The University Of Sheffield.

Investigating effect of dietary lipids on jejunal afferent sensitivity in the Mouse

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PhD Thesis

By

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Publications

Papers

1. T. Lubbers, J.J. De Haan, M. Hadfoune, **Y. Zhang**, M.D. Luyer, D. Grundy, W.A. Buurman, J.W. Greve "Lipid-enriched enteral nutrition controls the inflammatory response in murine Gram-negative sepsis". Crit Care Med. 2010 Oct;38(10):1996-2002.

2. Tim Lubbers, Jacco-Juri de Haan, M'hamed hadfoune, Yiren Zhang, David Grundy, Jan Willem Greve, Wim A. Buurman. "Chylomicron formation and glucagon-like peptide 1 receptor are involved in activation of the nutritional anti-inflammatory pathway."J Nutr Biochem. 2011 Dec;22(12):1105-11

Abstracts

1. **Yiren Zhang**, David Grundy, "Altered nutrient sensing in the Transient Receptor Potential Vanilloid 1 knockout mouse" Joint international Neurogastroenterology and Motility Meeting, Sep 06-08, 2012 in Bologna, Italy (award as best abstract)

2. **Yiren Zhang**, Christopher Keating, David Grundy, "Investigating effect of lipid-rich nutrient on jejunal afferent mechanosensitivity in aged murine model" Digestive Disease Week, DDW 2012, May 20 – 22, 2012 in San Diego Convention Centre, San Diego, CA, US

3. **Yiren Zhang**, Tim Lubbers, Wim A. Buurman & David Grundy, "Investigating the effect of lipid-containing nutrient on jejunal afferent mechanosensitivity" Joint international Neurogastroenterology and Motility Meeting, Aug 26-29,2010 in Boston, Massachusetts, US (selective as oral presentation)

Summary of thesis

Enteral nutrients, especially lipid, have been well recognized as signalling molecules which control various cellular processes and play an important role in food intake, metabolisms, inflammation and pain. Loss of appetite and altered functional property of TRPV1 ion channel have been found ageing. However, the transduction mechanism of extrinsic intestinal afferent nerves in lipid sensing from the gut remains poorly understood. Thus, the primary objective of this thesis was to investigate effect of lipid-containing nutrients on different subpopulations of jejunal afferents using an in vitro nerve-gut preparation from adult and aged mice using a sophisticated and comprehensive single unit analysis. The contribution of TRPV1 in aged related changes was also examined.

In adult mice, both lipid-low and lipid-rich containing nutrients were found to increase intraluminal distension-induced afferent response in three functional different subpopulations; Low threshold (LT), wide dynamic range (WDR) and high threshold (HT) units. The effects were lipid concentration dependent. Only the response of LT unit to lipids was significantly attenuated by CCK-1 antagonist devazepide which also effectively inhibited exogenous applied CCK, strongly suggesting that lipids-induced enhanced mechanosensitivity was predominately mediated by CCK-1 vagal nerve pathway.

Lipid rich-induced mechanosensitivity was further examined in aged mice. Response of both LT and HT was gradually reduced with ageing compared with adult. The reduced mechanosensitivity was restored in 12 months TRPV1-knockout mice, suggesting down-regulation role of TRPV1 in gut ageing.

Finally, the role of lipid in modulating jejunal afferent mechanosensitivity, its changing and function of TRPV1 as a sensor during ageing process was discussed. Thus this study has advanced our understanding of biological mechanisms underlying gut afferent signalling in nutrient sensing at single nerve fibre level.

Chapter 1

General Introduction

1.1 General anatomy and function of the gastrointestinal tract

The gastrointestinal (GI) system is essentially a muscular tube together with various accessory organs. It plays the major role in breaking down food for absorption to maintain the basic body function. The GI tract is divided into two parts; namely the upper and lower GI tracts. The upper GI tract consists of the oesophagus, stomach and duodenum. The main function of the stomach is to mechanically breakdown large food particles into smaller pieces, which provide a larger surface area for further enzymatic breakdown and absorption. The pH of the stomach is very low (~pH2), that serves as a potential defence system to kill any harmful bacteria that may enter the body via the oral cavity. The lower GI system consists of the small and large intestine, as well as the anus.

The small intestine contributes an important part of the lower GI tract being where most of the absorption takes place. It is divided into three parts including duodenum, jejunum and ileum, each of which has distinct anatomical features. The jejunum is the middle segment of the small intestine, which is approximately 2.5m long in humans. It has specific features called plicae circulars in its submucosa, which distinguish it from the rest of the small intestine, such as the Brunner's gland found in the duodenum and Peyer's patches in the ileum.

The gut wall exhibits four distinct functional layers, which are mucosa, submucosa, muscularis propria and serosa. The mucus membrane shows regional differences along the GI system reflecting the functional variations from mouth to anus. The mucosa is the innermost layer of the gut wall that consists of three components including an epithelium, a supporting lamina propria and a smooth muscle layer called muscularis mucosae. The muscularis mucosae generates sufficient force necessary for the production of the local movement and is also responsible for causing the folding of the mucosa. The submucosa is mainly composed of loose collagen fibres that form a supportive network around the mucosa. The submucosa is enriched with blood vessels, lymphatic vessels, and the extensive nerve innervations. The muscularis propria is made from two smooth muscle layers, with the outer longitudinal

muscles surrounding the inner circular smooth muscle. The muscular layer generates the essential peristaltic contractions to push the food along the GI tract during digestion. Lastly, the serosa is the outermost layer of the gut wall that mainly consists of the connective tissues.

The GI tract is controlled and coordinated by the activity of the intrinsic and extrinsic nerve fibres. The intrinsic nerve system is known as the enteric nerve system (ENS). It is often referred to as the 'little brain' in the gut (a term that was first coined by Dr. Gershon in 1996). The ENS includes the postganglionic sympathetic fibre, as well as the postganglionic fibres of the parasympathetic nervous system which are supplied by the vagus and pelvic nerve. The neurons of the ENS are located entirely within the gut wall and form the myenteric and submucosal plexus which play an important role for peristalsis during food digestion.

The extrinsic pathway is considered to be a part of the autonomic nerve system which plays an important role in the visceral reflexes. It contains both sympathetic and parasympathetic elements. The extrinsic nerve fibres are located within the mesentery which is a layer of connective tissue which is attached to the outer layer of the GI tract. Typically, there are two nerve bundles enclosed within the same mesentery arch which are located between blood vessels. These consist of the extrinsic afferent (sensory) nerve which carries the information away from the GI tract to the central nerve system (CNS) for processing and the extrinsic efferent (motor) nerve that brings the information back to the GI tract to perform the corresponding functions.

The general structure of the small intestine has been specially modified to enhance and maximise its absorptive ability. The mucosa of the small intestine forms finger-like protrusions known as the villi which project into the intestinal lumen. The villi are lined with numerous specialised epithelial cells such as enterocytes, goblets cells, paneth cells and enteroendocrine cells. Enteroendocrine cells secrete a variety of hormones such as cholecystokinin (CCK) and 5-hydroxytryptamine (5-HT) that have great influence on the modulation of on the intestinal function. Since the GI tract is in direct contact with the environment the villi will be subjected

to continuous damage caused by passage of food particles. Therefore, the epithelial lining of the villi undergoes rapid turnover with newly formed epithelial cells moving from the crypt to the top of the villi to replace the damaged cells.

1.2 Extrinsic nerve innervation of the gastrointestinal tract

The extrinsic nerve innervation provides a link between the gastrointestinal (GI) tract and the central nerve system (CNS). It plays a central role in the establishment of the spinal and brainstem reflex mechanisms which are essential for the regulation of the digestive functions for, feeding and illness behaviours, and also the cause of painful and non-painful sensations, such as fullness, bloating and nausea (Beyak et al., 2006).The extrinsic nerve system consists of both vagal and spinal fibres.

The nature of the extrinsic afferents is different from the vagal pathway to the spinal pathway. Vagal afferents play significant roles in conveying physiological information including that involved in emotional and behavioural aspects of human lives. However, spinal afferent pathways provide major routes for mediating pain perception (Grundy, 2002), because these afferents can also encode information beyond the normal physiological range. For example, the spinal afferents can respond to noxious levels of stimulation. The vagal and spinal afferents have peripheral terminals within the gut wall. They are mainly composed of non-specialised bare endings (Grundy et al., 2006), which are localized within the musculature, mucosal epithelium and also the ganglia of the ENS (Furness et al., 1999).

The extrinsic afferent nerves of the GI tract are sensitive to both mechanical and chemical stimuli. Two types of mechanism have been proposed to explain how afferents will respond to mechanical forces. While most of the molecular basis of these ion channels remains unknown, a physical mechanism depending on mechanosensitive ion channels has been proposed (Beyak et al., 2006). The other is a chemical mechanism that relies on the chemical mediators released from the gut wall following mechanical stimulation. Since most of the extrinsic afferents has terminals enclosed in the gut wall, it is not surprising that they can either directly or indirectly monitor the chemical nature of the local luminal contents. The luminal nutrients (i.e., fatty acids, amino acids etc.), before their absorption through the epithelium are able to trigger the release of messenger molecules (e.g., serotonin and CCK) from the enteroendocrine cells. The

released messenger molecules then influence the sensitivity of the afferents via the activation of both ligand-gated ion channels and G protein-coupled receptors (membrane-bounded receptors).

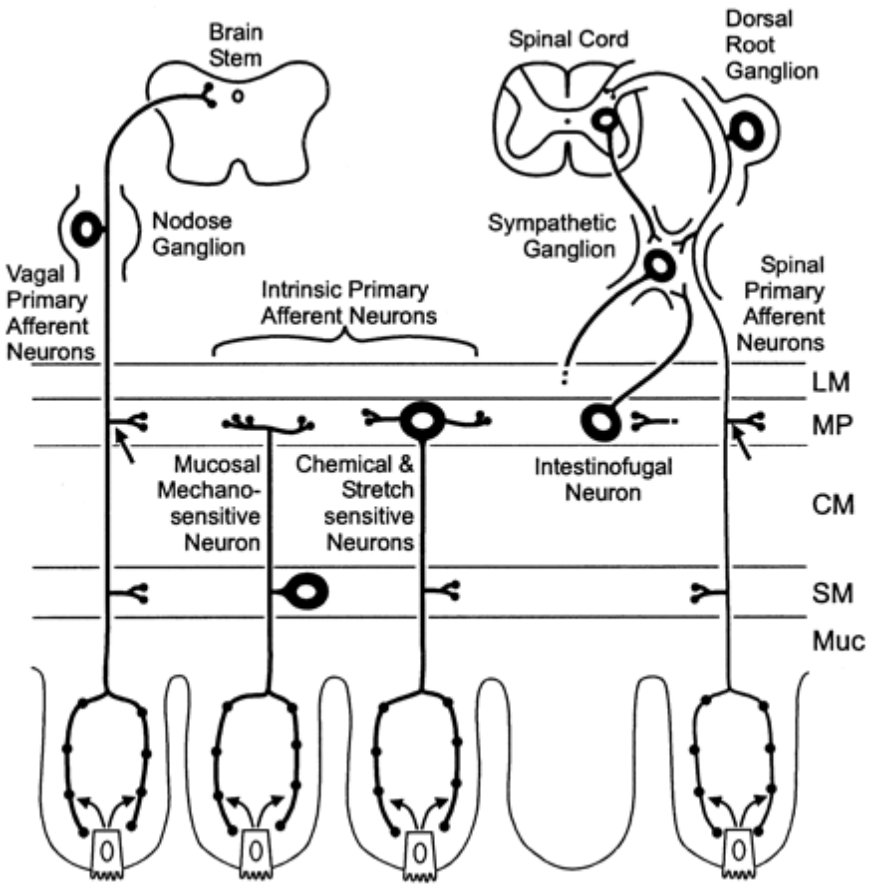


Figure 1.2.1 A simple illustration showing the arrangement of the extrinsic and intrinsic afferents within the GI tract. Taken from Furness, 1999 (Furness et al., 1999).

Vagal afferents

In the central nerve system (CNS), the cell bodies of the vagal afferents are located within the jugular and nodose (superior and inferior) ganglia. From here, the vagal afferents project into the brainstem primarily in the nucleus tracti solitarii (NTS) in the dorsal medulla. Peripherally, the majority of the thoracic and abdominal viscera are innervated by afferents travelling via the thoracic and cervical vagi. The small intestine is innervated by the celiac branch of the vagus nerve. The vagal afferents contribute the largest proportion of the fibres found in the vagus nerve by having a ratio of 9:1 with the efferent nerve fibres (Beyak et al., 2006). The majority of the vagal afferents are unmyelinated C fibres (Grundy and Scratcherd, 1989). The vagal afferent nerves are both chemo- and mechanosensitive.

Early studies using retrograde and anterograde neuronal tracing techniques, including horseradish peroxidise (HPR) and Dil demonstrated the presence of mucosal vagal afferents which had their endings embedded in the lamina propria of the mucosal layer (Berthoud et al., 1995). They showed that many branches of Dil-labelled vagal terminals in the mucosa came in close contact with the basal lamina but did not appear to penetrate it so as to make direct contacts with epithelial cells (Berthoud et al., 1995). In the other words, vagal afferents form an extensive network that is in a close anatomical apposition with the hormone releasing enteroendocrine cells, such as, I cells which releases CCK (Berthoud and Patterson, 1996). The mucosal afferents are mainly composed of unmyelinated C-fibres with only a small proportion of myelinated A^{δ} fibres (Clarke and Davison, 1978). They constitute the presumable chemoreceptors which will response to nutrient signals in a paracrine fashion (Berthoud and Patterson, 1996). The mucosal terminals are most abundant in the proximal part of the small intestine. Previous electrophysiological studies indicated that mucosa endings were sensitive to low threshold mucosal distortion, such as light stroking with a von Frey hair, but they were relatively insensitive to distension and contraction (Beyak et al., 2006). The mucosal afferent firing is described as a burst of impulses occurring every time the stimulus is applied to their

receptive field. Early electrophysiological studies also provided compelling evidence that demonstrated the existence of the mucosal afferent terminals. The experiments were performed by either removing the intestinal mucosa or treating the mucosal surface with local anaesthetic. This showed the loss of the afferent response to both mechanical (light stoking) and chemical (application of chemical mediator) stimuli (Iggo, 1957, Davison, 1972, Bitar et al., 1975).

Another group of vagal afferents are known as the muscle afferent because of the location of their receptive field. They are identified using similar methodological approaches same as the identification of the mucosa afferents. The muscle afferent terminals are embedded between the circular and longitudinal muscle layers of the muscularis propria (Phillips and Powley, 2000), and their discharge frequency was generated as long as the distension has been maintained (Beyak et al., 2006). Early electrophysiological studies showed that physical removal of the mucosal and serosal layer of the GI tract would not abolish activities of the muscle layer. They behave functionally as in-series tension receptors (Iggo, 1955, Cottrell and Iggo, 1984) but show regional variation. For example, the vagal afferent endings found in the corpus and antrum of the stomach show different responses toward gastric filling (Andrews et al., 1980, Beyak et al., 2006). They showed that there was a significant increase when the corpus vagal afferent endings where subjected to a passive stretch. The low-frequency, irregular and spontaneous activity of the corpus mechanoreceptors was elevated dramatically following gastric filling. However, the antral vagal afferent endings behave differently. They only responded to contraction by generating a burst of impulses during each wave of peristalsis.

There are two main types of vagal afferent endings in the GI wall. These have been classified as intramuscular arrays (IMAs) and intraganglionic laminar endings (IGLEs). IMAs and IGLEs are the putative vagal mechanoreceptors. The IMAs are dense throughout the stomach but are rare in the intestine (Powley and Phillips, 2002) which response to passive stretch and contraction of the muscle (Phillips and Powley, 2000).The IGLEs can be found throughout the

GI tract including the small intestine (Berthoud et al., 1997, Wang and Powley, 2000). They have a close association with the myenteric ganglia in the ENS (Neuhuber, 1987, Wang and Powley, 2000) suggesting a possible crosstalk between ENS and the extrinsic nerve system (Grundy et al., 2006). The IGLEs, (not IMAs) are classified as the mechanotransduction site of the low-threshold slow adapting vagal mechanoreceptors (Zagorodnyuk et al., 2001). The IGLEs can be directly exposed to the forces generated during muscle contraction and stretch within normal physiological range.

Spinal afferents

The cell bodies of the spinal afferents are located in the dorsal root ganglia. The spinal afferents project into the dorsal horn of the spinal cord. Later, the spinal afferent is subdivided further into the splanchnic and pelvic afferents which innervate the gut wall by following the routes of both sympathetic and parasympathetic efferents (Grundy et al., 2006). Unlike the vagus nerve, the spinal afferents only occupy a relatively small proportion of the overall fibres (10% to 20% within splanchnic nerve and 30% to 50% within pelvic nerve) present within the spinal nerve and its subdivisions (Beyak et al., 2006). Spinal afferent nerves project into four layers of the gut wall; namely the mucosa, muscle and serosa. The majority of the spinal afferents is small diameter unmyelinated C fibres, and contains a relatively larger proportion of thin myelinated A^{δ} fibres compared with the vagal afferents. The spinal nerves are mainly made from efferent nerve fibres. Therefore, the vagus nerves provide the largest afferent innervations to the gut wall, especially, in the upper GI tract. However, the spinal afferent innervations increase towards rectum with diminished innervations of vagus nerve rostrocaudally (Beyak et al., 2006).

Spinal afferent endings have multiple receptive fields that widely spread across the bowel (Bessou and Perl, 1966, Morrison, 1973). They are also considered as the serosal afferents which usually generate a short burst of impulses when stimulated by probing (Beyak et al., 2006). The spinal afferents consist of two sets of different afferent endings in the gut wall. One set is responsible for detecting distortions of the bowel, while the other can sense changes beyond normal physiological conditions. The spinal afferents are also considered to be mechanoreceptors. They are divided into three different classes. These are the low threshold, the high threshold and the silent afferents respectively. Both low and high threshold afferents can sense painful stimuli, but at a different level. However, the silent afferents can only be activated following inflammation or injury (Gebhart, 2000). They are sensitive to chemicals released during pathology, such as, bradykinin and prostaglandins. This is because the

inflammation can reduce the level of threshold and magnify the responses of the same given stimulus in a normal physiological circumstance to a higher level (Vergnolle, 2008). This is thought to contribute to the phenomenon known as peripheral sensitization, for example, hypersensitivity in irritable bowel syndrome (IBS).

1.3 Luminal nutrient sensation

Nutrient sensation in the gastrointestinal (GI) tract plays an important role in maintaining basic metabolic homeostasis. The passage of luminal contents, such as, macronutrients (carbohydrates, lipids/fatty acids, proteins/amino acids) trigger the release of a vast variety of hormones/chemical mediators from the specialized 'taste cells' located in the epithelium of the GI tract to the intestinal lumen. The released mediators are then detected by the potential sensors in a close anatomical apposition, of which one of them is the terminal of the extrinsic nerve afferents. The sensory afferents of the GI tract serve as a link to transmit signals to the central nerve system (CNS), which play a vital role in the regulation of GI function via a well-studied gut-brain axis. The vagal nerve is the key component of afferent pathway involved in the gut brain axis (Berthoud, 2008). This review will focus on the discussion of nutrient sensation of lipid/fatty acids in the GI tract.

Lipids

Lipids/fats are well-known stimuli for a gut hormone called CCK (Liddle, 1997). The breakdown of lipids is started in the duodenum in the form of gastric chyme. This is done by mixing fats with a digestive enzyme called lipase which is supplied from the pancreas. Bile secreted from the liver and stored in the gallbladder enter the duodenum via the bile duct emulsified fats by which mean they are disperse into small droplets. The emulsification process allows lipase to gain easier access to fat molecules. The digestive products of the large molecule of triglycerides are glycerol and 3 fatty acids. This is the essential step by which endogenous CCK can be released from the vesicles located in the basal side of the gut wall. CCK is released from enteroendocrine I cells by the luminal passage of fats and proteins.

Fatty acids receptors are expressed at the apical side of I cells located in the mucosa layer of GI tract. They are G-protein coupled receptors which have 7 transmembrane spanning helical bundles. The long–chain fatty acid receptors, G-protein-coupled receptor 40 (GPR40/FFAR1)

and GPR120 have been implicated in the chemo-sensation of dietary fats. A previous study showed that by using a purified population of I cells from duodenal mucosa isolated from a transgenic mice expressed green fluorescent protein under the control of the CCK promoter, they demonstrated that GPR40 directly induced the secretion of CCK by I cells in response to dietary fat (Liou et al., 2011b). A more recent study using semi-quantitative RT-PCR demonstrated the presence of mRNA transcripts encoding for the long chain fatty acid receptors (GPR40/FFAR1, GPR120/O3FAR1) and short chain fatty acid receptors (GPR41/FFAR3, GPR43/FFAR2) in the native I-cells (Sykaras et al., 2012).

The fatty acids of a chain length less than C10 diffuse out of enterocytes into the blood, while the fatty acids of a chain length more than C10 are absorbed via chylomicron formation into the lymph (Tso and Balint, 1986). Chylomicrons are delivery vehicles for fats and cholesterol from the small intestine to other locations in the body such as, the liver, adipose, cardiac and skeletal muscle tissue (Tso and Balint, 1986). An early study indicated that lipid-induced delay in gastric emptying was dependent on the formation of chylomicron (Raybould et al., 1998). Later, a study from the same group presented some direct evidence to show that chylomicron or their products can trigger the release of endogenous CCK which would reverse the effect of lipid induced inhibition of gastric motility (Glatzle et al., 2003).

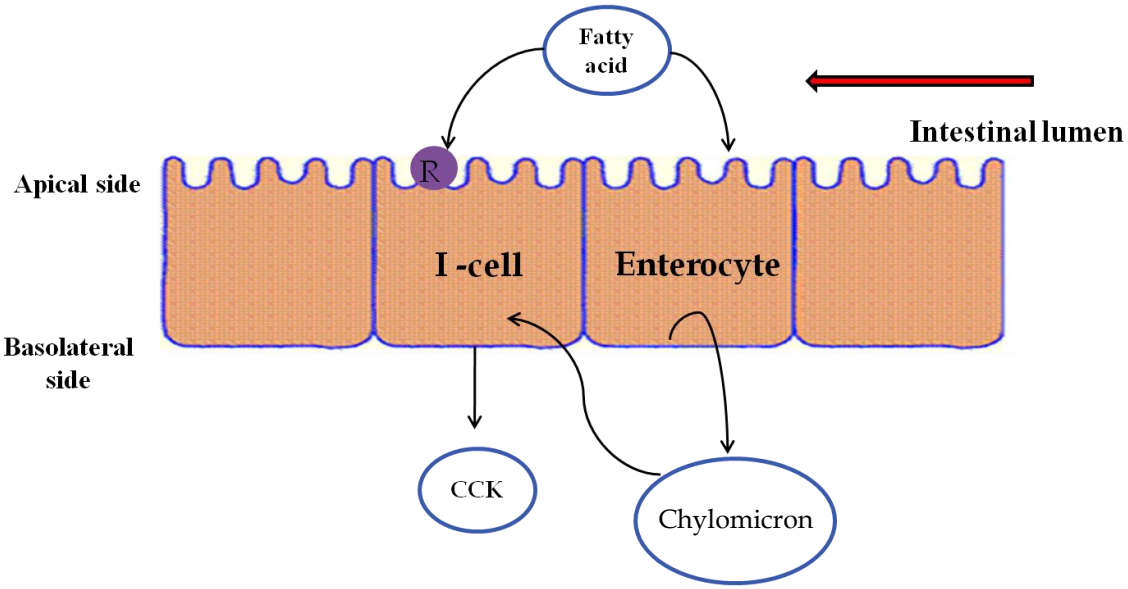


Figure 1.4.1 Schematic diagram showing the releasing mechanism of endogenous CCK by the passage of luminal lipids. It can be released either through the direct activation of receptors located on I cells, or through the formation of chylomicrons. R indicates the receptors for short chain/long chain fatty acids.

Lipid/fat is well known for its role as a potent inhibitor of gastric secretomotor function (Raybould et al., 1998). Mélone et al. 1990 demonstrated that cats have two types of lipid-sensitive vagal receptors in the small intestine which had an inhibitory effect on the regulation of gastric empty (Mélone and Mei, 1991). Vagal afferents responded to different lipids according to their chain length. *Lal et al.* 2001 showed that only the long chain fatty acids activated vagal afferents via a CCK-dependent mechanism while the short chain fatty acids appeared to directly act on the vagal afferent terminals (Lal et al., 2001). Earlier studies from an enteroendocrine cell line of STC-1(a mouse's intestinal endocrine tumour cell line) showed that only fatty acids of a chain length C10 or above and also with a free carboxy group would trigger the release of CCK from the intestinal endocrine cells (McLaughlin et al., 1998, Sidhu et al., 2000). Human study also showed a similar observation which 12C-long chain fatty acids

significantly elevated plasma CCK level that reduced the proximal gastric tone (McLaughlin et al., 1999). In rats, ablation of vagal afferents or pre-treating the animal with devazepide (CCK-1 receptor antagonist) significantly reversed the inhibitory effect of lipid on gastric empty (Holzer et al., 1994). This was further demonstrated using CCK-1 receptor knockout (CCK1R$^{-/-}$) mice. With ingestion of a lipid-containing meal, gastric empty was significantly faster in CCK-1R$^{-/-}$ mice. This is also true with gastric acid secretion, in which the inhibitory effect of lipid was abolished in CCK-1R$^{-/-}$ mice (Whited et al., 2006). Altogether these data showed that lipid exerts its effect on the GI tract via CCK though CCK-1 receptor mediated pathways.

Cholecystokinin and its receptors

CCK is a hormone that was discovered in the small intestine. It is secreted from the enteroendocrine I cells (Polak et al., 1975, Buchan et al., 1978, Liddle, 1997), which are mostly concentrated in the proximal small intestine. CCK plays a key role in the activation of intestinal feedback control of gastrointestinal (GI) function. In this way, it coordinates the entrance of nutrients with function of small intestine to maximise its digestive and absorptive capacity. It was initially identified as the stimulus of gallbladder contraction and pancreatic enzyme secretion (Ivy and Oldberg, 1928, Mutt and Jorpes, 1968). Later, it has also been found to be involved in the control of food intake (Gibbs et al., 1973) and the regulation of short-term inhibition of gastric empty and acid secretion (Debas et al., 1975 , Ishikawa et al., 1985).

CCK receptors are expressed on the vagal afferent. They were first detected in the rat vagus nerve using in vitro receptor autoradiography in the 1980s (Zarbin et al., 1981). Molecular cloning studies have identified the presence of two types of CCK receptors; namely CCK-1/A and CCK-2/B receptors which differ in their distribution and affinity to peptides (Wank et al., 1992a, Wank et al., 1992b, Wank, 1995, Moriarty et al., 1997).The CCK receptors are a group of G-protein coupled receptors. The CCK-A receptor is predominantly expressed in the peripheral tissue such as GI tract (Hill et al., 1987). The CCK-B receptor is expressed throughout the central nerve system (Crawley, 1985, Corp et al., 1993).

Over the last few decades numerous studies have provided compelling evidence uncovering the neural pathways underlying CCK mediated action on intestinal feedback. It is mainly mediated by the activation of extrinsic nerve pathways particularly through the vagal afferent nerves. CCK is considered as a master regulator of vagal afferent nerve function. It is well established that CCK stimulated the discharge of vagal afferent neurons. Blackshaw *et al.*1990 showed that in the ferret, vagal mucosal receptors were directly sensitive to exogenously applied CCK with a remarkable increase in firing rate (Blackshaw and Grundy, 1990). Another

sets of *in vivo* experiments showed that a group of gastric vagal mechanoreceptors were sensitive to an integrated action of gastric loads and CCK-8 (Schwartz et al., 1991, 1993, 1994). They showed that the discharge rate of those fibres which were responsive to gastric load nearly doubled their response with prior exposure of CCK (Schwartz et al., 1991).

Nutrient induced anti-inflammatory pathway via CCK-1 receptor

The immune system consists of two individual systems, one is the innate immune system and the other is the adaptive immune system. The innate immune system is non-specific which forms the first line of defence that defend the host from foreign intrusion by other organisms, such as, bacteria and viruses (Chaplin, 2010). The adaptive immune system consists of specialized antigen-recognition cells which are able to generate memory and recall responses to previously encountered antigens (Chaplin, 2010). A well-orchestrated immune response is essential for survive which requires a balanced simultaneous activation of both pro-and anti-inflammatory mediator; any dysregulation and malfunction in the immune response will lead to severe clinic conditions, such as sepsis, resulting in multiple organ failure and systemic inflammation which carries a high risk of mortality. The net effect of inflammation response in sepsis can be described using a biphasic model; a hyper-inflammatory phase that is initiated by the cytokine storm generated by the innate immune system, then followed by a hypo-inflammatory response known as immune-paralysis which may be activated to dampen systemic inflammation (Boomer et al., 2013).There is clinic evidence showing that malnutrition is closely associated with the development of systemic inflammation, particularly in the intensive care unit (Krenitsky, 1996, Delgado et al., 2008, Kenneth et al., 2013). These demonstrated the potential crosstalk between nutrient signalling and immune response in the diseased state which leads to the requirement of the carefully planned supplementation of nutritional intervention to be provided during hospitalization. Enteral nutrition is introduced as an alternative treatment instead of the traditional parenteral nutrition for critically ill patients, which has shown to exhibit certain benefits that may improve nutritional status (Shikora and Ogawa, 1996).

Borovikova *et al*. 2000 gave the first demonstration showed that the release of the inflammatory cytokine TNF-α was inhibited by the electrical stimulation of the peripheral vagus nerve in a mouse model of lipopolysaccharide (LPS)-induced sepsis (Borovikova et al.,

2000). Later, they characterized this pathway as a vagal, cholinergic anti-inflammatory pathway (Tracey, 2007). Acetylcholine receptors (AChRs) were expressed on macrophages and other cytokine-producing cells (Wang et al., 2003, Wang et al., 2004). They showed that activation of the AChRs transduced an intracellular signal that would then supress the synthesis of cytokines.

The idea of using dietary lipid/fat to prevent systemic inflammation was first brought out and demonstrated by a team based in Nederland (Luyer et al., 2004). They showed that high fat enteral nutrient reduced endotoxemia and prevented bacterial translocation. Following this study, they later demonstrated that this nutrient induced anti-inflammatory pathway was mediated via cholecystokinin (CCK) receptor through vagal nerves (Luyer et al., 2005). Therefore, this was also referred to as a vagal activated anti-inflammatory pathway. They proposed that nutrient in the gut activated vagal afferents which then through a reflex pathway in the brainstem lead to an efferent outflow to modulate activities of macrophages via the cholinergic (AChRs) receptors.

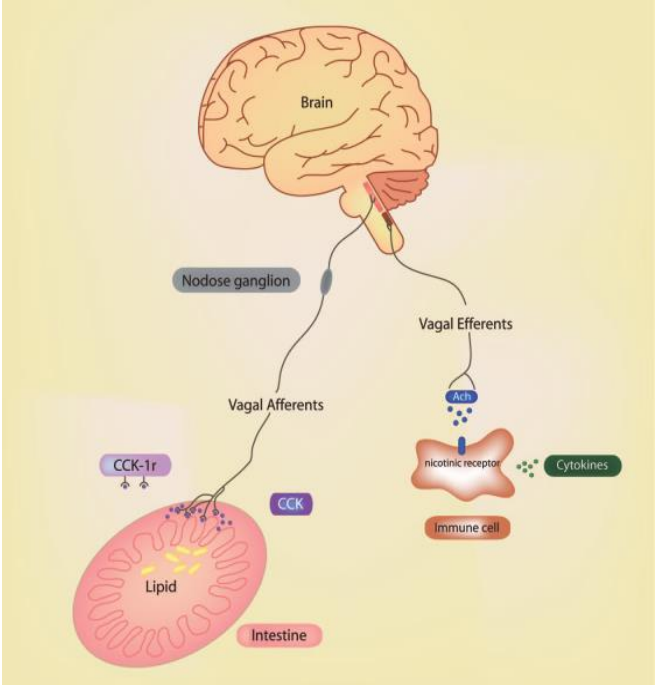


Figure 1.4.2 Schematic diagram showing the proposed nutritional anti-inflammatory pathways. Infusion of luminal lipid triggered release of CCK from endocrine cells, and then activated CCK-1R containing vagal afferents. It then activated vagal efferent pathway causing release of Ach which acted on macrophages to prevent release of pro-inflammatory mediators, such as TNF-α and IL-6. Therefore, it reduced inflammation that might cause organ damage. Taken from Lubbers 2010 (Lubbers et al., 2010c).

1.4 Aging bowel

The maintenance of health and nutrition is important in supporting longevity. In recent years, there have been growing concerns about the rising proportion of aged elderly population in society. According to the latest projections published by the government 10 million people in the UK are over 65 years old, and this will have nearly doubled to 19 million by 2050. This will then create a dramatic challenge in the healthcare system.

The Gastrointestinal (GI) tract is innervated by the intrinsic neurons of the enteric nerve system (ENS) and axons of the extrinsic nerve system including the sympathetic, parasympathetic and visceral afferent neurons. Both nerve innervations are affected by age to a certain extent. This aging is closely associated with multiple GI disorders observed in the elderly population, such as dysphagia, gastrointestinal reflux and constipation. Increasing studies have been conducted to increase understanding about how ENS changes with age (Wade, 2002, Saffrey, 2004, Phillips and Powley, 2007). It has been shown that functional properties, general structures of ENS, as well as the number of enteric neurons were altered in both aged human and rodents' populations. However, little is known about how ageing will affect afferent innervations of GI tract. Limited studies have showed that extrinsic afferent innervations also undergo age-related dystrophic or regressive changes (Vega et al., 1993, Phillips et al., 2010) and both mucosal afferent terminals, IMAs and IGLEs undergo age-related morphological changes at the target tissue.

1.5 Transient receptor potential vaniloid 1 (TRPV1)

The mammalian transient receptor potential (TRP) proteins are encoded by at least 28 different TRP subunit genes (Clapham, 2003, Moran et al., 2004). The primary structures of the TRP channel are made of six transmembrane domains with a pore domain between the fifth and sixth transmembrane (Vannier et al., 1998, Clapham et al., 2005, Holzer, 2011).The TRP channel is one of the largest groups of ion channels. This is broadly divided into two groups based on their sequence and topological differences (Venkatachalam and Montell, 2007). It is then further divided into seven subfamilies. The group-1 TRP channels include 5 subfamilies, which are the classical TRPC (1-7), the melastatin TRPM (1-8), the ankyrin TRPA (1), TRPN (not present in mammals) and the vanilloid TRPV (1-6). The group-2 TRP channels consist of the polycystin TRPP (1-4) and the mucolipin TRPML (1-3).

TRPV1 is the prototypical receptor of the TRPV subfamily which is activated in a voltage dependent manner upon depolarisation. TRPV1 was first cloned from a cDNA library of rat sensory neurons and it was activated by capsaicin and high temperature (Caterina et al., 1997). Even though virtually all tissues are supplied by TRPV1-positive nerve fibres, the TRPV1 receptor is predominately expressed in the small diameter afferent neurons involved in pain perception (Rong et al., 2004). The TRPV1 channel was initially considered as an integrator for physical and chemical noxious stimuli. A morphological study showed that only 32% of vagal afferents supplying the mouse jejunum contain TRPV1-positive fibres (Tan et al., 2009). Electrophysiological studies have suggested a role for TRPV1 involved in the modulation of gut sensitivity. TRPV1 knockout mice had an attenuated afferent sensitivity to jejunal distension, which is similar to the observation from wide type mice pre-treated with TRPV1 antagonist capsazepine (Rong et al., 2004).

A number of studies report a role of TRPV1 in gut pathophysiology, inflammation and pain. The upregulation of TRPV1 receptors was detected in human bowels which were inflamed and hypersensitive (Yiangou et al., 2001, Chan et al., 2003, Akbar et al., 2008), which is strongly

correlated with pain severity. TRPV1 also has a potential role in the protection of systemic inflammation. Several studies showed that local inflammation was enhanced, and the onset of the systemic endotoxemia was accelerated in TRPV1-null mice (Clark et al., 2007, Fernandes et al., 2012).

1.6 General aim and objectives

The sensory afferents of the gastrointestinal (GI) tract serve as a conductor to transmit signals to the central nerve system (CNS), which then allow for the detection and discrimination of the quantities and qualities of the luminal contents. A correspondent efferent output is generated to produce an appropriate action, which is in close association with the afferent input to control and regulate the coordinated GI activities. Together, these constitute the well-known gut-brain reflex axis, which establishes an important role in modulation of various GI functions, such as digestion, feeding, illness behaviours and pain sensation (Beyak et al., 2006). There is also evidence showing that peripheral afferent fibres activated by inflammatory mediators (Goehler et al., 1995, Watkins et al., 1995) or microbes (Gaykema et al., 1998, Goehler et al., 1998, Hosoi et al., 2005), behaved as potent modulators to monitor the function of the immune system (Olofsson et al., 2012).

The location of these extrinsic afferent nerves' endings in the different layers of gut wall influences their sensitivities to intraluminal nutrients and tension applied on the GI wall. Lipid/fatty acids detected by free fatty acids (FFAs) and GRP120 receptors located in the enteroendocrine cells (EEC) triggers nutrient absorption (Liou et al., 2011a, Rasoamanana et al., 2012). This leads to the release of numerous gastro-intestinal peptides, including cholecystokinin (CCK), Glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) into the lamina propria. These peptides then bind to the corresponding receptors, which are expressed predominantly in the vagal nerve terminals in the gut wall, to produce their regulatory effects on nutrient signalling through the gut-brain reflex axis. CCK as a satiety signalling molecule has been well known for its role in the regulation of feeding and reflex behaviours (Dockray, 2012, Joost et al., 2012).

In recent years, there has been growing evidence that enteral nutrients, particularly lipids, act as stimuli to activate a nutrient induced anti-inflammatory pathway, and the outcome of this pathway was the efferent modulation of macrophages via the cholinergic receptors to regulate

the release of pro-inflammatory mediators to control the degree of inflammation. The reflex is initiated via endogenously released CCK due to the passage of enteral lipids, which then act on peripheral CCK-1 receptors (CCK-1Rs) through a vagal afferent pathway (Luyer et al., 2004, Luyer et al., 2005). This was first demonstrated by Luyer et al., which in a rat model of sepsis, the attenuation in inflammatory response induced by enteral lipids was absent by vagotomy and application of CCK receptor antagonist (Luyer et al., 2005), indicating the involvement of mesenteric afferents and CCK receptors. This pathway was further investigated by Lubbers T et al., in a mice model of sepsis, showing a similar observation, which the protective effect of enteral lipid-rich nutrient was abrogated following the administration of CCK receptor antagonist (Lubbers et al., 2010b).

Together, this evidence introduces the possibility that the enteral nutrients may exert the potential influential effects on the immune system via CCK signalling mechanism on mesenteric afferent of the gut-brain axis, and hence could have therapeutic relevance in the treatment of clinic injury and infection. Indeed, these potentials was investigated previously using animal models that mimic the clinic relevant conditions; a rat model of either trauma or postoperative ileus, induced by hemorrhagic shock (de Haan et al., 2008) or intestinal manipulation (Lubbers et al., 2009, Lubbers et al., 2010a) respectively, was applied. They demonstrated that inflammatory response following both experimental conditions was significantly reduced upon enteral nutrient administration and these was induced via CCK signalling pathway, presenting a novel therapeutic approach to lower and limit the risk of clinic systemic inflammation. However, there is little direct electrophysiological data that support the hypothesis that whether enteral nutrients will activate afferent nerve via CCK-1Rs of this nutrient induced anti-inflammatory pathway. Therefore, for this reason, the overall aim of this thesis was to learn more about the mechanism involved in jejunal afferent transduction and mechanosensitivity to nutrient sensation, and how it will be affected with age.

This thesis consists of three investigations:

1. Investigate the effect of dietary lipids on jejunal sensitivity in adult animals
2. Examine the possible changes of dietary lipid sensation with age
3. Explore the potential role of TRPV1 channel in dietary lipid sensation

Chapter 2

Material and Methods

2.1 Recording intraluminal pressure and afferent activity

Animals

Experiments were performed using adult male mice (~3-4months old) with C57/BL6 genetic background. All animals were allowed free access to food and water and were housed under standard conditions with 12 hrs light: dark cycle. All experiments were conducted in accordance with UK Animals (Scientific Procedures) Act 1986. Mice were sacrificed by cervical dislocation in accordance with UK home office regulations covering schedule one procedures.

Tissue preparation

Segments of jejunum (2-3cm long) were removed 10-20cm proximal to the ileocaecal junction following the mid-line laparotomy of the abdominal cavity. Each piece of the jejunum was dissected out in a manner that a non-bifurcating mesenteric bundle could be emanated centrally. The jejunal segments were immediately put into the oxygenated (5% carbon dioxide + 95% oxygen) Krebs solution (in mM: NaCl 118; NaH2PO4 1 ; KCl 4.8; NaHCO3 25; MgSO4 1.2; glucose 11; CaCl2 2.5) that was perfusing constantly through the Sylgard-lined tissue chamber (20ml) at a flow rate of 15ml min^{-1} to maintain the temperature at 34^{o}C to prevent degradation of the tissue. The segments were securely cannulated at each end of the tissue chamber with one side (the input port) attached to a Perfusor VI syringe pump (Perfusor Secura, B.Braun, Germany) and the other (the outlet port) to a pressure transducer (plus DT-XX, Becton Dickson, Singapore). The outlet port was also attached to a drainage tube, which was required for the release of intraluminal content once tap was open. The intraluminal pressure was recorded via a pressure amplifier (NL 108, Digitimer, UK).

Nerve preparation and Recording

Mice had two parallel mesenteric nerves running together between vein and artery of mesenteric arcade that pinned at the base of the chamber. A single nerve bundle was carefully

teased out from its surrounding fat and connective tissue using fine forceps under a dissection microscope (Nikon, SMZ645). The nerve bundle was cut and then drawn in to a suction electrode for recording. Multi-fibres nerve activities were recorded with a Neurolog headstage (NL100, Digitimer, Ltd, UK), then amplified (NL104, Neurolog system, ×10,000), and filtered (NL125, Neurolog system and Hum Bug, Quest Scientific, band pass 200-3000Hz). It was acquired (20 kHz sampling rate) to a computer through a Micro 1401 analogue-to-digital interface and Spike2 software (Version 5.16, Cambridge Electronic Design, UK). Multiunit afferent nerve activities were quantified by counting the number of action potentials (AP) crossing a pre-set threshold (Digitimer D130). A threshold level for spike counting was set to pick up the smallest identifiable spikes, roughly twice the baseline noise level. The whole set up is illustrated in the schematic diagram in figure 2.1.

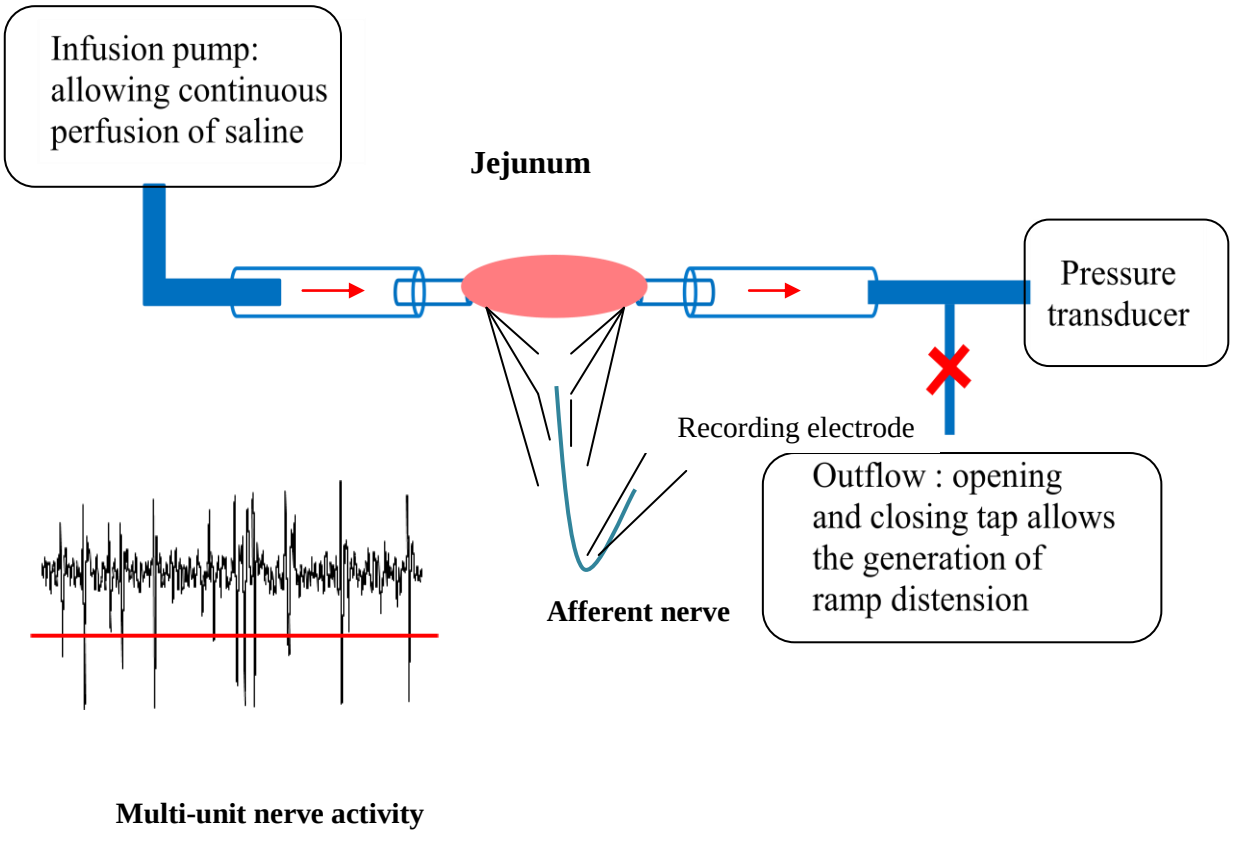


Figure 2.1 In vitro murine model to measure jejunal afferent nerve activity and intraluminal pressure. The jejunal segment was attached to the each end of the tissue chamber with one side connected a perfusion pump allowing continuous infusion of saline/drugs through the lumen of the jejunum. The other side attached to a pressure transducer to measure the intraluminal pressures during the jejunum distension. Multi-unit afferent nerve bundle was identified, dissected and held in a glass suction electrode. Action potentials captured from the nerve bundles were amplified, filtered and recorded using computer software. The multi-unit nerve activity was quantified by counting the number of action potentials crossing a pre-set threshold.

2.2 General experimental protocols

This section provided details of basic experimental protocols used throughout this thesis. Detailed experimental procedures for each experiment could be found in the method section of individual chapters.

Control distension and reproducibility of response

Multiunit afferent nerve activities were stabilised for a period of 40-60 minutes to generate a stabilised baseline firing. Continuous intraluminal perfusion of isotonic saline (0.9% NaCl) at 200µl min^{-1} was maintained by the syringe pump through the input port when the outlet port was open, but allowing the generation of periodic distensions upon the closure of the outlet port. The gut was distended until an intraluminal pressure of 55mmHg was reached. Responses of afferent nerve activities to ramp control distensions were captured by concurrent nerve recordings. Unless otherwise stated the repeated control distensions were carried out every 15 minutes to ensure reproducibility of response. The afferent nerve was required to generate at least three reproducible responses to control distension before any experimental procedures could be carried out.

2.3 Analysis of data

2.3.1 General analysis

Data were presented as a mean of either the absolute afferent discharge (spike s^{-1}), or the change in afferent discharge (response – baseline firing). Data were expressed as mean ±SEM from n values. Unless other stated, n was the number of animals used in each group. Analysis of individual experimental protocol would be discussed in relevant chapters.

The chemical effect of a drug on baseline discharge was investigated by comparing the mean response (i.e. during drug incubation) with mean baseline activity before drug application. The responses of afferent activities to ramp distensions were expressed as the change in afferent firing above baseline, calculated as the mean firing frequency in 2 second periods at each level of distending pressure subtracting baseline firing. Baseline firing was defined as the mean firing frequency in the 1 minute period preceding distension. The data was derived from multiunit nerve recordings, which the number of afferent fibres in nerve bundles was varied. Therefore, this type of analysis was very effective to minimize the natural variation that existed within each individual experimental group.

Several statistical tests were used appropriately and the differences were considered statistically significant at $p < 0.05$. GraphPad Prism (Version 5.02 for windows, GraphPad Software inc, San Diego, California USA) were used to perform the graphical and statistical analysis throughout this thesis.

Ramp distension of jejunal segments evokes a biphasic increase in afferent discharge, which represents the activation of low threshold (LT) and high threshold (HT) mechanosensitive afferent fibres. The LT component is represented as the increase in discharge between baseline

and 20mmHg. The HT component is represented as the increase in discharge between 20mmHg and 55mmHg. A sample trace is presented in figure 2.3.1

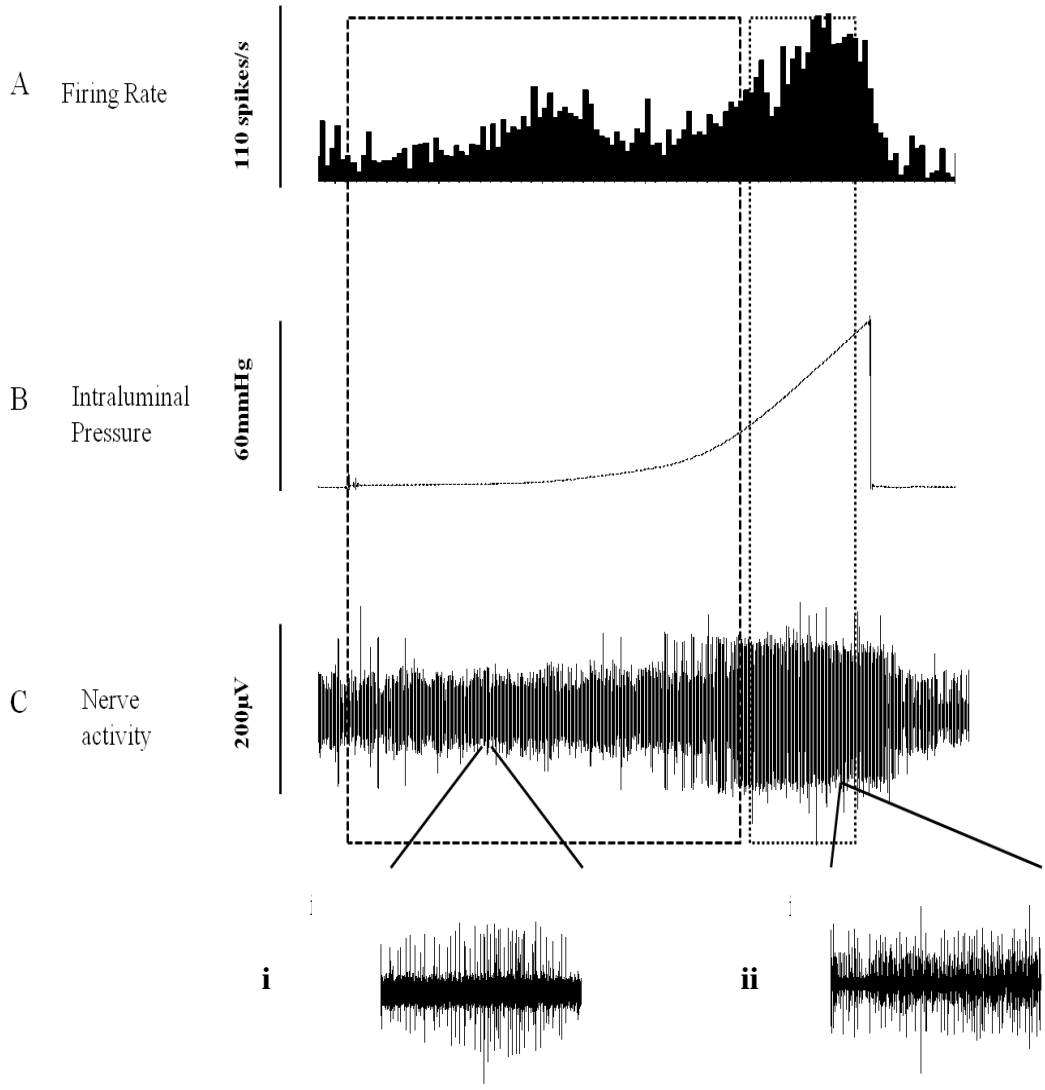


Figure 2.3.1 The response to jejunal ramp distension. A) The sequential rate histogram depicting the increase in whole jejunal afferent nerve firing rate evoked by saline distension. B) the rise in intraluminal pressure during ramp distension with isotonic saline (0.9% NaCl) at 200µl/min to a maximal intraluminal pressure of 55mmHg. C) raw nerve trace showing the increased afferent activity in response to distension. Below are examples of actual nerve recordings taken from low threshold level (i) and high threshold level of distension (Lutz et al.)

2.3.2 Single unit analysis

Single unit discrimination was performed offline using the spike sorting function of Spike 2 software (version 7). It allows the identification of individual single unit in each preparation. Each afferent unit from within the whole nerve recording has its own distinct spike shape with characterised waveform and amplitude. After re-scanning the raw nerve trace with a new established threshold, the software could then create a "wavemark" channel that separated the discharge frequency of single afferent units into individual bins. The formation of the wavemark channel is dependent on the creation of templates for each afferent unit according to their own distinctive waveform and amplitude. Afferent nerve activity was sampled at 25,000HZ. This was used to construct the spike templates which consisted of both positive and negative excursion of the action potential. Individual spikes were classified to a particular template, that with less than 10% variability in amplitude and 60% more than the data points within the template boundary. Principal component analysis (PCA) was carried out following the waveform analysis. It was used to identify the principle variations in the spike shape/action potential. The main purpose of this analysis is to ensure that the single unit identified in the single unit analysis are indeed different from each other and is able to be classified as distinctive nerve fibres. Once the number and profile of each unit was identified, further analysis and classification could take place.

Classification of single unit nerve fibres

Single unit analysis revealed three functional groups of afferent fibres with distinctive activation threshold and pressure-response characteristics: 1) low threshold (LT) afferent units which were activated only at low distension pressure (<20mmHg) within the physiological range; 2) Wide dynamic range (WDR) afferent units which was activated at low distension pressure, but encoding intensity up to the pathophysiological range; 3) High threshold (HT) afferent units that were activated and recruited at high distension pressure (>20mmHg) within

the pathophysiological range (Rong et al., 2004). The objective classification is based on the calculation: the response at 20mmHg expressed as a percentage of the maximum response at 55mmHg, a parameter referred to as LT%. A value of %LT > 55% are defined as LT unit and <15% were classified as HT unit. The afferents with a more linear increase in discharge over the range of distension (<55%, >15%) were termed as WDR unit (Booth et al., 2008, Keating et al., 2008).

2.4 Drugs and solutions

Pharmacological agents' application

Several methods were used in the application of pharmacological agents:

1) Intraluminal application of agonist to access their effects on afferent discharge in baseline activity or in response to distension

2) Extraluminal application of agents (agonist and antagonist) to study their direct effects on the activation of afferent nerve discharge

Intraluminal application of agonist

Drugs were preheated to 34ºC before application. Agonists were applied to the intraluminal surface of the small intestine (jejunum) by hand injection via a 2nd syringe attaching to the input port while the outlet port was open. The drug was pushed through carefully at a rate which intraluminal pressure was not altered (for most jejunums, the injection period was 2 minutes). The drugs were then left within the segments of jejunum for 15 minutes when the outlet port was closed. Changes in afferent activities during drug injection and incubation were recorded. Afferent responses to distension during (i.e. distension with drugs) and after drugs application (1.e. washout) were also recorded.

Extraluminal application of agents

Drugs were dissolved into stock solutions and then applied to bath via a pipette. Before application, circulation of Krebs solution was stopped (3-6 minutes) to allow drugs to disperse throughout tissue chamber (20ml) to achieve a desired concentration and also prevent the washout of drugs. Control experiments were carried out to exam whether there was any effect on afferent discharge during the period of non-perfusion.

Solubility

The solubility of a drug was determined from the information sheets provided by the drug manufacturer or provider. Unless otherwise stated all drugs were firstly dissolved in isotonic saline (0.9% NaCl) to form a stock solution. Drugs which were to be applied to the bath were subsequently diluted into Krebs solution. Cholecystokinin (CCK, Octapeptide, sulphated) were first dissolved in 2% BSA (Bovine Serum Albumin) and devazepide was first dissolved in a mixed vehicle of Tween 80, DMSO, saline with a ratio of 1:1:8. There drugs were then further diluted to a desired concentration in either saline or Krebs solution. A vehicle control experiment was performed to study the effects of solvents in every case. Dietary lipid nutrients were gifts from Dr. Tim Lubbers and Prof. Wim A. Buurman in the Maastricht University, Netherland. They were manufactured and provided by Danone.

Drugs

The concentration of the drugs is chosen either based on the preliminary studies or derived from the concentration used in the literatures. All pharmacological tools used in this thesis were summarised in table 2.1. The compositions of nutrients used were summarised in table 2.2 as energy percentage. The proteins were derived from lean milk; the carbohydrate source was a mixture of sucrose and corn-starch. The lipid source was vegetable oil with a fatty acid composition of 8.1% saturated fatty acids; 58.9% monounsaturated fatty acids, of which oleic acid was the main source (57.4%); 28.2% consisted of polyunsaturated fatty acids, of which linoeic acid was the main source (23%); the amount of n-3 and n-6 fatty acids in the high-fat nutrition was <5% of the total fat content. The types of carbohydrates and fat used in both nutrients were identical.

Drug name	Manufacturer	Vehicle	Main action
Cholecystokinin (CCK, Octapeptide, sulphated)	Sigma	2% BSA	CCK receptor agonist
Devazepide	Tocris	Tween80:DMSO: saline	Selective CCK1 receptor antagonist
mannitol	Oxoid	0.9% saline	osmolarity

Table 2.1 Summary of pharmacological tools used in this thesis

	Fat	Protein	Carbohydrates
Lipid-rich nutrient	52.2%	6.9%	40.9%
Low-lipid nutrient	16.7%	6.9%	75.4%

Table 2.2 Summary of composition of lipid nutrients used in the thesis

Chapter 3

Effect of lipid nutrients on jejunal afferent sensitivity in adult murine bowel

3.1 Introduction

Nutrients are detected by 'taste' cells located at the gut wall. They are defined as any cell types that can elicit responses to nutrient stimuli (Chandrashekar et al., 2006). Enteroendocrine and enterochromaffin cells are specialized 'taste' receptors in the mucosa. They are chemosensitive cells which can be activated by the passage of macronutrient, such as carbohydrates, proteins and lipids (Raybould et al., 2006). The release of endogenous CCK from enteroendocrine I cells is triggered by the passage of luminal fat/lipid or protein (Buchan, 1999) stimulating vagal afferents nearby (Berthoud and Patterson, 1996).

The effect of a high fat (HF) diet on the GI tract has been evaluated predominately in animals. In rats, exposure to a HF diet leads to altered GI tract morphology such as increased intestinal villus height, mostly observed in jejunum and ileum, (Sagher et al., 1991). Plasma CCK level was significantly elevated resulting in a marked increase in pancreatic secretion (Spannagel et al., 1996). Interestingly, HF nutrients also have an inhibitory effect on the sensitivity of satiety signals, for example, CCK (Covasa and Ritter, 1998, Covasa et al., 2001). A limited number of human studies have been performed. Boyd et al showed that in healthy adults, the plasma CCK level was not altered after exposure to a HF diet (Boyd et al., 2003). However, a later study performed by Little et al showed that the consumption of HF diet significantly increased fasting plasma CCK but had no impact on the sensitivity of CCK (Little et al., 2008).This is different to what has been observed in animals.

In recent years, a nutrient (lipid/fat) activated anti-inflammatory pathway has been described. In the clinic, development of new treatments to prevent and minimise the risk of post-surgery inflammation has always been a challenge. Patients suffering from sepsis or going through surgical operation experience a risk period known as the state of immune paralysis (Angele and Faist, 2002). This was especially crucial for critically ill patients in intensive care who were more vulnerable to bacteria and virus infection than healthy patients (Limaye et al., 2008). The pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL- 1β)

played an important role in the systemic inflammatory response and secondary tissue damage that occurs in patients with sepsis. The nutrient mediated anti-inflammatory pathway is regulated via the brain gut axis by the CCK-1 receptor mediated pathway (Luyer et al., 2005) in which vagal afferents provide a link between the GI tract and the brain. This pathway resulted in the release of pro-inflammatory cytokines, such as TNF-α and IL-1β, being inhibited in rats fed a HF diet during hemorrhagic shock. The protective effect of HF nutrients was significantly reduced in either vagotomised or CCK receptor antagonist treated animals.

This chapter focuses on the afferent mechanisms underlying this anti-inflammatory pathway. The aim of this study is to investigate the effect of dietary fat (lipid-rich and low-lipid) nutrients on jejunal afferent sensitivity. We hypothesize that lipid-rich and low lipid nutrients will exert differential effects on both chemosensitive and mechanosensitive afferents innervating mice jejunum. In order to examine this hypothesis; we also investigated the direct effect of exogenous CCK on jejunal chemo and mechanosensitivities.

3.2 Experimental protocol and analysis

The *in vitro* preparation was set up as described in chapter 2.

Measurement of afferent activity

All the experiments were performed on wild type C57BL6 mice aged 3 months old. Recordings were made from mesenteric afferent nerves innervating the jejunum. All preparations were left for 40-60 minutes for stabilisation followed by ramp distensions every 15 minutes until the response became reproducible (3-4 reproducible responses to distension) before the commencement of any protocol. Two different types of nutrients were used. They were the same as the nutrients used in the inflammation studies. One comprised a lipid-rich nutrient and the other a low-lipid nutrient. Their compositions vary largely in ratio. The detailed composition of these two types of nutrients is shown in table 2.2 in chapter 2.

Effect of CCK-8 on baseline and distension induced afferent discharge

To investigate the effect of exogenous CCK on jejunal afferent sensitivity, two different concentrations of CCK-8 were applied directly into the organ bath. (Figure 3.2.2A) Circulation of Krebs solution was stopped (3-6 minutes) to allow drugs to disperse throughout the organ chamber to achieve a desired concentration and also prevent the washout of drugs. The final bath concentration of CCK (5/100nM) was chosen based on previous studies from literature (Criddle et al., 2009, Daly et al., 2011).

Effect of lipid-containing (lipid-rich/low lipid) nutrients on baseline response

The lipid-rich / low lipid nutrient (volume) was infused by hand over a period of 2 minutes. The chemical (direct) effect of the lipid-rich/low lipid nutrient on afferent firing was measured by comparing the mean afferent firing (~5 minutes) before and with the lipid-rich/low lipid nutrient treatment. Control experiments were performed with saline (0.9% NaCl). This was to investigate whether any fluctuations in afferent discharge were a consequence of the mechanical effects of

infusion. In a separate set of experiments, 700mOsm/L mannitol was used as an osmotic control since both lipid-rich and low lipid nutrients were hyperosmotic solutions (697mOsm/l and 724mOsm/l respectively).

Effect of lipid-containing (lipid-rich/low lipid) nutrients on afferent sensitivity in response to distension

The effect of lipid-rich/low lipid nutrients on mechanosensory response to jejunal distension was determined during ramp distension up to an intraluminal pressure of 55mmHg. Jejunal afferent discharge, expressed as change in afferent discharge above the baseline, was calculated as the mean firing frequency in a period of 2 seconds at each level of distending pressure. It was then plotted against pressure. The experimental protocol for this study with lipid-containing nutrients is shown in figure 3.2.1 (A). Responses to distension before, during and after nutrient infusion were compared. The whole data was normalised using single unit analysis. Three groups of afferent units were identified using single unit analyses which were low threshold (LT), wild dynamic range (WDR) and high threshold (HT) units. Details of single unit analysis and unit classification were described and explained in Chapter 2.

Afferent response to CCK or nutrients (lipid-rich/low-lipid) in the presence of devazepide

Devazepide was added directly into the tissue chamber as indicated in figure 3.2.1B and 3.2.2B. The circulation of Krebs solution was stopped during this 3 min period in order to maintain exposure at a constant drug concentration. It was calculated on the basis of the bath volume. Afferent response to lipid nutrients/CCK-8 was compared in the absence and presence of devazepide, which added three minutes prior to lipid nutrients/CCK-8 application.

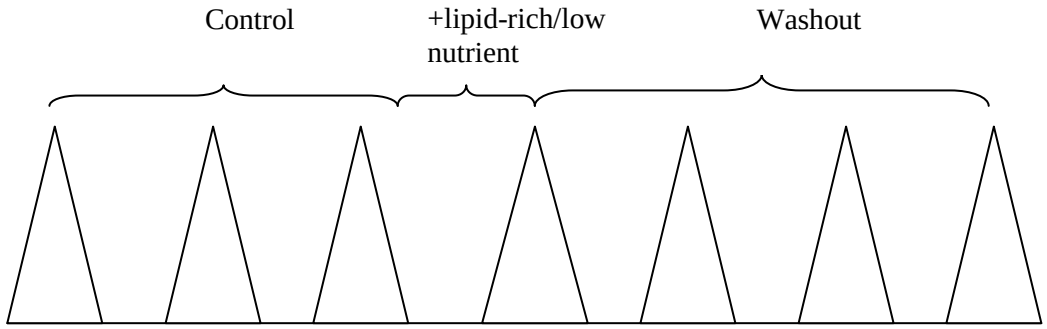
Jejunal compliance and intraluminal volume

Since infusion rate was always kept stable through ramp distension (200µl/min), intraluminal volume was calculated using the rate of distension and time taken to reach a given intraluminal pressure (55mmHg). The effect of treatment on jejunal compliance was evaluated as the final volume reaching a distending pressure of 55mmHg. It was compared with the control which is before any treatment was applied.

Statistical significance

Statistical analysis was carried out using the Student's t-test (paired/unpaired), repeated measured one way ANOVA with Dunnett's Multiple Comparison Test or two-way ANOVA with Bonferroni post-tests as appropriate. Data were presented as mean ± SEM, where $p < 0.05$ was considered significant.

A



B

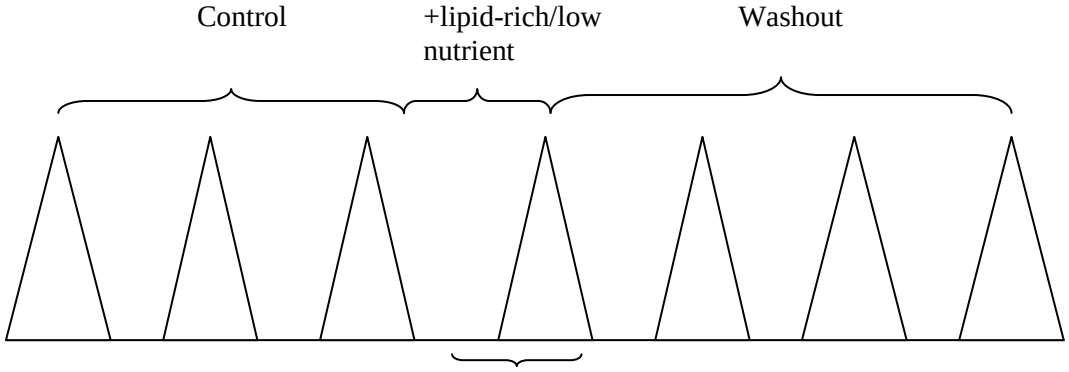


Figure 3.2.1 A) schematic diagram showing the protocol used for examining chemo- and mechanosensitivity of jejunal afferents to the lipid-containing nutrient (lipid rich/low) before and during intraluminal distension. Each triangle represents a single distension. B) is the protocol for the investigation of the effect of devazepide on afferent responses to nutrient.

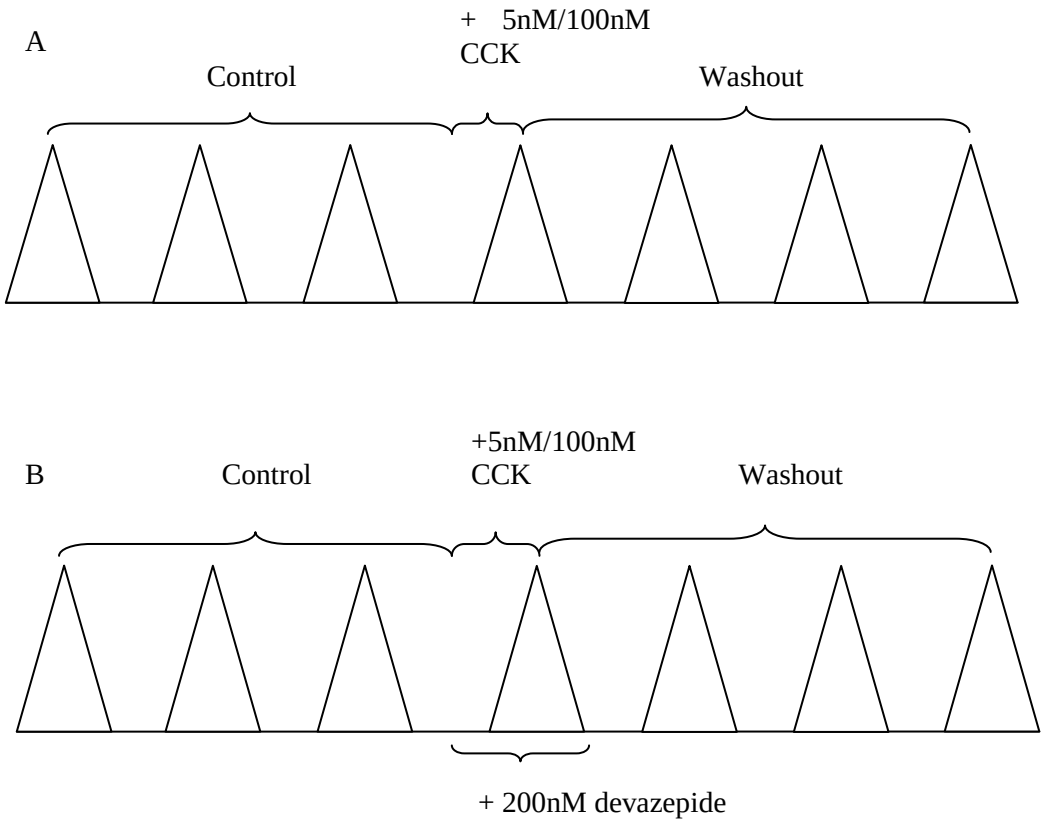


Figure 3.2.2 A) schematic diagram showing the protocol used for examining afferent response to exogenous CCK before and during intraluminal distension. Each triangle represents a single distension. B) is the protocol for investigating the underlying mechanism of CCK signalling pathway using CCK-1 antagonist, devazepide.

3.3 Effect of sulphated CCK-8 (5nM/100nM) on baseline discharge

Baseline discharge was measured as the mean firing response of nerve activity over a period of 3 minutes. It was compared in the absence and presence of CCK-8 (sulphated). The vehicle (2% BSA) for CCK-8 had no effect on baseline discharge (figure 3.3.1A). CCK-8 (5nM) stimulation caused a robust excitation of the jejunal afferents (figure 3.3.2A). The baseline discharge was 20.4±3.2 spikes s^{-1}. This was increased to 30.2 ± 7.3 spikes s^{-1} with bath applied 5nM CCK-8. Following a sufficient washout period of 45 minutes, response to CCK-8 stimulation was reproducible. There was no significant difference between the afferent responses to baseline discharge of these two separate applications (figure 3.3.2B). Effects of higher concentration of CCK-8 (100nM) on afferent response were examined using the same protocol. Similarly, 100nM CCK-8 also significantly augmented afferent response on baseline firing (figure 3.3.2C). Baseline firing was 18.8±3.4 spikes s^{-1}.This was increased to 35.7±7.4 spikes s^{-1} with bath applied 100nM CCK-8. The response to the 2nd 100nM CCK-8 application following a period of 45 minutes washout was also reproducible (figure 3.3.2D).

In a separate set of experiments, CCK-8 induced excitation on baseline discharge was further examined using a selective CCK_A receptor antagonist, devazepide. Vehicle for devazepide (a mixture of Tween 80, DMSO, and saline) has no effect on baseline nerve firing, (figure 3.3.1B). The response to bath applied 5nM CCK-8 was completely abolished by application of devazepide (figure 3.3.3A). The control baseline firing (before any drug treatment) was 37.2±4.9 spikes s^{-1}. This was increased to 49.4±7.0 spikes s^{-1} with bath applied 5nM CCK-8 which was then significantly reduced to 34.0 ±4.4 spikes s^{-1} with addition of devazepide. 100nM CCK-8 induced excitation on baseline firing was not completely abolished by application of devazepide (figure 3.3.3B). The baseline discharge was elevated significantly from 28.7±5.0 spikes s^{-1} to 45.7±6.6 spikes s^{-1} with bath applied 100nM CCK-8. This was only partially blocked by devazepide, the baseline firing reduced to 35.0±5.4 spikes s^{-1}. It was still significantly augmented compared with control baseline discharge.

In order to apply exogenous CCK into the organ bath, the perfusion pump was stopped for 3 minutes. A set of control experiments was carried out showing that 3-min absence of perfusion did not have any effect on baseline nerve activity or distension induced mechanosensitivity.

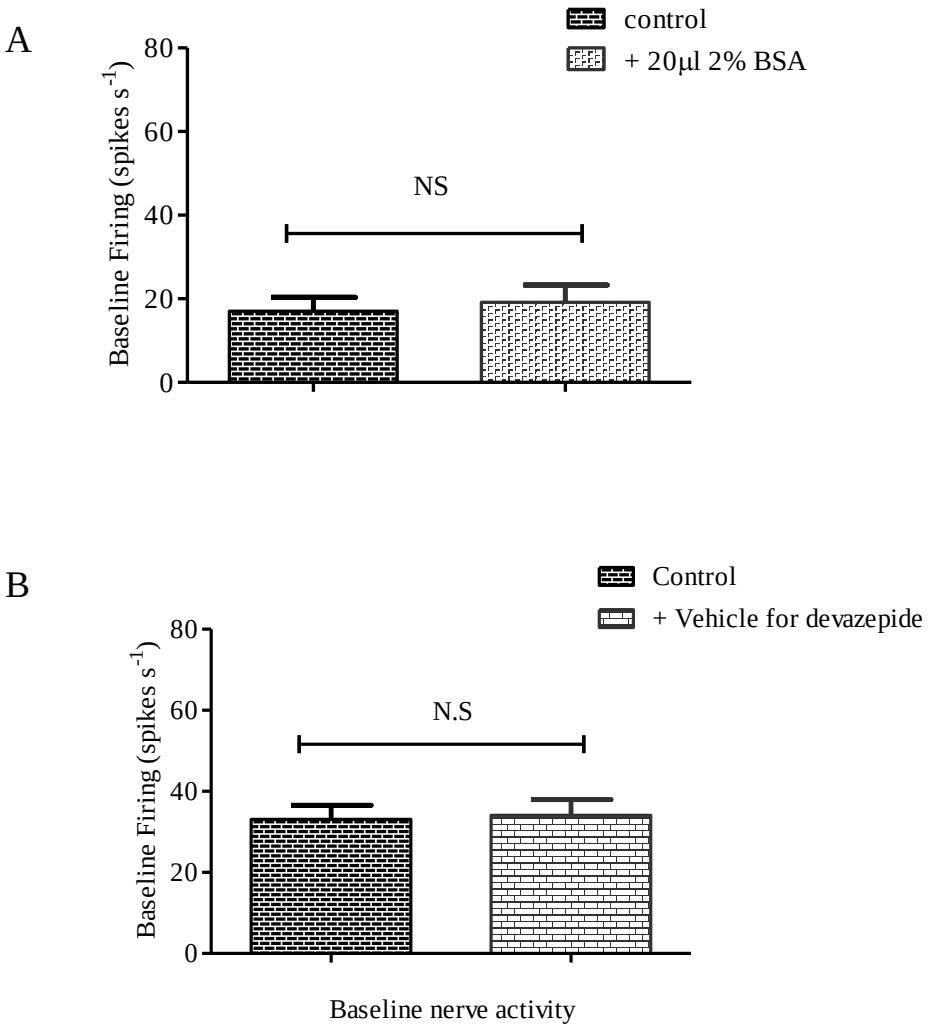


Figure 3.3.1 Vehicle of CCK (n=6) and devazepide (n=12) has no significant effect on baseline nerve activity, $p>0.05$, paired Student's t-test, n=12.

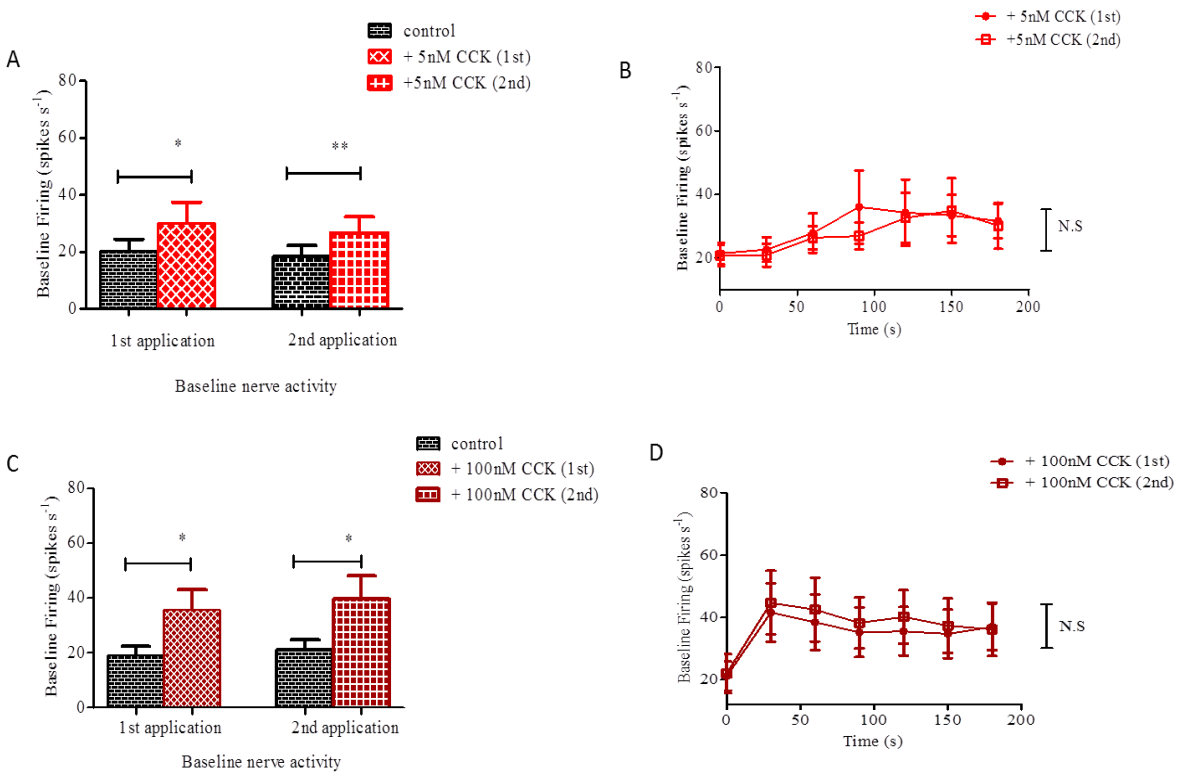
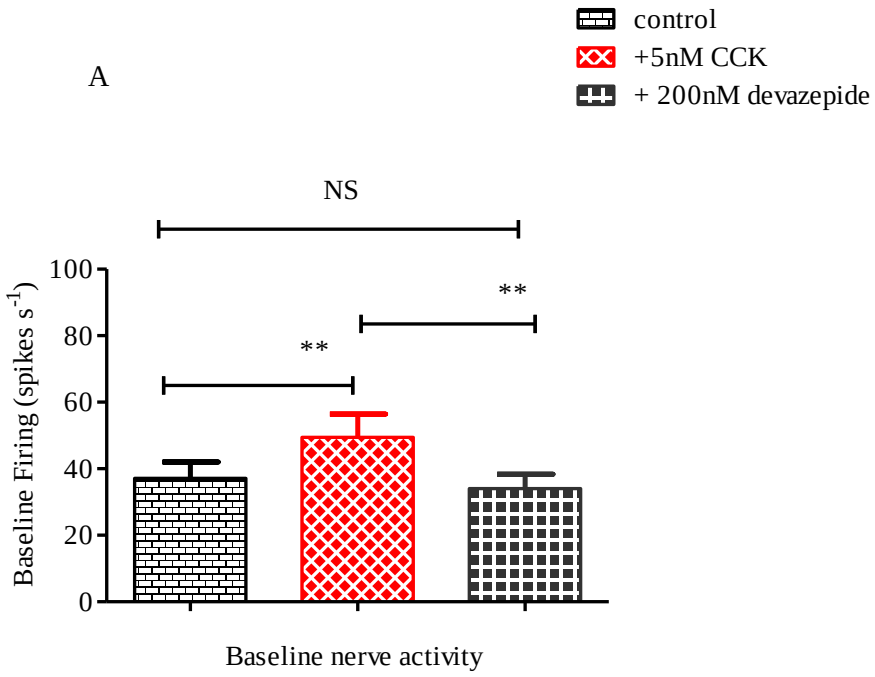
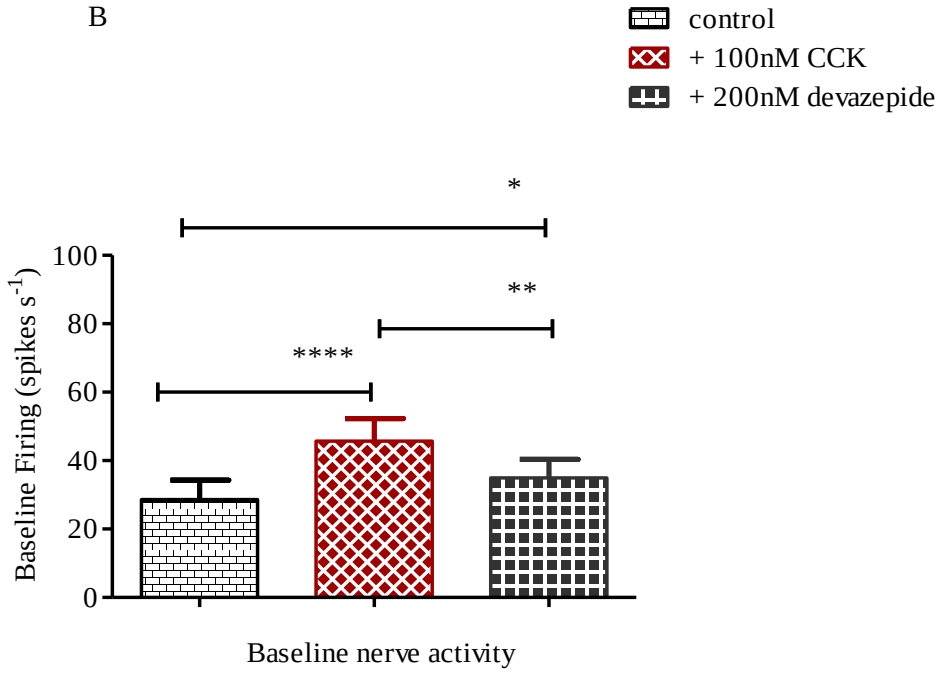


Figure 3.3.2 A) and C) histograms showing that consecutive application of 5nM/100nM CCK produces a reproducible excitation of baseline nerve activity. 5nM/100nM CCK significantly augmented baseline nerve activity, $p < 0.05$*, paired student t-test, n=6. B) and D), the baseline nerve response to 5nM/100nM CCK over time. There is no significant difference in baseline nerve response between two separate applications of CCK, (two-way ANOVA).





3.3.3 Effect of CCK on baseline discharge in presence of devazepide (200nM). A, 5nM CCK induced significant augmentation in baseline firing, which was has significantly reduced with the application of devazepide, $p<0.05$**, repeated measures ANOVA, n=6. B, Application of devazepide also significantly reversed the effect of 100nM CCK on baseline firing, $p <0.05$**, but it cannot be completely abolished, $p <0.05$*, (Repeated measured ANOVA).

3.4 Effect of sulphated CCK-8 (5nM/100nM) on distension induced afferent discharge

Effect of sulphated CCK-8 on distension induced afferent response was also investigated. CCK-8 was bath applied 3 minutes prior to distension. Vehicle (2% BSA) for CCK had no significant effect on the afferent response to distension (figure 3.4.1). Even though concentrations of CCK (5nM/100nM) caused a robust excitation in baseline nerve activity (figure 3.3.2A and 3.3.2C), they had no significant effect on distension induced afferent discharge. These data are summarised in figure 3.4.2. They also had no effect on jejunal compliance (figure 3.4.3)

To determine whether bath applied CCK-8 (5nM/100nM) had any effect on low threshold (baseline-20mmHg) and high threshold (20-55mmHg) components of the afferent response to distension, the mean change of firing frequency in those two phases was analysed. 5nM/100nM CCK has no significant effect on afferent firing at low (figure 3.4.2B) and high threshold level of distension (figure 3.4.2C) respectively.

Combining the data from the previous section, these results show that CCK-8 bath application was able to stimulate jejunal baseline afferent fibres via a CCK_A receptor mediated mechanism. However, distension-evoked afferent response was not altered by CCK-8.

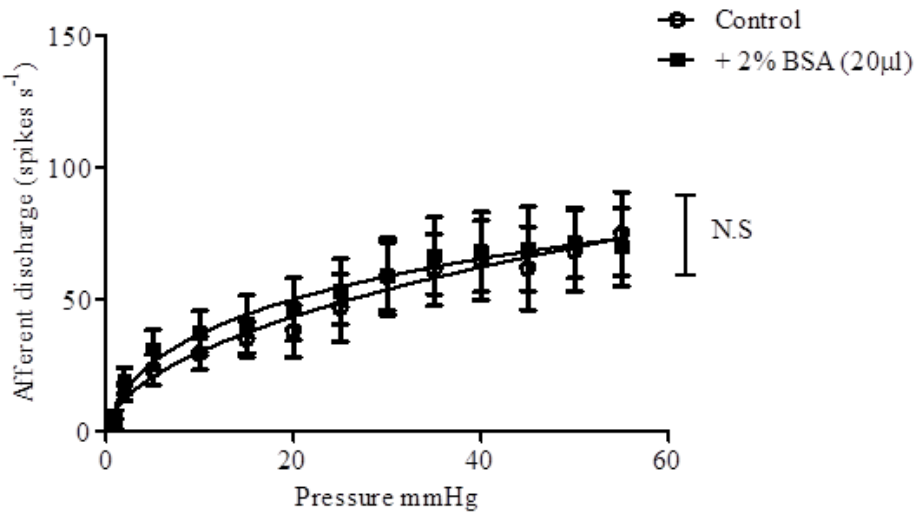


Figure 3.4.1 Vehicle (2% BSA) for CCK has no effect on distension induced afferent discharge, p= 0.4, two way ANOVA, n=6.

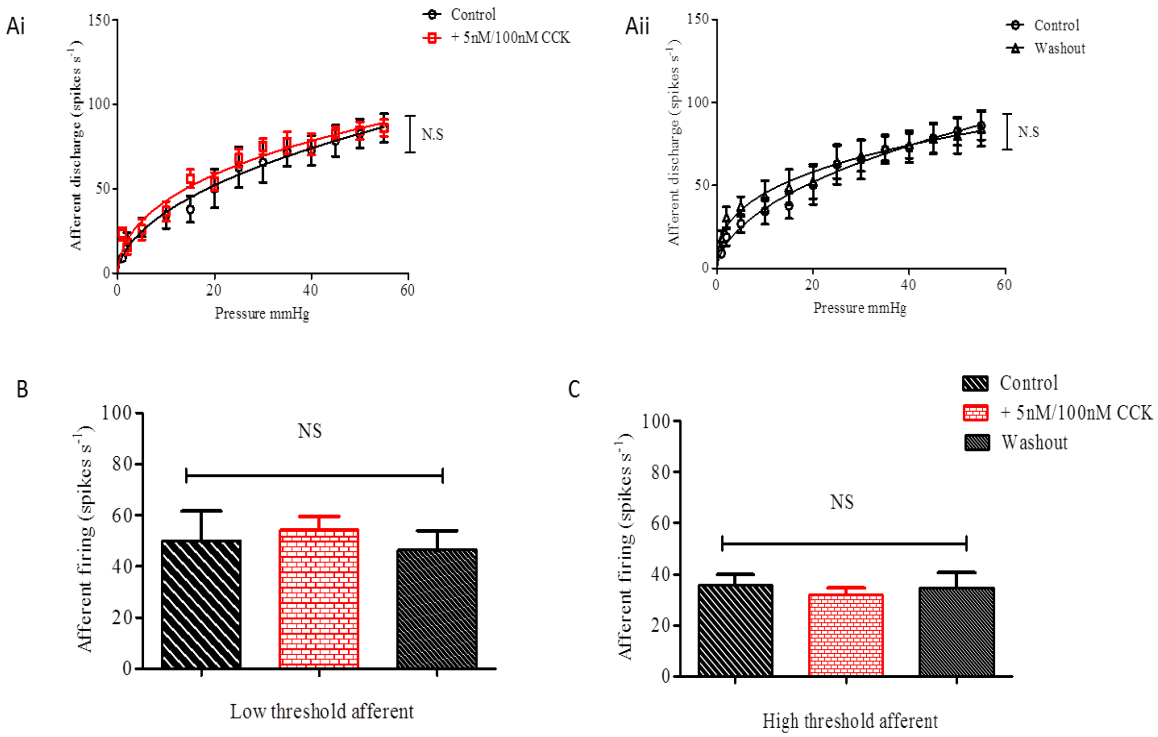


Figure 3.4.2 Mean afferent response to ramp distension up to 55mmHg is plotted. Ai) and Aii) 5nM/100nM CCK-8 has no effect on afferent sensitivity to distension, $p > 0.05$, two-way ANOVA, $n \geq 5$. B) and C) show the data for the low threshold (LT; baseline to 20 mmHg) and high threshold (HT; 20 to 55 mmHg) components of the response to distension. There is no effect on both the LT and HT mechanosensitivity; $p > 0.05$, repeated measured ANOVA.

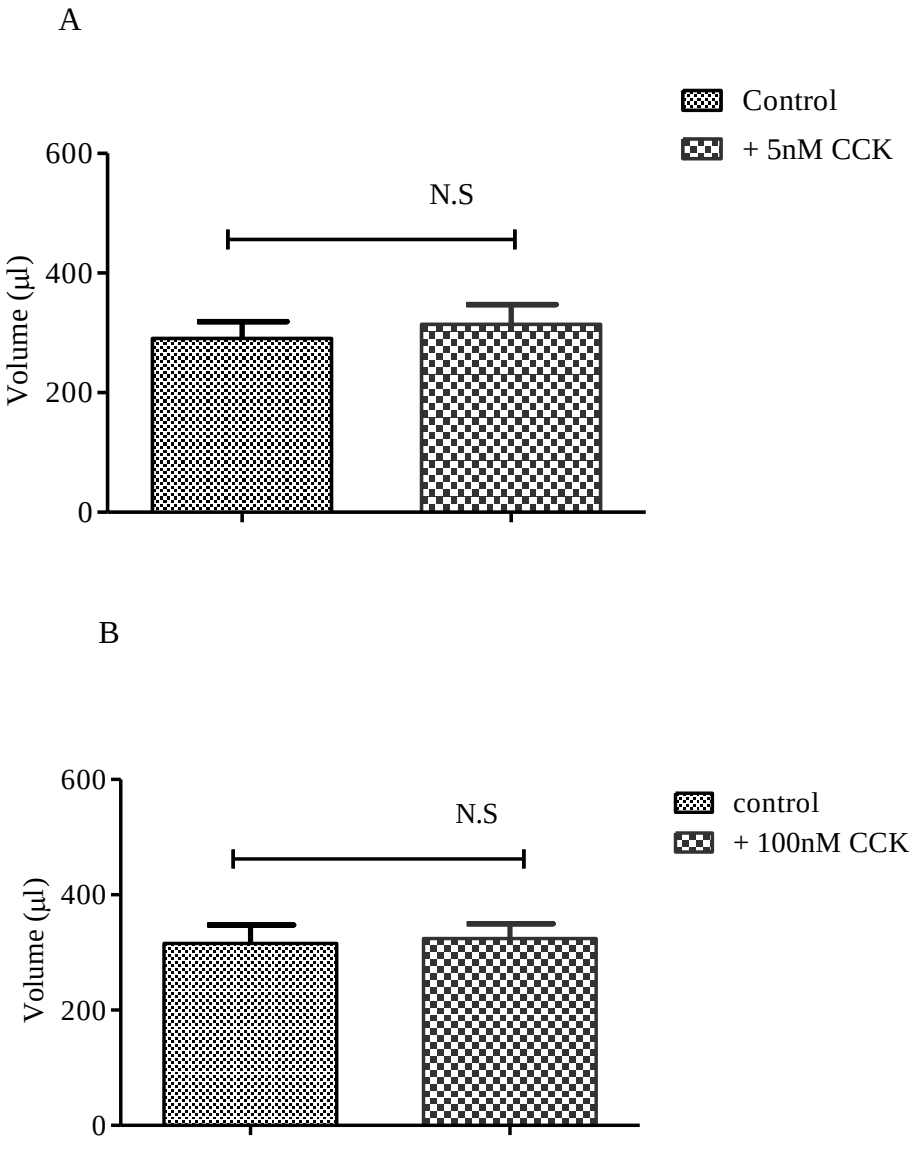


Figure 3.4.3 effect of exogenous CCK on jejunal compliance. A and B show that neither 5nM (p=0.06, n=5) nor 100nM CCK (p=0.6, n=6) have any effects on jejunal compliance, paired t-test.

3.5 Effect of lipid nutrients on baseline discharge and intraluminal compliance

Baseline discharge was measured as the mean afferent firing over a period of 5 minutes (~300s) immediately after nutrient infusion. The data is summarized in figure 3.5.1. Baseline discharge was unchanged by either lipid-rich or low lipid nutrient exposure. The data for the lipid-rich nutrient was obtained from 8 experiments conducted. In a separate set of experiments, osmolarity control experiments were carried out using 700mOsm/L mannitol. In these experiments there was a small but significant increase in baseline discharge which was absent in control experiments using isotonic saline (0.9% NaCl). The data from figure 3.5.2 showed that none of the stimuli (lipid nutrients, hyperosmotic mannitol and saline) had any effect on jejunal compliance.

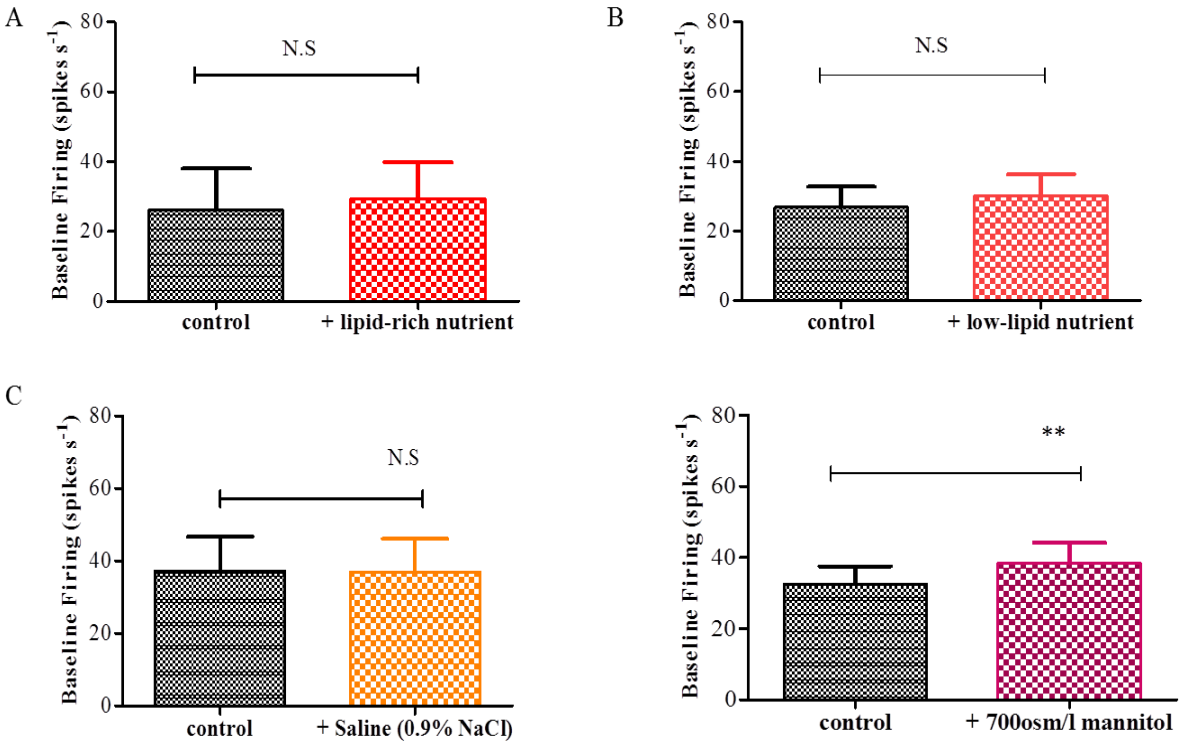


Figure 3.5.1 the mean response of baseline firing in a period of 5 minutes before and with application of 1ml of (A) lipid-rich, (B) low-lipid emulsion, (C) saline and (D) 700Mosm/l mannitol. (A) Lipid–rich nutrient (n=8) and (B) low lipid nutrient (n=7) have no effect on baseline firing. (C) There is also no change on baseline firing induced by saline supplementation (n=6). (D) Incubation of 700osm/l mannitol (n=7) significantly augments baseline firing, p= 0.006**, paired t-test.

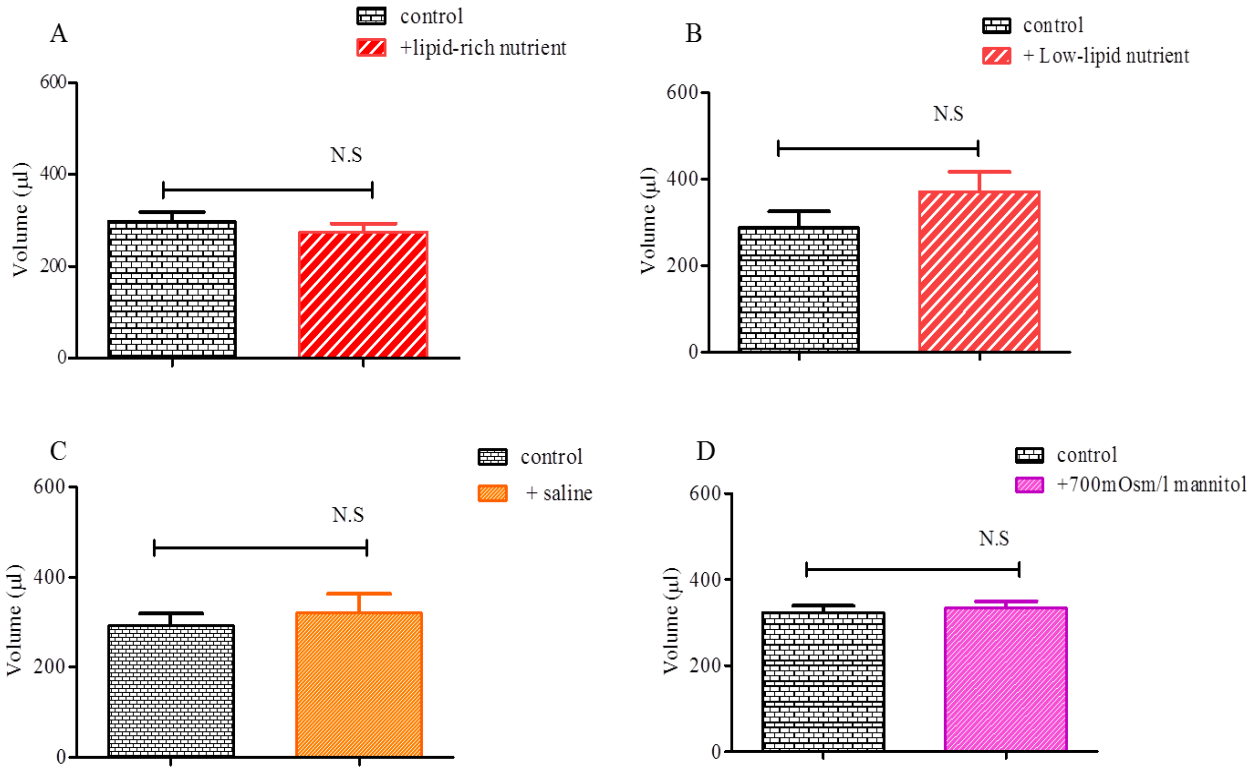


Figure 3.5.2 There is no change on jejunal compliance with any of the following treatments A, Lipid-rich nutrient, p=0.2, n=10. B, Low lipid nutrient, p=0.14, n=7. C, saline, p = 0.38, n=6 and D, hypertonic mannitol (700mOsm/l), p=0.1, n=6 (paired t-test).

3.6 Effect of lipid-rich nutrient on distension-induced afferent discharge

In these experiments, ramp distension was performed every 15 minutes until 3-4 reproducible responses had been obtained. Lipid-rich emulsion (1ml) was then applied and the afferent response to distension compared to that before (control) and after (washout) lipid-rich nutrient application. These data are summarised in figure 3.6.1 Ai and Aii. It shows that in the presence of the lipid rich nutrient there is an increase in overall mechanosensitivity.

To determine whether the lipid-rich emulsion had any differential effect on low threshold (baseline-20mmHg) and high threshold (20-55mmHg) components of the afferent response to distension, the mean change of firing frequency in those two phases was analyzed separately. The lipid-rich nutrient significantly augmented low threshold afferent firing (figure 3.6.1B). The increase in afferent discharge at 20mmHg was 44.8±9.0 spike s^{-1} before application of the lipid-rich emulsion, but increased to 72.2±11.6 spike s^{-1} during lipid-rich nutrient infusion, and recovered immediately to 45.4±7.4 spike s^{-1} during the first washout period. Interestingly, the lipid-rich emulsion had an inhibitory effect on high threshold afferent firing (figure 3.6.1C) resulting in a flattening of the pressure-discharge response profile (figure 3.6.1Ai). The afferent firing was reduced to 27.1±5.9 spike s^{-1} from 40.7±5.8 spike s^{-1} upon lipid-rich emulsion application. The inhibitory effect on high threshold afferent firing also washed out rapidly.

Single unit analysis (as described in chapter 2) was conducted. It corrected the whole nerve data to the number of active single units identified in each preparation. This was to minimize the variations caused by the presence of different numbers of single units in each afferent bundle. Following this analysis there was still a significant augmentation on afferent mechanosensitivity with the lipid-rich nutrient (figure 3.6.2A). However, the statistical significance was more robust when comparing the whole data (figure 3.6.1Ai).

A total of 88 single fibres were characterized from 8 preparations by their activation threshold. The other two preparations were excluded from single unit analysis, because the high frequency of firing precluded single unit analysis. Three types of fibres were identified in the whole nerve bundle (as described in chapter 2). Low threshold (LT) units, wide dynamic range (WDR) units and high threshold (HT) units (figure 3.6.2 B, C and D). The majority of single units identified were low threshold afferent fibres. The data showed that the lipid-rich nutrient significantly potentiated afferent firings in all three types of nerve units. Distinctive afferent response profiles to the lipid-rich nutrient were observed among these three afferent groups. Unlike LT units, there was a flattening in the pressure response profiles of both WDR and HT fibres. This indicated that afferent discharge was gradually attenuated in the high pressure level following an initial augmentation in the low pressure level. This was in agreement with the finding that afferent firing in the high threshold component had been attenuated (figure 3.6.1C)

To investigate the receptor mechanism underlying lipid-rich nutrient mediated activation of afferent mechanosensitivity to distension, a selective CCK-1 receptor antagonist, devazepide, was applied. As shown in CCK studies, 200nM devazepide was determined to be sufficient to block the effect of exogenous 100nM CCK and was therefore used in these experiments. The whole data was normalized by single unit analysis. 41 single fibres were identified from 4 preparations. Data from the other two experiments was excluded since they were too active. It showed that devazepide itself had no effect on distension induced afferent mechanosensitivity (figure 3.6.3).

The augmented mechanosensitivity following lipid-rich nutrient was significantly attenuated in the presence of 200nM devazepide (figure 3.6.4A). Devazepide caused a significant reduction in the low threshold afferent unit (figure 3.6.4B), but not in the wide dynamic range and high threshold afferent units (figure 3.6.4C and D). This was consistent with the finding that it was the low threshold component that was augmented by lipid and this was reversed in the

presence of devazepide. Previous studies demonstrated that the low threshold afferents mainly composed of vagal nerve fibres (Booth et al., 2001, Rong et al., 2004, Booth et al., 2008). Therefore, these data suggested that the lipid-rich nutrient had a direct effect on jejunal vagal mechanosensitive afferents in mice.

These data showed that lipid caused CCK-1 sensitive and insensitive effects on jejunal afferent response. Infusion of the lipid-rich nutrient triggered the release of endogenous CCK, which then activated on nearby CCK-1 receptor containing low threshold vagal afferents. It was highly likely that the effects of lipid-rich nutrient on the WDR and HT units were mediated by factors other than CCK, such as osmolarity or other components within the lipid mixture.

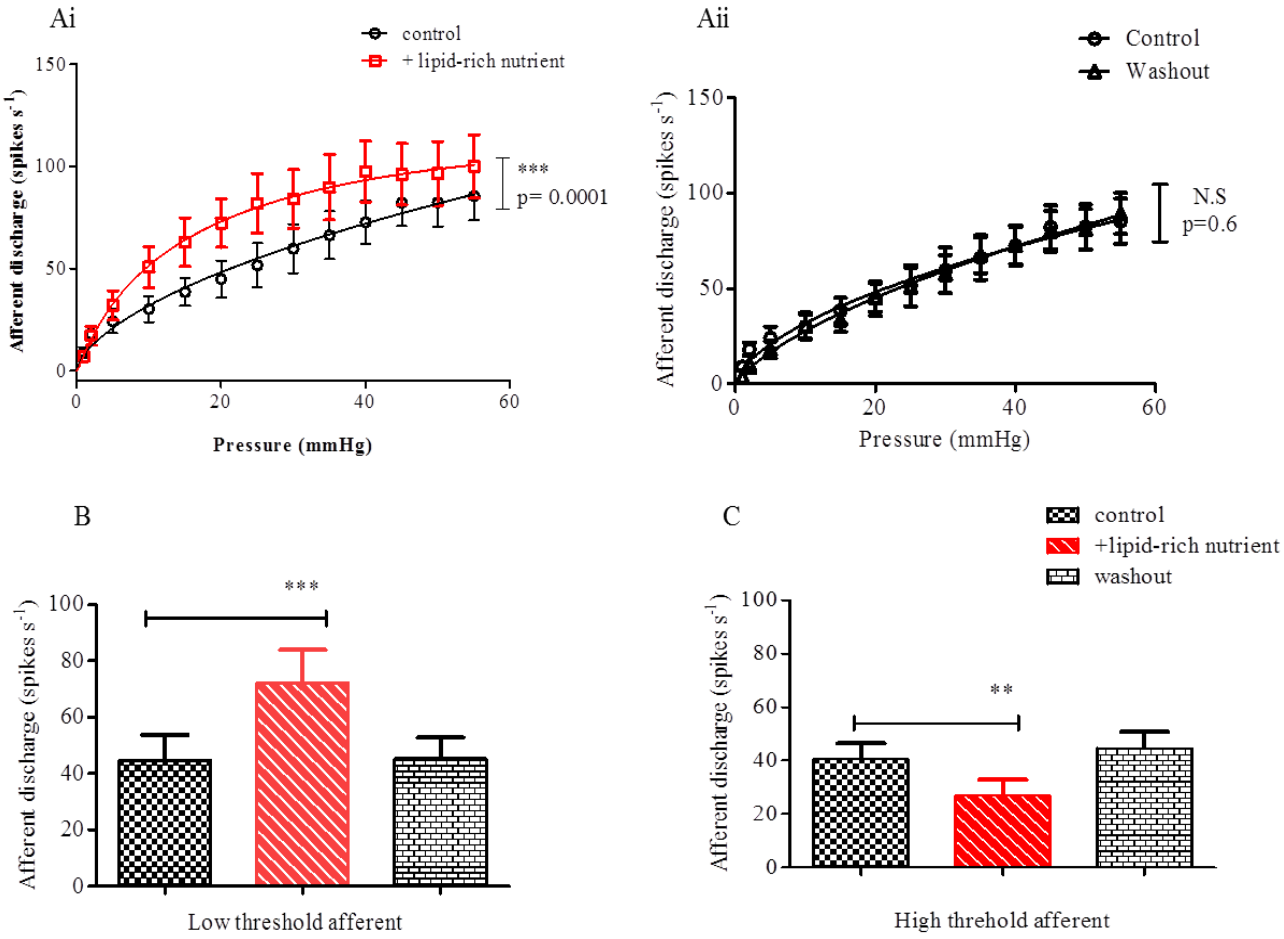


Figure 3.6.1 Mean afferent response to ramp distension up to 55mmHg is plotted. Ai) and Aii) the lipid-rich nutrient significantly augments afferent sensitivity to distension. p = 0.0001***. It is recovered rapidly upon washout, p = 0.6 (two way ANOVA, n=10). B) and C) show the data for the low threshold (LT; baseline to 20 mmHg) and high threshold (HT; 20 to 55 mmHg) components of the response to distension. The LT mechanosensitivity is significantly increased, p<0.001*** (repeated measured ANOVA). In contrast there is a significant attenuation in the HT component resulting in a flattening of the pressure-response profile at high pressure, p<0.001**.

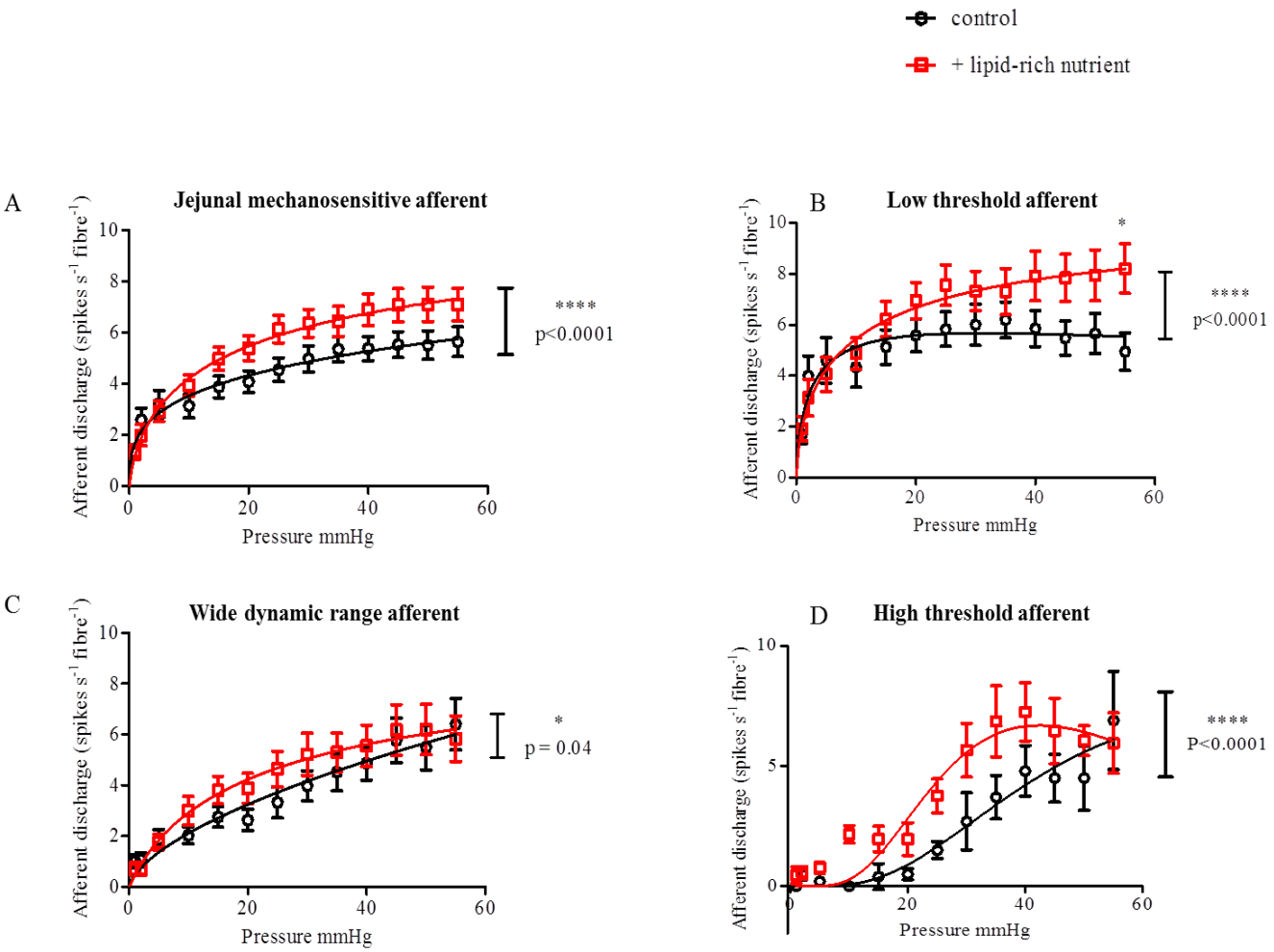


Figure 3.6.2 Single–unit responses to the lipid-rich nutrient on distension induced afferent mechanosensitivity. A, The whole nerve response has been normalised to the number of single units in the afferent bundles (see method). It produces a more robust effect indicating that the lipid rich nutrient significantly increases jejunal afferent mechanosensitivity, p<0.0001**** (two way ANOVA, n=88). B, low threshold afferent fibres (LT), shows LT mechanosensitivity is significantly augmented by the lipid-rich nutrient, p<0.0001**** (n=47), especially evident at a distending pressure of 55mmHg (Bonferroni post-tests, p<0.05*). C and D show the data for the wide dynamic range (WDR) and high threshold (HT) afferent fibres. Mechanosensitivities of both WDR and HT are significantly increased upon lipid-rich nutrient application, p=0.04* (n=36) and p<0.0001**** (n=5) respectively.

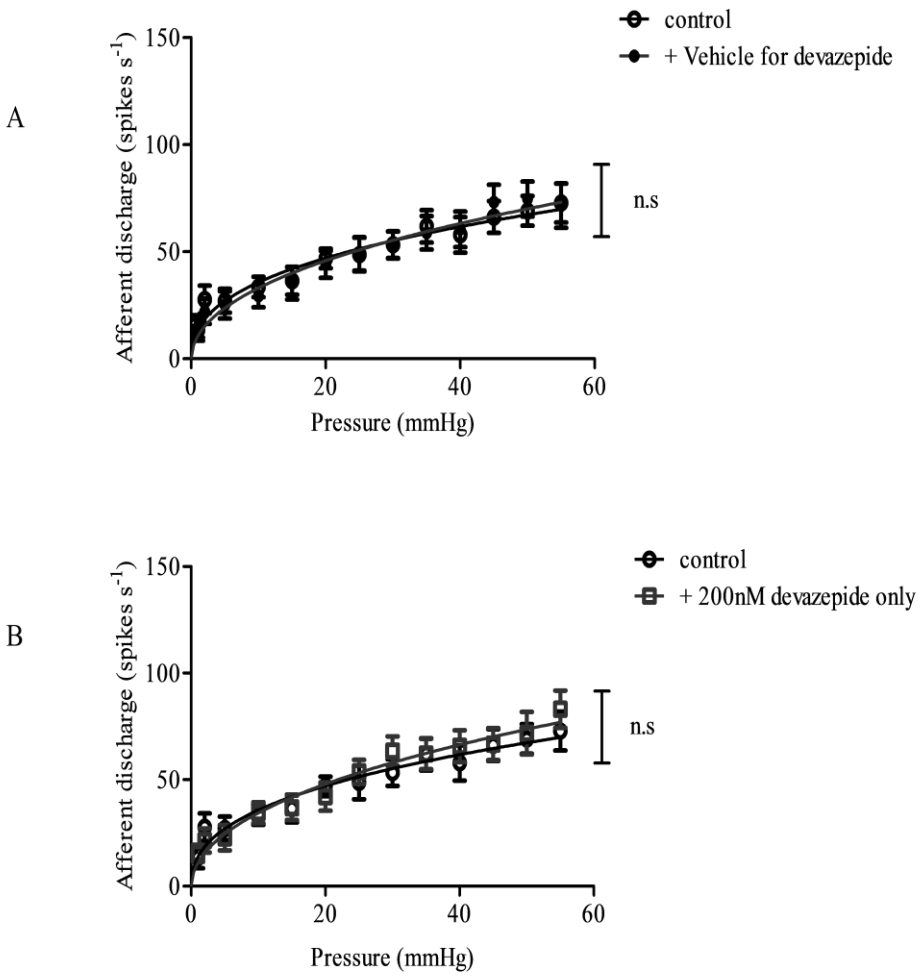


Figure 3.6.3 Effect of devazepide on distension induced afferent mechanosensitivity. A) Vehicle for devazepide (Tween 80: DMSO: saline) had no effect on distension induced mechanosensitivity, p = 0.93, (two way ANOVA, n=6). B shows the data that devazepide (200nM) itself had no effect on mechanosensitivity, p = 0.48 (n=7)

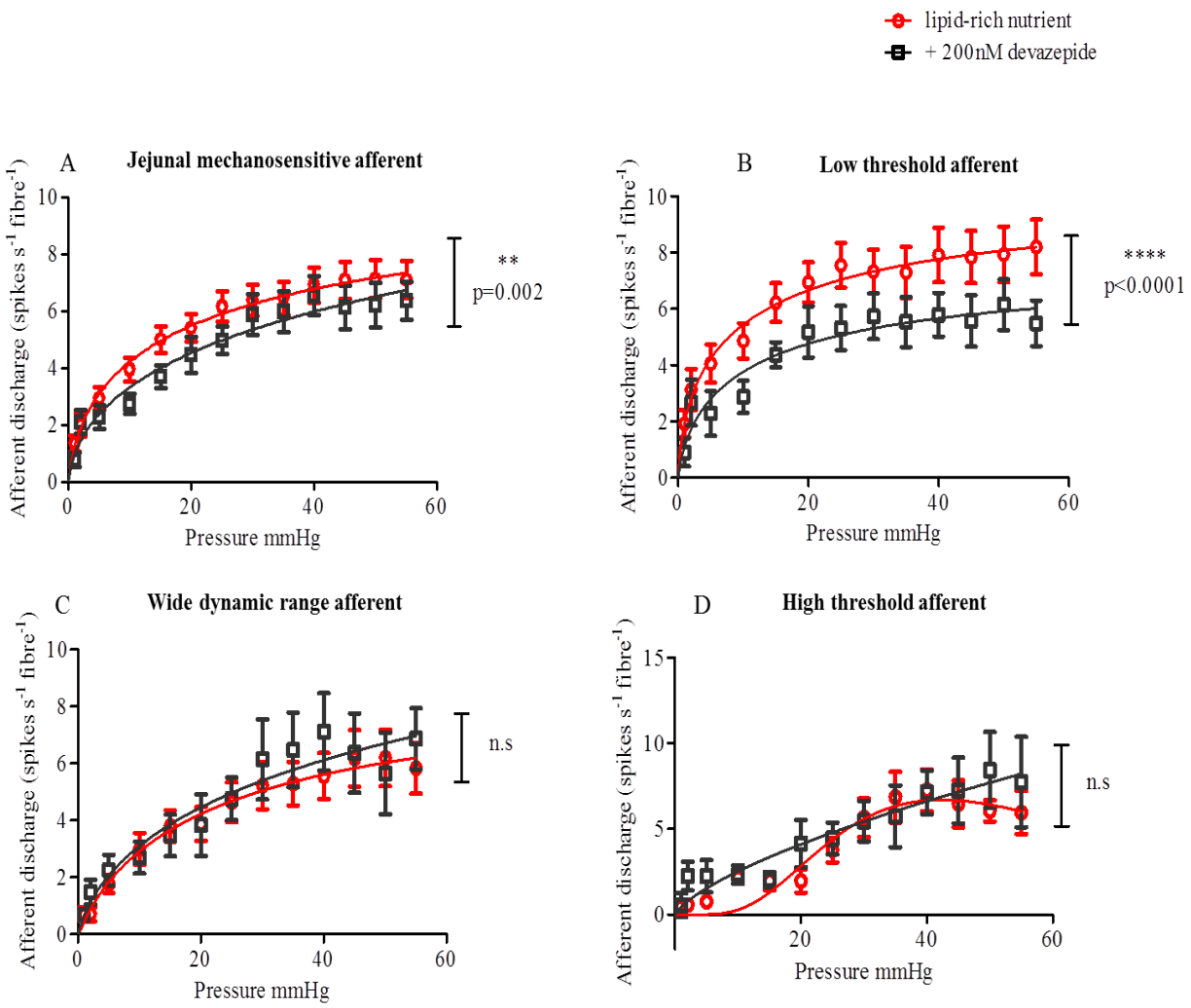


Figure 3.6.4 Single unit responses to the lipid-rich nutrient on distension induced mechanosensitivity are plotted in the presence and absence of devazepide (200nM). A, Lipid-rich nutrient induced augmentation in afferent mechanosensitivity is significantly reduced with devazepide application, p = 0.002** (two way ANOVA, n≥41). B, Low threshold afferent fibres (LT), shows that LT mechanosensitivity has been significantly attenuated by application of devazepide, p<0.0001**** (n ≥18). C and D show the data for wide dynamic range (WDR) and high threshold (HT) afferent fibres. No significant change on mechanosensitivities of WDR or HT fibres, p=0.3 (n ≥18) and p=0.1 (n=5) respectively.

3.7 Effect of low lipid nutrient on distension induced afferent discharge

The effect of low lipid nutrient on the mechanosensitivity to distension was investigated using the same method as the lipid-rich nutrient studies (figure 3.2.1A). Like with lipid-rich nutrient, afferent mechanosensitivity was increased in the presence of low-lipid nutrient. This was a transient effect which immediately recovered following washout. These data are summarized in figure 3.7.1.

To determine the extent to which low lipid emulsion had any effect on low threshold (baseline-20mmHg) and high threshold (20-55mmHg) components of afferent response to distension, the mean increase in firing frequency in those two phases was analysed separately. Low lipid nutrient significantly augmented the low threshold afferent firing (figure 3.7.1B). The increase in afferent discharge was 43.7±8.8 spike s^{-1} before application of low-lipid emulsion, but this was increased to 68.8±13.0 spike s^{-1} during low-lipid nutrient incubation, and the effect was reduced to 46.1±12.2 spike s^{-1} during washout. In figure 3.7.1C, even though it showed that there was a reduction in high threshold afferent firing, it was not statistically significant.

Single unit analysis had also been performed with low lipid nutrient studies. A total number of 58 single afferent nerve fibres were obtained from 7 preparations. It further showed that jejunal afferent mechanosensitivity was significantly potentiated by the low-lipid nutrient (figure 3.7.2A). However, unlike the lipid-rich nutrient, the augmentation was caused by low (LT) and high threshold (HT) afferent units only (figure 3.7.2B and D). The low lipid nutrient had no effect on the firing frequency of the wide dynamic range afferent fibres (figure 3.7.2C).

Devazepide (200nM) was used to investigate the contribution of CCK_A-receptor mechanism to the low-lipid nutrient mediated increase in afferent mechanosensitivity to distension. The whole data was normalized by single unit analysis. 49 single fibres were characterized from 5 preparations. As seen in figure 3.7.3A, the profile of the afferent response to distension was attenuated by devazepide compared to low-lipid alone. It was the firing frequency of LT unit

but not the HT units that had been significantly inhibited by devazepide (figure 3.7.3B and C). This was consistent with the finding that it was the low threshold component that was significantly augmented by lipid. This suggested that the augmented afferent firing caused by the low-lipid infusion depended, at least in part, on the release of CCK and subsequent activation of CCK_A receptors.

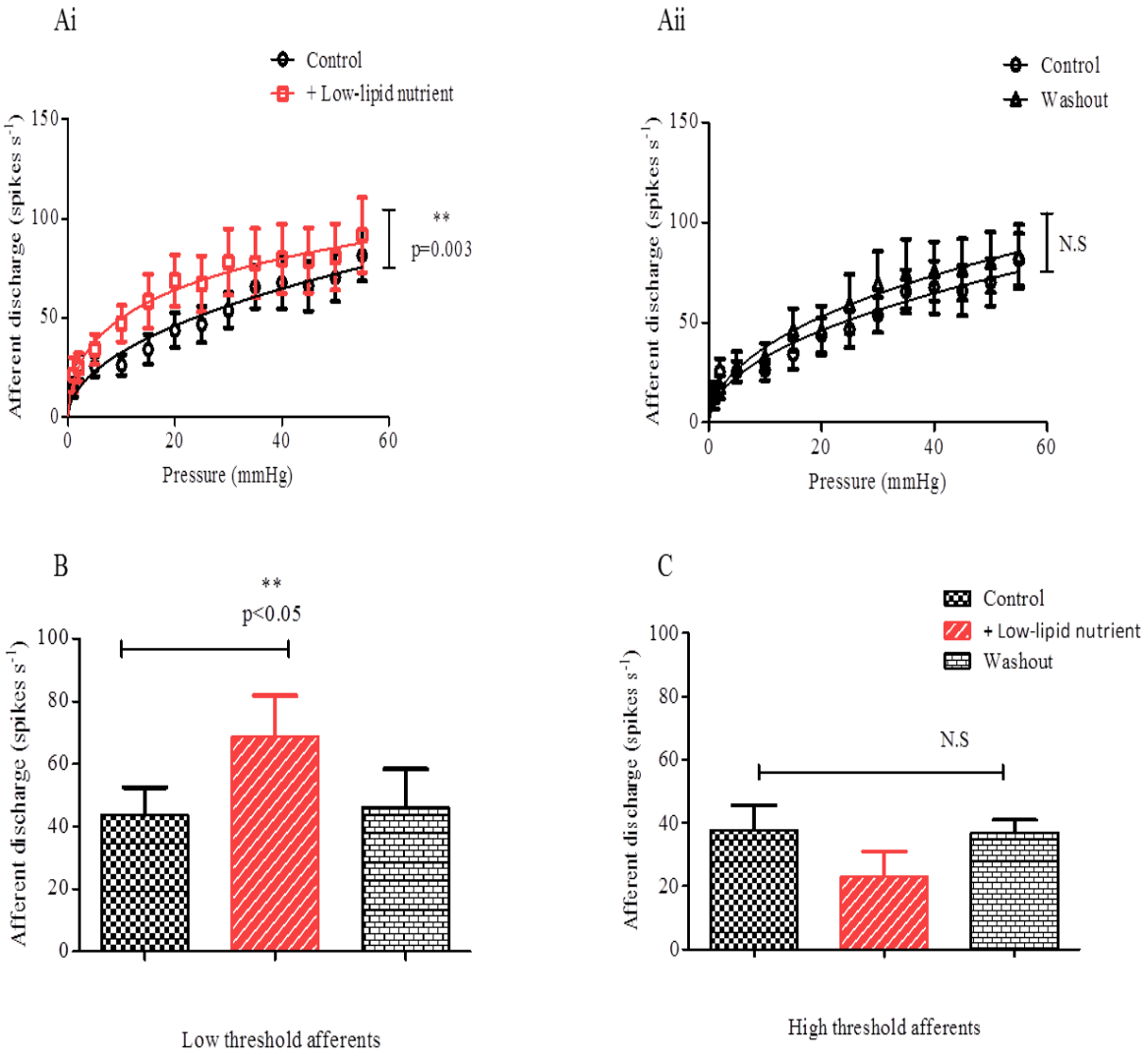


Figure 3.7.1 Mean afferent response to ramp distension up to 55mmHg is plotted. Ai) and Aii) Low-lipid nutrient significantly augments afferent sensitivity to distension which is recovered rapidly upon washout (two way ANOVA, n=7). B) and C) show the data for the low threshold (LT; baseline to 20 mmHg) and high threshold (HT; 20 to 55 mmHg) components of the response to distension. The LT mechanosensitivity is significantly increased (repeated measured ANOVA). There is no significant effect on HT mechanosensitivity.

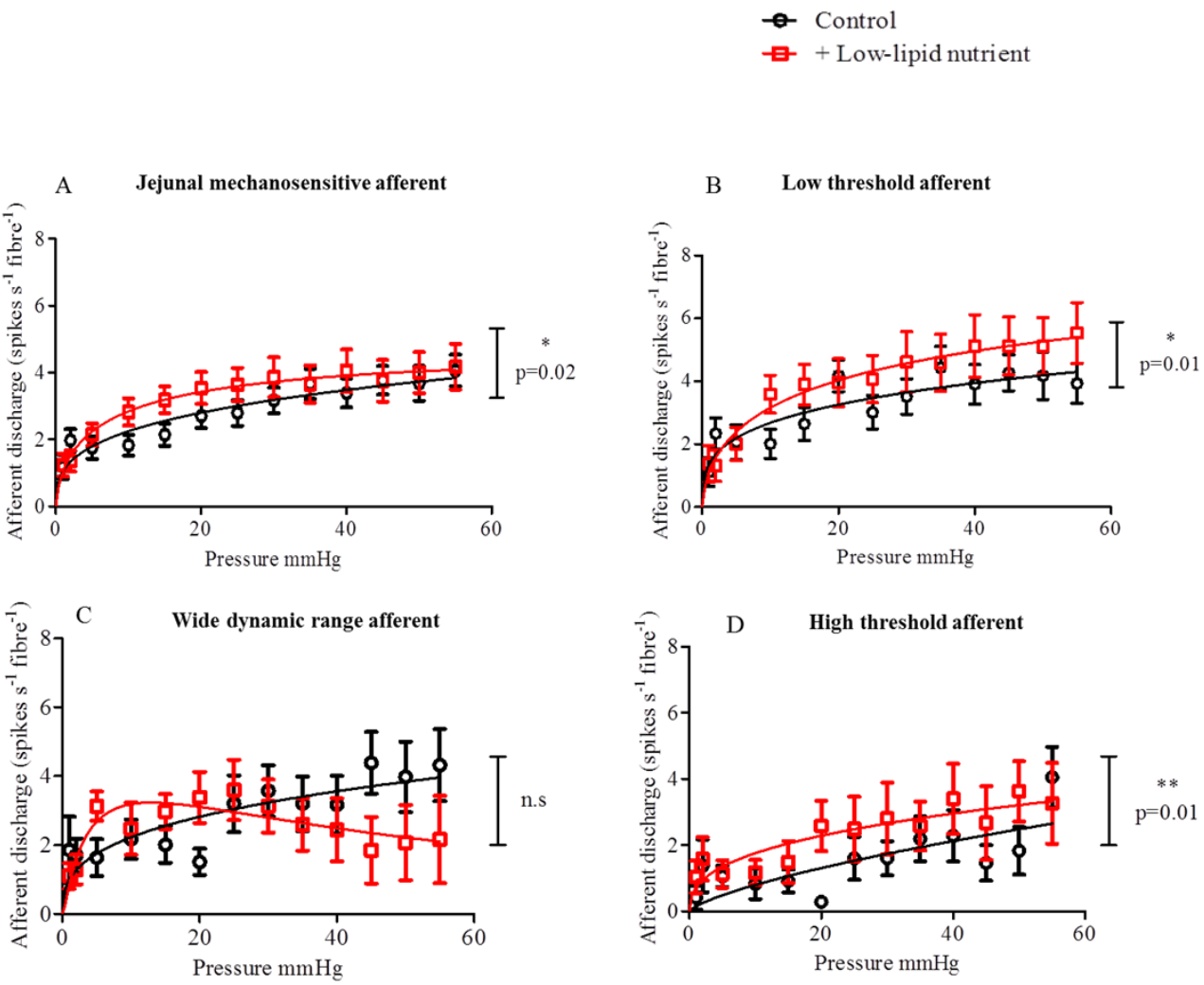


Figure 3.7.2 Single–unit responses to the low lipid nutrient on distension induced afferent mechanosensitivity. A, The whole nerve response has been normalised to the number of single units in the afferent bundles. Low lipid nutrient significantly increases jejunal afferent mechanosensitivity, p=0.02* (two way ANOVA, n=58). B, low threshold afferent fibres (LT), shows LT mechanosensitivity is significantly augmented by low lipid nutrient, p=0.01*(n=31). C, wild dynamic range afferent fibres (WDR), low lipid nutrient has no effect on WDR mechanosensitivity, p=0.3 (n=16). D, High threshold afferent fibres (HT), shows HT mechanosensitivity has also been significantly increased by low lipid nutrient, p=0.01** (n=11)

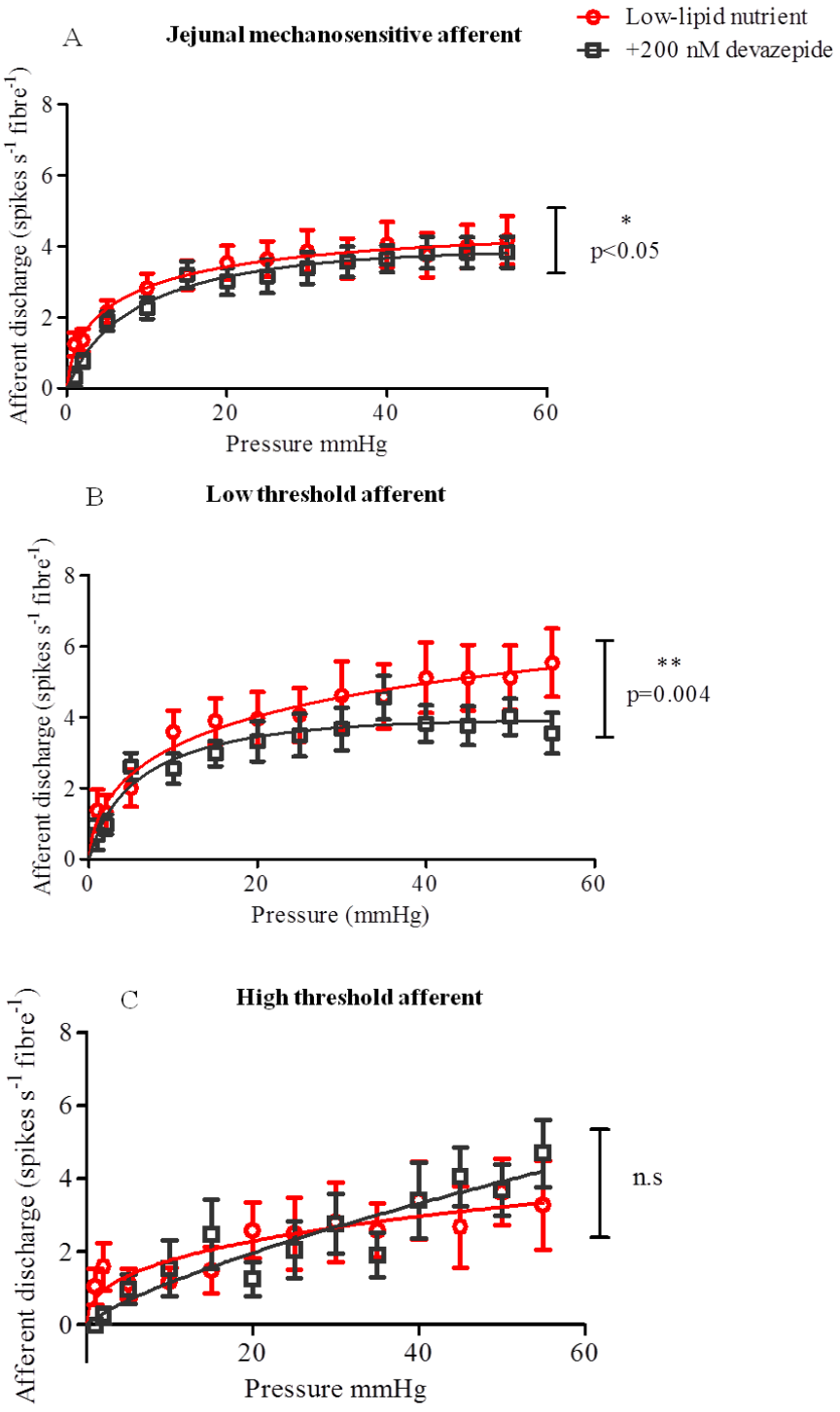


Figure 3.7.3 Single unit responses to low lipid nutrient on distension induced mechanosensitivity are plotted in the presence and absence of devazepide (200nM). A, low lipid nutrient induced increase in afferent mechanosensitivity is attenuated by application of devazepide, p = 0.045 (two way ANOVA, n≥49). B and C are data for low threshold (LT) and High threshold (HT) afferent fibres. Firing frequency of the LT unit is significantly reduced in the presence of devazepide, p = 0.004** (n≥26), but no change in the HT unit, p = 0.8 (n≥7).

3.8 Comparing effects of lipid-rich and low-lipid nutrients on afferent discharge to distension

Both lipid-rich and low lipid nutrients caused augmentations on distension induced afferent mechanosensitivity (figure 3.6.1Ai and 3.7.1Ai). In order to compare the profile of the response, the whole data were normalized by single unit analysis. The single unit analysis data from these 2 sets of experiments are plotted together in figure 3.8, which clearly from shows that these two types of nutrients give rise to similar pressure-response profiles (figure 3.8A) but the lipid-rich nutrient causes a much stronger effect on jejunal mechanosensitivity in comparison with the low-lipid nutrient. Since the interaction of the statistical analysis between those two sets of data was also significant, it was difficult to interpret the p value of significance. Instead, it was able to confirm that lipid-rich nutrient indeed produced stronger augmentations than low-lipid at several distending pressure points starting from 25mmHg up to 55mmHg.

Firing frequency of low threshold (LT), wild dynamic range (WDR) and high threshold (HT) afferent units were also compared between the two nutrient infusions. Lipid-rich and low lipid nutrients shared similar profiles of response on LT units (figure 3.8B). From previous sections, it demonstrated that the effects of lipids (two types) on LT units' mechanosensitivities were mediated via CCK-1receptor signalling pathway (figure 3.6.4B and 3.7.3B), suggesting that the lipid-rich nutrient triggers the release of a larger amount of endogenous CCK than low-lipid nutrient. In the other words, it was concentration dependent.

Interestingly, the response profiles of both WDR and HT units were distinctively different between the two nutrient infusions (figure 3.8C and D). It seemed that the lipid-rich nutrient give a larger augmentation than the low-lipid nutrient, especially at the high pressure level of response. However, those data sets were difficult to interpret as the interaction of the statistical analysis was also significant. Therefore, what could be sure was that the lipid-rich nutrient did produce a larger effect on WDR and HT units, but on certain distending pressure points only.

Taken together, these data supported our hypothesis that lipid-rich and low-lipid nutrient have differential effects on afferent discharge to mechanical stimulation.

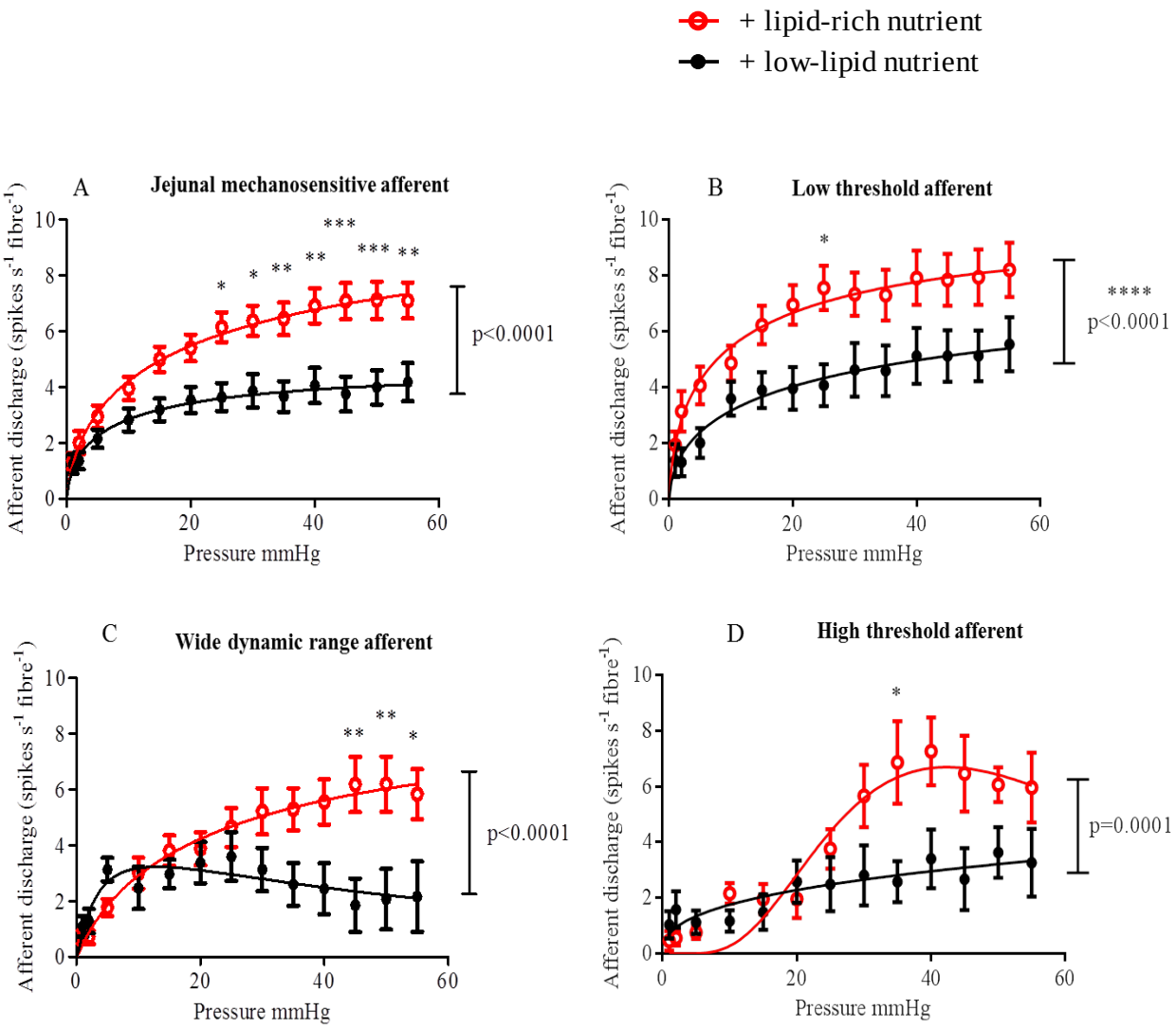


Figure 3.8. Comparison of effects of lipid-rich and low lipid nutrients on distension induced afferent mechanosensitivity**.** The whole data is normalised by single unit analysis. A, overall mean effect, p<0.0001, n ≥58 (two way ANOVA with Bonferroni post-tests, p<0.05*, p<0.01**, p<0.001***), evident at distending pressure from 25mmHg to 55mmHg. B, low threshold afferent units, p<0.0001****, n ≥31, especially at pressure 25mmHg. C, wide dynamic range unit, p<0.0001, n≥16, at pressure 40mmHg, 45mmHg and 55mmHg. D, high threshold unit, p=0.0001, n≥5, at pressure 35mmHg.

3.9 Effect of hyperosmotic mannitol on distension induced afferent discharge

Both lipid-rich and low-lipid nutrients are hyperosmotic emulsions of 697mOsm/L and 724mOsm/L respectively. It was important therefore to determine the potential contribution of osmolarity to the afferent responses to lipid. A series of osmotic control experiments were carried out using 700Mosm/L mannitol solution.

The afferent response profile was unchanged by the hyperosmotic mannitol. However, when the LT and HT components were examined separately it appeared that the HT component was attenuated (Figure 3.9.1C). This is apparent as a slight flattening in the pressure-afferent response profile (Figure 3.9.1Ai).

Single unit analysis has revealed that mechanosensitivity of WDR and HT, but not LT afferent units showed reduced response to hyperosmotic mannitol distension (61 single units were obtained from 5 out of 6 experiments), particularly at the high pressure level of distension (figure 3.9.2). This flattening of the response was also observed with lipid. However, unlike with lipid, there was no augmentation in the LT component of the distension response. This suggested that the augmented effect of lipid on the low threshold compartment was independent of hyper-osmolarity.

In a separate set of experiments, saline was used to control any non-nutrient effects on mechanosensitivity arising from luminal perfusion which had no effect on any of the measured parameters.

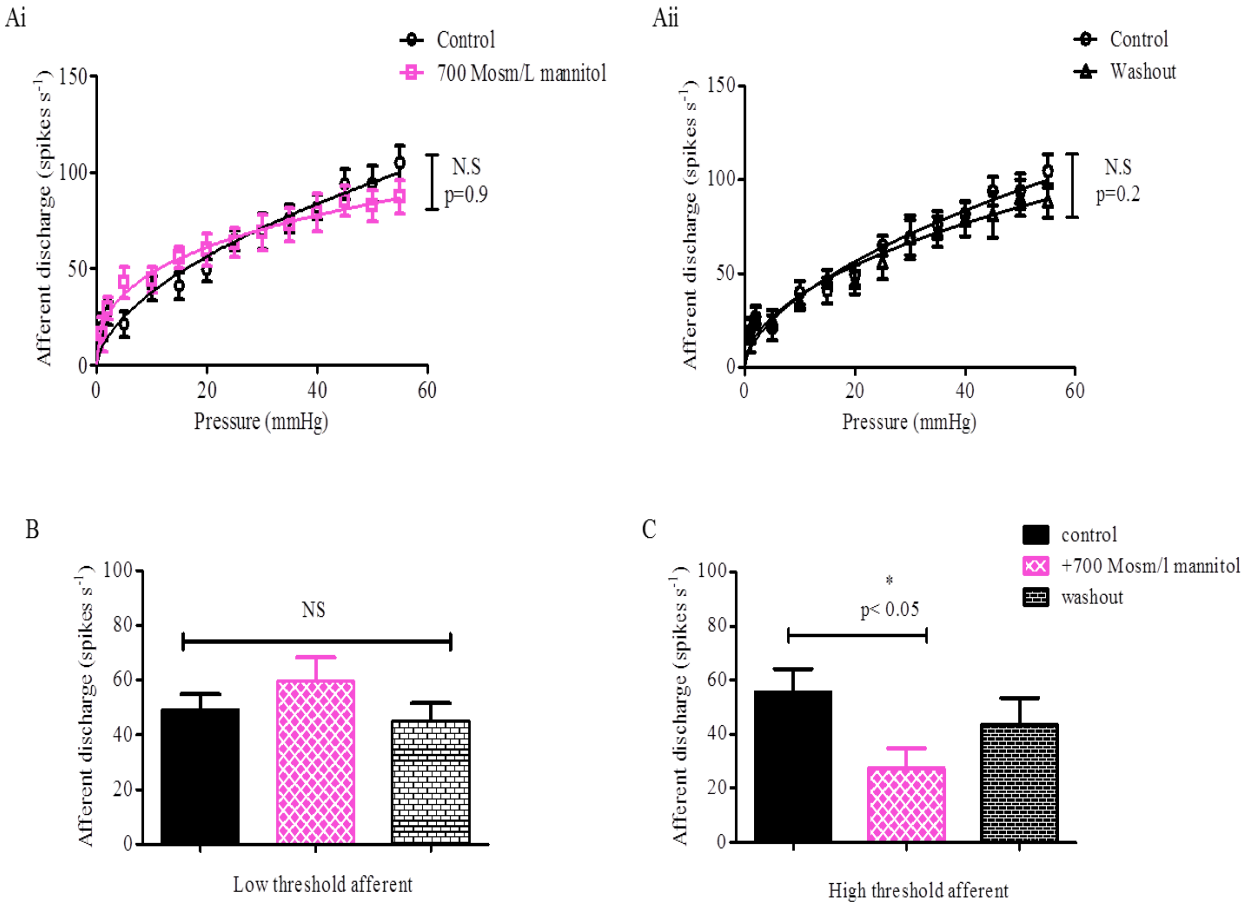


Figure 3.9.1 Mean afferent response to ramp distension up to 55mmHg is plotted. Ai) and Aii) 700mOsm/l mannitol has no effect on afferent sensitivity to distension (two way ANOVA, n=6). B) and C) shows the data for the low threshold (LT; baseline to 20 mmHg) and high threshold (HT; 20 to 55 mmHg) components of the response to distension. There is no effect on the LT mechanosensitivity (repeated measured ANOVA). However, there is a significant attenuation in the HT component resulting in a flattening of the pressure-response profile at high pressure.

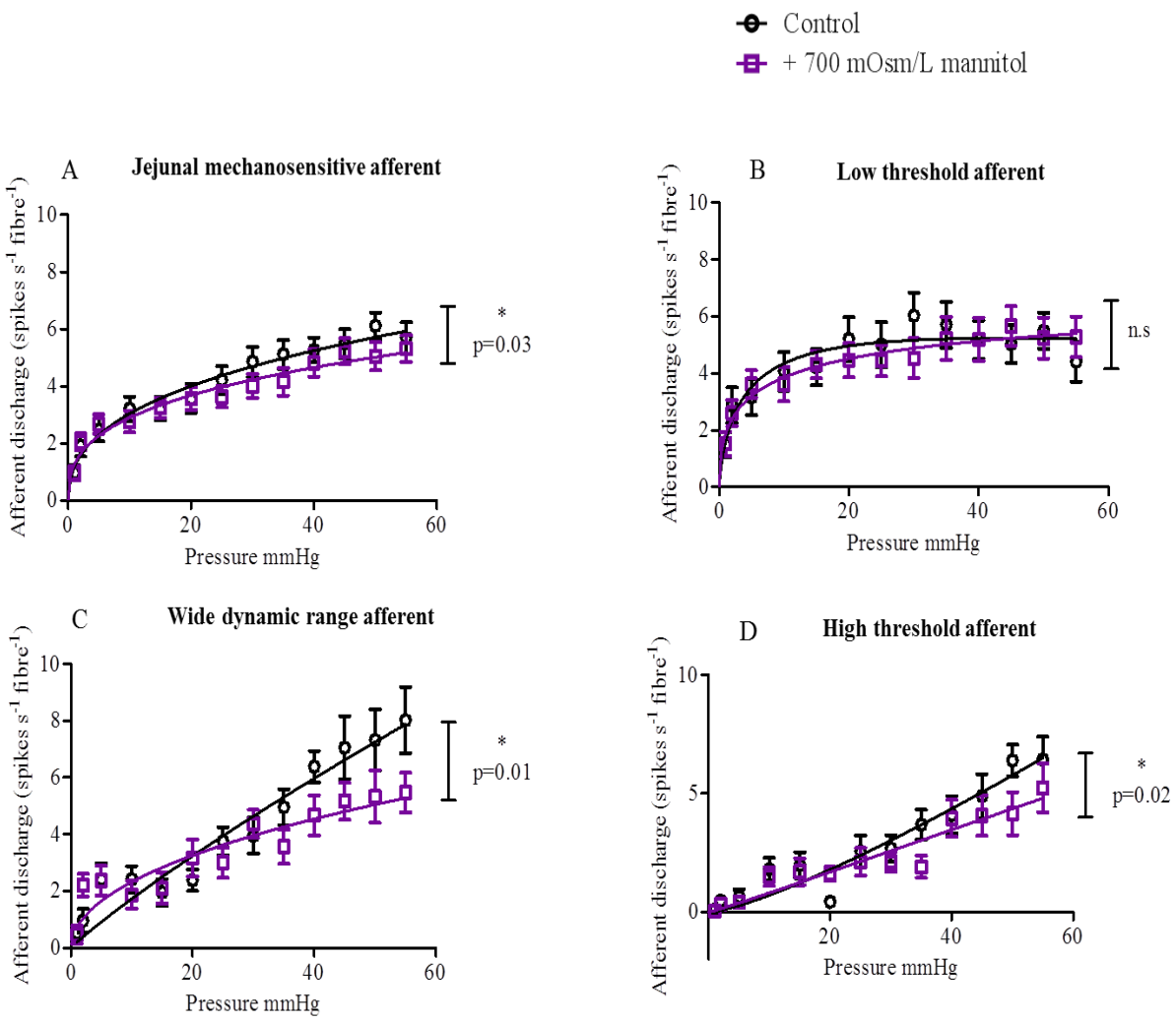


Figure 3.9.2 Single–unit responses to 700Mosm/L mannitol on distension induced afferent mechanosensitivity**.** A, Single unit analysis gives a more robust effect indicating that hypertonic mannitol inhibits jejunal afferent mechanosensitivity, p=0.03* (two way ANOVA, n=61). B, low threshold afferent fibres (LT), shows LT mechanosensitivity is unaffected by hypertonic mannitol, p=0.5 (n=34). C and D show the data for wide dynamic range (WDR) and high threshold (HT) afferent fibres. The mechanosensitivities of both WDR and HT are significantly attenuated upon hypertonic mannitol application, p=0.01*(n=15) and p=0.02*(n=12) respectively.

3.10 DISCUSSION

The major finding of the present study was that the lipid sensitivity was observed in mechanosensitive fibres. Subpopulations of these jejunal afferents respond differentially to nutrients containing different concentration of lipids via CCK-mediated signalling pathway. This discussion will focus first on assessment of the jejunal afferent neurophysiological response to exogenously administered CCK, followed by consideration of the contribution of endogenously released CCK in nutrient elicited mechanosensitivity. The last part of discussion will concentrate on the possible interaction between nutrient sensitive and osmosensitive receptors and their contribution to mechanosensitivity.

Exogenous CCK stimulated basal afferent discharge

It was well established that the action of CCK on neural elements was mainly on the vagus, which in turn caused a reflex pathway to modulate functions of the targeting tissues. A previous study in our lab showed that afferent sensitivity to CCK was completely abolished after chronic subdiaphragmatic vagotomy (Richards et al., 1996), indicating CCK action was mediated exclusively via a vagal pathway to the CNS. There are two types of vagal afferent fibres that have been classified according to their distinct nerve terminals. One was the mucosal afferent fibres which had their nerve terminals embedded in the lamina propria beneath the mucosal epithelium. The other one was the muscular afferent fibres terminating within the external muscle layer of the GI tract (Berthoud et al., 2004).

In the present study, jejunal afferents responded rapidly and transiently to exogenous CCK-8 before luminal distension. However, this action was not persisted or enhanced following jejunal distension. This indicated that those afferents were CCK chemosensitive fibres which were independent from mechanosensitivity. It suggested that this subpopulation of jejunal afferents might have their terminal receptive fields located in the mucosal layer rather than in the muscle layer. This observation was in agreement with previous studies from rat and ferret.

They showed that only vagal receptors located in the mucosa layer of GI wall were directly sensitive to CCK, irrespective of mechanical stimulation (Blackshaw and Grundy, 1990, Richards et al., 1996). Since there was no change on jejunal compliance following CCK administration, it ruled out possibility that the action of CCK on vagal mucosal afferents were secondary to changes in muscle tone.

However, other studies demonstrated that vagal mechanoreceptors were able to be directly activated by CCK. Back in the 1990s, a series of studies was carried out by Schwartz's team. They had identified a population of gastro-duodenal vagal mechanoreceptors which was sensitive to exogenously administered CCK (Schwartz et al., 1991, 1993, 1994, Schwartz and Moran, 1994, Schwartz et al., 1995). They showed that CCK and mechanical stimulation (gastric/duodenal loads) acted synergistically to enhance activity of those mechanosensitive vagal afferents. They showed that the discharge rate of those fibres which were responsive to gastric load nearly doubled their response with prior exposure of CCK. This was in contrast with the present study which mechanosensitive afferents were insensitive to exogenously applied CCK. However, this might be due to the regional variation along the GI tract.

Lipids enhanced afferent mechanosensitivity via endogenous CCK1 signalling pathway

It was well known that infusion of luminal nutrients, particularly lipids and proteins, would trigger release of endogenous CCK from enteroendocrine I cells in a paracrine fashion (Berthoud et al., 1995, Raybould, 2007, 2010). However, in the present study, both lipid-rich and low lipid nutrients had no effect on baseline nerve discharge. This negative response could be due to the following reasons. One possibility is that lipid-induced response on basal activity is counter-balanced by non-specific effect of lipid such as hyperosmolarity which is supported by excitatory effect of mannitol on the basal afferent activity. Osmoreceptors expressed in the epithelial cells or the mucosal sensory nerve terminals has been shown to be activated by hyperosmotic solution, suggesting luminal infusion with lipid in the absence of distension may

primarily trigger the release of inhibitory mediators or gastrointestinal peptides such as orexin, ghrelin and somatostatin rather than just CCK. These peptides can inhibit CCK action. Another possibility could be derived from the *in vitro* experimental setup used in the current study. The lipids used in these studies were not purified fatty acids, but a mixture of liquid lipid nutrients. Without bile and lipase, very limited lipids could be enzymatically breakdown to free fatty acids *in vitro* environment. In order for dietary lipids to release CCK, complex lipid component must be hydrolysed to fatty acids at a certain acyl chain length (McLaughlin et al., 1998). Taken together, intraluminal applied lipids may not be appropriately metabolised into biological active substrates in a short period of time prior to distension, thus extremely lower or no nutrition absorption could take place.

However, both lipid-rich and low–lipid nutrients significantly enhanced luminal distension-induced mechanosensitivity of three functional different subpopulations LT, WDR and HT units. A previous study showed that gastric vagal mechanosensitive afferents were able to be activated by duodenal infusion of proteins via endogenous CCK through CCK-1 receptor signalling pathways (Schwartz and Moran, 1996). They suggested this was an integrative effect which endogenously released CCK potentiated and amplified gastric vagal mechanosensitive afferent response to gastric-distending loads (Schwartz and Moran, 1998). This was also applicable in the present study. Of the three functional types of afferents, only the response of LT mechanosensitive units was markedly attenuated by devazepide. As LT afferent units were mainly composed of vagal nerves (Booth et al., 2001), these findings suggested a role of lipid-elicited endogenous CCK in the activation of jejunal vagal mechanosensitive afferents.

Mechanosensitivity of WDR and HT units were also augmented by lipid nutrients, especially with lipid-rich nutrient. The current data showed that they were CCK insensitive. A previous study from our lab showed that vagal afferents responded to fatty acids via different mechanism based on their chain size (Lal et al., 2001). Lipid-derived fatty acids could act

directly on I and L-cells at the apical side of I or L-enteroendocrine cells to release CCK or other peptides from the basal side via acting G-protein coupled receptors GPR40, 43 and 120 (Liou et al., 2011b, Sykaras et al., 2012). It might be that (1) other than CCK, gastro-intestinal peptides such as 5-HT, GLP-1 and PYY were released by lipid distension which then act on 5HT or GLP-1R mediated signalling pathways (Dockray, 2009); (2) the majority of HT afferents were spinal nerves which were mainly involved in pain and inflammation signalling. Since lipid nutrients did not induce any changes in jejunal compliance, it was unlikely that those augmentations were due to changes in muscle tone.

This part of my project was in collaboration with Prof. Buurman's lab. They investigated lipid induced anti-inflammatory pathway in a mice model of sepsis using LPS to induce inflammation (Lubbers et al., 2010b). Their work was focused on the efferent pathway. They measured the level of TNF-α. They showed that in fasted animals, there was an increase in TNF-α which had been attenuated when nutrients were delivered into gastrointestinal tract. The effect was nutrient and dosage specific. This was in agreement with the electrophysiological recordings showing that both lipid-rich and low lipid nutrients had effects on distension induced mechanosensitivity, of which lipid-rich nutrient exerted a significant bigger effect.

Lipid-induced mechanosensitivity is independent from osmotic effect

In the present study, both lipid-rich and low lipid nutrients were hyperosmotic solutions. Previous studies showed that osmotic signals could be detected by afferent fibres in the hepatic vagus with increased firing frequency of spike discharge in response to higher osmotic pressure (Adachi et al., 1976). Osmosensitive vagal receptors were also identified in the small intestine in cat (Mei and Garnier, 1986) which could trigger an inhibitory effect on enterogastric reflex that constituted an important part in the regulation of gastric emptying (Garnier et al., 1986). Those suggested that osmosensitive afferents could be activated by

hyperosmolarity and also had a possible role in regulation of gastrointestinal function. Therefore, the contribution of osmosensitive afferents in the present study must be considered.

Single unit analysis revealed that hyperosmotic mannitol had no effects on LT afferent (mainly composed of vagus nerve) firings, but had inhibitory effects on WDR and HT (mainly composed of spinal nerves) afferents. Previous studies showed that osmosensory information collected from the splanchnic mesentery is partially via spinal pathway (Vallet and Baertschi, 1982, King and Baertschi, 1991, Bourque et al., 2007). Therefore, this might suggest some interactions between hyper-osmosensitive and mechanosensitive afferents in spinal pathway. In this study, mannitol distension may cause the release of inhibitory mediators (somatostatin, ƙ-opioids or nitric oxide) from epithelial cells or enteric neurons, leading to reduced mechanosensitivity.

However, the response of the three subpopulation of afferents to lipid is distinctively different from mannitol (Table 1), strongly suggesting that lipid-evoked mechanosensitivity is independent from osmotic effect.

	BF	LT	WDR	HT
Lipid nutrients	-	↑↑	↑	↑
Mannitol	↑	-	↓	↓

Table 1: afferent response to hyper-mannitol and dietary lipids

Conclusion

In this chapter, I showed that lipid-containing nutrients-evoked low threshold jejunal afferents is primarily mediated by CCK-1 vagal pathways whereas WDR and HT units mediated by CCK-1 insensitive pathways. The results are in agreement with my hypothesis that lipid-rich and low lipid nutrient exerted differential effects on jejunal mechanosensitivity.

Chapter 4

Effect of lipid-rich nutrient on jejunal sensitivity in aged murine bowel

4.1 Introduction

Ageing has a profound effect on gastrointestinal function. "Anorexia of ageing" is defined as the physio-pathological decline in appetite and food intake with age, which leads to severe protein-energy malnutrition in the elderly (Di Francesco et al., 2007). CCK is one of the gut hormones that may account for changes in appetite with ageing, since it has a great impact on satiety and reduce hunger (Moss et al., 2012). Immunohistochemical analysis demonstrated that there were an increased number of CCK-containing endocrine cells from aged human duodenum (Sandström and El-Salhy, 1999). Other studies also showed a significant elevation in plasma CCK concentration in elderly subjects (MacIntosh et al., 1999, MacIntosh et al., 2001, Di Francesco et al., 2005). All together they would result a greater extent in suppression of food intake in aged than adult population.

Ageing is also associated with multiple dysfunctions in the immune system (Saltzman and Peterson, 1987). It will then put the elderly in a much more vulnerable situation to infection. Back in the 1990s, it was already shown that the fatality rate associated with hospitalizations for infectious disease doubled in elderly (Simonsen L, 1998). Previous evidence demonstrated that the level of pro-inflammatory cytokines, such as TNF-α, was significantly higher in the elderly patients (Marik et al., 2001) during septic shock. This will have a dramatic impact on the welfare and lifespan of the elderly population. Enteral nutrients, lipids in particular, have a potent effect on satiety and have been shown to attenuate inflammation triggered by factors such as surgery, LPS and haemorrhagic shock via a cholecystokinin-mediated vago-vagal reflex (Lubbers et al., 2010b). They showed that in the presence of lipid nutrients, the level of TNF-α was significantly reduced. They investigated this pathway using a mouse model of sepsis with a health and adult population. However, it is not sure whether this nutrient induced anti-inflammatory effect will be preserved in the aged population.

In the previous chapter, I examined the effects of lipid containing nutrients on intestinal afferent sensitivity in adult mice. I showed that lipids directly activated jejunal

mechanosensitivity via endogenously released CCK through a CCK-1 receptor mediated vagal pathway. However, I do not know whether this sensitivity to lipid nutrients will be altered with age. Therefore, the aim of this chapter is to investigate the possible changes in jejunal sensitivity to the lipid-rich nutrient. I hypothesized that the sensitivity to the lipid-rich nutrient would be reversed in aged mice (18 and 24 months) compared to adult animals (3 months).

4.2 Methods and analysis

Effect of the lipid-rich nutrient on baseline and distension induced afferent discharge in aged tissue

The mechanical effect (subjected to ramp distension up to 55mmHg) of the lipid-rich nutrient on jejunal afferent discharge was expressed as the change in afferent discharge above the baseline, calculated as the mean firing frequency in 2 second periods at each level of distending pressure subtracting the baseline firing which is 1 min prior to distension. It was then plotted against pressure. It was the same protocol as in Chapter 3 which was described in detail in the schematic diagram in figure 3.2.1 (A). It was compared with before and after nutrient treatment. Ramp distension of jejunal segments evoked a biphasic increase in afferent discharge, which represented the activation of low threshold (LT) and high threshold (HT) mechanosensitive afferent fibres. Details of single unit analysis and identification of different populations of mesenteric afferent fibres were described in Chapter 2.

Jejunal compliance and intraluminal volume

Since infusion rate was always kept stable through ramp distension (200µl/min), intraluminal volume was calculated using the rate of distension and time taken to reach a given intraluminal pressure (55mmHg). The effect of treatment on jejunal compliance was evaluated as the final volume reaching a distending pressure of 55mmHg. It was compared with control which is before any treatment took place.

Statistical significance

Statistical analysis was carried out using a Student's t-test (paired/unpaired), repeated measured one way ANOVA with Dunnett's Multiple Comparison Test and two-way ANOVA with Bonferroni post-tests. Data was presented as mean ± SEM, where $p < 0.05$ was considered as significant.

4.3 Effect of the lipid-rich nutrient on baseline and distension induced afferent discharge in 18 month-old mice

In these experiments, ramp distension was performed every 15 minutes until 3-4 reproducible responses had been obtained. The lipid-rich emulsion (1ml) was then applied and the afferent response to distension compared to that before (control) and after (washout) the lipid-rich nutrient. These data are summarised in figure 4.3.1. In the pressure-response profile (figure 4.3.1Ai), it shows that there is an initial increase in afferent discharge, especially in the low threshold compartment. The curve becomes flattened when it reaches the high threshold compartment. The overall effect of the lipid-rich nutrient on the afferent response to distension is not statistically significant. Therefore, it shows that the lipid-rich nutrient has no effect on jejunal mechanosensitivity in aged murine bowel. However, mechanosensitivity was attenuated during the washout period.

The effect of the lipid-rich nutrient on low threshold (baseline-20mmHg) and high threshold (20-55mmHg) levels of distension were analysed in aged mice. The augmentation in afferent firings at the low threshold level of distension was absent in 18M mice. Even though it appeared that there was a small increase in the firing rate, it was not statistically significant (figure 4.3.1B). The high threshold afferent firing rate was significantly inhibited and it was not fully recovered during washout (figure 4.3.1C). The firing rate was 37.4±5.5 spikes s^{-1}. It was reduced to 11.2 ±3.7 spikes s^{-1} with the lipid-rich nutrient application and partially recovered to 23.9±6.0 spikes s^{-1} during washout. The lipid-rich nutrient also had no effect on jejunal compliance in 18M mice. The intraluminal volume up to 55mmHg was 320.6±15.1µl before, and was 322.8±17.7µl upon lipid-rich nutrient infusion.

Single unit analysis (as described in chapter 2) was conducted. A total of 69 single fibres were characterised by their activation threshold. The overall mechanosensitivity upon lipid-rich nutrient infusion was not altered in 18M mice (figure 4.3.2A). Interestingly, the lipid-rich nutrient caused attenuation on firing discharge of the low threshold afferent units. It showed

that this reduction mainly appeared at the high threshold level of distension (figure 4.3.2B). The firing discharge of the wide dynamic range and high threshold afferent units was significantly augmented by the lipid-rich nutrient. These augmentation were mainly observed at the low range of distension pressure, but became plateau at the high range of pressure (figure 4.3.2C and D).

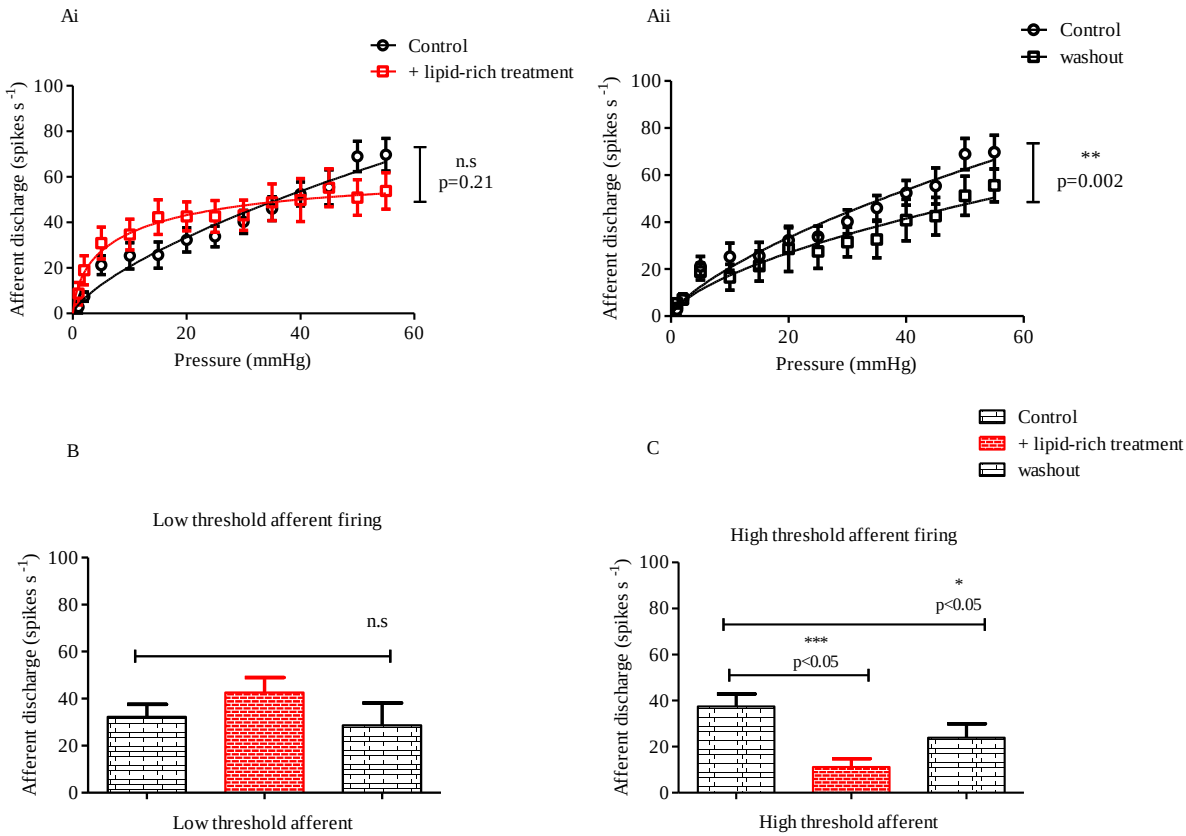


Figure 4.3.1 Effect of the lipid-rich nutrient on jejunal afferent mechanosensitivity in 18 months old mice. Mean afferent response to ramp distension up to 55mmHg is plotted. Ai) and Aii) The lipid-rich nutrient has no effect on afferent sensitivity to distension. p = 0.21. However, jejunal mechanosensitivity was significantly reduced during the period of washout, p = 0.002** (two way ANOVA, n= 6). B) and C) shows the data for the low threshold (LT; baseline to 20 mmHg) and high threshold (HT; 20 to 55 mmHg) component of the response to distension. The LT mechanosensitivity is not altered, p = 0.2 (repeated measured ANOVA). In contrast there is a significant attenuation in the HT component resulting in a flattening of the pressure-response profile at high pressure, p<0.001***, with Dunnett's Multiple comparison Test, p<0.05**. The response is not recovered during washout, p<0.05*.

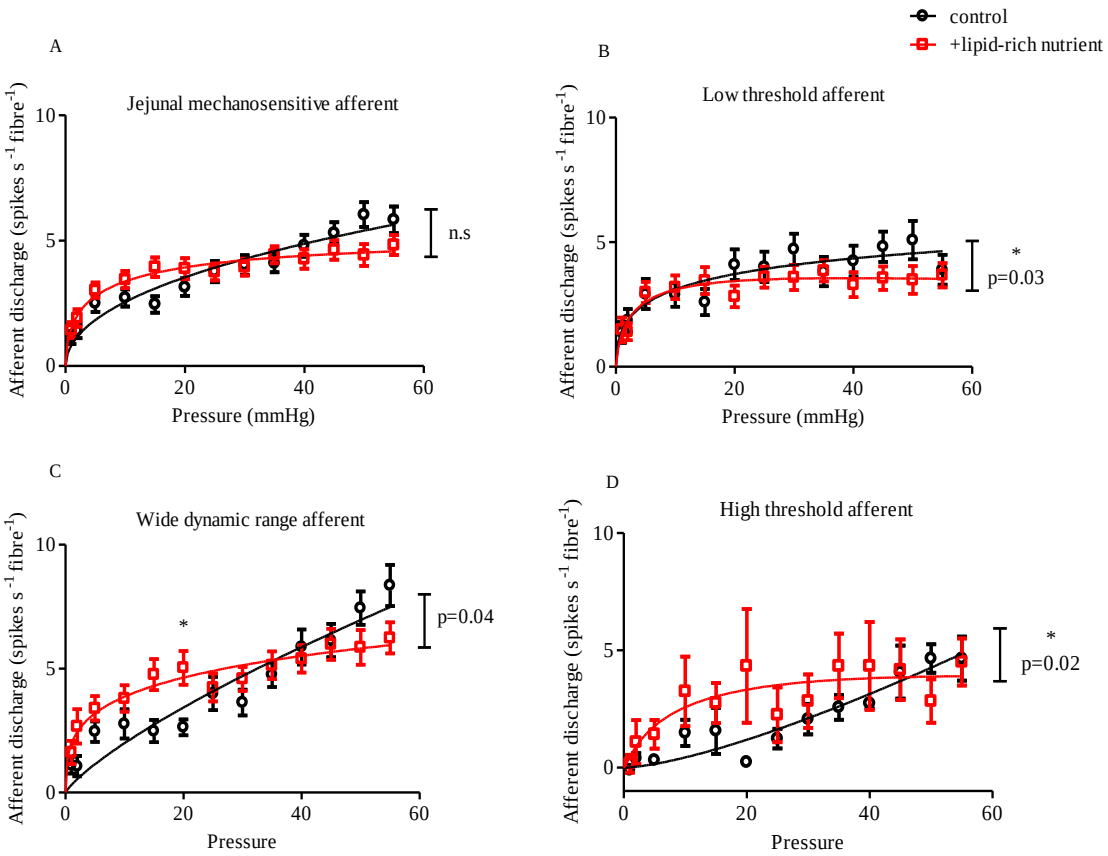


Figure 4.3.2 Single–unit responses to the lipid-rich nutrient on distension induced afferent mechanosensitivity in 18 month-old mice. A**,** It shows that the lipid-rich nutrient had no effect on jejunal afferent mechanosensitivity, p=0.7 (two way ANOVA, n=69). B, low threshold afferent fibres (LT), shows LT mechanosensitivity is slightly attenuated by the lipid-rich nutrient, p=0.03* (two way ANOVA, n=34). C and D show the data for wide dynamic range (WDR) and high threshold (HT) afferent fibres. The mechanosensitivities of WDR and HT units are significantly increased upon lipid-rich nutrient application, p=0.04 (two way ANOVA, n =29) and p=0.02* (two way ANOVA, n=6) respectively.

4.4 Effect of the lipid-rich nutrient on baseline and distension induced afferent discharge in 24 month-old mice

The lipid-rich nutrient produced no effect on distension induced mechanosensitivity. The data is summarized in figure 4.4.1. In the pressure-discharge response profile, even though it appeared that there was an augmentation in afferent firing at the low threshold (baseline-20mmHg) level of distension, the overall effect of the lipid-rich nutrient on afferent response to distension was not statistically significant (figure 4.4.1Ai). Unlike in 18 month-old mice, afferent discharge to distension during the period of washout was not altered (figure 4.4.1Aii). The effect of the lipid-rich nutrient on afferent discharge at the low threshold and high threshold levels of distension was also analysed in 24M mice. It produced a similar effect as in the 18 month-old animal. The lipid-rich nutrient caused a small increase in low threshold afferent firing, but it was not statistically significant (figure 4.4.1B). It also had an inhibitory effect on firing discharge at the high threshold level of distension (figure 4.4.1C). It was reduced from 33.2± 6.4 spikes s^{-1} to 12.3±4.1 spikes s^{-1} upon infusion of the lipid-rich nutrient. The attenuation in high threshold afferent firing is recovered during washout. The lipid-rich nutrient also had no effect on jejunal compliance in 24M mice. The intraluminal volume was 286.3±26.5µl beforehand. Even though it was reduced to 251.8±25.2µl upon lipid-rich nutrient infusion, it did not reach the level of significance.

Single unit analysis (as described in chapter 2) was conducted. A total of 76 single fibres were characterized by their activation threshold. The overall mechanosensitivity upon lipid-rich nutrient infusion was significantly increased in 24M mice (figure 4.4.2A). Since the interaction of the statistical analysis between those two sets of data was also significant, it was difficult to interpret the p value of significance. Instead, it could be confirmed that the lipid-rich nutrient indeed produced a significant augmentation in jejunal mechanosensitivity at distending pressure of 10mmHg and 15mmHg. The firing discharge of the wide dynamic range (WDR) units was significantly elevated by the lipid-rich nutrient. However, this data set was also

difficult to interpret as the interaction of statistical analysis was significant. Therefore, what could be deduced was that the lipid-rich nutrient did augment WDR afferent mechanosensitivity, but at distending pressure of 10mmHg and 15mmHg only. The lipid-rich nutrient also caused an increase on firing discharge of low threshold afferent (LT) units. The lipid-rich nutrient induced augmentation in LT and WDR units were mainly observed at the low threshold level of distension, but became plateau at the high threshold level resulting a flattening in the pressure-response profile (figure 4.4.2B and C). The mechanosensitivity of high threshold afferent unit was not changed by the lipid-rich nutrient (figure 4.4.2D).

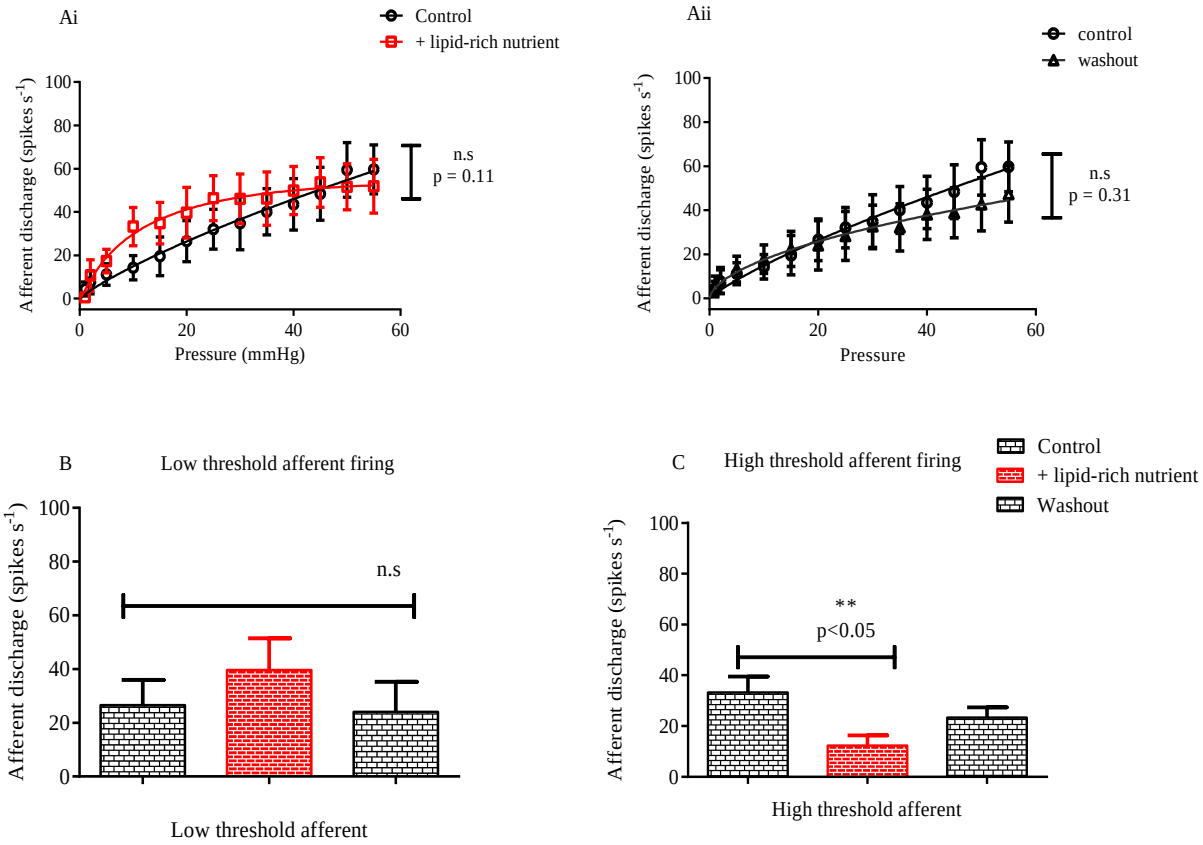


Figure 4.4.1 Effect of the lipid-rich nutrient on jejunal afferent mechanosensitivity in 24 month-old mice. Mean afferent response to ramp distension up to 55mmHg is plotted. Ai) and Aii) the lipid-rich nutrient has no effect on afferent sensitivity to distension. p = 0.11 (two way ANOVA, n= 6). B) and C) show the data for the low threshold (LT; baseline to 20 mmHg) and high threshold (HT; 20 to 55 mmHg) components of the response to distension. The LT mechanosensitivity is not altered, p = 0.5 (repeated measured ANOVA). In contrast there is a significant attenuation in the HT component resulting in a flattening of the pressure-response profile at high pressure, p=0.002**, with Dunnett's Multiple comparison Test, p<0.05**.

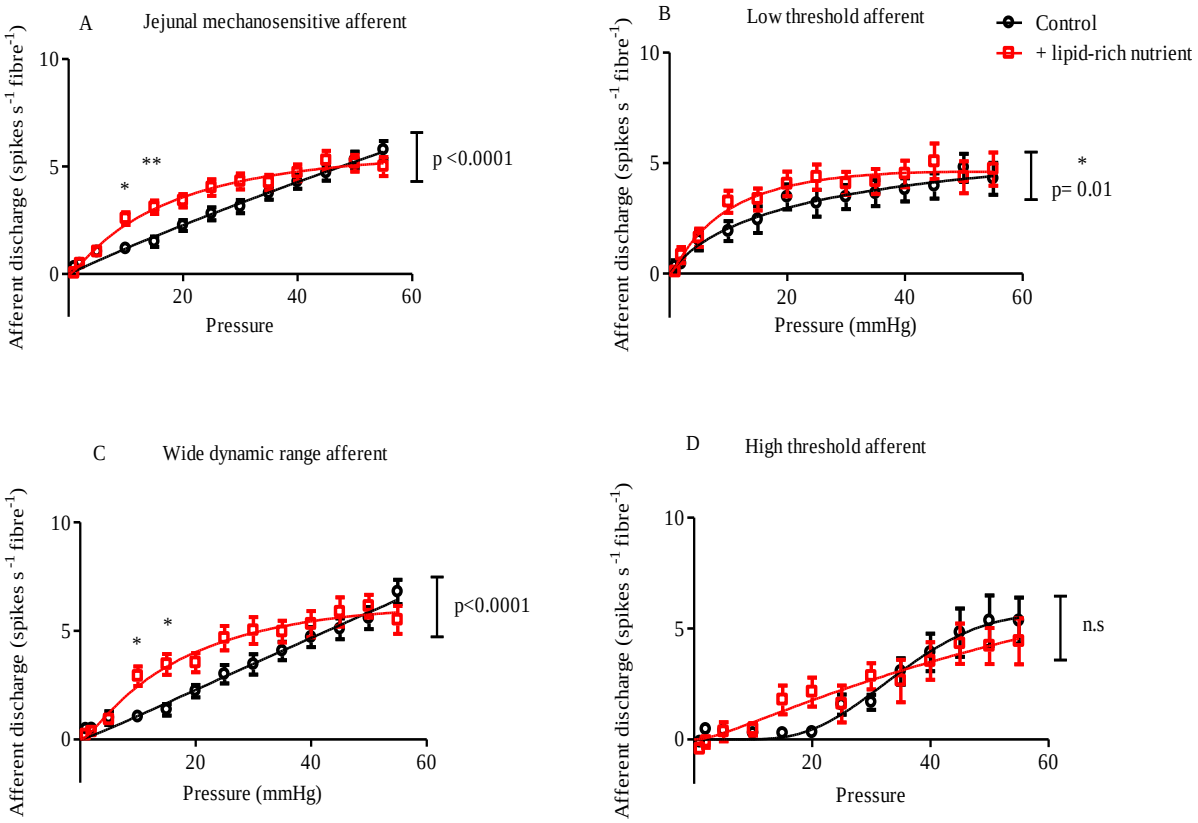


Figure 4.4.2 Single–unit responses to the lipid-rich nutrient on distension induced afferent mechanosensitivity in 24 months old mice. A**,** It shows that the lipid-rich nutrient significantly augments jejunal afferent mechanosensitivity, p<0.0001 (two way ANOVA with Bonferroni post-tests, p<0.05*, p<0.01**, n=7), specifically at distension pressure of 10mmHg and 15mmHg. B, low threshold afferent fibres (LT), shows LT mechanosensitivity is slightly increased by the lipid-rich nutrient, p=0.01* (two way ANOVA, n=23). C and D show the data for wide dynamic range (WDR) and high threshold (HT) afferent fibres. The mechanosensitivity of WDR units is significantly increased upon lipid-rich nutrient application, p<0.0001 (two way ANOVA, n =39), especially at pressure of 10mmHg and 15mmHg. However, high threshold mechanosensitivity is not altered, p = 0.9 (two way ANOVA, n=14).

4.5 Effect of aging process on baseline discharge and mechanosensitivity to distension adult (3 months) and aged (18 and 24months) mice

Ageing had a profound effect on gastrointestinal function. Jejunal afferent mechanosensitivities to ramp distension were well characterised in adult mice (3 months old). However, little was known about the effect of the ageing process on sensory signalling. In this part of the study, baseline discharge and mechanosensitivities were investigated in aged mice. Data from 18 and 24 months (18M and 24M) was compared with the control animal which was 3 months old (3M). It is summarized in figure 4.5.1.

Baseline firing rate was progressively attenuated with age from 23.6 ±4.3 spikes s^{-1} at 3M to 8.6 ±2.6 spikes s^{-1} at 24M (figure 4.5.1A). There was also a significant attenuation on jejunal afferent sensitivities to distension at 24M, especially evident at distending pressure of 30mmHg, 35mmHg and 45mmHg, although the data from 18M was comparable to the control as indicated in figure 4.5.1B. Afferent discharge at low threshold (figure 4.5.1C) and high threshold (figure 4.5.1D) levels of distension in aged animals was also investigated. Interestingly, the ageing process had no effect on both pressure ranges. Even though it did appear that there was a progressive reduction in low threshold afferent discharge from the 3M to the 24M animal, it did not reach the level of statistical significance.

Single unit analysis was performed to investigate the effect of age on jejunal mechanosensitivity to distension on three functional subgroups of fibres: low threshold (LT) afferent fibres, wide dynamic range (WDR) afferent fibres and high threshold (HT) afferent fibres. The jejunal mechanosensitivity to distension was significantly attenuated with age, especially in 24M mice which was most evident at distending pressure of 2mmHg to 20mmHg and also at 30mmHg (figure 4.5.2A). The mechanosensitivity of LT fibres was progressively reduced with age (figure 4.5.2B). This was consistent with the finding that there appeared to be a reduction in firing discharge at the low threshold level of distension (figure 4.5.1C). However, the mechanosensitivity of WDR fibres to distension was significantly augmented in

18M mice, but attenuated in 24M. The sensitivity of HT mechanoreceptors was not altered with age (figure 4.5.2D).

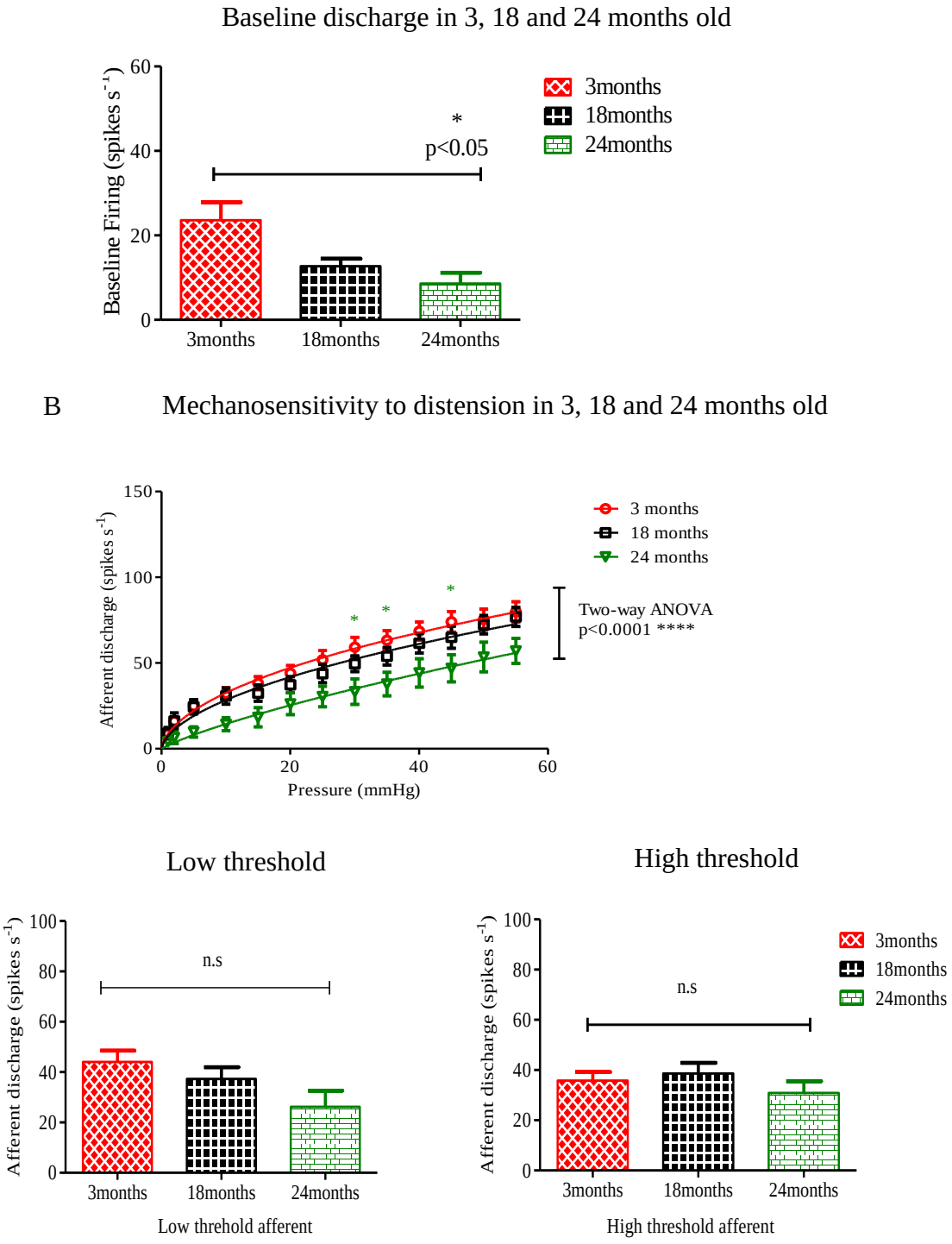


Figure 4.5.1 Effect of age on baseline discharge and mechanosensitivities to distension in 3-, 18- and 24-month old mice. A is a comparison of baseline discharge across 3 aged groups showing there was a significant reduction on baseline firing, particularly with 24M mice, p=0.03* (one-way analysis of variance, n≥10). B is the pressure-discharge response profile of afferent mechanosensitivities to distension in 3 aged groups showing a significant attenuation with age, p<0.0001**** (two way ANOVA, p≥10). C and D show that mechanosensitivities at LT and HT level of distension were not affected by the aging process, p = 0.07 and p=0.5 respectively. Note the green star represents the comparison between 3M and 24M mice.

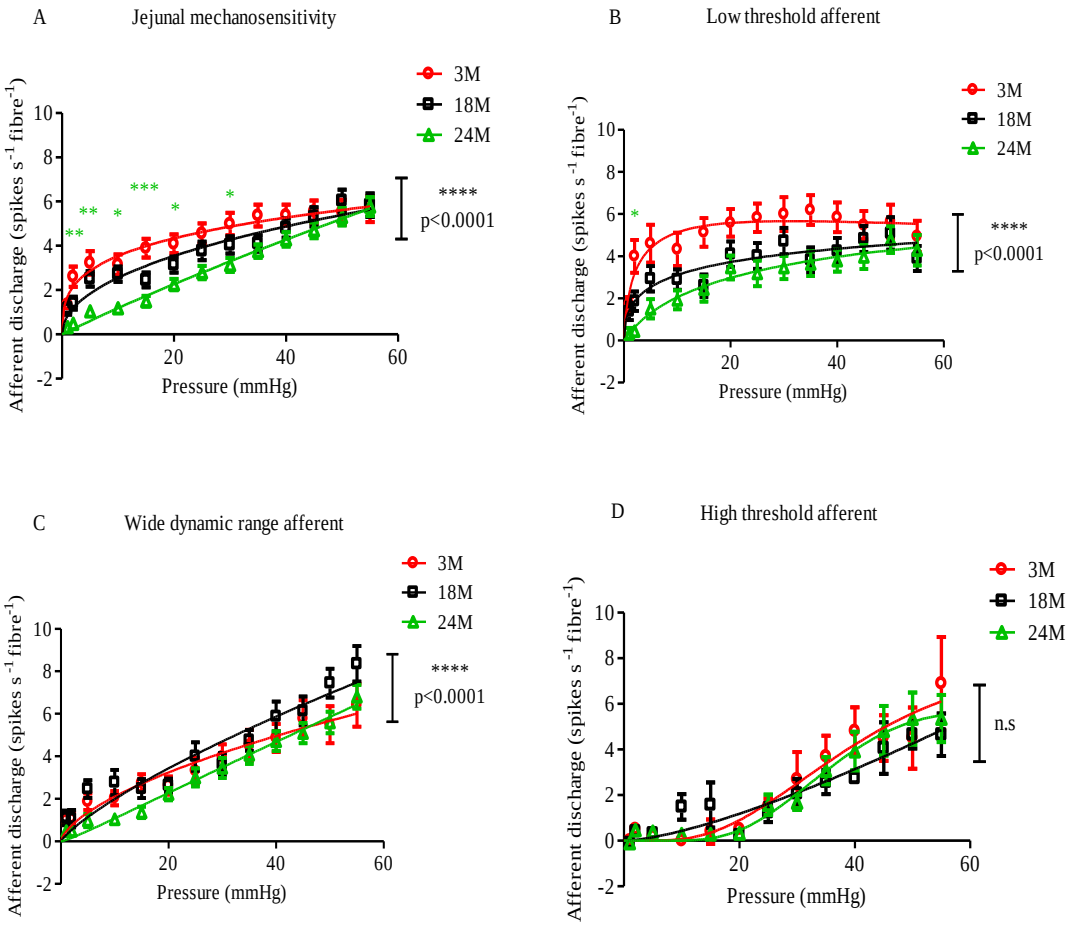


Figure 4.5.2 single unit response to distension induced afferent mechanosensitivity across 3 age groups. Data obtained from the lipid-rich studies only. A, pressure-response curve of jejunal mechanosensitivity to distension showing it was significantly attenuated with age, p<0.0001**** (two way ANOVA, n≥69). B-D, pressure-response profiles of three functional types of fibres: Low threshold units (LT fibres, n≥23), wide dynamic range units (WDR, n≥29) and high threshold units (HT fibres, n≥5). It shows that the mechanosensitivities of LT and WDR fibres are significantly different across the three age groups, p<0.0001****, but not for HT fibres, p = 0.57. Note the green star represents the comparison between 3M and 24M mice.

4.6 Effect of age on lipid-rich nutrient sensation in adult and aged mice

There was strong evidence for a loss of appetite with aging (Parker and Chapman, 2004). However, the effect of aging on the nutrient sensory signalling pathway has not been examined. In the following studies, nutrient sensation was examined across 3 age groups.

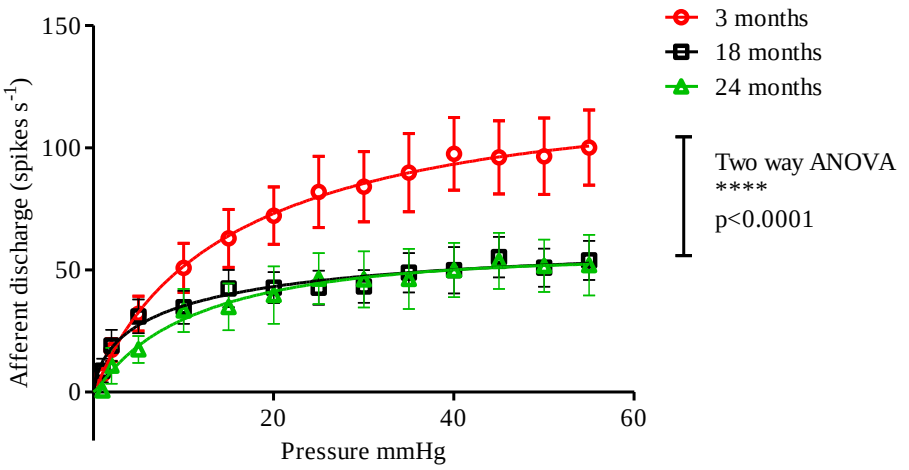
The lipid-rich nutrient induced augmentation on afferent discharge to distension was significantly attenuated in aged animal (figure 4.6.1A). It showed that the two curves representing 18- and 24-month old animals almost overlap with each other. This indicated that there was no significant difference in lipid-rich nutrient induced afferent discharge to distension in aged animals. Therefore, data from 18M and 24M animal was pooled together for the following studies.

To determine whether the effect of the lipid-rich nutrient on afferent discharge at low and high threshold level of distension would change with age, the mean firing frequency in those two compartments was analysed. They both significantly altered in aged animals (figure 4.6.1B). In adult animals, the lipid-rich nutrient induced low threshold afferent discharge was 72.2±11.8 spikes s^{-1}. This was reduced to 41.2±6.4 spikes s^{-1} in aged animal. The inhibitory effect of the lipid-rich nutrient in firing discharge at the high threshold level of distension was significantly attenuated in aged animals. It was 27.1±5.8 spikes s^{-1} in adult animals, but this was reduced to 11.7±2.6 spikes s^{-1} in aged animals.

Single unit analysis was performed to investigate the effect of age on lipid-rich nutrient sensation. Jejunal mechanosensitivity to the lipid-rich nutrient was dramatically reduced in aged mice, in both 18 and 24M mice (figure 4.6.2A). Since the interaction of the statistical analysis between those two sets of data was also significant, it was difficult to interpret the p value of significance. Instead, it could be confirmed that lipid-rich nutrient indeed produced a significant augmentation in jejunal mechanosensitivity at a distending pressure of 25mmHg up to 55mmHg in 18M mice, and at a distending pressure of 20mmHg up to 40mmHg and

50mmHg up to 55mmHg in 24M mice. However, there was no difference in the lipid-rich nutrient sensation at any distending pressure between 2 aged groups. The data was pooled together for the following analysis. LT and HT mechanosensitivity to the lipid-rich nutrient was markedly attenuated in aged group (figure 4.6.2B and D). This was consistent with the finding that lipid-rich nutrient sensation at both low and high threshold component was reduced with age (figure 4.6.1C). However, the mechanosensitivity of WDR fibres to the lipid-rich nutrient was reserved in aged animals (figure 4.6.2C).

A



B

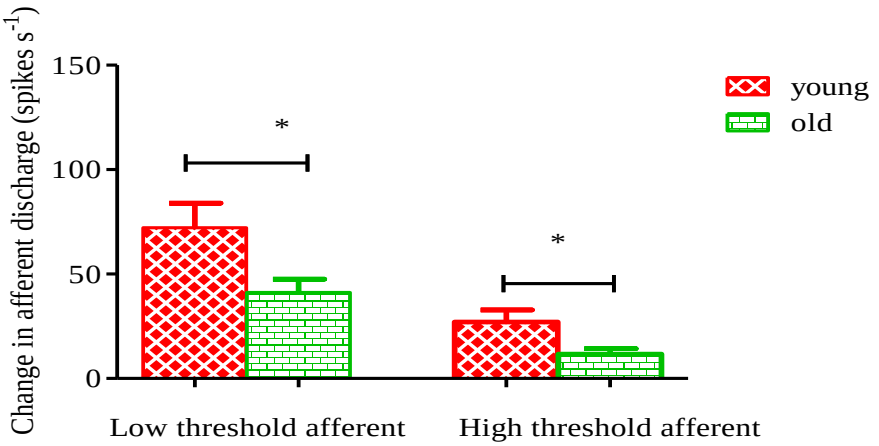


Figure 4.6.1 Effect of age on lipid-rich nutrient sensation. A is the pressure-response profile of the effect of the lipid-rich nutrient on afferent discharge to distension. It shows that jejunal mechanosensitivity to the lipid-rich nutrient was significantly attenuated with age, p<0.0001****, two way ANOVA, n≥6. B is the comparison of low and high threshold afferent firing between adult and aged animal. LT and HT mechanosensitivities were significantly reduced in aged mice, p=0.02*, (unpaired student's T test, n≥6).

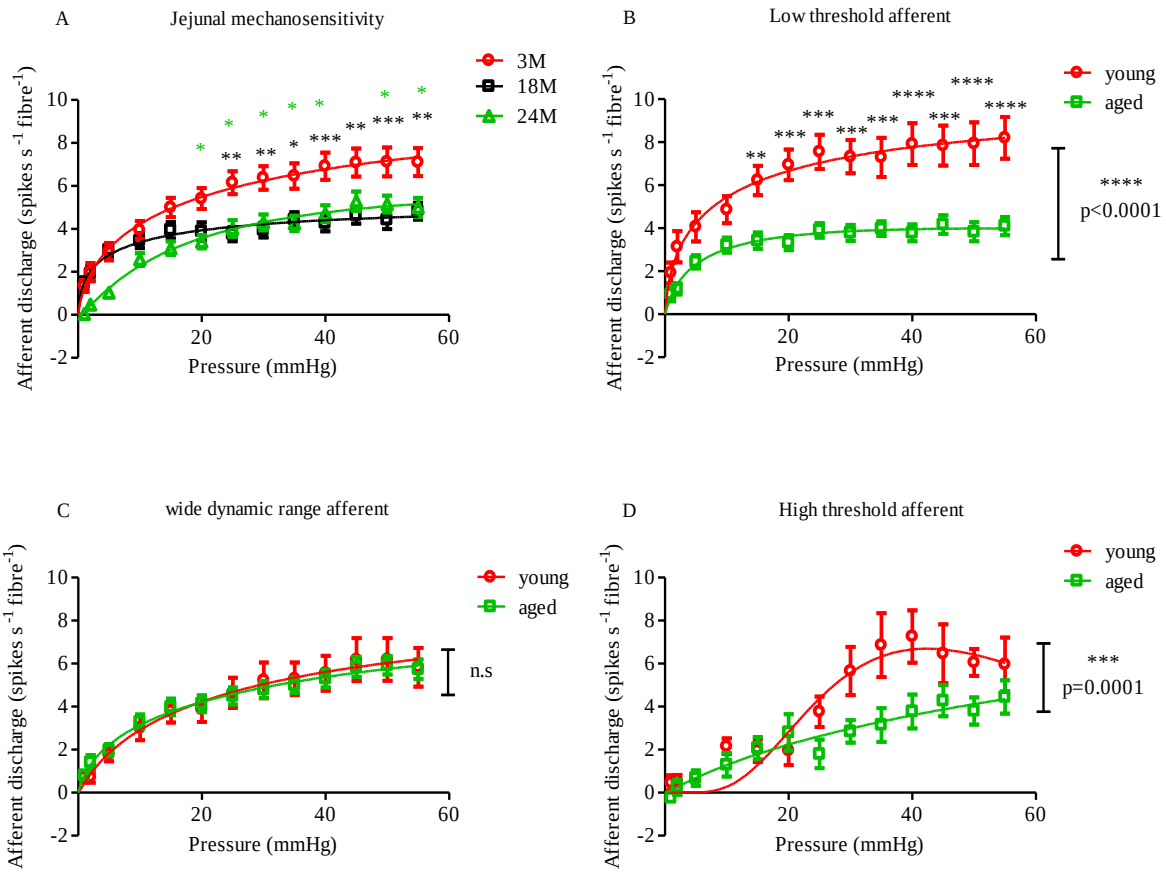


Figure 4.6.2 Single unit analysis of the lipid-rich nutrient induced afferent response to distension between adult and aged mice. A, the pressure-response profile shows that the lipid-rich nutrient induced augmentation on afferent mechanosensitivity to distension was significantly attenuated in 18M and 24M mice, $p<0.0001$ (two-way ANOVA, $n\geq69$), with Bonferroni post-tests ($p<0.05$*, $p<0.01$**, $p<0.001$*** and $p<0.00001$****). Note: in graph A, the green star represents the comparison between 3M and 24M mice, while the black star marks the difference between 3M and 18M mice. B-D, pressure-response curves represent comparison of the effect of the lipid-rich nutrient on 3 functional types of fibres between adult and aged mice. B, low threshold unit mechanosensitivity is significantly reduced in aged animal, $p<0.0001$**** ($n\geq47$). C, there is no change in the mechanosensitivity of WDR fibres, $p=0.97$ ($n\geq36$). D shows the mechanosensitivity of high threshold fibres to the lipid-rich nutrient also decreased with age, $p=0.0001$***, $n\geq5$.

4.7 Discussion

This is the first study to investigate the effect of dietary nutrients (lipid-rich) on jejunal extrinsic afferent activity in aged population of mice. The major finding of the present study was that the baseline discharge and mechanosensitivity to distension was attenuated in aged mice. Moreover, the lipid-rich nutrient evoked an augmented sensitivity of mesenteric afferents to distension in 3 months old animals only. The ability of intraluminal lipids to augment mechanosensitivity was significantly reduced with age.

Sensitivity of mesenteric jejunal afferent attenuated with age

In the present study, baseline discharge and jejunal afferent response to distension was progressively reduced with age. This suggested sensitivity of jejunal chemo- and mechanoreceptors was significantly altered in the aged population. Three functional groups of mechanosensitive afferents were identified by single unit analysis. It was the sensitivity of the low threshold vagal mechanoreceptors that was severely affected by the insults of age. The wide dynamic range afferent fibres displayed an adaptive effect on discharge frequency at different ages. The high threshold mechanoreceptors, however, were spared with age. A similar observation was made from a recent human study, which showed the chemo- and mechanosensitivity of esophageal visceral afferent were inversely correlated with age (Yamasaki et al., 2013). They showed both low (initial perception) threshold and high (pain) threshold sensitivity was reduced in an aged population. In my study, sensitivity of high threshold afferent was not altered with age. This is probably due to variations among different species and organs.

The underlying mechanism controlling all these changes is currently unknown. One possibility would be that they undergo age-related morphological changes, which in turn has impacts on their functional properties. Mucosal afferents form branching varicose endings that respond to both mechanical and chemical stimulation. They constitute the putative chemoreceptors.

Intraganglionic laminar endings (IGLEs) are located in the muscular wall and are defined as tension receptors, which are excited by distension and muscle contraction (Iggo, 1955, Cottrell, 1984, Phillips and Powley, 2000). Recent studies using an aged model of rat suggested, as the gut and its innervations age, dystrophic or regressive changes were observed at the vagal afferent terminals (Phillips et al., 2010). They showed both mucosal afferent and IGLEs undergo age-related morphological changes at the target tissue. Density of chemo- and mechanoreceptors expressed on these nerve terminals would be reduced as a result of aging related progressive loss of sensory nerves in the gut. This might contribute to the loss of jejunal chemo- and mechanosensitivity in aged mice.

Mechanosensitivity to the lipid-rich nutrient was dramatically reduced with age

Lipid-rich nutrient induced augmentation on jejunal mesenteric afferents to distension was blunted in aged animals. In the previous chapter, we showed that it was the low threshold (LT) vagal mechanoreceptors that responded to the lipid-rich nutrient via the CCK1 receptor mechanism. However, this augmentation was not observed in the aged population, suggesting the sensitivity of jejunal LT afferent fibres to endogenously released CCK is reduced with age. In adult animals, I suggested it was an integrative effect, which endogenous CCK potentiated and amplified jejunal mechanosensitivity or vice versa. There must be age-related changes affecting this synergistical action.

One possibility could be disruptions at the vagal afferent endings, such as the potential reduction on the receptive fields and varicosity at the terminals. Vagal afferents express CCK receptors (Zarbin et al., 1981). Its endings are in a close anatomical apposition with the CCK releasing enteroendocrine I cells in the gut wall (Berthoud and Patterson, 1996). There might be age-correlated alterations on the orientation of this arrangement, or a diminished number of either participating component. Any of these changes would have impacts on the paracrine function of endogenous CCK in the vagus pathway to CNS. Of the three identified types of afferents, LT afferents fibres are known to terminate as specialized intraganglionic laminar

endings (IGLEs) in the myenteric plexus (Zagorodnyuk et al., 2001). However, the location of the terminals of WDR and HT afferent fibres are currently unclear. Age-related morphological changes were observed at the IGLEs (Phillips et al., 2010). They were either deformed or had undergone dystrophic changes. We speculated that it could cause disruptions on this anatomical arrangement between endocrine I cells and the vagus nerve

The loss of vagus afferents with age has been investigated. Most of these studies were performed using visceral afferent neuron cell bodies located in the nodose ganglia of the vagus nerve, or the DRGs (Vega et al., 1993). The results were controversial, which failed to established a patterned change in neuronal number or size (Phillips et al., 2010). They contained a mixed population of cell bodies, which was difficult to determine the origin of the target tissue. Therefore, it was difficult to conclude whether there was a change in the number of vagus afferents innervating the GI tract.

Diminished LT mechanosensitivity to endogenous CCK was unlikely to be caused by a reduction in the endocrine I cells. A previous study showed an elevated number of the CCK secreting cells in aged human duodenum (Sandström and El-Salhy, 1999). This was consistent with the observation showed an increased concentration of mucosal CCK in aged guinea pigs (Poston et al., 1988b). Another possibility was a presumptive diminished number of CCK-1 receptors on the vagus sensory afferent. It was unknown at the moment, however, there was evidence showing that in other organs, an age-correlated reduction on CCK-1 receptors was observed at gallbladder and pancreas (Poston et al., 1988a, Poston et al., 1988b).

In the previous chapter, I showed that in adult animals, the lipid-rich nutrient also augmented the mechanosensitivity of wide dynamic range (WDR) and high threshold (HT) afferent fibres via a CCK-insensitive pathway. However, the physiological role or the underlying mechanisms controlling these observations is as yet unclear. In contrast, the lipid-rich nutrient-induced augmentation in the HT units' mechanosensitivity was disappeared with age. This suggested that the HT mechanosensitivity to lipid-rich nutrients was desensitized with age. On the other

hand, the WDR mechanosensitivity to the lipid-rich nutrient was preserved in an aged population. WDR afferents detected changes not only within the physiological range, but also extended into the pathological level. The unaltered sensitivity of the WDR units to the lipid-rich nutrient might be an adaptive mechanism, which is used to compensate the profound loss of nutrient sensation of both LT and HT afferent fibres.

Conclusion

To summaries, the data presented in this study reported the first direct evidence to suggest an age-associated impairment in nutrient (lipid-rich) sensory signalling, which may have impacts on feeding and reflex behaviour including aspects of neuro-immune modulation. These data suggested that the anti-inflammatory effect of nutrient (lipid-rich) diminished with ageing population especially in the elderly patients.

Chapter 5

The contribution of TRPV1 on nutrient sensation in aged murine bowel

5.1 Introduction

The transient receptor potential (TRP) channels are a superfamily of ion channels which were first identified and cloned in Drosophila in 1989 (Montell and Rubin, 1989). The vertebrae homologues of the TRP gene were revealed in 1995 by PCR cloning in mouse brain and Xenopus oocytes (Petersen et al., 1995). In mammals, it was subdivided into 6 groups including the transient receptor potential vanilloid (TRPV1-TRPV6) family of which the TRPV1 channel was the most studied and well characterised (Clapham et al., 2001).

TRPV1, a non-specific cation channel highly permeable to calcium, was first isolated from a cDNA library of rat sensory neurons (Caterina et al., 1997). It had six transmembrane (TM) domains that assemble to multimers with a homotetramer as a predominant form (Caterina et al., 1997, Kedei et al., 2001). The existence of a heterotetramer form has also been proposed (Smith et al., 2002, Hellwig et al., 2005). TRPV1 was viewed as a molecular integrator of physical and chemical stimuli in the peripheral pain perception (Tominaga et al., 1998, Ferrer-Montiel et al., 2004). It functioned as a polymodal receptor located at the small diameter primary afferent fibres (Tominaga et al., 1998). TRPV1 was robustly activated by low pH (5.2), noxious heat (>43°C), vanilloid compounds such as capsaicin (the pungent ingredient in the hot chilli pepper) and resiniferatoxin (RTX) (Szallasi and Blumberg, 1989, Szallasi et al., 1989, Caterina et al., 1997, Tominaga et al., 1998, Caterina and Julius, 2001). It was also potentiated by voltage and endovanilloids such as anandamide (Piper et al., 1999, Szolcsányi, 2000).

In chapter 3, it had been shown that the lipid-rich nutrient caused augmented mechanosensitivity in afferents supplying the mouse jejunum via a CCK-1 receptor mediated mechanism. In rat, TRP channels were implicated in the response to CCK since treatment with ruthenium red (RuR) attenuates CCK-evoked calcium entry into nodose ganglion cell (Zhao and Simasko, 2010). However, the channel mediating this effect was unclear. A recent study has shown that this linkage between CCK receptor activation and calcium influx through a

RuR-sensitive conductance in nodose neurons is preserved between rat and mice (Kinch et al., 2012).

The mice used in this study were 11-12 months old. The aim of this study therefore was to investigate whether TRPV1 may contribute to nutrient sensing by comparing the mesenteric afferent sensitivity to lipid-rich nutrients in TRPV1-/- and wild type mice.

5.2 Experimental protocol and analysis

The in vitro preparation was set up as outlined in chapter 2.

TRPV1 knockout mice

TRPV1 knockout mice were generated by GlaxoSmithKline (Harlow, UK). Trans-membrane domain 2-4 of mouse TRPV1 gene, encoding amino acid 460-555, was replaced by the neo gene (Davis et al., 2000). Mating pairs of $TRPV1^{-/-}$ and $TRPV1^{+/+}$ N1F1 littermates were obtained to generate separate colonies of TRPV1 wild type and KO mice at the University of Sheffield according to the UK Animals (Scientific procedures) Act 1986. Throughout the course of studies, genotyping was carried out periodically to confirm the absence of TRPV1 gene in $TRPV1^{-/-}$ mice.

All the experiments were performed on $TRPV1^{-/-}$ mice and their aged-match wild type (Newton) littermates. Recordings were made from mesenteric afferent nerves innervating the jejunum. All preparations were left for 40-60 minutes for stabilization. It was followed by ramp distensions every 15 minutes until the response became reproducible (3-4 reproducible response to distension) before the commencement of any protocol. The lipid-rich nutrient (1ml) was carefully pushed through the lumen of the jejunum in a period of 2 minutes.

Effect of the lipid-rich nutrient on distension induced afferent discharge

The mechanical effect (subjected to ramp distension up to 55mmHg) of the lipid-rich nutrient on jejunal afferent discharge was expressed as the change in afferent discharge above the baseline, calculated as the mean firing frequency in 2 second periods at each level of distending pressure subtracting the baseline firing which is 1 min prior to distension. It was then plotted against pressure. It was the same protocol as in Chapter 3 which was described in details in the schematic diagram in figure 3.2.1 (A). It was compared with before and after nutrient treatment. Ramp distension of jejunal segments evoked a biphasic increase in afferent

discharge, which represented the activation of low threshold (LT) and high threshold (HT) mechanosensitive afferent fibres. The LT component was represented by the increase in discharge between the baseline and 20mmHg. The HT component was represented by the increase in discharge between 20mmHg and 55mmHg. It was compared with before and after nutrient treatment.

Details of single unit analysis and identification of different population of mesenteric afferent fibres were described in Chapter 2.

Jejunal compliance and intraluminal volume

Since infusion rate was always kept stable through ramp distension (200µl/min), intraluminal volume was calculated using the rate of distension and time taken to reach a given intraluminal pressure (55mmHg). The effect of treatment on jejunal compliance was evaluated as the final volume reaching a distending pressure of 55mmHg. It was compared with the control which is before any treatment took place.

Statistical significance

Statistical analysis was carried out using a Student's t-test (paired/unpaired), repeated measured one way ANOVA with Dunnett's Multiple Comparison Test and two-way ANOVA with Bonferroni post-tests. Data was presented as mean ± SEM, where $p < 0.05$ was considered as significant.

5.3 Effect of the lipid-rich nutrient on distension induced afferent discharge in TRPV1$^{-/-}$ mice

The effect of the lipid-rich nutrient on the mechanosensitivity of the afferent response to distension was investigated in TRPV1$^{-/-}$ mice. The lipid-rich emulsion (1ml) was applied and the afferent response to distension compared to that before (control) and after (washout) the lipid-rich nutrient. These data are summarised in figure 5.3.1 and show that the lipid-rich nutrient has a profound effect on afferent mechanosensitivity to distension. This was a transient effect in which the augmented afferent firing was immediately recovered during the period of washout.

There was no change in jejunal compliance upon lipid-rich nutrient application in TRPV1$^{-/-}$ mice. It was 289.2±22.8µl before any treatment. Even though it was slightly reduced to 265.9±20.2µl with the lipid-rich nutrient, it did not reach the level of significance.

The effect of the lipid-rich nutrient on afferent discharge at low threshold (baseline – 20mmHg) and high threshold (20-55mmHg) level of distension was also analysed in TRPV1$^{-/-}$ mice. The lipid-rich nutrient significantly augmented afferent firing at the low threshold level of distension (figure 5.3.1B). It was increased to 88.4±10.3 spikes s^{-1} from 52.0±11.0 spikes s^{-1} upon lipid–rich nutrient application. The low threshold afferent discharge was recovered to 53.7±9.8 spike s^{-1} during the washout. Lipid-rich nutrient also had an inhibitory effect on high threshold afferent firing (figure 5.3.1C). The high threshold afferent firing was reduced to 4.0±8.5 spikes s^{-1} from 31.9±6.6 spikes s^{-1} with lipid-rich nutrient supplementation. Its inhibitory effect was immediately eliminated during period of washout. The high threshold afferent firing was returned to 25.9±6.8 spikes s^{-1}. Since this information was signalled differentially via vagal and spinal afferent pathways, it suggested that vagal nutrient signalling was enhanced while nociceptive signalling was attenuated by the lipid-rich nutrient in aged TRPV1-/- mice.

Single unit analysis (as described in chapter 2) was conducted. It corrected the whole nerve data to the number of active single units identified in each preparation. This was to minimise the variations caused by the presence of different number of single units in each afferent bundle. A total number of 63 single fibres were characterised by their activation threshold. Following this analysis there was still a significant augmentation in afferent mechanosensitivity with the lipid-rich nutrient. The effects of the lipid-rich nutrient on mechanosensitivity of low threshold (LT), wide dynamic range (WDR) and high threshold (HT) afferent units were investigated independently. This data is summarised in figure 5.3.2.

It showed that in TRPV1$^{-/-}$ mice, lipid-rich nutrient induced augmentation in mechanosensitivity was only present at WDR and HT afferent units. The WDR and HT afferent mechanosensitivities were significantly increased upon lipid-rich nutrient infusion (figure 5.3.2C and D). From the pressure-response profile, it appeared that there was an increase in firing discharge of the LT afferent fibres, particularly at the low threshold level of distension (figure 5.3.2B). However, it did not reach the level of significance, at which the LT mechanoreceptors were insensitive to lipid-rich nutrient. Since those TRPV1$^{-/-}$ mice was middle aged, it was important to investigate the possible changes in afferent sensitivity with aging. Therefore, a set of age control experiments was carried out.

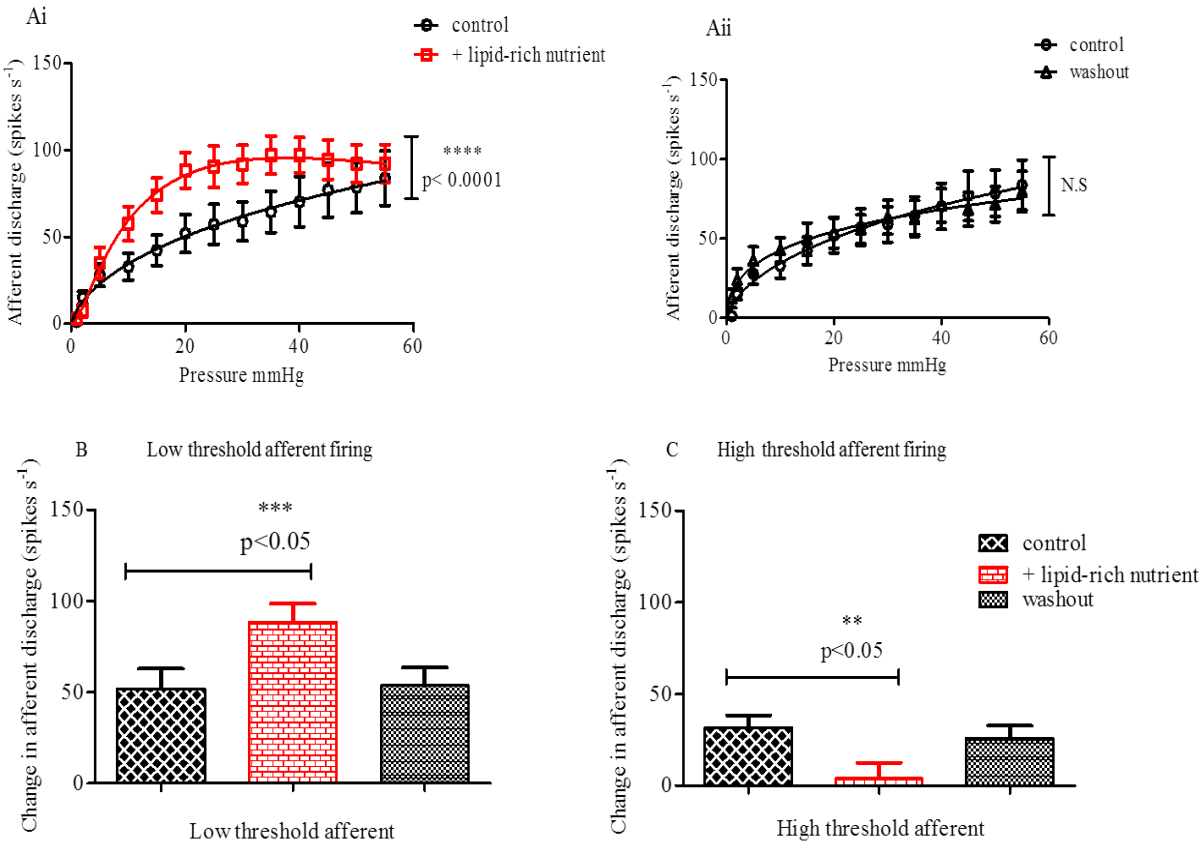


Figure 5.3.1 Mean afferent response to ramp distension up to 55mmHg is plotted for TRPV1 $^{-/-}$ mice. A) The lipid-rich nutrient significantly augments afferent sensitivity to distension, p<0.0001****, which recovered rapidly upon washout, p = 0.57 (two way ANOVA, N=7). B) and C) shows the data for the low threshold (LT; baseline to 20 mmHg) and high threshold (HT; 20 to 55 mmHg) components of the response to distension. The LT mechanosensitivity is significantly increased, p=0.0003*** (repeated measured ANOVA). In contrast there is a significant attenuation in the HT component resulting in a flattening of the pressure-response profile at high pressures, p=0.006**.

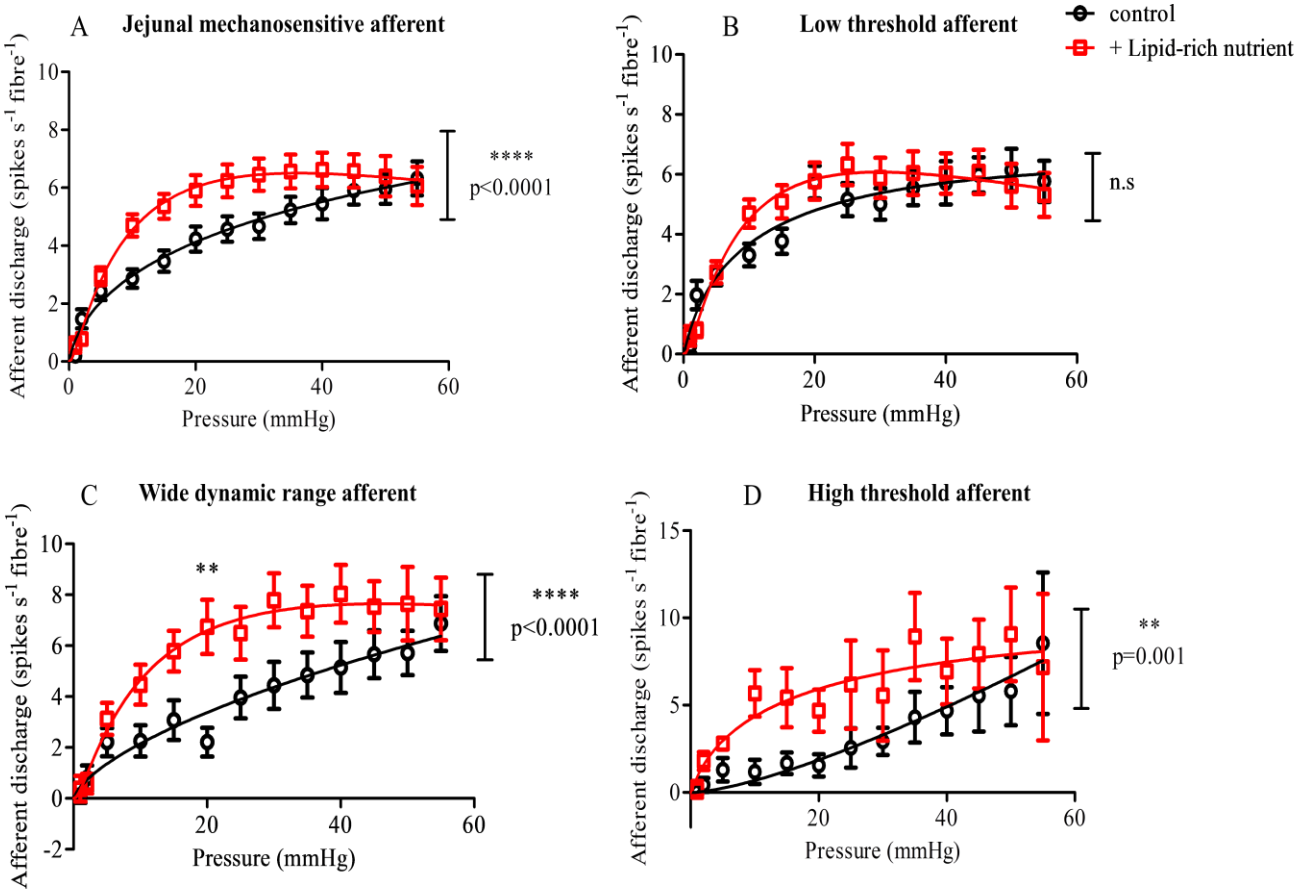


Figure 5.3.2 Single–unit responses to the lipid-rich nutrient on distension induced afferent mechanosensitivity in Trpv1$^{-/-}$ mice. A, The whole nerve response has been normalised to the number of single units in the afferent bundles (see method). It produces a more robust effect indicating that the lipid rich nutrient significantly increases jejunal afferent mechanosensitivity, p<0.0001**** (two way ANOVA, n=63), particularly at a distending pressure of 15mmHg (Bonferroni post-tests, p<0.05*). B, low threshold afferent fibres (LT), shows there is no change in LT mechanosensitivity, p = 0.18 (two way ANOVA, n=37). C, wild dynamic range afferent fibres (WDR), shows that WDR mechanosensitivity is significantly augmented by the lipid-rich nutrient, p<0.0001**** (two way ANOVA, n = 22), especially at a distending pressure of 20 mmHg (Bonferroni post-tests, p<0.01**). D shows the data for high threshold (HT) afferent fibres. The mechanosensitivity of HT is significantly increased upon lipid-rich nutrient application, p=0.001** (two way ANOVA, n =4).

5.4 Effect of the lipid-rich nutrient on distension induced afferent discharge in control wild type mice

The effect of the lipid-rich nutrient on afferent mechanosensitivity to distension was studied in age matched WT littermates of TRPV1$^{-/-}$ mice. The lipid-rich nutrient had no effect on distension induced afferent discharge rate. The jejunal compliance was not altered upon lipid-rich nutrient application. It was 340.5±28.4µl before treatment, and 323.4±23.8µl with lipid-rich nutrient infusion. The effect of the lipid-rich nutrient on afferent discharge at low threshold (baseline – 20mmHg) and high threshold (20-55mmHg) level of distension was also analysed in WT age control mice. It had no effect on either low threshold or high threshold afferent firings. This data is summarized in figure 5.4.1.

This is different from what had been discussed in chapter 3, in which the lipid-rich nutrient significantly augmented afferent mechanosensitivity to distension (figure 3.7.1). It showed that the lipid-rich nutrient induced augmentation on distension induced mechanosensitivity was attenuated even at the age of 11-12 months old. This suggests that LT and HT mechanoreceptors start to be insensitive to the lipid-rich nutrient from in middle aged mice.

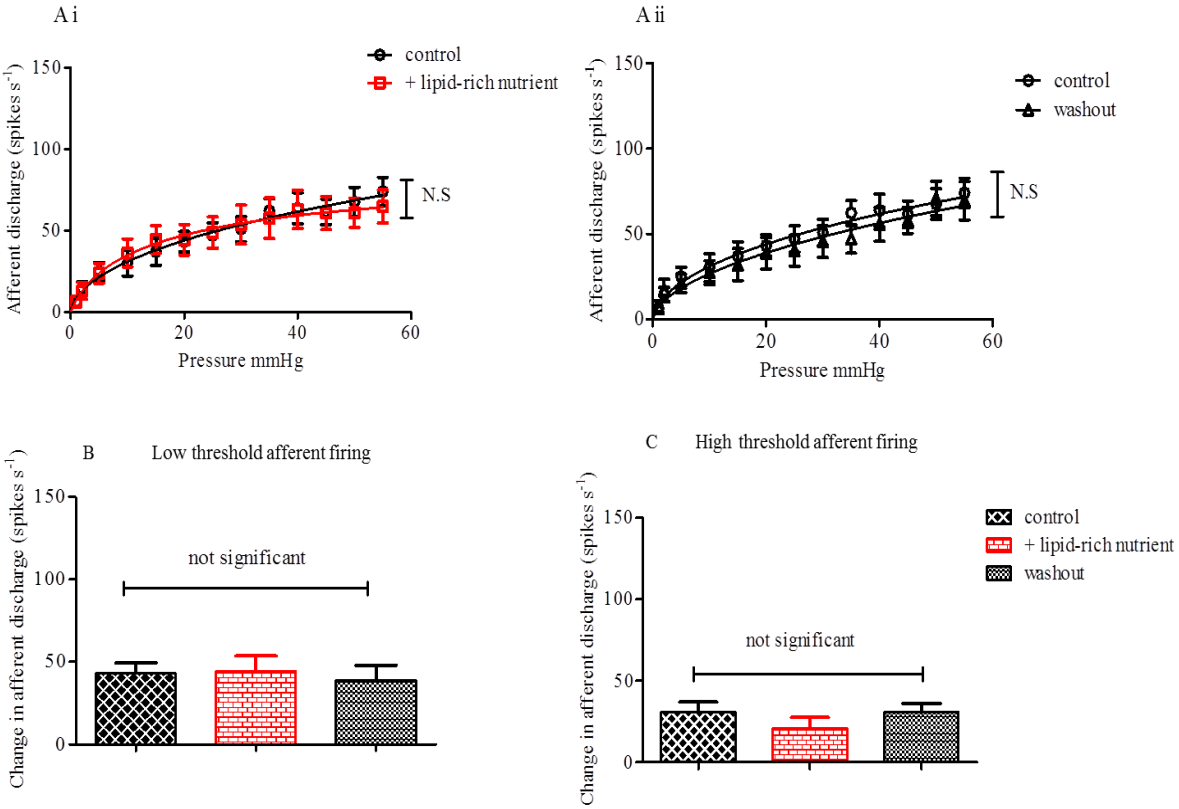


Figure 5.4.1 Mean afferent response to ramp distension up to 55mmHg is plotted for 11-12 month old mice. A) The lipid-rich nutrient has no effect on afferent sensitivity to distension (two way ANOVA, n=6). B) and C) show the data for the LT and HT component of the response to distension. The sensitivity to lipid-rich nutrient at LT and HT levels of distension is abolished in middle aged mice (repeated measured ANOVA).

5.5 Jejunal afferent sensitivity in one year old TRPV1$^{-/-}$ and wild type mice

The contribution of the TRPV1 receptor to jejunal afferent sensitivity in murine intestine was studied. It was investigated by comparing the baseline discharge and mechanosensitivity with their wild type littermates of the same age. These data are summarised in figure 5.5 and show that there is no change in either baseline discharge or distension induced afferent mechanosensitivity in TRPV1$^{-/-}$ mice. It appeared that there was a reduction in jejunal compliance in TRPV1$^{-/-}$ mice compared to WT. The intraluminal volume was 340.5 ± 28.4 µl in WT and this was 289.2 ± 22.8 µl in TRPV1 $^{-/-}$ mice. However, it did not reach the level of significance (figure 5.5.1C).

Single unit analysis identified a total number of 63 afferent units from WT mice. The number of different types of mesenteric afferent units identified from aged WT and TRPV1$^{-/-}$ mice was listed in table 1. It showed that there was no change between WT and TRPV1$^{-/-}$ mice. The mechanosensitivities of low threshold (LT) unit, wide dynamic range (WDR) unit and high threshold afferent (HT) unit were compared between WT and TRPV1$^{-/-}$ mice. The data is summarized in figure 5.5.2. It showed that there was no marked difference in the mechanosensitivity of LT units as the two curves from the pressure-response profile were almost overlapped with each other (figure 5.5.2B). However, the mechanosensitivity of the WDR unit was significantly attenuated in TRPV1$^{-/-}$ mice (figure 5.5.2C). Interestingly, the HT unit mechanosensitivity was markedly elevated in the KO mice compared to WT (figure 5.5.2D). However, the number of HT afferent fibres identified was very small ($n \leq 10$), so it was difficult to reach the conclusion that HT afferent mechanosensitivity was augmented in TRPV1$^{-/-}$ mice.

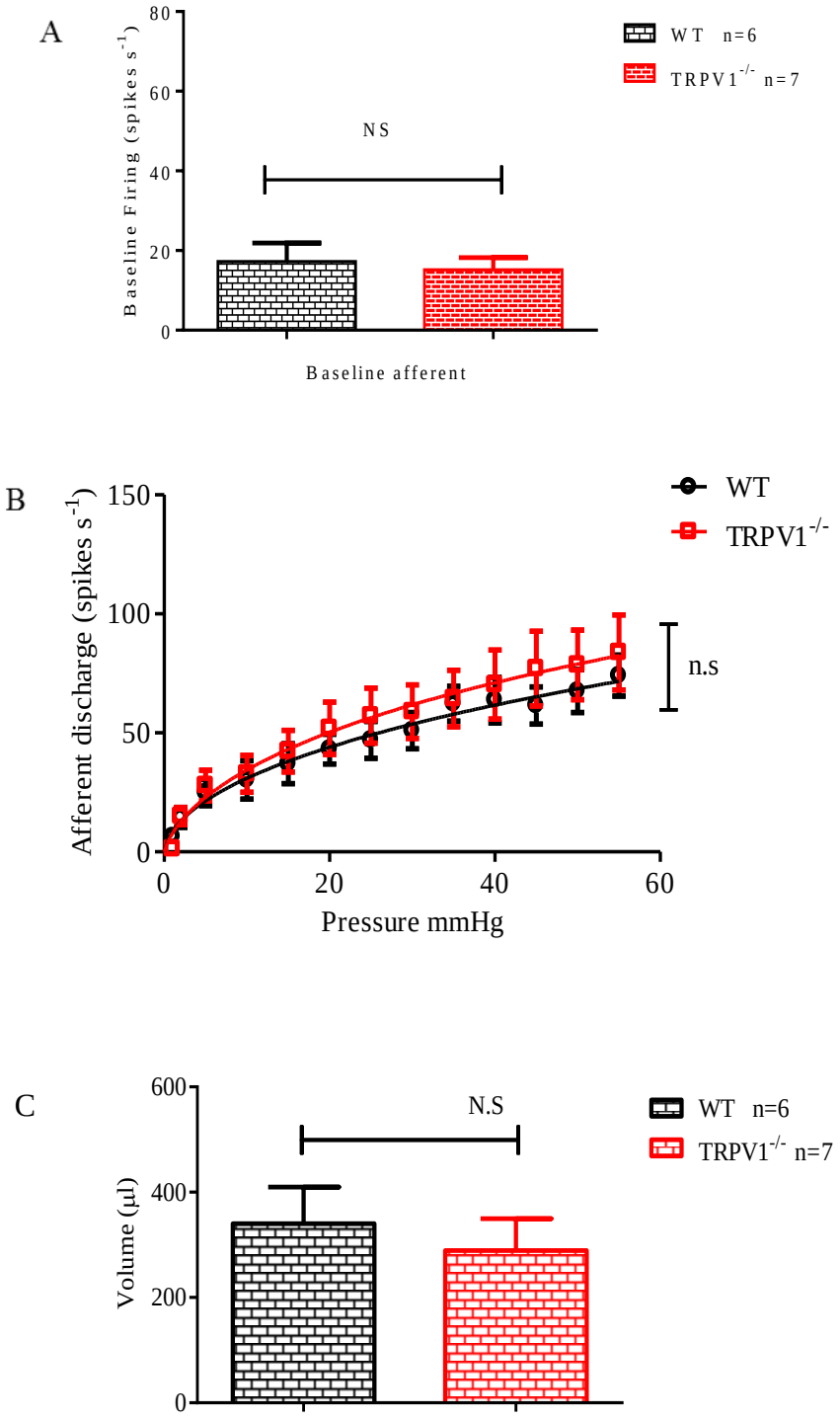


Figure 5.5.1 Mesenteric afferent response on baseline discharge and distension induced mechanosensitivity in wild type (Newton) and TRPV1$^{-/-}$ mice. A, the data shows that there is no difference in baseline discharge between WT and TRPV1$^{-/-}$ mice, p = 0.7, (unpaired t-test, n≥6). B, pressure-response relationship of multiunit activity between WT and TRPV1$^{-/-}$ mice indicates that there is no significant difference on distension induced mechanosensitivity, p = 0.12 (two-way ANOVA). C, Compliance of jejunal segments is not different between WT and TRPV1$^{-/-}$, p = 0.2 (unpaired t-test)

	TRPV1 WT	TRPV1 KO
Low threshold unit	34 (53.9%)	37 (58.7%)
Wide dynamic range unit	22 (34.9%)	22 (34.9%)
High threshold unit	7 (11.1%)	4 (6.3%)
Total	63 (100%)	63 (100%)

Table 5.5.1 Number of different types of mesenteric afferent fibres identified in age- marched Wild type (Newton) and TRPV1$^{-/-}$ mice

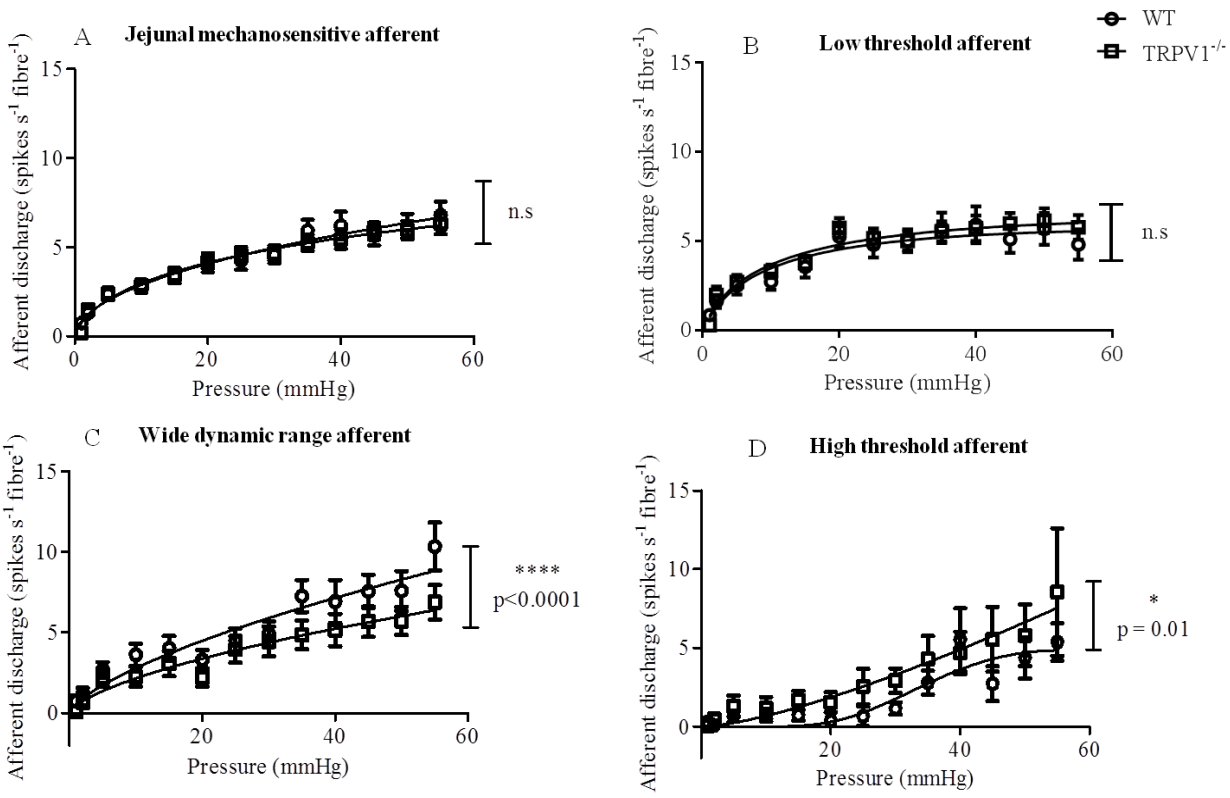


Figure 5.5.2 Single–unit responses to distension induced afferent mechanosensitivity in WT and TRPV1$^{-/-}$ preparations. A, pressure-response curve of jejunal mechanosensitivity to distension, n=63. The whole nerve response has been normalized to the number of single units in the afferent bundles (see method).There is no significant difference in mechanosensitivity between WT and TRPV1-/- mice. B-D, pressure-response curves of three different functional types of fibres: Low-threshold fibres (LT fibres, n≥34), wide dynamic range fibres (WDR fibres, n=22) and high threshold fibres (HT fibres, n≥4). Note that the curves for WDR and HT fibres are significantly different between WT and TRPV1-/- mice, but the curve for LT fibres is not (two-way ANOVA).

5.6 Potential role of the TRPV1 channel involved in lipid-rich nutrient sensation in one year old mice

In order to understand the potential role of the TRPV1 channel involved in nutrient sensation, lipid-rich nutrient induced baseline discharge and afferent mechanosensitivity to distension were investigated by comparing with their wild type littermates.

Jejunal afferent sensitivity to the lipid-rich nutrient on distension induced afferent discharge was significantly higher in TRPV1$^{-/-}$ than in WT mice (figure 5.6.1). Both curves show an increase in afferent discharge, but reach plateaus in the high threshold level of distension resulting in a flattening of the pressure-discharge response profile, especially in TRPV1$^{-/-}$ mice.

To determine whether there were any changes in lipid-rich nutrient induced afferent discharge at low (baseline-20mmHg) and high (20-55mmHg) threshold level of distension in TRPV1$^{-/-}$ mice, the mean change in firing frequency in those two phases was analysed. They were compared with their WT littermates. Low threshold afferent discharge to the lipid-rich nutrient was significantly augmented in TRPV1$^{-/-}$ compared with WT (figure 5.6.1B). It was 44.4±9.4 spikes s^{-1} in WT. This was increased to 88.4±10.3 spikes s^{-1} in TRPV1$^{-/-}$ mice. It showed that the high threshold afferent discharge to lipid-rich nutrient was attenuated in TRPV1$^{-/-}$ mice (figure 5.6.1B). Even though it was reduced to 4.0±8.5 spikes s^{-1} in TRPV1$^{-/-}$ mice compared with their WT littermates that is 20.6±7.0 spikes s^{-1}, it was not statistically significantly.

The afferent response of LT, WDR and HT units to the lipid-rich nutrient was also compared between WT and TRPV1$^{-/-}$ mice. The data is presented in figure 5.6.2. It suggested that the sensitivity of the LT mechanoreceptor (within the physiological range, <20mmHg)) to the lipid-rich nutrient was unchanged between WT and TRPV1$^{-/-}$ mice. In contrast, the sensitivities of WDR and HT mechanoreceptors to the lipid-rich nutrient were significantly elevated in TRPV1$^{-/-}$ compared with WT mice.

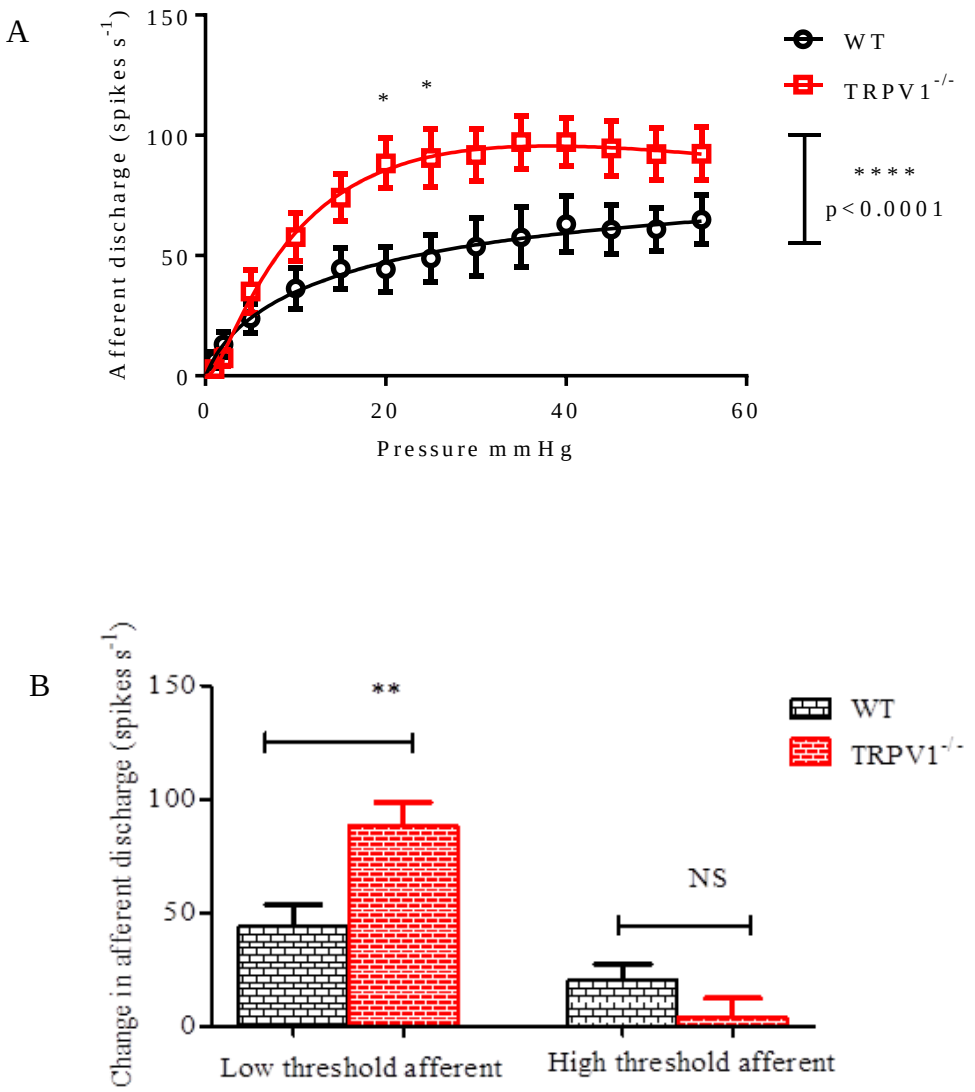


Figure 5.6.1 Effect of the lipid-rich nutrient on distension induced afferent discharge in TRPV1$^{-/-}$ mice and their WT littermates**.** Comparing 12 month-old animals afferent mechanosensitivity to the lipid-rich nutrient is significantly attenuated in WT mice compared to TRPV1 $^{-/-}$, p<0.0001**** (two way-ANOVA, n ≥6), especially evident at distending pressures of 20 and 25 mmHg (Bonferroni post-tests, p<0.05*) . B shows the data for the response of the low threshold (LT; baseline to 20 mmHg) and high threshold (HT; 20 to 55 mmHg) components to distension. The LT mechanosensitivity is significantly increased in TRPV1$^{-/-}$ p=0.01** (unpaired t-test, n ≥6). In contrast there is no significant difference in HT mechanosensitivity, p=0.2.

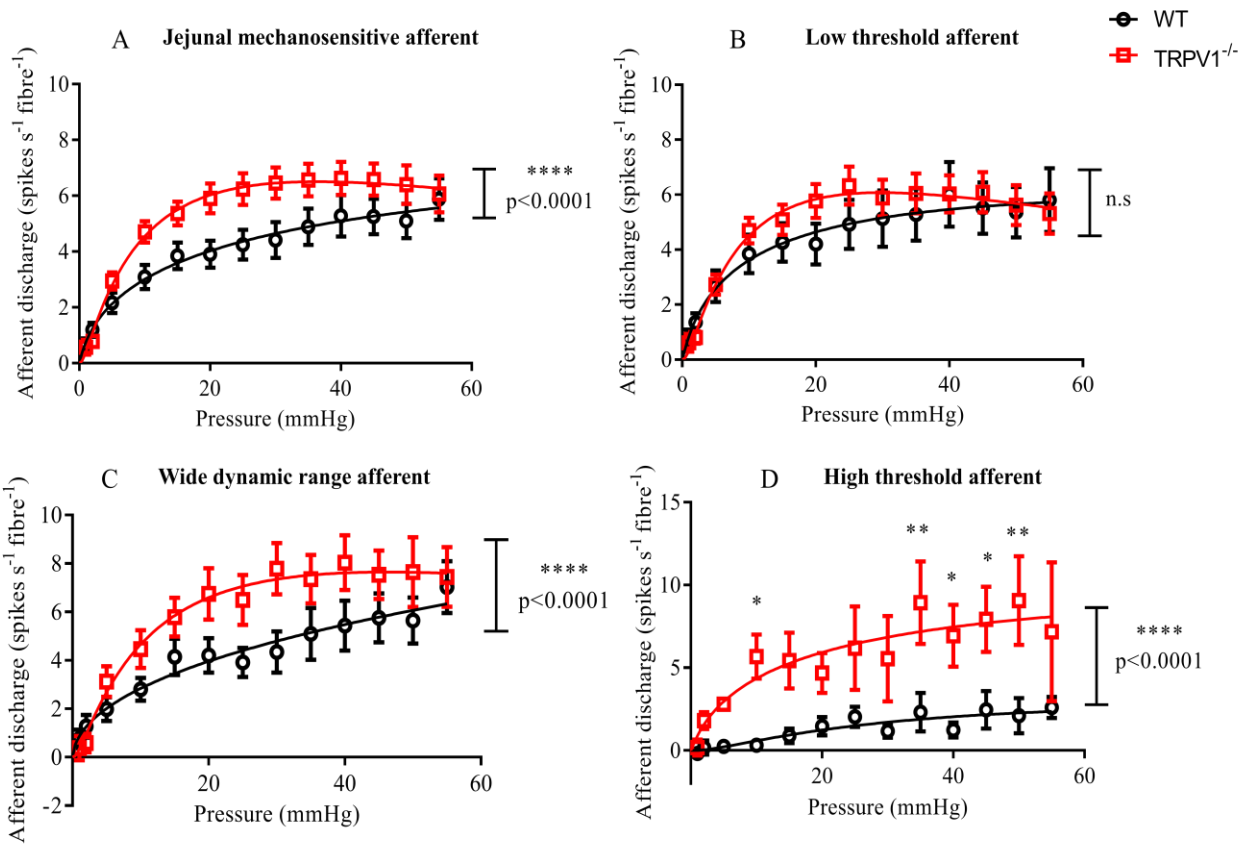


Figure 5.6.2 Comparison of the effects of the lipid-rich nutrient on distension induced afferent mechanosensitivity in WT and Trpv1$^{-/-}$ mice. The whole data is normalised by single unit analysis. A, overall mean effect, p<0.0001****, (two way ANOVA n = 63). B, low threshold (LT) afferent units, shows there is no significant difference in LT in Trpv1$^{-/-}$ mice and their wild type littermates, (two way ANOVA, n ≥34). C, wide dynamic range units (WDR), the lipid-rich nutrient induced a significantly larger augmentation in Trpv1$^{-/-}$ mice than in the wild type, p<0.0001****, (two way ANOVA, n=22). D represents the data for high threshold (HT) afferent units which is significantly increased by the lipid-rich nutrient in Trpv1$^{-/-}$ mi**ce** than in wild type, p<0.0001**** (two way ANOVA, n≥4), especially at pressure of 10 mmHg, 35 mmHg, 40mmHg, 45mmHg and 50mmHg (Bonferroni post-tests, p<0.05*, p<0.01**).

5.7 Discussion

This study is the first report of recordings from murine jejunal afferent nerves of one year old TRPV1$^{-/-}$ mice. In age-matched wild type preparations, combined with our previous data the current findings indicated that lipid-induced enhanced mechanosensitivity in 3 month mice disappeared in 12 month mice. In contrast, the response to the lipid rich nutrient was preserved in TRPV1$^{-/-}$ mice, such that compared to the age-matched controls there was an augmented response in altered mechanosensitivity. The baseline firing was also significantly elevated in aged TRPV1$^{-/-}$ mice. These data suggested that constitutive TRPV1 activity may down-regulate lipid-rich nutrient sensitivity, or that compensatory changes in excitability in TRPV1$^{-/-}$ neurones were present, resulting in enhanced sensitivity to the nutrient and CCK in one year old mice.

TRPV1 receptors do not modulate jejunal afferent sensitivity to distension in 12 months mice

I have already demonstrated that the jejunal chemo- and mechanosensitivity were progressively reduced with age (Chapter 4). In the WT preparation of 12 months old mice, this attenuation in jejunal sensitivity with age was not observed (data not shown). A morphological study showed that there was only a mild sign of age-related changes in mouse nerve of 12 months old (Ceballos et al., 1999). This is the time point in the mouse representing the end of the adult maturation period for the nerve fibres (Ceballos et al., 1999, Verdú et al., 2000). It was at this stage, which that the nerve fibres started to show age-related morphological changes that may start to have impacts on their functional properties. The mechanism underlying age-associated changes in afferent function is as yet unclear

Previous studies from our lab demonstrated that TRPV1 receptors were involved in modulating both gut and bladder sensitivity (Rong et al., 2004, Daly et al., 2007). They showed that TRPV1$^{-/-}$ mice had an attenuated afferent sensitivity to distension. This is in contrast with the current findings which showed that, in TRPV1$^{-/-}$ mice, the jejunal afferent

mechanosensitivity was unchanged. However, there is a crucial difference between these studies, in that the mice population used in the present study was one year old. The other studies were performed using a much younger population about 3 months old. Studies from these two age groups of mice suggest that functional role of TRPV1 in gut mechanosensitivity is altered in 12 months mice comparing with 3 months mice.

Of the three functional types of afferents identified by single unit analysis (details of classification was described in chapter 2), WDR fibres showed attenuated mechanosensitivity in one year old TRPV1$^{-/-}$ mice. The response of LT fibres was not significantly different between TRPV1$^{-/-}$ and their age-matched littermates. This was consistent with findings of a previous study from our lab, which showed that the mechanosensitivity of WDR and LT afferent fibres to distension in 3 months old TRPV1$^{-/-}$ mice are unchanged.(Rong et al., 2004). The LT fibres were mainly vagal afferents (Booth et al., 2001). Previous studies showed that in 3 months old animals, TRPV1 receptors were less likely to be detected on the vagal afferents innervating the small intestine (Patterson et al., 2003, Ward et al., 2003). Therefore, it may suggest the reason why the mechanosensitivity of WDR and LT afferent fibres to distension is not altered in TRPV1$^{-/-}$ mice. However, it is as yet unclear whether the distribution of TRPV1 receptors on the vagus nerve will be changed with age. The current study suggests that the functional property of TRPV1 receptors on vagal afferents was unaltered whereas TRPV1 receptors in spinal afferent were enhanced in 12 months old mice. It is likely that TRPV1 activity suppresses the mechanosensitivity of HT fibres in 12 months old mice. HT fibres are mainly spinal afferents (Booth et al., 2001), which involved in mediating pain perception (Grundy, 2002). TRPV1 channels were viewed as molecular integrators in the peripheral pain perception (Tominaga et al., 1998, Ferrer-Montiel et al., 2004). This might suggest the presence of possible compensatory changes in signalling pathways and also morphological structure during the development and ageing of TRPV1-null mutant mice.

TRPV1 receptor contributes to jejunal afferent mechanosensitivity to nutrient (lipid-rich), but independently from the CCK signalling pathway in aged mice

The nutrient-induced augmentation was not observed in the age-matched WT preparations. In the previous chapter, I have already demonstrated that nutrient sensation (the lipid-rich nutrient) was dramatically reduced with age. The findings in the current study showed that the decline in nutrient sensation with age started before 12 months in mouse afferents. Nutrient sensations of both LT and HT fibres declined with age, but with a preserved nutrient sensitivity of WDR fibres (data not included). No change in jejunal compliance was observed, therefore, all the changes were not secondary to the changes in muscle tone.

Jejunal mechanosensitivity to the lipid-rich nutrient was augmented in one year old TRPV1$^{-/-}$ mice compared to their age-matched counterparts. Of the three functional types of afferents, only LT fibres showed no change in mechanosensitivity to the lipid-rich nutrient in aged TRPV1$^{-/-}$ mice. In the previous chapter, I showed that this augmentation was mainly mediated via the CCK-1 receptor through the release of endogenous CCK, and also this responsiveness was declined with age. This suggested in an aged population, activity of the TRPV1 receptor was not involved in mediating nutrient sensation of LT fibres. In my opinion, an extra set of experiments using the CCK-1 receptor antagonist (devazepide) is very important. However, I was unable to perform this experiment since I only have a limited amount of aged TRPV1$^{-/-}$ tissue. A previous study showed that in adult mice, a linkage between the activation of the CCK receptor and a TRP channel was observed in vagal neurons isolated from nodose ganglion (Zhao and Simasko, 2010, Kinch et al., 2012), in which TRPV1 channel was not involved. Even though I showed a similar observation in the aged population, the contribution of TRPV1 receptors in LT firing to nutrient stimuli was still unclear in 3 months animals. In the present study, the WDR and HT fibres showed augmented mechanosensitivity to the lipid-rich nutrient in TRPV1$^{-/-}$ mice, which suggested the TRPV1 receptor might down regulate lipid sensitivity of WDR and HT mechanoreceptors in an aged population. This suggested that the

maintenance of the lipid-rich nutrient sensitivity in the aged TRPV1$^{-/-}$ mice was CCK independent. Also, in order to further prove that TRPV1 down regulates afferent mechanosensitivity to lipids in the 12 months old mice, the effect of lipid on 3 months old TRPV1$^{-/-}$ mice should be examined

Conclusion

To summarize, in this study, we showed that TRPV1 activity was incorporated into the nutrient sensation in 12 months mice. It suppressed the mechanosensitivity of jejunal afferents innervating the small intestine in a CCK independent manner. However, further investigation is required to determine its role in the nutrient signalling pathway in the adult population.

Chapter 6

General discussion

Dietary lipids in the lumen of the proximal GI tract have long been recognised as signalling molecules which control various cellular processes and play an important regulated role in physiological and pathophysiological conditions including food intake, metabolism, inflammation, pain and cancer (Raybould, 1999, de Lartigue et al., 2011, Tucker and Honn, 2013). A pioneer study carried out in our group a decade ago (Lal et al., 2001) has demonstrated that lipid-derived fatty acids directly and indirectly stimulate vagal afferent terminals in the rat small intestine *in vivo*, suggesting that nutrients signalling to the brain is transmitted by mesenteric afferents. In recent years, there has been growing evidence that enteral nutrients, particularly lipids, act as stimuli to activate a nutrient induced anti-inflammatory pathway via CCK receptors on the periphery nerve fibres (Luyer et al., 2004, Luyer et al., 2005). Luyer et al. 2005 showed that administration of enteral lipid-enriched nutrients caused attenuation in inflammatory response which was reversed by vagotomy or application of CCK antagonist, indicating involvement of mesenteric afferent and CCK receptors. There is however, little direct electrophysiological data supporting this hypothesis. Besides, it is also apparent from the literature that in vitro electrophysiological studies analysing the effect of lipid enriched nutrients on GI extrinsic afferents activities are lacking, especially in mice. For these reasons it was the aim of this thesis to further our understanding of how enteral nutrients, lipids, can be detected and subsequently influences afferent signalling, which in turn may affect the proposed nutrient-induced anti-inflammatory pathway in order to develop new therapies for systemic inflammation.

Lipid-containing nutrients augment jejunal mechanosensitivities via a CCK-1 mediated vagal pathway

This part of the project was undertaken in collaboration with Professor Buurman's lab in Masstricht. They further investigated the nutrient induced anti-inflammatory pathway described in the Luyer's study (Luyer et al., 2005) using a mice of model of sepsis. Lubbers et al. 2010 showed that pre-consumption of lipid-containing nutrients, especially lipid enrich

nutrient, significantly reduced systemic level of pro-inflammatory mediators (TNF-α and LI-1β) released by macrophages, indicating the protective effect of lipid nutrients on inflammation (Lubbers et al., 2010b). They also showed that in mice, this lipid nutrient induced anti-inflammatory pathway is mediated by CCK-1 receptor via peripheral activation of vagal afferents, showing that the protective effect of lipid nutrient was abolished by deafferentation with capsaicin or administration of CCK-1 receptor antagonist (Lubbers et al., 2010b, Lubbers et al., 2010c). In order to investigate this further, particularly to understand the underlying transduction mechanism of intestinal afferents in lipid sensing, in chapter 3 the direct effect of lipid enriched nutrients (same nutrients used in inflammatory study) on afferent chemo- and mechanosensitivities were investigated. Lipid containing nutrients-induced changes in basal afferent activity was used as a parameter to gauge chemo-sensitivity whereas intraluminal distension-evoked afferent responses were used to reflect any changes in mechanosensitivity. Intraluminal applications of lipid containing nutrients, notably lipid-rich nutrient, had a profound effect on extrinsic afferent mechanosensitivity, but surprisingly no direct effect on chemosensitivity. Using sophisticate and comprehensive single unit analysis indicated that lipid containing nutrients caused enhanced mechanosensitivity in three distinctly different subpopulations including low threshold (LT), wide dynamic range (WDR) and high threshold (HT) units. Chapter 3 also showed that lipid nutrients induced augmentation in afferents mechanosensitivity was mediated via endogenously released CCK in a paracrine fashion via a vagal pathway comprising mainly of the LT subpopulation. These data demonstrate for the first time that jejunal vagal mechanoreceptors are directly sensitive to lipid via a CCK signalling pathway.

The jejunal vagal mechanoreceptors described in chapter 3 actually resembles a set of vagal mechanoreceptors found in the upper GI system, described as gastric/duodenal vagal mechanoreceptors and were identified throughout a series of classic studies carried out by Schwartz's team back to the 1990s (Schwartz et al., 1991, 1993, 1994, Schwartz and Moran, 1994, Schwartz et al., 1995, Schwartz and Moran, 1996, 1998). They used a rat in vivo model

to examine the potential interaction of signals arising from gastric/duodenal load and CCK administration. They showed that this is an integrative effect in which the mechanosensitivity of gastric/duodenal vagal afferents was potentiated and amplified by application of exogenously CCK. A later study suggested that the integrated signals arising from CCK and duodenal loads might play an important role in the regulation of gastric empty and the control of food intake; they showed that nutrient (casein)-elicited endogenous CCK was able to synergized with duodenal load in order to potentiate the activities of gastric vagal mechanosensitive afferents (Schwartz and Moran, 1996). This correlates with the current study, in which jejunal vagal mechanosensitivity was amplified by endogenous CCK triggered by lipid nutrients. These results support the idea of the existence of the polymodal jejunal vagal afferents, which are sensitive to both endogenously released CCK and mechanical stimuli (distensions). They may act as 'safe guards' in the jejunum to ensure efficient digestion and absorption of lipid/fat. In the current study, only proximal jejunum, the segment immediately adjacent to duodenum, was used. This suggests they may mimic the behaviour of the duodenal mechanoreceptors to provide extra control on the amount of food entering the duodenum from stomach.

Chapter 3 also describes a series of experiments investigating the basic pharmacological effect of CCK (sulfated CCK-8) on GI afferent activity in vitro. The functional properties of CCK and its effect on GI functions, especially those related to nutrient absorption, have been studied for decades using *in vivo* methods. CCK receptors are widely distributed in periphery and also on the nearby organs. It has been shown that CCK can act directly on central vagal afferent terminals (Rogers and Hermann, 2008); CCK also exerts a role in circulatory control (Sartor and Verberne, 2008). Hence, a large number of confounding factors are present when using in vivo methods. In the current study, a well-established in vitro jejunum preparation has been used, which offers some advantages over in vivo methods. It is designed to target specific pathway of the gut-brain axis, which in this case is the afferent pathway, allowing for easier access to jejunal receptive fields; it prevents any possible blood-borne factors in any reflex

actions generated via CNS and neighbouring organs; it also gives a better control over the local environment as the medium can be tightly monitored. The tissue viability was evident since distension induced mechanosensitivity was reproducible throughout the experimental time span. Exogenously application of CCK caused a profound CCK-1 receptor mediated increase in baseline chemosensitivity, but it had no effect on afferent mechanosensitivity. These data are in agreement with the early in vivo studies, and fit into the characterized response profile of GI afferent activity to exogenous CCK. Blackshaw *et al*.1990 demonstrated that in the ferret gut, only vagal mucosal receptors were directly sensitive to CCK-8 (Blackshaw and Grundy, 1990). Later, Richard *et al*.1996 showed that in rat, CCK-sensitive afferents located from jejunum had mucosal receptive field, indicated by lack of response to luminal distension (Richards et al., 1996). Together, this shows that in the current CCK studies, only vagal mucosal receptors but not mechanoreceptors are directly sensitive to exogenous CCK. The mucosal afferents are sensitive to light stroking, but insensitive to distension, contraction and compression (Beyak et al., 2006). The mucosal afferents plays a major role in the reflex mechanism via the vagus to control GI functions and regulate food intake (Grundy, 2002). The release of CCK from enteroendocrine I cells is triggered by luminal passage of fats/lipids and proteins (Buchan, 1999). Eastwood et al. 1998 showed that casein acid hydrolysate (CAH, a type of protein) induced endogenous CCK only activate vagal mucosal afferents, but had no effect on mechanoreceptors; it was also via CCK-1 receptor in a paracrine fashion (Eastwood et al., 1998). Their findings are in contrast with the lipid study described in chapter 3. Those data suggested that the transduction mechanism of protein sensing is different from lipid-sensing. Data from the current lipid study suggests that lipid containing nutrient-induced endogenous CCK act directly on the nerve terminals of muscular afferents embedded within the gut wall. Those muscular nerve terminals response to passive stretch and active contraction of muscle (Beyak et al., 2006). IMAs and IGLEs are the proposed vagal mechanoreceptors in the GI tract (Grundy, 2002). Zagorodnyuk *et al*. 2001 identified IGLEs, but not IMAs as the mechanotransduction site of the low threshold, slow adapting vagal mechanoreceptors

(Zagorodnyuk et al., 2001). Therefore, it is likely that lipid-containing nutrients have a direct effect on the IGLEs.

Early studies showed that CCK occurs in multiple molecular forms; the major ones are CCK-58, CCK-33, CCK-22 and CCK-8 (Sayegh and Ya-Xiong, 2013). This might suggest that administration of enteral lipid and protein trigger release of different types of CCK molecules, which then activate different nerve fibres even though this may be via the same receptors. Several functional studies demonstrated that different molecular forms of CCK indeed cause differential physiological effects on animal/human behaviours. For example, in rats, even though CCK-58 and CCK-8 exhibit similar actions at a cellular level, such as calcium signalling, zymogen secretion and cell fate (Criddle et al., 2009), they differ in their effects on nocturnal solid meal pattern (Goebel-Stengel et al., 2012); CCK-33 was a more effective satiety peptide than CCK-8, but only CCK-33 reduced food intake by prolonging the intermeal interval (Washington et al., 2011). It has also been demonstrated that the existence of each molecule type of CCK varies significantly among species. Eberlein et al. 1998 did a thorough comparison with different subjects; they showed that CCK 33/39 and smaller CCK-forms (CCK-8) were dominant in rat, pig and beef intestinal mucosa, while CCK-58 was the most abundant peptide found in upper small intestinal mucosa of man, dog and cat (Eberlein et al., 1988). Their data actually collaborates with Eastwood's study. Eastwood et al. 1998 used segments of rat jejunum (Eastwood et al., 1998) where presumably the presence of smaller CCK form (e.g.CCK-8) in the mucosa is dominant. The data from the current lipid studies of a mice model is totally opposed from the CCK-8 and previous CAH studies, it might indicate that whatever lipid-induced endogenous CCK type is, it may not be CCK-8, but rather some other molecular type of CCK that could be predominant in the mice, and has a direct effect or act as a secondary signalling molecule on vagal jejunal mechanoreceptors. However, further studies are required to identify this group of CCK molecules in mice since there is little information in literature.

Lipid nutrient sensation disappeared with aging

Ageing is associated with multiple dysfunctions in the immune system which puts the vulnerable elderly population to a higher risk of infection (Saltzman and Peterson, 1987). In order to investigate whether ageing has an impact on the underlying mechanism of the previously described nutrient induced anti-inflammatory pathways (as illustrated in chapter 3), the effect of lipid enriched nutrients on jejunal afferent mechanosensitivity was investigated in an aged mice population of 18 and 24 months old. In chapter 4, two major pieces of evidence were obtained. (1) Jejunal afferent mechanosensitivity to distension was significantly reduced in aged mice. (2) The lipid enriched nutrient induced augmentation on jejunal mechanosensitivity was abolished in the aged population.

There are relatively few studies that have directly investigated the effect of ageing on the sensory pathway. One previous study found decreased perception of fullness, abdominal discomfort and bloating to gastric distension in older than younger human subjects (Rayner, 2000). Another study also reported an age-related reduction on both chemosensitivity and mechanosensitivity of human oesophageal visceral afferent (Yamasaki et al., 2013). Interestingly, a more recent study from our lab found increased bladder mechanosensitivity in aged mice than in younger controls (Daly et al., 2013). Those data indicates that ageing does have an effect on sensory nerve pathway and it varies among different species and organs. The small intestine is the major organ for nutrient absorption, and the innervated extrinsic afferents in the upper GI tract plays the central role in the regulation of various physiological and pathophysiological behaviours such as digestive function and pain perception (Beyak et al., 2006). However, there is currently no direct study within the literature to investigate any significant alterations on small bowel afferents nerve activities associated with ageing. The current study provides the first piece of electrophysiological evidence showing jejunal sensory pathways is altered with age and it has a significant impact on nutrient sensation.

There are several components that may contribute to this reduction in jejunal mechanosensitivity. (1) Age-related changes in sensory nerve morphology and function. One previous study found vagal afferent terminals in the gut undergo dystrophic and regressive changes with age (Powley et al., 2010). They reported that the organization of vagal muscular afferent terminals including both IGLEs and IMAs, as well as the vagal mucosal afferent terminal at the target tissue express age-related morphological alterations. (2) Loss of vagal afferents with age. In some previous studies loss of visceral afferent neuronal cells bodies in nodose ganglion and DRG have been described, however, the results are controversial (Vega et al., 1993, Phillips et al., 2010). Those changes in vagal afferents may also contribute to the diminished lipid-nutrient sensation found in the current ageing study. The changes of the vagal afferent terminals could lead to the potential anatomical disruption of the well-orchestrated signalling pathway in nutrient sensation, in which lipid nutrient induced endogenous CCK cannot activate on the nearby vagus nerve causing a significant reduction on the sensory nerve activity. This will cause disruptions on the regulation of general GI function such as gastric empty and food intake. Another possible outcome could be that extra endogenous CCK is released into the endocrine system causing an elevated plasma CCK level in aged population. Those predications actually fit into the classic physiological phenomenon known as the 'anorexia of aging'. It is described as a physiological decline in food intake with age, CCK levels are increased and elder populations are more sensitive to the satiating effects of CCK than younger generations (MacIntosh et al., 2001). Further studies required to confirm those predictions as it is not possible to determine those factors using the techniques in the current study.

The data obtained from the current lipid nutrient studies in aged mice is of particular significant in the development of new therapies for systemic inflammation. The current data suggests aged jejunal afferents are not responsive to lipid-rich nutrient. It shows that ageing indeed has a remarkable impact on the underlying mechanism of the lipid-nutrient induced anti-inflammatory pathways. The protective effects of lipid nutrients on systemic inflammation

disappear with ageing process. Therefore, this proposed new therapy for inflammation is ineffective for elderly patients. Instead, it may worsen their conditions, such as further reduction on food intake. This will result an increased risk of malnutrition.

The role of TRPV1 in lipid nutrient sensation in an aged population of mice (12 months old)

TRPV1 is viewed as a molecular integrator of physical and chemical stimuli in the peripheral pain perception (Tominaga et al., 1998). A previous study from our lab showed attenuated afferent sensitivity to mechanical distension in TRPV1 KO mice suggesting a role of TRPV1 receptors in control of gut sensitivity (Rong et al., 2004). In a later study, Phillis et al. 2009 reported an excitatory role for TRPV1 in modulating high-threshold colonic afferent mechanosensitivity only in an inflamed status (Phillis et al., 2009). TRPV1 receptors are well known for their role in anti-inflammation. Murai *et al*. 2008 showed that in adult animal, activation of TRPV1 on sensory nerve could potently inhibit production of LPS-induced TNF-α (Murai et al., 2008). Guptill *et al.* 2011 further confirmed the anti-inflammatory role of TRPV1 in adult mice using a combination of genetic manipulation and pharmacological blockade with capsazepine (Guptill et al., 2011). A more recent study reported ageing has a significant impact on the functionality of TRPV1 channels which they speculated that ageing could reverse the role of TRPV1 receptor in systemic inflammation from anti-inflammatory to pro-inflammatory (Wanner et al., 2012). In chapter 3 and 4, the transduction mechanisms of the lipid-nutrient induced anti-inflammatory pathways in adult and aged population were discussed. It is important to investigate whether TRPV1 receptors are involved and how they are being affected by age.

This study is the first report of recordings from murine jejunal afferent nerves in 12 months old TRPV1 KO mice. Three major findings are reported in chapter 5. (1) Lipid-nutrient sensation is attenuated in 12 months old mice. This finding is in agreement with data described in chapter 4. This indicates that aging has a profound impact on lipid sensing and it exerts its

effect as early as 12 months old. A previous morphological study reported nerve fibres start to show aged-related changes at this time point. However, there is currently no evidence in the literature describing how the afferent innervations are affected in 12 month old. It is unlikely to be caused by a reduction on jejunal mechanosensitivity to distension since the jejunal mechanosensitivity in 12 months old mice is unaltered comparing with younger adult mice. We speculate that, at the age of 12 months old, the vagal afferents innervations undergo mild aged-related morphological changes that are sufficient enough to have an impact on lipid nutrient sensation. (2) Jejunal afferent mechanosensitivity was not altered in 12 months old TRPV1 KO mice. This is in contrast to a previous study by Rong *et al*. 2004 reporting an attenuated afferent mechanosensitivity in TRPV1 KO mice (Rong et al., 2004). Rong *et al*. 2004 used a younger TRPV1 KO mice (3 month) comparing to the current study (18 months) suggesting ageing indeed altered the functionality of TRPV1 receptors in control of afferent sensitivity. (3) Lipid rich nutrient induced augmentation on jejunal mechanosensitivity was preserved in 12 months old TRPV1 KO mice. In this study, I found that this augmentation on afferent sensitivity to lipid–rich nutrient was mediated via a CCK independent mechanism, in which only WDR and HT fibres are responsive to lipid rich nutrients. It is important to bear in mind that TRPV1 receptors are mainly involved in mediating pain perception through spinal afferents. Booths et al. 2001 described HT fibres being mainly spinal afferents (Booth et al., 2001). These data suggests TRPV1 receptors may play an important role in mediating lipid sensing in the aged population. This finding implies the potential role of TRPV1 receptors into the proposed nutrient induced anti-inflammatory pathway for the elderly. We speculate that in the aged population, if we gave the patient a combined treatment of TRPV1 antagonist and a lipid-rich enteral nutrient, it may restore the protective effect of dietary nutrients in the elderly population.

Future studies and conclusion

All experiments were performed using non-inflamed mice tissue. It is important to examine if there are any changes of lipid-induced mechanosensitivity in inflamed mice tissue. An early clinical study showed that an elevated plasma concentration of CCK was detected associated with a human proximal gastrointestinal pathological infection known as Giardiasis (Leslie et al., 2003). Also, another study suggested that the secretion of CCK in an infected mouse model of Trichinella spiralis, was dependent on CD4+ T-cells which via the release of IL-4 and IL-13(McDermott et al., 2006). Moreover, a previous study from our lab using the Trichinella spiralis infected mice model showed that there was an altered jejunal chemo- and mechanosensitivity compared to the infected controls (Keating et al., 2008). From this study, an initial hyposensitivity was observed during acute infection period, and then followed by long-term hypersensitivity in the post infectious period. All those previous data suggested that the jejunal sensitivity and probability the bioavailability of CCK will be changed in the inflamed tissue. Unfortunately, I was unable to carry out this study using inflamed tissue, due to the unavailability of infected mice.

In conclusion, this study has advanced our understanding of biological mechanisms underlying gut afferent signalling in nutrient sensing at single nerve fibre level. Our data are consistent with the hypothesis that lipid nutrients have direct effects on jejunal afferent mechanosensitivity. In this study we show that lipid nutrient induced augmentation on mechanosensitivity is mediated via CCK-1 receptor signalling pathway. In this study, I also show that ageing causes diminished lipid nutrient sensation via low threshold vagal afferents. This is associated with attenuated jejunal afferent mechanosensitivity. Altered functionality of TRPV1 receptors is also reported in this study which indicates it may also contribute to the altered nutrient sensation in aged population. However, more studies are still required to fully validate this hypothesis. Understanding the underlying transduction mechanism of nutrient sensing and how it has been affected by aging is of particular important in future development of new treatments, such as new therapy for systemic inflammation for elder populations in hospital care.

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