Interplay between mast cells, enterochromaffin cells and afferent nerves innervating the gastrointestinal tract and influence of ageing

PhD thesis

By

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Abstracts:

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Yu, Y., Jiang, W. and Grundy, D. (2014) Involvement of 5-HT in TRPA1 sensory signaling from the GI tract and influence of ageing. The 1st Federation of Neurogastroenterology and Motility Meeting, Guangzhou, China. *Neurogastroenterology & Motility,* **26** (s1), 27.

Yu, Y., Nocchi, L., Rust, A., Keating, C., Jiang, W. and Grundy, D. (2013) Interplay between mast cells, 5-HT and TRPA1 in sensory signalling from the GI tract: Influence of ageing. 37th Congress of IUPS, Birmingham. *Proceedings of The Physiological Society,* PCD156.

Yu, Y., Wild, J., Kitsanta, P., Grundy, D., Adam, I. and Jiang, W. (2013) Mapping changes in TRP channel and enteroendocrine cell marker expression in aged human bowel. 37th Congress of IUPS, Birmingham.

Yu, Y., Adam, I., Kitsanta, P., Short-house, A., Grundy, D. and Jiang, W. (2012) Mast cell and enterochromaffin cell distribution in the aged human bowel. Joint International Neurogastroenterology and Motility Meeting, Bologna, Italy. *Neurogastroenterology & Motility, Volume 24, Issue Supplement s2,* pages 143.

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Keating, C., Nocchi, L., **Yu, Y.** and Grundy, D. (2012) Ageing and gastrointestinal sensory function: Altered colonic mechanosensory and chemosensory function in the aged mouse. Joint International Neurogastroenterology and Motility Meeting, Bologna, Italy. *Neurogastroenterology & Motility, Volume 24, Issue Supplement s2,* pages 74.

Keating, C., **Yu, Y.**, Zhang, Y., Jiang, W. and Grundy, D. (2012) Mechanical and chemical afferent nerve activity is attenuated in the aged mouse intestine. Digestive Disease Week, San Diego. *Gastroenterology Vol. 142, Issue 5, Supplement 1*, Page S-104.

Paper in preparation:

Ageing associated changes in the interplay between human mast cell, enterochromaffin cells and gastrointestinal afferents

Interplay between enterochromaffin cells and TRPA1 in sensory signaling from mouse gastrointestinal tract and influence of ageing

Acknowledgements

Summary of thesis

This thesis addressed the sensory functions of the gastrointestinal (GI) tract with an emphasis on the interaction between afferents nerves and other cell types such as enterochromaffin (EC) cells and mast cells, and also investigated the influence of ageing on these interactions.

Studies on mice focused on TRPA1, a central molecule in nociception and inflammation. TRPA1 signaling induced by allyl isothiocyanate (AITC) comprised direct activation of TRPA1 on extrinsic afferents, indirect activation of 5-HT$_3$ receptors on extrinsic afferents following 5-HT release form EC cells and indirect signals evoked by contractions. TRPA1 signaling was attenuated with advanced age. This could be attributed to reduced primary afferent innervation and decreased gene expression of 5-HT$_3$ in the gut wall.

Sensory functions of human GI tract were investigated morphologically and functionally. Some changes were identified in aged human bowel, including increased EC cells, mast cells, decreased sensory innervation and enhanced mast cell-afferent associations. Afferent activities were recorded from isolated human bowel using a novel *in vitro* model, allowing direct characterization of the mechanical and chemical sensitivities of human gastrointestinal afferents.

Abbreviation

4-HNE	4-hydroxy-2-nonenal		**IMAs**	intramuscular arrays
4-ONE	4-oxononenal		**LT**	low threshold
5-HIAA	5-hydroxyindoleacetic acid		**mMCP**	mouse mast cell protease
5-HT	5-hydroxytryptamine		**NG**	nodose ganglia
5-HTP	5-hydroxy-L-tryptophan		**NK1**	neurokinin 1
15d-PGJ$_2$	15-deoxy-prostaglandin J$_2$		**NMDA**	N-methyl-D-aspartate
AITC	allyl isothiocyanate		**NPY**	neuropeptide Y
ASIC	acid-sensing ion channel		**OCT**	optimal cutting temperature
B2	bradykinin receptor 2		**P2X**	purinergic receptor 2X
CgA	chromogranin A		**PAR2-AP**	protease-activated receptor-activating peptide
CNS	central nervous system			
DDC	amino acid decarboxylase		**PKA**	protein kinase A
DRG	dorsal root ganglia		**PLC**	phospholipase C
EC	enterochromaffin cell		**PYY**	peptide YY
ENS	enteric nervous system		**ROS**	reactive oxygen species
FITC	fluorescein isothiocyanate		**SERT**	serotonin reuptake transporter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase		**SP**	Substance P
			SynCAM	synaptic cell adhesion molecule
GI	gastrointestinal		**TNBS**	2,4,6-trinitrobenzene sulfonic acid
GPCRs	G protein-coupled receptors		**TNF**	tumor necrosis factor
HBSS	Hank's Balanced Salt Solution		**TPH**	tryptophan hydroxylase
HT	high threshold		**TRP**	transient receptor potential
IBS	Irritable Bowel Syndrome		**VIP**	vasoactive intestinal polypeptide
IGLE	intraganglionic laminar ending		**WDR**	wide dynamic range
IL	interleukin			

CHAPTER 1

General introduction

1.1 Anatomy and function of the GI tract

The gastrointestinal (GI) tract, also known as the digestive tract or the alimentary canal, a continuous, muscular digestive tube winding through the body, performs the function of digesting food and absorbing nutrients through its lining into the blood (Marieb, 2004). It is divided into the upper and lower GI tract by the Ligament of Treitz which suspends the duodenum from the diaphragm. The upper GI tract is composed of the esophagus, stomach and duodenum, while the lower GI tract consists of jejunum, ileum, caecum, colon and rectum (figure 1.1).

Each region of the GI tract is endowed with specialized structure to perform distinct function. The esophagus is a conduit that guides the chewed food from the mouth down to the stomach where it is stored. The stomach is not only a storage space, but also a secretory organ that produces gastric acid for digestion. The small intestine is divided into duodenum, jejunum and ileum. Enzymes from the pancreas and bile from the liver enter the duodenum to breakdown the food. The jejunum continues the digestive process and absorbs the majority of fatty acids, glucose, amino acids, vitamin, sodium and water into the bloodstream. The ileum is the final section of the small intestine where the remaining nutrients are absorbed, such as some amino acids, vitamin B-12 and most of bile salts. The lining of the small intestine is characterized by a large number of folds called plicae circulares as well as smaller finger-like projections called villi, which greatly increase the absorptive areas. Waste products of the digestive process are passed to the colon; by that time, most nutrients and 90% of the water have been absorbed. In humans, the colon is divided into four sections: ascending colon, transverse colon, descending colon and sigmoid colon. While the watery waste is moving forward along the colon, the excess water is gradually absorbed. When moving into the descending colon, the stools are solidified and stored as feces. Finally, fecal matter is evacuated through the rectum and the anal canal.
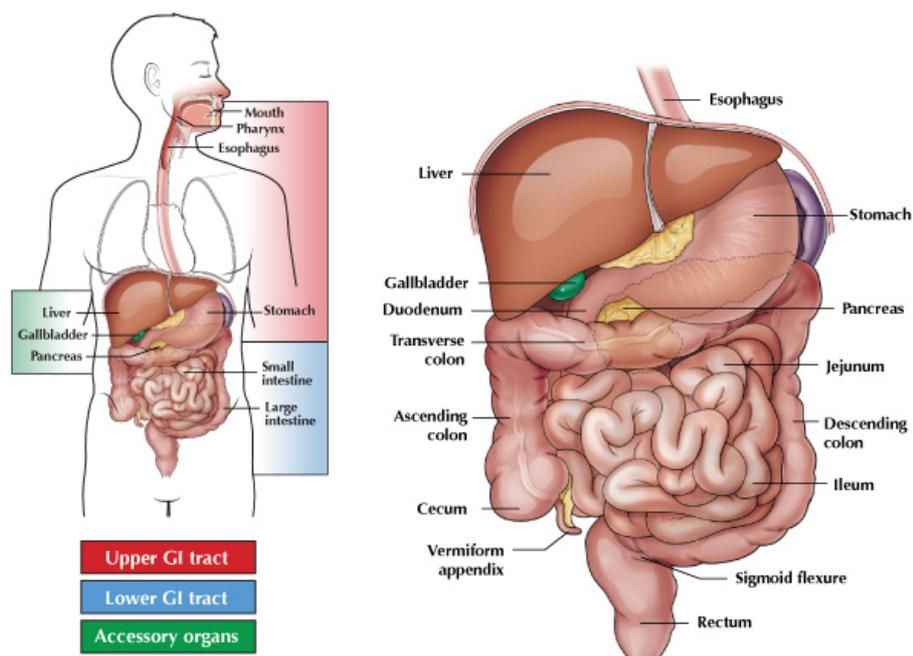
Figure 1.1 Representative graph showing the anatomy of human gastrointestinal system (Cawthray et al., 2012).

In association with accessory organs, all parts of the GI tract are well coordinated to perform the digestion and absorption effectively. Below the organ level, different types of tissues join in a structural unit and work collectively to implement specific functions. Typical gut wall can be divided into distinct layers in the following order: mucosa, submucosa, muscularis externa and serosa (figure 1.2).

The mucosa is the innermost layer of the GI tract, composed of epithelium, lamina propria and muscularis mucosae. Epithelium, lining the surface of mucosa, is a single layer of distinct types of epithelial cells, such as enterocytes, goblet cells, paneth cells, columnar cells and enteroendocrine cells, which contribute to the secretion and absorption. As epithelium is in direct contact with intraluminal contents, epithelial cells are joined together by tight junctions to form a barrier to harmful materials from the lumen. The laminar propria is a layer of loose connective tissue, lining beneath the epithelium. It contains capillaries and a central lacteal (lymph vessel), and is rich in immune cells. The muscularis mucosae is a thin layer of smooth muscle that separates laminar propria from the submucosa. In the small intestine, the mucosal surface protrudes into the lumen to form a number of finger-like projections called

villi to increase absorptive area. Differently, the mucosa of the large intestine appears smooth because there are no villi. Instead, numerous straight, tubular glands extend downward from the surface to the muscularis mucosae.

The submucosa is a layer of loose connective tissue that supports the mucosa and joins the mucosa to the underlying smooth muscle. Numerous blood vessels, lymphatic vessels and nerves are running through here.
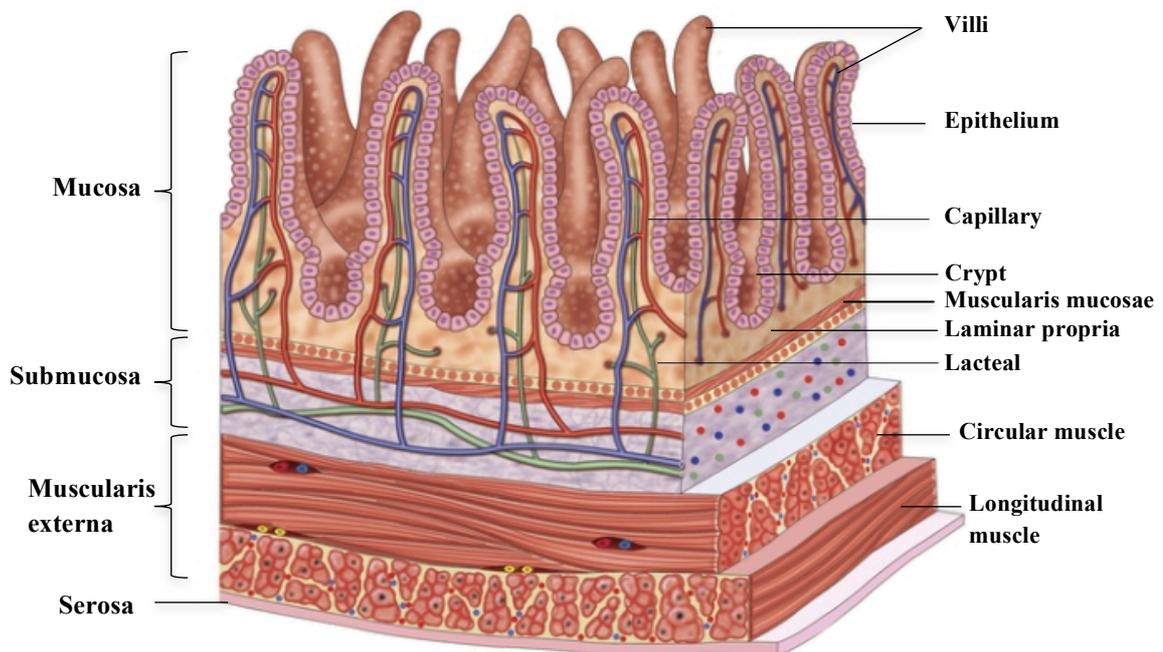
Figure 1.2 Diagram showing different layers of the small intestine (Wilkin and Brainard, 2012).

The muscularis externa is composed of an inner circular muscle layer and an outer longitudinal layer. The contraction and relaxation of these muscles are responsible for the segmentation and movement of intraluminal contents.

The serosa is a thin serous membrane covering the muscularis externa. In the small and large intestine, serosa is part of visceral peritoneum, which contains mesentery and mesocolon to attach intestines to the rear of the abdominal wall.

1.2 Sensory innervation of the GI tract

All the GI functions are controlled and coordinated by the autonomic nervous system. In addition to digestion and absorption, the GI tract must protect itself from potential harmful antigens and pathogens in the lumen. Essential to these functions is the ability to sense the stimuli in the lumen and gut wall in order to initiate appropriate responses. Consequently, the GI tract is endowed with an extensive sensory innervation, composed of intrinsic neurons of the enteric nervous system (ENS) and extrinsic nerves from the central nervous system (CNS) (figure 1.3).
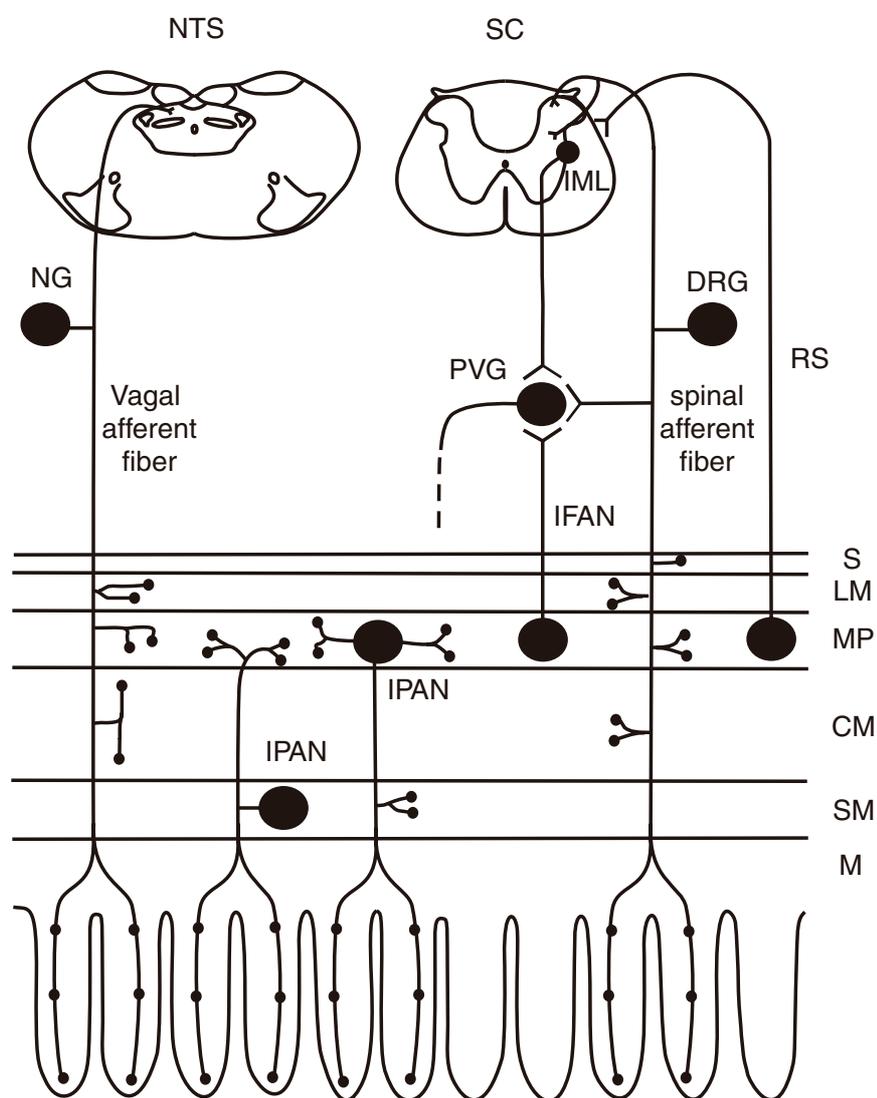
Figure 1.3 Schematic representation of the sensory innervation of the gut wall (Beyak et al., 2006). CM, circular muscle; DRG, dorsal root ganglia; IFAN, intestinofugal afferent neuron; IML, intermediolateral column; IPAN, intrinsic primary afferent neuron; NG, nodose ganglia; NTS, nucleus tracti, solitarii; PVG, prevertebral ganglia; RS, rectospinal; S, serosa; SC, spinal cord; SM, submucosa.

Enteric nervous system

The neurons of the ENS are distributed throughout the entire GI tract in two types of ganglia: myenteric plexuses located between the circular and longitudinal muscle layers and submucosal plexuses located in the submucosa. They play an important role in the control of gut motility including segmentation and peristalsis, regulation of local blood flow, fluid exchange and secretion.

The ENS has been described as a second brain for several reasons (Gershon, 1999). It is capable of coordinating reflexes independently of the brain and the spinal cord. The ENS comprises primary sensory neurons sensitive to mechanical and chemical stimuli, interneurons that form a connection between other neurons and motor neurons targeting on different effectors including smooth muscle, pacemaker cells, blood vessels, epithelia and mucosal glands (Costa et al., 2000).

Extrinsic sensory innervation of the GI tract

Although enteric neurons perform complex reflexes independently, the extrinsic sensory nerves convey signals of noxious and non-noxious events to the CNS, allowing the CNS to regulate crucial GI functions and enabling awareness of GI events.

Origins and projections

The extrinsic sensory nerves innervating the GI tract are derived from two sources: vagal and spinal nerves (figure 1.3). The vagal afferent nerves with their cell bodies located in jugular/nodose ganglia (NG), project centrally to the brainstem and peripherally to the visceral organs from the pharynx to the proximal colon. The cell bodies of spinal sensory neurons are located in the lower thoracic, lumbar and sacral segments of dorsal root ganglia (DRG), which relay sensory signals to the dorsal horn. The spinal afferents are divided into two subpopulations: splanchnic and pelvic. The splanchnic afferents innervate the entire length of the GI tract, whereas the pelvic afferents only supply the distal large intestine. These afferents run in mixed-nerve bundles that contain both afferent and efferent nerves. Vagal afferents are predominant over efferent fibers, accounting for 90% of total vagal nerves;

conversely, spinal sensory afferent nerves occupy a much smaller proportion, about 10~20% for the splanchnic nerve and 30~50% for the pelvic nerve. Furthermore, a greater proportion of afferent innervation of the GI tract is provided by the vagus nerve (Sengupta and Gebhart, 1994); however, this predomination diminishes rostrocaudally along the GI tract, especially in the distal colon and rectum where spinal afferents via pelvic nerve are predominant.
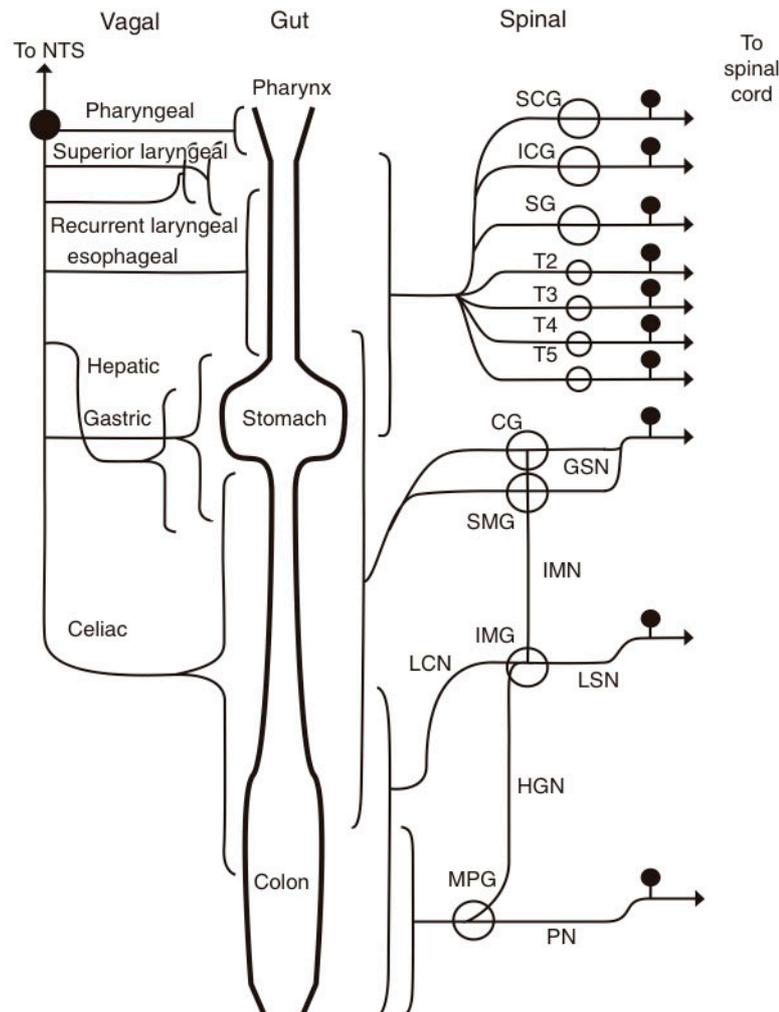
Figure 1.4 Schematic representation of the vagal and spinal innervation of the GI tract (Beyak et al., 2006). CG, celiac ganglia; GSN, greater splanchnic nerve; HGN, hypogastric nerve; ICG, intermediate cervical ganglia; IMG, inferior mesenteric ganglia; IMN, intermesenteric nerve; LCN, lumbar colonic nerve; LSN, lumbar splanchnic nerve; MPG, major pelvic ganglia; NTS, nucleus tracti solitarii; PN, pelvic nerve; SCG, superior cervical ganglia; SG, stellate ganglia; SMG, superior mesenteric ganglia.

Distribution of peripheral afferent terminals

Studies employing electrophysiologic and anatomic tracing techniques have demonstrated the presence of afferent nerve terminals within different layers of gut wall.

In the rat mucosa, a network of vagal afferent fibers have been shown extending through the laminar propria, with some terminals close to the basal laminar but not extending through the epithelial cells (Berthoud et al., 1995). Morphological studies on spinal mucosa afferent are limited, although their response to mucosal stroking has been documented by a few functional studies in the rodent (Lynn and Blackshaw, 1999; Brierley et al., 2004).

Most rat vagal afferent terminals in the muscle layers consist of two types of specialized endings, intraganglionic laminar endings (IGLEs) and intramuscular arrays (IMAs) (Wang and Powley, 2000); conversely, most spinal afferents terminating within the muscle layers have been suggested to be bare nerve endings (Mazzia and Clerc, 1997; Blackshaw and Gebhart, 2002), although some studies have described a type of rectal IGLEs resembling vagal IGLEs supplied by pelvic nerve in the guinea pig (Lynn et al., 2003; Olsson et al., 2004). IGLEs comprise a distinctive cluster of terminal puncta derived from a single afferent fiber, which encapsulate a myenteric ganglion or pole of ganglia. IGLEs have been demonstrated to be mechano-transduction sites of vagal tension receptors in the guinea pig (Zagorodnyuk and Brookes, 2000; Zagorodnyuk et al., 2001). IMAs innervating both circular and longitudinal muscle layers are composed of arrays of parallel terminal processes running in close proximity to each other and to muscle fibers. The structural characteristics of IMAs imply their role as mechanoreceptors; however, further investigation is required to confirm this hypothesis.

The sensory innervation of the serosa and mesentery is supplied by spinal afferents (Sengupta and Gebhart, 1994), and most of their endings are thought to be bare nerve endings.

Mechanosensitivity

Sensory afferent nerves innervating the GI tract can be classified according to the locations of their receptive fields as mucosal, muscular, serosal and mesenteric afferents. This approach of classification is based on in vitro recording on rat flat-sheet preparations and afferent responses to distinct mechanical stimulation including probing, stretch and mucosal stroking (Lynn and Blackshaw, 1999).

All mechano-sensitive afferent subtypes are responsive to probing, allowing accurate location of their receptive fields. In addition to probing, mucosal afferents are sensitive to light stroking of the mucosa and insensitive to distension or compression. Muscular afferents are characterized by their ability to response to stretch. Serosal and mesenteric afferents show responses to probing only.

Studies on mouse colonic spinal afferents have identified five different subtypes of afferents, three of which (mucosal, muscular and serosal) are conserved between splanchnic and pelvic nerves. Each pathway has a unique subtype: mesenteric afferent in the splanchnic nerve and muscular/mucosal in the pelvic nerve (Brierley et al., 2004).

Another commonly used mechanical stimulation is distension of tubular preparations by balloon inflation or saline infusion. Afferents can also be classified based on their response threshold to distension and adaptability. Studies on mouse small intestine have characterized three functional types of afferents by discriminating their responses to distension using spike sorting analysis (Rong et al., 2004). The first type is low-threshold unit whose discharges reach a peak rapidly in the first few millimeters of Mercury increase in pressure, but without further increase when pressure rises up to 60 mmHg. The second subtype, named as wide dynamic range afferents, also have a low threshold, whereas their discharges increase continuously following the increase of pressure (figure 1.5). The third type has a threshold more than 20 mmHg and thus classified as high-threshold unit. It is widely accepted that low-threshold units might originate from both vagal and spinal afferents, whereas high-threshold units are exclusively of spinal origin (Ozaki and Gebhart, 2001; Booth et al., 2001).

The molecular identity of the ion channels responsible for mechanical transduction has not been fully understood. Increasing evidence based on mutant rodent has suggested voltage gated sodium channel $Na_v1.7$ and $Na_v1.8$ might be the mechanotransduction sites (Nassar et al., 2005; Schuelert and McDougall, 2012). A recent study suggested that $Na_v1.9$ might be another candidate (Hockley et al., 2014). In addition, transient receptor potential (TRP) channels have been found to play an essential role in a number of mechanosensory systems. TRPV4 (Suzuki et al., 2003) and TRPA1 (Brierley et al., 2009) knockout mice appeared to have some deficits in the mechanical sensitivity.
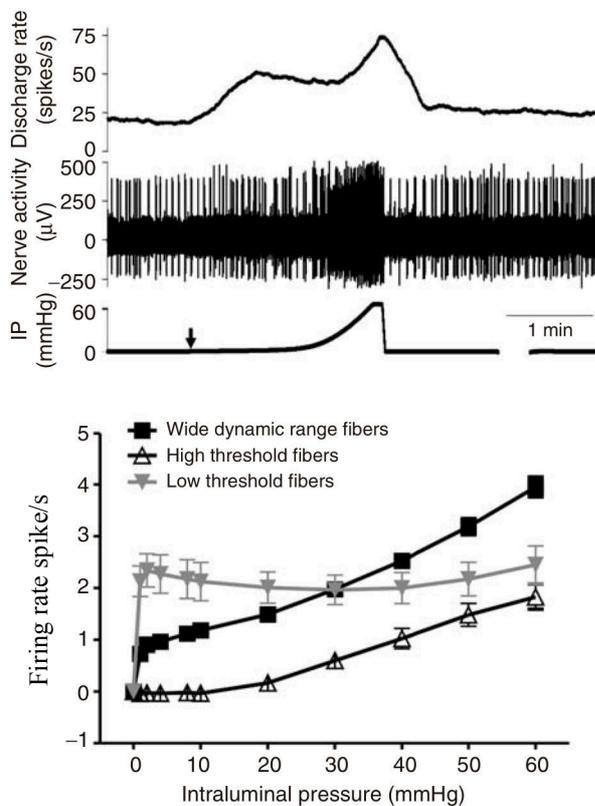
Figure 1.5 Example of multiunit mesenteric afferent response to ramp distension in mouse jejunum. The upper channel displays the overall afferent discharges in response to distension, whereas the lower diagram shows pressure-response relations of three functional classes of afferents. Reproduce from (Rong et al., 2004).

Chemosensitivity

Chemical sensitivity to nutrients, toxins and inflammatory mediators present in the lumen and gut wall are crucial functions of sensory afferents innervating the GI tract. Most of these actions are mediated by activation of ligand-gated ion channels and other membrane-bound receptors such as G protein-coupled receptors (GPCRs).

Ligand-gated ion channels, present on both vagal and spinal afferent, are gated by binding of various compounds, transmitters and protons. Some well-studied channels include 5-HT$_3$, TRP, purinergic receptor 2X (P2X), N-methyl-D-aspartate (NMDA), acid-sensing ion channels (ASIC) and nicotinic receptors. For example, the TRP family of ion channels plays a primary role in chemosensitivity. TRPV1 is gated by capsaicin, the pungent ingredient in chili peppers, and TRPM8, a cold-sensitive channel, is the receptor of menthol (Jordt et al., 2003). In addition, TRPA1 is gated by pungent natural compounds in cinnamon oil, mustard oil, garlic, wintergreen oil, clove oil and ginger (Bandell et al., 2004).

In addition to modulating afferent activity through ligand-gated ion channels, chemical stimuli may also act on plasma membrane protein receptors such as GPCRs that subsequently activate intracellular secondary signaling pathways. Increasing evidence has suggested an important role of GPCRs in regulating afferent signaling in response to nutrient-derived stimuli (Beyak et al., 2006), inflammatory mediators (Grundy, 2002) as well as mechanosensitivity (Page and Blackshaw, 1999). These modulations in concert with ligand-gated ion channels fine-tune afferent sensitivity to adapt with different conditions.

1.3 GI-related disorders

GI disorders such as gastritis, ulcerative colitis, Crohn's disease, irritable bowel syndrome (IBS) and diverticulosis are very common medical conditions. In the past decade, a large number of studies have addressed the pathogenesis of IBS.

IBS is defined as a functional bowel disorder characterized by chronic abdominal pain, discomfort, bloating and alteration of bowel habits, which cannot be explained by a visible structural or biochemical abnormality. However, some genetic, cellular and biochemical abnormalities in IBS have been reported, suggesting it is no longer purely functional (Barbara et al., 2004a; Spiller et al., 2007). Based on the predominant symptoms, it is classified into three subtypes: constipation-predominant IBS (IBS-C), diarrhea predominant IBS (IBS-D) and alternative IBS (IBS-A). IBS is diagnosed using either the Manning or the Rome criteria. The most recent Rome III criteria includes recurrent abdominal pain or discomfort at least 3 days a month in the past 3 months in association with two or more of the following: improvement with defecation, onset associated with a change in frequency of stool or onset associated with a change in form of stool (Longstreth et al., 2006).

Prevalence rates of IBS usually range from 5% to 20%, but vary markedly between countries and depend on the diagnostic criteria used (Spiller et al., 2007). An international survey shows that the overall prevalence of IBS is 11.5% in eight European countries (Hungin et al., 2003). This rate in the UK is 10.5%; furthermore, IBS accounts for 20~50% of referrals to gastroenterology clinics and thus is a substantial burden on primary healthcare services (Wilson et al., 2004).

Despite the large unmet medical needs, the pathogenesis of IBS hasn't been fully understood. One of the hypotheses is visceral hypersensitivity based on the observation that IBS patients have a lower pain threshold to colorectal distension or electrical stimuli (Ritchie, 1973; Whitehead et al., 1990; Sinhamahapatra et al., 2001; Verne et al., 2001; Dong et al., 2004). An abnormal bi-directional interaction between the immune system and nervous system is believed to be crucial in the pathophysiology of IBS. Increasing evidence has suggested an important role of mast cells and enterochromaffin cells (ECs) in IBS hypersensitivity.

Increased mast cells, EC cells and T lymph cells are observed in the colonic biopsies from IBS patients (Lee et al., 2008). The severity of abdominal pain in IBS patients has been found positively correlated with serotonin release (Cremon et al., 2011) and anatomical mast cell-nerve association (Barbara et al., 2004b).

2,4,6-trinitrobenzene sulfonic acid (TNBS) induced visceral hypersensitivity is abolished in mast cell deficient rats (Ohashi et al., 2008). Treatment with mast cell stabilizer, disodium cromoglycate, improves the symptoms in a large proportion of IBS-D patients (Leri et al., 1997).

Upon stimulation, EC cells and mast cells release 5-HT, which is an important neurotransmitter involved in signal transduction of visceral stimuli (Grundy, 2008). The contribution of serotonin in the pathology of IBS is demonstrated by the therapeutic effect of 5-HT_3 antagonists and 5-HT_4 receptor agonists on many IBS symptoms (De Giorgio et al., 2007; Ford et al., 2009). Animal models of visceral hypersensitivity are associated with increased EC cells, enhanced serotonin release and impaired uptake (Linden et al., 2003; O'Hara et al., 2004). In addition, mucosal supernatants from IBS patients significantly increased afferent firing recorded from rat jejunal nerves (Barbara et al., 2007), which could be blunted by 5-HT_3 receptor antagonist granisetron (Cremon et al., 2011).

1.4 Mast cells

Mast cells are long-lived tissue resident cells, extensively distributed at sites where host tissue in contact with external allergens and microorganisms, such as upper dermis, the respiratory tract and the GI tract (Beil et al., 2000). They belong to the immune system and are best known for their role in allergy and anaphylaxis. The most striking morphological characteristic of mast cells is numerous cytoplasmic secretory granules filled with preformed mediators, such as histamine, serotonin, protease, heparin, proteoglycans and cathepsin; in addition, many cytokines, lipid mediators and growth factors are newly synthesized after activation (Buhner and Schemann, 2011). The prominent diversity of mediators released from mast cells indicates their broad involvements in a wide variety of physiological and pathological activities.

Mast cells are derived from bone marrow and leave bone marrow as immature precursors. The sites where mast cells settle and mature largely determine their characteristics. Rodent mast cells can be divided based on their locations into mucosal mast cells and connective tissue mast cells. In humans, mast cells are classified by their protease profiles, one subtype expressing tryptase only and the other expressing both tryptase and chymase.

Interaction between mast cells and nerves has been a hot topic in the past two decades for its potential involvement in the pathogenesis of functional bowel disorders. It is estimated that 70% of intestinal mucosal mast cells are in direct contact with nerves with another 20% within 2 μm (Stead et al., 1989; Stead, 1992). Furthermore, the severity of abdominal pain in IBS patients is positively correlated with anatomical mast cell-nerve association (Barbara et al., 2004b). In animal model of stress-induced hypersensitivity, the number of mast cells in close proximity to PGP 9.5 or CGRP-positive nerve fibers was increased in rat colon (Barreau et al., 2008). This anatomical association has functional relevance as crosstalk between mast cells and nerves might modulate afferent sensitivity and alter mucosal homeostasis.

Based on animal experiments, upon stimulation mast cells release mediators that stimulate or sensitize afferent nerves, resulting in release of neuropeptides, which, in turn, further excite mast cells (Shanahan et al., 1985; Repke and Bienert, 1987; Purcell and Atterwill, 1995). As shown in figure 1.6, a number of signaling molecules and receptors involved in this crosstalk have been identified (Buhner and Schemann, 2011). This bidirectional communication forms a positive feedback loop, which might amplify inflammatory response within the GI tract. Imbalanced or exaggerated amplification is a likely mechanism contributing to visceral hypersensitivity.

Mast cell to nerve signalling	
Histamine	H1, H2, H3, H4
Proteases	PAR1, PAR2, PAR4
Cytokines	?
Leukotrienes	?
Prostaglandins	?
(Serotonin)	$5\text{-}HT_3$, $5\text{-}HT_{1p}$

Nerve to mast cell signalling	
Substance P	↑
CGRP	?
Acetylcholine	(↑)
Noradrenalin	↓
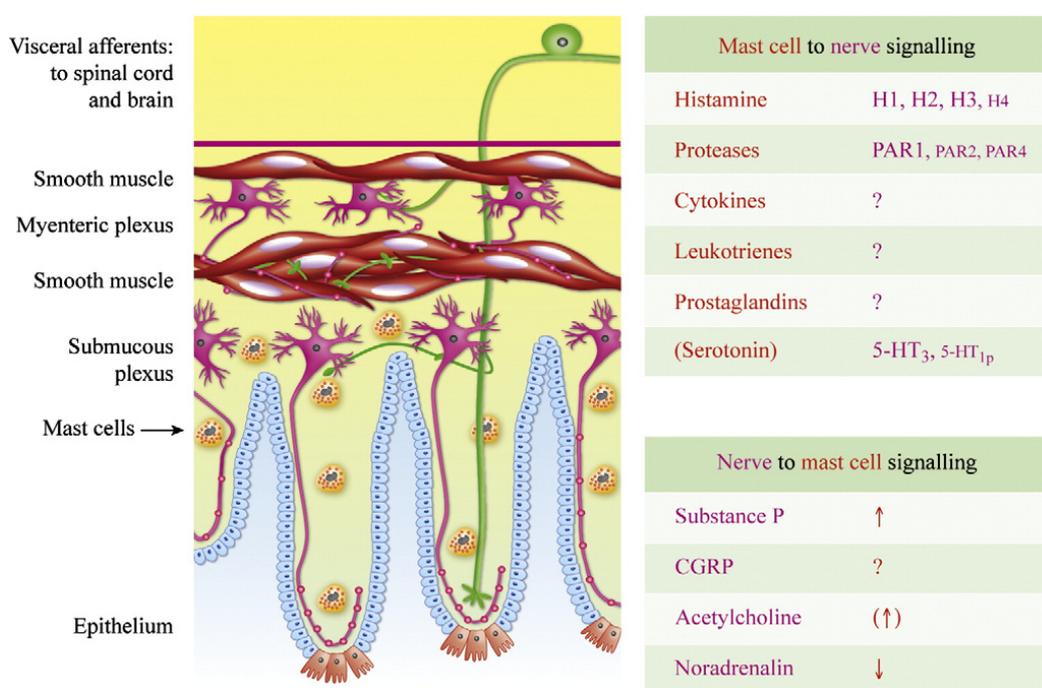
Figure 1.6 Schematic illustration of mast cell-nerve interactions in human gut and a summary of involved signaling pathways. Smaller font indicates smaller contribution. Image from (Buhner and Schemann, 2011).

1.5 Enterochromaffin cells

Enterochromaffin (EC) cells are a subtype of endocrine cells that reside in the epithelia lining of the GI tract. This name reflects their straining via a chromium salt reaction that they share with chromaffin cells in the adrenal medulla (Rindi et al., 2010). EC cells have apical microvilli exposed to the intestinal lumen to monitor intraluminal contents, which might activate EC cells to release mediators like serotonin (5-HT) across the basolateral membrane into the lamina propria (Raybould, 1999).

Although 5-HT is present in the CNS and platelets, about 90% of the body's 5-HT resides in the gut (Gershon and Tack, 2007), where it is synthesized by EC cells and to a lesser extent by serotonergic neurons within the myenteric plexus in the mouse (Gershon et al., 1965). It is generally believed that 5-HT is also present in murine mucosal mast cells but not in human, although some studies suggest a very low amount of 5-HT in human mast cells (Kushnir-Sukhov et al., 2007). 5-HT can be synthesized from L-tryptophan via 5-hydroxy-L-tryptophan (5-HTP) by a metabolic pathway composed of two enzymes: tryptophan hydroxylase (TPH) and amino acid decarboxylase (DDC). EC cells contain one form of enzyme for serotonin's biosynthesis, tryptophan hydroxylse-1 (TPH-1), whereas enteric serotonergic neurons have a different enzyme TPH-2 (Cote et al., 2003). The impact of mucosal 5-HT is terminated by a serotonin reuptake transporter (SERT) that transports 5-HT into epithelial cells.

5-HT activates a group of G-protein-coupled receptors (5-HT_1, 5-HT_2, $5\text{-HT}_{4\text{-}7}$) and only one exceptional ligand-gated ion channel (5-HT_3) to influence various biological and neurological processes. Many of these receptors are distributed in the GI tract. In terms of sensory signaling, most attention has focused on 5-HT_3 and 5-HT_4. In particular, immuno-labeled 5-HT_3 receptors are present on both NG and DRG neurons innervating rat GI tract (Hicks et al., 2002; Raybould et al., 2003), and 5-HT_3 expressions in the murine NG are much higher than DRG (Peeters et al., 2006). 5-HT activates vagal afferents in the upper GI tract exclusively via 5-HT_3 receptors in the ferret (Blackshaw and Grundy, 1993), whereas it activates spinal afferents through both 5-HT_3 and non-5-HT_3 receptors in the rat (Hicks et al., 2002).

In rat small intestine, afferent discharges triggered by 5-HT are composed of a direct excitation through $5-HT_3$ and indirect actions through increased motility, which is likely to arise from 5-HT receptors on enteric neurons and muscles (Hillsley et al., 1998).

EC cells are thought to be an important interface between the hostile environment of the gut lumen and the afferent nerves that are unable to penetrate into the epithelium. They function as taste cells of the GI tract by releasing 5-HT in response to a variety of stimuli including mucosal stroking, nutrients (e.g., glucose), toxin (e.g., cholera toxin, chemotherapeutics) and adenosine (Racke and Schworer, 1991; Kellum et al., 1999; Grundy, 2008). Under physiological conditions, released serotonin may interact with the closely apposed sensory nerves to orchestrate proper motor and secretion reflexes to facilitate the transition of luminal contents as well as digestion and absorption of nutrients. However, in pathophysiological state, 5-HT, in conjunction with other inflammatory mediators, may induce abnormal secretory and motor responses and result in nausea, vomiting, diarrhea, constipation or visceral pain. 5-HT has been demonstrated to be a major mediator of emetic and diarrhoea responses to luminal toxins; for example, intravenous cisplatin, a chemotherapeutic agent used in the treatment of tumors, (Andrews et al., 1990) and radiation (King et al., 1999)-induced emesis is significantly attenuated by $5-HT_3$ receptor antagonist.

Increasing evidence has revealed the involvement of 5-HT bioavailability in GI inflammation and functional disorders. Increased EC cells, enhanced serotonin release and impaired SERT activity have been shown in clinical (Coleman et al., 2006; Minderhoud et al., 2007) and animal models of inflammatory bowel diseases (Linden et al., 2003; Coates et al., 2004; O'Hara et al., 2004). Similarly, increased EC cells with enhanced spontaneous release of 5-HT have been reported in IBS patients (Spiller et al., 2000; Cremon et al., 2011). Furthermore, a positive correlation between 5-HT release and the severity of abdominal pain has been found in IBS patients (Cremon et al., 2011). These observations suggest a role of serotonin in the pathology of IBS. Indeed, $5-HT_3$ antagonists such as alosetron have been proved to relieve symptoms in female patients with non-constipated IBS, and $5-HT_4$ receptor agonist tegaserod has been found effective in IBS-C (De Giorgio et al., 2007; Ford et al., 2009).

1.6 Transient receptor potential ankyrin 1 (TRPA1)

Transient receptor potential (TRP) channels are a superfamily of ion channels mostly located on the plasma membrane of numerous cell types and conserved across a wide range of species. TRP channels were initially discovered in a *trp*-mutant strain of *Drosophila* characterized by a transient response to steady light, and as such were subsequently named as "transient receptor potential". Based on sequence homology, TRP channels are classified into seven subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin) and TRPN (NO mechanoreceptor potential C). Most TRP channels are composed of 6 transmembrane segments (S1-S6) with a pore loop between S5 and S6 and intracellular N- and C-terminal (Nilius and Owsianik, 2011).

The fundamental goal toward understanding the mechanisms of sensation or nociception is to identify the molecules that are responsible for stimulus detection and excitation or sensitization of primary sensory nerves. TRP channels have attracted attention as some members serve as receptors for a variety of natural plant compounds that excite nociceptors to elicit pain and inflammation (Clapham, 2003; Wang and Woolf, 2005). For example, noxious heat-sensitive TRPV1 is the receptor for capsaicin, the pungent ingredient in chili peppers, and TRPM8, a cold-sensitive channel, can be activated by menthol. In the past decade, increasing evidence has suggested that TRPA1, the sole member of the TRPA subfamily, is another candidate essential for neurogenic inflammation and nociception.

TRPA1, defined by the ankyrin repeats in the N-terminal, was first discovered in cultured fibroblasts by Jaquemar (1999) and coworkers. Similar to most TRP channels, TRPA1 consists of 6 transmembrane domains with intracellular N- and C-terminals (figure 1.7). Several cysteine residues in the N-terminal are implicated in TRPA1 activation by reactive agonists.
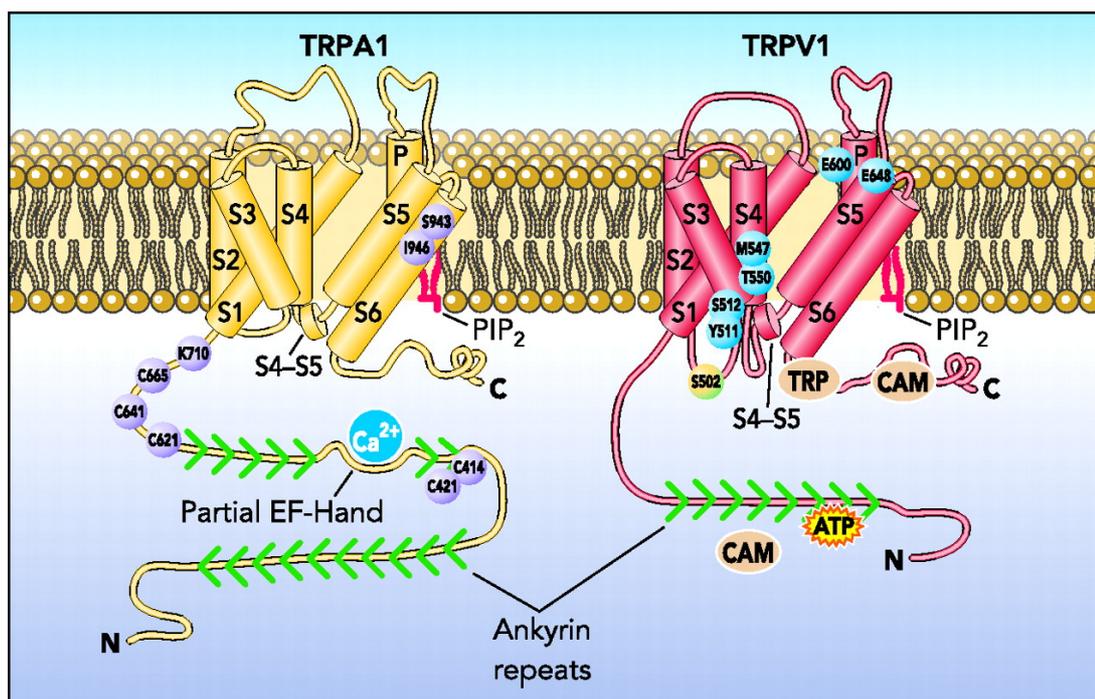
Figure 1.7 Schematic image showing the structures of TRPA1 (left) and TRPV1 (right) monomers. The intracellular N-terminal of TRPA1 has about 14 ankyrin repeats, containing some cysteine and lysine residues that are responsible for activation by agonists as well as a partial EF-hand domain involved in calcium-dependent gating. TRPV1 is gated by capsaicin binding to multiple intracellular regions, and also gated by protons interacting with acidic residues at the extracellular end of S5 and within the pore loop. N- and C-terminals have interaction sites for calmodulin (Cam) and ATP. Domains with positive charge in the C-terminal of TRPA1 and TRPV1 may serve as interaction sites of phosphatidylinositol-4,5-bisphosphate (PIP2), the substrate of phospholipase C. (Bessac and Jordt, 2008)

Activation of TRPA1

TRPA1 can be activated by pungent natural compounds in cinnamon oil, mustard oil, garlic, wintergreen oil, clove oil and ginger (figure 1.8), among which allyl isothiocyanate (AITC, mustard oil), cinnamaldehyde and allicin are specific to TRPA1 (Bandell et al., 2004; Jordt et al., 2004; Bautista et al., 2006). These compounds activate TRPA1 through covalent modification of reactive cysteine residues in the ankyrin repeat domains (Macpherson et al., 2007). Topical application of these agents has long been known to excite sensory nerve afferents and thus result in acute pain and neurogenic inflammation through release of neuropeptide, purines and other transmitters in the mouse (Bautista et al., 2006).

In addition to environmental irritants, a few endogenous TRPA1 agonist have been identified in the rodent, such as 4-hydroxy-2-nonenal (4-HNE) and 4-oxononenal (4-ONE) produced through peroxidation of membrane phospholipids by reactive oxygen species in response to oxidative insults during tissue injury and inflammation (Trevisani et al., 2007; Graepel et al., 2011). Additionally, a cyclopentane PGD_2 metabolite, 15-deoxy-prostaglandin J_2 (15d-PGJ_2) (Cruz-Orengo et al., 2008) and nitrooleic acid (Taylor-Clark et al., 2009) produced by reactive nitrogen species acid also activate TRPA1.

The role of TRPA1 as a direct transducer for noxious cold is controversial. It has been suggested that TRPA1 functions as a detector of noxious cold based on the observation that cells heterologously expressing TRPA1 could be activated by temperature below 17 °C (Story et al., 2003; Bandell et al., 2004). Conversely, other studies have shown that TRPA1 is not implicated in cold sensitivity (Jordt et al., 2004; Bautista et al., 2006). A recent study has revealed that most cold sensitive DRGs from rat are responsive to AITC, whereas only a small proportion of mouse thermosensors respond to AITC (Teichert et al., 2014). Similarly, it is controversial if TRPA1 is required for mechanosensitivity (Bautista et al., 2006; Kwan et al., 2006; Brierley et al., 2009; Cattaruzza et al., 2010).

Another mechanism of TRPA1 activation is modulation by G protein-coupled receptors through secondary messenger signaling. For example, bradykinin, a proalgesic and proinflammatory mediator, indirectly activates TRPA1 through the Gq and phospholipase C (PLC) coupled bradykinin receptor 2 (B2) (Bandell et al., 2004; Bautista et al., 2006; Wang et al., 2008).
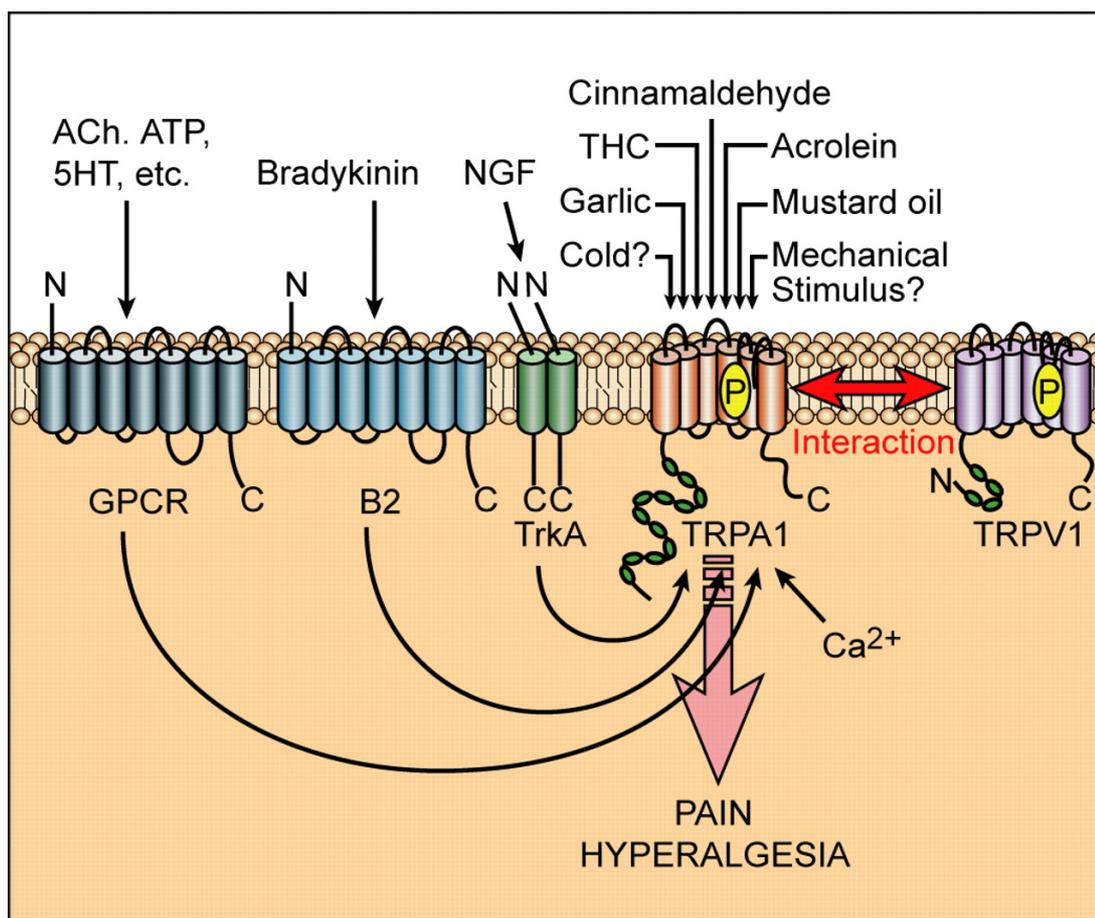
Figure 1.8 Schematic illustration of TRPA1 activation (Garrity, 2011).

TRPA1 expression in the GI tract

The GI tract is innervated by both intrinsic neurons and extrinsic nerves that originate from the NG and DRG. Combination of retrograde tracing and in situ hybridization has revealed TRPA1 expression in a high proportion of NG and DRG neurons specifically innervating mouse intestine (Brierley et al., 2009). In addition to extrinsic neurons, TRPA1 mRNA has been detected in whole-thickness intestinal specimens (Penuelas et al., 2007), muscularis externa and mucosa (Poole et al., 2011) in the mouse. Furthermore, a study has demonstrated that TRPA1 is highly expressed in human and rat EC cells (Nozawa et al., 2009).

Immunoreactivity for TRPA1 has been identified in a subset of mouse colonic DRG neurons containing sensory neuropeptide SP and CGRP (Cattaruzza et al., 2010). It is also found in peripheral nerve endings in different layers of mouse colon frequently colocalized with CGRP (Brierley et al., 2009). Another study has shown that TRPA1 immunoreactivity is prominently localized to myenteric and submucosal

ganglia and also present throughout the surface epithelial cells in the mucosa of mouse intestine (Poole et al., 2011). These inconsistent reports about TRPA1 distribution patterns within the gut wall may be attributed to the lack of specific antibodies.

TRPA1 in visceral pain and inflammation

TRPA1 is expressed in sensory neurons that serve as polymodal nociceptors because they can be activated by pungent irritants, noxious cold and endogenous proalgesic mediators. Activation of TRPA1 elicits acute nociceptive behavior in mice, followed by neurogenic inflammation and marked hypersensitivity to thermal and mechanical stimuli, which is absent in TRPA1 knockout animals (Bandell et al., 2004; Bautista et al., 2006). In line with these behavior studies, introcolonic administration of TRPA1 agonists, mustard oil and 4-HNE, augments visceromotor responses to colorectal distention in the mouse (Cattaruzza et al., 2010). Similarly, TRPA1 agonists ATIC and cinnamaldehyde as well as the inflammatory mediator bradykinin induce mechanical hypersensitivity to probing, which is absent in TRPA1 knockout mice (Brierley et al., 2009).

In animal models of colitis, TNBS induces a TRPA1-dependent hypersensitivity to colorectal distention accompanied with an up-regulation of TRPA1 in DRG neurons innervating the colon; this hypersensitivity is suppressed either by knockout of TRPA1 (Cattaruzza et al., 2010) or reducing TRPA1 expression using antisense oligoxynucleotide (Yang et al., 2008). Another study on mosue has demonstrated that TNBS directly activates TRPA1 on colonic DRG neurons by modification of cysteine and lysine residues in the N-terminal, which induces massive release of CGRP and SP to promote neurogenic inflammation; these effects can be prevented by either knockout of TRPA1 or pretreatment with TRPA1 antagonist (Engel et al., 2011).

These observations suggest that TRPA1 plays a critical role in the sensation and modulation of abdominal pain and inflammation, and thus it is a very promising therapeutic target for many gastrointestinal diseases characterized by abdominal pain.

1.7 Ageing and the GI tract

The world population is ageing at a very rapid rate, which boosts emerging concerns in age-related disorders. Comparing with cardiovascular, musculoskeletal and nervous system, the GI tract is generally less affected by ageing. However, a variety of gastrointestinal disorders have a major impact on the quality of life in the elderly, such as dysphagia, dyspepsia, anorexia, constipation and fecal incontinence (Johanson et al., 1989; Wald, 1990; Lindgren and Janzon, 1991; Morley, 1997; Firth and Prather, 2002). Most commonly, the incidence of motility or transit problems is increasing with age, including delays in gastric emptying and intestinal transit associated with fecal stasis (Jost, 1997; O'Mahony et al., 2002; Hays and Roberts, 2006; Norton, 2006). The immune system within the GI tract is impaired with ageing, resulting in increased susceptibility to infections in the elderly (Ogra, 2010). These conditions, individually or collectively, contribute to malnutrition and thus increased frailty and vulnerability of elderly population.

As part of the process of ageing, dramatic changes arise within most organs and tissues, and the GI-tract has no exception. These changes can help the understanding of the causes underlying the increased incidence of GI conditions with advanced age. Although motility problems are prevalent in the GI tract of the elder people, it is unknown whether this is due to impaired function of smooth muscles themselves or the neurons that regulate their activities. It has been demonstrated that smooth muscle contractility in response to electrical stimuli is not impaired in aged rat (Hoyle and Saffrey, 2012). However, some changes in the cellular level have been described. Isolated colonic smooth muscle cells from aged rat display a decrease in calcium channel currents and intracellular Ca^{2+} levels (Xiong et al., 1993). Abnormalities of mitochondrial structure and apoptosis of smooth muscle cells have been identified in the colon of aged animals. In addition, a reduction in the number and network of interstitial cells of Cajal has been reported in aged human stomach and colon (Gomez-Pinilla et al., 2011).

Table 1.1 Summary of age-related changes in gastrointestinal innervation

	Species	Findings	Reference
Intrinsic neural pathways			
Myenteric plexus	Rat	Reduced neurons	(Santer and Baker, 1988; Phillips et al., 2003)
	Guinea pig	Reduced neurons	(Gabella, 1989; Peck et al., 2009)
	Mice	Reduced neurons	(El-Salhy et al., 1999)
	Human	Reduced neurons	(de Souza et al., 1993; Gomes et al., 1997; Bernard et al., 2009)
	Human	Increased morphological abnormalities	(Hanani et al., 2004)
Submucosal plexus	Rat	Reduced neurons	(Phillips et al., 2007)
	Mouse	Reduced neurons	(El-Salhy et al., 1999)
	Human	Reduced number of ganglion	(Bernard et al., 2009)
Extrinsic neural pathways			
Motor innervation	Rat	Deterioration such as swollen axons, decreased tyrosine hydroxylase	(Phillips et al., 2007)
Sensory innervation	Human	Reduced primary sensory neurons	(Nagashima and Oota, 1974)
	Rat	No change in the number of sensory neurons	(Soltanpour et al., 1996; Bergman and Ulfhake, 1998)
	Rat	Dystrophic and regressive alterations in vagal afferents	(Phillips et al., 2010)

The myenteric plexus, the outer of the two ENS plexuses, is primarily involved in the control of smooth muscle motility. The reduction in myenteric neurons in aged small and large intestine was first described in rat (Santer and Baker, 1988), and verified in other species including guinea pig (Gabella, 1989; Peck et al., 2009), mice (El-Salhy et al., 1999) and human (de Souza et al., 1993; Gomes et al., 1997; Bernard et al., 2009), suggesting this is a general phenomenon in aged intestine. Another study on human has revealed that myenteric ganglia abnormalities (cavities in ganglia) are augmented with advanced age (Hanani et al., 2004). The submucosal plexus, located in the submucosa between the muscle layers and mucosa, regulates secretion and absorption as well as motility. Similar to myenteric plexus, neuron loss in submucosal plexus with advanced age has been found in rat (Phillips et al., 2007) and mouse (El-Salhy et al., 1999). A study on human colon did not find any significant change in the density of neurons or neurons per ganglion in aged

submucosal plexus, although the number of ganglion per unit declined with age (Bernard et al., 2009).

Multiple studies on rat have revealed that the neuronal loss starts in adulthood and progresses in a linear manner over the lifespan, with similar patterns in myenteric and submucosal plexuses (Phillips and Powley, 2001; Phillips et al., 2003; Thrasivoulou et al., 2006; Phillips et al., 2007; Phillips and Powley, 2007). Conversely, study on mouse has shown a significant decrease in the number of neurons per ganglion in 12-month-old mice, whereas no additional reduction was found in the 24-month group (El-Salhy et al., 1999).

It has been thoroughly studied that different functional classes of enteric neurons can be classified based on their chemical coding (Furness, 2000). Neurons in the myenteric plexus are composed of two subpopulations: nitrergic neurons and cholinergic neurons (Mann et al., 1999; Nakajima et al., 2000; Phillips et al., 2003). Multiple studies have demonstrated that there is no loss of nitrergic neurons in aged rat myenteric plexus (Belai et al., 1995; Cowen et al., 2000; Phillips et al., 2003). On the contrary, cholinergic neurons are significantly reduced with advanced age in rat ileum (Cowen et al., 2000). In agreement with animal studies, a study on human colon confirmed the reduction of cholinergic neurons in aged myenteric plexus, accompanied with unchanged nitrergic neurons (Bernard et al., 2009). Selective age-related loss of cholinergic neurons associated with sparing of nitrergic neurons might result in an accumulative augment of the proportion of inhibitory neurons, which might contribute to the motility dysfunction in the elderly. The reason why cholinergic neurons are more vulnerable in ageing process is not clear; however, Thrasivoulou (2006) proposed that this might be due to calcium dyshomeostasis in association with increased intra-neuronal level of reactive oxygen species (ROS). In addition, caloric restriction diet that reduces the accumulation of ROS has been demonstrated to rescue enteric neuron loss in aged rat (Cowen et al., 2000).

In addition to intrinsic neurons, sympathetic innervations of the myenteric plexus and submucosa plexus in the rat deteriorate with advanced age, as indicated by the presence of observable swollen axons and decreased expression of tyrosine hydroxylase; similar swollen varicosities are observed in the sensory terminals of

vagal innervation, whereas spinal afferents are not dramatically affected by senescence (Phillips and Powley, 2007). Some studies have described decreases in the size or number of primary neurons in the mammalian nodose ganglia and DRGs (Vega et al., 1993), while other studies haven't observed a significant change in the rat (Soltanpour et al., 1996; Bergman and Ulfhake, 1998). Although this neuronal loss with age is controversial, a number of evidences have revealed metabolic changes of these primary neuron cell bodies, such as reduced basophilic Nissl material, accumulated lipofuscin, translucent vacuoles, inclusions, granules, filamentous bodies, masses and tangles (Brizzee and Ordy, 1979; Koistinaho, 1986; Vega et al., 1993; Soltanpour et al., 1996). A study demonstrated that vagal afferent terminals innervating the rat GI tract displayed dystrophic and regressive alterations with ageing (Phillips et al., 2010).

In addition to the changes in the GI innervation, some other alterations might also contribute to the GI conditions in the elderly. It has been proposed that ageing is a chronic inflammatory process with a shift toward a proinflammatory cytokine profile evidenced by increased circulating levels of IL-1, IL-6, TNF-α and IL-12, IFN- α/β, which promote a low-grade chronic systemic pro-inflammatory state in the elderly (Fagiolo et al., 1993; Franceschi et al., 1995; Bruunsgaard and Pedersen, 2003; Csiszar et al., 2003; Giunta, 2008). It is generally considered that ageing is associated with immunosenescence with deficient innate and adaptive immune response (Solana et al., 2006; Gomez et al., 2008). However, due to the complication of ageing process, the basic mechanisms underlying ageing remain poorly understood.

The accurate relationship between the ages of the model animals compared with the ages of humans is still subject to controversy. There is no simple equation to convert animal age to human. At a rough estimate, 24 months in mice may be equivalent to 70-75 years in humans (Horgan et al., 2007), and 24 months in rat is comparable with 60 years in humans (Andreollo et al., 2012).

1.8 General aims and objectives

This thesis focused on the interactions between mast cells, EC cells and sensory innervation of the GI tract as well as the effect of ageing on these interactions. The objectives comprise:

1) to examine age-related changes in the density of mast cells, EC cells and sensory nerves as well as anatomical association between mast cells and afferent nerves in human bowel;

2) to characterize the mechanical and chemical sensitivity of human intestinal afferents using a recently established *in vitro* recording model;

3) to investigate the involvement of mast cells and EC cells in TRPA1 signaling induced by TRPA1 agonists in mouse jejunum;

4) to examine the influence of ageing on TRPA1 signaling and underlying mechanisms in mouse jejunum.

Hypotheses:

1) density of mast cell, EC cells and sensory nerves as well as anatomical association between mast cells and afferent nerves is altered in aged human bowel;

2) a predominant subpopulation of human intestinal afferents is nociceptors responding to both noxious mechanical and chemical stimulation;

3) mediators released from EC cells and mast cells amplify peripheral TRPA1 signaling in mouse jejunum;

4) peripheral TRPA1 signaling is attenuated in aged mouse jejunum.

CHAPTER 2

Materials and methods

2.1 Animals

The majority of animal experiments were carried out in tissue or cells freshly obtained from 3-4 months (adult) or 24 months old (aged) male mice with a C57/BL6 background purchased from Charles River (Margate, UK). Studies on TRPV1 knockout mice (generated in GlaxoSmithKline, Harlow, UK) were compared to their wildtype littermates under the same maintenance conditions. All mice had free access to food and water before they were humanly killed by cervical dislocation in accordance with Schedule 1 procedures of UK Animals (Scientific Procedures) Act 1986.

Once the mice were sacrificed, a midline incision of the abdomen was carried out to allow access to intestine specimens. The entire small intestines from duodenum to caecum were harvested in ice-cold Krebs solution (composition, in mM: NaCl 120, KCl 5.9, MgSO4 1.2, NaH$_2$PO4 1.2, NaHCO$_3$ 15.4, glucose 11.5 and CaCl$_2$ 1.2). Following further dissections, jejunum segments were collected 5 cm distal to the stomach and 10 cm proximal to the caecum.

2.2 Human bowel specimens

All experimental procedures comply with ethical approval obtained from South Humber Research Ethics Committee. Human bowel specimens were non-diseased surplus tissue obtained from consented patients undergoing bowel resection for surgical treatment in Northern General Hospital, Sheffield. A pathologist consultant was present at the theatre to assure that the specimens that we were given were not proximal to the tumors and free of morphological abnormalities or inflammation. The specimens were transported from the theatre to the research lab in pre-oxygenated ice-cold Krebs within less than an hour. Histological staining (H&E or toluidine blue) was performed to make sure that the morphology was normal.

Information of patients was confidential and anonymous. Only age, gender and diagnosis of the patents were known (listed in table 2.1). Detail medical history or other possible gastrointestinal symptoms of the patients were unavailable. 59

specimens in total were obtained from 35 male and 24 female patients aged between 24 and 88 with a median at 67.

As the specimens obtained were morphologically normal, no criteria were made to exclude any specimen. Exceptionally, 5 specimens were excluded for afferent recording due to limited mesentery, as required by dissection of nerve bundles from mesentery. Roughly, specimens obtained in the first two years were used for immunohistochemistry and histological staining, and specimens in the second and third year were used for afferent recording.

Table 2.1 List of human bowel specimens

Date	Age	Gender	Region	Diagnose
21.10.10	76	M	Sigmoid colon	Adenocarcinoma
24.11.10	65	M	Ascending colon	Adenocarcinoma
15.12.10	43	F	Sigmoid colon	Adenocarcinoma
26.01.11	72	F	Sigmoid colon	Adenocarcinoma
24.02.11	77	M	Sigmoid colon	Adenocarcinoma
02.03.11	68	F	Sigmoid colon	Diverticular disease
15.03.11	59	F	Sigmoid colon	Adenocarcinoma
23.03.11	84	F	Ascending colon	Adenocarcinoma
11.05.11	72	M	Rectum	Adenocarcinoma
18.05.11	31	M	Ascending colon	Pseudomembranous colitis
06.06.11	68	M	Sigmoid colon	Adenocarcinoma
14.06.11	46	M	Ileum	Crohn's disease
21.06.11	87	F	Ileum	Endocrine carcinoma
23.06.11	82	M	Ileum	Adenocarcinoma
30.08.11	67	M	Ascending colon	Appendicitis perforated
20.09.11	72	F	Sigmoid colon	Adenocarcinoma
27.09.11	67	M	Rectum	Adenocarcinoma
04.10.11	70	M	Sigmoid colon	Adenocarcinoma
11.10.11	24	F	Ileum	Crohn's and endometriosis
15.11.11	70	M	Sigmoid colon	Adenocarcinoma
29.11.11	77	M	Ascending colon	Tubullovillous adenoma
06.12.11	49	M	Sigmoid colon	Diverticular disease
20.12.11	55	F	Ascending colon	Adenocarcinoma
10.01.12	80	M	Descending colon	Carcinoma
17.01.12	50	F	Ileum	Crohn's disease
24.01.12	72	F	Ascending colon	Adenocarcinoma
31.01.12	38	M	Sigmoid colon	Proctitis cystica profunda
28.02.12	58	F	Ileum	Endocrine carcinoma
06.03.12	62	F	Ileum	Adenocarcinoma and lymphocytic

				lymphoma
11.05.12	80	M	Transverse colon	Adenocarcinoma
18.05.12	54	M	Sigmoid colon	Diverticular disease
22.05.12	54	M	Sigmoid colon	Adenocarcinoma
29.05.12	48	F	Sigmoid colon	Diversion colitis, previous ulcerative colitis
18.06.12	56	M	Sigmoid colon	Adenocarcinoma
04.07.12	66	F	Ileum	Tubulovillous adenoma
18.09.12	32	F	Rectum	Adenocarcinoma
19.09.12	64	M	Ileum	Tubulovillous adenoma
25.09.12	57	F	Ileum	Crohn's disease
12.10.12	78	F	Sigmoid colon	Adenocarcinoma
16.10.12	53	M	Ileum	Adenocarcinoma
17.10.12	88	F	Transverse colon	Adenocarcinoma
30.10.12	74	M	Sigmoid colon	Adenocarcinoma
31.10.12	78	M	Ileum	Adenocarcinoma
07.11.12	64	M	Ileum	Adenocarcinoma
13.11.12	63	M	Sigmoid colon	Adenocarcinoma
19.02.13	67	M	Descending colon	Adenocarcinoma
16.04.13	56	F	Ileum	Crohn's disease
23.04.13	66	M	Descending colon	Adenocarcinoma
24.04.13	62	M	Descending colon	Adenocarcinoma
08.05.13	42	M	Sigmoid colon	Adenocarcinoma
15.05.13	70	M	Descending colon	Adenocarcinoma
04.06.13	51	F	Sigmoid colon	Adenocarcinoma
05.06.13	72	M	Ileum	Tubulovillous adenoma
10.06.13	68	F	Ascending colon	Adenocarcinoma
11.06.13	73	M	Sigmoid colon	Adenocarcinoma
25.06.13	72	M	Descending colon	Adenocarcinoma
26.06.13	71	F	Ileum	Adenocarcinoma
02.07.13	49	M	Descending colon	Adenocarcinoma
10.07.13	67	F	Sigmoid colon	Adenocarcinoma

2.3 Histological staining and immunohistochemistry

All human (or mouse) specimens were only labeled with the date of harvest, so that the information that might lead to bias (such as age) was concealed. The codes were consistently labeled on corresponding tissue tubes, frozen blocks, slides and images. When acquiring images for immunohistochemistry, random fields were selected under DAPI filter so that the immunoreactivities were blinded.

2.3.1 Fixation

Different fixatives were used in different experiments. Unless otherwise stated, the fixation time was either overnight at 4 °C or 2~3 hours at room temperature. After fixation, specimens were rinsed using phosphate buffer (0.01M PBS, made from tablet, Sigma P4417) for 3 times.

Paraformaldehyde (PFA)

To make 4% PFA, 4g PFA was added into 100 ml phosphate buffer. The solution was heated while stirring to 60 °C under the hood until transparent. If solution did not get clear, NaOH was added drop by drop. The pH of solution was 7.2-7.4.

Carnoy's fixative

Carnoy's fixative was composed of 60% ethanol, 30% chloroform and 10% glacial acetic acid.

Zamboni's Fixative

125 ml PFA (from 16% stock solution) and 150 ml picric acid (saturated aqueous, Sigma, 239801) were combined and made up to 1 liter with 0.1 M phosphate buffer. The pH was adjusted to 7.3 using 10 M NaOH.

2.3.2 Embedding and sectioning

Prior to process of embedding, specimens were trimmed accordingly to fit the embedding mold used in different experiments.

JB4 resin embedding

Fixed specimens were dehydrated in graded ethanol (70%, 90%, 100%, and dried 100%), one hour for each concentration. Following removal of all ethanol, tissue was infiltrated in freshly made catalyzed JB4 solution A (JB4 solution A 100 ml combined with 0.9 gram Benzoyl Peroxide, Polysciences 00226-1) at least overnight. Infiltrated tissue was then embedded in molds filled with fresh catalyzed JB4 solution A plus accelerator solution B (JB4 solution A 50ml, Benzoyl peroxide 0.45g and solution B 0.8ml) and mounted on an aluminum stub. The mold was kept in fridge overnight at the minimum. Once resin solution set, the resin blocks were taken out of the molds, rinsed with 70% alcohol to remove excess unpolymerised resin and left dry in a fume hood for a couple of hours.

Microtome sectioning

Resin blocks were sectioned at 2 μm using a LKB 2218 Historange microtome (LKB-Produkter, Bromma, Sweden) equipped with a glass knife. Sections were placed onto distilled water to expand, collected using a microscope slide and dried on a hotplate.

Cryo-embedding

Fixed tissue was incubated in 30% sucrose (Sigma-Aldrich, S5016) overnight at 4°C until tissue sank down to the bottom of container. Thereafter, specimens were placed into molds filled with optimal cutting temperature compound (OCT, Bright Instrument Company, 53581) and orientated appropriately. The molds were kept on dry ice until OCT was fully frozen. OCT block with tissue could be either sectioned directly or stored at -80°C.

Cryo-sectioning

OCT blocks containing specimen were sectioned in a cryostat (Bright Instrument, OTF5000, Huntingdon, UK) at 10 μm. The cryostat was set up at -20°C for specimen temperature and -15°C for the chamber. Sections were collected discontinuously to avoid repetition using pre-coated glass slides (Thermo Scientific MNJ-700) and stored at -20°C.

2.3.3 Histological staining

H&E staining

Slides with sections were stained with *GILLS II Haematoxylin* (Surgipath, 01521E) for 5 minutes and rinsed within running water to remove excess dye. Sections were then counterstained in 1% Eosin Y /PBS for 1 minutes and washed in running water. After dried on a hotplate, sections were mounted with glass coverslips using DPX mounting medium.

Toluidine blue staining

Toluidine blue is one of the most commonly used metachromatic dyes for mast cells labeling in both clinic and research, based to its ability to stain acid mucopolysaccharide and glycoaminoglycans components within mast cell granules in red-purple (metachromatic staining) and the background in blue (orthochromatic staining). To make a stock solution, 1 gram toluidine blue O (Sigma, T-3260) was added into 100 ml 70% ethanol and mixed well. Stock solution was stable for 6 months. Fresh working solution was diluted by 10 times from stock using 1% sodium chloride.

Slides with sections were incubated within 0.1% toluidine blue for 3 minutes, rinsed in distilled water, dried on a hotplate and mounted with coverslips using DPX mounting medium.

2.3.4 Immunohistochemistry and immunocytochemistry

Slides with cryo-sections were placed in a closed humid box. Prior to application of primary antibodies, slides were washed with PBS for 5 minutes to remove OCT and blocking solution for 20 minutes to block unspecific binding. Treatment with 0.1% Triton X-100 (Sigma-aldrich, X100-100ML) was optional, depending on the location of epitope. Blocking solution was usually 5% normal serum from the same species in which the secondary antibodies were raised. After overnight incubation of primary antibodies at 4°C, slides were rinsed in PBS for three times, 5 minutes each. Following incubation of fluorophores conjugated secondary antibodies for 2 hours at room temperature and rinse with PBS, slides were mounted with coverslips using VECTASHIELD Mounting Medium with DAPI (Vector, H1200). Unless otherwise

stated, antibodies were diluted in corresponding blocking solution. Negative control was performed by omitting the primary antibodies.

In terms of double staining, two primary antibodies raised in different species were mixed and diluted accordingly prior to application. Similarly, two secondary antibodies were applied as a mixture.

For staining on cell culture, cells were seeded on coverslips pre-coated with polylysine (100 μl each) and left in incubator for 30 minutes. Prior to blocking using 2% normal serum (from the same species as secondary antibodies), cells were fixed in 4% PFA for 20 minutes and rinsed with PBS thoroughly. Treatment with 0.1% Triton X-100 for 1 minute was optional for intracellular antigens. Cells were incubated with primary antibodies and secondary antibodies separately at room temperature, each for one hour and followed with PBS rinse. Finally, coverslips were mounted on microscope slides using VECTASHIELD Mounting Medium with DAPI.

Table 2.2 Summary of primary antibodies used in this thesis

Antigen	Company	Catalogue	Source Species	Target species	Dilution
CGRP	PENINSULA LABORATORIES	T4239	Rabbit	Human	1:1000
CGRP	Abcam	ab47027	Rabbit	Human, rat, Predict: mouse	-
Tryptase	Santa Cruz	sc-271095	Mouse	Human, mouse & rat	1:1000 IHC 1:100 ICC
Serotonin	AbD serotec	AHP522	Rabbit	Human	1:50
Substance P	Abcam	ab24126	Rabbit	Human, Rat	1:50
TRPA1	Abcam	ab58844	Rabbit	Mouse, Rat,	-
TRPA1	Abcam	ab62053	Rabbit	Human, mouse	-
TRPA1	Abcam	ab68847	Rabbit	Mouse	-
TRPA1	Thermo	PA1-46159	Rabbit	Human, mouse	-
Tryptase	Santa Cruz	sc-32474	Goat	Mouse	1:200
Tryptase	Santa Cruz	sc-32889	Rabbit	Mouse	1:200

- indicates that antibody did not work convincingly.

Table 2.3 Summary of secondary antibodies used in this thesis

Species	Company	Catalogue	Fluorophore	Dilution
Goat anti-rabbit	Vector	FI-1000	FITC	1:200-1:400
Goat-anti-mouse	Jackson ImmunoResearch	115-166-003	Cy3	1:200-1:400
Goat anti-rabbit	Jackson ImmunoResearch	111-165-144	Cy3	1:200-1:400
Fab Fragment Goat anti- Rabbit	Jackson ImmunoResearch	111-297-003	Rhodamine Red-X	1:200-1:400

2.3.5 Microscopy and image analysis

Stained sections were observed under an Olympus BX51 microscope (Tokyo, Japan). Images were acquired using an Olympus ColorView II digital camera.

Image analysis with ImageJ

Image analysis was performed with ImageJ software (1.43u, National Institutes of Health, USA) if applicable. Prior to any measurement, pixel size was calibrated using a 1mm stage micrometer with 100 divisions.

Area measurement

The region of interest was selected using the Freehand Selection tool in the Startup toolbar. The area of selected region was measured using the Measure function in the Analyze menu.

Distance measurement

A straight line was drawn across the distance of interest using the Line tool in the Startup toolbar. The length of the line was measured using the Measure function in the Analyze menu.

Particle analysis

An intensity threshold was set up using the Adjust command in the Image menu to discriminate the immunoreactivities from the background. The range of particle size was set up in the Analyze Particles menu to exclude unspecific staining, and then the number of targeted particles and the area occupied by those particles was measured.

2.4 Extracellular recording of afferent activity

2.4.1 Dissection, nerve preparation and afferent recording

Mouse jejunum

After removed from mice, intestine specimens were placed and maintained in ice-cold Krebs solution bubbled with carbogen (95% O_2 and 5% CO_2). Following further dissection, a 2-3 cm jejunum segment with mesenteric neurovascular bundle in the middle was placed into recording chamber, which was constantly perfused with oxygenized Krebs solution and maintained at a stable temperature of 34°C. Two ends of the segment were cannulated and tied firmly. One end was connected to a perfusion pump (PERFUSOR®secura, Braun, Netherlands), allowing continual perfusion of Krebs through the lumen of jejunum at a rate of 10 ml per hour; the other side was attached to a pressure transducer (DTXPlus^{TM}, BD, Singapore) through a three-way connecter catheter. By controlling this three-way tap, segments were allowed to be distended with intraluminal pressure monitored (usually up to 60 mmHg). The neurovascular bundle was pinned onto a dissection platform made by sylgard (SYLGARD® 184 Silicone elastomer kit).

Under a dissection microscope (Nikon, SMZ-1B, Japan), afferent nerves in the neurovascular bundle were identified and pulled out gently using fine forceps. After cleaning up fat and connective tissue around, a nerve bundle was sucked into a suction electrode attached to a Neorolog headstage (NL 100, Digitimer, Ltd, UK) and an AC amplifier (NL 104). Electronic signals were amplified by 10,000 times, filtered properly (NL 125, band pass filter) and recorded on a computer via a power 1401 analogue-to-digital interface and Spike 2 software (Version 7.10, Cambridge Electronic Design, UK). Multi-unit afferent nerve activities were quantified by a spike processor (Digitimer D130) that counted the number of spikes over a pre-set threshold. The threshold was usually set at the peak of smallest identifiable action potentials.
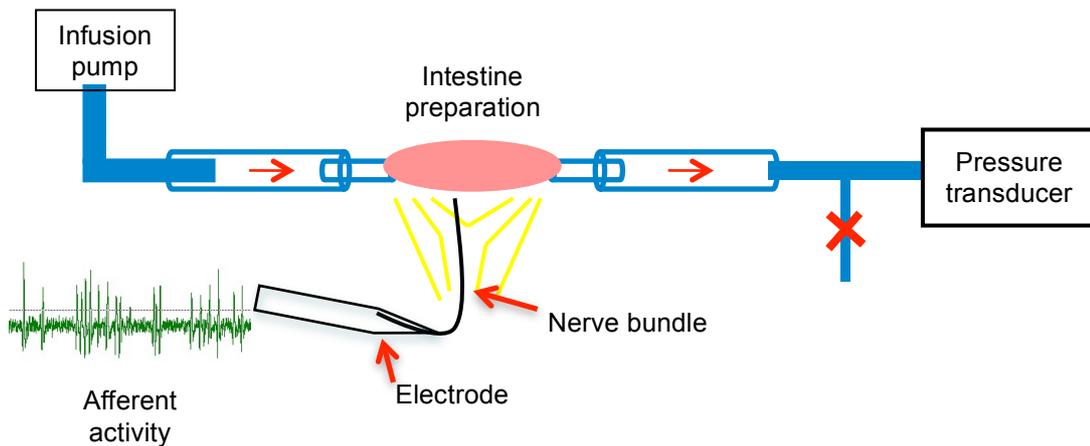
Figure 2.1 A schematic *in vitro* model to monitor the afferent nerve activity and intraluminal pressure of mouse jejunum. A jejunum segment was connected to an infusion pump allowing perfusion of Krebs into the lumen and a pressure transducer, which monitored intraluminal pressure after closing the three-way tap between the segment and the transducer. Mesenteric nerve bundle was identified, dissected and sucked in a glass electrode. Action potentials from the afferents were amplified, filtered and recorded through corresponding equipment and software.

Human bowel

In comparison with mouse intestine, human bowel was much larger in size with thicker mesentery attachment. As such, recording chamber for human bowel (volume at about 100 ml) was markedly larger than that for mouse (10 ml). Specimens were cut open along the middle of mesentery and pinned flat on the bottom of the organ bath with mucosa uppermost (as shown in figure 2.2). The mesentery was also divided into two parts, so that nerve bundles could be identified from both sides of the preparation. As the nerve bundles were deeply embedded among dense adipose tissue and connective tissue, identification of them significantly consumed more time and efforts than mouse. In addition, more dissection was required to expose clean nerve fibers. Nerve activities were then recorded from the dissected fibers as described above.
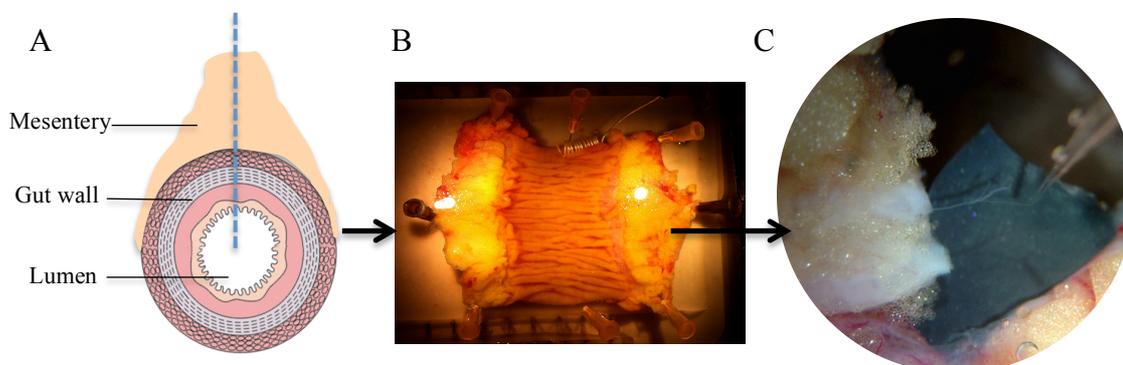
Figure 2.2 Schematic representation of afferent recording setup of human bowel specimens. (A) Tubular human bowel tissue was cut open along the dash line. (B) Flat-sheet specimen was pinned onto the bottom of organ bath with mucosa side up. (C) Nerve bundle was identified from either side of mesentery, dissected clean and sucked into a glass electrode. Action potentials from the afferents were amplified, filtered and recorded through corresponding equipment and software.

2.4.2 Experimental protocols

Prior to application of any protocol, the preparation was left stabilized in the recording chamber for 30~60 minutes until the afferent activities were stable.

Control distension

Distension of the intestine preparations refers to an intraluminal infusion of Krebs at a rate of 10 ml/h. The outflow tap between preparation and pressure transducer was closed to allow the segment to be filled until the intraluminal pressure reached 60 mmHg. Unless otherwise stated, the preparation was distended every 15-20 minutes. Chemical agents were not applied until three reproducible responses to distension achieved.

Extraluminal application of agonist

In most experiments, agonists or other chemical stimuli diluted in Krebs were constantly perfused into organ bath by a peristaltic pump (Gilson MINIPLUS 3, USA). In other cases, the drug was directed injected into the organ bath using a

pipette with the perfusion of Krebs closed. The volume of agonist solution and time interval between repeated applications varied in different groups of experiments.

Intraluminal application of agonist

Agonist or other chemical stimuli diluted in Krebs were continually applied into the lumen of preparations using a syringe driver (Genie Kent, mutil-phaser^{TM} NE-1000, USA). The timespan of perfusion might vary in different sets of experiments.

Extraluminal application of antagonist

Antagonists diluted in Krebs were perfused into organ bath 10-15 minutes before the application of agonists, which were diluted in antagonist-Krebs solution to make sure the concentration of antagonist unaltered. After the response to agonist finished, antagonist solution was replaced by fresh Krebs.

2.4.3 Analysis of data

Data are presented as either frequency of afferent discharge (number of action potentials per second) or total number of spikes in certain period. All data are expressed as mean ± SEM from the N values, where N refers to the number of animals or human specimens.

The response of afferent nerves to a pharmacological agent was analyzed in two ways, peak response and response profile against time. Peak response was quantified as maximum frequency in a 30-second bin subtracted by baseline activity using a script. Response profile showing the change of mean frequency over time was obtained from XY View measurements of Spike 2.

The response to ramp distension was plotted as mean afferent discharge per second against increasing intraluminal pressure from 0 to 60 mmHg using a script.

Statistical analysis

All graphical and statistical analysis was performed using GraphPad Prism (Version 6.0, San Diego, USA). Student paired & unpaired t-test, 1-way and 2-way ANOVA

were used as appropriate. $P<0.05$ was considered as significant between compared groups. * in a figure refers to $P<0.05$; **, $P<0.01$ and ***, $P<0.001$.

Single unit analysis

Single unit analysis was performed offline using the spike sorting function of Spike 2 (version 7.10) to discriminate afferent nerve activities of individual units. Afferent activity was sampled at 20,000 Hz. A 2.5 millisecond period was used to construct a template that was composed of 50 data points, so that the whole profile of a spike was analyzed. Following generation of the first template, each new spike was compared against this template. Spikes that did not match the first template would create new templates. As such, spikes were classified into different single units according to their waveform. An example of this process is seen in figure 2.3, in which 4 different units were discriminated.
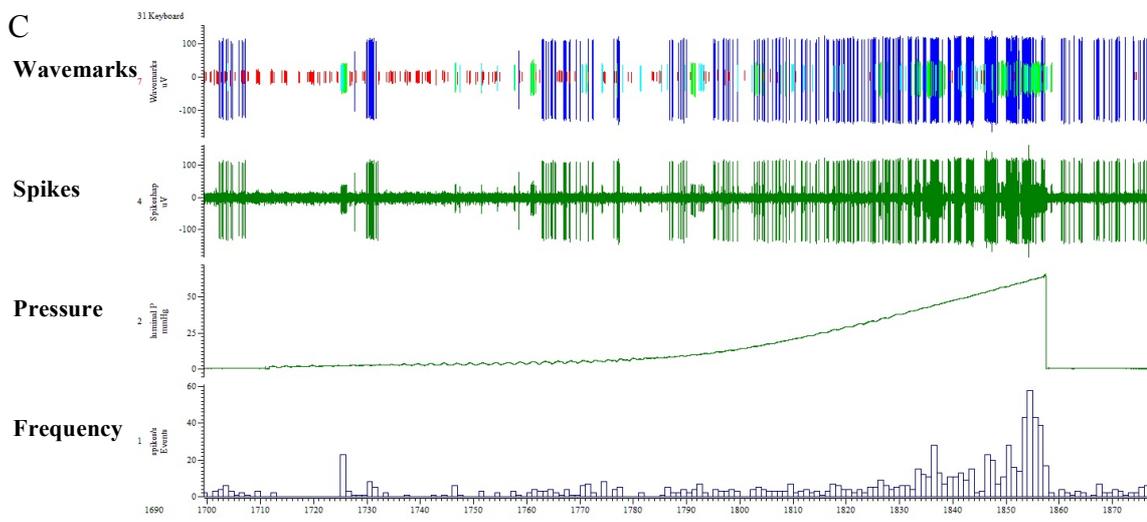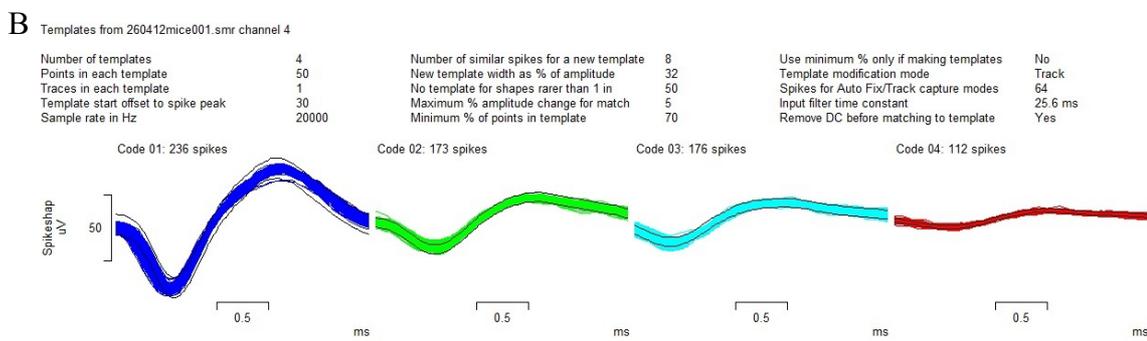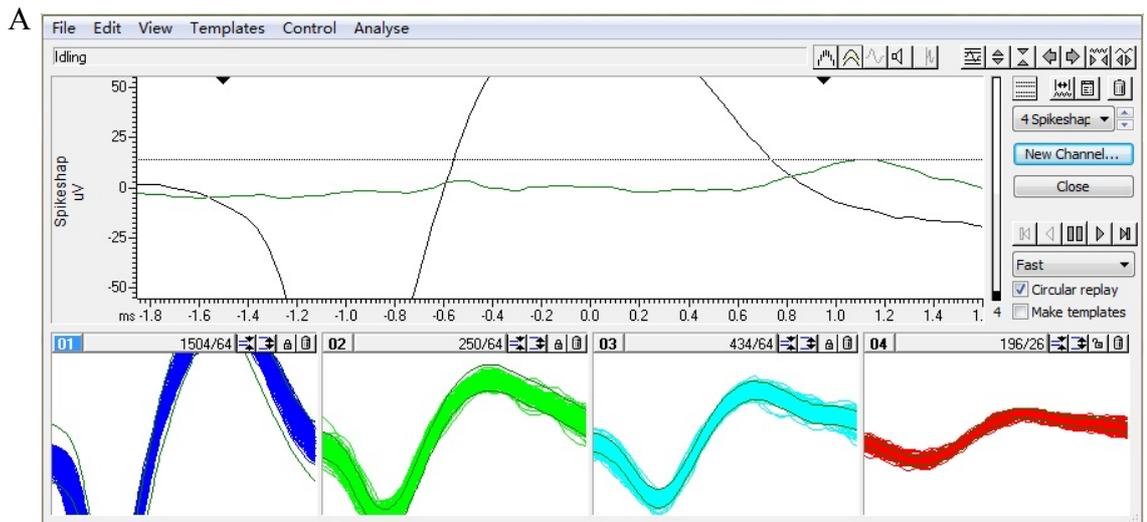
Figure 2.3 Diagrammatic representation of single unit analysis. (A) An example wavemark window of Spike 2 showing template generation and spike matching. (B) Output information about templates with setup parameters. (C) Example traces showing afferent response to distension. The wavemark trace reveals the activities of individual single units during the distension using the templates generated from figure A.

Principal component analysis was used to ensure that the single units identified by the wavemask analysis were different enough to be classified as distinct nerve fibers. It worked by disassociating the shapes of individual single units based on measurements on each spike shape, such as amplitude, latency, area and slope. These data were normalized, shifted and scaled in a 3-dimensional manner (figure 2.4A is a two-dimensional representation of this clustering). The amount of overlap between the clusters indicated the similarities of two units. For example, as shown in figure 2.4 A, unit 01 (blue) and unit 04 (red) were well discriminated from others, convincingly suggesting that they were two distinct units. Whereas, some overlaps existed between unit 02 (green) and unit 03 (cyan), indicating that they shared some similar properties and might be generated by the same afferent fiber. However, in the raw trace of spikes (figure 2.4B), they appeared to be slightly different. In this equivocal case, their functional characteristics would be considered. If they showed similar patterns in response to the same mechanical or chemical stimulations, they were likely to be the same unit.

This example exposed the limitations of single unit analysis. Since this recording technique detected and recorded the field potentials from the electrode, the profile of spikes would be largely influenced by the noise, which was the major source of variations in spike profile. If two distinct units generated action potentials with some tiny similarities, the addition of variations made it difficult to discriminate them. It was even worse if there were a large number of units in one nerve bundle, which usually showed a higher chance of similar and equivocal units. In addition, large number of units accompanied with high firing frequency resulted in frequent summations of spike profiles, which could not be discriminated.
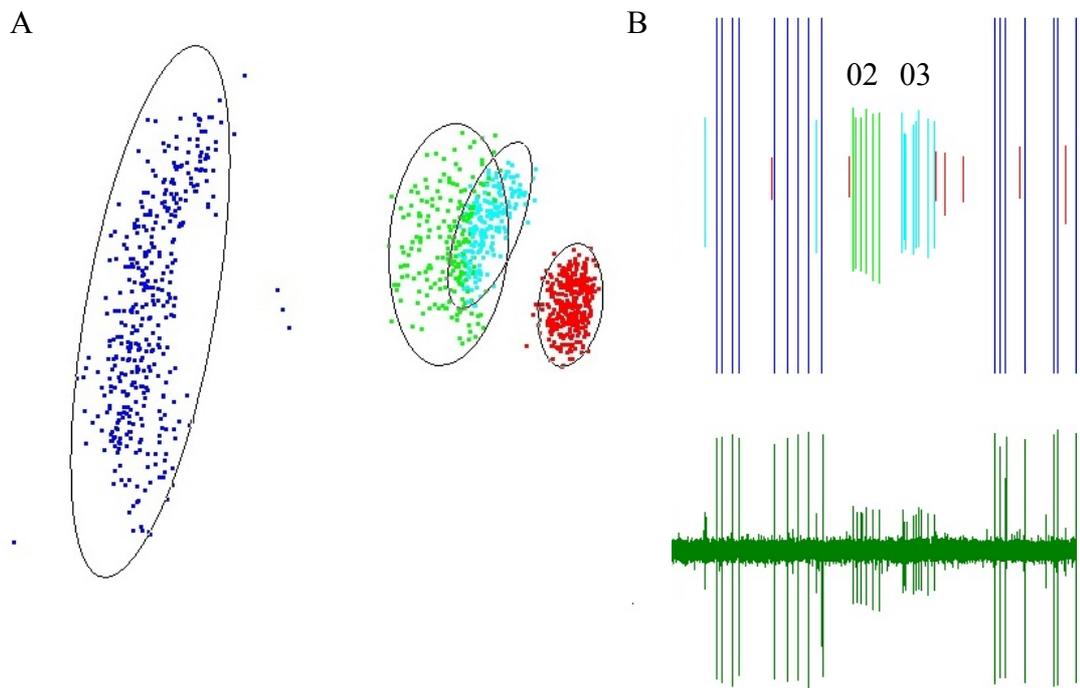
Figure 2.4 Example of principle component analysis. (A) Representative graph showing the clusters of individual units sorted in figure 2.3. Each colour represents a template. Unit 01 (blue) and unit 04 (red) are well discriminated from the other units. Some overlaps exist between unit 02 (green) and unit 03 (cyan). (B) Wavemark and raw spike traces comparing unit (02) and unit (03).

2.5 Investigation of gene expression

2.5.1 RNA extraction

Mouse or human specimens used for gene expression studies were immediately placed in at least 10 volumes of RNAlater Stabilization Reagent (QIAGEN, 76106) after harvested and stored at -20°C. RNA purification was performed using RNeasy Mini Kit (QIAGEN 74104) according to manufacturer's instructions. Up to 20 mg stabilized tissue was disrupted and homogenized in 1.5 mL Pestle and Microtube (VWR Labshop, 47747-366) using a Pestle Motor (VWR Labshop, 47747-370). Isolated RNA was eluted in RNase-free water and stored at -80°C.

2.5.2 Reverse transcription (RT)

Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, 4374966) according to manufacturer's instructions. $2\times$ Reverse Transcription Master Mix was prepared as shown in table 2.4 and distributed into PCR tubes, with 1 volume of total RNA added to create a $1\times$ mix. RT reaction was performed in a thermal cycler (TECHNE TC-3000X, Stone, UK). A summary of RT programme was shown in table 2.5.

Table 2.4 Component of RT reaction mix (for 20 μl reaction)

Component	Volume/Reaction (μl)
10× RT Buffer	2.0
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2.0
MultiScribe^{TM} Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H_2O	3.2
Total RNA	10.0

Table 2.5 RT reaction thermal cycle

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	

2.5.3 Quantitive real-time PCR

All probes were predesigned and verified by mass spectrometry by Integrated DNA Technologies. Probes were diluted using IDTE buffer, containing 10mM TRIS (Melford Laboratories Ltd., B2005) and 0.1mM EDTA (Sigma-aldrich, E9984).

Prior to use, the TaqMan Gene Expression Master Mix (Applied Biosystems 4369016) was thoroughly mixed by swirling the bottle. The components for 20 μl reaction mix in Hard-Shell® Thin-Wall 96-Well Skirted PCR Plates (BIO-RAD, HSP-9665) were listed in table 2.6. Reactions for each sample were run in duplicate.

Table 2.6 Component of q-PCR reaction mix (for 20 μl reaction)

Component	Volume/Reaction (μl)
TaqMan Gene Expression Master Mix	10
Probe mix	1
cDNA	1
RNAase-free water	8

Prior to run reactions, the plates were covered with MicroAmp^{TM} Optical Adhesive Film for 96-Well Plates (Applied Biosystems, 43111971) and centrifuged briefly to spin down the contents and eliminate any air bubbles from the solutions. PCR reactions were performed in BIO-RAD CFX96 Touch^{TM} Real-time system (C1000 Touch^{TM} Thermal Cycler, Bio-Rad Laboratories Ltd. Hercules, USA) with the thermal cycling programme set up as follows:

Table 2.7 Summary of q-PCR cycles

Step	Temperature (°C)	Duration	Cycles
UDG incubation	50	2 min	HOLD
AmpliTaq Gold®, UP Enzyme Activation	95	10 min	HOLD
Denature	95	15 sec	40
Anneal/Extend	60	1 min	

2.5.4 Analysis of data

Data obtained from the PCR machine were Ct value, which referred to the cycle number where the PCR curve reached the threshold in the linear part of the curve. Higher Ct value indicates less mRNA detected. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), one of the most commonly used housekeeping gene, was used as endogenous reference, and thus expression of tested gene were normalized against GAPDH expression. ΔCt referred to relative gene expression compared with GAPDH expression.

$$\Delta Ct = Ct_{\text{tested gene}} - Ct_{\text{housekeeping gene}}$$

To examine the change of gene expression between different samples, fold change was introduced and calculated as follows:

$$\Delta Ct_{\text{sample 1}} = Ct_{\text{tested gene in sample 1}} - Ct_{\text{housekeeping gene in sample 1}}$$

$$\Delta Ct_{\text{sample 2}} = Ct_{\text{tested gene in sample 2}} - Ct_{\text{housekeeping gene in sample 2}}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{sample 1}} - \Delta Ct_{\text{sample 2}}$$

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

In ageing study,

$$\Delta\Delta Ct = \Delta Ct_{\text{aged samples}} - \Delta Ct_{\text{control}}$$

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Fold change indicated change of gene expression in aged samples compared to control group. For example, if ΔΔCt was 1, fold change would be -2, suggesting this gene was down regulated by half in aged group; if ΔΔCt was -1, fold change would be 2, indicating this gene expression doubled in aged group. Fold change >2 or <-2 was considered as significant.

2.6 Calcium imaging

2.6.1 Dissociation of DRG and nodose ganglion

Culture media used for DRG and nodose neuron preparation was composed of 90% DMEM/F12 (Invitrogen, 31330-038), 10% fetal calf serum (Invitrogen, 10500-064) and 1% PenStrep (Sigma, P4333). Prior to cell plating, autoclaved coverslips were coated with matrigel (1:6 diluted in ice-cold culture media, 50 μl for each coverslip, BD Matrigel™ Basement Membrane Matrix, 356234) and left in incubator for 30 minutes.

Mice were sacrificed by decollation after anesthetization. T7-L2 DRG and nodose ganglion were quickly isolated under a dissecting microscope and collected into Hank's Balanced Salt Solution (HBSS, Sigma-aldrich, H1641). The ganglions were incubated in HBSS containing 2.0 mg/ml papain (Sigma-aldrich, P4762) and 0.3 mg/ml L-cysteine (Sigma-aldrich, C7352) at 37°C for 20 minutes. After gently removing all papain solution, 4.0 mg/ml collagenase (Sigma-aldrich, C0130) and 4.7mg/ml dispase (Sigma-aldrich, D4693) were added into the tube. Prior to incubation in 37°C water bath for 20 minutes, the tube was flicked gently to agitate ganglia. Cells were then washed in culture media and plated on metrigel-coated coverslips (50 μl per coverslip). Following 2-hour incubation, cells were flooded with culture media (1 ml per well) and put back into incubator.

2.6.2 Calcium imaging

After overnight incubation, cells were loaded with Fura-2 AM (4μM, Life technologies, F1201) for 30 minutes. Coverslips with cells were then sealed on a chamber platform (Series 20, Warner Instruments, Hamden, CT, USA) and continuously perfused with HEPES buffer (in mM: NaCl 135, KCl 5, HEPES 10, glucose 10, MgCl$_2$ 1 and CaCl$_2$ 2) at room temperature. Excitation lights were generated by OptoLED light source (Cairn Research Limited, Kent, UK). Fluorescent intensity of Fura2 was monitored and recorded using a digital camera (C4742-95 Hamamatsu Corporation, Sewickley, PA, USA), a Hamamatsu camera controller and a computer with SimplePCI software (Version 6.6.0.0, Hamamatsu Corporation).

2.6.3 Protocol and analysis

Prior to application of drug, 5-minute baseline intensity was recorded. Drug diluted in HEPES buffer was perfused into the recording chamber by gravity for 1 minute, followed by HEPES washout. At the end of each experiment, 5 μM ionomycin was applied as a positive control.

Intracellular calcium concentration was indicated by the ratio of emission fluorescence under 340 nm and 380 nm excitation light. Changes of calcium concentration in response to a drug were calculated as the net difference between baseline 340/380 ratio and the peak ratio during drug application. Data were expressed as mean±SE.

CHAPTER 3

Changes of EC cells, mast cells and afferent nerves in aged human bowel

3.1 Introduction

Ageing and GI innervation

Ageing is associated with a variety of GI disorders, such as dysphagia, dyspepsia, anorexia, constipation and fecal incontinence (Johanson et al., 1989; Wald, 1990; Lindgren and Janzon, 1991; Morley, 1997; Firth and Prather, 2002). Most commonly, the incidence of motility or transit problems is increasing with age (Jost, 1997; O'Mahony et al., 2002; Hays and Roberts, 2006; Norton, 2006). Although the mechanisms underlying those GI disorders in the elderly remain poorly understood, the symptoms suggest that the causes might be the loss or functional alteration of the neural signaling in the gut that regulates the gastrointestinal functions.

As introduced in chapter 1, a large number of studies on aged small or large intestine have described reductions in myenteric neurons (Santer and Baker, 1988; Gabella, 1989; de Souza et al., 1993; Gomes et al., 1997; El-Salhy et al., 1999; Bernard et al., 2009; Peck et al., 2009) and submucosal neurons (El-Salhy et al., 1999; Phillips et al., 2007). In addition to intrinsic neurons, signs of deterioration with advanced age have been observed in sympathetic and vagal innervation, whereas spinal afferents are not dramatically affected by senescence (Phillips and Powley, 2007). Some studies have described decreases in the size or number of primary neurons in the nodose ganglia and DRGs (Vega et al., 1993), while other studies did not observe a significant change (Soltanpour et al., 1996; Bergman and Ulfhake, 1998). Despite this discrepancy, a number of evidences have revealed metabolic changes of these primary neuron cell bodies, such as reduced basophilic Nissl material, accumulated lipofuscin, translucent vacuoles, inclusions, granules, filamentous bodies, masses and tangles (Brizzee and Ordy, 1979; Koistinaho, 1986; Vega et al., 1993; Soltanpour et al., 1996). A study demonstrated age-related dystrophic and regressive alterations on vagal afferent terminals innervating the GI tract (Phillips et al., 2010).

Neuropeptides transmit neuronal signal to neighboring cells to regulate physiological functions; therefore, they have been frequently used as markers for functional alterations in ageing studies. Some age-related changes in neuropeptide have been reported. Substance P (SP), vasoactive intestinal polypeptide (VIP) and somatostatin positive nerve fibers are decreased in the small intestine of senile rat in comparison

with young animals (Feher and Penzes, 1987). Concentrations of VIP, neuropeptide Y (NPY) and galanin in tissue extracts from mouse duodenum decrease in both 12 and 24 months old animals compared with 3-month group, whereas SP and neurotensin decrease in 12-month-old mice but not in 24-month group; in mouse colon, peptide YY (PYY), soatostatin, VIP, galanin and neurotensin decrease in 12-month-old animals, while SP increase in 24-month group (El-Salhy and Sandström, 1999).

SP is an important neuropeptide, participating a variety of physiological and pathological activities. Most importantly, SP has multiple proinflammatory effects on many different cell types including lymphocytes, monocytes, neutrophils, mast cells and eosinophils (Shanahan et al., 1985; Repke and Bienert, 1987; Haines et al., 1993; Ho et al., 1996; O'Connor et al., 2004). In addition, NK1 (SP receptor) mutant mice showed significant reduced nociceptive response when the stimuli were moderate to intense, suggesting SP is involved in the transmission of pain signal to the CNS (De Felipe et al., 1998).

Some studies have revealed age-related alterations in SP. Perivascular SP innervating rat aorta declined from 6 months old and disappeared after 12 months old (Connat et al., 2001). Density of SP content in some areas of rat spinal cord significantly declined with age (Goettl et al., 1999; Ranson et al., 2005). Density of SP-positive nerve fibers was decreased in the circular muscle of aged guinea-pig colon (Peck et al., 2009). SP concentration in mouse tissue extracts decreased in aged antrum and duodenum, but increased in aged colon (El-Salhy and Sandström, 1999).

Ageing and non-neuronal changes in the GI tract

Apart from the changes in the GI innervation, some other alterations might contribute to the GI disorders in the elderly as well. Two studies have demonstrated a severe weakening of the lymphatic pump including diminished contraction amplitude and frequency in aged mesenteric lymphatic vessels, implying lowered ability to adapt the situations with increased volumetric loads which might occur during inflammation and local edema in the gut (Akl et al., 2011; Nagai et al., 2011). Some reports proposed that ageing is a chronic inflammatory process with a shift toward a proinflammatory cytokine profile in tissue that may account for numerous

deleterious vascular changes associated with ageing (Csiszar et al., 2003; Bruunsgaard and Pedersen, 2003; Giunta, 2008). Ageing has been thought to involve immunosenescence with deficient innate as well as adaptive immune response (Solana et al., 2006; Gomez et al., 2008)

Interactions between nerves and other cell types like EC cells and mast cells not only regulate normal GI physiology but also contribute to some functional disorders, such as the hypersensitivity in IBS, as has been introduced previously. Extensive studies have testified to the close association between mast cells and nerves, whereas few have focused on sensory nerves specifically. A study on rat dura mater provided evidence that mast cells were in close apposition (less than 100 nm) to SP and CGRP positive nerve fibers (Dimitriadou et al., 1997). In addition, a large proportion of rat intestinal mucosal mast cells directly contact with nerves, some of which contain SP or CGRP (Stead et al., 1987; Pang et al., 1996). It has been documented that the contact values of SP and CGRP with mast cells increased in the developing lesions of psoriatic skin (Naukkarinen et al., 1996). However, limited information can be found from the literature about the effect of ageing on the anatomic relationship between mast cells and SP.

Although the alterations of gastrointestinal innervation with advancing age have been extensively studied, it is poorly understood if the densities of EC cells and mast cells in the GI tract are changed in the elderly.

EC cells are increased in rat stomach during ageing (Khomeriki, 1989). A study employing chromatographic analysis found an increase in the synthesis and release of serotonin in aged mouse distal ileum (Parmar et al., 2012). A study on human duodenum found that serotonin positive cells appeared to increase with age (Sandstrom and El-Salhy, 1999). Conversely, staining for somatostatin and chromogranin, markers for EC cells, did not reveal any change in aged rat colon (Sweet et al., 1996).

Table 3.1 Summary of age-related changes in the density of EC cells and mast cells

Species	Region	Findings	Reference
EC cells			
Rat	Stomach	Increased EC cells and enhanced serotonin production	(Khomeriki, 1989)
Rat	Colon	No change	(Sweet et al., 1996)
Human	Duodenum	Increased EC cells from infant to adult, no further significant increase	(Sandstrom and El-Salhy, 1999)
Guinea pig	Distal colon	No change	(Bertrand et al., 2010)
Human	Rectum	No change	(Dunlop et al., 2004)
Mouse	Ileum & colon	Increased 5-HT release	(Bertrand et al., 2010)
Mouse	Distal ileum	Increased synthesis and release of 5-HT	(Parmar et al., 2012)
Mast cells			
Rat	Mesentery	Increased mast cells	(Banerjee and Dasgupta, 1981; Chatterjee and Gashev, 2012)
Rat	Lung	Increase	(Migally et al., 1983)
Human	Jejunum	No change	(Arranz et al., 1992)
Mouse	Tibial nerve	Increase	(Ceballos et al., 1999)
Human	Rectum	Reduction	(Dunlop et al., 2004)
Human	Dermis	Increase	(Gunin et al., 2011; Petrov et al., 2013)

Some studies have demonstrated that the number of mast cell are increased with ageing in human dermis and suggested that mast cells could be potentially involved in tissue damage and ageing decay in the skin (Gunin et al., 2011; Petrov et al., 2013). Another study in rat lung has revealed a greater perivascular mast cell density in aged group, comparing with young control after exposure to and recovery from chronic hypoxia (Migally et al., 1983).

Studies on mast cells in ageing have been limited to derma and lung in context of allergy or asthma. In the GI tract, two reports revealed increased mast cells in the mesenteric tissue of aged rat (Banerjee and Dasgupta, 1981; Chatterjee and Gashev, 2012). A study quantified mast cells from jejunal biopsy and did not found dramatic difference between aged (>70) and adult group (20-50) (Arranz et al., 1992). Circulating monocyte chemoattractant protein-1, a chemoattractant for monocytes, T cells, mast cells and basophils, has been demonstrated positively correlated with advanced age in healthy humans (Inadera et al., 1999). In addition, production of stem cell factor that regulates mast cell formation is augmented with age (Hart et al.,

1999). These independent lines of evidence might suggest increases in mast cells in the elderly.

As introduced in chapter 1, transient receptor potential (TRP) channels play an important role in detecting and responding to a variety of stimulations. It has been shown that TRPV1, TRPA1, TRPM8, TRPC6, TRPM2 are involved in nociceptive visceral sensitivity (Boesmans et al., 2011). TRPM4 has been demonstrated to be a critical regulator of Ca^{2+} entry in mast cells (Vennekens et al., 2007). TRPC4 and TRPC6 are implicated in smooth muscle contractility (Walker et al., 2002; Boesmans et al., 2011). Potential alterations of these ion channels with advanced age might contribute to age-related dysfunctions; however, limited studies have addressed these potential changes. Expression level of TRPC6 was not changed in aged rat aorta (Erac et al., 2010). No change was found for TRPV1 expression in aged mice DRG (Wang et al., 2006). Changes in gene expression of TRP channels in aged human bowel are largely unknown. Similarly, effect of ageing on the expression of TPH1 and chromogranin A (CgA), markers for EC cells, hasn't been investigated in human bowel.

Objectives

This chapter examined intestinal specimens from patients at different ages using morphological and molecular techniques to evaluate possible age-related changes in:

1) the density of EC cells;
2) the density of mast cells;
3) the density of sensory afferents nerves;
4) the anatomical relationship between mast cells and afferent nerves;
5) the gene expression of selected TRP channels and EC cell markers

3.2 Experimental protocol and analysis

Quantification of EC cells density in human bowel mucosa

Human bowel specimens were fixed in 4% PFA, embedded in OCT and sectioned in a cryostat as described in section 2.3. EC cells were labeled by a polyclonal rabbit anti serotonin antibody (1:50, AbD Serotec, AHP522) and visualized by a goat anti-rabbit secondary antibody conjugated with Cyanine 3 (Cy3, 1:400, Jakson ImmunoResearch, 111-165-144).

10 random images were acquired from one specimen under 20 times objective for offline quantification. EC cells density was calculated as number of EC cells divided by the area of mucosa. EC cells were counted blindly, and area of mucosa was measured using ImageJ software.

Quantification of mast cells density in human bowel

Previous studies showed a critical influence of the fixative on the number of mast cells identified by staining, and Carnoy's fixative was recommended as the best one to evaluate human intestinal mucosal mast cells (Strobel et al., 1981). We did a brief test using human bowel specimens to compare Carnoy's fixative with PFA, and no significant difference was found in the densities of toluidine blue stained mast cells. As such, for mast cells quantification, all specimens were fixed in 4% PFA, embedded in resin, sectioned using a microtome and stained in toluidine blue as described in section 2.3.

Three populations of mast cells (mucosa, submucosa and serosa) were blindly quantified under 20 times objective of an Olympus BX51 light microscope using an eyepiece graticule (area= 0.35mm*0.35mm= 0.1225mm^2). As shown in figure 3.1, the eyepiece graticule was orientated along the muscularis mucosa or muscle layer at the left edge of sections to select the first field and then moved horizontally to select the remaining fields. Ten random microscope fields in mucosa and serosa respectively as well as 20 random fields in submucosa were counted for each section. The average density of a sample was calculated from 5 sections. Data were expressed as number of mast cells per square millimeter.
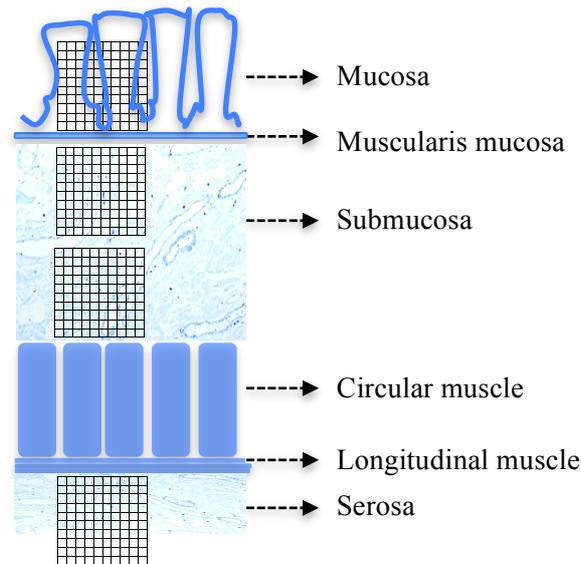
Figure 3.1 Schematic representation of the counting method for mast cells in human bowel. Prior to counting mast cells in the mucosa, one edge of the eyepiece graticule was orientated along the muscularis mucosa. By moving the graticule in parallel, ten random microscope fields in the mucosa were selected and counted. Similarly, 10 random fields in the serosa and 20 random fields in the submucosa were quantified. Area of graticule= 0.35mm×0.35mm= 0.1225mm^2.

Quantification of Substance P (SP) density in human bowel

Specimens were fixed in 4% PFA, embedded in OCT and sectioned in a cryostat as described in section 2.3. SP positive varicosities were labeled by a polyclonal rabbit anti SP antibody (1:50, Abcam, ab24126) and visualized by a goat anti-rabbit secondary antibody conjugated with fluorescein isothiocyanate (FITC, 1:400, Vector, FI-1000).

Images were acquired under a 20 times objective from 5 random fields for each specimen. Areas occupied by SP immunoreactivities and total areas of mucosa were measured using ImageJ software. SP density was expressed as percentage of SP occupied area in total area of mucosa.

Anatomical relationship between mast cells and afferent nerves

Specimens were fixed in 4% PFA, embedded in OCT and sectioned in a cryostat as described in section 2.3. Double immunostaining was performed by applying a mixture of monoclonal mouse anti mast cell tryptase antibody (1:1000, Santa Cruz, sc-271095) and polyclonal rabbit anti SP antibody (1:50, Abcam, ab24126). Staining

was revealed by a mixture of goat anti-mouse (Cy3, 1:400, Jackson ImmunoResearch, 115-166-003) and goat anti-rabbit secondary antibody (FITC, 1:400, Vector, FI-1000).

Images of double staining were acquired under a 20 times objective from 5 random areas for each specimen. The distance between a mast cell and its nearest SP varicosity was measured using ImageJ software. Considering the fact that the thickness of the cryo-section was 10 μm, if the distance between mast cell and SP was more than 10 μm, it was unclear whether there would be a closer SP varicosity in adjacent sections. As such, distances over 10 μm were excluded for distribution analysis.

Data analysis

Linear regression was used to evaluate the potential associations between age and EC cells, mast cells, SP and mast cell-SP relationship. The value of r^2 reflects the goodness-of-fit of linear regression. P value indicates the probability that randomly selected points would result in a regression line if there were no linear relationship between X and Y. P<0.05 was taken as significant. The solid line in a linear regression figure is the best-fit line and the dotted lines represent 95% confidence intervals.

Gene expression in human bowel mucosa

Up to 20 mg mucosa tissue dissected from human bowel specimens was immediately placed in at least 10 volumes of RNAlater Stabilization Reagent (QIAGEN, 76106) after harvested and stored at -20°C. RNA isolation, reverse transcription and quantitive real-time PCR were performed as described in section 2.5. Fluorescent probes used in RT-PCR were listed in table 3.2.

Table 3.2 Summary of probe sequences used in RT-PCR

All probes were against Homo sapiens genes.

Genes	Accession No.		Sequence	Expected Size (bp)
TRPA1	NM_007332	Forward	5' GCCACTGAGATTGTTAAACTGATG 3'	129
		Reverse	5' GTCTGCTAGCTCATGGTGAT 3'	
TRPC4	NM_016179	Forward	5' CGTCGAGTGGATGATATTACCG 3'	117
		Reverse	5' ACAAAGTCCATTAGATTCCACCA 3'	
TRPC6	NM_004621	Forward	5' AGGACTATCTGCTCATGGACT 3'	113
		Reverse	5' GTGAGCCAGTCTGTTGTCAG 3'	
TRPM2	NM_003307	Forward	5' TCCTCCTACTCTGCCTCTAC 3'	111
		Reverse	5' AAATCTGGTCCGTGTGCTC 3'	
TRPM4	NM_001195227	Forward	5' CAGTGACTTCCCAAGTATCCTG 3'	111
		Reverse	5' CAGTTGCTGTGCTCCATGA 3'	
TRPM8	NM_024080	Forward	5' GCTGGCTAATGAGTACGAGAC 3'	126
		Reverse	5' TCCAGACAGTTGCTTCCAC 3'	
TRPV1	NM_080705	Forward	5' TCCTGCTCAACATGCTCATC 3'	132
		Reverse	5' AGCTCTTCTCCGTGTCCA 3'	
TPH1	NM_004179	Forward	5' CCATTGTGCCAACAGAGTTC 3'	115
		Reverse	5' GTTCATAGCCAAGTCCGCAA 3'	
CgA	NM_001275	Forward	5' GATCCTTTCCATTCTGAGACATCA 3'	128
		Reverse	5' GAACCTCTGAGAGTTCATCTTCA 3'	
GAPDH	NM_002046	Forward	5' ACATCGCTCAGACACCATG 3'	143
		Reverse	5' TGTAGTTGAGGTCAATGAAGGG 3'	
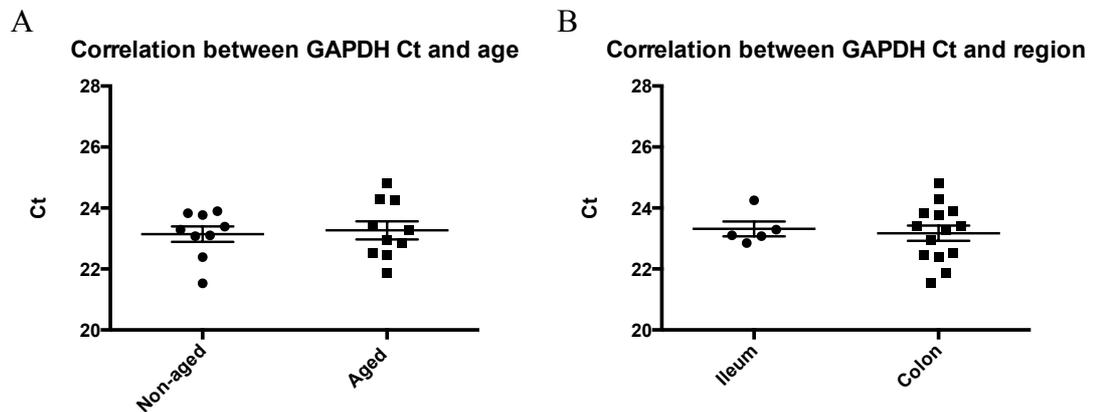
Figure 3.2 GAPDH expression levels in human bowel were not affected by age or region. Quantitive RT-PCR of GAPDH was performed with the same amount of starting RNA. Ct value indicated the expression level of GAPDH. (A) No significant difference was found between aged group (>65) and non-aged group (<65). P=0.75, unpaired t-test, N=9 for non-aged, N=10 for aged. (B) Expression levels of GAPDH in the ileum were comparable to that in the colon. P=0.75, unpaired t-test, N=5 for ileum, N=14 for colon.

GAPDH was used as endogenous reference gene after evaluating the influence of age and region on its expression. As shown in figure 3.2, no significant age or region-related change was found in GAPDH gene expression. To examine the changes of gene expression with advanced age, two analysis methods were employed. One method was linear regression to assess the relationship between relative gene expression ($1/2^{\Delta Ct}$) and age. The other was fold change analysis, as introduced in section 2.5. As such, specimens were classified into two groups based on the age of patients, aged (≥ 65) and non-aged (<65). Fold change >2 or <-2 was considered as significant.

3.3 Age-related changes in EC cell density of human bowel

EC cells, labeled by an anti-serotonin antibody, were distributed throughout the epithelia lining of mucosa in both ileum and colon. Figure 3.3 (A) and (B) showed the lateral view and apical view of serotonin immunoreactivities respectively. This conical distribution indicated that serotonin was concentrated at the base of EC cells, facing the laminar propria of the mucosa.
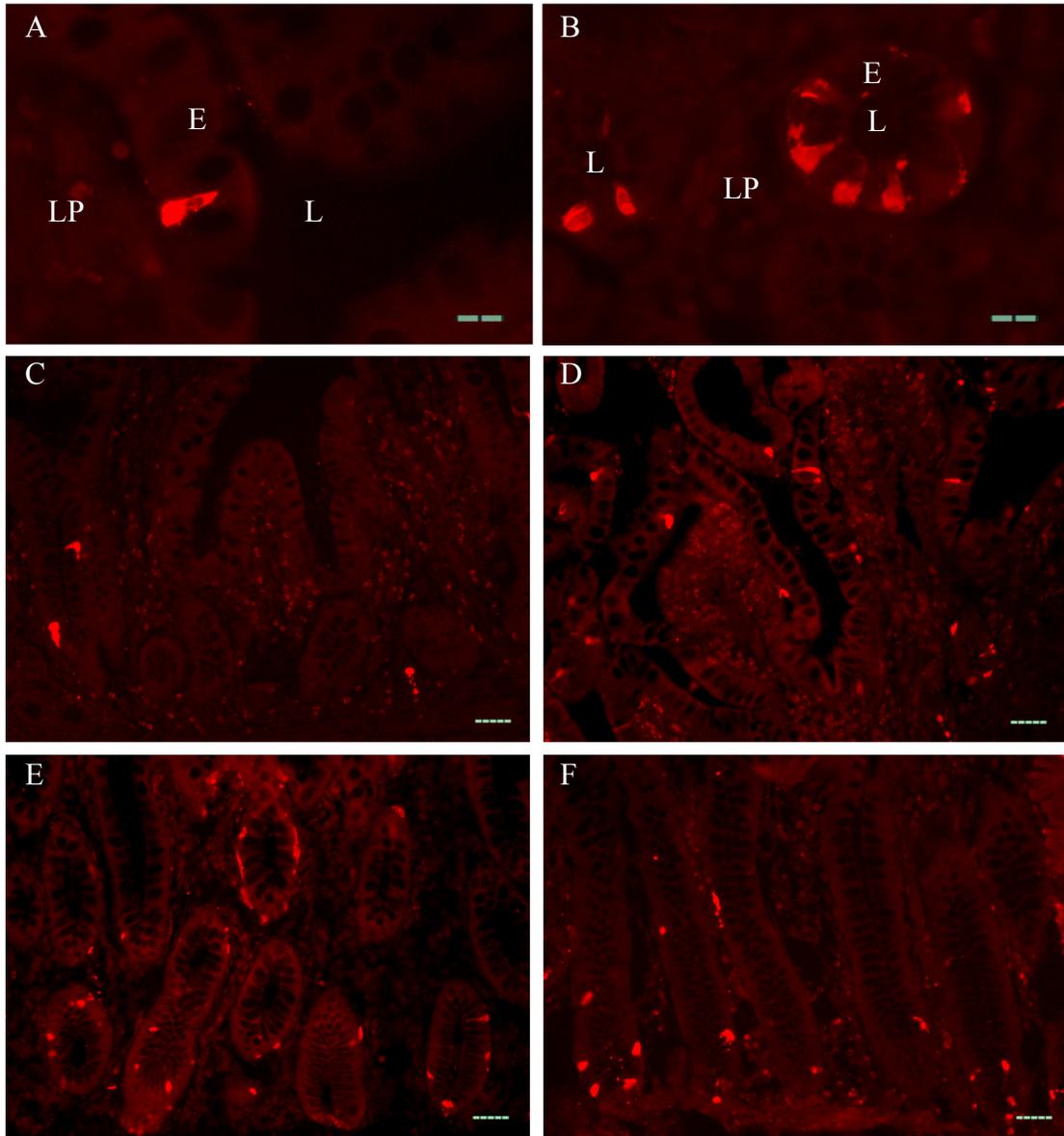
Figure 3.3 Representative images of human EC cells labeled by an anti-serotonin antibody. (A) Lateral view of an EC cell in the epithelium of a villus. (B) Apical view of colonic mucosa. Several EC cells were found in the epithelium surrounding a pit (crypt). (C) and (D) showed two examples of EC cell staining in the ileum from a 50-year-old patient (C) and a 87-year-old patient (D) respectively. (E) and (F) were two sample images of EC cells in the colon from two patients at age 49 (E) and 80 (F). E: epithelium, L: lumen, LP: laminar propria. Scale bar = 20 μm in A and B; 50 μm in C-F.

IHC was performed on 26 bowel specimens from patients of different ages, ranging form 38 to 87. The densities of EC cells were blindly quantified. EC cell density was increased with advancing age in human ileum (P<0.05, r^2=0.528, linear regression, N=10); conversely, such a correlation was not found in the colon (figure 3.4A&B). Furthermore, if male and female patients were separately analyzed, EC cells in the male ileum, female ileum and female colon tended to increase with age; only male colon showed no alteration with age (figure 3.4C-F).

The distribution patterns of EC cells throughout the lower GI tract were compared between both genders (figure 3.5). In specimens from male patients, EC cell density was augmented in a rostral-caudal direction (P<0.05, one-way ANOVA). On the contrary, in female specimens, EC cell density did not show any significant alteration across different regions.

These data indicated that EC cell distribution pattern along the GI tract was both region and gender related. To sum up, male patients had a marked higher density of EC cell in their colon compared with ileum, and that density in the colon was not increased with advanced age like ileum. In female patients, EC cell density did not show any regional difference, and was increased with age in both ileum and colon.
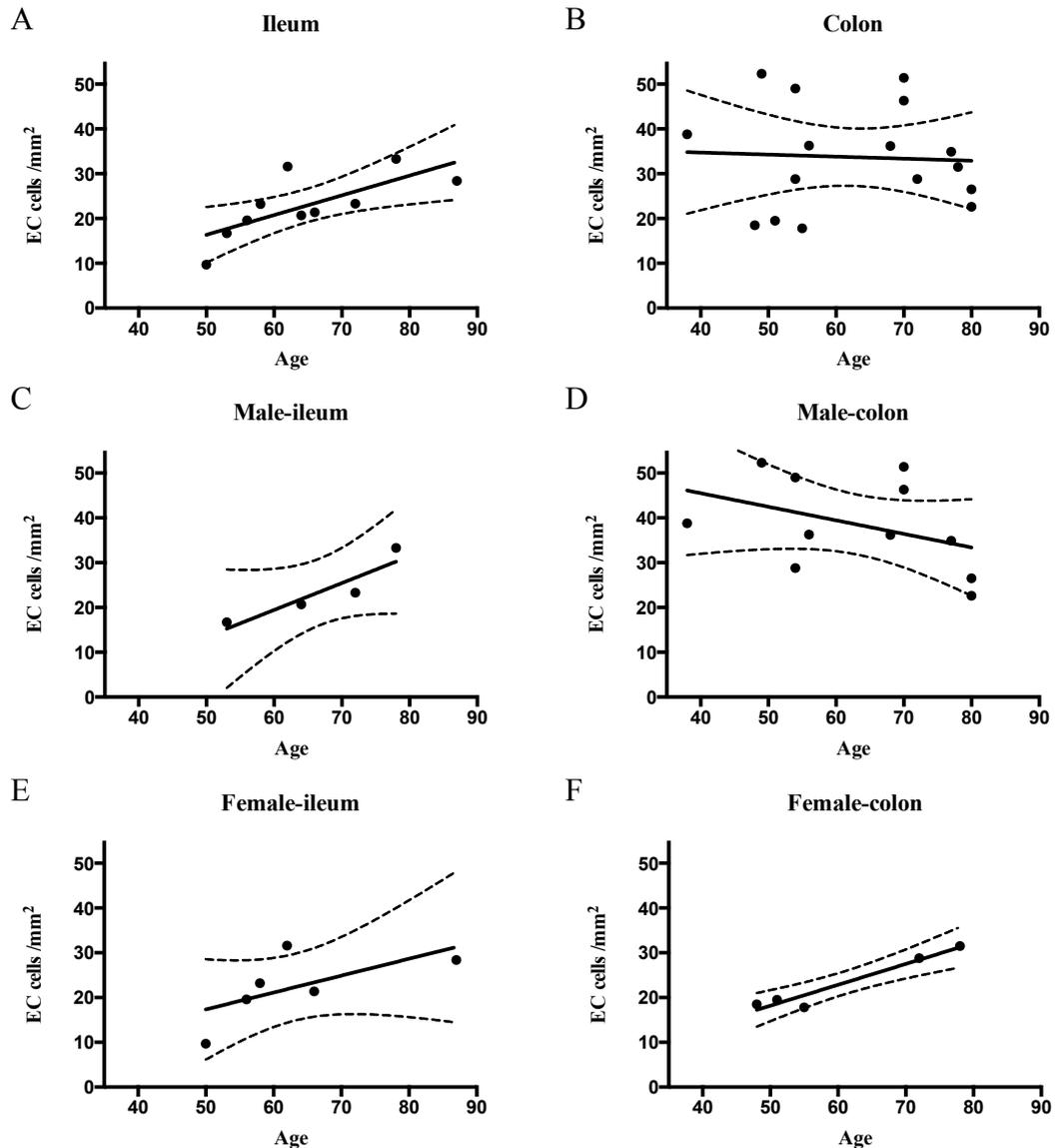
Figure 3.4 Changes of EC cell density with age in human ileum and colon. (A) EC cells density was increased with age in the ileum. $P<0.05$, $r^2=0.528$, linear regression, N=10. (B) EC cell density in the colon was not changed with age. $P=0.848$, $r^2=0.003$, N=16. (C, E and F) showed the relationship between EC cells and age in male ileum (C, $P=0.083$, $r^2=0.841$, N=4), female ileum (E, $P=0.175$, $r^2=0.405$, N=6) and female colon (F, $P<0.01$, $r^2=0.940$, N=5). (D) In male colon, EC cell density was not altered by ageing, $P=0.208$, $r^2=0.170$, N=11. EC cell density was expressed as number of EC cells per square millimeter of mucosa.
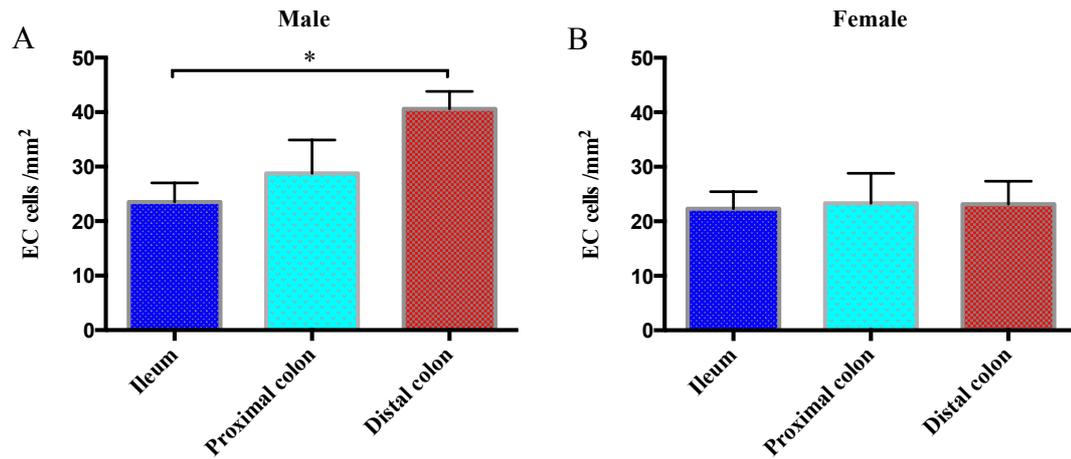
Figure 3.5 Distribution of EC cells throughout human intestine. (A) EC cell density was graded from ileum to distal colon in specimens from male patients. $P < 0.05$, one-way ANOVA. N=4 for ileum, 2 for proximal colon and 9 for distal colon. (B) In female patients, no significant difference was found throughout the intestine. N=6 for ileum, 2 for proximal colon and 3 for distal colon. Proximal colon was defined as ascending colon and transverse colon; distal colon included descending colon and sigmoid colon. * $P < 0.05$.

3.4 Age-related changes of mast cell density in human bowel

Due to containing granules rich in heparin and histamine, mast cells were stained red-purple (metachromatic staining) in the cytoplasm and blue (orthochromatic staining) in the nuclei by toluidine blue (as illustrated in figure 3.6A). Based on their location, three populations of mast cells were quantified, i.e., mucosal, submucosal and serosal mast cell.
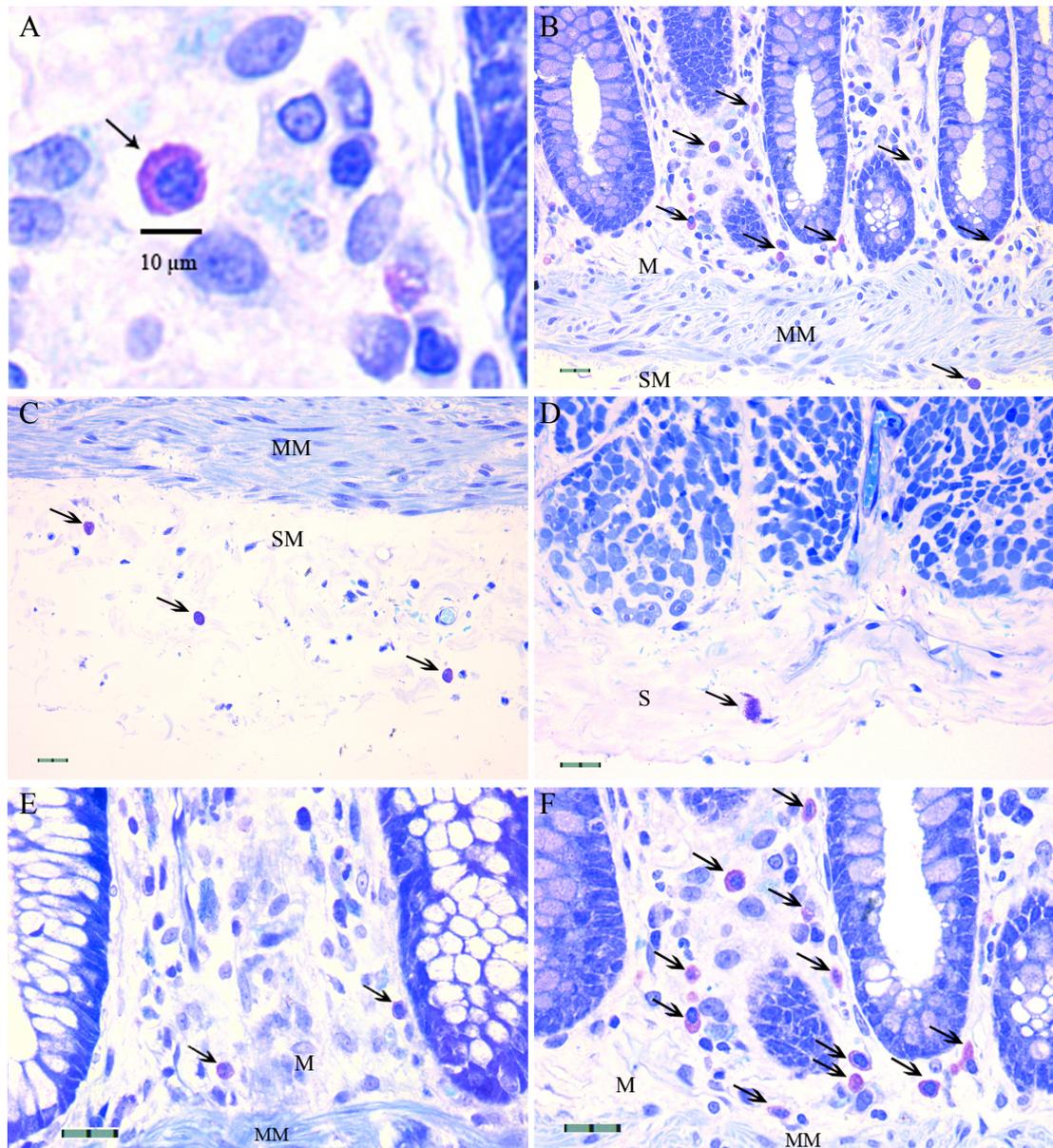
Figure 3.6 Sample images of human intestinal mast cells labeled by toluidine blue. (A) A high magnification image of mast cell stained in red-purple. (B-D) Sample staining of mast cells in the mucosa (B), submucosa (C) and serosa (D). (E-F) were two sample images of mucosal mast cells in the colon from two patients at age 43 (E) and 84 (F) respectively. M: mucosa, MM: muscularis mucosae, S: serosa, SM: submucosa. Scale bar = 10 μm in A; 20 μm in B-F.

Although mast cells could be detected throughout the gut wall from mucosa lamina propria to serosa/mesentery in human bowel, they were extensively distributed next to the muscularis mucosa, indicating their role in innate immune defense. Mucosa, as the innermost layer that directly contacts with the external environment, contained the largest density of mast cells (figure 3.7) in both small and large intestine. Mast cells were very occasionally found in the muscle layer, or to be accurate, in the connective tissue and blood vessels running through muscle layer.

Mast cell densities in different locations were plotted against the ages of patients (figure 3.8). Mucosal mast cell densities were significantly increased with advancing age in both ileum ($P<0.05$, $r^2=0.606$, linear regression, N=8) and colon ($P<0.05$, $r^2=0.413$, N=14). Such a relationship was not found in the submucosa and serosa.
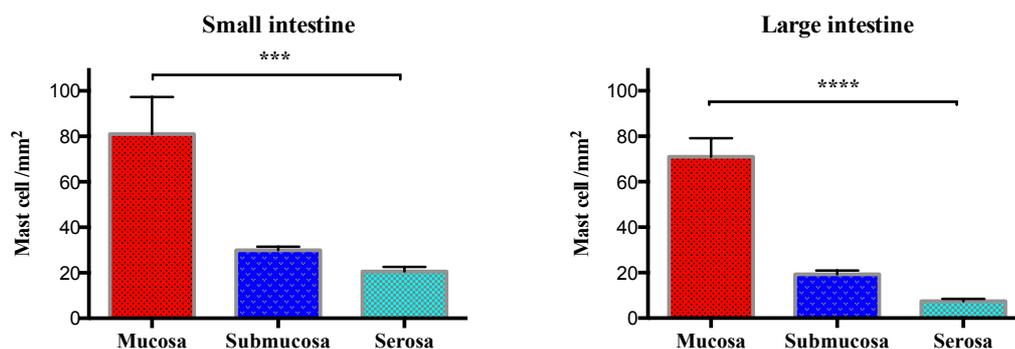
Figure 3.7 Distribution of mast cells throughout the gut wall of human small and large intestine. (A) Mast cell density in the mucosa was significantly greater than that in submucosa and serosa in small intestine. $P<0.001$, one-way ANOVA. Mucosa vs. submucosa, $P<0.01$; mucosa vs. serosa, $P<0.001$, N=9. (B) Similarly, in the large intestine, mucosa contained more mast cells than submucosa and serosa. $P<0.0001$, one-way ANOVA. Mucosa vs. submucosa, $P<0.0001$; mucosa vs. serosa, $P<0.0001$, N=15. *** $P<0.001$, **** $P<0.0001$.
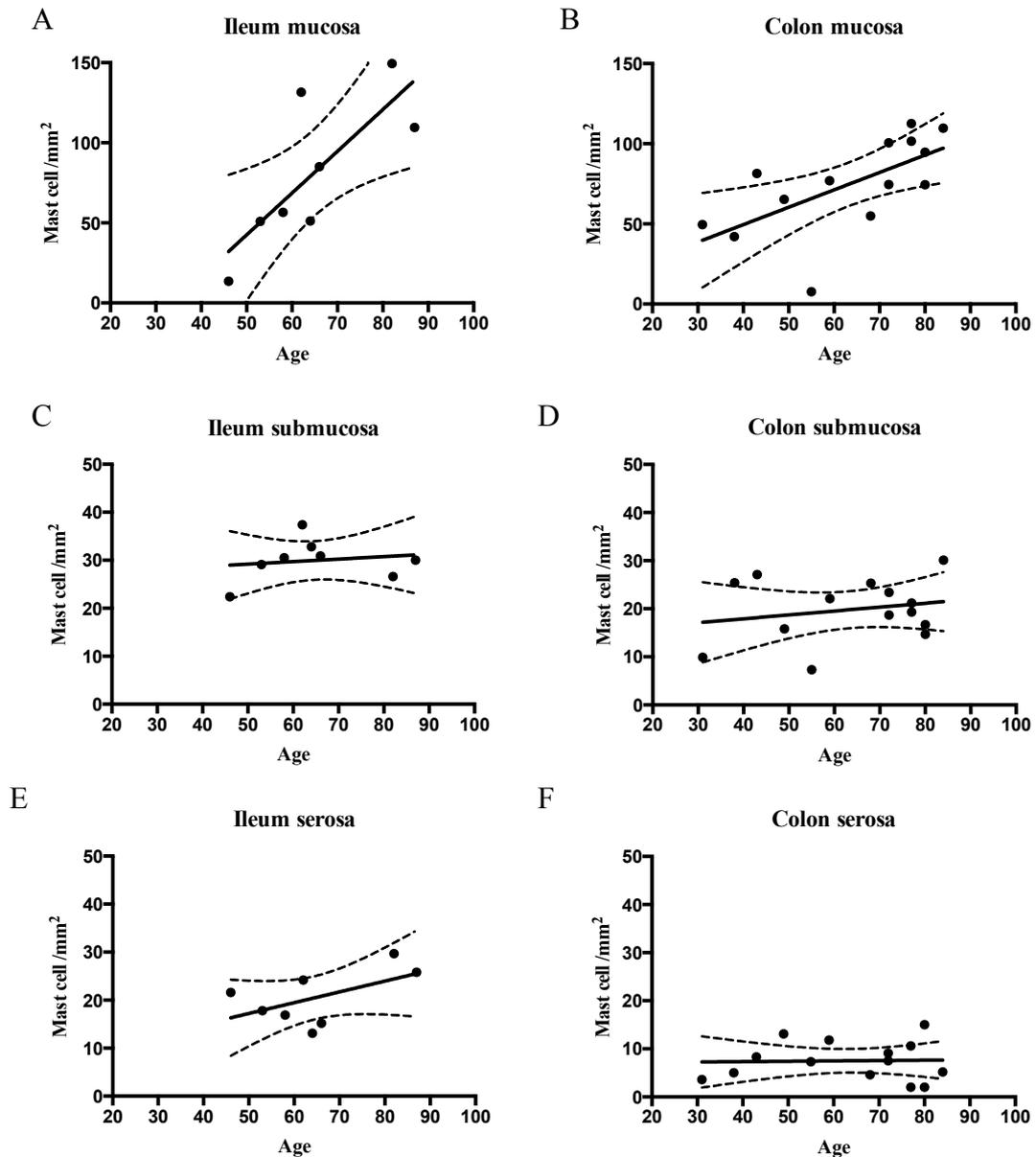
Figure 3.8 Changes of mast cell density with age in human ileum and colon. Mucosal mast cell densities were increased with age in both ileum (A, P<0.05, r^2=0.606) and colon (B, P<0.05, r^2=0.413). No significant correlation was found in ileal submucosa (C, P=0.697, r^2=0.027), ileal serosa (E, P=0.166, r^2=0.293), colonic submucosa (D, P=0.458, r^2=0.047) and colonic serosa (F, P=0.914, r^2=0.001). N=8 for ileum, 14 for colon, linear regression. Mast cell density was calculated as number of mast cells per square millimeter of mucosa.

Our data revealed a positive significant correlation between EC cell density and mucosal mast cell density in the ileum but not colon. This observation was not contrary to the age related changes in the densities of EC cells and mast cells. In the ileum, both EC cells and mucosal mast cells were increased with advanced age, whereas in the colon, EC cells were not changed with ageing.
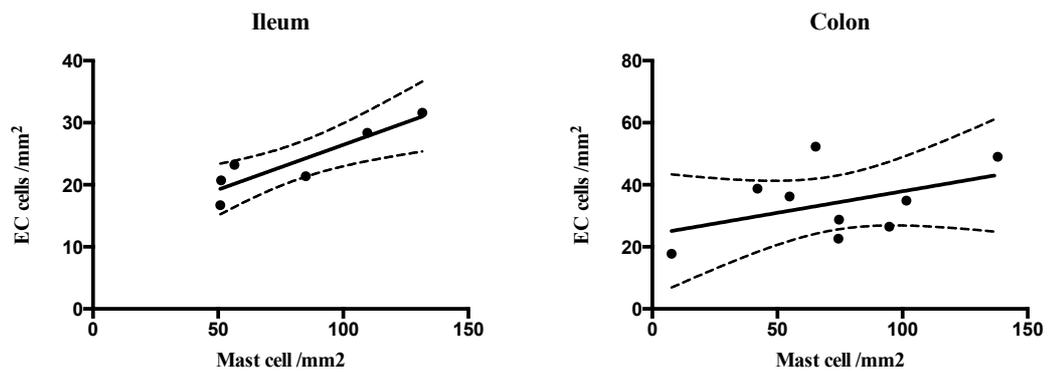
Figure 3.9 Relationship between EC cells density and mucosal mast cell density. A significant linear relationship was found between EC cell density and mucosal mast cell density in the ileum (A, P<0.05, r^2=0.818, linear regression, N=6) but not colon (B, P=0.226, r^2=0.201, linear regression, N=9).

3.5 Age-related changes of SP density in human colon

Vesicle-like SP positive immunoreactivities could be detected in myenteric plexuses, submucosa plexuses, nerve terminals and blood vessels. Very short SP positive nerve fibers were occasionally found in the submucosa and muscle layers, while continuous fibers were extensively distributed in the lamina propria of mucosa. This might be resulted from the angle between nerve pathway and sectioning direction. Another reason might be that SP contained vesicles were not visible at this magnification unless they formed varicosities close to the nerve terminals.

Diameter of SP positive varicosities was about 1~2 μm. Areas occupied by these varicosities in colonic mucosa were measured using ImageJ software. SP density tended to reduce with advanced age, although this was not statistically significant (figure 3.10C, P=0.058, r^2=0.344, N=11). This suggests a possible neurodegeneration of sensory innervation in aged human colon.
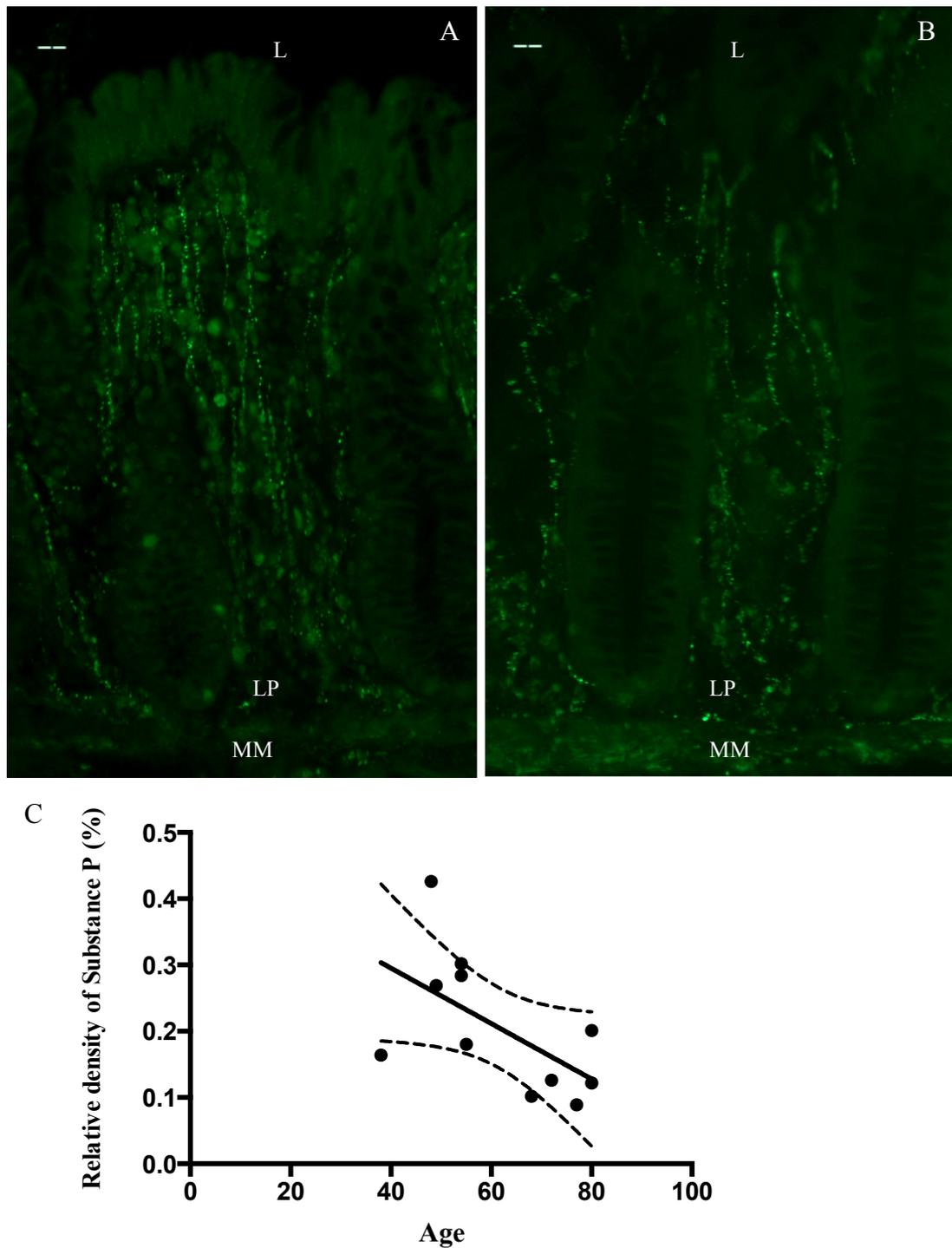
Figure 3.10 Changes of SP positive varicosities in aged human colonic mucosa. A and B illustrated SP positive nerve fibers running through the laminar propria of colonic mucosa in specimens obtained from two patients at age 54 (A) and 80 (B) respectively. L: lumen, LP: laminar propria, MM: muscularis mucosae. Scale bar = 20 μm. (C) SP density in colonic mucosa tended to reduce with advanced age, P=0.058, r^2=0.344, linear regression, N=11. Relative SP density was calculated as the percentage of areas occupied by SP immunoreactivities in the areas of mucosa.

3.6 Age-related changes of anatomical relationship between mast cells and afferent nerves in human colon

To examine the anatomical relationship between mast cells and sensory afferent nerves, double immunostaining of mast cell tryptase and SP was performed. A number of mast cells (stained in red) were closely associated with SP-positive varicosities (stained in green) (figure 3.11A&B). To quantify this relationship, the distance between mast cell and its closest SP varicosity was measured using ImageJ software. The distribution pattern of the distances between mast cells and SP was shown by a histogram (figure 3.11C). More than 50% of mast cells were located within only 1 μm away from SP-positive nerve fibers. The percent of mast cells within 2 μm (1~2 μm) from SP was about 14%, and this percentage reduced gradually with the distance increasing.

Two representative distribution patterns of this anatomic relationship from adult and aged patients respectively were shown in figure 3.11D. The distribution pattern in aged specimen shifted to the left remarkably, suggesting more mast cells in close apposition with SP. A significant positive linear relationship was found between the percent of mast cells within 1 μm from SP with advanced age (figure 3.11E, P<0.05, r^2=0.508). From 4 μm, the percentage tended to reduced with age, although only 8 μm (P<0.05) and 9 μm (P<0.01) groups reached significant level.

To examine whether this close association between mast cells and SP was random, a flipped control was perform. Prior to superimposing red channel (mast cell staining) and green channel (SP staining) together, one of the channels was flipped over horizontally (figure 3.12). Measurement of mast cells-SP distances and distribution analysis was performed consistently. As a result, the distribution pattern was completely broken in the flipped image, suggesting that the close associations that we observed were not accidental.

A

B

C

D

E

F
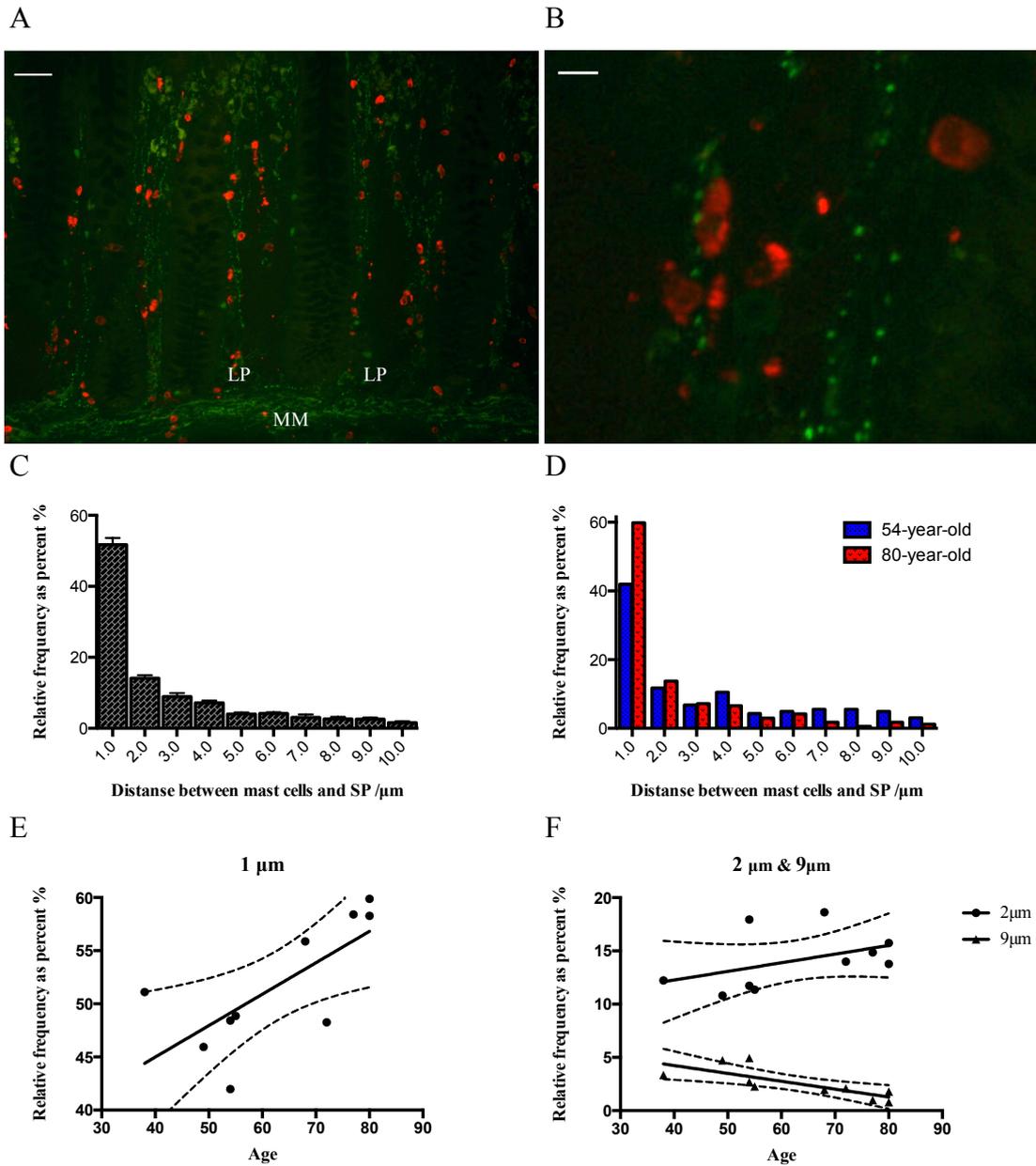
Figure 3.11 Changes of the anatomical relationship between mast cells and sensory nerves in aged human colonic mucosa. (A) Double immunostaining revealed the spatial relationship between mast cells (in red) and SP (in green) in colonic mucosa. (B) An amplified image showing the close association between mast cells and SP positive nerve fibers. (C) A histogram showing the distribution pattern of the mast cell (MC)-SP distances. Relative frequency indicated the percentage of mast cells located within certain distance. N=10. (D) Two sample distribution patterns of the MC-SP distance obtained from two patients at age 54 and 80 respectively. (E) The percentage of mast cells within 1μm from SP was increased with age, P<0.05, r^2=0.508, linear regression, N=10. (F) Percent of mast cells within 2 μm (1-2μm) from SP showed an increasing tendency, but not significant, P=0.205, r^2=0.192. For 3 μm group, no obvious tendency was found, r^2=0.008. From 4 μm to 10 μm, the percentages tended to reduce, r^2=0.120, 0.079, 0.350, 0.084, 0.453, 0.589 and 0.336 respectively; among these groups, a significant relationship was found only in 8 μm (P<0.05) and 9 μm (P<0.01) groups, linear regression, N=10. LP: laminar propria, MM: muscularis mucosae. Scale bar = 50 μm in A, 10 μm in B.
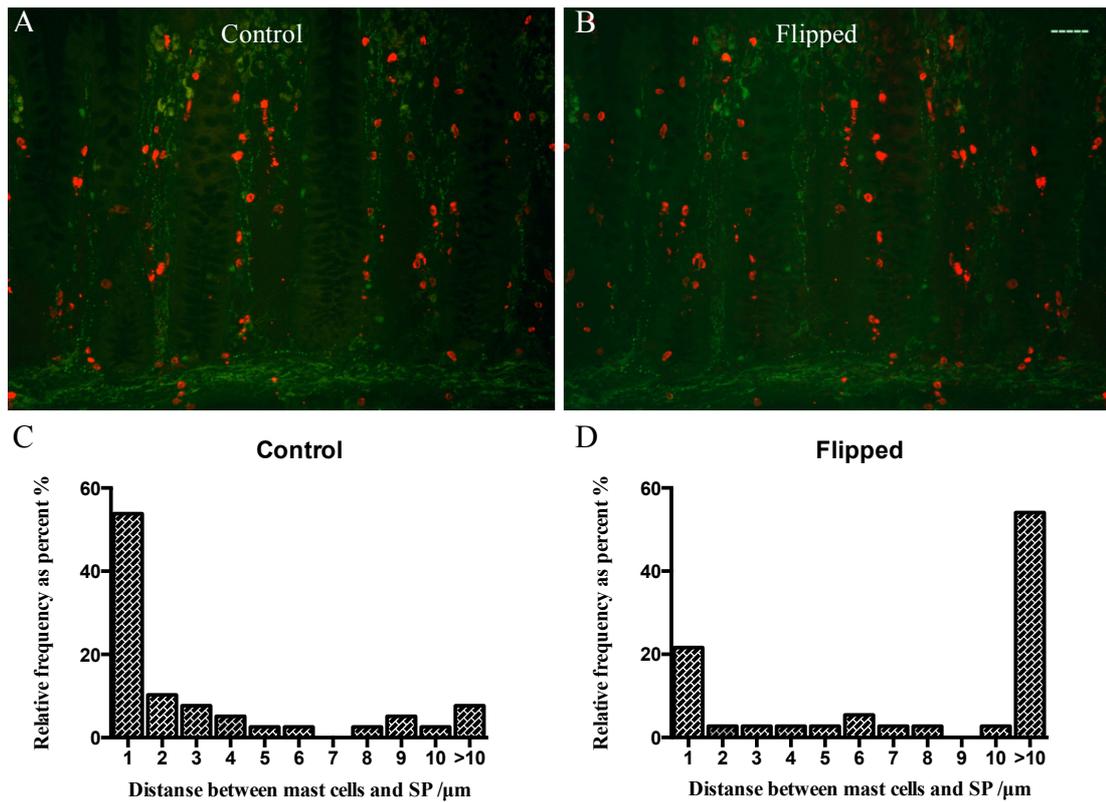
Figure 3.12 A flip control demonstrating that the close association observed above was not random. (A) A normal superimposed image showing the spatial relationship between mast cells (in red) and SP (in green) in colonic mucosa. (B) With the same image in (A), prior to merging red channel (mast cell staining) and green channel (SP staining), red channel was flipped over horizontally. Comparing with a control distribution in (C), the distribution pattern in flipped image was completely broken. Scale bar =50 μm.

3.7 Gene expression in human bowel mucosa

Quantitive RT-PCR was performed on total RNA isolated from the mucosa of human bowel specimens. All the examined genes were present in the mucosa of both ileum and colon, as confirmed by gel electrophoresis (figure 3.13A&B). Among these genes, TRPA1, TRPM4 and CgA were highly expressed, comparing with others (figure 3.13C&D).

Comparison of gene expression was made between ileum and colon. Based on t-test of relative gene expression ($1/2^{\Delta Ct}$), colonic mucosa showed a significant lower expression in TRPA1 ($P<0.05$) and TRPV1 ($P<0.01$) than ileum (figure 3.13C, N=5 for ileum, N=15 for colon). Fold changes were calculated as described in methods section of this chapter. Significant differences could be detected for TRPA1 (-2.19±0.26), TRPC4 (2.64±0.37), TRPC6 (2.00±0.30), TRPM2 (-2.62±0.39), and TRPV1 (-2.76±0.25) (figure 3.13E).

Ileum and colon specimens were classified into aged group (≥ 65, N=11) and non-aged group (<65, N=9). Unpaired t-test of the relative gene expression ($1/2^{\Delta Ct}$) did not reveal any significant differences (figure 3.13D) between the aged and the non-aged. Fold changes (aged vs. non-aged, figure 3.13F) revealed that only TRPC4 showed a significant up-regulation in the aged bowel with a fold change more than 2 (2.01±0.40).

The age-related comparison above was based on 5 ileum specimens and 15 colon specimens. Based on colon only, a similar significant increase of gene expression was detected for TRPC4 (figure 3.14A, fold change 2.40±0.30). However, there was no significant linear relationship between the relative gene expression ($1/2^{\Delta Ct}$) and age for TRPC4 (figure 3.14B) and all the other genes.
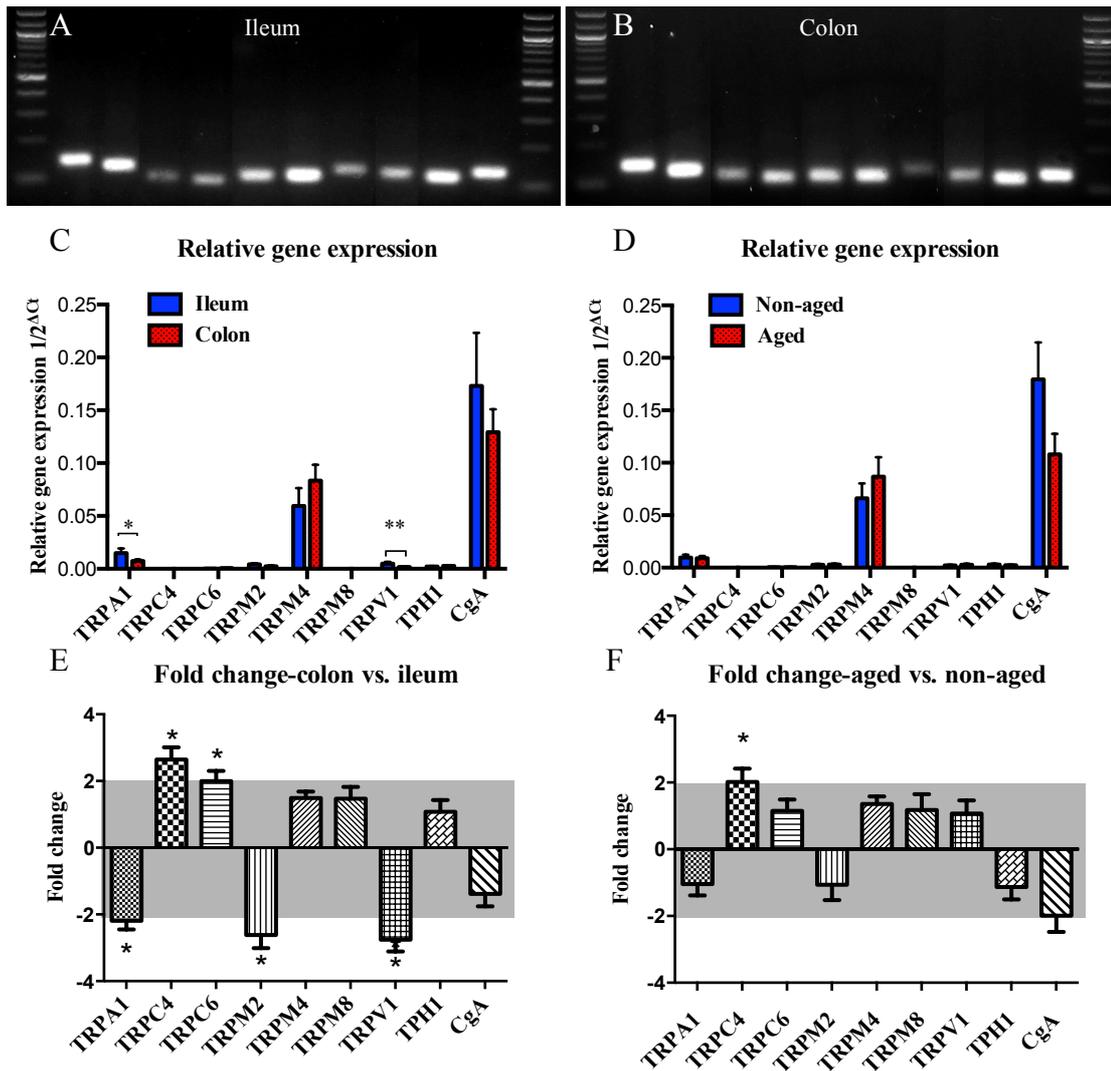
Figure 3.13 Gene expression in the mucosa of human ileum and colon. (A and B) Two electrophoresis images showing the presence of PCR products for examined genes in ileum (A) and colon (B). Bands from left to right were GAPDH, TRPA1, TRPC4, TRPC6, TRPM2, TRPM4, TRPM8, TRPV1, TPH1 and CgA. (C) Relative gene expression ($1/2^{\Delta Ct}$) in human ileum and colon. Comparing with ileum, colon showed a significant lower expression of TRPA1 ($P<0.05$) and TRPV1 ($P<0.01$), unpaired t-test, N=5 for ileum, N=15 for colon. (D) Relative gene expression ($1/2^{\Delta Ct}$) in aged and non-aged human bowel. Unpaired t-test did not reveal any significant differences, N=9 for non-aged group (3 ileum and 6 colon), N=11 for aged group (2 ileum, 9 colon). (E) Fold change of gene expression in the colon vs. ileum. Fold change = -2.19±0.26 for TRPA1, 2.64±0.37 for TRPC4, 2.00±0.30 for TRPC6, -2.62±0.39 for TRPM2, -1.49±0.19 for TRPM4, 1.47±0.35 for TRPM8, -2.76±0.25 for TRPV1, 1.08±0.35 for TPH1 and --1.38±0.38 for CgA. (F) Fold change of gene expression in aged group against non-aged group. Fold change= -1.04±0.34 for TRPA1, 2.01±0.40 for TRPC4, 1.15±0.34 for TRPC6, -1.06±0.46 for TRPM2, 1.36±0.23 for TRPM4, 1.18±0.47 for TRPM8, 1.07±0.39 for TRPV1, -1.13±0.37 for TPH1 and -1.99±0.49 for CgA.
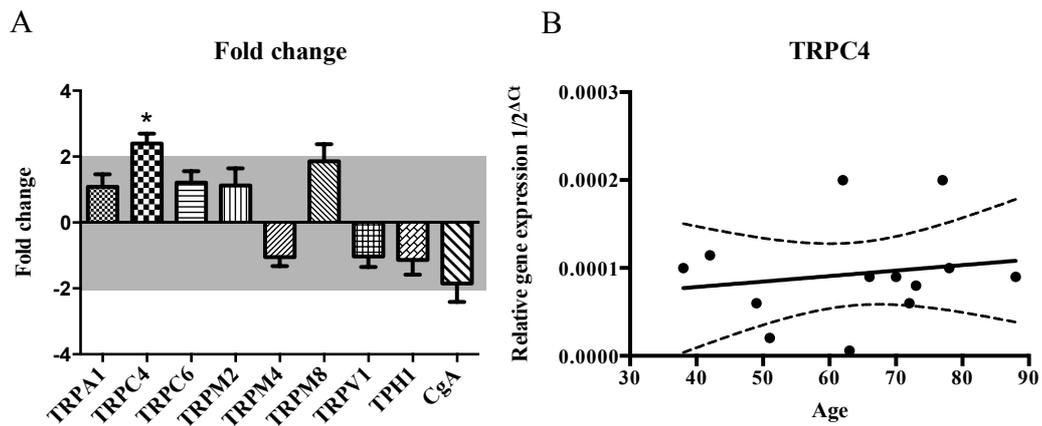
Figure 3.14 Gene expression in the mucosa of human colon. (A) Fold change of gene expression in aged colonic mucosa compared with non-aged group. Fold change= 1.08±0.38 for TRPA1, 2.40±0.30 for TRPC4, 1.21±0.35 for TRPC6, 1.12±0.52 for TRPM2, -1.05±0.27 for TRPM4, 1.86±0.52 for TRPM8, -1.03±0.32 for TRPV1, -1.14±0.45 for TPH1 and -1.85±0.56 for CgA. Only TRPC4 showed a significant up-regulation. N=9 for aged group, N=6 for non-aged group. (B) No significant linear relationship was found between relative gene expression ($1/2^{\Delta Ct}$) and age for TRPC4 and all the other examined genes in colonic mucosa. TRPA1, r^2=0.038, TRPC4, r^2=0.027, TRPC6, r^2=0.093, TRPM2, r^2=0.006, TRPM4, r^2=0.019, TRPM8, r^2=0.162, TRPV1, r^2= 0.009, TPH1, r^2=0.114 and CgA, r^2=0.216, linear regression, N=14.

3.8 Discussion

This chapter evaluated the effects of ageing on the density of EC cell, mast cell, SP, the anatomical relationship between mast cells and SP as well as gene expression of selected TRP channels in human bowel.

Effects of ageing on EC cell density in human bowel

The distribution of EC cells in the epithelium of the gut wall facilitates their function as the sensors of the luminal contents. Serotonin released from EC cells activates intrinsic and extrinsic sensory nerve terminals to initiate peristaltic and secretory reflexes as well as to transmit signals to the CNS; furthermore, inhibition of 5-HT$_3$ receptor has been shown to attenuate visceral afferent sensitivity and blunt abdominal pain in IBS patients (Prior and Read, 1993; Delvaux et al., 1998; De Ponti, 2004; Tonini, 2005; De Giorgio et al., 2007; Gershon and Tack, 2007; Spiller, 2007; Ford et al., 2009).

Our data suggest that EC cell density is increased with advanced age in human ileum but not in the colon. Further analysis demonstrated that this difference was because EC cells density in male colon was not changed with age.

In line with the EC cell distribution pattern observed in male patients, immunohistochemical studies on mouse have revealed that serotonin positive cells increase from small intestine to colon (Lee et al., 2010; Wheatcroft et al., 2005). Moreover, it has been demonstrated that mucosal serotonin concentrations in the lower GI tract of both IBS patients and healthy control display an ascending rostral-caudal gradient (Miwa et al., 2001). These studies prove a regional difference for EC cell distribution and serotonin availability; however these mixed-gender studies might largely reflect the pattern of the male, since the female shows an even distribution that might be masked by the male.

A study that revealed increased 5-HT positive EC cells in IBS patients compared with healthy control did not find any gender related differences in both groups (Cremon et al., 2011), although their data appeared to show a higher EC cell density in female control than male (0.42±0.19% vs. 0.3±0.1%, P=0.279). The difference

between their data and ours may be due to that they collected biopsies from the proximal descending colon, where the gender related difference is not as prominent as distal colon. Another conflicting report on rectal biopsy from healthy volunteers did not find any gender differences in EC cells distribution (Dunlop et al., 2004). It has been demonstrated that expression of $5-HT_3$ receptor in the colon of ovariectomized rats is significantly augmented, supporting a gender-related difference in serotonergic signaling (Li et al., 2004).

In addition to region and gender, our data suggest an age-related increase of EC cell in the ileum but not colon, or to be accurate, male colon. A chromatographic assay revealed elevated synthesis and release of serotonin in aged mouse ileum and suggested that was due to enhancement of the activity or expression of the enzyme L-amino acid decarboxylase, based on a significant increase in the ratio of 5-HT: 5-HTP (Parmar et al., 2012). In agreement with our data, a study on aged rat colon did not find any change in staining for somatostatin and chromogranin, markers for EC cells (Sweet et al., 1996). In addition, human rectal biopsy did not show any age-related alteration in EC cell density (Dunlop et al., 2004).

A study has reported a positive correlation between 5-HT release and mucosal mast cell infiltration in IBS colon and suggested that immune activation could drive 5-HT release (Cremon et al., 2011). Although this is reasonable inference, there is no sufficient evidence to rule out the possibility that 5-HT release drives mast cell infiltration or possibly both 5-HT release and mast cell infiltration are consequence of other factors. Our data revealed a positive correlation between EC cell density and mast cell infiltration in the ileum but not in the colon (figure 3.9). This may be not contrary to previous report, as EC cell density may not accurately reflect the release of serotonin. Alternatively, our data might suggest that some other mechanisms instead of mast cell infiltration accounted for the increase of EC cells, as the density of EC cells in the colon maintained at a high level across the age spectrum, despite increased mast cells with advanced age.

Effects of ageing on mast cell density in human bowel

Mast cells are classified into mucosal mast cells and connective tissue mast cells based on their locations. Our data suggest that mucosal mast cells predominate in human small and large intestine. Furthermore, densities of mucosal mast cell are augmented with advanced age in both ileum and colon. No significant alteration was detected in the densities of submucosal and serosal mast cells.

A study has reported a 27% increase in the total number of mast cells and 400% increase in the number of activated mast cells in aged rat mesenteric tissue and suggested that the presence of extensive degranulated mast cells in resting conditions might promote a chronic inflammatory environment in aged mesentery (Chatterjee and Gashev, 2012). Conversely, a study on human rectal biopsy reported a reduction in mast cell density in the aged population (Dunlop et al., 2004). It is unknown why this study is contrary to our data. It may be attributed to regional (ileum, colon vs. rectum) or methodological differences (whole-thickness specimen vs. biopsy). Another study did not find dramatic difference in mast cell density quantified from jejunal biopsy between aged (>70) and younger group (20-50) (Arranz et al., 1992). However, their criteria for screening suitable experimental objects was that the final diagnose was of functional gastrointestinal symptoms or of a minor clinical problem without evidence of immunological, infectious, neoplastic, or allergic disease. It is possible that mast cells were abnormal on those patients of functional GI disorders.

Some studies have proposed that increased circulating levels of pro-inflammatory cytokines including interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-alpha and IL-12, IFN-alpha/beta up-regulate the inflammatory response in the elderly, and promote a low-grade chronic systemic pro-inflammatory state (Fagiolo et al., 1993; Franceschi et al., 1995; Bruunsgaard and Pedersen, 2003; Csiszar et al., 2003; Giunta, 2008). This low-grade inflammatory state may be associated with enhanced immune cell infiltration including mast cells.

Effects of ageing on density of sensory nerves in human colon

SP is present at extrinsic primary sensory afferents innervating the GI tract (Holzer, 1998; Chang et al., 1996). Although a proportion of SP positive myenteric neurons are excitatory motor neurons (Costa et al., 1996), the majority of SP positive neurons in the submucosa are sensory neurons (Bornstein et al., 1989). As such, SP is widely used as a marker for sensory nerve fibers (Miller et al., 2000; Sulaiman et al., 2001; Arnold et al., 2012).

SP-positive nerve fibers ramify throughout the laminar propria to form a dense network underneath the epithelium in human colonic mucosa (Keast et al., 1985). This distribution pattern facilitates their function to detect external stimulation, collect signal from other cells (e.g., EC cells, mast cells) and interact with them.

As introduced in the beginning of this chapter, a number of studies revealed a significant loss of enteric neurons in the elderly. Furthermore, the ganglionic areas were found decreased in the submucosal plexus in association with reduced neuropil spaces (Phillips et al., 2007; Phillips and Powley, 2007). Normally neurons within the ganglion are surrounded by small interstices of neuropil composed of axons, dendrites and glia cells. Based on the evidence of collapsed ganglia with loss of neuropil, it can be inferred that the nerve fibers that innervate or project from the ganglia might be reduced. A supporting evidence has revealed that the density of SP-positive nerve fibers is decreased in the circular muscle of aged guinea-pig colon (Peck et al., 2009). In addition, a decrease in SP level has been described in the DRG neurons of aged rat (Bergman et al., 1996).

In agreement with these studies, our data indicates that SP density tends to decrease in the mucosa of aged human colon. Reduction of sensory innervation may result in impaired initiation of motility reflex and consequently delayed or diminished motility, which might contribute to the prevalence of constipation in the elderly.

Effect of ageing on the close association between mast cells and sensory nerves

Mast cells have been reported in close proximity to SP positive afferent nerve fibers in a variety of rodent organs (Stead et al., 1987; Dimitriadou et al., 1997) and in human skin (Naukkarinen et al., 1996), bile duct (Gulubova and Vlaykova, 2004)

and IBS colon (Pang et al., 1996). Our data confirmed this association in the colonic mucosa of non-diseased specimens from cancer patients.

The close anatomical connections between mast cell and SP facilitate their communications. SP can induce degranulation of mast cells and release of histamine, tryptase and serotonin (Shanahan et al., 1985; Repke and Bienert, 1987), which in turn, activate afferent nerve through corresponding receptors on the terminals (Corvera et al., 1999; Nozdrachev et al., 1999; Breunig et al., 2007). These bi-directional interactions might form a positive feedback loop and amplify inflammatory responses. Evidences have revealed a significant correlation between abdominal pain and the association of mast cells and nerves in IBS patients (Barbara et al., 2004b).

The effects of ageing on the close morphological association between mast cells and nerves haven't been investigated. This study revealed an increased proportion of mast cells in close apposition to SP afferent nerves with advanced age. Although we reported an increased density of mast cells with ageing, that is not likely to be the main cause of the enhanced mast cell-SP association. The first reason is that we analyzed the relative distribution as a percentage instead of absolute number. Increased number of mast cells may not necessarily change the distribution pattern. Additionally, in our flipped control, the mast cell-SP association is completely broken, indicating that the pattern is not random. It has been identified that both mast cells and neurons express the same adhesion molecule, named synaptic cell adhesion molecule (SynCAM), which is believed to be responsible for the connection between mast cells and nerves (Biederer et al., 2002; Ito and Oonuma, 2006).

The elevated anatomical mast cell-SP associations may be consistent with the low-grade pro-inflammatory state in the elderly. Since closer associations increase the severity of abdominal pain in IBS, whether it is the case in the elderly will be further discussed later by comparison of the alterations in IBS and ageing.

In comparison with mast cells, EC cells are not closely apposed to afferent nerves. Although EC cells are able to shift along the epithelium from the crypt to the top and

finally peel off in the lumen, they are restricted in the epithelium. Even though SP in the laminar propria might approach the base of epithelium, the grade of apposition is difficult to reach nanometer level.

Effect of ageing on gene expression of TRP channels in human colonic mucosa

Our data demonstrate that presence of TRPA1, TRPC4, TRPC6, TRPM2, TRPM4, TRPM8, TPH1 and CgA mRNA in the mucosa of human ileum and colon. Among these genes, TRPA1, TRPM4 and CgA are highly expressed, comparing with others. We examined the differences in gene expression between ileum and colon. Colonic mucosa showed a lower expression in TRPA1, TRPM2 and TRPV1 but a higher expression of TRPC4 and TRPC6 than ileum.

Specimens were classified into aged group ($\geq$65) and non-aged group ($<$65) to calculate fold changes (aged vs. non-aged) of gene expression. As a result, only TRPC4 showed a significant up-regulation in the aged bowel with a fold change more than 2, although correlation test between the relative gene expression ($1/2^{\Delta Ct}$) of TRPC4 and age did not reveal any significance. The involvements of TRPC4 in the regulation of visceral contractility have been extensively studied (Walker et al., 2002; Lee et al., 2005; Boesmans et al., 2011); in addition, a recent study on TRPC4-knockout rat demonstrated that it's also implicated in visceral pain sensation (Westlund et al., 2014). Since TRPC4 plays multiple roles in the GI physiology, the interpretation of up-regulation of TRPC4 in the elderly requires further investigation.

Ageing and IBS

As introduced in previous chapters, IBS is a common gastrointestinal disorder characterized by abdominal pain, bloating and disturbed defecation. The etiopathogenesis underlying IBS has not been fully understood. One of the hypotheses is end organ visceral hypersensitivity mediated by mast cell and EC cells. In comparison with many previous reports, current study notes some interesting similarities between aged bowel and IBS bowel, such as increased EC cells, mast cells and enhanced mast cell-nerve apposition.

Table 3.3 Summary of changes in EC cells and mast cells in IBS patients

Region	Gender (M: F)	Clinical subtype	Findings	Magnitude of change	Reference
EC cells					
Ileum	17:21	IBS-C +IBS-D	No change		(Wang et al., 2007)
	9:16	IBS-D	No change		(Park et al., 2006a)
Colon	7:18	IBS-C +IBS-D	Increase	51%	(Cremon et al., 2011)
	5:2	IBS-D	No change		(Kim et al., 2010)
	7:3	IBS-D	Increase	64%	(Bian et al., 2009)
	9:16	IBS-D	No change		(Park et al., 2006a)
Mast cells					
Ileum	17:21	IBS-C +IBS-D	Increase	25%	(Wang et al., 2007)
	10:8	IBS-D	Increase	30%	(Park et al., 2006b)
	4:16	IBS-C +IBS-D	Increase	243%	(Weston et al., 1993)
Colon		IBS-D	Increase	68%	(Sohn et al., 2014a)
	9:16	IBS-C +IBS-D	Increase	45%	(Coeffier et al., 2010)
	5:2	IBS-D	No change		(Kim et al., 2010)
	7:3	IBS-D	Increase	94%	(Bian et al., 2009)
	4:7	IBS-C +IBS-D	Increase	164%	(Buhner et al., 2009)
	13:35	IBS-C +IBS-D	Increase	116%	(Cremon et al., 2009)
	3:20	IBS	Increase	38%	(Akbar et al., 2008)
	10:8	IBS-D	Increase	28%	(Park et al., 2006b)
	13:31	IBS	Increase	179%	(Barbara et al., 2004b)
	2:12	IBS	No change		(O'Sullivan et al., 2000)

To examine if the magnitude of increase in EC cells and mast cells with advanced age was pathophysiologically significant, their magnitude of change in IBS was roughly calculated based on literature (table 3.3). The average increasing rates of EC cells and mast cells in IBS were 58% and 94% respectively. To make a comparison, the current study divided human specimens into aged (>65) and non-aged group (<65). The increasing rate by ageing for EC cells and mast cells were about 31% and 80%, which were comparable to the rates in IBS.

It has been proposed that the hypersensitivity in IBS might be attributed to a low-grade inflammation present in IBS bowel (Barbara et al., 2002; Spiller, 2003), evidenced by increased number of T cells (Spiller et al., 2000; Chadwick et al., 2002; Tornblom et al., 2002) and increased proinflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α (Dinan et al., 2006; Liebregts et al., 2007). In line with IBS, ageing is associated with increased circulating levels of pro-inflammatory cytokines

including IL-1, IL-6, TNF-α and IL-12, IFN- α/β, which promote a low-grade chronic systemic pro-inflammatory state in the elderly (Fagiolo et al., 1993; Franceschi et al., 1995; Bruunsgaard and Pedersen, 2003; Csiszar et al., 2003; Giunta, 2008).

In line with the low-grade inflammation in IBS, increased mast cells have been frequently reported in IBS bowel. Although conflicting results exist, as summarized in a review, 29 in 40 studies reported increases in mast cells with only one reporting a decrease (Matricon et al., 2012). In agreement with the majority, our data reveal that mast cells increase with advanced age. The causes of increased mast cells in IBS patients remain unknown. It has been suggested previous episodes of infectious enteritis (Gwee et al., 1999) and food allergies (Smout et al., 2000) might contribute independently or in combination. Whether the age-related increases in mast cells are accelerated by previous inflammatory events requires further investigation.

A study has reported a significant correlation between abdominal pain and vicinity of mast cells to nerves in IBS patients (Barbara et al., 2004b). Similarly, we found closer anatomical associations between mast cells and sensory nerve fibers in the elderly.

It is very controversial whether EC cells are augmented in IBS bowel. About half of previous studies have reported increases in EC cell, while the rest found no change (Matricon et al., 2012). This study revealed an increase of EC cells in the ileum and female colon with age, but not in the male colon. Since our data suggest that EC cell distribution depends on age, gender and region, the conflicting reports in IBS might be owing to ignorance of age or gender related differences.

These similarities between IBS and ageing suggest that they might share some common mechanisms. However, it has been shown that IBS is less prevalent in aged population and the severity of pain is decreased (Kay, 1994; Wilson et al., 2004; Tang et al., 2012). A difference between our data on age-related changes and IBS-related changes might explain this contradiction. Numerous studies have reported an increase in SP in IBS bowel (Mazumdar and Das, 1992; Dong et al., 2004; Sohn et al., 2014b); whereas, our data suggest an age-related decrease of SP positive sensory

nerve fibers. Since IBS is characterized as abdominal pain and discomfort, reduction of sensory innervation is likely to attenuate these sensation-based symptoms.

It is unknown whether the reduction of SP nerve fibers contributes to the increase of mast cells. Capsaicin or dihydrocapsaicin treatment that depletes peptidergic nerve fibers results in an increase in mast cells in neonatal rat dura mater (Dimitriadou et al., 1997) and neonatal sheep lung (Ramirez-Romero et al., 2000). A possible explanation is that one or more mediators released from afferent C-fibers might have a negative regulative effect on mast cell proliferation. Alternatively, as a compensative mechanism, reduction of sensory innervation results in augmented mast cells to maintain the sensory input from the immune system to the nervous system.

Mechanisms underlying ageing process in the GI tract, especially factors that promote ageing, are poorly understood. Diet habit is believed to play an essential role. Caloric restriction that is known to extend lifespan in rodents and other species has been demonstrated to prevent neurons loss in myenteric plexus of aged rat (Cowen, 2002). Further investigations are required to address these mechanisms.

Limitations

Studies on human specimens must confront variations in the patient age, gender, genetic background, medical history and so on. This variability is difficult to control by the researcher. An elegant review has discussed some basic principles in functional studies with human specimens, such as influence of disease, choice of control, timing of experiment, influence from surgical process, age/gender related variability and sample size (Sanger et al., 2013). Considering these factors might minimize variations.

One limitation of the current study is that inadequate clinical information was available, such as the genetic background, medical history and other gastrointestinal symptoms. For example, it was unknown if the patients were suffering from abdominal pain and changes in stools, which would suggest IBS. Since a lot of evidence has revealed altered densities of mast cell, EC cells and SP in IBS patient, ignorance of this information might influence the evaluation of age-related changes

in those cells. It is unclear whether the diseases of patients or the treatment they received could affect our observation. Although these "safe margins" were morphologically normal, it could not be assured that the changes that we observed in the elderly were pure effect of ageing. The huge variability in human specimens was difficult to control. When investigating the age-related alterations, we tried to control the gender and region. However, this was only feasible if there were a large number of tissue supplies.

In the introduction and discussion above, a number of conflicting and controversial results in the literature have been frequently mentioned. Ageing is a very complicated and comprehensive process. A variety of factors might contribute to this confliction, such as experimental models, co-morbidities, sampling strategies, experimental techniques and analysis methods.

Differences in experimental models including species, strains and different regions of the GI tract might account for most of the contradictory results obtained from different models. It has been noted that a large number of species differences do exist and knowledge obtained from one model sometimes translates poorly to another. In addition, nutrition might influences the ageing patterns as well, which is supported by the evidence that caloric restriction diet prevented neuronal loss in the myenteric plexus of aged rat ileum (Cowen et al., 2000). Perhaps other maintenance or environmental factors can potentially affect the patterns of ageing in the gastrointestinal innervation. Ageing study on human specimens is more complicated and difficult. In addition to genetic varieties, differences in living environments, ways of life, especially eating habits would largely affect the health conditions and further influence the patterns of ageing in the GI tract. Moreover, many diseases are more common in the aged population, such as cardiopulmonary disorders, degenerative disorder of the CNS, rheumatoid arthritis and degenerative joint disease, and the therapy aiming to treat them may directly or indirectly influence GI physiology. Last but not least, access to human specimens, especially "health" objects is very limited.

Experimental techniques might influence the results. An obvious example is different choice of markers, which might have different sensitivity and selectivity, leading to

variable labeling. Another possible example is that most studies that quantify certain type of cells in the elderly often employ immunohistochemistry or chemical staining. The results of cell labeling could be influenced by changes in tissue permeability to staining agents that might be one of the effects of ageing. In this case, these labeling techniques cannot truly reflect the changes of cells induced by age.

Another limitation in our study is that we sampled through a series of consecutive sections of a three-dimensional specimen when we analyzed the spatial relationship between mast cells and nerves. The problem is that nerve fibers and mast cells might overlay with each other or at different depth within the section, which preclude the measurement of the three-dimensional distances. Considering the fact that the thickness of the section is smaller than the diameter of mast cell, this limitation should be tiny. However, a powerful stereological technique would definitely be more reliable for this type of studies.

In addition to sampling and technique, comparisons between specimens varying in age are still based on analysis methods, which is crucial for interpreting the raw data. Some animal studies proposed that as animals grow, changes in organ size and surface area might result in a dilution of the density of neurons or other elements interested; to address this dilution, they developed a age-specific correction factor, which was the ratio of cross-sectional areas of samples in different age groups (Gabella, 1989; Cowen et al., 2000; Phillips et al., 2003; Phillips and Powley, 2007). The purpose of this normalization is that they tried to conclude a neuronal loss in aged animals. If they use the term neuronal loss instead of decreased density, they have to validate that the reduction they observed are not due to increase of organ size. In our study, the density of examined targets is simply expressed as number of cells per unit area.

CHAPTER 4

Extracellular afferent recording from isolated human bowel

4.1 Introduction

Significance of human afferent recording

Abdominal pain is a common character of many gastrointestinal disorders. Some of these disorders are defined as functional GI disorders, such as IBS, on the basis of unexplained recurrent pain in association with other GI symptoms. The prevalence of IBS reported by many epidemiological studies is quite variable due to diverse diagnostic criteria. It is widely accepted that an estimated prevalence rate in industrialized countries is about 10-15% (Maxion-Bergemann et al., 2006; Spiller et al., 2007). Although IBS is not a life-threatening disease, it consumes a large proportion of healthcare resource and thus imposes a substantial economic burden on its sufferers and the society (Fullerton, 1998; Hulisz, 2004; Maxion-Bergemann et al., 2006).

IBS is associated with a large unmet medical need, which can be attributed to poor understanding of the underlying pathophysiology of this disorder. Since IBS is defined as a functional bowel disorder, lack of marked pathological alterations make it difficult to build a mirrorlike animal model. Instead, animal models of visceral hypersensitivity have been developed based on the observations of hypersensitivity to colorectal distension in a large proportion of IBS patients (Whitehead et al., 1990; Verne et al., 2001; Dong et al., 2004). Studies on these models have developed some potential treatments for abdominal pain. For example, alosetron, a 5-HT$_3$ receptor antagonist, has been shown to inhibit the response to colorectal distension in the rodents (Scott et al., 1994; Clayton et al., 1996; Kozlowski et al., 2000). In 2000, alosetron was approved for marketing to release symptoms of abdominal pain in IBS patients; however, it was withdrawn from the market 9 months later owing to serious life-threatening adverse effects including ischaemic colitis and severe constipation accompanied with 7 death cases (U.S. Food and Drug Administration, 2002). Although alosetron was reintroduced in 2002, it was restricted to treat women with diarrhea-predominate IBS. In addition, Some other drugs failed to show efficacy against abdominal pain in IBS clinical trials, such as talnetant (NK3 receptor antagonist), GW876008 (corticotropin-releasing factor-1 receptor antagonist) and AZD7371 (5-HT$_{1A}$ receptor antagonist)(Drossman et al., 2008; Camilleri, 2010), in

spite of promising data from animal studies (Fioramonti et al., 2003; Schwetz et al., 2004; Lindstrom et al., 2009).

These translational failures highlight a demand for more thorough research on human directly. Indeed, a large number of studies using biopsies and supernatant from IBS patients have revealed the alterations in the immune infiltration (Spiller et al., 2000; Chadwick et al., 2002; Tornblom et al., 2002; Barbara et al., 2004b; Cremon et al., 2009), chemical environment (Barbara et al., 2004b; Dinan et al., 2006; Liebregts et al., 2007; Cremon et al., 2011), innervation patterns and receptor expression (Akbar et al., 2008) in IBS bowel. Furthermore, studies on human enteric neurons have provided evidence on hypersensitivity involving mast cells (Breunig et al., 2007; Buhner et al., 2009). Nevertheless, these studies are limited to intrinsic nervous system at the level of single cell type.

Studies on human visceral afferent signaling have been limited to *in vivo* studies based on symptom reporting or brain imaging. For example, colorectal distention in humans using a barostat device is a useful method to investigate the visceral mechanosensitivity in IBS patients. However, it is limited by subjective perception and thus may be not reliable. In addition, artificially evoked visceral pain may not truly reflect the pathophysiology of spontaneous pain in IBS (Bulmer and Grundy, 2011). Although these techniques have improved our understanding of visceral pain signaling in humans, they are less capable to provide deep insight into the underlying mechanisms compared with *in vitro* models at the level of end organ.

Although *in vitro* recordings of sensory afferent nerve activity from the rodent GI tract have been well established, the application of such recordings on human bowel has been a blank area in the literature. Until recently, our group and another group reported independent preliminary data on afferent recording from isolated human bowel (Jiang et al., 2011; Peiris et al., 2011). This will allow directly mirroring the studies that have been performed in animal models addressing mechanisms underlying visceral pain. Human visceral afferent recording has enormous advantage over animal model in the light of avoiding translational problems that have been discussed above. Another remarkable advantage of this *in vitro* human model is the

ability to test the efficacy of drugs against visceral pain or other symptoms, which will greatly reduce the risks of *in vivo* trials in patients or healthy volunteers.

Afferent subtypes determined by mechanosensitivity

Afferents can be classified according to their terminal distribution and sensitivity to different stimuli. A classification method has been established on flat sheet preparations of rodent GI tract to facilitate studies on specific afferent subpopulations (Lynn and Blackshaw, 1999; Brierley et al., 2004). As summarized in table 4.1, spinal afferents innervating mouse colon can be classified into five distinct classes based on their sensitivity to three types of mechanical stimuli: probing, mucosal stroking and stretch. All mechanosensitive afferents are able to response to probing, allowing accurate locating of their receptive fields. In addition to probing, mucosal afferents are also responsive to light mucosal stroking without disturbance to underneath layers. Muscular afferents are defined by their ability to respond readily to circumferential stretch of the tissue. Serosal and mesenteric afferents are identified by sensitivity to probing on serosa or mesentery respectively and lack of response to mucosal stroking and stretch. Additionally, muscular/ mucosal afferents are sensitive to all three distinct stimuli. It remains unknown whether afferents innervating human bowel are consist of similar subpopulations.

Table 4.1 Afferent subtypes determined by mechanosensitivity on mouse colon
Summarized from (Brierley et al., 2004)

Stimuli	Probing	Stretch	Mucosa stroking
Mesenteric	✔		
Serosal	✔		
Muscular	✔	✔	
Mucosal	✔		✔
Muscular/ mucosal	✔	✔	✔

Mediators and ion channels involved in hypersensitivity

It has been demonstrated that supernatants derived from IBS biopsies significantly elevate the excitability of rat visceral afferents (Barbara et al., 2007) and human enteric neurons (Buhner et al., 2009), which is attributed to increased levels of 5-HT

(via 5-HT$_3$ receptor), histamine (via H1-H3 receptor) and proteases (via PAR2 receptor) in IBS supernatants. Furthermore, supernatants from hypersensitive IBS patients induced a greater response on guinea pig submucosal neurons compared with non-hypersensitive IBS biopsies (Buhner et al., 2011).

Therapeutic treatments for visceral pain have been developed under two strategies. One approach is to prevent the effects of mediators that elicit pain or sensitize nociceptive pathways by modulating their synthesis and release or by blocking their target receptors. The other approach is to suppress the signaling within the nociceptive pathways directly. A number of mediators including 5-HT, histamine and proteases have been shown to contribute to the hypersensitivity in IBS, whereas it is unclear which subpopulation of afferents interact with these mediators or which ion channels are responsible for transducing noxious stimuli and transmitting nociceptive signals to the CNS. TRP channels have been shown to serve as direct transducer for noxious stimuli including heat, cold, acidity and mechanical pressure (Basbaum et al., 2009). Thereinto, TRPV1 has been given the most attention. Studies on TRPV1 knockout mice have revealed attenuated jejunal afferent response to noxious distension (Rong et al., 2004). Additionally, in animal models of visceral hypersensitivity, a TRPV1 antagonist attenuated the visceromotor response to colorectal distension (Miranda et al., 2007; Winston et al., 2007). Increasing evidence has suggested that TRPA1 might be another candidate based on the attenuation effects of TRPA1 antagonist on hypersensitivity induced by mast cell activation (Yu et al., 2009), bradykinin (Yu and Ouyang, 2009) and diabetes (Wei et al., 2009). On the other hand, TRPA1 agonists allylisothiocyanate (AITC) and cinnamaldehyde have been demonstrated to induce mechanical hypersensitivity of serosal afferents to probing in mouse colon (Brierley et al., 2009).

Bradykinin is one of the chemical stimuli most physiologically relevant to tissue injury, and has been revealed to be an important mediator in signaling of pain (Mizumura et al., 1990; Shin et al., 2002). In addition, bradykinin has been demonstrated to induce mechanical hypersensitivity in mice colon (Brierley et al., 2005; Brierley et al., 2009) and guinea pig esophagus (Yu and Ouyang, 2009) via bradykinin receptor B2. In flat sheet preparations of mouse colon, bradykinin

induced mechanical hypersensitivity to probing is absent in TRPA1 knockout mice (Brierley et al., 2009).

Ageing and human bowel afferent sensitivity

The incidence of motility or transit problems is increasing with age (Jost, 1997; O'Mahony et al., 2002; Hays and Roberts, 2006; Norton, 2006). Since detection of intraluminal content is a precondition to initiate motor reflexes, impaired afferent sensitivity might contribute to the reduced motility in the elderly. However, it is largely unknown whether the afferent sensitivity in aged human bowel is impaired or not.

Objectives

This study aimed to investigate the mechanical and chemical sensitivity of afferent nerves innervating human bowel using a recent established *in vitro* recording model. Objectives include:

1) to identify afferent subtypes by mechanosensitivity;

2) to examine chemical sensitivity of identified afferent subpopulations to capsaicin, AITC, bradykinin, 5-HT and histamine;

3) to test the effects of AITC and bradykinin on mechanosensitivity;

4) to examine the influence of ageing on afferent sensitivity.

4.2 Experimental protocol and analysis

Sources and collection of human bowel specimens were described in chapter 2. Within two years, 50 specimens were used for afferent recording; thereinto, 5 were excluded due to limited mesentery. As illustrated in figure 4.1, these specimens were obtained from patients of various age groups (varying from 24 to 88) and covered most regions of the lower GI tract (from ileum to rectum), among which sigmoid colon and ileum were the most abundant sources.

As illustrated in figure 2.2, full-thickness tissue was pinned flat with the mucosa uppermost in an organ bath, which was perfused with Krebs buffer at 34 °C. Nerve bundles were identified from the attached mesentery and sucked into a glass electrode. Afferent activity was recorded as described in section 2.4.
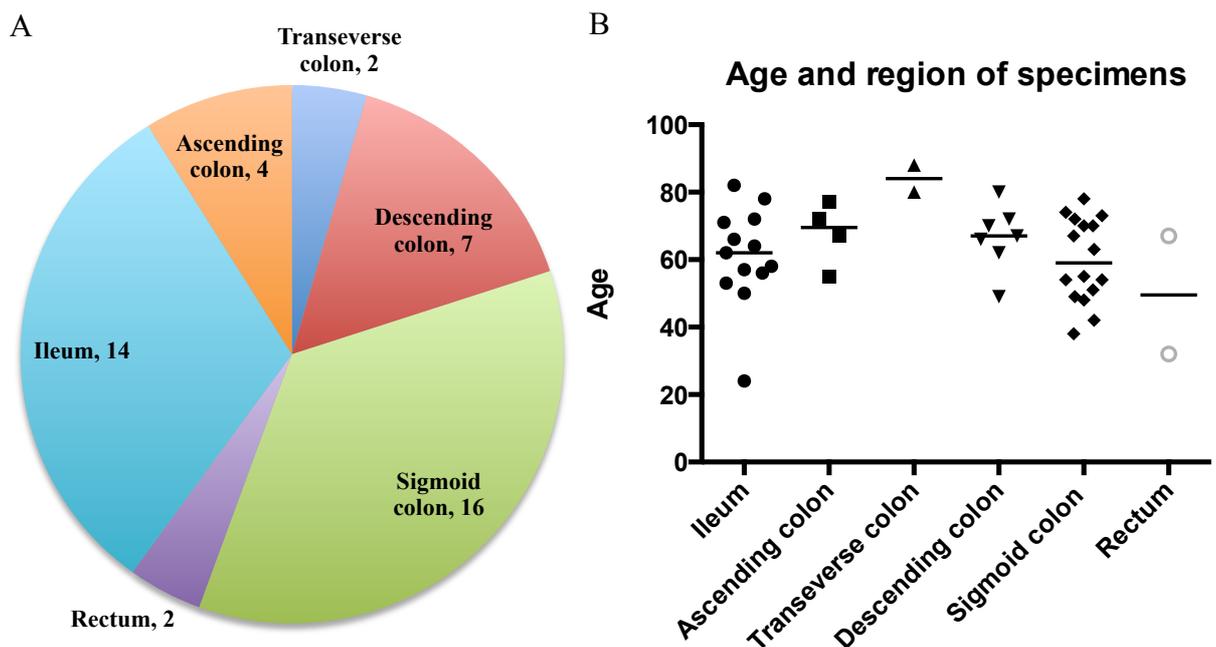
Figure 4.1 Age and region distribution of human bowel specimens used for afferent recording. (A) A pie chart showing the proportion of different regional sources of specimens. Sigmoid colon and ileum accounted for about one third of total specimens respectively. (B) Age and region information was plotted for each specimen.

After nerve activity stabilized, the mechanosensitive hotspot was identified by systematical probing using the blunt end of glassware. Once identified, the mechanosensitivity was examined by applying three distinct types of stimuli. Probing was applied on the hotspot with calibrated von Frey hair (4g, 10g and 60g force; each probing applied at least 5 times for a period of 2 seconds with a 3-second interval). Similarly, fine mucosal stroking was applied on the receptive field with calibrated von-Frey hair (1g or 4g force). In addition to probing and stroking, stretch was applied circumferentially and longitudinally using forceps.

Following mechanical stimulation, a variety of chemical stimuli were applied directly onto the receptive fields using a pipette, including bradykinin (10 μM), capsaicin (0.1~10 μM), AITC (10~1000 μM), 5-HT (10~100 μM) and histamine (10~300 μM). Volume of capsaicin applied was 0.2 ml, while volume of the other drugs was 1 ml. The total volume of the organ bath was about 60 ml. During the application of drug, the perfusion pump was left on so that the drug could be washed out quickly. Time interval between two drugs was at least 10 minutes.

Data were analyzed offline using Spike 2 software. In the case that activities of multiple units were recorded from the same nerve bundle, single unit analysis was performed to discriminate activities of each unit. Data were expressed as mean afferent discharge frequency ± SEM.

4.3 Success rate of recording

The overall success rate of human afferent recording in current study is 45% (20 in 45 specimens). Success here was defined as nerve activities recorded. Among the 20 successful recordings, 8 specimens were responsive to the stimuli applied, whereas the remaining 12 either did not show prominent responses or stopped firing before application of any stimuli. Figure 4.2B illustrated the possible correlation between success rate and the age and region of specimens. Descending colon appeared to have a higher chance of successful recording, whereas ascending and transverse colon showed the least chance. Age seemed to have no effects on the success rate of recording.
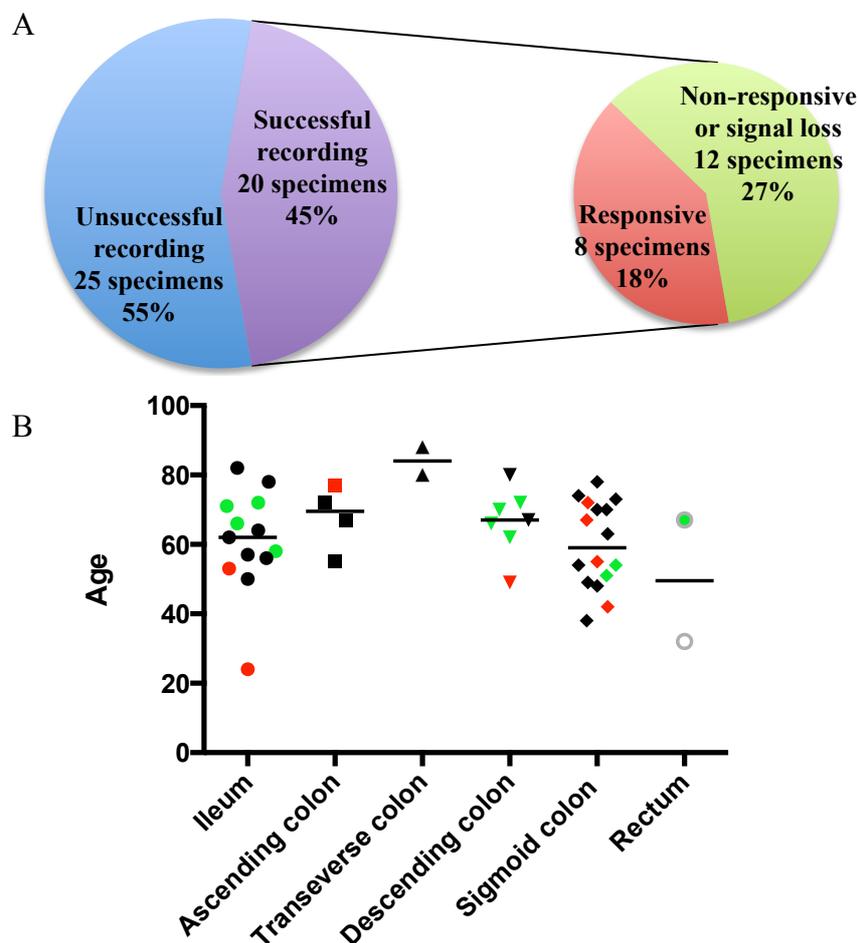
Figure 4.2 Success rates of recording and its correlation with the age and region of specimens. (A) A pie chart shows that 45% recording were successful, which is defined as nerve signal recorded. Among these recordings, only 8 in 20 were responsive. (B) Correlation between success rate and the age and region of specimens. Red dots indicate responsive recordings, and green dots label non-responsive recordings with spontaneous nerve activities.

4.3 Afferent subtypes determined by mechanosensitivity

As introduced above, afferents can be classified based on their responses to three kinds of mechanical stimuli: probing, mucosal stroking and stretch. Representative traces of afferent responses to these stimuli were shown in figure 4.3. Spontaneous burst firing might be a character of certain nerve subtype, and thus was noted as well.

As summarized in table 4.2, about 29% (10 in 34) of identified units innervating human bowel were mechanosensitive afferents, whereas 71% (24 in 34) were mechanically-insensitive afferents (or silent afferents). Among the mechanically-sensitive units, four types of afferents were identified: mesenteric, serosal, muscular and mucosal afferent. Serosal afferents were the most abundant class, accounting for 50% (5 in 10) of total mechanosensitive units. The other subtypes were relatively scarce. Spontaneous burst firing appeared to be a common character shared by various afferent subtypes.
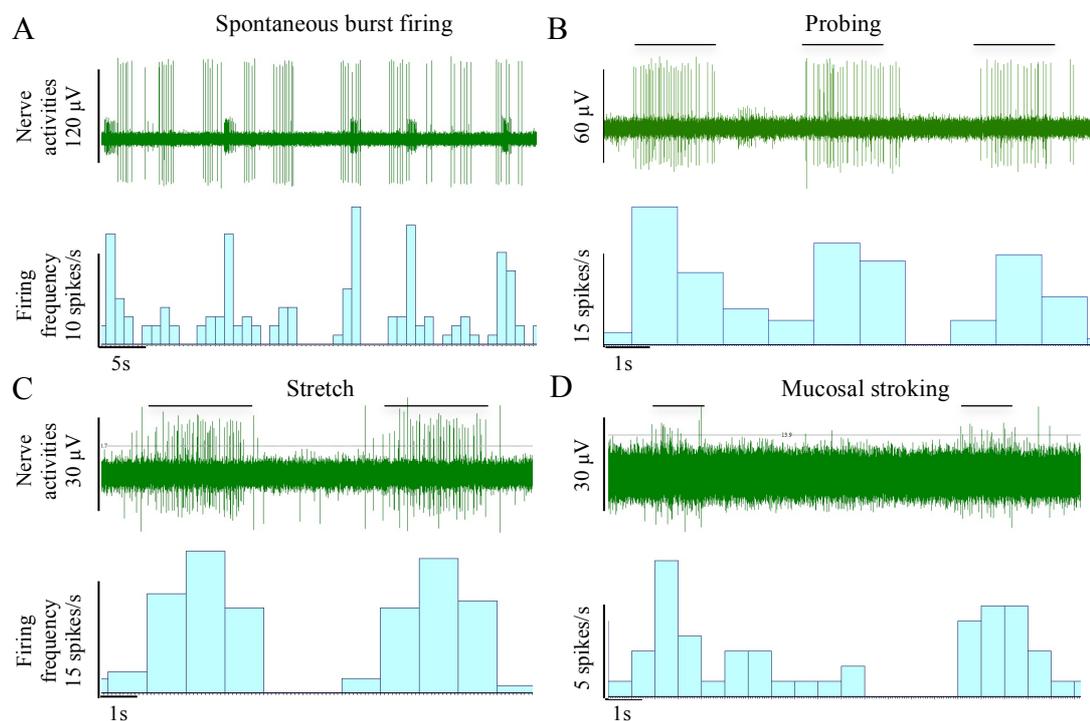
Figure 4.3 Sample traces to show spontaneous burst firing (A) and afferent responses to probing (B), stretch (C) and mucosal stroking (D). Upper channels in each trace showed the nerve activities, and lower channel displayed corresponding firing frequency. Age, gender and region of the sources of these specimens were 58-female ileum (A), 77-male ascending colon (B), 77-male ascending colon (C) and 42-male sigmoid colon (D).

Table 4.2 Afferent subtypes identified in human bowel

Classification	Number of units	Spontaneous burst firing	Probing	Mucosal stroking	Stretch
Mesenteric afferent	2	Yes	Yes	No	No
Serosal afferent	5	1: Yes 4: No	Yes	No	No
Muscular afferent	2	1: Yes 1: No	Yes	No	Yes
Mucosal afferent	1	Yes	Yes	Yes	No
Unclassified	24	7: Yes 17: No	No	No	No

Afferent discharges in response to graded probings (4g, 10g and 60g) were dramatically increased in a serosal afferent (figure 4.4A). However, repeated probing with constant force resulted in gradually decreased afferent responses (figure 4.4B). This might be due to either adaptation or desensitization.
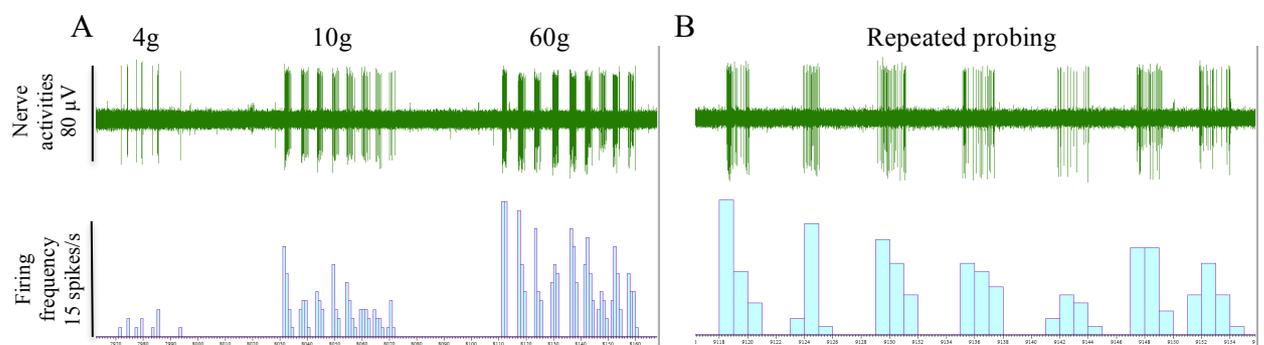
Figure 4.4 Sample traces to show afferent responses to graded probing force (A) and adaptation in repeated probing (B) on a serosal afferent. (A) Probing with increased force (4g, 10g and 60g) was applied with calibrated von Frey hair. Each probing lasted for 2 seconds with a 3-second time interval from another. Graded probing resulted in increased afferent discharges. (B) Afferent responses to repeated probing (10g) were gradually decreased. Upper channels showed the nerve activities, and lower channel displayed corresponding firing frequency. Age, gender and region of the source of this specimen were 67-female sigmoid colon.

4.4 Chemical sensitivity

A variety of chemical stimuli were applied directly onto the receptive fields identified by probing. Our results demonstrated that bradykinin (10 μM), capsaicin (0.3~1 μM), AITC (300 μM) and 5-HT (10 μM) were able to trigger afferent responses in human bowel. Single unit analysis was performed to discriminate responsive units and non-responsive units. Representative traces to show these responses were displayed in figure 4.5.
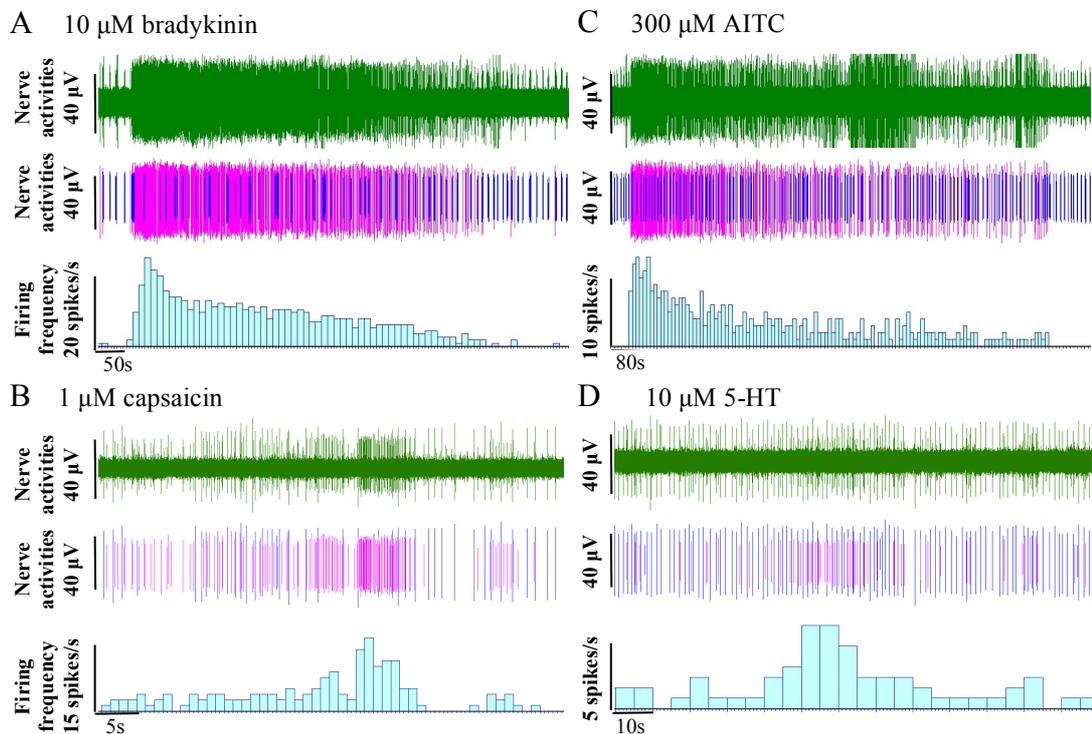
Figure 4.5 Representative traces to show afferent response to 10 μM bradykinin (A), 1 μM capsaicin (B), 300 μM AITC (C) and 10 μM 5-HT (D). Top channels (green waveform) displayed raw nerve activities. The middle channels showed discriminated activities of responsive units (in purple) and non-responsive units (in blue) separated by single unit analysis. Bottom channels displayed firing frequency of the responsive units only. Age, gender and region of the sources of these specimens were 42-male sigmoid colon (A), 77-male ascending colon (B), 42-male sigmoid colon (C) and 77-male ascending colon (D).

Characterisations of chemosensitive afferents were summarized in table 4.3. The most potent chemical stimulus in this study was bradykinin, which triggered marked afferent discharges from 4 in 17 examined units (those 4 units from different experiments). Bradykinin sensitive units were composed of two subpopulations: one was mechanosensitive serosal afferent associated with co-response to AITC, whereas the other was mechanically-insensitive unit without co-response to AITC. 2 in 24 units (those 2 units from different experiments) were capsaicin sensitive, including one muscular afferent and one serosal afferent. AITC induced increased afferent discharge from 2 out of 19 units (those 2 units from different experiments), both of which were mechanosensitive serosal afferent with co-response to bradykinin but without co-response to capsaicin. Only 1 in 8 units was responsive to 5-HT, and it was a muscular afferent that was capsaicin sensitive.

Table 4.3 Characterization of chemosensitive units

Drug	Responsive proportion	Unit Number	Co-response to				Burst	Probing	Mucosal stroking	Stretch	Afferent subtype
			BK	CAP	AITC	5-HT					
BK 10μM	4/17	2	-	No	Yes	1: no 1: NT	No	Yes	No	No	Serosal afferent
		2	-	No	No	NT	No	No	No	No	Silent afferent
CAP 1 μM	2/24	1	NT	-	NT	Yes	No	Yes	No	Yes	Muscular afferent
		1	No	-	No	NT	Yes	Yes	No	No	Serosal afferent
AITC 300 μM	2/19	2	Yes	No	-	1: no 1: NT	No	Yes	No	No	Serosal afferent
5-HT 10 μM	1/8	1	NT	Yes	NT	-	No	Yes	No	Yes	Muscular afferent

BK: bradykinin, burst: spontaneous burst firing, CAP: capsaicin, NT: not tested, -: not applicable.

Afferent discharges in response to bradykinin were compared among three sigmoid colon obtained from patients of different ages. Based on these three recording, afferent responses to bradykinin in the elderly appeared to be blunted and possibly delayed (figure 4.6).
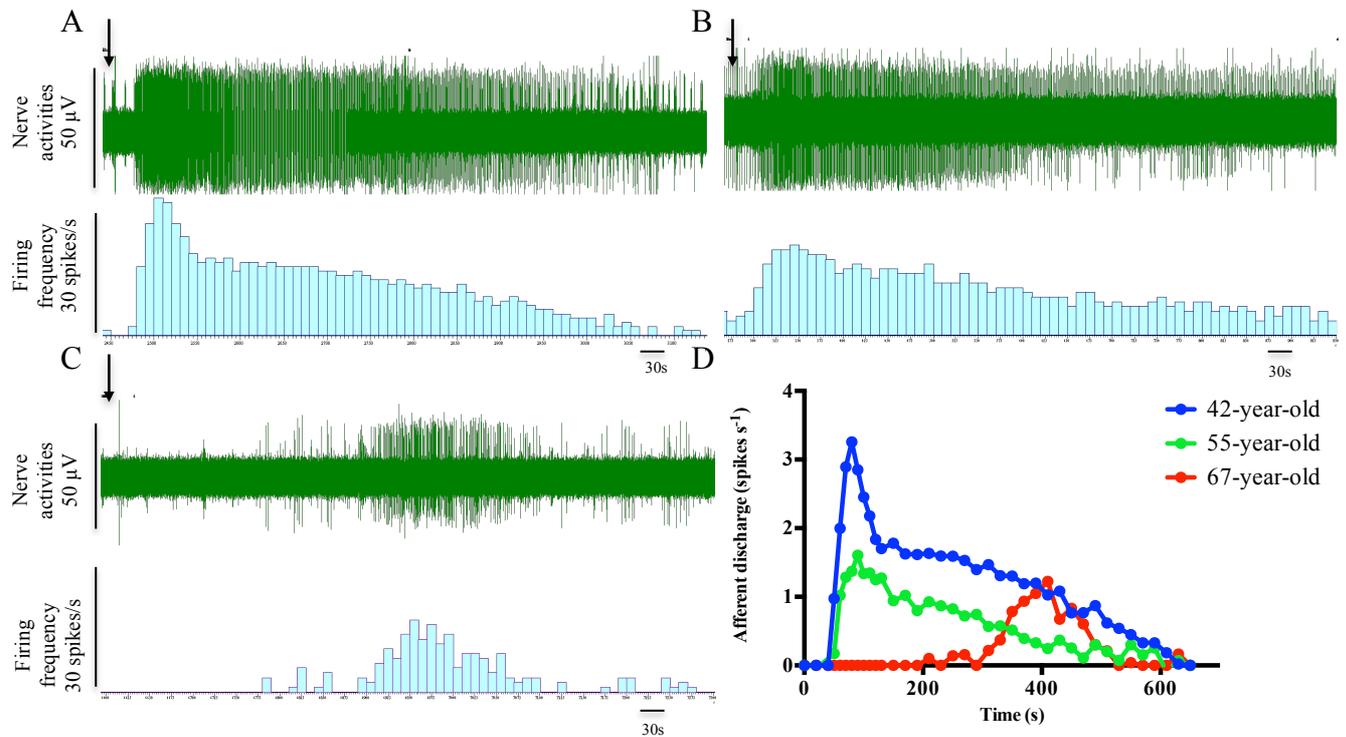
Figure 4.6 Afferent responses to bradykinin were decreased with advanced age in human sigmoid colon. Representative responses to 10 μM bradykinin in sigmoid colon from 42 years old (A), 55 years old (B), and 67 years old (C) patients. Bottom channels displayed firing frequency of the responsive units only. (D) Response profile revealed that bradykinin induced afferent discharges were blunted and delayed in the elderly.

4.5 Hypersensitivity induced by bradykinin and AITC

Effects of bradykinin treatment on mechanosensitivity were investigated by comparing afferent responses to probing after BK treatment with control. Based on one recording, afferent discharges in response to probing after bradykinin treatment were greater than control (18.9 ± 5.2 vs. 9.5 ± 2.3, figure 4.7C). Despite a 2-fold augment, it was not statistically significant (P =0.13), which was due to the large variations in treatment group. As revealed in the representative trace (figure 4.7B), the first three probing responses were markedly increased in comparison with control, whereas the following three responses were greatly reduced due to either adaptation or desensitization.
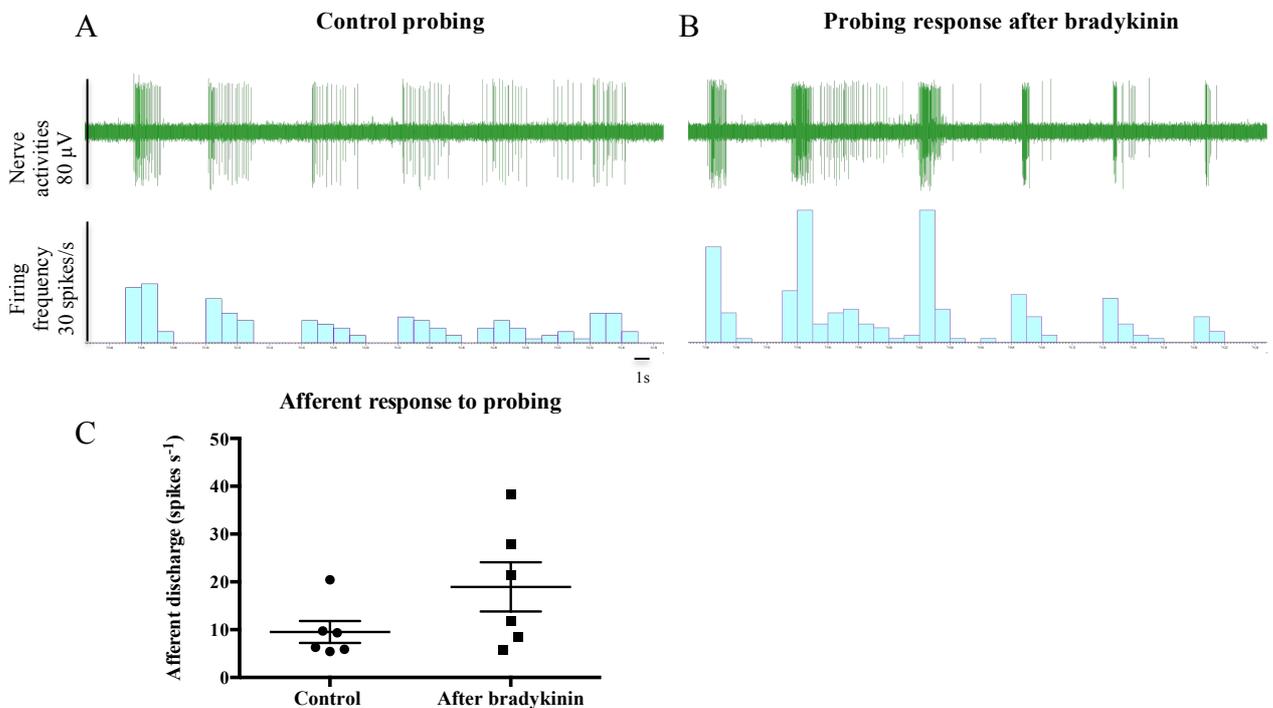
Figure 4.7 Afferent responses to probing might be augmented by bradykinin treatment. Representative traces showed afferent discharge induced by probing with 60g von Frey hair before (A) and immediately after (B) bradykinin response. 6 probing were applied for both groups. (C) Afferent discharge in response to probing after bradykinin treatment was greater than control (18.9 ± 5.2, N=6 vs. 9.5 ± 2.3, N=6), although this was not statistically significant (P =0.13, unpaired t-test). Data were obtained from one recording experiment on a 67-female sigmoid colon.

Similar to bradykinin, based on one recording, AITC treatment significantly enhanced afferent discharges induced by probing (12.4 ± 1.7, N=6 vs. 5.9 ± 0.6, N=6, P <0.01, unpaired t-test). It was noteworthy that AITC itself did not cause any change of afferent discharge.
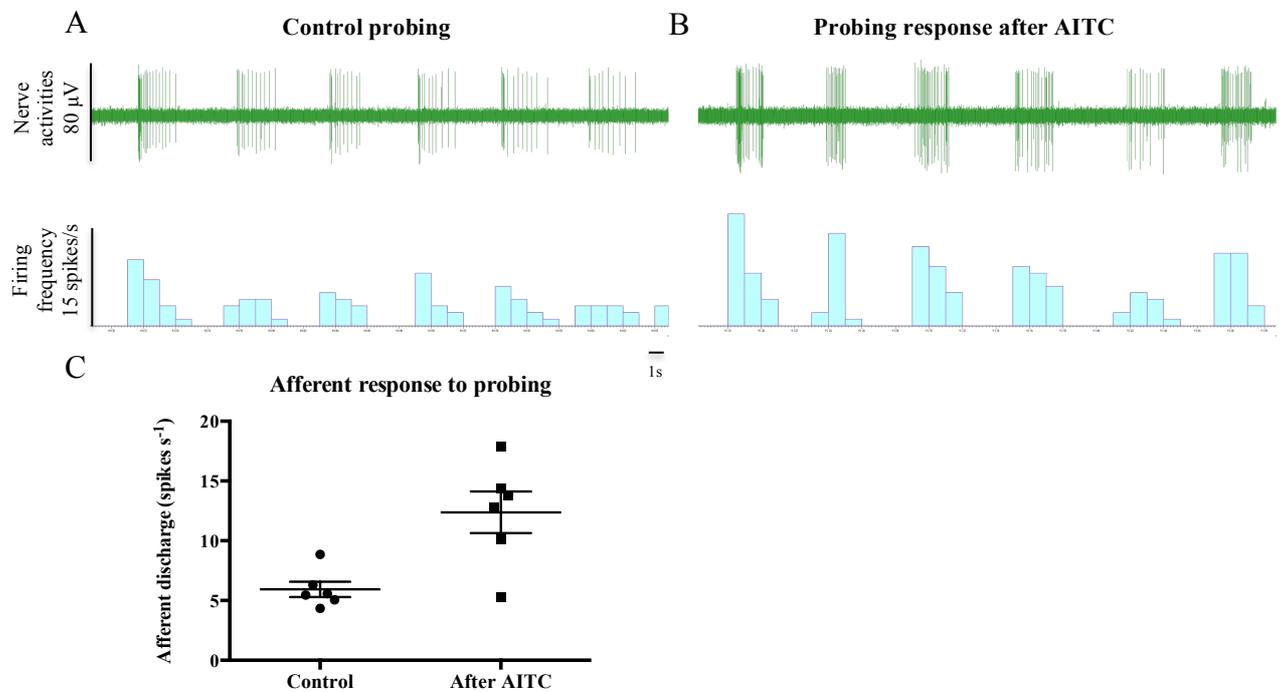
Figure 4.8 Afferent responses to probing are elevated by AITC treatment. Sample traces showed afferent discharge triggered by probing with 10g von Frey hair before (A) and 10 minutes after (B) AITC application. 6 probing were applied for both groups. (C) Afferent discharge in response to probing after AITC treatment was significantly greater than control (12.4 ± 1.7, N=6 vs. 5.9 ± 0.6, N=6, P <0.01, unpaired t-test). Data were obtained from one recording experiment on a 67-female sigmoid colon.

4.6 Discussion

Afferent subtypes determined by mechanosensitivity

This study identified four subtypes of afferents innervating human bowel including mesenteric, serosal, muscular and mucosal afferents, based on their sensitivity to probing, stretch and mucosal stroking. In combination with recently published preliminary data from our group (Jiang et al., 2011), 15 mechanosensitive units can be classified into five subtypes: serosal (46%), muscular (27%), mesenteric (13%), mucosal (7%) and muscular/ mucosal (7%) afferent. Although the number of classified unit is relative small, to our knowledge, this is the first report about classification of human bowel afferent. The other group who also managed to record nerve activities from isolated human bowel set up preparations in a different way by pinning colon flat with serosal side up, which did not allow application of mucosal stroking and thus identification of afferent subtypes (Peiris et al., 2011).

Similar studies have been performed on flat sheet preparations of mouse colon; however, such studies on mice allow discrimination of lumbar splanchnic nerves and pelvic nerves, and indeed these two pathways have been shown to contain distinct populations of mechanosensitive afferents (Brierley et al., 2004; Feng and Gebhart, 2011). Our *in vitro* recording model on isolated human bowel doesn't allow identification of nerve pathways, as the nerve recording sites are extremely close to the gut wall. Since the specimens which we record from cover the most regions of lower GI tract from ileum to rectum, afferents identified might belong to lumber splanchnic, pelvic or even vagal pathways. As such, direct comparison to studies on mouse is not reliable. However, there is one obvious similarity in the proportion of serosal afferent in total mechanosensitive units. Based on two studies on mouse colon, serosal afferents are usually the most abundant subtypes, occupying 47% or 36% in lumbar splanchnic afferents and 37% or 33% in pelvic pathways (Brierley et al., 2004; Feng and Gebhart, 2011). In line with these reports, our data indicate that 50% of mechanosensitive afferents innervating human bowel are serosal afferent. One of the limitations with this classification system is the lack of specific stimulation for submucosal, therefore it is possible that some of the units classified as serosal afferents actually terminate in the submucosa.

It is noteworthy that we found a high proportion of mechanical insensitive afferents (71%) innervating human lower GI tract. A study employing an electrical search strategy has revealed that about 23% of the pelvic afferents innervating mouse colorectum are normally mechanically insensitive (silent afferents), 71% of which are able to acquire mechanosensitivity after application of inflammatory soup to their receptive fields; whereas 33% of lumbar splanchnic afferents are mechanically-insensitive units, only 23% of which acquire mechanosensitivity after exposure to inflammatory soup (Feng and Gebhart, 2011). Our data indicate a much higher proportion of silent afferents. This might be attributed to species-related difference between human and mouse, considering the huge distinction in terms of the size of bowel. Another possibility is that some of these unclassified units might belong to mesenteric afferents. Considering the thickness and flexibility of human mesentery, probing on mesentery was often associated with high risks of losing the nerve, as such some of the specimens did not allow systematical application of probing on their mesentery.

Based on limited data, we observed some characteristics of serosal afferent mechanosensitivity. In agreement with animal studies (Brierley et al., 2004), afferent discharges in response to probing with graded von Frey hair force (4g, 10g and 60g) were markedly increased. In addition, their responses to repeated consecutive probing adapted very fast.

Chemical sensitivity

Chemical sensitivity of visceral afferents innervating human bowel is nearly blank in the literature. Our recently published report has shown evidence of afferent response to capsaicin in human colon (Jiang et al., 2011). The other preliminary report on human visceral recording has revealed a robust increase in afferent discharge on human appendix elicited by capsaicin (10 μM) and inflammatory soup (PGE2 10 μM, histamine 10 μM, serotonin 1 μM, ATP 5 μM, adenosine 1mM and bradykinin 1 μM) (Peiris et al., 2011). In addition to capsaicin, this current study is the first one to provide direct evidence that afferent discharges could be elicited by bradykinin (10 μM), AITC (300 μM) and 5-HT (10 μM) in isolated human ileum and colon.

Bradykinin is one of the most potent algogenic substances released from tissue injury and inflammation (Couture et al., 2001). A study using flat sheet preparations of mouse colon has reported that bradykinin elicits afferent discharges in 66% of lumbar splanchnic nerves, among which 10 in 27 are mechanically-insensitive afferents recruited during the application of bradykinin; conversely, only 2 in 19 pelvic nerve afferents respond to bradykinin and both are mechanically sensitive (Brierley et al., 2005).

In this study bradykinin was the most effective chemical stimulus that trigger marked afferent discharges from 4 in 17 tested units. Among the 4 responsive units, two units were mechanosensitive serosal afferents accompanied with the ability to co-response to AITC, whereas the other two were mechanically-insensitive units without co-response to AITC. The two silent afferents did not acquire mechanosensitivity after responses to bradykinin. A study addressing mouse colorectal silent afferents has revealed that 71% of the silent pelvic afferents are able to acquire mechanosensitivity after application of inflammatory soup to their receptive fields; whereas only 23% of silent lumbar splanchnic afferents acquire mechanosensitivity after exposure to inflammatory soup (Feng and Gebhart, 2011). On the other hand, AITC induced increased afferent discharges from 2 out of 19 units, both of which were mechanosensitive serosal afferent with co-response to bradykinin. Previous studies have demonstrated that bradykinin is able to activate TRPA1 indirectly via bradykinin receptor 2 (B2), which is modulated by phospholipase C (PLC) and protein kinase A (PKA) pathways (Bandell et al., 2004; Bautista et al., 2006; Wang et al., 2008). In association with these studies, our data suggested that TRPA1 might be expressed in a subpopulation of B2 receptor-expressing afferents. Neither bradykinin sensitive nor AITC sensitive afferents co-responded to capsaicin, which is contrary to previous reports suggesting that TRPA1 is exclusively expressed in a subpopulation of TRPV1-present afferents (Story et al., 2003; Kobayashi et al., 2005; Bautista et al., 2005). However, some functional studies revealed that a proportion of mustard oil-sensitive neurons did not response to capsaicin (Kwan et al., 2006; Hjerling-Leffler et al., 2007). It is possible that some TRPV1 channels that are co-expressed with TRPA1 are not functionally active.

In flat sheet preparations of mouse colon, afferent responses to probing are significantly increased after the chemosensory response to bradykinin (Brierley et al., 2005; Brierley et al., 2009). Similarly, TRPA1 agonists AITC and cinnamaldehyde induce mechanical hypersensitivity of serosal afferents to probing (Brierley et al., 2009). In agreements with these studies, we observed a short-term mechanical sensitization of serosal afferent to probing by bradykinin and AITC, in spite of fast adaptation to repeated probing. AITC treatment increased mechanosensitivity to probing whereas AITC itself did not elicit afferent discharges, which is consistent with mouse studies (Brierley et al., 2009). One possible explanation is that AITC activated TRPA1 on non-neuronal cells, such as EC cells, which release mediators to depolarize or sensitize afferent nerves.

Studies on mouse showed that 14 in 40 serosal afferents respond to capsaicin, and mechanosensitivity was significantly reduced after chemical response to capsaicin (Brierley et al., 2009). Current study only identified 2 (out of 24) capsaicin sensitive units, including one muscular afferent and one serosal afferent. The effects of capsaicin on mechanosensitivity were not tested. Only 1 in 8 units was responsive to 5-HT, and it was a muscular afferent that was capsaicin sensitive. Limited comparable data about serotonin sensitivity in flat sheet animal models can be seen, as it is often collectively applied with other mediators as inflammatory soup.

In the current study, chemical stimuli were topically administrated onto the receptive field with a pipette. Thus the concentration of drug hitting the receptor might be lower than stated due to diffusion. In addition, manual application of drug reduced the accuracy. However, considering the size of human specimen and organ bath, perfusion into the bath brought more limitations, such as large consumption of drug, difficulty in washing out, delayed response and potential indirect effects.

Ageing on afferent sensitivity

Efforts have been made to examine the influence of ageing on human bowel afferent sensitivity. However, since human bowel specimens were obtained from various regions (ileum, colon and rectum) of patients within different age groups (from 24 to 88) and of both genders, so many variables required a large number of successful recordings to make variable-controlled comparisons. In addition, afferents classified

into different subtypes (serosal, mesenteric, muscular, mucosal and muscular/ mucosal) might be not comparable directly.

Based on very limited data, afferent discharges in response to bradykinin in human sigmoid colon appeared to be blunted in the elderly, which is consistent with our unpublished data on mouse. This indicates an attenuated sensitivity to inflammatory events in the elderly. The underlying mechanisms are not fully understood. Our studies on mice have identified some possible contributors, such as reduced primary innervation with advanced age (chapter 6). Whether this applies to human requires further investigation. In addition, changes in the expression of bradykinin receptor and some secondary messengers in bradykinin signaling pathway might contribute to the reduced afferent response to bradykinin.

Success rate

Current study managed to record afferent nerve activities from 45% of human bowel specimens, which is higher than a 15% success rate in our preliminary report (Jiang et al., 2011) and similar to 48% of the other group (Peiris et al., 2011). All these studies are greatly limited by the low success rate, reflecting the inherent difficulties of *in vitro* recording on isolated human bowel. The difficulties might be owing to short tissue supply, low specimen viability, adverse surgical influence and technical problems. However, accurate causes for the failure are not clear.

It is suggested that some factors could affect functional studies with human tissues, such as the type of surgery (laparoscopic or open), ischaemia time, time of equilibration in the organ bath and possible influence by drugs given during surgery (Sanger et al., 2013). We tried to find out the possible influence of surgery (type of surgery, surgeon, ischaemia time and use of cauterization) when possible. Based on limited information from a small proportion of cases, it seemed that these factors were not decisive. Although it is possible that these factors might influence the chance of successful recording, there is not sufficient information to make systematical analysis. We were not aware about the drugs given during the surgery. Overcoming of these limitations might help to figure out potential ways of increasing success rate. We did not found any obvious influences by time of equilibration, diagnosis of patients, transferring time from theatre to organ bath and preparation

setup. In addition, there was no obvious correlation between success rate and the ages of patients. Regarding to the region, descending colon appeared to have a higher probability for successful recording, whereas ascending and transverse colon showed the least chance. It is likely that the success rate of human bowel recording is determined by summation of a variety of factors.

Even though nerve activities were successfully recorded, 60% of these specimens did not show any significant response to mechanical and chemical stimuli applied. This might be due to a smaller proportion of sensory afferents in the nerve bundles identified comparing with efferent or a substantial proportion of mechanically-insensitive units. It is difficult to apply chemical stimuli if the receptive fields are not identified, as the huge size of organ bath consumes too much drugs and time to wash out. Another reason might be that many specimens did not allow systematical probing, which is associated with high risk of losing the nerve.

A prominent difference between human and mouse afferent recording is the "level" of recording sites, which refers to the distance away from the terminals and the volume of afferent fibers. In a typical recording on mouse jejunum, recording site is far away from the gut wall and usually about 10-15 afferent units can be recorded. Whereas in human specimens, recording sites might be very close to the gut wall considering the relative size of human bowel, and typically only 1 or 2 units can be identified. This difference decreases the chance to get responsive units in human bowel.

Despite obvious technical challenges, this human *in vitro* afferent recording model at the peripheral afferent level has enormous advantages, such as avoiding translational failures, test of compounds for clinical use and better understanding of the mechanisms underlying human bowel disorders.

Chapter 5

Interplay between EC cells, mast cells and TRPA1 in afferent signaling from mouse intestine

5.1 Introduction

Inflammation is a normal biological response to harmful stimuli, such as pathogens, tissue injury or irritants, which activate neuronal, immune and epithelial cells to initiate protective and healing process. In response to harmful stimuli, a variety of cells in the damaged area release inflammatory mediators including bradykinin, prostaglandins, ATP, adenosine, leukotrienes, histamine, proteases, tumor necrosis factor-α, interleukin-1β and glutamate, some of which activate a subpopulation of primary afferents that release inflammatory neuropeptides such as substance P, CGRP and neurokinin A.

The link between TRPA1 and inflammatory pain was first indicated by the observation that AITC (mustard oil), an inducer of neurogenic inflammation and hypersensitivity, is a specific agonist of TRPA1 (Jordt et al., 2004). Further evidence has revealed that activation of TRPA1 elicits acute nociceptive behavior in mice, followed by neurogenic inflammation and marked hypersensitivity to thermal and mechanical stimuli, which is absent in TRPA1 knockout animals (Bandell et al., 2004; Bautista et al., 2006).

In addition to generating nociceptive sensation, TRPA1 expressing afferents release neuropeptides to communicate with other cell types and modulate inflammatory response. In animal model of colitis induced by TNBS and DSS, activation of TRPA1 elicits marked release of SP and CGRP; furthermore, SP knockout mice are protected from colitis, while CGRP mutants show a more severe course of TNBS colitis, indicating a proinflammatory effect for SP and a protective role for CGRP (Engel et al., 2011). This is in agreement with observations that pharmacologic block of the SP receptor using a neurokinin 1 antagonist significantly attenuates TNBS and DSS colitis (Di Sebastiano et al., 1999; Stucchi et al., 2000); on the other hand, treatment with a CGRP receptor antagonist augments the severity of inflammation (Reinshagen et al., 1998), and exogenous administration of CGRP markedly ameliorates TNB colitis (Evangelista and Tramontana, 1993). Interestingly, CGRP/SP double-knockout mice are completely protected against TNBS colitis, suggesting that the proinflammatory effect of SP is predominant over the protective effect of CGRP (Engel et al., 2011). Moreover, activation of neurokinin 1 (NK1)

receptors contributes to somatic hypergesia and stress-induced visceral hyperalgesia (Mantyh et al., 1997; Bradesi et al., 2006).

TRPA1 and mast cells

Some neuropeptides released by TRPA1 activation promote communications between nerves and mast cells. SP can induce degranulation of mast cells accompanied with release of histamine, tryptase and serotonin (Shanahan et al., 1985; Repke and Bienert, 1987), which in turn, activate afferent nerve through corresponding receptors on the terminals (Corvera et al., 1999; Nozdrachev et al., 1999; Breunig et al., 2007). These bi-directional interactions might form a positive feedback loop to amplify inflammatory responses.

As IBS patients display a lower pain threshold to stimulation in colon-rectum (Sinhamahapatra et al., 2001; Dong et al., 2004) and also skin (Verne et al., 2001), mast cell induced hypersensitivity has been thought the most likely mechanism accounting for the recurrent pain and discomfort in IBS patients. As introduced in previous chapters, many independent lines of evidence have provided support for this mechanism. However, it hasn't been fully understood what mediators from mast cells and what subtypes of afferents nerves are involved.

Intracolonic administration of protease-activated receptor-activating peptide (PAR2-AP) augments visceromotor responses to graded colorectal distension (Cattaruzza et al., 2010). PAR2-AP can induce mechanical hypersensitivity of vagal afferents to esophageal distension, which is inhibited by TRPA1 antagonist (Yu et al., 2009). These observations suggest that protease released from mast cells might sensitize TRPA1 expressing afferent nerves through PAR2 receptors. In addition to protease, evidence has implicated histamine in the interaction between TRPA1 and mast cells (Andrade et al., 2008; Hox et al., 2012).

TRPA1 and EC cells

TRPA1 expression has been described in both myenteric neurons (Penuelas et al., 2007; Poole et al., 2011) and extrinsic neurons innervating mouse intestine (Brierley et al., 2009). Most interestingly, it has been demonstrated that TRPA1 is highly expressed in EC cells, and activation of TRPA1 significantly increases serotonin

release from enriched rat EC cell fraction, a rat endocrine cell line RIN14B (Nozawa et al., 2009) and a human pancreatic endocrine cell line QGP-1 (Doihara et al., 2009). This observation provides a crucial link between TRPA1 and serotonergic signaling. Serotonin, as the major transmitter molecule within the GI tract, plays an essential role in the orchestration of motility and secretion reflexes to facilitate intestinal transition and digestion under physiological conditions and in the initiation of inappropriate secretomotor activity and sensation under pathophysiological state, such as nausea or pain.

Serotonin can act on a number of G-protein coupled receptors as well as one ligand-gated channel known as 5-HT$_3$, which has been extensively studied in the context of sensory signaling. Serotonin directly activates nodose ganglion neurons (Peters et al., 1993) and DRG neurons (Todorovic et al., 1997) via 5-HT$_3$ receptor. In addition, serotonin excites human enteric neurons via 5-HT$_3$ receptors, which may comprise both 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits (Michel et al., 2005). 5-HT$_{3B}$ subunit has been shown to be a major determinant of receptor function (Davies et al., 1999).

A recent study on TPH1 deficient mouse has observed a much slower propulsion with larger faecal pellets as well as reduced colonic migrating motor complexes and reduced responses to intraluminal balloon distension (Heredia et al., 2013). Additionally, the spontaneous afferent activity in small intestine is attenuated by 5-HT$_3$ antagonist granisetron (Hillsley et al., 1998). These observations suggest an essential role of mucosal 5-HT in normal gastrointestinal motor reflexes and mechanosensitivity.

In addition to physiological activities, serotonin is also implicated in pathogenesis of multiple GI disorders. Study on rat model of inflammatory pain has revealed an antinociceptive role of 5-HT3 receptor antagonist ondansetron (Butkevich et al., 2007). Intrathecally administered 2-methyl-5-HT produced antinociception against formalin-induced inflammatory pain in neonatal rat (Giordano, 1997). In clinic, 5-HT$_3$ receptor antagonists represent a class of antiemetics that are commonly used on cancer patients undergoing chemotherapy and radiotherapy. Moreover, 5-HT3 receptor antagonists are effective in relieving symptoms in patients with diarrhoea-predominant IBS (Camilleri et al., 2001)

Since TRPA1 is present on EC cells, which is the major source of 5-HT within the gut, TRPA1 might be implicated in the various physiological and pathological process mediated by 5-HT.

Bradykinin

Bradykinin, one of the most potent algogenic substances released from tissue injury and inflammation (Couture et al., 2001; Bandell et al., 2004), elicits acute pain through immediate excitation of nociceptors, followed by a lasting sensitization to mechanical and thermal stimulations (Dray and Perkins, 1993). Many studies have addressed the mechanism underlying bradykinin-induced hyperalgesia. It has been revealed that bradykinin sensitizes TRPV1 through B2 receptor and PLC pathway (Chuang et al., 2001), whereas some evidence has implied that this pathway is not predominant based on the observation that afferent response to bradykinin in TRPV1 knockout mice is not altered (Rong et al., 2004). However, studies on TRPA1 deficient mice have reported a significant attenuation in bradykinin induced nociceptors excitation and pain hypersensitivity (Bautista et al., 2006; Kwan et al., 2006).

Similar to TRPA1, Bradykinin receptor 2 (B2) is expressed in a subpopulation of TRPV1-expressing nociceptors (Kasai et al., 1998). Bradykinin activates 78% of cinnamaldehyde-responsive rat DRG neurons (Bandell et al., 2004). AITC activates 31.67% of rat DRG neurons and 65.22% of bradykinin-responsive neurons (Wang et al., 2008). 97% of mouse trigeminal neurons that exhibit large response to bradykinin are mustard oil sensitive (Bautista et al., 2006). These independent lines of evidence suggest a large overlap of TRPA1 expression and bradykinin receptor expression in primary sensory neurons. Based on heterologous-expression studies, TRPA1 might be functionally coupled to B2 receptor, as suggested by the acute current response to bradykinin on cells expressing both TRPA1 and B2 receptors (Bandell et al., 2004). However, another *in vitro* recording study has revealed that TRPA1 antagonist does not affect BK-evoked discharges in nodose and jugular C fibers (Yu and Ouyang, 2009). In addition, afferent response to bradykinin is not altered in TRPA1 knockout mouse colon (Brierley et al., 2009), making it still controversial whether bradykinin can activate TRPA1.
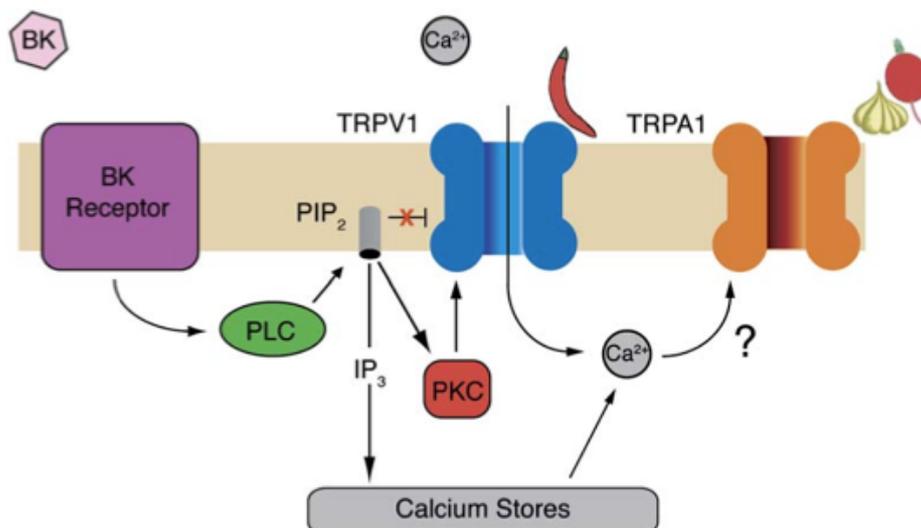
Figure 5.1 A schematic graph illustrating functional interactions between bradykinin receptors, TRPA1 and TRPV1 (Bautista et al., 2006). Binding of bradykinin to its corresponding G protein-coupled receptors on primary afferents activates phospholipase C (PLC), which results in the hydrolysis of membrane phosphatidylinositol bisphosphate (PIP_2), activation of protein kinase C (PKC), and release of calcium from intracellular stores. Consequently, TRPV1 is sensitized and open. TRPA1 might open in response to both calcium release from intracellular stores and calcium influx via TRPV1.

TRPA1 and TRPV1

TRPA1 and TRPV1 channels both belong to the TRP superfamily and share some structural similarity. Increasing evidence has suggested that TRPA1 and TRPV1 interlink with each other to a considerable extent, especially in the context of pain and neurogenic inflammation. TRPA1 has been shown to be exclusively expressed in a subpopulation of unmyelinated, peptidergic nociceptors that also express TRPV1 (Story et al., 2003; Kobayashi et al., 2005; Bautista et al., 2005), although some functional studies have argued that a proportion of mustard oil-sensitive neurons did not response to capsaicin (Kwan et al., 2006; Hjerling-Leffler et al., 2007). Due to co-expression of TRPV1/A1 in intestinal afferents, activation of either channel results in similar downstream effects, such as neuropeptide release and hyperalgesia. It has been shown that afferent responses to capsaicin tend to increase in TRPA1 knockout mouse colon (Brierley et al., 2009).

Objectives

Preliminary data from our group has shown that AITC induces increase in afferent discharge from mesenteric afferents innervating mouse jejunum in a dose dependent manner (Jiang et al., 2012). This chapter further addressed following objectives:

1) to examine the involvement of bradykinin in TRPA1 signaling;
2) to assess the mechanical hypersensitivity induced by AITC and bradykinin;
3) to examine the effect of 5-HT$_3$ receptor antagonist on TRPA1 signaling;
4) to examine the effect of mast cell stabilizer on TRPA1 signaling;
5) to test the effect of TRPA1 agonist AITC on intestinal motility;
6) to evaluate TRPA1 signaling on TRPV1 knockout mice.

5.2 Experimental protocol and analysis

Extracellular recording on mouse jejunum was performed as described in chapter 2. Once the nerve activities were stabilized, a number of distensions were applied at an interval of 15~20 minutes. Prior to the commencement of any protocol, at lease three reproducible responses to distension were achieved.

Afferent response to AITC and bradykinin

AITC working solution (final volume at 20 ml) was diluted from fresh-made stock (10 mM) with Krebs and perfused into the organ both (volume at about 9 ml) at a rate of 10 ml/min. To investigate the dose-dependent responses to AITC, a range of concentrations (100 μM, 300 μM and 1mM) were applied orderly with an interval at 20 minutes.

Bradykinin stock (10 mM) was diluted to 10 μM working solution with Krebs. 1 ml working solution was administered into a corner of the organ bath (about 9 ml) using a pipette to make the final concentration at 1 μM.

Effects of antagonists or inhibitors on TRPA1 signaling

Control experiments were performed by applying two repeated doses of AITC or bradykinin with time intervals at about 40 minutes. In treatment groups, HC-030031 (30 μM), granisetron (10 μM) or cromolyn (100 μM) were perfused into organ bath 10~15 minutes prior to the second application of agonists so that Krebs in the bath was replaced by antagonist solution. Time interval between applications of agonists was consistent with control. After responses to agonists finished, antagonist solution was washed out by fresh Krebs.

TRPA1 and contractility

Mouse jejunum segments were set up and nerve activities were recorded in the same way as routine afferent recording. To assess the contractility of the jejunal preparation, the tissue was slightly distended to 5 mmHg. The infusion pump was then closed, whereas the connection to the pressure transducer was maintained to allow the real-time detection of the intraluminal pressure that was used to reflect the contractility. After the pressure stabilized, AITC (300 μM) were perfused into the

organ bath consistently. To examine the effect of 5-HT$_3$ antagonist on AITC induced contraction, granisetron (30 μM) was perfused into organ bath 10~15 minutes prior to the second application of AITC. At the end of each experiment, 1 μM carbachol was applied as a positive control. The contractions induced by AITC were calculated as the peak intraluminal pressure subtracted by baseline pressure within 30 second bins. These changes in pressure induced by AITC were normalized against carbachol-induced contractions.

TRPA1 signaling in TRPV1 knockout mice

TRPA1 signaling induced by AITC in TRPV1 knockout mice was assessed and compared with their wildtype littermates. All experimental procedures were consistent with that on normal wildtype mice.

Single unit analysis

Afferent units were classified into low threshold (LT), wide dynamic range (WDR) and high threshold (HT) based on their response profile to graded pressure. An objective classification method that introduced a parameter referred to as LT% was described previously (Raybould, 1998). LT% is the ratio of the afferent discharges at 20 mmHg in the response at 50 mmHg. A value around 40% indicates a linear increase in afferent discharges in response to ramp distension. <15% was used as the criteria for the HT and >55% for the LT. However, these criteria were too strict for high threshold units. In this study, units with a threshold>5 mmHg were classified into high threshold units.

Data analysis

Data are presented as frequency of afferent discharges (number of action potentials per second). All data are expressed as mean ± SEM from the N values, where N refers to the number of animals or preparations.

The changes in afferent discharges in response to AITC or bradykinin were analyzed in two ways, peak response and response time course. Peak response was quantified as maximum firing frequency in a 30-second bin subtracted by baseline frequency before the application of drugs. To compare two or more peak responses, student's t-test or one-way ANOVA were performed. P value <0.05 was considered as

significant. Response profile showing the time course of changes in mean firing frequency was obtained from XY View measurements of Spike 2. Two-way ANOVA was used to compare two or more groups of changes over time.

The afferent response to ramp distension was plotted as increase in afferent firing frequency against increase in intraluminal pressure from 0 to 60 mmHg. Data were extracted using a script. To analyze whether distension responses were significantly changed, two-way ANOVA with Sidak multiple comparisons test was used.

Some representative images of afferent recording are shown in this and next chapter. Unless otherwise stated, the three channels as shown in the below image display nerve activities, pressure and firing frequency, respectively.
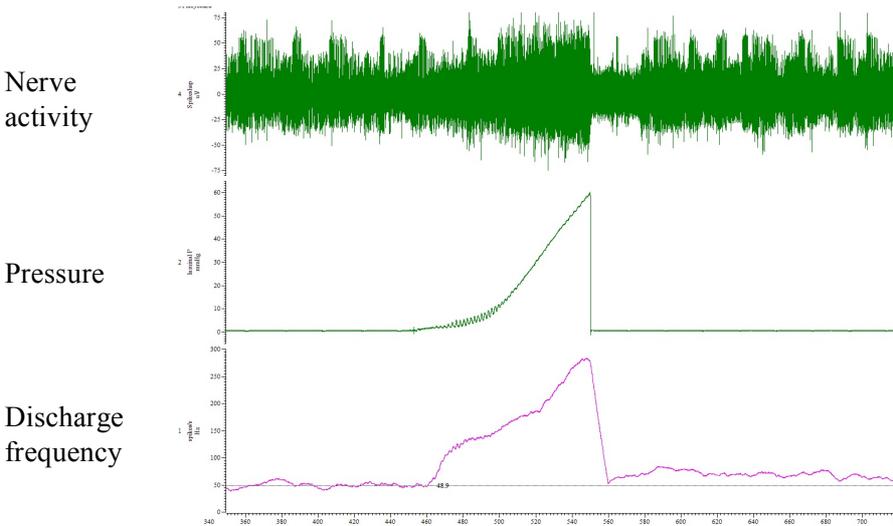
5.3 TRPA1 signaling induced by AITC and bradykinin

Perfusion of AITC into organ bath consistently induced increase in afferent discharge from mesenteric afferents innervating mouse jejunum. These afferent responses were mediated via TRPA1, as they were significantly attenuated by HC-030031, a selective TRPA1 antagonist (P<0.01, figure5.2 D&E). By discrimination of different afferent subtypes, low threshold (LT) units, wide dynamic range (WDR) units and high threshold (HT) units were all implicated in AITC induced TRPA1 signaling, and LT afferents appeared to contribute more than HT (figure 5.2F). In addition, HC-030031 appeared to be effective on all three subpopulations, although only WDR and HT units achieved significance (figure 5.2G).
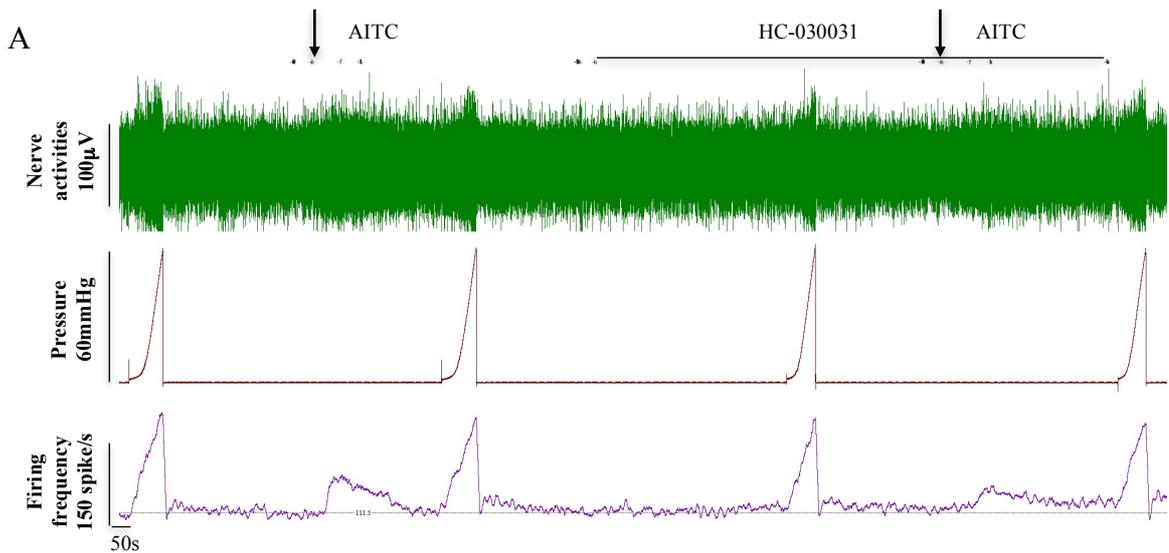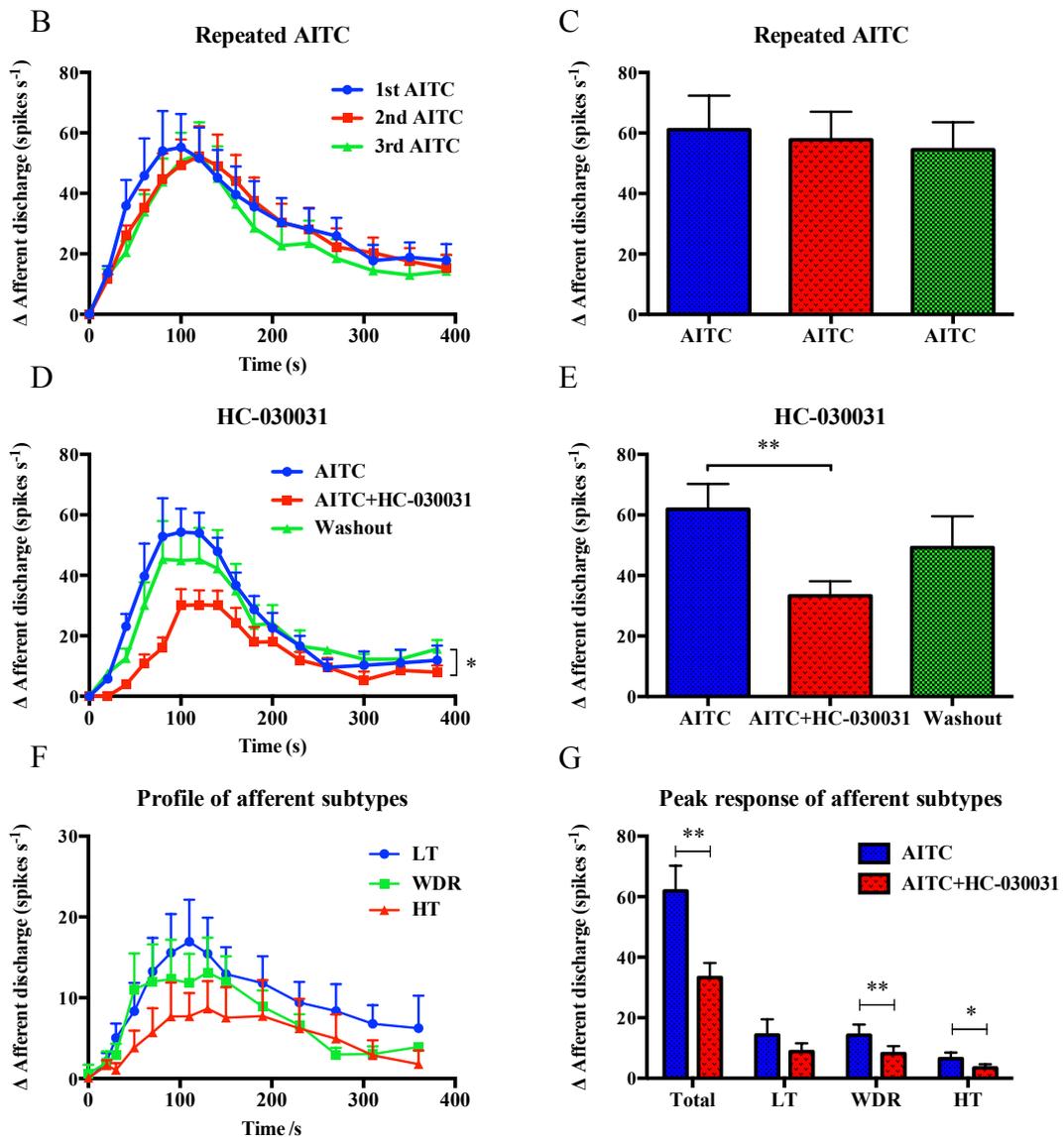
Figure 5.2 AITC induced afferent response was mediated by TRPA1. (A) Sample trace to show afferent response to 300 μM AITC was attenuated by TRPA1 antagonist HC-030031 (30 μM). (B&C) Repeated application of 300 μM AITC induced reproducible peak responses (61.1 ± 11.2 vs. 57.8 ± 9.3 vs.54.5 ± 9.1, P=0.895, N=8, one-way ANOVA) and response profiles (P=0.854, two-way ANOVA). (D&E) Response profile to 300 μM AITC was blunted by HC-030031 (P< 0.05, two-way ANOVA) with a reduced peak response (61.9 ± 8.4 vs. 33.3 ± 4.8, P<0.01, paired t-test), N=6. (F) Response profiles of different afferent subtypes to 300 μM AITC, P= 0.396, N=6 two-way ANOVA. (G) Peak response of high threshold (6.5 ± 1.9 vs. 3.4 ± 1.2, P<0.05) and wide dynamic range (14.2 ± 3.6 vs. 8.1 ± 2.5, P<0.01) units were significantly attenuated by HC-030031, P=0.151 for low threshold units (14.3 ± 5.2 vs. 8.8 ± 2.8). N=6, paired t-test.

Bradykinin (1 μM) triggered potent afferent response from jejunal afferent nerves. These responses were significantly inhibited by HC-030031 ($P<0.01$, figure 5.3B), suggesting TRPA1 activation was involved. Similar to AITC, bradykinin evoked all three afferent subpopulations, and LT units contributed more than HT ($P<0.05$, figure 5.3D). Inhibition of HC-030031 on bradykinin response was not selective for any afferent subpopulation (figure 5.3E).

At a rough estimate, more than 80% and 90% units participated afferent signaling induced by AITC and bradykinin, respectively. The ratio between direct activation and secondary activation was not clear.
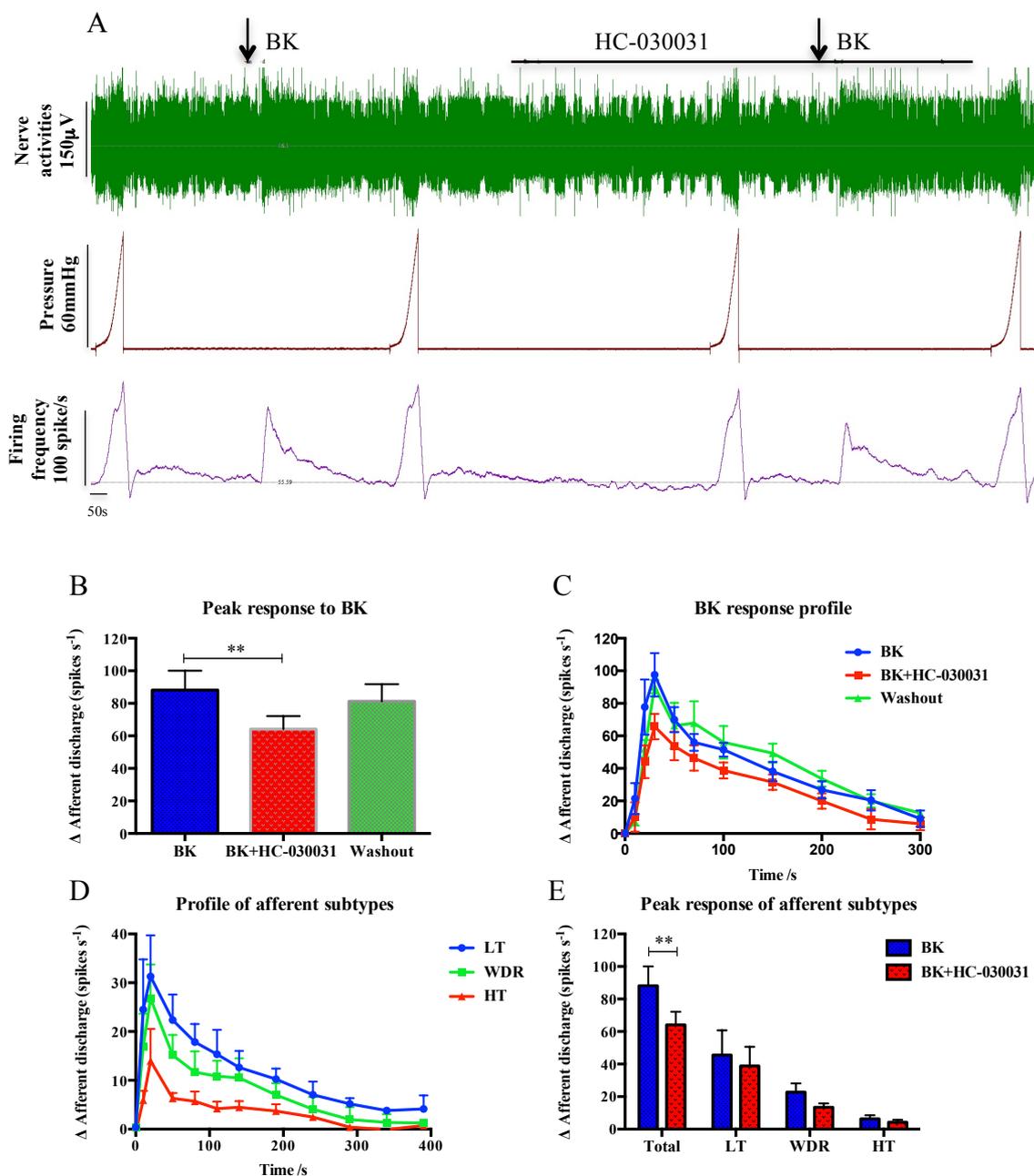
Figure 5.3 Bradykinin induced afferent discharges involved TRPA1 activation. (A) Representative trace to show afferent response to bradykinin (1 μM) was attenuated by HC-030031 (30 μM). (B) Peak responses to BK were significantly attenuated by HC-030031 from 88.1±11.9 to 64.1±8.0, P<0.01, N=5, paired t-test. (C) Response profile to BK, BK with HC-030031 and washout. BK vs. BK+HC-030031, P= 0.053, N=5, two-way ANOVA. P<0.05 at time points of 20 and 30 seconds. (D) Response profile of different afferent subtypes in response to BK, P= 0.101 for all. LT vs. HT, P<0.05, two-way ANOVA. (E) Peak responses of different afferent subtypes to BK. P= 0.212, 0.062 and 0.076 for low threshold (45.5±15.3 vs.38.9±11.8), wide dynamic range (22.7±5.5 vs. 13.4±2.4) and high threshold units (6.3±2.2 vs. 4.2±1.5), N= 5, paired t-test.

To investigate if AITC and bradykinin induce hypersensitivity to mechanical stimuli in this *in vitro* recording model, afferent responses to ramp distension before and after the application of AITC or bradykinin were analyzed. As a result, no obvious mechanical sensitization caused by AITC (300 μM) or BK (1 μM) was observed for total firing (figure 5.4 & 5.5). After further discrimination of different afferent subtypes, there were no significant alterations for low threshold, wide dynamic range and high threshold units as well.
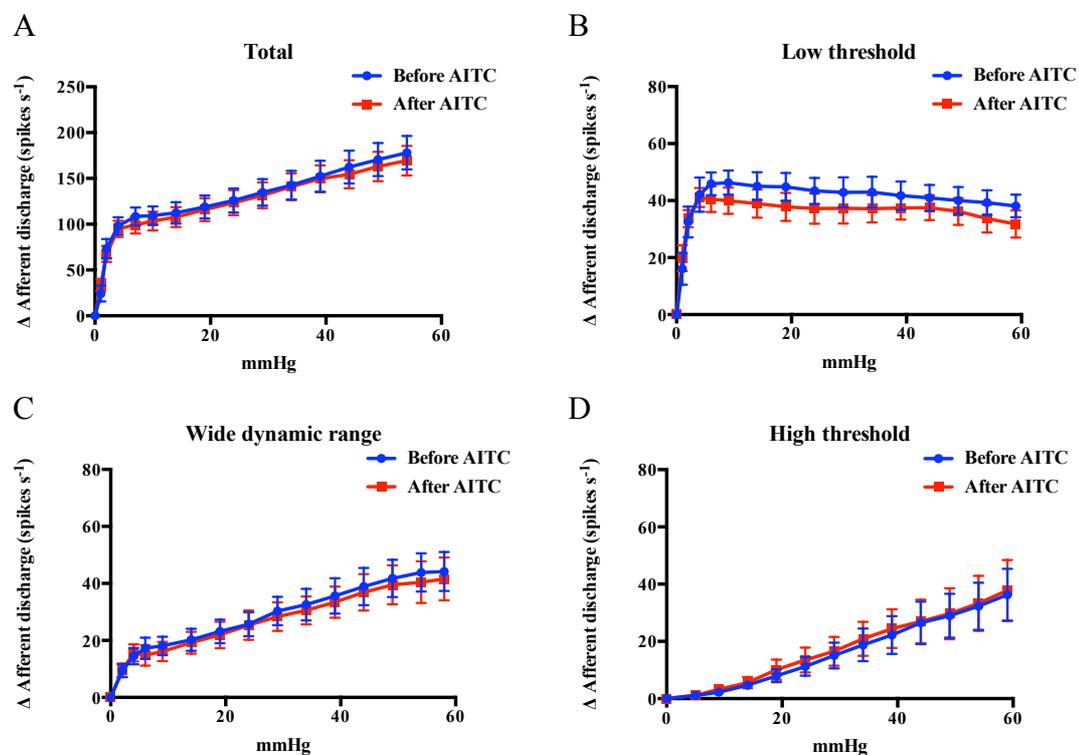
Figure 5.4 Effects of AITC on afferent responses to ramp distension. (A) Total afferent responses to ramp distension up to 60 mmHg before and after AITC treatment (300 μM) did not show any dramatic difference. P= 0.817. (B-D) No significant alterations were observed for low threshold units (P= 0.478), wide dynamic range units (P= 0.812) and high threshold units (P= 0.854). N= 10 for each group, two-way ANOVA.
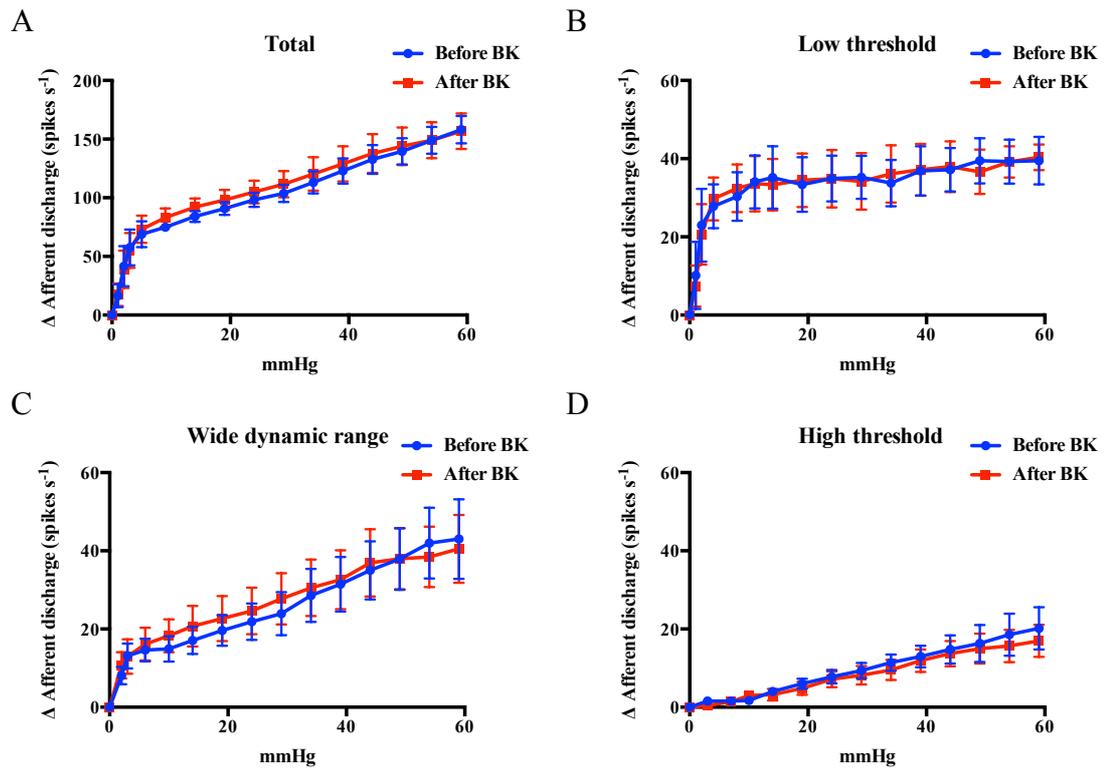
Figure 5.5 Effects of bradykinin on afferent response to ramp distension. (A) Total afferent response to ramp distension up to 60 mmHg was not significantly changed by bradykinin treatment (1 μM), P= 0.795. (B-D) No significant changes were observed for low threshold units (P= 0.987), wide dynamic range (P= 0.866) and high threshold units (P= 0.716). N= 10 for each group, two-way ANOVA.

5.4 Effect of granisetron on TRPA1 signaling

As shown in figure 5.6, the increase in afferent discharge in repsonse to AITC (300 μM) was significantly attenuated by granisetron (10 μM), a selective 5-HT$_3$ receptor antagonist, indicating that serotonin was implicated in AITC-induced TRPA1 signaling. Single unit analysis revealed that responses of LT, WDR and HT all appeared to be inhibited by granisetron, although WDR and HT were not statistically significant.

The washout response to AITC did not recover, which might be due to insufficient washing time.
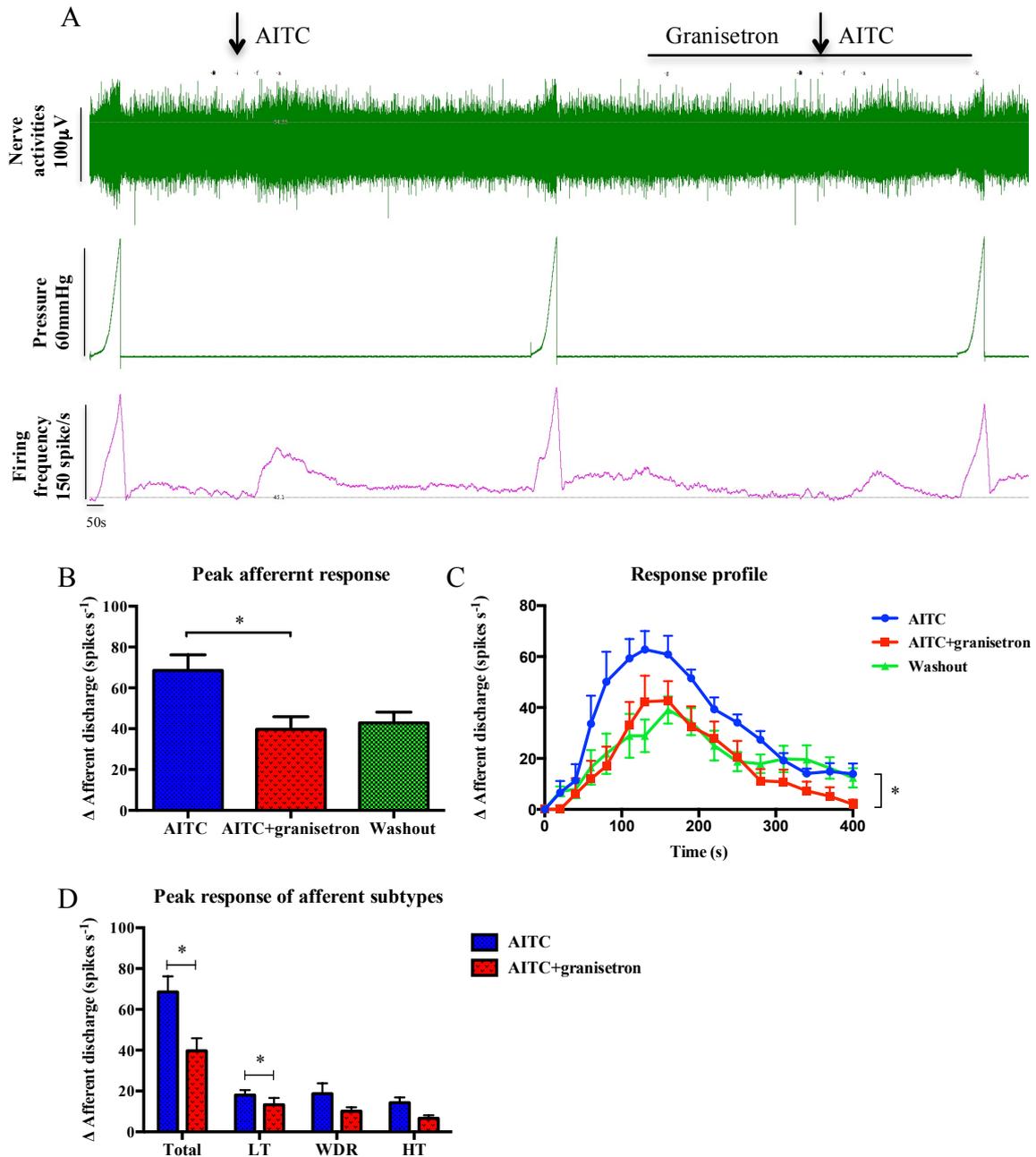
Figure 5.6 Granisetron attenuated afferent response to AITC. (A) A representative trace to show that afferent response to 300 μM AITC was attenuated by 10 μM granisetron. (B) Peak afferent discharge in response to 300 μM AITC was significantly reduced by granisetron from 68.5 ± 7.7 to 39.7 ± 6.1, $P<0.05$, N=7, paired t-test. (C) Time course of the response to AITC with or without granisetron. Granisetron significantly blunted afferent response to AITC, $P<0.05$, two-way ANOVA. (C) Response of all afferent subpopulations appeared to be reduced by granisetron, whereas only low threshold units achieved significance, 18.0 ± 2.4 vs. 13.3 ± 3.3, $P<0.05$. WDR: 18.7 ± 5.1 vs. 10.1 ± 1.9, $P= 0.115$; HT: 14.3 ± 2.5 vs. 6.6 ± 1.5, $P= 0.089$, paired t-test.

5.5 Effect of cromolyn on TRPA1 signaling

As shown in figure 5.7, the increase in afferent discharge in repsonse to AITC (300 μM) was significantly attenuated by mast cell stabilizer cromolyn sodium (100 μM), suggesting that mast cells might be involved in AITC-induced TRPA1 signaling. Similarly, single unit analysis did not find prominent selectivity for cromolyn's inhibition effect on LT, WDR and HT units, although only WDR units were statistically significant.
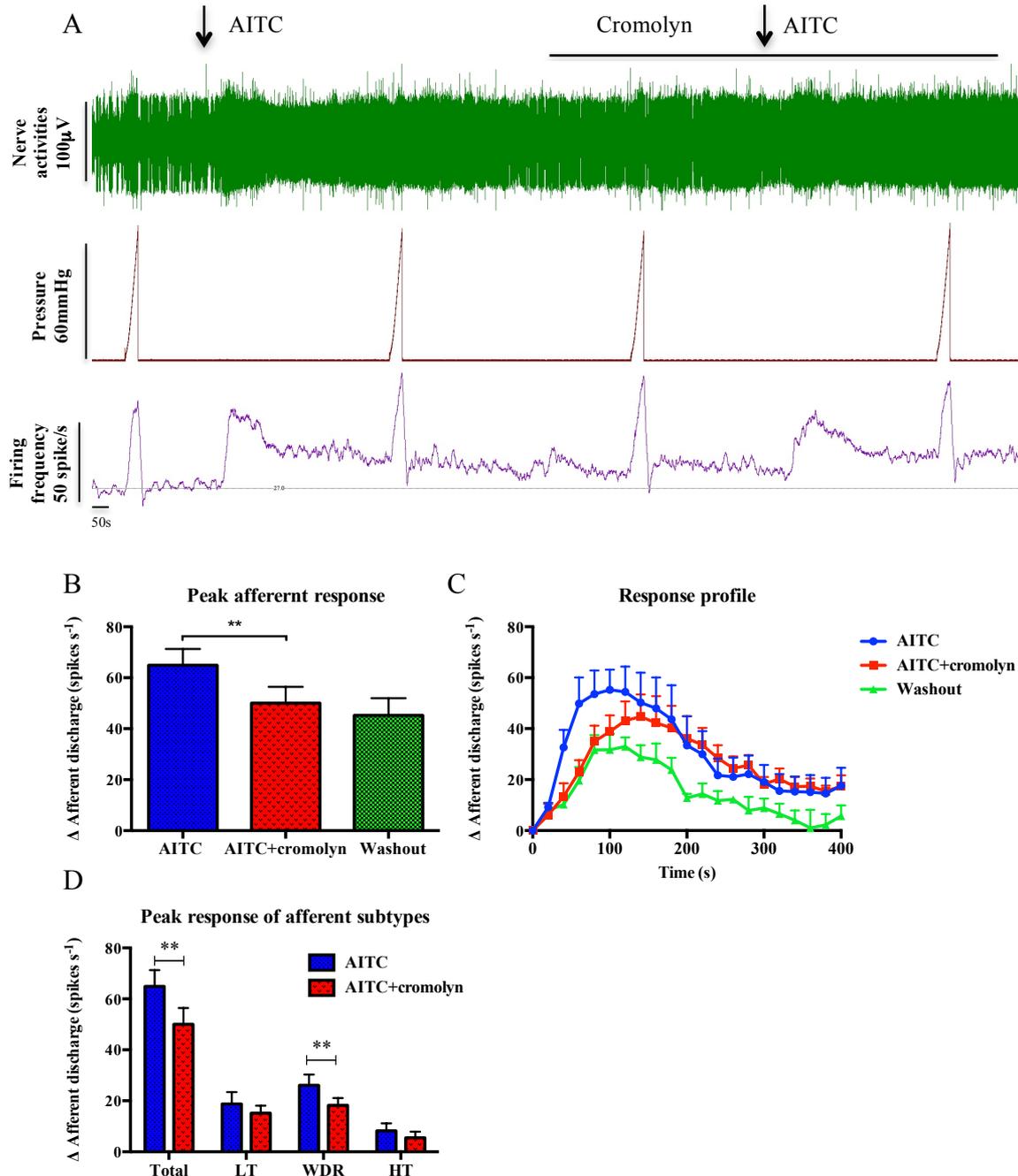
Figure 5.7 Cromolyn inhibited afferent response to AITC. (A) Example recording trace to show the inhibition of cromolyn sodium (100 μM) on AITC (300 μM) induced afferent discharges. (B) Peak afferent response to AITC was significantly attenuated by cromolyn, 64.9±6.4 vs. 50.1±6.4, P<0.01, N=6, paired t-test. (C) Response profile to AITC was blunted by cromolyn, P= 0.619, two-way ANONVA. (D) Responses of all three subpopulations appeared to be inhibited by cromolyn, while only wide dynamic range units reached significant level, 26.1±4.2 vs. 18.3±2.9, P<0.01. LT: 18.8±4.7 vs. 15.2±3.0, P=0.154; HT: 8.3±2.9 vs. 5.5±2.4, P= 0.051, N=6, paired t-test.

5.6 TRPA1 and contractility

Although interfered by spontaneous contractions sometimes, in most jejunum preparations, AITC (300 μM) elicited prominent increases in intraluminal pressure which reflected increased contractile activity. This observation suggested that AITC induced TRPA1 signaling comprised indirect activation secondary to contraction. In addition, AITC induced contractions were abolished by 5-HT$_3$ receptor antagonist granisetron (figure 5.8), indicating that these contractions were evoked through a 5-HT$_3$ receptor mediated pathway.
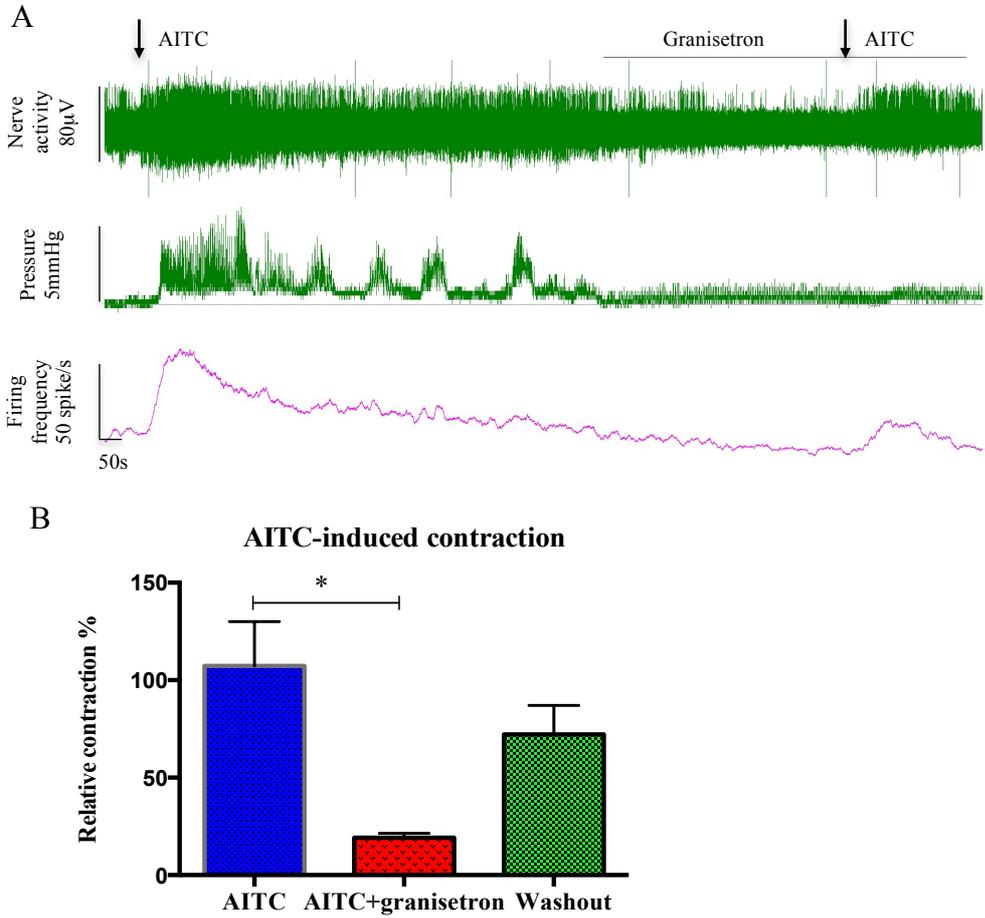
Figure 5.8 AITC elicited contractions of mouse jejunum through a 5-HT$_3$ pathway. (A) Sample recording trace to show AITC induced contractions and the inhibition of granisetron on this contraction. (B) AITC (300 μM) induced contraction were significantly suppressed by granisetron (30 μM), 107.3±22.8 vs. 19.2±2.2, P<0.05, N=5, paired t-test. Data were expressed as relative contraction normalized by 1 μM carbachol.

5.7 TRPA1 signaling in TRPV1 knockout mice

To assess TRPA1 signaling in the absence of TRPV1, afferent responses to AITC in the TRPV1 knockout mice were examined and compared with their wild type littermates. Jejunal mesenteric afferents displayed augmented responses to 100 μM, 300 μM and 1 mM AITC in the knockout mice, although only peak response to 300 μM achieved significance (P<0.05). Time courses of response shown in figure 5.9 revealed that TRPV1 knockout mice had a shorter latency time before afferent discharges rose up.
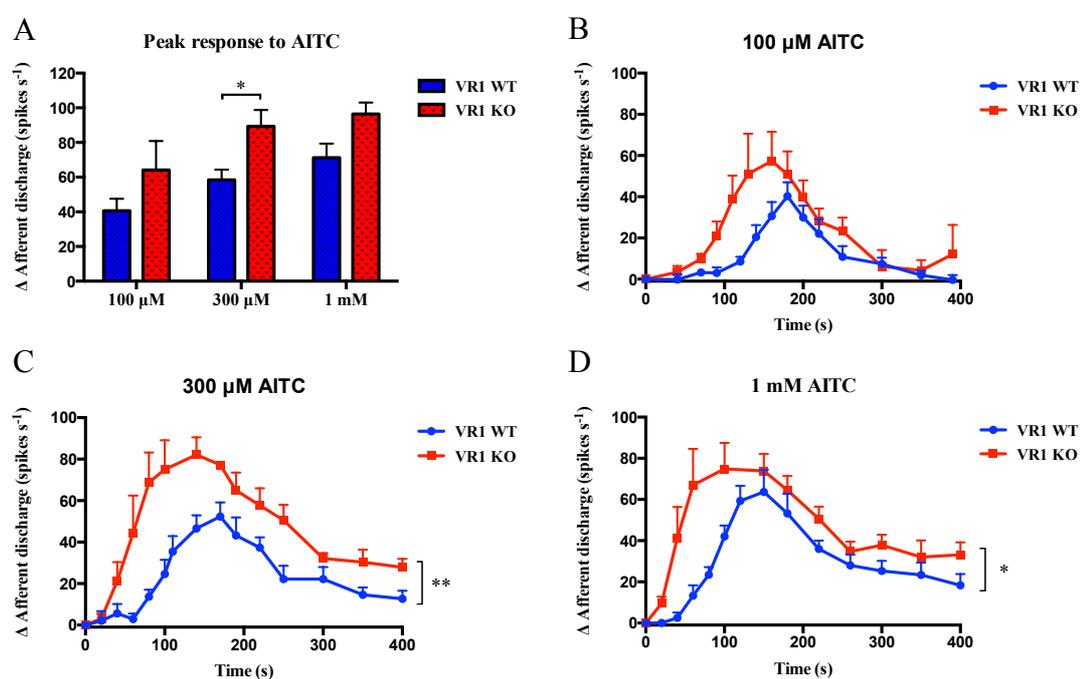
Figure 5.9 TRPA1 signaling in the TRPV1 knockout mice was augmented. (A) Peak responses to 100 μM, 300 μM and 1 mM AITC inTRPV1 knockout mice and wildtype littermates. WT vs. KO, 100 μM AITC: 40.6±6.9 vs. 64.2±16.6, P= 0.195; 300 μM: 58.5±5.9 vs. 89.3±9.4, P< 0.05; 1 mM: 71.3±8.1 vs. 96.4±6.7, P= 0.059; N=6 for WT, N=5 for KO, unpaired t-test. (B-D) Time course of response to 100 μM, 300 μM and 1 mM AITC in TRPV1 WT and KO mice. P< 0.01 for 300 μM, P<0.05 for 1mM, P= 0.119 for 100 μM, two-way ANOVA.

5.8 Discussion

This study employed *in vitro* afferent recording to assess TRPA1 sensory signaling at the level of a segment of mouse small intestine. Main findings were summarized as follows:

> 1) afferent responses to AITC and bradykinin were mediated by TRPA1;
>
> 2) 5-HT$_3$ receptor antagonist granisetron attenuated TRPA1 signaling;
>
> 3) mast cell stabilizer cromolyn attenuated TRPA1 signaling;
>
> 4) AITC elicited contractile activities were inhibited by granisetron;
>
> 5) TRPA1 signaling was augmented in TRPV1 knockout mice.

TRPA1 signaling induced by AITC and bradykinin

Increases in afferent discharge elicited by TRPA1 agonist AITC were significantly inhibited by a selective TRPA1 antagonist, HC-030031. By discrimination of individual afferent units, more than 80% of afferents were implicated in TRPA1 signaling, and low threshold units contributed more than high threshold units. However, different from wide dynamic range and high threshold units, low threshold afferents were not significantly inhibited by HC-030031.

As introduced in the introduction of this chapter, it is controversial whether bradykinin can activate TRPA1. This study demonstrated that bradykinin induced increase in afferent discharge was significantly attenuated by TRPA1 antagonist HC-030031. This is in agreement with the observation that TRPA1 knockout mice exhibit a reduced nociceptive behavior to subcutaneous bradykinin (Kwan et al., 2006).

Our preliminary data indicated that AITC did not induce any change in mechanosensitivity (Jiang et al., 2012). This study further discriminated different afferent subpopulations, and no significant alteration was observed for all three afferent subtypes. This is in agreement with a study on guinea pig that AITC (1 mM) perfusion for 30 minutes did not change esophageal distension-evoked vagal nerve action potentials (Yu and Ouyang, 2009). It has been demonstrated on mouse colonic flat sheet preparations that TRPA1 agonist AITC (40 μM) or cinnamaldehyde (100

μM) potentiate mechanosensitivity to probing in mesenteric, serosal and mucosal afferents instead of muscular afferents characterized by their responses to stretch (Brierley et al., 2009). The type of mechanical stimulation might account for this disagreement.

In line with AITC, bradykinin (1 μM) did not significantly sensitize afferent responses to ramp distension, which is not consistent with some evidence in the literature. Bradykinin (1 μM) has been shown to induce a slight sensitization to probing in splanchnic afferents but not pelvic afferents (Brierley et al., 2005). Perfusion of bradykinin (10 μM) for 30 minutes significantly augments vagal nerve response to esophageal distention in guinea pig. Bradykinin injection into mouse paw causes hypersensitivity to mechanical stimuli by measuring plantar withdrawal response 2 hours after injection(Kwan et al., 2006). In addition, a study has suggested that bradykinin at low doses (20 nM) doesn't activate TRPA1 but sensitize this channel to agonist stimulation through PLC and PKA (protein kinase A) pathways (Wang et al., 2008). Hence the concentration of bradykinin, incubation time and target afferent subpopulation might produce variable results.

Effects of granisetron on TRPA1 signaling

The current study demonstrated that TRPA1 sensory signaling induced by AITC was significantly attenuated by 5-HT$_3$ antagonist granisetron, which provided functional evidence that serotonin was implicated in TRPA1 signaling. Unpublished data from our group have demonstrated that AITC induced increased serotonin release from mouse jejunum preparations and BON cells (human EC cell model), which was in agreement with studies on enriched rat EC cells and rat endocrine cell line RIN14B (Nozawa et al., 2009). Combining these observations suggests that EC cells play an important role in the amplification of TRPA1 signaling following 5-HT release. As such, TRPA1 signaling might not only transmit nociceptive signals to the CNS but also mediate inflammatory response by the interaction between afferent nerves and non-neuronal cells.

Increasing evidence has demonstrated the important role of TRPA1 in visceral inflammation and pain (Lapointe and Altier, 2011; Bautista et al., 2012). Considering the antinociceptive role of 5-HT$_3$ receptor antagonists on a rat model of

inflammatory pain (Butkevich et al., 2007) and the relieving effect of 5-HT$_3$ receptor antagonists in patients with diarrhoea-predominant IBS (Camilleri et al., 2001), it is likely that TRPA1 is the central molecular responsible for the pain and discomfort in IBS. Consequently, TRPA1 might be a promising pharmacological target in treating functional bowel disorders such as IBS. Indeed, a recent study has suggested that ASP7663, a novel TRPA1 agonist, can improve the symptoms in an animal model of constipation (Kojima et al., 2013).

Rodent mast cells also contain 5-HT. It is possible that a proportion of released serotonin in TRPA1 signaling is originated from mast cells. However, serotonin antibody revealed intensive immunoreactivities in EC cells of mouse intestine, whereas no mast cells were labeled. As such, serotonin released from mast cells might be marginal.

TRPA1 and contractility

In the small intestine, afferent discharges triggered by 5-HT are composed of a direct excitation through 5-HT3 receptors and an indirect action through increased motility, which is likely to arise from 5-HT receptors on enteric neurons and muscles (Hillsley et al., 1998). Evidence has shown that serotonin released from EC cells activates enteric nerves system to control gastrointestinal motility and secretion (Yamano and Miyata, 1996; Gershon, 2003). A very recent study on TPH1 deficient mouse has observed a much slower propulsion with larger faecal pellets as well as reduced responses to intraluminal balloon distension, suggesting an essential role mucosal 5-HT in gastrointestinal motility (Heredia et al., 2013). Since literature and our unpublished data have demonstrated AITC's ability to induce serotonin release, whether AITC could induce gastrointestinal contractions was determined on isolate mouse jejunum. Despite interference from spontaneous contractions, AITC induced prominent contractions in most preparations, and granisetron abolished these contractions, indicating a 5-HT$_3$ receptor-mediated mechanism. This is in agreement with a study on guinea pig isolated ileum, showing that AITC induces contractions in a dose-dependent manner and these contractions are inhibited by ruthenium red and 5-HT$_3$ antagonist ramosetron (Nozawa et al., 2009).

However, it remains controversial whether TRPA1 agonist can elicit contractions of isolated intestine. Evidence has shown that AITC dose-dependently induced contractions in mouse proximal and distal colon, whereas in the jejunum and ileum AITC elicits only small contractions (Penuelas et al., 2007). Nevertheless, a study has suggested that cinnamaldehyde has no effect on mouse duodenum and ileum, but inhibits contractility in the colon, which might be attributed to co-expression of TRPA1 with inhibitory motor neurons (Poole et al., 2011). As such, it is possible that TRPA1 signaling orchestrates a bidirectional modulation on the motility through multiply pathways.

Effects of cromolyn on TRPA1 signaling

Mast cells can release a variety of mediators to orchestrate allergic and inflammatory responses, such as histamine, protease, serotonin and TNF-α. Intracolonic administration of protease-activated receptor-activating peptide (PAR2-AP) augments visceromotor responses to graded colorectal distension (Cattaruzza et al., 2010). Activation of mast cells by antigen or perfusion of PAR2-AP both can induce mechanical hypersensitivity of vagal afferents to esophageal distension, which is inhibited by TRPA1 antagonist; furthermore, TRPA1 is highly co-expressed with PAR2 receptors (Yu et al., 2009). PAR2 activation potentiates TRPA1 currents evoked by AITC or cinnamaldehyde in DRG neurons and HEK293 cells transfected with TRPA1 (Dai et al., 2007). These observations suggest that protease released from mast cells might sensitize TRPA1 expressing afferent nerves through PAR2 receptor that is coupled with TRPA1. In addition to protease, TRPA1 has been suggested an essential downstream pathway in histamine-dependent itch (Wilson et al., 2011). Pre-evacuation of mast cell mediators or histamine receptor H1 antagonist pyrilamine significantly inhibit AITC induced nociception in mice (Andrade et al., 2008).

Current study demonstrated that TRPA1 signaling induced by AITC was significantly attenuated by cromolyn sodium, a mast cell stabilizer, suggesting that mast cells might be involved in TRPA1 signaling. Cromolyn sodium has been used in clinic to treat asthma for more than 30 years. Inhalation of cromolyn by patients with allergic asthma blocks allergen-induced bronchospasm, presumably by

suppressing the release of mediators from mast cells, as supported by the drug's inhibition effect on the degranulation of rat peritoneal mast cells in response to challenge with IgE and specific antigen (Kusner et al., 1973). In addition to IgE dependent activation, evidence has shown that cromolyn (100 μM) significantly inhibits mast cell degranulation induced by a variety of stimuli such as substance P, thapsigargin, ionomycin and compound48/80; however, its specificity and efficacy have been controversial, especially in the mouse (Oka et al., 2012; Weng et al., 2012). Hence further studies are required to validate this observation.

TRPA1 signaling in TRPV1 knockout mice

Interestingly, our afferent recording data indicated that TRPA1 signaling in the TRPV1 knockout mice was significantly augmented. This may be a longtime consequence of compensative mechanisms. Loss of TRPV1 results in a deficiency in nociceptive signaling, and TRPA1 is up regulated to compensate this deficiency. Alternatively, other non-neuronal cell types might adapt to up-regulate communications with afferent nerves.

In line with our observation, afferent responses to capsaicin tend to increase in TRPA1 knockout mouse colon (Brierley et al., 2009). Conversely, another study has suggested that there is no significant change in capsaicin sensitivity in TRPA1 mutant neurons, and similarly no prominent alteration of mustard oil sensitivity in TRPV1-deficient neurons (Bautista et al., 2006). This study may be not contradicted with our observation, since our in vitro recording comprises the contribution of non-neuronal cells. For example, EC cell might be up-regulated in the knockout mice with enhanced serotonin release to amplify sensory signaling. The exact mechanisms underlying this observation require further investigations.

Deconstruction of TRPA1 signaling pathways

A study employed microarray hybridization and laser-capture microdissection to compare gene expression profiles of DRG and NG neurons projecting to mouse viscera, and revealed that DRG neurons had much higher expressions of TRPA1 but much lower expressions of 5-HT$_{3A}$ and 5-HT$_{3B}$ receptors, compared with nodose neurons (Peeters et al., 2006). Interestingly, a recent study on animal model of constipation has revealed that ASP7663, a novel TRPA1 agonist, significantly

improves loperamide (opioid receptor agonist)-induced constipation, and this effect can be reversed by either pretreatment with TPRA1 antagonist or vagotomy (Kojima et al., 2013). This suggests that physiological motor reflexes are mainly mediated via vagus nerve, possibly through serotonin release and $5-HT_3$ receptors on vagal nerve terminals. It is widely accepted that vagal afferents only have low threshold units and regulate normal physiological activities, whereas spinal afferents comprise both low and high threshold units to detect noxious stimuli. The current study observed that more than 80% of afferent units participating AITC induced TRPA1 signaling, which maybe consist of direct activation of spinal afferents through TRPA1 and indirect activation on vagal afferents through $5-HT_3$ following 5-HT release. This is consistent with afferent sorting result that response of low threshold units to AITC was significantly inhibited by $5-HT_3$ antagonist, whereas high threshold units were not. This pattern is relative, as TRPA1 and $5-HT_3$ are present on both DRG and NG neurons.

In addition to primary sensory neurons and EC cells, TRPA1 is expressed in myenteric ganglia where it localizes to inhibitory motorneurons, descending interneurons, cholinergic neurons and intrinsic primary afferent neurons (Poole et al., 2011), suggesting that the modulation effects of TRPA1 signaling could be diverse. The above study has provided functional evidence that AITC can activate myenteric neurons directly. This chapter described the possible pathways involved in TRPA1 signaling (figure 5.11). AITC directly excite extrinsic and intrinsic neurons to elicit afferent discharge as well as to modulate motor and secretion reflexes. Activation of TRPA1 on EC cells results in 5-HT release, which also either directly depolarizes extrinsic afferents or act on enteric neurons to regulate motility. Altered motility or secretion provides feedback to extrinsic afferents. Activation of afferent nerves releases neuropeptides, which can degranulate mast cells; mediators released from mast cells, in turn, depolarize or sensitize afferent nerves.

The interaction between EC cells, mast cells and TRPA1 modulates afferent sensitvity to noxious stimuli or inflammatory mediators. Imbalanced interaction might result in inapropriate sensitivity, such as hypersensitivity, a hall marker for IBS. As such, TRPA1 is a potential therapeutic target for treating funtional bowel disorders associated with abdominal pain.
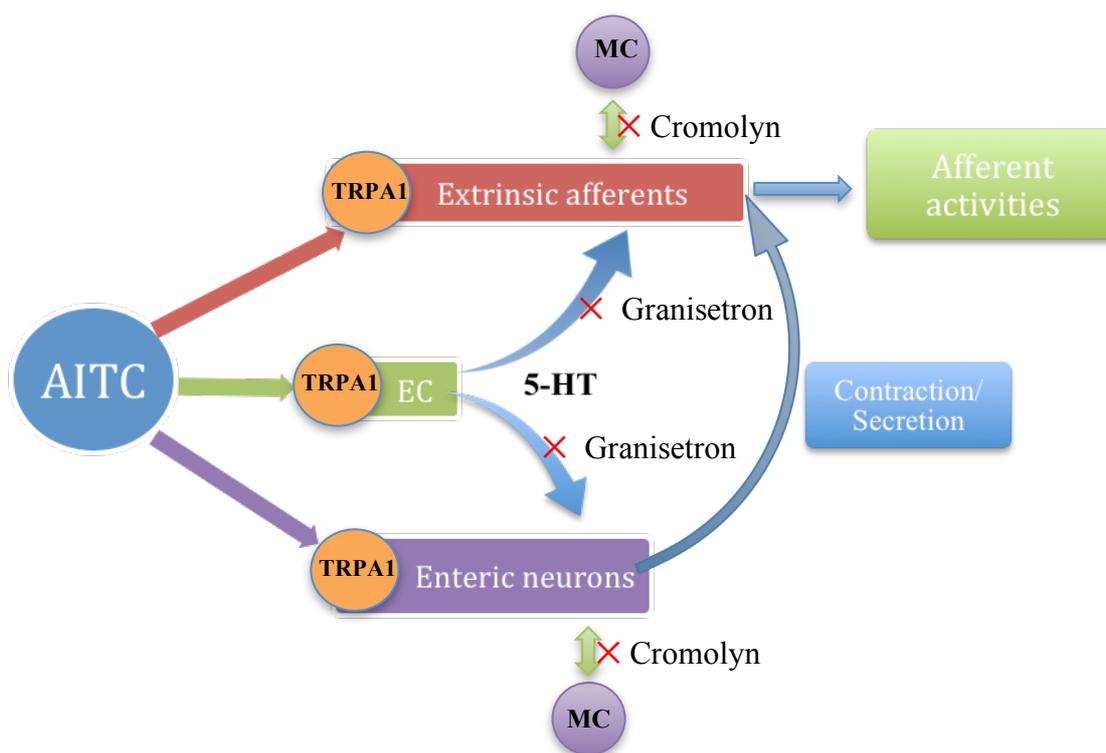
Figure 5.11 Schematic illustration of TRPA1 signaling pathways induced by AITC. AITC could directly activate TRPA1 on extrinsic afferents to elicit afferent discharge. Activation of TRPA1 on EC cells results in 5-HT release, which on one hand target on 5-HT receptors on extrinsic afferent to depolarize primary neurons, and on the other hand act on enteric neurons to initiate contractions and secretions. AITC activation on the intrinsic neurons excites or inhibits motilities and secretions, which provides feedback to extrinsic afferents. Activation of afferent nerves might release neuropeptides to degranulate mast cells, and mediators released from mast cells in turn depolarize or sensitize afferent nerves. MC: mast cells.

The distribution of TRPA1 protein within the gut wall remains controversial. TRPA1 immunoreactivities have been shown colocalized with CGRP in afferent endings throughout different layers of mouse colon (Brierley et al., 2009). Another study has reported that TRPA1 is detected not only in enteric ganglia but also uniformly in surface epithelial cells in the mucosa at a low level (Poole et al., 2011). In situ hybridization on rat and human duodenum has shown that TRPA1 mRNA is restricted to epithelial cells with frequent colocalization with serotonin immunoreactivities (Nozawa et al., 2009). Efforts were made to locate TRPA1 on the gut wall using immunohistochemistry. However, due to the lack of reliable TRPA1 antibodies, no conclusive data were achieved. As such, whether TRPA1 proteins are present in EC cells and whether TRPA1-expressing afferent nerves are closely apposed to mast cells require further investigations.

Chapter 6

Influence of ageing on TRPA1 afferent signaling from mouse intestine

6.1 Introduction

As introduced in section 1.7 and 3.1, a variety of age-related changes occur in the GI tract. In terms of nervous system, a large number of studies have reported a reduction in myenteric neurons in aged intestine of rat (Santer and Baker, 1988), guinea pig (Gabella, 1989; Peck et al., 2009), mice (El-Salhy et al., 1999) and human (de Souza et al., 1993; Gomes et al., 1997; Bernard et al., 2009). Similar to myenteric plexus, neuron loss in submucosal plexus with advanced age has been found in rat (Phillips et al., 2007) and mouse (El-Salhy et al., 1999). In addition to intrinsic neurons, sympathetic innervations within the gut wall display some signs of neurodegeneration with advanced age, as indicated by the presence of observable swollen axons and decreased expression of tyrosine hydroxylase; similar swollen varicosities are observed in the sensory terminals of vagal innervation, whereas spinal afferents are not dramatically affected by senescence (Phillips and Powley, 2007). Some studies have described decreases in the size or number of primary neurons in the nodose ganglia and DRGs (Vega et al., 1993), whereas other studies did not observe a significant change (Soltanpour et al., 1996; Bergman and Ulfhake, 1998). In addition, changes in neuropeptides and neurotrophin receptors have been described in DRGs of aged rat, such as increased NPY and galanin as well as decreased CGRP and SP (Bergman et al., 1996).

In addition to morphological changes, some functional studies have described alterations in the sensitivity of aged GI tract. Intraesophageal balloon distension on human subjects have revealed an increased pain threshold with advanced age (Lasch et al., 1997). In line with this study, rectal sensory thresholds have been found significantly higher in aged subjects. (Lagier et al., 1999).

It is poorly understood whether the expressions and functions of TRP channels are influenced by advanced age. It has been revealed that TRPV1 mRNA is not changed in aged mouse DRG whereas TRPV1 protein appeared to be reduced in the DRG and peripheral nerves (Wang et al., 2006; Wang and Albers, 2009). Another study reported an increased expression of TRPV1 in human epidermis of elderly subjects (Lee et al., 2009). Regarding to TRPA1, it is largely unknown whether its expression and function in the GI tract are altered by ageing.

As demonstrated in chapter 5, EC cells and serotonin played an important role in the amplification of TRPA1 signaling. Consequently, the alteration of EC cells density or serotonin release with advanced age might change TRPA1 signaling. EC cell has been found increased in rat stomach during ageing (Khomeriki, 1989). A study employing chromatographic analysis found an increase in the synthesis and release of serotonin in aged mouse distal ileum (Parmar et al., 2012). A study on human duodenum found that serotonin positive cells tend to increase with age (Sandstrom and El-Salhy, 1999). Conversely, staining for somatostatin and chromogranin, markers for EC cells, did not reveal any change in aged rat colon (Sweet et al., 1996); moreover, EC cells in the guinea pig distal colon are spared from the influence of ageing (Wade et al., 2003). In addition, serotonin receptors measured by positron tomography in the living human brain decline with advanced age (Wong et al., 1984). However, it is largely unknown if 5-HT receptor expressions in the primary sensory neurons or cells within the gut wall are influenced by ageing.

Hypothesis and objectives

This chapter hypothesized that TRPA1 signaling was attenuated in the intestine of aged mice, and this attenuation might be attributed to reduced sensory innervation, down regulation of TRPA1 or 5-HT$_3$ receptors, reduced EC cells or serotonin release and impaired sensitivity of sensory neurons.

Parallel studies were performed on aged mice (24 months) and control (3 months), aiming to investigate potential age-related changes in

1) mechanosensitivity of jejunal innervation;
2) TRPA1 signaling induced by AITC and bradykinin;
3) expression of TRPA1 and 5-HT$_3$ receptors in DRG and jejunum;
4) density of EC cells in the jejunum;
5) TRPA1 mediated calcium signaling in primary sensory neurons.

6.2 Experimental protocol and analysis

Afferent recording

Extracellular recording on jejunal segments from 24 months old mouse was performed as described in section 2.4 and 5.2. All experimental procedures and analysis methods were consistent with adult mice. Student's t-test or two-way ANOVA were used to compare the peak response or time course of response between 3 and 24 months old mice.

Changes of gene expression in aged mice

RNA was isolated from jejunal segments (whole-thickness) and DRG neurons of 3 and 24 months old mice. RNA isolation, reverse transcription and quantitive real-time PCR were performed as described in section 2.5. Probes for genes encoding TRPA1, 5-HT$_{3A}$ and 5HT$_{3B}$ were commercially available from Integrated DNA Technologies. Data analysis was described in section 2.5 and 3.2. Probe sequences for mouse GAPDH and TRPA1 were as follow.

Genes	Accession No.		Sequence	Expected Band Size (bp)
TRPA1	NM_177781	Forward	5' ACCATCGTGTATCCAAATAGACC 3'	91
		Reverse	5' GTACTTCTTGTCGTGTTTCTTGC 3'	
GAPDH	NM_008084	Forward	5' AATGGTGAAGGTCGGTGTG 3'	150
		Reverse	5' GTGGAGTCATACTGGAACATGTAG 3'	

Quantification of EC cells density in mouse jejunum

Jejunum segments were fixed in 4% PFA, embedded in OCT and sectioned in a cryostat as described in section 2.3. EC cells were labeled by a polyclonal rabbit anti serotonin antibody (1:50, AbD Serotec, AHP522) and visualized by a goat anti-rabbit secondary antibody conjugated with Cyanine 3 (Cy3, 1:400, Jakson ImmunoResearch, 111-165-144).

10 random images were acquired from one tissue under 20 times objective for offline quantification. EC cells density was calculated as number of EC cells divided by the area of mucosa. EC cells were counted blindly, and area of mucosa was measured using ImageJ software.

Calcium imaging

Dissociation, culture and preparation of mouse DRG (T7-L2) and nodose ganglion was performed as described in section 2.6.

Prior to application of AITC (30 or 300 μM) or bradykinin (1 μM), 5-minute baseline intensity was recorded. Drug diluted in HEPES buffer was perfused into recording chamber by gravity for 1 minute and then washed out by HEPES. At the end of each experiment, 5 μM ionomycin was applied for 1 minute as a positive control.

Intracellular calcium concentration was indicated by the ratio of emission fluorescence under 340 nm and 380 nm excitation light. Raw data exported to Excel were emission fluorescent intensities at a series of time points. Ratio between 340 nm and 380 nm channels was calculated prior or to any analysis. A time window covering a period of baseline activity and the response profile of the drug was selected for analysis. Changes of calcium concentration in response to a drug were calculated as the net difference between baseline ratio (minimum value within the time window) and the peak ratio (maximum value within the time window) during drug application. Data were expressed as mean±SE.

6.3 Mechanical sensitivity in aged mouse

To investigate if mechanosensitivity in aged mouse intestine was impaired, afferent recording was performed on jejunal preparations from 3 and 24 months old mice. Ramp distension up to 60 mmHg produced a biphasic increase in afferent discharges, corresponding to the activation of low threshold and high threshold afferents. In comparison with adult mice, total afferent responses to distension were significantly reduced in the 24 months old mice ($P<0.01$, figure 6.1B). By further discrimination of different afferent subtypes, this reduction was selective for low threshold units ($P<0.01$, figure 6.1C). This is consistent with the quantification of the number of units per nerve bundle, which revealed that average number of low threshold units detected by recording was significantly decreased (4.5 ± 0.4 vs. 2.7 ± 0.5, $P<0.01$, figure 6.3B).

The total number of units per nerve bundle was significantly reduced in aged mice (11.8 ± 0.4 vs. 8.8 ± 0.5, $P<0.001$). LT units were the major contributor to this reduction, whereas HT units were unchanged. WDR units might be decreased, but not statistically significant. In addition, the proportions of different afferent subtypes were altered in aged mice. As shown in the pie charts (figure 6.2A), proportion of LT was decreased from 35.7% to 28.5%, whereas HT was increased from 27.6% to 36.8%. Besides, WDR units remain unchanged, and mechano-insensitive units were slightly decreased.
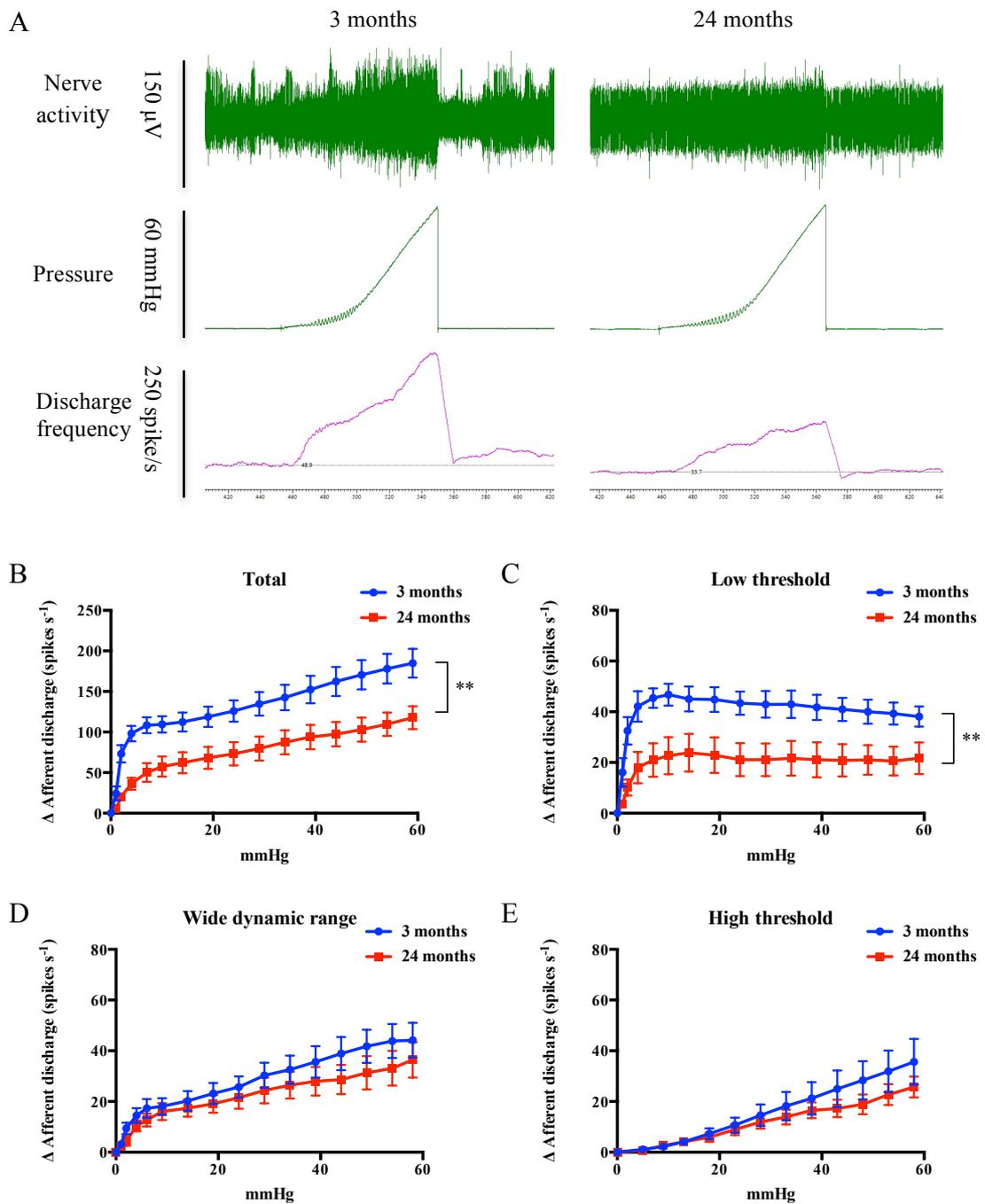
Figure 6.1 Afferent responses to ramp distension were attenuated in aged mouse. (A) Sample traces of afferent recording to compare responses to distension up to 60 mmHg in aged and control mice. (B-E) Comparison of increase in afferent discharge in response to distension in 24 and 3 months old mice. P<0.01 for total units, P<0.01 for LT units, P= 0.345 for WDR, P= 0.435 for HT. N= 10 for both groups, two-way ANOVA.
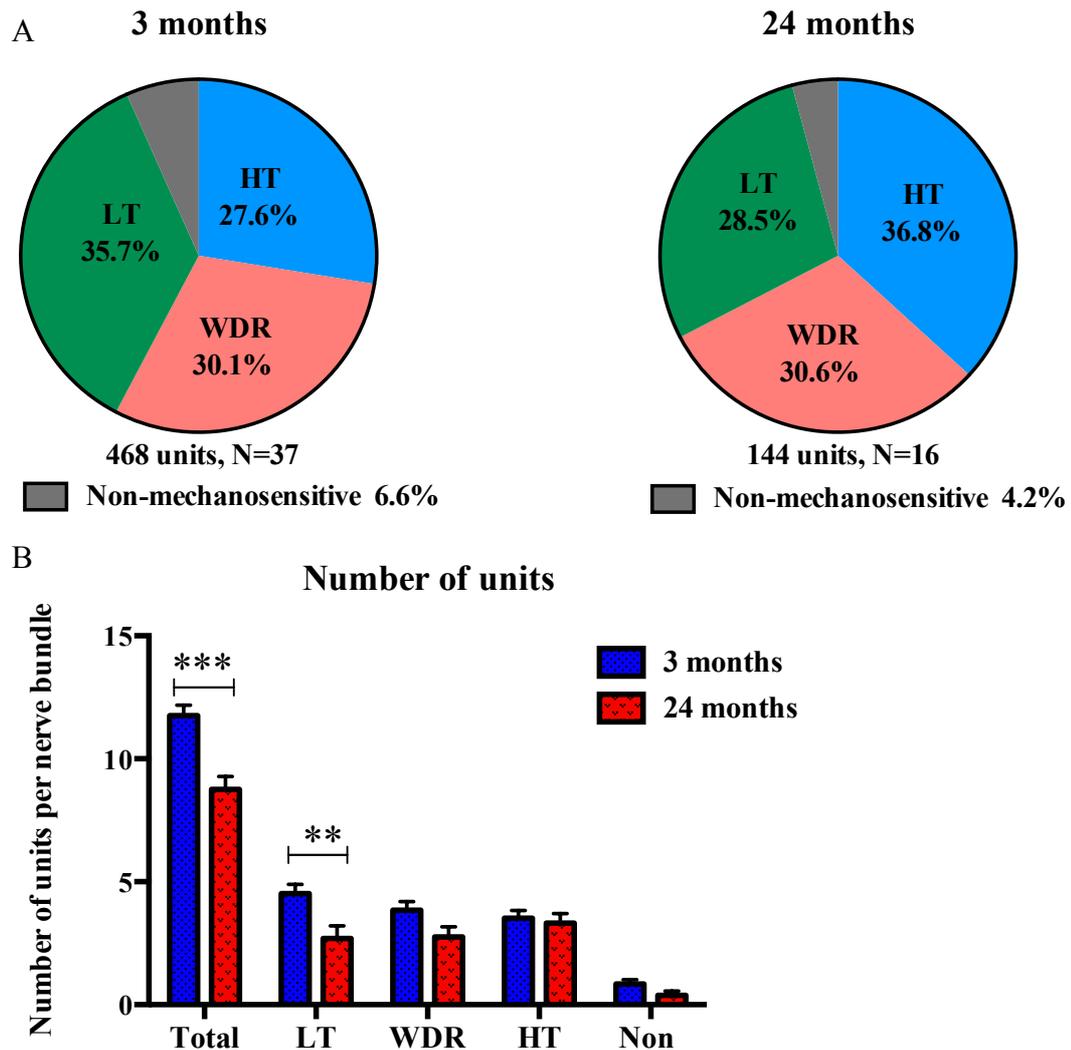
Figure 6.2 Number of low threshold afferents was decreased in aged mouse. (A) Pie charts to show the percentage of afferent subtypes in control and aged mice. LT, WDR, HT and non-mechanosensitive units account for 35.7%, 30.1%, 27.6% and 6.6% of total units in 3 months old mice, 28.5%, 30.6%, 36.8% and 4.2% in 24 months old animals. (B) Quantification of average number of units per nerve bundle innervating a segment of jejunum from aged and control mice. 3 months vs. 24 months: 11.8±0.4 vs. 8.8±0.5, P<0.001 for total; 4.5±0.4 vs. 2.7±0.5, P<0.01 for low threshold units; 3.8±0.4 vs. 2.8 ±0.4, P= 0.078 for wide dynamic range; 3.5±0.3 vs. 3.3±0.4, P= 0.717 for high threshold; 0.8±0.5 vs. 0.4±0.3, P= 0.127 for non-mechanosensitive units. N= 37 for 3 months old mice, N= 16 for 24 months group.

6.4 TRPA1 signaling in aged mouse

In comparison with adult mice, changes in afferent discharge in response to AITC at a range of concentrations were all significantly attenuated in aged mouse jejunum (P<0.05 for 100 and 300 µM, P< 0.01 for 1mM, figure 6.3). By discrimination of activities from different afferent subtypes, low threshold and wide dynamic range units were responsible for this age-related attenuation, whereas high threshold units were spared (figure 6.4). This is consistent with the attenuation of mechanosensitivity and reduced number of low threshold units per nerve bundle.

In line with the attenuation in AITC-induced TRPA1 signaling, afferent response to bradykinin was significantly decreased (P<0.05, figure 6.5) in aged mice. LT and WDR appeared to be responsible for this attenuation.
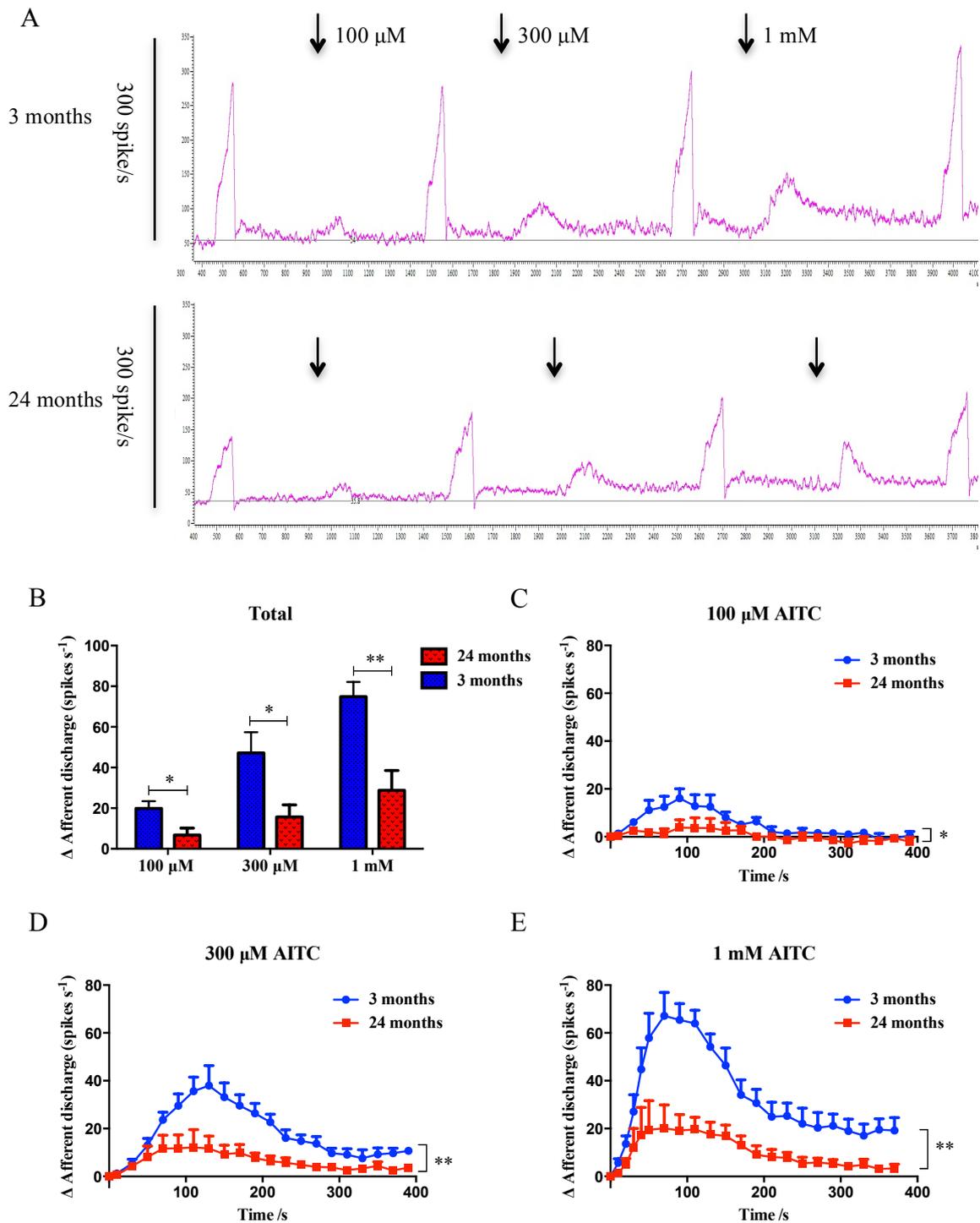
Figure 6. 3 Increases in afferent discharge in response to AITC were attenuated in aged mice. (A) Example recording trace to show afferent responses to AITC in 3 and 24 months old mice. (B) Peak responses to 100 μM, 300 μM and 1 mM AITC in 24 months old mice were all significantly reduced, comparing with 3 months group. 3 months vs. 24 months, 100 μM AITC: 19.9±63.6 vs. 6.8±3.4, P<0.05; 300 μM: 47.2±10.2 vs. 15.7±5.9, P<0.05; 1 mM: 74.9±7.3 vs. 28.8±9.7, P<0.01; N=5 for both groups, unpaired t-test. (C-E) Time course of increase in afferent discharge in response to AITC in aged and control mice, P<0.05 for 100 μM AITC, P<0.01 for 300 μM and 1 mM AITC, two-way ANOVA.
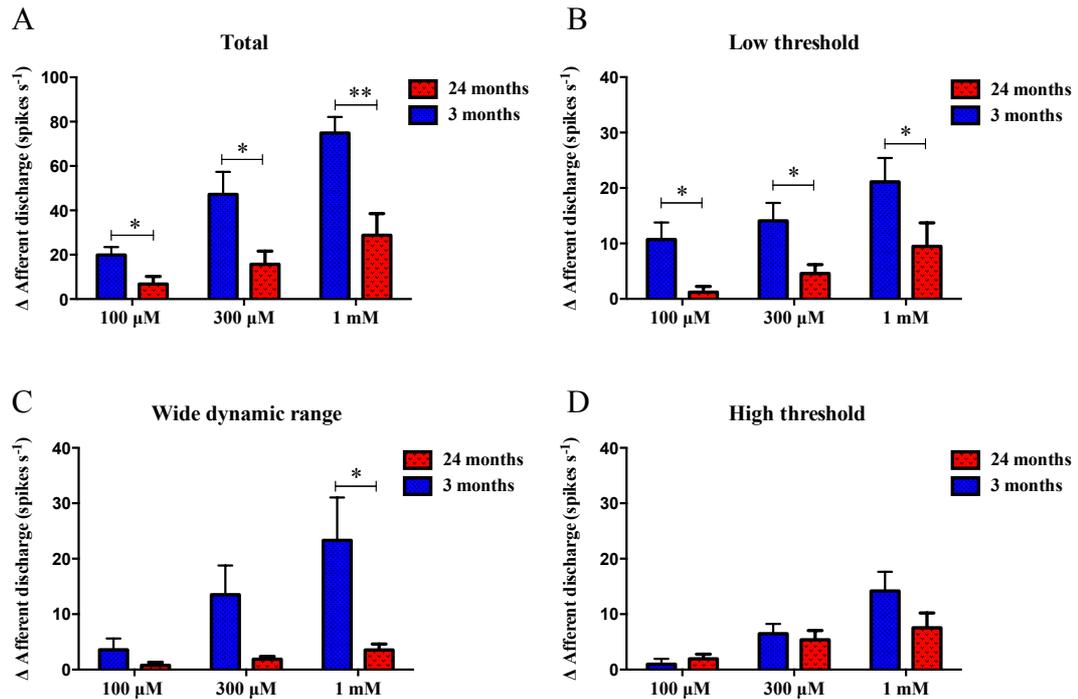
Figure 6.4 Low threshold and wide dynamic range units contributed to the age-related attenuation of afferent responses to AITC. (A) Total afferent responses to 100 μM, 300 μM and 1 mM AITC were attenuated in the 24 months old mice. (B) Responses of low threshold units to 100 μM, 300 μM and 1 mM AITC were all significantly reduced in aged mice, 3 months vs. 24 months, 100 μM AITC: 10.7±3.1 vs. 1.0±0.8, P<0.05; 300 μM: 14.1±3.2 vs. 3.7±1.5, P<0.05; 1 mM: 21.1±4.3 vs. 7.6±3.8, P<0.01; N=5 for both groups, unpaired t-test. P<0.05. (C) Wide dynamic range units displayed a similar pattern, whereas only response to 1 mM AITC achieved significance (23.3±7.7 vs. 3.5±1.1, P<0.05). P= 0.212 and 0.061 for 100 μM (3.6±2.0 vs. 0.8±0.5) and 300 μM (13.5±5.3 vs. 1.9±0.5). (D) No significant reductions were observed in high threshold units. P= 0.477, 0.667 and 0.166 for 100 μM (1.0±1.0 vs. 1.9±0.8), 300 μM (6.5±1.8 vs. 5.4±1.7) and 1 mM AITC (14.2±3.5 vs. 7.6±2.7). N=5 for both groups, unpaired t-test.
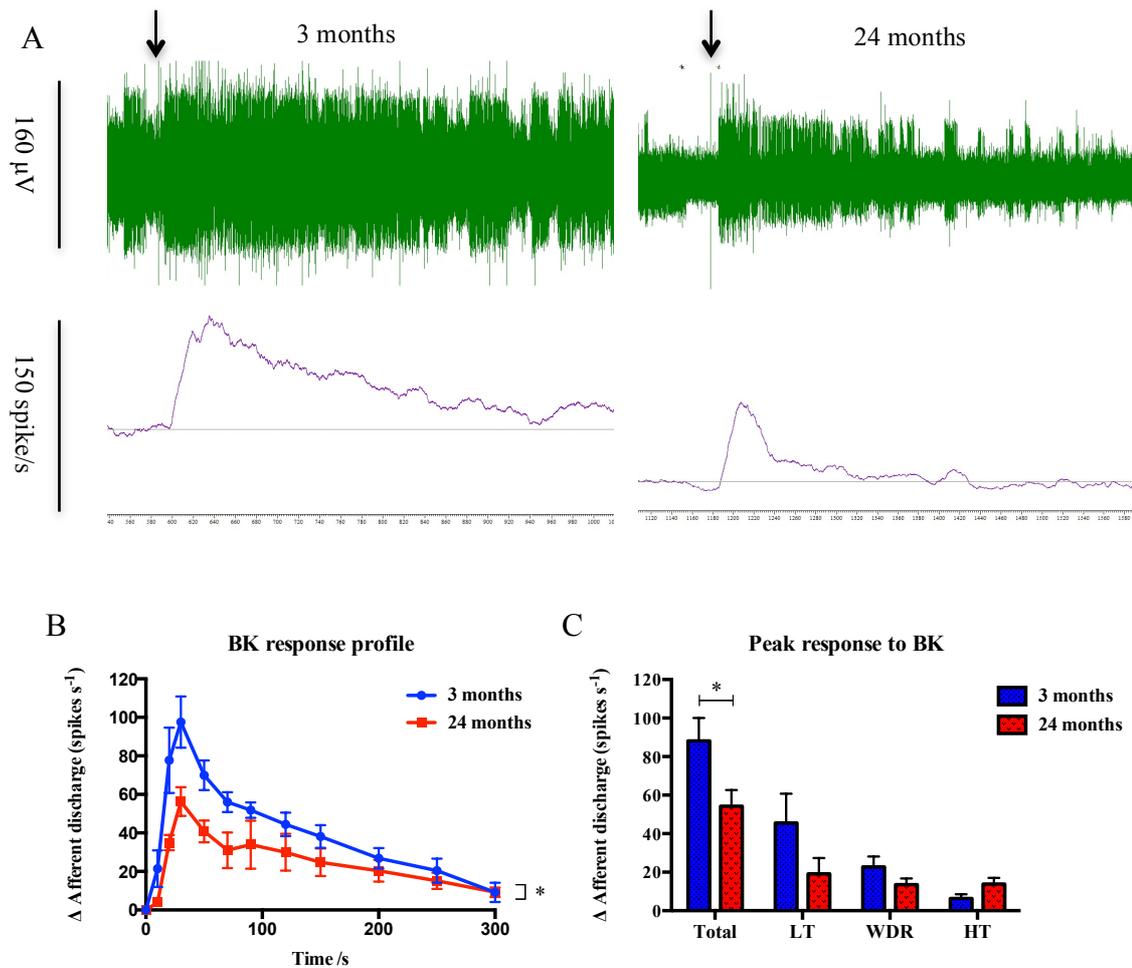
Figure 6.5 Afferent responses to bradykinin were significantly reduced in aged mice. (A) Example traces of afferent responses to bradykinin in 3 and 24 months old mice. (B) Response profile to 1 μM bradykinin in 3 and 24 months old mice. P<0.05, N=5, two-way ANOVA. (C) Peak responses of total units and subpopulations to 1 μM bradykinin. Total response was significantly attenuated in 24 months mice (54.3±8.4), comparing with 3 months group (88.1±11.9), P<0.05. P= 0.167, 0.186 and 0.086 for LT (45.5±15.3 vs.19.1±8.3), WDR (22.7±5.5 vs. 13.5±3.2) and HT (6.3±2.2 vs. 13.9±3.2). N=5 for both groups, unpaired t-test.

6.5 Gene expression of TRPA1 and 5-HT$_3$

One of the hypotheses for the attenuation of TRPA1 signaling in the aged mice is reduced expression in corresponding genes, such as genes that encode TRPA1 and 5-HT$_3$ receptors. Quantitive PCR was performed to compare the gene expression of TRPA1, 5HT$_{3A}$ and 5HT$_{3B}$ in 3 and 24 months old mice.

Relative gene expression ($1/2^{\Delta Ct}$) reflects the percentage of tested gene expression in the housekeeping gene expression (GAPDH in this study). Overall, all examined genes were highly expressed in the DRG, comparing with jejunum (revealed by the difference in scale in figure 6.6A&B), which might be due to that only a small proportion of cell types in the jejunum expressed these genes. Furthermore, TRPA1 expression appeared to be higher than 5HT$_{3A}$ and 5HT$_{3B}$ in both DRG and jejunum segment.

Comparison of the relative gene expression between aged mice and control did not reveal any significant alteration. Fold change of TRPA1 and 5-HT$_{3A}$ were not significant in both DRG and jejunum. 5-HT$_{3B}$ expressions were not changed in the DRG, but decreased in the jejunum with a significant fold change (-2.11, figure 6.6).
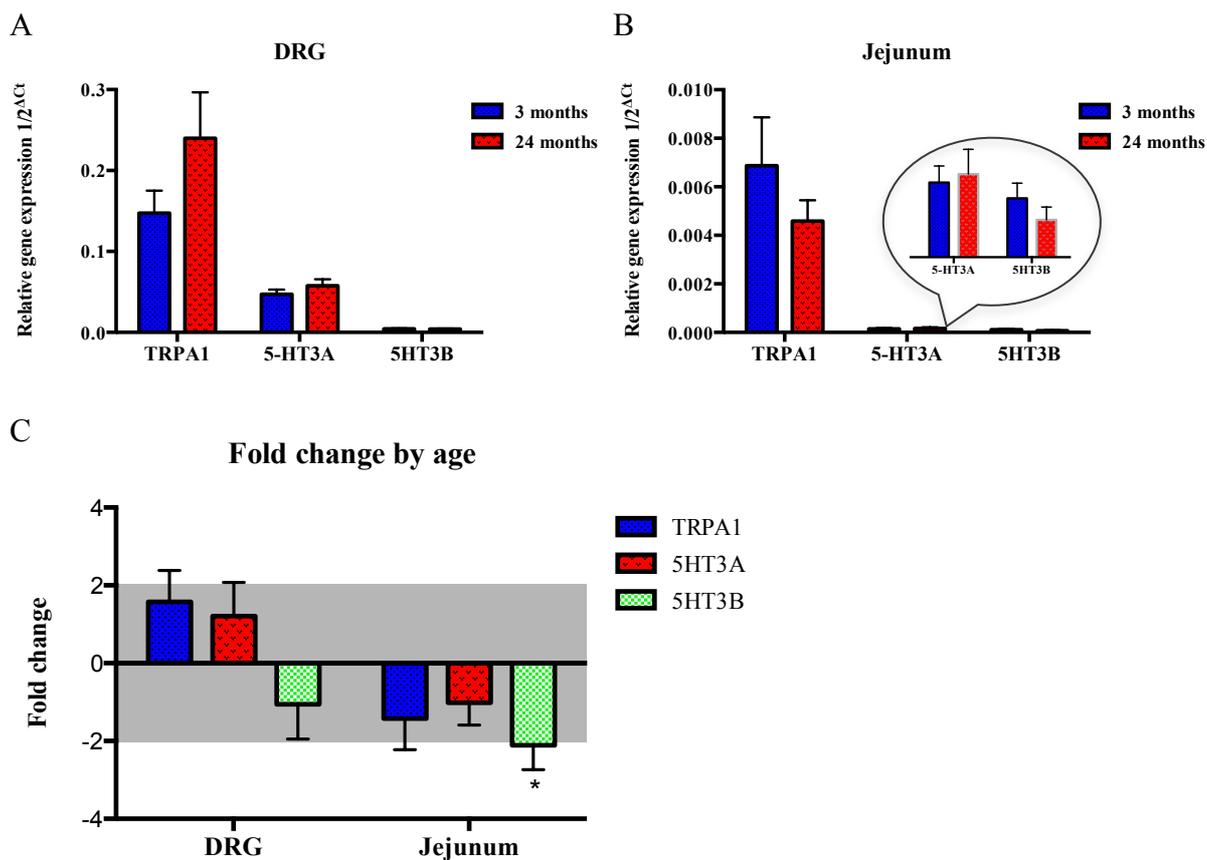
Figure 6.6 Changes in the expression of TRPA1 and 5-HT$_3$ receptor in the aged mouse. Comparison of relative gene expression ($1/2^{\Delta Ct}$) of TRPA1 and 5-HT$_3$ receptors in the DRG (A) and jejunum (B) from aged mice and control. DRG: P=0.182, 0.362 and 0.767 for TRPA1 (N=5), 5-HT$_{3A}$ (N=4) and 5HT$_{3B}$ (N=4); Jejunum: P= 0.245, 0.795 and 0.296 for TRPA1 (N=10), 5-HT$_{3A}$ (N=7) and 5HT$_{3B}$ (N=7), unpaired t-test. (C) Fold changes of gene expression in 24 months mice compared with 3 months group. Fold changes for TRPA1 in DRG (1.58±0.80, N=5) and jejunum (-1.42±0.80, N= 10) were not significant. Expression of 5-HT$_{3A}$ in DRG (1.21±0.87, N= 4) and jejunum (-1.02±0.57, N= 7) were not significantly changed. 5-HT$_{3B}$ expressions was unaltered in DRG (-1.06±0.89, N=4), but significantly down regulated in jejunum (-2.11±0.63, N=7). Gene expression of 5-HT$_3$ was investigated by L. Nocchi and C. Keating.

6.5 Changes of EC cell density in aged mouse intestine

EC cells, labeled by an anti-serotonin antibody, were located in the epithelium of mucosa in mouse jejunum. The conical distribution of serotonin was similar to human EC cells. Densities of EC cells were quantified blindly on jejunal sections of 3 and 24 months old mice. As a result, EC cells were significantly increased in aged mouse jejunum (P<0.01, figure 6.7B).
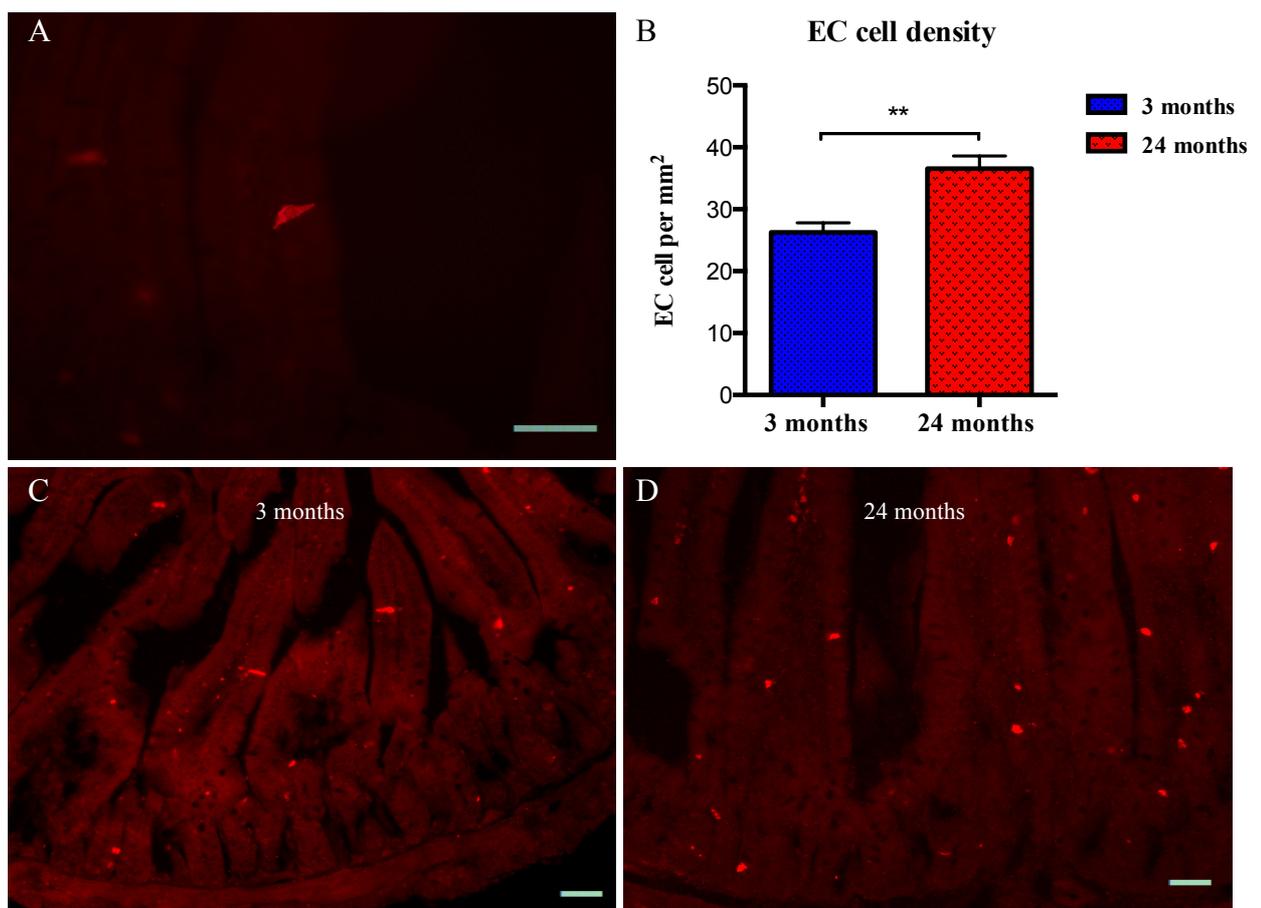
Figure 6.7 EC cell density in the jejunum was augmented in the aged mice. (A) Lateral view of an EC cell in the epithelium of a villus. (B) Density of EC cells were significantly increased in the jejunum of 24 months old mice, 26.3±1.5 vs. 36.5±2.1, P<0.01, N=5 for 3 months, N=7 for 24 months, unpaired t-test. EC cell density is calculated as number of EC cells per mm^2 of mucosa. (C and D) Representative staining of EC cells in the jejunum from 3 and 24 months old mice. Scale bar= 50 μm.

6.7 Effect of granisetron on TRPA1 signaling in aged mouse

It has been previously demonstrated that TRPA1 signaling was attenuated by 5-HT$_3$ receptor antagonist granisetron in the adult mice. Parallel experiments were performed on aged mice. Unlike in adult mice, granisetron failed to block afferent response to AITC significantly (figure 6.8A). The inhibition efficiency of granisetron was significantly decreased in the aged mice (60.0±4.2% vs. 33.4±10.6%, P<0.05). This is consistent with the reduced expression of 5-HT$_3$ receptor in aged jejunum. All three afferent subtypes in aged group appeared to show a lower inhibition by granisetron (figure 6.8B).
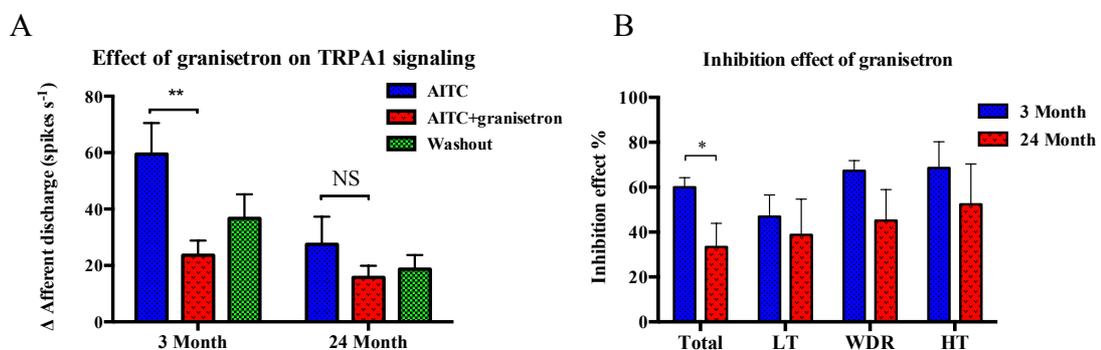
Figure 6.8 Inhibition of TRPA1 signaling by granisetron was lost in the aged mice. (A) Comparison of the effect of granisetron on peak response to AITC in the jejunum from 3 and 24 months old mice. In adult mice, afferent responses to 300 μM AITC were significantly reduced from 59.5±11.0 to 23.6±5.2, P<0.01, N=7, paired t-test. In aged mice, this effect was not significant, 33.0±9.9 vs. 19.0±3.2, P= 0.124, N=5, paired t-test. (B) Inhibition effect of granisetron on TRPA1 signaling of total units and subpopulations. For total afferents, the inhibition of granisetron was significantly decreased in the elderly, 60.0±4.2% vs. 33.4±10.6%, P<0.05; P= 0.664 (46.9±9.7 vs. 38.8±15.9), 0.242 (67.3±4.5 vs. 45.1±13.8) and 0.457 (68.6±11.6 vs. 52.4±17.9) for LT, WDR and HT. N=7 and 5 for 3 and 24 months group, unpaired t-test. Inhibition effect referred to the percentage of afferent discharges blocked by granisetron.

6.8 TRPA1 mediated calcium signal in aged DRG and NG

Afferent recording is capable of evaluating the sensory signaling at an integrated level. To exclude influence of non-neuronal cells and examine TRPA1 signaling of primary sensory neurons directly, calcium imaging on cultured DRG and NG neurons were performed in aged and control mice in parallel.

About 52.1% and 64.6% of DRG neurons from control mice were responsive to 30 and 300 μM AITC, and these proportions were not altered in the aged mice (50.9% and 62.5%). 35.1% of adult DRG neurons responded to 1 μM bradykinin, whereas this probability was only 6.9% in aged DRG (P= 0.08, figure 6.9C). In comparison with DRG, a much smaller proportion of nodose neurons were responsive to AITC (P<0.05). As such, the responsive proportions were quite variable between aged and control nodose neurons.

Relative increases of Ca^{2+} in response to AITC and bradykinin were not significantly altered in the aged DRG and NG (figure 6.9D).

Calcium imaging on DRGs from both aged and control mice was performed at room temperature in parallel. It has been suggested that temperature doesn't significantly affect the response to TRPA1 agonist cinnamaldehyde, based on the comparison between the cinnamaldehyde concentration for half-maximal activation of TRPA1-expressing cells at 23°C and 35°C (Bandell et al., 2004).
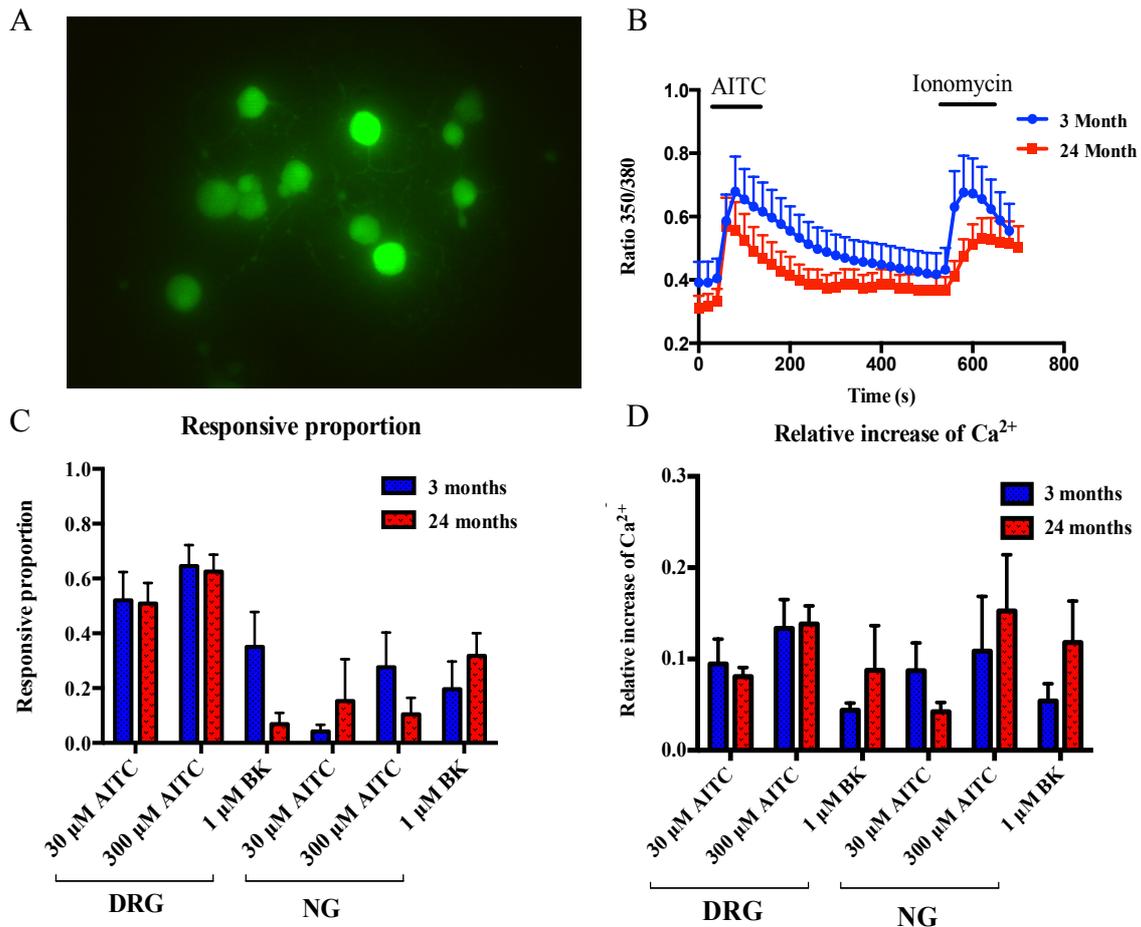
Figure 6.9 Calcium imaging of DRG and NG neurons from aged and adult mice. (A) an representative image to show DRG neurons labeled by fura-2. (B) Example trace to show increase of Ca^{2+} in response to AITC and ionomycin. (C) Responsive proportions to 30 and 300 μM AITC were 52.1% (N=6, n=141) and 64.6% (N=6, n=111) in adult DRGs, and were not dramatically changed in the aged mice (50.9%, N=4, n=108 and 62.5%, N=4, n=245). Responsive proportion to bradykinin was much lower in the aged DRG (6.9% vs. 35.1%, P= 0.08, N=6, n=107 for adult mice, N=4, n=107 for aged group). Responsive proportions to 30 and 300 μM AITC were 4.2% (N=4, n=73) and 27.7% (N=4, n=64) in adult nodose and 15.3% (N=4, n=37) and 10.4% (N=4, n=69) in aged nodose. Bradykinin responders occupied 19.6% (N=4, n=49) in adult nodose and 31.8% (N=4, n=38) in aged nodose neurons. (D) Relative increase of Ca^{2+} (Ratio 340/380) in response to AITC and bradykinin. 3 months vs. 24 months: DRG, 30 μM AITC, 0.095 vs. 0.081, P=0.659; DRG, 300 μM AITC, 0.133 vs. 0.138, P= 0.891; DRG, bradykinin, 0.044 vs. 0.088, P= 0.430; NG, 30 μM AITC, 0.087 vs. 0.042, P= 0.279; NG, 300 μM AITC, 0.109 vs. 0.153, P=0.637; NG, bradykinin, 0.054 vs. 0.118, P= 0.308, unpaired t-test. N refers to the number of animal and n indicates number of cells.

6.9 Discussion

Ageing and mechanosensitivity

In vitro extracellular recording is capable of examining sensory signaling at the level of end organ. With this technique, the current study demonstrated that ageing is associated with attenuated mechanosensitivity in the small intestine of mice, and low threshold afferent components appeared the most susceptible to ageing. Furthermore, the number of functional low threshold afferents detected by recording was reduced in the nerve bundles supplying the small intestine of aged mice.

One straightforward explanation is that some of the primary sensory neurons or their fibers declined with advanced age or lost their function due to possible structural deteriorations or loss of mechanosensitive receptors on their terminals. Some studies have described decreases in the size or number of primary sensory neurons in the nodose ganglia and DRGs (Vega et al., 1993), whereas contrary study did not observe a significant change in nodose ganglion of aged rat (Soltanpour et al., 1996). Another study reported a tiny decrease (12%) in the number of cervical and lumbar DRG neurons in aged rat and a significant cell body atrophy among myelinated primary afferents (Bergman and Ulfhake, 1998). Similarly, a down-regulation of neurofilaments accompanied with reduced cross-sectional area and circularity of myelinated fibers has been observed in aged rat DRG (Parhad et al., 1995). It has also been demonstrated that collateral sprouting of nociceptive axons is impaired by ageing in the rat (Kovacic et al., 2010).

In addition, vagal sensory terminals innervating the myenteric plexus display markedly swollen varicosities in aged rat, and vagal free nerve endings in the villi contract into smaller plates of dystrophic neuritis; conversely, spinal afferents within the gut wall are not severely affected by ageing (Wade and Cowen, 2004). This more severe susceptibility to ageing induced deteriorations in vagal innervation is in agreement with the selective functional loss of low threshold units in aged mouse intestine observed in the current study, as it is widely accepted that low-threshold units might originate from both vagal and spinal afferents, whereas high-threshold units are exclusively of spinal origin.

The impaired mechanosensitivity is also likely due to the neuronal loss occurring in the enteric nervous system. As introduced previously, a large number of studies have described age related loss in enteric neurons. It has been suggested that enteric sensory neurons are particularly susceptible to neurodegeneration with age (Wade, 2002; Wade and Cowen, 2004). Enteric neurons provide sensory output via viscerofugal afferents, which are often recorded in the mixed nerve bundles, and thus contribute to the sensory signaling.

Another possibility is that some primary afferents respond to distention indirectly through the action of some transmitters, and reduction of those transmitters or their receptors might result in impaired sensitivity to distension. For example, stroking on the mucosa of human jejunum results in a significant increase in serotonin release (Kellum et al., 1999), which might directly excite primary sensory neurons through 5-HT receptors on the afferents terminals (Peters et al., 1993; Klein et al., 2011). In addition, serotonin has been shown to induce contraction of guinea pig distal colon via 5-HT$_3$ receptors in the enteric nervous system, which might involve the stimulation of acetylcholine and substance P release (Yamano and Miyata, 1996). This study has revealed down regulation of 5-HT$_3$ receptors, which might contribute to the reduced mechanosensitivity.

Ageing and TRPA1 signaling

Similar to mechanical sensory signaling, TRPA1 signaling is a complicated pathway involving both extrinsic and intrinsic nervous system as well as non-neuronal cells. Mechanosensitivity in TRPA1 knockout mice is significantly impaired (Brierley et al., 2009), suggesting that they share some common pathways. As revealed in chapter 5, TRPA1 signaling comprises direct activation of TRPA1 on extrinsic afferents, indirect activation through 5-HT$_3$ receptors on extrinsic afferents and signaling from enteric neurons following the effects of 5-HT.

As far as we know, this study is the first one to report the influence of ageing on the gene expressions and functions of TRPA1 in mouse GI tract. Based on results from afferent recording, TRPA1 signaling induced by AITC and bradykinin was significantly attenuated at the level of peripheral afferent in aged mouse intestine. Furthermore, this attenuation was more dramatic in low threshold afferents in

comparison with high threshold components, which is consistent with the decrease in mechanosensitivity. As such, one of the explanations for this attenuation is age-related decline in some low threshold primary sensory neurons, especially vagal afferents, considering their susceptibility in structural deterioration caused by ageing (Phillips and Powley, 2007).

One of the hypotheses for the attenuation of TRPA1 signaling with advanced age is reduced expression in corresponding genes, such as genes that encode TRPA1 and 5-HT$_3$ receptors. Quantitive PCR was performed to compare the gene expression of TRPA1, 5HT$_{3A}$ and 5HT$_{3B}$ in 3 and 24 months old mice. Expressions of TRPA1 and 5-HT$_{3A}$ were not significantly changed in both DRG and jejunum. 5-HT$_{3B}$ expressions were not changed in the DRG, but decreased in the jejunum with a significant fold change (-2.11). These data indicate that no significant change was found at the level of primary sensory neurons, whereas 5-HT$_3$ receptors in the cells within the gut wall were declined with advanced age. In agreement with this observation, our unpublished data have demonstrated that peak afferent responses to superfusion of 2-Me-5-HT, a selective 5-HT$_3$ agonist, were significantly reduced in the small intestine of 18 and 24 months old mice. In addition, 5-HT$_3$ receptor antagonist granisetron significantly inhibited TRPA1 signaling in the adult mice, whereas its inhibitory effect was reduced in the aged mice, which was consistent with the decrease in 5-HT$_3$ receptors. Moreover, microarray hybridization study on DRG and NG neurons supplying mouse jejunum has revealed that gene expressions of 5-HT$_{3A}$ and 5-HT$_{3B}$ in nodose are much higher than DRG (Peeters et al., 2006). As such, the possible functional loss of low threshold vagal afferents in aged mice might be associated with reduction in total 5-HT$_3$ receptors, which could also explain the reduced inhibition effect of granisetron.

Interestingly, as the major source of 5-HT in the gut, EC cells were significantly increased in the jejunum of aged mice. It is possible that as 5-HT$_3$ receptors declined with ageing process, EC cells were up-regulated to compensate the impaired signaling pathway mediated by 5-HT$_3$ receptors. This is similar to the observation that serum levels of bradykinin are increased with advanced age, whereas the responsiveness of target cells is reduced (Perez et al., 2005). However, 5-HT release and reuptake in this ageing model remain to be assessed.

In vitro afferent recording facilitates investigation of integrated sensory signaling at the level of a segment of intestine, while calcium imaging on cultured DRG and NG neurons evaluates their sensitivity directly. AITC elicited increase in intracellular Ca^{2+} in about 64.6% of DRG neurons from adult mice, and this proportion was not dramatically altered in the aged mice (62.5%). The magnitude of calcium signaling induced by AITC was not significantly changed in the aged DRG. In comparison with DRG, a much smaller proportion of NG neurons were responsive to AITC, and thus the responsive proportions were quite variable. However, the magnitude of calcium response in aged NG was also preserved. These data suggested that TRPA1 expressing primary sensory neurons were not prominently influenced by ageing, which was in line with the unaltered TRPA1 gene expression in aged DRG.

The proportion of DRG neurons responding to bradykinin appeared to be decreased in aged animals. Whether bradykinin receptors on these neurons were reduced with ageing require further investigation. Nevertheless, bradykinin induced calcium increases were not significantly changed by ageing. Conversely, a study has reported that calcium increase in response to bradykinin is significantly attenuated in senescent fibroblast cells (Muramoto et al., 2011), which may be not directly comparable with current study.

To sum up, this chapter revealed a significant attenuation in the mechanosensitivity and TRPA1 signaling in aged mouse intestine, and low threshold afferents were most susceptible to ageing. Functional loss of some low threshold afferents in aged mice contributed to this attenuation. TRPA1 expressions in primary sensory neurons and jejunum segments were not significantly changed with ageing, which was in agreement with unaltered calcium signaling induced by TRPA1 agonist on aged DRG and NG neurons. Although EC cells were found increased in aged jejunum, its compensatory effect might be masked by the reduction of 5-HT$_3$ receptors within the gut wall.

CHAPTER 7

Discussion

This thesis addressed the sensory functions of the GI tract with an emphasis on the interaction between afferents nerves and other cell types such as EC cells and mast cells, and also investigated the influence of ageing on these interactions.

Understanding the mechanisms underlying diseases is the ultimate objective of most research in life science, including the current study. As one of the most common GI disorders, the pathogenesis of IBS hasn't been fully understood. IBS is a functional bowel disorder characterized by chronic abdominal pain, discomfort, bloating and alteration of bowel habits. Prevalence rates of IBS usually range from 5% to 20%, but vary between countries and depend on the diagnostic criteria used (Spiller et al., 2007). This rate is about 10.5% in the UK; moreover, IBS accounts for 20~50% of referrals to gastroenterology clinics and thus is a substantial burden on primary healthcare services (Wilson et al., 2004). One of the theories accounting for the pathogenesis of IBS is visceral hypersensitivity based on the observation that IBS patients have a lower pain threshold to colorectal distension or electrical stimuli (Whitehead et al., 1990; Sinhamahapatra et al., 2001; Verne et al., 2001; Dong et al., 2004).

Increasing evidence has suggested an important role of mast cells and EC cells in IBS hypersensitivity. Despite of many discrepant reports, increased mast cells and EC cells are often associated with IBS (Barbara et al., 2004b; Lee et al., 2008; Cremon et al., 2011; Matricon et al., 2012). More importantly, the severity of abdominal pain in IBS patients has been found positively correlated with serotonin release (Cremon et al., 2011) and anatomical mast cell-nerve association (Barbara et al., 2004b). Moreover, TNBS induced visceral hypersensitivity is abolished in mast cell deficient rats (Ohashi et al., 2008). Treatment with mast cell stabilizer, disodium cromoglycate, improves the symptoms in a large proportion of IBS-D patients (Leri et al., 1997). $5-HT_3$ receptor antagonists are effective in relieving symptoms in patients with diarrhoea-predominant IBS (Camilleri et al., 2001), whereas $5-HT_4$ agonists improve symptoms in constipated IBS patients due to prokinetic effects (Novick et al., 2002). In addition, mucosal supernatants from IBS patients significantly increased afferent firing recorded from rat jejunal nerves (Barbara et al., 2007), which could be blunted by $5-HT_3$ receptor antagonist granisetron (Cremon et al., 2011).

A large number of studies have provided evidence on the interaction between nerves and mast cells, including close anatomical association and functional crosstalk. However, what mediators from mast cells are involved in hypersensitivity and especially what subtypes of afferent nerves are depolarized or sensitized by those mediators require further investigation. Some members of TRP channels are possible candidates due to their role in nociception. Increasing evidence has revealed the important role of TRPA1 in visceral inflammation and pain (Lapointe and Altier, 2011; Bautista et al., 2012).

Activation of TRPA1 elicits acute nociceptive behavior in mice, followed by neurogenic inflammation and marked hypersensitivity to thermal and mechanical stimuli (Bandell et al., 2004; Bautista et al., 2006). Pharmacological blockage or genetic knockout of TRPA1 significantly attenuates hypersensitivity to mechanical and thermal stimuli (Bautista et al., 2006; Kwan et al., 2006). Therefore, TRPA1 might be the key molecule responsible for the pain and discomfort in IBS patients.

TRPA1 mediated sensory signaling in mouse small intestine

Although TRPA1 signaling might be a possible determinant of abdominal pain, it hasn't been thoroughly investigated at the level of peripheral afferent nerves. This study employed in vitro afferent recording to assess peripheral TRPA1 signaling induced by TRPA1 agonist and the potential involvement of EC cells and mast cells.

TRPA1 agonist AITC elicited increase in afferent discharge in a dose dependent manner. AITC induced afferent response was significantly attenuated by 5-HT$_3$ receptor antagonist granisetron, which provided a functional link between serotonin and TRPA1 mediated sensory signaling. Unpublished data from our group have demonstrated that AITC induced increased serotonin release from mouse jejunum preparations and BON cells (human EC cell model), which was in agreement with a study on enriched rat EC cells and rat endocrine cell line RIN14B (Nozawa et al., 2009). The above study also demonstrated that TRPA1 was highly expressed in EC cells. Although rodent mast cells also contain 5-HT, in this context, serotonin released from mast cells might be marginal, based on our observation that serotonin immunoreactivities were not detectable in mast cells. Combination of these data indicated that EC cells amplify TRPA1 signaling by 5-HT release.

Afferent response to AITC was also inhibited by cyomolyn, a mast cell stabilizer, suggesting that mast cells might play a similar role in the amplification of TRPA1 signaling. Activation of mast cells by antigen or perfuse of PAR2-AP both can induce mechanical hypersensitivity of vagal afferents to esophageal distension, which is inhibited by TRPA1 antagonist (Yu et al., 2009). In addition to protease, pre-evacuation of mast cell mediators or histamine receptor H1 antagonist pyrilamine significantly inhibit AITC induced nociception in mice (Andrade et al., 2008). These observations suggest that protease or histamine released from mast cells might sensitize TRPA1 expressing afferent nerves.

Current study demonstrated that AITC induced increased motility in mouse jejunum through $5-HT_3$ receptors. Since activation of TRPA1 on EC cells increased 5-HT release, this is in agreement with a recent study on TPH1 deficient mice that exhibit a much slower propulsion as well as reduced responses to intraluminal balloon distension (Heredia et al., 2013). Nevertheless, it has been shown that activation of TRPA1 inhibits contractility in mouse colon, which might be attributed to co-expression of TRPA1 with inhibitory motor neurons (Poole et al., 2011), These observations suggest that the modulation effects of TRPA1 signaling on GI motility could be diverse.

Combination of current study and recent publications can reveal the complicated pathways of TRPA1 mediated signaling recorded from primary afferent nerves innervating a segment of jejunum. As illustrated in figure 5.11, TRPA1 signaling induced by AITC comprises direct activation of TRPA1 on extrinsic afferents, indirect activation of $5-HT_3$ receptors on extrinsic afferents following 5-HT release from EC cells and indirect signals evoked by contractions, which are either induced by action of 5-HT on enteric neurons or by activation of TRPA1 expressed on enteric neurons. In addition, communications between mast cells and afferents might result in depolarization or sensitization of afferent nerves. Since TRPA1 mediated sensory signaling is such a complicated network including the amplification and modulation by mast cells and EC cells, abnormal or imbalanced interactions might result in hypersensitivity, a hall marker for IBS. As such, TRPA1 is a promising pharmacological target for IBS or other bowel disorders. A recent study has

suggested that ASP7663, a novel TRPA1 agonist, can improve the symptoms in animal model of constipation (Kojima et al., 2013).

Influence of ageing on sensory functions in the GI tract

This study identified some age-related changes involving the sensory functions of the GI tract in both human and mouse models. In humans, EC cells and mast cells appeared to be increased with advanced age, whereas sensory afferent nerves labeled by SP were decreased. In addition, the close anatomical association between afferents and mast cells were enhanced in the elderly. RT-PCR did not reveal any change with ageing in the expression of TRPA1 and TPH1 in the mucosa of human bowel.

Interestingly, ageing and IBS share some common alterations, for example, IBS is often associated with increased EC cells, mast cells and enhanced mast cell- nerve association. However, one striking difference is that SP has been shown increased in IBS bowel (Mazumdar and Das, 1992; Dong et al., 2004; Sohn et al., 2014b), whereas we observed reduced SP positive sensory afferents with ageing. This might explain that IBS is less prevalent in aged population and the severity of pain is decreased (Kay, 1994; Wilson et al., 2004; Tang et al., 2012). As previously discussed, peptidergic TRPA1 afferents might be responsible for the abdominal pain in IBS. Therefore, the less prevalence of IBS in the elderly might be attributed to attenuated TRPA1 signaling.

Hence, this study examined peripheral TRPA1 signaling in aged mouse intestine. As a result, AITC or bradykinin-induced TRPA1 signaling was significantly attenuated in aged mouse. After discrimination of individual afferent units by spike sorting, this attenuation was found more dramatic on low threshold units, which was consistent with the decrease in mechanosensitivity with ageing. Reduction in the number of functional distension-sensitive low threshold units detected by recording might account for this attenuation. Gene expressions of TRPA1 and 5-HT_{3A} were not changed in both DRG and jejunum. Expressions of 5-HT_{3B} subunits were not altered in aged DRG, but decreased in aged jejunum. In agreement with this observation, our unpublished data have demonstrated that peak afferent responses to superfusion of 2-Me-5-HT, a selective 5-HT_3 agonist, were significantly reduced in the small intestine

of 18 and 24 months old mice. In addition, 5-HT$_3$ receptor antagonist granisetron significantly inhibited TRPA1 signaling in the adult mice, whereas its inhibitory effect was reduced in the aged mice, which was consistent with the decrease in 5-HT$_3$ receptors. Interestingly, as the major source of 5-HT in the gut, EC cells were significantly increased in the jejunum of aged mice. It is possible that as 5-HT$_3$ receptors declined with ageing process, EC cells were up-regulated to compensate the impaired signaling pathway mediated by 5-HT$_3$ receptors. *In vitro* afferent recording facilitates investigation of integrated sensory signaling at the level of a segment of intestine, while calcium imaging on cultured DRG and NG neurons evaluates their sensitivity directly. As a result, sensitivity of primary neurons to TRPA1 agonist was not significantly altered by ageing.

If the attenuation of TRPA1 signaling in aged mice could be translated to humans, the less prevalence of IBS in the elderly might be attributed to impaired TRPA1 signaling. This could be a key direction for future studies.

Efforts were made to investigate potential age-related changes in the density of mouse intestinal mast cells by immunohistochemistry. Antibodies against mouse mast cell tryptase and CD34 occasionally revealed sporadic mast cells in the submucosa of intestine from both adult and aged mouse. The density of mast cells was too small to make a systematic quantification. A number of studies have shown that mast cells are dramatically increased after helminth-infection, while their data indicate that the density of mast cells in noninfected mice is fairly small (Friend et al., 1996; Scudamore et al., 1997; Friend et al., 1998; De Jonge et al., 2002). In comparison with larger mammals, mice have fewer resident mast cells, as suggested by the observation that human and rat have substantial mast cells in the lung parenchyma, whereas mast cells in the mouse are located predominantly adjacent to the major airways (Miller and Pemberton, 2002). In addition, mouse mast cells comprise a variety of phonotypes: chymase (mMCP-1, -2 and -4), elastase (mMCP-5) and tryptase (mMCP-6 and -7). Certain markers can only detect a subpopulation of mast cells, which further decreases the chance to visualize mast cells.

This study addressed the influence of "normal" ageing on the sensory function of the GI tract. However, some alterations in bowel functions in the elderly are likely to be

consequences of accumulative medical conditions or GI side effects of medications used to treat those conditions. These limitations require strict sampling strategy and cautious interpretation.

Another limitation is that a senescence marker was not used in age-related gene expression study. Some quantitative indicators of senescence have been proposed, such as senescence-associated β-Galactosidase (Sen-β-Gal) and Ki67 (Lawless et al., 2010). These markers would be a control to evaluate the degree of senescence in aged human or mouse specimens. However, it is also suggested that no marker or hallmark of senescence identified so far is entirely specific to the senescent state (Rodier and Campisi, 2011).

In vitro afferent recording on human bowel

Most of our knowledge about sensory functions of the GI tract was obtained form animal studies. However, some of the knowledge translates poorly to humans, which highlight the demand for more thorough research on human directly. In vitro afferent recording on human bowel is nearly blank in the literature; until recently, our group and another group reported independent preliminary data on afferent recording from isolated human bowel (Jiang et al., 2011; Peiris et al., 2011). This study further improved this *in vitro* recording model and increased success rate of recording.

Current study examined mechanosensitivity and chemosensitivity on afferent nerves innervating human bowel. Based on the sensitivity to probing, stretch and mucosal stroking, 15 mechanosensitive units can be classified into five subtypes: serosal (46%), muscular (27%), mesenteric (13%), mucosal (7%) and muscular/ mucosal (7%) afferent. Afferent discharge induced by capsaicin has been shown in the two preliminary reports. In addition to capsaicin, this current study is the first one to provide direct evidence that afferent discharges could be elicited by bradykinin, AITC and 5-HT in isolated human ileum and colon.

Based on limited data, afferent discharges in response to bradykinin in human sigmoid colon appeared to be blunted in the elderly, which is consistent with our observation on mouse. This indicates an attenuated sensitivity to inflammatory

events in the elderly. In addition, as our study on mouse jejunal afferent suggested that bradykinin induced afferent response is mediated via TR`PA1, this might imply a possible attenuation of TRPA1 signaling in aged human bowel.

Lots of efforts have been made to improve the success rate for human afferent recording. Current study managed to record afferent nerve activities from 45% of human bowel specimens, which is higher than a 15% success rate in our preliminary report (Jiang et al., 2011) and similar to 48% of the other group (Peiris et al., 2011). All these studies are greatly limited by the low success rate, reflecting the inherent difficulties of extracellular recording on isolated human bowel. Despite technical challenges, this *in vitro* afferent recording model at the level of peripheral afferent has enormous advantages, such as avoiding translational failures, testing compounds for clinical use and better understanding of the mechanisms underlying human bowel disorders.

The use of *in vitro* afferent recording to investigate sensory signaling

Extracellular recording on *in vitro* preparations were used throughout this thesis. This technique is extremely robust, efficient and reliable. Peripheral sensitivity to mechanical and chemical stimuli could be examined straightforward and immediately. Pharmacological effects of agonist or antagonist on afferent discharge could be easily assessed.

Many investigators have used *in vivo* models as they are assumed to closely resemble living tissue. However, there are a number of aspects making it difficult to interpret *in vivo* data. *In vitro* afferent recording employed in this study facilitates investigation of integrated sensory signaling at the level of a segment of intestine. Its prominent advantage over cellular and molecular studies is the inclusion of secondary effects of various cell types, and it thus better reflects the physiological process in organisms. Nevertheless, the advantage is disadvantage at the same time, because the summation of multiple mechanisms increases the difficulties in interpretation. As such, in addition to afferent recording, this thesis also employed histochemistry, immunohistochemistry, RT-PCR and calcium imaging. Combination of these techniques allows investigation of sensory signaling from different aspects and thus deconstruction of complicated mechanisms.

Future studies

Similar to all courses of scientific research, there are still a number of undetermined issues arising from current study for future investigations.

Whether attenuation of TRPA1 signaling in aged mice can be translated to humans remains to be investigated. Preliminary data on human bowel recording have shown a promising attenuation with ageing in bradykinin-induced afferent signaling. Gene expression of TRPA1 has been examined on mucosa of human bowel, while expression of 5-HT$_3$ receptor would be an interesting target to test. This study quantified the changes in the density of EC cells with advanced age on both human and mouse specimens, whereas the influence of ageing on 5-HT release and reuptake remains to be assessed.

Current study identified a functional loss of some low threshold afferents innervating mouse jejunum, which were possibly vagal afferents originated from nodose neurons, considering the susceptibility of vagal nerves in structural deterioration caused by ageing (Phillips and Powley, 2007). This hypothesis can be further tested by quantification of the low threshold units on vagotomized aged mice. In addition, gene expressions for TRPA1 and 5-HT receptor on aged nodose remain to be examined.

Since the specificity of cromolyn as a mast cell stabilizer is controversial, the involvements of mast cells in the TRPA1 signaling need further validation. As mast cells can release so many types of mediators and each mediator may act on a few subtypes of receptors, a comprehensive antagonist test would be difficult. Study on mast cell deficient mouse is an alternative way to examine the role of mast cells in TRPA1 involved signaling.

This study also focused on 5-HT$_3$ receptor, as it is highly expressed in primary sensory neurons innervating the GI tract. However, there are totally 7 subfamilies in 5-HT receptors, in addition to 5-HT$_3$, at least 5-HT$_2$, 5-HT$_4$ and 5-HT$_7$ receptors are present in the GI tract. Since activation of TRPA1 on EC cells results in serotonin

release, which can act on corresponding 5-HT receptors. Whether those 5-HT receptors beside 5-HT$_3$ are involved in TRPA1 signaling remains to be clarified.

It is controversial if activation of TRPA1 induces mechanical hypersensitivity. In mouse model of *in vitro* recording, afferent response to ramp distension before and after AITC or bradykinin treatment were reproducible. However, based on limited data from human recording, AITC and bradykinin of the same concentration appeared to induce mechanosensitivity to probing. It requires further studies to explain this discrepancy.

Many independent lines of evidence have supported the essential role of TRPA1 in visceral hypersensitivity and pathogenesis of IBS. It thus becomes a very promising target for the treatment of IBS. Examination of the pharmacological effects of TRPA1 agonists or antagonists on IBS symptoms or other bowel disorders would be very useful.

Investigation of these issues would achieve a better understanding of the sensory functions of the GI tract and pathogenesis of GI-related disorders and ultimately promote development of specific and effective treatments.

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