turn hydrolyses membrane phosphoinositides resulting in the formation of inositol phosphates and diacylglycerol (DAG), which can then cause Ca^{2+} release and activate the protein kinase C (PKC) pathway, respectively (Nichols and Nichols, 2008).

Enzyme linked receptors are a separate type of metabotropic receptors. Their intracellular domains are linked to enzymes, most notably protein kinases e.g. tyrosine kinase. The activity of the linked enzyme is regulated by the binding of chemical mediators to the receptors extracellular binding site. Upon activation, protein kinases can phosphorylate target proteins facilitating the binding of further signalling molecules and enzymes to the receptor, which in turn triggers their intracellular signalling cascades (Purves, 2012).

1.4.2 Membrane potential

The resting membrane potential of a neuron arises from the difference in the concentration and movement of charged ions between the intracellular cytoplasmic side of the cell

membrane and the extracellular matrix. Typically in DRGs the resting membrane potential is approximately -55 to -50 millivolts (mV). Intracellular concentrations of ions such as potassium (K^+), sodium (Na^+), chloride (Cl^-) and calcium (Ca^{2+}) and there movement through respective ion channels open at the resting membrane potential (so called "leak currents") dictate the resting membrane potential of the cell. In addition the activity of the sodium/potassium ATPase pump, which pumps a ratio of $3K^+$:$2Na^+$ into versus out of the cell helps set the resting membrane potential. As a consequence of the pump's activity potassium ions are more concentrated inside the cell, sometimes being 30 times higher than the external concentration. By contrast, sodium, chloride and calcium ions are more highly concentrated outside the cell (Alberts, 2008, McCormick, 2008).

1.4.3 Generation of action potentials in sensory nerves

The generation of action potentials (AP) in sensory nerves begins with a stimulus, for example mechanical, chemical, or thermal stimuli. These stimuli are transduced via different receptors on the nerve terminal. These receptors can either be ionotropic ion channels, or metabotropic G protein coupled receptors, which when activated depolarise the neuron by allowing the entry of cations into the cell (ionotropic) or by releasing stores of intracellular calcium or altering the activity of other receptors (metabotropic) (Siegelbaum, 2000). This stimulus evoked depolarisation is referred to as a "generator potential". If the generator potential produced by a given stimulus is large enough to depolarise the membrane potential to the threshold for action potential generation (~-30mV in DRGs), then voltage gated Na^+ channels (VGSC) involved in the action potential up-stroke will open causing an influx of Na^+ ions down their electrochemical gradient (Alberts, 2008). The influx of these positive charged Na^+ ions depolarises the membrane potential further, thereby opening additional voltage gated Na^+ channels resulting in more Na^+ ion entry. This feedback loop continues until the membrane potential reaches ~+30mV, close to Na^+ equilibrium potential (McCormick, 2008). At this point the voltage gated Na^+ channels inactivation is such that the net influx of positive

Na^+ ions into the cell begins to fall, and continues to decline until the influx of sodium return to baseline levels. At the same time voltage gated K^+ channels also open in response to the depolarisation of the membrane potential, however they exhibit much slower activation kinetics. Once opened, K^+ flows out of the cell down its electrochemical gradient. The combination of the cessation of Na^+ entry and the rapid outflow of K^+ ions decreases the permeability of the cell membrane to Na^+ relative to K^+ and quickly brings the membrane potential of the neuron back towards resting levels (Alberts, 2008). Indeed the rapid efflux of K^+ ions causes the neuron to hyperpolarise falling below its resting membrane potential. Hyperpolarisation quickly equilibrates as voltage gated K^+ channels close and inwardly rectifying K^+ channels open allowing K^+ ions to flow back into the cell, restoring the resting membrane potential (McCormick, 2008). When voltage gated Na^+ channels are completely inactivated, no stimulus regardless of strength can induce an AP. This is called the absolute refractory period and it occurs from depolarisation until hyperpolarisation (Alberts, 2008, McCormick, 2008).During hyperpolarisation a period exists where a stronger than normal stimulus is required to generate an AP. This is called the relative refractory period (Alberts, 2008, McCormick, 2008).

1.5 VISCERAL PAIN

Visceral pain is the commonest pain produced by disease, and is a major symptom of both IBS and IBD. The characteristics of visceral pain differ to that of pain originating in somatic structures (Robinson and Gebhart, 2008). Despite this, the majority of information about pain comes from experiments on somatic, non-visceral systems, and as a result our understanding of the mechanisms involved in visceral pain is less extensive compared to those of somatic pain (Grundy, 2004, Robinson and Gebhart, 2008). In response to peripheral disease, visceral pain arises from the activation of the pain sensitive nerves that innervate the gut. These signals are then relayed to the spinal cord, where they may be amplified as part of a process referred to as central sensitisation or inhibited by descending inhibitory input from the CNS.

Responses to the spinal cord are then relayed to a number of brain regions (e.g. thalamus, limbic system, somatosensory cortex and prefrontal cortex) collectively known as the pain processing matrix, where the conscious perception of pain including the discriminatory, emotional, and cognitive aspects occur.

As pain is a conscious complex experience it is difficult to measure even in clinical studies. As a result, it is common to measure the activation of pain processing pathways instead, for example in conscious animal studies, behavioural responses such as paw withdrawal may be used as a surrogate for pain. While in *in vitro* studies it is common to measure electrical activity in nerves thought to be involved in the processing of pain. The term nociception was developed to describe these experiments in which the activation of sensory pathways by noxious (tissue damaging) stimuli is studied rather than pain itself, and nociceptors for sensory nerve endings, which respond to noxious stimuli.

1.5.1 Mechanisms of visceral pain

Transduction of mechanical stimuli is essential for the normal GI functioning, e.g. bolus sensation and peristalsis. This is normally a subconscious process, controlled by the ENS with inputs from vagal and spinal extrinsic nerves that signal to the CNS (Furness, 2006). Vagal nerve endings in the mucosa and in the muscle layers of the gut, IMAs and IGLEs, are predominantly low threshold afferents sensing physiological levels of mechanical and chemical stimuli (Powley and Phillips, 2002, Lynn et al., 2003). Spinal nerve endings in the serosa, mesentery, and mucosa tend to be tonic, high threshold or “silent” afferents signalling noxious stimuli, hence have been implicated in visceral pain (Cervero, 1994). In uninflamed conditions, noxious mechanical, chemical and thermal stimuli are thought to be sensed by transducing channels, such as transient receptor potential (TRP) channels, acid sensing ion channels (ASIC) and purinoceptors, expressed on afferent nerves (Knowles and Aziz, 2009). As discussed above the activation of these channels leads to the formation of generator potentials and ultimately

the firing of an action potential (Knowles and Aziz, 2009). During inflammation, conditions under which pain signals are transduced are altered.

1.5.1.1 Peripheral sensitisation

Peripheral visceral afferent hypersensitivity is an established mechanism causing GI pain (Bueno and Fioramonti, 2002). A myriad of inflammatory chemical mediators have been suggested to play a role in visceral peripheral sensitisation. These mediators can exert their effects by direct activation of visceral afferents, sensitisation of visceral afferents with concomitant alteration of visceral afferent phenotype, or inducing neurogenic inflammation (Kirkup et al., 2001). Upon insult or injury, e.g. mechanical stimuli, toxins etc., cells become damaged causing a migration of inflammatory cells to the area. Cells such as mucosal epithelial cells, enteroendocrine cells, enterochromaffin cells, macrophages, degranulating mast cells and other immune cells release mediators such as ATP, BK, 5-HT, histamine, PGE_2, NGF etc. These mediators have been shown to directly activate visceral afferents; for example adenosine (Kirkup et al., 2001), ATP (Wynn and Burnstock, 2006), BK (Brunsden and Grundy, 1999, Brierley et al., 2005b), histamine (Kreis et al., 1998), and 5-HT (Hicks et al., 2002). These mediators, through activation of their receptors, GPCRs, and ligand gated ion channels ,recruit a range of intracellular signalling pathways such as PLC, PKA, PKC, mitogen activated protein kinases (MAPK), pERK, adenylate cyclase (Woolf and Ma, 2007). These signalling pathways can subsequently modulate, frequently by phosphorylation, existing tonic inhibitions, activation, kinetics, internalisation and trafficking of receptors e.g. TRP channels, P2X receptors, and ion channels e.g. VGSC, and under chronic inflammatory conditions can cause longer term changes in gene transcription and expression (McMahon, 2004, Zhang et al., 2005) (figure 1.04). These changes describe the plasticity of visceral afferents, which can result in sensitisation of the nerve (Knowles and Aziz, 2009).

Indeed, the expression of a number of channels/receptors is increased by inflammation; ASICs, voltage gated sodium channel (Na_V) 1.8 and $Na_V1.9$, TRPV1, $P2X_3$, which may influence sensitisation (Yiangou et al., 2001a, Yiangou et al., 2001d, Yiangou et al., 2001c, Yiangou et al., 2001b). Changes in the distribution and size of nociceptor endings may also contribute to the peripheral sensitisation of afferent nerves (Bueno and Fioramonti, 2002). Together, this results in prolonged nerve stimulation, lowering of the threshold for activation of afferent nerves including nociceptors, and causing a greater activation of afferents in response to a given stimulus (McMahon, 2004). The activation of afferents by normally non-noxious stimuli to activate the pain pathway is called allodynia (Woolf and Ma, 2007). Additionally, responses to noxious stimuli can be exaggerated, known as hyperalgesia (Anand et al., 2007). Collectively the occurrence of these 2 phenomena is referred to as hypersensitivity.

1.5.1.1.1 Neurogenic inflammation and sensitisation

The milieu of mediators released during an inflammatory event, especially biogenic amines e.g. histamine and 5-HT, can stimulate nerves to release neuropeptides such as calcitonin gene related peptide (CGRP) and substance P. These in turn promote the release of nerve growth factor (NGF) from immune cells such as lymphocytes and mast cells (Barouch et al., 2000). The release of NGF in turn augments mast cell degranulation and stimulates the release of the neuropeptides substance P and CGRP from neurons, which in turn promote the release of more NGF, hence exhibiting a self-sustaining loop (Bueno and Fioramonti, 2002). CGRP and substance P are expressed by neurons in the ENS. It is conceivable that enteric neurons can release these neuropeptides in response to noxious stimuli, hence augmenting neurogenic inflammation (Knowles and Aziz, 2009). A summary of peripheral sensitisation can be found in figure 1.04.

1.5.1.1.2 Potential causes of peripheral sensitisation in bowel diseases

Peripheral sensitisation is a contributing factor to the visceral pain reported by IBS and IBD patients. Changes in the microbiota of the gut are thought to be a contributing factor in the pathogenesis of IBS (Ohman and Simren, 2013). Indeed, probiotic formulas, which included either lactobacilli or bifidobacteria, aimed at restoring healthy microbiota were analgesic in IBS patients (Halpern et al., 1996, O'Mahony et al., 2005, McKernan et al., 2010). Proteins and products released by bacteria can activate afferent nerves in the colon, which may also activate both the innate and adaptive immune responses (Liu et al., 2005a, Xu et al., 2009, Ochoa-Cortes et al., 2010).

Similarly, a compromised intestinal epithelial barrier in the lumen of the gut, which is evident in IBS patients (Dunlop et al., 2006, Aerssens et al., 2008, Zhou et al., 2009, Gecse et al., 2012), may allow easier access of the contents of the gut to the wall of the intestine. This in turn promotes activation of the immune system and the development of inflammation, which can activate and alter the sensitivity of afferent nerves as described above (Hughes et al., 2013).

In IBS, the role of the innate immune system (mast cells, macrophages, dendritic cells etc.) has been studied more extensively than the adaptive immune system (T cells, B cells). Mast cells are of particular interest, with studies in IBS patients, with studies showing either increased or no change in mast cell numbers e.g. (O'Sullivan et al., 2000, Barbara et al., 2004, Barbara et al., 2007, Park et al., 2006, Braak et al., 2012). Of importance are the reports of mast cells, which are in closer proximity to the terminals of afferent nerves in the colon of IBS patients (Barbara et al., 2004). Moreover, in IBS, mucosal mast cells release excessive amounts of mediators (histamine, 5-HT and tryptase) which activate ENS neurons and extrinsic sensory neurons, and produce hypersensitivty (Bueno et al., 1997, Vergnolle et al., 2003, Barbara et al., 2006). Similarly, cytokines released by immune cells can sensitise colonic afferents (Xia et al.,

1999, Ibeakanma and Vanner, 2010, O'Malley et al., 2011). Taken together this demonstrates a role for microbiota, and a dysfunctional epithelium in the activation of the immune system which can subsequently cause sensitisation of peripheral nerves, a likely mechanism for abdominal pain in IBS.

1.5.1.1.3 "Silent" nociceptors in peripheral sensitisation

The existence of a separate class of unmyelinated visceral afferents that only respond to stimuli during inflammation, and not under normal conditions, has been speculated (Cervero and Janig, 1992). These "silent" (aka MIAs) nociceptors are proposed to be functionally distinct to other visceral afferents, focused more on injury and inflammation (Cervero, 1994). Evidence for the existence of these "silent" nociceptors comes from the observation that only a small proportion of sacral afferents responded to colonic distension, suggesting some redundancy in the system (Janig and Koltzenburg, 1991). Furthermore, a subset of afferents only responded to mechanical stimuli following the induction of inflammation (Habler et al., 1988, Habler et al., 1990). However, methodological issues such as restricted levels of pre-inflammation distension pressures, just reaching activation threshold for high threshold mechanoreceptors, make a definitive conclusion impossible (Cervero, 1994). For example, these fibres could represent a population of very high threshold afferents not activated by the levels of noxious stimuli present in the viscera. Inflammation and subsequent sensitisation may reduce their activation threshold and increasing their excitability. Hence their functionality may be similar when compared to other high threshold afferents (Cervero, 1994). "Silent" nociceptors are discussed further in chapter 2 part 1.

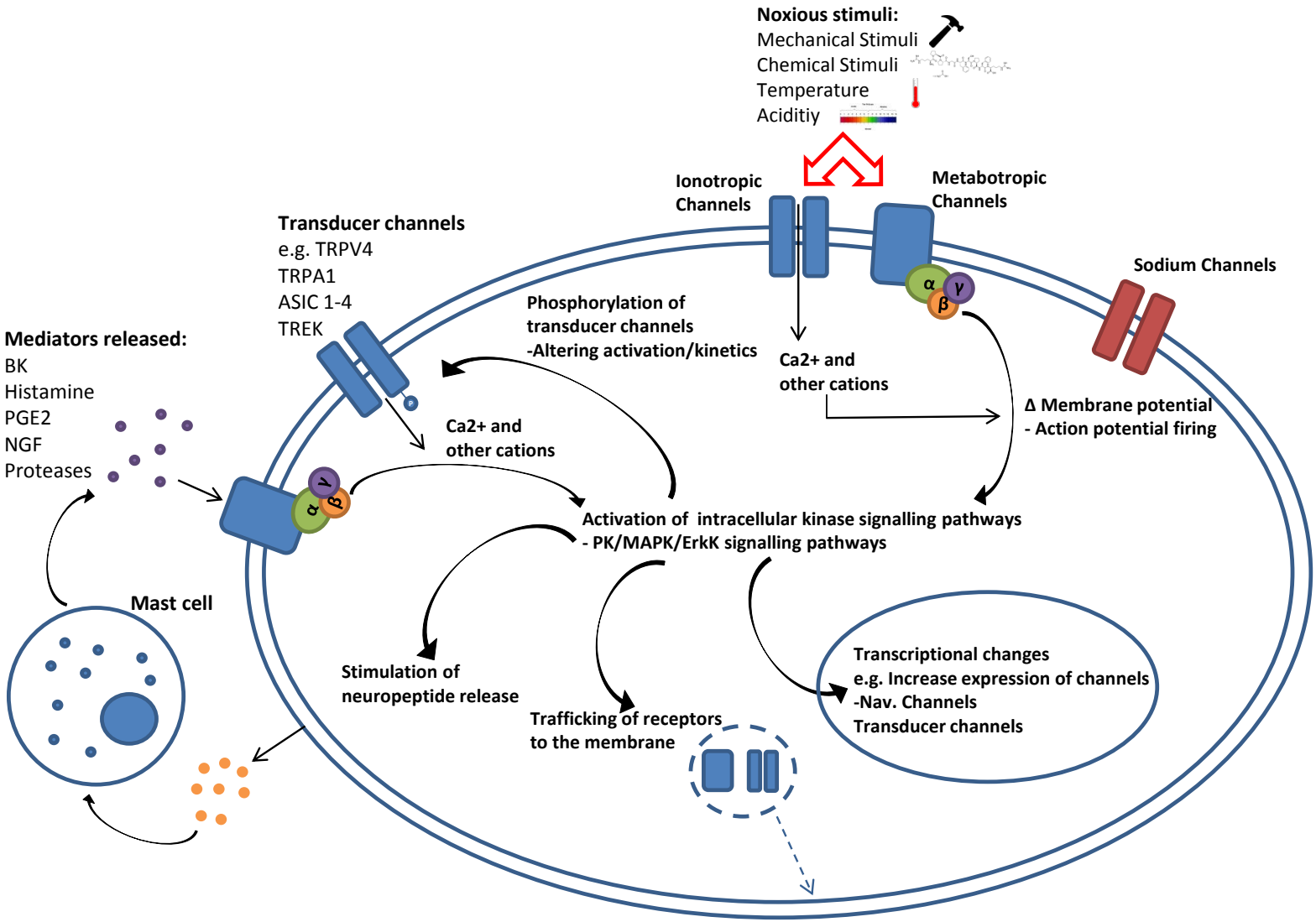

Noxious stimuli:
Mechanical Stimuli
Chemical Stimuli
Temperature
Aciditiy
Ionotropic Channels
Metabotropic Channels
Transducer channels
e.g. TRPV4
TRPA1
ASIC 1-4
TREK
Sodium Channels
Phosphorylation of transducer channels
-Altering activation/kinetics
Ca2+ and other cations
Δ Membrane potential
- Action potential firing
Mediators released:
BK
Histamine
PGE2
NGF
Proteases
Ca2+ and other cations
Activation of intracellular kinase signalling pathways
- PK/MAPK/ErkK signalling pathways
Mast cell
Stimulation of neuropeptide release
Trafficking of receptors to the membrane
Transcriptional changes
e.g. Increase expression of channels
-Nav. Channels
Transducer channels

Figure 1.04: Overview of peripheral sensitisation. Persistent noxious stimuli, such as the release of algogenic mediators or noxious distension, leads to activation of ionotropic and metabotropic channels and continued action potential firing. Ionotropic channels allow the release of cations into the cell increasing neuron excitability. Metabotropic receptors activate downstream intracellular kinase signalling pathways through second messenger systems e.g. protein kinase C, mitogen activated protein kinase. These kinases can alter gene transcription, control the trafficking of receptors to the membrane, and alter activation and kinetics of transducer channels all leading to a change to a more excitable neuronal phenotype. The release of neuropeptides such as calcitonin gene related peptide and substance P is stimulation by kinase signalling pathways. These in turn activate mast cells which release bioamines and growth factors e.g. bradykinin, prostaglandin E_2, and nerve growth factor. These mediators subsequently activate metabotropic channels, leading to the activation of kinase signalling pathways, hence creating a self-sustaining loop.

1.5.1.2 Central sensitisation

Central sensitisation is a process where afferent signalling is modified and augmented in the spinal cord and brain, to produce a greater perception of pain (Vermeulen et al., 2014). Central sensitisation is thought to contribute to the visceral pain reported by IBS and IBD patients. Briefly the sensitisation of visceral afferents in the periphery is thought to trigger an increase in action potential firing sufficient to cause the release of excess neurotransmitters such as glutamate, substance P, at their central terminals in addition to enhancing prostaglandin production and other trophic factors such as brain derived neurotrophic factor (BDNF) (Vermeulen et al., 2014). The excess of mediators leading to activation of N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (AMPA), kainate receptors, mGlu receptors, tyrosine kinase receptors and neurokinin receptors, which in turn causes significant elevation of intracellular Ca^{2+} levels, and hence amplified activation of signalling pathways PKA and PKC and additional downstream events in post-synaptic neurons (Kawasaki et al., 2004). The NMDA glutamate receptor in particular is thought to play a pivotal role in central sensitisation. As a result of prolonged activation within the dorsal horn the NMDA receptor undergoes aberrant phosphorylation, releasing the properties of its voltage dependent magnesium block, and increasing its activity to future synaptic glutamate (Woolf and Ma, 2007). In addition, the trafficking and insertion of the AMPA glutamate receptor may be augmented, increasing the responsiveness of neurons to glutamate (Galan et al., 2004).

Blockade of inhibitory influences can also contribute to sensitisation. For example, prostaglandin E_2 (PGE_2) can block the transmission of glycinergic neurons, which in turn removes the inhibitory control these neurons have on nociceptors in the dorsal horn (Vermeulen et al., 2014). In addition, centrally sensitised neurons can induce secondary hyperalgesia, whereby adjacent neurons are affected leading to hypersensitivity in uninvolved areas of the periphery (Knowles and Aziz, 2009). Alterations in the levels of transcription of certain proteins e.g. substance P in DRGs can also contribute to long lasting central

sensitisation (Neumann et al., 1996, Anand et al., 2007). Furthermore, in IBS, impairment of the ability of the descending pathways to exert their inhibitory effects on sensory pathways, may contribute to central afferent sensitisation (Mayer et al., 2005).

Psychosocial factors may also contribute to central sensitisation. A particular stressful life event, or negative past experience such as childhood or sexual abuse can lead to the development of chronic hypervigilance to normal physiological stimuli, and the development of allodynia and hyperalgesia (Anand et al., 2007). Indeed, this is the case in IBS and other FGID patients, who demonstrate long term hypervigilance of the viscera (Labus et al., 2004). Symptoms of IBS often develop in personally stressful times (Mertz, 2002, Dickhaus et al., 2003). Consistent with these clinical observations, animal models of stress have reported visceral hypersensitivity to colorectal distension (CRD) paradigms (Stam, 1996, Coutinho, 2002, Schwetz, 2004). Excess cortisol, released through activation of the hypothalamic-pituitary-adrenal (HPA) axis, during stress, is likely to play a role in visceral hypersensitivity (Lembo et al., 1996, Lechner et al., 1997). Mast cell degranulation (stress), 5-HT_3 receptors and prostaglandins may also be involved, although a thorough understanding of the mechanisms remains elusive (Gue et al., 1997, Botella et al., 1998). A summary of central sensitisation can be found in figure 1.05.

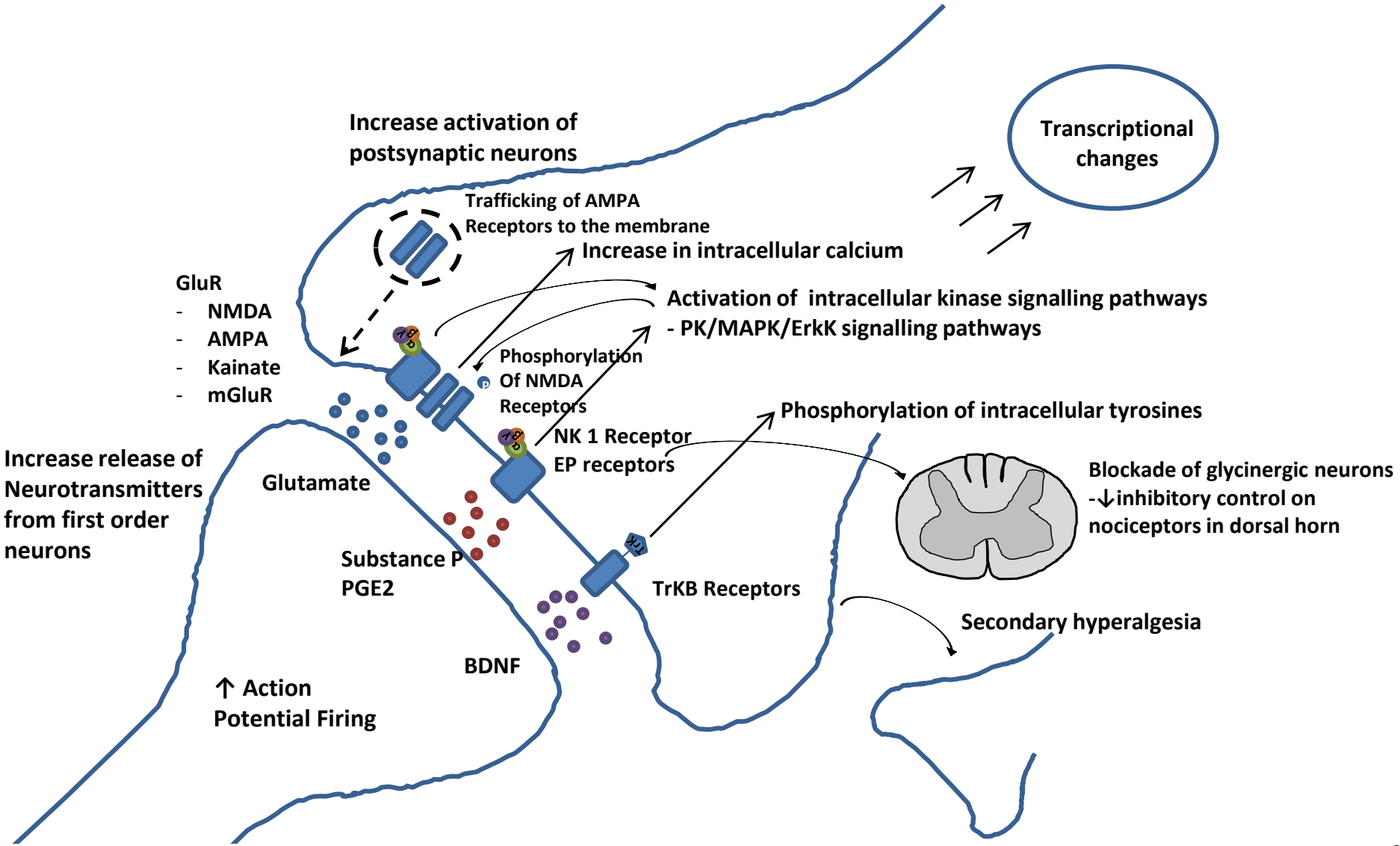

Increase activation of postsynaptic neurons
Transcriptional changes
Trafficking of AMPA Receptors to the membrane
Increase in intracellular calcium
GluR
- NMDA
- AMPA
- Kainate
- mGluR
Activation of intracellular kinase signalling pathways
- PK/MAPK/ErkK signalling pathways
Phosphorylation Of NMDA Receptors
Phosphorylation of intracellular tyrosines
NK 1 Receptor
EP receptors
Increase release of Neurotransmitters from first order neurons
Glutamate
Blockade of glycinergic neurons
-↓inhibitory control on nociceptors in dorsal horn
Substance P
PGE2
TrKB Receptors
Secondary hyperalgesia
BDNF
↑ Action Potential Firing

Figure 1.05: Overview of central sensitisation. Increased action potential firing in presynaptic neurons leads to increase neurotransmitter release e.g. glutamate, substance P, and brain derived neurotrophic factor (BDNF). The subsequent activation of ionotropic and metabotropic receptors leads to increased intracellular calcium, the activation of kinase signalling pathways, and the phosphorylation of intracellular tyrosines, all of which lead to increased neuronal excitability. The phosphorylation and subsequent release of the magnesium block in N-methyl-D-aspartate (NMDA) receptors, and the increase trafficking of α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (AMPA) receptors to the membrane result in an increase sensitivity to synaptic glutamate. Long term central sensitisation can occur when continued activation of these channels leads to alterations in gene transcription. The activation of EP receptors by prostaglandin E_2, can lead to a blockade of glycinergic neurons, and the subsequent removal of their inhibitory influence on nociceptors in the dorsal horn. Centrally sensitised neurons can induce secondary hyperalgesia, whereby adjacent neurons are affected leading to hypersensitivity in uninvolved areas of the periphery.

1.6 TRANSLATION

Treating visceral pain will address one of the primary symptoms of both IBS and IBD, and significantly improve the quality of life of patients. Recently, considerable investment has been expended in an attempt to develop novel compounds for the treatment of visceral pain (Mayer et al., 2008). Although some effective compounds were developed e.g. alosetron and tegaserod, many other compounds failed to show efficacy in clinical trials, notably the kappa opioid agonist fedotozine, and the neurokinin 3 receptor (NK3-R) and M3 muscarinic receptor antagonists talnetant and darifenacin respectively (Mayer et al., 2008, Blackshaw, 2014). Indeed, unwanted side effects from both alosetron and tegaserod meant they were subsequently withdrawn from the market. Given the vast incidence of abdominal pain, this area represents a great unmet clinical need, which also has substantial economic impact (Blackshaw, 2014).

One reason for the failure of these novel analgesics is a lack of translation studies that can bridge the gap between our findings from animal studies and human disease pathophysiology prior to embarking on clinical trials. Although, the use of animal models has clearly facilitated our understanding of the pathogenesis of many diseases, the ability of animal experiments to forecast the efficacy of novel treatments in the human condition is a contentious issue (Hackam and Redelmeier, 2006, Hackam, 2007, Perel et al., 2007). For example several putative visceral analgesics that have failed in clinical trials have shown efficacy in animal experiments.

There are many possible explanations for this poor translation such as imperfect clinical trial design, lax methodologies in animal studies and publication bias (van der Worp et al., 2010). However, perhaps the most fundamental reason is the biological species difference between animals and humans. For example there are clear genetic, physiological and phenotypic differences between mice and humans that must be taken into consideration.

These include differences in the regulation of gene transcription (Odom et al., 2007), physiological parameters such as heartbeat, and body size. Indeed, the differential expression of mediators and use of distinct transduction pathways and signalling cascades in various species underlies the translational problems between animal and man (Schemann, 2011). Collectively, these translational issues highlight the potential usefulness for human pre-clinical models of disease.

Bringing a compound all the way from pre-clinical studies to the marketplace is a long and complicated process. To identify a compound with potential, hundreds of thousands of others may first need to be tested (Chaplan et al., 2010). This is confounded by the estimated 90% failure rate of these identified compounds during the 3 main phases of clinical trials. Furthermore, this process is extremely expensive, with the current estimated cost of between US $800 million and US $1.7 billion for getting a drug to the market (DiMasi et al., 2003, Adams and Brantner, 2006, Collier, 2009). Expenditure on clinical trials is a significant portion of this sum (Chaplan et al., 2010). The main reasons for the failure of a drug in clinical trials are; clinical safety; human pharmacokinetics, and poor efficacy (Fredheim et al., 2008, Hermann and Ruschitzka, 2009). Indeed, as mentioned, a number of drugs for visceral pain have succumbed to this fate, e.g. talnetant (Houghton et al., 2007).

The selection of novel targets as potential treatments for visceral pain has largely focused on either centrally modulating the pain pathway itself (central) or blocking mediator driven activation of visceral nociceptors (Bulmer and Grundy, 2011). Compounds targeting central mechanisms can be more efficacious compared to peripheral targets, but generally cause more side effects hindering their progression in clinical trials. Recently there has been more focus on modulating receptors and ion channels, on visceral afferent endings themselves, potentially combining the advantages of central and peripheral based targets (Bulmer and Grundy, 2011).

1.6.1 A pre-clinical model of visceral pain

The translational limitations of animal research can be addressed by using isolated human tissue, in which potential therapeutics, and disease mechanisms may be studied in a physiologically relevant model. Isolated human tissue approaches are widely used in gastroenterology to study motility, secretion and more recently the enteric nervous system, with great success (Cox and Tough, 2002, Banks et al., 2005, Schemann et al., 2005, Buhner et al., 2009, Broad et al., 2012, Broad and Sanger, 2013, Cirillo et al., 2013). A pre-clinical human model of visceral pain directly focused on afferents innervating the intestines has been recently pioneered (Peiris et al., 2011). This preliminary report describes the electrophysiological recording of spontaneous afferent activity from human appendix and colon (Peiris et al., 2011). Chemical mediators and novel compounds can be applied to this preparation to assess their effect on human visceral afferent (HVA) nerves. The report describes increased afferent activity in appendix preparations treated with an inflammatory soup of chemical mediators ATP, adenosine, BK, histamine, 5-HT and PGE_2 or with capsaicin. Colonic afferent responded to blunt probing with a 0.8 mm VFH. Another brief study, demonstrated spontaneous activity and HVA responses to capsaicin (Jiang et al., 2011). In addition, this study reported HVA responses to mechanical stimuli, including, VFHs, circumferential and longitudinal stretch and mucosal stroking.

These findings demonstrate the feasibility of recording from afferent nerves in human viscera. This pre-clinical human model of visceral pain could be used to help test the pharmacokinetic properties and the efficacy of potential therapeutics in humans, many years before they are entered into costly clinical trials (Peiris et al., 2011). Sensory GI specific side effects could be tested for drugs not intended for GI diseases, examining their capability to alter extrinsic visceral afferent firing patterns (Schemann, 2011). Furthermore, this model could be used to elucidate the signal transduction mechanisms in human tissue and the properties of ionotropic and metabotropic receptors in human GI nociception (Peiris et al.,

2011, Jiang et al., 2011). Alterations in these mechanisms could then be identified in diseased states (Schemann, 2011). However, a robust characterisation of this model is of foremost importance. Splanchnic and pelvic afferents from murine models have been characterised according to their response to different mechanical stimuli, VFH probing, mucosal stoking, and circumferential stretch. Five types of colonic afferents were reported; mesenteric, serosal, muscular, muscular/mucosal, and mucosal (Lynn and Blackshaw, 1999, Brierley et al., 2004). Serosal and mesenteric afferent terminals have also been described as one subset of afferent terminals termed vascular afferents (Zagorodnyuk et al., 2010). The HVA model requires similar characterisation in these terms. Indeed, preliminary mechanical characterisation has been reported although with low n numbers (Jiang et al., 2011). The mechanical thresholds of these populations of terminals also need to be delineated.

The responses to inflammatory mediators in visceral afferents in animal models are well characterised in murine models e.g. (Haupt et al., 1983, Kreis et al., 1998, Lynn and Blackshaw, 1999, Brunsden and Grundy, 1999, Hicks et al., 2002, Brierley et al., 2005a, Wynn and Burnstock, 2006, Song et al., 2009). Preliminary characterisation of HVA responses to an inflammatory soup and to capsaicin has been demonstrated (Peiris et al., 2011, Jiang et al., 2011). Of importance is the characterisation of the responses of HVAs to individual inflammatory mediators, and their respective receptor involvement (Schemann, 2011). In addition, examination of the application of inflammatory mediators or transducer channels (e.g. TRP channels) agonists and antagonist on the subsequent responsiveness of HVAs to mechanical and chemical stimuli will be possible.

1.7 AIMS

- Develop a model of human visceral pain
    - Characterise functional subtypes of HVAs based on their response to mechanical and chemical stimuli. Particular emphasis will be put on identifying the subtypes of HVAs involved in the processing of pain, termed visceral nociceptors.
    - Develop a chemosensitivity protocol suitable for mechanistic studies and for investigating the potential effects of therapeutic drugs on HVA chemosensitivity.
    - Additionally, we sought further evidence for a role of these characterised visceral nociceptors in pain by examining their response to clinically effective visceral analgesics.

Note: This project was funded by the Dr. Hadwen trust for humane research. This organisation funds projects that directly aim to reduce or replace animal experiments. The use of animal tissue, animal cell lines, human fetal cell lines, embryonic tissues, embryonic cells or cell lines, certain monoclonal antibodies and tissue culture serums for experimentation are forbidden. Therefore, all experiments carried out in this report are conducted on ethically obtained resected human tissue.

CHAPTER 2 PART 1: CHARACTERISATION OF SUBTYPES OF AFFERENTS INNERVATING THE HUMAN INTESTINE

The primary aim of this chapter was to characterise HVAs innervating intestinal flat sheet and appendix preparations. Furthermore, the involvement of transient receptor potential vanilloid 4 (TRPV4) receptors on the mechanosensitivity of HVAs is also examined. This chapter is split into 2 parts. Part 1 describes the characterisation of different subtypes of HVAs innervating a flat sheet intestinal preparation, based on their response to mechanical stimuli. In addition, the role of TRPV4 receptors on response of serosal HVAs to VFH probing is investigated. Part 2 outlines the characterisation of distension sensitive afferents innervating the human appendix, based on their pressure threshold for activation, their firing rate, and the pressure at which the firing rate plateaus. The involvement of TRPV4 receptors in the mechanotransduction of appendix distension in HVAs is investigated.

2.1.1 INTRODUCTION

2.1.1.1 OVERVIEW OF THE EXTRINSIC INNERVATION OF THE GUT

Chapter 1 describes in detail the different subpopulations of afferents innervating the intestine of rodents. Six main functional subtypes of afferent nerves have been described, namely, mesenteric, serosal, “silent”, muscular, muscular-mucosal, and mucosal afferents.

Mesenteric afferents are located in the mesentery, are restricted to the splanchnic nerve (not found in the pelvic nerve), and respond to probing of the mesentery and to high intensity stretch, which is transduced to the mesentery via longitudinal forces (Hughes et al., 2009a). Serosal afferents originate from the splanchnic or pelvic nerves and terminate in the

serosa. They respond to direct probing, and also to high intensity stretch. Both mesenteric and serosal afferents are often found in close association with blood vessels leading some authors to refer to them as vascular afferents. In addition a third population of vascular afferents have been proposed. “Silent” afferents are normally unresponsive to any mode of mechanical stimuli, however, following the release of inflammatory mediators such as BK, PGE_2, and 5-HT, these afferent fibres become sensitised and may subsequently respond to probing, but not mucosal stroking or stretch. Hence, the majority of “silent” afferents are likely to have vascular endings.

Muscular afferents are found in the smooth muscle wall of the gut and are found within the vagal, splanchnic and pelvic nerves. Vagal muscular afferents have 2 distinct endings, IMAs and IGLEs. IMAs consist of long axons tracking parallel to the respective muscle layer, with shorter perpendicular branches and transmit information on muscle stretch and length. IGLEs have endings parallel to the muscle fibres in the wall of the intestine, with fine branching endings that extend into the myenteric plexus, allowing them to respond in-series to mechanical tension (Brookes et al., 2013). Splanchnic and pelvic muscular afferents are responsive to stretch and blunt probing of the tissue, but not to mucosal stroking. Pelvic afferents may also have endings in the muscle of the rectum, similar to those of IGLEs, which respond to distension and contraction and are so called rIGLEs (Lynn et al., 2003).

Muscular-mucosal afferents can originate from the pelvic nerve, or from the vagal nerve (termed tension-mucosal afferents). They are thought to be located in either the muscularis externa, and the laminar propria (Page and Blackshaw, 1998, Blackshaw and Gebhart, 2002, Brierley et al., 2004) or in the subepithelial plexus (Zagorodnyuk et al., 2010, Brookes et al., 2013). They are responsive to stroking of the mucosa and to stretch.

Mucosal afferents are located in the mucosa of the gut. Vagal mucosal afferents respond to mucosal stroking but not distension or stretch, and display chemosensitivity. Spinal

mucosal afferents can originate from the splanchnic or pelvic nerve and are responsive to mucosal stroking but not stretch. Spinal mucosal afferents are also chemosensitive. Mucosal afferents constitute a much higher proportion of afferents in the vagal compared to the spinal pathway.

2.1.1.2 GRADED RESPONSES TO VFH PROBING

All subtypes of mouse splanchnic and pelvic afferents exhibit graded responses to increasing weighted VFHs (Brierley et al., 2004). It has been suggested that splanchnic afferents may require a larger stimulus for activation. Indeed, both serosal and muscular pelvic afferents demonstrated significantly higher rates of afferent firing in response to each VFH probing compared to their respective subtypes in the splanchnic pathway. In addition, although comparisons across pathways for each respective afferent subtype, serosal and muscular, reveal similar proportions are activated by 0.07g, the lowest weight VFH used, a heavier VFH was required to activate 100% of splanchnic serosal or muscular afferents compared to the pelvic pathway (Brierley et al., 2004, Brierley et al., 2005b, Brierley et al., 2009, Hughes et al., 2009a).

Only 1 study has presented data, allowing comparisons between the mechanical sensitivity profiles of serosal and muscular afferents. A lower proportion of serosal afferents were activated at each VFH weight, compared to muscular afferents (up to 2g splanchnic and 1g pelvic, the weight at which 100% of fibres were activated) (Brierley et al., 2004). There was no difference in the rate of action potential firing in response to any VFH weight between serosal and muscular afferents in the pelvic pathway. In the splanchnic pathway, the response rate to VFH probing was similar in serosal and muscular subtypes, except at the heaviest VFH (4g) at which serosal afferents displayed higher response rates (Brierley et al., 2004). Taken together, the lower responsiveness to lighter VFHs and greater firing rate upon heavier VFH

probing may tentatively suggest a greater role for serosal afferents in transmitting noxious stimuli, especially in the splanchnic pathway, compared to muscular afferents.

To date only 1 paper has examined subtypes of visceral afferents innervating the human gut (Jiang et al., 2011). This study described 2 serosal, 2 muscular, and 1 muscular-mucosal afferent innervating the human colon. The aim of the present report is to expand on these initial findings, and describe the different subtypes of afferents innervating the human intestine based on their response to mechanical stimuli. Furthermore, differences in the intensity of the stimulus required for activation of each subtype will be examined.

2.1.1.3 SPONTANEOUS ACTIVITY

Spontaneous activity has previously been reported in HVAs (Peiris et al., 2011, Jiang et al., 2011). Two different types have been described, an irregular firing pattern, and a burst firing pattern. The regular firing pattern consists of infrequent firing often combined with long periods of quiescence of up to 60 seconds, where no action potential firing was evident (Peiris et al., 2011). This type of activity was evident in colon and appendix specimens, with comparable firing rates of 2.0 and 2.4 spikes s^{-1}, respectively. The bursting firing pattern was evident in HVA recordings and was characterised by bursts of action potentials separated by short lag periods of 10-15 seconds (Jiang et al., 2011). The bursting pattern was theorised to be related to ongoing contractile activity of the smooth muscle of the gut, since this burst firing pattern was only evident in units sensitive to stretch (Jiang et al., 2011). Similar bursting firing patterns have been demonstrated in both vagal and spinal pathways and in viscerofugal afferents (Page and Blackshaw, 1998, Page et al., 2002, Jiang et al., 2011, Hibberd et al., 2012). This bursting pattern was evident in tension-mucosal afferents in the oesophagus of the ferret and mouse (Page and Blackshaw, 1998, Page et al., 2002).

Reports on the proportions of each afferent subtype exhibiting spontaneous activity in splanchnic pathway are varied and conflicting, with substantially different proportions

described between studies and species. For example, 27%, 37%, 40% of mesenteric, serosal and muscular mouse splanchnic afferents, respectively, exhibited spontaneous activity (Brierley et al., 2004). However, a study on the same afferent pathway in the same species, by the same group some years later stated that no spontaneous activity was evident in any afferent subtype (Hughes et al., 2009a). Up to 80% of splanchnic distension sensitive afferents have been reported to exhibit spontaneous activity (Haupt et al., 1983), while a number of studies have reported a lack of spontaneous activity in all subtypes of splanchnic afferents innervating mouse and rat colon (Hicks et al., 2002, Page et al., 2005, Hughes et al., 2009a). However, a consensus exists on the spontaneous activity of pelvic afferents. Afferents of any subtype in the pelvic pathway do not exhibit spontaneous activity in murine models (Bahns et al., 1987, Janig and Koltzenburg, 1991, Brierley et al., 2004, Hughes et al., 2009a). The only exception is pelvic distension sensitive afferents, of which up to 96% are spontaneously active (Sengupta and Gebhart, 1994, Su and Gebhart, 1998).

The proportion of each subtype of vagal afferent exhibiting spontaneous activity, although seemingly measured are not often mentioned in the literature. One study, suggests 33.3% of mucosal and 64.1% of tension-mucosal vagal afferents displayed spontaneous activity (Page et al., 2002). Vagal tension receptors also display spontaneous activity (Page and Blackshaw, 1999).

The rate of spontaneous activity, when evident in splanchnic nerves is remarkably consistent between afferent subtypes. Spontaneous activity is very low in all subtypes ranging between 0.1 – 0.7 spikes s^{-1} in mesenteric, serosal, muscular, mucosal and distension sensitive splanchnic afferents (Blumberg et al., 1983, Lynn and Blackshaw, 1999, Brierley et al., 2004). Distension sensitive afferents, the only spontaneously active subtype in the pelvic pathway, displayed rates of 3-10 spikes s^{-1}, considerably higher than any subtype of splanchnic afferent (Janig and Koltzenburg, 1991, Sengupta and Gebhart, 1994). In the vagal pathway, mucosal afferents exhibited the lowest spontaneous activity rates, <1 spike s^{-1}. Both tension-mucosal,

~3 spikes s^{-1}, and tension sensitive afferents, 3-10 spikes s^{-1}, displayed higher rates of spontaneous activity (Page and Blackshaw, 1999, Page et al., 2002, Zagorodnyuk et al., 2003, Page et al., 2005).

Many studies have reported a reduction in the spontaneous firing rate immediately after the cessation of a mechanical stimulus. A brief inhibition of spontaneous activity was evident after the cessation of VFH probing of a receptive field in rat colon (Lynn and Blackshaw, 1999). In addition, in HVAs a reduction of the spontaneous firing rate was obvious after blunt probing of the mucosa (Jiang et al., 2011). Similarly, following the cessation of stretching of the colon wall in rats and humans, by either circumferential or longitudinal stretch or distension, spontaneous firing rate was transiently reduced (Lynn and Blackshaw, 1999, Andrew and Blackshaw, 2001, Zagorodnyuk et al., 2003, Jiang et al., 2011). These studies have not examined the mechanism or importance of this phenomenon.

2.1.1.4 TRP CHANNELS

TRP channels are a diverse superfamily of cation channels (Montell and Rubin, 1989, Wong et al., 1989, Hardie and Minke, 1992, Zhu et al., 1995). Seven subfamilies of TRP channels have now been identified; TRPC (TRP cation channel canonical), TRPV, TRPM (TRP cation channel melastatin), TRPA, TRPP (TRP cation channel polycystin), TRPML (TRP cation channel mucolipin), TRPN (TRP cation channel no mechanoreceptor potential C) (Montell and Rubin, 1989, Walker et al., 2000). TRP channels share a similar basic structure consisting of 4 identical subunits each with 6 transmembrane (S1-S6) domains. Both the N and C termini are in the cytoplasm (Gaudet, 2007). The S5-S6 domains of each subunit face centrally and together form the pore and selectivity filter. The pore spans the membrane to form a passage from the extracellular matrix to the cytoplasm. The selectivity filter, dictates which ions can pass by its electrostatic and stereochemical properties. A gate is formed by the cytoplasmic region of the S6 domain. The gate receives its information from the sensor, comprised of S1-S4 domains,

which can sense voltage changes (Gaudet, 2007). A number of protein interaction motifs have been identified on both the N and C termini of TRP channels including; ankyrin repeats, homology regions, TRP box, PDZ domain, phospholipase-C-interacting kinase, and endoplasmic reticulum retention domains. The combination of these cytoplasmic motifs varies considerably between TRP subfamilies, often determining sensitivity to various stimuli as well as structural properties such as the assembly of subunits into a functional channel (Clapham, 2003, Gaudet, 2007).

The majority of TRP channels conduct cations non-selectively with the exception of TRPM3a1/4/5 (sodium-selective) and TRPM3a2/TRPV5/6 (calcium-selective) (Wu et al., 2010). Therefore, upon activation of TRP channels, cells depolarise, causing a myriad of downstream signals. TRPs can be regulated by calcium, phosphatidylinositol 4, 5-bisphosphate (PIP2) and phosphorylation (Voets and Nilius, 2007, Wu et al., 2010). Most TRP channels can be activated by a variety of means including receptors such as receptor tyrosine kinases and GPCRs, various ligands including endogenous and exogenous molecules, calcium and magnesium ions and directly by temperature and mechanical stimuli (Ramsey et al., 2006). This polymodality suggests a role for TRPs as cell sensors (Clapham, 2003). Furthermore, TRPs are expressed in all cell types (Wu et al., 2010). TRP channel sensitivity to stimuli will therefore be within the context of a particular cell and its environment, including the concentration of ions, ligands, and proteins (Ramsey et al., 2006).

2.1.1.4.1 TRPV channels

There are 6 members of the TRPV family, TRPV1-6. TRPV channels are divided into 2 TRPV subgroups, TRPV1-4, which are cation channels, marginally selective to calcium, and sensitive to small changes in temperature (Caterina et al., 1997, Caterina et al., 1999, Peier et al., 2002, Guler et al., 2002), and TRPV5-6, which are cation channels, highly selective for calcium and which do not respond to changes in temperature (Vennekens et al., 2000, Yue et al., 2001).

2.1.1.4.1.1 TRPV4 channels

TRPV4, originally called OTRPC4, TRP12, VRL2, or VR-OAC, was first discovered using murine cDNA encoding the TRPV channels to search genomic libraries for similar sequences (Liedtke et al., 2000, Strotmann et al., 2000). TRPV4 was initially described as an osmosensor, opening upon small decreases in osmolarity (Liedtke et al., 2000, Strotmann et al., 2000). It has subsequently been demonstrated that TRPV4 exhibits gating promiscuity and can be activated by warm temperatures (27-35°C), phorbol compounds, lipid derivatives, metabolites e.g. 5,6-epoxyeicosatrienoic acid (EET), mechanical stimuli, as well as the small molecule GSK1016790A (Guler et al., 2002, Watanabe et al., 2002, Watanabe et al., 2003, Brierley et al., 2008, Jin et al., 2011).

TRPV4 is 871 amino acids long and has 40% homology to TRPV1 and TRPV2 (Liedtke et al., 2000, Strotmann et al., 2000, Everaerts et al., 2010). TRPV4 shares a basic structure with the other TRP channels, consisting of 6 transmembrane domains in each of its 4 subunits that combine to form a tetramer. TRPV4 contains 3 ankyrin repeat domains (ARD) on its N terminus (Liedtke et al., 2000). A proline rich domain (PRD) resides close to the first ARD. These 2 N terminal motifs are thought to be important in TRPV4 formation into a tetramer and its mechanical sensitivity respectively (Gaudet, 2007, D'Hoedt et al., 2008, Everaerts et al., 2010). A sequence of 6 highly conserved amino acids make up the TRP box located on the C terminus of TRPV4. Further C terminal protein interaction motifs are present on TRPV4 including a PDZ domain and a calmodulin binding domain, which is critical in the calcium-dependent regulation of TRPV4 (Strotmann et al., 2000, Garcia-Elias et al., 2008). TRPV4 is widely expressed in tissues including the renal system (Tian et al., 2004), cornea (Pan et al., 2008), skin (Chung et al., 2003), DRG (Facer et al., 2007, Cenac et al., 2008), peripheral nerves (Alessandri-Haber et al., 2003, Facer et al., 2007) and sensory nerves innervating the gut (Zhang et al., 2005, Brierley et al., 2008). Indeed, TRPV4 IR has been demonstrated around serosal vessels in human colon (Brierley et al., 2008).

2.1.1.4.1.1.1 TRPV4 channels in visceral pain

There is evidence for the involvement of TRPV4 in various types of somatic pain (Suzuki et al., 2003, Alessandri-Haber et al., 2003, Alessandri-Haber et al., 2004, Alessandri-Haber et al., 2005, Alessandri-Haber et al., 2006, Grant et al., 2007). TRPV4 has been shown to be a transducer of hypo and hyper-tonicity induced somatic pain in behavioural and electrophysiological experiments. Similarly, TRPV4 mediated somatic pain is potentiated by the application of inflammatory mediators, suggesting a role for TRPV4 in somatic inflammatory pain (Alessandri-Haber et al., 2003, Alessandri-Haber et al., 2005, Alessandri-Haber et al., 2006). In a paw withdrawal paradigm, intraplantar injection of a PAR-2 agonist induces mechanical hyperalgesia in wild type but not TRPV4 KO mice suggesting an involvement of TRPV4 in this phenomenon (Grant et al., 2007).

Recently, TRPV4 has been implicated in visceral pain (Brierley et al., 2008, Cenac et al., 2008, Sipe et al., 2008, Ceppa et al., 2010, Cenac et al., 2010). Injection of the TRPV4 agonist 4-a- phorbol 12,13- idecanoate (4αPDD) into the pancreatic duct induced spinal neuron activation in $TRPV4^{+/+}$ mice but not $TRPV4^{-/-}$ mice, as measured by the expression of the transcription factor c-Fos (Ceppa et al., 2010). Moreover, $TRPV4^{-/-}$ mice exhibit less painful behaviours compared to $TRPV4^{+/+}$ mice after the induction of pancreatitis by abdominal cerulein injections (Ceppa et al., 2010).

The role of TRPV4 in gut sensation is of particular interest, where sensitivity to mechanical stimuli, such as hollow organ distension or traction of the mesentery, is often a cause of pain (Brierley et al., 2008). The TRPV4 agonist EET potentiates afferent firing in response to VFH probing in a mouse colonic electrophysiological preparation. This potentiation is abolished in $TRPV4^{-/-}$ mice (Brierley et al., 2008). Similarly, application of the non-selective TRP antagonist ruthenium red (RR) reduced the afferent firing rate in response to VFH probing in $TRPV4^{+/+}$ mice but not $TRPV4^{-/-}$ mice (Brierley et al., 2008, Sipe et al., 2008). In addition, the

TRPV4$^{-/-}$ mice demonstrated a reduced afferent firing rate (~50%) in response to VFH probing (Sipe et al., 2008). These data suggest a role for TRPV4 in the transduction of intense mechanical stimuli in colonic afferents.

In contrast to these reports, in a behavioural paradigm where VMR are measured using electromyography in response to CRD, TRPV4$^{-/-}$ mice and TRPV4$^{+/+}$ mice have been shown to exhibit similar baseline visceral motor response (VMR) to CRD pressures of 15, 30, 45, 60 mm Hg (Sipe et al., 2008). The authors suggest that this discrepancy may be explained by the high threshold nature of serosal afferents. These afferents may respond to high intensity VFH probing but may not be activated by the pressures reached during CRD. However, it must be noted that 30-60mm Hg are considered noxious pressures, and 60mm Hg is likely enough to activate serosal afferents (Cenac et al., 2008). Furthermore, another study found that mice pre-treated with inter-vertebral injections of TRPV4 targeted silencing ribonucleic acid (siRNA), to eliminate TRPV4 expression, exhibited lower VMR to the noxious 30, 45 and 60 mm Hg CRD pressures compared to mice treated with mismatched siRNA. However, there was no difference in VMR to the innocuous 15mm Hg stimulus (Cenac et al., 2008). This suggests that TRPV4 channels may transduce nociceptive rather than physiological stimuli in gut sensory nerves.

Further evidence implicates TRPV4 channels in the transduction of mechanical stimuli in the presence of inflammation or inflammatory mediators. Colonic afferents from TRPV4$^{+/+}$ mice but not TRPV4$^{-/-}$ mice responded to the pro-inflammatory mediator protease activated receptor 2 activating peptide (PAR2-AP) (Sipe et al., 2008). In addition, TRPV4$^{+/+}$ mice that underwent intra-colonic administration of the pro-inflammatory PAR2-AP prior to CRD showed significantly increased VMR compared to baseline. VMR in TRPV4$^{-/-}$ mice remained unchanged (Sipe et al., 2008). These data imply a role for TRPV4 in inflammation induced afferent sensitisation to mechanical stimuli. Indeed, a number of studies have suggested a role for TRPV4 in the development of hyperalgesia and allodynia, as induced by various inflammatory

mediators (Cenac et al., 2008, Sipe et al., 2008, Cenac et al., 2010). Intra-colonic administration of the pro-inflammatory PAR-AP produced both allodynia and hyperalgesia in a CRD paradigm (Cenac et al., 2008). However, mice pre-treated with TRPV4 targeting siRNA did not develop allodynia or hyperalgesia. Similarly, intra-colonic administration of the TRPV4 agonist 4αPDD induced allodynia and hyperalgesia in $TRPV4^{+/+}$ mice, but not in $TRPV4^{-/-}$ mice or mice pre-treated with TRPV4 targeted siRNA (Cenac et al., 2008). A summary of these findings can be found in table 2.01.

TRPV4 may also mediate hyperalgesia induced by the inflammatory mediators histamine and 5-HT. Hyperalgesia to CRD induced by the intra-colonic administration of histamine or 5-HT was eliminated by pre-treatment with TRPV4 targeted siRNA. In addition, 5-HT but not histamine induced allodynia was inhibited in mice pre-treated with TRPV4 targeted siRNA (Cenac et al., 2010). Taken together these data indicate a clear role for TRPV4 in inflammatory visceral pain.

The role of TRPV4 channels in the transduction of mechanical stimuli in human afferent nerves innervating the bowel has not been studied, although TRPV4 receptors have been shown to be localised around human vessels in the serosa (Brierley et al., 2008). This report will investigate the involvement of TRPV4 channels in the transduction of mechanical stimuli, both distension of the appendix, and VFH probing of the serosal surface, in afferents innervating the human bowel.

Method	Result	Paper
VFH probing in presence/absence of TRPV4 agonist or TRPV4 antagonist	Increased and decreased response to probing in TRPV4$^{+/+}$ but not TRPV4$^{-/-}$ mice in presence of TRPV4 agonist and antagonist, respectively	Brierley et al, 2008
VFH probing in TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice	Decreased response to probing in TRPV4$^{-/-}$ mice	Sipe et al, 2008
Inter-vertebral injections of TRPV4 siRNA or mismatched siRNA	Decreased VMR to noxious CRD when mice were injected with TRPV4 siRNA but not mismatched siRNA	Cenac et al, 2008
Injection of TRPV4 agonist in pancreatic duct	Increased spinal neuron c-fos expression in TRPV4$^{+/+}$ but not TRPV4 $^{-/-}$ mice	Ceppa et al, 2010

Table 2.01: Describes recent findings implicating TRPV4 receptors as transducers of mechanical stimuli in mouse visceral afferents.

2.1.1.5 AIMS

- Examine the sensitivity of HVAs to mechanical stimuli, namely VFH probing, circumferential and longitudinal stretch, and mucosal stroking
- Describe the different subtypes of afferents innervating the human intestine based on their response profile to various mechanical stimuli
    - The intensity of the stimulus required for activation of each subtype will also be examined
- Describe the spontaneous activity in each subtype of HVA
- Ensure repeated VFH probing is reproducible by conducting time matched controls
- Examine the role of TRPV4 channels in the transduction of mechanical stimuli, specifically VFH probing, in human afferent nerves innervating the intestine

2.1.2 METHODS

2.1.2.1 PATIENTS

All experiments were performed in accordance with human ethics regulations (NREC 09/H0704/2). Resected human ileum, colon, and rectum were collected after written consent from patients undergoing elective surgery for cancer, polyps, familial adenomatous polyposis, CD, UC, diverticular disease (DD), trauma, chronic constipation at the Royal London Hospital or Whipps Cross University Hospital (London, UK) (figure 2.01). All tissues were cut by a trained histopathologist following macroscopic examination. “Normal tissue” was obtained from patients with non-obstructive tumours at least 10cm away from the tumour or lymphatic drainage field and from patients with diverticular disease or polyps in areas without evidence of these pathologies (figure 2.06). Tissue from trauma cases was also considered “normal”. Inflamed tissue was collected from patients with UC, CD.

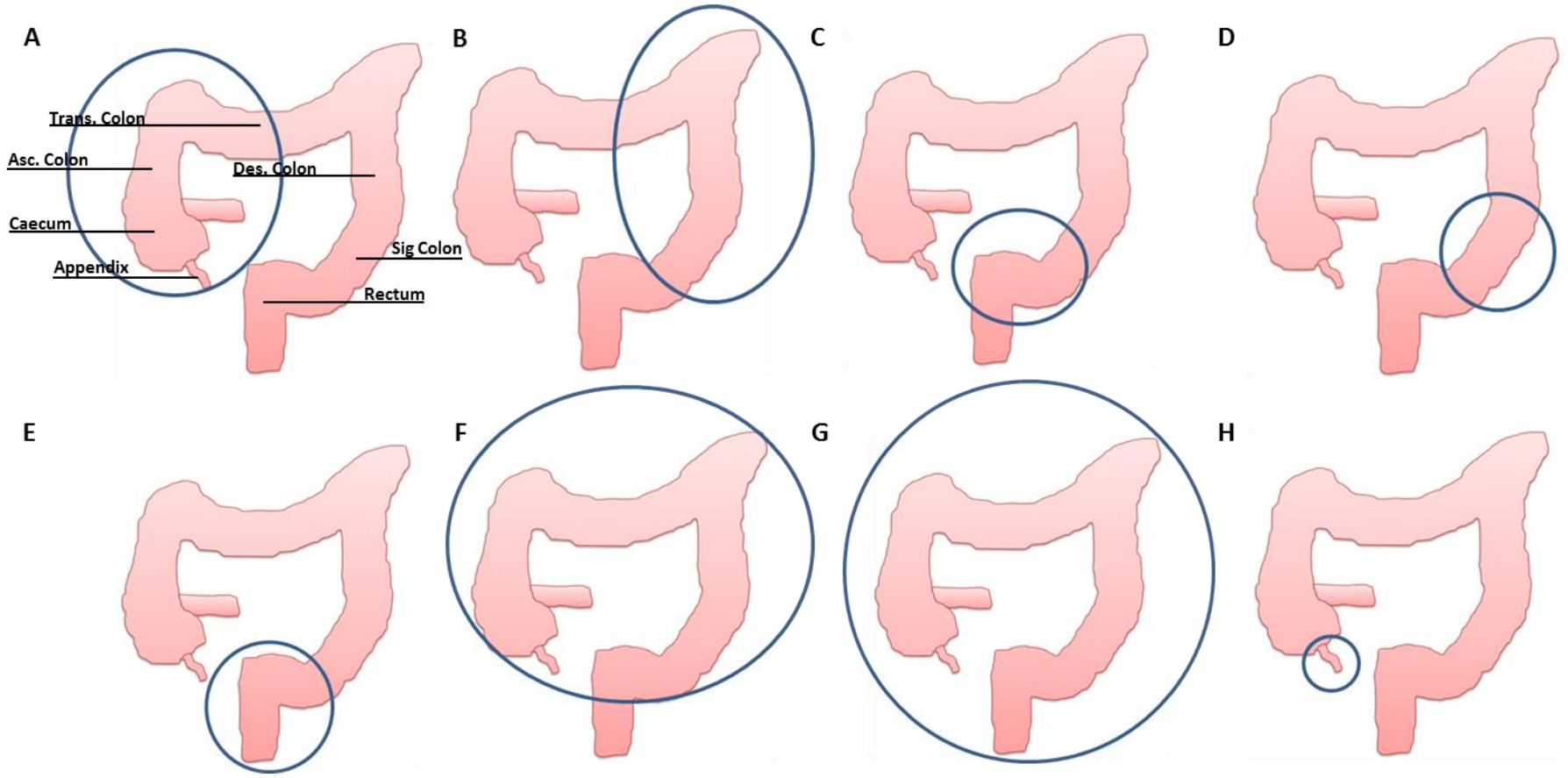


Figure 2.01: Outlines the area of tissue resected during 8 of the most common surgeries from which tissue was collected. A) Right-hemicolectomy, B) left-hemicolectomy, C) anterior resection, D) sigmoid colectomy, E) abdomino perineal of rectum (APER), F) subtotal colectomy, G) panproctocolectomy, H) appendicectomy.

2.1.2.2 ELECTROPHYSIOLOGY RECORDINGS

The majority of experiments took place on the day of surgery. However, in some circumstances tissues were placed in carbongenated Krebs buffer and stored overnight at 4°C (chapter 5 part 2). Firstly, the tissue was grossly examined using a stereomicroscope (M5A, Wild Heerbrugg) and blood vessel arcades identified. Excess mesentery was removed before the tissue was transferred to the tissue bath and pinned out, serosal side up (figure 2.02). The tissue was then superfused with carbongenated (95% O2, 5% CO2) Krebs buffer (6ml/min; 32-34°C; pH 7.4; 124mM NaCl, 4.8mM KCl, 1.3mM NaH_2PO_4, 1.2mM $MgSO_4.7H_2O$, 2.5mM $CaCl_2$, 11.1mM Glucose, 25.0mM $NaHCO_3$). Nerves running in close proximity to the blood vessel arcades were finely dissected using a microscope (SZ40, Olympus). Nerves were then sucked into a boroscilicate glass suction electrode (Harvard Apparatus), which was filled with Krebs buffer and neuronal activity recorded using a differential amplifier (headstage and AC/DC amplifier (gain 5K) (Neurolog Ltd). The analogue signal was then band pass filtered (100-2000Hz; digitally filter using a humbug 50Hz filter(Quest Scientific) following which the resultant signal was digitised at a sampling rate of 20KHz using a Micro 1401 MKII (Cambridge Electronic Design) and displayed a desktop computer running Spike2 software in a chart recorder format. Data was stored for further off line analysis (Cambridge Electronic Design). Additionally neuronal activity was also simultaneously counted from the filtered and amplified signal using a spike processor (Digitimer). The threshold for spike counting was set at twice the background noise and the output from the spike processor sent to the events channel on the 1401 for processing and relay to the desk top computer were it was displayed alongside the raw trace on spike 2. Nerve activity was expressed as a rate histogram as either spikes/$20s^{-1}$, $5s^{-1}$, or $1s^{-1}$ (Peiris et al., 2011). The description of electrophysiological recordings given here is consistent with the recordings in flat sheet preparations in chapters 2, 3, 4 and 5.

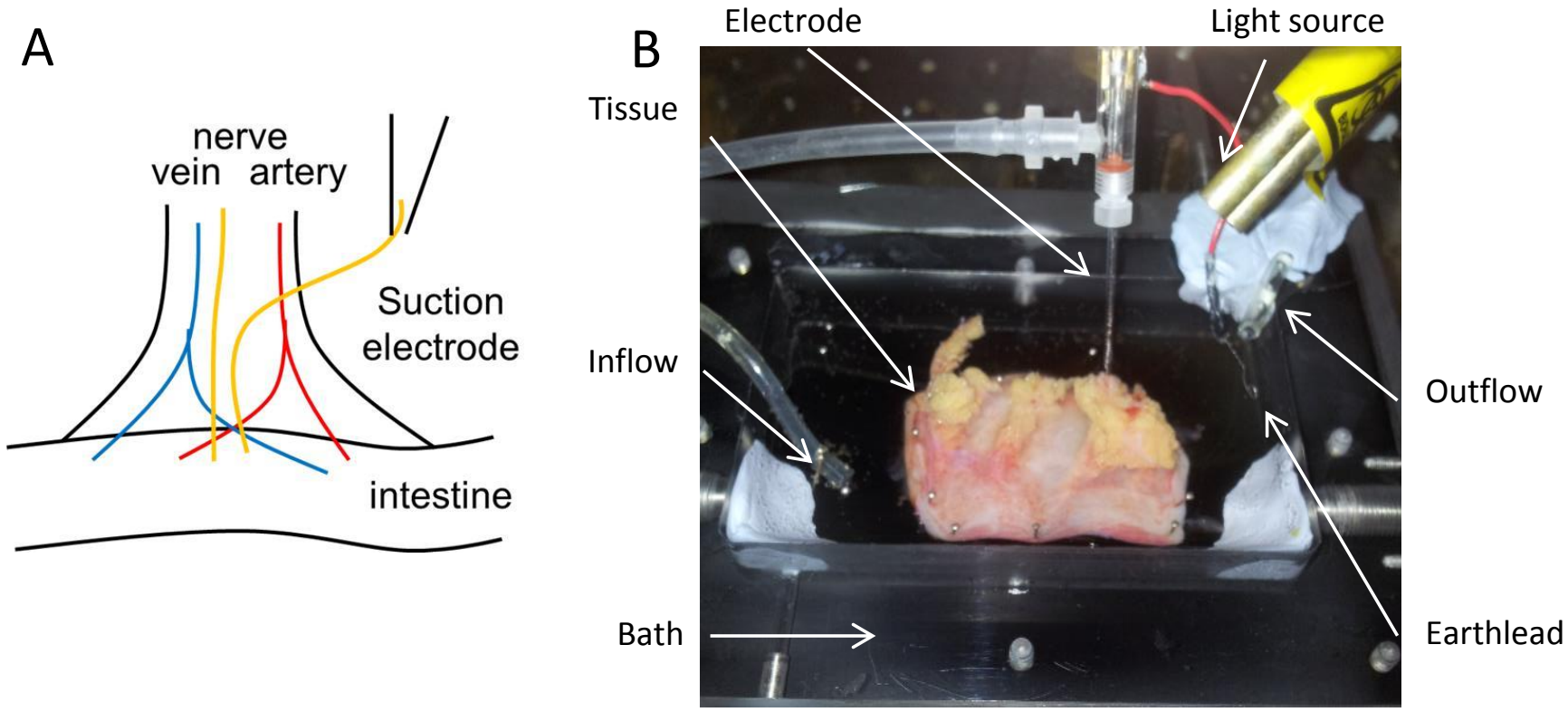


Figure 2.02: A) A schematic of an intestinal nerve in yellow being sucked up by a suction electrode. B) Shows a HVA recording from a piece of human colon, which is pinned in a tissue bath

2.1.2.3 MECHANOSENSITIVITY

2.1.2.3.1 VFH Probing, circumferential and longitudinal stretch, and mucosal stroking

Once a viable recording was attained, baseline firing was recorded for 15 minutes. The tissue was then unpinned at the proximal end and at both sides to allow access to the mucosal surface. To examine the presence of mucosal afferents, a rod, was used to stroke the mucosa systematically to activate mucosal afferent endings. Mucosal stroking was repeated twice more, each repetition separated by 5 minutes. A stretching protocol consisting of both longitudinal (side to side) and circumferential (top to bottom) stretch, was then performed. Stretching was performed by holding the tissue with a rounded forceps and applying a stretching force. Both longitudinal and circumferential stretch were repeated twice more, with 5 minutes in between each stimulus. The tissue was then repinned in the tissue bath. Using a grid based system a cotton bud was used to probe the serosal surface and mesentery to search for a receptive field. Once a receptive area was identified, a 2g VFH (Ugo Basile) was used to isolate the receptive field more specifically (figure 2.03). If the receptive field gave a consistent response to probing, 2 stimulus response curves, using 0.02g, 0.04g, 0.07g, 0.16g, 0.4g, 0.6g, 1.0g, 1.4g, 2.0g, 4.0g VFHs, each curve separated by 5 minutes, were generated (figure 2.04). Awkward tissue contours or problematic locations of the receptive field (e.g. in the mesentery close to the nerve) sometimes hindered the acquisition of consistent responses to probing. A number of preparations could not be tested for all mechanical stimuli, but were deemed to be serosal based on their sensitivity to very low weight VFHs.

A proportion of HVAs were unresponsive to VFH probing. In a small number of recordings (n=5), BK (approximate bath concentration (BC) 20nM or 2µM, 20ml of 100nM or 10uM) was superfused into the tissue bath to sensitise the units to mechanical stimuli. Using a grid based system, a 2g VFH was then used to search for any new receptive fields, after the cessation of any acute excitatory afferent fibre response to BK.

2.1.2.3.2 VFH time matched controls

To determine to reproducibility and stability of repeated 2g VFH probing's, time matched control experiments were performed. In these experiments, no drug was added, but probing continued every 5 minutes as with other experiments. The average of the first 3 sets of probes were then compared to average of the subsequent consecutive sets, i.e. sets 4,5,6, sets 5,6,7 etc.

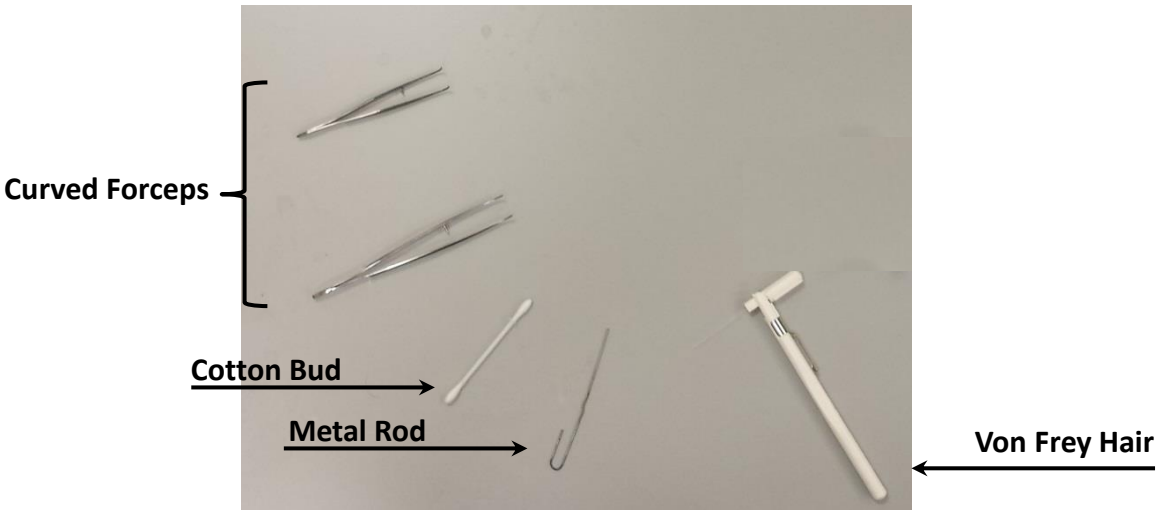


Figure 2.03: Shows the instruments that were used to produce the various modes of mechanical stimuli. The cotton bud and VFHs were used as the probing stimuli. The tissue was stretched using the curved forceps. The metal rod was used to stroke the mucosa.

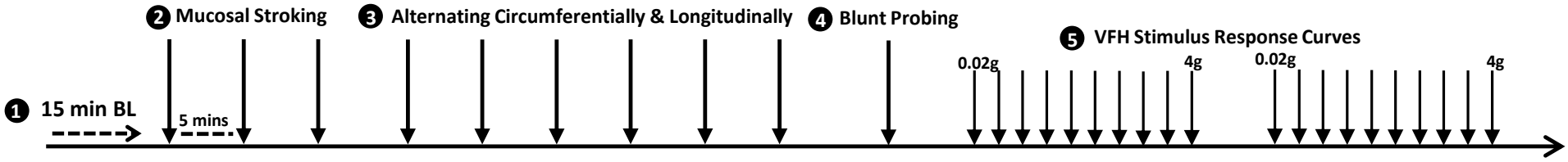


Figure 2.04: Flat sheet characterisation protocol

1) Fifteen minutes of baseline afferent firing is recorded.

2) The mucosa is stroked using a metal rod. This repeated 3 times with 5 minutes between each stroking.

3) The tissue is then stretched both circumferentially and longitudinally, each 3 times. Each stretch is separated by 5 minutes.

4) A cotton bud is then used to search for a receptive field.

5) If a receptive field is found, 2 stimulus response curves are performed using 0.02g, 0.04g, 0.07g, 0.16g, 0.4g, 0.6g, 1g, 1.4g, 2g, and 4g VFH. Five minutes is left between stimulus response curves.

2.1.2.4 MECHANOSENSITIVITY PROTOCOLS

2.1.2.4.1 VFH probing protocol

After mechanical characterisation of afferents, atropine (10µM) and nifedipine (10µM) were added to the Krebs buffer and given 30 minutes to take effect. Once a receptive field was identified and a stimulus responses curve had been attained, the receptive field was probed using either a 0.4g or 2.0g VFH, for 3 sets of 3 x 3 second probes, each set separated by 5 minutes. During TRPV4 experiments, the bath was subsequently superfused with a vehicle solution (0.1% DMSO, 10-20ml) or GSK1016790A (TRPV4 agonist, BC 2µM, 10-20ml of 10µM,) or HC067047 (TRPV4 antagonist, BC 20µM, 10-20ml of 10 µM). In all experiments this was followed with 6-9 sets of 3 x 3 second probes, each set separated by 5 minutes. For analysis, the 2 probes with the highest firing rate in each set of 3 probes were averaged. This was done as accurate probing of the receptive field can be difficult, and 3/3 direct hits is not always achieved. The 3 sets of baseline probes were averaged and compared to the average of the 2 sets of probes at which the drug is at its highest bath concentration i.e. the average of post drug sets 1 and 2 (figure 2.05). The data were analysed using a 2 tailed paired t test, $p<0.05$.

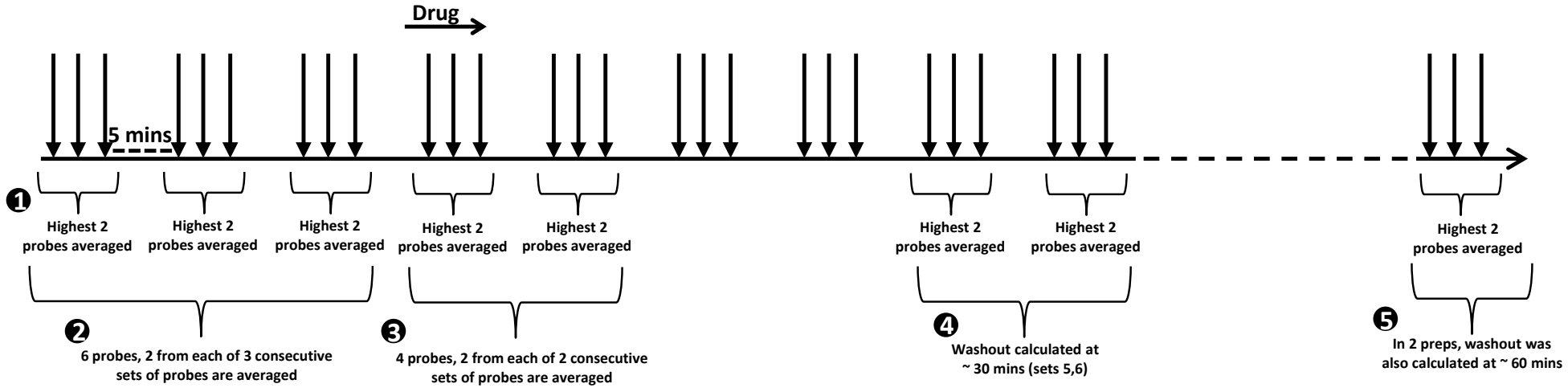


Figure 2.05: TRPV4 VFH probing protocol

1) Average of the 2 highest 2 second probes in each set are averaged

2) The average of the 6 probes, 2 from each of the first 3 consecutive sets is used as baseline.

3) The average of the 4 probes from the first 2 consecutive sets after drug application is then averaged. For drug effect comparisons the average of the baseline probes are compared to the average of the 1st and 2nd post drug sets of probing.

4) For drug vs. washout comparisons the 1st and 2nd post drug sets of probing are compared to the average of the 5th and 6th post drug sets of probing.

5) In 2 experiments enough probes were done so that washout could also be calculated at 60 mins.

2.1.2.5 OFFLINE WAVEFORM ANALYSIS

The HVA recordings were often few fibre recordings made up of action potentials with different shaped waveforms, each distinctive waveform representing the firing from an individual afferent fibre. Using spike 2 waveform analysis software, action potentials that passed a set amplitude threshold could be accurately discriminated using waveform templates. To generate a template, each action potential in the HVA recording was averaged, the DC offset from any incurring noise was removed, before it was either assigned to a relevant template, used to make a new template, or left unassigned. Waveform analysis was also checked by eye by comparing action potentials assigned to different templates together on the raw trace to ensure accurate assignment. Parameters could be tweaked and wave-form analysis repeated if spikes were not accurately discriminated. New nerve waveform channels were then created with the relevant templates. All analysis was performed on these waveform template channels, allowing for the frequency of individual unit firing to be calculated and plotted. Typical parameters were set at 8% for the maximum amplitude change for match to a template, and at least 60% for minimum percentage points in the template. All analysis was done on a HP Compaq computer running Spike 2 5.03 software. A maximum of 18 templates could be accommodated by the software; however typical HVA recordings had 1-5 units, with appendix preparations usually having more than flat sheet preparations. This technique has been previously reported (Richards et al., 1996, Hillsley and Grundy, 1998, Hillsley et al., 1998). The above protocol has been used when conducting waveform analysis on any electrophysiological recording in this thesis.

2.1.2.6 DRUGS

Drugs in powder form were made up using the recommended solutions, aliquoted and frozen at 20°C. When needed, aliquots were diluted in Krebs to make the final working concentration

and vortexed to mix. GSK1016790A was obtained from Sigma Aldrich (St Louis, MO, USA). HC067047 was purchased from Tocris Bioscience (Bristol, UK).

2.1.3 RESULTS

2.1.3.1 OVERALL TISSUE COLLECTION – FLAT SHEET

Consent was obtained from 213 patients. 135 tissues were collected, of which, 108 were used for electrophysiological recordings. Failure to collect a specimen was usually for one of the following reasons; the specimen was put in formalin by theatre staff, the type of operation was changed, surgery was cancelled, or there was no tissue available for research (e.g. large tumour). In total, electrophysiological recordings were successfully made from 84 resected human tissues (95 recordings); ileum (n=15), caecum (n=2), ascending colon (n=8), transverse colon (n=4), descending colon (n=4), sigmoid colon (n=53), rectum (n=9) (figure 2.06). A summary of how tissues were designated to experiments is shown in figure 2.07.

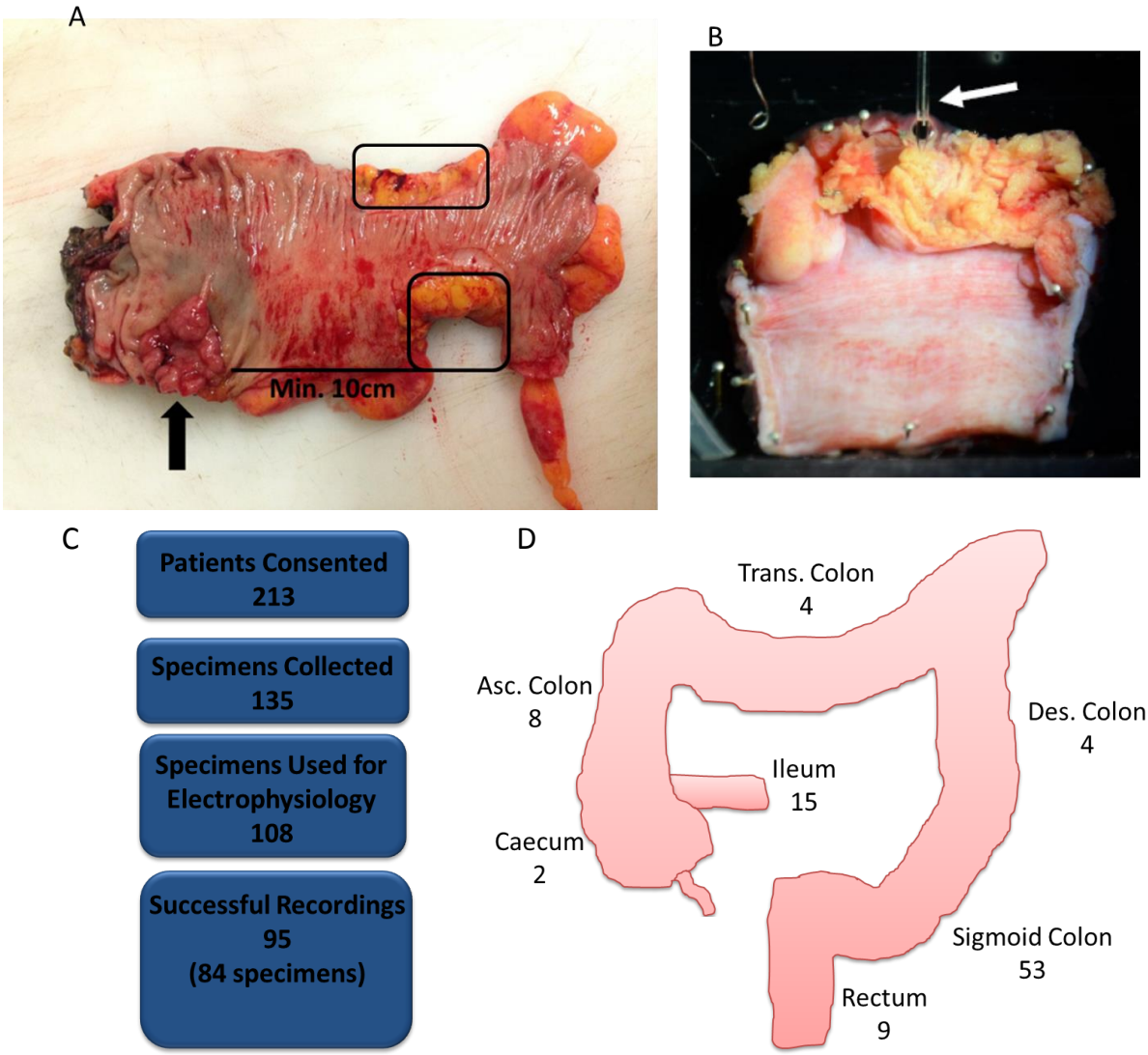


Figure 2.06: A) An example of a colon cancer specimen, cut open along the anti-mesenteric border and lying mucosa side up. The black arrow indicates the tumour. The black boxes indicate where segments of colon were removed for research. Samples are always taken at least 10cm away from the tumour. Continuity of the specimen is always preserved. B) Shows a segment of colon pinned out in a tissue bath with the serosal side up. The white arrow indicates the glass electrode into which the nerve is sucked. C) Diagram illustrating the total numbers of patients consented, specimens collected and recordings made in flat sheet preparations. The numbers of each tissue type collected is also displayed.

2.1.3.2 TISSUE – FLAT SHEET CHARACTERISATION

Twenty-three tissues, 20 normal, 2CD, 1UC, were used for flat sheet characterisation experiments, 18 sigmoid colon, 2 rectum, 2 transverse colon, 1 descending colon (M:F 1:0.39, median age 64).

2.1.3.3 TISSUE – TRPV4 VFH PROBING EXPERIMENTS

Twelve tissues, 10 normal, 2 CD, were used for TRPV4 VFH probing protocols, 6 sigmoid colon, 2 rectum, 2 ileum, 1 transverse colon, 1 descending colon (M:F 1:1.4, median age 53). Further details on the tissues use in each set of experiments can be seen in table 2.02.

Protocol	Sex M:F	Age	Appendix	Ileum	Asc. Colon	Tran. Colon	Des. Colon	Sig. Colon	Rectum
Flat Sheet Characterisation	1:0.39	64 (20-85)				2	1	18 (1)	2 (2)
Distension Characterisation	1:1.22	51 (16-84)	20 (4 ,2, 2)						
TRPV4 (Probes)	1:1.4	53 (20-78)		2 (1)		1	1	6	2 (1)
TRPV4 (Distension)	1:0.5	34 (16-84)	9 (2, 2, 1)						
BK Pharmacology									
- Repeats	1:2	57 (19-63)	1 (1)	2 (1)		1		2	
- HOE140 (300nM)	1:0.5	65 (27-78)	3 (1)				1	1	1
- HOE140 (1µM)	1:0	57 (48-87)						4	
- R715	1:0.2	77 (57-85)	2		1			2	1
ATP Pharmacology									
- Repeats	1:1	46.5 (27-72)	1					2 (1)	
- PPADS	1:0.2	72 (57-85)	2					3	1
- RO4	1:0	56 (55-64)					1	1	1
- CGS 15943	1:1	51.5 (26-85)	2 (1, 1)					4	
5-HT Pharmacology									
- Repeats	1:0.5	24 (24)			1	1		1	
- α-methyl-5-HT maleate	1:1.33	57 (35-78)			1		2	2	2 (1)
- methyl chlorophenylbiguanide hydrochloride	1:0.75	39 (27-84)	2 (1)	1 (1)		1	1	2 (1)	
Histamine									
- Repeats	1:1	43.5 (19-93)	1 (1)					1	
Tegaserod (Probes)	1:0.5	57.5 (45-72)						4	2
Tegaserod (Distension)	1:1	38 (20-76)	6 (2, 1)						
ICI 204, 448 (Probes)	1:2	51 (45-72)						2	1
STa (Distension)	1:1.33	50 (20-82)	7 (1, 1)						
Disease									
- Probes	1:1	54 (20-82)		2 (1)	1		1	10	1 (1)
- Distension	1:1.1	52 (16-84)	19 (3, 2, 2)						
- Chemosensitivity	1:0.76	60 (16-87)	22 (3, 3, 5)	7 (4)	6	4	2	35	8 (1, 1)
Viability									
- Probes	1:1	54 (20-82)		2 (1)	1		1	10	1 (1)
- Distension	1:1.1	52 (16-84)	15 (2, 2, 1)						
- Chemosensitivity	1:1.3	57 (16-87)	20 (3, 3, 6)	7 (4)	5	1	2	35 (1)	8 (1, 1)

Table 2.02: Tissue details for each set of experiments described in this report. Age data expressed as medians (range in parenthesis). Numbers of each tissue type are expressed in each column with an "of which" number in parenthesis, i.e. 4 (of which 1, 1, 1). Colour code; Crohn's disease=RED, ulcerative colitis=GREEN, appendicitis=ORANGE. Other tissues were considered "normal".

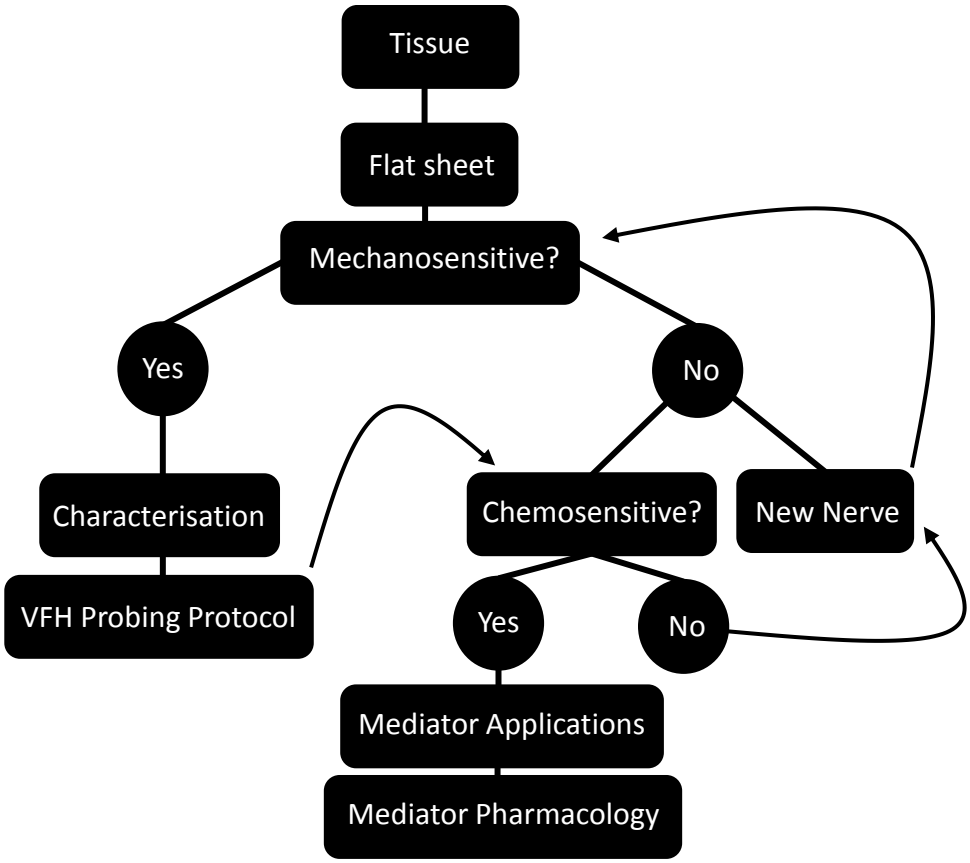


Figure 2.07: Given the limited supply of human tissue, every effort was made to make the most out of each piece. In each preparation, mechanosensitivity of the afferent was tested first. If the nerve was mechanosensitive the nerve was characterised based on their response to mechanical stimuli. A VFH protocol was then performed if the preparation was deemed suitable. If the nerve was not mechanosensitive the nerve was discarded and a new recording from a separate nerve was attained. If no other suitable nerves were available or the 3 consecutive mechanically insensitive nerves were found, a chemosensitivity protocol was performed.

2.1.3.4 BASIC MECHANOSENSORY PROPERTIES OF HUMAN VISCERAL AFFERENTS

Of the 95 successful flat sheet recordings, 36 were robustly responsive to either VFH probing, circumferential or longitudinal stretch, or mucosal stroking (42 units). Note, many preparations were mechanically sensitive, but deemed unsuitable for characterisation or mechanosensitive protocols for reasons including, weak response, response was unstable/not reproducible, nerve location and position in the electrode was delicate thereby making mechanical stimulation impractical. Very few preparations were mechanically insensitive. A response to VFH probing of an afferent's receptive field was characterised by a burst of action potentials above that of spontaneous activity, which dissipates immediately after the removal of the probing stimulus (figure 2.08). On occasion, and particularly relating to VFHs of high force, the responding unit would transiently continue at a higher activity rate even after the cessation of the VFH stimulus. The firing rate would usually revert back to pre-stimulus spontaneous activity levels within seconds of removing the probe. Responses to circumferential and longitudinal stretch exhibited similar qualities. Upon tissue stretching, an increase in afferent firing was evident for the duration of the stimulus (figure 2.08). No adaptation was evident for either mode of stretch, although this could be due to the brief stretching period of ~3-5 seconds used compared to longer stretching stimuli (1 min) previously reported (Brierley et al., 2004). The increased firing rate disappeared once stretching was stopped. A response to mucosal stroking was described by a subtle increase in baseline firing, which upon cessation of the stroke quickly subsided.

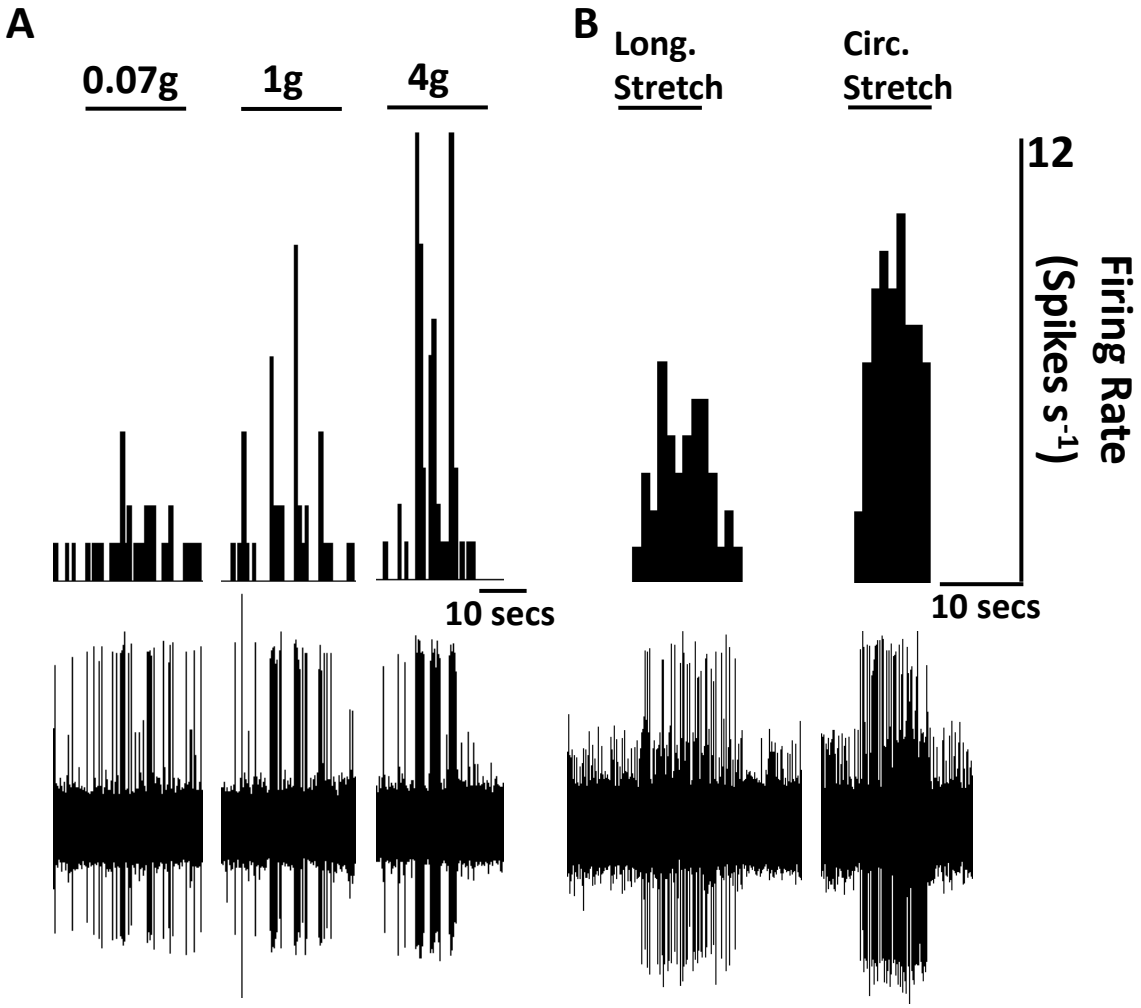


Figure 2.08: HVAs responded to a variety of mechanical stimuli. A) Probing of receptive fields located on the serosal surface of the tissue with different weighted VFHs produced a graded stimulus response curve. Some HVAs responded to probing stimuli as low as 0.02g. B) Stretching the tissue using curved forceps in a longitudinal or circumferential direction produced a marked increase in HVA firing in a separate unit that responded to light VFH probing.

Of the fibres which displayed mechanosensitivity, 4 subtypes could be identified based on their anatomical location and response to different mechanical stimuli. These were *mesenteric*, *serosal*, *muscular,* and *muscular mucosal* (figure 2.10). In addition, a group of "*silent*" afferents were identified. Each of these subtypes was responsive to 2g von Frey hair (VFH) probing. Due to nature of whole nerve recordings, HVAs often had more than 1 receptive field. Receptive fields were usually small, although occasionally covered a more extensive portion of the tissue. Serosal receptive fields were often associated with blood vessels on the serosal surface. The strength of the VFH probing required (20mg-4g) to illicit a response, the response profile to other mechanical stimuli (circumferential and longitudinal stretch, mucosal stroking), and the location of their receptive field were required to characterise each subtype.

Serosal afferents (n=22) had receptive fields in the wall of the intestine. Serosal afferents responded in a graded manner to VFH probing starting at very light weight probes (min threshold 20mg), but not to circumferential or longitudinal stretch, or mucosal stroking (figure 2.10). A proportion of these afferents were not tested with all stimuli, but were considered serosal afferents due to their sensitivity to very light VFH probing of the serosal surface (<600mg). Eight out of 22 serosal afferents exhibited spontaneous activity (0.8±0.2 spikes s^{-1}, 36.4%). Muscular afferents (n=20) had focal areas in the wall of the intestine that were responsive to strong VFH probing (min threshold 1g). Twelve out of 17 muscular afferents tested for both circumferential and longitudinal stretch, responded to both (3 muscular afferents were not tested for both modes of stretch). Three out of 17 and 2/17 muscular afferents only responded to circumferential or longitudinal stretch, respectively. All muscular afferents failed to respond to mucosal stroking. Sixteen out of 20 muscular afferents displayed spontaneous activity (4.2±0.9 spikes $2s^{-1}$, 80%). The receptive field of mesenteric afferents (n=2) were located in the mesentery attached to the intestinal tissue. These afferents responded in a graded fashion to probing of their receptive field with increasing weights of

calibrated VFHs (min threshold 20mg). Since the location of these afferents was in the mesentery, only 1 of the mesenteric afferents was tested for responsiveness to other mechanical stimuli. Comparable to serosal afferents, this mesenteric afferent did not respond to circumferential stretch, or to mucosal stroking. One out of 2 mesenteric afferents were spontaneously active (0.5 spikes s^{-1}, 50%). A response to mucosal stroking was evident in only 1/28 preparations tested. This responsive preparation also responded to circumferential and longitudinal stretch, and to 1g VFH probing, and may therefore represent the identification of a muscular-mucosal afferent (n=1). This muscular-mucosal afferent was spontaneously active (1.6 spikes s^{-1}, 100%).

"Silent" afferent fibres (n=2) initially had no discernible receptive field when probed with 2g VFH. However, after the application of BK (20nM or 2µM) a receptive field became apparent, which was responsive to either 2g VFH probing or to probing with a cotton bud (figure 2.09). "Silent" afferents were not tested for their responsiveness to VFHs under 2g, stretch stimuli, or to mucosal stroking. Therefore, no comment can be made on the location of "silent" afferent terminals. "Silent" afferents did not demonstrate any spontaneous activity.

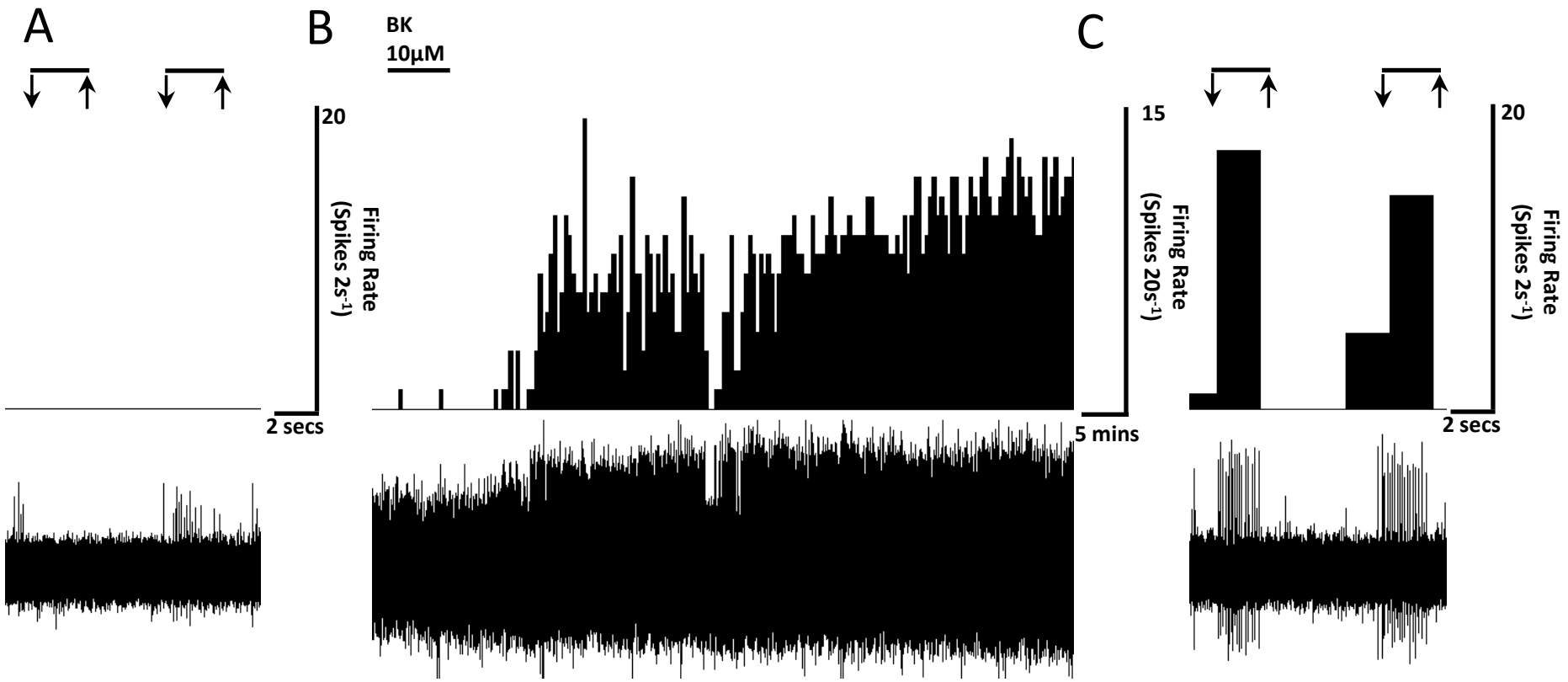


Figure 2.09: "Silent" afferents were evoked after the application of the algogenic mediator BK (n=2). A) VFH probing before the application of BK did not elicit a HVA response. B) Application of BK activated HVAs in 1/2 preparations. C) VFH probing after the application of BK produced a response in HVAs.

2.1.3.5 GRADED RESPONSES TO VFH PROBING IN SEROSAL/MESENTERIC AND MUSCULAR AFFERENTS

Graded responses to VFHs of increasing weight were evident in serosal, mesenteric and muscular afferents. Serosal and mesenteric afferents will henceforth be combined under the term serosal/mesenteric afferents, due to their similar responses to mechanical and chemical stimuli and low mesenteric n numbers, as has been done previously (Lynn and Blackshaw, 1999, Hicks et al., 2002). Different response characteristics were observed when serosal/mesenteric afferents were compared to muscular afferents. The minimum threshold for activation by VFH probes was lower for serosal/mesenteric afferents compared to muscular afferents (20mg vs. 1g VFH probes). Serosal/mesenteric afferents tended to have a higher firing rate compared to muscular afferents when probed with 1g (20.4±3.0 vs. 11.5±2.5 spikes/$2s^{-1}$), 2g (21.0±2.7 vs. 12.2±4.2 spikes/$2s^{-1}$), and 4g (24.3±7.3 vs. 11.0±2.0 spikes/$2s^{-1}$) VFHs (figure 2.10). The minimum VFH probe (20mg) excited 12.5% (2/16) of serosal/mesenteric afferents. The proportion of serosal/mesenteric afferents that were excited by VFH probing increased until 600mg probes, and all subsequent probes (1g, 1.4g, 2g, 4g), activated 100% of afferents. In contrast, the minimum threshold for activating muscular afferents was 1g, which activated 15.4% (2/13) of afferents. The proportion of muscular afferents activated by VFH probing increased with VFH weight, however even 4g VFH probes only activated 46.2% (6/13) of afferents (figure 2.10). Furthermore, the 1g VFH hair elicited similar rates of action potential firing compared to 2g, or 4g (11.5±2.5 vs. 12.2±4.2 vs. 11±2.0 spikes $2s^{-1}$, respectively). A cotton bud, used to find the receptive field, activated 81.3% (13/16) of muscular afferents. No receptive field could be located, defined as a lack of response to any probing using VFHs or a cotton bud, for 18.7% (3/16) of muscular afferents. The identified muscular-mucosal afferent had a minimum activation threshold of 2g VFH. “Silent” afferents were only tested with either a 2g VFH probe or a cotton bud; hence their activation threshold could not be determined.

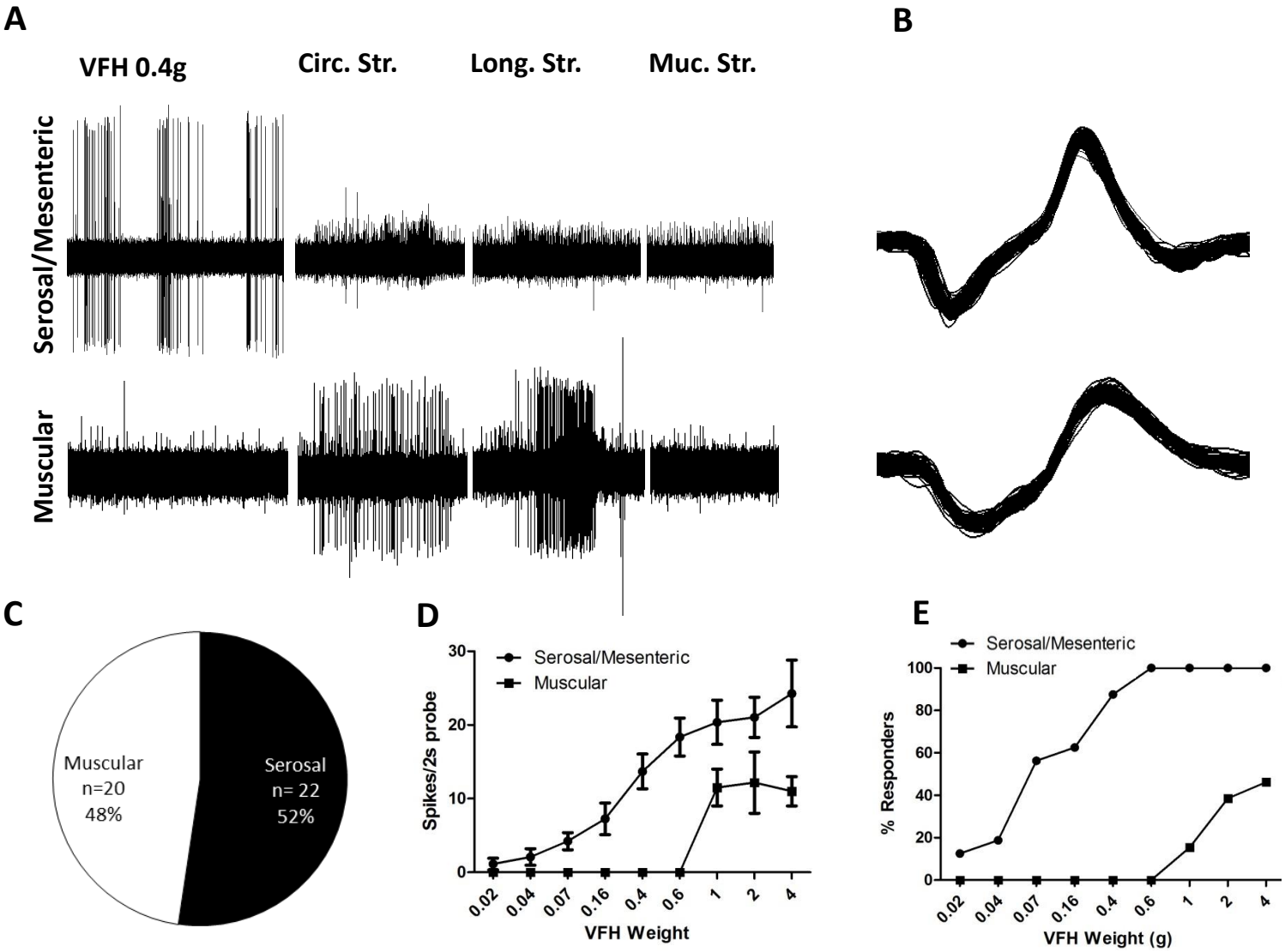

A
VFH 0.4g
Circ. Str.
Long. Str.
Muc. Str.
Serosal/Mesenteric
Muscular
B
C
Muscular
n=20
48%
Serosal
n= 22
52%
D
Serosal/Mesenteric
Muscular
Spikes/2s probe
VFH Weight
E
Serosal/Mesenteric
Muscular
% Responders
VFH Weight (g)

Figure 2.10: Characterisation of subtypes of HVAs based on their response to mechanical stimuli. A) Shows the mechanical response profile of each subpopulation. B) The shapes of the respective action potentials, used to discriminate between the different units as analysed by waveform analysis software within spike2. C) Displays the number of muscular and serosal afferents found in HVA preparations. Mesenteric and muscular-mucosal were not included as they were not searched for in every preparation. D) Describes the rate of afferent firing in response to VFH probing in serosal/mesenteric and muscular preparations. Serosal/mesenteric units responded to lower weight VFHs, and also had a higher firing rate at 1g, 2g, and 4g VFHs compared to muscular units. E) Shows the proportion of serosal/mesenteric and muscular units that responded to each VFH probe. Muscular units did not respond to VFH probes lower than 1g. However, >90% and 100% of serosal/mesenteric units responded to 400mg and 600mg VFH, respectively, potentially allowing subpopulations to be discriminated by VFH probe alone.

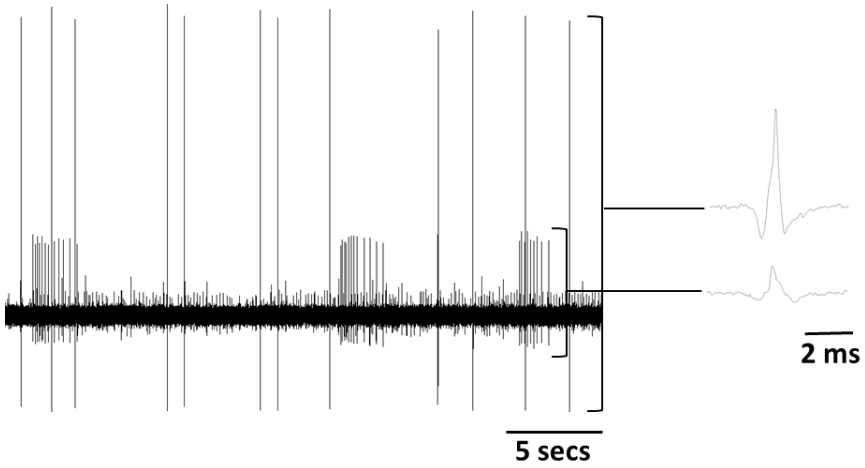
2.1.3.6 SPONTANEOUS ACTIVITY

In HVA recordings, spontaneous activity took 2 forms, a regular firing pattern, characterised by a continuous firing of single action potentials, and a burst firing pattern, characterised by burst of action potentials separated by a lag period. All subtypes of HVAs displayed spontaneous activity. Eight out of 22 serosal fibres exhibited spontaneous activity (0.8±0.2 spikes s^{-1}, 36.4%) (figure 2.11). All spontaneously active serosal units exhibited a regular firing pattern. Sixteen out of 20 muscular afferents displayed spontaneous activity (4.2±0.9 spikes s^{-1}, 80%). Of these 16 afferents, 5 displayed both burst and regular types of spontaneous activity. The remaining 11 spontaneously active units had a regular firing pattern. No muscular unit had exclusively bursting spontaneous activity. One out of 2 mesenteric afferents was spontaneously active, displaying a regular firing rate (0.5 spikes s^{-1}, 50%). The only identified muscular-mucosal afferent displayed a regular spontaneous activity rate (1.6 spikes s^{-1}, 100%). The rate of spontaneous activity in serosal afferents was significantly lower than exhibited by muscular afferents (0.8±0.2 vs. 4.2±0.9 spikes s^{-1}, $p<0.05$) (figure 2.11).

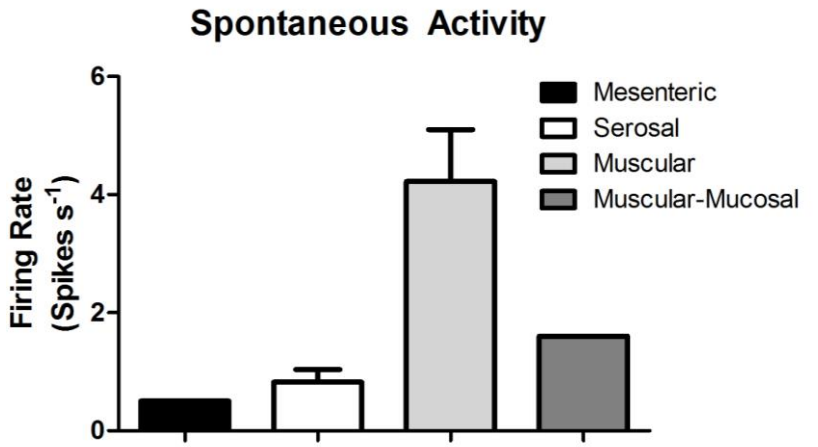
In preparations that exhibited baseline spontaneous activity, a transient inhibition or abolishment of spontaneous firing was evident immediately after the cessation of mechanical stimuli. After removal of the longitudinal or circumferential stretch stimulus, spontaneous activity was transiently inhibited (longitudinal: 8/18 preparations; recovery average ± SEM, 12.3 ±3.3 seconds; range 1.0-27.9 seconds; circumferential: 10/16 preparations; recovery average ± SEM 9.7 ± 1.4 seconds; range, 3.4-18.1 seconds) or transiently abolished (longitudinal: 1/18 preps; recovery 2.9 seconds, circumferential: 2/16 preparations, recovery 2.4 seconds). The spontaneous activity of 4 preparations was not changed after the cessation of either longitudinal or circumferential stretch. The withdrawal of the last VFH probe in each set transiently abolished spontaneous activity in 8/8 preparations that exhibited spontaneous activity (average ± SEM; 24.8 ± 5.2 seconds; range 1.0-70.8 seconds). This was evident in both serosal and mesenteric afferents. Similarly, the release of luminal pressure in distension

preparations transiently abolished spontaneous activity in 19/19 preparations (average ± SEM; 3.0 ± 0.50 seconds; range 0.1-13.8 seconds).

A


2 ms
5 secs

B


Spontaneous Activity
Firing Rate (Spikes s-1)
6
4
2
0
Mesenteric
Serosal
Muscular
Muscular-Mucosal

C

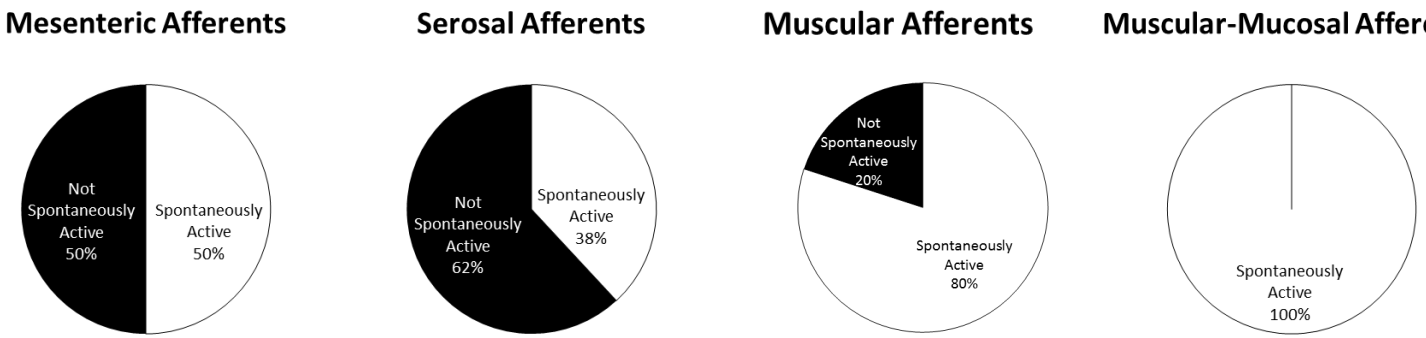

Mesenteric Afferents
Not Spontaneously Active 50%
Spontaneously Active 50%
Serosal Afferents
Not Spontaneously Active 62%
Spontaneously Active 38%
Muscular Afferents
Not Spontaneously Active 20%
Spontaneously Active 80%
Muscular-Mucosal Afferents
Spontaneously Active 100%

Figure 2.11: Spontaneous activity in HVAs was evident in the majority of preparations. A) Spontaneous activity took 2 forms, a regular firing pattern, characterised by a continuous firing of single action potentials, and a burst firing pattern, characterised by burst of action potentials separated by a lag period. B) Rates of spontaneous activity differed between the subtypes of HVAs. C) Displays the proportion of different HVA subtypes that exhibited spontaneous activity.

2.1.3.7 VFH TIME MATCHED CONTROLS

Two out of 2 preparations used for probing time matched control experiments were spontaneously active (1.3±0.3 spikes s^{-1}). The HVA responses to 2g VFH probing were very similar over a time period of ~60 minutes, with no group of probes differing by more than 11% (Baseline probes 1,2,3 100%, probes 4,5,6 105.9%, probes 5,6,7 98.4% (sets of probes normally considered post drug probes), probes 6,7,8 93.4%, probes 7,8,9 95.4%, probes 8,9,10 94.7%, probes 9,10,11 93.1%, probes 10,11,12 89.4%) (figure 2.12).

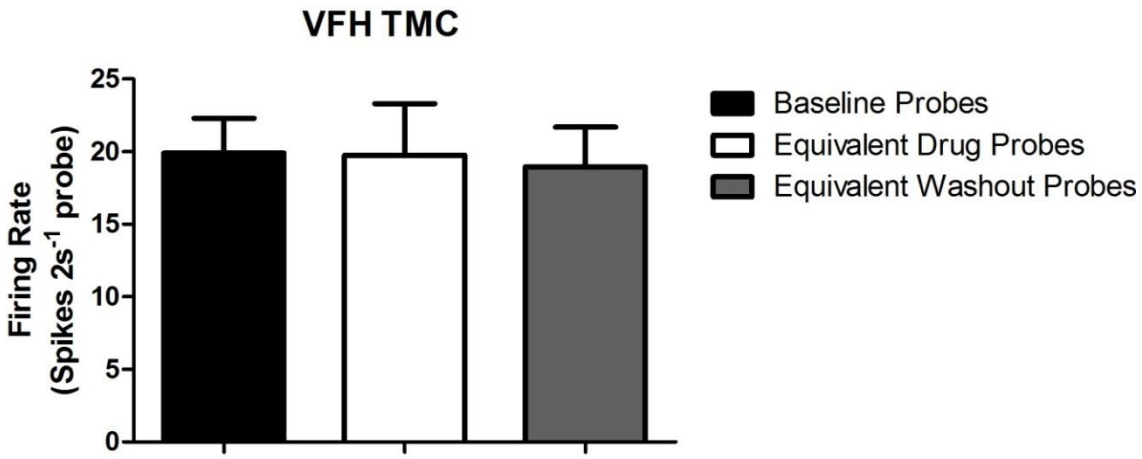


Figure 2.12: Bar graph showing VFH time matched controls. The HVA responses to 2g VFH probing were very similar over a time period of ~60 minutes. The black bar is the average of the baseline probes. The white bar is the average of the probes that are equivalent to the drug effect probes normally compared to the baseline probes in drug studies. The grey bar is the average of the probes that are equivalent to the washout probes normally compared to the baseline probes in drug studies.

2.1.3.8 TRPV4 VFH PROBING PROTOCOL

Considering the significant body of evidence for a role of TRPV4 in the transduction of mechanical stimuli in murine colonic afferents (Brierley et al., 2008, Cenac et al., 2008, Sipe et al., 2008, Cenac et al., 2010, Ceppa et al., 2010), we investigated the functional role of TRPV4 in mechanosensitivity in HVAs. Mechanotransduction was examined using VFHs in flat sheet intestinal tissue. HVAs responded incrementally to increasing weight VFHs as described. Five out of 12 units used for VFH studies exhibited spontaneous activity (1.3±0.8 spikes s^{-1}). Three out of 14 serosal HVAs responded directly to the application of GSK1016790A (BC 2μM, 20ml of 10μM, average Δ firing rate 18.3±4.1 spikes $20s^{-1}$) (figure 2.13). In those fibres that responded directly to GSK1016790A, there was a trend for an augmented HVA response to VFH probing (17.9±1.4 vs. 21.3±1.8 spikes $2s^{-1}$ probe, 19.1%, n=3, p>0.05), but this did not reach significance. In afferents that did not respond directly to GSK1016790A, there was no augmentation of the HVA response to probing by the TRPV4 agonist. Furthermore, when fibres that did not respond directly to GSK1016790A were split into groups based on VFH stimulus, HVA responses to both 2g (20.5±4.0 vs. 19.5±1.3 spikes $2s^{-1}$ probe, -2.4%, n=2, p>0.05) and 400mg (21.6±3.7 vs. 20.6±3.0 spikes $2s^{-1}$ probe, -2.0%, n=6, p>0.05) remained unaffected.

In contrast, the potent and selective TRPV4 channel antagonist HC067047 significantly attenuated the response of serosal HVAs to VFH probing (22.5±2.9 vs. 17.5±2.7 spikes $2s^{-1}$, -23.9%, n=9, p<0.05) (figure 2.14). After a 30 minute washout of HC067047, all preparations failed to return towards baseline mechanosensitivty to VFH probing. However, after a 60 minute washout 2/5 preparations studied over this period recovered towards baseline mechanosensitivity to VFH probing (baseline 25.3±10.8; vs. HC067047 13.8±13.8; vs. washout 20.5±12.0 spikes $2s^{-1}$ probe) (figure 2.14). When experiments were split based on their VFH stimulus, HC067047 did not reduce the response of HVAs to 2g (22.0±8.5 vs. 19.8±6.0 spikes $2s^{-1}$ probe, -6.9%, n=2) VFH probing, although this may be an n number issue. However, the

response of HVAs to 400mg VFH probing was significantly reduced after HC067047 application (22.7±3.3 vs. 16.8±3.2 spikes $2s^{-1}$, -28.7%, n=7, $p<0.05$).

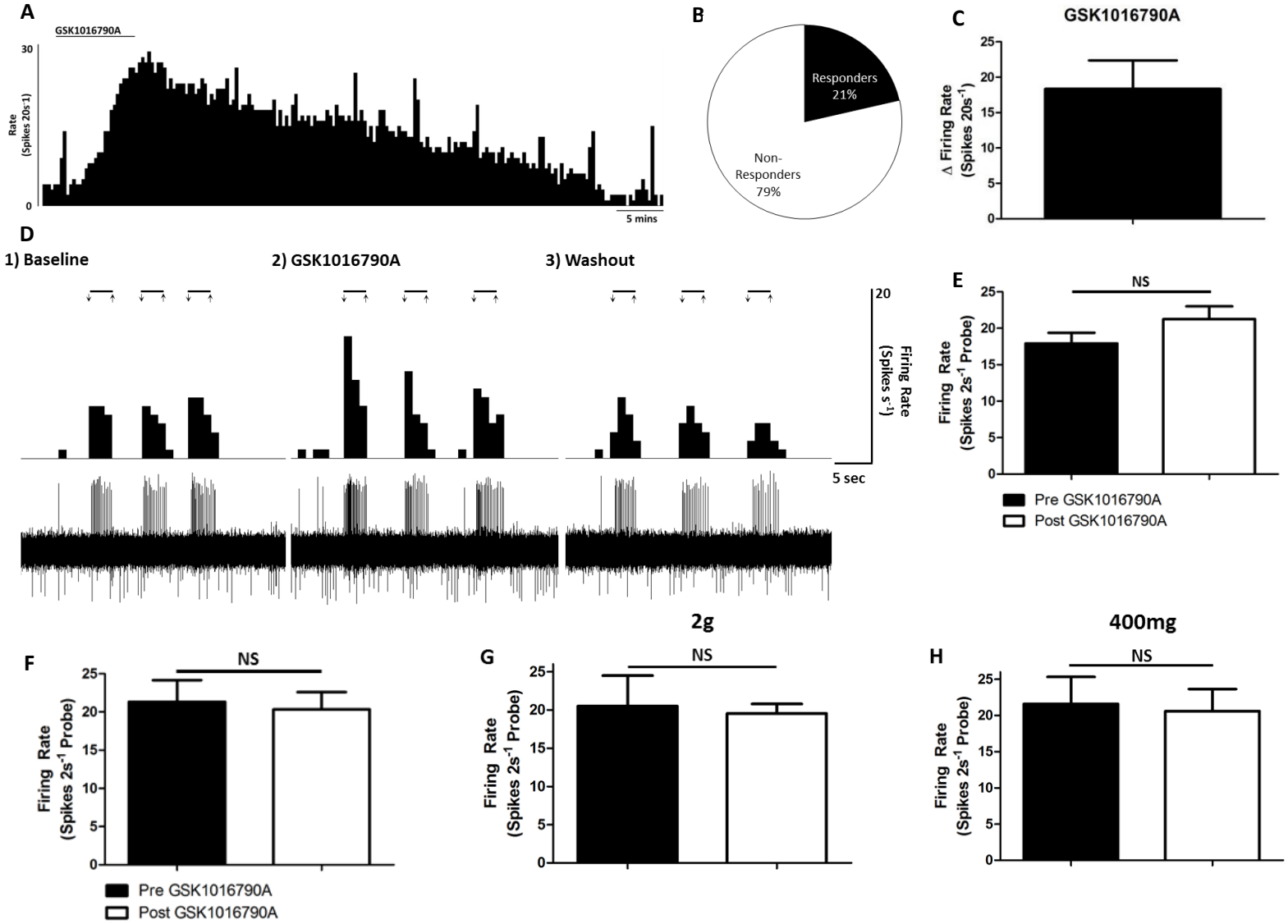

A
GSK1016790A
30
Rate (Spikes 20s-1)
0
5 mins
B
Responders 21%
Non-Responders 79%
C
GSK1016790A
Δ Firing Rate (Spikes 20s-1)
D
1) Baseline
2) GSK1016790A
3) Washout
20
Firing Rate (Spikes s-1)
5 sec
E
NS
Firing Rate (Spikes 2s-1 Probe)
Pre GSK1016790A
Post GSK1016790A
2g
400mg
F
NS
Firing Rate (Spikes 2s-1 Probe)
Pre GSK1016790A
Post GSK1016790A
G
NS
Firing Rate (Spikes 2s-1 Probe)
H
NS
Firing Rate (Spikes 2s-1 Probe)

Figure 2.13: The effect of the TRPV4 agonist GSK1016790A on the mechanosensitivity of HVAs. A-C) Three out of 14 serosal HVAs responded directly to the application of the TRPV4 agonist GSK1016790A. A) Shows a rate histogram of an example of the activation of a HVA after application of GSK1016790A. B) Displays the number of proportion of afferents that responded to GSK1016790A. C) A bar graph demonstrating the average change in afferent firing in the 3 activated afferents. D-E) In contrast, GSK1016790A failed to potentiate the human serosal afferent response to VFH probing, $p>0.05$. D) Shows the raw data and rate histograms for a set of 3 probes, before the addition of GSK1016790A, a set after the drug has been applied, and a set after it has been washed out. E) There was a trend towards a slight potentiation of the response to VFH probing in the 3 preparations that also directly responded to GSK1016790A; however this did not reach significance. F) In preparations that did not respond directly to GSK1016790A, there was no augmentation of the HVA response to VFH probing. G-H) When the direct GSK1016790A non-responders are split into experiments based on VFH stimulus, HVA responses to both 2g (20.5±4.0 vs. 19.5±1.3 spikes $2s^{-1}$ probe, -2.4%, n=2, $p>0.05$) and 400mg (21.6±3.7 vs. 20.6±3.0 spikes $2s^{-1}$ probe, -2.0%, n=6, $p>0.05$) show no augmentation. Data was analysed using a 2 tailed paired t test, $p<0.05$.

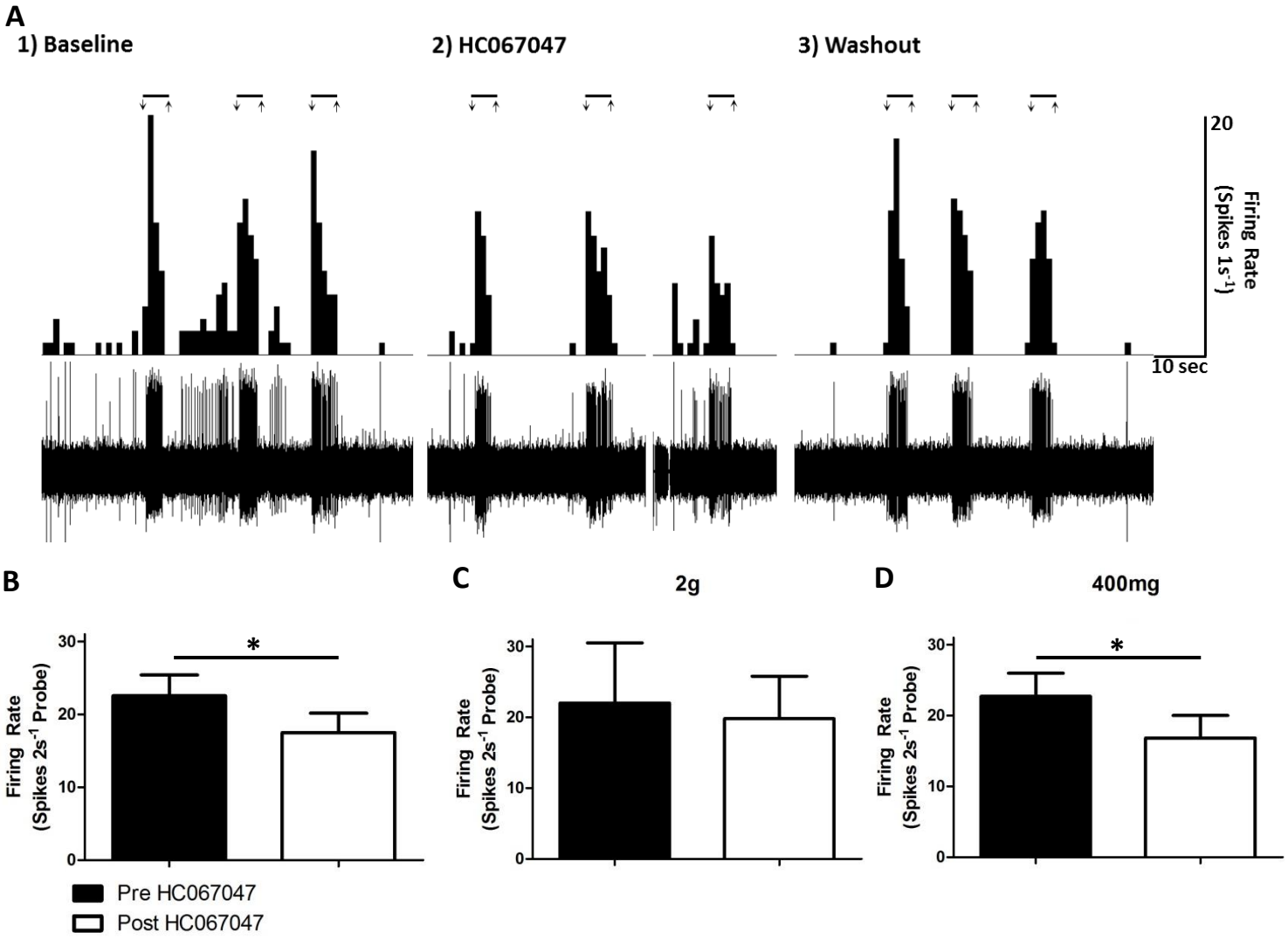

A
1) Baseline
2) HC067047
3) Washout
20
Firing Rate
(Spikes 1s-1)
10 sec
B
C
2g
D
400mg
Firing Rate
(Spikes 2s-1 Probe)
30
20
10
0
*
Pre HC067047
Post HC067047

Figure 2.14: TRPV4 modulates the transduction of mechanical stimuli in HVAs. A) Shows the raw data and rate histograms for a set of 3 probes, before the addition of HC067047, a set after the drug has been applied, and a set after it has been washed out. A-B) Application of the TRPV4 antagonist HC067047 significantly attenuated the human serosal afferent response to VFH probing (22.5±2.9 vs. 17.5±2.7 spikes $2s^{-1}$ probe, -23.9%, n=9, $p<0.05$). C-D) When experiments are split based on their VFH stimulus, HC067047 did not reduce the response of HVAs to 2g VFH probing (22.0±8.5 vs. 19.8±6.0 spikes $2s^{-1}$ probe, -6.9%, n=2), although this may be a n number issue, but did significantly reduce the response to 400mg probing (22.7±3.3 vs. 16.8±3.2 spikes $2s^{-1}$, -28.7%, n=7, $p<0.05$). Data was analysed using a 2 tailed paired t test, $p<0.05$.

2.1.3.9 SUMMARY OF RESULTS

- HVAs respond to VFH probing of their receptive fields, longitudinal and circumferential stretch, and stroking of the mucosa
- Five subtypes of HVA were characterised based on their response to mechanical stimuli, mesenteric, serosal, muscular, muscular-mucosal, and “silent” afferents
- Spontaneous activity was evident in all subtypes, and was greatest in muscular afferents
- The TRPV4 agonist, GSK1016790A, activated 3/14 HVAs, but failed to augment the HVA response to VFH probing.
- The TRPV4 receptor antagonist HC067047 significantly attenuated the response of serosal HVAs to VFH probing.

2.1.4 DISCUSSION

2.1.4.1 MECHANOSENSITIVITY AND HVA CHARACTERISATION

The present study describes the 5 different subtypes of afferents terminating in the human gut based on their sensitivity to specific types and intensities of mechanical stimuli (see table 2.03 for comparison to animal literature). The first *in vitro* electrophysiological characterisation of colonic afferents in any species, described afferent endings in the serosa, muscle layers and mucosa of the rat colon (Lynn and Blackshaw, 1999). The 5 subtypes identified in this study, "silent", mesenteric, serosal, muscular, and muscular-mucosal have been previously identified in mouse colonic afferents (Brierley et al., 2004, Page et al., 2004, Page et al., 2005, Brierley et al., 2005a, Brierley et al., 2008, Brierley et al., 2009, Hughes et al., 2009a, Hughes et al., 2009b, Jones et al., 2005, Jones et al., 2007, Feng et al., 2012b, Feng et al., 2012a, Feng et al., 2013). Indeed, these functional subtypes have been described in 2 separate spinal nerve pathways innervating the mouse colon, the pelvic and splanchnic nerves (Brierley et al., 2004). Initial reports of potential subtypes of HVAs have been previously published (Jiang et al., 2011). This study represents the first extensive characterisation of subtypes of afferent terminals in the human gut using an *in vitro* model.

2.1.4.2 SEROSAL AND MESENTERIC AFFERENTS

Serosal HVAs were the most abundant subtype of HVAs, comprising 45.8% of the population. Serosal afferents were found in comparable proportions in both mouse splanchnic and pelvic afferents constituting 36-48% and 24-37% , respectively (Brierley et al., 2004, Hughes et al., 2009a, Feng and Gebhart, 2011) and in rat splanchnic afferents between 51.9-81.4% (Lynn and Blackshaw, 1999, Hicks et al., 2002). They are also represented in the cat colon (Blumberg et al., 1983, Haupt et al., 1983). Only 2 human mesenteric units were identified in the present investigation. Mesenteric afferents are only found in the murine splanchnic afferent pathway, but they constitute up to 50% of these afferents (Brierley et al., 2004, Page et al., 2004, Page et

al., 2005, Brierley et al., 2005a, Brierley et al., 2009, Feng and Gebhart, 2011). Mesenteric afferents were not systematically looked for due to the inherent technical difficulties, or studied in the present report therefore no comparisons can be made. However, an important point to consider in the future is the distinct difference in the quantity and composition of the mesentery between mice and humans. Human mesentery is fatty, thick, and fibrous due to the constituent connective tissue, and the quantity available varies from patient to patient.

Human serosal afferents were only activated by VFH probing, and were unresponsive to both modes of stretch and to mucosal stroking, a finding that is supported by work in both mouse and rat serosal afferents (Hicks et al., 2002, Brierley et al., 2004, Feng and Gebhart, 2011). In animal studies, serosal afferents have been shown to have a role in nociception based on their lack of response to innocuous stretch or mucosal stroking, and their responsiveness to noxious mediators such capsaicin, BK, ATP etc. (Maubach and Grundy, 1999, Hicks et al., 2002, Brierley et al., 2004, Brierley et al., 2005b, Wynn and Burnstock, 2006, Feng and Gebhart, 2011). Importantly, serosal afferents can respond to strong or dynamic stretching of the gut wall such as the initial phase, or levels of stretch which are supra-threshold to that required to activate muscular afferents (Blumberg et al., 1983, Haupt et al., 1983, Lynn and Blackshaw, 1999, Brierley et al., 2008)(Hughes et al., 2009a). Some have suggested that this could be accounted for by friction on the serosal caused by the underlying bath (Blumberg et al., 1983, Haupt et al., 1983, Lynn and Blackshaw, 1999). The large size, the thickness and the orientation, serosal side up, of the human tissues preparations likely eliminated the occurrence of this.

Serosal and mesenteric afferents have previously been shown to sometimes have multiple receptive fields, often associated with blood vessels and capillaries (Morrison, 1973, Lynn and Blackshaw, 1999, Brierley et al., 2004). Similar observations were made in human serosal and mesenteric afferents, although we did not attempt to map their locations.

Mapping the location of each receptive field should be a standard procedure in future experiments.

2.1.4.3 MUSCULAR, MUSCULAR-MUCOSAL AND MUCOSAL AFFERENTS

All human muscular afferents responded to at least 1 mode of stretch, and were activated by VFH probing at much greater strengths than serosal afferents (minimum threshold of 1g). These response characteristics are comparable with muscular afferents in murine models which respond to stretch and VFH probing stimuli Stretch sensitive human muscular afferents made up 41.7% of the fibres we recorded from, which even accounting for the absence of a systematic assessment for mesenteric afferents is considerably greater than the proportion of muscular afferents were found in both mouse (splanchnic 10-12%; pelvic 12-22%) and rat (splanchnic 5-19%) visceral afferents (Lynn and Blackshaw, 1999, Hicks et al., 2002, Brierley et al., 2004, Hughes et al., 2009a, Feng and Gebhart, 2011). Brierley et al (2004) considered that muscular afferents may be under reported in *in vitro* preparations not utilising both circumferential and longitudinal stretch. Every attempt is made to test both modes of stretch in HVAs; however, the position of the nerve and electrode, as well as the size of the tissue does not always allow this. However, 17/20 muscular afferents were tested for both modes of stretch, with ~29% (5/17) of afferents only responding to 1 distinctive mode of stretch suggesting that there may be distinct populations of afferent sensitive to a particular direction of stretch. The lack of testing both stretch modes in murine models may account for the higher proportions of muscular afferents found in HVAs, which may more accurately reflect all the muscular populations. However, it must be taken into account that, at least in the mouse pelvic pathway, the proportion of stretch sensitive afferents is actually high, ~38-44%, but the majority of these were also responsive to mucosal stroking and hence classified as muscular-mucosal afferents (Brierley et al., 2004, Hughes et al., 2009a, Feng and Gebhart, 2011). We have not reported muscular mucosal afferents in great abundance; however this may be due

to the serosa up orientation of our preparation. In order to better study mucosal afferents, a preparation using a mucosa up orientation may need to be used.

Despite testing 17 HVAs for responses to mucosal stroking, no mucosal afferents were identified. In mouse splanchnic afferents, the number of mucosal afferents was very low at 4-5%, however in rat splanchnic (14-23%) and mouse pelvic nerves (20-38%), mucosal afferent proportions were substantially higher (Lynn and Blackshaw, 1999, Hicks et al., 2002, Brierley et al., 2004, Hughes et al., 2009a, Feng and Gebhart, 2011). One human afferent was responsive to mucosal stroking, but it was also responsive to both longitudinal and circumferential stretch, and a 1g VFH probe. This was deemed to be a human muscular-mucosal afferent. The single human muscular-mucosal afferent identified from 17 preparations, is markedly less than the 22-26% reported in rodent models (Brierley et al., 2004, Hughes et al., 2009a, Feng and Gebhart, 2011). The orientation of the tissue in the bath must be considered when comparing the relative proportions of afferents subtypes characterised in human and animal *in vitro* preparations. For example, in the HVA model, the mucosa faces down. Although the tissue is partially unpinned to allow for mucosal stroking, a portion of the mucosa remains unavailable for stroking. This may lead to the underreporting of human mucosal and muscular-mucosal afferents. Human tissue recordings with the mucosa up have previously been reported (Jiang et al., 2011), and may reveal a greater innervation of the mucosa by extrinsic afferent fibres. Furthermore, characterisation of muscular-mucosal and mucosal in the mouse revealed a clustering of these subtypes in the distal colon (Brierley et al., 2004, Hughes et al., 2009a). Animal *in vitro* electrophysiological preparations involve studying the entire, or at least a large portion of the colon in 1 experiment. This is not reflected in human studies, where a piece of colon wall ~3cm^2 from a 1.5 meter colon, represents the entire experimental tissue. Furthermore, although the majority of the tissue we used in these experiments was from the distal colon, tissue from more proximal intestine was also used.

Although the human intestinal tissue is kept in carbongenated Krebs buffer during collection and experimentation, some mucosal degradation is possible considering its vulnerability to ischaemic damage (Park et al., 1990, Park and Haglund, 1992). This may account for the small number of afferents sensitive to mucosal stroking. Therefore, when studying these afferents it is important to record from the tissue as soon as possible to ensure a healthy mucosa. Furthermore, a mucosa side up orientation, would not only allow greater access to the mucosa for stroking, but allow better perfusion of the Krebs buffer over the mucosa.

2.1.4.4 "SILENT" AFFERENTS

"Silent" afferents, traditionally display no spontaneous activity, and are not responsive to mechanical stimuli, until sensitised in inflammatory conditions (Cervero, 1994, Feng and Gebhart, 2011). We found 2 mechanically insensitive afferents, which after BK application, become responsive to probing of their receptive fields. These data represent the first demonstration of "silent" afferents in the human intestine. These may represent an important class of human nociceptors, which only become sensitive to mechanical stimuli after sensitisation. "Silent" afferents have previously been reported in many species including cat, rat, and mouse (Habler et al., 1988, Habler et al., 1990, Janig and Koltzenburg, 1991, Sengupta and Gebhart, 1994, Lynn and Blackshaw, 1999). Serosal afferents have been proposed to be the main source of "silent" afferents (Brookes et al., 2013). Indeed, after sensitisation "silent" afferents only respond to VFH probing and not to circumferential stretch or mucosal stroking (Feng and Gebhart, 2011). We only tested HVAs for responses to probing, and so cannot comment on the potential location of these "silent" afferents. The minority of "silent" mouse colonic afferents (33%) were directly activated by an inflammatory stimulus (a soup of BK, 5-HT, histamine and PGE_2) (Feng and Gebhart, 2011). Similarly, only ½ of HVAs were directly activated by the application of BK. However, this may be explained by the relatively low dose of BK, 20nM, applied in ½ experiments.

Paper	Species	Organ	Afferent Type	Terminals Identified
Page and Blackshaw, 1998	Ferret	Oesophagus, Stomach	Vagal	Tension, Mucosal, Tension-mucosal
Lynn and Blackshaw, 1999	Rat	Colon	Splanchnic	Serosal, Muscular, Mucosal
Hicks et al, 2002	Rat	Colon	Splanchnic	Serosal, Muscular, Mucosal
Brierley et al, 2004 Either splanchnic or pelvic: Page et al, 2004; Page et al; 2005; Brierley et al, 2005b; 2008; 2009; Jones et al, 2005; Hughes et al, 2009a, Feng and Gebhart, 2011. etc.	Mouse	Colon	Spinal	Splanchnic: Mesenteric, Serosal, Muscular, Mucosal Pelvic: Serosal, Muscular, Muscular-mucosal, Mucosal
Jiang et al, 2011	Human	Colon	Unknown	Serosal, Muscular, Muscular-mucosal
Present Report	Human	Ileum, Colon	Unknown	Mesenteric, Serosal, Muscular, Muscular-mucosal

Table 2.03: Details reports describing the different subtypes of vagal and spinal afferents based on their response to mechanical stimuli using VFH probing, stretching, and mucosal stroking in rodent and human tissue.

2.1.4.5 GRADED RESPONSES TO VFH PROBING

Only 1 group has produced VFH stimulus response curves in colonic afferents, limiting the qualitative comparisons between animal and human preparations (Brierley et al., 2004, Page et al., 2004, Brierley et al., 2005a, Page et al., 2005, Brierley et al., 2009, Hughes et al., 2009a). Human serosal and mesenteric afferents were the most sensitive to probing, responding to VFHs as low as 20mg. This contrasted to the robust minimal probing stimulus ≥1g VFH required to activate human muscular afferents. This is in contrast to mouse pelvic and splanchnic afferents, which demonstrate similar submaximal stimulus response curves, with up upwards of 20% of afferents activated by the lowest VFH probe, 0.07g. Indeed, a heavier VFH probe was needed to active 100% of serosal afferents, compared to muscular afferents (Brierley et al., 2004, Hughes et al., 2009a). This difference is most likely due to the thickness of the human tissue, which is many times that of the mouse and hence the activation of deeper muscular afferents requires stronger stimuli for excitation. This is supported by the observation that less than half of human muscular afferents are activated by the strongest VFH probe (4g), while 1-2g VFH probes are enough to activate 100% of mouse pelvic and splanchnic muscular afferents, respectively (Brierley et al., 2004, Brierley et al., 2005b, Hughes et al., 2009a).

Similarly, the thickness of human tissue may also explain the higher firing rates to all VFH probes in serosa compared to muscular afferents, whereby lighter VFHs are not sufficient to fully activate deeper receptive fields. Firing rates in response to probing across all VFH weights is substantially higher in mouse colonic afferents compared to human afferents (Brierley et al., 2004, Brierley et al., 2008, Brierley et al., 2009, Hughes et al., 2009a). Again this may reflect the relatively thin mouse colonic tissue, whereby each VFH weight represents a greater relative stimulus. On the other hand, mouse colonic afferents tend to exhibit higher spontaneous firing frequencies compared to HVAs, which may account for the seemingly increased response to VFH probing (Brierley et al., 2004, Brierley et al., 2005b).

A greater sensitivity of human serosal afferents is evident when compared to mouse serosal afferents in both pathways. One hundred percent of human serosal afferents are activated by 600mg VFHs, considerably lighter than the required stimulus, 1-2g, to activate all mouse pelvic and splanchnic serosal afferents, respectively. The greater sensitivity displayed by human serosal afferents may reflect the serosa side up orientation of the preparation, compared to mucosa side up in mouse experiments. Indeed, a number of studies have reported an increased sensitivity of serosal afferents to VFH probing when the experiment was performed with the serosa up, allowing direct access to the receptive field (Lynn and Blackshaw, 1999, Berthoud et al., 2001, Hicks et al., 2002). Considering this, the generation of serosal stimulus response curves in murine tissue by probing the mucosal surface, as demonstrated by a number of groups, is likely to be different to those generated in human tissue.

The use of isolated segments of human gut, from the ileum to the rectum, means our HVA recordings could potentially be from vagal, splanchnic, or pelvic nerves and represents a limitation of this model. Using distinct areas of intestine in future experimental sets can go some way to addressing this issue. The majority of studies performed in this report were on sigmoid colon, by far the most abundant type of tissue available. Hence using only sigmoid colon would be the most experimentally feasible idea to pursue in the future. Furthermore, as with all electrophysiological recordings from intestinal afferents, the possibility that some fibres may be enteric viscerofugal neurons cannot be ruled out.

Advantages of pinning the tissue mucosal side up include, allowing the mucosa access to the nutrients, oxygen and pH of the Krebs buffer, thereby optimising the protection of its integrity, allowing stroking of the mucosa and hence identification of mucosal afferents, and even allowing for improved quality of HVA recordings (Jiang et al., 2011). However, while the former 2 points have merit, the latter theory is contradicted by the high signal to noise ratios evident in the HVA recordings in this report. A serosa up orientation allows for superior access

to all parts of the mesentery, and indeed the serosal surface, and is therefore more amenable to accurate identification of serosal and mesenteric afferents, and generation of meaningful stimulus response curves in these afferent subtypes.

A number of studies report that afferents responsive to mucosal stroking are also sensitive to blunt probing of the mucosa (Lynn and Blackshaw, 1999, Hicks et al., 2002, Brierley et al., 2004, Feng and Gebhart, 2011). Indeed, this report demonstrates a response to 1g probing in a purposed muscular-mucosal afferent. In thick human colonic tissue, it is perhaps unlikely that a 1g VFH could directly activate a distinct site in the mucosa following application to the serosa. This may suggest that the ending of some muscular-mucosal afferents which respond to mucosal stroking may terminate in deeper layers of the gut than the mucosa. Indeed, is has previously been postulated that some muscular-mucosal afferents may terminate in the muscularis externa (Page and Blackshaw, 1998, Blackshaw and Gebhart, 2002, Brierley et al., 2004). Alternatively agitation of the mucosal surface by the tissue bath during probing may account for the response to a 1g VFH.

2.1.4.6 SPONTANEOUS ACTIVITY

The present study found 2 types of spontaneous activity in HVAs, regular and burst firing. Bursting spontaneous activity was evident in 31% of spontaneous active muscular units, consistent with previous reports in muscular, and in tension-mucosal afferents in the human, ferret and mouse GI tract, but never in any stretch insensitive afferents (Page and Blackshaw, 1999, Page et al., 2002, Jiang et al., 2011). Indeed, in the current study no burst like spontaneous activity was evident in any other HVA subtype. It is possible that ongoing contractile activity in the muscle drives this burst like activity, as has been previously suggested (Jiang et al., 2011). A muscular involvement is supported by the restriction of bursting spontaneous activity to stretch-sensitive afferents. Indeed, some cases of burst firing in tension-mucosal receptors was induced by excessive stretching of the tissue during pinning.

Furthermore, the authors comment on the co-incidence of burst like spontaneous activity with contractions in the muscularis-mucosae (Page and Blackshaw, 1999). However, any contractile activity would have to overcome the presence of both atropine and nifedipine in the krebs buffer. Burst firing may also represent firing from injured nerve fibres as previously suggested by Lord Adrian.

Spontaneous activity was evident in a proportion of each HVA subtype; mesenteric (1/2, 50%), serosal (8/22, 36.4%), muscular (16/20, 80%), muscular-mucosal (1/1, 100%). The proportion of serosal fibres displaying spontaneous activity is similar to previous reports in mice (Brierley et al., 2004). However, muscular HVAs were more likely to be spontaneously active compared to previous studies; HVAs 80% vs. 40% mouse models (Brierley et al., 2004). Nociceptors in the skin do not exhibit spontaneous activity, until they have been challenged by a noxious stimulus (Burgess, 1973, Cervero, 1994). Similarly in the heart it has been argued that nociceptors exhibit no spontaneous activity, and it was postulated that this may extend to all viscera (Malliani, 1989). However, without direct evidence, visceral nociceptors, in animal or humans, cannot be identified by a lack of spontaneous activity, as has been proven in cutaneous nociceptors (Cervero, 1994). Indeed, it is not necessarily true that every action potential from a primary afferent will activate a second order neuron in the spinal cord (Sengupta and Gebhart, 1994). Furthermore, it is possible that spontaneous activity in nociceptors is not physiological, and is caused by factors such as trauma during surgery/excision and ischemia (Cervero and Sann, 1989, Longhurst et al., 1991).

Rates of spontaneous activity in serosal afferents was lower (0.8±0.2 spikes s^{-1}), compared with muscular afferents (4.2±0.9 spikes s^{-1}), consistent with a putative noxious and non-noxious role for these 2 different fibre types. Low levels of spontaneous activity in HVAs have been previous reported, with both appendix (2.4±0.6 spikes s^{-1}) and colon (2.0±0.4 spikes s^{-1}) preparations displaying similar rates (Peiris et al., 2011). The spontaneous activity rates of both serosal and mesenteric afferents are very similar to those reported in mice, rat and cat

splanchnic afferent pathways, HVA: 0.5 and 0.8 spikes s^{-1} vs. animal 0.5 and 0.38 spikes s^{-1}, respectively (Lynn and Blackshaw, 1999, Brierley et al., 2004). In contrast, the spontaneous activity rates of muscular and muscular-mucosal HVAs are more similar to those reported in tension and tension-mucosal afferents in vagal afferent pathways; HVAs 4.2 and 1.6 spikes s^{-1} vs. animal 10 and 3 spikes s^{-1} (Page and Blackshaw, 1999, Page et al., 2002, Zagorodnyuk et al., 2003).

Spontaneous activity rates tended to vary in long HVA experiments. This event has been previously reported to be 5-HT_3 mediated, at least in vagal mucosal afferents (Blackshaw and Grundy, 1993, Hillsley et al., 1998). Further study of this phenomenon in HVAs is warranted.

This project reports a transient cessation of spontaneous activity after the removal of a mechanical stimulus, i.e. VFH probing, circumferential and longitudinal stretch, and distension. Indeed, the withdrawal of any of these stimuli can cause a temporary interruption of spontaneous activity in a number of animal models (Lynn and Blackshaw, 1999, Andrew and Blackshaw, 2001, Zagorodnyuk et al., 2003) and in HVAs (Jiang et al., 2011). This was not studied in depth however, an enteric occult reflex described recently (Dickson et al., 2007) has been theorised to play a role in this phenomenon (Schemann, 2011). This reflex describes the release of nitric oxide from descending interneurons, which subsequently causes muscle relaxation on the anal side, which may affect the action potential discharge from any adjacent afferent fibres (Dickson et al., 2007, Schemann, 2011). This theory could be investigated in HVAs using nitric oxide synthase inhibitors such as L-NG-Nitroarginine Methyl Ester (L-NAME).

2.1.4.7 TRPV4

This report has demonstrated a role for TRPV4 in the transduction of mechanical stimuli. Only serosal and mesenteric HVAs were used in flat sheet studies, identified by their response to <1g VFH probes. TRPV4 has previously been implicated as a transducer of noxious mechanical

stimuli specifically in mouse serosal and mesenteric afferents (Brierley et al., 2008). The results presented in this report corroborate the putative role of TRPV4 as a transducer of mechanical stimuli, likely to include noxious stimuli, given the location of the terminals in the serosa and mesentery. Responses in muscular afferents in flat sheet preparations were not tested.

Application of GSK1016790A increased activity in 3 serosal afferents. Although this may be a direct effect on the afferents, an indirect action, whereby GSK1016790A activates another cell type, which subsequently releases another mediator that then excites the afferent nerves cannot be ruled out. These responding afferents also exhibited a trend towards increased mechanosensitivty to VFH probing after GSK1016790A application, with percentage increases of 5.2%, 13.7%, and 38.5%, although this did not reach significance. Furthermore, with the exception of the latter afferent, both positive and negative percentage changes in mechanosensitivity occur to a similar degree in afferents that did not directly respond to GSK1016790A. The former 2 values are within, or close to, the natural variance exhibited by VFH time matched controls (figure 2.12). Therefore, the significance of the percentage changes in the former 2 afferents is minimal. GSK1016790A also did not alter mechanosensitivity in afferents that did not respond to GSK1016790A. An excitation of intestinal afferents has not previously been demonstrated in rodent models. This may reflect the use of alternative agonists such as EET and 4αPDD, the latter of which may not be a true TRPV4 agonist (Alexander et al., 2013).

In contrast, consistent with mouse data the TRPV4 antagonist HC067047 significantly reduced the HVA response to VFH probing, an effect which showed partial recovery in a proportion of preparations. To explain this result, after the first 4 experiments it was postulated that a 2g VFH was an excessive stimulus, allowing no room for system redundancy and hence reducing the potential for GSK1016790A to augment the response to probing, but preserving the inhibitory potential of HC067047. However, the remaining experiments using a 400mg VFH, which elicits a substantially lower HVA response, produced similar results.

High levels of constituent activation of TRPV4 channels in human intestinal afferents could potentially explain the lack of effect GSK1016790A had on afferent mechanosensitivity, despite the clear reduction induced by the antagonist, HC067047. However, this is difficult to explain given the role TRPV4 receptors play as cell sensors, transducing various stimuli. The specificity of HC067047 must be considered, as high doses have been shown to antagonise the hERG and TRPM8 receptors, which could be potentially contributing to the reduced mechanosensitivity exhibited in this report. However, these receptors are not generally considered transducers of mechanical stimuli, although there is very limited somatic evidence for some involvement in mechanosensitivity (Brignell et al., 2008, Angus et al., 2011).

In this report no distinction was made between vagal, pelvic and splanchnic nerves. Previous reports in mice, have demonstrated augmented responses to VFHs in serosal and mesenteric afferents from the splanchnic nerve and serosal afferents in the pelvic nerve (Brierley et al., 2008). However, TRPV4 receptors seemed to have no role in the mechanotransduction of mechanical stimuli in splanchnic, pelvic or vagal muscular, muscular-mucosal, or mucosal afferents. Indeed, vagal afferents may be present in certain regions of the human intestinal tissue used in these experiments especially in the ileum, but do not constitute a proportion of serosal afferents.

2.1.5 CONCLUSION

The subtypes of colonic afferents innervating the mouse colon have only recently been elucidated (Brierley et al., 2004). This report represents the expansion of the preliminary characterisations of human visceral afferents described by Jiang et al (2011). Our data demonstrates the existence of subtypes of afferents that terminate in the mesentery, serosa, muscle and muscular-mucosal layers. Each subtype responds to a distinct subset of mechanical stimuli, with specific activation thresholds. Examination of the role of these subtypes in normal conditions and in disease states is warranted.

Intestinal nociceptors have been postulated to terminate in the serosa and the mesentery, both of which have been characterised in this HVA model. Further confirmation of their role in nociception will be examined in the next chapter. “Silent” nociceptors that are only responsive to mechanical stimuli after sensitisation by inflammatory mediators have also been described.

This report has demonstrated a higher spontaneous activity firing rate in muscular compared to other HVA subtypes. This may reflect may reflect a greater role in transmitting physiological information to the CNS. An enteric occult reflex may explain the transient inhibition of spontaneous activity following the cessation of a mechanical stimulus, which could be examined in the future.

A proportion of fibres responded directly to the TRPV4 agonist GSK1016790A. In these afferents there was a trend towards an augmented response to VFH probing, although this wasn’t significant. There was no alteration in mechanosensitivity to VFH probing after GSK1016790A in afferents that did not respond directly to the drug. The TRPV4 antagonist HC067047 attenuated the response of HVAs to VFH probing, specifically in experiments using 400mg VFHs. This indicates a role for TRPV4 in the transduction of mechanical stimuli in HVAs, as has been previously demonstrated in mice.

CHAPTER 2 PART 2: CHARACTERISATION OF SUBTYPES OF AFFERENTS INNERVATING THE HUMAN APPENDIX

2.2.1 INTRODUCTION

2.2.1.1 DISTENSION SENSITIVE AFFERENTS

The stimulus that best represents perceived sensation, both physiological and nociceptive is a rise in intraluminal pressure of the colon (Lipkin and Sleisenger, 1958, Ness and Gebhart, 1990). Indeed, distension of the colon by raising intraluminal pressure is a classical stimulus to investigate the function of extrinsic afferent fibres (Janig and Koltzenburg, 1991). Experiments from cats, rats and mice, demonstrate that distension of the gut wall can activate splanchnic, pelvic and vagal afferents (Blumberg et al., 1983, Ness and Gebhart, 1987, Ness and Gebhart, 1988a, Ness and Gebhart, 1988b, Janig and Koltzenburg, 1991, Sengupta and Gebhart, 1994, Brooks and Tracey, 2005).

Different types of distension responses have been reported including rapidly adapting, an initial burst of activity followed by a complete adaptation, and slowly adapting, a gradual adaptation to a tonic level of distension induced activity (Blumberg et al., 1983, Janig and Koltzenburg, 1991). Similarly, afferents have also been characterised based on their threshold of activation (Sengupta and Gebhart, 1994). Splanchnic nerves innervating the ferret gallbladder, the oesophagus of the opossum, and the colon of the cat, and pelvic nerves innervating the mouse bladder, cat bladder, and rat colon, have all been shown to have both low threshold (LT) and high threshold (HT) afferent fibres (Cervero, 1982, Blumberg et al., 1983, Sengupta et al., 1990, Habler et al., 1990, Sengupta and Gebhart, 1994, Sengupta et al., 1999, Rong et al., 2002). LT afferents have low thresholds for activation and tend to saturate at

low distension pressures. HT afferents are not activated until higher pressures are reached, and continue to signal into the noxious range of distension pressures. However, the threshold pressure for LT and HT afferents are not equivocally defined, with LT being defined as 3 to <25mm Hg, and HT as >20 to ≥33mm Hg, although these differences are partially explained by the different pressures required to produce pain in different species (Sengupta and Gebhart, 1994). Furthermore, some studies have demonstrated the existence of WDR units, which have low thresholds for activation, but continue to signal into the noxious range of distension pressures, in addition to LT and HT fibres (Sengupta et al., 1990, Rong et al., 2004).

Most studies dividing distension sensitive afferents between LT and HT report a higher proportion of LT fibres (2/3 to 3/4), with high threshold afferents making up the rest. Other studies, for example, Rong et al, (2004) reported very high proportions of WDR afferents (67%) compared to LT (14%) or HT (19%) when recording from mesenteric nerves innervating the jejunum of the mouse, although these included recordings from both spinal and vagal fibres. Furthermore, when considering just the threshold for activation, LT fibres may still constitute the majority of afferents. It is only when the saturation point of afferent firing is considered do LT and WDR afferents differentiate, a concept not applied across all papers reporting just LT and HT fibres.

In the colon, LT fibres tended to respond with higher firing rates than HT fibres to all distension pressures (Sengupta and Gebhart, 1994). The threshold of afferent nerves in the splanchnic and pelvic pathways is likely to be affected by the location of their terminals in the colon wall. Indeed, muscular, and muscular-mucosal afferents tend to have lower distension thresholds compared to serosal afferents e.g. (Zagorodnyuk and Spencer, 2011).

To date, the thresholds and characteristics of distension sensitive human afferents have not been investigated. This report aims to examine the different types of distension

sensitive afferents innervating the human appendix, based on their response threshold and firing frequency to luminal distension.

2.2.1.2 TRPV4

TRPV4 channels and their involvement in the transduction of mechanical stimuli are discussed in chapter 2 part 1. The aim of this report was to examine the role of TRPV4 receptors in the transduction of mechanical stimuli, specifically luminal distension of the appendix.

2.2.1.3 AIMS

- Examine the effect of luminal distension of the appendix on HVAs
- Examine the different types of distension sensitive afferents innervating the human appendix, based on their response threshold to luminal distension, their firing rate and the pressure at which the firing rate plateaus
- Ensure repeated luminal distension are reproducible by conducting time matched controls
- Examine the role of TRPV4 channels in the transduction of mechanical stimuli, specifically luminal distension of the appendix, in human afferent nerves

2.2.2 METHODS

2.2.2.1 PATIENTS

Resected human appendix were collected after written consent from patients undergoing elective surgery for cancer, polyps, CD, UC, DD, trauma, chronic constipation, or appendicitis at the Royal London Hospital or Whipps Cross University Hospital (London, UK). Normal appendices were usually collected from right hemicolectomies, undertaken as an intervention for colon cancer. Acutely inflamed appendices were collected from appendicitis cases. Furthermore, appendices from subtotal colectomies, and panproctocolectomies, undertaken as an intervention for IBD were also collected. Appendices were returned to the pathology department after experimentation.

2.2.2.2 APPENDIX DISTENSION

The majority of experiments took place on the day of surgery. However, in some circumstances tissues were placed in carbongenated Krebs buffer and stored overnight at 4°C (see chapter 5 part 2). Firstly, the tissue was grossly examined using a stereomicroscope (M5A, Wild Heerbrugg) and blood vessel arcades identified. The lumen of appendix preparations was flushed using Krebs buffer. Excess mesentery was removed before the tissue was transferred to the tissue bath and cannulated. The tissue was then superfused with carbongenated (95% O2, 5% CO2) Krebs buffer (6ml/min; 32-34°C; pH 7.4; 124mM NaCl, 4.8mM KCl, 1.3mM NaH_2PO_4, 1.2mM $MgSO_4.7H_2O$, 2.5mM $CaCl_2$, 11.1mM Glucose, 25.0mM $NaHCO_3$), supplemented with atropine (10µM) and nifedipine (10µM). Nerves running in close proximity to the blood vessel arcades were finely dissected using a microscope (SZ40, Olympus). Nerves were then sucked into a boroscilicate glass suction electrode (Harvard Apparatus), which was filled with Krebs buffer and neuronal activity recorded using a differential amplifier (headstage and AC/DC amplifier (gain 5K) (Neurolog Ltd). The analogue signal was then band pass filtered (100-2000Hz; digitally filter using a humbug 50Hz filter(Quest Scientific) following which the

resultant signal was digitised at a sampling rate of 20KHz using a Micro 1401 MKII (Cambridge Electronic Design) and displayed a desktop computer running Spike2 software in a chart recorder format. Data was stored for further off line analysis (Cambridge Electronic Design). Additionally neuronal activity was also simultaneously counted from the filtered and amplified signal using a spike processor (Digitimer). The threshold for spike counting was set at twice the background noise and the output from the spike processor sent to the events channel on the 1401 for processing and relay to the desk top computer were it was displayed alongside the raw trace on spike 2. Nerve activity was expressed as a rate histogram as either spikes/$20s^{-1}$, $5s^{-1}$, or $1s^{-1}$ (Peiris et al., 2011).

Cannulated appendix preparations were distended using a mechanical driven perfusion pump (Genie Touch, Kent Scientific Corporation), which delivered 1 or 1.5ml/min of Krebs buffer into a sealed system, depending on the size of the appendix, to ensure distension from 0 to 60mm Hg took 80-100 seconds (figure 2.15).

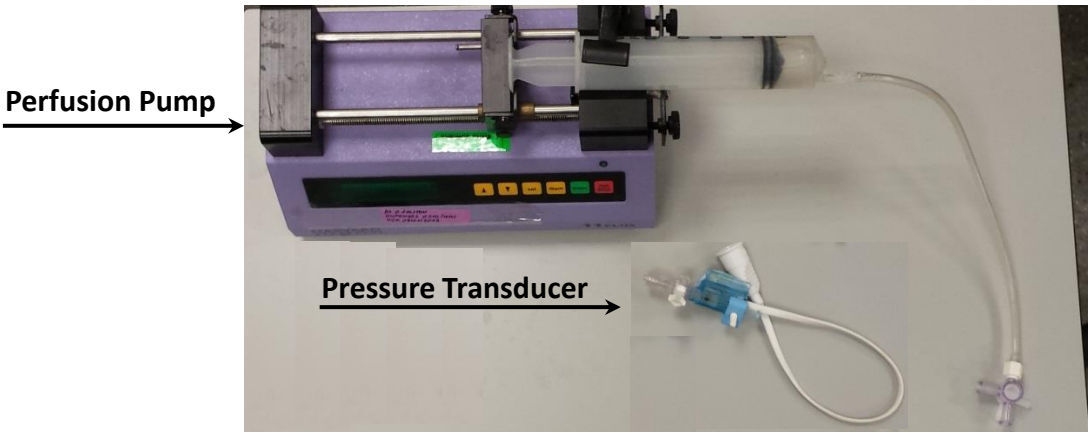


Figure 2.15: A perfusion pump and pressure transducer were used to luminally distend appendices.

2.2.2.2.1 Characterisation of distension sensitive afferents

The 3 baseline distensions from each preparation were used to characterise distension sensitive afferents based on their threshold for activation, their firing rate, and the pressure at which their firing plateaus. Firstly, single units were identified using waveform analysis, as previously described in chapter 2 part 1. The response of each unit to each of the 3 baseline distensions, at pressure points, 10, 20, 30, 40, 50, 60 mm Hg were recorded. The responses at each of these pressure points were then averaged, i.e. the 3 values at 10mm Hg were averaged etc. These values at each pressure point were then used to characterise the distension sensitive units (figure 2.16).

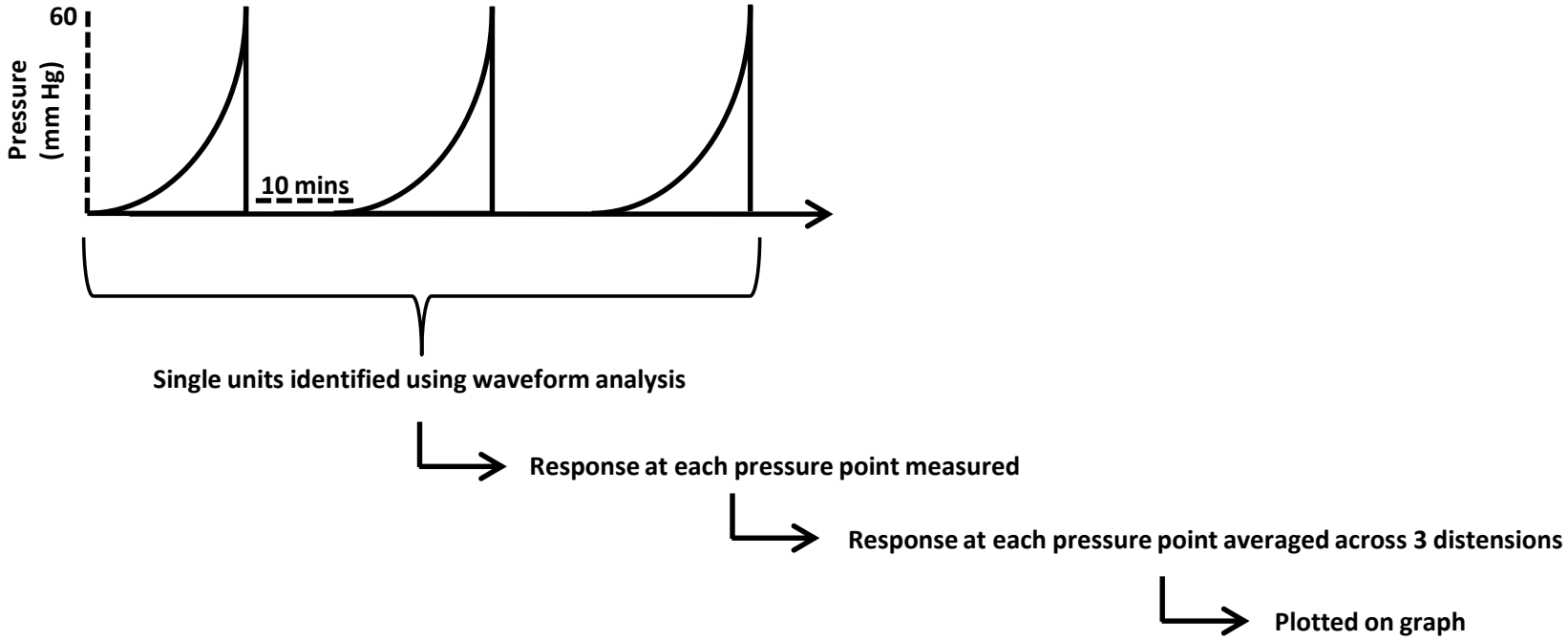


Figure 2.16: Distension characterisation protocol

1) Single units identified.
2) Responses at pressure point, in 10mm Hg increments, were measured.
3) The responses at each pressure point were then averaged across the 3 distension i.e. response at 10mm Hg at distension 1, 2 and 3 were averaged.
4) The averages were then plotted on a graph and used to characterise the distension sensitive afferents.

2.2.2.2.2 Distension Time Matched Controls

To investigate the reproducibility and stability of repeated appendix distensions, time matched control experiments were performed. This involved appendix distension every 10 minutes as normal, but no drug was perfused into the tissue bath or through the lumen. The average of the responses to the first 3 distensions were then compared to the average of the subsequent 2 consecutive distensions i.e. distensions 4, 5 distension 5, 6 etc. at each 10 mm Hg pressure point. These were then compared using a 2 way ANOVA, $p<0.05$.

2.2.2.3 APPENDIX DISTENSION PROTOCOLS

Three distensions, each separated by 10 minutes, were used as baseline responses. Drugs were added immediately after the 3rd baseline distension. For TRPV4 experiments either GSK1016790A (BC 2µM, 20ml of 10µM) or HC067047 (BC 20µM, 20ml of 100µM) was subsequently superfused into the bath. In a subset of experiments HC067047 was superfused into the bath and through the lumen (BC 20µM, 100ml of 20µM, 20ml of 20µM luminal perfusion). In all the experiments, distensions were continued every 10 minutes after the 3 baseline distensions (figure 2.17). For analysis, firing frequency (spikes $5s^{-1}$) was measured at each 10 mm Hg pressure increments up to 60mm Hg. The firing frequency at each pressure point was averaged across the 3 baseline distensions. These baseline values at each pressure point were then compared to their respective pressures in distensions performed when the concentration of the drug in the bath was at its highest, i.e. the 1st post drug distension for low volume experiments (20ml), and the 2nd post drug distension for high volume experiments (100ml). Responses were analysed using a 2 way ANOVA, $p<0.05$.

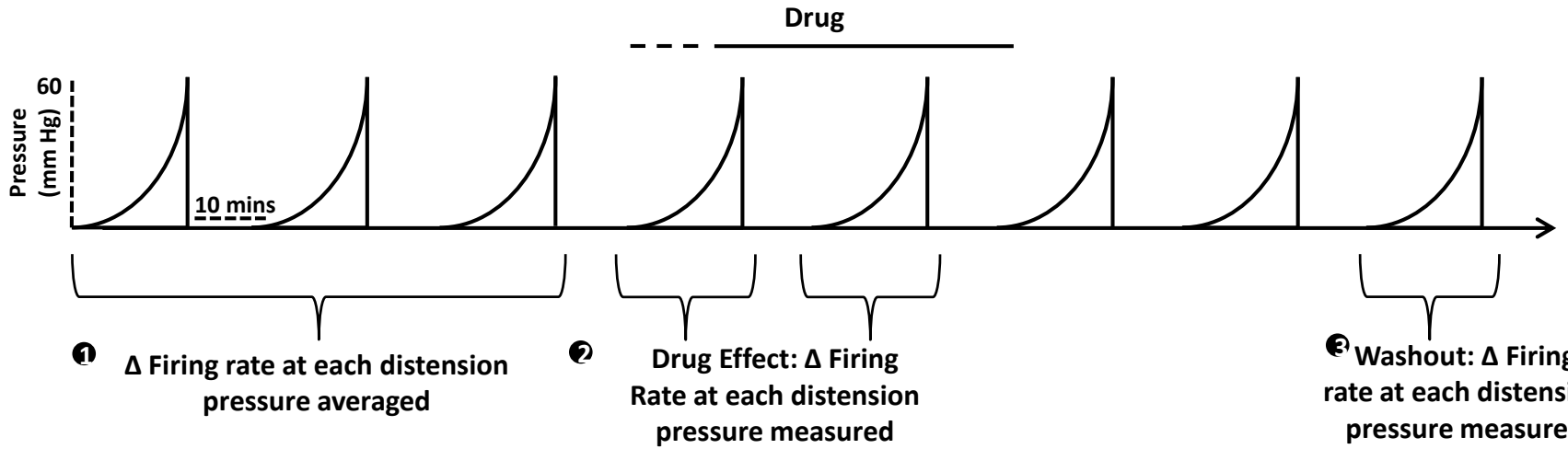


Figure 2.17: TRPV4 distension protocol

1) The Δ in firing rate at each 10mm Hg pressure point was averaged for the 3 baseline distensions.

2) In experiments with low drug volumes (20ml, dotted line), the 1st post drug distension is then compared to the average of the baseline distensions. The 2nd post drug distension was compared to the average of the baseline distensions in experiments using higher drug volumes (100ml, dotted + solid line).

3) For drug vs. washout comparisons, the 1st (20ml) or the 2nd (100ml) post drug distensions were compared to the 5th post drug distension.

2.2.2.4 DRUGS

Drugs in powder form were made up using the recommended solutions, aliquoted and frozen at 20°C. When needed, aliquots were diluted in Krebs to make the final working concentration and vortexed to mix. GSK1016790A was obtained from Sigma Aldrich (St Louis, MO, USA). HC067047 was purchased from Tocris Bioscience (Bristol, UK).

2.2.3 RESULTS

2.2.3.1 OVERALL TISSUE COLLECTION – APPENDIX

Eighty-three appendix tissues were collected from surgery. Of these, 66 were used for electrophysiological recordings. Fifty of these appendices were cannulated; although not all were suitable for distension protocols (figure 2.18). A summary of how appendices were assigned experiments is in figure 2.19.

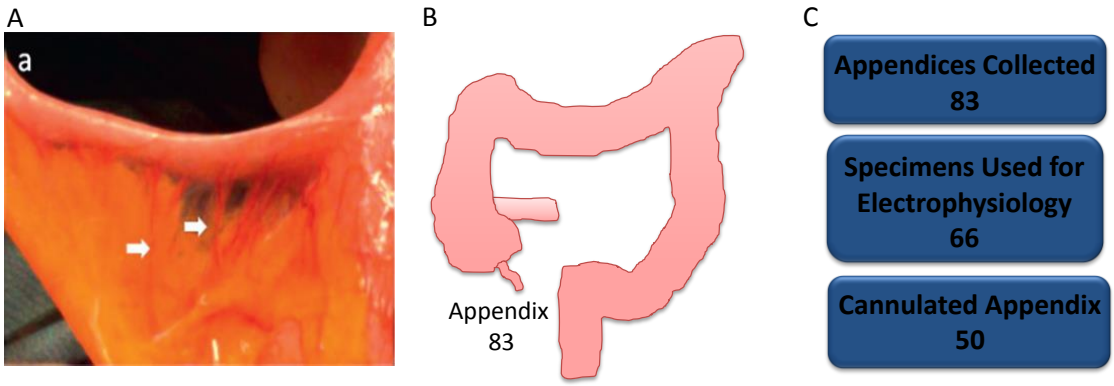


Figure 2.18: A) Shows an appendix and attached mesentery. The white arrows indicate the blood vessel arcades, with which the nerves track (Peiris et al, 2011). B) A diagram of the human colon indicating the number of appendices collected for used in research. C) Details the number of appendices collected, and subsequently used for electrophysiology, as well as the number that were cannulated during experimentation.

2.2.3.2 TISSUE – DISTENSIONS SENSITIVE AFFERENT CHARACTERISATION

Twenty appendices, 12 normal, 4 CD, 2 UC, 2 appendicitis, were used for distension sensitive afferent characterisation (M:F 1:1.22, median age 51).

2.2.3.3 TISSUE – TRPV4 DISTENSION EXPERIMENTS

Nine appendices, 4 normal, 2 CD, 2 UC, 1 appendicitis, were used for TRPV4 distension experiments (M:F 1:0.5, median age 34). Further details on the tissues use in each set of experiments can be seen in table 2.02.

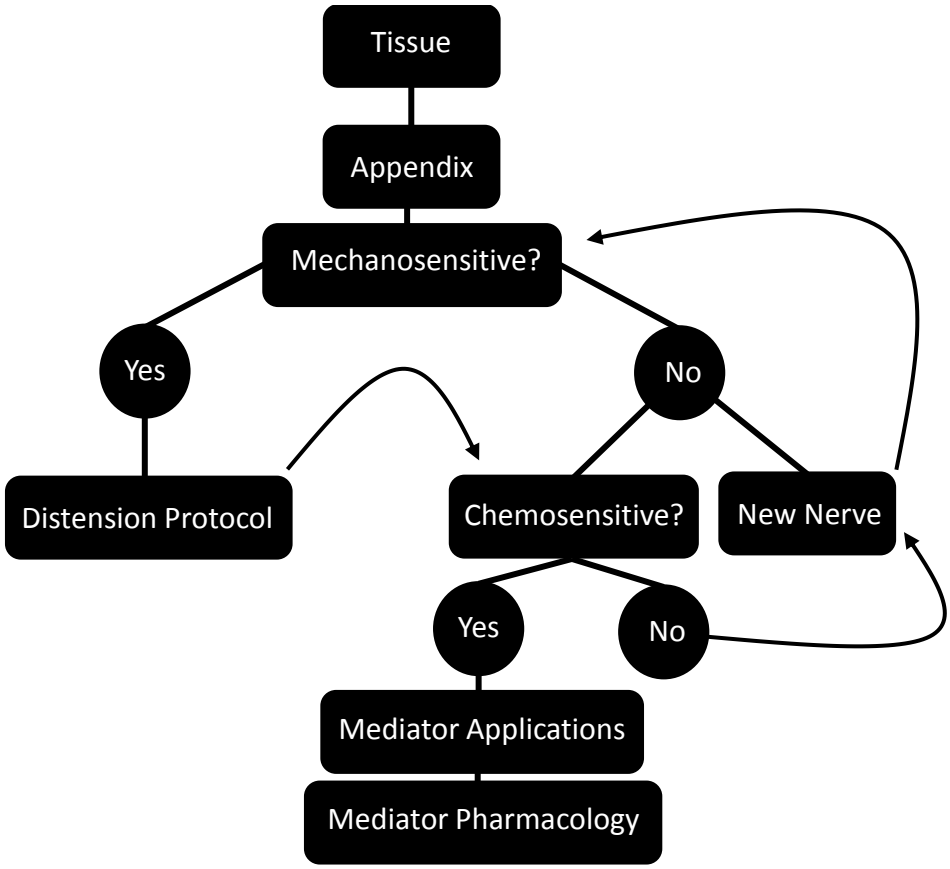


Figure 2.19: Given the limited supply of human tissue, every effort was made to make the most out of each piece. In each preparation, mechanosensitivity of the afferent was tested first by distending the appendix. If the nerve was mechanosensitive the nerve was characterised based on its threshold for response to luminal distension. If the preparation was deemed suitable, a distension protocol was then performed. If the nerve was not mechanosensitive, or deemed unsuitable, the nerve was discarded and a new recording from a separate nerve was attained. If no other suitable nerves were available or the 3 consecutive unsuitable nerves were found, a chemosensitivity protocol was performed.

2.2.3.4 DISTENSION SENSITIVE AFFERENTS

Distension sensitive afferents responded in a graded manner to rising intra-luminal pressures in the appendix. This could begin at pressures as low as 10mmHg and continued well into noxious pressures (60 mm Hg). Twenty appendices were used for characterisation from which 46 discriminated units were identified. Three different types of distension sensitive units could be identified based on the pressure threshold at which they start responding to distension, their firing frequency, and the pressure at which their firing starts to plateau (figure 2.10, table 2.04). Thirteen distension sensitive units were classified as low threshold units (28.3%), based on their high firing rate, in response to a small rise in luminal pressure, starting at 10mm Hg (>20 spikes /5s^{-1}), the lowest pressure measured. These units generally reached peak firing rate at around 30mm Hg, after which the firing rate saturated despite increasing luminal pressure. Twenty-seven distension sensitive units were classified as wide dynamic range units (WDR) (58.7%) based on their gradually increasing response to incremental luminal pressure. This started at low pressures 10mm Hg, with a moderate firing rate (<20 spikes/5s^{-1}) and usually continuing to increase up to 60 mm Hg. Six distension sensitive units were classified as high threshold units (13.0%) based on their lack of response to distension at 10mm Hg, and low firing rate (<10 spikes/5s^{-1}) to distension at 20 mm Hg. These units tended to responded incrementally to pressures of 30 to 60 mm Hg (figure 2.20). Of note, low threshold (0/4 BK, 0/4 ATP, 1/2 capsaicin mediators elicited a response), wide dynamic range (1/1 BK, 1/1 capsaicin) and high threshold (0/2 BK, 1/1 ATP) distension sensitive afferents were chemosensitive to bath application of BK, ATP or capsaicin.

The firing rate of low threshold units in response to luminal distension were significantly higher at all pressures compared to WDR or high threshold units ($p<0.001$). The firing rates of WDR and high threshold units in response to distension were similar between 30-60 mm Hg luminal pressure range ($p>0.05$). The average change in afferent firing rate in

response to each pressure for low threshold, WDR, and high threshold units are shown in table 2.04.

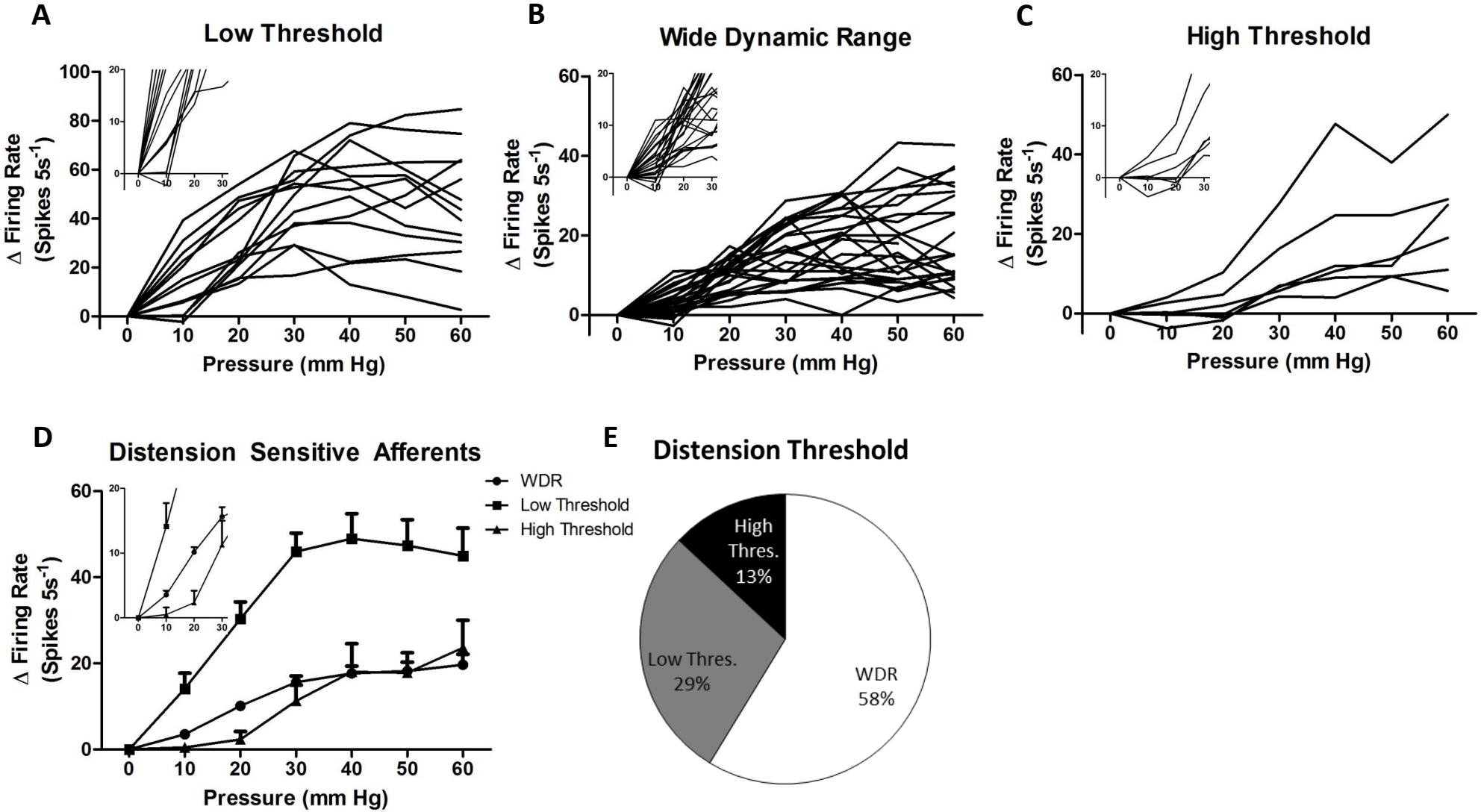

A
Low Threshold
Δ Firing Rate (Spikes 5s-1)
Pressure (mm Hg)
B
Wide Dynamic Range
Δ Firing Rate (Spikes 5s-1)
Pressure (mm Hg)
C
High Threshold
Δ Firing Rate (Spikes 5s-1)
Pressure (mm Hg)
D
Distension Sensitive Afferents
WDR
Low Threshold
High Threshold
Δ Firing Rate (Spikes 5s-1)
Pressure (mm Hg)
E
Distension Threshold
High Thres. 13%
Low Thres. 29%
WDR 58%

Figure 2.20: Characterisation of distension sensitive HVAs. Three different types of distension sensitive HVAs were identified based on their pressure threshold for activation, A) low threshold (LT), B) wide dynamic range (WDR), C) high threshold (HT). D) shows the mean firing rates of each of these units over the pressure range. LT units had a higher firing rate in response to distension at all pressures, compared to WDR and HT units (see table 2.04). E) displays the proportion of each subtype of distension sensitive afferent. WDR units were the most prevalent, making up 58% of distension sensitive afferents.

Pressure (mm Hg)	Low Threshold	WDR	High Threshold
10	14.1 ± 3.6	3.3 ± 0.6	0.5 ± 1.1
20	30.3 ± 4.0	9.5 ± 0.8	2.3 ± 1.9
30	46.0 ± 4.3	14.8 ± 1.4	11.3 ± 3.7
40	49.0 ± 5.8	16.9 ± 1.7	18.0 ± 6.6
50	47.4 ± 6.0	17.5 ± 2.0	17.8 ± 4.7
60	45.0 ± 6.5	18.4 ± 2.2	23.6 ± 6.4

Table 2.04: Table showing average firing rates (spikes $5s^{-1}$) at pressures 10-60 mm Hg for low threshold, wide dynamic range, and high threshold distension sensitive afferents.

2.2.3.5 DISTENSION TIME MATCHED CONTROLS

Distension time matched control experiments were firstly analysed as whole nerve recordings. The line graph produced over pressure 10-60 mm Hg for the first 3 baseline distensions closely matched the line graphs representing the subsequent sets of consecutive distensions (n=2, figure 2.21). Furthermore, when these experiments were analysed using individual units, there was no significant change in the HVA response to distension between the first 3 baseline distensions compared to any subsequent set of 2 consecutive distensions in WDR units (n=3, $p>0.05$, figure 2.21). One LT unit was also identified with no obvious variations in HVA response to luminal distension across the pressure ranges between baseline and subsequent sets of distensions.

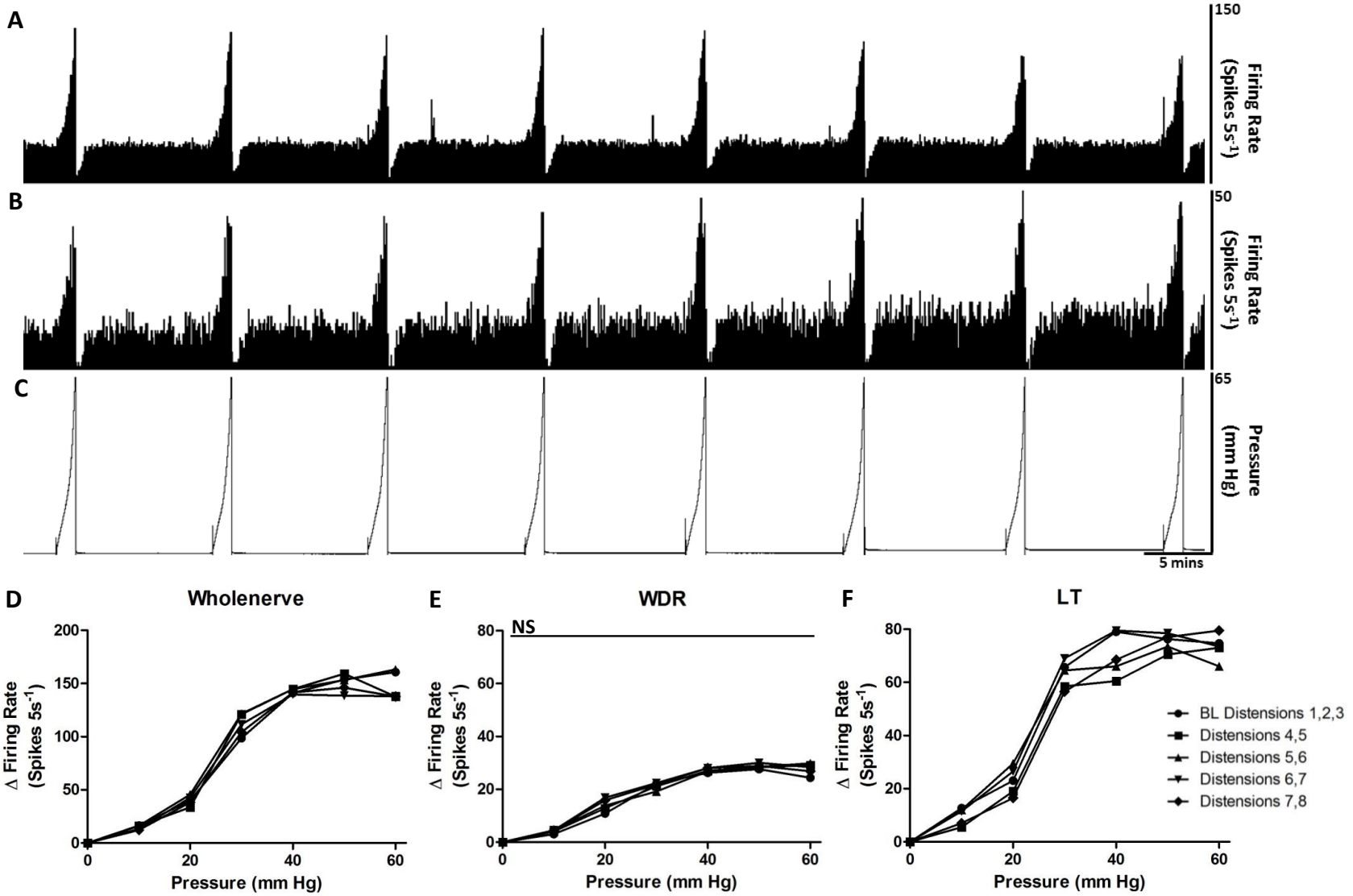

A
150
Firing Rate
(Spikes 5s-1)
B
50
Firing Rate
(Spikes 5s-1)
C
65
Pressure
(mm Hg)
5 mins
D
Wholenerve
Δ Firing Rate
(Spikes 5s-1)
Pressure (mm Hg)
E
WDR
NS
Δ Firing Rate
(Spikes 5s-1)
Pressure (mm Hg)
F
LT
Δ Firing Rate
(Spikes 5s-1)
Pressure (mm Hg)
BL Distensions 1,2,3
Distensions 4,5
Distensions 5,6
Distensions 6,7
Distensions 7,8

Figure 2.21: Distension time matched controls. Repeated luminal distension of the appendix every 10 minutes produced consistent HVA responses over an 80-90 minute period. A-B) Shows an example of repeated whole nerve (A) and wide dynamic range (WDR) (B) HVA responses to luminal distension of the appendix in rate histogram form. C) Shows the pressure curve for each distension. D, E, F) Displays the whole nerve (n=3) (D), WDR (n=2) (E), and low threshold (n=1) (LT) (F) line graphs for each set of distensions. Baseline distensions in WDR units were not significantly different to any subsequent set of 2 consecutive distensions at any pressure, $p>0.05$

2.2.3.6 TRPV4 DISTENSION PROTOCOL

GSK1016790A (n=4) did not activate any HVAs. Bath superfusion of GSK1016790A did not alter the whole nerve HVA response to luminal distension, at any pressure point, or across the pressure ranges (n=4, $p>0.05$). Similarly, when the recordings were analysed as individual units, GSK did not change the response of WDR afferents (n=3, $p>0.05$, figure 2.22). One LT unit was also identified, but more of these units are needed before comment can be made.

Bath superfusion of HC067047 did not alter the whole nerve HVA response to luminal distension at any pressure point or across the pressure range (n=6, $p>0.05$). HC067047 did not alter the mechanosensitivity of the 4 WDR units identified in these whole nerve recordings (n=4, $p>0.05$, figure 2.23). Two LT units were also identified, but their pressure line graphs almost completely overlaid those of baseline.

Combined bath superfusion (higher volume, lower dose than previous experiments) and luminal perfusion of HC067047 failed to alter the response of whole nerve HVAs to luminal distension, at any pressure point or across the pressure range (n=5, $p>0.05$). In addition, there was no change in the response of WDR units to distension after combined bath and luminal application of HC067047 (n=8, $p>0.05$, figure 2.23). The data was also pooled for analysis regardless of drug application method, however, HC067047 did not significantly alter the whole nerve (n=11), WDR (n=12) or LT (n=3) HVA response to luminal distension at any pressure (data not shown in figure form, $p>0.05$).

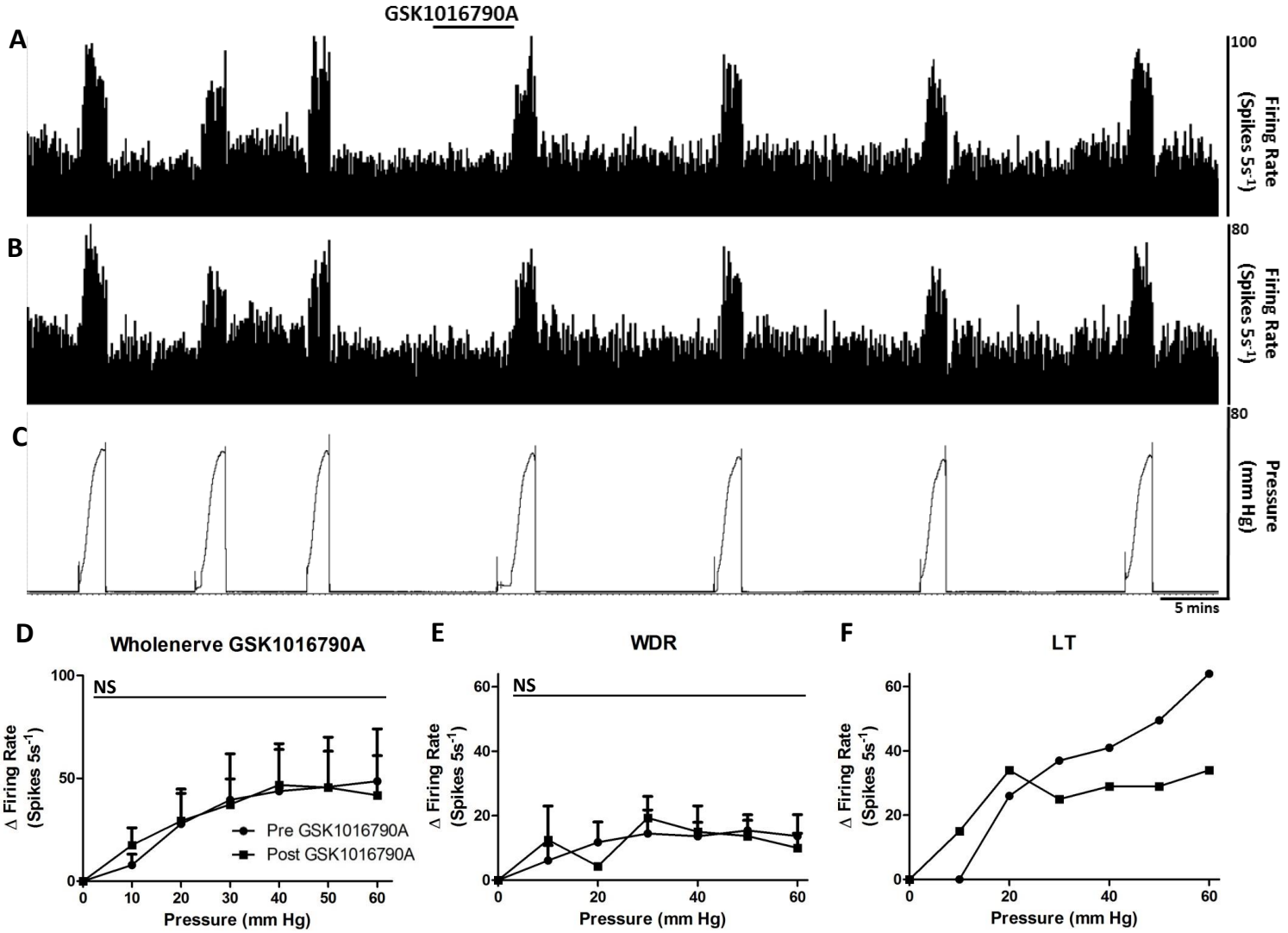

GSK1016790A
A
100
Firing Rate (Spikes 5s-1)
B
80
Firing Rate (Spikes 5s-1)
C
80
Pressure (mm Hg)
5 mins
D
Wholenerve GSK1016790A
NS
Δ Firing Rate (Spikes 5s-1)
Pre GSK1016790A
Post GSK1016790A
Pressure (mm Hg)
E
WDR
NS
Δ Firing Rate (Spikes 5s-1)
Pressure (mm Hg)
F
LT
Δ Firing Rate (Spikes 5s-1)
Pressure (mm Hg)

Figure 2.22: Application of the TRPV4 agonist GSK1016790A did not alter the HVA response to luminal distension. A-B) Shows an example of repeated whole nerve (A) and WDR (B) HVA responses to luminal distension of the appendix in rate histogram form. GSK1016790A application is noted by the solid black line. C) Shows the pressure curve for each distension. D-E) Bath application of GSK1016790A did not alter the whole nerve (n=4) (D) or WDR (n=3) (E) HVA response to distension, $p>0.05$. F) One LT units was also identified in these recordings. Data were analysed using a 2 way ANOVA, $p<0.05$.

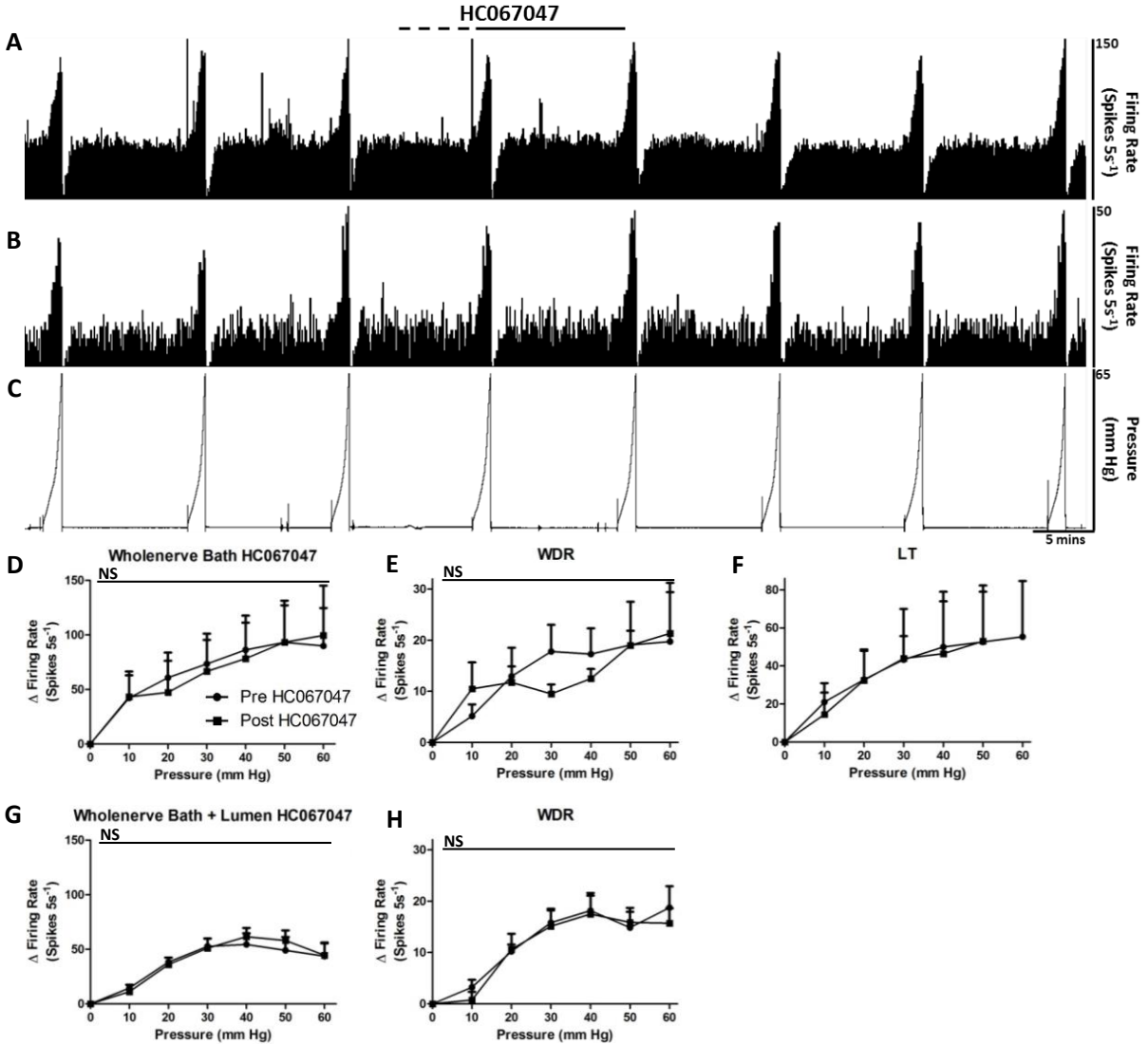

HC067047
A
150
Firing Rate (Spikes 5s-1)
B
50
Firing Rate (Spikes 5s-1)
C
65
Pressure (mm Hg)
5 mins
D
Wholenerve Bath HC067047
NS
Δ Firing Rate (Spikes 5s-1)
Pre HC067047
Post HC067047
Pressure (mm Hg)
E
WDR
NS
Δ Firing Rate (Spikes 5s-1)
Pressure (mm Hg)
F
LT
Δ Firing Rate (Spikes 5s-1)
Pressure (mm Hg)
G
Wholenerve Bath + Lumen HC067047
NS
Δ Firing Rate (Spikes 5s-1)
Pressure (mm Hg)
H
WDR
NS
Δ Firing Rate (Spikes 5s-1)
Pressure (mm Hg)

Figure 2.23: Application of the TRPV4 antagonist HC067047 did not change the HVA response to luminal distension. A-B) Shows an example of repeated whole nerve (A) and wide dynamic range (WDR) (B) HVA responses to luminal distension of the appendix in rate histogram form. HC067047 addition to the bath is marked by the dotted line, while its application to the bath and lumen in together is the dotted and solid line together. C) Shows the pressure curve for each distension. D-E) Bath application of HC067047 did not alter the whole nerve (n=6) (D) or WDR (n=4) (E) HVA response to distension, $p>0.05$. F) Two low threshold (LT) units were also identified in these recordings, and seemed to be unaffected by bath application of HC067047. G) Combined bath and luminal application of HC067047 also failed to change the whole nerve (n=5) (G) or WDR (n=8) (H) HVA response to distension, $p>0.05$. Data were analysed using a 2 way ANOVA, $p<0.05$.

2.2.3.7 SPONTANEOUS ACTIVITY

All distension sensitive afferents, regardless of threshold for activation displayed spontaneous activity. LT distension sensitive afferents had a higher spontaneous activity rate compared to WDR and HT afferents (11.3±3.6 vs. 3.1±0.6 vs. 6.3±4.0 spikes s^{-1}) (figure 2.24).

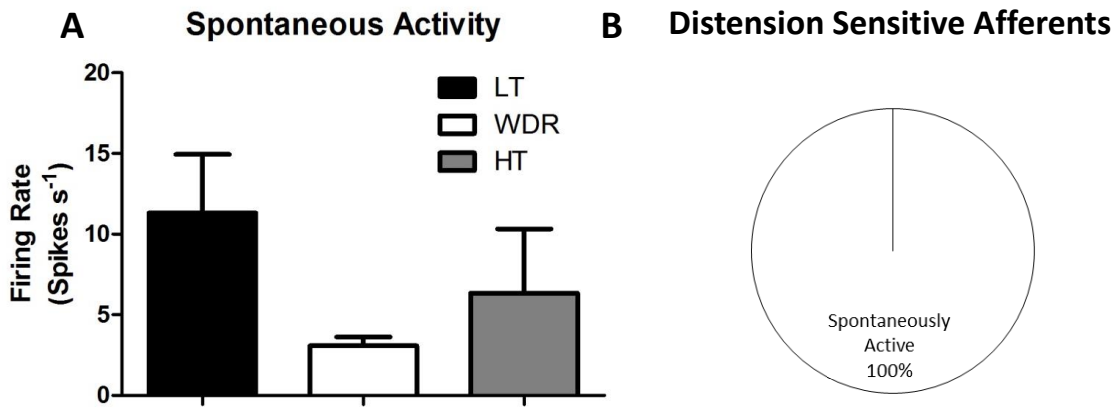


Figure 2.24: A) Spontaneous activity in the different subtypes of distension sensitive afferents. Low threshold (LT) afferents had higher spontaneous activity than wide dynamic range (WDR) units and high threshold units (11.3±3.6 vs. 3.1±0.6 vs. 6.3±4.0 spikes s^{-1}). B) All distension sensitive afferents, regardless of their subtype, displayed spontaneous activity.

2.2.3.8 SUMMARY OF RESULTS

- HVAs innervating the appendix were sensitive to luminal distension of the appendix
- Three subtypes of distension sensitive afferents could be characterised based on their threshold to luminal distension, their firing frequency, and the pressure point at which afferent firing saturates. These subtypes are low threshold, wide dynamic range, and high threshold afferents.
- Repeated luminal distension elicits reproducible responses in whole nerve and WDR units.
- The mechanosensitivity of whole nerve HVAs was not altered by bath application of GSK1016790A. Furthermore, GSK1016790A did not change the WDR HVA response to luminal distension.
- Neither bath application of HC067047 alone or in combination with luminal application of HC067047 alters the response of whole nerve HVAs to distension. In addition, the mechanosensitivity of WDR units was not altered by either treatment.

2.24 DISCUSSION

2.2.4.1 DISTENSION SENSITIVE AFFERENTS

This report demonstrates the presence of 3 functionally distinct distension sensitive afferent fibres, 2 of which are characterised by their threshold for activation, LT, <10mm Hg, and HT >20mm Hg. A further subgroup of WDR distension sensitive afferents also have low thresholds, 10mm Hg, but exhibit an incremental afferent discharge into the noxious distension range. The majority of human distension sensitive afferents are LT/WDR, ~87%, with HT making up the remaining ~13%. This is consistent with previously reported values in animal studies where LT afferents are more prevalent than HT afferents, constituting 66-75% of fibres in the rat colonic pelvic nerves, cat colonic splanchnic nerves, and mesenteric nerves innervating the mouse jejunum (Blumberg et al., 1983, Sengupta and Gebhart, 1994, Rong et al., 2004, Rong et al., 2007). LT HVAs exhibited a significantly higher firing rate at all pressures compared to WDR and HT afferents. Similarly, LT afferents are the fastest firing afferents innervating the rat colon (Sengupta and Gebhart, 1994).

Whether HT afferents are involved in nociception is a contentious issue. In this report the term HT may be considered a relative rather than a descriptive term, since human pain threshold to distension of the colon has been estimated at between 40-60mm Hg (Lipkin and Sleisenger, 1958, Ness et al., 1990), while HVA HT afferents started to respond at 30mm Hg. However, previous studies have characterised afferents as HT when they responded to pressures as low as 20 mm Hg (Cervero, 1982, Blumberg et al., 1983, Sengupta et al., 1990, Habler et al., 1990, Sengupta and Gebhart, 1994, Sengupta et al., 1999, Rong et al., 2002, Rong et al., 2004). Furthermore, given the small size of the appendix in relation to the colon, it is possible that lower pressures may be noxious. Indeed, in the rat colon, which is a similar size to the human appendix, distension of 30-40 mm Hg produces pain related behaviours (Ness and Gebhart, 1988a). Pain threshold may be more related to species than the size of the tissue

being distended. However, since HT HVAs respond in a graded manner into high distension pressure, they are likely to transmit noxious information, at least to the spinal cord. Here it is the coding and processing of the signal that may ultimately decide if it contributes to pain (Sengupta and Gebhart, 1994). Similarly, WDR HVAs responded in a graded manner into high distension pressures, and are potentially nociceptive. LT HVAs are unlikely to transmit nociceptive information, since their firing tends to plateau around 30mm Hg. A caveat in recording from human distension sensitive afferents is the inability to distinguish between afferents from different pathways e.g. vagal vs. splanchnic. This limits the ability to define the role of each afferent pathway in disease mechanisms or drug efficacy. However, the general physiology of afferents that are likely to be nociceptors can be studied. Future studies using appendix distension preparations may need to control for the use of different threshold units.

2.2.4.2 TRPV4 IN DISTENSION SENSITIVE AFFERENTS

Whole nerve, LT, or WDR HVA responses to appendix distension to any pressure were not altered by simple superfusion of the bath with GSK1016790A or HC067047. The human appendix is substantially thicker than rodent colon, and remains cannulated for distension preparations. Therefore it was postulated that bath applied drugs were not able to penetrate to the terminals of distension sensitive afferents, some of which may be deep in the muscular layers of the appendix wall. To examine this hypothesis, a combined bath and luminal application of HC067047 was also tested (n=8), thereby allowing easy access to mucosal afferents, and allowing permeation from 2 sides of the appendix. However, bath and luminal application of HC067047 did not alter whole nerve or WDR HVA responses to distension at any pressure. Considering luminal and serosal exposure, and the longer drug perfusion time in this subset of experiments it is unlikely that poor tissue penetration could account for the lack HC067047 effect. Future studies should examine a small concentration range of HC067047 and GSK1016790A.

HVA distension sensitive afferents used for TRPV4 studies are likely to have terminals in either the serosa or gut musculature. Indeed, there were no HT distension sensitive afferents in any appendix preparation used for TRPV4 experiments, which means the inclusion of any serosal afferents is unlikely, since they, at least in animal models, require high distension pressures to be activated (Brierley et al., 2008). In contrast, in VFH probing protocols, only serosal and mesenteric afferents were tested. Therefore the type of methodology used in an experiment, e.g. distension vs. VFH probing, will determine the subtype of afferents studied, which in turn could influence the efficacy of the drug. The data in this report suggests that TRPV4 antagonism reduces mechanosensitivity of serosal afferents (VFH experiments), but not WDR distension sensitive units, which are likely to muscular afferents. Similarly, in animal studies, TRPV4 receptors were not involved in the transduction of mechanical stimuli in muscular afferents (Brierley et al., 2008). Furthermore, 1 study reported no difference in the VMR to CRD between TRPV4 $^{-/-}$ and TRPV4$^{+/+}$ mice, even at low pressures. However, in mice in which expression of TRPV4 was eliminated by siRNA, there was a reduced VMR response to CRD starting at the lowest pressure measured, 15mm Hg (Cenac et al., 2008). The low pressure at which the TRPV4 siRNA takes effect suggests the afferents are muscular, or muscular-mucosal.

2.2.4.3 SPONTANEOUS ACTIVITY

HVAs from appendix specimens have previously been shown to exhibit spontaneous activity (2.4 spikes s^{-1}). This report outlines the spontaneous activity of distension sensitive afferents based on their thresholds for activation, their firing rate, and the pressure at which their firing rate plateaus. LT afferents exhibited the highest rates of spontaneous activity, considerably higher than muscular units found in flat sheet preparations (11.3±3.6 vs. 4.2±0.9 spikes s^{-1}). Spontaneous activity in WDR units was most similar to muscular afferents (3.1±0.6 vs. 4.2±0.9 spikes s^{-1}). LT and WDR distension sensitive units as well as muscular HVAs had a significantly higher spontaneous firing rate compared to serosal afferents ($p<0.01$). Spontaneous activity of

HT units and serosal afferents was significantly different ($p>0.05$), although this is likely due to low numbers of HT fibres. This is concurrent with the literature, which generally reports higher spontaneous firing rates in afferents responding to stretch of the gut wall, at least in pelvic and vagal pathways.

The higher spontaneous activity rate may reflect a greater role in transmitting physiological information to the CNS. However, it is important not to draw too many conclusions, given the myriad of conditions that likely contribute to the rate of spontaneous activity, e.g. temperature, ischaemic time of the tissue, trauma during surgery, tension on the tissue upon pinning etc.

2.2.5 CONCLUSION

Subtypes of distension sensitive afferents have been described in murine models of colonic distension, based on their distension threshold for activation. This report describes 3 different subtypes of distension sensitive HVAs from appendix specimens, LT, HT, and WDR, the latter being the most prevalent. Although briefly mentioned in this report, further research needs to be done on the chemosensitivity of these different subtypes of HVA.

Bath application of GSK1016790A did not alter the whole nerve or WDR HVA response to distension at any pressure. Similarly, bath perfusion of HC067047 alone or in combination with luminal application did not reduce the whole nerve or WDR HVA response to distension at any pressure. TRPV4 receptors may have a greater role as mechanotransducer channels in serosal HVAs.

CHAPTER 3 PART 1: CHEMOSENSITIVITY OF HUMAN VISCERAL AFFERENTS TO ALGOGENIC MEDIATORS

The primary aim of the studies described in chapter 3 was to investigate the chemosensitivity of the different subtypes of HVAs characterised in chapter 2 part 1. Furthermore, this report aimed to develop chemosensitivity protocols suitable for mechanistic studies and for investigations into therapeutic approaches to treating visceral pain. This chapter is divided into 2 parts. In part 1, the noxious inflammatory mediators BK, and ATP were used to delineate the role of serosal and muscular afferents in visceral nociception. In addition, the responses of whole nerves to BK, ATP, and capsaicin, and the development of a repeated mediator application protocol are described. Part 2 examines the chemosensitivity of whole nerves to mediators involved in IBS, 5-HT, histamine, and PGE_2.

3.1.1 INTRODUCTION

3.1.1.1 MEDIATORS

During inflammation chemical mediators including BK, ATP, 5-HT, and histamine can be released by a myriad of cells, including mast cells, mucosal epithelial cells, and neurons themselves, leading to the direct activation of afferent nerves (Kreis et al., 1998, Brunsden and Grundy, 1999, Hicks et al., 2002, Brierley et al., 2005a, Wynn and Burnstock, 2006). Mediators such as ATP can also be released in large amounts by intestinal epithelial cells during noxious distension of the gut (Burnstock, 2001). These mediators through activation of their receptors recruit a range of intracellular signalling pathways such as PKA, PKC and adenylate cyclase, which in turn modulate, often through phosphorylation, the activation, kinetics, and trafficking of receptors and ion channels, and under chronic inflammatory conditions, gene transcription and expression. This peripheral sensitisation leads to a greater activation of afferents in

response to a certain stimulus (McMahon, 2004). It is therefore of interest to investigate these mediators in the context of nociception.

3.1.1.2 BRADYKININ

BK, kallidin, and T-kinin are a group of blood derived peptides 9-11 amino acids long, collectively known as kinins (Marceau et al., 1998, Couture et al., 2001). Kinins can be rapidly broken down into their metabolites by a number of enzymes present in human tissues (Couture et al., 2001). Kinins and their metabolites stimulate endothelial cell release of nitric oxide and other factors, as well as relaxing arterial and contracting venous smooth muscle cells in the peripheral circulation to induce vasodilation and venoconstriction, respectively (Regoli and Barabe, 1980, Gaudreau et al., 1981). In addition, kinins promote cell migration into tissue from the bloodstream (Bhoola et al., 1992).

There are 2 kinin receptors, known as BK B1 (B1), and BK B2 (B2) (Regoli and Barabe, 1980). Kinins have higher affinity for B2, while their active metabolites des-Arg9-bradykinin (des-Arg9-BK) and des-Arg10-kallidin (des-Arg10-K) exhibit higher affinity for B1 (Marceau et al., 1998). The human B1 and B2 receptors are 353 and 364 amino acids long, respectively. Interestingly, there is only 36% homology between the 2 human receptors (Menke et al., 1994). BK receptors are members of the rhodopsin superfamily of GPCRs, each with 7 transmembrane domains (Burch and Axelrod, 1987). B1 receptors can couple with $G_{i/o}$ or $G_{q/11}$ proteins (Austin et al., 1997), while B2 receptors can couple to all types of G proteins, $G_{i/o}$, $G_{q/11}$ or G_s proteins e.g. (Ewald et al., 1989).

B2 is widely and constituently expressed in human tissues (Marceau et al., 1998). In contrast, B1 is not expressed under normal conditions, but is inducible by infection, treatment with toxins or certain cytokines and upon tissue injury (Marceau et al., 1998, Siebeck et al., 1998). The BK receptors have markedly different internalisation profiles. Ligand activation of human B1 receptors in a Chinese hamster ovary (CHO) cells resulted in minimal receptor

internalisation. In contrast, significant B2 receptor internalisation and sequestering was evident resulting in reduced ligand binding at the membrane (Austin et al., 1997). These profiles describe a quickly desensitising B2 response to B2 agonists, with B1 exhibiting far less desensitisation (Marceau et al., 1998).

3.1.1.2.1 Bradykinin in visceral pain

BK is a potent algogenic inflammatory mediator produced during tissue injury and inflammation. The activation of B2 by BK, and B1 by its metabolites des-Arg9-BK and des-Arg10-K have been implicated in inflammatory visceral pain. The rapid desensitisation and down-regulation profile of B2 suggests it's involvement in the acute inflammatory phase (Marceau et al., 2001). Since B1 does not readily desensitise, it is likely to be more important in chronic inflammatory pain states. Furthermore, the long half-life of B1s endogenous ligands, des-Arg9-BK and des-Arg10-BK, facilitate B1 upregulation and contribution to inflammatory pain (Dray and Perkins, 1993, Dray, 1997, Austin et al., 1997).

B1 and B2 receptors are expressed on visceral afferent neurons and small diameter nociceptive DRGs (Steranka et al., 1988, Vellani et al., 2004). Higher quantities of BK are found in injured tissues (Leme et al., 1978). BK receptor mRNA is upregulated in DRGs from a mouse model of caerulein induced painful acute pancreatitis (Takemura et al., 2011). Similarly, intestinal inflammation induced by indomethacin in rats upregulates B2 receptors (Stadnicki, 2011). Furthermore, in patients with IBD, both B1 and B2 receptor expression and localisation was altered in surgically resected inflamed colonic tissue compared to healthy controls (Stadnicki et al., 2004). These expression data suggest a role for BK receptors in visceral pain.

A number of electrophysiological studies have demonstrated the ability of BK to directly activate visceral afferent nerves (Longhurst et al., 1984, Lew and Longhurst, 1986, Longhurst and Dittman, 1987, Tjen-A-Looi et al., 1998), including intestinal and colonic afferents (Haupt et al., 1983, Maubach and Grundy, 1999, Brunsden and Grundy, 1999,

Brierley et al., 2005b). However, electrophysiological evidence of direct BK stimulation of human colonic afferents is lacking. In the mouse colon, BK excited a higher proportion of splanchnic serosal afferents and to a greater degree compared to pelvic serosal afferents (Brierley et al., 2005b). The response to BK in serosal afferents from rat jejunum was blocked by the B2 antagonist HOE140. However, the B1 antagonist [Des Arg^{10}] HOE140 had no effect on jejunal serosal afferent firing (Maubach and Grundy, 1999).

A number of studies using a visceral pain model have reported reduced writhings in response to acetic acid in B2 KO mice, but not B1 KO mice (Cayla et al., 2012). Furthermore, the B2 antagonist HOE140 reduced abdominal constrictions in response to intraperitoneal acetic acid administration (Heapy et al., 1993). However, in both of these studies, the inhibition was incomplete, suggesting that there may still be a role for B1 receptors in acute visceral pain.

BK can stimulate endothelial cells, mast cells, immune cells and afferent nerve endings to release other algogenic mediators such as 5-HT, histamine, NGF, CGRP, and substance P (Dray and Perkins, 1993, Kennedy and Leff, 1995, Purcell and Atterwill, 1995, Geppetti, 1993). As a result, afferent nerve endings become sensitised and demonstrate visceral allodynia and hyperalgesia (Bueno and Fioramonti, 2002). Indeed, B2 receptor knockout mice do not develop thermal or mechanical hyperalgesia induced by carrageenan injection into the hindpaw (Boyce et al., 1996, Rupniak et al., 1997).

B1 receptors have yet to be conclusively linked to acute visceral pain. For example, the B1 receptor agonists des-Arg-9-BK and des-Arg-10-K, failed to depolarise cultured rat DRGs under control or acute inflammatory conditions (Davis et al., 1996). Furthermore, the B1 antagonist, des-Arg9-[Leu8]-BK, did not suppress the response of afferent fibres to the application of BK in a dog testis-spermatic nerve preparation (Mizumura et al., 1990). However, there is evidence for the involvement of B1 receptors in persistent inflammatory

visceral pain (Jaggar et al., 1997, Couture et al., 2001). In a rat cystitis model of inflammatory visceral pain, B1 antagonists only demonstrated analgesic properties after an extended period of inflammation (Jaggar et al., 1997). Indeed, the expression ratio of B1 to B2 receptors increases in the colon of IBD patients, a chronic inflammatory disease, compared to healthy controls (Stadnicki et al., 2004). B1 may therefore be important in chronic visceral inflammatory pain (Dray and Perkins, 1993).

3.1.1.3 PURINES AND PYRIMIDINES

ATP, adenosine 5' diphosphate (ADP), adenosine 5'-monophosphate (AMP), and adenosine belong to a group of endogenous purines. Uridine 5'-triphosphate (UTP), uridine 5'diphosphate (UDP), and UDP-sugars, belong to a group of endogenous pyrimidines (Ralevic and Burnstock, 1998, Abbracchio et al., 2006). ATP consists of an adenine attached to the sugar ribose at 1' carbon. This sugar is connected to 3 phosphate groups at the 5' carbon. Uridine forms the backbone of UTP, in an otherwise comparable structure to that of ATP. ATP and UTP are rapidly hydrolysed by a group of enzymes known as ectonucleotidases to produce ADP, AMP, adenosine, and UDP, UMP and uracil (Zimmermann, 2006). Purinergic signalling is involved in neurotransmission in both the CNS and PNS (Burnstock, 2007). Hence, purinergic signalling has a multitude of functions including a role in inflammatory pain in the viscera (McMahon, 2004, Burnstock, 2006).

There are 3 receptor subtypes through which purines and pyrimidines exert their effects, P1, P2X and P2Y receptors. Adenosine is the endogenous ligand for the 4 P1 receptors, A1, A2a, A2b, and A3. P1 receptors are GPCRs with 7 transmembrane domains, an extracellular N terminus and an intracellular C terminus (Ralevic and Burnstock, 1998). Human P1 receptors are between 318-409 amino acids in length, demonstrating 40-61% homology between subtypes (Fredholm et al., 2011). A1 and A3 receptor subtypes are linked with $G_{i/o}$ proteins, while A2a and A2b signal through Gs proteins (Londos et al., 1980). P1 receptors exhibit a

broad expression profile, including in the CNS, heart, and on sensory nerves in the viscera (Pierce et al., 1992, Kirkup et al., 1998). The evidence for the involvement of P1 receptors in visceral pain will be discussed (Sawynok, 1998).

P2X receptors are ligand gated cation channels, exhibiting greatest permeability to Ca^{2+} (Abbracchio and Burnstock, 1994). P2X receptors form trimers from 7 distinct subunits each encoded by a separate gene and named $P2X_{1\text{-}7}$ (Nicke et al., 1998, North, 2002, Roberts et al., 2006). $P2X_{1\text{-}5}$ and $P2X_7$ can form homotrimers, while there are 7 other heterotrimer combinations ($P2X_{1/2}$, $P2X_{1/4}$, $P2X_{1/5}$, $P2X_{2/3}$, $P2X_{2/6}$, $P2X_{4/6}$ and $P2X_{4/7}$) (Roberts et al., 2006, Guo et al., 2007). P2X receptor subunits have between 379-472 amino acids sharing 30-50% sequence homology (Ralevic and Burnstock, 1998). P2X receptors are activated by ATP>ADP>AMP>adenosine (Fredholm et al., 1994). Upon activation, a flow of cations through the channel pore depolarises the cell membrane. A secondary influx of Ca^{2+} through voltage gated Ca^{2+} channels is initiated by this depolarisation, which in the case of neurons, increases the likelihood of action potential firing (Bean, 1992, Ralevic and Burnstock, 1998). Neural cells express every P2X subunit with the exception of $P2X_7$, however $P2X_{2/3}$ and $P2X_3$ are the predominant forms in sensory neurons, and have been strongly implicated in pain pathways (Burnstock, 2007, Abbracchio et al., 2009).

P2Y receptors are metabotropic GPCRs containing 7 transmembrane domains and 308-377 amino acids (Abbracchio et al., 2006). There are 8 human P2Y receptors, $P2Y_{1,\ 2,\ 4,\ 6,\ 11,\ 12,\ 13}$ and $P2Y_{14}$ (Abbracchio et al., 2003). P2Y receptors can be classified into 2 subgroups based on their phylogenetics, amino acid sequences and the type of G protein with which they are coupled (Abbracchio et al., 2003, Abbracchio et al., 2009). $P2Y_{1,\ 2,\ 4,\ 6}$, and $P2Y_{11}$ coupled with $G_{q/11}$ proteins (Verkhratsky, 2005, Abbracchio et al., 2009). $P2Y_{12,\ 13}$ and $P2Y_{14}$ are linked to $G_{i/o}$ proteins. Through their respective signalling pathways P2Y receptors can regulate the activity of ion channels (Abbracchio et al., 2003). P2Y receptors are widely expressed in the human body, from bone to brain (Bowler et al., 1995, Schachter et al., 1996). P2Y receptors are

expressed on sensory neurons, with $P2Y_1$ and $P2Y_2$ evident in small diameter nociceptive neurons (Gerevich and Illes, 2004). P2Y receptor involvement in visceral pain will be discussed.

3.1.1.3.1 Purines and pyrimidines in visceral pain

3.1.1.3.1.1 P1 receptors in visceral pain

ATP can be rapidly broken down to adenosine by endogenous ectonucleotidases. Adenosine can activate P1 receptors which are present on peripheral nerve endings (Sohn et al., 2008). The involvement of adenosine receptors in somatic pain is complex. Activation of A1 on peripheral nerve terminals has an analgesic effect, while A3 stimulation produced nociception (Sawynok, 1998). However, in somatic pain models, activation of A2a and A2b reveal differential functions, algesic or analgesic, depending on the dose and location, nerve terminals vs. spinal cord, of agonist application (Sawynok, 1998).

There is limited data on adenosine receptors in visceral pain. Adenosine receptors are coupled to sodium channels in visceral afferent terminals (Bueno and Fioramonti, 2002). Adenosine itself can activate mesenteric afferent innervating the rat jejunum (Kirkup et al., 1998). Altered expression of A1, A2 and A3 receptors has been demonstrated in animal models of GI inflammation (Sundaram et al., 2003, Guzman et al., 2006). One study induced colitis by injecting zymosan into anesthesised rats (Sohn et al., 2008). They then administered an A1-A2 agonist, 5'-N-ethylcarboxyamidoadenosine (NECA), an A1 agonist, R(-)-N6-(2- phenylisopropyl)-adenosine (R-PIA) or an A2a agonist CGS 21680 hydrochloride (CGS 21680) either subcutaneously or intrathecally and examined their effects on the visceromotor reflex (VMR) response to colorectal distension (CRD) in their hyperalgesic rats. Each agonist attenuated the VMR response to CRD, indicating an analgesic function for adenosine receptors A1 and A2 in visceral pain (Sohn et al., 2008). In contrast, in rat mesenteric afferents innervating the jejunum intravenous administration of NECA or the A1 agonist GR 79236 increased afferent activity (Kirkup et al., 1998). In addition, activation of A1 and A2 adenosine receptors excites

cardiac afferents in dogs (Dibner-Dunlap et al., 1993, Huang et al., 1995). The site of action may therefore determine the effect of adenosine receptor agonist on nociception. In visceral afferents, activation of A1 and A2 receptors seems to be nociceptive.

3.1.1.3.1.2 P2X receptors in visceral pain

With the exception of $P2X_7$ all P2X subunit proteins are expressed in sensory neurons. However, $P2X_3$ and $P2X_{2/3}$ are the main P2X receptor subtypes implicated in visceral pain (Burnstock, 2007, Abbracchio et al., 2009). $P2X_3$ and $P2X_{2/3}$ receptors are mainly expressed on a subpopulation of small diameter, IB4+, visceral nociceptors (Bradbury et al., 1998). Indeed, in a rat model of TNBS induced colitis, DRGs supplying the colorectum exhibited enhanced P_{2X3} immunoreactivity (IR) (Wynn et al., 2004). Similarly, immunohistochemistry (IHC) on colonic tissue from pain predominant IBD patients, revealed increased $P2X_3$ IR compared to controls (Yiangou et al., 2001a). This suggests a role for $P2X_3$ in inflammatory pain in humans.

A theory for the purinergic transduction of mechanical stimuli in the viscera has been proposed (Burnstock, 1996). Mechanosensory epithelial cells sense mechanical stimuli and release ATP. This ATP subsequently activates $P2X_3$ and $P2X_{2/3}$ receptors on the extrinsic afferents, which terminate nearby (Burnstock, 1996). Furthermore, noxious distension of hollow organs such as the gut would lead to large amounts of ATP release and increased activation of extrinsic afferent $P2X_3$ and $P2X_{2/3}$ receptors (Burnstock, 2001). Indeed, ATP is released in proportion to extent of bladder distension in mice (Vlaskovska et al., 2001). Furthermore, in $P2X_3$ KO mice, the pelvic afferent nerve response to bladder distension even at noxious pressures was significantly reduced (Vlaskovska et al., 2001). The addition of ATP or the $P2X_{1,\ 2/3,\ 3}$ agonist α, β methylene ATP (meATP) augmented the WT but not $P2X_3$ KO mouse pelvic afferent response to bladder distension. Another study also showed a reduced pelvic afferent nerve response to bladder distension in $P2X_3$ KO mice. However, this study also showed an inhibition of distension related pelvic afferent firing, even at noxious pressures, in

$P2X_2$ and also $P2X_{2/3}$ double KO mice (Cockayne et al., 2005). These data together suggest a role for $P2X_2$, $_3$, and $P2X_{2/3}$ receptors in the ATP mediated activation of afferent nerves to distension, even at noxious pressures.

Inflammatory mediators can augment the response of cells to ATP application. This interaction can arise during inflammation. The co-application of ATP with capsaicin, 5-HT or protons increased rat colonic afferent nerve firing by more than ATP alone (Wynn and Burnstock, 2006). Substance P can sensitise the effect of ATP application on small diameter DRGs (Hu and Li, 1996). ATP induced currents in P2X expressing Xenopus oocytes, are potentiated by a range of mediators including 5-HT, adenosine, BK, CGRP, and substance P (Wildman et al., 1997, Paukert et al., 2001). Injection of the hindpaw of rats with PGE_2 or carrageenan potentiated the time spent with the hindpaw lifted after the application of meATP (Hamilton et al., 1999). These data suggest a role for P2X receptors, especially the $P2X_{2/3}$, $P2X_3$ subtypes in inflammation induced visceral nociception.

$P2X_{2/3}$ receptors are sensitive to changes in pH (Stoop et al., 1997). Indeed, an acidic environment can augment the response of rat sensory neurons to ATP application. This suggests a role for $P2X_{2/3}$ receptors in proton induced sensitisation during inflammation (Li et al., 1996). Indeed, pH can drop to 5.5 in an inflammatory environment (Reeh and Steen, 1996). Taken together, these reports have spurred interest in $P2X_3$ and $P2X_{2/3}$ antagonism for the treatment of pain. Indeed, $P2X_3$, $P2X_{2/3}$ antagonist compounds such as A-317491 (Abbott Laboratories), RO3 (Roche Palo Alto) and AF219 (Afferent Pharmaceuticals) have been developed, the latter 2 compounds are currently in clinical trials (Abbracchio et al., 2009, Shi et al., 2012).

In humans, ATP has been shown to activate isolated peripheral sural nerve preparations (Lang et al., 2002). However, the effect of ATP on HVA firing has not yet been demonstrated in an electrophysiological preparation. Studies have suggested that $P2X_3$ may be

important in signalling pain in the viscera. Electrophysiological studies in murine models have shown that ATP can directly activate visceral afferents from organs such as the bladder (Rong et al., 2002), small intestine (Kirkup et al., 1999, Rong et al., 2009) and the colon (Wynn and Burnstock, 2006). Indeed, the $P2X_{1,\ 2/3,\ 3}$ agonist meATP and $P2X_{1,\ 2/3,\ 3}$ antagonist 2' 3' –O-trinitrophenyl- ATP (TNP-ATP) have been shown to potentiate and reduce afferent firing induced by innocuous and noxious levels of bladder distensions, respectively (up to 60mm Hg) (Namasivayam et al., 1999, Rong et al., 2002). In a rat *in vitro* model, noxious distension of the colorectum caused mucosal epithelial ATP release. In addition, sensory nerve firing during this distension (50mm Hg) increased when ATP or meATP was applied. The P2X antagonists TNP-ATP or PPADS reduced the response to colorectal distension (Wynn et al., 2004). These effects were not replicated in another study on normal mouse jejunal afferents (Rong et al., 2009). However, after recovery from inflammation, PPADS significantly attenuated the afferent response to distension (Rong et al., 2009). Similarly, the effects of purinergic ligands on distension responses were augmented in a hypersensitive colitis model (Wynn et al., 2004), suggesting a role for P2X signalling in visceral hypersensitivity. The response evoked in pelvic afferent nerves from rat bladder by ATP, was abolished by PPADS (Yu and de Groat, 2008). Furthermore, meATP can activate both splanchnic and pelvic afferents innervating the mouse colon, an effect blocked by PPADS (Brierley et al., 2005a). Similarly, the P2X antagonist PPADS inhibited the activation of rat jejunal afferents by meATP *in vivo* (Kirkup et al., 1999). These data suggests a role for $P2X_3$ and $P2X_{2/3}$ receptors in mechanosensory transduction and nociception in the viscera.

Recently, another highly potent P2X receptor antagonist has been discovered, RO-4 (aka AF353) (Gever et al., 2010). It has been shown to block Ca^{2+} signals evoked by the application of meATP in CHO-K1 cells transfected with the human $P2X_3$ and $P2X_{2/3}$ receptors (Gever et al., 2010). Indeed, one study suggests that RO-4 may be analgesic when administered to rats with bone cancer (Kaan et al., 2010). Similarly, RO-4 attenuated the

activation of nociceptive pathways in rat bladder spinal cord neurons (Munoz et al., 2012). This compound has not been tested on colonic afferents. The aim of this report was to test the involvement of P2X receptors in the activation of HVAs by ATP. The P2X antagonists, PPADS and RO-4 will be tested.

3.1.1.3.1.3 P2Y receptors in visceral pain

Sensory nerves express P2Y receptors. $P2Y_1$ and $P2Y_2$ receptors, while expressed on medium and large diameter neurons, exhibited greater IR in small diameter sensory neurons (Ruan and Burnstock, 2003). Interestingly, co-localisation studies on rat DRG revealed that $P2Y_1$ receptors were co-expressed with both $P2X_3$ and TRPV1 in 80% of small diameter neurons, the latter considered a marker for nociceptive afferents (Gerevich et al., 2004). Similarly, $P2Y_2$ mRNA was co-expressed with TRPV1 mRNA in a considerable number of neurons in rat DRG (Moriyama et al., 2003). However, the same study reported only 1.6% co-expression of P2Y1 mRNA in TRV1 mRNA positive rat DRGs (Moriyama et al., 2003). The expression of a number of P2Y receptors including $P2Y_{1, 2, 4, 6}$ are altered in hypersensitive rats induced by TNBS (Guzman et al., 2006).

The application of the $P2Y_{1, 12}$, and $P2Y_{13}$ agonist adenosine 5' –O-(2-thiodiphosphate) (ADP-β-S) to nociceptive rat DRGs (small diameter, IB4+), induced release of intracellular Ca^{2+}, which was antagonised by the $P2Y_1$ antagonist MRS 2179 (Borvendeg et al., 2003). This indicates a role for $P2Y_1$ receptors in the activation of nociceptive neurons. However, signalling through P2Y receptors may also have an analgesic function. The application of UTP, ADP, or ATP on cultured small diameter rat DRGs has been shown to inhibit voltage gated Ca^{2+} channels, which are important in pain transmission (Gerevich et al., 2004). The ADP induced inhibition was mediated through $P2Y_1$, and is likely to act on central sensory afferent terminals to reduce neurotransmitter release (Gerevich and Illes, 2004).

P2Y receptors may be involved in the sensitisation of nociceptive signals through other transducer channels. Patch clamp recordings of human embryonic kidney cells (HEK293)

demonstrated the potentiation of capsaicin evoked currents by ATP, ADP, and 2-methythio ATP (2-meSATP) acting through the P2Y receptors, particularly $P2Y_1$ (Tominaga et al., 2001). Furthermore, ATP lowered the temperature activation of TRPV1 receptors, via phosphorylation, from the noxious 42°C to 35°C. This suggests TRPV1 could be activated and can produce pain at body temperature when P2Y receptors are activated by ATP, such as during inflammation (Tominaga et al., 2001, Gerevich and Illes, 2004). However, in another study the potentiating effect of ATP on capsaicin induced currents in WT and $P2Y_1$ KO mouse DRGs were similar, indicating the lack of $P2Y_1$ involvement (Moriyama et al., 2003).

The $P2Y_2$ and $P2Y_4$ agonist UTP augmented the capsaicin induced current to a similar extent as ATP. However, the P2 antagonist suramin, which blocks $P2Y_2$ but not $P2Y_4$ receptors, inhibited any potentiation by UTP, suggesting the effect was meditated through $P2Y_2$ receptors (Moriyama et al., 2003). In cultured rat sensory neurons, ATP and UTP augmented the release of the neuropeptides CGRP and substance P, involved in neurogenic inflammation, by capsaicin through P2Y receptors (Huang et al., 2003). UTP can also evoke depolarisation and cause AP firing in rat sensory neurons (Molliver et al., 2002). There is also evidence for UTP induced, $P2Y_2$ mediated, CGRP release from rat DRG neurons (Sanada et al., 2002). Taken together these data indicate an important role for $P2Y_1$ and $P2Y_2$ in nociceptive signalling.

3.1.1.4 CAPSAICIN

Capsaicin is a natural pungent compound found in chilli peppers. It is the natural ligand to TRPV1, a member of the TRPV family of cation channels. TRPV1 consists of 4 identical subunits each with 6 transmembrane (S1-S6) domains, which form the pore and selectivity filter for cations as well as a sensor detecting changes in voltage. Both the N and C termini are in the cytoplasm (Gaudet, 2007). TRPV1 channels are also sensitive to temperature >43°C, low pH <6 and a number of spices (Caterina et al., 1997, Holzer, 2008).

Capsaicin is expressed mainly on small and medium diameter DRGs, and historically has been suggested to be a marker for nociceptive neurons (Holzer, 1991). Indeed, a large proportion of pelvic (40-50%) and splanchnic (~80%) spinal afferents express TRPV1 receptors (Berthoud et al., 1995, Robinson et al., 2004, Christianson et al., 2006), however they are also expressed on up to 40+% of non-nociceptive vagal afferents (Patterson et al., 2003).

Expression of TRPV1 receptors are often altered in disease models and in patients with painful bowel disorders. TNBS induced colitis leads to upregulation of TRPV1 receptors and mRNA in both thoracolumbar and lumbarsacral DRGs (Miranda et al., 2007, De Schepper et al., 2008). TRPV1 receptor expression and mRNA content have been shown to be upregulated in mucosal nerve fibres from IBD patients (Yiangou et al., 2001b). In addition, in mucosal biopsies from patients with quiescent UC but ongoing abdominal pain, TRPV1 receptor expression and mRNA remained upregulated compared to patients without pain and correlated with abdominal pain scores (Akbar et al., 2010, Keszthelyi et al., 2013). Similarly, higher levels of TRPV1 immunoreactivity and mRNA have been demonstrated in mucosal biopsies from patients with IBS (Akbar et al., 2008, Keszthelyi et al., 2013). This implicates TRPV1 as a potential contributor to abdominal pain and hypersensitivity in both IBD and IBS patients. However, these findings are not universal. A recent study did not find upregulation of either TRPV1 receptors or mRNA in mucosal biopsies from patients with IBS, even in those that exhibited hypersensitivity to rectal distension (van Wanrooij et al., 2014). Furthermore, TRPV1 expression levels did not correlate with IBS symptoms.

In the gut TRPV1 KO mice exhibit a reduced VMR to CRD at all pressures, indicating a role for TRPV1 in both physiological and noxious mechanotransduction (Jones et al., 2005). There is substantial evidence describing a role for TRPV1 in inflammation and hypersensitivity in a number of viscera including the pancreas, oesophagus, and gut (Rong et al., 2004, Winston et al., 2007). For example in the gut, when neonatal mice are sensitised with a low dose of acetic acid, they demonstrate chronic visceral hypersensitivity to CRD. This was attenuated by

the TRPV1 antagonist SB-366791, when applied before the acetic acid administration or after the development of hypersensitivity (Winston et al., 2007). Similarly, the development of hypersensitivity to CRD induced by water-avoidance stress could be prevented by intraperitoneal injection of the TRPV1 antagonist capsazepine (Hong et al., 2009). Indeed, it has been established that a variety of inflammatory and hyperalgesic mediators e.g. BK, ATP, 5-HT can sensitise TRPV1 receptors, increasing their likelihood of firing due to thermal and other stimuli and causing pain (Holzer, 2008).

Capsaicin can excite extrinsic afferent nerves from animal models and from human tissue. Approximately 30% of tension, mucosal and tension/mucosal vagal afferents innervating the stomach, oesophagus, and duodenum were directly activated by capsaicin (Blackshaw et al., 2000). About 50% of both splanchnic and pelvic serosal afferents were activated by capsaicin. Capsaicin also evoked activity in MIAs in the spinal pathways (Brierley et al., 2005a). Slightly higher proportions (~67%) of splanchnic afferents responded to capsaicin *in vivo* (Longhurst et al., 1984). Capsaicin has also been shown to activate HVAs in from colon and appendices (Peiris et al., 2011, Jiang et al., 2011). Capsaicin activated muscular and muscular-mucosal HVAs but not serosal afferents, although only 1 application was tested (Jiang et al., 2011).

3.1.1.5 AIMS

- To investigate the chemosensitivity of the different subtypes of HVAs identified in Chapter 2, Part 1, in order to delineate their role in nociception
- To examine the chemosensitivity of whole nerve HVAs to BK, ATP and capsaicin
- To develop repeated mediator application protocols using BK, and ATP, which may be used for mechanistic studies and for investigations using potential visceral analgesics.
- To study the receptor pharmacology involved in BK and ATP activation of single unit HVAs using the repeated mediator application protocol

3.1.2 METHODS

3.1.2.1 CHEMOSENSITIVITY OF MECHANICALLY CHARACTERISED AFFERENTS

After afferents had been characterised based on their response to mechanical stimuli, atropine (10µM) and nifedipine (10µM) were added to the krebs buffer and given 30 minutes to take effect. Drugs were applied to the tissue bath by superfusion of a 20ml volume: BK (BC 2µM, 20ml of 10µM), ATP (BC 2mM, 20ml of 10mM), adenosine (BC 200µM, 20ml of 1mM), or capsaicin (BC 2µM, 20ml of 10µM). An effort was made to keep the mediator applications in the same order in each preparation. If a mediator failed to elicit a response, the next mediator was added 30 minutes later. If an effect was evident, a washout period of 60 minutes was observed.

3.1.2.2 CHEMOSENSITIVITY OF NON-MECHANICALLY CHARACTERISED AFFERENTS

All preparations were tested for mechanosensitivity. Given the limited supply of tissue available, and the occurrence of some HVAs that were mechanically insensitive, or that were deemed unsuitable for mechanical characterisation and mechanical protocols, chemosensitivity protocols were performed to ensure the tissue was used to some degree. After testing for mechanosensitivty, atropine (10µM) and nifedipine (10µM) were added to the krebs buffer and given 30 minutes to take effect. Drugs were then applied to the tissue bath by superfusion of a 20ml volume to make up the final bath concentrations of: BK (BC 2µM, 20ml of 10µM), ATP (BC 2mM, 20ml of 10mM), adenosine (BC 200µM, 20ml of 1mM), or capsaicin (BC 2µM, 20ml of 10µM). An effort was made to keep the mediator applications in the same order in each preparation. If a mediator failed to elicit a response, the next mediator was added 30 minutes later, otherwise a washout period of 60 minutes was observed.

3.1.2.3 MEDIATOR PHARMACOLOGY

For repeat application protocols, either BK (BC 2μM, 20ml of 10μM), ATP (BC 2mM, 20ml of 10mM) was superfused into the bath 3 times consecutively, with a washout period of 60 minutes between applications (figure 3.01). For pharmacological protocols involving BK and ATP, the first application of the mediator was superfused as normal. The preparation was then pre-treated by superfusion of an antagonist before the second application of the mediator. These were antagonists to the B1 receptor (R715, 300nM, 100ml), or the B2 receptor (HOE140, 300nM, 100ml or 1μM, 100ml) for BK or antagonists to the $P2X_{1,\ 2,\ 3,\ 5}$ receptors (PPADS, 30μM, 100ml) or $P2X_{2/3,\ 3}$ receptors (RO4, 10μM, 100ml) or adenosine receptors (CGS 15943, 10μM, 100ml) for ATP. A second application of the appropriate mediator was superfused with the last 20ml of the antagonist. This was followed by a 60 minute washout period before the third application of the mediator was superfused into the bath. The HVA response to the second and third mediator applications were then compared using a 2 tailed paired t test, $p<0.05$ (figure 3.01). To test if activation of P2Y receptors could activate HVAs, the $P2Y_{1,\ 12}$ and $P2Y_{2,\ 4,\ 6}$ receptor agonists ADP (2mM), and UTP (2mM), respectively, were superfused in the tissue bath. If both agonists were given in the same experiment a washout period of at least 60 minutes was observed between applications. Data were analysed using a 2 tailed paired t test, $p<0.05$.

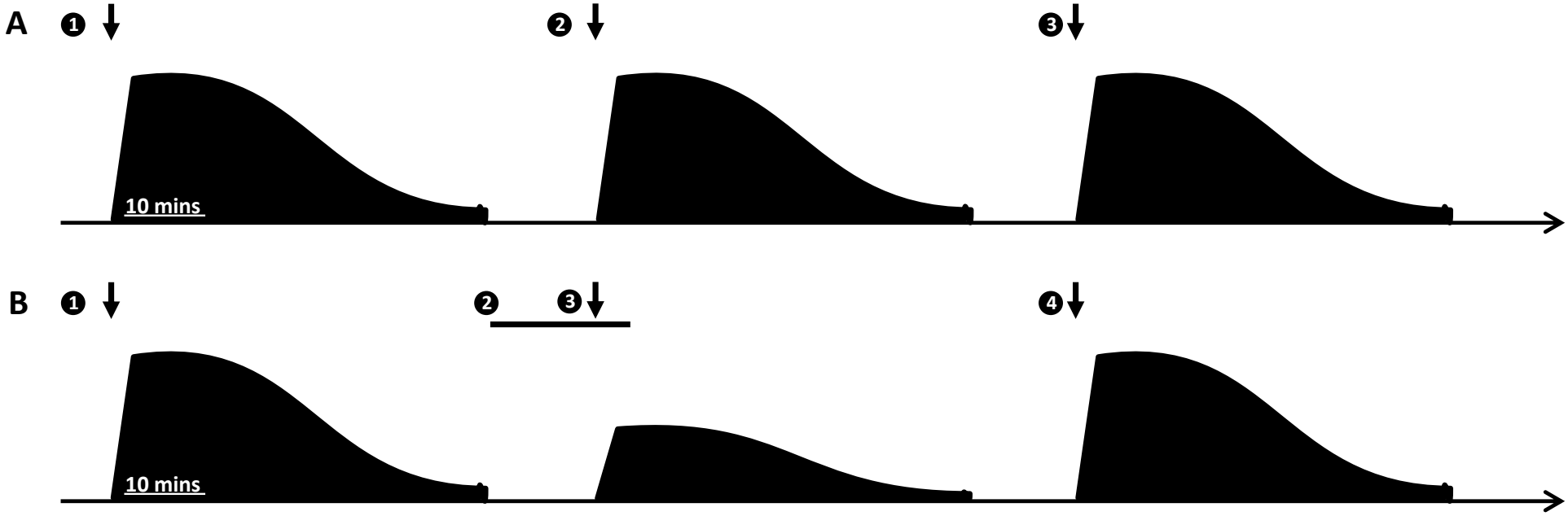

A
1
2
3
10 mins
B
1
2
3
4
10 mins

Figure 3.01: Repeat mediator application and chemosensitivity protocol

A) 1. The first application of the mediator (↓) e.g. BK is superfused into the bath.

2. An hour later the second application of the mediator (↓) is applied.

3. After another hour the third application of the mediator (↓) is added.

B) 1. The first application of the mediator (↓) e.g. BK is superfused into the bath.

2. An hour later the antagonist (—) to a specific receptor subtype is added e.g. HOE140 B2 receptor antagonist.

3. The second application of the mediator is added in the presence of the antagonist.

4. An hour later the third and final application of the mediator is added.

3.1.2.4 DRUGS

Drugs in powder form were made up using the recommended solutions, aliquoted and frozen at -20°C. When needed, aliquots were diluted in Krebs to make the final working concentration and vortexed to mix. Bradykinin, ATP, ADP, UTP, and Capsaicin were obtained from Sigma Aldrich (St Louis, MO, USA). HOE140, R715, PPADS, and CGS 15943, were purchased from Tocris Bioscience (Bristol, UK). RO4 was a gift from Neusentis (Cambridge, UK).

3.1.3 RESULTS

3.1.3.1 TISSUES – BK PHARMACOLOGY

3.1.3.1.1 Repeat BK Applications

Six tissues, 4 normal, 1CD, 1 appendicitis, were used for repeat BK application experiments, 2 ileum, 2 sigmoid colon, 1 appendix, 1 transverse colon, (M:F 1:2, median age 57).

3.1.3.1.2 B1 antagonist (R715)

Six tissues, all normal, were used for B1 antagonist studies, 2 appendix, 2 sigmoid colon, 1 ascending colon, 1 rectum (M:F 1:0.2, median age 77).

3.1.3.1.3 B2 antagonist (HOE140)

Ten tissues, 9 normal, 1 UC, were used for B2 antagonist studies, 5 sigmoid colon, 3 appendix, 1 descending colon, 1 rectum (M:F 1:0.25, median age 62).

3.1.3.2 TISSUES – ATP PHARMACOLOGY

3.1.3.2.1 Repeat ATP Applications

Five tissues, 2 normal, 1 CD, 1 UC, 1 appendicitis, were used for repeat ATP application experiments, 2 appendix, 2 sigmoid colon, 1 ileum (M:F 1:0.66, median age 39).

3.1.3.2.2 P2X antagonist (PPADS and RO4)

Nine tissues, all normal, were used for P2X antagonist experiments, 4 sigmoid colon, 2 appendix, 2 rectum, 1 descending colon (M:F 1:0, median age 64).

3.1.3.2.3 P1 antagonist (CGS 15943)

Six tissues, 4 normal, were used for P1 antagonist studies, 4 sigmoid colon, 2 appendices (M:F 1:1, median age 51.5). Further details on the tissues use in each set of experiments can be seen in table 2.02.

3.1.3.3 CHEMOSENSITIVITY IN CHARACTERISED HUMAN VISCERAL AFFERENTS

BK and ATP were applied to a proportion of HVAs, after they had been mechanically characterised. Serosal afferents responded to the application of the chemical mediators BK (BC 2µM, 6/14 responded, 43%, Δ firing rate 40.3 spikes $20s^{-1}$) and ATP (2mM, 4/10 responded, 40%, Δ firing rate 42.8 spikes $20s^{-1}$) (figure 3.02). In contrast, muscular afferents did not respond to the application of BK (2µM, 0/11 responded) (figure 3.03) and responded with much less frequency to the application of ATP (2mM, 1/10 responded, 10%, Δ firing rate 45.8 spikes $20s^{-1}$) (figure 3.02). Two out of 8 (25%) serosal afferents responded to both BK and ATP. The mesenteric afferent that was challenged with chemical mediators was co-sensitive to both BK and ATP. In contrast, no muscular afferent was sensitive to both mediators. The identified muscular-mucosal unit responded to the application of ATP but not BK. “Silent” units, became responsive to either 2g VFH or cotton bud probing, after the application of BK (BC 20nM or 2µM), with 1/2 (50%) preparations responding directly to the mediator (figure 3.02).

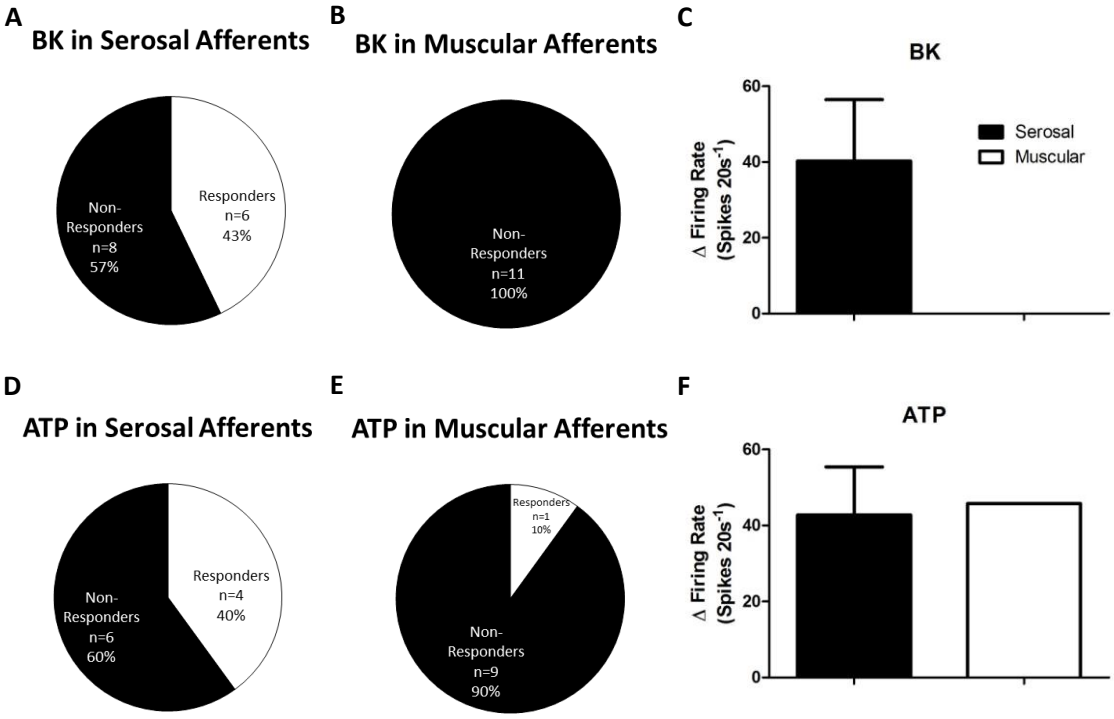


Figure 3.02: Chemosensitivity in characterised HVAs in flat sheet preparations. A-B) Bradykinin (BK) responds in a proportion of serosal afferents (6/14) (A), but does not respond in any muscular afferents (0/11). C) Displays the average change in serosal HVA firing in response to BK. D-E) Adenosine trisphosphate (ATP) responds with much more regularity in serosal HVAs (4/10) (D) compared to muscular HVAs (1/10) (E). F) Shows the average change in serosal and muscular HVA firing in response to the bath application of ATP.

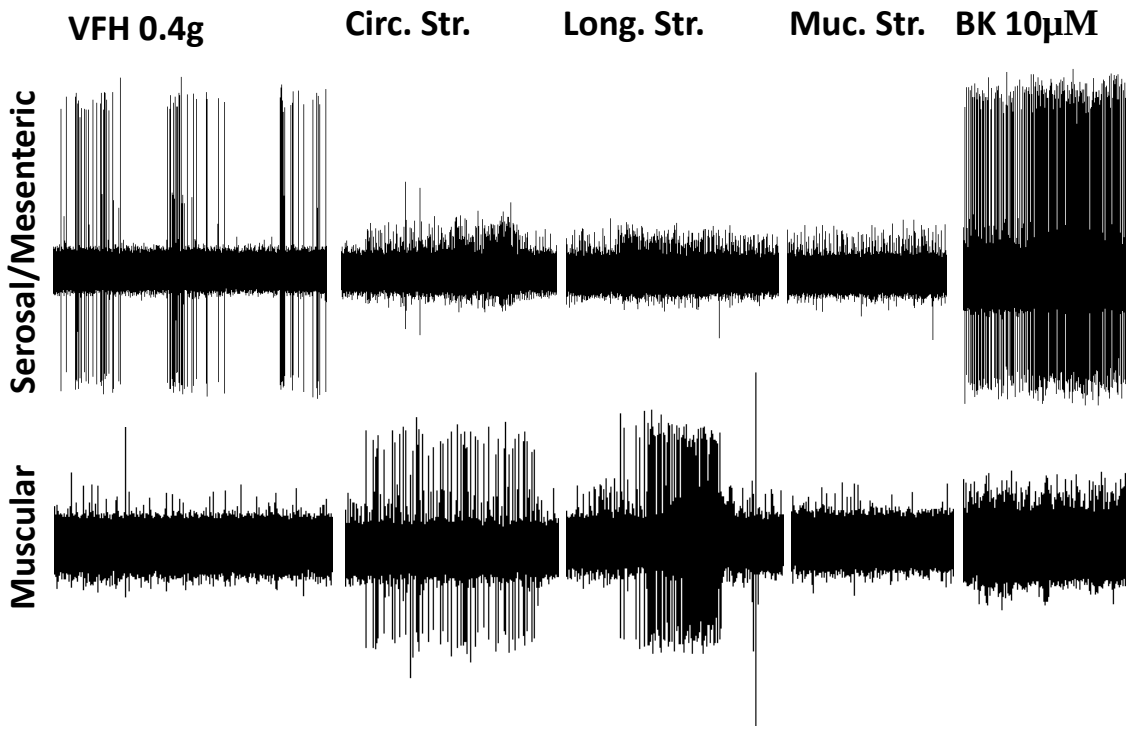


Figure 3.03: Subtypes of HVAs can be characterised based on their response to mechanical and chemical stimuli. Serosal and mesenteric afferents respond to light VFH probing <1g, but not to circumferential or longitudinal stretch. Muscular afferents do not respond to VFHs of <1g weight, but respond to circumferential and longitudinal stretch. Furthermore, serosal and mesenteric afferents are much more likely to respond to chemical mediators such as bradykinin, or adenosine trisphosphate (ATP) compared to muscular afferents.

3.1.3.4 CHEMOSENSITIVITY IN WHOLE NERVE RECORDINGS

The algogenic mediators BK, ATP, and capsaicin caused robust action potential firing in flat sheet whole nerve HVA recordings, BK (BC 2μM, 35/66 responded, 53.0%, Δ firing rate 69.7±14.2 spikes $20s^{-1}$), ATP (BC 2mM, 31/43 responded, 72.1%, Δ firing rate 68.4±7.8 spikes $20s^{-1}$), capsaicin (BC 2μM, 19/35 responded, 54.3%, Δ firing rate 100.5±21.3 spikes $20s^{-1}$) (figure 3.04). A subset of ATP responses were biphasic in that they exhibited 2 peaks, as has been previously described in rat mesenteric afferents *in vivo* (Kirkup et al., 1999). Responses to capsaicin were fast to peak and were generally shorter in duration that other mediators.

Flat sheet whole nerve preparations exhibited polymodality to chemical mediators. Fourteen out of 24 whole nerves (58.3%) were responsive to >1 mediator when at least 2 mediators were tested (BK, ATP, or capsaicin). Indeed, in preparations in which all 3 mediators, BK, ATP, and capsaicin, were added, 4/12 (33.3%) responded to all 3.

BK, ATP, and capsaicin were also added to appendix whole nerve preparations; BK (2μM, 11/18 responded, 61.1%, Δ firing rate 178.8±44.8 spikes $20s^{-1}$), ATP (2mM, 13/16 responded, 81.25%, Δ firing rate 179.5±43.7 spikes $20s^{-1}$), capsaicin (2μM, 10/13 responded, 76.9%, Δ firing rate 36.1±38.6 spikes $20s^{-1}$).

Whole nerve recordings from appendices exhibited polymodality to chemical mediators. Eight out of 18 whole nerves (44.4%) were responsive to >1 mediator when at least 2 mediators were tested (BK, ATP, or capsaicin). In addition, in appendix preparations in which all 3 mediators, BK, ATP, and capsaicin, were added, 4/5 (80.0%) responded to all 3.

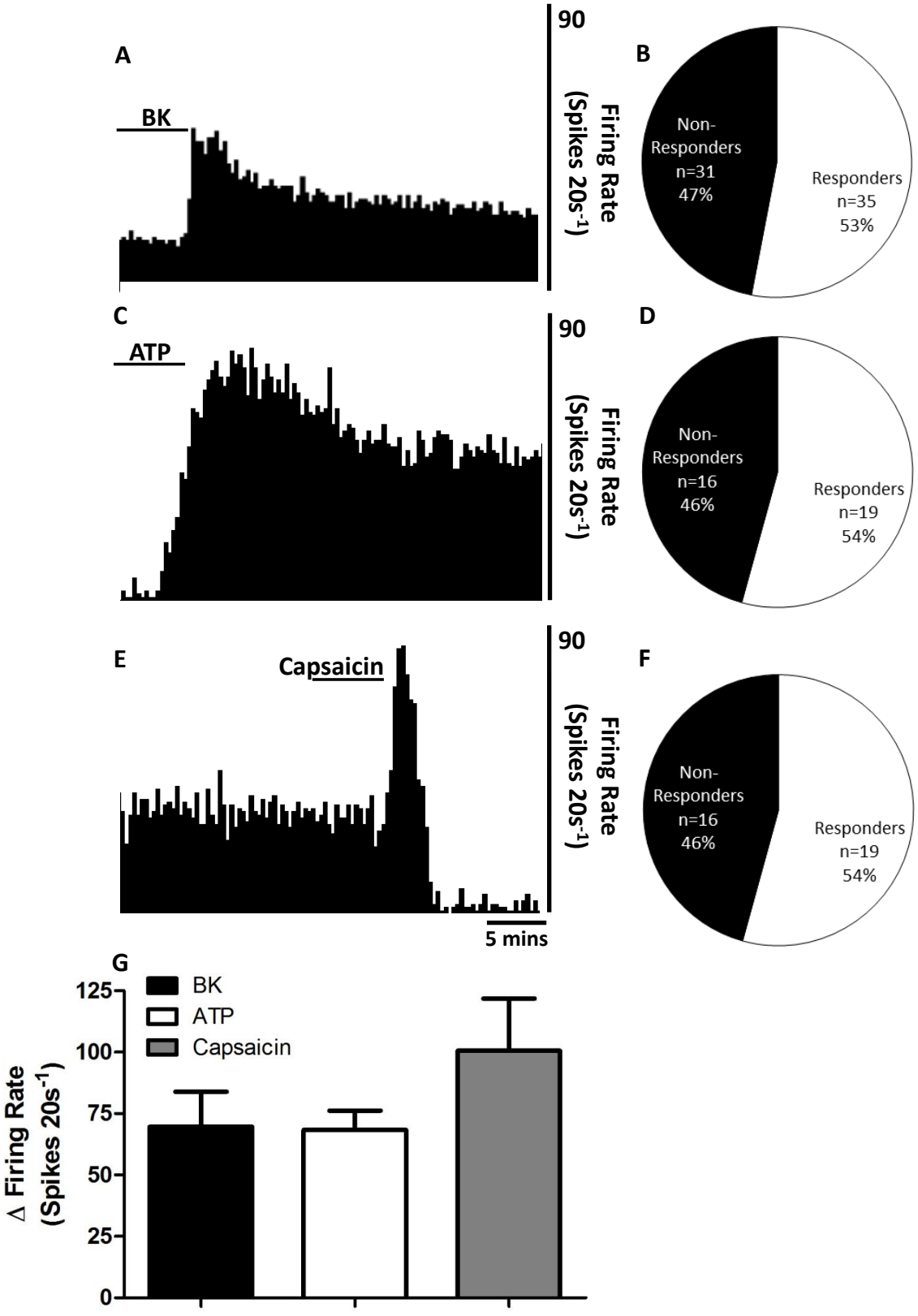


Figure 3.04: Flatsheet whole nerve HVAs respond to the application of bradykinin, adenosine trisphosphate (ATP), or capsaicin. A, C, E) Shows an example of a HVA response to BK (A), ATP (C), and capsaicin (E) in rate histogram form. B, D, F) Displays the proportion of whole nerve

recordings that responded to BK (B), ATP (D) or capsaicin (F) application. G) A bar graph indicating the average change in HVA firing rate after the application of BK, ATP, or capsaicin.

3.1.3.5 BK PHARMACOLOGY

Six out of 6 units (6 preparations) responded repeatedly to the application of BK (figure 3.05). Responses to the first application of BK tended to be larger than the subsequent applications, as has been previously reported (Δ firing rate 1st 37.5±11.6, normalised 100%, vs. 2nd 20.5±5.8, 65.2%±9.3%, vs. 3rd 19.3±5.2 spikes 20s^{-1}, 61.1%±9.9%, n=6) (Brunsden and Grundy, 1999). The responses to the second and third applications of BK were the same ($p>0.05$). This demonstrates the suitability of the HVA model for pharmacological manipulation studies, whereby the second application of a mediator, after the pre-treatment with a compound of interest, is compared to the third application of a mediator. This type of experiment has been previously used in animal models (Maubach and Grundy, 1999, Brunsden and Grundy, 1999). Pre-treatment before the second application of BK with the selective B2 receptor antagonist HOE140 (300nM) significantly attenuated the HVA response to the second BK application compared to the third BK application, given after an hour washout (Δ firing rate; treatment 22.9 ± 6.2 spikes 20s^{-1} vs. washout 44.0 ± 7.9 spikes 20s^{-1}, n=6, $p<0.05$) (figure 3.06). When the change in afferent firing rate in response to BK was normalised to the first BK application, and the treatment and washout BK responses compared, antagonism by HOE140 (300nM) was also significant (baseline BK 100%, treatment BK 27.2%±6.2% vs. washout BK 57.6%±9.0%, $p<0.05$). An even greater antagonism was evident after pre-treatment with a higher dose of HOE140 (1μM) (Δ firing rate; treatment 0.5±1.4 spikes 20s^{-1} vs. washout 22.8±7.3 spikes 20s^{-1}, n=4, $p>0.05$). Once responses were normalised to the baseline BK application and the treatment and washout BK responses compared, antagonism by HOE140 (1μM) was significant (baseline BK 100%, treatment 9.3%±8.6% vs. 85.4%±12.1%, $p<0.01$) (figure 3.06).

In contrast, the selective B1 antagonist R715 had no effect on HVA response to BK (Δ firing rate treatment 33.1 ± 1.7 spikes 20s^{-1} vs. washout 29.7 ± 1.5 spikes 20s^{-1}, n=6, $p>0.05$). When responses were normalised to the baseline BK application, there was still no effect of

R715 application (baseline BK 100%, treatment 81.6%±14.9% vs. washout 69.1%±8.4%, p>0.05) (figure 3.06).

3.1.3.6 ATP PHARMACOLOGY

Repeat applications of ATP, produced repeated responses in 4/5 Units (5 preparations) (figure 3.05). One unit did not respond to the second application of ATP. Responses to the first application of ATP tended to be larger than the subsequent applications comparable (average Δ firing rate 1st 55.2±12.1, normalised 100%, vs. 2nd 33.9±12.6, 53.8%±12.3%, vs. 3rd 28.4±8.9 spikes 20s^{-1}, 47.7%±10.1%, n=4). HVA responses to the second and third applications of ATP were not different (p>0.05). The $P2X_{1,\ 2,\ 3,\ 5}$ antagonist PPADS, when given before the second application of ATP, modestly attenuated the HVA response, compared to the third ATP application give an hour later, however this did not reach significance (treatment 50.1 ± 14.8 spikes 20s^{-1} vs. washout 56.2 ± 14.7 spikes 20s^{-1}, n=6, p=0.060)(figure 3.07). When the change in afferent firing was normalised to the baseline ATP application, PPADS still did not have a significant effect (baseline 100%, treatment 65.1%±3.5% vs. washout 76.6%±7.7%, p=0.095). The $P2X_{2/3,\ 3}$ antagonist RO4, when applied before the second application of ATP, did not significantly alter the response of HVAs to ATP, compared to the third application of ATP given after an hour washout period (treatment 20.4±6.5 vs. washout 21.4±9.7 spikes 20s^{-1}, n=3, p>0.05). When the response was normalised to the baseline ATP application, there was still no effect of RO4 (baseline 100%, treatment 100.7%±39.7% vs. washout 106.5%±55.6%, p>0.05) (figure 3.07).

To examine the theory that ATP was being degraded to adenosine by ATP-endonucleotidases, and subsequently activating HVAs, we first applied adenosine to confirm its ability to activate HVAs. Application of adenosine caused an increase in action potential firing in whole nerve HVA recordings (BC 200μM, 2/9 responded, 22.2%, Δ firing rate 66.7±51.9 spikes 20s^{-1}). To continue this investigation we used a similar repeat ATP application protocol

as described above. HVAs pre-treated before the second application of ATP with the pan adenosine receptor antagonist CGS 15943, did not reduce the response to the second ATP compared to the post washout ATP application (treatment 61.5 ± 14.2 spikes $20s^{-1}$ vs. washout 61.5 ± 17.1 spikes $20s^{-1}$, n=6, p>0.05) (figure 3.07). Similarly, when the response to ATP was normalised to the first ATP application, CGS 15943, had no effect on the HVA response to ATP (baseline 100%, treatment 81.8%±10.6% vs washout 75.8%±12.0%, p>0.05).

To demonstrate that activation of P2Y receptors could augment afferent firing in HVAs the $P2Y_{1,\ 12}$ and $P2Y_{2,\ 4,\ 6}$ agonists ADP and UTP, respectively, were applied to preparations. ADP activated 13/18 (72.2%) preparations, from which 14 responding single units could be discriminated (average Δ firing rate 32.0.1±5.9 spikes $20s^{-1}$) (figure 3.08). UTP activated 10/20 (50.0%) preparations, from which 19 responding units could be discriminated (average Δ firing rate 50.4±6.1 spikes $20s^{-1}$) (figure 3.08). Eight out of 14 (57.1%) units were co-sensitive to both ADP and UTP. Three out of 4 units responded to both ADP and ATP, while 4/4 responded to both UTP and ATP. Two units were tested for co-sensitivity to ADP, UTP and ATP, from which 1 responded to all 3 mediators.

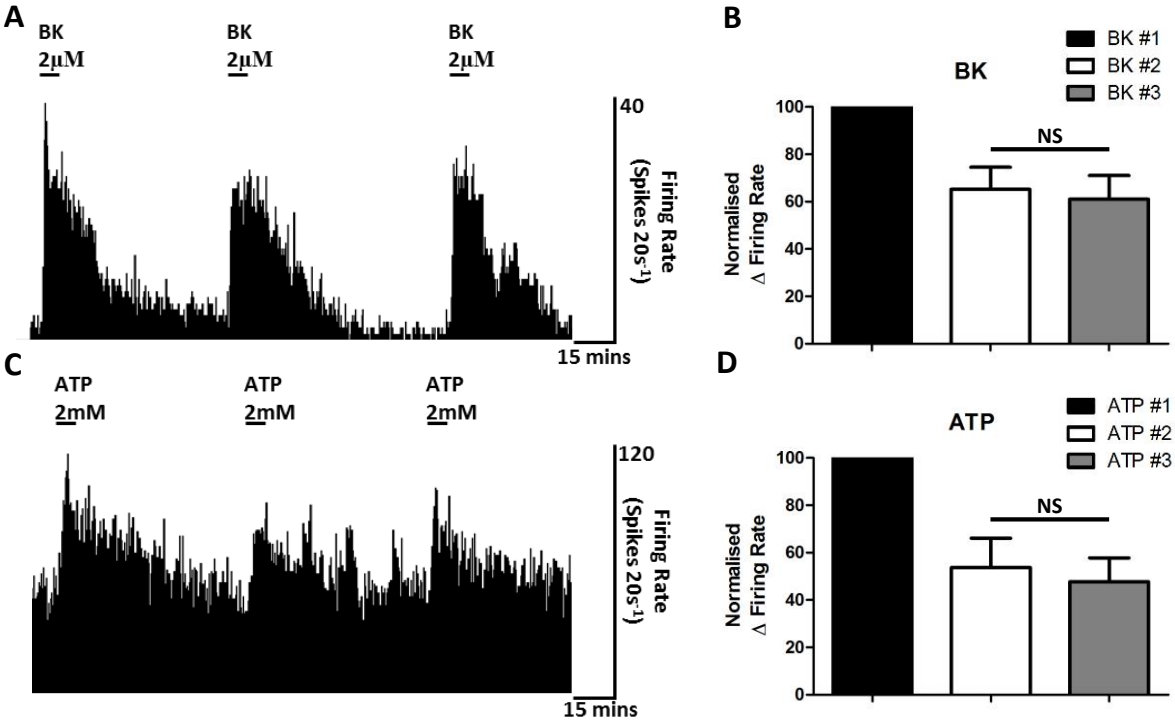


Figure 3.05: HVAs responded to repeated applications of bradykinin (BK) and adenosine trisphosphate (ATP). A-B) Shows the reproducibility of repeat BK responses, both as a rate histogram on the left (A), and displayed as a bar graph (B), which has been normalised to the 1st BK application, on the right (n=6). C-D) Shows the equivalent panels for ATP (n=4). This suggests the HVA model is suitable for pharmacological manipulation studies targeting the second application of these mediators. Data were analysed using a 2 tailed paired t test, $p<0.05$.

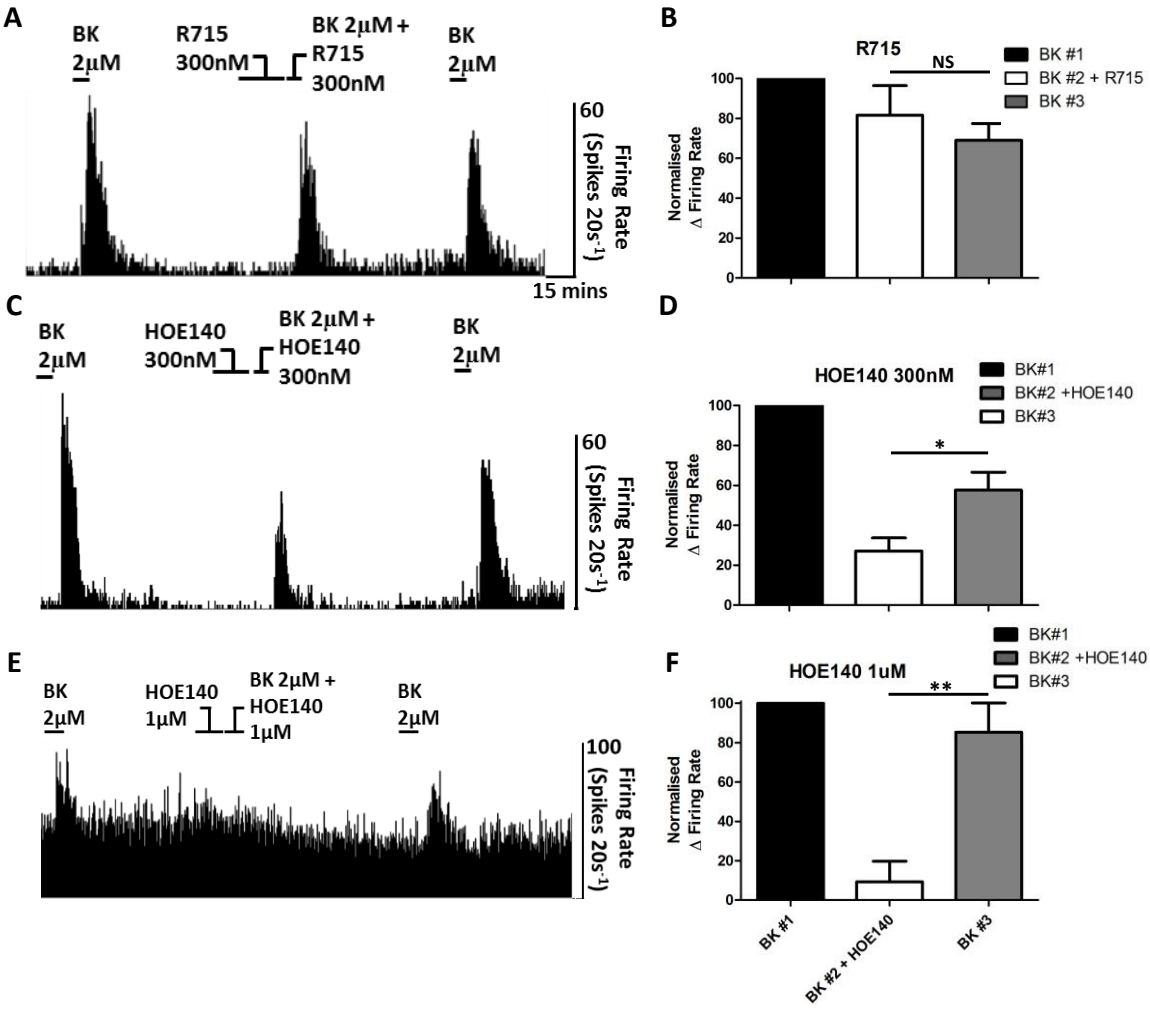


Figure 3.06: The HVA response to bradykinin (BK) is mediated by B2 receptors, with limited B1 involvement. A-B) The HVA response to BK was not attenuated when a bradykinin B1 receptor antagonist R715 was used (n=6, p>0.05), as shown here as a rate histogram (A) and a bar graph (B). C-D) Pre-treatment before the second application of BK with the selective B2 bradykinin receptor antagonist, HOE140 (300nM), significantly attenuated the HVA response to BK application compared to the BK application given after an hour of washout (n=6, p<0.05). E-F) When the second BK application was pre-treated with a higher dose of HOE140 (1µM), the response to BK was abolished, but recovers after an hour of washout (n=4, p<0.01). This suggests that in HVAs, BK signals through the B2 receptor, with seemingly limited involvement of the bradykinin B1 receptors. Data were analysed using a paired 2 tailed t test, p<0.05.

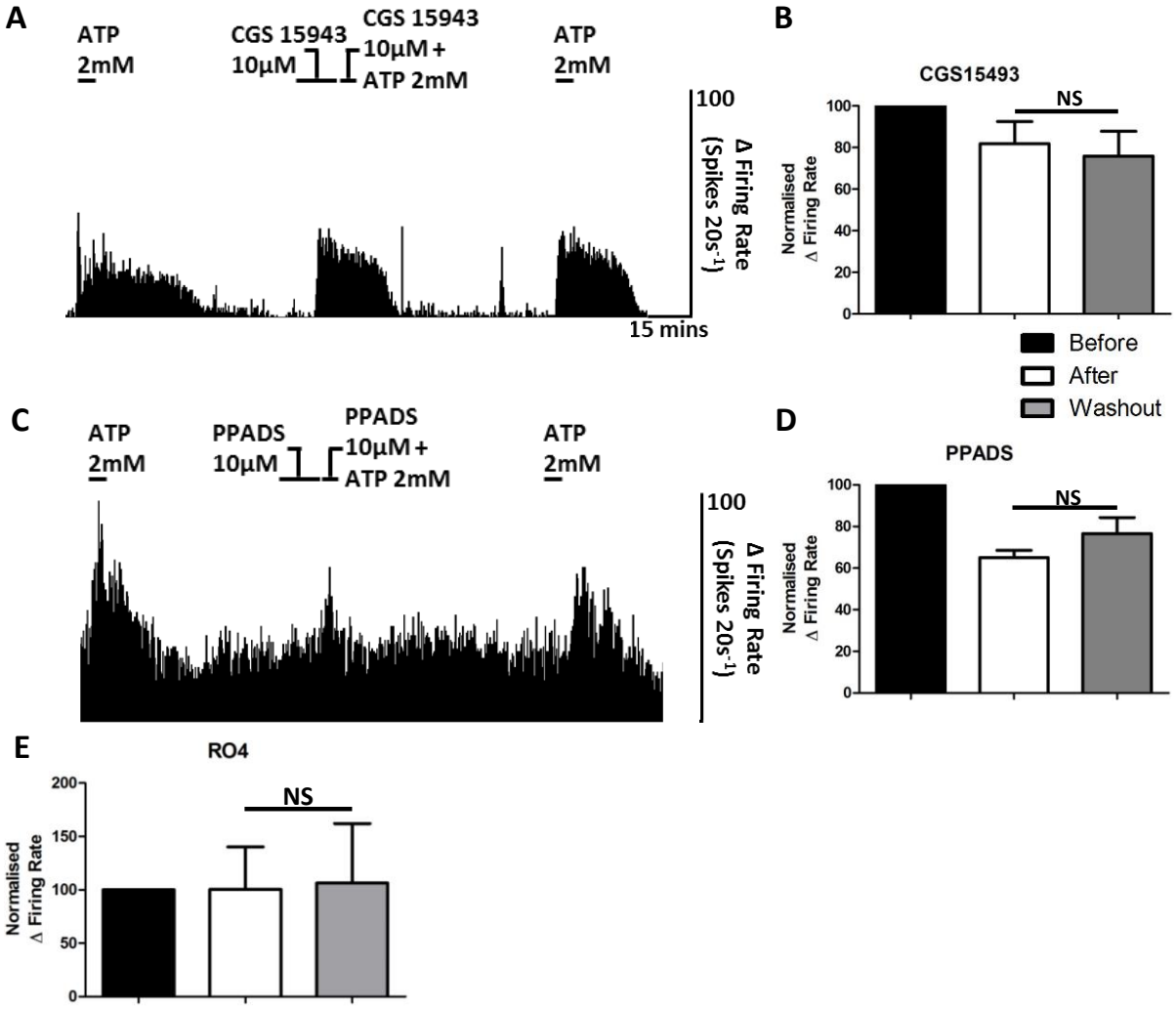


Figure 3.07: The HVA response to adenosine trisphosphate (ATP) is not altered by pre-treatment with the P2X antagonist PPADS or RO4, or the adenosine antagonist CGS 15493. A-B) Pre-treatment with the pan adenosine antagonist CGS 15943 before the second application of ATP did not attenuate the HVA response when compared to the ATP application after an hour of washout (n=6, p>0.05), as shown here as a histogram (A), and a bar graph (B). C-D) Pre-treatment before the second application of ATP with the $P2X_{1,\ 2,\ 3}$ and $_{5}$ antagonist PPADS (30µM) did not alter the HVA response to ATP compared to the ATP application given after an hour of washout (n=6, p>0.05). E) Similarly, pre-treatment with RO4 a $P2X_{2/3,\ 3}$ antagonist (10µM) did not significantly change the HVA response to ATP (n=3, p>0.05). Data were analysed using a paired 2 tailed t test, p<0.05.

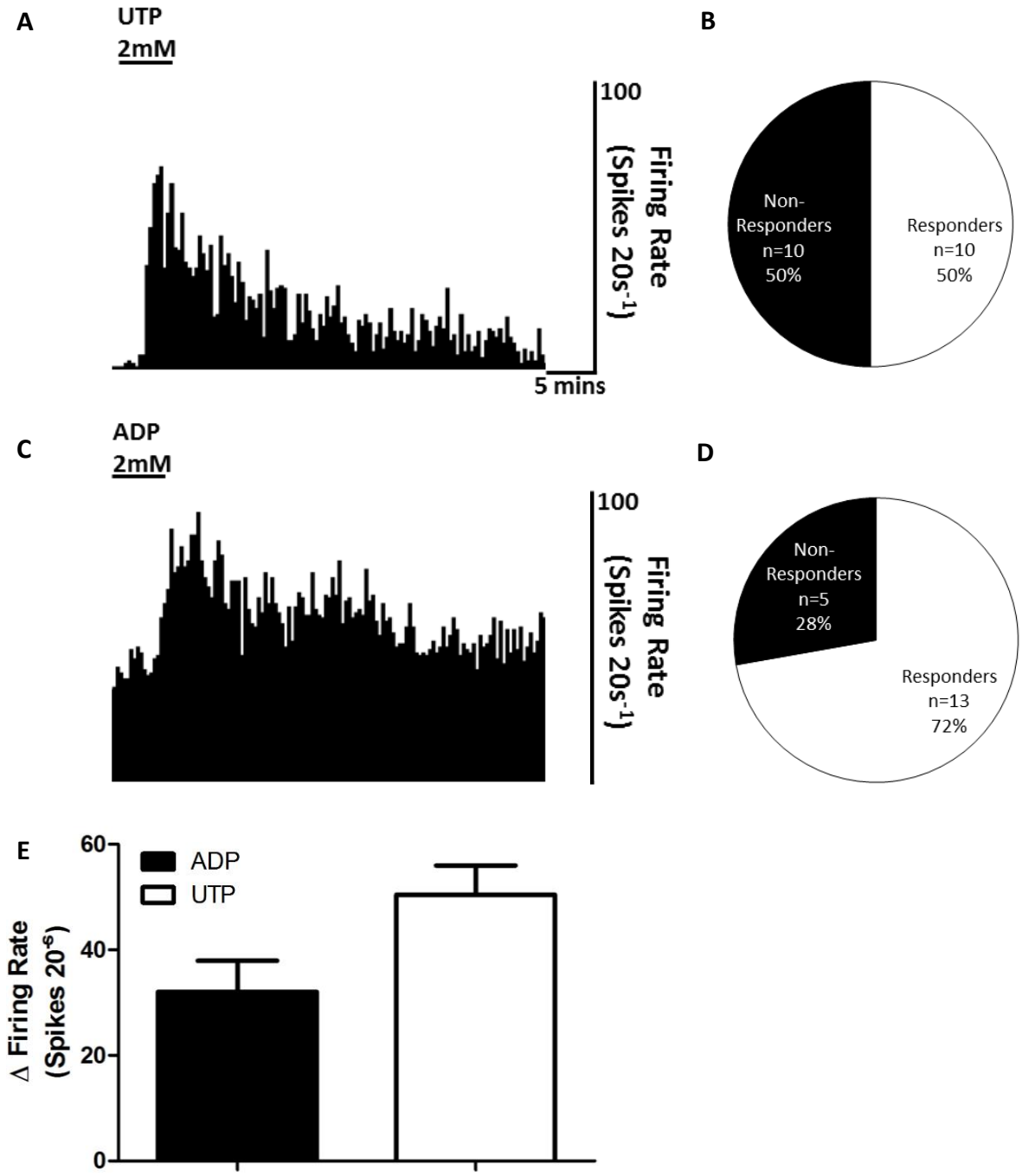


Figure 3.08: A) HVAs responded to the application of the $P2Y_{1,\ 12}$ receptor agonist ADP and C) the $P2Y_{2,\ 4,\ 6}$ agonist uridine trisphosphate (UTP), supporting a potential role for P2Y receptors in the mediation of HVA activation by adenosine trisphosphate (ATP). An example of an adenosine diphosphate (ADP) (A) and a UTP (C) response are illustrated in rate histogram form. B, D) Displays the proportions of HVAs that responded to UTP (10/20) (B) and ADP (13/18) (D). E) Shows the average change in afferent firing elicited by each agonist.

3.1.3.7 SUMMARY OF RESULTS

- Human serosal afferents are considerably more likely to respond to the algogenic mediators BK, or ATP compared to muscular afferents and represent a population of human visceral nociceptors

- Whole nerve HVA recordings are sensitive to a number of chemical stimuli, namely BK, ATP, and capsaicin

- Repeated applications of BK, or ATP produce reproducible responses in HVAs and represent a potentially useful experimental protocol

- B2 receptors are responsible for the BK induced activation of HVAs

- Adenosine can activate HVAs. A P1 adenosine receptor antagonist (CGS 15493) did not attenuate the HVA response to ATP

- Two separate P2X antagonists, PPADS and RO4, did not significantly reduce the HVA response to ATP

- The $P2Y_{1,\ 12}$ receptor agonist ADP, and the $P2Y_{2,\ 4,\ 6}$ receptor agonist UTP excited HVAs

3.1.4 DISCUSSION

3.1.4.1 SEROSAL/MESENTERIC VS. MUSCULAR CHEMOSENSITIVITY

We have demonstrated that human mesenteric and serosal afferents respond to the algogenic mediators BK and ATP, with more frequency than afferents terminating in the muscle layers (BK, 43% vs. 0%, ATP, 40% vs. 10%). Previous reports from animal studies have suggested that mesenteric and serosal afferents are the main nociceptive afferents innervating the gut (Blackshaw and Gebhart, 2002, Knowles and Aziz, 2009). The finding in this report that human mesenteric and serosal afferents are very responsive to the algogenic mediators BK, and ATP, implies certain functional roles and substantiates the evidence that suggests these afferents are nociceptive. Furthermore, the limited sensitivity of muscular afferents to painful mediators suggests that the majority of these afferents are not nociceptive, at least chemo-nociceptive.

However, a small proportion of muscular and distension sensitive afferents did respond to chemical mediators. Indeed, the response of some muscular and distension sensitive afferents to the application of chemicals has been previously reported in animals (Sengupta and Gebhart, 1994, Lynn and Blackshaw, 1999). Responses were taken to be directly activating muscular afferent fibres due to a lack of accompanying muscular contraction. Similarly, our human tissue preparations are treated with the calcium channel blocker, nifedipine, before the application of any mediators, inhibiting muscle contractions. Taken together, these results suggest that the response evident in muscular afferents is direct, and implies the ability of a small proportion of muscular afferents to transduce noxious chemical stimuli.

3.1.4.2 BK AND ATP IN CHARACTERISED AFFERENTS

Nearly half of human serosal afferents responded to the application of BK. This compares to 11% and 66% of serosal afferents from the pelvic and splanchnic pathways in the

mouse, respectively (Brierley et al., 2005b). The HVA model does not discriminate between pelvic and splanchnic afferents. Spinal pathways taken together, BK activates ~48% of mouse serosal afferents (Brierley et al., 2005b), which is comparable to HVAs. However the potential to record from vagal fibres in HVA recordings, especially in small intestine, must not be overlooked. In contrast, all rat serosal afferents were activated by BK (Maubach and Grundy, 1999). The marked differences in responders could reflect a fundamental species difference. Rat experiments involved removing the serosa and studying it in isolation, which may also account for these discrepancies when compared to full thickness human preparations.

This study represents one of the only times ATP has been applied to fully characterised serosal afferents in any species. Half of human serosal afferents were activated by ATP. This is higher than the 32% of mouse splanchnic serosal afferents activated by the $P2X_3$ receptor agonist meATP (Brierley et al., 2005a). ATP activated 1/5 distension sensitive units. However, when analysed for single units, 0/4 LT units, and 1/1 HT units respond to the application of ATP suggesting a functional afferent subtype split in ATP sensitivity, although clearly more work is needed. Indeed, ATP has been previously been shown to activate vagal and pelvic distension sensitive afferents innervating the rodent GI tract and bladder (Rong et al., 2002, Zagorodnyuk et al., 2003).

It has been postulated that, organ distension releases ATP which activates afferent nerves giving rise to distension sensation and nociception (Burnstock, 1996). This report certainly, demonstrates the activation of serosal and HT distension sensitive units to ATP. The addition of either BK or ATP activated around half of serosal afferents. The activation of serosal afferents by these algogenic mediators supports the proposed notion that serosal afferents are nociceptors, signalling noxious information in the human gut. Indeed, the lack of response to BK and the lower proportion that were responsive to ATP (10%) suggests muscular afferents play a smaller role in nociception.

3.1.4.3 CHEMOSENSITIVITY IN WHOLE NERVE RECORDINGS

This report has demonstrated that a variety of chemical mediators including, BK, ATP, adenosine, and capsaicin activate whole nerve HVAs from both flat sheet preparations of small intestine and colon, and from cannulated appendices. This is the first time that individual mediators have been shown to activate HVAs with the exception of capsaicin (Peiris et al., 2011, Jiang et al., 2011).

Whole nerve preparations often responded to multiple mediators, with upwards of 58.3% and 44.4% of flat sheet and appendix preparations responding to >1 mediators, respectively. Nerve bundles containing multiple HVAs are clearly responsive to multi chemical stimuli. HVA bundles are almost always responsive to at least 1 mediator, 80.8% and 92.3% in flat sheet and appendix preparations, respectively. Indeed, HVA bundles are likely to contain a proportion of nociceptors given their response to a least 1, and often multiple, algogenic mediators. Indeed, a proportion of mesenteric and serosal afferents are chemosensitive and have been shown to respond to mediators such as capsaicin, 5-HT, BK and histamine (Berthoud et al., 2001, Coldwell and Blackshaw, 2002, Hicks et al., 2002, Brierley et al., 2005b, Feng and Gebhart, 2011).

The concentration of mediators used in this report is similar to those used in animal studies examining intestinal afferent activation. The approximate final bath concentration of BK (2µM) is similar to the concentrations used in animal studies (1µM) (Brunsden and Grundy, 1999, Maubach and Grundy, 1999, Brierley et al., 2005b). ATP has been used in rodent intestinal and colonic afferent preparations at concentrations up to 1mM (Wynn and Burnstock, 2006, Rong et al., 2009), similar to the 2mM final bath concentration of ATP used in this study. Furthermore, ATP can be stored in nerves and other cells at millimolar concentrations (Hamilton and McMahon, 2000). 5-HT has been used up to 1mM in animal afferent preparations, although 100µM was enough to activate almost 100% of preparations

(Hicks et al., 2002, Coldwell et al., 2007). This is similar to the 200µM final bath concentration used in this report. Doses of histamine up to 1mM, was administered in a cat intestinal afferent preparation (Akoev et al, 1996). This is comparable to the 600µM final bath concentration used in this report. In addition, the application of 20µM PGE_2 final bath concentration to HVAs in this report is a similar concentration to that used in a rat pelvic nerve preparation (Su and Gebhart, 1998) and previously in an inflammatory soup applied to HVAs (Peiris et al., 2011). A 2µM dose of capsaicin was applied to HVAs in this study, which is comparable to that given to a mouse colonic afferent preparation 3µM, (Brierley et al., 2005a) and previously in a HVA preparation 10µM (Peiris et al., 2011).

3.1.4.4 BK PHARMACOLOGY

This report has shown that reproducible responses to repeated applications of certain mediators in HVAs, establishing the model's suitability for pharmacological manipulation studies targeting the second mediator application. This type of experiment has previously been used by a number of studies e.g. (Brunsden and Grundy, 1999). Using this protocol, we have demonstrated that BK acts through B2 receptors, with a seemingly limited involvement of B1 receptors. The HVA response to BK was only partly abolished by 300nM HOE140. However, when a higher dose, 1µM, was used, HOE140 abolished the HVA response to BK. However, 10nM of HOE140 was enough to almost eliminate the response splanchnic afferents to BK in mice and rats, although this was against a lower dose of BK, 1µM (Maubach and Grundy, 1999, Brierley et al., 2005b). This may suggest a greater potency in murine afferents over human afferents.

The present study corroborates previous work in animal models reporting B2 receptors as the main receptor involved in bradykinin signalling in colonic and other visceral afferents (Rangachari et al., 1993, Pan et al., 1994, Maubach and Grundy, 1999, Brierley et al., 2005b). The B1 receptor antagonist [Des-Arg^{10}] HOE140 had no effect on the response of rat

serosal afferents. Similarly, the B1 antagonist R715 did not affect the response of HVAs to BK. This represents the first data implicating B2 receptors as the main mediators of BK signalling in HVAs from normal tissue.

3.1.4.5 ATP PHARMACOLOGY

Using a similar protocol, we have shown a modest attenuation of the ATP response in the presence of the P2X antagonist PPADS, although this did not reach significance. Similarly, the P2X antagonist RO4 did not have a significant effect on the HVA response to ATP. In contrast, the response to ATP was abolished in rat pelvic balder afferents by treatment with the same dose of PPADS (30μM) (Yu and DeGroat, 2008). However, another study used 100μM of PPADS on colonic afferents in order to block the response to meATP (1mM) (Brierley et al., 2005a). Given that the inhibitory effect of 30μM of PPADS on the HVA response to ATP came close to significance ($p=0.067$), a higher dose may have been effective. Indeed, meATP can activate both splanchnic and pelvic afferents innervating the mouse colon, and rat jejunal afferents, effects that are blocked by PPADS, again demonstrating a role for P2X receptors in murine afferent signalling (Kirkup et al., 1999, Brierley et al., 2005a). meATP was not tested in HVAs, mainly due to the prohibitive expense. For this experiment to be feasible, the drug would have to be applied locally to the receptive field, using a metal ring. The present study is the first to show a potential P2X involvement in HVAs. Delineation of specific P2X receptors involved is warranted in future studies, but will require methods to reduce the volume of drug needed in order to be economically feasible e.g. ring application, or reduced bath volume.

ATP responses in murine afferent nerves (Kirkup et al., 1999) and in HVAs in this report are sometimes biphasic, exhibiting 2 peaks. This report hypothesised that ATP was activating P1 receptors after it had been broken down to adenosine by endogenous ATP-endonucleotidases, and was responsible for the second peak. Indeed, this report has demonstrated the activation of HVAs by adenosine. However, the P1 receptor antagonist CGS

15943 had no effect on the response of HVAs to ATP, suggesting that adenosine receptors are not involved in the activation of HVAs by ATP.

This report demonstrates that UTP and ADP, agonists to $P2Y_{2,\ 4,\ 6}$ and $P2Y_{1,\ 12}$ receptors, respectively, activate HVAs. P2Y antagonists were deemed not to be suitably efficacious for study in HVAs. This is the first study to show that ADP and UTP can activate afferent nerves. Indeed, the co-sensitivity of a single unit HVAs to ATP and either ADP (3/4 units) or UTP (4/4 units) or to all 3 mediators (1/3 units) demonstrates that a number of P2Y receptors are expressed on ATP sensitive afferents. This suggests a role for P2Y signalling in HVAs. Indeed, more efficacious P2Y antagonists will allow the elucidation of the involvement of P2Y receptors in the activation of HVAs by ATP.

3.1.5 CONCLUSION

Serosal HVAs are much more likely to respond to the algogenic mediators BK, and ATP. This substantiates the evidence for serosal afferents playing a nociceptive role in HVAs. Furthermore, the uncommon nature of responses to painful mediators in muscular units suggests that these mediators may be useful in confirming the location of an afferent terminal, in addition to confirming its role in nociception. This report also describes the broad chemosensitivity of HVAs, demonstrating whole nerve and single unit HVA responses to BK, ATP, adenosine and capsaicin.

In HVAs, BK exerts it's activation through the B2 receptor as demonstrated by blockade of HVA firing in response to BK by a B2 receptor antagonist HOE140. Multiple receptors are likely to be involved in the activation of HVAs by ATP, including P2Y receptors and even P2X receptors, but with limited involvement of P1 receptors.

CHAPTER 3 PART 2: CHEMOSENSITIVITY OF HUMAN VISCERAL AFFERENTS TO DISEASE MEDIATORS

3.2.1 INTRODUCTION

3.2.1.1 SEROTONIN

5-HT is a monoamine neurotransmitter and hormone. It is synthesised from its precursor L-tryptophan, in a 2-step process involving the enzymes tryptophan hydroxylase (TpH1 and TpH2) and aromatic amino acid decarboxylase. Serotonin is subsequently degraded by various isoforms of the monoamine oxidase enzyme (Nichols and Nichols, 2008). Serotonin has a role in a myriad of physiological processes such as appetite, sleep, gastrointestinal function, and pain (Nichols and Nichols, 2008). Indeed, most of the 5-HT in the body is found in the intestines (Gershon and Tack, 2007).

There are 14 different 5-HT receptors, encoded by 14 separate genes, and have been grouped into 7 families, 5-HT_{1-7} (Hoyer et al., 1994). All but the 5-HT_3, are part of the rhodopsin superfamily of GPCRs, exhibiting 7 transmembrane domains, displaying 3 intracellular and 3 extracellular loops, with a cytosolic carboxy terminal and extracellular amino group (Baez et al., 1995). Ligand binding induces conformational changes in the heteromeric G proteins and subsequent involvement in downstream signalling pathways (Gray and Roth, 2001). The 5-HT receptor families are linked to different types of G proteins, $G_{i/0}$, $G_{q/11}$ or, G_s, which govern their effects on cell signalling.

The 5-HT_3 receptor is a ligand gated cation channel, which upon activation unselectively allows the entry of monovalent cations and also Ca^{2+} to varying degrees, which is dependent on the receptor subunit composition (Lambert et al., 1989, Sugita et al., 1992,

Yang, 1990, Yang et al., 1992, Davies et al., 1999). The current report will focus on 5-HT_2, 5-HT_3 and 5-HT_4 receptor families.

3.2.1.1.1 5-HT_2 RECEPTORS

The 5-HT_2 receptor family is comprised of 3 distinct receptors, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}, which display 42-57% homology (Hoyer et al., 2002). They range in length, between 458-481 amino acids, and exhibit individual expression patterns. All 5-HT_2 receptors are linked to $G_{q/11}$ proteins, which upon activation result in membrane phosphoinositide hydrolysis and the formation of signalling molecules such as inositol phosphates and diacylglycerol (DAG), which can then alter downstream signalling pathways e.g. PKC pathway (Nichols and Nichols, 2008).

5-HT_{2A} receptors are 471 amino acids in length (Stam et al., 1992) and are expressed throughout peripheral and central tissues including the heart, the dorsal horn of the spinal cord and DRG (Andrade, 2014). They are involved in a number of processes including the contraction of smooth muscle and regulation of mood (Gray and Roth, 2001, Andrade, 2014). 5-HT_{2A} receptors undergo desensitisation, interestingly this occurs upon agonism and antagonism (Gray and Roth, 2001).

5-HT_{2B} receptors are comprised of 481 amino acids (Andrade, 2014), and exhibit a scattered expression pattern. It is found in central areas such as the cerebellum and hypothalamus and in most viscera and endothelial cells in the periphery (Duxon et al., 1997, Andrade, 2014). 5-HT_{2B} receptors are involved in the contractile and relaxatory properties of the stomach and blood vessels (Andrade, 2014).

The 5-HT_{2C} receptor is made up of 458 amino acids and its expression is mainly restricted to the CNS and choroid plexus, although their presence has been demonstrated on rat DRGs (Pierce et al., 1996, Andrade, 2014). There are 14 known distinct isoforms of the 5-HT_{2C} receptor, which are produced by RNA editing. These isoforms have different receptor

activation and desensitisation kinetics, suggesting a multiple roles for the 5-HT_{2C} receptor subtypes (Burns et al., 1997, Fitzgerald et al., 1999). 5-HT_{2C} has a role in a number of physiological processes including regulation of sleep (Frank et al., 2002) and food intake (Fone et al., 1998), as well as anxiety (Bagdy et al., 2001) and nociception (Chojnacka-Wojcik et al., 1994). As mentioned, dimerization is required for a functional 5-HT_{2C} receptor (Herrick-Davis et al., 2005).

3.2.1.1.2 5-HT_3 RECEPTORS

5-HT_3 receptors are the only ionotropic receptors in the 5-HT receptor family. They unselectively allow the passage of monovalent cations, as well as the divalent Ca^{2+} ion (Humphrey et al., 1993, Hoyer et al., 1994). 5-HT_3 receptors are comprised of 5 transmembrane subunits organised around a central pore region (Boess et al., 1995). Each subunit consists of a large N terminus and short C terminus, both of which are extracellular, and are separated by 4 transmembrane domains, which are connected by 1 extracellular and 2 intracellular loops (Nichols and Nichols, 2008). The diversity of the 5-HT_3 receptor is only beginning to be understood. To date, 5 distinct 5-HT_3 subunits have been discovered; 5-HT_{3A}, $_{3B}$, $_{3C}$, $_{3D}$, and 5-HT_{3E} (Barnes, 2014). The hetero-pentamer of the 5-HT_{3A}/5-HT_{3B} (ratio 2:3) subunits is currently the only known combination that grants full 5-HT receptor functionality (Dubin et al., 1999, Hanna et al., 2000). Single nucleotide polymorphisms (SNPs) and alternative splicing of 5-HT receptor subunits can influence functionality and hence further augment diversity (Nichols and Nichols, 2008). 5-HT_3 receptors are expressed in various brain regions as well as on peripheral nerves, including sensory neurons innervating the gut (Bufton et al., 1993, Holbrook et al., 2009).

3.2.1.1.3 5-HT IN VISCERAL PAIN

5-HT has distinct functions peripherally compared to centrally. Intrathecal administration of 5-HT can be analgesic (Bardin et al., 1997, Bardin et al., 2000). However, in

the periphery 5-HT is involved in inflammatory processes and diseases, such as IBS, and can activate intestinal afferent nerves (Hillsley and Grundy, 1998, Coldwell et al., 2007, Cremon et al., 2011). 5-HT is found in and released by EC cells and ENS neurons in close proximity to 5-HT sensitive mucosal extrinsic afferents, which are unlikely to be nociceptive. However, 5-HT can also activate splanchnic afferents, which are involved in nociception (Coldwell et al., 2007). This report will concentrate on peripheral actions of 5-HT.

In a guinea-pig TNBS model of colitis, used as a model for visceral hypersensitivity, EC cell numbers, the primary source of 5-HT in the gut, were increased along with a twofold increase in 5-HT levels. This was coupled with the decreased expression of, and mRNA transcripts for, the serotonin transporter (SERT), which removes 5-HT from the interstitial space (Linden and El-Fakahany, 2002). Alterations in the presence of 5-HT are also described during painful diseases of the bowel, such as IBS and IBD. 5-HT immunoreactivity in the myenteric plexus of patient's with CD was shown to be higher than in controls (Sakurai-Yamashita et al., 2000). However, when rectal mucosal biopsies from UC and IBS patients were compared to controls, 5-HT content and EC cell numbers were reduced. SERT mRNA and immunoreactivity were also decreased (Coates et al., 2004). Although 5-HT levels were decreased, the simultaneous decrease in SERT and hence a reduced capability to remove 5-HT from the interstitial space or synapse may lead to an increased activation of 5-HT receptors. In contrast a number of other studies have demonstrated increased mucosal 5-HT levels and 5-HT positive mast cells in the intestines (Kerckhoffs et al., 2008, Cremon et al., 2011). Furthermore, mucosal release of 5-HT was 10 fold higher in IBS patients compared to controls. Indeed, the amount of mucosal 5-HT release was correlated with abdominal pain scores (Cremon et al., 2011). These alternations in 5-HT could play a role in visceral hypersensitivity (Grundy, 2008). Indeed, in somatic tissue elevated levels of 5-HT have been associated with allodynia and increased pain (Kopp, 1998, Ernberg et al., 1999). Similarly, the addition of 5-HT with other mediators to cultured DRG enhanced a proton induced current, whereas 5-HT alone

had no effect (Kress et al., 1997). Taken together, this evidence suggests a major role for 5-HT in the pathophysiology of disease such as IBS.

The ability of 5-HT to activate vagal afferents innervating the jejunum, demonstrate the involvement of 5-HT in physiological sensory signalling (Hillsley and Grundy, 1998). However, it has also been demonstrated in electrophysiological experiments that 5-HT can activate splanchnic afferent fibres innervating the rat colon, which constitute the principle pathway in colonic nociception (Hicks et al., 2002, Coldwell et al., 2007). Furthermore, the percentage of nerves that respond to 5-HT increases, the response is larger and the EC50 is reduced following the acute and recovery phase of inflammation induced by DSS, suggesting a greater role for 5-HT during inflammation (Coldwell et al., 2007).

3.2.1.1.3.1 5-HT_2 Receptors in Visceral Pain

5-HT_2 receptors are expressed on sensory apparatus. 5-HT_{2A}, $_{2B}$ and 5-HT_{2C} receptor mRNA has been found on lumbar DRGs suggesting they are expressed on afferent nerves (Pierce et al., 1996, Nicholson et al., 2003). Indeed, 5-HT_{2A} receptors are found on afferents innervating rat skin (Carlton and Coggeshall, 1997). There is no IHC data on the expression of 5-HT_2 receptors on afferents innervating the viscera. However, there is some functional evidence for 5-HT_2 receptors in hyperalgesia in the somatic and visceral nociceptive pathways. Co-administration of various 5-HT_2 agonists increased pain behaviours in response to injection of PGE_2 or noreadrenaline into the paw of a rat. Similarly the pain behaviour response to 5-HT and PGE_2 or a 5-HT_2 agonist, α-methyl-5-HT and PGE_2, was antagonised by the 5-HT antagonist ketanserin (Abbott et al., 1996). Similarly, the 5-HT_2 antagonists ketanserin and sarpogrelate reduced pain behaviours to the injection of formalin (Abbott et al., 1997, Obata et al., 2000).

The role of 5-HT_2 receptors in mediating visceral pain, is complex, evoking questions on the involvement of different 5-HT_2 subunits, if they exert nociceptive or analgesic effects and, whether these effects are peripherally or centrally mediated. For example, peripheral

administration of the 5-HT antagonist ketanserin augmented the anti-nociceptive effect of imipramine, a tricyclic antidepressant and analgesic agent, on VMR to CRD in rats (Ilkaya et al., 2014). Similarly, peripheral 5-HT_2 receptors may be involved in nociception caused by chemical stimuli. For example, the antinociceptive effect of the selective serotonin reuptake inhibitor (SSRI) paroxetine on intraperitoneal acetic acid application was potentiated by the 5-HT_2 antagonist ketanserin (Kesim et al., 2005). However, when administered intrathecally (centrally), 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI) a 5-HT_2 agonist reduces the VMR and vigorous pressor response (VPR) to CRD in rats either on its own or by augmenting the anti-nociceptive effect of other analgesics e.g. the α_2 adrenergic agonist clonidine (Danzebrink and Gebhart, 1991b, Danzebrink and Gebhart, 1991a). Therefore it seems that the site of administration, peripheral vs. central determines the role of 5-HT_2 receptors in visceral nociception.

5-HT_{2B} receptors have been implicated in visceral hypersensitivity. A number of studies have demonstrated that peripheral or central administration of the 5-HT_{2B} antagonist RS-127445 reduced visceral hypersensitivity, both noxious pressure threshold and number of pain behaviours, induced by either TNBS or restraint stress or in a hypersensitive strain of rats (Wistar Kyoto) (Ohashi-Doi et al., 2010, O'Mahony et al., 2010). However, RS-127445 had no effect in reducing visceral sensitivity in normal rats (Ohashi-Doi et al., 2010). In addition, activation of 5-HT_{2C} receptors may be involved in the central antinociceptive effects of fear and other adverse emotions (Baptista et al., 2012). Taken together these data suggests role for 5-HT_2 receptors in nociception in the periphery, whereby antagonism of these receptors produces or augments visceral analgesia to both mechanical and chemical stimuli.

3.2.1.1.3.2 5-HT_3 Receptors in Visceral Pain

5-HT_3 receptors are present on ganglia from both vagal and spinal pathways and on their related extrinsic afferents innervating the gut (Rosenberg et al., 1997, Hicks et al., 2002,

Raybould et al., 2003). However, the vagal nodose ganglia express considerably more than spinal DRGs, suggesting a greater physiological role for 5-HT_3 receptors (Peeters et al., 2006). Indeed, 5-HT has been shown to activate mesenteric vagal afferents innervating the jejunum of anesthetised rats. When 5-HT was administered intravenously, afferent firing rates increased. This activation was abolished by the 5-HT_3 antagonist granisetron (Hillsley et al., 1998, Hillsley and Grundy, 1998). Moreover, intravenous administration of the 5-HT_3 agonist 2-methyl-5-HT mimicked this afferent activation (Hillsley et al., 1998).

5-HT_3 is clearly involved in vagal afferent signalling in the small intestine. Although vagal afferents are likely to play a small role in nociception, they are not considered part of the major pain signalling pathway (Grundy, 2008). However, 5-HT_3 is also involved in the transduction of nociceptive signals in the major pain pathway. Application of the 5-HT_3 agonist 2-methyl 5-HT activates splanchnic afferent nerves innervating the rat colon (Hicks et al., 2002). Furthermore, the 5-HT_3 receptor antagonist alosetron inhibited the response to 5-HT by about a 1/2 to 2/3s in rat colonic splanchnic nerves in 2 separate studies (Hicks et al., 2002, Coldwell et al., 2007). Taken together these data suggest a major role for 5-HT_3 receptors in 5-HT signalling in the main nociceptive pathway in the colon. However, this incomplete inhibition suggests that other 5-HT receptor subtypes are also involved, at least in splanchnic afferents.

5-HT_3 receptors may contribute to visceral pain in IBS, a hallmark of the disease. Supernatants generated from mucosal biopsies from IBS patients, activated both DRGs, and mesenteric afferents innervating the terminal jejunum in rats, while no effect was evident when using control supernatants (Barbara et al., 2007). Granisetron attenuated this IBS supernatant activation of rat mesenteric afferent by ~20%. Indeed, a number of 5-HT_3 antagonist, such as granisetron, ramosetron, cilansetron, alosetron, have demonstrated antinociceptive effects in preclinical models (Kozlowski et al., 2000, Barbara et al., 2007, Hirata et al., 2008, Cremon et al., 2011). For example, treatment with alosetron, ramosetron or

cilansetron increased the nociceptive threshold to CRD in rats. Furthermore, these compounds also significantly inhibited the hypersensitivity to CRD induced by stress (Hirata et al., 2008).

Clinical trials have demonstrated the efficacy of the 5-HT_3 anatagonists, ramosetron, cilansetron, alosetron in treating visceral pain associated with IBS (Camilleri et al., 1999, Matsueda et al., 2008a, Matsueda et al., 2008b). For example, a number of randomised, double blind, placebo controlled trials have demonstrated the efficacy of a 5-HT_3 antagonist alosetron in reducing abdominal pain. A greater proportion of IBS patents reported adequate pain relief with oral alosetron compared to a placebo control (Camilleri et al., 1999, Camilleri et al., 2000, Camilleri et al., 2001). In addition, another clinical trial described an increased proportion of pain free days in IBS patients treated with alosetron vs. placebo (Bardhan et al., 2000). Furthermore, when compared to the antispasmotic, smooth muscle relaxant, mebeverine, a great proportion of IBS patients treated with alosetron reported adequate pain relief (Jones et al., 1999). However, these clinical trials offer no evidence for a direct effect of 5-HT_3 antagonists on sensory nerves. A relief of IBS symptoms including abdominal pain may be due to the effects of these compounds on motility, transit time, bloating etc. However, the electrophysiological data presented in this report suggests that 5-HT_3 antagonists have some function at the site of afferent terminals. All these data together implicate 5-HT_3 receptors in visceral pain associated with diseases such as IBS.

5-HT_4 receptors are discussed in Chapter 4, Part 1.

3.2.1.2 HISTAMINE

Histamine is a biogenic amine and exerts its effects through 4 receptors H_1, H_2, H_3 and H_4, all of which are members of the 7 transmembrane domain containing rhodopsin-family of GPCRs (Bongers et al., 2010). H_1 receptors are linked to $G_{q/11}$ proteins, H_2 receptors are linked to G_s proteins, and H_3 and H_4 receptors are linked to $G_{i/o}$ proteins (Bongers et al., 2010). Histamine is involved in the development of visceral hypersensitivity and neurogenic inflammation.

Histamine receptors are widely expressed on cells in the intestine including endocrine cells, immune cells and nerves (Repka-Ramirez, 2003). Canine DRGs contain mRNA for each of the histamine receptors (Rossbach and Baumer, 2014). H_1 receptor mRNA are expressed on a proportion of nociceptive DRGs in guinea pigs (Kashiba et al., 1999). Furthermore, intestinal biopsies from IBS patients had higher levels of histamine receptor expression compared to controls (Sander et al., 2006).

Histamine can directly activate enteric nerves from animals (Tamura and Wood, 1992) and humans (Breunig et al., 2007). Histamine can also activate extrinsic afferent neurons innervating the thoracic and abdominal viscera. Histamine injected into the left atrium activated cat cardiac spinal afferents. The injection of a H_1 agonist also activated these spinal afferents. The H_1 antagonist but not H_2 or H_3 antagonists attenuated the cardiac spinal afferent response to histamine (Fu et al., 1997). Similarly, intra-arterial administered histamine can activate mesenteric afferents innervating cat intestines. Antagonists to either H_1 or H_2 receptors antagonised the intestinal afferent response to histamine (Akoev et al., 1996). Another group demonstrated jejunal afferent activation after intravenous application of histamine (Kreis et al., 1998, Kreis et al., 2002). Again, a H_1 antagonist, pyrilamine, but not a H_2 or H_3 antagonist attenuated the excitation of jejunal afferents by histamine.

H_1 receptors are also involved in visceral hypersensitivity. A recent study induced colitis in rats with rectally administered TNBS (Deiteren et al., 2014). After recovery, mast cell numbers and histamine release were increased, and rats demonstrated hypersensitive VMR responses to CRD. These enhanced VMR responses were inhibited by either the H_1 antagonist levocetirizine or the H_4 antagonist JNJ7777120 (Deiteren et al., 2014). Similarly, the H_1 antagonists fexofenadine and ebastine were effective in reducing the augmented VMR response to CRD in rats stressed by maternal separation and water avoidance (Stanisor et al., 2013). These studies demonstrate a role for both mast cells, but specifically histamine in visceral hypersensitivity.

Mediators released by mast cells, which are the main source of histamine in the gut (Buhner and Schemann, 2012), can activate afferent nerves. Intraluminal injections of a mast cell degranulator 48/80 activated mesenteric nerves innervating rat intestine *in vivo*. Indeed, these afferent responses could be blocked by the H_1 agonist clemastine (Nozdrachev et al., 1999). This suggests that degranulating mast cells can activate extrinsic sensory nerves by releasing histamine. Indeed, the ~90% of intestinal mucosal mast cells are touching or in very close proximity to nerves in the intestine, and can release histamine upon degranulation (Stead et al., 1989, Stead, 1992, Metcalfe et al., 1997). Furthermore, in IBS, elevated levels of histamine and tryptase were released by the markedly increased numbers of degranulating mast cells, which correlated to abdominal pain scores (Barbara et al., 2004). Similarly, supernatants generated from mucosal biopsies from IBS patients with visceral hypersensitivity contain more mediators released by mast cells, such as histamine and proteases (Buhner et al., 2012). Indeed, 2 clinical trials demonstrated the effectiveness of the mast cell stabilizers and H_1 antagonists ketotifen and ebastin in improving abdominal pain and other IBS symptoms (Klooker et al., 2010, van Wanrooij et al., 2014). These data taken together clearly establish a role for histamine and its receptors in visceral pain and hypersensitivity in diseases such as IBS.

3.2.1.3 PGE_2

PGE_2 is a pro-inflammatory lipid metabolite produced when arachidonic acid goes through the cyclooxygenase (COX) pathway. PGE_2 is a key mediator in both somatic and visceral inflammation and hypersensitivity at both peripheral and central levels (Lin et al., 2006). Indeed, non-steroidal anti-inflammatories (NSAIDS), which block COX enzymes, are the most widely used analgesics (Lin et al., 2006). PGE_2 has 4 GPCRs, $EP_{1, 2, 3}$, and EP_4 each containing 7 transmembrane domains (Kawabata, 2011). EP_1 receptors are linked to $G_{q/11}$ proteins, EP_2 and EP_4 receptors are linked to G_s proteins and depending on the splice variant EP_3 receptors can couple with G_s or G_i proteins (Lin et al., 2006). This suggests that EP_3 receptors may actually

have analgesic effects, by inhibiting the production of cAMP synthesis, and indeed there is some evidence for this e.g. (Natura et al., 2013).

All EP receptors are expressed on DRGs (Southall and Vasko, 2001, Natura et al., 2013). EP receptors are likely expressed on visceral afferents, since the application of PGE_2 can activate intestinal afferents, including those from nociceptive pathways (Longhurst and Dittman, 1987, Haupt et al., 2000). In addition, PGE_2 can sensitise nociceptive afferent nerves innervating the intestines, such that their subsequent response to algesic mediators such as BK is enhanced (Longhurst and Dittman, 1987, Maubach and Grundy, 1999, Brunsden and Grundy, 1999). There is some evidence that PGE_2 can sensitise subsequent responses of pelvic afferents to colorectal distension *in vitro* (Su and Gebhart, 1998). However, in this case PGE_2 was applied as part of an inflammatory soup, which also contained BK, histamine, 5-HT and KCl, all of which may have contributed to this sensitisation. Similarly, PGE_2, as part of an inflammatory soup, can activate afferents innervating the human intestine (Peiris et al., 2011). PGE_2 alone can depolarise trunks of isolated human visceral vagus nerve (Belvisi et al., 2008, Maher et al., 2009). In addition, PGE_2 can activate mesenteric afferents innervating the small intestine of the cats (Akoev et al., 1996). Similarly, PGE_2 and EP_1 (17-phenyl-ω-trinor-PGE_2) and $_2$ (misoprostol) agonists caused activation of rat jejunal afferents *in vivo* (Haupt et al., 2000). The EP_2 receptor seemed to be involved in a gradual increase in afferent activity, while EP_1 receptor agonists caused an early peak response.

PGE_2 is involved in visceral hyperalgesia. PGE_2 levels are elevated in patients with IBS or IBD (Jones et al., 1982, Hommes et al., 1996). Noxious events such colonic distension to painful pressures releases large quantities of PGE_2 (Roza and Reeh, 2001), which may sensitise afferents to subsequent mechanical events. In patch clamping experiments colonic DRGs are sensitised by PGE_2, such that their threshold for activation is reduced, and upon excitation more action potentials are fired (Gold and Traub, 2004). Furthermore, EP receptors, specifically EP_1 may mediate the behavioural response to nociceptive chemical stimuli since

EP_1 KO mice demonstrated a reduced number of writhings in response to intraperitoneal injection of acetic acid (Stock et al., 2001).

Application of an EP antagonist, ZD6416, inhibits hyperalgesia in the upper oesophagus as a result of lower oesophageal acid infusion (Sarkar et al., 2003). This suggests a role for PGE_2 in human visceral hyperalgesia. Importantly, PGE_2 may also be involved in the modulation of TTX resistant (TTX-R) sodium channels in hyperalgesia. The application of PGE_2 induced a quick increase in sodium current and altered the biophysical properties of the TTX-R sodium channels in both splanchnic and pelvic rat colonic DRGs (Gold et al., 2002). Taken together, this data suggests an involvement of PGE_2 in visceral hyperalgesia and pain in disease such as IBS.

3.2.1.4 AIMS

- To further examine the chemosensitivity of whole nerve HVAs to a number of mediators implicated in IBS, i.e. 5-HT, histamine, PGE_2
- Determine if 5-HT and histamine are suitable for using in repeat mediator application protocols, by examining the reproducibility of the response to repeated applications of these mediators
- Examine the receptor pharmacology underlying HVA activation by 5-HT

3.2.2 METHODS

3.2.2.1 CHEMOSENSITIVITY

After any mechanical characterisation protocols, atropine (10μM) and nifedipine (10μM) were added to the Krebs buffer and given 30 minutes to take effect. Some preparations were used solely for chemosensitivity protocols. Drugs were applied to the tissue bath by superfusion of a 20ml volume to make up the final bath concentrations of: 5-HT (BC 200μM, 20ml of 1mM), histamine (BC 600μM, 20ml of 3mM), PGE_2 (BC 20μM, 20ml of 100 μM). An effort was made to keep the mediator applications in the same order in each preparation. If a mediator failed to elicit a response, the next mediator was added 30 minutes later, otherwise a washout period of 60 minutes was observed.

3.2.2.2 MEDIATOR PHARMACOLOGY

For repeat application protocols, either 5-HT (BC 200μM, 20ml of 1mM) or histamine (BC 600μM, 20ml of 3mM) was superfused into the bath 3 times consecutively, with a washout period of 60 minutes between applications (figure 3.09). Pharmacological protocols using 5-HT and specific 5-HT receptor ligands were also performed. A selective agonist for either the $5\text{-}HT_2$ (α-methyl-5-HT maleate, BC 20μM, 20ml of100μM) or $5\text{-}HT_3$ (methyl-chlorophenylbiguanide hydrochloride, BC 20μM, 20ml of 100μM) receptor was superfused into the bath. If given in the same preparation, a washout period of at least 60 minutes was observed between the applications of the 5-HT receptor agonists. Data were analysed using a 2 tailed paired t test, $p<0.05$.

Figure 3.09: Repeat mediator application protocol

1) The first application of the mediator (↓) e.g. 5-HT is superfused into the bath.

2) An hour later the second application of the mediator (↓) is applied.

3) After another hour the third application of the mediator (↓) is added.

3.2.2.3 DRUGS

Drugs in powder form were made up using the recommended solutions, aliquoted and frozen at 20°C. When needed, aliquots were diluted in Krebs to make the final working concentration and vortexed to mix. 5-HT and histamine were obtained from Sigma Aldrich (St Louis, MO, USA). Prostaglandin E_2, α-methyl-5-HT maleate, and methyl-chlorophenylbiguanide hydrochloride were purchased from Tocris Bioscience (Bristol, UK).

3.2.3 RESULTS

3.2.3.1 TISSUES – 5-HT PHARMACOLOGY

3.2.3.1.1 Repeat 5-HT applications

Three tissues, all normal, were used for repeat 5-HT application experiments, 1 ascending colon,, 1 transverse colon, 1 sigmoid colon, (M:F 1:0.05, median age 24).

3.2.3.1.2 5-HT_2 agonist (α-methyl-5-HT maleate)

Seven tissues, 6 normal, 1 UC, were used for 5-HT_2 agonist experiments 2 descending colon, 2 sigmoid colon, 2 rectum, 1 ascending colon (M:F 1:1.33, median age 57).

3.2.3.1.3 5-HT_3 agonist (methyl-chlorophenylbiguanide hydrochloride)

Seven tissues, 4 normal, 2 UC, 1CD, were used for 5-HT_3 agonist experiments, 2 sigmoid colon, 2 appendix, 1 ileum, 1 transverse colon, 1 descending colon (M:F 1:0.75, median age 39).

3.2.3.2 TISSUES – HISTAMINE

3.2.3.2.1 Repeat histamine applications

Two tissues, 1 normal, 1 appendicitis, were used for histamine repeat experiments, 1 sigmoid colon, 1 appendix (M:F 1:1, median age (43.5). Further details on the tissues use in each set of experiments can be seen in table 2.02.

3.2.3.3 CHEMOSENSITIVITY IN WHOLE NERVE HVA RECORDINGS

The disease mediators 5-HT, histamine, and PGE_2 activated flat sheet whole nerve HVAs, 5-HT (BC 200µM, 7/15, 46.7%, Δ firing rate 42.2±23.4 spikes $20s^{-1}$), histamine (BC 600µM, 13/17, 76.5%, Δ firing rate 30.5±6.4), PGE_2 (BC 20µM, 7/8, 87.5%, Δ firing rate 41.0±14.8 spikes $20s^{-1}$). Responses to PGE_2 were generally gradual, HVA activity increasing over a period of time (figure

3.10). 5-HT (BC 200µM, 2/6, 33.3%, Δ firing rate 84.0±32.8 spikes $20s^{-1}$), histamine (BC 600µM, 3/4, 75.0%, Δ firing rate 55.6±16.1 spikes $20s^{-1}$), and PGE_2 (BC 20µM, 2/3 -, 66.6%, Δ firing rate spikes 48.2±3.0 $20s^{-1}$) also activated whole nerve HVA innervating the appendix.

3.2.3.4 5-Hydroxytryptamine

Repeat applications of 5-HT produced repeated responses in 2/4 units (3 preparations) (figure 3.11). The responding units only responded to 2 applications of 5-HT. The response to the first addition was considerably larger than the response to the second (Δ firing rate 1^{st} 116.5±23.1, normalised 100%, vs. 2^{nd} 48.0±11.0 spikes $20s^{-1}$, 44.8%). The $5\text{-}HT_3$ agonist methyl-chlorophenylbiguanide hydrochloride was applied to 8 preparations from which 17 single units could be identified. No activation of HVAs was evident. A gradual decrease was evident in 10 /17 units (58.8%), whereby activity had been reduced by at least 20% 45 minutes after $5\text{-}HT_3$ agonist application ($p<0.05$). Four out of 17 and 7/17 demonstrated reduced activity (min. 20%) at 15 and 30 minutes post 5-HT3 agonist application, respectively ($p<0.05$). Seven preparations were treated with the $5\text{-}HT_2$ α-methyl-5-HT maleate, from which 13 single units could be distinguished. Two out of 13 units (15.4%) responded to the $5\text{-}HT_2$ agonist (average Δ firing rate 16.7±4.3 spikes $20s^{-1}$) (figure 3.11).

3.2.3.5 HISTAMINE

Repeat applications of histamine produced repeated responses in 1/3 units (2 preparations) (figure 3.11). The responding unit only responded to 2 applications of histamine. The response to the first addition was considerably larger than the response to the second (Δ firing rate 1^{st} 58, normalised 100%, vs. 2^{nd} 33 spikes $20s^{-1}$, 56.9%).

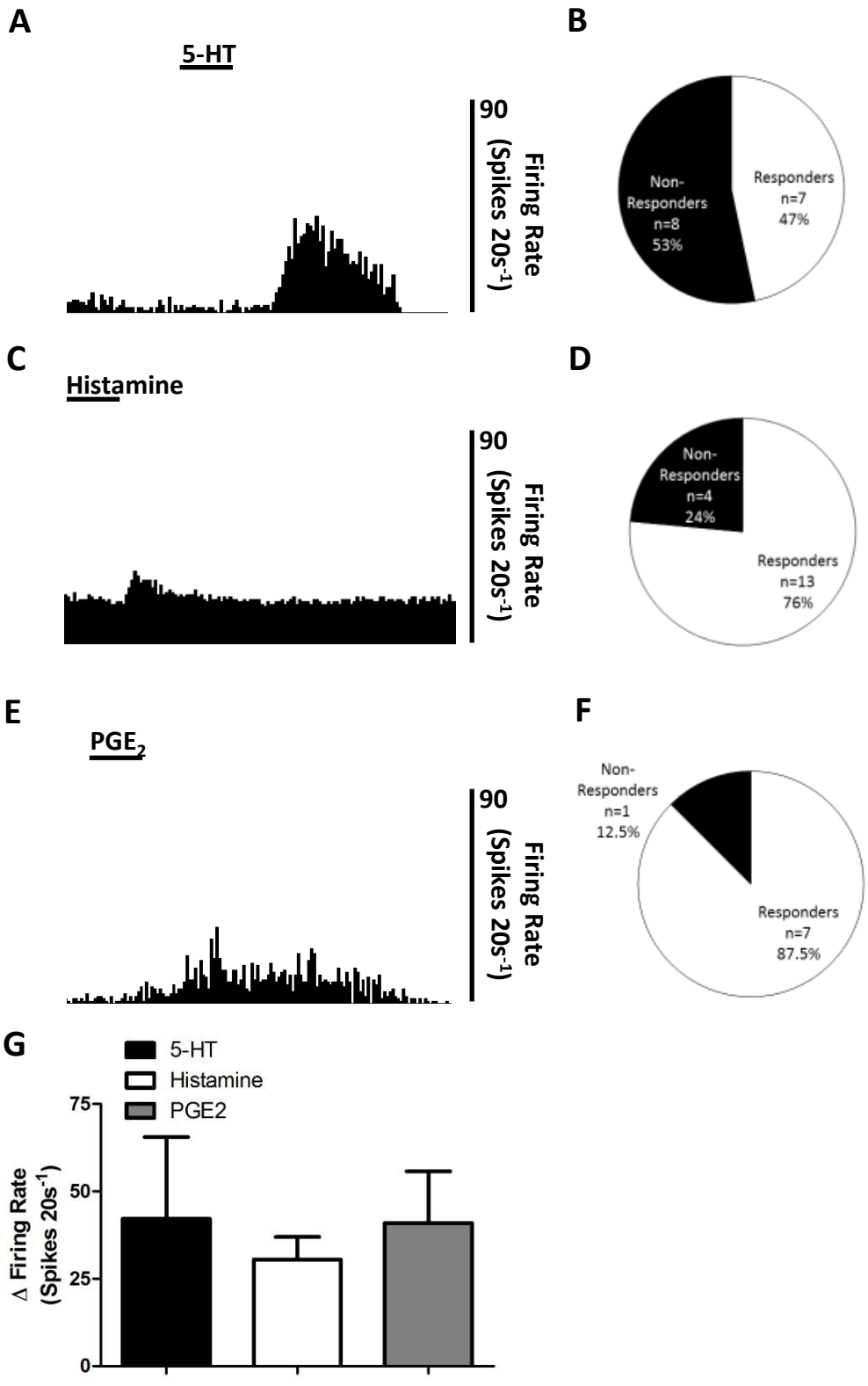


Figure 3.10: Wholenerve HVAs respond to the application of serotonin (5-HT), histamine and prostaglandin E_2 (PGE_2). A, C, E) Shows an example of a HVA response to 5-HT (A), histamine (C), and PGE_2 (E), in the form of a rate histogram. B, D, F) Displays the proportion of wholenerve recordings that responded to 5-HT (B), histamine (D) or PGE_2 (F). G) A bar graph indicating the average change in HVA firing after the application of 5-HT, histamine or PGE_2.

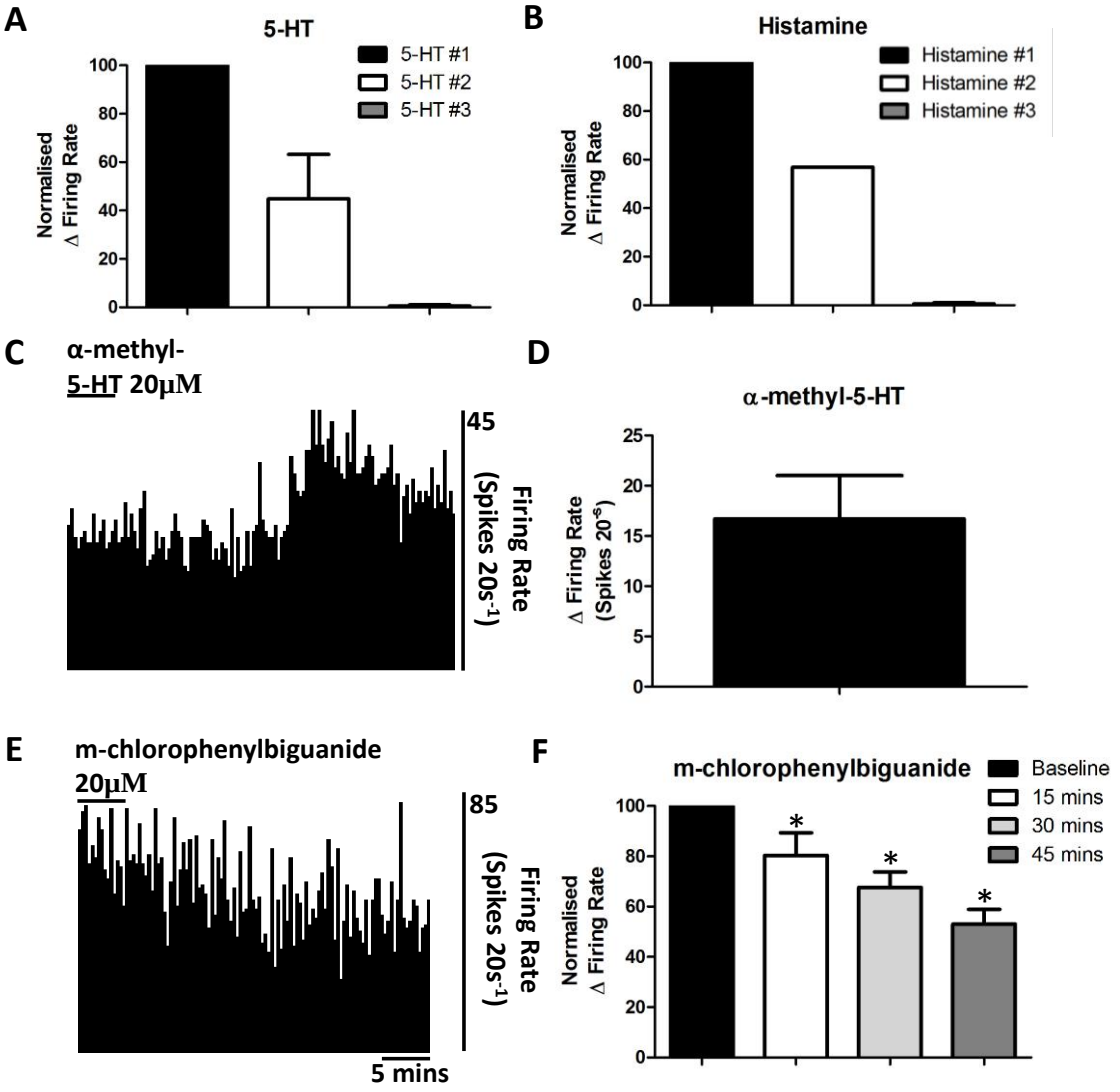


Figure 3.11: Application of 5-hydroxytryptamine or histamine desensitise HVAs to subsequent applications of the same mediator. A) HVAs were activated by the 1st and 2nd applications of 5-HT in 2/4 preparations tested. There was no response to the third 5-HT application. B) A robust HVA response was evident after the 1st and 2nd application of histamine in 1/3 units tested. However, this preparation then failed to respond to the third application of histamine. C-D) A small proportion of HVAs responded to the 5-HT_2 agonist α-methyl-5-HT maleate (2/13, 15.4%). An example of this activation is shown as a rate histogram (C), with the average rate of activation presented as a bar graph (D). E-F) The addition of the 5-HT_3 agonist methyl-chlorophenylbiguanide hydrochloride reduced afferent firing in 10/17 units (58.8%) ($p<0.05$ at 15, 30 and 45 mins). An example reduction is shown as a rate histogram (E), with the average reduction over 15, 30 and 45 minutes shown with a bar graph (F). Values are normalised to baseline firing. Data were analysed using a paired 2 tailed t test, $p<0.05$.

3.2.3.6 SUMMARY OF RESULTS

- Whole nerve recordings are sensitive to a number of chemical stimuli, namely 5-HT, histamine, and PGE_2
- Application of 5-HT, or histamine desensitised afferents to subsequent applications, and are therefore not suitable for repeated mediator application protocols, at least using this dose and drug application method.
- A small proportion of HVAs responded to the $5\text{-}HT_2$ agonist α-methyl-5-HT maleate
- The $5\text{-}HT_3$ agonist methyl-chlorophenylbiguanide hydrochloride gradually reduced HVA firing

3.2.4 DISCUSSION

3.2.4.1 SEROTONIN PHARMACOLOGY

This report has demonstrated the response of HVAs to 5-HT. In a subset of preparations, these mediators responded to a second application, but not a third. Pharmacological manipulation studies were therefore not attempted. This could be revisited in the future using lower concentrations of the mediators using different application methods as discussed in chapter 6. Instead selective 5-HT agonists were tested.

A small proportion of afferents ~15% responded to the application of the 5-HT_2 receptor agonist, α-methyl-5-HT. There is no animal electrophysiological data available for comparison. However, the role of 5-HT_2 receptors in visceral nociception, at least when applied peripherally, suggests that agonism is pro-nociceptive (Kesim et al., 2005, Ohashi-Doi et al., 2010, O'Mahony et al., 2010), to which our results concur. The addition of the 5-HT_3 agonist, methyl-chlorophenylbiguanide hydrochloride, actually gradually reduced the HVA firing rate in nearly 60% of units, with some units exhibiting a 20% reduction in firing as early as 15 minutes after application. In contrast, the 5-HT_3 agonist 2-methyl-5-HT (me5-HT) can activate both vagal and splanchnic afferents innervating rat intestine (Hillsley et al., 1998, Hillsley and Grundy, 1998, Hicks et al., 2002). Similarly, the activation of these afferents by 5-HT can be reduced by antagonising the 5-HT_3 receptor (Hicks et al., 2002, Coldwell et al., 2007). Furthermore, antagonism of the 5-HT_3 receptor has been shown to be anti-nociceptive in IBS patients exhibiting abdominal pain symptoms (Camilleri et al., 1999, Caras et al., 2001, Matsueda et al., 2008b). Although 5-HT has different effects centrally and peripherally, in the periphery evidence supports an analgesic effect upon 5-HT_3 antagonism. The results shown in this report are therefore hard to explain. No vehicle effect was observed since the drug was dissolved in distilled H_2O, which constituted just 0.1% of the final drug volume added to the tissue bath. A suitable time matched control would involve allowing a unit to fire as regular

Krebs buffer was being perfused for the same time as the protocol, ~1 hour. This was done often to allow the preparation to rest or after a protocol has finished, with no evident reduction in HVA firing in the preparation as seen in methyl-chlorophenylbiguanide hydrochloride experiments. Methyl-chlorophenylbiguanide hydrochloride has a similar affinity at the 5-HT_3 receptor as the more commonly used me5-HT (pk*i* 5.4-5.8) (Alexander, 2011). It would be of interest to try me5-HT on HVAs to investigate if the same reduction in activity occurs.

In afferents innervating the rat small bowel and the upper GI tract of ferrets, 5-HT3 antagonism reduced spontaneous activity, indicating a role for 5-HT in ongoing afferent discharge (Blackshaw and Grundy, 1993, Hillsley et al., 1998). However, 5-HT_3 antagonism did not have an effect on the spontaneous activity in rat colonic afferents. The authors suggested this was potentially due to low rates of existing spontaneous activity (Hicks et al., 2002). HVA preparations often exhibit spontaneous activity. It would be interesting to examine the role of endogenous 5-HT in this spontaneous activity using specific 5-HT receptor subtype antagonists.

Investigating the sensitising effect of the various mediators used in this report on the HVA response to chemical and mechanical stimuli would be of interest. These mediators given as an inflammatory soup (Su and Gebhart, 1998) or individually e.g. PGE_2 or histamine (Brunsden and Grundy, 1999) can sensitise rodent intestinal afferents to subsequent mechanical and chemical stimuli. Indeed, the sensitising effects of 5-HT and histamine have been shown to be mediated by TRPV4 receptors (Cenac et al., 2010). It would be interesting to examine this concept further in HVAs using, 5-HT, histamine, and other sensitising mediators.

3.2.4.2 Histamine

This report has demonstrated the activation of HVAs by histamine. This is the first time histamine alone has been shown to activate HVAs. Histamine as part of an inflammatory soup activated HVAs innervating the human colon (Peiris et al, 2011). Histamine can activate visceral

afferents including cat spinal afferents innervating the heart (Fu et al, 1997) and mesenteric afferents innervating the jejunum of the rat (Kreis et al, 1998; 2002). The latter study concluded that the effect of histamine was likely a direct effect on visceral afferents. Indeed, histamine can modulate muscle contractility which could indirectly activate HVA (Sakai, 1979). However, muscle contractility was inhibited in this report through the presence of atropine, a muscarinic acetylcholine antagonist, and nifedipine, an L type calcium channel blocker, in the Krebs buffer. Therefore it is likely that histamine is directly activating HVAs, although the release of other mediators from cells in response to histamine application, which subsequently activate HVAs cannot be ruled out.

The responses to histamine in jejunal mesenteric rat afferents have been shown to be mediated by the H1 receptor (Kreis et al, 1998). The lack of reproducible responses to repeat histamine applications did not allow for further evaluation of histamine pharmacology in HVAs using the dose and drug application method described in this report. Indeed, a desensitisation of the response to repeated histamine application was evident in *in vivo* recordings from rat jejunal afferents (Kreis et al, 1998; 2000). Altering the drug concentration and/or the application method of histamine may allow for evaluation of histamine pharmacology.

3.2.4.3 PGE_2

This report demonstrates the activation of HVAs by PGE_2. PGE_2 has previously been shown to activate HVAs as part of an inflammatory soup (Peiris et al, 2011), however this represents the first time that PGE_2 alone can activate HVAs. Repeated applications of PGE_2 were not attempted. PGE_2 can activate visceral afferents (Akoev et al, 1996; Haupt et al, 2000) and sensitise them to subsequent other mediators such as BK (Maubach and Grundy, 1999; Brunsden and Grundy, 1999). Furthermore, prostaglandins have been shown to be important in the activation of rat jejunal afferents by BK (Maubach and Grundy, 1999; Brunsden and Grundy, 1999). In the future it would be interesting to examine the role of PGE_2 and other prostaglandins in the activation and sensitising of HVAs to other mediators.

3.2.5 CONCLUSION

This report describes the broad chemosensitivity of HVAs, demonstrating responses to the disease mediators 5-HT, histamine, PGE_2, which is in addition to responses to BK, ATP, adenosine and capsaicin that have been shown in chapter 3 part 1. In contrast to BK and ATP, 5-HT and histamine are not suitable for the described repeated mediator application protocol; however, this could be revisited in the future using different doses and/or application methods. Furthermore responses to a $5\text{-}HT_2$ receptor agonist, α-methyl-5-HT maleate, have been demonstrated in HVAs. The decrease in afferent firing induced by the $5\text{-}HT_3$ agonist methyl-chlorophenylbiguanide hydrochloride application requires further investigation.

CHAPTER 4 PART 1: THE EFFECT OF TEGASEROD ON THE MECHANOSENSITIVITY OF HUMAN VISCERAL AFFERENTS

This chapter utilises drugs that have shown both preclinical and clinical efficacy in reducing nociception or pain (tegaserod), drugs that mimic the effects of another drug currently in clinical trials clinical trials (STa – Linaclotide), or drugs targeting the same receptor as another drug currently in clinical trials (ICI 204, 448 – kappa opioid receptor). The primary aim of this chapter was to examine the effects of these drugs on the transduction of mechanical stimuli in HVAs. This chapter is split into 3 parts. Each part examines the effect of a different drug, tegaserod (part 1), STa (part 2), and ICI 204, 448 (part 3) on the HVA responses to VFH probing, and/or luminal distension of the appendix.

4.1.1 INTRODUCTION

4.1.1.1 TEGASEROD

4.1.1.1.1 5-HT_4 receptors

Serotonin receptor families have previously been introduced in chapter 2. Briefly, 5-HT_4 receptors are G protein coupled receptors, 387 amino acids in length (Andrade, 2014). They couple to G_s proteins that enhance the production of cAMP from ATP by stimulating the enzyme adenylate cyclase, which then acts as a signalling molecule for subsequent cellular events. They have been shown to be expressed in various tissues including the heart, many regions of the brain and the smooth muscle and myenteric plexus of the intestine (Andrade, 2014). The 5-HT_4 receptor is constitutively expressed as a dimer, with 8 distinct splice variants (Bockaert et al., 2004, Maillet et al., 2005), different combinations of which may determine the

biological effect of the receptor upon activation (Berthouze et al., 2007). 5-HT_4 has a myriad of functions including stimulation of peristalsis and the relaxation and contraction of the small intestine and colon (Andrade, 2014).

4.1.1.1.1.1 5-HT_4 receptors in visceral pain

5-HT_4 receptors are expressed on both nodose and dorsal root ganglia, albeit to a lesser extent than other 5-HT receptors such as 5-HT_3 (Grundy, 2008). During inflammation, 5-HT_4 mRNA may be upregulated, as reported in rat DRGs after inflammation of the hindpaw (Bockaert et al., 2004). 5-HT_4 receptors are also expressed on pre-synaptic cholinergic IPANs, and upon activation stimulate the release of acetylcholine from intrinsic nerves. This augments the amplitude of fast excitatory postsynaptic potentials strengthening the transmission of synaptic signals and promoting smooth muscle contractions, which has an overall pro-kinetic effect on the bowel (Pan et al., 1994, Galligan et al., 2003, Galligan and Vanner, 2005, Liu et al., 2005b). Indeed, 5-HT_4 agonists exhibit prokinetic effects e.g. tegaserod, cisapride, prucalopride etc. However, the role of 5-HT_4 receptors in visceral pain is more controversial.

Tegaserod, a partial 5-HT_4 agonist, is one of the most studied 5-HT_4 agonists, and will be the focus of this 5-HT_4 section. Tegaserod is also a 5-HT_{2B} receptor antagonist, with a similar pKi to 5-HT_4 receptors (7.5-8) (Beattie et al., 2004).Tegaserod may act as a partial agonist, or alternatively by antagonising the effects of endogenous 5-HT on the 5-HT_4, or the 5-HT_{2B}receptor (Bockaert et al., 2004). Therefore, whether analgesia is induced by the activation or inhibition of the 5-HT_4 receptor is controversial. For example, activation of 5-HT_4 receptors modulates tetrodotoxin – resistant (TTX-R) sodium channels, increasing the excitability of nociceptive like neurons (Cardenas et al., 2001). However, the majority of studies report analgesia upon 5-HT_4 activation. For example, treatment with tegaserod for 8 days has been shown to reduce nociception in response to slow rectal distensions in healthy women (Coffin et al., 2003). The same authors went on to demonstrate a similar effect in women with IBS-C

(Sabate et al., 2005). The analgesic effect of tegaserod is also evident in animal experiments. Tegaserod increases pain threshold, as evident by a reduced number of abdominal contractions, to CRD in rats (Coelho et al., 2000). Similarly, intraperitoneal administration of tegaserod reduced the VMR response to CRD in both normal and TNBS treated rats. This analgesic effect was inhibited by a 5-HT_4 antagonist (Greenwood-Van Meerveld et al., 2006). In addition, the reduction of VMR responses to CRD evident after intracolonic administration of tegaserod or the 5-HT_4 agonist naronapridem was inhibited by the 5-HT_4 antagonist GR113808 (Hoffman et al., 2012). Indeed, activation of 5-HT_4 receptors using full 5-HT_4 agonists, such as mosipride citrate, naronapride and prucalopride, produce analgesic effects in both animal experiments (Seto et al., 2011, Lee et al., 2012, Hoffman et al., 2012), and in clinical trials (Camilleri, 2008, Quigley et al., 2009, Tack, 2009). These results taken together strongly suggest that 5-HT_4 agonism is analgesic.

The efficacy of tegaserod has been examined in a large number of clinical trials, both randomised controls trials (RCT) (Lefkowitz, 1999, Muller-Lissner et al., 2001, Novick et al., 2002, Kellow et al., 2003, Nyhlin et al., 2004, Tack et al., 2005, Chey et al., 2008), and open labelled trials (Bardhan et al., 2004, Layer et al., 2005, Muller-Lissner et al., 2005). These trials have mainly been performed on IBS-C patients with some studies including patients with alternating IBS. Generally, the primary end point used was a global relief of IBS symptoms in all or most weeks during the treatment period. Of importance to this report, secondary endpoints included relief from abdominal pain and discomfort.

The majority of RCTs reported a statistically significant reduction in abdominal pain and discomfort during tegaserod treatment (Lefkowitz, 1999, Muller-Lissner et al., 2001, Novick et al., 2002, Kellow et al., 2003, Tack et al., 2005). Symptoms returned quickly after cessation of treatment with tegaserod (Novick et al., 2002). In contrast, in some clinical trials no relief from abdominal pain was reported (Nyhlin et al., 2004, Chey et al., 2008). Similarly, open labelled trials reported a significant reduction in abdominal pain, which returned after

cessation of tegaserod treatment (Bardhan et al., 2004, Layer et al., 2005, Muller-Lissner et al., 2005). Indeed, abdominal pain was again relieved after retreatment with tegaserod (Muller-Lissner et al., 2005). Taken together these clinical trials demonstrated the effectiveness of tegaserod in reducing abdominal pain.

However, tegaserod, and indeed another 5-HT_4 receptor agonist cisapride, were taken off the market due to a small number of cardiovascular (CV) side effects (De Maeyer et al., 2008). These adverse CV events may not be related to the 5-HT_4 receptor, since both tegaserod (5-HT_1, 5-HT_2) and cisapride (5-HT_2, 5-HT_3) have affinity for other 5-HT receptors (De Maeyer et al., 2008). This has raised the possibility that a more selective 5-HT_4 agonist, which does not have cardiovascular side effects, may be useful clinically. Of these compounds, the most work has been done on prucalopride, which has so far demonstrated efficacy in normalising bowel function and reducing symptoms including abdominal pain scores in chronically constipated patients (Camilleri, 2008, Quigley et al., 2009, Tack, 2009).

The prokinetic effects of 5-HT_4 agonists raise the question of whether the relief of abdominal pain symptoms in IBS-C, and chronic constipation patients treated with tegaserod is direct on extrinsic afferents or simply due to improved bowel function, and the concomitant decrease in bloating and abdominal discomfort. A study on pelvic afferents innervating the cat rectum demonstrates 5-HT_4 activity nerve terminals. Afferent action potential discharge in response to distension of the rectum in conscious cats was inhibited by the intravenous application of tegaserod. This inhibition was partially inhibited by the 5-HT_4 antagonist SB203186 (Schikowski et al., 2002). These studies together with the analgesic effects demonstrated in both animal and human experiments utilising colorectal distension paradigms (Coelho et al., 2000, Coffin et al., 2003, Greenwood-Van Meerveld et al., 2006) indicate that 5-HT_4 receptors can acutely and directly exert analgesic effects, which does not require improved bowel functioning.

In summary, tegaserod is clinically effective in relieving abdominal pain. This may be due to agonism of the 5-HT_4 receptor, rather than a competitive antagonist effect on endogenous 5-HT on the 5-HT_4 receptor, although an effect due to 5-HT_{2B} antagonist cannot be ruled out. Similarly, the analgesia produced is likely to be independent of the associated changes in motility. Indeed, tegaserod can inhibit afferent discharge in response to rectal distension in cats (Schikowski et al., 2002). The direct effect of tegaserod on the response of human afferents to mechanical stimuli has not been previously investigated. This report aims to examine the effect of tegaserod on the HVA response to both VFH probing and distension of the human appendix. Furthermore, these experiments will also reveal the effectiveness of a clinically efficacious visceral analgesic on directly reducing activity in HVAs, thus testing the model's capacity to provide insight into the likely efficacy of drugs before they enter clinical trials.

4.1.1.2 AIMS

- Examine the effects of tegaserod on the transduction of mechanical stimuli
    - VFH probing
    - Luminal distension of the appendix

4.1.2 METHODS

4.1.2.1 VFH PROBING PROTOCOL

The VFH probing protocol has been previously described in chapter 2, part 1. Briefly, after the initial baseline 1g VFH probes, 3 sets of 3 x 3 second probes, each set separated by 5 minutes, were attained, the bath was superfused with tegaserod (30µM, 100ml). In all experiments this was followed with 6-9 sets of 3 x3 second probes, each set separated by 5 minutes (figure 4.01). Data were analysed using a 2 tailed paired t test, $p<0.05$.

4.1.2.2 APPENDIX DISTENSION PROTOCOL

The appendix distension protocol has been previously described in chapter 2, part 2. Briefly, after the initial 3 baseline distensions (0-60 mm Hg), the bath was superfused with tegaserod (30µM, 100ml, or 30µM, 100ml superfusion, 20ml luminal perfusion). In all the experiments, distensions were continued every 10 minutes after the 3 baseline distensions (figure 4.02). Data were analysed using a 2 way ANOVA, $p<0.05$.

4.1.2.3 DRUGS

Drugs in powder form were made up using the recommended solutions, aliquoted and frozen at -20°C. When needed, aliquots were diluted in Krebs to make the final working concentration and vortexed to mix. Tegaserod was purchased from Tocris Bioscience (Bristol, UK).

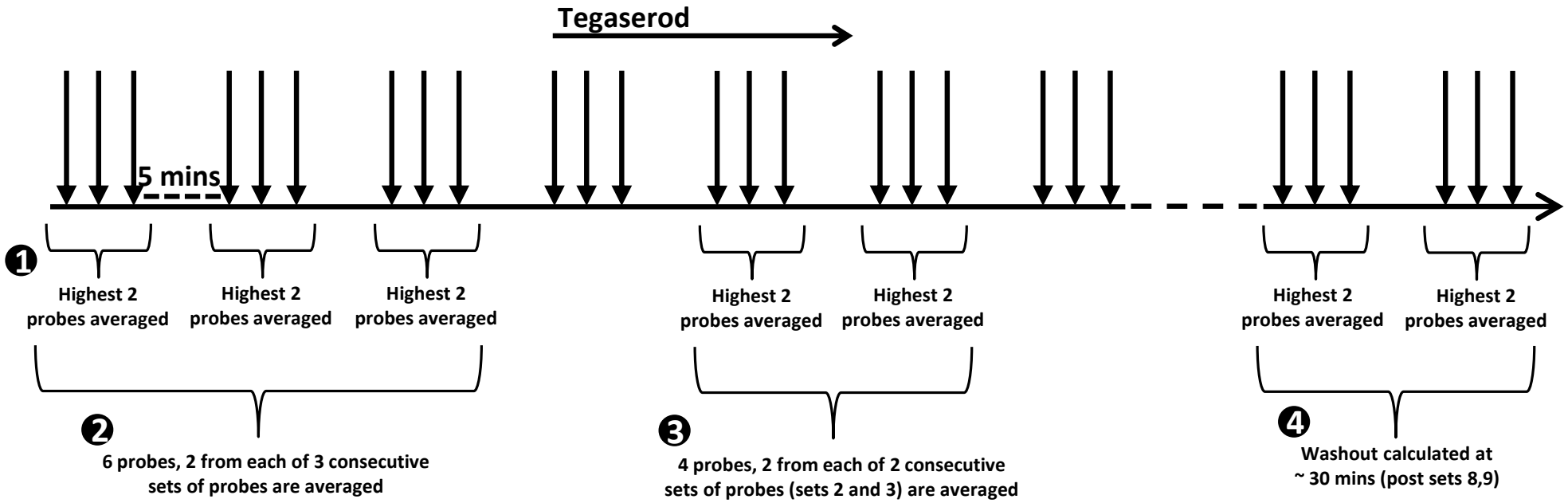

Tegaserod
5 mins
1
Highest 2 probes averaged
Highest 2 probes averaged
Highest 2 probes averaged
Highest 2 probes averaged
Highest 2 probes averaged
Highest 2 probes averaged
Highest 2 probes averaged
Highest 2 probes averaged
2
6 probes, 2 from each of 3 consecutive sets of probes are averaged
3
4 probes, 2 from each of 2 consecutive sets of probes (sets 2 and 3) are averaged
4
Washout calculated at ~ 30 mins (post sets 8,9)

Figure 4.01: Tegaserod VFH probing protocol

1) Average of the 2 highest 2 second probes in each set are averaged

2) The average of the 6 probes, 2 from each of the first 3 consecutive sets is used as baseline.

3) The average of the 4 probes from 2 consecutive sets after tegaserod application is then averaged. For drug effect comparisons the average of the baseline probes are compared to the average of the 2nd and 3rd post tegaserod sets of probing.

4) For drug vs. washout comparisons the 2nd and 3rd post tegaserod sets of probing are compared to the average of the 8th and 9th post tegaserod sets of probing.

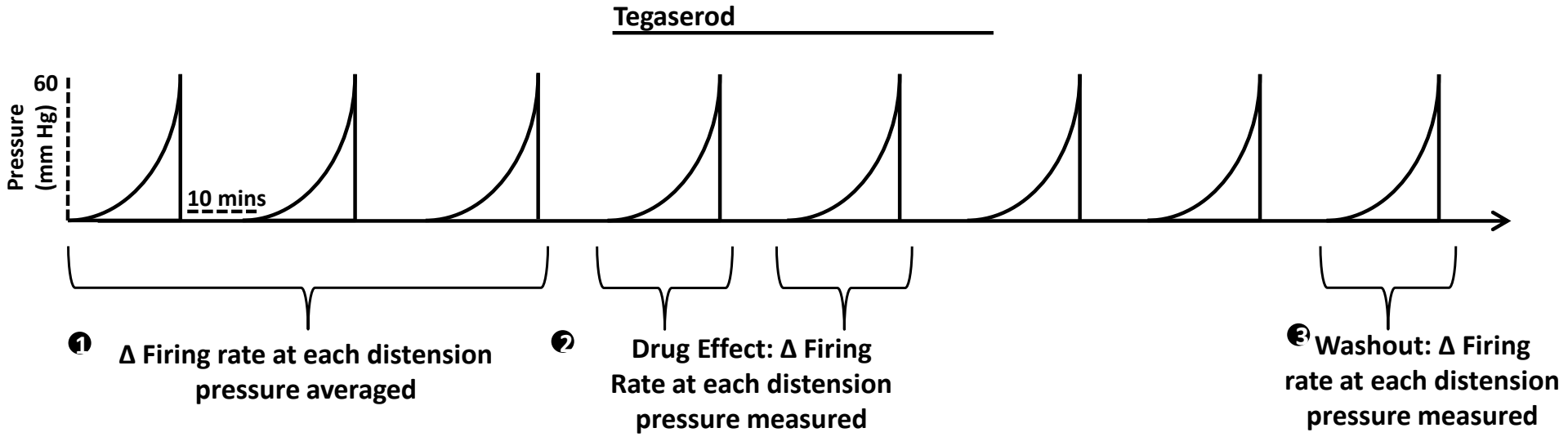


Figure 4.02: Tegaserod distension protocol

1) The Δ in firing rate at each 10mm Hg pressure point was averaged for the 3 baseline distensions.

2) For analysis the 2nd post tegaserod distension was compared to the average of the baseline distensions.

3) For tegaserod vs. washout comparisons the 2nd post drug distensions were compared to the 5th post drug distension.

4.1.3 RESULTS

4.1.3.1 TISSUE – TEGASEROD EXPERIMENTS

Six tissues, all normal, were used for tegaserod probing experiments, 4 sigmoid colon and 2 rectum (M:F 1:0.5, median age 57.5). Six appendices, 3 normal, 2 CD, 1 appendicitis, were used for tegaserod distension experiments (M:F 1:1, median age 38). Further details on the tissues use in each set of experiments can be seen in table 2.02.

4.1.3.2 TEGASEROD VFH PROTOCOL

Tegaserod, a selective partial $5HT_4$ agonist, has been shown to be anti-nociceptive against mechanical stimuli in both *in vitro* and *in vivo* pre-clinical experiments (Schikowski et al., 2002, Coffin et al., 2003, Yan et al., 2012) and in clinical trials (Muller-Lissner et al., 2001, Novick et al., 2002). In view of these findings, we aimed to investigate the efficacy of tegaserod in reducing mechanosensitivity in our HVAs, a potential target for their anti-nociceptive properties, and to use them as clinical standards for the validation of our model. A mechanosensation protocol using flat sheet VFH probes was used, as described previously. Tegaserod did not directly activate any serosal units (n=6). Three out of 6 units used for tegaserod VFH studies exhibited spontaneous activity (1.1±0.4 spikes s^{-1}). Tegaserod significantly reduced HVA firing in response to 1g VFH probing (24.1±4.0 vs. 19.3±3.6 spikes $2s^{-1}$, -20.8%, n=6, $p<0.05$) (figure 4.03). After a washout of at least 20 minutes, mechanosensitivity to VFH probing returned towards baseline in 3/6 preparations (baseline 19.1±6.7; vs. tegaserod 13.1±7.1; vs. washout 16.1±5.3 spikes $2s^{-1}$ probe).

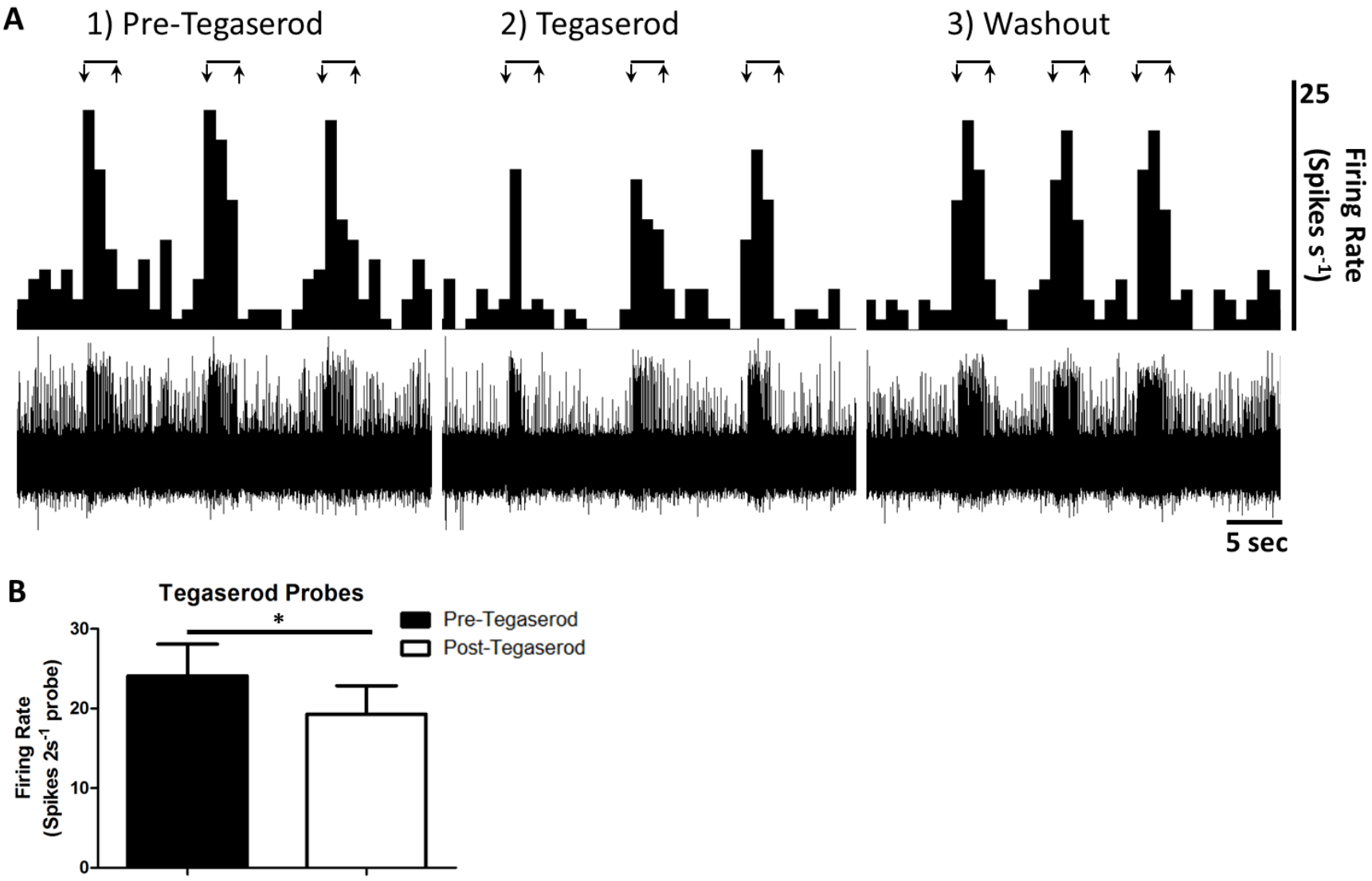

A
1) Pre-Tegaserod
2) Tegaserod
3) Washout
25
Firing Rate
(Spikes s-1)
5 sec
B
Tegaserod Probes
*
Pre-Tegaserod
Post-Tegaserod
Firing Rate
(Spikes 2s-1 probe)
30
20
10
0

Figure 4.03: Tegaserod significantly reduces the HVA response to 1g VFH probing (n=6). A) Shows a set of 3 probes before the application of tegaserod (1), while tegaserod was in the tissue bath (2), and after it had been washout (3). B) Displays the firing rate of serosal HVAs in response to VFH probing before and after the application of tegaserod. Data were analysed using a 2 tailed paired t test, $p<0.05$.

4.1.3.3 TEGASEROD DISTENSION PROTOCOL

Tegaserod (n=7) did not directly activate any HVA units. Bath superfusion of tegaserod did not alter the whole nerve HVA response to luminal distension, at any pressure point, or across the pressure ranges (n=4, $p>0.05$). Similarly, when the recordings were analysed as individual units, the pressure response line graphs produced by LT (n=2) and WDR (n=1) units were very similar before and after bath application of tegaserod (figure 4.04). Similarly, the pressure response line graphs from whole nerve recordings were comparable before and after the combined bath and luminal application of tegaserod (n=2). When bath and luminal perfusion recordings were analysed as individual units, tegaserod did not alter the response of WDR HVAs to distension at any pressure compared to pre tegaserod distensions (n=3, $p>0.05$). One LT unit was also identified and is shown in figure 4.04. The data was also pooled for analysis regardless of drug application method, however, tegaserod did not significantly alter the whole nerve (n=6), WDR (n=4) or LT (n=3) HVA response to luminal distension at any pressure (data not shown in figure form, $p>0.05$).

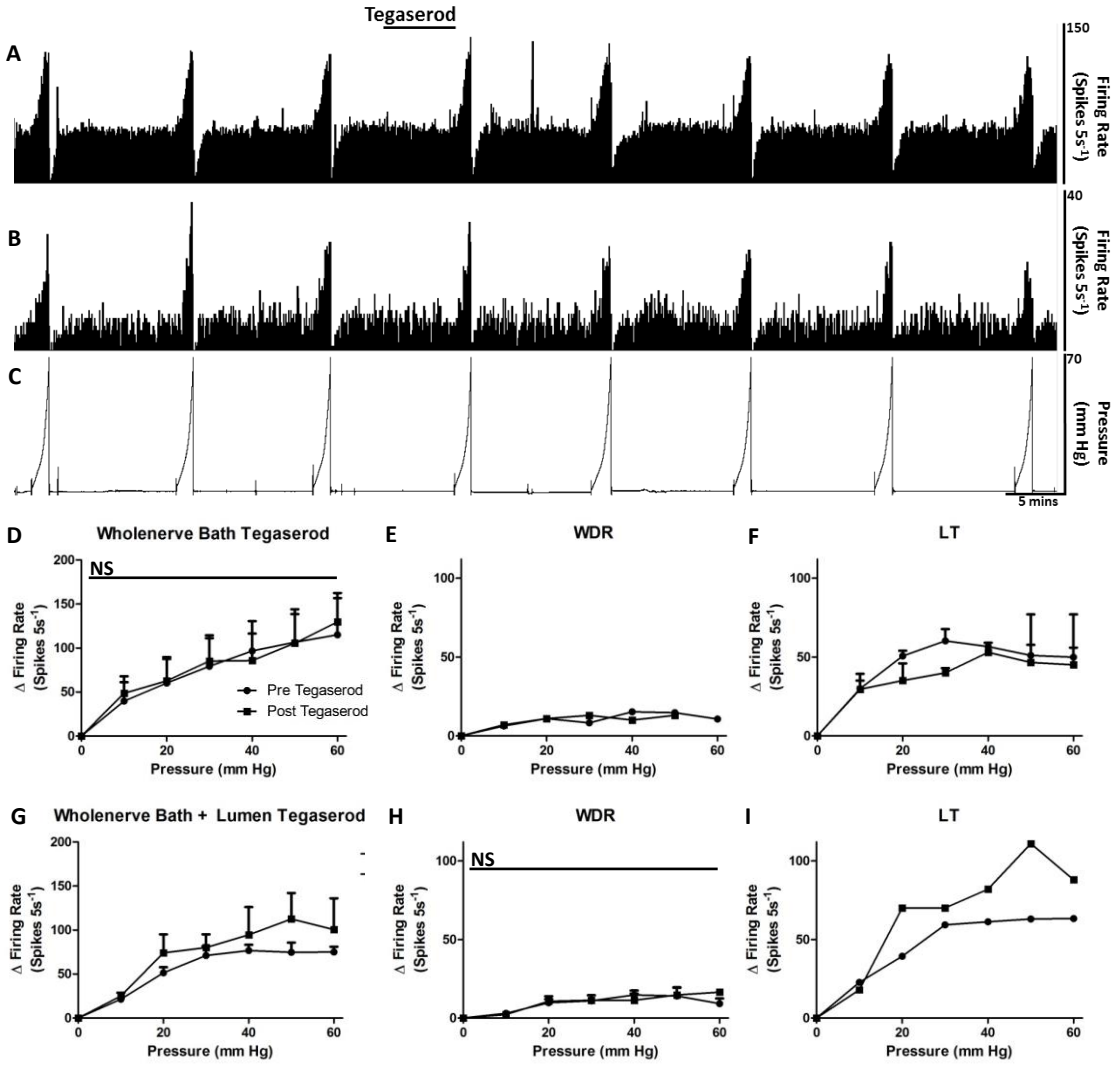


Figure 4.04: Tegaserod does not alter the HVA response to luminal distension. A-B) Shows an example of repeated whole nerve (A) and wide dynamic range (WDR) (B) HVA responses to luminal distension of the appendix in rate histogram form. Tegaserod application is marked by the solid black line. C) Shows the pressure curve for each distension. D) Bath application of Tegaserod did not alter the whole nerve HVA response to distension (n=4, $p>0.05$). E-F) Similarly, pressure response line graphs are similar before and after bath application of tegaserod in WDR (N=1) and LT (n=2) units. G, I) Pressure response line graphs were also similar in whole nerve recordings (n=2) and low threshold units (n=1) before and after the combined application of tegaserod into the bath and the lumen of the appendix. H) In addition, combined bath and luminal tegaserod application did not alter the response of WDR HVAs to distension at any pressure (n=3, $p>0.05$). Data were analysed using a 2 way ANOVA, $p<0.05$.

4.1.3.4 SUMMARY OF RESULTS

- Tegaserod significantly reduced HVA firing in response to VFH probing
- Tegaserod did not reduce the HVA firing rate in response to luminal distension of the appendix at low, medium, or high pressures

4.1.4 DISCUSSION

4.1.4.1 TEGASEROD

This report demonstrates that administration of tegaserod can reduce mechanosensitivity in HVAs. This is the first report to demonstrate the efficacy of tegaserod specifically in serosal afferents in any species and is consistent with the analgesic effect of tegaserod treatment reported in humans undergoing barostat balloon distension of the rectum (Coffin et al., 2003, Sabate et al., 2005).

Despite this observation in serosal units there was no effect of tegaserod on whole nerve or single unit HVA (LT and WDR) responses to distension of the human appendix at any pressure. One possible explanation for this observation is that we have only studied the activation of LT or WDR units in these appendix preparations. We were unable to discriminate any HT units which are likely to be comparable to the serosal units studied in the flat sheet intestinal preparations due to their activation by higher levels of stretch (Brierley et al., 2009, Hughes et al., 2009a). Serosal like HT units in appendix preparations may be difficult to activate given the thickness of the appendix and its tough fibrous outer layers. These features may potentially reduce the stretch on the tissue at higher distension pressures used in this report, hence reducing stretch on the serosa and as a consequence reduced activation of serosal afferents. Luminal distension protocols may therefore be biased towards the activation of LT and WDR afferents, which contrasts the flat sheet VFH protocol which targets serosal afferents, likely to be comparable to HT appendix afferents.

Consistent with the hypothesis that tegaserod's effects are restricted to serosal or HT afferents, tegaserod inhibited cat pelvic afferent responses to rectal distension at pressures consistent with that seen for HT units in our appendix preparations (30mm Hg) (Schikowski et al., 2002). Similarly, a range of behavioural animal studies using pain surrogates such as VMR, pain behaviours in response to colorectal distension are reduced after the administration of

tegaserod, especially at higher distension pressures consistent with the activation of HT units (Coelho et al., 2000, Greenwood-Van Meerveld et al., 2006, Hoffman et al., 2012).

The innervation of the type of colonic tissue used in VFH experiments and in the appendices used in distension preparations must be considered. Appendices are mainly innervated by splanchnic and vagal afferent pathways. In contrast, the vast majority of VFH tegaserod studies were conducted in the distal bowel, mainly sigmoid colon, which is predominantly supplied by splanchnic and pelvic pathways. This means that pelvic afferents may have constituted a portion of the recordings used for VFH probing protocols, but not in distension preparations, and vice versa for vagal afferents.

One further technical explanation for the lack of efficacy of tegaserod in the appendix preparations is that a difficulties penetrating into the deeper layers of the relatively thick human appendix (especially by comparison with rodent tissue) prevented the drug from reaching the endings of LT and WDR fibres. To help counter this we examined the effects of tegaserod after combined bath and luminal perfusion to allow diffusion from both surfaces of the appendix, however no effect of tegaserod was evident in these preparations. Furthermore excitatory responses were seen following bath application of capsaicin or BK in LT or WDR populations of appendix afferents suggesting tissue penetration is not an issue.

Tegaserod is not a selective drug possessing antagonist activity at 5-$HT2_B$ receptors and agonist activity at 5-HT_{1A} receptors. Further experiments are now warranted to determine the receptor subtype mediating the effects of tegaserod on human serosal afferent fibre mechanosensitivity

4.1.5 CONCLUSION

The 5-HT_4 partial agonist, tegaserod, significantly reduced the serosal HVA response to VFH probing. In contrast, tegaserod did not affect whole nerve or single unit (LT, WDR) HVA response to distension at any pressure. Potential explanations for the lack of effect in appendix preparations include 1) a lack of HT fibre activation following appendix distension, 2) a potential difference in the afferent pathways studies i.e. splanchnic and vagal in distension preparations vs. splanchnic and pelvic in VFH experiments 3) reduced diffusion of tegaserod into the muscle and mucosa layers of the appendix sufficient to inhibit activity in LT and WDR units.

CHAPTER 4 PART 2: THE EFFECT OF STa ENDOTOXIN ON THE MECHANOSENSITIVITY OF HUMAN VISCERAL AFFERENTS

4.2.1 INTRODUCTION

4.2.1.1 STa ENDOTOXIN AND GUANYLATE CYCLASE-C SIGNALLING

Guanylate cyclase-C (GC-C) is a transmembrane receptor, predominantly expressed on the apical surface of epithelial cells in the intestine (Hannig et al., 2014). It was first identified as the receptor to STa, a heat stable enterotoxin, which is produced by various bacteria including Escherichia coli (E. coli) (Lin et al., 2010). The endogenous ligands to GC-C were subsequently identified as guanylin and uroguanylin, members of the guanylin family of peptide hormones (Bryant et al., 2010, Busby et al., 2010, Hannig et al., 2014). Guanylin and uroguanylin are secreted as pro-peptides and are subsequently proteolytically cleaved into their active form, which act as agonists to the GC-C receptor (Martin et al., 1999, Moss et al., 2008). In this sense, STa is a super agonist to the GC-C receptor, having a similar structure, but 10-100 times the affinity compared to guanylin and uroguanylin (Potter, 2011). Upon activation, GC-C catalyses the breakdown of guanosine triphosphate (GTP) to cGMP in the cytosol of the cell (Lucas et al., 2000, Vaandrager, 2002). cGMP accumulates in these intestinal epithelial cells and exerts its effect through interaction with proteins linked to different signalling pathways, the most common of which are the c-GMP dependent protein kinases (Sager, 2004). For example, the accumulation of cGMP activates the cGMP-dependent protein kinase II (PKG-II), which controls the activity of the ion channel cystic fibrosis transmembrane conductance regulator (CFTR) through phosphorylation (Pfeifer et al., 1996, Vaandrager et al., 1998, Schlossmann et al., 2005). Upon activation CFTR secretes chloride and bicarbonate into

the lumen of the gut. Simultaneous direct inhibition of isoform 3 of the sodium hydrogen exchanger by cGMP leads to intracellular sodium and excess water secretion into gut (Fawcus et al., 1997, Vaandrager et al., 1997, Forte, 1999, Vaandrager et al., 2000, Vaandrager, 2002). Through this mechanism, guanylin and uroguanylin control fluid and electrolyte homeostasis (Pfeifer et al., 1996). In contrast, the release of STa by bacteria is pathologic, causing massive supra-physiological accumulation of cGMP, which subsequently releases excess quantities of water and electrolytes into the lumen of the gut causing diarrhoea (Brierley, 2012). However, the exploitation of the GC-C signalling pathway could potentially be useful in treating diseases such as IBS-C and constipation, given the potential pro-motility and pro-secretory characteristics of these agonists.

4.2.1.2 LINACLOTIDE

Linaclotide is a synthetic peptide 14 amino acids long and has a similar structure to guanylin and uroguanylin (Bryant et al., 2010). Linaclotide is a GC-C agonist, which upon binding activates downstream signalling cascades as described for the other guanylin peptides, controlling fluid and electrolyte secretion. It was postulated that linaclotide may be a potential treatment for the symptoms of IBS-C and chronic constipation, including bloating, constipation, discomfort and abdominal pain (Cada et al., 2013). Linaclotide has been shown to increase gastrointestinal transit rates in pre-clinical rodent models, in a cGMP dependent manner, since the effect was abolished in GC-C KO mice (Bryant et al., 2010, Busby et al., 2010). Correspondingly, clinical trials have demonstrated the effectiveness of linaclotide in increasing frequency of bowel movements, decreasing time to first bowel movement, changing stool consistency, and easing of passage in both healthy controls and IBS-C patients(Currie et al., 2005, Andresen et al., 2007, Johnston et al., 2010). Furthermore, in patients with chronic constipation, linaclotide reduced straining and the severity of constipation while increasing spontaneous complete bowel movements and stool consistency (Johnston et al., 2009, Lembo et al., 2010).

In some of these clinical trials linaclotide also demonstrated analgesic potential, reducing abdominal pain and discomfort in both patients with chronic constipation (Johnston et al., 2009, Lembo et al., 2010, Lembo et al., 2011) and those with IBS-C (Johnston et al., 2010). However, whether analgesia is a result of improved bowel function or due to a direct effect on afferent nerves is unclear and has recently become a topic of interest (Brierley, 2012). Indeed, linaclotide has been shown to reduce VMR responses to CRD in rats with visceral hyperalgesia induced by either restraint stress, or by TNBS induced colonic inflammation (Eutamene et al., 2010). In an additional model, rats with water stress induced visceral hyperalgesia exhibited reduced EMG responses to CRD after linaclotide treatment. However, linaclotide did not affect the number of abdominal contractions in normal, control rats (Eutamene et al., 2010). Two further studies demonstrate that linaclotide or uroguanylin can indirectly alter the mechanosensitivity of afferent fibres (Castro et al., 2013, Feng et al., 2013). Application of linaclotide or uroguanylin reduced splanchnic serosal afferent responses to VFH probing in healthy mice and to a greater extent in mice with visceral hypersensitivity (Castro et al., 2013). The same study reported reduced levels of pERK positive neurons in the dorsal horn of the spinal cord in regions projecting to the gut in response to CRD after intracolonic administration of linaclotide (Castro et al., 2013). Uroguanylin has also been shown to inhibit the response of both muscular and muscular-mucosal pelvic afferents to stretch, when applied to their receptive fields (Feng et al., 2013). However, this study reported no changes in pelvic serosal afferent or mucosal afferent sensitivity to VFH probing after uroguanylin application (Feng et al., 2013). This seemingly contradictory finding when compared with Castro et al (2013) may be explained by the use of pelvic and splanchnic nerves in these studies, respectively. These studies together suggest that guanylin peptides, such as linaclotide and uroguanylin may reduce sensitivity to mechanical stimuli in normal and especially in sensitised afferents, by a mechanism which directly involves the nerves, and which is unrelated to the improvement of bowel function.

4.2.1.3 cGMP AS A MECHANISM OF ACTION OF GC-C AGONISTS

Stimulation of the production of cGMP, its release from the basolateral side of intestinal epithelial cells and its subsequent interaction with extrinsic afferent nerves is the likely mechanism by which linaclotide and uroguanylin exert their effects (Castro et al., 2011, Castro et al., 2012, Feng et al., 2013). The application of cGMP to splanchnic serosal afferents reduces their response to VFH probing, similar to the effect exerted by the guanylin peptides (Castro et al., 2011). Similarly, cGMP applied to low threshold or high threshold muscular or muscular-mucosal afferents reduces their response to stretch (Feng et al., 2013). These studies demonstrate that cGMP can have the same effect as linaclotide or uroguanylin on the mechanosensitivity of extrinsic afferents. Evidence that the effects of linaclotide and uroguanylin are mediated by cGMP, come from the use of probenecid, an inhibitor to the cGMP transporter. Two studies have shown that probenecid blocks the inhibitory effect of linaclotide and uroguanylin on afferent mechanosensitivity (Castro et al., 2013, Feng et al., 2013). Furthermore, when the mucosa, the source of cGMP, was stripped from the mouse colon preparation prior to the experiment, the ability of linaclotide to reduce mechanosensitivity was greatly impaired (Castro et al., 2013). Taken together these results indicate cGMP as the mediator of the inhibitory effect of linaclotide and uroguanylin on afferent mechanosensitivity, by directly acting on the extrinsic nerve terminals. Investigating the effect of GC-C signalling and cGMP release on mechanosensitivity of human afferent nerves is of great interest, since cGMP producing drugs reduce visceral pain in the clinic. This report aims to examine the effect of GC-C signalling on the mechanosensitivity of extrinsic afferents innervating the human appendix, using the enterotoxin STa to release cGMP from the lumen.

4.2.1.4 AIMS

- Examine the effects of STa endotoxin on the transduction of mechanical stimuli
    - Luminal distension of the appendix

4.2.2 METHODS

4.2.2.1 APPENDIX DISTENSION PROTOCOL

The appendix distension protocol has been previously described in chapter 1 part 2. Briefly, after the initial 3 baseline distensions, the bath was superfused with STa endotoxin (100nM, 120ml superfusion, 20ml luminal perfusion. In all the experiments, distensions were continued every 10 minutes after the 3 baseline distensions (figure 4.05). Data were analysed using a 2 way ANOVA, $p<0.05$.

4.2.2.2 DRUGS

Drugs in powder form were made up using the recommended solutions, aliquoted and frozen at -20°C. When needed, aliquots were diluted in Krebs to make the final working concentration and vortexed to mix. STa endotoxin was a gift from Ironwood Pharmaceuticals.

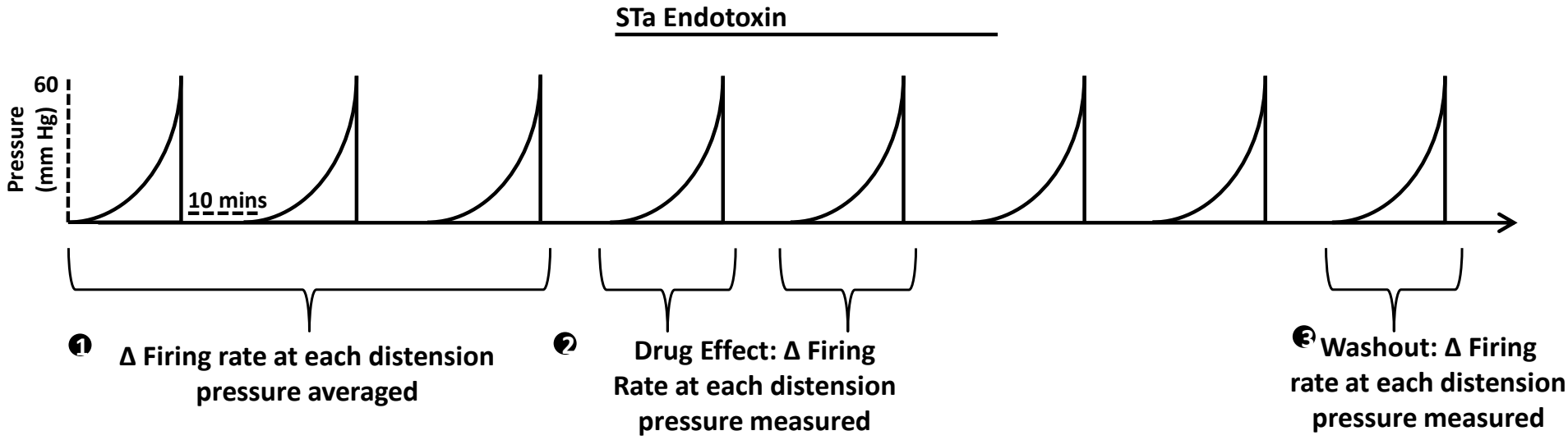


Figure 4.05: STa Distension Protocol

1) The Δ in firing rate at each 10mm Hg pressure point was averaged for the 3 baseline distensions.
2) For analysis the 2nd post STa distension was compared to the average of the baseline distensions in experiments
3) For STa vs. washout comparisons, the 2nd (120ml) post drug distensions were compared to the 5th post drug distension.

4.2.3 RESULTS

4.2.3.1 TISSUE – STa ENDOTOXIN EXPERIMENTS

Seven appendices, 5 normal, 1CD, 1 appendicitis, were used for STa luminal distension experiments (M:F 1:1.33, median age 50). Further details on the tissues use in each set of experiments can be seen in table 2.02.

4.2.3.2 STa ENDOTOXIN DISTENSION PROTOCOL

STa endotoxin can release cGMP from the mucosa of the gut, which may subsequently reduce the sensitivity of extrinsic afferent fibres to mechanical stimuli (Castro et al., 2011, Castro et al., 2012, Feng et al., 2013). Indeed, linaclotide, a drug that works in a similar way has shown efficacy in reducing abdominal pain in clinical trials (Johnston et al., 2009, Johnston et al., 2010, Lembo et al., 2010, Lembo et al., 2011). In this report, the effect of STa endotoxin on the HVA response to distension of the appendix was tested. The combined application of STa to the bath and lumen of the appendix produced a significant reduction in whole nerve activity to distension across the pressure range ($n=7$, $p<0.05$), although no significant difference was seen for individual pressure. When the recordings was analysed as single units, luminal and bath application of STa failed to change the response in LT ($n=4$) or HT ($n=4$) HVAs to distension at any pressure ($p>0.05$). No WDR range units could be discriminated in this study (figure 4.06).

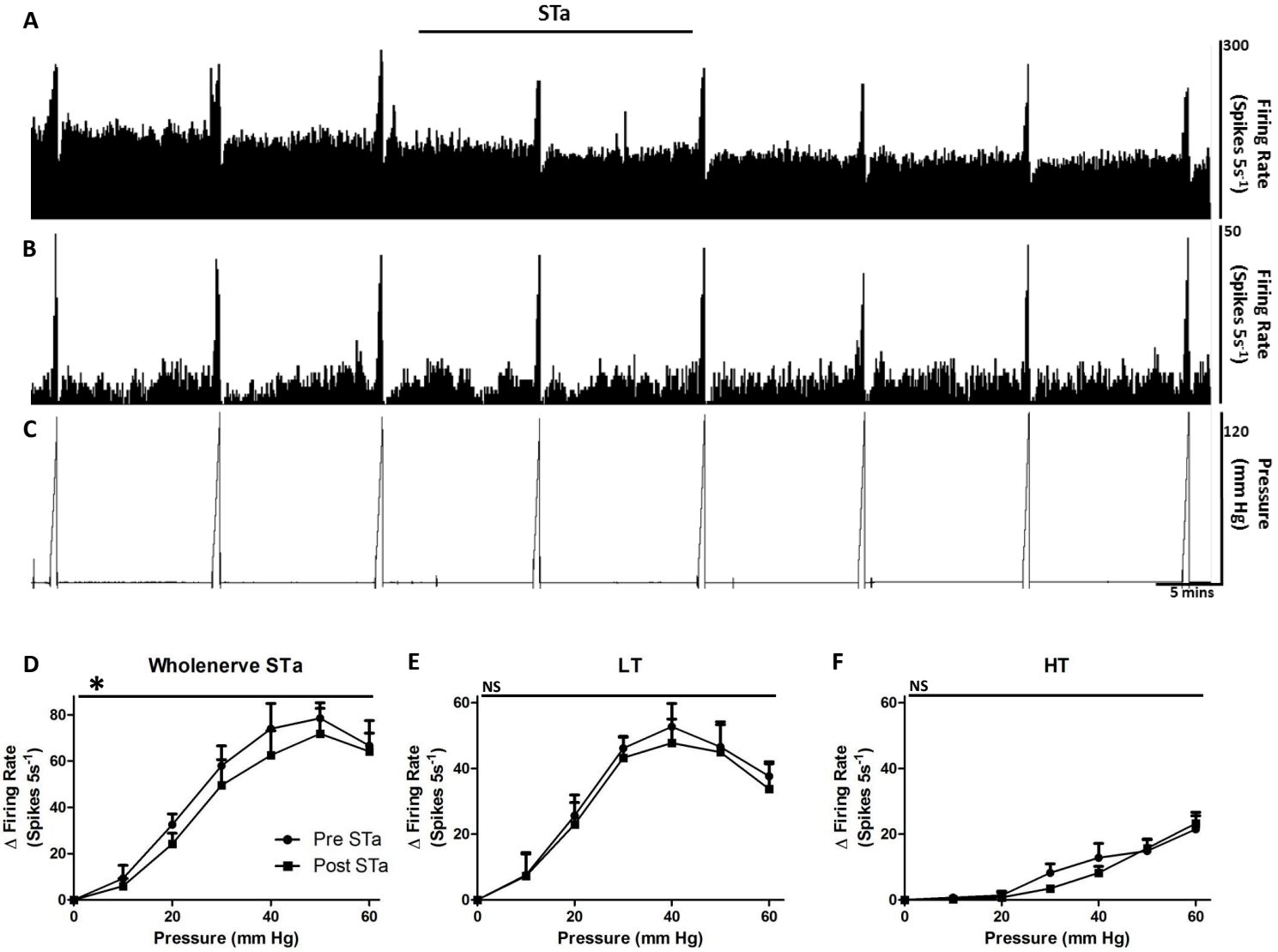

A
STa
300
Firing Rate
(Spikes 5s-1)
B
50
Firing Rate
(Spikes 5s-1)
C
120
Pressure
(mm Hg)
5 mins
D
Wholenerve STa
*
Δ Firing Rate
(Spikes 5s-1)
Pre STa
Post STa
Pressure (mm Hg)
E
LT
NS
Δ Firing Rate
(Spikes 5s-1)
Pressure (mm Hg)
F
HT
NS
Δ Firing Rate
(Spikes 5s-1)
Pressure (mm Hg)

Figure 4.06: STa inhibits the whole nerve HVA response to luminal distension. A-B) Shows an example of repeated whole nerve (A) and high threshold (HT) (B) HVA responses to luminal distension of the appendix in rate histogram form. Combined STa application into the bath and through the lumen is marked by the solid black line. C) Shows the pressure curve for each distension. D) Simultaneous application of STa into the bath and through the lumen of the appendix significantly reduced the whole nerve afferent response to distension across the pressure range ($n=7$, $p<0.05$). E-F) When single units were analysed separately, low threshold (LT) ($n=4$) (E), or HT ($n=4$) (F) HVA response to distension was not altered by STa ($p>0.05$). Data were analysed using a 2 way ANOVA, $p<0.05$.

4.2.3.3 SUMMARY OF RESULTS

- Bath and luminal perfusion of STa inhibited the whole nerve HVA response to distension of the appendix across the pressure range although no effect was seen in discriminated populations of LT or HT units

4.2.4 DISCUSSION

4.2.4.1 STa ENDOTOXIN

Combined bath and luminal application of the GC-C receptor superagonist STa significantly reduced the whole nerve afferent response to distension across the pressure range. When characterised by activation threshold, neither discriminated populations of LT nor HT units were inhibited by STa.

Consistent with whole nerve data presented in this report previous studies have also demonstrated the efficacy of another GC-C receptor agonist, linaclotide, in reducing the VMR to CRD across the range of pressures tested (stating at the lowest measured pressure, 15 mm Hg, and continuing up to the highest pressure 60 mm Hg), in mice with hyperalgesia (Eutamene et al., 2010). Similarly, the response of pelvic afferents to colonic distension is inhibited by the application of cGMP, the molecule released after STa and linaclotide binding to GC-C (Feng et al., 2013, Silos-Santiago et al., 2013).

In the present report, HT units were not inhibited by STa. Similarly, there was no effect on the response of pelvic serosal afferents to probing after cGMP application, which are likely to be comparable to HT distension sensitive units (Feng et al., 2013). However, a recent study demonstrated that HT nociceptive splanchnic serosal afferent activity in response to VFH probing was inhibited by linaclotide (Castro et al., 2013). One possible explanation for this difference may be the concentration of agent used. In the present study, 100nm of STa was used, while in the linaclotide study, although efficacy was evident at 100nM of linaclotide, greater efficacy was seen at higher doses, either 300nm or 1000nm. Although not the same compound, it may be warranted to try a higher dose of STa in HVA distension preparations.

Another explanation for the discrepancy may be related to the relative exposure of the mucosa to the drug. For example in a previous study in mouse colon, efficacy of linaclotide

in reducing mechanosensitivity to VFH probing was seen after 5 minutes of mucosal exposure (Castro et al., 2013). Considering the human appendix is much thicker than the mouse colon it may require a longer exposure time. Indeed, this report used a 20 minute luminal exposure, which is likely to be enough time to compensate for the thicker nature of human tissue.

In addition, studies have also reported a greater efficacy of linaclotide during inflammation (Eutamene et al., 2010, Castro et al., 2013). The current study used mainly normal, uninflamed appendix, although 1 appendix was removed due to appendicitis, and another as part of a CD resection. However, it is not known if these latter 2 appendices were inflamed. In the future, appendices with proven inflammation should be tested to determine the effects of STa in inflamed states. This could be achieved by collection of appendix tissue supernatants prior to HVA recordings, and their subsequent content analysis for markers of inflammation, such as IL-8.

A final difference is that the current study did not examine the effect of STa on the responsiveness of HVAs to VFH probing, and so it would be important to examine the effect of STa on serosal afferents using a VFH protocol, since evidence in this report suggests that they are involved in visceral nociception in the human gut. In the flat sheet preparations, were the mucosa is pinned downwards in the bath, it would be necessary to make sure the mucosa was exposed to the STa agonist. Finally, to confirm the mechanism of action, the effect of cGMP on afferent mechanosensitivity should also be investigated, and in addition, the effect of probenecid on the efficacy of STa.

4.2.5 CONCLUSION

Bath and luminal perfusion of the GC-C agonist STa inhibited the whole nerve HVA response to distension of the appendix across the pressure range. This effect was not observed in discriminated populations of LT or HT units. Further studies are needed using higher doses of STa on specific HT units innervating the appendix, which likely represent the nociceptor population, to confirm a role for STa in modulating nociceptor activity. The effects of STa on distension in appendices with proven inflammation should also be investigated. Additional work confirming cGMP production as the mechanism of action of GC-C agonists on HVA mechanosensitivity is also warranted.

CHAPTER 4 PART 3: THE EFFECT OF ICI 204, 448 ON THE MECHANOSENSITIVITY OF HUMAN VISCERAL AFFERENTS

4.3.1 INTRODUCTION

4.3.1.1 OPIOID RECEPTORS

To date, 4 opioid receptors, mu (μ), kappa (κ), delta (δ), and nociceptin receptor, have been identified (Waldhoer et al., 2004). Opioid receptors are GPCRs, with 7 transmembrane domains and are usually linked intracellularly with $G_{i/o}$ proteins, which inhibit calcium channels, activate potassium channels and inhibit the production of cAMP, which combined serve to reduce neuronal excitability (Jordan and Devi, 1998, Waldhoer et al., 2004). Opioid receptors are expressed on a number of neuronal types including extrinsic afferents innervating the intestines (Danzebrink et al., 1995, Sengupta et al., 1996, Su et al., 1997). Opioid receptors can be activated by both endogenous and exogenous opiates, the latter producing remarkable analgesia (Hughes and Kosterlitz, 1983). Indeed, opiates, especially μ-opioid receptor agonists such as morphine are used for reducing severe pain, and in palliative care (Gebhart et al., 1999). The effects of opioid receptor agonists can be mediated centrally or peripherally. However, the central actions of many of these drugs cause unwanted side effects including respiratory depression, tolerance and dependence, which limits their use to certain types of pain (Mangel and Hicks, 2012). Indeed, morphine and other μ-opioid receptor agonists have been shown to be effective visceral analgesics, reducing pain indicators in various animal models of visceral pain, but are not usually used for the treatment of abdominal pain in disease such as IBS (Ness and Gebhart, 1988a, Danzebrink et al., 1995,

Harada et al., 1995, Borgbjerg et al., 1996). Peripherally restricted opioid receptor agonists are therefore sought after.

4.3.1.1.1 Kappa opioid receptors in visceral pain

Indeed κ opioid receptor agonists have shown some promise in this sense. Early κ agonists did not show side effects such as addiction and constipation, but were centrally active and caused dysphoria and sedation (Riviere, 2000). This led to the creation of further generations of κ opioid receptor agonists which had fewer side effects (Riviere, 2004). A number of *in vivo* experiments reported that systemic but not intrathecal administration of κ-opioid receptor agonists reduced VMR to CRD in conscious rats, suggesting that their site of analgesic action is in the periphery (Langlois et al., 1994, Danzebrink et al., 1995, Harada et al., 1995, Burton and Gebhart, 1998). In contrast, both μ and δ opioid receptor agonists were effective in reducing nociceptive behaviours after intrathecal injection. Furthermore, *in vitro* pelvic afferent electrophysiological studies, containing only the peripheral aspects of sensory pathways, revealed that, μ and δ opioid receptor agonists have no effect on the afferent response to CRD (Sengupta et al., 1996, Su et al., 1997). In contrast, a number of κ opioid receptor agonists, including EMD 61, 753 (asimadoline), were effective in reducing the afferent response to noxious CRD, demonstrating the efficacy of κ opioid receptor agonists in reducing surrogates of visceral nociception mediated through peripherally based sites of action (Sengupta et al., 1996, Su et al., 1997). Furthermore, in humans the κ opioid receptor agonist fedotozine relieved the hypersensitivity to colonic distension in patients with IBS, without any central effects (Delvaux et al., 1999). This report will focus on the peripherally restricted κ opioid receptor agonist ICI 204, 448.

A number of selective κ opioid receptor agonists exist, including ICI 204, 448 and asimadoline, which have low permeability across the blood brain barrier, and thus are peripherally restricted. ICI204, 448 and asimadoline demonstrates analgesic potential in

preclinical animal models of visceral pain (Sengupta et al., 1996, Su et al., 1997, Burton and Gebhart, 1998, Sengupta et al., 1999, Joshi et al., 2000). Intravenous injection of ICI 204, 448 asimadoline, reduced the VMR to CRD in normal conscious rats, rats with acute colonic inflammation induced by acetic acid injection, and rats with chronic inflammation induced by TNBS (Burton and Gebhart, 1998, Sengupta et al., 1999). Furthermore, asimadoline but not ICI 204, 448 exhibited a greater potency in reducing the VMR response in the chronically inflamed TNBS treated rats (Sengupta et al., 1999).

In addition, a number of studies using *in vivo* electrophysiological recordings made from pelvic nerves innervating the colon, devoid of any central sensory input, showed a reduced pelvic afferent nerve response to CRD in normal rats after pre-treatment with asimadoline (Sengupta et al., 1996, Su et al., 1997, Sengupta et al., 1999). In addition, the inhibitory effect was greater in the presence of acute (acetic acid) or chronic (TNBS) inflammation. ICI 204, 448 had no effect in normal rats, but reduced the afferent response to CRD in acutely and chronically inflamed rats (Sengupta et al., 1999). Furthermore, the inhibitory effect of asimadoline on either the VMR or the afferent response to CRD in TNBS treated rats was inhibited by pre-treatment with naloxone, an opioid receptor antagonist (Sengupta et al., 1999). These results suggest the upregulation of κ opioid receptors in the periphery during inflammation.

One study conducted *in vivo* electrophysiological experiments on pelvic nerve of rats that underwent intrathecal administration of antisense oligodeoxynucleotides, to knock down the expression of κ opioid receptors (Joshi et al., 2000). This experiment demonstrated the effectiveness of asimadoline in reducing the afferent response to CRD even in rats in which the central κ opioid receptor was not present. This suggested that there is a distinct peripherally expressed κ opioid receptor, localised to the colon, through which asimadoline can exert its effects (Joshi et al., 2000, Camilleri, 2008).

Of note, kappa receptor antagonists, at high doses, can inhibit sodium channels (Su et al., 2002). This is unlikely to contribute to the efficacy of asimadoline in reducing surrogates of visceral pain, since it is 500-1000 times less potent at sodium channels compared to the κ opioid receptor (Joshi et al., 2003). Additionally, ICI 204, 448 has no activity on sodium channels, and therefore is a suitable compound to use in experiments (Su et al., 2009).

Taken altogether, these studies indicate that asimadoline and ICI 204, 448 are effective in reducing surrogate visceral pain responses to noxious mechanical stimuli, specifically colorectal distension, especially under inflammatory or post inflammatory conditions. Furthermore, the inhibitory effects of asimadoline and ICI 204, 448 are mediated by opioid receptors expressed on peripheral afferent nerves innervating the colon (Camilleri, 2008).

4.3.1.2 CLINICAL EFFICACY OF A PERIPHERALLY RESTRICTED K RECEPTOR AGONIST

Preclinical studies on human subjects also demonstrate the efficacy of κ agonists, especially asimadoline in reducing visceral pain. The effectiveness of 1 dose of asimadoline on pain intensity ratings in response to stepwise colonic distension was examined in IBS patients with proven visceral hyperalgesia (Delvaux et al., 2004). Asimadoline reduced the area under the curve ratings of pain intensity at each distension step, and increased the pain threshold, although this did not quite reach significance (Delvaux et al., 2004). Another study was performed on healthy subjects, receiving asimadoline twice daily for 9 days (Delgado-Aros et al., 2003). Subjects on the lowest dose of asimadoline showed decreased pain across all distension pressures although this was only significant at the lowest pressure (Delgado-Aros et al., 2003). In contrast, higher doses of asimadoline increased pain scores in healthy subjects at low and moderate pressures, the reason for which remains unclear (Delgado-Aros et al., 2003). The results of these studies are in line with the preclinical animal studies, suggesting an increased efficacy of asimadoline after sensitisation.

Based on the efficacy of asimadoline in preclinical animal and human experiments, asimadoline was tested in clinical trials (Szarka et al., 2007, Mangel et al., 2008, Mangel and Williams, 2010). A small, short term study examined the effect of 1mg of asimadoline taken as needed up to 4 times a day for 4 weeks in 100 IBS-D, IBS-C or IBS-M female patients (Szarka et al., 2007). Most patients had moderate abdominal pain based on a visual analogue scale on at least 4 out of 14 days at baseline. This study failed to show any improvement on the primary endpoint, the average reduction in severity of pain 2 hours after asimadoline, or on any of the secondary endpoints, including adequate relief from pain and discomfort (Szarka et al., 2007). IBS-M patients seemed to respond better to treatment. The authors note that the study design and sample size, are best suited to hypothesis generation rather than hypothesis testing (Szarka et al., 2007). In a larger, longer double blind, placebo controlled trial, 595 patients with IBS-C, IBS-D or IBS-M, were randomised to placebo or asimadoline at 1 of 3 doses, b.i.d 0.15mg, 0.5mg and 1mg, for 12 weeks (Mangel et al., 2008). The primary endpoint was the number of months with adequate relief from IBS pain and discomfort, while a number of other pain related endpoints were also evaluated (Mangel et al., 2008). In contrast to Szarka et al (2007), when IBS-D patients with at least moderate levels of abdominal pain were analysed as a subgroup, asimadoline treatment, 0.5mg, caused a significant improvement in the primary endpoint, and in pain scores, pain free days, adequate relief from the symptoms of IBS, urgency, and stool frequency (Mangel et al., 2008). These results highlight the potential of asimadoline for treating abdominal pain in IBS-D, and warrants further clinical trials. Indeed, based on the results of the latter clinical trial, a further phase 3, 12 week, double blind, randomised, placebo controlled clinical trial in IBS-D patients has been completed with using b.i.d 0.5mg asimadoline. However, the results have yet to be published.

4.3.1.3 AIMS

- Examine the effects of ICI 204, 448 on the transduction of mechanical stimuli
    - VFH Probing

4.3.2 METHODS

4.3.2.1 VFH PROBING PROTOCOL

The VFH probing protocol has been previously described in chapter 1 part 2. Briefly, after the initial baseline 1g VFH probes, 3 sets of 3 x 3 second probes, each set separated by 5 minutes, were attained, the bath was superfused with ICI 204, 448 (300nM, 100ml). In all experiments this was followed with 6-9 sets of 3 x3 second probes, each set separated by 5 minutes (figure 4.07). Data were analysed using a 2 tailed paired t test, $p<0.05$.

4.3.2.2 DRUGS

Drugs in powder form were made up using the recommended solutions, aliquoted and frozen at -20°C. When needed, aliquots were diluted in Krebs to make the final working concentration and vortexed to mix. ICI 204, 448 was purchased from Tocris Bioscience (Bristol, UK).

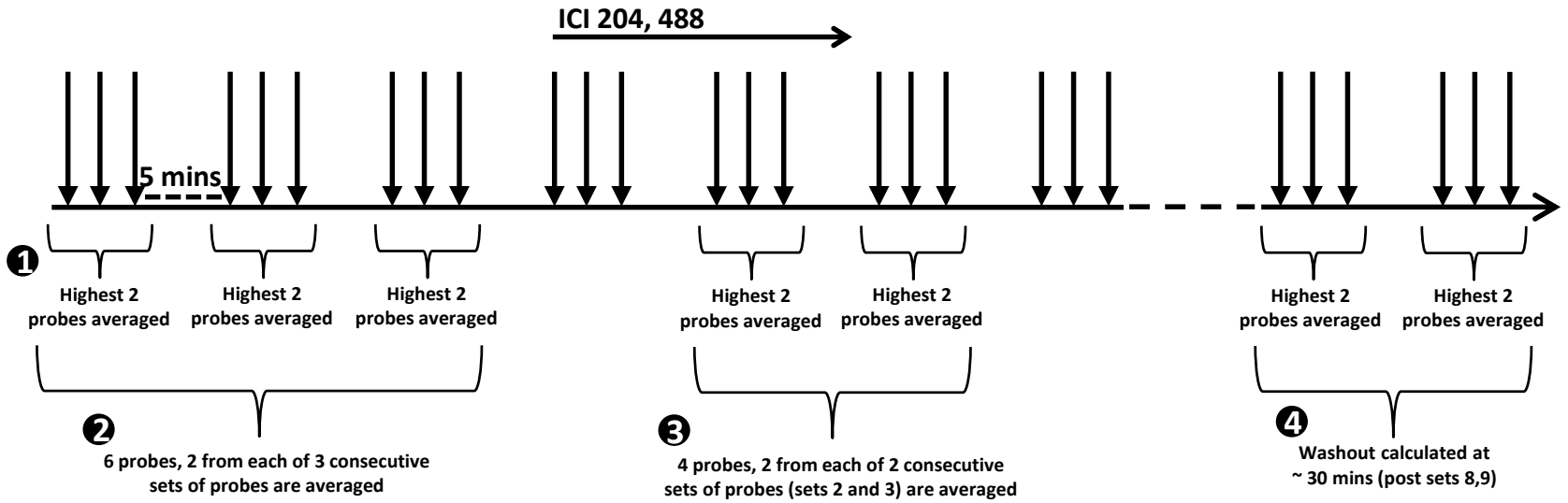


Figure 4.07: ICI 204, 448 probing protocol

1) Average of the 2 highest 2 second probes in each set are averaged

2) The average of the 6 probes, 2 from each of the first 3 consecutive sets is used as baseline.

3) The average of the 4 probes from 2 consecutive sets after drug application is then averaged. For drug effect comparisons the average of the baseline probes are compared to the average of the 2^{nd} and 3^{rd} post ICI 204, 448 sets of probing.

4) For ICI 204, 448 vs. washout comparisons the 2^{nd} and 3^{rd} post drug sets of probing are compared to the average of the 8^{th} and 9^{th} post drug sets of probing.

4.3.3 RESULTS

4.3.3.1 TISSUE - ICI 204, 448 VFH PROBING EXPERIMENTS

Three tissues, all normal, were used for ICI 204, 448 probing protocols, 2 sigmoid colon, 1 rectum (M:F 1:2, median age 51). Further details on the tissues use in each set of experiments can be seen in table 2.02.

4.3.3.2 ICI 204, 448 VFH PROTOCOL

ICI 204,448 is a peripherally acting selective κ opioid receptor agonist, which demonstrates analgesic potential in preclinical animal models (Sengupta et al., 1996, Su et al., 1997, Burton and Gebhart, 1998, Sengupta et al., 1999, Joshi et al., 2000). Two out of 3 units used for ICI 204, 448 VFH studies exhibited spontaneous activity (0.8±0.5 spikes s^{-1} probe). The application of ICI 204, 448 reduced the response of HVAs to 1g VFH probing, but this did not reach significance (17.9±4.5 vs. 12.9±1.5 spikes $2s^{-1}$, 21.8%, n=3, $p>0.05$) (figure 4.08).

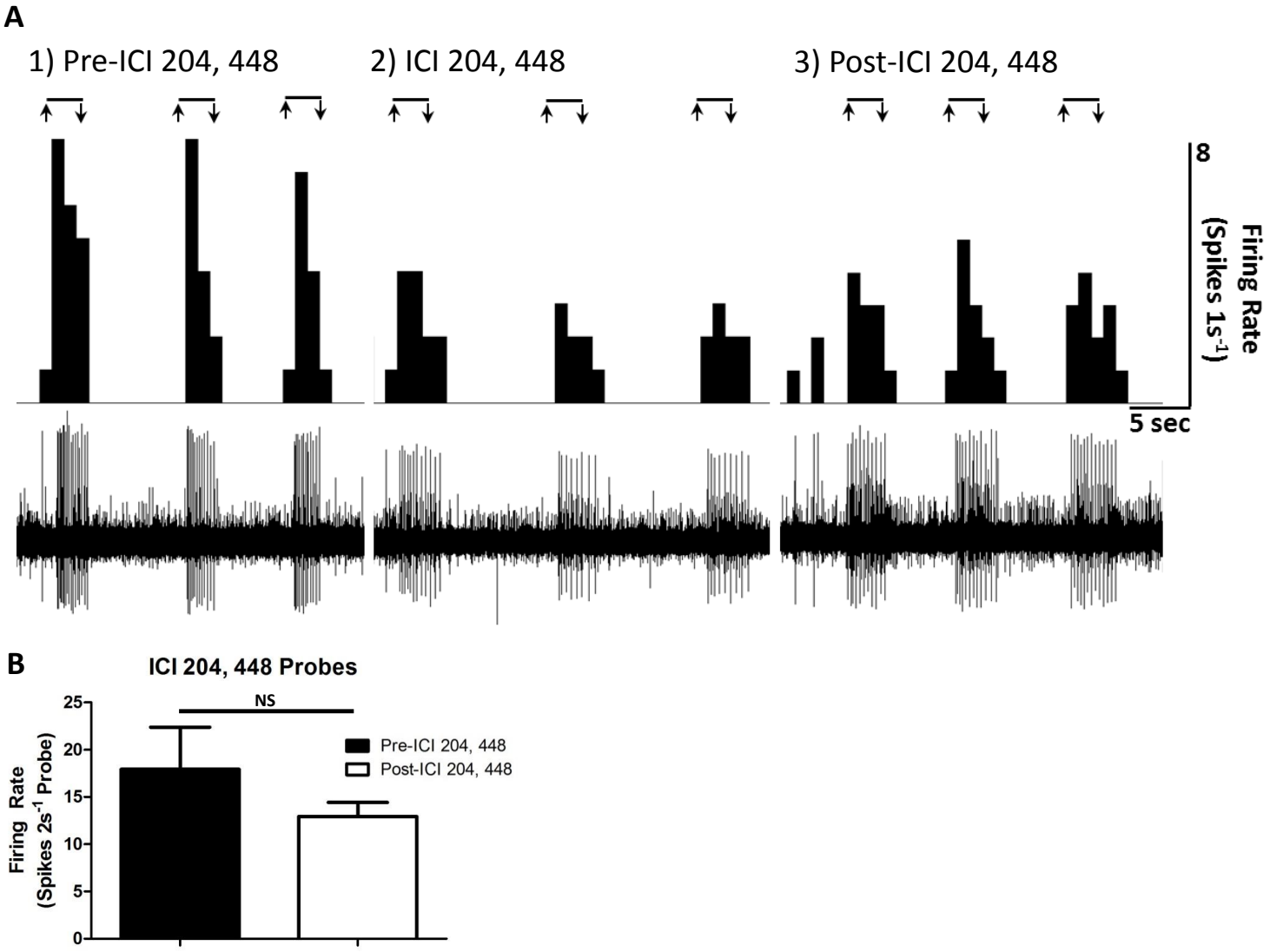

A
1) Pre-ICI 204, 448
2) ICI 204, 448
3) Post-ICI 204, 448
8
Firing Rate
(Spikes 1s-1)
5 sec
B
ICI 204, 448 Probes
NS
Pre-ICI 204, 448
Post-ICI 204, 448
Firing Rate
(Spikes 2s-1 Probe)
25
20
15
10
5
0

Figure 4.08: The effect of ICI 204, 448 on the mechanosensitivity of HVAs. A-B) ICI 204, 448 did not reduce the response of HVAs to 1g VFH probing of the serosa (n=3, $p>0.05$). However there was a trend for reduced mechanosensitivity. A) Shows a set of 3 probes before the application of ICI 204, 448 (1), while ICI 204, 448 was in the tissue bath (2), and after it had been washout (3). B) Displays the firing rate of serosal HVAs in response to VFH probing before and after the application of ICI 204, 448. Data were analysed using a 2 tailed paired t test, $p<0.05$.

4.3.3.3 SUMMARY OF RESULTS

- ICI 204, 448 did not alter serosal HVA firing in response to VFH probing

4.3.4 DISCUSSION

4.3.4.1 ICI 204, 448

The HVA response to VFH probing was not reduced by ICI 204, 448. However there was a trend for reduced mechanosensitivity that requires further investigation. Previous studies in animals using surrogates for pain such as VMR to CRD have demonstrated the efficacy of ICI 240, 488 in reducing response to mechanical stimuli (Burton and Gebhart, 1998). All recordings used for ICI 204, 448 probing protocols were made from normal tissue collected from the uninvolved part of non-inflammatory conditions. Previous studies using *in vivo* recordings from pelvic afferents innervating the rat colon reported no effect of IC 204, 488 on reducing the afferent response to colonic distension in uninflamed tissues. However, in colonic afferents from rats in which inflammation had been induced either acutely (acetic acid) or chronically (TNBS), ICI 204, 448 significantly reduced firing in response to colonic distension (Sengupta et al., 1999). This suggests ICI 204, 448 and other κ agonists may be more efficacious in inflammatory conditions. This would be consistent with suggestions that k opioid receptors are upregulated in the periphery during inflammation (Sengupta et al., 1999).

In the current report, only a small preliminary sample size of 3 units was used for ICI 204, 448 probing protocols, due to the limitation of tissue availability. However, the results of this report suggest that further studies using ICI 204, 448 are warranted, both in normal uninflamed tissues, and also in tissues from patients with both acute and chronic colonic inflammation. Furthermore, ICI 204, 448 should be tested using a HVA appendix distension preparation.

4.3.5 CONCLUSION

The kappa peripherally restricted selective κ opioid agonist ICI 204, 448 did not significantly alter the response of serosal HVAs to VFH probing, although a trend was evident. Further studies using ICI 204, 448 are warranted in HVAs from both normal and inflamed human intestinal tissue.

CHAPTER 5 PART 1: POST HOC ANALYSIS: THE EFFECTS OF INFLAMMATORY DISEASE ON THE MECHANOSENSITIVITY AND CHEMOSENSITIVITY OF HUMAN VISCERAL AFFERENTS

Data collected as part of the studies described in chapters 2-4 was pooled and analysed post hoc to determine trends in the response profile to mechanical and chemical stimuli based on 1) the presence of inflammatory disease in the tissue (inflammatory bowel disease and appendicitis) (Part 1) or 2) the overnight cold storage of tissue (Part 2). It should be stressed that these studies were performed post-hoc and therefore study protocols have not been optimised to produce definitive experiments. Instead these data sets provide pilot data to inform and guide future detailed studies in these areas, and will be discussed in that context.

5.1.1 INTRODUCTION

Colonic inflammation is known to cause abdominal pain and hypersensitivity. However, the exact role of extrinsic afferents in this hypersensitivity is not fully understood (Feng et al., 2012b). There is evidence that colonic inflammation can affect the sensitivity of extrinsic colonic nerves to mechanical and chemical stimuli.

5.1.1.1 MECHANOSENSITIVITY

IBD is characterised by chronic recurring inflammation that causes among other things abdominal pain (Srinath et al., 2012). This pain may be caused by hypersensitivity of extrinsic afferents to mechanical and chemical stimuli. A number of studies have demonstrated that during active UC, patients sense rectal distension earlier and exhibit lower maximal distension

pressures compared to controls or UC patients in remission (Rao et al., 1987, Rao and Read, 1990, Drewes et al., 2006). In contrast, another study reported that patients with UC exhibiting mild on-going inflammation demonstrated less sensitivity to colorectal distension presenting in higher distension pressure required to elicit pain (Chang et al., 2000). Furthermore, UC patients in remission exhibit decrease rectal sensitivity to distension (Rao and Read, 1990). Patients with CD where inflammation is only in the ileum, exhibit a lower sensitivity to rectal distension (Jehle et al., 1993, Bernstein et al., 1996). Together, this evidence from colorectal balloon distension paradigms in patients with IBD and controls suggest that factors such as the type of IBD, CD vs. UC, and the duration, degree and current state of inflammation may determine the effect of inflammation on colonic nerve sensitivity (De Schepper et al., 2008).

The mechanosensitivity of all subtypes of afferents in both spinal pathways during the acute and recovery phases of inflammation, induced by a number of methods, has been studied in rodent models. There was no change in proportion of afferents activated by mechanical stimuli in either the splanchnic or pelvic nerve after the induction of inflammation by TNBS (Hughes et al., 2009a, Feng et al., 2012b). However another study from one of the same groups reported a significant increase in pelvic serosal afferents after zymosan treatment in mice and attributed this to a recruitment of previously "silent" MIAs or "silent" afferents, as the increase in pelvic serosal afferents occurred with a decrease in the number of MIAs (Feng et al., 2012a). The mechanosensitivity of splanchnic afferents during acute inflammation (7 days) and after recovery from inflammation (28 days) induced by TNBS has been studied in mice (Hughes et al., 2009a). The authors reported reduced thresholds for activation and an increased firing rate in response to VFH probing in splanchnic serosal and mesenteric afferents during both acute inflammation and recovery. Moreover, the activation threshold was reduced and rate of firing enhanced in response to stretching of the gut wall in splanchnic serosal afferents after recovery from inflammation, but not in the acute phase. However, another study found that neither the threshold for activation nor the firing rate upon VFH probing were

altered in splanchnic afferents after colitis induced by (dextran sulphate sodium) DSS in rats, suggesting that the mechanism by which colitis is induced experimentally may be important (Coldwell et al., 2007).

Additionally the spinal afferent pathway studied may be important. Pelvic afferents were not sensitised to any mechanical stimulus during acute inflammation, but serosal afferents exhibited reduced activation thresholds and increased firing rates to VFH probing after recovery from inflammation induced by TNBS (Hughes et al., 2009a). In contrast, another study on the mechanosensitivty of the subtypes of afferents in the mouse pelvic pathway reported a reduction in both the response rate to VFH probing at 14 days post TNBS induced inflammation, and an increased activation threshold to a 0.4g VFH at day 14 and 28 post inflammation (Feng et al., 2012b). The increased activation threshold at 0.4g was also evident in mice recovering from zymosan induced inflammation, from the same lab (Feng et al., 2012a). In addition, pelvic muscular afferents exhibited augmented responses to high intensity stretch at post inflammation day 14 (Feng et al., 2012b). However, the same group report no changes in the responsiveness of pelvic muscular afferents to stretch in mice after treatment with zymosan (Feng et al., 2012a). Pelvic muscular-mucosal afferent did show augmented responses to stretch.

Other *in vitro* studies have demonstrated sensitisation of rat pelvic serosal afferents to distension after the induction of inflammation by TNBS (Wynn et al., 2004). Indeed, similar results were attained from *in vivo* recordings of pelvic afferents in rats undergoing colorectal distension (De Schepper et al., 2008). However, this concept is far from clear cut, as a study of *in vivo* recordings from rat in pelvic nerves has previously demonstrated a lack of sensitisation to colorectal distension by TNBS induced inflammation in rats (Sengupta et al., 1999). Similarly, the response to stretch was unaltered in mice treated with zymosan to induced colonic inflammation (Jones et al., 2007).

A simpler model of sensitisation, in which the effect of individual algogenic and inflammatory mediators on mechanosensitivity is tested, has also been studied. Indeed, a number of mediators including BK, ATP etc. have been shown to sensitise afferent fibres to mechanical stimuli (Wynn et al., 2004, Brierley et al., 2005b). A 2 minute application of 1μM of BK to the receptive field (mucosal side) of a splanchnic serosal afferent sensitised the subsequent response to a 2g VFH (Brierley et al., 2005b). Similarly serosal application of ATP increased responsiveness of pelvic afferents to *in vivo* luminal distension of the colorectum. This augmentation was even greater in TNBS treated rats (Wynn et al., 2004).

5.1.1.2 CHEMOSENSITIVITY

Inflammation can sensitise afferent nerves innervating many areas, increasing their response to chemical stimuli (Kocher et al., 1987, Habler et al., 1990). Indeed, inflammation can also sensitise spinal afferents innervating the gut to chemical mediators. For example, in rats, an increased proportion of splanchnic serosal and mesenteric afferents responded to 5-HT, and fired at a higher rate, after acute inflammation induced by DSS and during recovery (Coldwell et al., 2007). The response to ATP in pelvic nerves on the other hand was not augmented in rats, in which inflammation had been induced by TNBS (Wynn et al., 2004). Individual mediators have also been shown to sensitive intestinal afferents to subsequent chemical stimuli. For example, in a rat mesenteric nerve preparation, PGE_2, histamine, and adenosine were shown to sensitise the afferent response to subsequent application of BK (Brunsden and Grundy, 1999).

The spontaneous activity of murine splanchnic nerves seems to be unchanged after the induction of inflammation by either TNBS or DSS (Coldwell et al., 2007, Hughes et al., 2009a). In addition a number of studies have reported no change in either the proportion or rate of spontaneously active pelvic nerves in rodent models after the induction of inflammation, either TNBS or zymosan (Hughes et al., 2009a, Feng et al., 2012a). However,

there is also evidence for an increased proportion of spontaneous active pelvic afferents, which also fire at a higher rate after inflammation induced by TNBS (Sengupta et al., 1999, Wynn et al., 2004, De Schepper et al., 2008).

5.1.1.3 AIMS

- Compare the responsiveness of HVA from normal and inflamed tissues (CD, UC, appendicitis) to a range of mechanical (VFH probing, luminal distension) and chemical (BK, ATP) stimuli.

5.1.2 METHODS

5.1.2.1 PROTOCOLS

Protocols included in this chapter have been previously described, VFH probing (chapter 2, part 1), distension (chapter 2, part 2), and chemosensitivity (chapter 3, part 1).

5.1.2.1 NORMAL VS. DISEASE

To examine whether different diseases affected the chemosensitivity (proportion of responders, change in firing rate) or mechanosensitivity (change in firing rate) of HVAs, the response functions to the application of chemical (BK, ATP) and mechanical (VFH probing, and distension) stimuli were compared between “normal” and diseased tissues (CD, UC, appendicitis). No distinction was made between overnight storage, age, or gender. Chemosensitivity data were analysed using a paired t-test, $p<0.05$. Distension data were analysed using a 2 way ANOVA, $p<0.05$.

5.1.3 RESULTS

5.1.3.1 TISSUE – VFH PROBING

Sixteen tissues, 14 normal, 2 CD, were used for disease VFH analysis, 10 sigmoid colon, 2 ileum, 2 rectum, 1 ascending colon, 1 descending colon (M:F 1:1, median age 54).

5.1.3.2 TISSUE – DISTENSION

Nineteen appendices, 12 normal, 3 CD, 2 UC, 2 appendicitis, were used for disease distension analysis (M:F 1:1.1, median age 52).

5.1.3.3 TISSUE – CHEMOSENSITIVITY

Eighty-six tissues, 65 normal and 19 inflamed (8 CD, 6 UC, 5 appendicitis) were used for disease chemosensitivity analysis, 35 sigmoid colon, 22 appendix, 8 rectum, 7 ileum, 6 ascending colon, 4 transverse colon, 2 descending colon, 2 “unspecified colon” (M:F 1:0.76, median age 60). Further details on the tissues use in each set of experiments can be seen in table 2.02.

5.1.3.4 NORMAL VS. DISEASE

5.1.3.4.1 Mechanosensitivity

Whole nerve recordings from IBD appendices (n=5) had a significantly reduced response to luminal distension across the pressure range and at the individual pressures 40, 50, and 60 mm Hg compared to normal appendices (n=12) ($p<0.05$). Comparable with the whole nerve response a trend towards a reduced afferent to distension was also observed in WDR units discriminated from IBD appendices (n=4) compared with WDR units discriminated from normal appendices (n=5), although this did not reach significance at any pressure ($p>0.05$) (no LT or HT units were discriminated from normal or inflamed appendix). Similarly, there was a trend for serosal afferents from CD patients (n=2) to have lower responses to VFH probing compared to serosal afferents from normal tissue (n=14), although n numbers were too small for statistical

analysis. In contrast, whole nerve recordings from appendices collected from appendicitis cases (n=2) tended to have a greater response to luminal distension of the appendix compared to normal appendices (n=12) (figure 5.01).

5.1.3.4.2 Chemosensitivity

HVAs from IBD tissues (6/12, 50.0%) were as likely to respond to BK compared to normal (35/59, 59.3% tissues. In addition, the proportion of HVAs from IBD tissues (5/9, 55.6%) that responded to ATP was lower compared to normal (34/42, 81%) tissues, but this may reflect low n numbers. Similar HVA responses were observed in single unit recordings, distinguished from whole nerve flat sheet preparations, after the application of BK or ATP, whether the tissue was normal or from patients with IBD (BK IBD 35.9±6.3 (n=13) vs. normal 24.5±17.5 spikes $20s^{-1}$ (n=3), $p>0.05$; ATP IBD 51.3±6.1 (n=9) vs. normal 55.8±26.8 (n=2) spikes $20s^{-1}$) (figure 5.01).

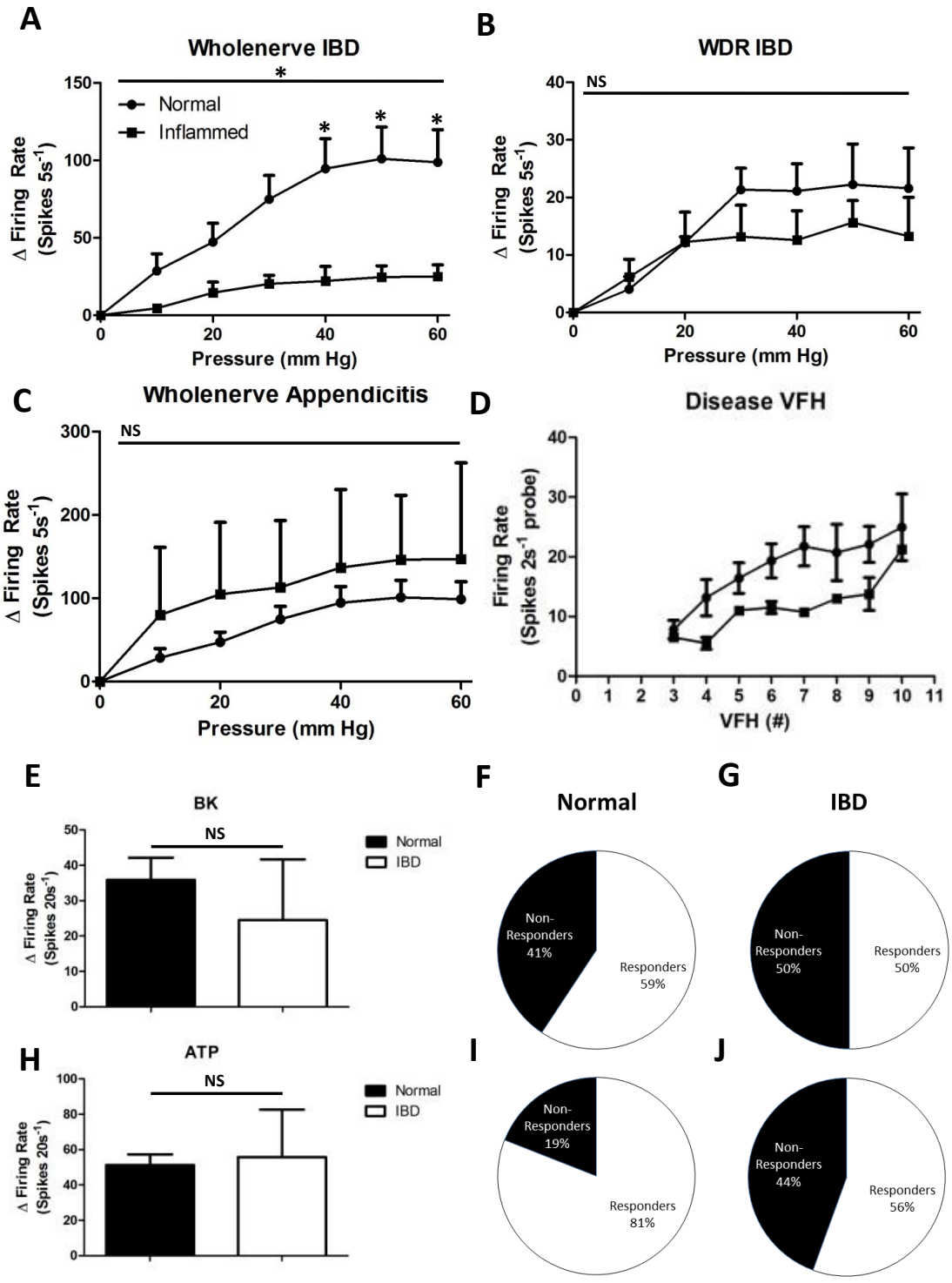


Figure 5.01: Comparison of mechano- and chemosensitivity of HVAs from inflamed and uninflamed tissue. A) Whole nerve recordings from inflammatory bowel disease appendices (n=5) had a significantly reduced response to luminal distension across the pressure range, and at the individual pressures 40, 50, and 60 mm Hg, compared to normal appendices (n=12) ($p<0.05$). B) When these recordings were analysed for single units, it was found that wide dynamic range (WDR) units from IBD appendices (n=4) also exhibited a trend for reduced

mechanosensitivity compared to WDR units from normal appendices (n=5), but this did not reach significance at any pressure ($p>0.05$). C) In contrast, whole nerve recordings from appendices collected from appendicitis cases (n=2) exhibited a trend for hypersensitivity to luminal distension of the appendix compared to normal appendices (n=12). D) Shows a trend for hyposensitivity of Crohn's disease serosal afferents (n=2) to VFH probing compared to normal serosal afferents (n=14). E) Similar increases in HVA firing rates in response to bradykinin were evident between normal (n=13, 35.9±6.3 spikes $20s^{-1}$) and IBD tissue (n=3, 24.5±17.5 spikes $20s^{-1}$, $p>0.05$). F-G) HVAs from IBD tissues (6/12, 50.0%) were as likely to respond to BK compared to normal (35/59, 59.3% tissues. H) Similarly, adenosine trisphosphate (ATP) caused a comparable increases in HVA firing in normal (n=9, 51.3±6.1 spikes $20s^{-1}$) and IBD tissues (n=2, 55.8±26.8). I-J) The proportion of HVAs from IBD tissues (5/9, 55.6%) that responded to ATP was lower compared to normal (34/42, 81%), but may reflect the low IBD n numbers. VFH #s 1=20mg, 2=40mg, 3=70mg, 4=160mg, 5=400mg, 6=600mg, 7=1g, 8=1.4g, 9=2g, 10=4g. Chemosensitivity data were analysed using a paired t-test, $p<0.05$. Distension data were analysed using a 2 way ANOVA, $p<0.05$.

5.1.3.5 SUMMARY OF RESULTS

- Whole nerve recordings from IBD appendices exhibited a significantly reduced firing rate in response to distension at 40, 50, and 60 mm Hg compared to normal appendices
- There was a trend for hyposensitivity to VFH probing in HVAs from CD patients compared to HVAs from normal tissue, but further study is needed
- Similar proportions of HVAs from IBD tissues responded to BK compared to normal tissues
- Although the proportions of IBD tissues that responded to ATP was lower compared to normal tissue, this may reflect low IBD tissue n numbers. More study on the chemosensitivity of HVAs from inflamed tissues is therefore needed.

5.1.4 DISCUSSION

5.1.4.1 DISEASE

This report has presented preliminary evidence for reduced HVA mechanosensation in tissues from IBD patients. Responses in whole nerve HVAs to luminal distension were significantly reduced across the pressure range and at the individual pressures, 40, 50, and 60 mm Hg in IBD appendices compared to normal appendices. Consistent with this observation, there was a trend for reduced response to VFH probing in serosal units from CD compared with normal tissues. This corroborates previously published data showing that patients with CD exhibit reduced responses to balloon distension of the rectum (Jehle et al., 1993, Bernstein et al., 1996). Indeed, patients with UC have also demonstrated a reduced sensitivity to rectal distension (Rao and Read, 1990, Chang et al., 2000).

There was no change in the proportion of HVAs responding to BK in tissues from IBD patients compared to normal tissues. ATP did respond less often in IBD tissues; however more study is need before definitive conclusion can be made. In addition, a similar magnitude of HVA activation was seen in IBD and normal tissues. The concept that mediators applied afferents from inflamed tissues would respond more often and to a greater degree than afferents from normal tissue has some logic, given the sensitisation of afferents in inflammatory states. Indeed, the responsiveness of a proportion of splanchnic serosal and mesenteric afferents to 5-HT was potentiated in rats after DSS induced colitis in rats (Coldwell et al., 2007). However, this sensitisation is not always reported, for example, response to ATP in pelvic nerves was not altered by colitis induced by TNBS in rats (Wynn et al., 2004). The data in this report shows no increase in proportion of responders to BK or ATP, or in the magnitude of response to these mediators. Indeed, chronic inflammatory conditions may lead to the downregulation of receptors response to prolonged exposure to inflammatory mediators. This

may act to counter the effect of this prolonged exposure to mediators on afferent nerve fibres and may explain the findings in this study.

Studies using balloon distension paradigms in patients with IBD highlight the influence of current inflammatory state, i.e. remission vs. on-going inflammation, on mechanosensitivity to distension (Rao et al., 1987, Rao and Read, 1990, Chang et al., 2000, Drewes et al., 2006). Indeed, the current inflammatory state in the tissues used in this report is not known. This could be done by collecting clinical data on each patient. Indeed, gathering as much clinical data on each patient will allow for more accurate comparisons between diseases and disease states, and should become part of the standard operating procedure when carrying out HVA experiments in the future. In addition, to further confirm the immediate inflammatory state of the tissue, supernatants could be generated from the inflamed tissues in order to measure inflammatory markers.

Many animal models have been developed to study colitis including inflammation induced by TNBS, DSS, or zymosan. The specificity of these types of colitis to human diseases such as IBD is uncertain. There is a spread of these experimentally induced colitis models across the literature. In addition, there are inconsistencies between studies on the definition of terms such as acute inflammation, and recovery from inflammation. This makes comparisons between animal studies, and between studies using patients with disease such as IBD difficult to compare. A more simplistic and potentially more useful paradigm for investigating the effects of inflammation in animals is examining the effects of individual inflammatory mediators on responses of spinal nerves to mechanical stimuli. Indeed, it would be of interest to test this kind of paradigm in HVAs. This has previously been done in a rat model, where the application of PGE_2, histamine, or adenosine could sensitisation the subsequent mesenteric afferent responses to BK (Brunsden and Grundy, 1999).

5.1.5 CONCLUSION

Preliminary evidence has been presented for a hyposensitivity of HVAs to mechanical stimuli in patients with CD. Responses in whole nerve HVAs to luminal distension were significantly reduced across the pressure range and at the individual pressures, 40, 50, and 60 mm Hg in IBD appendices compared to normal appendices. There was also a trend for a reduced response to VFH probing in HVAs from CD tissues. Indeed, this compliments both *in vivo* human data using colonic distension paradigms, and the clinical experience. This mechanical hyposensitivity should be investigated further. The magnitude of the response and proportion of responders to BK was similar between IBD and normal tissues. The proportion responders to ATP, was lower in IBD tissues, but this may reflect the low number of studies in this report. This report presents a post hoc analysis data from experiments to investigate another question. The availability of resected human intestine that has a specific disease warrants the use of these tissues in specifically designed experiments.

CHAPTER 5 PART 2: POST HOC ANALYSIS: THE EFFECT OF COLD TISSUE STORAGE ON THE MECHANOSENSITIVITY AND CHEMOSENSITIVITY OF HUMAN VISCERAL AFFERENTS

5.2.1 INTRODUCTION

5.2.1.1 COLD STORAGE

The development of successful organ transplantation encouraged scientists and clinicians to investigate how to progress the field. Preservation solutions, to keep the organ healthy, for as long as possible were of keen interest (Voigt and DeLario, 2013). The first effective solution was developed in 1969 (Collins et al., 1969). Since, then a number of organ specific physiological solutions, have been developed including the University of Wisconsin (UW) solution, and the Histidine-tryptophan-ketoglutarate (HTK)/Custodiol solution. In addition, to slow down metabolism, organs are cold stored at 4°C. Tissues stored for prolonged length of time are subject to cell swelling, acidosis, the activation of caspases in the apoptotic pathway, and reduced function after transplantation (Salahudeen et al., 2004). Indeed, transplant failure rates are higher when using kidneys stored for >24 hours (Ojo et al., 1997, Salahudeen et al., 2004).

Taking the kidney as an example, the average cold storage is ~21 hours, according to the United Network of Organ Sharing registry (Salahudeen et al., 2004). The vast majority of cadaveric kidneys are cold stored for 10-30 hours, although ~15% of the kidneys are cold stored for 30-50 hours (Salahudeen et al., 2004). The survival time of the transplanted kidneys was not significantly worse (p=0.79) between the 0-10 hours and 11-20 hours cold storage

time, but was significant at >30 hours (p=0.011). Taken together, this data suggests it is desirable to minimise the cold storage time. However, it also suggests that kept under the right condition, tissues are quite viable and can stay healthy and functional for long periods. Less information is available on intestinal transplantation, since it is a more recently developed and less common surgery. A noteworthy study removed a portion of a dog's small bowel and stored at 5°C in an oxygen free saline solution for 4-5 hours. The bowel was then anastomosed back into the dog. The dogs were allowed to recover and were subsequently sacrificed. At autopsy, all bowel remained viable (Lillehei et al., 1959). This study demonstrates the extraordinary viability of the bowel.

Electrophysiology requires healthy, functional tissue; hence in the past experiments were performed on the day of tissue collection, once tissue was back in the lab. However, the nature of experimenting on resected human tissue means an irregular supply of specimens. Often, tissue will not be available for a few days of the week, followed by a day with a number of procedures yielding a significant amount of human intestinal tissue. It is important to maximise the use of this tissue. Knowledge concerning the viability of human tissue may allow functional electrophysiological experimentation on the day after tissue collection, provided the tissue remains healthy. This would allow more experiments to be performed and make the HVA model more practical. It would also demonstrate the feasibility of collecting and experimenting on tissue from hospitals further away from the laboratory.

This report aimed to assess the viability of surgically resected human intestinal tissue by storing it in favourable conditions overnight before experimentation. The responsiveness of tissues to mechanical and chemical stimuli was compared between fresh specimens and those stored overnight. To reduce the damaging effects of cold storage, and to maximise the health of the tissue, cold storage times were kept to a minimum, usually between 12-16 hours. Furthermore, the tissue was stored in a physiological solution, Krebs buffer, which was carbongenated for at least 30 minutes prior to cooling.

5.2.1.2 AIMS

- Assess the viability of surgically resected human intestinal tissue
    - Examine responses to mechanical and chemical stimuli after overnight storage

5.2.2 METHODS

5.2.2.1 PROTOCOLS

Protocols included in this chapter have been previously described, VFH probing (chapter 2, part 1), distension (chapter 2, part 2), and chemosensitivity (chapter 3, part 1).

5.2.2.2 COLD STORAGE

To investigate the viability of resected human tissue, samples were stored in a 1 litre bottle of Krebs buffer and bubbled with carbogen (95% oxygen, 5% carbon dioxide) for 30 minutes. The bottle was then stored overnight at 4°C. The next morning electrophysiological recordings were attained and the HVAs challenged with a number of chemical (BK, ATP) and mechanical stimuli (VFH probing, and distension). Spontaneous activity, chemosensitivity (proportion of responders, change in firing rate) and mechanosensitivity (change in firing rate) were then compared to HVAs recorded on the same day as surgery. Appendix specimens were not included in whole nerve change of firing rate calculations due to their inherent higher firing rates. No distinction was made between different diseases, ages, or genders. Chemosensitivity data were analysed using a paired t-test, $p<0.05$. Mechanosensitivity data were analysed using a 2 way ANOVA, $p<0.05$.

5.2.3 RESULTS

5.2.3.1 TISSUE – VFH PROBING

Sixteen tissues, 12 were used the day of surgery, and 4 were stored overnight and used the day after surgery, 10 sigmoid colon, 2 ileum, 2 rectum, 1 ascending colon, 1 descending colon (M:F 1:1, median age 54).

5.2.3.2 TISSUE – DISTENSION

Fifteen appendices, 9 were used the day of surgery, and 6 were stored overnight and used the day after surgery (M:F 1:1.1, median age 52).

5.2.3.3 TISSUE – CHEMOSENSITIVITY

Eighty-one tissues, 47 were used the day of surgery, and 34 were stored overnight and used the day after surgery, 35 sigmoid colon, 20 appendices, 8 rectum, 7 ileum, 5 ascending colon, 3 “unspecified colon”, 2 descending colon, 1 transverse colon (M:F 1:1.3, median age 57). Further details on the tissues use in each set of experiments can be seen in table 2.02.

5.2.3.3 COLD STORAGE

Recording from HVAs was no more difficult the day after (DA) surgery, compared to recording the day of (DO) surgery. DO and DA recordings did not differ in their mechanosensitivity. There was no difference in the response of HVAs to VFH probing of any weight, or overall between DO (n=12) and DA (n=4) tissues ($p>0.05$). Similarly, in whole nerve recordings, whether the experiment was done the day of surgery (n=9) or the day after surgery (n=6), did not change the HVA response to distension at any pressure ($p>0.05$). When these recordings were analysed as single units, WDR (DO – n=6, DA – n=2), LT (DO – n=2, DA – n=3), and HT (DO – n=1, DA – n=3) pressure response line graphs were similar for tissues that were stored overnight or that were recorded from immediately (figure 5.02).

The chemosensitivity of HVAs were similar in DO and DA recordings. Single unit responses in flat sheet preparations to the algogenic mediators BK and ATP were not different between groups (Δ firing rate BK DO (n=22) 36.9±9.3 vs. DA (n=18) 28.7±5.4 spikes $20s^{-1}$, $p>0.05$; ATP DO (n=22) 46.1±11.6 vs. DA (n=19) 54.7±7.8 spikes $20s^{-1}$, $p>0.05$; excluding appendix preparations). The proportions of HVA responding to BK and ATP was comparable between DO and DA recordings; BK 53.7% (22/41) vs. 64.3% (18/28); ATP 78.6% (22/28) vs. 73.1% (19/26), respectively (figure 5.02).

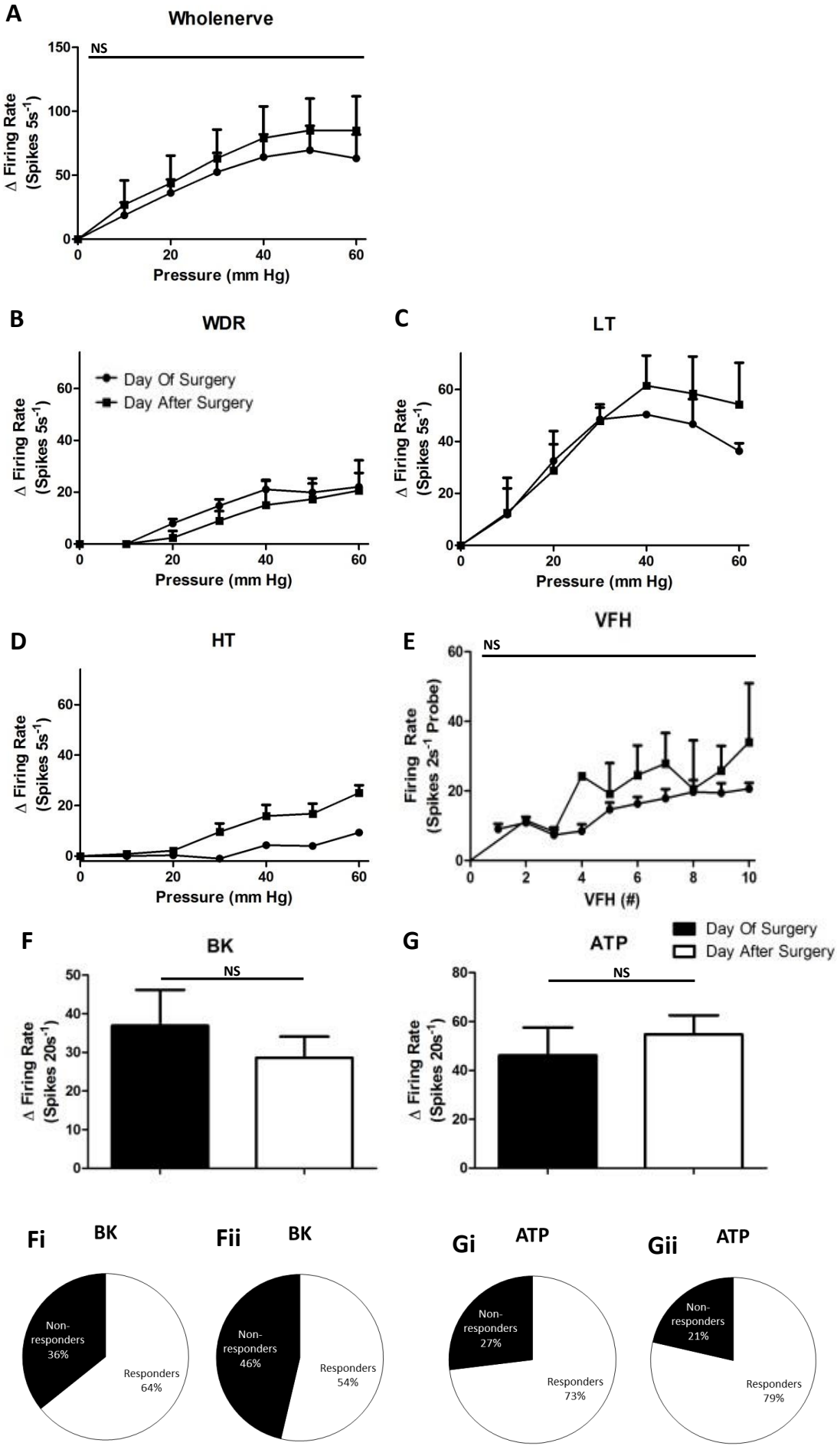

A
Wholenerve
NS
Δ Firing Rate (Spikes 5s-1)
Pressure (mm Hg)
B
WDR
Day Of Surgery
Day After Surgery
C
LT
D
HT
E
VFH
Firing Rate (Spikes 2s-1 Probe)
VFH (#)
F
BK
G
ATP
Day Of Surgery
Day After Surgery
Δ Firing Rate (Spikes 20s-1)
Fi BK
Non-responders 36%
Responders 64%
Fii BK
Non-responders 46%
Responders 54%
Gi ATP
Non-responders 27%
Responders 73%
Gii ATP
Non-responders 21%
Responders 79%

Figure 5.02: Comparison of HVAs from tissues recorded on the day of surgery (DO) or the day after surgery (DA) in their sensitivity to mechanical and chemical stimuli. A) There was no significant difference in the whole nerve HVA response to distension at any pressure if the recording was done on the day of (n=9) surgery or the day after surgery (n=6) ($p>0.05$). B-D) In addition, pressure response line graphs were very similar between DO and DA recordings in wide dynamic range (WDR) (B), low threshold (LT) (C), and high threshold (HT) (D) HVAs. E) There was no difference in the response of HVAs at any VFH weight, or overall between DO (n=12) and DA (n=4) tissues ($p>0.05$). F) HVAs recorded the DO and the DA surgery exhibited comparable firing rates to the application of bradykinin (BK) (DO (n=22) 36.9±9.3 vs. DA (n=18) 28.7±5.4 spikes $20s^{-}1$, $p>0.05$). A similar proportion of DO (Fi, 22/41) and DA (Fii, 18/28) HVAs responded to BK. G) The activation rates of HVAs recorded on the DO and DA surgery were similar after the application of Adenosine trisphosphate (ATP) (DO (n=22) 46.1±11.6 vs. DA (n=26) 54.7±7.8 spikes $20s^{-1}$, $p>0.05$). In addition, the likelihood of DO (Gi, 22/28) or DA (Gii, 19/26). HVAs responding to ATP was similar. VFH #s 1=20mg, 2=40mg, 3=70mg, 4=160mg, 5=400mg, 6=600mg, 7=1g, 8=1.4g, 9=2g, 10=4g. Chemosensitivity data were analysed using a paired t-test, $p<0.05$. Mechanosensitivity data were analysed using a 2 way ANOVA, $p<0.05$.

5.2.3.4 SUMMARY OF RESULTS

- Whole nerve HVA firing in response to distension did not differ after storage compared to same day experimentation. Similarly, pressure response line graphs produced by WDR, LT, and HT single units were similar whether the tissue was stored overnight or used immediately. There was no difference in HVA responses to individual VFH probes of any weight or over the entire range together.
- Whole nerve responses, and the proportion of nerves responding, in flat sheet preparations to the algogenic mediators BK and ATP were not different between recordings done on the day tissue was collected or after overnight storage.

5.2.4 DISCUSSION

5.2.4.1 COLD STORAGE

We have demonstrated that the HVA response to mechanical and chemical stimuli, are comparable between DO and DA recordings. In addition, we have demonstrated that the HVA response to the chemical mediators BK and ATP is not different between DO and DA recordings. This suggests that the tissue remained viable and healthy during overnight storage. Indeed, cadaveric organs can be stored for up to 50 hours in preservation medium, although with some associated damage and reduced transplantation success rates (Salahudeen et al., 2004). The bowel is an extremely viable organ, as canine intestinal transplantation studies demonstrate, whereby intestines remained viable after 5 hour storage in just a chilled saline solution.

Improved viability may be possible if better preservation mediums are used. This study used carbongenated krebs solution chilled to 4°C, which seemed to be suitable. Apart from slight mucosal degradation, the human tissue remained macroscopically healthy. Indeed, its mechano- and chemosensitivity remained unchanged compared to “fresh” tissue. In the future, it would be of interest to test some preservation mediums, e.g. UW medium, used in organ transplantation to seem if they increase the longevity of the tissue. This would be useful to know for any institution looking to collect human tissue for research purposes from hospitals that are not close by. Using the optimal preservation medium during long journeys from the hospital to the lab, could help keep the tissue healthy and viable. The observation in this report has the potential to increase the yield of human tissue data, both from collecting specimens from hospital further away, and making use of tissue that cannot be used on the day of research due to researcher commitments, large supply of tissue in 1 day etc. The introduction of this storage variable can be avoided by designating certain groups of

experiments for same day or next day recordings. Indeed, experiments on mucosal afferents should be done on the same day of surgery.

5.2.5 CONCLUSION

The viability of HVAs has been described. Resected colonic tissue remains viable and healthy, aside from some mucosal degradation, after overnight storage in a simple carbongenated Krebs solution. Other media, normally used to preserve cadaveric organs due for transplantation, could be tested in the future. The viability and longevity of human intestinal tissue suggests that human tissue can be collected from hospitals further afield of the lab. This would allow more tissue to be collected for our lab, and may encourage other labs, not in close proximity to a hospital, to collect human tissue, and do electrophysiological experiments using HVAs.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

6.1 GENERAL DISCUSSION

This report has demonstrated the feasibility and practicality of human visceral afferent recordings. There has been a significant improvement in both the collection process and the number of tissues collected since the beginning of this project. In the 2nd half of 2011, 13-14 specimens were collected in each 3 month period. This number has increased to 21-22 in the each 3 month period by the end of 2013 (figure App.03 in appendix). Over 130 recordings were made from 8 distinct areas of the human GI tract, ileum, appendix, caecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum. The success rate of HVA recordings has dramatically improved, from ~40% in 2011 to over 95% in 2013. These results demonstrate the practicality of this technique and the potential to expand it to other laboratories.

Importantly, this report has demonstrated that HVAs are sensitive to both mechanical and chemical stimuli. Indeed, an afferent's response profiles to these stimuli can help determine the location of their terminals. Furthermore, a number of protocols based on these stimuli have been developed, including VFH probing protocols, appendix luminal distension protocols, and repeat application of chemical mediators. This report tested several drugs or their surrogates that have been or are in clinical trials to determine their effects on HVA responses to mechanical stimuli. The results of this report also suggest it is feasible to use this model to study the mechanisms behind the transduction of both mechanical and chemical stimuli, and demonstrates the potential capacity of HVA experiments to substantiate findings from animal studies examining the efficacy of novel potentially analgesic compounds.

6.1.1 MECHANICAL STIMULI AND THE CHARACTERISATION OF SUBTYPES OF HVAs

6.1.1.1 Distension

Pain originating in the bowel can be elicited clinically by traction of the mesentery or noxious levels of distension. Indeed, many preclinical studies use rectal, colonic or colorectal distension as a physiologically relevant noxious mechanical stimulus. This report demonstrates the reproducibility of HVA responses to repeated appendix distension demonstrating the presence of three functional subtypes of distension sensitive afferents, LT, WDR, and HT, distinguishable by their pressure activation threshold, rate of firing, and the pressure at which their firing saturates. This is comparable with studies in animals, which have also shown that repeated distensions of the rat or cat colo-rectum *in vivo* evokes reproducible pelvic afferent responses (Blumberg et al., 1983, Sengupta and Gebhart, 1994).

Importantly, the extrinsic nerves innervating the appendix are embryologically identical to those innervating the caecum, being derived from the same nerve and progenitors (Peiris et al., 2011). Therefore the appendix distension model may provide a useful insight into the mechanisms of pain in the intestine. Additionally, the appendix is a practical resource to study both acute inflammatory pain, given the high incidence of appendicitis and subsequent appendicectomies, and afferent function under normal conditions, given the large number of right hemicolectomy surgeries for colorectal cancer. Furthermore, the lack of any translational issues makes this an attractive and useful model.

6.1.1.2 VFH probing

VFH probing is another common mechanical stimulus used in preclinical animal studies, and involves probing the receptive field with a calibrated nylon or optical glass filaments. This stimulus has less physiological relevance in visceral pain than distension, but is very practical

and easy to use. VFHs have other limitations, which are important to note. The straight cut end of the filament lends itself to a change in surface area in contact with the tissue upon bending, hence the pressure applied may change (Bove, 2006). Indeed, how compliant the tissue is to probing will also influence the pressure exerted, by determining the indentation depth (Bove, 2006). This is just one shortcoming, with many others being cited, including, researcher bias (Wallas et al., 2003), incorrect filament calibration (Bell-Krotoski and Tomancik, 1987), the rate of force development, which is determined by the speed of filament bending (Bove, 2006), and both temperature and relative humidity can affect the stiffness and therefore calibration of the filament (Andrews, 1993). Nonetheless, VFH filaments are a useful tool in visceral pain models, especially when distension or stretching is impractical, as is sometimes the case in human intestinal tissue. Indeed, a number of papers recently published in high impact journals, have used this methodology e.g. (Brierley et al., 2004, Brierley et al., 2008, Feng et al., 2012a, Castro et al., 2013).

A proportion of HVA receptive fields exhibited desensitisation after prolonged probing protocols, but not before 15 sets of x3 probes, as observed throughout various probing protocols. This desensitisation has also been reported in guinea pig ileum (Song et al., 2009, Brookes et al., 2013). However, VFH probing time matched control experiments in this report demonstrates that the majority of HVA receptive fields evoke consistent and reproducible responses over a period of 1+ hours, with probing every 5 minutes. Studies in guinea pigs report the desensitisation of the receptive field is irreversible (Song et al., 2009). Therefore further evidence that the reduction in HVA response to VFH probing after application of various drugs (e.g. HC067047) is drug related and not desensitisation is demonstrated by the recovery of the response after drug washout in a proportion of the HVAs. Decreasing the magnitude of the stimulus would likely extend the life of the receptive field, hence the lowest VFH capable of eliciting reproducible responses were chosen for use in the majority of experiments.

Serosal and mesenteric afferents are thought to be the main nociceptive afferents innervating the gut, as they have high distension pressure thresholds, and respond to noxious mediators such as BK and ATP (Brierley et al., 2005b, Wynn and Burnstock, 2006, Andresen et al., 2007, Johnston et al., 2009, Lin et al., 2010). It was therefore postulated that serosal and mesenteric HVAs were also nociceptive, although this is not immediately evident from their response to VFH probing. For example, serosal and mesenteric HVAs respond to light weight VFHs, sometimes as low as 20mg. A VFH of this weight is certainly not noxious, since it barely breaks the fluid tension in the tissue bath. However, a low VFH activation threshold does not exclude them from a classification of nociceptor, which are classically high threshold. VFH probing represents a non-physiological stimulus and does not mimic any event that happens *in vivo*. It is possible that these afferents are more prone to activation by direct probing of their receptive filed, but require high distension pressures to be activated, a physiological stimulus. Indeed, both serosal and mesenteric mouse colonic afferents respond to low VFH probing in mouse models, despite only responding to high distension pressures (Brierley et al., 2004, Brierley et al., 2008). This report did not find any response to serosal or mesenteric units in response to tissue stretch in our studies suggesting high levels of stretch may be required to activate these afferents. In contrast, muscular units are readily activated by tissue stretch.

Finally, this report confirmed a role for serosal afferents in nociception, by demonstrating their responsiveness to noxious inflammatory mediators such as BK and ATP. In contrast, muscular afferents had little or no response to these mediators, suggesting they play a more physiological role in the gut.

6.1.1.3 VFH time matched controls

This report demonstrates the reproducibility of repeated VFH probing over the course of a standard protocol of ~60 minutes. This data suggests that VFH probing is a suitable protocol in which to investigate the mechanisms of, and drugs that may affect the transduction of

mechanical stimuli. Greater n numbers would be desirable and necessary to consolidate this finding. It is important to ensure that the receptive field of the HVA is in a suitable location and can be probed repeated without any obstructions e.g. mesentery. It is often the case that the receptive field can be probed, but not consistently because of its relatively inaccessible location e.g. under a fold in the mesentery. Accessibility can sometimes be improved by carefully manipulating, and re-pinning the tissue, while taking care not to interfere with the electrode. Early stability in VFH probing is of key importance, and any HVAs with early erratic responses should not be used. These 2 issues make receptive fields fit for purpose hard to come by, hence decreasing the number of experiments that can be performed.

6.1.1.4 Distension time matched controls

This report demonstrates the reproducibility of repeated luminal distension of the appendix over the time course of a standard protocol of 60-80 minutes. This data confirms that appendix distension is a suitable protocol in which to investigate the mechanisms of, and drugs that may affect the transduction of mechanical stimuli. However, it is important to ensure the HVAs are responding robustly to distension before starting the protocol, as weak responses have a tendency to decline after a few distensions. Furthermore, care must be taken to distend the appendix to the same pressure each time, as changing the distension pressure mid protocol could change the recovery time and hence could affect the HVA response to the next distension.

6.1.1.5 Stretching

Methods to standardise stretch have been considered. A cantilever system as used previously in rodents e.g. (Page and Blackshaw, 1998), may be used, but would require large weights to stretch the tissue. Similarly a force transducer attached to a force actuator could be used (Feng et al., 2012a). Stretching the tissue, a certain distance as marked by pins in the bath would only serve as standardisation if each piece of human tissue was the same size, which is not the

case. Standardisation of stretching stimuli is necessary before protocols using this stimulus can be carried out.

6.1.1.6 Comparison of afferent subtypes innervating colonic and appendix

This report describes 4 distinct subtypes of HVA innervating the intestine based on their response to mechanical stimuli; mesenteric, serosal, muscular, and muscular mucosal. Three different subtypes of HVAs innervating the appendix based on their distension pressure threshold for activation, HT, WDR, and LT, have also been described. High intensity distension, ~45 mm Hg (Brierley et al., 2008) or stretch, ~ 9-10g (Brierley et al., 2008, Hughes et al., 2009a) is required to activate serosal or mesenteric afferents in murine models. The activation of mesenteric afferents by distension, despite the absence of terminals in the gut wall, has been explained previously as the translation of longitudinal forces onto the mesentery (Hughes et al., 2009a). In contrast, light stretching, ~2g, akin to low distension pressures activates muscular afferents (Hughes et al., 2009a). These data suggest that in murine models HT distension sensitive afferents are likely to be mesenteric or serosal afferents, while WDR and LT afferents are likely to be muscular or muscular mucosal afferents. This could potentially be extrapolated to HVAs innervating the human colon and the appendix, given that afferents innervating the appendix and right colon are branches of the same nerve and are from the same embryological background (Peiris et al., 2011).

6.1.2 VALUE OF HVA RECORDINGS

Using electrophysiological recordings of HVAs to add to existing animal data, and establish some *in vitro* human data, on the efficacy of a potential visceral analgesics before they enter into clinical trials is seen as an important step in basic drug development research (Schemann, 2011). This report describes the efficacy of one compound, which has previously been approved for clinical use (subsequently withdrawn for safety reasons), tegaserod, a κ agonist, ICI 204, 448 used as a replacement for one compound currently in clinical trials (asimadoline),

and one compound which mimics the action of linaclotide, a drug currently in clinical trials, STa endotoxin. In clinical trials, all three have reduced abdominal pain (asimadoline not ICI 204, 448 and linaclotide not STa) (Lefkowitz, 1999, Muller-Lissner et al., 2001, Novick et al., 2002, Kellow et al., 2003, Tack et al., 2005, Mangel et al., 2008, Johnston et al., 2009, Lembo et al., 2010, Lembo et al., 2011). In this report tegaserod significantly attenuated the HVA response to VFH probing, and STa reduced the whole nerve HVAs to distension of the appendix. ICI 204, 448 attenuated but not significantly the HVA response to VFH probing even with low n number (n=3). Therefore, this report demonstrates the first evidence that this model may be useful in pre-clinical trial drug development research as a means to build more confidence on drugs showing potential in animal experiments. However, the inability of tegaserod to reduce the HVA response to distension of the appendix highlights the need for careful consideration when selecting, the methodology, and the area of the gut used in experiments, as this will determine both the afferent pathways examined i.e. vagal, splanchnic, and pelvic, and the subtypes of afferents used i.e. serosal/mesenteric vs. muscular/muscular-mucosal. Experiments using HVAs therefore need to be specific and well designed to reduce the variability and potential for false negative or false positive results.

6.1.3 EXPERIMENTAL DESIGN

A disadvantage of using human tissue is the inherent variability in the tissue available for experimentation, including type of tissue e.g. ascending vs. sigmoid colon, and disease e.g. “normal” cancer tissue vs. IBD tissue. Initially we used all tissue types as the development of the HVA recording technique was the key goal. However, it is important to try to reduce these variables in future HVA experiments. Multiple projects using different tissue types and diseases should be ongoing simultaneously, such that when, for example, a piece of normal sigmoid colon is collected, a different experimental protocol is performed than if a piece of ascending Crohn’s disease tissue is collected.

In addition, now that the practicality of characterising HVAs has been demonstrated, it should be standard procedure to determine the location of the afferent terminal before commencing an experiment. This will allow further experimental specificity, whereby, for example, only serosal afferents are used for a particular protocol. These measures will reduce variability and incite greater confidence in the experimental results.

6.1.4 IMPROVING THE PROTOCOL

This report has used a pharmacological intervention, targeting the second application of a mediator. There are aspects of this experiment which would benefit from review. These experiments lasted up to 4 hours, allowing for at least 60 minutes between mediator applications. Although not excessive, this long experimental protocol had negative consequences. 1) A gradual change in action potential shape and size, as has been known to occur over long recording periods (Berthoud et al., 2001). This can make offline waveform analysis more difficult, time consuming and less accurate. 2) Increased attrition rate with longer experiments. On occasion, action potential firing from a HVA will substantially drop over a period of hours, or activity will cease completely. Although rare, the incidence of this phenomenon increases with longer experimental protocols.

These problems can be addressed by reviewing the concentration of drugs added and their delivery method. The concentration of BK and ATP used in these experiments were chosen so that reasonable amount of HVAs would be responsive, whereby lower doses activate a small proportion of HVA fibres. Similarly, a 20ml volume allows the mediator to be superfused into the bath in ~5 minutes. Changing the concentration of the drug added or the application method may shorten the protocol, allowing for reproducible responses with shorter washout periods. For example, it is possible that lower final bath concentrations of mediators could be used if the addition of the drug was immediate e.g. squirted into the bath using a pipette. This could be followed by applying a small volume of the mediator at final bath

concentration into the bath to ensure the bath concentration remains stable for a given amount of time. This should be explored in the future. Another potential solution would be to apply the drugs locally to a receptive field using a metal ring and silicone grease. However, the relative number of suitable receptive fields found in HVAs would make this difficult.

6.1.5 PATIENT DETAILS

An important issue not addressed in this report is the inherent variability in patients from which tissue is collected. Patients differ in the treatment they have received; they may or may not have undergone chemotherapy or radiotherapy for cancer, or may or may not have been given infliximab, azathioprine, or steroids to treat their IBD, or may have received different anaesthetics before surgery. Similarly, some patients may have gut related co-morbidities, for example, patients undergoing a colonic resection for cancer may also have chronic constipation or another FGID, which is important information when designing an experiment. Furthermore, issues such as the time the patient has been suffering the disease, whether the disease is active or in remission, as well as ethnicity, gender and age are all factors that require consideration.

Certainly, it is ideal to collect as much information as possible about each patient from which tissue is collected. If necessary this information should help dictate which experiments the tissues are most suited for, or could be used retrospectively to develop hypothesis about or explain erroneous results. However, given that patients and the disease they suffer from are inherently heterogeneous, experimentation on heterogeneous tissue may not actually confer any disadvantage, rather it may be more applicable to the real life situation. Routine collection of this kind of patient information has only begun. Retrospective collection of patient information is possible and is planned for the future.

6.1.6 USES OF THE HVA MODEL

Recording the activity of human afferent nerves innervating the gut is useful translational model. This report has confirmed the existence of afferent terminals in the human gut that have previously only be described in animal models i.e. *mesenteric, serosal, muscular, and muscular-mucosal*, which will allow the study of distinct functional subpopulations of HVAs including a population of afferents likely to be human nociceptors. Furthermore, the function and importance of various receptors and channels as well as their respected intracellular pathways in nociception, previously only described in animal models, could be elucidate in human afferents. Alterations in these mechanisms could then be identified in diseased states using IBD and appendicitis tissues (Schemann, 2011). For example, this report demonstrates the importance of B2 but not B1 receptors in the activation of HVAs by BK in normal tissue. However, there is evidence that the expression of B1 receptors is induced during inflammation and injury (Stadnicki et al., 2004), and that they may play a role in chronic visceral inflammatory pain (Jaggar et al., 1997). Hence, it would be interesting to investigate the role of B1 receptors in afferents from diseased (IBD or appendicitis) human tissue.

This HVA model allows the study of a number of mechanical stimuli e.g. VFH probing, stretch, mucosal stroking, and luminal distension (appendix). Distension of the appendix allows the study of stimuli that is likely to be physiological and non-noxious (i.e. low pressures) and noxious stimuli, likely to induce pain (high pressures), and to study the receptors, channels, mediators and intracellular mechanisms involved. Indeed, the involvement of mechanotransducer channels (e.g. TRP channels, ASICs) in afferents from normal and diseased tissue could be examined.

Similarly, a number of chemical stimuli can be tested in this HVA model e.g. algogenic mediators, inflammatory mediators, cytokines etc. Supernatants can be generated from normal and diseased tissue (see APP 1.2.1) and applied to HVAs from normal or diseased tissue

to test their ability to activate the nerves. Multiplex analysis of the noxious supernatants could reveal mediators involved in the supernatant induced activation of nerves hence, substantiating evidence of their involvement in visceral pain, or revealing novel mediators and potential clinical targets.

This report has described a number of protocols that can be used in HVA experiments e.g. repeated mediator application, VFH probing, luminal distension of the appendix. Furthermore, the present report has described the efficacy of a number of drugs or drug surrogates in reducing the mechanosensitivity of HVAs to either VFH probing (tegaserod) or luminal distension (STa endotoxin). Indeed, these HVA protocols could be used to substantiate animal data on novel therapeutics to increase confidence in their efficacy before they are brought forward into clinical trials. Furthermore, the model could be used to help predict afferent nerve specific side effects e.g. changes in visceral sensation (Schemann, 2011), from other drugs about to enter clinical trials.

6.1.7 DIFFICULTIES IN HUMAN TISSUE RESEARCH AND USING THE HVA MODEL

Working with human tissue and particular the HVA model has inherent difficulties. Establishing the infrastructure to collect human tissue requires a lot of work. Ethics needs to be obtained, which can be a frustrating process. Furthermore successful collection requires the commitment of a number of teams, where research is not a priority, for example surgeons, theatre staff and pathology staff. A tissue collection and division procedure needs to be implemented which can involve some delicate politics between respective labs collecting human tissue. Furthermore, if the collection hospital is far away from the lab suitable transportation and a storage solution must be used, and agreed on by each collecting group.

Human tissue collection is also time consuming, involving consenting the patient, collection of the tissue, gathering of patient information, and database generation and management. A relatively low number of specimens, compared to animal models where tissue

is readily available, means collecting sets of data takes longer, especially when controlling for tissue type and disease. Planning experiments can also be made difficult by unpredictable surgery schedules, time of surgery, cancellations etc. The initial difficulty of learning the preparation is confounded by the limited tissue supply leading to longer training time for new scientists hoping to use the technique. Electrophysiological training on animal models prior to using human tissue is therefore desirable.

The HVA model also has a number of limitations. For example the model is devoid of any central processing which is likely to be important in visceral nociception. Furthermore, it is not possible to determine exactly which pathway the nerve you are recording from is in i.e. splanchnic vs. pelvic vs. vagal. There is also the small possibility of recording from an IFAN. In addition, the predictive capacity of the model extends only to the efficacy of drugs reducing the signalling of either chemical or mechanical stimuli, or afferent specific side effects. Therefore, it does not have a predictive capacity for all drugs.

6.1.8 FUTURE PLANS

Continued work is required to improve the HVA model. Stretch responses should be standardised such that a given force can be applied to stretch each tissue. Electrical stimulation for identification of the receptive fields of “silent” nociceptors is of particular interest. Furthermore, electrical stimulation will allow for the characterisation of afferents based on their conduction velocity. The lack of mucosal afferents evident in this report could be investigated by using a mucosa up orientation of the tissue to allow better access to the mucosal receptive fields. A new drug application method, such as metal ring application will allow for specific concentration of drugs to be added to specific receptive fields. This will allow experiments with expensive compounds as well as opening the door for experimentation on multiple afferents with distinct receptive fields from the same human preparation.

Since mesenteric and serosal afferents are of particular interest, due to their putative involvement in visceral nociception, increasing the yield of these afferents would be of interest. Mesentery only recordings have been postulated and should be attempted in the future. In addition studies examining the receptors involved in nociception in both health and disease should be continued e.g. B1 vs. B2 BK receptors, the mechanotransducer channels e.g. TRPs, ASICs etc. Finally the predictive capacity of this model should be tested further with other potential visceral analgesics.

6.2 GENERAL CONCLUSIONS

Our data demonstrates the existence of subtypes of afferents that terminate in the mesentery, serosa, muscle and muscular-mucosal layers. Each subtype responds to a distinct subset of mechanical stimuli, with specific activation thresholds. Indeed, this report has identified a likely population of human visceral nociceptors, namely serosal afferents, which have previously shown to be nociceptors in mice. Human serosal afferents do not respond to stretch at low intensities, but respond to algogenic mediators such as BK and ATP.

Furthermore, this report has demonstrated evidence for a "silent" nociceptor population in HVAs. Three distinct subtypes of distension sensitive afferents, LT, WDR, and HT, have also been characterised based on their pressure activation threshold, firing frequency, and the pressure at which their afferent activity plateaus. HT afferents are likely comparable to serosal afferents, since they require higher pressures for activation. LT and WDR are likely to be comparable to stretch sensitive afferents namely muscular and muscular-mucosa afferents, given their responsiveness to lower pressures.

This report demonstrates the use of two mechanical protocols in HVAs, VFH probing of serosal afferents, and luminal distension of the appendix. The TRPV4 antagonist HC067047 significantly attenuated the HVA response to VFH probing. This suggests that TRPV4 receptors are important in the transduction of mechanical stimuli in serosal HVAs, the likely nociceptive

population. In contrast, there was no effect on the whole nerve, LT, or WDR HVA response to distension after the application of the TRPV4 agonist, GSK1016790A, or antagonist, HC067047. Indeed, no HT afferents were discriminated in these experiments, which may explain these results.

Furthermore, this report demonstrated that application of tegaserod, shown to reduce pain in clinical trials, reduced the HVA response to VFH of serosal HVAs. Another compound, ICI 204, 448, used as a surrogate for another drug in clinical trials, asimadoline, exhibited a trend for a reduced HVA response to VFH probing of the serosa; however this was only a set of 3 experiments. Furthermore the whole nerve HVA response to distension was reduced after application of the GC-C superagonist STa endotoxin, a compound that mimics the effect of linaclotide, which is currently in clinical trials. These results suggest that the HVA recordings may be useful in substantiating animal data on the pre-clinical efficacy of a drug, before they go into clinical trials.

This report demonstrates the broad chemosensitivity of HVAs, showing responses to BK, ATP, adenosine, capsaicin, 5-HT, histamine, and PGE_2. Indeed, a proportion of afferents demonstrate responsiveness to a multitude of these mediators. In addition, repeat mediator application protocols for BK and ATP have been developed. These protocols have been used to show the involvement of B2 receptors in the activation of HVAs by BK, and the lack of a role of adenosine P1 receptors in the activation of HVAs by ATP. P2X receptor antagonists failed to reduce the HVA response to ATP suggesting it may not play a dominant role in the activation of HVAs by ATP.

Post hoc analysis of data revealed a potential hyposensitivity of IBD tissues to mechanical stimuli such as luminal distension and VFH probing. Furthermore, post hoc analysis revealed that there were no differences in the mechano- and chemosensitivity of HVAs whether the tissue was stored overnight at 4°C, or used immediately after surgery. However,

some experiments, e.g. studies examining mucosal and muscular-mucosal afferents, may be best done on the day of the surgery.

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APPENDIX PART 1: THE EFFECT OF APPENDICITIS SUPERNATANTS ON HUMAN VISCERAL AFFERENTS

APP1.1 INTRODUCTION

APP 1.1.1 SUPERNATANTS

Abdominal pain is a major symptom of diseases such as IBS and IBD. However the exact mechanism for this abdominal pain is not fully understood in IBS or IBD, but may be due to neuronal activation and the induction of hypersensitivity (Drossman et al., 2002). In IBS, excessive mucosal mast cells release mediators which activate ENS neurons and extrinsic sensory neurons, which lie in close proximity, and subsequently produce hypersensitivty (Bueno et al., 1997, Vergnolle, 2003, Barbara et al., 2006). During active IBD pro-inflammatory mediators are released from the colon wall, including the mucosa, and can activate extrinsic sensory fibres, either directly or through the modulation of other receptors and channels e.g. TRP and Na_v channels, causing increasing neuronal excitability and hypersensitivity (Ibeakanma and Vanner, 2010). Supernatants generated from colonic mucosal biopsies taken from IBS and IBD patients, and containing naturally occurring disease mediators, have been investigated for their effects on neuronal firing and excitability (Nasser et al., 2014).

Using a voltage sensitive dye it was demonstrated that IBS supernatants can activate enteric nerves (Buhner et al., 2009, Buhner and Schemann, 2012). IBS supernatants can also activate extrinsic afferent nerves. Two separate studies showed that IBS supernatants applied to isolated nociceptive DRG neurons mobilised the release of calcium in a calcium imaging assay (Barbara et al., 2007, Cenac et al., 2007). In addition, IBS-D supernatants increased action potential firing in patch clamped mouse nociceptive DRGs (Valdez-Morales et al., 2013). IBS supernatants applied to extrinsic afferent nerves innervating the rat jejunum caused a robust

increase in action potential discharge (Barbara et al., 2007). Rat jejunal extrinsic afferents were also activated by mucosal 5-HT isolated from IBS colonic mucosal biopsies (Cremon et al., 2011). Similarly, supernatants generated from patients with active UC increased neuronal excitability and spike discharge when applied to isolated DRGs (Ibeakanma and Vanner, 2010). This data suggests that mediators released by the mucosa in both IBS and IBD can directly activate sensory neurons. Furthermore, intracolonic administration of IBS supernatants induced both allodynia and hyperalgesia to colorectal distension in mice (Cenac et al., 2007).

To date no studies have examined the effect of supernatants generated from acutely inflamed appendices on neuronal activity. Appendicitis is painful and usually represents an acute inflammatory event, one which may be governed by separate mechanisms to those involved in more chronic visceral pain states associated with IBS and IBD.

APP 1.1.2 AIMS

- Examine the effects of supernatants generated from an acutely inflamed appendix on a human afferent nerve recorded from a naïve, uninflamed appendix specimen

APP 1.2 METHODS

APP 1.2.1 GENERATION OF SUPERNATANTS

Once in the laboratory, the tissue was weighed (EK-600, A & D Instruments Ltd). The tissue was then incubated in Krebs buffer (NaCl 124mM, KCl 4.8mM, NaH_2PO_4 1.3mM, $MgSO_4.7H_2O$ 1.2mM, $CaCl_2$ 2.5mM, Glucose 11.1mM, $NaHCO_3$ 25.0mM), 2.5ml for each gram of tissue, at 37°C for 25 minutes, while being continuously carbongenated (95% O_2, 5% CO_2) (Raithel et al., 1999, Barbara et al., 2004, Barbara et al., 2007). The tissue was then removed and the supernatants were centrifuged at 2000rpm for 10 minutes, and subsequently decanted into a new falcon tube. The supernatants were then aliquoted and stored or stored in the falcon tubes, at -80°C until needed.

APP 1.2.2 APPLICATION OF SUPERNATANTS

In 1 experiment, human appendicitis supernatants were applied to a “normal” human appendix. The limited quantity of appendix supernatants that were available for use restricted the volume of supernatant that could be used. The human tissue bath was therefore reduced in volume from ~100ml to ~30ml, by pinning a block of styrofoam at either side of the appendix in the bath. In flow and out flow tubing was adjusted accordingly. The voltage of the inline heater was turned down, and a thermometer was used to ensure an appropriate temperature was maintained. The human appendicitis supernatants (6ml) were then superfused into the bath. The supernatants were applied to a naïve preparation that had not received any prior chemical or mechanical stimuli.

APP 1.3 RESULTS

APP 1.3.1 HUMAN APPENDICITIS SUPERNATANTS

Supernatants that were generated from a human inflamed appendix were applied to a HVA recording made from an appendix removed during colon cancer surgery. These supernatants gradually increased the HVA firing rate (average Δ firing rate 15 minutes after application 17.4 spikes $20s^{-1}$ vs. 30 mins 29.0 spikes $20s^{-1}$ vs. 45 mins 36.8 spikes $20s^{-1}$) (figure App.01).

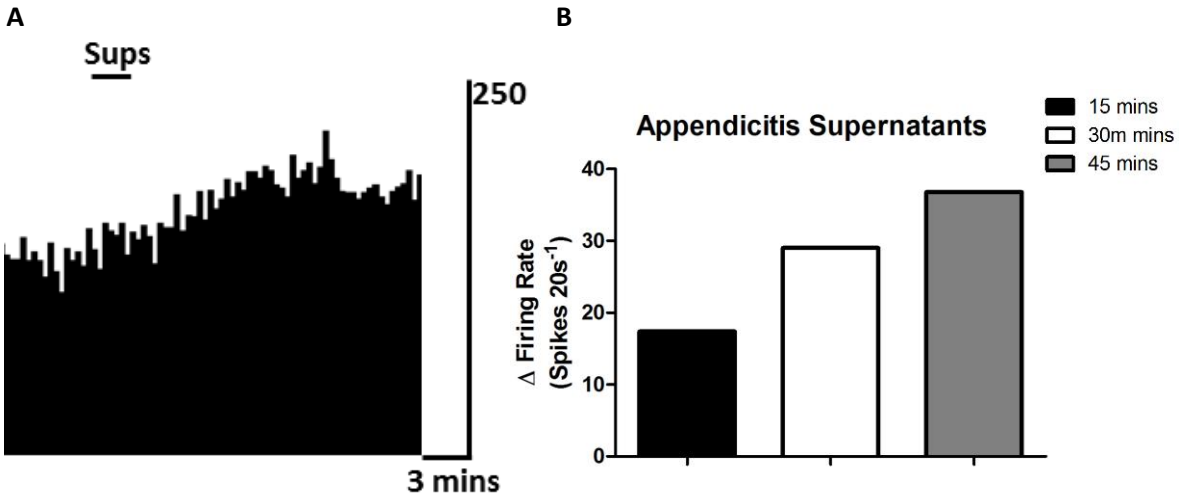

Figure App.01: Human appendicitis supernatants generated from an inflamed appendix activate HVAs. Firstly, the bath volume was reduced (to ~30ml). Human appendicitis supernatants (6ml) were applied to a HVA recording from a "normal" naïve appendix from a cancer resection specimen. The supernatants gradually increased HVA firing. A) A rate histogram showing the increase in afferent activity after appendicitis supernatant application. B) A bar graph demonstrating the increase in afferent activity over time.

APP 1.3.2 SUMMARY OF RESULTS

- Application of human appendicitis supernatants activated HVAs from a naïve uninflamed human appendix

APP 1.4 DISCUSSION

APP 1.4.1 SUPERNATANTS

This report demonstrates that supernatants generated from acutely inflamed appendices gradually increase the activity of HVAs from a naïve uninflamed appendix. To our knowledge this is the first time supernatants from appendicitis have been shown to activate extrinsic afferent nerves in any species. The gradual increase of HVA activity evident is similar to the gradual increasing activity in the plateau phase of the rat jejunal afferent response to PGE_2, supposedly mediated by the EP_2 receptor (Haupt et al., 2000). In addition PGE_2 gradually increases HVA activity. Appendicitis supernatants may well contain high levels of PGE_2, indeed PGE_2 release is elevated in chronic inflammatory conditions e.g. IBD (Hommes et al., 1996). However, the activation of HVAs by appendicitis supernatants is likely to be due to a myriad of mediators and cytokines. Determining what is contained in such supernatants would be of interest. Supernatants generated from IBD and IBS tissues could also be tested. Furthermore, examining effect of these supernatants on the HVA response to mechanical stimuli is important.

This report has demonstrated the activation of HVAs by supernatants generated from an acutely inflamed appendix. The use of supernatants from inflamed human tissue could be a useful tool to elucidate the mediators that are upregulated in inflammatory conditions, and their direct and sensitising effects on extrinsic afferent nerves innervating the human gut. Indeed, it is quite feasible to acquire supernatants from tissues from a variety of diseases including UC, CD, IBS, and appendicitis.

APP 1.5 CONCLUSION

Appendicitis supernatants can activate HVAs. Further studies examining the effects of supernatants, generated from diseased tissues such as appendicitis, CD, and UC, on HVAs are warranted.

APPENDIX PART 2: THE EFFECT OF TEMPERATURE ON HUMAN VISCERAL AFFERENTS

APP 2.1 INTRODUCTION

APP 2.1.1 TEMPERATURE

Afferent fibres from a number of nerves, e.g. vagus, splanchnic, saphenous, have been shown to be thermosensitive responding to shifts in both axonal and nerve ending temperature (Paintal, 1963, Paintal, 1965b, Paintal, 1965a, Franz and Iggo, 1968, Gurin and Itina, 1992, Li et al., 2002). One study demonstrated a distinction between the splanchnic and vagus nerves of a cat in their response to *in vivo* cooling of the gastric mucosa (Gurin and Itina, 1992). The spontaneous activity of the splanchnic nerve increased as the temperature of the mucosa decreased. Conversely, mucosal cooling led to a decrease in spontaneous discharge from the vagus nerve (Gurin and Itina, 1992). In another study, microfiber recordings were made from peripheral nerves projecting from excised L4 and L5 rat DRGs *in vitro* (Li et al., 2002). They found that ~95.7% of L4/5 DRG neurons were thermosensitive, with the majority (83.7%), responding to a decrease in temperature with a reduced spontaneous firing rate. A minority responded to a reduced temperature with an increase in their spontaneous discharge (12%) (Li et al., 2002). This project aimed to determine whether HVAs were responsive to gradual changes in tissue bath temperature.

APP 2.12 AIMS

- Examine the effect of temperature on HVA firing

APP 2.2 METHODS

APP 2.2.1 THERMOSENSITIVITY

To determine if HVAs were sensitive to a change in the temperature of the krebs solution in the bath, the in-line heater was turned off and the bath allowed to cool down to close to room temperature (24°C). Once the bath had reached 24°C, the inline heater was switched back on. The HVA firing rate was recorded at each degree point as the bath temperature decreased and while the preparation heated back up to 32°C. A response to temperature was defined as at least a 30% change in spontaneous activity rate (spikes s^{-1}) (Xie et al., 1995). The mean firing rate per second over the previous 60 seconds was calculated for each unit at each degree point. The means of the highest temperature (30-32°C) and the means of the lowest temperature (24°C) were then compared using a paired t-test.

APP 2.3 RESULTS

APP 2.3.1 THERMOSENSITIVITY

HVAs were sensitive to a change in bath temperature (N=3, n=7). 7/7 units responded to a gradual decrease in bath temperature with a decrease in their spontaneous activity rate. The highest HVA firing rate was evident at 32°C, 4.72±2.53 spikes s^{-1}. At the lowest temperature, 24°C, the mean unit firing rate dropped significantly to 1.22±0.91 spikes s^{-1}, $p<0.05$. 7/7 units responded to a gradual return of bath temperature to 32°C with a recovery of their spontaneous activity rate back towards baseline levels (before the bath temperature was decreased). The peak HVA firing rate after temperature recovery was evident at 32°C, 3.51±1.90 spikes s^{-1} (figure App.02).

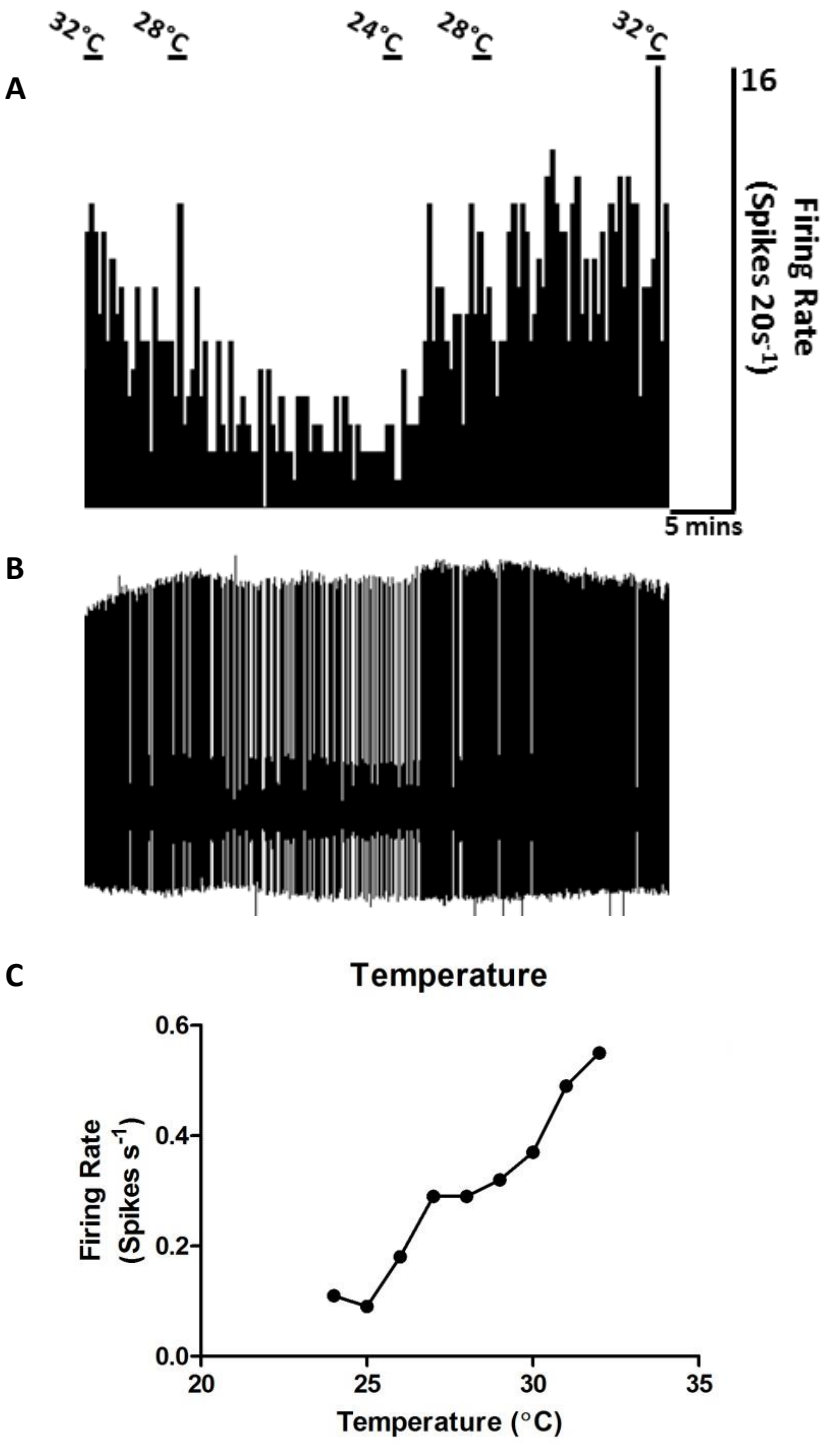

Figure App.02: HVAs were sensitive to changes in Krebs temperature in the tissue bath. A-B) The HVA firing rate reduced as the temperature of the Krebs in the tissue bath fell from 32°C to 24°C. As the Krebs was heated back up to 32°C the rate of HVA firing rate recovered back to baseline, as is evident in the rate histogram (A), and raw trace (B). C) Shows the recovery of the HVA firing rate as the temperature increases.

APP 2.3.2 SUMMARY OF RESULTS

- HVAs are sensitive to a change in bath temperature

APP 2.4 DISCUSSION

APP 2.4.1 TEMPERATURE

This project has demonstrated that HVAs are thermosensitive, and in this report they all fall under the previously described category of “warm sensitive” afferents. That is they respond to a gradual decrease in tissue bath temperature with a reduction in spontaneous activity rate, and vice versa. In rat DRG microfiber recordings a separate population of “cold sensitive” afferents were described, with response characteristics opposite to that of “warm sensitive” afferents. No “cold sensitive” HVAs were identified. In the present study, the temperature of the HVA terminals was reduced. However, “cold sensitive” afferents were identified by cooling the somata of rat DRG neurons (Li et al., 2002). However, this may not be an issue since, both axons and terminals are both sensitive to changes in temperature (Teliban et al., 2011). It is more likely that the low HVAs tested for thermosensitivity (N=3, n=7), and the relative rarity of these “cold sensitive” afferents in rat DRG (12%) would account for the lack of “cold sensitive” HVAs.

TRP channels, including TRPV1 (heat) and TRPA1/TRPM8 (cold) have been implicated in the sensing and transduction of thermal stimuli (Caterina et al., 1997, Story et al., 2003). In the future, it would be of interest to examine the role of these channels in the transduction of thermal stimuli in HVAs.

APP 2.5 CONCLUSION

HVAs respond to changes in bath temperature. In the future, the role of various heat sensitive channels on the transduction of thermal stimuli in HVAs could be examined.

APPENDIX PART 3: NOTES ON TISSUE COLLECTION

APP 3.1 ETHICS AND DATA STORAGE

Considerable time and effort goes into collecting human tissue before an experiment can begin. A number of procedures need to be in place for the efficient collection and use of human tissue from surgery. These range from obtaining ethics approval to collection of surgery schedules and tissue disposal. It requires basic understanding and continual communication between researchers, surgeons, pathologists and theatre staff. The human tissue act in 2004 outlined the need for both ethical approval and the patient's informed consent before tissue can be used for research. The patients consent also provides the rights to ownership of the data acquired from the tissue from experimentation (Sanger et al., 2013). Researchers should ensure that there is no direct link between any particular piece of tissue used during experimentation, or any resulting piece of data, and the identity of any patient (Sanger et al., 2013).

APP 3.2 OPERATING SCHEDULE

A fundamental aspect of collecting human tissue is knowledge of operating schedules. Elective colo-rectal surgeries operate on a weekly schedule, with most surgeons having 1 list each week, some having 1 list every 2 weeks. Operating schedules can be obtained from the NHS database by a NHS staff member and passed onto the research team. The operation schedule is subject to considerable change throughout the course of a week. Some reasons for changes in the operating schedule were, lack of high dependence unit, (HDU) beds, patients cancelling their procedures, and patients not being fit for surgery. Where possible, to minimise these changes, operating schedules were collected 1 day in advance. Surgical procedures of interest include, right and left hemicolectomy, anterior resection, small bowel resection, subtotal

colectomy, completion colectomy, proctocolectomy, APER, ileocecal resection, appendectomy for cancer, CD, UC, DD, trauma, chronic constipation, appendicitis. The operating schedule was then distributed via email to all researchers who hold ethical approval and are interested in collecting human tissue. No patient identifiable information was revealed. This gave researchers sufficient notice to plan experiments, ready their tissue collection kits and organise a party to consent the patient the morning of the surgery.

APP 3.3 CONSENT

A rota for consenting patients was adhered to, which included members of each lab interested in human tissue collection. Researchers needed to collaborate fully in this sense as each group may have a different consent form, meaning multiple consent forms may need to be signed by the patient. Researchers must have obtained a research governance framework certificate from the college, and must have shadowed a doctor consenting a number of times, before they are deemed fit to consent themselves. Researchers unfamiliar with working in a hospital environment may require time before becoming comfortable with interacting with patients about to undergo major surgical procedures. In the majority of cases patients were consented on the morning of their surgery, as this is when they arrived for surgery. On occasion in-patients could be consented the day before their surgery. Patients arrive into a ward at around 7am. Researchers aim to visit them by 8am given the first patient on the list is sent for by theatres at 8:30am. A basic explanation of the research and the procedure of how we obtain tissue after the operation is given and any patient questions answered, before the patients decides to whether to consent. After consent, a copy of the consent form as well as an information sheet containing a lay explanation of the research is given to the patient.

APP 3.4 EMERGENCY OPERATIONS

Tissue was also collected from emergency operations. These are usually restricted to suspected appendicitis cases but can include bowel resections. To find out about such

operations, researchers must obtain the emergency operating list each morning from theatre reception. The process is similar to elective procedures henceforth. However, emergency operations are less predictable as more serious cases can be intermittently admitted and given higher priority than the procedure of interest. Furthermore, and not to be underestimated is the turnover of emergency theatre staff, both from day to day and shift to shift. This means unfamiliarity with researcher needs i.e. fresh unfixed tissue, and a higher likelihood of forgetting to contact researchers when the tissue has been removed. Researchers must intermittently ring theatres to remind them of their interest in a particular procedure.

APP 3.5 INFORMING THEATRES

After the patient has given consent, an email is sent to inform the other researchers interested in the tissue. At approximately 10am, a phone call is placed to the appropriate theatre. Theatre staff are asked to ring researchers on a dedicated “tissue hotline” phone when the tissue specimen is ready for collection. It is requested that the specimen is not put into formalin prior to ringing researchers. A laminated sign with the “tissue hotline” phone number is given to each operating theatre to minimise confusion. Theatre staff and surgeons must be aware that the earlier they call after tissue removal the healthier the tissue is likely to be, avoiding unnecessary damage due to autolysis, ischemia etc.. Surgeries of interest mainly happen in the same theatres each week. This means that the researchers are dealing with the same theatre staff each week. The importance of this cannot be understated, since theatre staff and researchers get to know each other and their respective requirements. This leads to a much more reliable collection procedure, whereby very few specimens are missed or put into formalin, which was a considerable problem at the beginning of this project.

APP 3.6 EVOLUTION OF TISSUE COLLECTION PROCEDURE

After researchers receive a call on the “tissue hotline”, each lab is called to inform them the specimen is ready for collection. Tissue collection used to involve a representative from each

research group as well as a research surgeon scrubbing up and visiting theatres. Here, a forceps and scissors are borrowed from theatre and the specimen was dissected and divided between research groups in the sluice. A surgeon was necessary to cut the tissue as often cancers have to be identified and appropriate margins defined. An agreement with the pathology department, allowing researchers dissect the tissue in the sluice before the specimen reaches the pathologist was in place. On occasions when the cancer could not be identified by the research surgeon, the operating surgeon was required to demonstrated what portions of specimen can be taken. Again this highlights the communication and understanding required between the research team and the surgical team. The specimen is then stitched up and placed in formalin. Tissue collection details were filled out on the pathology form.

However, a meeting in late 2012 between pathologists, surgeons and researchers led to a change in the tissue collection process. Pathologists wanted to put in place their own standard operating procedure to ensure each specimen was dealt with appropriately and accordingly based on the type of tissue and the disease in question. All specimens were now to go through the core pathology department. Furthermore, pathologist requested photographs of some specimens before any tissue was taken, which could only be done using the core pathology imaging system. The core pathology department therefore had a much greater role in the new (and current) tissue collection procedure.

Once again, when researchers receive a call on the “tissue hotline” from theatre, each lab is called to inform them the specimen is ready for collection. A further call is placed to the core-pathology department to notify them of the incoming tissue. One or two representatives from the core collection groups then go to theatres to collect the specimen. At theatres the researchers stand in a hallway next to the doors that lead to theatres. This hallway does not require researchers to be scrubbed, and hence saves time. A member of the theatre staff bring the specimen in a labelled pot, along with a completed pathology form, to the researchers in

the hallway. The specimen is then brought to pathology, where a member of each research group is waiting. A trained biomedical scientist, working in the core-pathology department, was on-hand to dissect the tissue. The specimen was macroscopically examined and the area where the mesentery was cut from the patient during surgery marked with black ink. On occasion a photograph of the specimen was obtained. The specimen was then opened on the anti-mesenteric border, and cleaned out with water. The tumour or other disease was then identified by the biomedical scientist. Another photograph was taken if necessary. Areas of tissue available for research were then identified by the biomedical scientist and cut appropriately. Tissue was taken at least 10cm from any tumour. In addition, the specimen had to have continuity after tissue was taken, and a margin of about 3-4cm was left intact at either side of the tissue. Tissue removed for electrophysiology was taken carefully to ensure the mesenteric attachment to the ileum/colon/appendix wall was undisturbed, to ensure intact blood vessel arcades. After dissection tissue is placed in ice cold carbongenated Krebs buffer and transported to the laboratory on ice.

Tissue yield was noticeably lower during the first few months of this new collection system, however, after the initial slow down, tissue yield return back to normal levels. The new system also had the advantage of taking the responsibility of cutting the tissue away from the researcher, and returned it to the appropriately trained core-pathology staff. Of note was the marked difference between the amount of research tissue received from each biomedical scientist. This was due to a number of reasons including the biomedical scientists self-perceived lack of training/knowledge of the tissue pathology and therefore confidence in taking research specimens, especially related to cancer specimens, the researchers level of priority in the eyes of the biomedical scientist compared to their other daily tasks, and the relationship between the researcher and biomedical scientist. In addition, core pathology working hours can sometimes be a limitation to the collection of human tissue. Specimens occasionally came out late in the evening i.e. after normal working hours and usually between

17:30-20:00. The core pathology team could sometimes provide cover for these specimens; however, in some cases nobody was available to dissect the specimen, leaving the tissue uncollected. Communication and a good working relationship helped reduce the number of specimens lost for this reason.

The number of patients consented was consistent for the 1st and 2nd years of experimentation, 97 and 93 respectively. Similarly the collection rate was comparable between the 1st and 2nd years, 62 (63.9%) and 61 (65.6%) respectively. The last year has seen an increase in both the number of patients consented (119) and the number of specimens collected (80), and a comparable collection rate to previous years (67.2%) (figure App.03). This is primarily due to a focus on collecting more specimens from a second hospital, Whipps Cross University hospital, and better communication between researchers and pathologist/biomedical scientists regarding research needs.

APP 3.7 ADVANTAGES OF GETTING SURGEONS ON-BOARD

Although signing multiple research consent forms is not the ideal situation for a patient about to undergo surgery, it is the reality until a combined ethical approval is obtained. An enhanced involvement of surgeons in the research environment would be desirable. A more vested interest by surgeons could lead to, improved knowledge of operation schedules, including last minute changes to the operation schedule, consent of the relevant patients on presentation to the surgical clinic, which would reduce patient stress the day of the procedure. Furthermore, a greater knowledge of un-tapped emergency cases could be obtained from surgeons sending patients for surgery while on-call. Indeed, there has been considerable improvement in co-operation with surgeons, due mostly to the opening of the National Centre for Bowel Research and Surgical Innovation, which houses researchers and surgeons together, in an institute with state-of-the-art research labs. Bi-weekly meetings by the heads of each research and surgical

group ensure deep understanding of the needs and interest of each group, which inspires further co-operation.

APP 3.8 TISSUE DISPOSAL

Disposal of human tissue was done in accordance with the human tissue act 2004. After experimentation, ileum and colon was fixed in formalin and kept in the fridge. For disposal, intestinal tissue was triple contained and placed in biohazard bags for incineration. Appendix specimens were returned to pathology after experimentation. A full pathology form must be filled out, and the appendix fixed in a special labelled pot of formalin supplied by pathology.

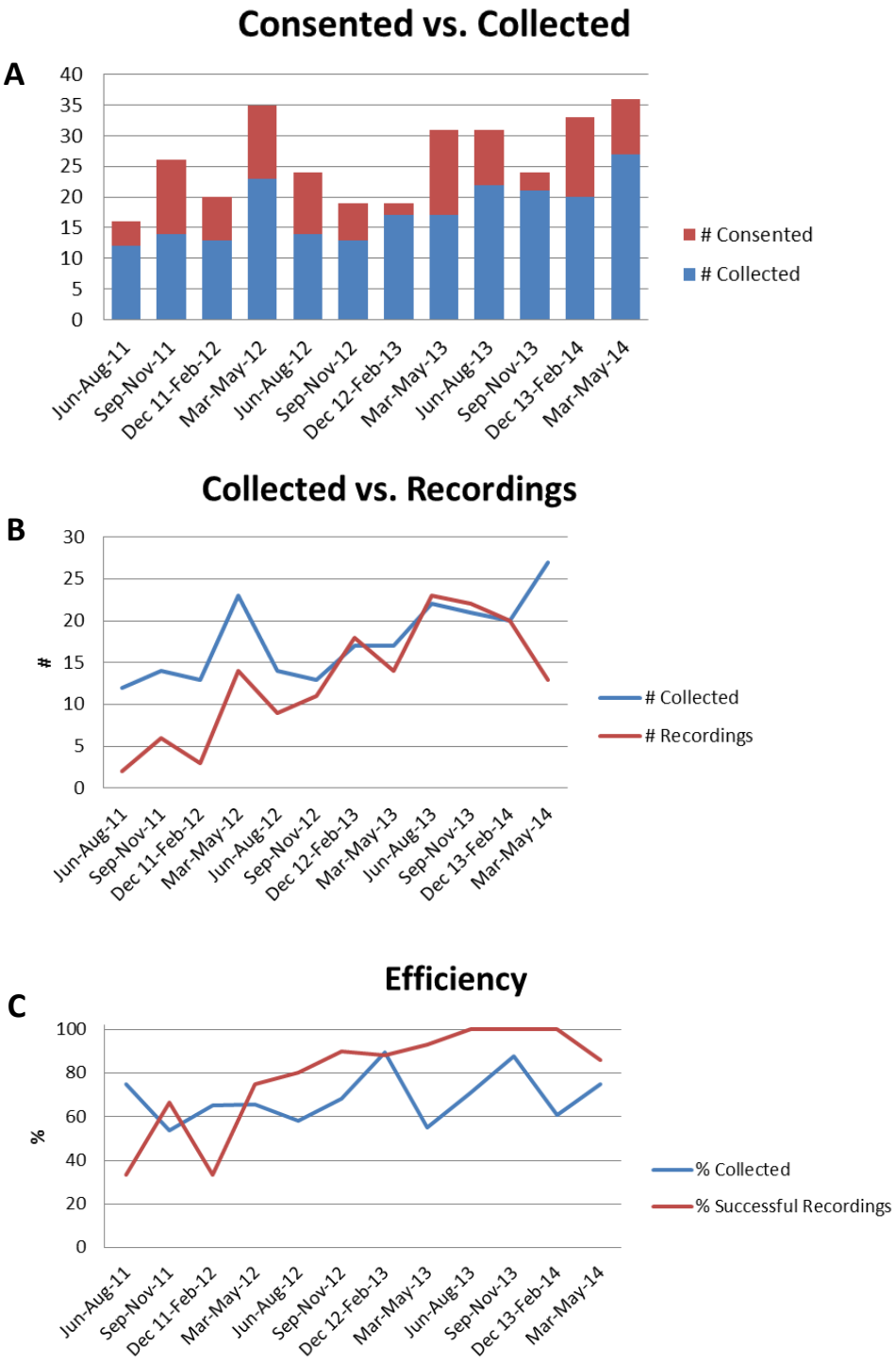

Figure App.03: Illustration of numbers of patients consented, tissues collected and recordings made over 3 month periods starting from June 2011 to August 2011. A) Demonstrates the number of patients consented and the number of specimens successfully collected. B) Tracks the percentage of consented patients from which tissue was collected and the percentage of successful recordings made from these tissues. C) Shows the number of collected tissues and the number of successful recordings made from these tissues.

APPENDIX PART 4: NOTES ON HUMAN VISCERAL AFFERENT RECORDINGS

Recording from HVAs was initially quite difficult and problematic. Twenty-five recordings were attained in the first year, a success rate of 57.5%. Furthermore, recordings routinely took 3-5 hours to attain. Year 2 represented a significant improvement, recordings were made from 45 tissues (52 recordings), and a success rate of 88.2%. Recordings also became more routine and took less time to acquire. This improvement continued into the third year with a 98.7% success rate and a total of 80 recordings from 74 tissues. The overall recording rate for 3 years was 85.5%.

The gradual refinement of this technique can be largely attributed to better identification of the nerve bundles in the mesentery. A general scan of the cut edge of the mesentery, mainly in the proximity of blood vessel arcades, was generally a better approach to finding a nerve, than immediate dissection. A scan of the mesentery often revealed the tips of nerves poking out, made more visible by ensuring a black background behind the relevant area of mesentery. Excessive dissection causes fat to be released into the bath, decreasing visibility, and liberating strands of connective tissue, which proceed to plot their invasion of your electrode. Excess fat was siphoned off by increasing pump speed during dissection. Indeed, the location of nerve bundles could be predicted in some tissues. For example, in the appendix, nerve bundles were located 3 quarters of the way up the caecal side of the mesentery, beside the biggest blood vessel arcade. Dense white spots of tissue near the cut end of the blood vessels in the mesentery invariably marked the location of a nerve bundle, and often as many as 3-4.

Initially dissecting a length of nerve was mostly unnecessary. Instead the sheath on the visible nerve needed to be removed, by grabbing the top of the nerve and pushing the whiter “fluffy” connective tissue down, like taking off a sock. This method revealed multiple

millimetres of the nerve, as it pushed the connective tissue and fat away from the base of the nerve, enough for 2-3 recording attempts. This unveil was usually limited by branching of the nerve which halted the retreat of the connective tissue sheath. When dissection was necessary and more of the nerve needed to be revealed, a method of dissecting around the nerve was employed. This involved dissecting away fat and connective tissue all around the nerve, such that a thick strand composed of nerve, connective tissue and fat remained isolated from the rest of the mesentery. Only then should the mesenteric fat and connective tissue be removed from the nerve (mainly using the "sock" method). A helpful teaching analogy used was digging a deep moat around a tree and its roots, before carefully stripping back the bark to reveal the wood underneath. Generally a few millimetres of "naïve" nerve (nerve that had not been previously sucked) was dissected out of the mesenteric fat and connective tissue sheath, before recordings were attempted. On occasion, if after a prolonged search, active nerves were not evident, a crude last chance dissection method was used. This involved using reverse dissection to pull and tease apart thick strands of clumped connective tissues, intermittent with a microscope scan to try and identify any new nerves freed up by the process.

Nerve bundles were translucent, with a white tiger stripe pattern, not to be confused with the similar looking but whiter connective tissue strands. Tiny pieces of thin black plastic for colour contrast were used to help with nerve bundle visibility and in distinguishing between the different strands in the bath. Nerve bundles exhibited considerable branching, both convergent and divergent, usually the latter, as the nerve tracks towards the wall of the gut. Thicker and thinner strands of these nerve bundles demonstrated activity, with no clear relationship between diameter and firing rate, although no hard data was obtained. A policy of sucking up the larger nerve first was employed.

The importance of tissue positioning and pinning in the bath was initially underappreciated. There must be enough space on each side of the tissue to apply stretching stimuli. Similarly the tissue needed to be robustly pinned on 1 side if it was to withstand any

force encountered during nerve characterisation protocols, mainly circumferential stretch. If only pinned at the corners, the tissue would shorten gradually overtime. Insufficient pinning, coupled with long protocols meant the tissue shortened pulling the nerve out of the electrode and ruining the recording and protocol. Using mosquito pins around the sides of the tissue can help avoid this, without damaging extensive parts of the tissue. It was important to ensure the full thickness of the bowel wall was pinned, as mucosal pinning did not prevent tissue shortening.

The practice of cauterising the tissue during laparoscopic surgeries, and thereby damaging the nerves, was thought to be a reason for the initially low recording rates. Tissue from open procedures, where cautery was not practiced, was therefore preferred. However, a number of successful recordings from cauterised tissue proved this not to be the case. Nerves were found to be active just millimetres away from the cauterised region (figure App.04).

Initially a small volume (~20ml) tissue bath was used for HVA recordings. This was only suitable for small pieces of human tissue with thin mesentery. If the tissue was too thick a lot of the mesentery protruded above the top level of the Krebs. In addition, the bath did not have facility to cannulate appendix specimens for distension preparations. Smaller volumes of drugs were added to preparations done in this bath, usually applied using a pipette straight over the tissue, but occasionally superfused into the bath. A bigger tissue bath (~100ml) was custom made, with accompanying cannula attachments. The bath was wider, longer and deeper and allowed even thick preparations to be completely submerged in Krebs. Drugs from then on were almost exclusive applied by bath superfusion.

Local application of drugs to a nerve's receptive field using a metal ring, as previously used in animal electrophysiological recordings was trialled. It proved difficult to place the ring steadily and securely on the tissue surface, due to the relatively unequal thickness and uneven

surface of the human tissue. However, better pinning and the use of silicone gel to seal the bottom of the metal ring has made using this method for future experiments a possibility.

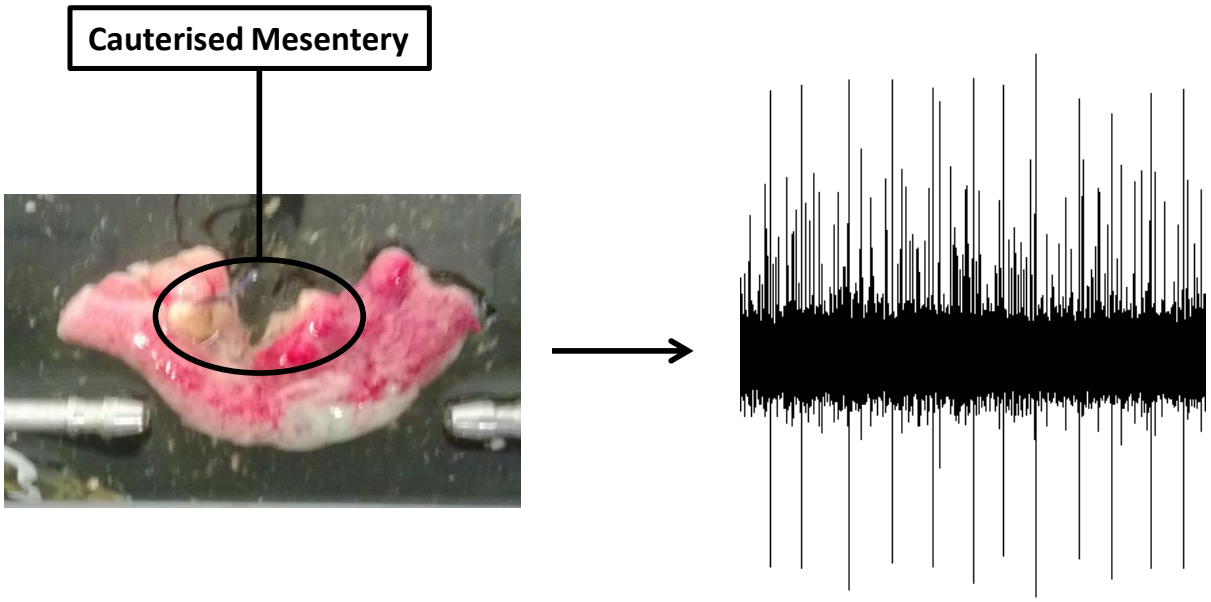

Figure App.04: Appendices from an emergency appendicectomies demonstrate HVA activity despite considerable cauterisation of its mesentery. A) Appendix in tissue bath. The black circle indicates the region of the mesentery that was cauterised during surgery. B) Raw trace of HVA activity from the same cauterised appendix. This demonstrates the feasibility of recordings from even heavily cauterised tissue, provided some intact mesentery remains.