Effect of oxidative stress on afferent nerve activity from small intestine and colon in young and aged mouse

PhD thesis

By

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Summary

This thesis addressed the sensory functions of the gastrointestinal (GI) tract with a focus on the effect of oxidative stress on afferent nerve activity from small intestine and colon in young and aged mouse.

Oxidative stress appears to be involved in the pathogenesis of many gastrointestinal conditions, such as inflammatory bowel disease (IBD), colon cancer, and may contribute to the gut dysfunction in ageing. How diverse regions of the gut react to, and handle, elevated levels of ROS in young and aged is unclear. Here, I investigated the effect of oxidative stress on afferent nerve activity in young and aged mice, and if it contributes to age associated changes.

The study used in vitro afferent nerve recordings from jejunum and colon of male mice and concluded that colonic sensory neurons are more sensitive to oxidative stress than jejunum sensory neurons. In the aged group, decreased afferent mechanosensitivity associated with a greater oxidative status was observed only in the aged colonic mucosa. Findings obtained by RNA microarray analysis suggested that the difference between the mouse jejunum and colon in ROS production genes and secondary antioxidant genes may have contributed to the colonic afferent being more sensitive to oxidative stress. In addition, upregulation of inflammatory related genes associated with long-life exposure to high endogenous ROS level are possible factors for colon being more inclined to develop diseases or decreased function as a result of normal ageing.

Abbreviations

5-HT	5-hydroxytryptamine	**IGLEs**	Intraganglionic laminar endings
8-OHdG	8-hydroxy-2'-deoxyguanosine	**IMAs**	Intramuscular arrays
ATP	Adenosine triphosphate	**IPANs**	Intrinsic primary afferents
AITC	Allyl isothiocyanate	**IBS**	Irritable bowel syndrome
α-syn	Alpha-synuclein	**LT**	Low-threshold
IFANs	Intestinofugal afferent neurons	**Nav**	Na^+ channels
BK	Bradykinin	**NGF**	Nerve growth factor
BDNF	Brain-derived neurotrophic factor	**NKA**	Neurokinin A
CGRP	Calcitonin-related peptide	**NADPH**	Nicotinamide adenine dinucleotide phosphate
CAT	Catalase	**NO**	Nitric oxide
CHO	Chinese hamster ovary	**NOS**	Nitric oxide synthase
CCK	Cholecystokinin	**NG**	Nodose ganglia
ChAT	Choline acetyltransferase	**NANC**	Non-adrenergic, non-cholinergic
Caco	Colorectal adenocarcinoma cells	**NTS**	Nucleus tractus solitarius
CD	Crohn's disease	**PMCs**	Peristaltic motor complexes
DHE	Dihydroethidium	**Prx**	Peroxiredoxin
DNBS	Dinitrobenzene sulphonic acid	**ONOO⁻**	Peroxynitrite anion
DRG	Dorsal root ganglia	**PACAP**	Pituitary adenylyl cyclase activating peptide
EEC	Enterendocrine cells	**PVG**	Prevertebral ganglion
ENS	Enteric nervous system	**IPANs**	Primary afferent neurons
ECC	Enterochromaffin cell (ECC)	**PGs**	Prostaglandins
Fe2+	Ferrous	**P2X**	Purinergic receptor2 X
GABA	Gamma-amino butyric acid	**P2Y**	Purinergic receptor2 Y
GI	Gastrointestinal	**RNS**	Reactive nitrogen species
Grx	Glutaredoxin	**ROS**	Reactive oxygen species
GPO	Glutathione peroxidase	**RSN**	Renal sympathetic nerve
HT	High-threshold	**SC**	Spinal cord
HEK	Human embryonic kidney cells	**SP**	Substance-P
H2O2	Hydrogen peroxide	**SMG**	Superior mesenteric ganglion
OH●	Hydroxyl radicals	**O₂⁻**	Superoxide anion
HOCl	Hypochlorous acid	**SOD**	Superoxide dismutase
HIFs	Hypoxia-inducible factors	**Trx**	Thioredoxin
IMG	Inferior mesenteric ganglion	**TRP**	Transient receptor potential channels

IBD	Inflammatory bowel disease	TNSB	Trinitrobenzenesulfonic acid
INFγ	Interferon-γ	TNFα	Tumour necrosis factor
IL-1	Interleukin 1	UC	Ulcerative colitis
ICC	Interstitial cell of Cajal	VIP	Vasoactive intestinal peptide
		WDT	Wild dynamic threshold

Chapter 1

General introduction

Introduction

The gastrointestinal (GI) tract functions are controlled by distinct populations of neurons with cell bodies resident within or outside of the gut wall. The neuronal control is coordinated by intrinsic and extrinsic pathways. The intrinsic element consists of the enteric nervous system with neurons that are contained within the gut wall, providing internal reflexes in addition to neurons that pass through to the prevertebral sympathetic ganglia and provide extraspinal reflexes. The extrinsic pathways provide reflexes that are transmitted to and from the central nervous system (CNS) and involve efferent nerves (sympathetic and parasympathetic) and afferent, sensory nerves. These neurons and their nerve endings locate in a challenging environment where they are exposed to the risks of mechanical distortion during digestion and contraction in addition to complex signalling pathways from internal elements such as nutrients, gut microflora and immune system.

1.1 A brief overview of the structure and functions of the human gastrointestinal (GI) tract

The GI tract is a long muscular tube that extends through the body and is associated with other organs including the liver, pancreas and gall bladder. The GI tract is divided into upper and lower GI and the main subdivisions are the oesophagus, stomach, and the small and large intestine. The sphincters control the passage of gut contents between the main areas of the GI tract. However, functionally specialized regions exist within subdivisions,

and the small intestine is divided into: 1) duodenum where pancreatic digestive enzymes and bile acids from the liver are secreted. 2) jejunum where glucose, amino acids, fatty acids, vitamins, and electrolytes are absorbed across the epithelium and subsequently into the bloodstream. 3) ileum which is responsible of absorb the remaining nutrients, vitamins and reuptake most of the bile acids. The large intestine is also divided into 3 parts: ascending, transverse colon are responsible for absorbing the excess water and descending colon where the stools are solidified and stored as faeces in the sigmoid colon.

Basic structure of the gut wall

Although each section of the GI tract has a specific function, a similar basic structure is shared by the entire tract with some regional differences. The gut wall is divided into 4 major layers: mucosa, submucosa, muscularis externa and serosa (Figure1).

A. The mucosa is the innermost layer of the GI tract and consists of epithelium, supported by an underlying layer of connective tissue called the lamina propria. Epithelium is a layer of single cells coating the surface of mucosa which contributes to the function of secretion and absorption. It consists of distinct types of cells including enterocytes, enteroendocrine cells, goblet cells, Paneth cells, and columnar cells. As they are in constant exposure to luminal contents, they are sealed together by a tight junction to form a barrier against pathogens, ingested materials and bacteria in the lumen. The submucosa is the underlying fibrous connective tissue that contains blood vessels, nerve plexus, glands and is rich in immune cells (such as mast cells and macrophages).

B. The muscularis externa has inner circular and outer longitudinal layers of smooth muscle fibres and the myenteric plexus is located in-between the two layers. The

motor function of these muscles - called peristalsis - is control by the enteric nervous system.

C. The outer layer of the GI tract is formed by a thin serosal membrane consisting of fat and a thin layer of epithelial cells. The serosa is part of the visceral peritoneum contains the mesentery (in small intestine) and mesocolon (in colon) to attach the gut to the rear of the abdominal wall.
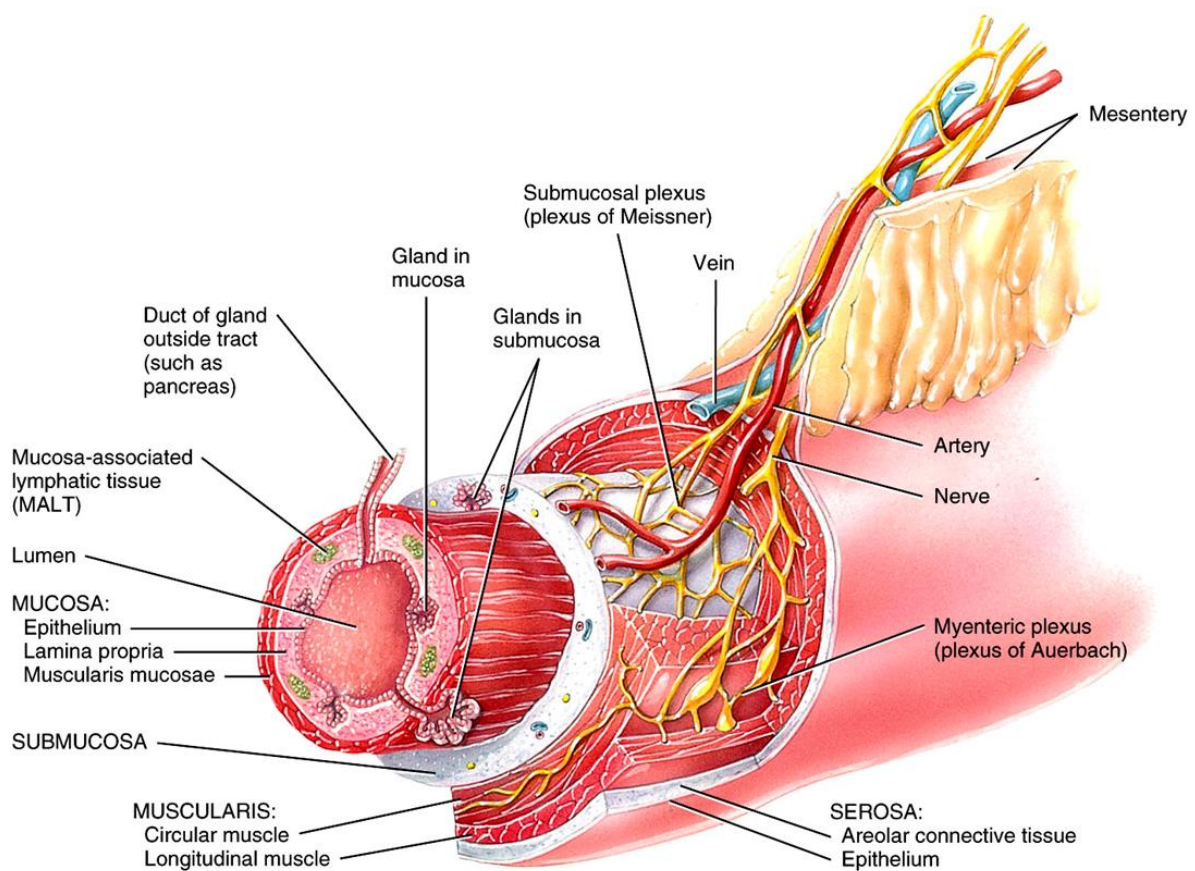
Figure 1 Diagram shows different layers of the gut wall

(Tortora and Derrickson, 2009)

Specialised structures of the intestine

The main function of the small intestine is nutrient absorption, which requires a large area to provide an optimal exposure to the nutrients in the lumen. Therefore, a series of large folds known as plicae are formed within the submucosa layer in addition to massive numbers of finger-like projections (villi) that extend to the lumen to further maximize the absorptive area. The villi are lined with a single layer of enterocytes consisting of columnar cells with microvilli at their apical surface. The microvilli are coated by a brush boarder membrane (a glycoprotein rich coat called glycocalyx containing several enzymes).

The crypt of the villi contains stem cells that replicate and differentiate into various epithelial cells. Below the intestinal stem cells are Paneth cells, responsible for protection via secretion of definsins enzymes. Scattered between enterocytes are enteroendocrine and goblet cells; they are responsible for important hormones/transmitters and mucus secretions respectively. The epithelial cells lining the villi undergo a rapid turnover (3-4 days) where cells at the top are shed and new cells move up from the crypt.

In the colon, the mucosa mainly consists of absorptive and goblet cells. There are no circular folds or villi, but microvilli are present.

1.2 Sensory innervation of the GI tract

Along with digested food, the GI lumen is exposed to a variety of toxic, pathogenic substances and ingested material. The digested food activates a cascade of physiochemical stimuli in the lumen that determine the course of digestion and its precise control. Therefore, the GI needs a well-organised nutrients absorption in balance with effective protection which is provided by a rich sensory network. Primary sensory neurons, immune cells and endocrine cells are together synchronised to form the sensory system. The primary sensory neurons can be divided into 3 types:

A. Intrinsic primary afferents (IPANs) are restricted within the gut wall where their cells bodies in the myenteric and submucosal plexus and their axons reach the mucosa. They are important mechanosensors for local intestinal reflexes.

B. Viscerofugal (also known as intestinofugal afferent neurons, IFANs) are a unique subset of enteric neurons which function as length-sensitive mechanosensors. Their cell bodies lie within the gut wall while their processes extend to the prevertebral ganglion (PVG) and modulate the motor activity in the intestine.

C. Extrinsic primary afferents which represent an essential connection to pass signals of the GI status to the CNS, allowing the CNS to modulate GI functions according to the needs of digestion (known as gut-brain axis).

1.2.1 Enteric nervous system (ENS)

The intrinsic innervation of the GI tract involves the enteric nervous system (ENS), which consists of enteric neurons organised in two interconnected networks of enteric ganglia: the myenteric and submucosal plexus.

The myenteric plexus contains most of the enteric neurons involved in motor functions of the GI with some intestinal primary afferent neurons (IPANs) located in the submucosa plexus. Depending on their function, individual enteric neurons can be divided into: 1- primary afferent neurons, activated by chemical and mechanical stimuli, 2- interneurons and 3- motorneurons that influence different GI functions including smooth muscle contraction, hormone secretion, blood flow and immune responses.

Excitatory circular muscle motor neurones use choline acetyltransferase (ChAT) and tachykinins while inhibitory circular muscle motor neurones use nitric oxide synthase (NOS), vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase activating peptide (PACAP) to elicit smooth muscle relaxation. Ascending interneurones contain ChAT and tachykinins. They transmit to each other via acetylcholine, but to excitatory motor neurones via both acetylcholine and tachykinins (Bornstein et al., 2004).

1.2.2 Viscerofugal neurons

Viscerofugal neurons receive direct input or indirect (from IPANs), and project to the PVG which is a cluster of three ganglions: inferior mesenteric ganglion (IMG), superior mesenteric ganglion (SMG) and the coeliac ganglion. It is the site in which the preganglionic sympathetic efferent fibres synapse with the cell bodies of postganglionic sympathetic fibres. Also, where spinal afferents synapse and offer short reflex arcs. The

reflex is inhibitory and can be initiated in the distal part of the GI by sensing chemicals (nutrients products) or mechanical stimulus, and results in reduced intestinal transit and secretion to the same GI area or more proximal ones. Most of the viscerofugal projecting to the PVG come from the colon and rectum and respond to circular muscle stretch but not tension. The reflex arc is functionally very important because the smooth muscles of the colon wall tend to depolarise and contracts as a result of filling with luminal contents, but this reflex helps to keep the wall relaxed during this period. This controls the motor activity of the intestine and helps to maintain a suitable amount of luminal materials in the proximal regions of the GI tract, and hence arrive "on time" to the colon (Furness, 2003). Extracellular recordings from colonic nerves showed that all viscerofugal neurons are directly length-sensitive mechanosensitive (Hibberd et al., 2012).

1.2.3. Extrinsic sensory afferents

Signals about the bowel events is conveyed to the CNS via vagal and spinal afferents, which allows the CNS to provide the efferent limb of sympathetic and parasympathetic reflexes to control feeding behaviour and mediate sensations including pain and discomfort (Beyak et al., 2006). Under physiological conditions, pain can be caused by chemical irritation, strong contraction and sudden distension while these stimuli provoke a hypersensitive response in pathological conditions.

The visceral afferents innervating the GI tract originate from two sources vagal and spinal nerves. They run in mixed population bundles of efferent nerves (sympathetic and parasympathetic nerves). The vagal afferents with their cell bodies, located in the jugular/nodose ganglia (NG), project centrally to the nucleus tractus solitarius (NTS) in

the brain and peripherally to the visceral organs from the pharynx to the proximal colon. The neurons from the NTS project within the brain stem to reach the hypothalamus and structures related to behavioural/emotional aspects of sensory processing. The spinal fibres are divided into two subpopulations, splanchnic and pelvic. They have their cell bodies in the spinal thoracolumbar and lumbosacral dorsal root ganglia (DRG) which convey sensory signals to the spinal cord. The splanchnic afferents supply the entire GI tract while the pelvic afferent innervations are limited to the distal large intestine. The peripheral projections of the vagal afferents are denser in the proximal gut and they become less dominant toward the distal parts of the GI while the spinal afferents are, in contrast, predominant in the distal parts.

A variety of afferents have been identified and described over the entire GI tract, using numerous experimental techniques and mechanical stimuli to classify their function. The afferent endings were classified into five subtypes according to their response to chemical or mechanical stimuli and the suggested location of their mechanoreceptive fields. These subtypes are termed mucosal, muscular, muscular/mucosal, serosal and mesenteric afferents (Brierley et al., 2004 , Page et al., 2002, Brierley et al., 2010), see Figure 2.
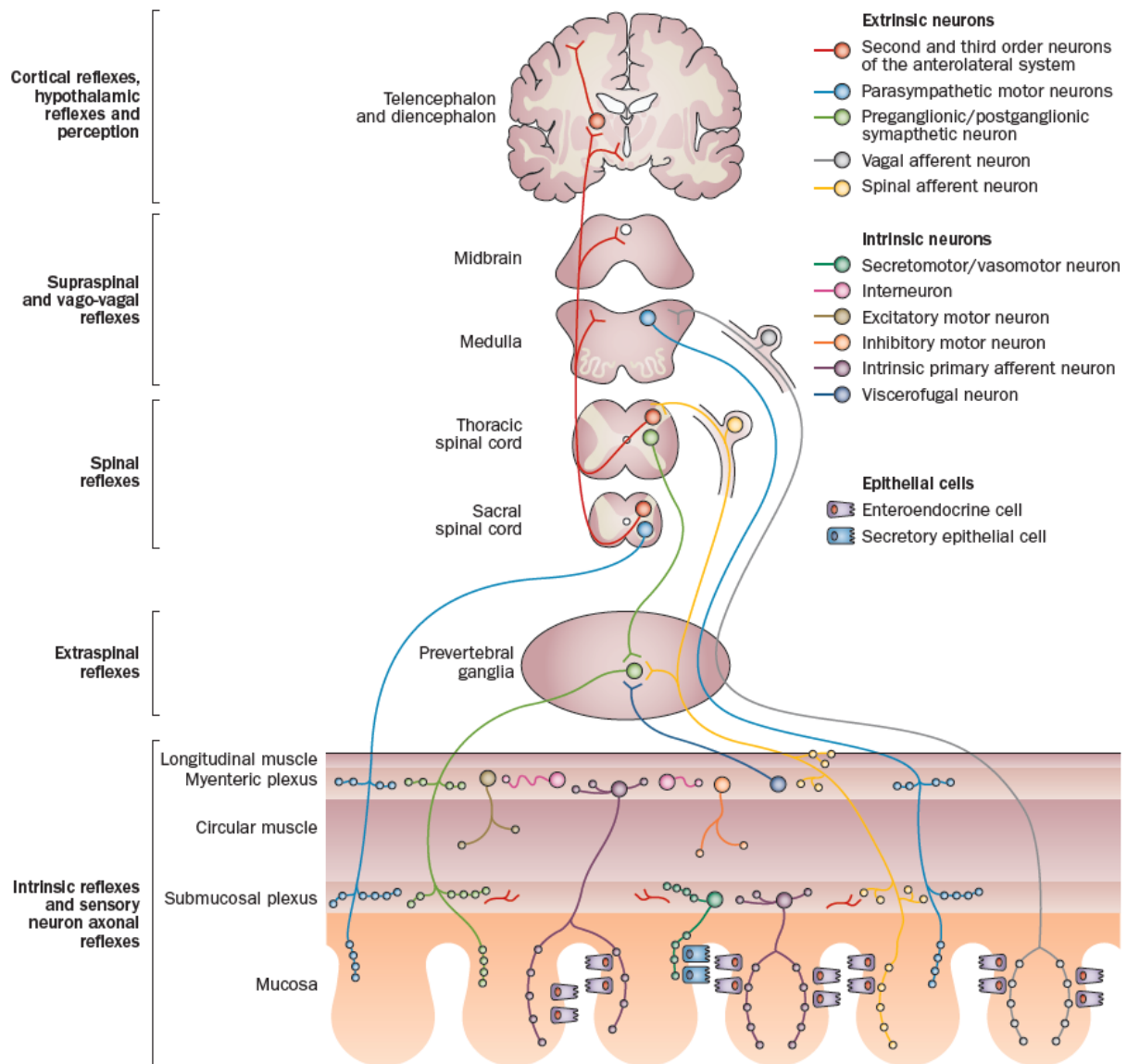
Figure 2 Neural innervation of the gastrointestinal tract.

Intrinsic neurons (Enteric nervous system) have their cell bodies and terminals contained within the gut wall. Viscerofugal neurons are a subtype of ENS that project to postganglionic sympathetic visceromotor neurons in the prevertebral ganglia. Postganglionic sympathetic neurons project to the gut wall and contribute in modulating gut functions, and receive input from preganglionic sympathetic neurons in the spinal cord which provide the efferent limb of CNS reflexes. The second efferent limb comes from parasympathetic motor neurons in the sacral spinal cord and brainstem. Vagal afferents have their cell bodies located in the nodose and jugular ganglia and project centrally to the nucleus of the solitary tract, while spinal afferents (splanchnic and pelvic nerves) cell bodies are in the thoracolumbar and lumbosacral dorsal root ganglia and project centrally to the dorsal horn of the spinal cord. Extrinsic afferents contribute to conscious perception and have efferent function via local or extraspinal reflexes. From (Brierley and Linden, 2014).

Afferent endings in the mucosa

Within the mucosa layer, morphological studies on the vagal afferents have indicated that they project all over the laminar propria, close to the basal laminar but not through the epithelial cells (Berthoud et al., 2004). They form a close association with different types of cells including epithelial cells, immune cells and other neurons which allow them to detect absorbed nutrients, hormones and peptides (Brookes et al., 2013).

It has always been believed that endocrine cells stimulate the sensory nerve terminals in a paracrine fashion, but recently it has been shown that these cells possess an axon-like basal process that can be over 50 μm long. They are cytoplasmic processes that look like axons with synaptic-like endings, which suggests that they are able to convey chemical information through cell–cell connections to other cells in the lamina propria or nerve endings (Bohórquez et al., 2015).

Spinal mucosa afferents are activated by stroking the mucosa (Brierley et al., 2004) and are excited by 5-HT or 5-HT$_3$ agonists (Hicks et al., 2002).

Afferent endings in the muscle and serosa layers

Nerve terminals in the muscle convey mechanosensory information related to distension and contraction of the bowel wall. In the muscle layer, vagal afferent terminals are divided into two types, intraganglionic laminar endings (IGLEs) and intramuscular arrays (IMAs). IGLEs are basket like structures located surrounding the myenteric ganglion. Their responsiveness to distension quickly saturates at around ~20 mm Hg. whilst spinal muscular afferents respond to wider distension pressures (10–60 mm Hg). Studies have

shown that in the colon, muscular afferents are prevalent in the pelvic pathway but rare in the splanchnic pathway (Brierley et al., 2004 , Lynn et al., 2003).

IMAs are mainly in the stomach but present in the small intestine as well. IMAs are spread in both circular and longitudinal muscle layers and are believed to detect changes in the length and stretch of the muscle as they have long axons that run in parallel with the muscle layer. They can respond to passive stretch such as stomach accommodation besides active contractions such as peristalsis (Beyak et al., 2006).

Serosal and mesenteric afferents respond at noxious levels of distension (>35 mm Hg) (Brierley et al., 2004) or higher intensity circular stretch (Brierley et al., 2008, Hughes et al., 2009). A distinct mechanically-insensitive fibres have also been described to respond only to chemical stimuli (Brierley et al., 2005).

1.2.4 Mechanosensitivity

Mechanotransduction can arise from physical opening of mechanosensitive ion channels such as transient potential channels (TRPA1 and TRPV4) which can be caused by direct deformation of the membrane and its associated proteins. In addition, mechanical forces can be transmitted via the release of chemical mediators from neighbouring cells. ATP is the most identified chemical which is released from epithelial cells upon mechanical stimulation and immune cells during inflammation. It acts through purinergic receptors (P2X and P2Y). $P2X_2$ and $P2X_3$ receptor contribute to the visceral hypersensitivity (Shinoda et al., 2009). Activation of P2Y receptors excites human visceral afferents and mouse colonic nociceptors, and expression of voltage gated sodium channel ($Na_V1.9$) is required for this effect (Hockley et al., 2016a).

1.2.5 Chemosensitivity

Extrinsic afferents of the GI tract are sensitive to a variety of chemical stimuli within the lumen such as nutrients, toxins and inflammatory mediators. Chemical sensitivity is mediated via activation of both ligand-gated ion channels and other membrane-bound receptors, for example G protein-coupled receptors (GPCRs).

Ligand-gated ion channels are present on both vagal and spinal afferent fibres. They are gated by binding of various transmitters and mechanical stimuli. Among these are 5-HT$_3$, TRP channels, nicotinic receptors, nmethyl- D-aspartate (NMDA), purinergic receptor 2X (P2X) and acid-sensing ion channels (ASIC). Chemical stimuli can also affect nerve firing via action on membrane receptors such as GPCRs that subsequently stimulate intracellular signalling pathways. Studies have shown an important role of GPCRs in regulating afferent signalling in response to nutrients, inflammatory mediators as well as modulation of mechanosensitivity (reviewed in (Beyak et al., 2006).

1.3 Reactive oxygen species

Reactive oxygen species (ROS) including superoxide anion ($O2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH●) are by-products of aerobic metabolism. Each ROS possess specific chemical properties that confer reactivity to a special biological target. Excessive release of ROS is associated with oxidative stress which is one of the main mechanisms of tissue damage.

1.3.1 Biochemistry and properties

Superoxide anion *(O_2^-)*. The primary ROS is superoxide anion (O_2^-), formed by single-electron reduction of molecular oxygen. It is an oxygen free radicals as it has an unpaired electron and is generated in both physiological and pathophysiological conditions by many sources. It's generation by neutrophils and macrophages is most relevant to the pathogenesis of intestinal inflammation (Glover and Colgan, 2011).

Superoxide is also produced in non-inflammatory conditions, intracellularly by mitochondria as electrons are continuously leaked from their carriers within the respiratory chain. It has been estimated that 1–5% of the overall oxygen consumption by normal tissues could be transformed into superoxide (Kruidenier and Verspaget, 2002). In addition, substantial amounts of superoxide can be produced endogenously by the peroxisomal enzyme xanthine oxidase upon re-induction of oxygen after a period of hypoxia (Du et al., 1998). Superoxide is contained within the cell as it does not quickly cross the lipid membrane bilayers, plus it spontaneously dismutase at physiological pH by the superoxide dismutases (SOD) and produces Hydrogen peroxide (H_2O_2) (reaction

1). The importance of the superoxide anion is that it is the first of a chain of oxidant reactions that can produce more reactive ROS, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and the most active hydroxyl radicals (OH•).

$$\textit{Reaction (1): } 2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$
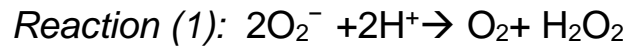
Hydrogen peroxide (H_2O_2) H_2O_2 is commonly considered as a weak ROS even though it has been shown to directly cause permanent damage to epithelial cells (Du et al., 1998). Inflammatory phagocytes produce and release significant amounts of H_2O_2. Part of H_2O_2 high reactivity is due to its stability and the fact that it can diffuse freely through the cell membrane. The cell is provided with antioxidants enzymes where H_2O_2 can be catalysed into water by catalase (CAT) or glutathione peroxidase (GPO) (reactions 2 and 3 respectively).

$$\textit{Reaction (2): } 2H_2O_2 + O_2 \xrightarrow{\text{CAT}} 2H_2O$$

$$\textit{Reaction (3): } 2H_2O_2 + H^+ + NADPH \xrightarrow{\text{CPO}} 2H_2O + NADP^+$$
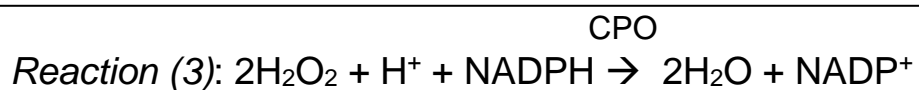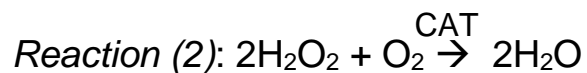
However, the most harmful property of H_2O_2 is that it can react (in so called Fenton reaction) with metal ions, such as ferrous (Fe^{2+}) to form hydroxyl radicals OH• (reaction 4).

$$\textit{Reaction (4):} \ H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^- + HO\bullet$$
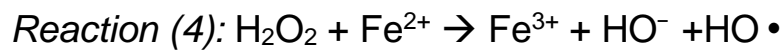
Hypochlorous acid (HOCl). H_2O_2 can also be metabolized by the enzyme myeloperoxidase to form the powerful oxidizing agent HOCl (known as bleach) which is more toxic than O_2^- or H_2O_2 (reaction 5). Phagocytes are very rich with the haemoprotein myeloperoxidase protein as it is expressed in macrophages, monocytes and neutrophils (Dröge, 2002). Therefore, this reaction is essential in an inflammatory response after infection to induce microbial killing.

$$\textit{Reaction (5):} \ H_2O_2 + Cl^- \rightarrow HOCl^- + H_2O$$
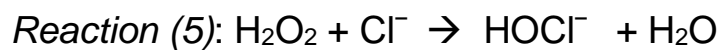
Hypochlorous acid is capable of producing irreversible damage to enzymes, membrane lipids and proteins or even reducing the adhesive properties of some extracellular matrix elements. Unfortunately, there is no specific HOCl deactivating enzyme for HOCl, but it can be eliminated by albumin and ascorbic acid (Kruidenier and Verspaget, 2002).

Hydroxyl radicals (OH•). OH• is the most reactive forms of ROS, it can directly causes DNA damage, deactivates essential enzymes (mitochondrial pyruvate dehydrogenase), and depolymerizes the GI mucin (Kruidenier and Verspaget, 2002). This radicals is formed from H_2O_2 by the Fenton reaction (reaction 4) or by another $O_2^-\bullet$ (reaction 6).

$$\textit{Reaction (6):} \ H_2O_2 + O_2^-\bullet \rightarrow O_2 + OH^- + OH\bullet$$
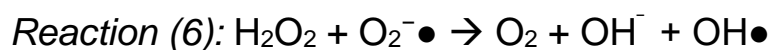
Reactive nitrogen species (RNS). Reactive nitrogen species (RNS) are derivatives of nitrogen, and include NO and peroxynitrite. NO is not noxious, in contrast has protective and inflammation-reducing influences. NO has been reported to protect epithelial cells from H_2O_2-mediated toxicity (Wink et al., 1995). However, NO can rapidly interact with superoxide and producing peroxynitrite anion ($ONOO^-$) which is considerably more reactive and damaging than the supeoxide itself plus it has a longer lifetime and passes through lipid bilayers much easier (reaction 7) (Dröge, 2002) .

$$\textit{Reaction (7): } NO + O_2^-\bullet \rightarrow H^+ + ONOO^-$$
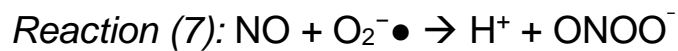
1.3.2. Oxidative damage

Excessive production of ROS and RNS can result in oxidation or nitration of cellular macromolecules and extracellular components (Figure 4). The cellular targets of the oxidative damage are membrane lipid, proteins and DNA.

Cell's membrane lipids. The polyunsaturated fatty acids reside within the cell membrane are a favorite target for hydroxyle radicalss and by that initiating lipid peroxidation process which once started, it continues as a chain reaction that produces lipid hydroperoxides and aldehydes. Therefore, one oxidative event can change several lipid molecules. The build-up of hydroperoxides in the cell membrane cause transformations in the membrane flexibility; thereby, changing many constituents (such as transporters, transmembrane enzymes, receptors and membrane proteins). Hence, causing alteration in the permeability and selectivity of the cell membrane which eventually leads to changes in cellular homeostasis and metabolism. Additionally, hydroperoxides and aldehydes are directly toxic to cell's components. Luckily, the lipid

peroxidation reaction is accelerated only if the cell fails to remove precursors of hydroxyle radicals - that would be H_2O_2. However, if started, the reaction could be broken down by lipid-soluble antioxidants (e.g vitamin E).

Cell's proteins. Proteins are the most rich components in the cell, thereby, they are significant targets of ROS. Most importantly, a slightest damage (oxidative) in the structure of a protein, can cause a significant change in it's biological activity. Similar to membrane lipids, hydroxyle radicalss can target cell proteins and cause protein oxidation. In this process, new functional groups can be constructed, which contributes to the following permenant modifications:

- Function of the protein

- Degredation

- Protein fragmentation, cross-linking and unfolding.

Tyrosines peroxynitrite (nitration of protein-bound) is another permanent alteration that can badly modify protein function. As tyrosine is a main amino acid that is involved in many cell functions (such as signal transduction pathways), its nitration can have severe effects on cellular regulation. The enzymes (SOD) (which catalyse H_2O_2 to water) are particular targets of nitration.

Cell DNA. Both mitochondrial and nuclear DNA are identified targets of oxidation. The most known DNA modification is base hydroxylations and strand cleavage which leads to ATP depletion and gene mutations which cause malignant transformation or death. The superoxide is rather unreactive with DNA, but other RNS implicated in DNA damage are peroxynitrite and NO, which are able to directly damage the chromatin. Moreover, H_2O_2 from cells deactivating enzymes (SOD) can reach the nucleus, react with chromatin-

bound iron (or copper) where hydroxyle radicals can be produced, which in turn attack close DNA residues.

It has to be mentioned that ROS have been implicated in cell death which can occur in two pathways: necrosis or apototosis. Necrosis is a disorganised cell death due to injury while apoptosis is an organised cell death. This process has been investigated in vitro and exposure to ROS or a depleting of cellular antioxidants, has been found to cause apoptosis (Kruidenier and Verspaget, 2002).

1.3.3. Reactive oxygen species and regulation of inflammation

ROS is usually related to oxidative stress which propose that ROS cause pathology by damaging lipids, proteins, and DNA. Redox signaling is the second "good" face of ROS and it has become clear in the past years that these metabolic products are important signaling molecules that regulate many biological and physiological processes.

The redox signaling mechanism involves H_2O_2-induced oxidation of cysteine residues within the proteins. At physiological pH, cysteine residues are a thiolate anion (Cys-S-) which are more inclined to oxidation in contrast to protonated cysteine thiol (Cys-SH). In redox signaling, H_2O_2 oxidizes the thiolate anion to the sulfenic structure (Cys-SOH) modifing the protein's function. The sulfenic structure can be reduced by disulfide reductases thioredoxin (Trx) and glutaredoxin (Grx) to thiolate anions which return the protein function to its initial state. Thus, first step oxidation of cysteine residues in the proteins works as a reversible signal transduction mechanism. The thiolate oxidation in living cells is estimated to take place in nM range of H_2O_2, whereas greater levels of the H_2O_2 induce further oxidization of thiolate anions to sulfinic (SO2H) or sulfonic (SO3H)

structures. These structures are irreversible changes within the protein and results in permanent damage (Schieber and Chandel, 2014).

The importance of ROS as immunomodulators is that the variety of kinases and transcription factors, activation and regulation, are ROS-dependent. In particular, the transcription factors: nuclear factor-ƙB and activator protein-1. These factors are activated in inflamed epithelia during intestinal inflammation and they cause the up-regulation of complex cytokine, chemokine, and adhesion molecule expression. Once the upregulation started, a continuous circle of activations occur as inflammatory mediators interleukin 1 (IL-1) and TNFα are known to stimulate ROS production (Mittal et al., 2013).

1.3.4 Intestinal inflammation and reactive oxygen species

Inflammation of the GI tract triggers an adaptive immune response which is characterized by excessive proliferation of T- and B-cells and high demands for great amounts of fuel for the oxidative phosphorylation (glucose, amino acids, and lipids). In contrast to resident lymphocytes, innate immune cells such as neutrophils, macrophages, and dendritic cells need to be actively summoned to the site of inflammation. Therefore, multiple signalling mediators such as cytokine and chemokine which are regulated by redox signalling are necessary to induce cell migration to the inflammatory site (Glover and Colgan, 2011). Energy and oxygen requirements for the arriving immune cells are increased to assist phagocytosis. Phagocytic functions are controlled by complex molecules including antimicrobial peptides, proteases, and ROS.

Rapid generation of ROS (respiratory burst) occur by phagocytes as they interact with bacterial products, which increases the oxygen and glucose consumption that in turn

activate further ROS. The respiratory burst mechanism involves a rapid activation of the membrane-bound enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which in turn stimulates the release of great amounts of superoxide. Although ROS production by immune cells during bacterial infection is fundamental for an effective host defence, excessive ROS production during inflammatory processes contribute to tissue damage and hypoxia development at the inflammatory site (Mittal et al., 2013).

1.4 Oxidative stress in inflammatory bowel disease (IBD)

The idiopathic inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is generally thought to depend on multiple factors: immunological, environmental and genetic. It is considered to result from an inappropriate and uncontrolled inflammatory response to luminal microbes in a genetically susceptible host. Crohn's disease can affect any part of the GI tract, whereas ulcerative colitis is more restricted to the colon and rectum. In Crohn's disease, the inflammation can occur to any layer of the gut wall while in ulcerative colitis the inflammation is limited within the mucosa layer. Some structures (such as fistulas) are associated with Crohn's disease but are not usually observed in ulcerative colitis. Genetic background was suggested to have a more obvious role in Crohn's disease than in ulcerative colitis (Abraham and Cho, 2009).

Uncontrolled and persistent host immune response is implicated in IBD as it is correlated with a widespread inflammatory infiltrate in the lamina propria, involving polymorphonuclear neutrophils and eosinophils [reviewed in (Fournier and Parkos, 2012)]. ROS is one of the factors responsible of chronic inflammation and tissue damage observed in IBD as studies have reported elevated levels of ROS in the colonic biopsy specimens of UC and CD patients compared to controls. The increase of ROS production was positively correlated with the disease activity and considered to be driven by neutrophils. Within the same line, levels of NO were significantly higher in colonic samples from UC and CD patients [see review (Kruidenier and Verspaget, 2002)].

Markers of oxidative stress including lipid peroxidation, protein and DNA damage have been used by several studies to evaluate the level of oxidative damage in samples from

IBD patients. Evidence of increased lipid peroxidation reactions (measured by Ethan in breath) was reported in UC patients, which was positively correlated with the severity of the disease (Sedghi et al., 1994). In colonic samples from UC patients, the protein carbonyl content which indicate protein damage and the DNA oxidation product, 8-hydroxy-2'-deoxyguanosine (8-OHdG), were found to be increased when compared to control samples (Lih-Brody et al., 1996). The study also reported a significant decrease in the superoxide dismutase (Cu-Zn SOD) antioxidant activity in inflamed tissue.

The first report of hypoxia formation in IBD was in mice by using 2-nitroimidazole dye which is metabolised intracellularly depending on the oxygen presence. The study reported a basal level of hypoxia in normal epithelial cells, specifically in the colonic epithelium that when compared with a colitis animal, showed greater levels of hypoxia at inflammatory mucosal lesions. Clinical studies followed to report the incidence of hypoxia in IBD patients (Mariani et al., 2009, Giatromanolaki et al., 2003). Immunohistochemical staining of the hypoxia-inducible factors (HIFs) HIF-1 and HIF-2 have shown that they are more pronounced in inflamed colonic biopsies from IBD patients (Giatromanolaki et al., 2003). Moreover, in an experimental model of cancer, Hif1α deficient mice had significantly lower colon inflammation compared to genotype control mice (Mladenova et al., 2015). It must be mentioned that microvascular deficits in IBD patients and reduced intestinal blood supply and oxygen delivery is also important contributing factors to mucosal hypoxia.

1.4.1 Visceral hyperactivity in functional GI disorders

Visceral hypersensitivity is an important factor underlying abdominal pain in functional gastrointestinal disorders such as irritable bowel syndrome (IBS) and IBD (Azpiroz et al., 2007). Therefore, several animal models have been established to mimic these diseases, see review (Fioramonti and Gebhart, 2007).

Behavioural studies have indicated that chemically induced colitis produces a significant decrease in pain threshold to colonic distension in both rats and mice (Lamb et al., 2006, Delafoy et al., 2006) which continued after the inflammation resolved.

In humans, rectal hypersensitivity to mechanical stimuli with increased smooth muscle tone was reported in UC-IBD patients (Drewes et al., 2006). Furthermore, in a more detailed study that was performed on UC active patients and IBS patients, UC patients were found to have higher discomfort thresholds to rectum tonic distension compared to IBS patients and healthy controls. The study found that severity of inflammation was inversely correlated with pain thresholds to noxious sigmoid stimulus (Chang et al., 2000).

Behavioural responses are important to study pain perception but they reflect changes in both CNS and peripheral sensory neurons and cannot identify the role of afferents sensitization. Therefore, researchers have used and developed a variety of in vitro methods (including extracellular nerve recordings, patch clamp and calcium imaging) to examine exact changes in the sensory neurons and identify which afferent subtypes are altered during and after inflammation.

Supernatants from mucosal biopsies of IBS patients have been shown to activate both submucosa and DRG neurons in animals which was correlated with the individual discomfort/pain threshold pressure values (Buhner et al., 2014).

Although there is a general consent between researchers that inflammation does alter the function of afferents, controversy exist about the exact effect on the colonic low-threshold mechanosensitive afferents. Some studies reported absent effects of inflammation (Hughes et al., 2009, Lynn et al., 2008) while others reported limited change (De Schepper et al., 2008) or a significant increase (Deiteren et al., 2015).

The picture is clearer in term of the effect of inflammation on high-threshold mechanosensitive nerves. In TNBS-induced colitis, inflammatory mechano-hypersensitivity was observed during acute phase (7 days post-TNBS administration) in colonic high-threshold splanchnic, mesenteric and serosal afferents. The extent of this hypersensitivity became even greater after recovery (30 days post-TNBS administration) when compared with acute conditions. On the other hand, pelvic afferents were not hypersensitive during acute inflammation and only serosal afferents showed hypersensitivity following recovery from inflammation (Hughes et al., 2009). These results conclude that splanchnic afferents are implicated in inflammatory hypersensitivity during both inflammation and recovery while pelvic afferents become involved after the recovery.

Furthermore, single unit analysis of splanchnic nerves indicated that rats with TNBS-colitis (acute phase), had an enhanced spontaneous activity of low-threshold and mechanically insensitive afferents, increased mechanosensitivity of low-threshold (LT), wide dynamic range (WDR) and high-threshold (HT) afferents. These activities in

inflamed tissue were associated with an increased proportion of both WDR and HT afferents, and a decrease in mechanically insensitive afferents (MIA) when compared with healthy tissues. After recovery, LT and MIA remained the spontaneous hyperactivity and WDR and HT continued to show mechanical hypersensitivity although the proportion of all afferent classes being normalized to control values (Deiteren et al., 2015).

Spinal afferents changes during and after resolution of inflammation, reviewed in (Brierley and Linden, 2014), are summarize in Table 1.

Table 1.

Spinal afferents	Inflammation	Postinflammation
Cell bodies	Hyperexcitability of colonic afferents neurons. Increased Na_V and decreased K_V current Altered channels and receptors expression	Continued hyperexcitability of colonic afferent neurons Continued altered Na_V and K_V currents. Altered channels and receptors expression
Nerve terminals	Sensitization of afferents Reduced mechanical activation threshold Mechanical hypersensitivity Altered receptors expression and pharmacology.	Further increase in hyperexcitability Continued reduction in mechanical threshold Hypersensitivity of additional afferent sets

It is clear now that afferents mechanical hypersensitivity during and after inflammation is attributed to changes in the expression and properties of certain ion channels (see details below).

TRP channels. TRP channels are 'cellular sensors' that respond to changes in the cellular environment, including temperature, stretch and pressure, oxidation/reduction, chemicals, osmolarity and pH. They are also stimulated by natural products, including spices and toxins. Mammalian TRP channels contain 28 members and are divided into 6 subfamilies including: TRPV (Vanilloid), TRPA (Ankyrin) TRPC (Canonical), TRPM (Melastatin), TRPP (Polycystin) and TRPML (Mucolipin) based on their homology of amino acid sequences. Many TRP channels are non-selective Ca^{2+} permeable channels excluding TRPM4 and TRPM5, are only permeable to monovalent cations and they do not conduct Ca^{2+} and Mg^{2+}. TRP channels are thought to play a diverse physiological role and have been implicated in many human diseases (Boesmans et al., 2010).

Several members of TRP channels are expressed in nociceptive sensory neurons and have attracted extensive research that used genetically modified animals and pharmacological agents to identify their role in the generation and transduction of pain (Kaneko and Szallasi, 2014). TRP channels are extensively expressed in the GI tract, with key functions in luminal taste, visceral sensation, and GI motility, in addition to absorptive and secretory functions (Blackshaw et al., 2010, Boesmans et al., 2010). TRPV1, V4 and TRPA1 have been shown to have an important role in the inflammatory afferent hypersensitivity and can also contribute to the inflammatory response themselves by stimulating neuropeptide release from afferent terminals and subsequent neurogenic inflammation (Brierley and Linden, 2014).

TRPV1. TRPV1 receptor can be activated by capsaicin and all classes of low and high-threshold afferents in vagal and spinal pathways are sensitive, in variable degrees, to capsaicin (Beyak et al., 2006). Activation of this channel induces neuronal activation that causes pain sensation and numerous mediators, such as prostaglandins (PGs), 5-HT, ATP and histamine can enhance the sensitivity of the TRPV1 receptor (Blackshaw et al., 2010).

In DSS-colitis mice, intracolonic infusions of capsaicin have been shown to increase visceral sensitivity assessed by behavioural response (licking, stretching and squashing the abdomen and abdominal retractions) (Eijkelkamp et al., 2007). TRPV1 channel involvement in the mechanical visceral hyperalgesia was indicated in rat-IBD, as TRPV1 antagonist (JYL1421) was found to suppress the visceromotor response to colorectal distension and improved colitis in rats (Miranda et al., 2007). However, opposed to these results, there are also suggestions of a protective role of TRPV1 in inflammatory models where TRPV1 activation improved dinitrobenzene sulphonic acid (DNBS)-induced colitis in mice (Martelli et al., 2007). In addition, (DNBS)-induced colitis was reported to be more severe in TRPV1$^{-/-}$ mice than in TRPV1$^{+/+}$ mice (Massa et al., 2006).

In humans, it has been recently indicated that histamine receptor H1 (HRH1)-mediated sensitization of TRPV1 is involved in IBS. The study showed that acute application of capsaicin on submucosa neurones causes a significantly higher Ca^{2+} influx in biopsy of IBS patients compared with healthy individuals which could be increased by pre-incubation with histamine; this effect was blocked by histamine receptor 1 (HRH1) antagonist. The double-blind trial performed on IBS-patients showed that the group that received Ebastine (an antagonist of HRH1) showed reduced visceral hypersensitivity, increased symptom relief and decreased abdominal pain compared with individuals given placebo (Wouters et al., 2016).

TRPV4. TRPV4 is expressed in the neurons as well as in epithelium cells. It is highly expressed in colonic sensory neurons compared with other visceral and somatic sensory neurons. In addition, TRPV4 protein was found in colonic nerve fibres from IBD-patients and it colocalized with the sensory neuropeptide CGRP in a subclass of fibres in mice (Brierley et al., 2008). Blocking this channel in animals with TNBS-induced colitis improves colitis and associated pain during inflammation (Fichna et al., 2012), therefore it is considered a high value target for the development of treatments in IBD (Vergnolle, 2014). It has been recently shown that application of a TRPV4 antagonist reduces the mechanosensitivity of serosal nociceptors from resected human tissues (McGuire et al., 2016).

TRPA1. This channel is well established as a pain sensor, and can be activated by a wide range of irritant natural products, including allyl isothiocyanate, cinnamaldehyde and allicin found in mustard oil, cinnamon and garlic. TRPA1 agonists induce neurogenic inflammation via the release of substance P and CGRP associated with macrophage and neutrophil activation and recruitment and increase in cytokine release (Kimball et al., 2007). TRPA1 expression is upregulated in the inflamed human and mouse colon (Kun et al., 2014) and chemically induced colitis (by dinitrobenzene sulphonic acid (DNBS) was decresed by pharmacological blockade TRPA1 antagonist (HC-030031) and genetic inactivation of TRPA1. DNBS was found to bind to cysteine residues in the intracytoplasmic N-terminus of the TRPA1 protein, identifying TRPA1 as a direct target in DNBS-induced colitis (Engel et al., 2011). TRPA1 is also targeted by environmental irritants and function as a receptor for ROS. Cyclopentenone prostaglandin (metabolites of prostaglandins) induce pain behaviour in wild-type mice, but not in TRPA1$^{-/-}$ mice (Trevisan et al., 2014) which indicates that prostaglandins are TRPA1 agonist.

Using TRPA1$^{-/-}$ mice has shown selective deficits in visceral afferent function in high-threshold colonic afferent specifically in splanchnic mesenteric afferents and splanchnic and pelvic serosal afferents. In addition, administration of TRPA1 agonists causes greater afferents hypersensitivity to mechanical stimuli in animals with acute TNBS-induced colitis compared with control mice (Brierley et al., 2009).

Voltage-Gated Sodium and Potassium Channels (Na$_V$ and K$_V$).

These channels are important in determining the neuronal excitability, with Na$_V$ channel mediating the rapid upstroke of the action potential, while K$_V$ channels act to repolarise the cell membrane and preventing repetitive firing. Na$_V$1.7 is a Tetrodotoxin (TTX)-sensitive current critical in setting action potential threshold. Na$_V$1.8 mediates a TTX-resistant current causing the upstroke of action potentials and firing activity during prolonged depolarisations (Dib-Hajj et al., 2009). Na$_V$1.9 mediates a persistent current important in setting resting membrane potential and function to transduce sensitizing stimuli such as inflammatory mediators (Hockley et al., 2016b). Voltage-gated Potassium channels (K$_V$) include various subunits and classed on their inactivation kinetics as either delayed rectifier (I$_K$) currents or transient outward (I$_A$) currents (Brierley et al., 2010). In afferent cell bodies, increases in Na$_V$ currents, particularly Na$_V$1.8 and suppression of K$_V$ currents, particularly I$_A$ and I$_K$ currents are implicated in afferents hyperexcitability during GI inflammation (Brierley and Linden, 2014).

Protein expression of Na$_V$1.7, Na$_V$1.8 and Na$_V$1.9 have been determined in colonic neurons from thoracolumbar DRG labelled with retrograde tracer but only Na$_V$1.8 protein levels was found to be increased in TNBS-induced colitis (day 7) while both Na$_V$1.7 and 1.9 levels were unchanged (King et al., 2009). Functionally, TTX-R (Na$_V$1.8) Na$^+$ current

was reported to be increased in labelled colonic and ileum neurons from TNBS-induced colitis and ileitis when compared with healthy animals (Beyak et al., 2004, Stewart et al., 2003).

A recent study showed that $Na_V1.7$ is not required for visceral pain processing although it is crucial for regulating somatic pain. Using knockout mice ($Na_V1.7^{Nav1.8}$), the afferent response to chemical stimuli (capsaicin, mustard oil, ATP and bradykinin) and to distension was reported unchanged when compared to controls. Inhibiting the channel by antagonist also showed no change in mesenteric nerve response to noxious pressure response in human tissue (Hockley et al., 2017).

More recently, attention is turning to $Na_V1.9$ channel after the identification of mutations in SCN11A (the gene encoding $Na_V1.9$) associated with insensitivity to pain and painful neuropathy in two Chinese families (Zhang et al., 2013, Hockley et al., 2016b). Study followed to show that $Na_V1.9^{-/-}$ mice have reduced mechanical hypersensitivity after application of inflammatory soup (including multiple inflammatory mediators such as ATP, histamine, bradykinin, PGE2 and 5-TH) to the serosa and mesentery nociceptors; and that colonic afferent responses to supernatants form IBD-patients is also significantly reduced (Hockley et al., 2014). This suggested a significant role for $Na_V1.9$ in the development of inflammatory visceral hypersensitivity in addition to its action on regulating the neuronal excitability of DRG and myenteric neurons (see review (Hockley et al., 2016b) which makes it a promising therapeutic target for management of pain and hyperalgesia.

Figure 3 shows several mediators that can directly activate gut afferents by binding to the numerous cell surface receptors and channels expressed on their peripheral terminals.
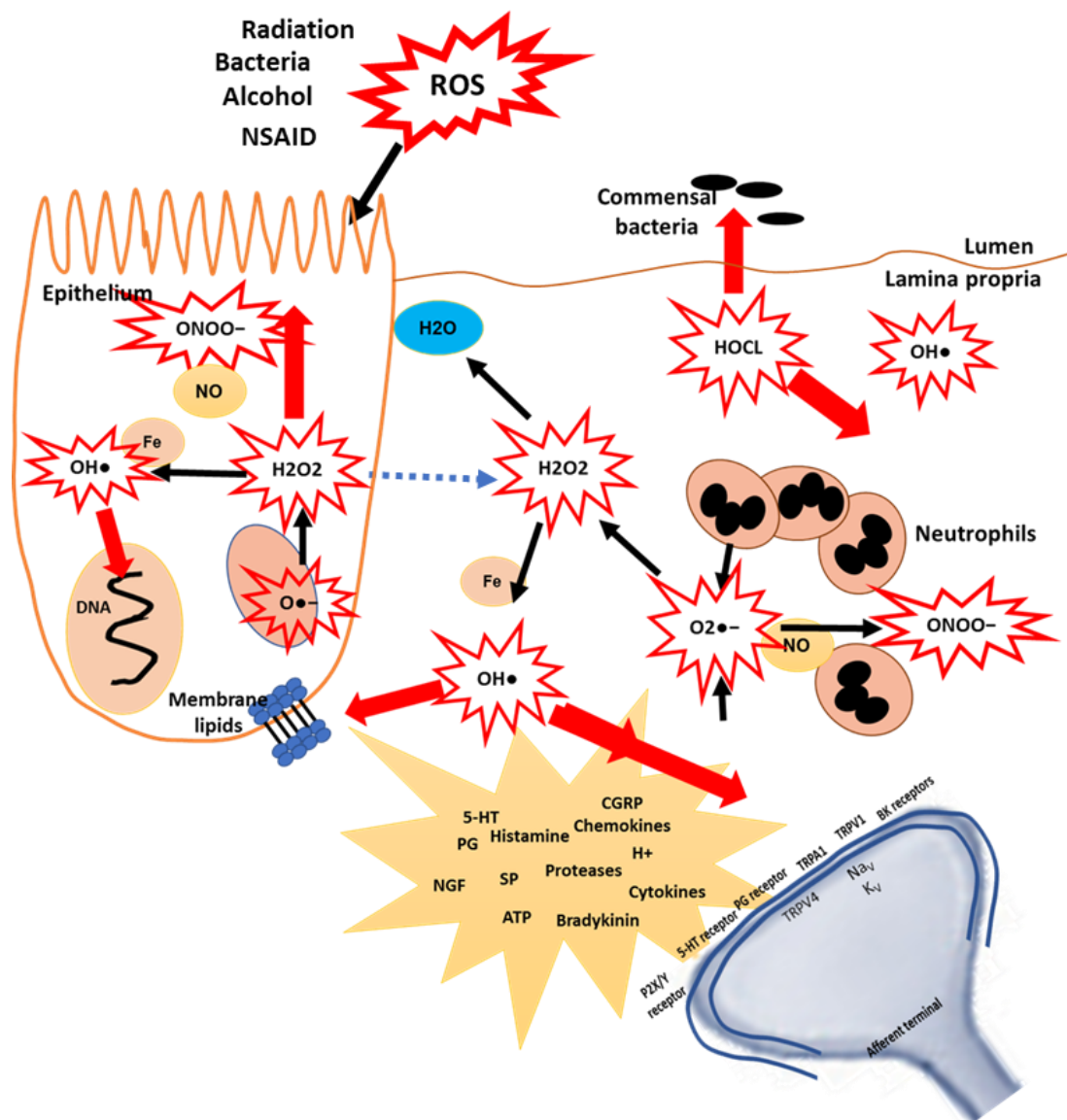
Figure 3 Simplified schematic of inflammation-induced hypersensitivity

Letters indicate the sources of ROS. **A)** neutrophil, **B**) endothelial and **C**) mitochondria. Red arrows indicate cellular targets of ROS (membrane lipids, proteins, DNA, matrix blood vessels and bacteria). Numerous mediators released, by many sources, can stimulate GI tract afferents directly by binding to cell surface receptors and channels expressed on their peripheral endings. Most channels and receptors expressed are implicated in afferent activation, hypersensitivity and hyperexcitability. In addition, the release of substance P and CGRP from nociceptors themselves upon activation induces neurogenic inflammation. TRPA1, TRPV1 and TRPV4 channels are involved in this process. **Abbreviations**: Fe, ferrous iron; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; MT, metallothionein; NO, nitric oxide; $O2^{-•}$, superoxide anion; OH•, hydroxyl radicals and $ONOO^-$, peroxynitrite; 5-HT, serotonin; CGRP, calcitoningene related peptide; TRPA1, transient receptor potential cation channel, subfamily A, member 1; TRPV, transient receptor potential vallinoid; PG: Prostaglandin; NGF: nerve growth factor. Figure adopted from (Kruidenier and Verspaget, 2002).

1.4.2 Sensory hyperactivity and oxidative stress

Oxidative stress occurs when ROS is produced in high rates that exceeds the ability of antioxidant enzymes to neutralize it. Several studies in animals using *in vitro* nerve recordings have demonstrated that ROS causes prolonged afferent hyperactivity.

In ischemia-sensitive abdominal C fibres, application of H_2O_2 at their receptor field has been shown to increase the discharge frequency, while ischemia-insensitive fibres were unresponsive to H_2O_2. Pre-treatment of ischemia and reperfusion-sensitive afferents with dimethylthiourea (DMTU), which scavenges H_2O_2 and hydroxyl radicals, was found to decrease their impulse activity to a repeated period of ischemia or reperfusion (Stahl et al., 1993). In rats, topical application of H_2O_2 to the heart has been shown to activate 50% of chemosensitive vagal afferents, but not mechanosensitive fibres. The H_2O_2 response is not mediated via superoxide anion as it wasn't blocked by SOD enzyme antioxidants that either prevent the formation of hydroxyl radicals or scavenge the radicals were found to abolish the H_2O_2-hyperactivity. The study also indicated that the cardiac afferents response to H_2O_2 wasn't mediated by prostaglandin synthesis as inhibition of cyclooxygenase activity by indomethacin had no effect on the H_2O_2 response (Ustinova and Schultz, 1994).

On the other hand, in a following study, prostaglandins were found to mediate the changes in renal sympathetic nerve (RSN) activity that occur at the onset of cardiac ischemia in rats. The study found that the vagal afferents are predominant during early ischemia while the sympathetic afferent activity predominates during prolonged ischemia and reperfusion. Changes in RSN activity after prolonged ischemia and during

reperfusion were abolished by ischemia pre-treatment with the antioxidant deferoxamine while indomethacin (a prostaglandin synthesis inhibitor) abolished the changes in RSN activity that occurred at the start of ischemia. These results indicated that changes in vagal and sympathetic afferents activity that occur at the onset of ischemia are mediated by prostaglandins while changes after prolonged ischemia and during reperfusion are mediated by ROS (Ustinova and Schultz, 1996).

Another study identified the sensitivity of splanchnic afferent C fibres to H_2O_2, found that 60% of splanchnic units with identified warm-receptive and/or mechano-receptive fields were responsive to H_2O_2 applied to their receptive fields. In warm-sensitive fibres only, the H_2O_2 response was observed in 66% of units and higher in both warm-sensitive and mechano-sensitive units (88%). A subset of Bradykinin-responsive fibres (62%) were responsive to H_2O_2. Most of responsive units had fast and single response to H_2O_2 which indicates that the response is a result of a direct sensitivity to H_2O_2. On the other hand, in some cases, the H_2O_2 response was found to be either delayed or ineffective, but triggered a substantial response by repeated applications of the initially ineffective concentration. This suggests that the afferent response to H_2O_2 can also be mediated by tissue response to H_2O_2 rather than by a direct sensitivity (Adelson et al., 1996).

Studies on several tissues have shown that ROS induced afferents excitability is mediated by ion channels TRPV1, TRPA1 and P2X purinoceptors present on nerve endings as described next.

TRPV1 blockers (capsazepine and ruthenium red) were found to inhibit vagal and sympathetic cardiac afferent response to H_2O_2 but not to bradykinin which indicated the ROS activate capsaicin-sensitive fibres (Schultz and Ustinova, 1998). Similar observation

was reported in the vagal lung afferent as nerve response to H_2O_2 was attenuated by hydroxyl radical's scavenger (dimethylthiourea) and blocked by antioxidant catalase. The study showed that H_2O_2 response was reduced by TRPV1 receptor antagonist, but also added that P2X receptor antagonist (iso-PPADS) was able to reduce the response to H_2O_2. Using a combination of TRPV1 blocker and P2X receptor antagonist was shown to further reduce H_2O_2 hyperactivity. The study's results indicate that both TRPV1 and P2X receptors are involved in the sensory transduction of ROS by capsaicin-sensitive vagal lung afferents (Ruan et al., 2005). TRPV1 channels were also shown to be involved in ROS transmission in renal afferents as perfusing the renal pelvis with H_2O_2 caused a significant increase in nerve activity that was reduced by co-application of antioxidant catalase and TRPV1 blocker (capsazepine) (Lin et al., 2015).

However, in the mouse DRG neurons, H_2O_2 was shown to act specifically via TRPA1 channel. Using calcium imaging, H_2O_2 evoked a strong increase in the Ca^{2+} signal in ~25% of small diameter (20–25 μm) rat DRG neurons. All the H_2O_2-sensitive DRG neurons responded to TRPA1 agonist AITC. Some neurons responded to H_2O_2 and TRPV1 agonist (capsaicin) but about 50% of capsaicin-sensitive DRG neurons did not respond to H_2O_2. Furthermore, H_2O_2- induced Ca^{2+} influx in mouse DRG neurons were greatly decreased in neurons from TRPA1−/− mice (Andersson et al., 2008).

In the same line, a recent study has investigated the H_2O_2 sensitivity in stretch-sensitive bladder afferents. The application of H_2O_2 was found to activate 72% of high threshold afferents, inducing prolonged firing while low thresholds were insensitive. When applied to the same fibres, 65% of capsaicin-sensitive high threshold afferents were activated by H_2O_2 and 82% by TRPA1 agonist, (AITC). TRPA1 antagonist (HC030031) significantly reduced H_2O_2-induced activation of high threshold afferents by 65% and the AITC-

induced activation by 70%. The TRPV1 antagonist, capsazepine had no effect on H_2O_2-induced activation of high threshold afferents while prostaglandin synthesis blocker (dexketoprofen) reduced the H_2O_2 response. These results clearly indicate that the long-lasting activation of the majority of capsaicin-sensitive high threshold afferent by H_2O_2 is mediated by TRPA1 channel and that prostaglandins are likely to contribute to the observed response (Nicholas et al., 2016).

That said, the data about the effect of oxidative stress on intestinal afferents is lacking, and although the gut is normally in contact to various types of ROS, we don't know how different regions of the GI tract respond to ROS. Therefore, Chapter 3 of this thesis was planned to answer these important 2 questions.

1.5 The GI tract and ageing

The world population is ageing at accelerated rates and the incidence of a number of GI tract disorders increases considerably with age (Saffrey, 2014). This section discusses what we know about the effect of ageing on the GI innervation.

1.5.1 Ageing and ENS

Several morphological studies have described a decrease in the density and number of myenteric neurons associated with age in animals (Johnson et al., 1998, Wu et al., 2003, Phillips et al., 2003, Phillips and Powley, 2001, Cowen et al., 2000b), and humans (Bernard et al., 2009b).

In rodents, the loss of enteric neurons with age appeared to be restricted to cholinergic neurons (Phillips et al., 2003). Similar findings were observed in the human colon where the number of ChAT-positive myenteric neurons declined with age but not nNOS-positive myenteric neurons. This alteration was not associated with a change in a total volume of neurons suggesting compensatory modifications in the remaining neurons (Bernard et al., 2009a). On the other hand, *Takahashi et al* reported a decrease in NOS expression, in the rat colon with increased age, indicating that these neurons may also experience an age-related change in this region of the gut (Takahashi et al., 2000).

Age-associated alterations in non-adrenergic, non-cholinergic (NANC) VIP and SP function have been examined in the jejunum of young and middle-aged Lewis rats. In the jejunum of middle-aged rats, contribution of VIP in functional NANC innervation was increased, while functional innervation with SP was decreased supporting age-related impairment in neuromuscular transmission (Kasparek et al., 2009). A significant

decrease in both substance P and VIP immunoreactive nerve fibre density was reported in the aged myenteric guinea pig and mouse mucosa respectively (Saffrey, 2014). Similarly, substance P- immunoreactivity was found to be reduced with age in human bowel intestine samples. (Yu et al., 2016b).

Interestingly, Phillips and colleague observed signs of neurodegeneration in middle-age and aged rats as accumulations of alpha-synuclein (α-syn) appeared regularly in axonal swellings and distorted terminal fields located within the myenteric ganglia (Phillips et al., 2009b).

1.5.2 Ageing and afferent sensitivity

In humans, a decrease in oesophageal pain perception to graded intraoesophageal balloon distension was reported in elderly patients compared to younger controls (Lasch et al., 1997a). Similarly, rectal sensitivity thresholds induced by distension were increased in aged healthy human volunteers, but the colorectal smooth muscle compliance and tone were unaffected (Lagier et al., 1999a). Another study assessed rectal sensation in 61 normal females (aged 44±2 years) has indicated an age-associated reduction in sensitivity (Fox et al., 2006). More recently, extracellular nerve recordings of intestinal afferents from human samples has reported that afferent nerve activity declines with age. In the study, mucosal mast cell density was positively correlated with increased age and double immunostaining with mast cell tryptase and SP antibodies showed that more than 50% of mast cells were located very close to SP-positive nerve fibres. This was suggested to result from a low-grade inflammatory state in the aged gut.

In animals, both colonic mechanosensory and chemosensory function was shown to be altered in the aged C57BL/6J mice. The study assessed the afferents activity in control (3-month), 12-month and 24-month and used ramp distension protocol to activate low and high-threshold mechanosensitive afferent fibres and showed a significant reduction in high-threshold mechanosensitive afferents in both the 12 and 24-month samples compared to 3-month preparations. The study also showed that afferent discharge response to TRPV1 agonist (capsaicin) was significantly attenuated at 12 and 24-months. The expression level of TRPV1 mRNA was unchanged in the 24-month DRG neurons as well the response to capsaicin was not altered compared to 3-month. This result indicates that the change in afferent chemosensitivity is a result of change at the level of the nerve endings within the gut wall. The study also indicated that levels of EC cells and serotonin were elevated in the aged colon compared to control (Keating et al., 2016).

Morphological investigations have focused on mechanoreceptors in the muscle wall and chemoreceptors in the mucosa. Dystrophic changes associated with ageing have been reported in the terminals of vagal afferents supplying the myenteric plexus and mucosal layer which may represent some loss of trophic support and stimulation for the visceral afferents (Phillips et al., 2009a). On the contrary, two histological studies have not reported any age-related changes in the spinal afferent endings innervating the gut (Phillips et al., 2008).

1.5.3 Ageing and oxidative stress

The data available in the literature about the levels of oxidative stress in the aged gut is very limited and was obtained from animal models. ROS have been shown to be greater

in myenteric neurons in the ileum of aged rats compared to young ones. The level of ROS was correlated with the onset of neuronal loss (Thrasivoulou et al., 2006). Another study indicated that the elevated oxidative status of the aged rat ileum (measured by lipid peroxidation) was found to be reversed by dietary caloric restrictions (Cirilo et al., 2013). In mouse bladder, aged urothelial cells showed high levels of oxidative stress markers (including superoxide, intracellular H_2O_2 and 8-OHdG) that was combined with upregulation of TRPM8 channel compared to young. Calcium imaging showed that response to oxidative stress induced by H_2O_2 is enhanced in the aged urothelial cells compared to control which was blocked by antioxidant NAC (Nocchi et al., 2014). These results suggest that tissue response to ROS is affected by ageing.

Although the intestine is in continuous exposure to pathogen and noxious material from the external environment in addition to luminal contents, it is unknown if the response to ROS is changed in the aged gut and, therefore, was examined in this thesis (chapter 4).

1.5.4 Using animals to study ageing

One of the fundamental issues about using animal models to study aged GI is that animals might not present age-associated changes of GI functional similar to those observed in elderly people. The common functional changes in the aged human gut are increased colonic transit time, decreased stool production and changed patterns of defecation (Saffrey, 2014). Similar changes must be observed in the aged animal to be considered as a good model of study which was addressed by very few studies. Evidence in the literature indicates that aged rats displayed delayed gastric emptying, increased colonic transit time and decreased stool formation (Smits and Lefebvre, 1996), in addition to

similar reports from aged C57BL/6 mice that indicated delayed colonic transit and decreased faecal pellet formation (Patel et al., 2012). Regarding sensory function, *in vitro* recordings from human bowel afferent have reported an age associated decrease in nerve sensitivity to chemical and mechanical stimuli (Yu et al., 2016b), which was also reported in aged C57BL/6 mice (Keating et al., 2016). Hence, some important age-associated alterations in the GI tract functions, in some aged animals, seems to be similar to those reported in humans.

1.6 Aim and objectives

The aim of this study was to investigate the effect of oxidative stress on sensory signalling from the small intestine and colon of young and aged mice and if it contributes to age associated changes observed in these parts of the GI tract.

The objectives were:

1- Examine the response of intestinal afferents to acute oxidative stress induced by H_2O_2 and mitochondrial inhibitor using *extracellular* nerve recordings; and whether there is a regional difference in afferents response to oxidative stress between the jejunum and colon.

2- Assess the oxidative status of the small intestine and colon in young and aged tissue using oxidative stress markers and examine if the sensory response to oxidative stress is altered in the aged intestine.

3- Examine the regional difference in ROS production and antioxidant genes between the jejunum and colon using mRNA microarray.

4- Scan for age-related changes in gene expression in the jejunum and colon of young and aged mice using mRNA microarray.

Chapter 2

Materials and methods

2.1 Animals

All experiments were performed in accordance with the University of Sheffield's Animal Care Committee under UK protocol and project licence following the UK Animals (Scientific Procedures) Act 1986. Wild-type C57BL/6 mice were used in the study at 3 months and 22–23 months of age. The mice were housed in a temperature-controlled environment (22.5°C) on a 12h light–dark cycle with access to standard laboratory rodent chow and tap water. The animals were given a visual inspection to look for obvious lesions and abnormalities, and were monitored for signs of ill health during their housing. Mice were killed by cervical dislocation (after Isoflurane [5% in combination with O_2]), a mid-line laparotomy was performed, and small intestines from duodenum to caecum were removed and placed in room-temperature Krebs solution (composition in mM: NaCl 120, KCl 5.9, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 15.4, glucose 11.5, and $CaCl_2$ 1.2) that was gassed with carbogen (95% O_2, 5% CO_2).

2.2 Electrophysiology experiments

2.2.1 Tissue preparation

For jejunum afferent recordings, segments of jejunum (3 cm long) with the mesenteric attachments (containing the neurovascular bundle in the middle) were harvested 10 cm proximal to the ileocaecal junction, and placed into a 20 ml organ bath. The bath was continuously perfused with gassed Krebs solution (10 ml/min) and maintained at 33–35°C. Segments were horizontally cannulated and tied firmly at both ends to an input and outlet port. The input port was connected to a syringe pump (Genie Kent, mutil-phaser TM NE-1000, USA) that allowed continuous intraluminal perfusion of Krebs solution through the segments (12 ml/hour). Closing the outlet port allowed to fill or distend the

segment. The anal end of the segment was attached to a pressure transducer (DTXPlusTM, BD, Singapore) to record intraluminal pressure connected in series with the input port. The mesenteric attachment (containing the neurovascular bundle) was pinned onto a dissection platform made by Sylgard (SYLGARD® 184 Silicone elastomer kit).

Under a dissecting microscope (Nikon, SMZ-1B, Japan), a nerve bundle was identified and dissected gently, cleaned from surrounding fats and connective tissue, and drawn into a suction electrode.

For colonic nerve recordings, the whole pelvic area was removed and placed in Krebs solution. Under the dissecting microscope, the terminal part of colon (4 cm) attached to the kidney and mesenteric artery were all gently removed with all mesenteric and neurovascular attachments intact (containing the lumber colonic nerve and inferior mesenteric ganglia) and placed in the organ bath (See Figure 4 below). The preparation was further dissected to liberate the lumbar colonic nerve from the neurovascular bundle and the nerve sheath around the nerve trunk was stripped away using fine forceps. The exposed nerve bundles were placed into a suction glass electrode (See Figure 5 below).

Nerve firing was recorded with Neurolog headstage (NL100, Digitimer) and electrical signals was amplified (NL104), filtered with a band width of 100–1000 Hz (NL125) and captured by a computer processor that counted the number of action potentials crossing a specific threshold through Micro 1401 MKII interface running Spike2 software (version 7, Cambridge Electronic Design). The threshold was usually set at twice the noise level.
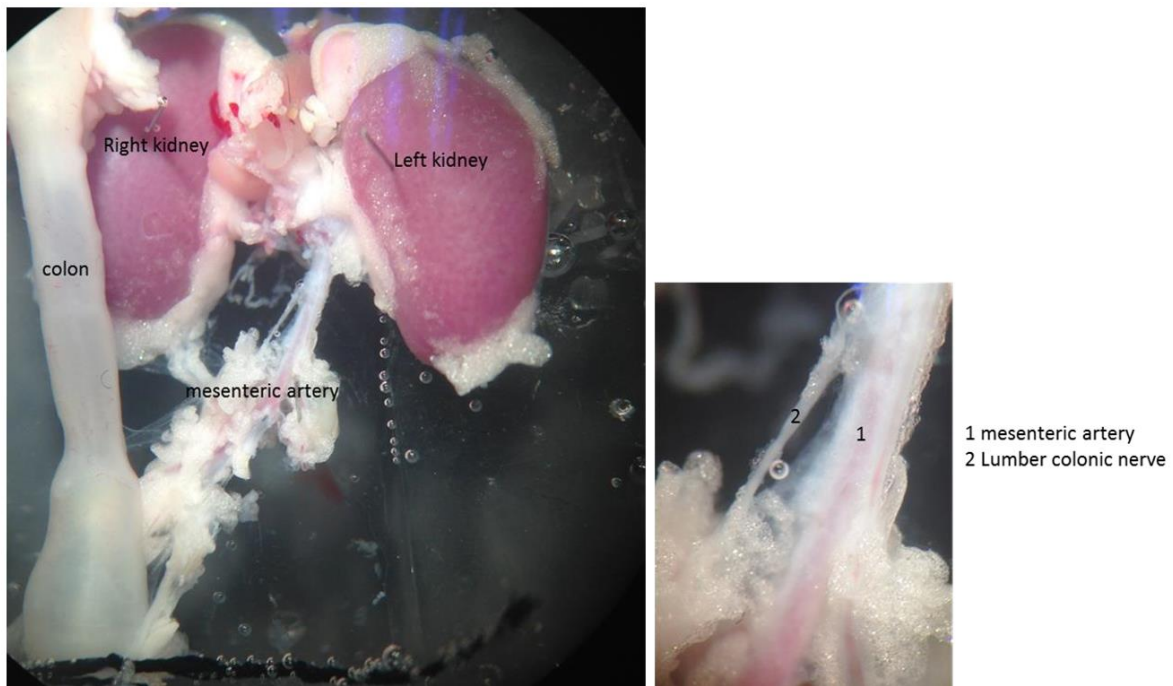
Figure 4: Afferent nerve recording of mouse colonic nerve.

Lumber colonic nerve (2) was dissected and liberated from mesenteric artery (1).
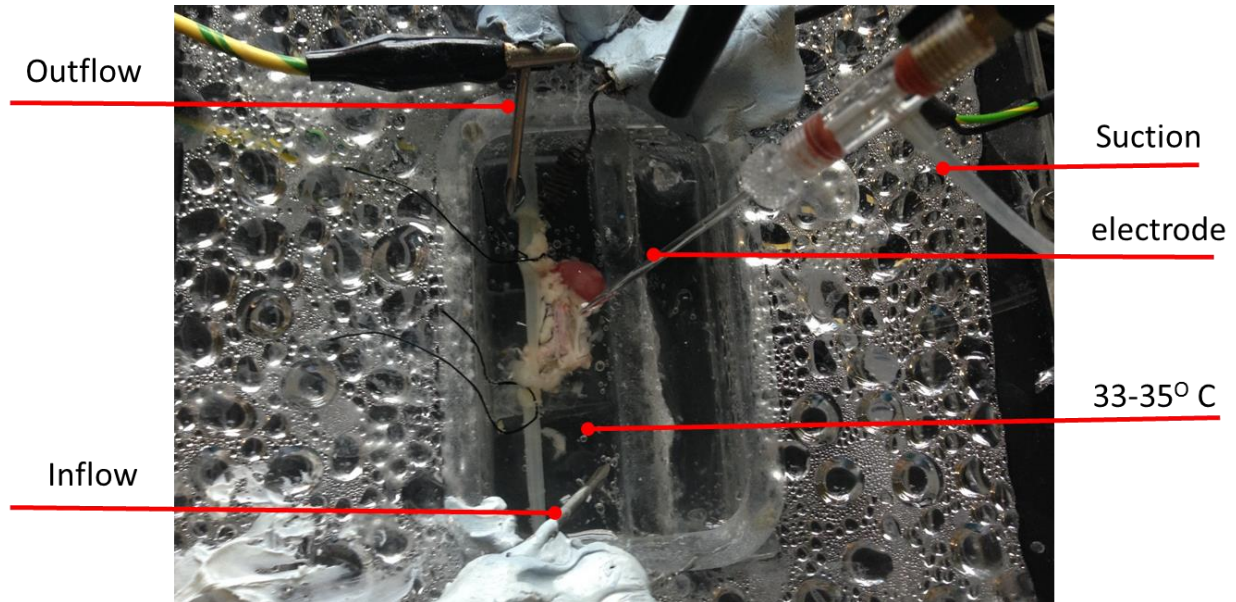
Figure 5: Preparation of nerve recording.

An isolated segment was fixed horizontally in the organ bath and catheterised from each end. The nerve was dissected from the neurovascular bundle and placed into the suction electrode. The organ bath was perfused continually with Krebs solution and maintained at 33–35 °C.

2.2.2 Experimental protocol

Both jejunum and colonic nerve recordings were stabilised for 30 and 60 min respectively to obtain a steady state spontaneous afferent nerve activity and when three repeated ramp distensions (with a 900 second interval) yield a reproducible multi-unit discharge.

Mechanosensitivity of sensory nerves

Nerves' mechanosensitivity was verified by distending the preparation at a rate of 12 ml/ml to an intraluminal pressure of 60 mmHg using a ramp-distension protocol. This protocol allowed for the activation of low-threshold (LT), wide dynamic (WDT) and high-threshold (HT) mechanosensitive fibres and was repeated every 900 seconds to test the reproducibility of the nerve response to ramp distensions. Chemical agents were applied after achieving three reproducible responses to distension.

The ex vivo nerve recordings technique in mouse was recently descried in the Journal of Visualized Experiments (JOVE) (Nullens et al., 2016).

2.2.3 Chemicals

Chemical agents were either applied directly to the Krebs solution perfused to the bath by a peristaltic pump (Gilson MINIPLUS 3, USA) or to the intraluminal perfusion. H_2O_2 (H1009), Antimycin A (A0149) were purchased from Sigma HC030031 (Tocris, 2896) and all salts were purchased from BDH.

2.2.4 Single unit analysis

In order to identify single units within multiunit afferent nerve bundle, single unit analysis was performed offline using spike sorting function of Spike 2 program. A template composed of 60 data points was constructed from 2.5 milliseconds period which allowed to analyze the whole profile of a spike signal. Following generation of the first

template, each new spike was compared to it and spikes that didn't match would create a new one. Spikes were then sorted into distinct units according to their waveform (Figure 6).

2.2.5 Principle component analysis

Principal component analysis was conducted to examine single units assigned to an individual template and identify and eliminate ambiguous waveforms. The analysis works by separating the shapes of individual single units based on parameters (such as amplitude, latency, area and slope). The data collected were normalized, shifted and scaled in 3-dimensional clusters. Overlap between the clusters indicated the similarities of their units (Figure 7).

2.2.6 Data analysis

Data is presented as the rate of afferent discharge (number of action potentials per second). The distension–response curves (mean afferent discharge plotted against intraluminal pressure) for whole-nerve or single-unit activities were calculated using a customised script program (Cambridge Electronic Design, Cambridge, UK). Spontaneous nerve activity was calculated before the distension starts (mean firing over 20 s).

Mechanosensitive single units were classified into 3 types according to their response profile during ramp distensions (0-60 mm Hg) as previously described in (Keating et al., 2016). High threshold (HT), wide dynamic range (WDR) and low threshold (LT) afferents. Briefly, the magnitude of the afferent response at 20 mmHg reflecting low-threshold (LT) was expressed as a percentage of the maximum response at 60 mmHg and this value is referred to as %LT. For a linear stimulus–response function, %LT would be 33%. Values

higher than this indicate low-threshold sensitivity, while values below this reflect high-threshold sensitivity. To classify afferent fibres, values for %LT more than 55 are described as LT. High threshold units are those responding with a %LT of less than 15% whereas afferents that exhibit more linear increase in discharge over the range of distension used (> 15%, < 55%) were identified as WDR units.

All data is expressed as mean ± SEM and N refers to the number of animals and (n) refers to the number of individual single units. All statistical analysis was performed using GraphPad Prism (Version 6.0, San Diego, USA). Student paired & unpaired t-test, 2-way ANOVA (with Bonferroni corrections) were used as appropriate. P<0.05 was considered as significant (*= P <0.05; **= P < 0.01 and ***= P < 0.001).
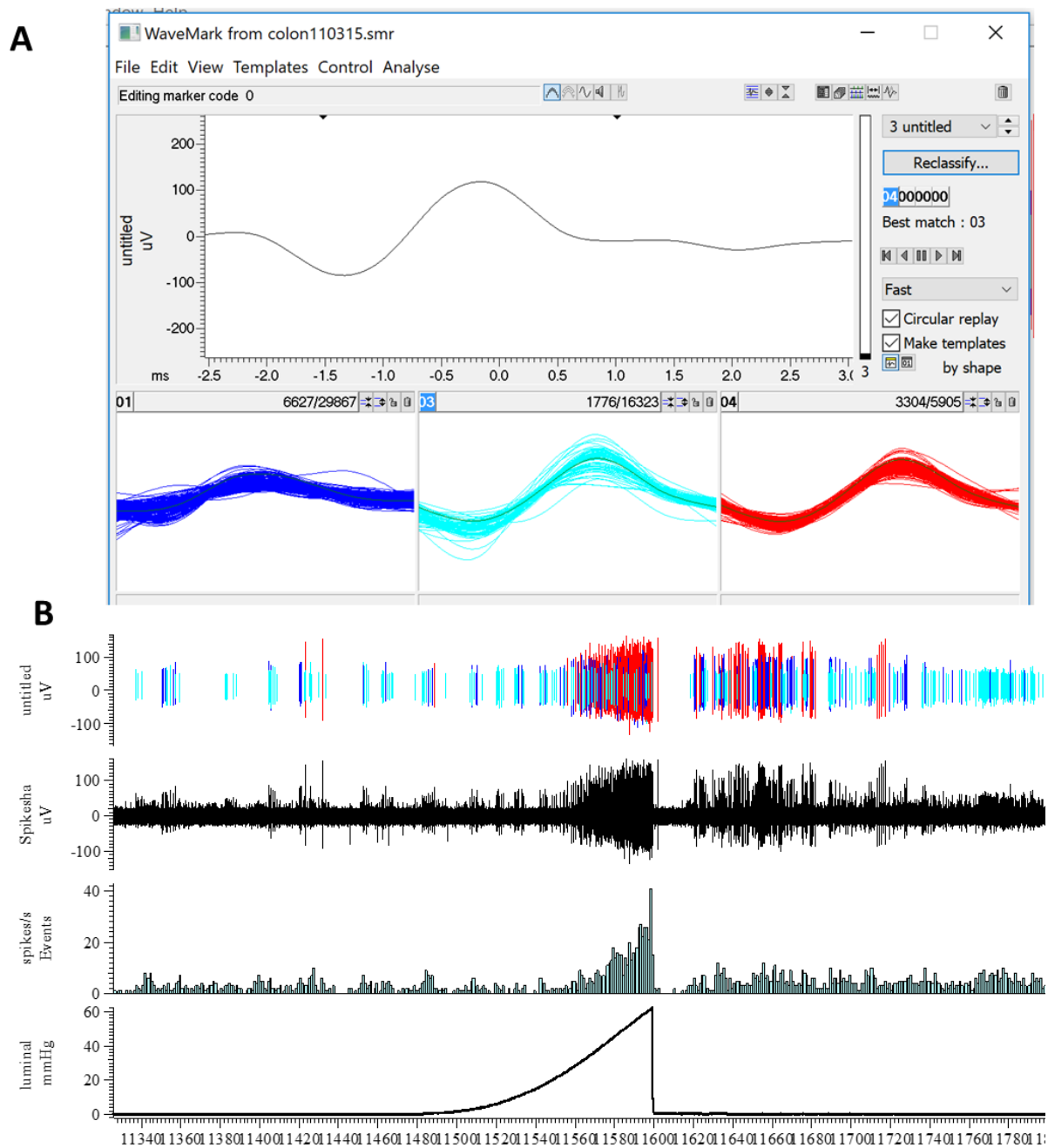
Figure 6 **Single unit analysis**.

A) Wavemark window of Spike 2 shows template generation and spike matching. **B**) Example trace shows the activities of individual single units during distension using the templates generated from figure A.
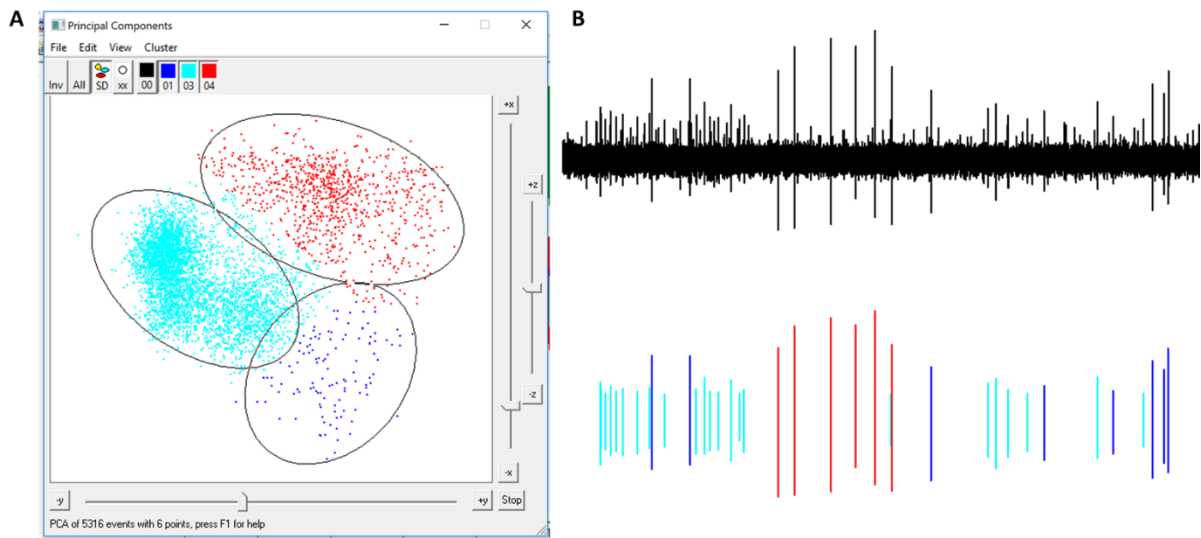
Figure 7 Principle component analysis.

A) Representative graph shows the clusters of individual units sorted in figure B. Each colour represents a template. Unit 01 (blue) and unit 04 (red) are well discriminated from each other. Some overlaps exist between unit 02 (light blue) and the other 2 units.

2.3 Muscle-contraction experiments

The muscle-contraction experiments were performed—under my close supervision—by Lisbeth Roelants, a visiting student from the Institute for European Studies (IES), Vrije Universiteit, Brussels.

An isolated colon was mounted horizontally in the organ bath, maintained at 33–35°C, and continuously perfused with Krebs solution (10 ml/min). The segments were allowed to equilibrate for at least 60 mins before any protocol started. The oral and anal ends of each segment were firmly catheterised and fixed and adjusted to maintain the segments at their resting length. As described in the electrophysiology experiments, the oral end was connected to the perfusion pump for infusion of Krebs solution and the anal end was attached to a pressure transducer to record contractile activity as changes in intraluminal pressure under isovolumetric conditions. Peristaltic motor complexes (PMCs) were evoked by infusing Krebs solution into the closed segment to an initial intraluminal pressure of 4–5 mmHg and could be maintained for 2–4 hours. H_2O_2 was added to Krebs solution and continuously perfused to the bath for 30 mins. The recording continued for a further 30 mins after washing H_2O_2.

2.3.1 Data analysis

The PMCs were quantified in terms of their peak amplitude above baseline and were expressed as mmHg, while interval between peaks was expressed in second (s), (Figure 8). The baseline, the mean values of the amplitude of the PMCs and the frequency (interval) of phasic contractions were determined 30 mins. before and after application of H_2O_2. All data was expressed as mean ± SEM and N refers to the number of animals. All statistical analysis was performed using GraphPad Prism. Student paired t-test was used as appropriate and $P<0.05$ was considered to be significant.
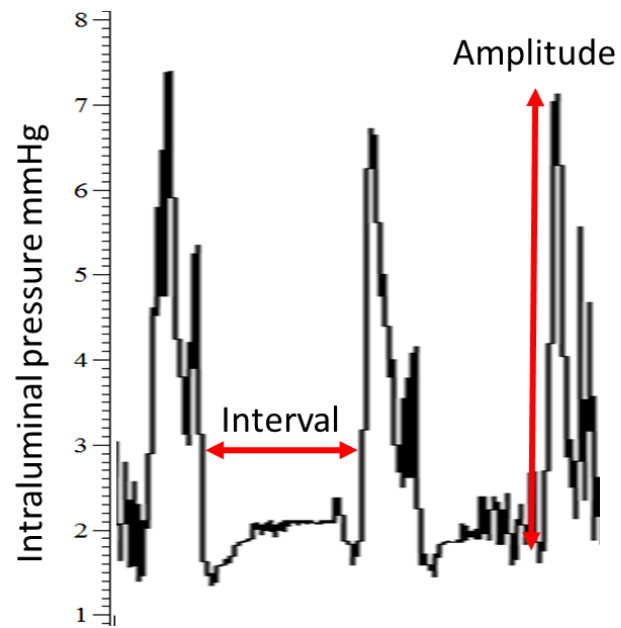
Figure 8 Analysis of peristaltic motor complexes

PMCs were measured in terms of their peak amplitude (mmHg) above the baseline and the intervals (seconds) between them.

2.4 Detection of reactive oxygen species levels

2.4.1 Tissue preparation

A sample approximately 1 cm long was cut from the proximal end of jejunum and colon of young and aged mice. Unfixed tissue samples were immediately placed into moulds filled with optimum cutting temperature embedding compound (OCT, Bright Instrument Company, 53581) and orientated appropriately. The moulds were kept on dry ice mixed with isopropanol until OCT was fully frozen and then kept at -80°C for later sectioning.

2.4.2 Cryo-sectioning

OCT blocks were sectioned in a cryostat (Bright Instrument, OTF5000, Huntingdon, UK) at 20 μm. The cryostat was set at -20°C for specimen and chamber temperature. 20 μ thick sections were collected using pre-coated glass slides (Thermo Scientific MNJ-700) and stored at -20°C.

2.4.3 Experimental protocol

On the day of experiment, two slides (young and aged) were defrosted to room temperature for 30 min. and the sections were washed three times with room temperature PBS and incubated in PBS containing DHE (20 μM) for 30 min. at 37°C in a humidified chamber. The slides were then washed with PBS, mounted with coverslips, using VECTASHIELD Mounting Medium with DAPI (Vector, H1200) and analysed immediately. Paired slides (young and aged) from jejunum and colon were processed one at a time to avoid a possible increase in fluorescent intensity over time. Dihydroethidium was purchased from Thermo Fisher (D23107).

2.4.4 ROS production in cell culture

Human Caco-2 cells were kindly provided by Claire Murzeau, a PhD student in Dr. Kai Erdmann's lab in the department of biomedical science. The cells were cultured in 96-well black clear-bottom plates for 12 days in minimum essential media (MEM. Gibco, 11095080) containing 10% FBS (Gibco) and 1% Penstrep (Sigma, P4333) at 37 °C humid CO_2 incubators. The cells were treated with vehicle, antimycin A (20 µM) and H_2O_2 (100 µM) for 3 hours, then washed (x2) with a fresh MEM media. DHE (20 µM) was added to the culture media and incubated for 30 mins. The media was then replaced with fresh PBS and cells were analysed immediately.

2.4.5 Microscopy and image analysis

Unfixed sections and living cells were examined under a Leica DMIRB Inverted Fluorescence Microscope. Images were captured using a digital camera (Olympus ColorView II).

2.4.6 Data analysis

The average integrated fluorescent intensity of three sections per part (jejunum, colon) was averaged from 6 young and 6 aged mice. Image analysis was performed with ImageJ software (1.43u, National Institutes of Health, USA). Pixel size was adjusted using a 1 mm stage micrometer with 100 divisions.

2.5 Gene expression experiments

2.5.1 Intestinal mucosal cells isolation

The whole gut from a mouse was immediately placed in cold PBS. Jejunum was identified at approximately 10 from caecum. Segments 4 cm long from jejunum and whole colon were used. Muscle layers were peeled off under the dissecting microscope and the mucosa was then longitudinally cut open and washed very gently 3 times with cold PBS. Tissue was diced into a fine paste with a sharp blade and transferred to a new tube containing collagenase (10 mg/40 ml) [C9891, sigma]. The tube was shaken vigorously for 10 seconds and incubated for 45 min at 37 °C. Liberated cell clumps were collected and washed with PBS for DNA, RNA, and protein extraction.

2.5.2 DNA, RNA, and protein extraction

DNA, RNA, and protein extraction and purification was performed using AllPrep DNA/RNA/Protein Mini Kit (QIAGEN 80004) according to the manufacturer's instructions. At the final stage of extraction, extracted DNA, RNA, and protein were eluted in RNase-free water and recommended buffers. All samples were stored at   -80°C for further investigation.

2.5.3 Reverse transcription (RT)

RT was performed using High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, 4387406) according to manufacturer's instructions. RT Master Mix (20 µl) was prepared and distributed into PCR tubes (Table 1) and reaction was performed in a Thermal Cycler (Table 2).

Table 1. Component of RT reaction mix (for 20 μl reaction)

Component	Volume (μL)	
	+RT	-RT
2X RT enzyme	10	10
20X RT enzyme mix	1.0	-
RNase free water	4	4
Sample (RNA)	5	5
Total per reaction	20	20

Table 2 RT reaction thermal cycle

	Step 1	Step 2	Step 3	Step 4
Temperature	25	37	85	4
Time	10 min	120 min	5 min	Hold

2.5.4 Quantitive real-time PCR

QPCR was performed using TaqMan® Genotyping Master Mix (4371355, Applied Biosystems). Components for 20 μl reaction mix were prepared in Hard-Shell® Thin-Wall 96-Well Skirted PCR Plates (BIO-RAD, HSP-9665). TaqMan Gene Expression Master Mix was obtained from Applied Biosystems (4369016) and mixed thoroughly before use. Each sample was run in duplicate (Table 3).

Before running, 96-Well plates were covered with MicroAmpTM Optical Adhesive Film (Applied Biosystems, 43111971), centrifuged for 15 secs. to mix, and the contents were

spun down. PCR reactions were performed in BIO-RAD CFX96 TouchTM Real-time system (C1000 TouchTM Thermal Cycler, Bio-Rad Laboratories Ltd. Hercules, USA) with programme set up as indicated in Table 4.

Table 3. 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

Table 4. Program set up for 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	95	15 sec	40
Anneal/Extend	60	1 min	

2.5.5 Data analysis

'Ct values' were obtained from the Thermal Cycler; they referred to the cycle numbers with which the PCR curve reached the threshold in the linear part of the curve. Ct value is inversely related to the amount of mRNA detected, meaning that high Ct value indicates less presence of mRNA in the sample. Glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) is commonly used as a housekeeping gene and was used in this experiment as reference. The expressions of tested gene were normalised against GAPDH expression.

ΔCt is calculated and referred to as relative gene expression compared with GAPDH expression.

ΔCt = Ct tested gene - Ct GAPDH

To examine the change of gene expression in aged samples compared with control group, fold change was calculated as follows:

ΔCt sample control = Ct tested gene in sample control - Ct GAPDH in sample control

ΔCt sample ageing = Ct tested gene in sample ageing - Ct GAPDH in sample ageing

 ΔΔCt = ΔCt sample ageing - ΔCt sample control

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

For example, if ΔΔCt value equals 1, the fold change would be -2, indicating that a gene was downregulated by half in ageing samples. If ΔΔCt was -1, fold change would be 2, suggesting that this gene expression was upregulated by double in ageing. Fold change >2 or -2 was considered to be significant.

2.6 RNA- Micro array investigation

The array was performed with the help of Dr Paul Heath in Sheffield Institute for Translational Neuroscience (SITraN). RNA concentration was measured on a NanoDrop 1000 spectrophotometer and RNA integrity was tested by bioanalyzer (2100, RNA 6000 Pico LabChip; Agilent, Palo Alto, CA). RNA was considered as suitable for the test only if samples showed intact bands of 18S and 28S ribosomal RNA subunits, displayed no chromosomal peaks or RNA degradation products, and had an RNA integrity number ≥ 9.0 (Figure 9).

2.6.1 RNA amplification

Equal amounts of total RNA of three young and three aged mice were used from the jejunum and colon. 100 ng of RNA was used for linear amplification of RNA (GeneChip® WT PLUS Reagent Kit). The Kit uses a RT priming method that primes the entire length of RNA, including both poly(A) and non-poly(A) mRNA, to provide complete and unbiased coverage of the transcriptome.

After generation of double-stranded cDNA, copy RNA (cRNA) is transcribed, which then constructs the RNA template for the second round of amplification. After the synthesis of double-stranded cDNA, in vitro transcription creates biotin labelled cRNA (GeneChip Expression 3′-Amplification reagents for in vitro transcription labelling; Affymetrix, Santa Clara, CA), that can be hybridised to the GeneChip. After two rounds of linear amplification, 60 ng of RNA produced 50 μg of labelled cRNA. The quality and quantity of amplified cRNA was analysed at the end of the second round.

2.6.2 Microarray hybridisation

Following the standard Affymetrix protocol (GeneChip reagents; Affymetrix), 10 µg of cRNA was fragmented and hybridised to Affymetrix GeneChip Mouse Gene 2.0 ST. Twelve chips (three young jejunums versus three aged jejunums and three young colons versus three colons) were hybridised. All arrays were hybridised together in one experiment. After overnight hybridisation at 42°C, the GeneChips were intensity washed in a GeneChip Fluidics Station 400 (Affymetrix) and scanned with a GeneChip Scanner (3000, Affymetrix), (see Figure 10 below).

Overall Results for sample 1 : __A15__

RNA Area:	31.5
RNA Concentration:	113 ng/µl
rRNA Ratio [28s / 18s]:	2.7
RNA Integrity Number (RIN):	9.3 (B.02.08)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.30

Fragment table for sample 1 : __A15__

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,724	2,244	4.0	12.7
28S	3,600	4,561	10.8	34.2
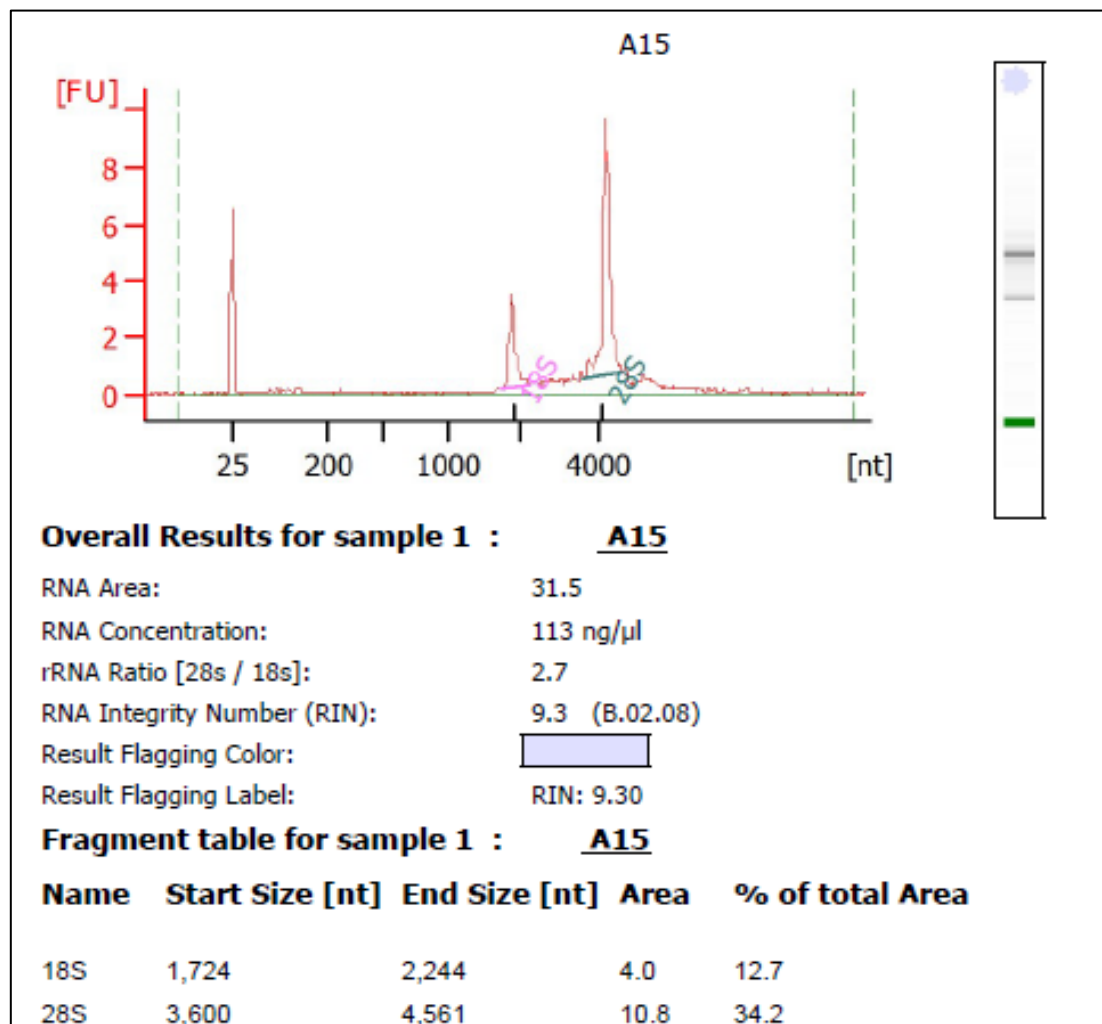
Figure 9: Selecting criteria of RNA sample.

RNA was judged according to ribosomal RNA ratio28S/18S and RNA integrity number > 9.0.

2.6.3 Data analysis

Samples were analysed using GeneChip Qlucore Omics Explorer operating software which is used for direct import and normalisation for Affymetrix files. The software automatically analyses data by calculating background points and setting an arbitrary threshold for the scan, then calculating an intensity value for each transcript. From there, data was exported to Microsoft Excel where it was subsequently processed to statistically analyse changes in gene expression in the samples. Changes in gene expression were calculated as folds changed between jejunum and colon or between young and aged mice. Transcripts were defined as differentially expressed if the difference in the gene-expression level was > 1.3-fold change and there was a P value < 0.05 (Multiple t-test with correction).
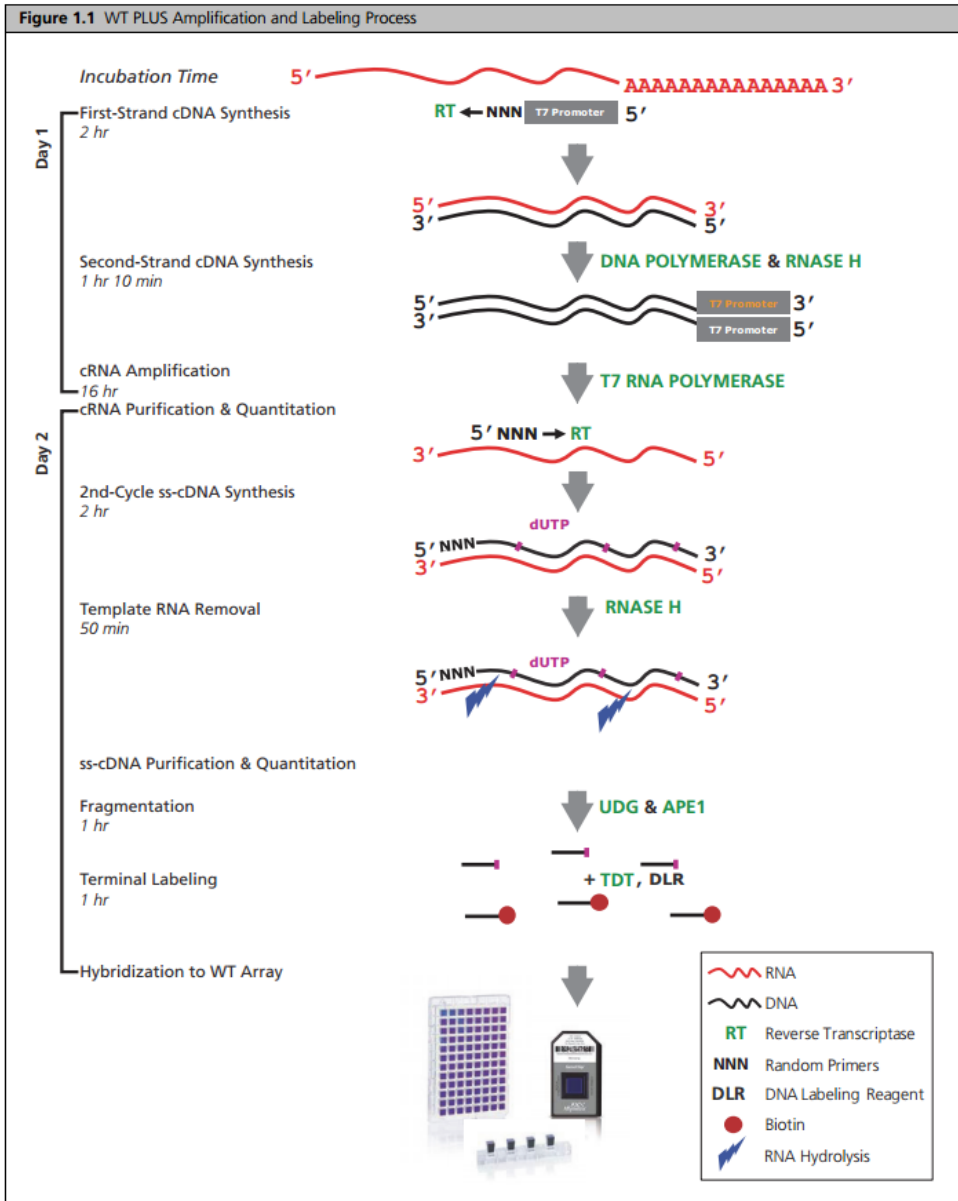
Figure 10: WT PLUS amplification and labelling process.

Image is adopted from the user guide of GeneChip whole transcript (WT) expression arrays plus Reagent Kit, Affymetrix.

2.7 Western blot

Protein from jejunum and colonic epithelial cells was quantified using a Bradford assay (Bio-Rad Laboratories Ltd, Hertfordshire, UK): a standard curve was created using the known concentration of bovine serum albumin and was run together with the cells lysates. Using a plate-reader set at 595 nm, the absorbance was read and the protein concentration was extrapolated from the standard curve. 20 µg protein was resolved in 12% SDS-PAGE, transferred to a nitrocellulose membrane (Protran; VWR Lutterworth, Leicestershire, UK) and incubated with rabbit anti-SOD2 (3:1000) (ab13533) and mouse anti-GAPDH (1:1000). The primary antibodies were detected with goat anti-rabbit (1:10,000) and goat anti-mouse (1:5000). Brain lysate was used as a positive control and GAPDH was used as loading control. The membranes were scanned by Odyssey Infrared Imaging System (LI-COR Ltd., UK). Band intensities were analysed by Odyssey software, and results are expressed as ratio, using GAPDH as the loading control.

2.8 Detection of 8-hydroxydeoxyguanosine (8-OHdG) level

DNA from young and aged intestinal epithelial cells was quantified using NanoDrop (Thermo Scientific, Wilmington, DE, USA). The level of 8-hydroxy-2′-deoxiguanosine (8-OHdG) was calculated using EpiQuick 8-OHdG DNA damage quantification direct kit (Epigentek, Cambridge Bioscience Ltd, Cambridge, UK) and determined by fluorescence at 530 excitation/590 emission nm. The results are expressed as relative quantification (%) to positive control as recommended by the kit manufacturer.

Chapter 3

Effect of oxidative stress on afferent nerve activity in mouse jejunum and colon

3.1 Introduction

Reactive oxygen species (ROS) including superoxide anion (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radicalss ($OH\cdot$) are by-products of aerobic metabolism. Each ROS possess specific chemical properties that confer reactivity to a special biological target. Excessive release of ROS is associated with oxidative stress which is one of the main mechanisms of tissue damage.

Most of the cell supply of ATP is generated in the mitochondria by food oxidation process. This process depends on oxygen but if it is restricted, a less efficient anaerobic respiration takes place where glycolytic products are metabolized in the cytosol. During normal respiration, a small percentage of electrons escape from oxidative phosphorylation, reducing molecule oxygen to form superoxide. Superoxide can also be produced outside the mitochondria by plasma membrane NADPH oxidases and lipid peroxidation. However, ROS formed inside the mitochondria presents the majority (90%) of the total ROS produced in the cell (Liu et al., 2002). Superoxide is quickly converted by superoxide dismutases 1&2 (SOD1 & SOD2) into H_2O_2. SOD1 is located in the cytosol and mitochondrial inter-membrane space, while SOD2 is in the mitochondrial matrix. Both SODs act to prevent the build-up of superoxide.

H_2O_2 is kept at a low level and in a steady state within the cell as extra H_2O_2 is catalysed by antioxidants, enzymes, glutathione peroxidase (GPx) and peroxiredoxin (Prx). This protection mechanism is essential because H_2O_2 reacts easily with metals such as intracellular iron and copper to generate hydroxyl radicals. These radicals are much more reactive and less discriminating than H_2O_2 itself and causes irreversible damage to DNA, proteins and membrane lipids (Liu et al., 2002).

ROS production, including H_2O_2 is important to the function of the immune system and to the tissue damage process associated with inflammation. After activation by noxious materials (such as bacterial products), polymorphonuclear neutrophils and macrophages undergo what is known as "respiratory burst" which involves the production of great amounts of superoxide via a membrane-bound NADPH oxidase which is converted then to H_2O_2. The production of ROS in higher rates that overpower the antioxidants activity causes oxidative stress.

ROS including H_2O_2 can directly act on sensory neurons innervating the GI tract which are devoted for the detection of endogenous noxious stimulus (chemical or mechanical). H_2O_2 was found to increase Ca^{+2} signal in isolated DRG neurons (Andersson et al., 2008), and produce neuronal hyperexcitability by changing cell membrane properties of nucleus tractus solitarii (nTS) neurons (Ostrowski et al., 2014). The nTS is the first central region in the brainstem that receives cardiorespiratory and GI vagal afferent information.

To perform the sensory function efficiently, numerous ion channels and receptors are expressed at the sensory nerve endings and they are key players in visceral sensation.

Treatment of tissues and cells with H_2O_2 is a common tool for studying the mechanism of action of oxidative stress on sensory neurons and substantial evidence has established that H_2O_2 activates TRPV1 and TRPA1 channels in animals. In mouse DRG neurons, H_2O_2 was indicated to induce neuronal excitability via TRPA1 channels. The H2O2-induced neuronal excitability was reversed by treatment with dithiothreitol indicating that H_2O_2 acts by promoting the formation of disulfide bonds (Andersson et al., 2008). In afferents from the heart and kidney, H_2O_2 was shown to act via TRPV1 channels on capsaicin-sensitive fibres (Schultz and Ustinova, 1998, Lin et al., 2015). More recently, it has been shown that H_2O_2 strongly activates capsaicin-sensitive high threshold afferents in guinea

pig bladder. H_2O_2 response was significantly inhibited by dexketoprofen which inhibits prostaglandin synthesis (Nicholas et al., 2016). Table 5 summarizes the concentrations of H_2O_2 than have been used in previous studies.

Visceral hypersensitivity is a hallmark in IBD and oxidative stress markers including DNA damage, lipid peroxidation and protein damage have been reported to be higher in samples from IBD patients compared to controls; also a greater production of ROS was positivity correlated with the severity of the inflammation (Kruidenier and Verspaget, 2002). Animal studies have shown that ROS contributes to colonic inflammation as scavengers of ROS were found to decrease inflammation in chemically induced colitis, (Seril et al., 2003, Roberts et al., 2013).

There is a good evidence of regional differences between the GI sites in response to oxidative stress. H_2O_2-induced oxidative stress was indicated to produce more DNA-damage to colonocytes than enterocytes from jejunum which indicates a difference in oxidative stress response (Sanders et al., 2004). However, it is unknown if this regional difference is also present in intestinal afferents response to oxidative stress.

Therefore, in the present study, H_2O_2 was used on the jejunum and colon to determine the effect of oxidative stress on chemosensitivity and mechanosensitivity of intestinal afferents and whether there is a regional difference between the jejunum and colon in sensitivity to oxidative stress.

Table 5 Concentrations of H₂O₂

Study	Afferent type	Concentration	Reverse H2O2 response
Stahl et al., 1993	Ischemia- and reperfusion-sensitive abdominal Visceral C fibre afferents	44 µM	dimethylthiourea (DMTU) scavenges H2O2 and hydroxyl radicals. iron chelator deferoxamine (DEF)
Ustinova and Schultz, 1994	Cardiac vagal afferents	3µM	deferoxamine and dimethylthiourea
Adelson et al., 1996	Single splanchnic afferent C fibres	0.88-880 mM	not used
Schultz and Ustinova, 1998	Cardiac afferents	1.5–30 µM	not used
Lin et al., 2015	Afferent renal nerve	1, 30 and 100 µM	not used
Andersson et al., 2008	DRG and nodose ganglion neurons	230 µM	dithiothreitol (inhibit the formation of disulfide bonds)
Ostrowski et al., 2014	nTS neurons	500 µM	not used
Nocchi et al., 2014	Bladder afferents	0.33 & 8 mM	not used
Nicholas et al., 2016	Bladder afferents	300 µM	TRPA1 antagonist, dexketoprofen (PGs synthesis inhibitor)

3.2 Experimental protocol

ROS production in cell culture

Human Caco-2 cells cultured in a Multi-Well plate and treated as described in chapter 2. Briefly, cells were treated with vehicle, antimycin A (20µM) and H_2O_2 (100µM) for 3 hours, then washed before adding DHE (20 µM) to the media. After 30 min of incubation, the media was replaced with fresh PBS and cells were analysed immediately. Live cells were examined under an Inverted Fluorescence Microscope. Images were captured using a digital camera (Olympus Color View II). Image analysis was performed with ImageJ software and integrated fluorescent intensity of cells was calculated. For each cell, outline was determined manually and the calculated fluorescent intensity was normalised to the background first and then to the cell's area to eliminate size difference. The values of fluorescent intensity were averaged per well and divided by the number of cells.

Hydrogen peroxide and sensory signalling

Extracellular recordings from mouse jejunum and colon were performed as described in chapter 2. Before starting any protocol, nerve activities were allowed to stabilize for 30 minutes in the jejunum and 1 hour in the colon. Then, distensions were performed at 900 s intervals and three reproducible responses to distension were achieved before start any protocol. After 30 min, nerve activity from jejunum displayed 3 reproducible response to distension, but the colonic nerve still exhibited change in activity and, therefore, required longer time to display a stable response to increase in pressure (result Figure 8 B&C).

Hydrogen peroxide was applied either directly to the bath or perfused into the lumen. For the bath application, a working solution (final volume is 300 ml) was diluted from purchased stock (8 M) with freshly made Krebs and perfused into the organ bath (volume

~20 ml) for 30 minutes at a rate of 10 ml/min. For the intraluminal application, a working solution was prepared at 100 ml final volume and perfused by a syringe pump at 12 ml/h rate for 45 min. Two concentrations of H_2O_2 were applied (100 and 500 μM). For both applications, H_2O_2 was applied only once to each segment and responses were calculated after 10 min of application. The PH of Krebs solution was unchanged by adding of H_2O_2 up to 500 μM (7.4 ± 0.01 vs 7.3 ± 0.02, $P > 0.05$, paired t-test, N=5).

A working solution of antimycin A (final volume is 40 ml) was diluted from pre-made stock (5 mM) with freshly made Krebs solution and perfused intraluminally.

Antagonist experiments

HC030031 (30 μM) was applied intraluminally for 45 mins. Antimycin A (20 μM) was applied in the presence of HC030031 for 45 mins, Figure 11 shows the protocol and identify the points where afferent mechanoresponse was measured.

Data analysis

Total nerve firing and single unit analysis was performed as described in Chapter 2. Data of nerve recording are presented as frequency of afferent discharges, which refers to number of spikes per second. All data are expressed as mean ± SEM from the N values, where N refers to the number of animals and n refers to the number of individual single unit. The nerve activity response to ramp distension was calculated as increase in the afferent firing frequency against increase in intraluminal pressure from 0 to 60 mmHg. Data were obtained using a custom-made script (Cambridge Electronic Design, Cambridge, UK). Spontaneous nerve activity was calculated from the nerve activity recorded before the start of the distension protocol (mean firing over 20 s). To analyse whether distension responses were significantly changed, two-way ANOVA with Bonferroni correction for multiple comparisons test was used.
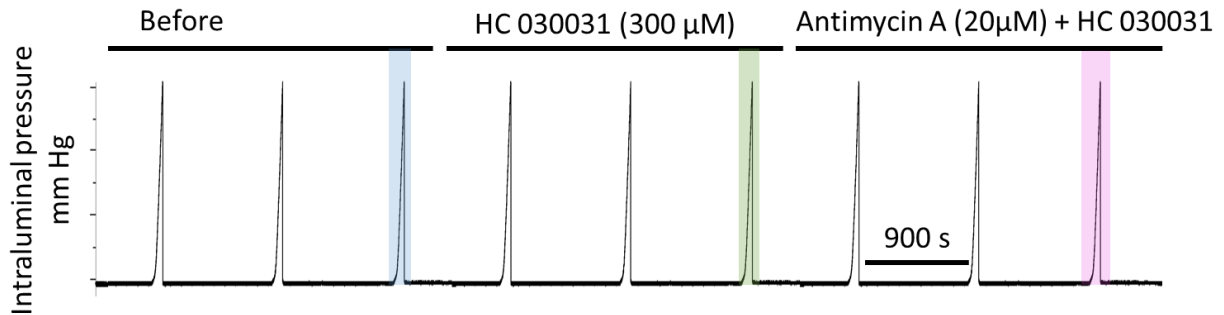
Figure 11 Protocol used for the antagonist experiment.

Pressure response profile was measured before (light blue area) and compared to after application of HC 030031 (green) and to Antimycin A in the presence of antagonist (purple).

Hydrogen peroxide and intestine contractions

To measure the contractions of intestine preparation, the tissue was distended up to 5 mmHg at a rate of 12 ml/h. The syringe pump was then stopped and the connection to the pressure transducer was sustained to detect the change in intraluminal pressure which reflects the muscle contractility. After the pressure had stabilized (30 min for jejunum and 1 hour for colon), H_2O_2 (100 and 500 µM) was continually perfused into the bath for 30 min. The recording continued for a further 30 min after washing out H_2O_2. The jejunum contractions and nerve activity were measured in the same experiment while colonic contractions were performed on separate experiments than the nerve recordings.

The colonic motor complex was calculated by the peak intraluminal pressure subtracted by baseline pressure within 30 second bins and the intervals between peaks in seconds.

Western blot

Protein was isolated from mucosa cells lysate of jejunum and colon as described in chapter 2. Twenty micrograms of protein were resolved in 12% SDS-polyacrylamide gel, transferred to membrane and incubated with rabbit anti-SOD2 (3:1000) and mouse anti-GAPDH (1:1000). The primary antibodies were detected with goat anti-rabbit (1:10,000) and goat anti-mouse (1:5000). Brain lysate was used as a positive control for SOD2 expression and GAPDH was used as loading control. The membrane was analysed with LI-COR Odyssey Imaging system. Band's intensity was measured using ImageJ software. Mean and standard error were calculated with Graphpad Prism 6 software.

3.3 Results

3.3.1 Afferents baseline nerve activity and mechanosensitivity in the mouse jejunum and colon.

Afferent mechanosensitivity was assessed in 3 month old animals by making whole nerve recordings using an in vitro preparation. Ramp distensions (0- 60 mmHg) of jejunum and colon segments induced biphasic increases in afferent nerve discharge corresponding to the activation of low and high threshold mechanosensitive afferent fibres (Figure 12A).

In order to examine the stability of the recordings for the time course of protocol, control experiments were first performed as recordings continued for several hours and distensions performed every 900 seconds. For recordings from the jejunum, the pressure response profile was measured at the 3rd distension (which represents the start of protocol) and compared with the response profile at the 6th distension which represents the end of experiment (Figure 13 A&B). For colonic recordings, the response from the 3rd distension was compared with the one at 9th distension (Figure 13 C&D).
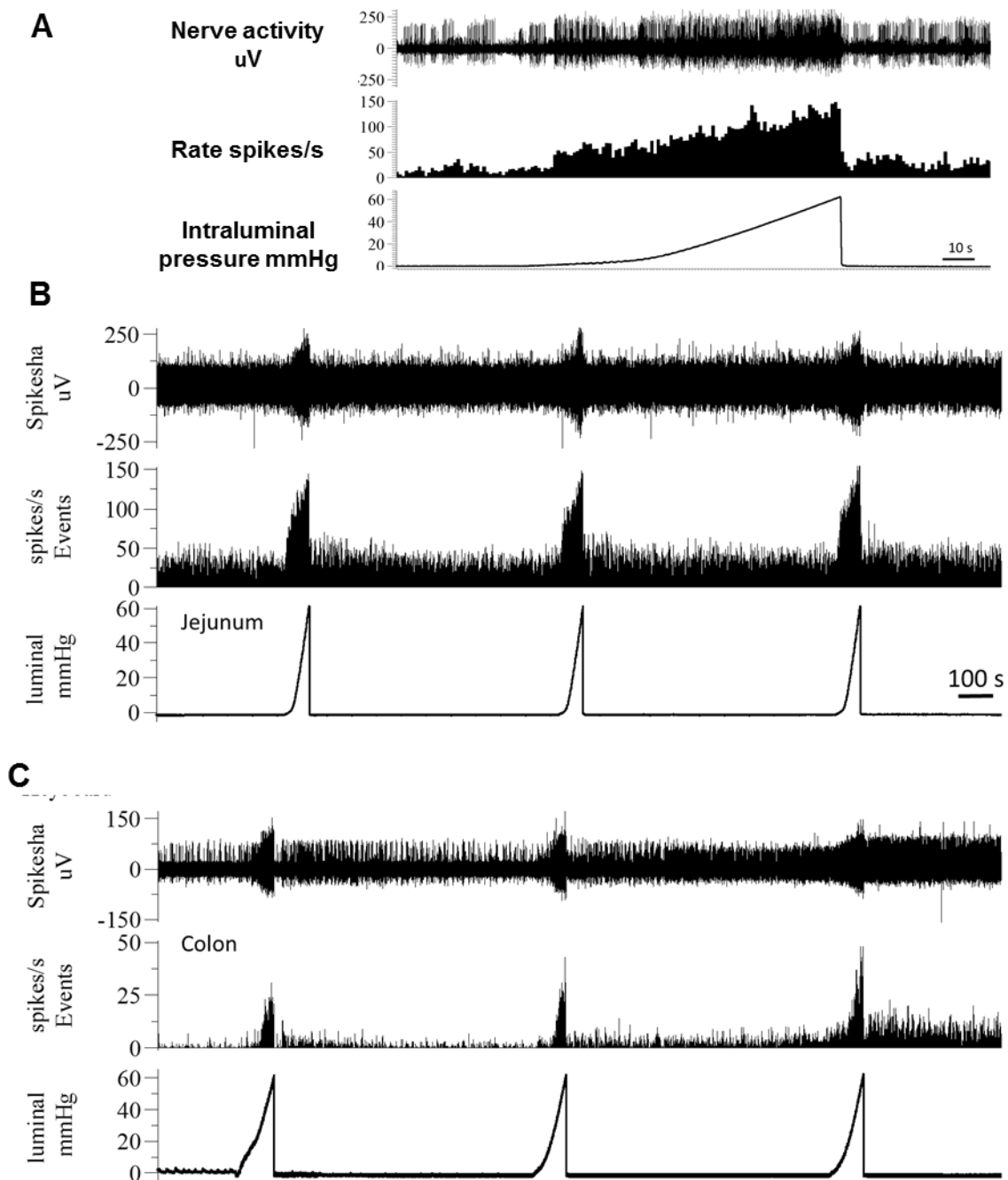
Figure 12 Example trace of nerve recordings from the jejunum and colon.

Ramp distension of the intestine evokes a biphasic increase of afferent nerve activity corresponding to the activation of low threshold and high threshold mechanosensitive afferent neurons. **A)** Each recording composes of 3 traces Top trace: raw recording of whole nerve activity. Second trace is nerve discharge rate and third one is distension response profile. **B)** recording of mesenteric nerve bundle innervating the jejunum and it can be seen that nerve activity is stable after 30 min and 3 reproducible distension is accomplished. **C)** recording shows that colonic nerve activity after 30 min stabilization, the nerve activity still exhibits changes and, therefore, needs longer time to be stable.

Jejunum
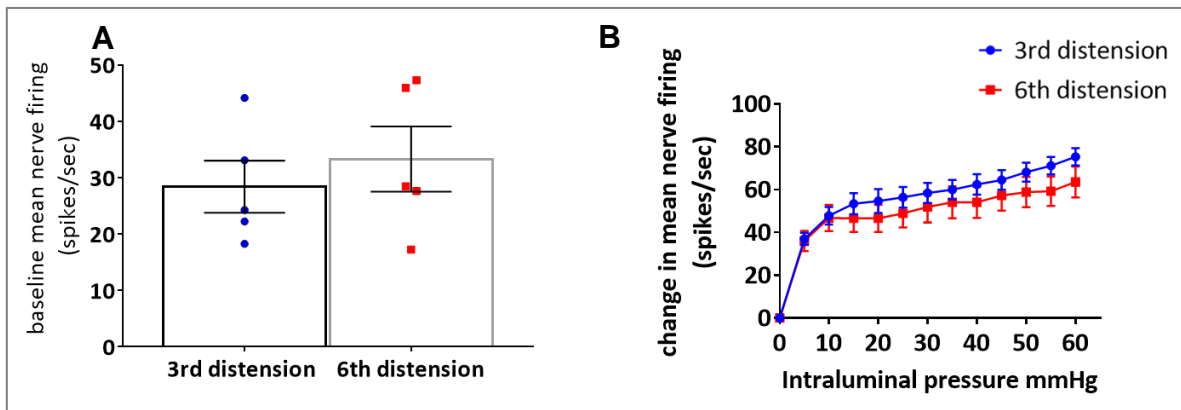
Colon
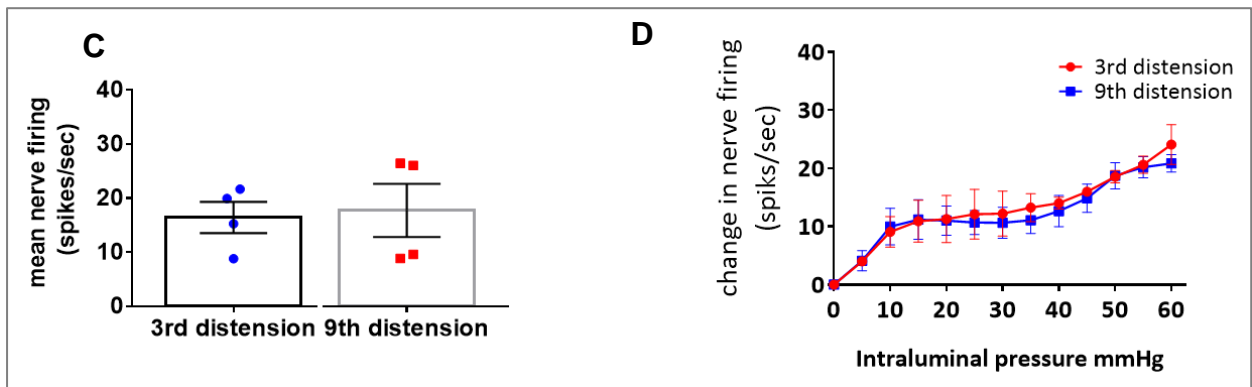
Figure 13 Nerve recording time control experiment

A) nerve recording from jejunum, N=5, mean baseline nerve activity measures before the start of the 3rd distension was not significantly different compared to baseline at the 6th distension. **B**) Pressure response profile was also unchanged at the end of experiment. **C & D)** the colonic nerve activity, N=4 spontaneous nerve activity and the response to distension was stable during 4 hours of recordings

3.3.2 Levels of ROS in culture are increased by H_2O_2 and antimycin A

Oxidative stress was induced in this study in one of two ways: hydrogen peroxide (H_2O_2) and antimycin A. H_2O_2 is one of the ROS that is excessively produced in inflamed tissue. Antimycin A is a mitochondrial inhibitor that causes an accumulation of superoxide in the tissue and increased oxidative stress. Concentrations were chosen based on previous data indicating that H_2O_2 in range from 100 to 1000 µM is related to oxidative stress (Schröder and Eaton, 2008). The same rationale for choosing antimycin A at (20 µM) concentration (Nesuashvili et al., 2013).

To determine if the chosen concentration was sufficient to produce oxidative stress, the oxidative marker Dihydroethidium (DHE) was used. DHE is a cell permeable dye that indicates intracellular ROS. The dye exhibits a blue-fluorescence in the cytosol until oxidized where it exhibits a bright red fluorescent colour. DHE can form two red fluorescent products, the first is 2-hydroxyethidium (2-OH-E$^+$) after being oxidized by cytosolic superoxide. The second is ethidium, formed by reactions with hydroxyl radicals ($^\bullet$OH) and peroxonitrite (ONOO$^-$). Ethidium interacts with the cell's DNA, staining its nucleus. The fluorescent spectra of 2-OH-E$^+$ and ethidium overlap, and therefore, cannot be distinguished (Wojtala et al., 2014). Therefore, I measured the total red fluorescence in the cells.

The fluorescent intensity was measured after treating Caco-2 cells with antimycin A and H_2O_2 (100 µM) which is the lowest concentration used in this study. Antimycin A (20 µM) produced a significant increase in the fluorescent intensity in the treated cells (14.32 ± 2.18) compared to control (6.27 ± 0.85), unpaired t-test, $P= 0.03$ (vehicle: 3 replicates, antimycin A: 5 replicates, Figure 14 A, B &E). H_2O_2 (100 µM) produced a greater increase

in the fluorescent intensity compared to the vehicle (6.28 ± 0.70 *vs* 27.54 ± 3.44, paired t-test, P =0.001, Figure 14 C, D &E).
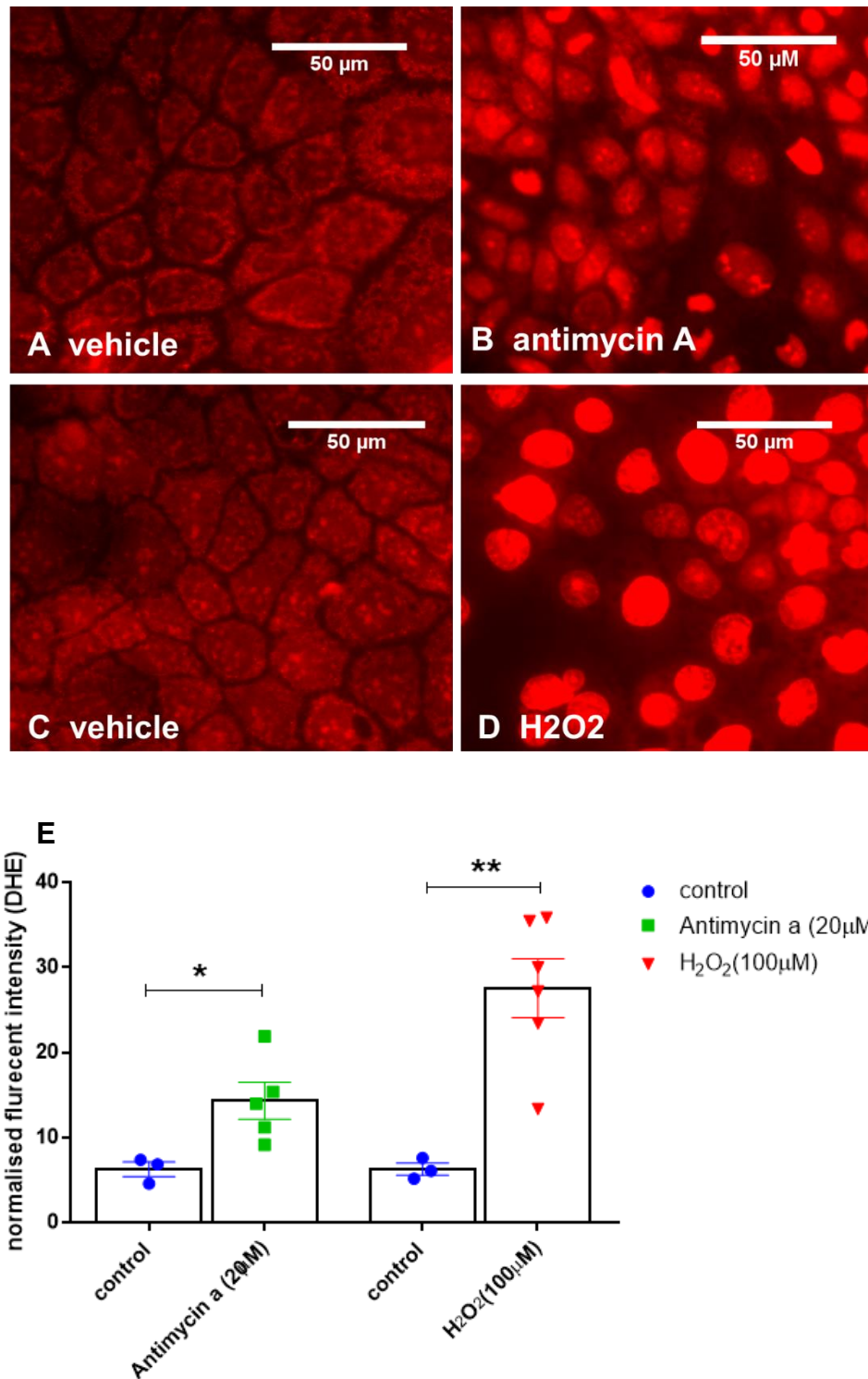
Figure 14 Levels of ROS in culture are increased by H_2O_2 and antimycin A

Images shows dihydroethidium (DHE) fluorescent intensity indicating intracellular ROS in Caco-2 cells. **A**) DHE indicating basal superoxide levels in vehicle (ethanol 0.01%) treated cells compared to (**B**) greater fluorescent intensity produced by antimycin A (20 μM). **D**) H_2O_2 (100 μM) produced a dramatic increase compared to (**C**) vehicle (H_2O). **E**) Histogram shows the calculated fluorescent intensity was higher by antimycin A (20μM) and H_2O_2 (100μM) (vehicle: 3 replicates, Unpaired t-test, * $P<0.05$, ** $P<0.01$).

3.3.3 Jejunum afferents activity is unchanged by intraluminal induction of oxidative stress.

Two concentrations of hydrogen peroxide low (100 µM) and high (500 µM) were applied into the lumen. Both low and high concentrations of H_2O_2 failed to produce any change in jejunal spontaneous nerve activity (paired t-test, P >0.05, N=5). The mechanosensitivity also was unchanged. Because the jejunum afferents resisted the chosen concentrations, I decided to examine an even higher concentration. H_2O_2 at (1000 µM) was also unable to produce any response in the jejunum (Figure 15).

Similarly, intraluminal application of antimycin A (20 µM) failed to trigger any response in the activity of the jejunal nerve as both baseline activity and response to distention remained unchanged after 30 min of application (Figure 16).
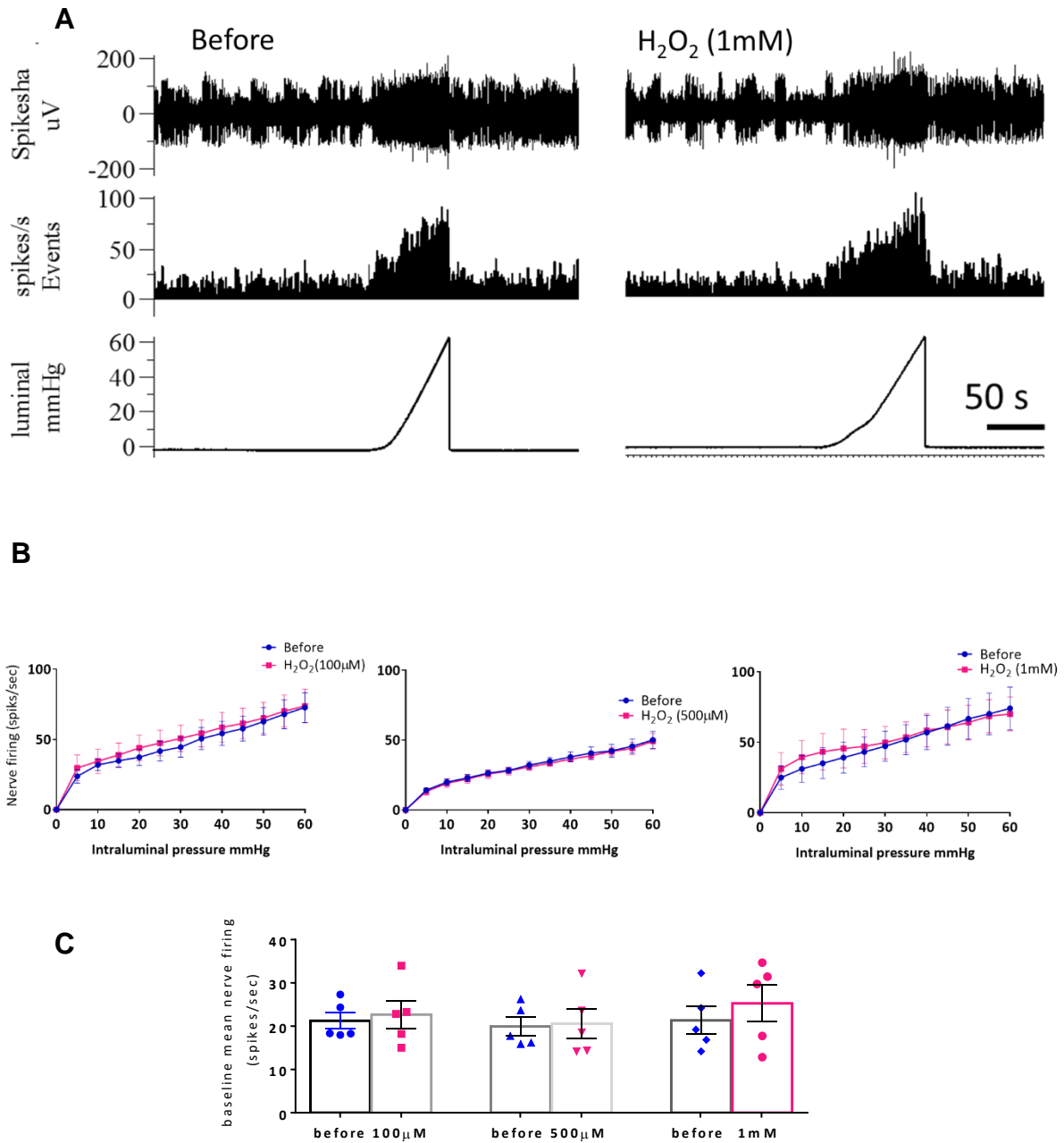
Figure 15 Jejunum nerve activity is unchanged by intraluminal application of H_2O_2

A) representative trace shows that even H_2O_2 (1mM) failed to produce any change on jejunum nerve activity. **B**) shows the unchanged pressure response profiles. Two-way ANOVA, $P>0.05$. **C**) histograms show unchanged spontaneous nerve activity after application of H_2O_2, paired t-test, $P>0.05$, N=5.
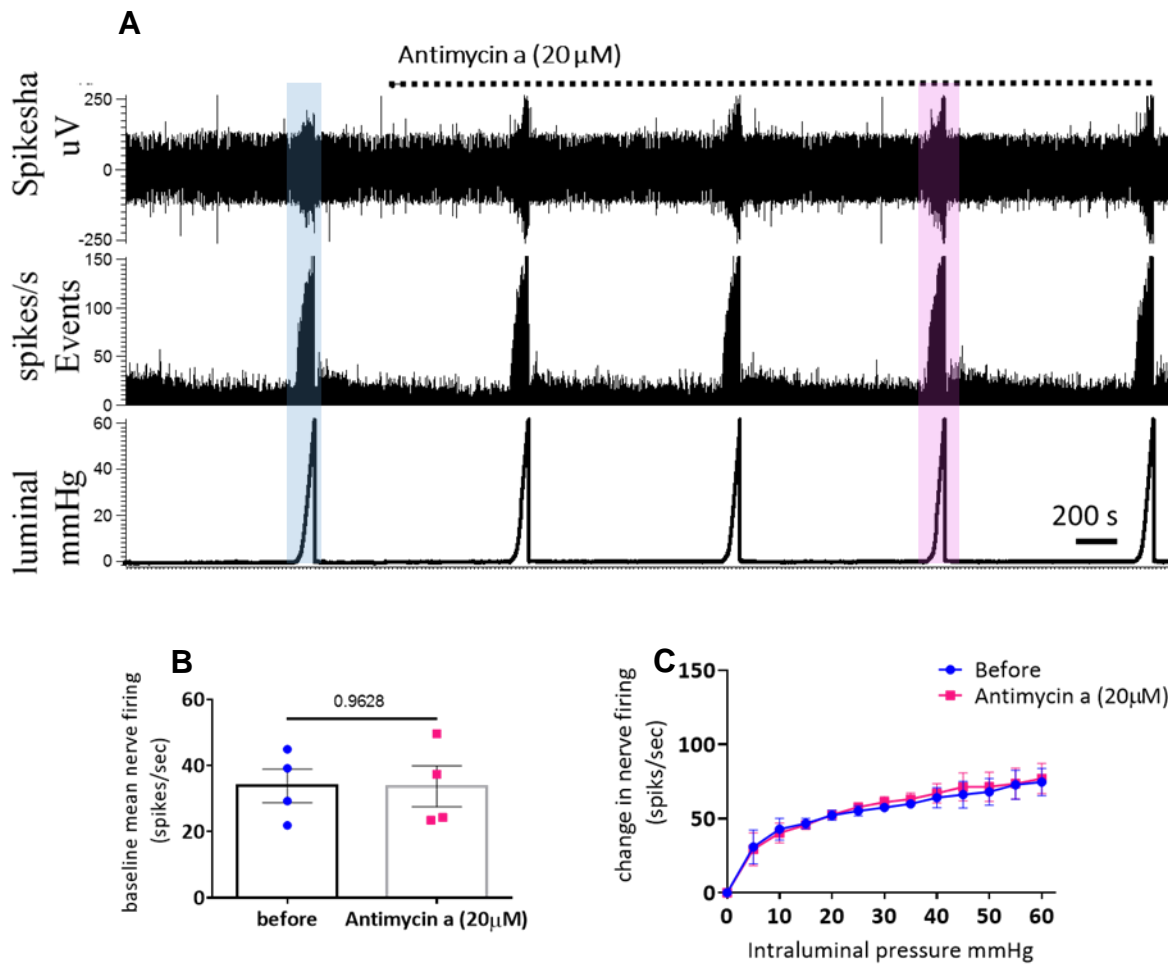
Figure 16 Jejunum afferents activity is unchanged by mitochondrial inhibitor

(**A**) Representative trace shows that intraluminal application of antimycin A (20 μM) did not produce any change in jejunal afferents (**B**) baseline nerve activity remained unchanged, paired t-test. **C**) response of distension was unchanged, Two-way ANOVA, N=4. Vehicle for antimycin A is ethanol (0.00025%).

3.3.4 Colonic mechanosensitive afferents are activated by intraluminal induction of oxidative stress

Unlike the jejunum, the colon was sensitive to even the lowest concentration of H_2O_2 which produced a significant increase in the colonic afferent mechanosensitivty, (two-way ANOVA, $P<0.05$, N=5). The H_2O_2-mechanohyperactivity was observed in multi unit nerve activity at high level of distension and was confirmed by single unit analysis. The spontaneous activity was significantly enhanced only by 1000 μM from 14.30 ± 0.65 to 20.38 ±1.77 spikes/s, paired t-test, $P < 0.01$, N=5 (Figure 17).

In addition, antimycin A 20 (μM) produced a significant increase in colonic spontaneous nerve firing from 14.43 ± 4.33 to 23.08 ± 5.78 spikes/s, paired t-test, P=0.018, N=6. The maximum effect was developed after 30 min of application. The response to distension was also enhanced after application of antimycin A and the significant effect was observed from 20 mmHg and single unit analysis showed a significant increase in the activity of WDR and HT afferents (Figure 18 A,B,C&D). This change was found to occur without any significant alteration in colonic compliance and total nerve activity immediately after distension which were unaffected after application of antimycin A Figure 18 E&G). This result indicates that the colon is more susceptible to oxidative stress.
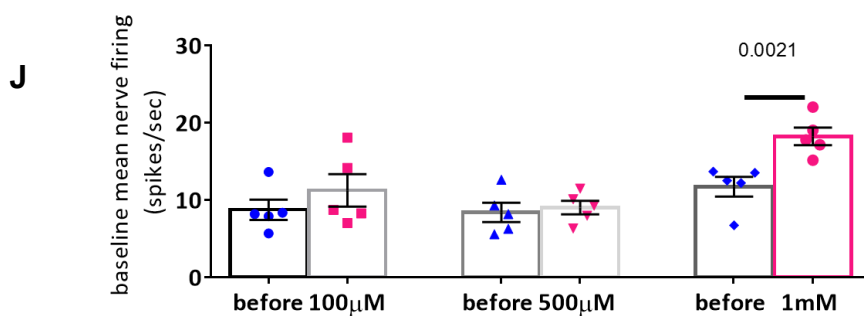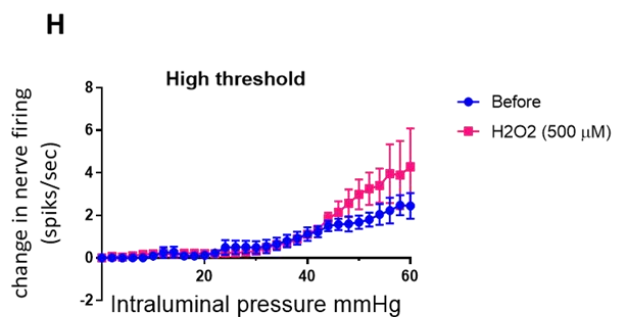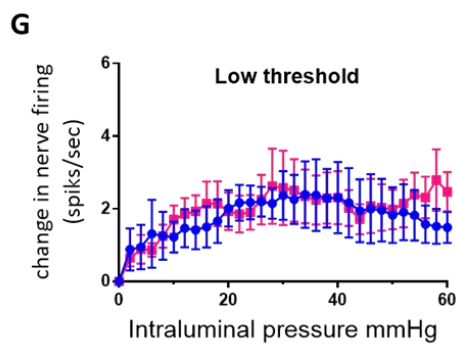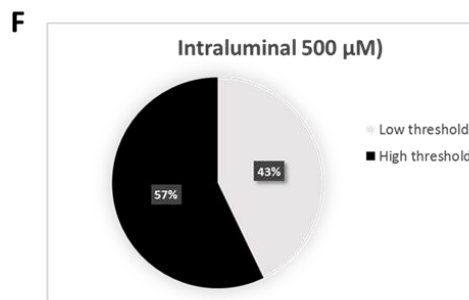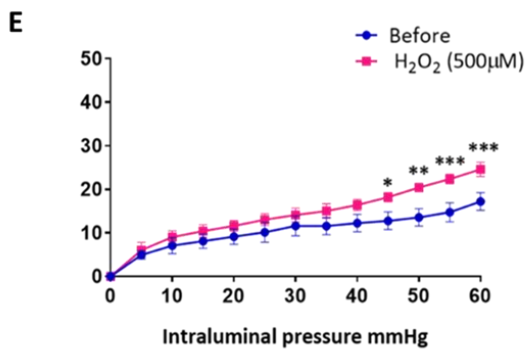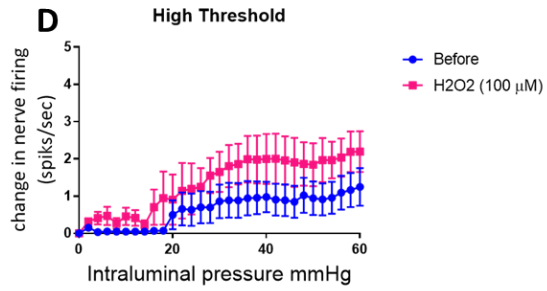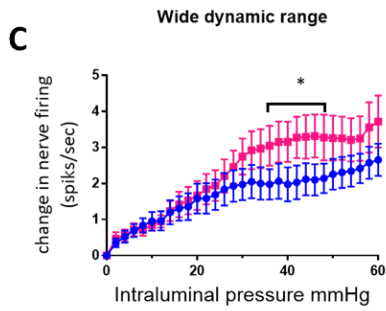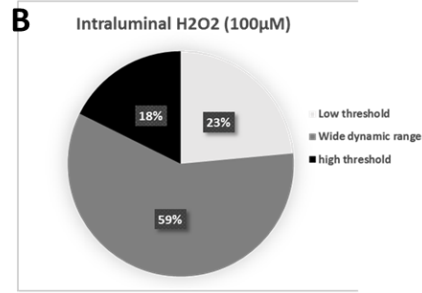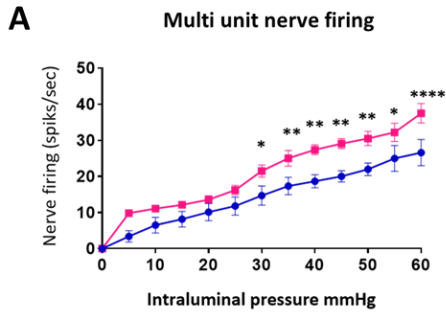
Figure 17 Colonic afferents are more sensitive to intraluminal H_2O_2

A) Pressure response profile of multi unit nerve activity shows that H_2O_2 (100μM) produced a significant increase in mechanosensitivity which was significant at high level of distensions, Two-way ANOVA, N=5. **B**) The relative percentages of unit subtypes identified (N=5, n=17). **C&D**) Curves shows significant difference was seen in WDR afferents (n=10) but not in HT (n-=3). **E**) H_2O_2 (500μM) produced a significant increase in multi unit nerve activity that was significant at high level of distensions, Two-way ANOVA, N=5. **F**) The relative percentages of unit subtypes identified (N=3, n=7). **G&H**) Curves shows no significant difference in LT (n=4) and HT afferents (n-=3), the number of units was not enough to perform statistical analysis. **J**) histograms show that significant change in spontaneous nerve activity was only observed in response to H_2O_2 (1mM), paired t-test, N=5. *, $P<0.05$. **, $P<0.01$. ***,$P<0.001$.
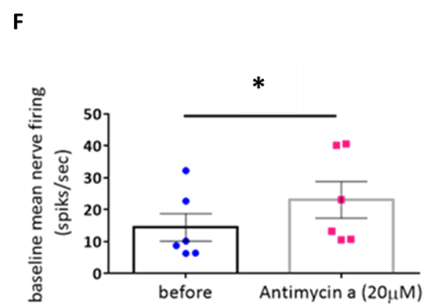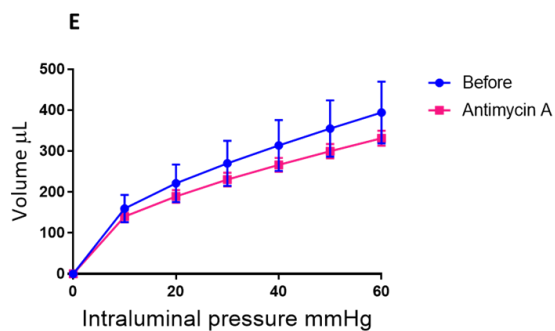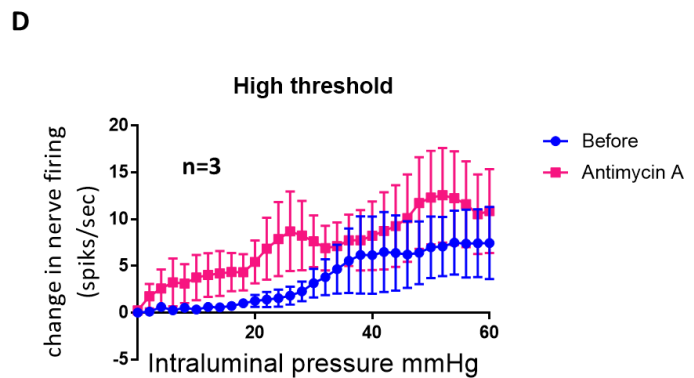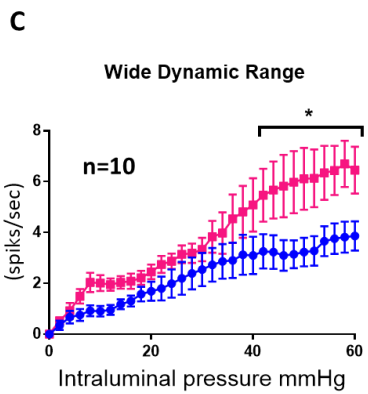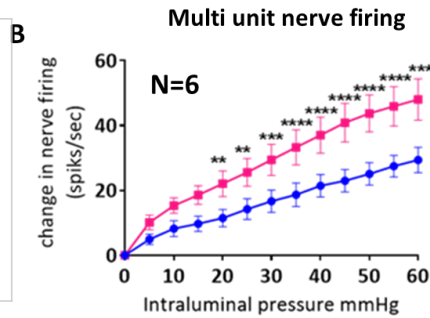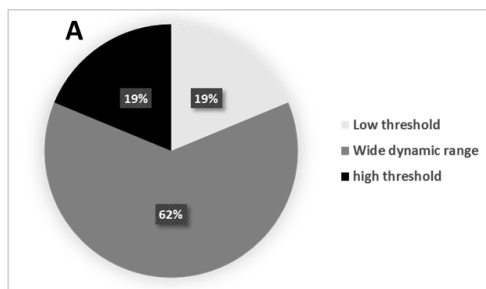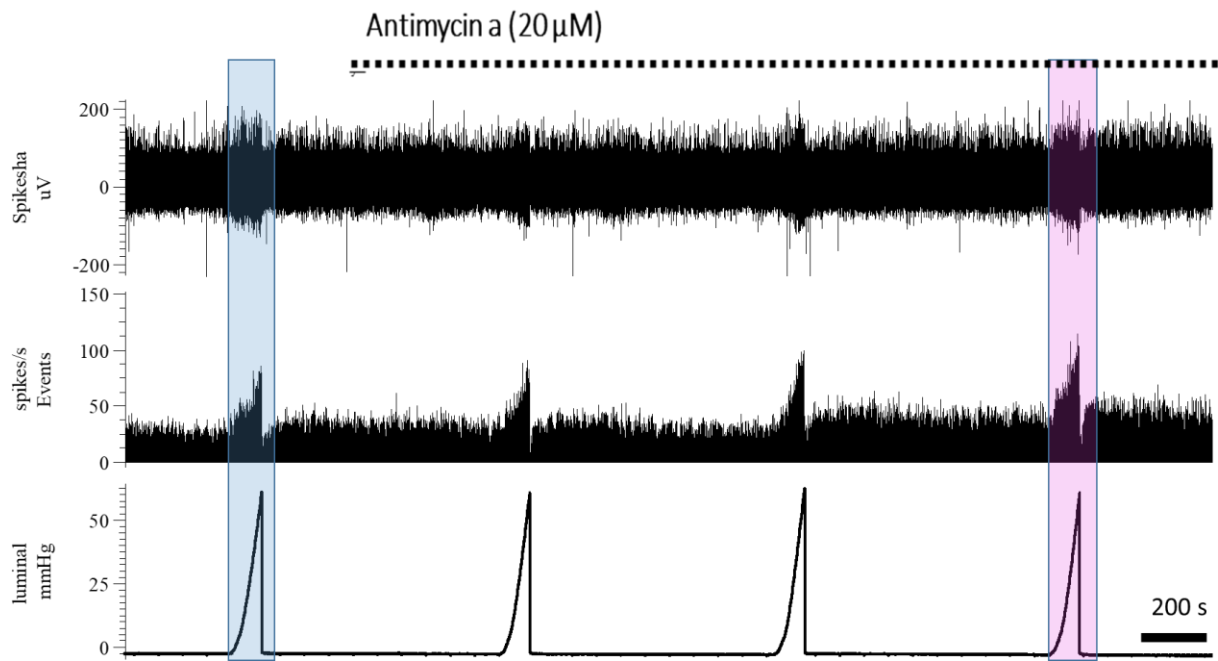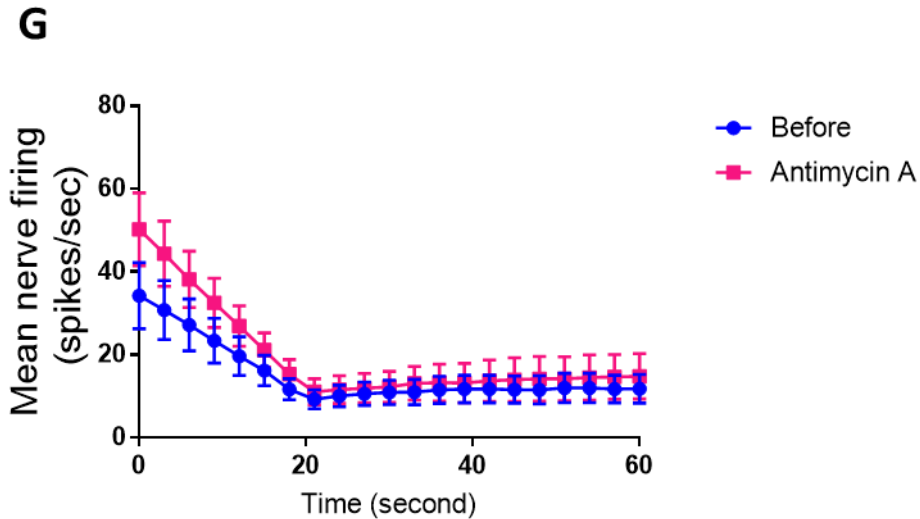
Figure 18 Colonic afferents are more sensitive to mitochondrial inhibitor.

On the TOP: Representative trace shows that antimycin A (20 µM) produced a gradual increase on colonic nerve activity which developed within 30 min after application. Blue area is where control measurements were taken and compared to purple area. **A**) The relative percentages of unit subtypes identified (N=6, n=16). **B**) Pressure response profile of the multi unit nerve activity shows that significant difference was seen at ≥20 mm Hg (Two-way ANOVA N=6.). (**C&D**) Curves shows significant difference was seen in WDR afferents (n=10) but not in HT (n-=3). **E**) The relationship between the perfusion volume and the intraluminal pressure of the colonic segment did not differ by antimycin A. **F**) Histograms shows that baseline activity was enhanced, paired t-test. **G**) Curve shows that the total mean firing following the end of distension was not changed by antimycin A.

*, $P<0.05$. **, $P<0.01$. ***,$P<0.001$. Vehicle for antimycin A is ethanol (0.00025%).

3.3.5 Possible reasons for the difference in response between jejunum and colon

The absence of response in the jejunum could be due to the difference in the mucosa barrier or antioxidant level between the jejunum and colon. The mucosa in the jejunum is more fragile and easily damaged during repeated distensions. Therefore, the lack of response in the jejunum could be due to a disruption of the mucosa layer. To eliminate this possibility, H_2O_2 was perfused into the lumen of jejunum without performing any distension. H_2O_2 (500 µM) applied for 30 min had no significant effect on the spontaneous nerve activity, $P > 0.05$, Two-way ANOVA, N=5, (Figure 19).

The next step was determining the levels of antioxidants in both regions. The protein expression of antioxidant SOD2 in the mucosal cells from the jejunum and colon was determined by western blot (Figure 20). The protein density normalised to GAPDH was 0.09 ± 0.01 in the jejunum and 0.10 ± 0.05 in the colon which was not significant (unpaired t-test, P=0. 0.85). It must be mentioned that a faint band were detected at 26 KD in samples from colon and jejunum. The band was more intense in the jejunum samples and was not detected in the control (brain) sample. The protein is encoded by SOD2 gene and alternative splicing of this gene results in multiple transcript variants from the same protein family which could be the reason behind the multiband that was observed. However, no confirmation experiment was performed.

The negative response to H_2O_2 and antimycin suggested that mucosa in the jejunum respond differentially than in the colon to oxidative stress. Therefore, in order to overcome the mucosal barrier and examine the response of sensory neurons directly, H_2O_2 was applied to the organ bath where it can be in direct contact with serosa and muscle afferents.
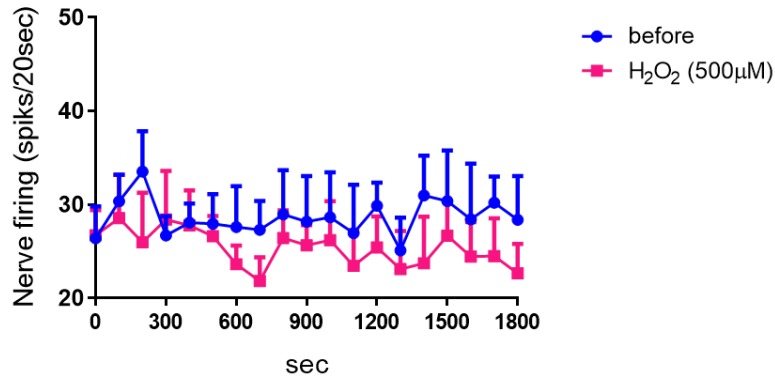
Figure 19 Jejunum spontaneous nerve activity is unchanged by H₂O₂

Representative trace shows that intraluminally H_2O_2 (500 μM) failed to produce a significant change in jejunum baseline nerve activity. Second graph shows a non-significant reduction in nerve activity Two-way ANOVA, N=5.
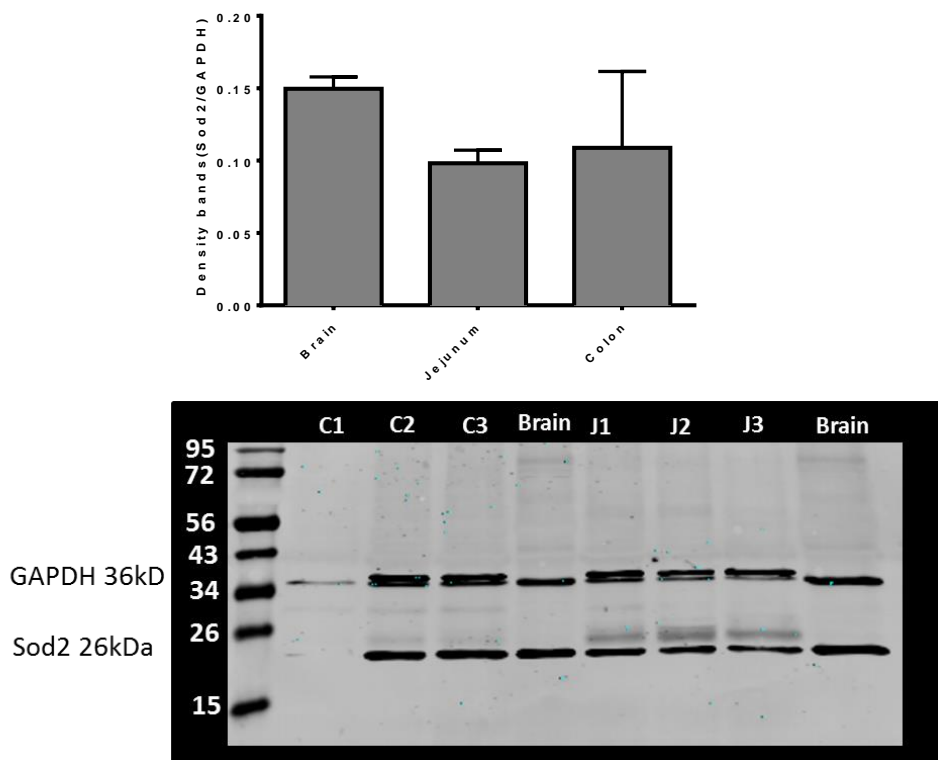
Figure 20. SOD2 protein level is the same in the jejunum and colon.

On the top: Histogram shows that the measured levels of SOD2 in the mucosal cells in mouse jejunum and colon was not significantly different ($P = 0.85$, unpaired t-test, N=3). Second: original western blot shows the intensity band which was not significantly different between jejunum and colon. The brain lysate was used as a positive control to assess the right band's size. C1, C2 and C3 are samples from colon. J1, J2 and J3 are samples from jejunum.

3.3.6 Only spontaneous nerve activity of jejunum afferents is activated by bath application of H_2O_2

Application of H_2O_2 (100 μM) directly to the bath produced an increase in spontaneous jejunal nerve activity but not in mechanosensitivity. The baseline nerve firing increased from 25.82 ± 4.06 to 33.81 ±7.55 spikes/s, paired t-test, p= 0.05, N=5. High concentration of H_2O_2 (500 μM) failed to produce any change in mechanosinsitivity in the jejunum, but the spontaneous nerve activity again increased from 22.95± 2.34 to 28.15 ± 2.69 spikes/s, paired t-test, P=0.04 (Figure 21).

3.3.7 Colonic afferents are activated by bath application of H_2O_2

In the colon, a small increase in baseline nerve activity was observed after H_2O_2 (100 μM) (15.22 ± 2.76 to 18.74 ± 2.64, paired t-test, P= 0.01, N=6). Colonic mechanosensitivity was unchanged, two-way ANOVA, P> 0.05. H_2O_2 (500 μM) produced a significant increase in colonic mechanosensitivity. The increase in nerve firing was observed at ≥ 20 mmHg mechanosensitive nerves and in spontaneous nerve activity (12.65 ± 2.34 to 23.53 ± 3.48 s. paired t-test, P=0.03, N=6). Single unit analysis indicated an increase in WDR afferent after application of H_2O_2 (100 and 500 μM) and the number of HT afferent was not enough to perform statistic test, see Figure 22.
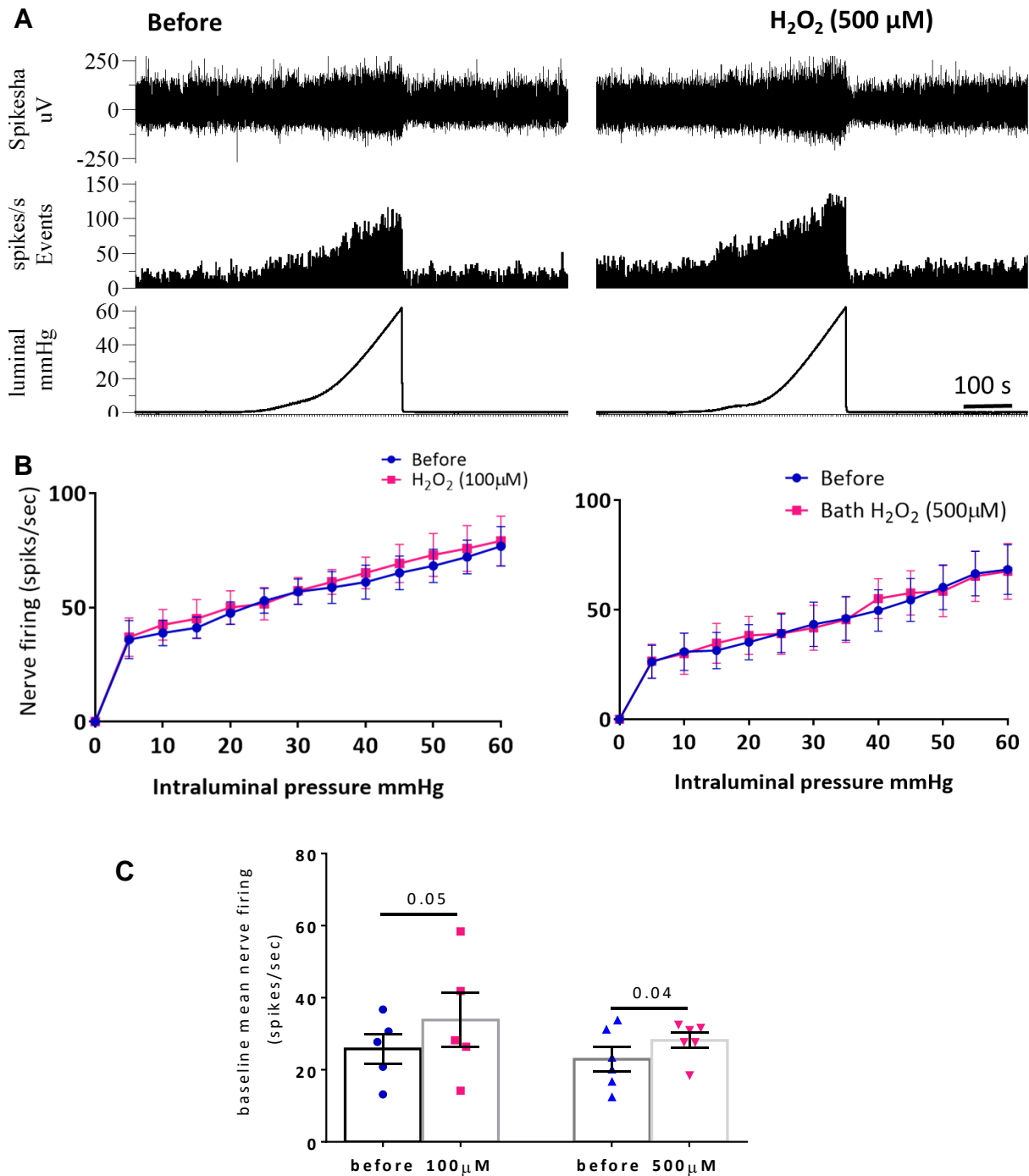
Figure 21. Increased spontaneous nerve activity in the jejunum by bath application of H₂O₂

A) example trace shows that H₂O₂ (500 μM) produced an increase on jejunum baseline nerve activity, but mechanosensitivity was not significantly changed. B) graph shows non-significant change in the response to pressure profile by H₂O₂ (100 μM N=5) and (500 μM, N=6), Two-way ANOVA. C) histograms show that mean baseline activity significantly increased after H₂O₂, paired t-test.

A Multi unit nerve firing

B Extraluminal H2O2 (100μM)
- Low threshold
- Wide dynamic range
- high threshold

C Wide dynamic range

D High threshold
- Before
- H2O2 (100 μM)

E Multi unit nerve firing

F Extraluminal H2O2 (500μM)
- Low threshold
- Wide dynamic range
- high threshold

G Wide dynamic range

H High threshold
- Before
- H2O2 (500 μM)
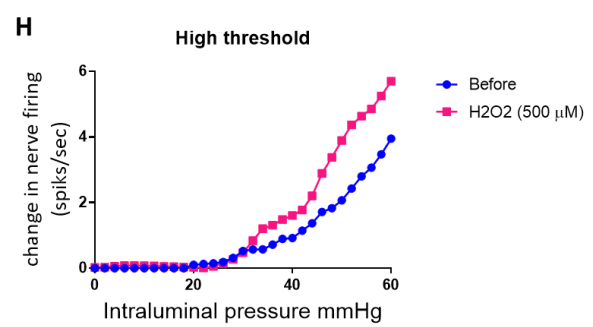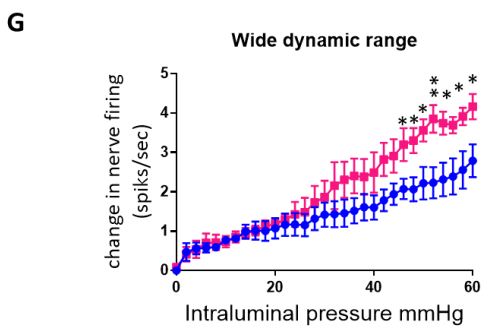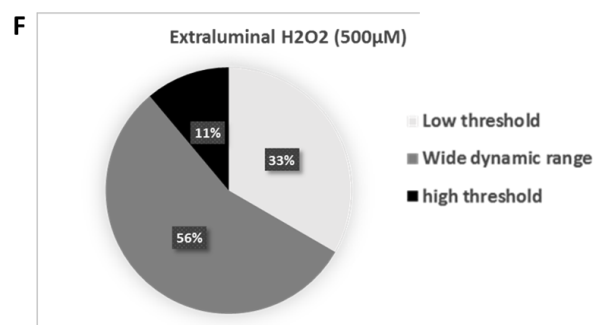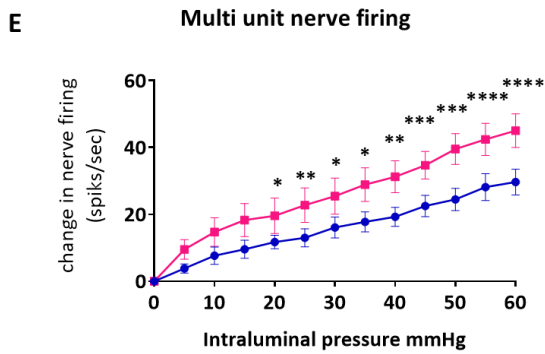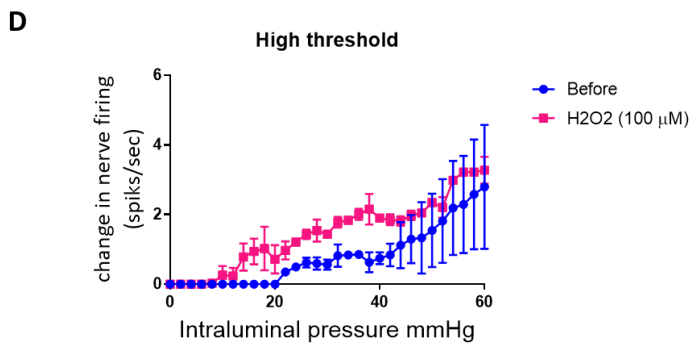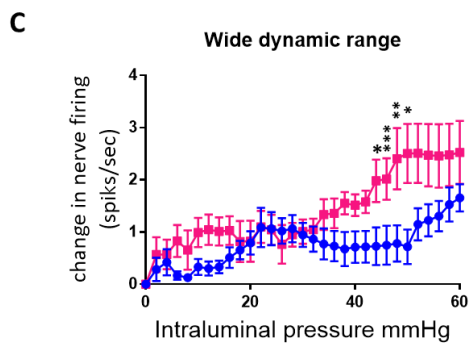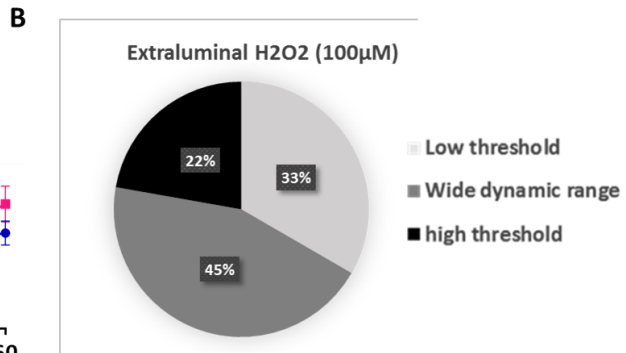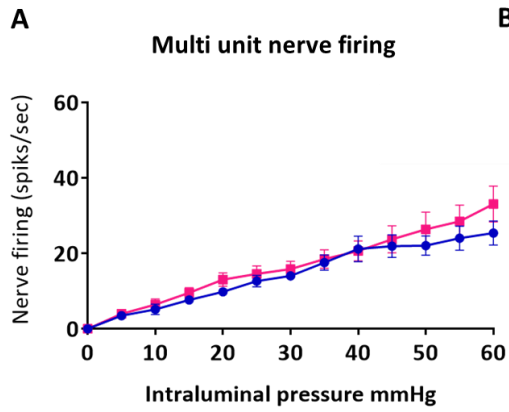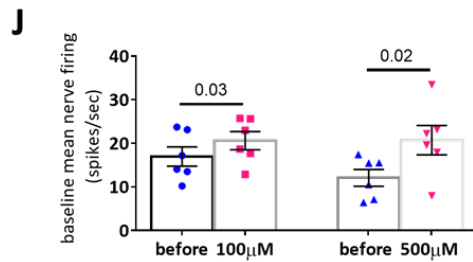
Figure 22 Increased colonic nerve activity by bath application of H₂O₂

(**A**) Pressure response profile of multi unit nerve activity shows that H_2O_2 (100µM) had no change on mechanosensitivity, Two-way ANOVA, N=5. **B**) The relative percentages of unit subtypes identified (N=3, n=9). **C&D**) Curves shows significant difference was seen in WDR afferents (n=4). HT afferents were only n=2. **E**) H_2O_2 (500µM) produced a significant increase in multi unit nerve activity that was significant at high level of distensions, Two-way ANOVA, N=5. **F**) The relative percentages of unit subtypes identified (N=5, n=9). **G&H**) Curves shows significant difference in WDR afferents (n=5), HT unit was only n=1. **J**) Histograms show significant change in spontaneous nerve activity in response to H_2O_2 , paired t-test, N=5. *, $P<0.05$. **, $P<0.01$. ***,$P<0.001$.

3.3.8 Jejunum muscle contractions are increased by H₂O₂ (100 µM) and abolished by (500 µM)

Because H_2O_2 hyperactivity was observed in the spontaneous nerve activity, I decided to examine if the effect was secondary to a change in muscle contractions. In the jejunum, the contractions and nerve recording was measured in the same experiments. H_2O_2 was perfused directly to the bath for 30 minutes after contractions had stabilized.

A low concentration of H_2O_2 produced an increase in the frequency of contraction reflected by decrease in the time of intervals between them. The intervals decreased from 191.7± 1.05 to 78.49±7.55 s, paired t-test, P= 0.006, n=4. The increase in the peak

contraction was not significant (1.81±0.31 to 2.91±0.55 mmHg, paired t-test, P= 0.12, N=4). The change in baseline pressure was also not significant (Figure 23).

H_2O_2 (500 μM) produced a strong and complete inhibition of contractions in the jejunum concurrent with significant increase in afferent activity, paired t-test, $P < 0.04$, N=4 (Figures 24). The inhibition developed within 10 min of application and continued after washing with no sign of contractions returning.
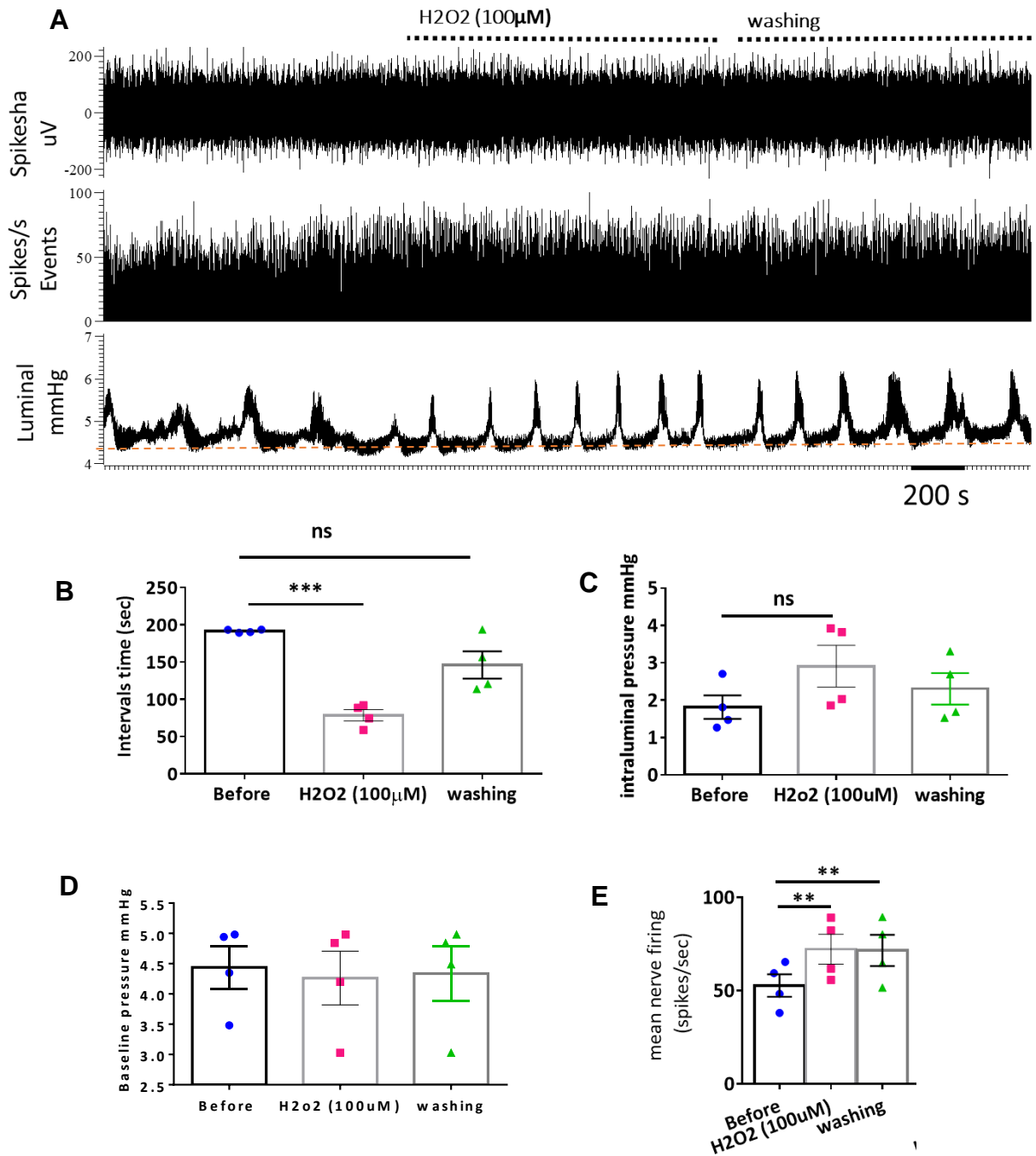
Figure 23 Increased jejunum muscle contractions and nerve activity by bath application of H₂O₂ (100 μM)

A) representative trace shows that H_2O_2 (100μM) applied directly to the organ bath produced an increase in nerve activity combined with an increase in jejunum contractions. **B)** histograms show that intervals between contractions decreased, which reflects increased contractions, the contractions recovered after washing. **C)** the effect on the amplitude of intraluminal pressure was not significant. **D)** H_2O_2 had no effect on baseline pressure. **E)** H_2O_2 produced a significant increase on spontaneous nerve activity. **, $P<0.01$, ***, $P< 0.001$, One way ANOVA. N=4.
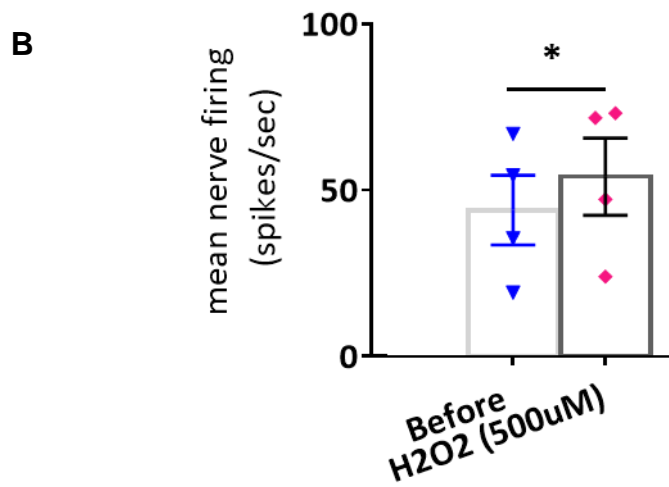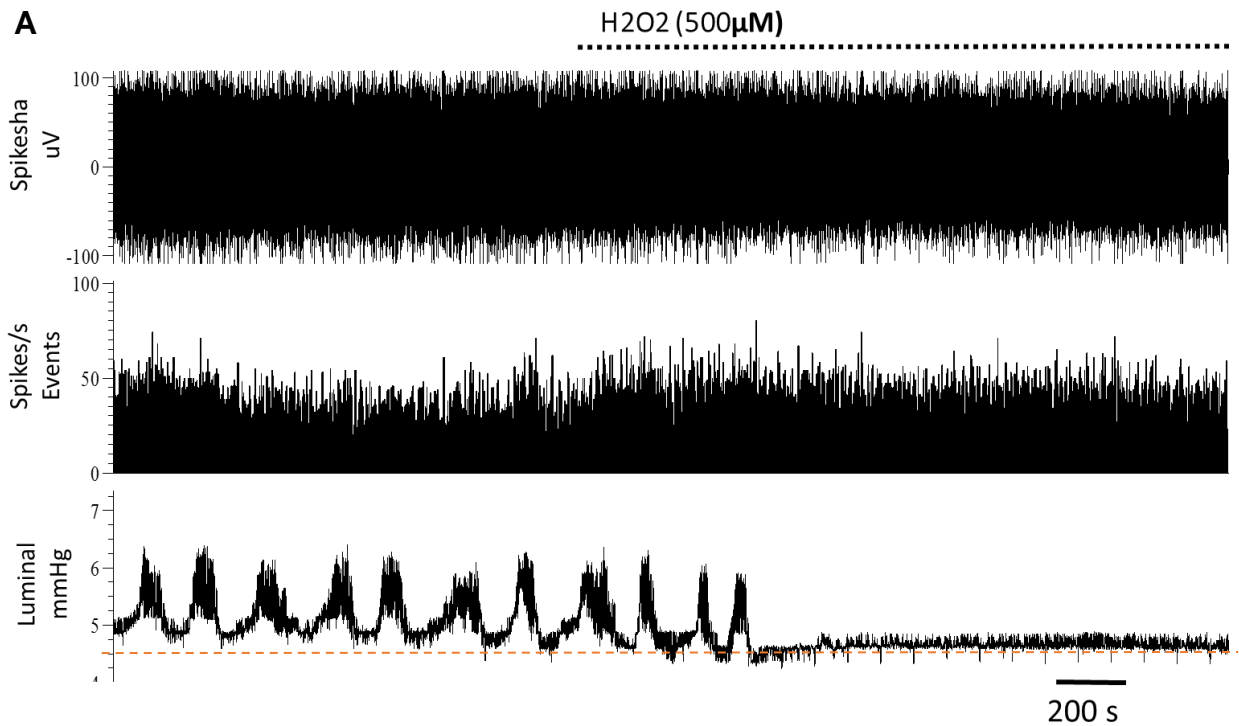
Figure 24 Abolished contractions and increased jejunum afferents spontaneous activity by H_2O_2 (500 µM).

A) representative trace shows that H_2O_2 (500 µM) applied directly to the organ bath produced a complete inhibition of jejunum contractile activity combined with (**B**) nerve hyperactivity, paired t-test, *, $P < 0.05$, N=4**.**

3.3.9 Colonic muscle contractions are inhibited by H_2O_2

The colonic contractions exhibit higher peaks and longer duration than in the jejunum. Therefore, it was difficult to maintain intact suction necessary for nerve recording in the colon. Accordingly, the colonic contractions study was performed on separate experiments using the same setup as nerve recordings preparation excluding only the nerve output.

The low concentration of H_2O_2 (100 μM) induced an inhibition on the peak of colonic contractions and increase in time intervals between them. The amplitude decreased from 10.71± 2.13 to 3.44 ± 0.60 mmHg (paired t-test, P=0.01, N=4). The inhibition did not recover after washing for 30 minutes. The baseline was significantly increased from 0.96 ± 0.44 to 2.02 ± 0.43 mmHg and did not recover after washing (2.02 ± 0.44 mmHg), paired t-test P= 0.03, N=4. The increase in time between contractions was not significant (from 93.20 ± 16.75 to 123.4 ± 15.65 s, paired t-test, P = 0.22, N=4). The effect of H_2O_2 was developed within 10 min of application (Figure 25). H_2O_2 at (500 μM) completely abolished the muscle contractions and the effect was immediate after application (Figure 26).
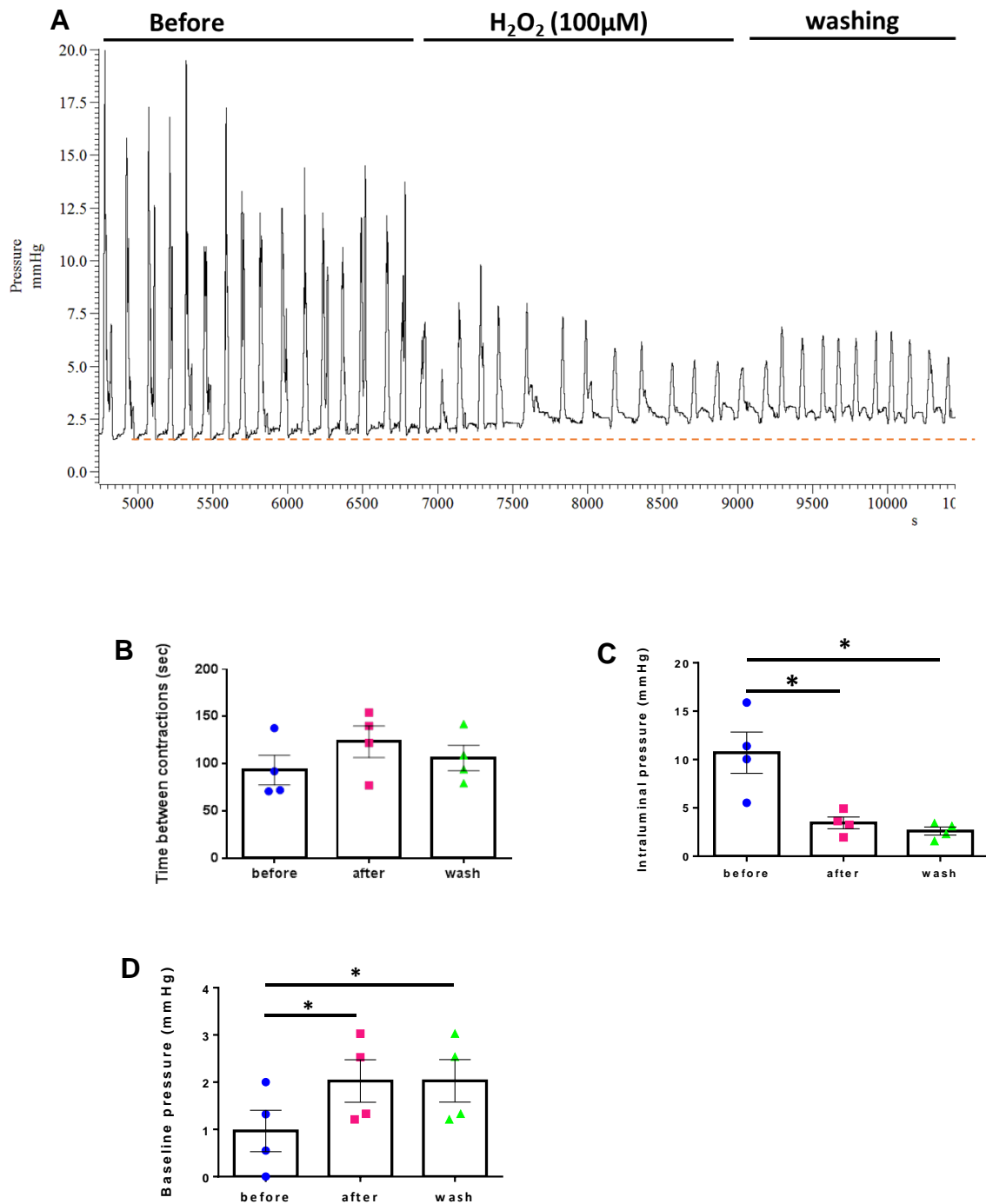
Figure 25 Decreased colonic muscle contractions by H$_2$O$_2$ (100 μM)

In **A**: representative trace shows that H$_2$O$_2$ (100μM) applied directly to the organ bath produced a reduction on the colonic contractions. **B:** decrease intervals between contractions was not significant. **C:** The amplitude of intraluminal pressure was significantly reduced and did not recover after washing. **D:** H$_2$O$_2$ produced an increase on baseline pressure which continued after washing (*, $P<0.05$, One way ANOVA). N=4.
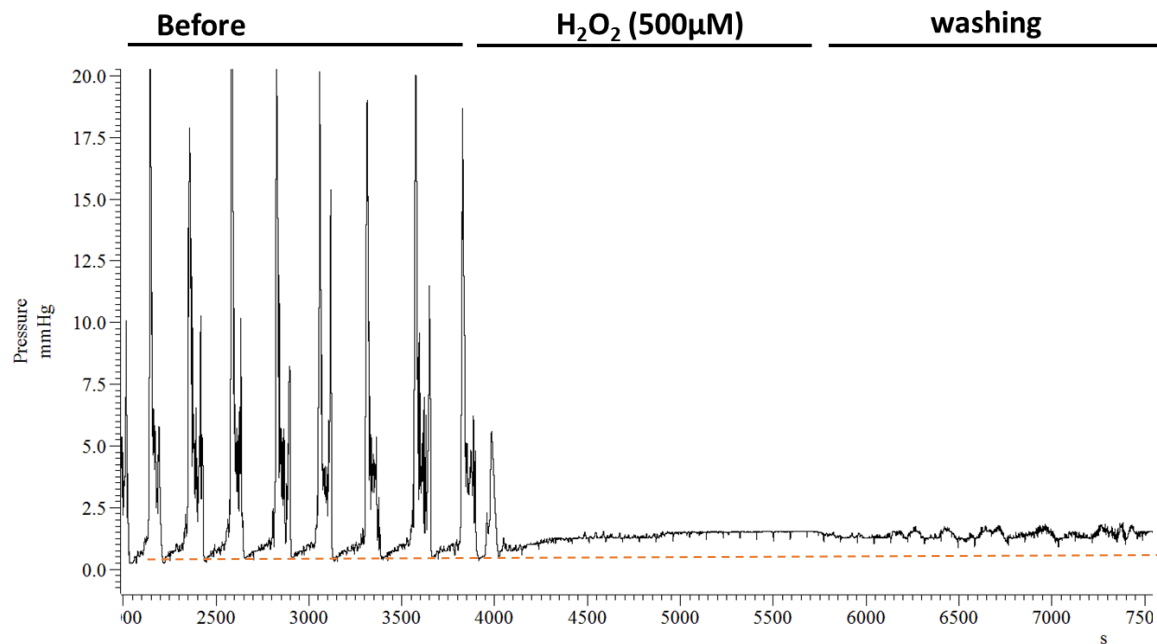
Figure 26 Abolished colonic muscle contractions by H₂O₂ (500 μM)

Representative trace shows that H₂O₂ (500 μM) applied directly to the organ bath produced a complete inhibition of colonic contractions, N=4.

3.3.10 TRPA1 antagonist blocks response to antimycin A in WDR and HT afferents

TRPA1 channel is an important sensor for several products of oxidative stress (Andersson et al., 2008). The information about the role of TRPA1 channel in oxidative stress-mediated hyperactivity on intestinal afferents is lacking. Therefore, to examine the potential role of TRPA1 in oxidative stress-mediated hyperactivity, TRPA1 blocker HC030031 was used in this study and the experiments were performed using 3-month animals.

Data presented in Figure 27 (A) shows that HC030031 (30 μM) blocked the antimycin A hyperexcitability after 30 mmHg pressure, which represents the high-threshold component of mechanosensitive colonic afferents. (Figure 27 C&D). The blocker failed to reduce the baseline-nerve hyperactivity in response to antimycin A (20μM) (One-way ANOVA. *, $P < 0.05$ & **, $P < 0.01$, N = 6), which suggests that other channels or mediators are involved in the response to oxidative stress.

Next, I compared the response to antimycin A with and without HC030031. Figure 27, (F&G) shows the inhibition of antimycin A response in the presence of HC030031 in both WDR (F= 1.8, $P<0.01$) and HT afferents (F=6.5, $P< 0.0001$).

A Multi unit nerve firing

B Normalised mean nerve firing (spikes/sec)

0.03

before
HC030031 (30µM)
Antimycin A

C Wide dynamic range

n=5

D High Threshold

n=6

- Before
- Hc030031
- HC030031+ Antimycin A

E Multi unit nerve firing

F Wide dynamic range

n=10

P<0.007

n=4

G High threshold

n=3

P<0.0001

n=6

- Antimycin A
- HC030031+ Antimycin A
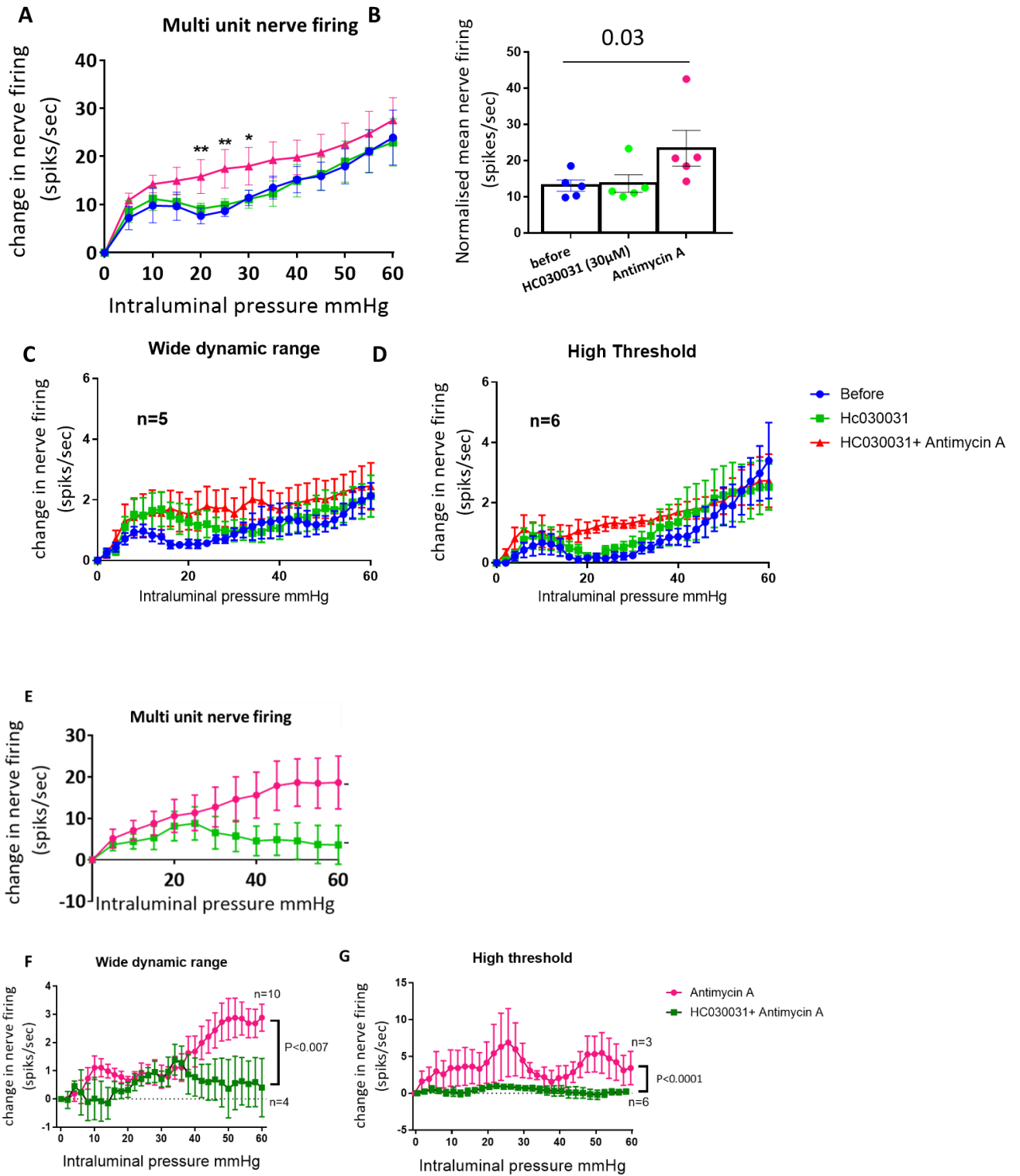
Figure 27: Effect of TRPA1 antagonist on antimycin A-response

A) pressure reponse profile of multi unit nerve activity shows that HC030031 (30 µM) was able to block the effect of antimycin A at very high levels of distensions (≥ 35 mmHg) **B)**: Significant response to antimycin in baseline-nerve activity was still observed after HC030031 (30 µM) (baseline nerve activity: paired t-test. Response to pressure: Two-way ANOVA. *, $P < 0.05$ & **, $P < 0.01$, N = 6). **C&D)** curves shows that Antimycin A in the presence of HC030031 had no significant effect on WDR and HT afferents. **E**) Curve shows the mutli unit nerve response to antimycin A (red line) compare with antimycin A+ HC030031 (green). (**F&G**): Curves shows the inhibition in antimycin A response in the presence of HC03003 in both WDR and HT afferents.

Pressure response profile (Two-way ANOVA, $P > 0.05$, N = 6). Vehicle for antimycin A is ethanol (0.00025%), for HC030031 is DMSO (0.002%)

3.4 Discussion

Inflammation causes excessive production of ROS and chronic inflammatory diseases such as IBD has been described as an "oxyradicals overload" disease (Hussain et al., 2003). H_2O_2-induced oxidative stress has been shown to cause afferent hypersensitivity in many organs such as heart, lung, kidney and bladder (Ustinova and Schultz, 1994, Lin et al., 2015, Nocchi et al., 2014, Nicholas et al., 2016). However, there are no reports about the effect of oxidative stress on sensory function from the intestine and whether there is a regional difference in response to oxidative stress between the small intestine and colon.

The current study demonstrates that induction of oxidative stress into the intestine mucosa by H_2O_2 at concentrations within the range that has been reported to be relevant to inflammatory conditions (Schröder and Eaton, 2008) evokes irreversible nerve hyperactivity of mechanosensory afferents fibres of the mouse colon. Luminal mitochondrial ROS (provoked by Antimycin A) also strongly activates colonic afferents. Activation of colonic-afferent mechanosensitivity induced by antimycin A is reduced by the TRPA1 channel antagonist. The jejunum exhibits a continuous resistance to oxidative stress as response to luminal H_2O_2 and antimycin A are absent in the jejunum afferents despite levels of the antioxidant protein SOD2 being similar in both tissues.

In addition, the study showed that extra-luminal H_2O_2 enhances spontaneous nerve activity of jejunum and colonic afferent fibres, and that colonic mechanosensitive fibres are more susceptible to H_2O_2 induced oxidative stress. Extra-luminal H_2O_2 causes an increased contractile response in the jejunum while it is inhibiting colonic contractions.

Induction of oxidative stress

In the present study, I used brief exposures of H_2O_2 and antimycin A to induce oxidative stress. H_2O_2 concentrations higher than 10 μM and up to 1 mM have been shown to be associated with oxidative stress. I confirmed here that H_2O_2 (at the lowest concentration chosen for this study) and antimycin A (20 μM) were both able to produce an increase in ROS production by using a marker of oxidative stress (DHE) on cultured Caco-2 cells. Ideally, this experiment would be performed using whole tissue sections on slides, but live tissues cannot tolerate incubation time and washing without wearing down the slide, therefore the cell culture was chosen as it handles the protocol very well. The DHE dye is used to stain the intracellular ROS and studies have used DHE staining as an oxidative marker (Nocchi et al., 2014, Roberts et al., 2013). *Nocchi et al* showed that treating mouse cultured urethelial cells for 10 min with H_2O_2 (0.003% =~300 μM) caused a significant increase in DHE intensity compared to control cells. *Roberts at al* used DHE (2 μM) to examine oxidative stress levels in TNBS-induced colitis and indicated that inflamed tissue had significantly higher DHE fluorescent intensity compared to control tissues. My data showed a very strong DHE fluorescent intensity observed in treated cells compared to vehicles which confirmed that H_2O_2 (100μM) and antimycin A (20 μM) both triggered oxidative stress.

A very common criticism of exogenous H_2O_2 application is that it does not mimic its endogenous generation. However, H_2O_2 does accumulate extracellularly in tissues (Dröge, 2002, Cao et al., 2005). The final intracellular H_2O_2 concentration is always considerably lower than that applied because the diffusion rate of extracellular H_2O_2 is limited by the cell membrane, to which the intracellular concentration can be 7 to 10-fold less than the outside the cells. The free diffusion depends on the cell type and composition

of membrane lipid (Antunes and Cadenas, 2000). Given this assumption, the final intracellular H_2O_2 used in the present study may range from 10 to 100 µM, but cannot accurately be determined because H_2O_2 is also spontaneously broken down by a range of antioxidant enzymes, especially in the intestinal tissue which is rich with antioxidants.

Afferents mechanosensitivity

This study used response to pressure in order to identify mechanosensory afferents. In this protocol, distension of intestinal segments was performed to stimulate mechanosensitive afferents and recording their response. This protocol takes into account muscle response to distension but does not identify the exact position of afferent nerve endings within the intestine wall (mucosal, muscular, mesenteric and serosal). Distending the intestine from 0 to 60 mmHg mimics physiological and superphysiological stimuli which activates non-nociceptive and nociceptive fibres. (Schröder and Eaton, 2008, Roberts et al., 2013).

Intraluminal versus bath application of H_2O_2

Applying H_2O_2 to the bath can directly activates serosal afferents present in the gut wall in addition to muscle afferents. Intraluminal application probably stimulate first mucosal afferents and other mucosal cells before the peroxide diffuses further to the muscle.

Response to intraluminal application of H_2O_2

The intestine mucosa consists of epithelial cells lining the lumen which help nutrients to be absorbed and act as a barrier to the absorption of macromolecules, toxic compounds and bacteria. The mucosa is dynamically interacting with sensory innervation and changes in the mucosa-sensory network is a key factor in visceral hypersensitivity in functional GI diseases such as IBD and IBS (Posserud et al., 2007, Vermeulen et al., 2014).

This study showed that the jejunum resisted all concentrations of H_2O_2 while the colonic mechanosensory was more susceptible. The response in the jejunum was lacking even after application of H_2O_2 (1 mM) and mitochondrial inhibitor antimycin A. These results indicate that induction of oxidative stress to the mucosa causes afferents hyperactivity only in the colon.

The absence of H_2O_2 response observed in the jejunum compared to the colon can reflect one or all of the following: more effective barrier of the mucosa, higher protection mechanism and less pro-oxidant environment. However, an important point to bear in mind is that the difference in the time course required for stabilization of recording between the jejunum and colonic afferent is one of the limitation of the experiment design which could have affected the afferent response to H_2O_2 and antimycin A.

I examined the hypothesis that a more effective mucosa barrier is present in the jejunum but found that the mucosa in the jejunum is more fragile and deteriorates faster and easier than the colonic mucosa. Accordingly, the H_2O_2 induced hyperactivity of colonic nerves perhaps was caused by a mucosal component that was lost in the jejunum because of repeated distensions. Therefore, and in attempt to maintain intact mucosa, I applied H_2O_2 intraluminally in the jejunum without performing any distension. Once again, no significant change was observed.

It can then be speculated that the jejunum is more equipped to handle oxidants than the colon because of more efficient protection machinery, which result in a less pro-oxidant environment and, therefore, tolerates high concentration of H_2O_2. This possibility was tested by measuring the protein expression of SOD2 in the mucosa from jejunum and colon. The antioxidant SOD2 works to convert superoxide to H_2O_2 and mucosa cells were selected because they are the first line in the face of peroxide and mitochondria inhibitor

as they were applied intraluminally. My data showed that protein levels of antioxidant Sod2 was the same in the jejunum and colon. Previously, basal intracellular ROS measured by (CM-H2DCFDA, a fluorescence probe) and antioxidants (CAT, GPx, and SOD) enzyme activity in the colon have been shown to be higher than in the small intestine. The higher level of antioxidant enzyme activity in the colon was a normal response to the observed ROS (Sanders et al., 2004). The difference between the study's findings and mine may relate to the methods used. The study used an assay kit to measure the enzyme activity while I used western blot which provides information limited to the total amount of protein in the cells/tissue and cannot indicate its functional activity.

Sanders's study also indicated that H_2O_2- induced oxidative stress caused more DNA damage and greater ROS to the colonocytes compared to enterocytes from the jejunum. My result indicated that colonic afferents were more affected by H_2O_2 which is consistent with the previous finding about regional difference in response to oxidative stress.

Although results from the western blot were negative, the electrophysiology study indicated a coherent difference between the two regions, which encouraged me to investigate this more using RNA microarray scanning. Chapter 5 in this thesis discusses the differences between the jejunum and colon at a molecular level.

Response to bath application of H_2O_2

Application of the low concentration of H_2O_2 (100 μM) to the serosa produced an increase in jejunum afferents spontaneous nerve activity combined with a significant increase in the frequency of muscle contractions. The opposite was found in the colon where H_2O_2 (100μM) produced a reduction of the contraction's amplitude concurrent with a small increase of baseline intraluminal pressure. Because the colonic contractions experiments

were performed independently to the nerve recordings, the effect of H_2O_2 on both cannot be considered within the same experiment like the jejunum. However, this result indicates a difference in the muscle response to low levels of H_2O_2 between the small and large intestine. Further studies are needed to substantiate this observation.

The effect of H_2O_2 on the contractions was reversible after washing in the jejunum but the nerve hyperactivity did not recover. Since mechanosensitive afferent responds to contraction and increased in muscle tone, it can be suggested that the observed afferents hyperactivity in the jejunum was partially affected by the muscle response.

The same concentration of H_2O_2 (100 µM) was found previously to reduce the amplitude of inhibitory junction potential of guinea pig colon (Roberts et al., 2013) which can explain the increase in the baseline intraluminal pressure in the colon, but not the decreased contractions. The decreased colonic contractions suggest that H_2O_2 hyperactivity is independent of muscle contractions. Interestingly, it has been shown that mucosa of UC patients releases significantly higher amounts of H_2O_2, interleukin -1β, and nitric oxide than normal mucosa. Incubating isolated sigmoid smooth muscles with solutions collected from UC-submucosa significantly reduced the muscle contraction induced by neurokinin A (NKA). The UC-solution also decreased NKA-and caffeine-induced Ca^{2+} signal which was partially reversed by interleukin-1β antibody and antioxidant catalase but not nitric oxide antagonist. These results suggested that H_2O_2 and IL-1β contribute to the disrupted Ca^{2+} signal and impaired muscle contractility (Cao et al., 2005). Furthermore, a global upregulation of Dual Oxidase 2 (DUOXA2) was reported in active CD and UC patients compared with both non-inflamed and healthy controls (Mirza et al., 2015). DUOX2 gene and its maturation factor DUOXA2 encode proteins that are part of the NADPH oxidase family of enzymes involved in release of

H_2O_2. Accordingly, the reduction in colonic contractions and nerve hyperactivity in response to H_2O_2 in the micro molar range observed in the present study can be related to IBD.

Bath application of H_2O_2 at high concentrations (500 μM) completely abolished the muscle contractility in the jejunum and colon. The effect was developed within 10 min in the jejunum but was immediate in the colon. Again, this observation emphasizes the regional difference in the muscle response. Also, it provides clearer evidence that the H_2O_2-induce hyperactivity of intestinal afferents was independent of the muscle response. One of the way to exclude the muscle response in the preparation is to supplement the Krebs solution with nifedipine and atropine to block smooth muscle contraction and paralyze the tissue. This is a standardised protocol used by many researchers however I was interested in the integrated response to oxidative stress and therefore sought not to use the contraction blockers.

Antimycin A mechanism of action

ROS are able to oxidize cysteine residues to promote the formation of disulphide bonds between the residues (Schieber and Chandel, 2014). This mechanism of activation can occur in the intracellular N-terminal domain of the TRPA1 channel. In Chinese hamster ovary (CHO) cells, the H_2O_2-induced TRPA1 activation was reversed by dithiothreitol (DTT), which reduced disulphide bonds (Andersson et al., 2008). DTT was also shown to inhibit antimycin A-induced TRPA1 activation in the HEK293 cell (Nesuashvili et al., 2013) which suggests that the effect of antimycin A is due to ROS (likely H_2O_2). In the same study, combinations of antioxidants (tempol SOD and catalase), which are scavengers of both superoxide and H_2O_2, were found to inhibit the antimycin A-induced

TRPA1 activation, which provides evidence that the effect of antimycin A is driven by H_2O_2.

In the present study, I tested whether antimycin A-hyperactivity of colonic afferents can be blocked by the TRPA1 antagonist. For this I used HC030031, which is a selective TRPA1 blocker and 30 µM was selected for two reasons. Firstly: previous evidence in the literature is that 30 µM is sufficient to block TRPA1 channel activity (Nesuashvili et al., 2013). Secondly: the work of my colleague Yang Yu (former PhD student in the laboratory of Professor David Grundy) who provided evidence that HC030031 at 30 µM significantly reduced allyl isothiocyanate (AITC)-induced hyperactivity in jejunal mesenteric afferents (Yu, 2014).

In the current study, HC030031 (30 µM) was able to block the antimycin A-response in WDR and HT afferents which is in line with a previous study that showed that HC030031 blocked H_2O_2-activation of capsaicin-sensitive high-threshold afferents in a guinea pig bladder (Nicholas et al., 2016). This suggests that this channel represents a potential target of ROS released during inflammation and related oxidative stress conditions in bladder and gut. HC030031 was expected to attenuate the afferent mechanosensitivity as it is pronounced in genetic ablation of TRPA1. However, this was not observed in the current study which could be due to the selectivity the antagonist.

It has to be mentioned that ROS, lipid peroxidation products, and some prostaglandins are TRPA1 agonists and in the current study, antimycin A was applied intraluminally to the colonic segment, for which it can affect not only the afferent nerve endings that are packed with mitochondria but also epithelial cells, enteric neurons, and other cells. Any/all of the previous components could have influenced the colonic hyperactivity by mitochondrial ROS. Therefore, a more detailed study is needed which is beyond the

limitation of this project. However, the results give a motivation to continue investigating the effect of oxidative stress on colonic sensory activity which is related to inflammatory diseases.

To conclude, both jejunum and colonic spontaneous nerve activity exhibited similar response when H_2O_2 was applied directly to the bath which in the jejunum could be partially due to the effect of the muscle tone. The colonic mechanosensory activity was more affected by H_2O_2 while jejunum mechanosensitivty was unchanged. These results provide evidence that colonic mechanosensitive afferents are more susceptible to H_2O_2-induced oxidative stress.

ROS and afferents activity

ROS and other noxious materials, released during inflammation and tissue damage can influence the nerve terminals by direct action on specific ion channels present on the nerve endings; and indirectly by stimulating the release of excitatory mediators from neighbouring cells such as 5-HT from EC cells (Kono et al., 2013), or histamine and 5-HT from mast cells. Here I discuss the possible explanations of ROS response.

Effect of oxidative stress on chemo and mechano-sensitive afferents

When applied into the lumen, H_2O_2 at (100-500 µM) had no effect on baseline nerve activity and only 1mM was able to produce a significant increase in colonic firing. This is most likely due to the mucosal barrier which limits the peroxide diffusions to reach nerve endings within the laminar propria and other layers. Overcoming this barrier by serosa application showed that H_2O_2-induced hyperactivity was observed in the jejunum and colon afferents at the lowest concentration used, which was - as discussed before -

independent of the muscle response. As no change was observed in mechanosensitivity after H_2O_2 at (100 µM) concentration, it is possible that it activated the chemosensitive afferents in the jejunum and colon. H_2O_2 at (500 µM) continued its effect on chemosensitivity in the jejunum while in the colon it also affected mechanosensitive afferents.

I showed that mitochondrial–ROS provoked by antimycin A was able to produce a significant activation of baseline nerve activity in the colon. This activation is driven by mitochondria inhibition of epithelial and smooth muscle cells which causes accumulation of ROS in the tissue. The effect of antimycin A was developed within 30 min of application which was considered logical because ROS accumulation resulting from mitochondria inhibition cannot be immediate and needed time to build-up intracellularly and then in the tissue. The mitochondria-driven ROS is related to inflammatory conditions as several inflammatory mediators (such as TNF and neurotrophins) increase the production of mitochondrial ROS by either inhibiting the respiratory chain, or downregulating the mitochondrial antioxidant genes (Nesuashvili et al., 2013).

It must be mentioned that inflammatory mediators (such ATP and prostaglandins) maybe involved in the H_2O_2 activation of intestinal afferents. These mediators released close to nerve endings can directly excite the sensory neurons or/and sensitize the afferent response to chemical or mechanical stimuli (Brunsden and Grundy, 1999). It has been recently shown that inhibiting prostaglandin synthesis (by dexketoprofen) can significantly reduce H_2O_2-induced activation of bladder high threshold afferents, which suggests that prostaglandins are involved in the excitatory effect of H_2O_2 on bladder sensory neurons. In addition, purinoceptors (P2X) antagonist (iso-PPADS) was shown to reduce the response to H_2O_2 in vagal lung afferents and when combined with TRPV1

blocker, it abolished the response (Ruan et al., 2005). Therefore, the involvement of inflammatory mediators in response to H_2O_2 is an interesting target for future investigation which will help to understand the mechanisms by which oxidative stress activate colonic sensory activity.

Effect of oxidative stress on low and high-threshold mechanosensitive afferents.

Previous studies have shown that H_2O_2 activates cardiac vagal afferents (Ustinova and Schultz, 1994) and high threshold mechanosensitive nerve of the bladder afferent (Nocchi et al., 2014, Nicholas et al., 2016). In line with these findings, my data showed that H_2O_2 produced activation of colonic afferents at high levels of distensions (≥ 30 mmHg). I also showed that mitochondrial-ROS activate the same type of mechanosensitive colonic afferents (≥ 20 mmHg). These results indicate that oxidative stress preferentially activates high threshold afferents compared to low threshold mechanosensitive fibres. These fibres are spinal afferents and their terminals present in the mucosa, muscle and serosa layers, and ion channels present on their endings are targeted by ROS as was shown by several studies.

In mouse DRG neurons, H_2O_2 was found to evoke a depolarizing inward current and an increase in Ca^{2+} in TRPA1 expressing neurons which was significantly decreased in neurons from trpa1$^{-/-}$ mice (Andersson et al., 2008). *Nicholas et al*, 2004 showed that H_2O_2 induced activation in capsaicin-sensitive high threshold afferents that were also activated by AITC (TRPA1 agonist). He also showed that H_2O_2 response was significantly inhibited by TRPA1 antagonist (HC-030031) (Nicholas et al., 2016). In bronchopulmonary fibres, mitochondrial-ROS provoked by antimycin A was shown to cause a strong activation of capsaicin sensitive sensory nerves that was inhibited by TRPA1 and TRPV1 antagonists HC030031 and capsazepine, respectively (Nesuashvili et al., 2013).

Therefore, it is tempting to suggest similar mechanisms underlying the effect of ROS on colonic mechanosensitive afferents which will be examined in the next chapter (4).

To summarize and conclude, my findings showed that H_2O_2 application in the concentration range that has been detected in inflamed tissue, produced activation of colonic mechanosensitive afferents while jejunum afferents were unchanged. The colonic sensory neurons are more sensitive to oxidative stress induction to the mucosa by H_2O_2 and mitochondrial-ROS. The TRPA1 channel located on the afferent terminals is a possible target for mitochondrial-derived ROS. Colonic mechanosensitive neurones are important for sensing physiological and super-physiological stimuli and change in their activity may contribute to the visceral hypersensitivity in IBD.

Chapter 4

Effect of oxidative stress on afferent nerve activity in aged jejunum and colon

4.1 Introduction

Like all organs, the gastrointestinal tract and its innervation do not escape the effect of age and the incidence of GI conditions, such as decreased peristalsis, constipation, and faecal incontinence, increases with age (Salles, 2007). These complications have a severe impact upon the quality of life of affected individuals. The mechanisms underlying the increased incidence of GI complications in the elderly population are as yet poorly understood.

Elderly Inflammatory Bowel Disease (IBD) patients are less likely to present abdominal pain which contributes to delay and misdiagnosing (Katz and Feldstein, 2008b). Clinical studies have demonstrated an age-associated reduction in sensory function in both the oesophagus and the rectum. In elderly patients, sensory thresholds for oesophagus balloon distensions were higher when compared to healthy subjects (Lagier et al., 1999b). Similar results were observed in the lower gut as elderly patients have been reported to experience decreased pain perception in response to rectal distension (Lasch et al., 1997b).

Recent study used in vitro nerve recordings from human intestine samples has indicated a decrease in afferent sensitivity that was correlated with advanced age, as both baseline nerve firing and response to inflammatory bradykinin were reduced (Yu et al., 2016b). Nevertheless, much remains to be understood about how ageing influences extrinsic gut innervation at a time when studies about age-related changes in humans are very limited. This is not surprising due to ethical issues and many factors that influence GI physiology such as lifestyle, which are difficult to control. Studying age-associated changes in humans can be very challenging. To avoid uncontrolled variables, animals have been used to investigate the effect of ageing on the GI function.

Although young and adult animals have been used extensively in research to study GI sensory function, only very few physiological studies were performed on ageing models. Some signs of neurodegeneration of extrinsic neurons innervating the intestine, such as swollen nerve fibres within the gut wall, have been indicated in aged rats (Phillips and Powley, 2007). In mice, both colonic and jejunal afferent mechanosensory function was found to be lower in 24-month-old animals compared to the 3-month-old and the colonic high-threshold afferents were the most affected (Keating et al., 2016). The study also reported a reduction in chemosensory function in aged animals as colonic nerve activity in response to 5-HT and capsaicin were significantly reduced. Despite that, the intestine is constantly exposed to noxious stimuli such as bacterial products. It is unknown whether the sensory response to oxidative stress is altered in advanced age.

Oxidative stress theory of ageing is an acceptable explanation of how ageing occurs at the molecular level. Accumulation of intracellular ROS generated by normal metabolic process causes oxidative damage to a cell's lipid, DNA, and protein (Kregel and Zhang, 2007). In rodents, DNA oxidation has been reported to be increased in several tissues, including the intestine (Bokov et al., 2004). Oxidative stress has been implicated in the pathogenesis of multiple GI diseases such as IBS, colitis, and cancer (Hussain et al., 2003). However, very little is known about the age-related changes of the oxidative status in the intestine.

Oxidative stress has been previously implicated in enteric neurodegeneration associated with age. A significant loss in myenteric neurons has been reported (50%) in 24-month-old rats that were fed a normal diet and it was prevented by caloric restriction, which is known to reduce oxidative-stress level but was not measured in the study (Cowen et al., 2000a). In a following one, the onset of myenteric neuronal-cell death in aged rats was

associated with elevated levels of intraneuronal ROS measured using ROS-sensitive dyes. Neurotrophic factors (NT3 and GDNF) were found to enhance the neuronal antioxidant defence in aged rats under caloric-restricted diet but not in aged rats fed a normal diet (Thrasivoulou et al., 2006). However, regarding afferent activity, the reduced sensitivity in the aged mice was not correlated with neuronal loss as the number of DRG neurons was not significantly changed in the aged group when compared to the young (Keating et al., 2016) which indicates that the reduction is related to a change at the level of nerve terminals. In addition, human intestine samples have shown reduced density of mucosal substance P-immunoreactive (SP-IR) nerve varicosities that was correlated with age (Yu et al., 2016b) which likely reflects neurodegeneration.

Taken together, one can suggest that chronic exposure to high levels of ROS may be behind the changes in afferents' function which are distributed in the same area as enteric neurons. It is possible that ROS-induced damage to nerve endings may contribute to the reduction in the GI sensory function. Therefore, this was a major motive to assess the oxidative status of aged intestines.

Several studies have reported that noxious stimuli including oxidative stress activate some members of TRP (transient receptor potential) channels by changing calcium influx (Miller and Zhang, 2011, Takahashi and Mori, 2011, Andersson et al., 2008). Within the GI tract, TRP channel subunits are expressed by many cells and neurons such as enterochromaffin cells, enteric neurons, ICC network, and smooth muscle. They play an important role in chemosensation, mechanosensation, and pain, and promote the regulation of many GI functions such as mucosal homeostasis, blood flow, absorption, secretion, and motility (Boesmans et al., 2010). Much evidence from clinical studies

supports the important contribution of TRP channels to GI sensation and nociception (for review see (Yu et al., 2016a).

TRPA1 channel has attracted significant attention in sensory research because of its important role in inflammation and pain. In dissociated DRG neurones, it was shown that H_2O_2 acts via TRPA1 channels (Andersson et al., 2008) to induce neuronal excitability. In airway sensory nerves, mitochondrially derived ROS (induced by antimycin A) was found to activate C-fibres. The response was reduced by H030031 (a TRPA1 antagonist) and completely abolished by using a combination of TRPA1 and TRPV1 blockers (Nesuashvili et al., 2013). In addition, H_2O_2-induced hyperactivity of high-threshold bladder afferents has been shown to be mediated specifically by TRPA1, which strongly indicates that this channel is a target for ROS (Nicholas et al., 2016). In the GI tract, TRPA1 expression is upregulated as a result of inflammation in human IBD and a mouse model of colitis (Kun et al., 2014). However, studies about TRPA1 channel expression in the aged gut are lacking, despite its important role in sensing luminal environment.

Chapter 3 has concluded that oxidative stress induction to the mucosa activates colonic afferents, but it is unknown if this response is changed in aged tissue and if oxidative stress contributes to age-associated changes in the mouse's intestine. It is also unclear whether TRPA1 is involved in oxidative stress-induced hyperactivity of colonic afferents. Therefore, the aims of this chapter are firstly: to examine if oxidative stress contributes to changes in the aged mouse and to examine if the sensory response to oxidative stress is changed in the aged group. Secondly: to assess gene expression of TRPA1 channel and whether it is involved in afferents' responses to oxidative stress.

4.2 Experimental protocol

8-hydroxydeoxyguanosine (8-OHdG) level detection

DNA samples from jejunum and colon mucosa at 3 months and 22 months were obtained using AllPrep kit; and the level of 8-hydroxy-20-deoxiguanosine (8-OHdG) was determined using EpiQuick 8-OHdG DNA damage quantification direct kit as described in Chapter 2. The results are presented relative (%) to a positive control and normalised to the input DNA as recommended by the kit manufacturer.

Detection of ROS levels

Jejunum and colon samples from 3 months and 22 months were collected, embedded in OCT, and sectioned in a cryostat as described in Chapter 2. The sections were incubated for 30 mins. with dihydroethidium (DHE) (20 μM) to measure the intracellular ROS levels.

Three sections were used from each mouse and four images were taken from each section at 10X objective for offline quantification. Using Image J, the mucosa area was manually indicated and the measured DHE fluorescent intensity was normalised to the background first and then divided by the mucosa area. The data is presented as mean ± SEM and the groups were compared using One-way ANOVA test.

Recording of colonic and jejunum afferent nerve activity

Extracellular recordings on mouse jejunum and colon were performed as described in Chapter 2. Before starting any protocol, the nerve activities were stabilised for 30 minutes in jejunum and 1 hour in colon (as described in detail in Chapter 3). After stabilisation, distensions were performed at an interval of 900 seconds. Three reproducible responses to distension were achieved before starting any subsequent protocol.

Antimycin A (20 µM) was prepared from premade stock (5 mM) with freshly made Krebs solution perfused intraluminally by a syringe pump at a rate of 12ml/h.

Data analysis

Data of nerve recordings are presented as nerve firing, which refers to the number of action potentials per second. All data is expressed as mean ± SEM from the N values, where N refers to the number of animals. The nerve-activity response to ramp distension was calculated as increasing in the afferent firing frequency against increase in intraluminal pressure from 0 to 60 mmHg. Data was obtained using a custom-made script (Cambridge Electronic Design, Cambridge, UK). Spontaneous nerve activity was calculated from the nerve activity recorded before the start of the distension protocol (mean firing over 20 secs.). To analyse whether distension responses were significantly changed, Two-way ANOVA with Bonferroni correction for multiple comparisons test was used.

4.3 Results

4.3.1 Ageing causes increased oxidative stress levels in the mouse colon

Higher DNA damage in the aged colonic mucosa

DNA oxidation leads to nucleoside-based modifications and the products such as 8-hydroxy-deoxyguanosine. 8-OHdG can be used as a marker to measure the level of oxidative DNA damage. In this study, 8-OHdG was detected in the young group and was undistinguished between jejunum and colon, which indicates baseline levels of DNA damage in young tissue. Greater 8-OHdG was found in the aged mucosa compared to young. However, the difference was not statistically significant ($P > 0.05$, Dunn's-test) likely due to the small sample size (N = 3, Figure 28). Unfortunately, it was not possible to repeat the experiment using more samples due to limited materials.
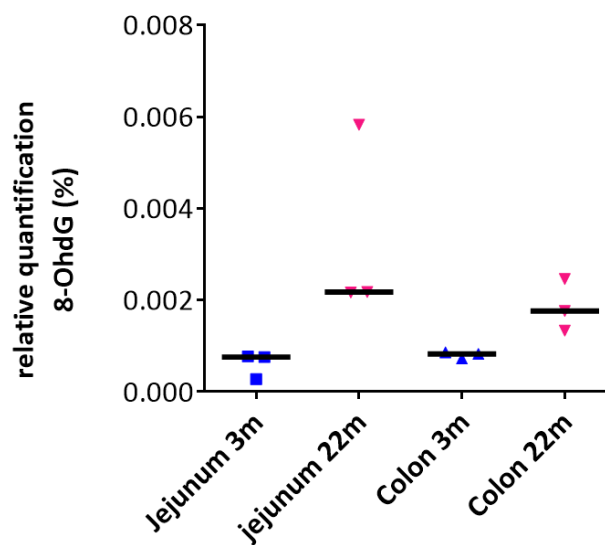
Figure 28: Oxidative DNA damage in young and aged mucosa.

Histograms show that the level of 8-hydroxy-2′-deoxiguanosine (8-OHdG) was similar between jejunum and colon at 3 months. Higher 8-OHdG levels were observed in the 22-month mucosa samples but the difference was not statistically significant compared to 3-month (P >0.05 Dunn's-test, N = 3). The lines represent the median values.

Higher intracellular ROS levels in the aged colonic mucosa

ROS-sensitive dye was assessed in sections of the jejunum and colon from both young and aged groups. The DHE red fluorescence was detected in all samples (Figure 29: **A, B, C, & D**). Baseline ROS levels can be seen similarly in the young group between jejunum and colon (A & C) which, when calculated, showed no significant difference (jejunum: 866.7 ± 52 vs colon: 871.0 ± 40, $P > 0.05$, One-way ANOVA, N = 6, Figure 30).

As can be seen in images from Figure 29 (A & B), the red fluorescent intensity was similar between 3-month and 22-month jejunum samples. The difference was not statistically insignificant as can be seen in Figure 30 (3 months: 866.7 ± 52 vs 22 months: 824.0 ± 70, One-way ANOVA, $P > 0.05$, N = 6). In contrast, higher red fluorescence can be seen in the 22-month colon section compared to the 3-month (Figure 29: C & D), which, when compared, showed significantly higher DHE intensity (1169 ± 40 vs 871.0 ± 80 respectively, *, $P < 0.05$, One-way ANOVA, N = 6, Figure 30). In addition, DHE intensity was significantly higher when jejunum and colon from the aged group were compared (824.0 ± 71 vs 1169 ± 80, One-way ANOVA, **, $P < 0.01$, N = 6).
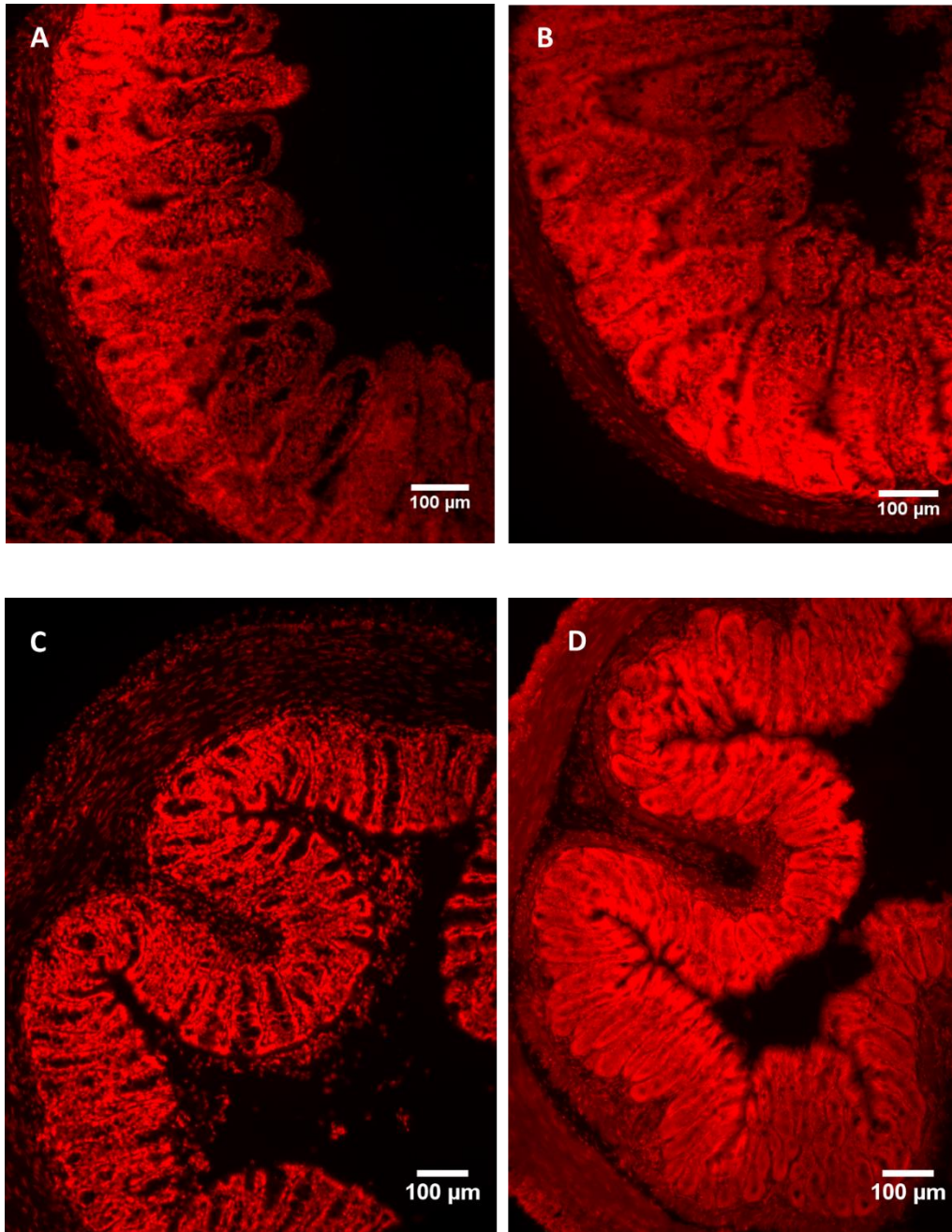
Figure 29: Oxidative marker in young and aged intestine tissues

Cross-sections of the jejunum and colon from the young and aged groups show fluorescent intenstity of DHE, which indicates the level of intracellular ROS in the tissue. Images from 3-month and 22-month jejunum samples showed no difference in the DHE fluorescence intensity (A & B). Higher DHE fluorescence intensity can be seen in the 22-month colon section compared to the 3-month (C & D).
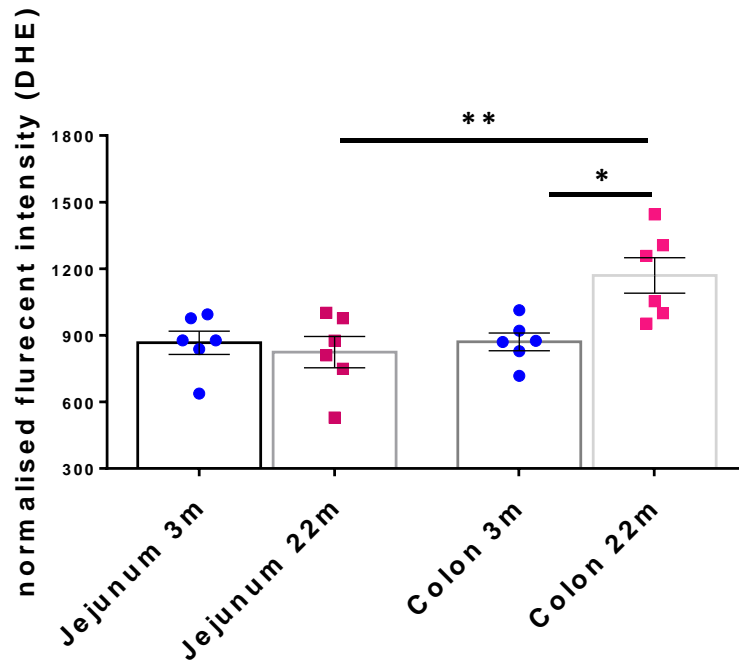
Figure 30: ROS levels in young and aged mucosa tissue.

Histograms show that at 3 months, DHE intensity was similar between jejunum and colon, which reflects no difference in the baseline level of ROS. At 22 months, DHE intensity can be seen to be greater in the 22-month colon compared to the 3-month colon, and to the 22-month jejunum, One-way ANOVA, * $P < 0.05$, ** $P < 0.01$, N=6.

4.3.2 Age-related reduction in sensory-nerve activity was observed only in the mouse colon

Nerve recording of jejunum afferents indicated no significant difference in the spontaneous activity or in pressure-response profile of mechanosensitive afferents. However, as can be seen in Figure 31 (A), animals in the aged group displayed more variability in spontaneous nerve activity, therefore larger group size is required to achieve clearer results.

In the absence of any changes in intraluminal pressure, colonic afferents exhibited a normal basal afferent activity (Figure 32 C&D). In the young animals, baseline afferent

activity was 15.29 ± 2.1 spike/s, which was indistinguishable from baseline activity

recorded at 22-months age (14.47 ± 1.8 spikes/s; unpaired t-test, P = 0.76, N = 14).

Afferent responses to distension were significantly lower in 22-month than 3-month

samples and the significant differences were observed at high level of distension (55 and

60 mmHg pressure, Two-way ANOVA, * P < 0.05, and ** P < 0.01 respectively).
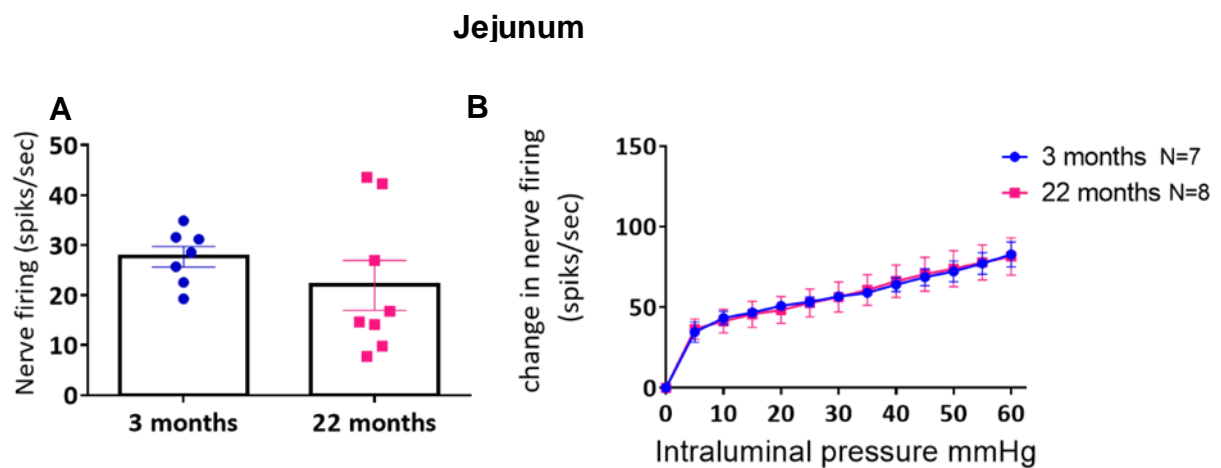
Figure 31: Age-related changes in jejunum afferent activity.

In the jejunum, **A & B** show difference between 3-month (N = 7) and 22-month (N = 8) animals was not significant in spontaneous nerve activity and pressure-response profile.

Colon

A Multi unit nerve activity

B Low threshold

C Wide dynamic range

D High Threshold

E 3-months

F 22-months

Low threshold
Wide dynamic range
high threshold
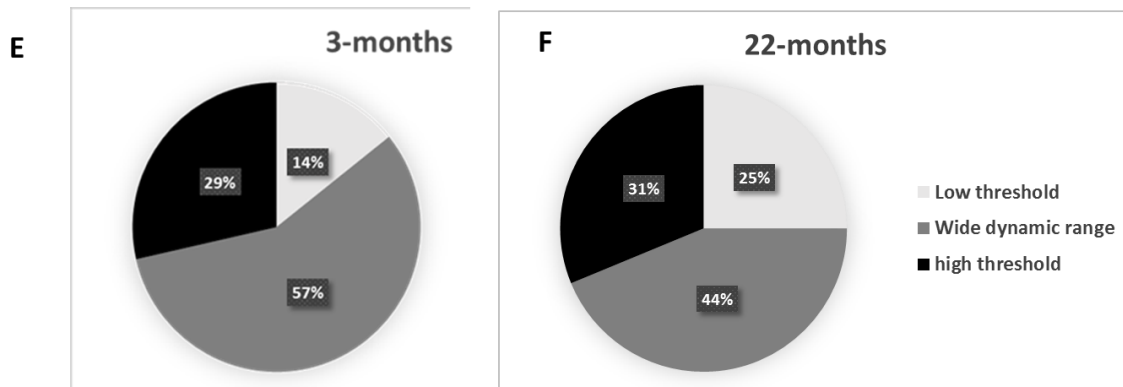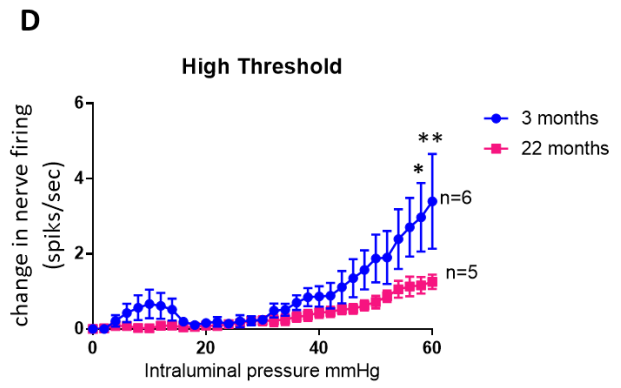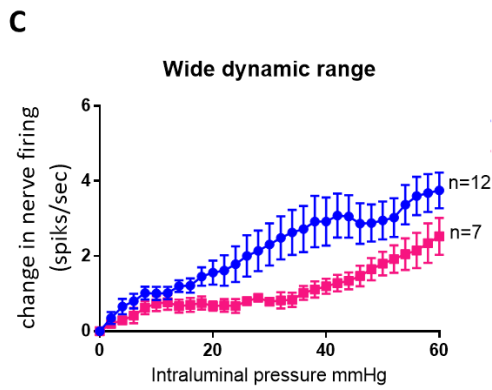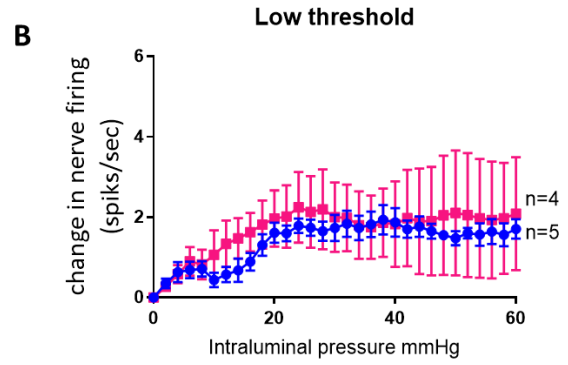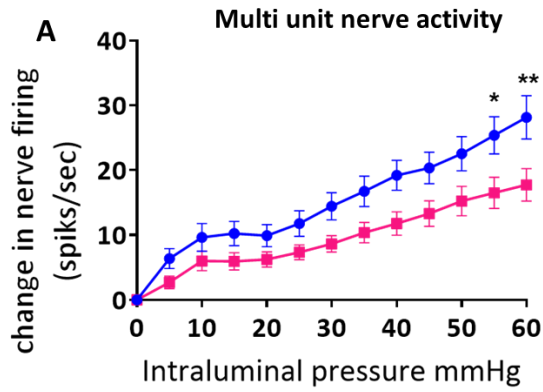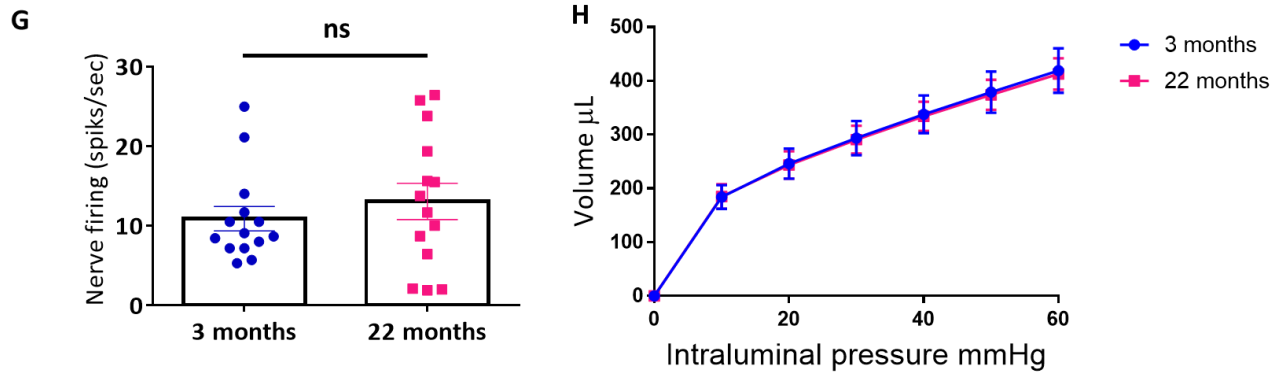
Figure 32. Age-related changes in jejunum afferent activity.

A) Pressure-response profile (N = 11) was attenuated in 22-month compared to 3-month mice, and significant values were detected at > 50 mmHg. **B**, **C & D)** Curves shows the response to distension in LT, WDR and HT unit. **E&F)** The relative percentages of unit subtypes identified in 3 -months (N=11, n=23) and 22-month animals (N=8, n=16). Afferent fibre type composition is unaltered in 3- months (N=11) and 22-months (N=8) samples. **G**) Histograms show no significant difference in spontaneous multi unit nerve activity between 3-month and 22-month (N = 14, unpaired t-test). **H)** The relationship between the perfusion volume and the intraluminal pressure of the colonic segment did not differ by age. Pressure response profile analysed by Two-way ANOVA, * $P < 0.05$, and **$P < 0.01$).

4.3.3 Jejunum and colonic afferents response to antimycin A is not altered in the aged mouse

Previously in Chapter 3, jejunum afferents of 3-month-old mice showed no response to antimycin A. Nerve recording was performed here to examine whether the response to oxidative stress is changed in the aged jejunum. As can be seen in Figure 33 (see below) the baseline nerve discharge was unchanged after intraluminal application of antimycin A (20 µM) (25.88 ± 7.5 vs 26.87 ± 7.9 spikes/s, paired t-test, $P = 0.4$, N = 6). Similarly, the pressure-response profile of mechanosensitive afferents was unchanged.

In the aged colon, antimycin A produced a significant increase in baseline afferent discharge from 14.14 ± 2.6 spikes/s, to 18.09 ± 3.0 spikes/s (paired t-test, $P = 0.001$, N =

6). The colonic mechanosensitivity was also enhanced after antimycin A (20 μM) which was significant in WDR and HT mechanosensitive fibres. (Two-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, N = 6, Figure 34 C&D).

To determine if the response to antimycin A was changed in the aged colon, I compared the response between young and aged animals. In 3-month-old animals, antimycin A produced an increase in the colonic afferents that was observed at high levels of distension as well as baseline nerve discharge. The response to antimycin A in 22-month-old mice was indistinguishable from the 3-month (Figure 34: E & F).
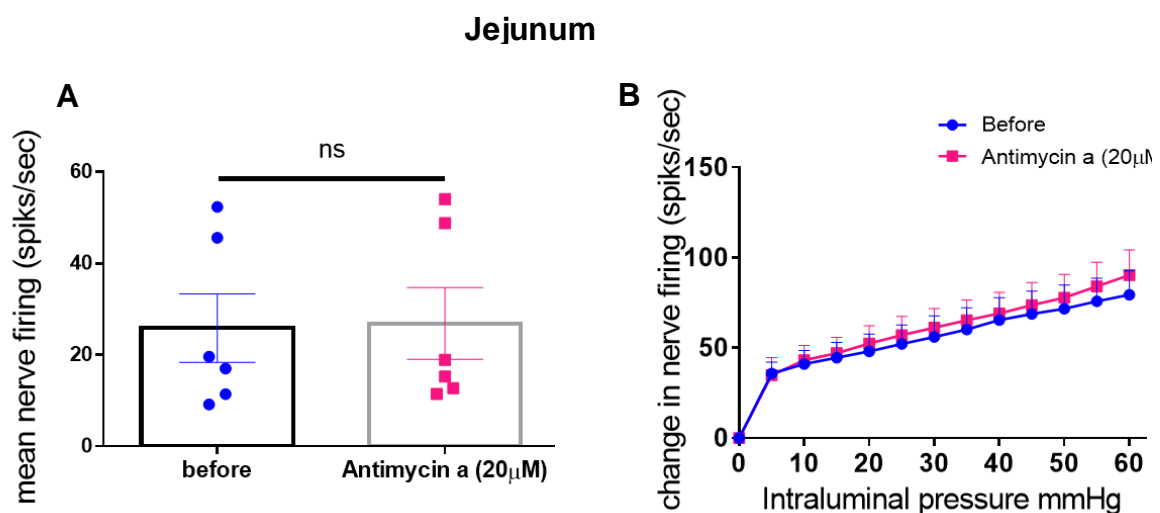
Figure 33: Antimycin A produced no change in afferent firing of aged jejunum

A & **B**) spontaneous nerve activity and pressure-response profile were unchanged by antimycin A (20 μM). Vehicle for antimycin A is ethanol (0.00025%).
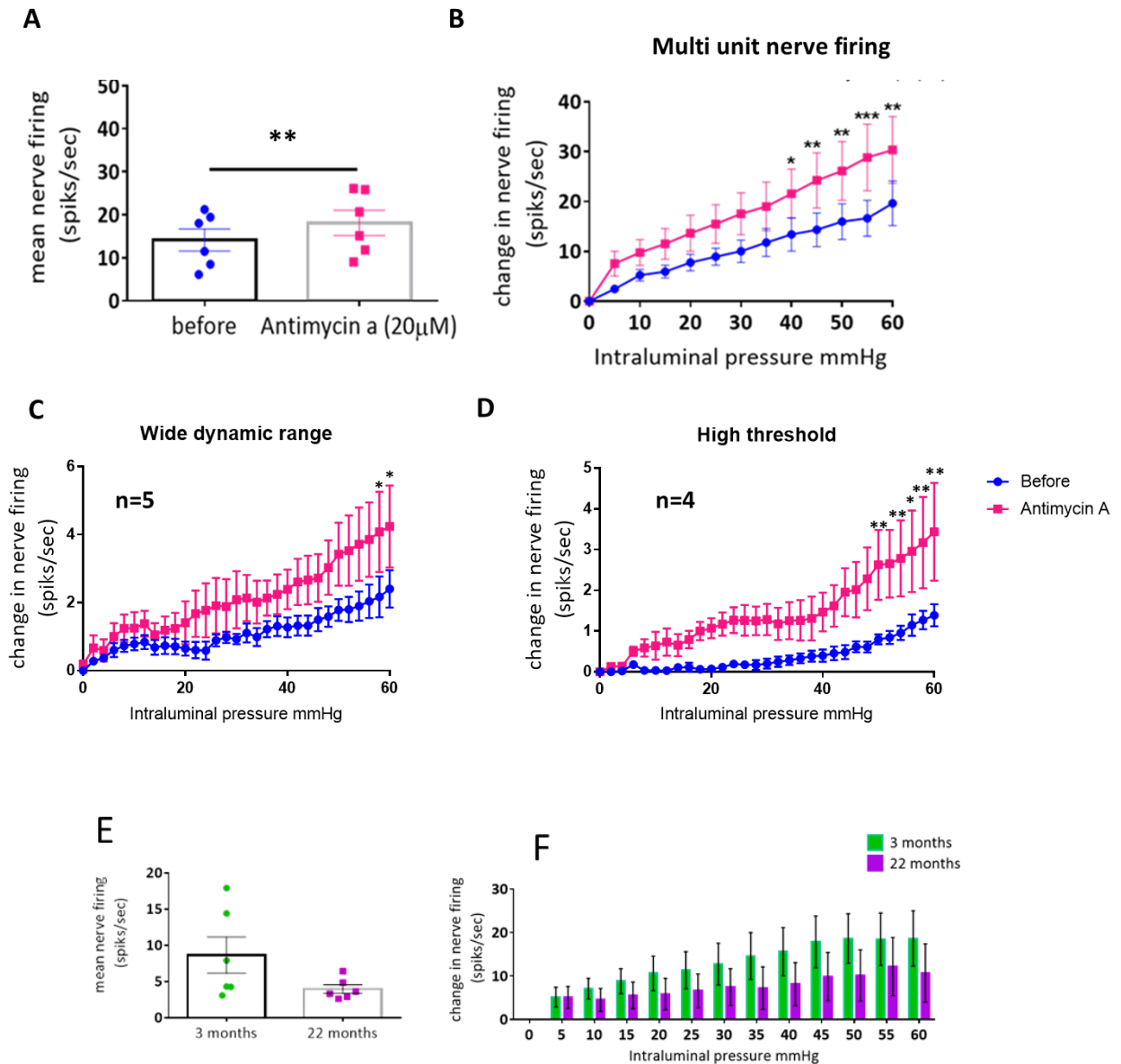
Figure 34 Colonic afferents response to antimycin A is not changed by age.

A & B) show that in 22-month animals, both spontaneous multi unit nerve activity and pressure-response profile were enhanced by antimycin A and the significant difference was seen after 40 mmHg. **C & D** Curves show the significant response to antimycin A observed in WDR and HT afferents. **E & F** Histograms show that the multi unit nerve response to antimycin A was indistingushable between 22-months and 3-months (for baseline activity: unpaired t-test. For pressure response: Two-way ANOVA. *, $P < 0.05$, **, $P < 0.01$ & ***, $P < 0.001$, N = 6. Vehicle for antimycin A is ethanol (0.00025%).

4.4 Discussion

The current chapter demonstrates that the mechanosensensory function of colonic afferent fibres is attenuated as a result of ageing, while the sensory activity of jejunum afferents is unchanged. Oxidative markers are elevated in the aged colonic mucosa. In addition, the data indicates that aged jejunum afferents resist the induction of oxidative stress by antimycin A while aged colonic afferents are more affected. The mechanosensory fibres play an essential role in the gut-brain axis and sensitise in response to injury and inflammation. The loss of mechanosensory function with advanced age may explain the propensity to lower awareness of gut injury in the elderly.

While the mechanisms underlying the complex process of ageing are not yet fully understood, evidence suggests that oxidative damage can be the cause of functional changes in advanced age (Hamilton et al., 2001). The GI tract is very susceptible to ageing and clinical studies have reported a reduced sensory function in the gut (Lasch et al., 1997b, Lagier et al., 1999b). In a recent study on animals, a significant reduction in afferents' mechanosensitivity was reported in the jejunum and colon of 24-month-old mice (Keating et al., 2016). However, much less is known about the effect of ageing on the GI tract and studies are still very limited.

Ageing causes elevated ROS levels in colon more than in jejunum

The present study indicates that ageing is correlated with a higher oxidative status in the aged intestine and that the colon was significantly affected. The results showed a significant increase in the cellular ROS marker (DHE) in the colonic mucosa as a result of ageing. The data provides evidence that jejunum mucosa is well protected with advanced age with no change in the oxidative status. The oxidative DNA damage can cause many functional changes, leading to an increased tendency to develop various diseases. DNA

damage has been shown to induce senescence-associated phenotype in mucosal epithelial cells located near the crypt (Wang et al., 2009). This phenotype is associated with higher generation of ROS. Accordingly, my data suggests that greater oxidative damage to the DNA occurs in the aged tissue compared to the young (in colon more than in jejunum) which is in line with previous studies that have reported an age-associated accumulation of oxidative DNA damage in several tissues in both animals (Hamilton et al., 2012) and humans (Meissner, 2007).

Previous studies that investigated ROS in the aged intestine have focused mainly on the myenteric neurones, as they control the important function of motility (for review see (Korsak et al., 2012). In rats' ileum, levels of intracellular ROS (detected by ROS-sensitive dyes) correlated with a significant myenteric neuronal loss have been found more in 24-month animals compared to 3-month (Thrasivoulou et al., 2006).

To my knowledge, the present study is the first to report the oxidative status in the aged intestinal mucosa and indicates a difference between jejunum and colon in age-associated accumulation of ROS. The current result comes in line with previous findings from the aged bladder, in which higher oxidative status was found in the urothelial tissue (Nocchi et al., 2014). In the study, Nocchi et al. indicated an increase in oxidative stress markers including intracellular H_2O_2, superoxide anion, and 8-OHdG levels in the aged mouse's urothelium. The study also found an upregulation of the antioxidant SOD2 protein in the aged urothelium tissue, which could have occurred in response to chronic high ROS.

Ageing causes a reduction in sensory activity only in the colon

The effect of age on the mechanosensory function of intestine afferents has been recently shown (Keating et al., 2016). My study was performed in the light of Keating's protocol

for nerve-activity recording. In Keating's study, a significant reduction of mechanosensitivity in the aged colonic afferents was reported from 12-month and 24-month mice. The reduction of sensory afferents innervating the jejunum was not significant until 24-months, which indicates that the jejunum is only affected at a very late age.

The study analysed in detail the difference in low-threshold, wide dynamic rage, and high-threshold mechanosensitive fibres, and the significant effect of ageing was found on the high-threshold component. In line with these findings, I showed that the mechanosensory function of the colonic nerve was attenuated with 22-month mice and the effect was significant in HT afferents. Regarding aged-related changes in the jejunum, Keating et al. reported a declined nerve activity in 24-month (but not 12 month) jejunal afferents. However, my data showed no significant difference at 22 months compared to 3 months. The difference in the animal group size should be taken into consideration, as Keating's study used N = 12 and a more advanced age (24-month) which could be behind the difference in findings. Particularly because of the variability in the aged group, as spontaneous nerve activity was normal in some animals, it was markedly reduction in others which suggests that animals may not age physiologically at the same rate.

Since ROS level and sensory function in the aged jejunum were found to be unchanged compare to the young, it is very tempting to propose that a protective mechanism is found in the small intestine to preserve the process of nutrient absorption. Following the same direction, it is possible that age-associated changes in the aged colon are caused by chronic exposure to oxidative stress.

It is unknown what is the effect of chronic exposure to high levels of ROS upon sensory nerves and it is very possible that accumulated harmful ROS in the tissue can damage the structure of sensory nerve fibres and endings within the gut wall, which participate to a lower response to stimuli. This explanation is very possible if we consider results from previous investigations. First, Keating's study showed that capsaicin response was attenuated in the colonic nerve but not in the DRG of aged mice. This observation strongly suggests that the decline in mechanosensory and chemosensory functions resulted from a change in properties at the level of afferent terminals within the gut wall and particularly that there was no evidence of age-associated neuronal loss in the DRG. Secondly, the density of mucosa substance P-immunoreactive nerve fibres was found to be significantly reduced with advanced age in humans, which suggests an age-associated neurodegeneration in the gut wall (Yu et al., 2016b). Thirdly, a morphological study that reported dystrophic age-associated changes in the terminals of vagal afferents supplying the myenteric plexus and mucosal layer indicates a loss of support and stimulation for the visceral afferents (Phillips et al., 2010). On the other hand, no age-related changes were reported in the spinal afferents' endings (Bergman and Ulfhake, 1998; Phillips et al., 2008). Clearly, further investigations are needed to examine how ageing alters innervation patterns in the gut and whether long-life oxidative stress is involved.

Jejunum and colonic afferents response to oxidative stress is unchanged in the aged mouse.

In all experiments, the jejunum seemed to be protected against oxidants, ROS, and ageing. Similar to that observed in young animals (Chapter 3), aged jejunum afferents showed no significant change after application of the mitochondrial inhibitor, which indicates that the jejunum's ability to handle ROS is continued in advanced aged. To my knowledge, this

is the first study to report this observation. Similarly, the response of colonic afferent to antimycin A was indistinguishable between the young and aged groups. Antimycin A is a mitochondrial inhibitor that causes accumulated ROS in the tissue (endogenous ROS) therefore, it is hard to form an opinion regarding how the aged tissue would respond to environmental ROS, (present in ingested food). Using an oxidant like H_2O_2 would have been a better tool to address this question, but this was not possible due to the timeframe of the study.

In conclusion, this chapter concludes that colonic sensory function is more susceptible to ageing. The reduction in mechanosensitivity is associated with higher ROS levels in the aged mucosa. The difference between the jejunum and colon in susceptibility to ageing could be because of long-life exposure to harmful ROS. Mechanosensitive nerves are important to respond to noxious stimuli such as injury, and the loss of their function may participate in the lower perception of GI injury or disease in the elderly, which can have devastating consequences upon the quality of life.

Chapter 5

mRNA of intestinal mucosa and the effect of ageing

5.1 Introduction

Food digestion and absorption is the main function of the small intestine. The colon is responsible for electrolytes' absorption and also provides a media for fermentation of undigested nutrients. The colon is the home of a wide variety of commensal bacteria that are responsible for fermentation of undigested nutrients, synthesis of important vitamins, and play an important role in shaping the immune system (Barbara et al., 2016).

High efficiency for digestion and absorption requires a specialised tissue structure. The intestine surface area is enlarged by intestinal folds, villi, and crypts. This large surface area enables an optimal and direct contact between food and digestive enzymes as well as providing enormous capacity for active and passive nutrients' uptake. At the same time, this large surface increases the risk of infections by pathogens form the external environment through consumed food or by enteric microorganisms. The intestine is protected against invasion of the enteric microorganism by several defence mechanisms: the apical junctional complex that keeps epithelial cells tightly sealed and regulates paracellular permeability together with a layer of mucus coating the top of the cells, which offers a first-line defence against pathogens and oxidants in the lumen, in addition to several immune cells within the mucosa and submucosal layers of the intestine (von Moltke et al., 2016, Barbara et al., 2016).

Although the jejunum and colon show structural and functional similarities, essential differences also exist in each segment. Differences between the small intestine and colon in ROS-production and antioxidants' enzyme activity may contribute to the variation in susceptibility to diseases such as cancer and IBD. Enhanced production of ROS has been reported in the colon. In response to high ROS, antioxidants' enzyme activity was found more in the colon than in the jejunum. However, colonocytes were more prone to

oxidative DNA damage induced by H_2O_2 (Sanders et al., 2004). Therefore, the colon may be unable to handle oxidative stress as effectively as the small intestine, which may lead to accumulative oxidative damage at this site of the GI tract. From this evidence, we can also suggest that the colon might be more susceptible to the ageing process.

Ageing and accompanying reduction in physiological functions affect all organs, including the GI tract. Changes in taste-sensation, dysphasia, impaired gastric emptying, diarrhoea, and constipation have been reported in the elderly (for review see (D'Souza, 2007). In the human gut, it has been shown that ageing promotes changes in microbiota composition which was associated with increased inflammatory status (Biagi et al., 2010). However, the GI system is very adaptive and therefore it is still not clear what molecular patterns contribute to age-associated changes in the gut.

Over the years, techniques have been developed to study and measure the expression of a large number of genes. At present, RNA microarray is a well-established approach, containing thousands of gene sequences synthesised to chips, to which labelled complementary cDNA samples are hybridised. Although microarray technique has been extensively used in cancer research and other gut diseases such as IBD, it has been used considerably less to scan for changes associated with non-pathological ageing.

In the aged rat, cDNA arrays have indicated age-associated changes in gene expression in the intestine, and they were seen more in the colon than in the small intestine. A study reported a downregulation in genes encoding lipids and carbohydrate enzymes in the duodenum. In contrast, genes encoding enzymes involved in fatty acids' oxidation and protein metabolism were upregulated in the colon. In the study, cancer genes were observed in the aged rat's colon, but not in the duodenum (Lee et al., 2001).

A more comprehensive study was conducted using C57BL/6J mice to examine the effect of ageing on the whole intestine. Based on gross energy intake and faecal out-put, the calculated digestible energy uptake was found to be similar between 3- and 22-month mice. Morphometric examination of the small intestine and colon showed no significant difference between the young and aged groups. Difference in gene expression was reported more in the aged colon than in the small intestine, to which immune and inflammatory related genes were upregulated. Using quantitative polymerase chain reaction (QPCR), the study confirmed the upregulation of pro-inflammatory cytokines interferon-γ (INFγ), tumour necrosis factor (TNFα) and interleukin1-β (IL-1β) in the 22-month mice, compared to 4-month controls. However, in contrast to what was observed in the rat, the study indicated no change in expression of genes involved in the metabolic process between 3-month and 21-month mice. Cubilin was the only lipid metabolic gene that was reported to have changed in the aged jejunum (Steegenga et al., 2012). Although the study provided details about the effect of different features of ageing on the mouse's intestine, additional studies to support the findings and provide more information are lacking at a time when the field of genes in sequencing and scanning is witnessing a significant progress. Such studies are necessary to increase our understanding of changes seen in non-pathological ageing.

It has been shown earlier in this thesis that colonic afferents were more affected by oxidative stress and ageing than jejunum afferents and that levels of ROS were greater in aged colonic mucosa compared to young, but not in jejunum mucosa. ROS acts as signalling molecules and chronic exposure to ROS in aged tissue could activate cascades of genes. Therefore, microarray-based genomics was used in this chapter to characterise the gene expression in adult male C57BL/6 mice using Affymetrix mouse gene 2.0 ST

arrays. I was particularly interested in examining oxidation and antioxidant genes in the mouse's jejunum and colon because of the regional difference in response to oxidative stress that was observed in previous chapters. In addition, changes in gene expression in the jejunum and colon of young and aged mice were examined to scan for molecular events associating the ageing process.

5.2 Experimental protocol

Quantitative RT-PCR

RNA samples were extracted and quantified using AllPrep kit, cDNA was synthesised, and qRT-PCR reactions were performed as described in Chapter 2. A complete list of primer sequences is provided in Table 1. Brain RNA samples were used as positive control.

Table 1. List of primers used in the study

Product	Accession number	Primer sequence (5'–3')
Gapdh	NM_008084	5'-GTGGAGTCATACTGGAACATGTAG-3' 5'-AATGGTGAAGGTCGGTGTG-3'
Trpa1	NM_177781	5'-GTACTTCTTGTCGTGTTTCTTGC-3' 5'-ACCATCGTGTATCCAAATAGACC-3'
Trpm2	NM_138301	5'-ACGCCATCTTCATTAGTCCAC-3' 5'-GCGAGTACCTCATCTACCTGT-3'
Trpc4	NM_001253682	5'-GAGAACTGTTTATCAAGGAGAATGG-3' 5'-AATGTCTATGTAGGCGATGCG-3'
Trpc5	NM_009428	5'-GCAAACTTCCACTCAATATCAGC-3' 5'-GACATACAACGTCATCTCCCT-3'
Trpc6	NM_013838	5'-TCAGCTGCATTCATGACGAG-3' 5'-CTGGCTCTCATATACTGGTGTG-3'

Microarray RNA

The microarray was performed on RNA extracted from mucosa of jejunum and colon from young and aged mice was performed using AllPrep isolating as described in Chapter 2. GeneChip® Mouse Gene 2.0 ST Arrays were purchased from Affymetrix UK Ltd. Total RefSeq transcripts in the array covered 35,240 genes. According to the product's description, the whole-transcript array was designed to include probes that measured the messenger (mRNA) as well as long intergenic non-coding RNA transcripts (lincRNA). This type of design (whole-transcript) offers a complete expression profile of mRNA and the intermediary lincRNA transcripts that influence the mRNA expression profile.

Data analysis

Samples were analysed using GeneChip Qlucore Omics Explorer operating software which is used for direct import and normalisation for Affymetrix files. The software automatically analyses data by calculating background points and setting an arbitrary threshold for the scan, then calculating an intensity value for each transcript. From there, data was exported to Microsoft Excel where it was subsequently processed to statistically analyse changes in gene expression in the samples. Changes in gene expression were calculated as folds changed between young and old mice or between jejunum and colon. Transcripts were defined as differentially expressed if the difference in the gene-expression level was > 1.3-fold change and there was a P value of <0.05 (Multiple t-test with Sidak-Bonferroni correction). Four comparisons were made and the generated lists of differentially expressed genes were as follows:

1. young jejunum vs young colon

2. aged jejunum vs aged colon

3. aged jejunum vs young jejunum

4.   aged colon vs young colon

Bioinformatics software and online database used in the analysis

Principal components analysis (PCA) is a statistical method used to outline the main variables in a multidimensional data group that explains the differences in the observations. The variables are identified by 2 or 3 directions (called principle components), in which each sample can be represented by fewer numbers. This way, a multidimensional data set is simplified to visually evaluate the similarities between samples and whether they can be grouped together. In this study, PCA was performed using the PCA function in Qlucore Omics Explorer software.

In order to understand in which pathways the genes were involved, Pathway Analysis (PA) was applied using PANTHER bioinformatics. PANTHER (Protein ANalysis ThHrough Evolutionary Relationships) is a free-access web-based classification system that was designed to classify proteins and their related genes (Mi et al., 2016). The system identifies genes according to their protein family and subfamily, molecular function, and also the biological process and pathway. The list of genes was submitted online, identified, and classified. The result folders were exported to an Excel sheet to generate graphs.

The intersection of the lists of genes in aged jejunum and colon was drawn as a Venn diagram using Venny2.1. Venny is an online diagram generator with which one can submit two/or more lists of genes, visualise, and export the result (Oliveros, 2005-2007). The National Center for Biotechnology Information (NCBI) which provides free access to genomic information including references was also used.

5.3 Results

5.3.1 Upregulation of TRPA1 mRNA in the aged-colon mucosa compared to young

Quantitative RT-PCR was used to assess the expression levels of TRP-channel transcripts in the colonic segments which showed elevated ROS level. I focused on several members of TRP channels because of information about their role in sensing oxidative stress (TRPA1, TRPM2, TRPC5, TRPC6) (Yamamoto and Shimizu, 2016). Quantitative PCR analysis indicated differential gene expressions of TRPA1 and TRPM2 (4-fold and 2-fold change respectively; Figure 26) in aged colonic mucosa compared to young. Although the change in TRPA1 expression was of over 4-fold increase, it was not statistically significant which is likely due to the small sample size, N = 3 (Kolmogorov-Smirnov test, P = 0.1; Figure 35).
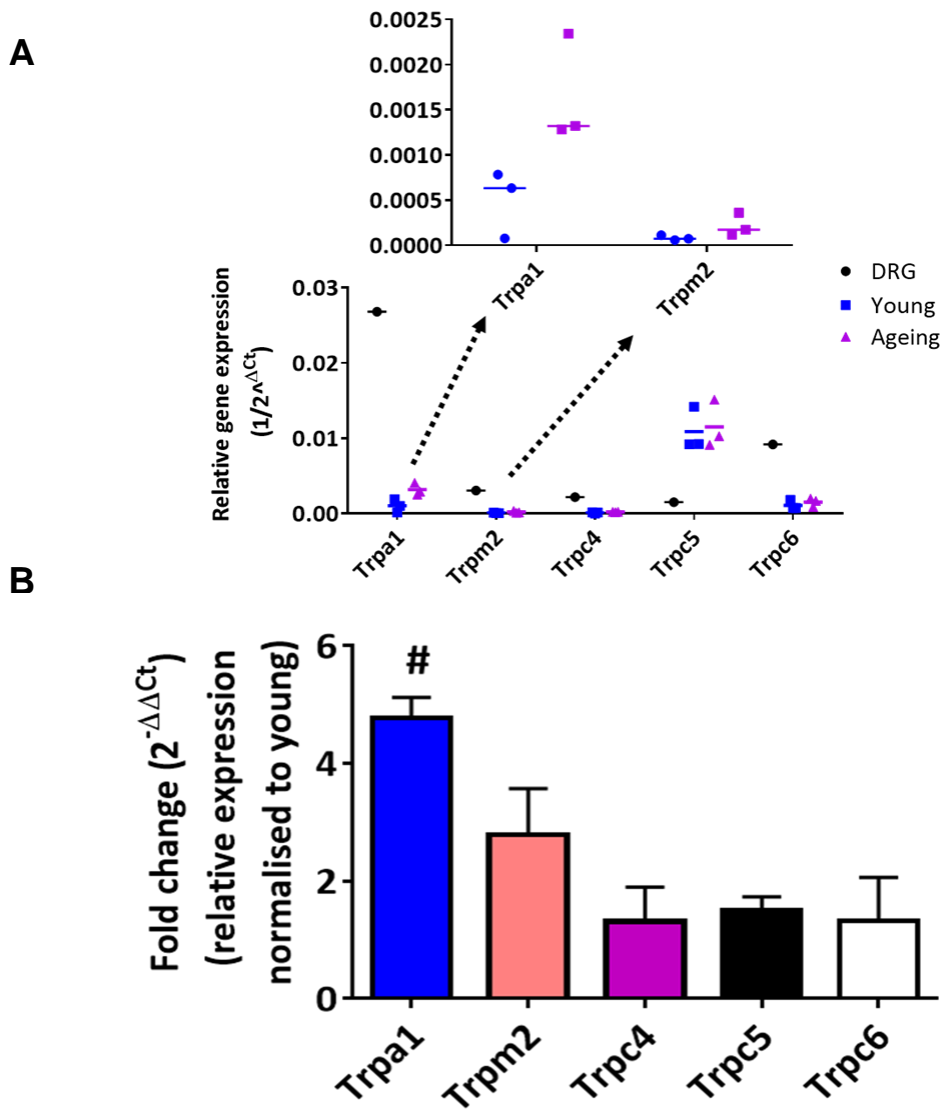
Figure 35: Upregulation of TRPA1 mRNA in the aged colon

A) Messenger RNA (mRNA) expression for TRPM4, TRPC5, TRPM2, and TRPA1 in 3-month and 22-month colonic mucosa relative to the housekeeping gene GAPDH. The lines represent the median values. **B)** The greater change (> 4-fold increase) was observed in TRPA1 mRNA expression in the 22-month colonic mucosa relative to the 3-month. DRG sample was used as positive control to test the primer's activity.

5.3.2 Determine the quality of the microarray run

Three confirmation steps were followed in order to know if the microarray scan indicated differences between samples. The first was the principle component analysis (PCA) and as can be seen in Figure 36 (see below) the PCA of young samples (jejunum and colon) indicated the similarities and differences between samples and grouped them into six groups which represented the six animals used (A). It can be seen that jejunum samples (yellow dots, N = 3) were plotted close to each other and were distinguished from the colon samples (blue dots, N = 3). The same was observed in every other comparison (B, C, & D).

Secondly, I identified a list of genes that are recognised as Paneth cells, well-known markers which are known to be intensively expressed more in jejunum than in colon. Jejunum mucosa expressed higher levels of antimicrobial peptides characteristic of Paneth cells, including alpha defensin family (Defa), lysozyme (Lyz), and regenerating islet-derived 3 alpha (Reg3A) (see Table 6 below) (Farin et al., 2014). The fold difference of gene expression was calculated in jejunum over colon and the values indicate that they are higher in jejunum.

The third step of confirmation was to identify colonocyte's markers. Colon expressed a higher expression of carbonic anhydrase 1 (Car1), colon SVA-like protein (Sval1), and mucosal pentraxin (Mptx1) (see Table 7 below). The fold difference of gene expression was calculated in *jejunum over colon*, therefore the negative values indicate that they are less in jejunum.

Lastly, a third list of genes that were involved in digestion/nutrients-transportation and known to be expressed more in the jejunum were also identified and compared to their level of expression in the colon to confirm the previous results (see supplement Table 1).
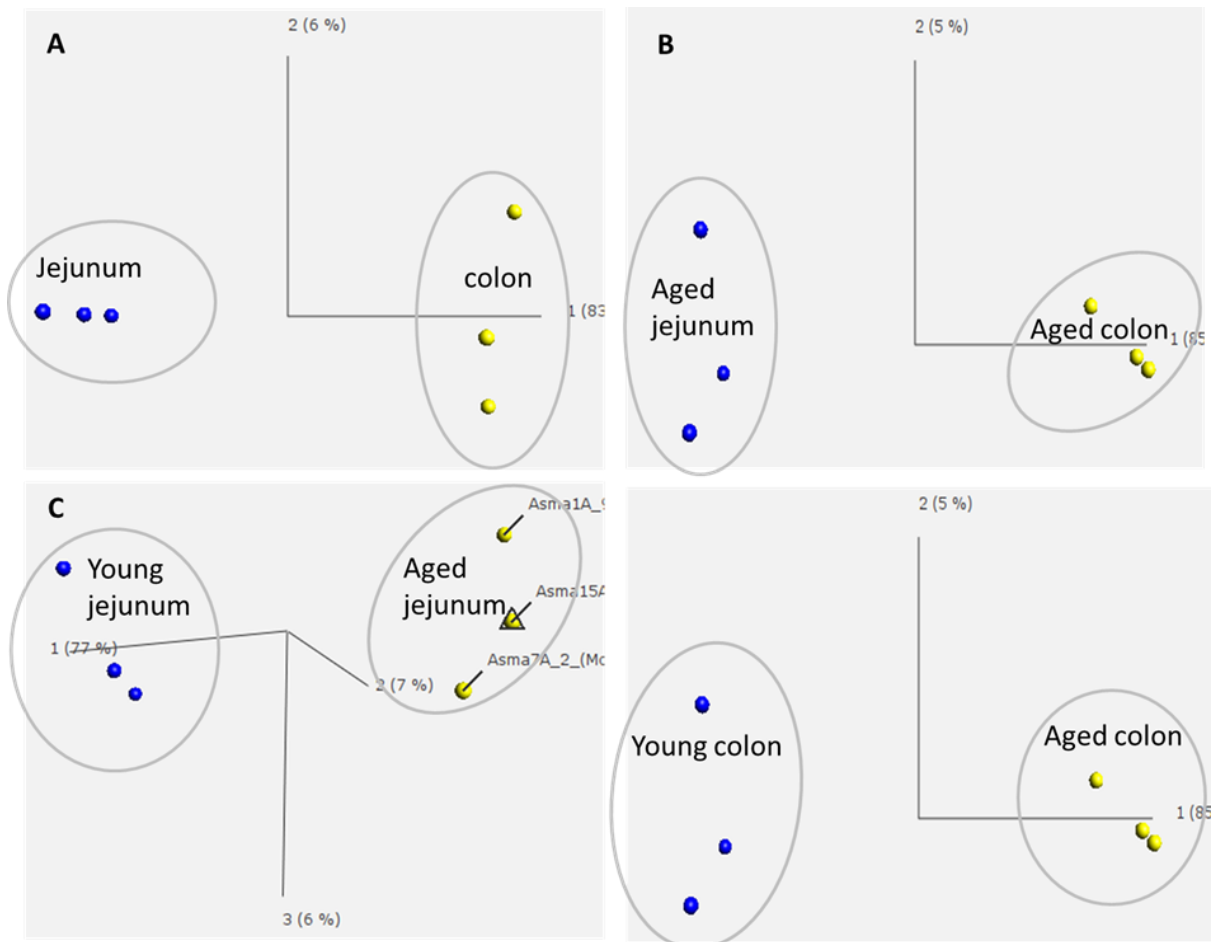
Figure 36: Principal component analysis (PCA) of microarray data:

Each dot represents samples from one animal. In **A,** looking at samples from jejunum and colon (young group), no overlapping was observed. In **B**, samples blotted from aged jejunum and aged colon showed also no overlapping between groups. Using only two dimensions (2 PC) for samples from young and aged jejunum was not enough to visualise the discrimination between groups, and therefore a third PC was used. In **C,** there is clear separation between clusters of young colon and aged colon.

Table 6: Expression of Paneth cell markers is higher in jejunum than in colon

(**Fold difference:** is the difference in gene expression in jejunum over colon)

Symbol	Gene Name	Gene Accession	Fold Difference
Defa29	Alpha-defensin 29	NM_001177482	121.7
Defa17	Alpha-defensin 17	NM_001167790	75.4
Lyz1	Lysozyme C-1	NM_013590	49.4
Defa22	Alpha-defensin 22	NM_207658	42.5
Defa3	Alpha-defensin 3	NM_007850	41.8
Defa23	Alpha-defensin 23	NM_001012307	40.5
Defa24	Alpha-defensin 24	NM_001024225	39.0
Defa21	Alpha-defensin 21	NM_183253	34.9
Defa26	Alpha-defensin 26	NM_001079933	28.2
Reg3a	Regenerating islet-derived protein 3-alpha	NM_011259	14.7

Table 7: Expression of colonocyte's markers is higher in colon than in jejunum.

(**Fold difference:** is the difference in gene expression in jejunum over colon)

Symbol	Gene Name	Gene Accession	Fold Difference
Slc6a14	Sodium- and chloride-dependent neutral and basic amino acid transporter B(0+)	NM_020049	-78.9
Car1	Carbonic anhydrase 1	NM_001083957	-69.6
Sval1	Colon SVA-like protein	NM_027832	-41.5
Mptx1	Mucosal pentraxin	NM_025470	-32.3

5.3.3 Young jejunum versus young colon

Deferentially expressed genes between jejunum and colon of young mouse

The analysis was applied on genes that were expressed in both regions and two criteria were taken into consideration in order to judge the difference on the levels of expression between jejunum and colon, fold difference thresholds of 1.3-fold and P value smaller than 0.05. The analysis indicated a significant difference in the level of expression of 3948 genes between the jejunum and colon. Then, two lists of genes were made according to their level of expression. List (jejunum) is for genes highly expressed in jejunum over colon and list (colon) is for genes that are highly expressed in the colon over jejunum.

In order to understand which pathways these genes are involved in, PA was applied on both lists, using PANTHER 11 and the number of genes in each protein class was calculated. The number of genes in all categories was exported but my interest was focused on oxidation and antioxidants genes.

Based on protein class, the analysis indicated that in the jejunum, more defence/ immunity genes are highly expressed in jejunum than in colon. Oppositely, in the colon, more oxidoreductase genes are highly expressed than in jejunum (see Figure 37 below) which indicates that in colon, there is more chance for ROS to be produced. As ROS is known to activate the expression of antioxidant genes. It is likely that they would be overexpressed in colon over jejunum. As expected, antioxidants' genes were overexpressed in the colon over jejunum (fold difference 2-4). However, the significant value was seen only in Prdx6 gene.

That said, three genes (Fabp1, Apoa4, and Ada) that participate in secondary antioxidant activity pathways were over 10-folds higher in jejunum over colon (Figure 38).
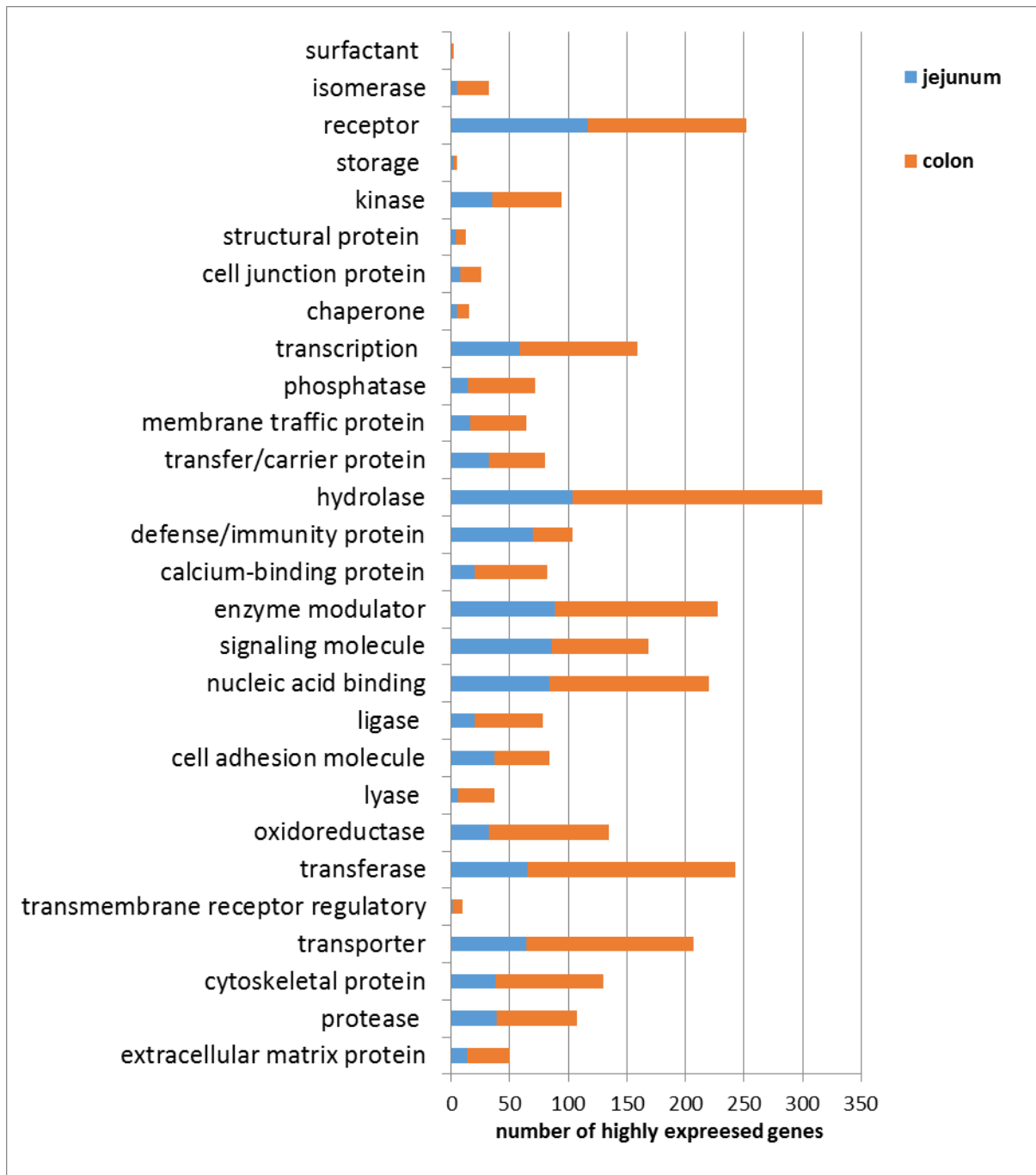
Figure 37: Numbers of highly expressed genes in the jejunum and colon of young mice

Histograms indicating the number of genes highly expressed in one region over the other in each category.
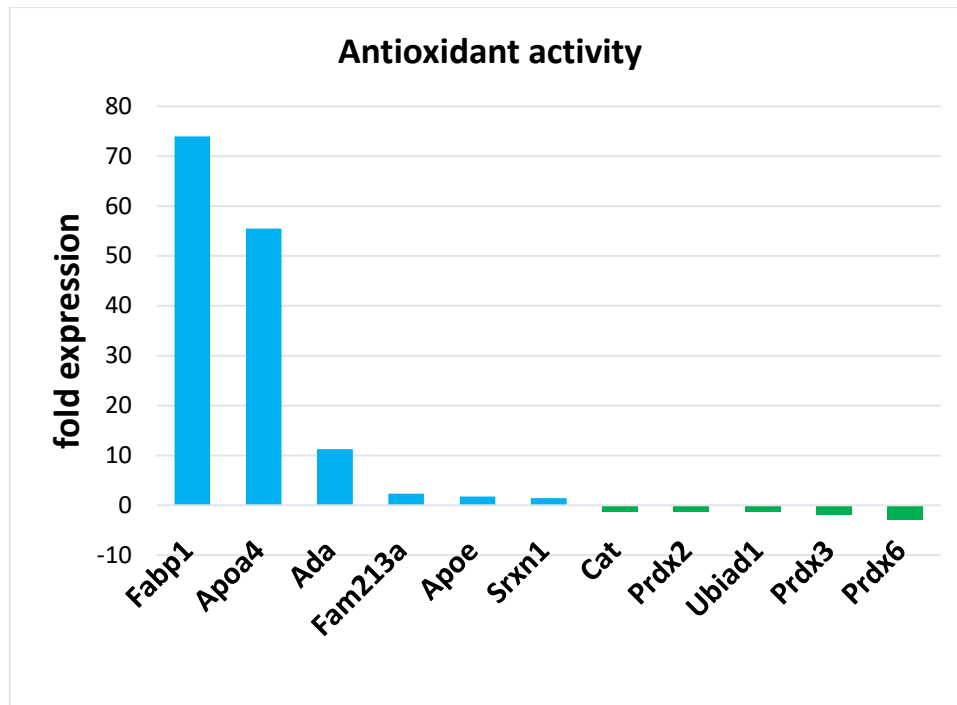
Figure 38: Genes involved in secondary antioxidant mechanism are overexpressed in jejunum over colon.

Histograms show that genes involved in direct antioxidant mechanisms were highly expressed in the colon (green) over jejunum (blue). The levels of expression of genes involved in indirect antioxidants' pathways (Fabp1, Apoa4, and Ada) were significantly higher ($P < 0.05$, multiple t-test) in jejunum over colon. Fold difference is the gene expression in jejunum over colon (negative value means that it is higher in the colon). Fab1: Fatty acid binding protein 1, Apoa4: Apolipoprotein A4, Ada: Adenosine deaminase, Apoe: Apolipoprotein E, Srxn1: Sulfiredoxin 1, Cat: Catalase, Prdx: Peroxiredoxin

5.3.4 Aged jejunum versus aged colon

5.3.4.1 Ageing-related changes in gene expression are more prominent in the colon than in the jejunum

To evaluate the molecular effects of ageing, the microarray RNA analysis was performed on young and aged groups. Fold change (aged over young) threshold of 1.3-fold was applied first to form a general picture about the age-related change in genes expression

in both regions. The results showed that ageing altered the expression of 274 genes in the jejunum and 524 genes in the colon (Figure 39). To identify the genes that were markedly changed in the aged group, a higher threshold (≥1.5-fold) was chosen in addition to *P* value < 0.05.
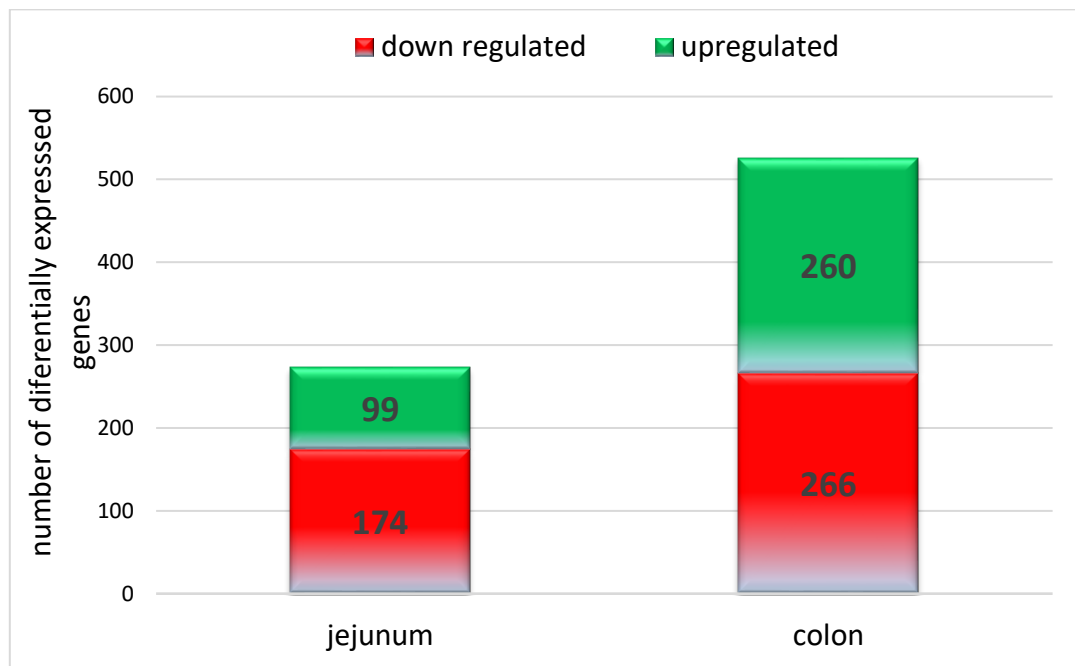
Figure 39: Ageing-related changes in gene expression are more prominent in the colon than in the jejunum.

Microarray data indicated that the number of genes changed as a result of ageing was higher in colon than in jejunum (aged vs young). (Fold change ≥ 1.3, *P* < 0.05, Multiple t-test with Sidak-Bonferroni correction, N = 3).

5.3.4.2 Ageing induced more changes in oxidoreductase and inflammatory genes expression in the colon

The data indicated that the number of oxidoreductase genes changed in aged compared to young was higher in colon than in jejunum. This could indicate a disruption in the

process of oxidation/reduction and ROS-production in the aged colonic tissue (see Table 9 below).

Because ROS is related to inflammation, I looked at inflammatory genes as well. Both aged jejunum and colon exhibited a downregulation (2-fold) in genes responsible for negative regulation of inflammatory response and protection response against viruses. This observation can suggest a low/mild increase in the inflammatory status in both regions as a result of ageing. However, the aged colon displayed an increase in genes related to positive regulation of inflammatory response and production/secretion of cytokines including the pro-inflammatory Interleukin 33 (IL-33) and one receptor type 1 (Il1r1) while aged jejunum displayed a downregulation in Il23a and Il18bp (Table 10).

This finding suggests that inflammation might be presented more in aged colon than in aged jejunum.

Table 8: Ageing induced more changes in the expression of oxidoreductase genes in the aged mouse colon.

Fold change (FC): is change in gene expression in aged compared to young

Aged jejunum			Aged colon		
Gene Symbol	FC	P value	Gene Symbol	FC	P value
Cyp2b13	-1.3	0.02	Cyp2d26	1.6	0.02
Aldh3b1	-1.3	0.002	Pipox	1.5	0.01
Aldh4a1	-1.8	0.04	Hpd	1.5	0.02
Rdh9	-1.9	0.03	Hsd17b13	1.4	0.01
Hsd3b3	-2.0	0.01	Far2	1.4	0.02
Cyp3a11	-2.2	0.01	Fads1	1.4	0.02
			Qsox1	1.4	0.02
			Sod3	1.4	0.04
			Aldh1l2	1.3	0.04
			Tet2	-1.3	0.02
			Prdx6	-1.3	0.01
			Cyp51	-1.4	0.03
			Sqle	-1.4	0.01
			Hmgcr	-1.5	0.02
			Msmo1	-1.5	0.05
			Nnt	-1.7	0.0004
			Dhrs9	-2.8	0.05

Table 9: Ageing induced more changes in the expression of inflammatory genes in colon than in jejunum.

Fold change (FC): is change in gene expression in aged compared to young.

Aged jejunum			Aged colon		
Gene Symbol	FC	P value	Gene Symbol	FC	P value
H2-M1	1.5	0.04	Iglv1	2.4	0.04
Cd1d2	1.5	0.04	H2-Aa	1.8	0.03
Tac1	1.4	0.04	H2-Ab1	1.8	0.01
Stk39	1.3	0.01	Cd74	1.7	0.03
H2-M10.1	1.3	0.01	Ccl11	1.6	0.04
Il18bp	-1.3	0.02	Nupr1	1.6	0.02
Fcgr1	-1.3	0.02	Cd4	1.5	0.001
Il23a	-1.4	0.04	Bcl2	1.4	0.001
Mbl2	-1.4	0.04	Bdkrb2	1.4	0.04
Lipa	-1.4	0.04	Fgr	1.4	0.05
H2-Q1	-1.8	0.04	Nkx2-3	1.4	0.004
Ggt1	-1.8	0.03	Ccl17	1.4	0.01
Vnn1	-1.9	0.03	Susd2	1.3	0.03
Susd2	-2.2	0.01	Trp53	1.3	0.03
Ifit1	-2.2	0.01	Ccrl2	1.3	0.02
Trim15	-2.2	0.01	Fcgrt	1.3	0.01
Nt5e	-2.5	0.002	Il33	1.3	0.04
Ada	-2.9	0.0003	Il1r1	1.3	0.02
			Agt	1.3	0.01
			Mx1	-1.3	0.01
			Trat1	-1.3	0.04
			Ccl25	-1.3	0.003
			Serpinc1	-1.5	0.01
			Syk	-1.5	0.004
			Trim15	-1.5	0.04
			Oas1g	-1.7	0.01
			Apoa1	-2.0	0.03

5.3.5 Limited number of genes exhibited similar age-associated changes in jejunum and colon

The lists of the differentially expressed genes in both regions were compared using Venny as described in the data-analysis section. Venny's analysis indicated 23 genes that were changed in both regions (Figure 40, see below). The pathway analysis (using PANTHER)

was able to identify 19 genes while four were unknown and therefore excluded. The genes are presented in Table 11. The results suggest the following:

1- Decreased lipid transport and metabolism in both jejunum and colon as significant reduction in Apol7a (apolipoprotein L 7a), a member of apoli-poprotein gene family.

2- Increase in susceptibly to cancer as tumour-suppressor gene FERM domain containing 3 (Frmd3) was significantly depressed in both regions.

3- Change in mucosa permeability as cell-adhesion genes showed a decrease in mRNA expression in aged jejunum and colon. The list includes Gprc5a, Lama3, Ceacam20, and Gzmb.

4- Decreased immunity in the aged GI as genes involved in innate immune response Trim15, Trim40, and RasGEF, were downregulated.

It has to be mentioned that data also showed downregulation in Mxd1 (MAX dimerization protein 1), an important regulator for transcription and DNA-templated.
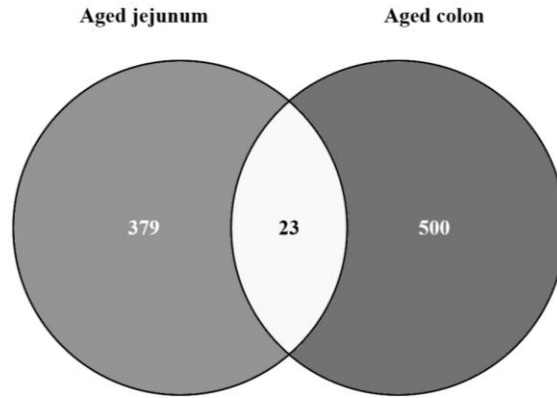
Figure 40: Overlapped genes between jejunum and colon of aged mouse

Venny's analysis indicated that 23 genes exhibit differential expression in both jejunum and colon of aged group.

Table 10: List of the intersection genes in the aged jejunum and colon

Fold change (FC): is change in gene expression in aged compared to young

Name	Symbol	protein class	Jejunum FC	Colon FC
Protein Igkv12-98	Igkv12-98	immunoglobulin	2.6	1.7
Tripartite motif-containing protein 40	Trim40		-1.9	-1.4
Protein Ighv1-54	Ighv1-54	immunoglobulin	-5.0	3.0
FERM domain-containing protein 3	Frmd3		-1.5	-1.3
Protein Trim15	Trim15		-2.2	-1.5
Apolipoprotein L 7a	Apol7a	apolipoprotein; transporter	-2.4	-1.4
Multimerin-1	Mmrn1	extracellular matrix glycoprotein	2.3	7.3
Retinoic acid-induced protein 3	Gprc5a	G-protein coupled receptor	-2.7	-1.6
SAGA-associated factor 29 homolog	Ccdc101		1.4	1.3
Protein Igkv9-124	Igkv9-124	immunoglobulin	-3.0	5.4
Phospholysine phosphohistidine inorganic pyrophosphate phosphatase	Lhpp	phosphatase	1.4	1.3
Ras-GEF domain-containing family member 1B	Rasgef1b	guanyl-nucleotide exchange factor	-2.1	-1.3
Solute carrier family 13 member 1	Slc13a1	cation transporter	-1.8	1.4
Laminin subunit alpha-3	Lama3	extracellular matrix linker protein; receptor	-1.9	-1.5
Protein Ceacam20	Ceacam20		-1.9	-1.3
Granzyme B(G,H)	Gzmb	serine protease	-2.1	1.3
Sushi domain-containing protein 2	Susd2		-2.2	1.3
Max dimerization protein 1	Mxd1	basic helix-loop-helix transcription factor	-1.8	-1.6

5.3.6 Aged jejunum versus young jejunum

Marked changes in immunoglobulins and metabolic genes in the aged mouse jejunum

The genes that exhibited more than 2-fold difference are presented in the supplements section, Table 2. Starting with the most upregulated genes, they were members of immunoglobulin kappa variable (Igkv, 18 genes) followed by immunoglobulin heavy family (Ighv, 14 genes). The human immunoglobulins (Igs) are the antigen-recognition molecules of B cells and involved in immune-response pathways. Solute-carrier family 1, member 3 (Slc1a3), showed a 1.8-fold increase in aged jejunum. This gene encodes a member of a high-affinity glutamate transporter family, essential for many cells' process, including nutrient transport.

Bottom of the list, immunoglobulin kava member Igkv12-89 showed the most downregulation (-5.8-fold) in aged jejunum followed by other immunoglobulins.

The microarray data indicated a marked decrease in the expression of genes involved in the lipid metabolic pathway and micronutrients' absorption (Vitamin B12, chloride, sodium, and phosphate. Fatty-acid binding protein 6 (Fabp6) showed the most downregulation (>4-fold). Fabp6 protein binds to long-chain fatty acids and bile acids and plays a vital role in fatty-acid uptake, transport, and metabolism. Serum amyloid A (Saa1) was significantly downregulated (>-3-fold). This gene functions in the cholesterol metabolic process (Figure 6, see below). Furthermore, Cubilin (Cubn) transcript showed more than 2.4-fold decrease in the aged group. Cubilin protein acts as a receptor for intrinsic factor-vitamin B12 complexes. The list also includes solute-carrier family 34 member 2 (Slc34a2), a pH-sensitive sodium-dependent phosphate transport, see Table

13. The analysis also showed more than 2.3-fold upregulation in Multimerin 1 (Mmrn1) which is considered a marker for tumour cells.

Table 11: Downregulation of metabolic genes in the aged jejunum.

Fold change (FC): is change in gene expression in aged compared to young.

Gene	Gene name	FC	P value	Biological Process Term
Slc27a4	Long-chain fatty acid transport protein 4	-2.0	0.02	lipid metabolic process
Apoc2	Apolipoprotein C-II	-2.0	0.02	lipid metabolic process // transport
Cubn	Cubilin	-2.4	0.003	lipid metabolic process // transport
Plb1	Phospholipase B1, membrane-associated	-2.4	0.002	lipid metabolic process // phospholipid metabolic process
Slc34a2	solute carrier family 34 member 2	-2.7	0.0006	sodium ion transport // phosphate ion transport
Clca6	chloride channel accessory 6	-3.9	4.0E-06	chloride transport // chloride transmembrane transport
Fabp6	Fatty acid binding protein 6	-4.2	9.5E-07	transport // bile acid metabolic process
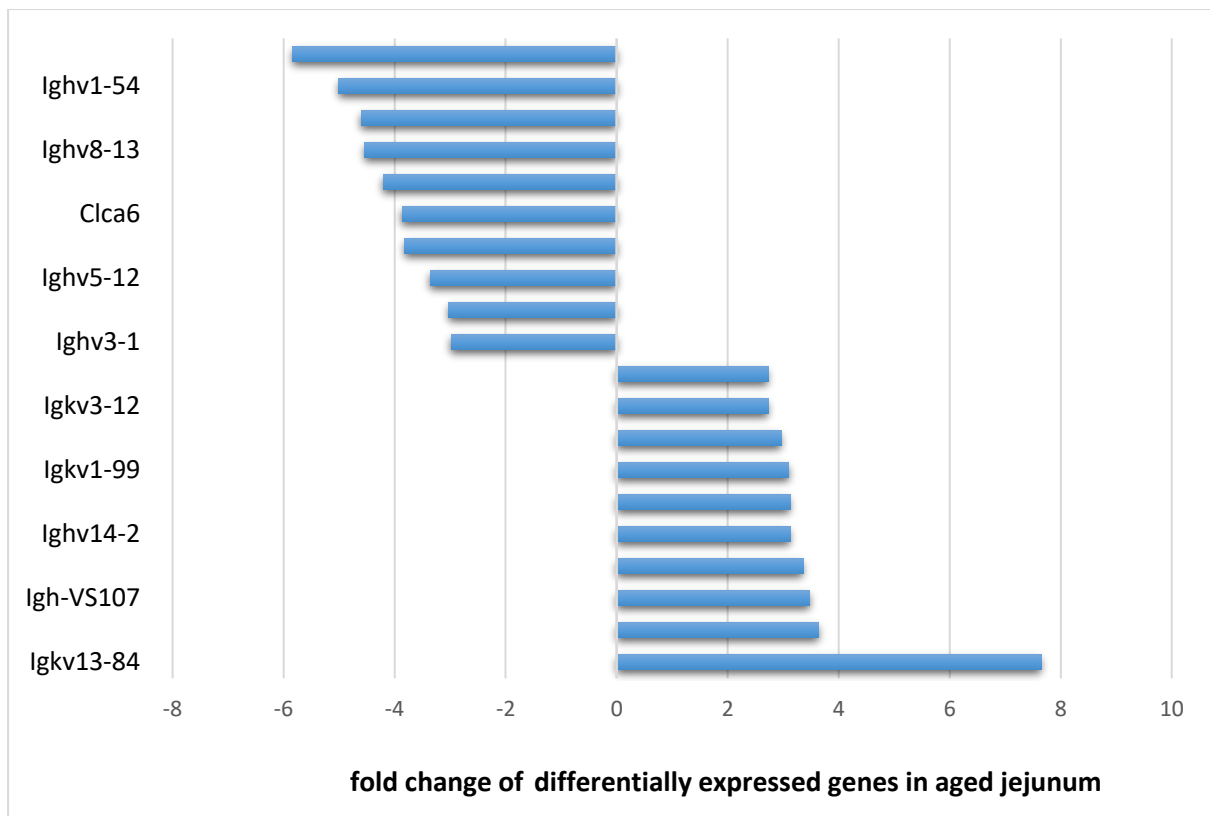
Figure 41: The most changed genes in aged mouse jejunum compared to young.

Histograms show the most upregulated and downregulated genes in aged colon compared to young. **Fold change:** is the gene expression in aged colon compared to young.

5.3.7 Aged colon versus young colon

Marked change in defence, metabolic and inflammation-related genes in the aged mouse colon.

Genes that exhibited the most change (more than 2-fold) in aged colon are presented in Figure 42.

Multimerin 1 (Mmrn1), which is expressed in tumour cells, appeared on the top of the upregulated genes with more than 7-fold increase. Not far from that is seen in jejunum;

members of the immunoglobulins (Igkv = 24, Ighv = 16) dominated the most upregulated area in the list.

At the far end of the list, the main downregulation (>14-fold) was observed in defensin alpha 22 (defa 22) gene. Other defence genes such as Intelectin 1 (Int1) which encodes the protein lectin and facilitates phagocytic clearance of microorganisms, Alpha defensin family members (Def21, 24, 23), and lysozyme 1, which are known to play a role in defence response to bacteria, showed also a marked decrease in their expression (>3-fold). Mucosal pentraxin 2 (Mptx2), which is involved in maintenance of the mucosal barrier, exhibited more than 3.9-fold decrease.

Concerning metabolic process, 2-fold reduction was observed in Apolipoprotein A-I (Apoa1) gene, which encoded high-density lipoprotein (HDL). The HDL is important for the removal of cholesterol and other fats from tissues by transporting them to the liver for excretion. Similarly, transferrin receptor (Tfrc), which encodes a cell-surface receptor necessary for cellular iron uptake, showed 2.1-fold decrease in the aged colon.

Two genes involved in pH regulation process displayed more than 2-fold decrease in the aged colon (FXYD domain containing ion transport regulator 4 [Fxyd4] and solute-carrier family 9 member 3 [Slc9a3]). They have been implicated in inflammatory conditions. More interesting, mucin 1, transmembrane (Muc1), showed a significant >1.8-fold increase. Mucin protein is known to be increased in response to hypoxia and important for maintenance of immune status. A complete list of genes that exhibited ≥ 2-fold changes is provided in supplement Table 3.
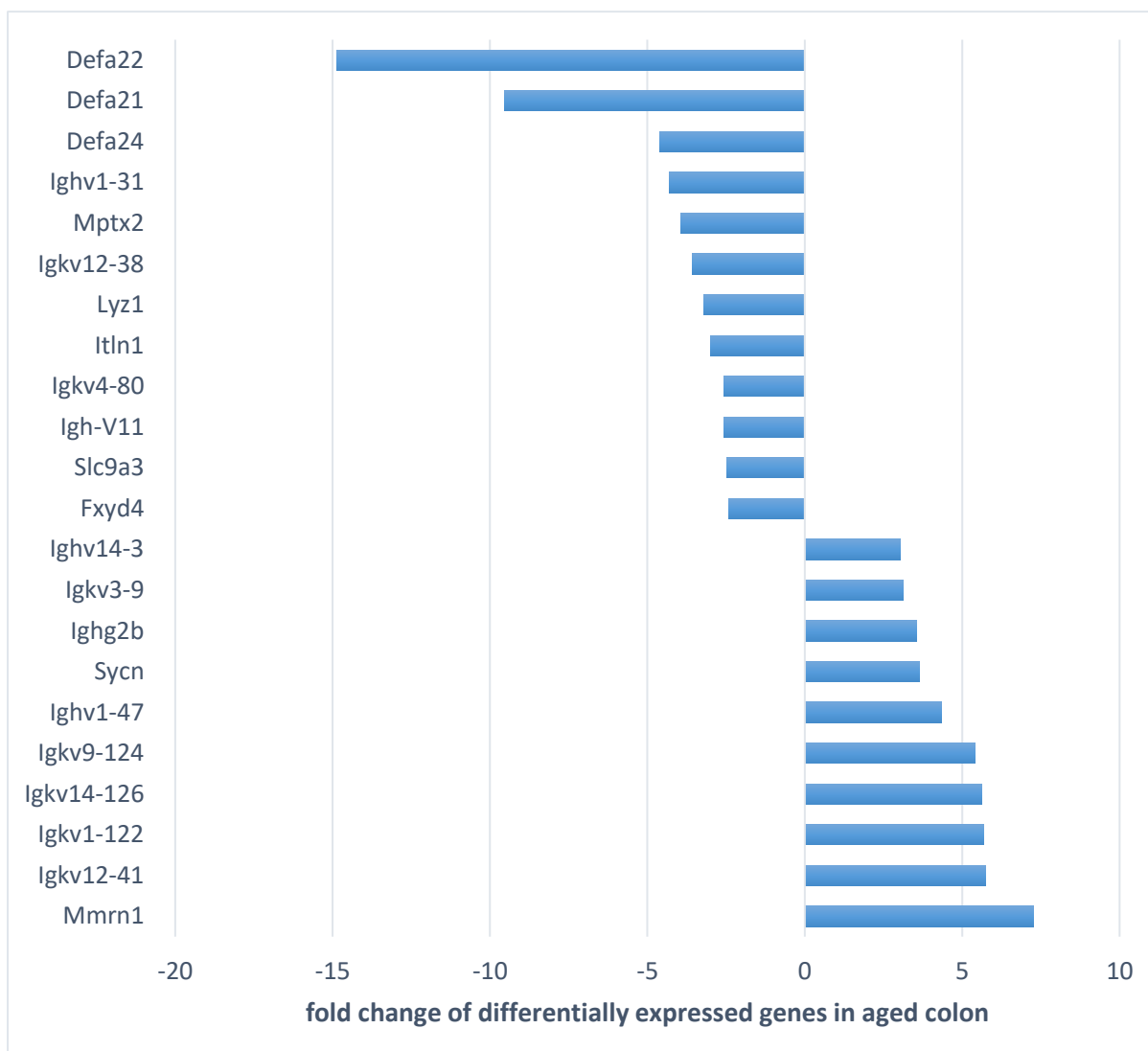
Figure 42: The most changed genes in aged mouse colon compared to young.

Histograms show the most upregulated and downregulated genes in aged colon compared to young. 2, P<0.05.

Fold change: is the gene expression in aged colon compared to young.

5.4 Discussion

In the present study, RNA microarray analysis was used to screen for regional differences in gene expression in the small intestine and colon of the mouse. My focus was on the expression of oxidoreductase and antioxidant genes in order to reach a better understanding of the difference seen in sensory-activity response to oxidative stress induction to the mucosa between the jejunum and colon. In addition, sensory function of the colonic nerves was affected more by ageing—therefore I screened for age-associated changes in both regions.

Many years ago, the molecular data of regional gene expression was lacking in GI biology. Unlike studying specific genes and mechanisms regulating regional gene expression, the microarray technology allows researchers to obtain genome-level information of regional specification of gene expression in the GI tract. The microarray technology offers analysis of the expression of thousands of genes at the same time and generates data about gene expression in tissues/cells of various sources and for gene discovery. Microarray has been used as a tool to study gene-expression profiles in normal tissue of the GI tract (Mariadason et al., 2005) and also to determine changes in gene expression in response to ageing (Englander, 2005, Steegenga et al., 2012), and inflammation and cancer (Avula et al., 2012).

Upregulation of TRPA1 mRNA in the aged colon and response to oxidative stress

The colon was found to be more susceptible to oxidative stress and ageing, therefore it was interesting to assess the mRNA expression of some members of TRP channels in the colonic mucosa. The QPCR results indicated an increase in TRPA1 mRNA in the aged mucosa which may have occurred as a result of greater oxidative status compared to the young. This result suggests that the response to oxidative stress may be higher in the aged

colon compared to the young. However, there are two points to be considered when discussing this result. The first is that the overexpression of genes does not necessarily mean that more protein is expressed and active in the tissue and since no protein-quantification was performed, I can only predict an increase in protein levels. Secondly, TRPA1 expression was measured in the entire mucosa and not specifically in sensory-neuron endings. It is possible that the gene's upregulation may have reflected the increase in the number of cells expressing this channel in the aged mucosa. This is very likely if we consider that within mucosa cells, TRPA1 expression is abundant in EC cells (Nozawa et al., 2009), and they release 5-HT which activates sensory neurons. EC cells' number has been recently reported to be increased significantly in aged colonic samples compared to young (Keating et al., 2016).

Pro-oxidant environment in the colon

Short-chain fatty acids (products of luminal bacterial fermentation) are the preferred energy source for colonocytes (Roediger, 1982). Therefore, it was not surprising to find that 98 oxidoreductase genes including fatty-acid oxidation genes were highly expressed in the colon over jejunum. As a consequence, one can suggest that more ROS and subsequently a higher level of antioxidants are normally produced by colonocytes. ROS has been shown to stimulate the gene expression of antioxidant peroxidase 6 (Prdx6) in the human epithelial cell line (Chowdhury et al., 2009) and the activity of antioxidant enzymes—superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)—in rat colonocytes (Sanders et al., 2004). In the present data, only Prdx6 was significantly higher (3-fold) in the colon over jejunum. This gene encodes the enzyme responsible for catalysing H_2O_2 into water and prevents its harmful accumulation in the cell.

The previous study by Sanders et al. showed that exogenous H_2O_2 causes more DNA damage to colonocytes despite their possession of higher antioxidant activity. The damage was suggested as accruing at a rate that exceeded the repair ability of antioxidants and therefore possibly explained the high risk of colorectal carcinogenesis (Sanders et al., 2004). Taking this evidence in addition to the present microarray data, it is very possible that the colonic pro-oxidant environment contributed to the susceptibility of colonic sensory activity to oxidative stress induced by H_2O_2 and antimycin A that was shown in Chapter 3.

Lipid metabolic genes' and antioxidants' properties in the jejunum

The present data indicated that the mouse jejunum showed high expression of lipid metabolic genes that participate in secondary antioxidant pathways. Liver fatty-acid binding protein (FABP1) was significantly overexpressed by more than 70-fold in the mouse jejunum. This protein has been described as having strong antioxidant properties and contributing to cellular response to H_2O_2. It was reported that ROS levels after treatment with H_2O_2 were lower in Chang FABP1 transfected cells compared to vector transfected cells (Wang et al., 2005).

Apolipoprotein A-1&4 genes were also overexpressed in the jejunum compared to colon. The proteins encoded by these genes are involved in the transport of triglycerides and cholesterol in the small intestine. The proteins also participate in the H_2O_2 catabolic process. In diabetic patients with cardiovascular disease, the reduced level of APoA4 was associated with decreased-plasma total-antioxidant status. The study offered a support for the antioxidant capacity of ApoA4 (Wong et al., 2004). The microarray data also showed that Adenosine Deaminase (Ada) was overexpressed by more than 10-fold in the jejunum over colon. The protein is mainly responsible for purine metabolism but

possesses many other properties, such as negative regulator of inflammatory response and response to H_2O_2 and hypoxia.

These results suggest that jejunum is better equipped to handle ROS, which could explain—to a certain degree—the lack of response in jejunum after induction of oxidative stress by H_2O_2 and antimycin A in electrophysiology experiments (see Chapter 3). It is possible that proteins encoded by fatty-acid genes which possess powerful secondary antioxidant activity were able to catalase H_2O_2 into water at a fast rate and therefore jejunum tolerated high concentration of the peroxide. At the same time, the overexpression of ROS-production genes in the colon provided a pro-oxidant environment and therefore was susceptible to any further increase in ROS. The secondary antioxidants, fatty acids, genes, and proteins represent a very attractive target for future direction to confirm the current results and examine how they can be relevant to the GI sensory function.

Impact of ageing on the mouse intestine

In the present study, microarray RNA expression analysis was used to screen for age-associated changes in gene expression in the mouse jejunum and colon. The two major findings of this screen are: firstly, that the colon is affected more by the ageing process, and secondly, that fatty-acid metabolism and transporting function are affected by age in both the jejunum and colon.

Tumorigenesis genes were more pronounced in the aged colon

Tumour-suppressor genes were downregulated in both jejunum and colon. However, tumour marker Mmrn1 was greatly pronounced in the aged colon. This provides more support to the commonly accepted fact that age represents a risk factor for colorectal cancer (Suzuki et al., 2006). It has to be mentioned that although the change in mRNA

expression was not striking in many genes, such as solute-carrier family 13 (Slc13a1) that has been suggested as playing an important role in cancer and longevity (Markovich et al., 2011). The function of the encoded protein is what determines eventually the importance of any size of change.

Cell-adhesion genes were more downregulated in the aged jejunum

Reduction in cell-adhesion genes can cause impaired mucosal permeability and may relate to a leaky gut and increase the proneness to microbial invasion. Although the degree of reduction was not profound, the significance of this function indeed requires more studies to address this change.

Metabolic genes in aged jejunum

The main function of the jejunum is nutrients' digestion and absorption, which requires a synchronised process of multiple cell types and a continuous energy supply.

The main finding obtained from comparing young jejunum with the aged is that no major changes accrue in the jejunum as a result of advanced age. The analysis showed no change in energy-production genes, which is in line with previous study that reported no change in the energy-generating genes in the aged mouse jejunum (Steegenga et al., 2012). Reduction in some metabolic genes was observed (fatty-acid binding proteins and lipid-transport genes). The reduced expression of these proteins can be translated to diminished capacity for lipid absorption and metabolism. Cubilin gene expression was among the ones that exhibited marked decrease. The reduction in this gene could relate to vitamin B12 deficiently in the elderly. This finding is consistent with previous study (Englander, 2005).

Reduction in bile-acid transporter genes was also observed, which can suggest an age-associated bile-acid malabsorption, which contributes to faecal incontinence. In humans though, it is still unknown whether the elderly people are more common to develop idiopathic diarrhoea due to bile-acid malabsorption. At molecular levels, it seems that jejunum mucosa is well preserved in advanced age, which provides more support to my previous finding that ageing had no significant effect on small-intestine sensory function.

Inflammation, ROS, and cancer in the aged colon

The main finding from comparing young and aged colons is that change in defence-, metabolic-, and inflammation-related genes occurs in non-pathological ageing.

Colonic inflammation causes reduction in ion transporters (Borenshtein et al., 2008). The observed increase in interleukins (IL-33 and Il1r1) combined with down-regulation of ion transporters Atp12a and Fxyd4 (which are required for nutrient uptake) can be translated to a decline in transport of a host of nutrients, including glucose, amino acids, and bile acids, and may also contribute to inflammation-associated diarrhoea. In addition, the analysis indicated a reduction in the expression of Slc9a3 gene in the aged colon. Solute-carrier family 9 (sodium/hydrogen exchanger), member 3, (also known as NHE3) expression, is important for the maintenance of mucosal integrity and NHE3 knockout mice were reported to develop spontaneous colitis and are very prone to dextran sulfate (DSS)-induced mucosal injury (Larmonier et al., 2013). The NHE3 expression is downregulated by pro-inflammatory cytokines (Barmeyer et al., 2004) and enteric infections (Hecht et al., 2004), while commensal bacteria increases NHE3 expression (Singh et al., 2012). This downregulation raises the possibility of a change in the microflora composition as we take into account the role of Slc9a3 in shaping the gut

microbiota. Age-associated change in gut microbiota has been indicated in healthy humans (Odamaki et al., 2016).

ROS and following oxidative damage are consequent of inflammation. The upregulation of inflammatory genes along with the observed higher oxidative stress markers (8-OHdG and DHE, see Chapter 4) in the aged colon indicate an increase in the inflammatory state in advanced aged that is not associated with sensory hyperactivity. This data suggests that chronic exposure to high endogenous ROS could contribute to the decline of sensory function. However, this explanation needs more detailed analysis that was beyond the limit of this project.

As chronic inflammation and related DNA oxidative damage is known to increase the risk of cancer. It was not surprising to observe overexpression in the tumorigenesis genes and downregulation in tumour suppressors. Mmrn1, A4gnt, and Muc1 were reported in myeloid leukaemia (Laszlo et al., 2015) gastric cancer (Karasawa et al., 2012) and during adenoma-adenocarcinoma progression (Krishn et al., 2016).

Dehydrogenase/reductase member 9 (Dhrs9) possesses important anti-tumour activity by inhibiting cell proliferation. Dhrs9 was considered a potential prognostic biomarker of human colorectal cancer where its decline was correlated with tumour progression (Hu et al., 2016). Another tumour-suppressor (Itln1) was downregulated in the aged colonic mucosa. This gene was found to suppress the growth and metastasis of human neuroblastoma cell lines (Li et al., 2015). The upregulation in tumorigenesis genes and reduction in the tumour-suppressor genes suggest a malignant transformation of colon preneoplastic lesions, which is an early sign for colon cancer.

Interesting findings for future studies

Immunoglobulins' genes

Immunoglobulins (Igs) Igkv9-124 and Ighv1-54 were markedly decreased in the jejunum while showing an upregulation in the colon.

The appearance of Igs in our microarray data presented a puzzle because of their well-known expression and function in B Lymphocytes. Their presence can be referred to contamination in the RNA samples with blood cells, but this explanation is unlikely for the following reasons: my followed method for isolating mucosal cells includes dissecting the muscle layer, digesting only the mucosa on three occasions, discarding the waste after the first and second digestion. Thus, it was designed to minimise the contamination with blood, mucus, faeces, and bacteria. Considering this strict method, the Igs resulting from very few remaining blood cells should have a very low density. However, their level of expression was above the microarray's threshold and enough to be detected. Therefore, the immunoglobulins' turnout in our data was more likely because of their sufficient density in the intestinal mucosa of the jejunum and colon. This conclusion is even stronger if we know that some low-density genes (such as members of TRP channels), known to be well expressed in the intestine mucosa, did not show up in the data due to their low expression.

For many years, Igs have been known to be produced from B-lymphocytes. According to their molecular weight, they are classified into heavy and light chains. Immunoglobulins have been found in human epithelial cells and mouse neurons. Immunoglobulin SNC73 gene was found to be expressed in non-cancerous colorectal mucosa tissue (Hu et al., 2016). IgG heavy chain and kappa light chain were reported in mice's normal neurons (Huang et al., 2008). The information about the expression of Igs in normal tissue is very

limited while their biological function is basically unknown yet. Therefore, it is difficult to discuss the present interesting finding regarding the age-related change in the expression of Igs due to the lack of information about their function in the intestinal mucosa.

The Ig heavy and light chains have been found also to be expressed in the carcinoma cells line. In human tissues, the mRNA of IgG was found in breast, colon, liver, and lung cancers. Unlike normal Ig (B-lymphcytes), the function of Ig expressed in cancer cells is uncertain. There is evidence that blocking tumour-derived IgG by anti-human IgG antibody can suppress the growth of cancer cells and increase cell death. The Ig kappa light chain was found to be expressed in uterine cervical and epithelia with dysphasia and suggested as serving as a marker for malignant cell transformation (for review on Ig in non-lymphocytes see (Chen et al., 2009).

Appreciating the previous observations, it was not totally surprising to observe a change in the Igs expression in response to ageing, as data showed an enhanced expression of tumour marker in the aged mouse mucosa. To my knowledge, this data is the first to report age-associated change in the expression of Igs in the mouse-intestine mucosa.

Validation of the previous results requires firstly proving the expression of Igs in the mouse mucosa. This could be done by several methods: performing single-cell PCR, detecting protein expression by western blot, and immunostaining. Performing the single-cell PCR represents a challenge because the type of cell/s expressing Igs is unknown in a heterogenic population. The western blot technique can indicate the protein expression but not identify the type of cell/s and it requires highly specific antibodies. The same case applies for the immunostaining for which highly specific

antibodies for many types of immunoglobulins are commercially unavailable, although this remains the best way to detect the expression at a cellular level.

Unfortunately, no validation was performed for the present microarray data due to timeframe and limited funds for this project.

Significant reduction in defensin genes

The microarray analysis indicated a significant decrease in 6 members of defensin alpha family and lysozomal 1 in the aged colon compared to the young. In fact, these genes were found to be the most significantly reduced genes that showed more than 3-fold decrease in the aged colon. Defensin alpha and lysozomal 1 genes are markers of Paneth cells and primarily found in the small intestine, but they also are expressed in very low density in the proximal and distal colon. In humans, colorectal paneth cell metaplasia (PCM) is considered a hallmark of idiopathic IBD. In both healthy subjects and IBD patients, PCM has frequently been found in the proximal colon and is described as a non-specific phenomenon. In the distal part of the colon, PCM was significantly increased with occurrence of IBD than in healthy subjects (Tanaka et al., 2001).

Appreciating the previous study, it is possible that inflammation drives the colonic PCM. In this case, one can speculate that the young mice used in the present study were under inflammation and therefore reduction in Paneth cells was observed in the aged group. However, all results indicated the opposite, as no sign of inflammation was observed in the young mice. Electrophysiology experiments did not show any functional abnormality regarding nerve activity. Oxidative stress, which is associated with inflammation, was found to be lower in the young mice as described in Chapter 4. In addition, inflammatory genes were upregulated in the aged group.

Taken together, the observed reduction in the Defa family and Lyz1 genes is more likely to be translated into an age-dependent loss of colonic Paneth cells if we accept that metaplasia occurs for unidentified reasons in the healthy state. Information that can support this opinion is lacking as the present study is the first to report this observation. Further studies are indeed needed to investigate this possibility.

Reduction in metabolic genes

The noticeable change among metabolic genes was observed in lipid metabolic and transporter genes. These genes are not only responsible for the metabolic process but also involved in negative regulation of cytokine secretion and in immune response, and therefore reduction in their expression might be related to the observed change in inflammatory genes. Enhanced lipids, protein metabolism, and short-chain fatty-acid oxidation has been previously reported in the aged colon (Lee et al., 2001). Our results contradict these observations, as we reported a reduction in the mtRNA expression of lipid metabolism genes. One very possible explanation for this conflict in findings is the difference in tissue or the RNA extraction method. The mentioned study used whole tissue of the distal part of the rat's colon, whereas we used RNA extracted only from mucosal cells of the complete mouse colon. Therefore, difference in the type of tissue used may have affected the results. Our finding also comes in contrast to a previous study that reported no change in metabolic genes in the aged colon of C57BL/6J (Steegenga et al., 2012). It could be that the difference was noticed in the present data because of the material used for the microarray scan. Mouse gene 2.0 ST array was used in the present scan, which covered more transcripts that the 1.0 ST array that was used by Steegenga et al.
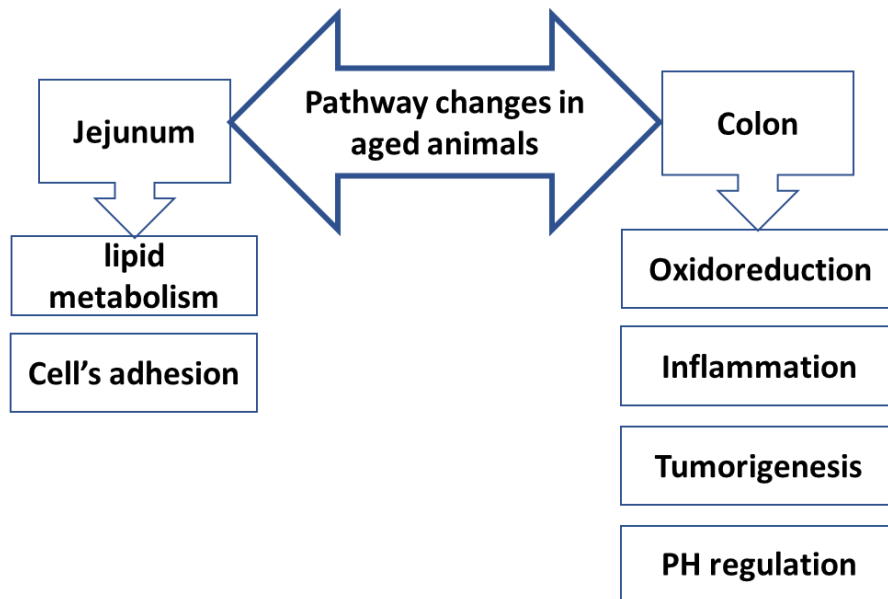
Figure 43. Diagram summarizes the pathway changes in aged jejunum and colon.

In conclusion, findings obtained by microarray analysis indicated an inherent difference in the expression of ROS production and secondary antioxidant genes between the mouse jejunum and colon. This difference may have led to colon tissue's inability to handle ROS as effectively as the small intestine. DNA damage caused by a long-life exposure to high endogenous ROS level may be the reason behind the colon's being more inclined to develop diseases or decline to function as a result of normal ageing. In addition, the microarray scan suggested that lipids' metabolism and ph regulation appeared to have deteriorated in the aged mice and the most marked effects of ageing were observed in the colon. The current findings provide indications of molecular events related to the decreased function and high prevalence of colon cancer in the elderly.

Chapter 6

Conclusion and future direction

Although oxidative stress is implicated in the pathogenesis of many GI diseases including IBD, ischemia and cancer, it is still not clear if it's involved in functional changes that accrue in non-pathological ageing. The GI tract is very susceptible to ageing as the incidences of a number of GI tract diseases are prevalent in the elderly. Importantly, 10-15% of new cases of IBD are diagnosed in elderly patients and 10-30% of IBD patients are over the age of 60 (Gisbert and Chaparro, 2014). Typical symptoms of IBD including anaemia, diarrhoea and abdominal pain are less likely to be presented by elderly patients (Katz and Feldstein, 2008a) and clinical studies have indicated a declined sensory function in the upper (Lasch et al., 1997b) and lower parts of GI tract (Lagier et al., 1999b) of healthy elderly subjects.

IBD has been described as "oxyradicals overload disease" and DNA oxidation in mucosal biopsies was reported to be greater in IBD patients than in normal individuals (Lih-Brody et al., 1996). Similarly, DNA oxidation was reported to be higher in samples from aged rat intestine compared to control (Bokov et al., 2004). It is unknown how oxidative stress influences sensory function of the small intestine and colon. We also don't know if the response to ROS is changed in advanced aged. This thesis came to address these questions.

Oxidative stress activates colonic mechanosensory function

Many pathological conditions such as inflammation and ischemia cause an excessive production of ROS which contributes to inflammation, ageing and cancer (Schieber and Chandel, 2014). The GI tract is an important source of ROS production.

Pathogens and ingested materials can cause inflammation by stimulating immune cells to produce H_2O_2, inflammatory cytokines and other mediators that activate further production of ROS. Increased ROS levels and oxidative DNA have been reported in IBD patients (Kruidenier and Verspaget, 2002). Harmful levels of H_2O_2 and inflammatory mediators within the mucosa can alter the sensitivity of gut afferents to become mechano/chemo-hypersensitive and for patients with IBD and IBS, altered GI motility, discomfort and pain are common symptoms (Brierley and Linden, 2014).

The current study in Chapter 3 indicates that mechanosensitivity of sensory neurons in the colon is more sensitive than in the jejunum to the induction of oxidative stress by H_2O_2 and mitochondrial inhibitor antimycin A. The jejunum resisted the luminal application of H_2O_2 (100-1000 μM) as well as antimycin A. The H_2O_2-hyperactivity was significant at high levels of distension (≥30 mmHg) and at ≥ 20 mmHg pressure for antimycin A in addition to spontaneous activity. Both vagal and spinal afferents can become sensitised by chemicals in the lumen but usually spinal afferents are the ones implicated in response to inflammation and injury and they respond to a wide dynamic range of distensions (Beyak et al., 2006). The observed hypersensitivity at high levels of distension indicates that ROS activates high threshold mechanosensitive spinal afferents.

In addition, afferents chemosensitivity was activated by direct application of H_2O_2 to the bath which was independent of muscle response as contractions were reduced in the colon. In line with the present findings, inflammatory cytokine IL-1β, H_2O_2, and NO have been shown to be higher in mucosa from ulcerative colitis (UC) patients compared to control (Cao et al., 2005). The study showed that treating normal sigmoid circular muscle cells with the solution collected from the UC submucosal side caused a decrease in muscle contraction and Ca^{2+} signals which was partially recovered by pre-treatment with IL-1β

antagonist or with antioxidant catalase. Therefore, the reduction in colonic muscle contraction in response to H_2O_2 that was observed in the present study can be related to IBD.

The current study demonstrated that sensory function of the jejunum is less affected by H_2O_2 and mitochordrial-ROS which suggests a protection mechanism in this region. To discuss this finding more, microarray data in Chapter 5 indicated that jejunum mucosa is provided with a dense expression of metabolic genes encoding proteins (Fabp6, Apoa4 and Ada) essential for the function of nutrients absorption in this site. These proteins possess powerful antioxidant properties as it has been shown that L-FABP-expressing cell line had lower intracellular ROS versus control (Wang et al., 2005). The human apoA-IV low expression was reported to be associated with increased oxidative stress status in diabetic and cardiovascular disease patients (Wong et al., 2004). Taken together, it can be considered that region specific secondary antioxidant proteins protect the jejunum against induction of oxidative stress.

At the same time, microarray data showed that more oxidoreductase genes were expressed in the colon over jejunum indicating a higher production of ROS in the colon which was associated with increased expression of the antioxidant Prdx6 gene. The human Prdx6 mRNA overexpression was reported in lung-derived cell line after treating the cells with H_2O_2 (Chowdhury et al., 2009). Hence, the pro-oxidant environment in colonic mucosa contributed to a less effective response to oxidative stress, therefore, sensory activity was more affected than the small intestine. The oxidative stress susceptibility is likely to increase the risk of developing inflammation and related afferents hypersensitivity and promote colorectal carcinoma caused by chronic inflammation at this part of the GI tract.

Ageing causes more effects in colon than in jejunum

Life expectancy has increased considerably over the past decades, and the world's population is ageing at an accelerated rate. The increase in life extent raises the risk of GI conditions such IBD, cancer, constipation and faecal incontinence. Therefore, there is a substantial need for a better knowledge of the biology of the changes associated with normal ageing and translates that understanding into therapeutics that can protect/improve GI function, and enhance the quality of life among elders. A highly significant positive correlation between oxidative markers and ageing has been reported In European healthy elders (Andriollo-Sanchez et al., 2005) which strongly predicts an increased risk of elevated oxidative stress in advanced age. Therefore, efforts were made here to examine the involvement of oxidative stress in age-associated changes in the small intestine and colon.

The data in Chapter 4 has concluded that there is a significant shift in baseline ROS levels combined with TRPA1 upregulation in the aged colon mucosa compared to young while the oxidative status of the aged jejunum mucosa was unchanged. TRPA1 channel expression can be found in all components of the gut wall (Boesmans et al., 2010) but is abundant in EC cells within the mucosa (Nozawa et al., 2009). The overexpression of this channel might indicate an increased EC density if we take into account recent data that reported a significantly higher number of EC cells in colonic samples from 24-month mice compared to 3-month (Keating et al., 2016). Increase in EC cells density and elevated levels of 5-HT are implicated in GI inflammatory disorders as was shown by clinical and experimental studies (Manocha and Khan, 2012) (Wheatcroft et al., 2005).

The accumulated oxidative stress and upregulated TRPA1 possibly resulted from low or mild chronic inflammation as microarray scanning (Chapter 5) showed a small

upregulation (1.3-fold) in inflammatory cytokine (IL1r1 and IL33) in the aged group compared to the young. Data also indicated a marked overexpression in mucin 1 (encodes glycoprotein) which is associated with infection and cancer; in addition to a downregulation of ion transporters (Atp12a, Fxyd4 and Slc9a3).

Although the microarray data is by no means definite evidence, as it was not confirmed by QPCR or specific protein measurements, these data combined with the rest (elevated levels of ROS and TRPA1 overexpression, which both known to be increased in inflammation) gives a strong indication that oxidative stress and inflammation are involved in normal ageing. This suggestion is supported by other investigations as several studies have previously proposed that increased circulating levels of several proinflammatory cytokines (including interleukin IL-1 and IL-6) promote a low-grade chronic systemic proinflammatory state in the elderly (see review by (Baylis et al., 2013, Biagi et al., 2010). In aged mouse colon, enhanced expression of TNFα and IL1β was reported (Steegenga et al., 2012). In addition, the density of mast cells and EC cells were reported to be increased with age in humans which suggests a shift in inflammatory state of the gut. Mast cells are the master regulators of the immune response and they were found in close association with afferent nerves. Clearly further investigations are needed to confirm and support these data.

Colonic mechanosensitivity function exhibited a significant decline at high levels of distension in aged mice compared to young while sensory function of the jejunum was unchanged. The response to mitochondrial-ROS in the aged colon was not significantly distinguished from the young. The jejunum continued to resist elevated ROS levels as sensory signalling in the aged group was unresponsive to intraluminal antimycin A.

Considering all, declined function of the colon in normal ageing could be due to a long-life exposure to endogenous ROS associated with chronic low or mild inflammation. The jejunum did not exhibit major changes at the molecular level as a result of ageing, which was reflected by normal oxidative levels and a well-preserved sensory activity. The mechanosensitive nerves are important to respond to noxious stimuli such as injury and inflammation and the decline in their function may participate in the lower perception of GI injury and IBD in the elderly, which can have devastating consequences upon the quality of life.

The present study also provided information about the involvement of TRPA1 channel in oxidative stress induced hyperactivity. TRPA1 channel present on colon mucosa and afferent endings are both possible targets of mitochondrial-ROS in the colon as current data showed that blocking this channel reduces antimycin A-mechanohyperactivity at high levels of distension. In DRG neurons, oxidizing agents like chloramine-T (ChT) has been shown to modulate the inactivation of K_V channels

Age-associated change in the mouse intestine

The current study indicated a change in gene expression of a very limited number of genes in the aged jejunum compared to young (chapter 5). Few metabolic genes exhibited a reduction in the aged mucosa samples; and the highest reduction (-4.2-fold) was in Fabp6 gene which is important for bile acid metabolic process and uptake. Reduction in this gene can contribute to bile acid malabsorption, consequently contributing to faecal incontinence, but there is no evidence yet if bile acid malabsorption induced diarrhoea is more common in elderly patients. Yet this finding represents a very attractive direction

for nutrients research as fatty acids are involved in potent inhibitory (satiety) signals and food intake has been reported to decrease with age as the elderly often present a decline in appetite which can promote malnutrition (Rémond et al., 2015).

More change was observed in the gene expression in the aged colon compared to young. As high levels of ROS were observed in addition to a high expression of inflammatory related genes in the colon, it was not surprising that the microarray scanning indicated an overexpression of tumorigenesis genes synchronized with downregulation of tumour suppressor genes, which represent early indication of developing cancer. The data also indicated a significant downregulation of defensin genes expressed by Paneth cells which suggests an age-associated loss of Paneth cells. Paneth cells metaplasia can accrue in the distal part of colon as a result of inflammation (Tanaka et al., 2001) and was reported in more proximal parts of the colon in children-IBD (Simmonds et al., 2014). However, there is no information about this phenomenon in the mouse. Because of their important function in innate mucosal defense, more studies are needed to investigate the significance of the loss of Paneth cells in advance age.
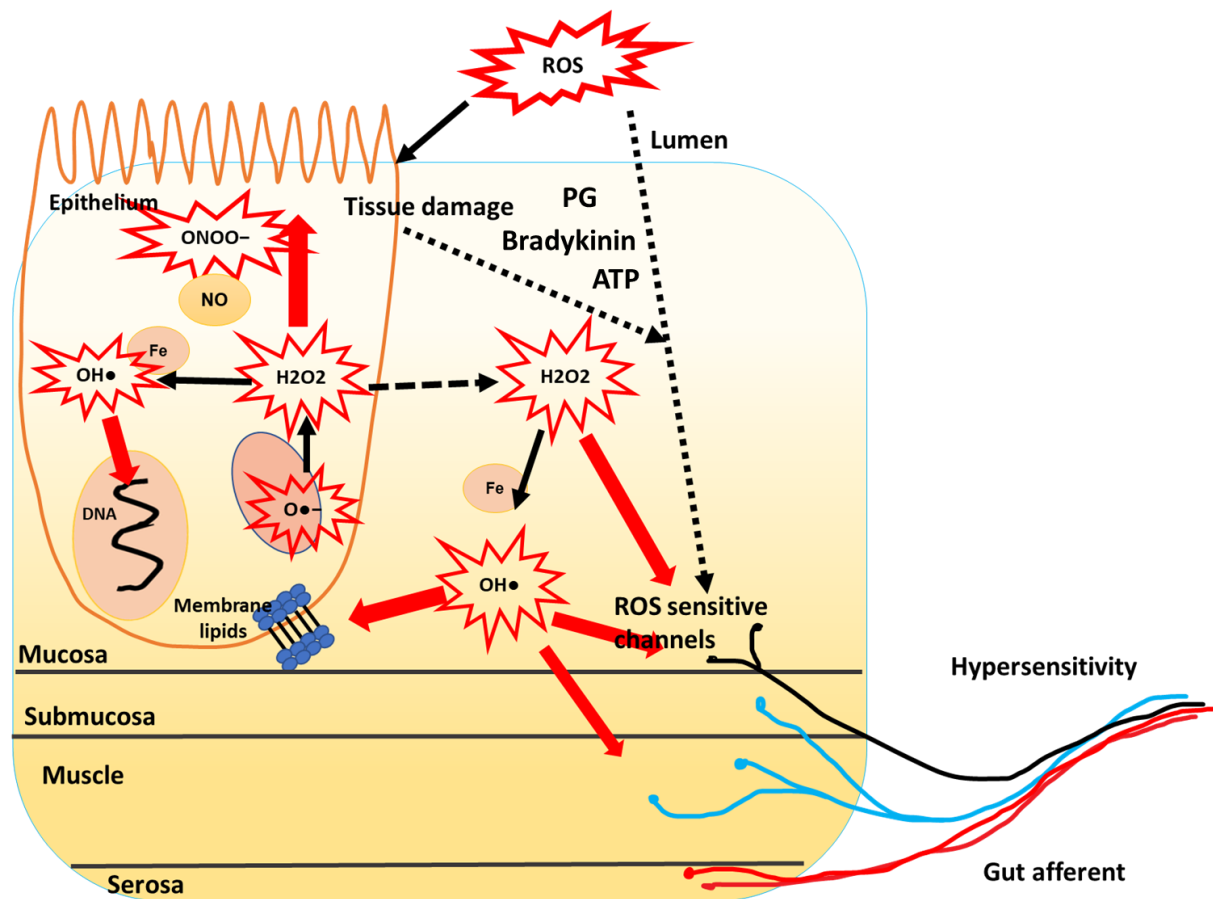
Figure 44 Summary of the effects of ROS on colonic afferent.

Luminal ROS can damage epithelial ROS which causes the production of more ROS and the release of inflammatory mediators. ROS and endogenous mediators can activate afferent terminals in the gut wall and causes hypersensitivity. Endogenous ROS may also participate to gut hypersensitivity via act on ROS sensitive channels. Abbreviations Fe, ferrous iron; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; NO, nitric oxide; $O_2^{-\bullet}$, superoxide anion; OH•, hydroxyl radicals and $ONOO^-$, peroxynitrite; PG: Prostaglandin.

Using in vitro nerve recording to study sensory function

The GI tract is a complex system, neurons, immune cells and various other cell types are arranged to deliver specific functions. Performing cellular and molecular studies to examine such a complex process can only provide limited information. Extracellular nerve recording was used throughout this study to investigate the sensory response of mouse small intestine and colon to oxidative stress and ageing. The advantage of this method is that it is a simple experimental approach that can be used to examine the response properties of peripheral afferents to mechanical stimulus and allowing pharmacological manipulation in the same time, which makes it an efficient and reproducible tool. The nerve recording technique from whole tissue provides a better understanding of physiological function than molecular and cellular studies as it takes into account all contributed effects from many cells. On the other hand, contribution of multiple mechanisms in physiological observation makes it harder to interpret; therefore, cellular and molecular studies can be more specific. In the current study, cellular QPCR, western blot and RNA microarray were used in addition to nerve recording to understand different aspects contributing to sensory activity.

Study limitation

The nerve recordings experiments (Chapters 3 and 4) were performed on mesenteric nerve bundles containing multiple vagal and spinal afferents in the jejunum and splanchnic nerve in the colon. Therefore, the information obtained here was limited to these populations of nerves.

In ageing studies (Chapter 4), two age groups, 3-months and 22-months old, were used to study the effect of oxidative stress and ageing on the intestine sensory function. The choice of using 3 months as the control group is mainly because of previous data provided by Grundy's group where afferent recordings were performed on mice aged between 3 and 6-months and considered as control as no significant changes were detected in their afferent chemosensitivity and mechanosensitivity to different stimuli. Hence, using mice at 3-months as control to start my experiment was reasonable. However, including more age points including beyond the age of 22 months to the study would have provide conclusions from more data about age-associated changes that may have happened throughout the animal's life. Using animal beyond the age of 22-months old, was not possible because after this age, animal care becomes more difficult and can be compromised and the study becomes even more expensive.

Because of the cost of the microarray technique (Chapter 5), the animal number was limited to 3 for each group which may contain the risk of selectivity if they were representing the whole group. Another limitation of this technique is RNA integrity, the tissue has to be processed very quickly to preserve the RNA integrity. It is a crucial step as degraded mRNA can generate false data and confirming the data is important to reach correct conclusions. Also, microarray scanning only provides information about the genes expression that are included on the array and further confirmation for protein levels or activity in the tissue needs to be added to the test.

Lastly, no autopsies were performed on the aged mice to confirm the absence of any diseases as they are likely to accrue at a late age. However, the aged group were monitored daily for wellbeing and visual examinations were undertaken to search for

any abnormalities, lesions and signs of pain or distress. That said, as far as I could tell the aged animals that were used throughout this study were healthy and active.

Future direction

Translating knowledge to humans is the ultimate goal for every physiologist and some aspects of this research are suitable to be repeated using samples from humans. Thanks to improvements in living tissue preserving techniques plus the knowledge and practical integration between researchers and clinicians, in vitro nerve recording from human GI samples has been recently started by some research groups (Hockley et al., 2014, Wouters et al., 2016, Hockley et al., 2017). We don't know what is the effect of ROS that are related to IBD on the human sensory function although from animal studies, we can speculate a similar effect, but difference between species may be present.

In addition, we know that TRPA1 channel is involved in oxidative stress-hyperactivity but other ion channels (such as TRPV1) and inflammatory mediators (such as prostaglandins) should also be looked at to fully understand the mechanism underlying the effect of oxidative stress. Sodium channels are also an attractive target as studies have provided evidence for the involvment of $Na_V1.8$ (Beyak et al., 2004) and $Na_V1.9$ (Hockley et al., 2014) in visceral nociceptor. $Na_V1.8$ was reported to be ROS-sensitive in the DRG neurons (Schink et al., 2016) but it is unknown if all Na_V channels are sensitive to oxidative stress.

In addition, biopsies from elderly patients cab be used to assess ROS levels specificly in the gut, and their supernatants to examine their effect on nerve activity in animals. We can use our current knowledge about the antioxidant properties of fatty acid proteins

that are normally rich in the small intestine and see if they can prevent or reduce the influence of oxidative stress on colonic sensory function. These fatty acid proteins provide a promising target for the development of therapeutic or preventive agents for oxidative stress related diseases. However, much work is first to be done on animal models to investigate the mechanism and test the precise applications.

Lastly, using RNA microarray to scan for age-associated changes that may occur in humans at different age points is a very useful tool especially because it is a well established technique. However, the use of larger scale studies such as RNA-sequencing and Proteomics that are available now would provide more efficient use of human samples.

Supplements

Supplement table 1: Nutrients/digestion genes' expression is higher in jejunum than in colon.

(Fold difference: is the difference of gene expression in jejunum over colon)

Symbol	Gene name	Fold difference	P-value	Protein class
Slc3a2	4F2 cell-surface antigen heavy chain	29.922	0.006	amylase
Akr1c12	Aldo-keto reductase a	26.599	0.002	reductase
Apoa1	Apolipoprotein A-I	67.911	0.072	
Apoa4	Apolipoprotein A-IV	79.366	0.015	
Aqp3	Aquaporin-3	28.208	0.016	transporter
Slc7a15	Aromatic-preferring amino acid transporter	42.477	0.107	amino acid transporter
Abca1	ATP-binding cassette sub-family A member 1	32.985	0.011	ATP-binding cassette (ABC) transporter
Abcg5	ATP-binding cassette sub-family G member 5	40.564	0.012	ATP-binding cassette (ABC) transporter
Abcg8	ATP-binding cassette sub-family G member 8	48.368	0.004	ATP-binding cassette (ABC) transporter
Slc7a9	B (0,+)-type amino acid transporter 1	44.275	0.000	amino acid transporter
Abcc2	Canalicular multispecific organic anion transporter 1	50.603	0.016	ATP-binding cassette (ABC) transporter
Cubn	Cubilin	41.837	0.020	Apolipoprotein, cell adhesion molecule enzyme modulator; extracellular matrix protein; membrane-bound signalling
Fabp2	Fatty acid-binding protein, intestinal	33.568	0.042	
Fabp1	Fatty acid-binding protein, liver	73.978		
Lgals2	Galectin-2	26.310	0.008	cell adhesion molecule; signaling molecule
Itln1	Intelectin-1a	49.514	0.035	
Slc7a8	Large neutral amino acids transporter small subunit 2	40.502	0.053	amino acid transporter
Reg1	Lithostathine-1	13.223	0.079	growth factor
Mgam	Maltase-glucoamylase	3.453	0.001	glucosidase
Mttp	Microsomal triglyceride transfer protein large subunit	75.391	0.017	apolipoprotein; transporter

Slc25a20	Mitochondrial carnitine/acylcarnitine carrier protein	24.272	0.002	amino acid transporter; calmodulin; mitochondrial carrier protein; ribosomal protein; transfer/carrier protein
Abcb1a	Multidrug resistance protein 1A	23.672	0.009	
Nomo1	Nodal modulator 1	2.130	0.003	
Sis	Platelet-derived growth factor subunit B	63.721	0.003	glucosidase
Lct	Protein Lct	73.978	0.031	Glucosidase; glycosidas
Reg3a	Regenerating islet-derived protein 3	14.712	0.009	growth factor
Reg3b	Regenerating islet-derived protein 3	8.101	0.001	growth factor
Reg3g	Regenerating islet-derived protein 3	12.342	0.001	growth factor
Rbp2	Retinol-binding protein 2	50.566	0.005	
Slc5a12	Sodium-coupled monocarboxylate transporter 2	49.351	0.067	carbohydrate transporter; cation transporter
Slc9a3	Sodium/hydrogen exchanger 3	24.978	0.016	
Slc13a2	Solute carrier family 13 member 2	24.354	0.001	cation transporter
Slc15a1	Solute carrier family 15 member 1	34.787	0.000	transporter
Slc22a18	Solute carrier family 22 member 18	26.979	0.054	
Slc23a1	Solute carrier family 23 member 1	37.831	0.016	transporter
Slc2a2	Solute carrier family 2, facilitated glucose transporter member 2	55.460	0.006	

Supplement table 2 Complete list of genes that exhibited more than 2-fold change in aged mouse jejunum compared to young jejunum.

Fold change (FC): is change in gene expression in aged compared to young.

Symbol	Gene name	Gene Accession	Fold change	P value
Igkv13-84	Protein Ighv1-13-84	ENSMUST00000103339	7.6	2.2E-06
Ighv5-9-1	Protein Ighv5-9-1	ENSMUST00000103452	3.6	0.002551
Igh-VS107	Protein Igh-VS107	ENSMUST00000103461	3.5	0.003638
Ighv10-3	Protein Ighv10-3	ENSMUST00000103495	3.4	0.00466
Ighv14-2	Protein Ighv14-2	ENSMUST00000103467	3.1	0.007768
Ighv1-12	Protein Ighv1-12	ENSMUST00000103500	3.1	0.007815
Igkv1-99	Protein Ighv1-99	ENSMUST00000103326	3.1	0.008161
Igkv9-123	Protein Igkv9-123	ENSMUST00000103313	3.0	0.011006
Igkv3-12	Protein Igkv3-12	ENSMUST00000103396	2.7	0.018797
Igkv9-129	Protein Igkv9-129	ENSMUST00000103308	2.7	0.018972
Igkv12-98	Protein Igkv12-98	ENSMUST00000103327	2.6	0.025812
Ighv9-1	Protein Ighv9-1	ENSMUST00000103471	2.6	0.028068
Igkv1-122	Protein Ighv1-122	ENSMUST00000103314	2.5	0.033717
Ighv1-4	Protein Ighv1-4	ENSMUST00000103493	2.4	0.045766
Mmrn1	Multimerin	NM_001163507	2.3	0.047705
Mptx1	Mucosal pentraxin1	NM_025470	-2.4	0.044021
Cubn	Cubn	NM_001081084	-2.4	0.04291
Ceacam18	Carcinoembryonic antigen-related cell adhesion molecule	NM_028236	-2.4	0.041058
Rhod	Rho-related GTP-binding protein RhoD	NM_007485	-2.4	0.039689
Isg15	Ubiquitin-like protein ISG15	NM_015783	-2.4	0.03947
Apol7a	Apolipoprotein L 7a	NM_001164640	-2.4	0.037687
Plb1	Phospholipase B1, membrane-associated	NM_001081407	-2.4	0.037495
Nt5e	5'-nucleotidase	NM_011851	-2.5	0.035799
Cldn4	Claudin-4	NM_009903	-2.5	0.033854
Tmigd1	Transmembrane and immunoglobulin domain-containing protein 1	NM_025655	-2.6	0.028699
Ighv8-9	Protein Ighv8-9	ENSMUST00000103533	-2.7	0.022602
Sprr2a2	small proline-rich protein 2A2	ENSMUST00000090872	-2.7	0.021331
Gprc5a	Retinoic acid-induced protein 3	NM_181444	-2.7	0.020695

Slc34a2	Sodium-dependent phosphate transport protein 2B	NM_011402	-2.7	0.020041
Ighv8-6	Protein Ighv8-9	ENSMUST00000103524	-2.7	0.01951
Car1	Carbonic anhydrase	NM_001083957	-2.7	0.018587
Sprr1a	Cornifin-A;Sprr1a	NM_009264	-2.8	0.017521
Ighv1-39	Protein Ighv1-39	ENSMUST00000103515	-2.9	0.014141
Ada	Adenosine deaminase	NM_001272052	-2.9	0.013958
Ighv3-1	Protein Ighv1-4	ENSMUST00000103478	-3.0	0.01068
Saa1	Serum amyloid A-1	NM_009117	-3.0	0.009626
Igkv9-124	Protein Igkv9-124	ENSMUST00000103312	-3.0	0.009608
Ighv5-12	Protein Ighv5-12	ENSMUST00000103457	-3.4	0.00464
Ighv1-49	Protein Ighv1-49	ENSMUST00000103519	-3.8	0.001752
Clca6	chloride channel calcium activated 6	NM_207208	-3.9	0.001613
Fabp6	fatty acid binding protein 6, ileal Gastrotropin	NM_008375	-4.2	0.000798
Ighv8-13	Protein Ighv8-13	ENSMUST00000103542	-4.5	0.000421
Ighv1-2	Protein Ighv1-2	ENSMUST00000109711	-4.6	0.000369
Ighv1-54	Protein Ighv1-54	ENSMUST00000103525	-5.0	0.000172
Igkv12-89	Protein Igkv12-89	ENSMUST00000103335	-5.8	3.96E-05

Supplement table 3: Complete list of genes that exhibited more than 2-fold change in the aged mouse colon

Fold change (FC): is change in gene expression in aged compared to young

Symbol	Gene name	Gene Accession	FC	P value
Mmrn1	Multimerin	NM_001163507	7.3	8.72E-18
Igkv12-38	Protein Igkv12-38	ENSMUST00000103369	5.7	3.41E-14
Igkv1-122	Protein Igkv1-122	ENSMUST00000103314	5.7	4.89E-14
Igkv14-126	Protein Igkv14-126	ENSMUST00000103310	5.6	6.62E-14
Igkv9-124	Protein Igkv9-124	ENSMUST00000103312	5.4	2.17E-13
Ighv1-47	Protein Ighv1-47	ENSMUST00000103518	4.3	1.83E-10
Sycn	Syncollin	NM_026716	3.7	1.62E-08
Ighg2b	Protein Ighg2b	ENSMUST00000103418	3.6	3.38E-08
Igkv3-9	Protein Igkv3-9	ENSMUST00000103398	3.1	6.27E-07
Ighv14-3	Protein Ighv14-3	ENSMUST00000103469	3.0	1.31E-06
Ighv3-3	Protein Ighv3-3	ENSMUST00000103476	3.0	1.54E-06
Ighv1-54	Protein Ighv1-54	ENSMUST00000103525	3.0	2.2E-06
Igkv14-130	Protein Igkv14-130	ENSMUST00000103307	2.9	2.9E-06
Tmem254b	protein Tmem254b	NM_001270495	2.8	5.04E-06
Plac9b	protein Plac9b	NM_001270503	2.5	4.56E-05
Igkv2-109	Protein Igkv2-109	ENSMUST00000103322	2.5	5.18E-05
A4gnt	Alpha-1,4-N-acetylglucosaminyltransferase	NM_001077424	2.5	6.65E-05
Iglv1	Protein Iglv1	BC046910	2.4	0.000181
B3gnt6	Beta-1,4-glucuronyltransferase 1	NM_001081167	2.2	0.000494
Entpd4	Ectonucleoside triphosphate diphosphohydrolase 4	NM_026174	2.2	0.000543
Samd5	Sterile alpha motif domain-containing protein 5	NM_177271	2.1	0.001461
Igkv14-111	protein Igkv14-111	ENSMUST00000103320	2.1	0.001545

Gm21464	protein Gm21464	ENSMUST00000183695	2.0	0.00236
Areg	Amphiregulin	NM_009704	-1.9	0.003816
Apoa1	Apolipoprotein A-I	NM_009692	-2.0	0.003426
Gm25905	protein Gm25905	ENSMUST00000158490	-2.0	0.002343
2310079G19 Rik	Protein 2310079G19Rik	NM_027173	-2.0	0.002113
Vmn1r143	Protein Vmn1r143	NM_001166750	-2.0	0.002044
Gm23521	Gm23521	ENSMUST00000177845	-2.1	0.001384
Tfrc	Transferrin receptor protein 1	NM_011638	-2.1	0.001125
Atp12a	Potassium-transporting ATPase alpha chain 2	NM_138652	-2.2	0.000659
Mir1938	Mir1938	NR_035459	-2.3	0.000213
Wdfy1	Protein Wdfy1	NM_001111279	-2.4	0.000111
Fxyd4	FXYD domain-containing ion transport regulator 4	NM_001173372	-2.4	0.000108
Slc9a3	Sodium/hydrogen exchanger 3	NM_001081060	-2.5	7.01E-05
2010109I03Rik	Protein 2010109I03Rik	NM_025929	-2.6	4.32E-05
Dhrs9	Dehydrogenase/reductase SDR family member 9	NM_175512	-2.8	6.97E-06
LOC672450	Protein LOC672450	ENSMUST00000103383	-2.9	2.89E-06
Itln1	Intelectin-1a	NM_010584	-3.0	1.61E-06
Lyz1	Lysozyme C-1	NM_013590	-3.2	3.99E-07
Defa23	Alpha-defensin 23	NM_001012307	-3.4	1.07E-07
Igkv12-38	protein Igkv12-38	ENSMUST00000103371	-3.6	3.07E-08
LOC238440	protein LOC238440	ENSMUST00000103529	-3.7	9.28E-09
Gm7849	Protein Gm7849	NM_001177518	-3.9	2.68E-09
Mptx2	protein Mptx2	NM_001205011	-3.9	2.64E-09
Gm10104	Protein Gm10104	NM_001177481	-3.9	2.49E-09
Ighv1-31	Protein Ighv1-31	ENSMUST00000103511	-4.3	6.42E-10
Gm15315	Protein Gm15315	NM_001177528	-4.4	2.04E-10
Gm15308	Protein Gm15308	NM_001177521	-4.5	1.14E-10
Defa24	Alpha-defensin 24	NM_001024225	-4.6	5.73E-11
Defa21	Alpha-defensin 21	NM_183253	-9.5	2.99E-11
Defa22	Alpha-defensin 22	NM_207658	-14.9	1.81E-22

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