THE IMPACT OF GENETIC BACKGROUND AND RESOLVED INFECTION WITH *TRICHURIS MURIS* ON THE ENTERIC NERVOUS SYSTEM IN MICE

A thesis submitted to The University of Manchester for the degree of

Doctor of Philosophy

in the Faculty of Biology, Medicine and Health

2017

VICTORIA J KINSLEY

SCHOOL OF BIOLOGICAL SCIENCES/ Division of Neuroscience and Experimental Psychology

List of Contents

Word count- 55,091

List of Tables

List of Figures

Abstract

The University of Manchester

Victoria J Kinsley

A thesis submitted to The University of Manchester for the degree of

Doctor of Philosophy in the Faculty of Biology, Medicine and Health

The impact of genetic background and resolved infection with *Trichuris muris* on the enteric nervous system in mice.

20th September 2017

Introduction

The gastrointestinal tract is so extensively innervated that it has been referred to as the 'mini brain' and can function independently of central nervous system control. It is also well known that there is a bidirectional link between the immune system and the nervous system that can affect response to pathogenic invasion. Despite the potential importance of the neuro-immune link in susceptibility to gut disease, the impact of host genetics and resolved infection on the enteric neural map is poorly understood. We investigated these factors using two mouse strains with known differences in peripheral nervous innervation (AKR and C57BL/6) and resolved infection with a Th1 or Th2 immune profile in the resistant C57BL/6 strain following infection with a high (Th2) or low (Th1) dose of *Trichuris muris*

Materials and Methods

We measured gross and microscopic anatomical features, such as gut length and muscle thickness, faecal pellet parameters such as number, weight, length, water content and transit time, and neuroreceptor expression, to examine differences between groups in gut physiology. Enteric neuronal density was quantified using histological and immunohistochemical techniques. Due to potential impact on neuronal cell survival, enteric glial cell number and nOS expression was quantified in infection groups.

Results

We identified significant differences in gut transit time, faecal pellet length and enteric neuronal density between AKR and C57BL/6 mouse strains. We also identified significant differences in transit time, faecal water content and enteric neuronal density in C57BL/6 mice following resolution of infection.

Conclusion

Results suggest that genetic background and resolved infection affect gut physiology and enteric neuronal density. This may be reflected in a human population affecting susceptibility to and recovery from infection-driven pathology.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

List of Abbreviations

AGE-	advanced glycation end products
ANS-	autonomic nervous system
BDNF-	brain derived neurotrophic factor
BMI-	body mass index
CCK-	cholecystokinin
CGRP-	calcitonin gene related peptide
ChAT-	choline acetyltransferase
CNS-	central nervous system
DiI-	1,1'-dioactadecyl-3,3,3',3'-tetramethylindocarbodyanine perchlorate
EAE-	experimental autoimmune encephalomyelitis
ELISA-	enzyme-linked immunosorbent assay
eNOS-	endothelial nitric oxide synthase
ENS-	enteric nervous system
FACS-	fluorescence-activated cell sorting
GABA-	gamma aminobutyric acid
GDNF-	glial cell-line derived neurotrophic factor
GFAP-	glial fibrillary acidic protein
GI-	gastro-intestinal
GPCR-	G protein coupled receptors
GWAS-	genome-wide association study
IBD-	inflammatory bowel disease
IBS-	irritable bowel syndrome
IFAN-	intestinofugal afferent neuron
IGLE-	intraganglionic laminar ending
IMSP-	intermediate submucosal plexus
iNOS-	inducible nitric oxide synthase
IPAN-	intrinsic primary afferent neuron
ISP-	inner submucosal plexus
MLCK-	myosin light-chain kinase
MP-	myenteric plexus
NADPH-	nicotinamide adenine dinucleotide phosphate

NADPH-D-	nicotinamide adenine dinucleotide phosphate- diaphorase
NFKB-	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF-	nerve growth factor
NMDA-	N-Methyl-D-aspartate
nNOS-	neuronal nitric oxide synthase
NO-	nitric oxide
NOS-	nitric oxide synthase
NPY-	neuropeptide Y
OCT-	optimal cutting temperature compound
OSP-	outer submucosal plexus
PGP9.5-	protein gene product 9.5
PNS-	peripheral nervous system
RAGE-	receptor for advanced glycation endproducts
RAMEN-	rapidly activating mechanosensitive enteric neurons
ROS-	reactive oxygen species
SCID-	severe combined immunodeficiency
SIM1-	single-minded homolog 1
SP-	substance P
SPF-	specific pathogen free
TLR-	toll-like receptor
TNBS-	2,4,6-trinitro benzesulfonic-acid
TRPV1-	transient receptor potential cation channel, subfamily V, member 1
VIP-	vasoactive intestinal peptide

Acknowledgements

With thanks to the Biotechnology and Biological Sciences Research Council and The University of Manchester for funding and supporting this award.

Many thanks for all of the tuition, advice and support to all of the following:
Dr Peter March, Dr Steve Marsden and Roger Meadows in the Bio-Imaging Department,
Pete Walker in the Histology Department,
Nic and Anthony Steel in the Stopford Labs,
Staff in the Biological Services Unit,
Everyone on the 4th floor, AV Hill Building.

Also, many thanks to my advisor, Dr Natalie Gardiner, and my supervisors, Dr Jaleel Miyan and Dr Joanne Pennock, who have been essential in both my own personal success and the success of the project. With great thanks and appreciation.

Dedication

This thesis is gratefully dedicated to the people in my life who have enabled me to complete it:

Firstly, to my advisor and supervisors, for tuition, guidance and support. Particular thanks goes to Dr Joanne Pennock for the encouragement and advice prior to entering PhD study, and who provided me with the chance to begin.

Secondly, to my family and friends for the many years of emotional, moral and financial support that I have been lucky enough to receive, and in anticipation of the many more yet to come. You are all invaluable.

Thirdly, to my little niece Lilly who is obviously wonderful and provides hours of endless joy and fun. Also to the new niece or nephew who will not be born until after this thesis is submitted- hello there!

Finally, to my children, for the constant encouragement, disinterest, fun, anger, support, ridicule, gossip, deep meaningful conversation and generally keeping life interesting. I can only aspire to give you as much inspiration as you give me. With constant and endless love.

Chapter 1
Introduction

1.1 The gut nervous system, the immune system and the microbiome

The nervous system, the immune system and the gut microbiome are vital in maintaining and sustaining a healthy functioning body. The nervous system acts as the main control centre of the body. The immune system combats pathogenic challenge and disease. The microbiome contributes towards a healthy gut environment. Each of these individual systems are required for normal function and maintenance of health. It is now emerging that these three body systems may be related and indeed, that each may be capable of influencing the others. This insight offers novel ideas about health, our understanding of immune challenge and subsequent recovery from gastro-intestinal disease and thereby contributing to an explanation of the wide variations found in susceptibility to disease between individuals of the same species. Here we aim to investigate the physiology and anatomy of the neural network of the gut in different strains of mouse and in the same strain of mouse following resolution of infection with two differing immune profiles (T helper 1 and T helper 2). It could be that enteric neural physiology and anatomy is predetermined by genetic factors that may vary between different strains of the same species, and this neural map may be altered epigenetically throughout life by environmental factors such as stress, ageing and the microbiome. If the neural network is linked with the immune response, environmental factors that change the neural map in the gut, such as previous infection, may therefore change host response to gastro-intestinal challenge. If this is the case, this research could greatly further our understanding of the influence of any variation within the enteric nervous system and possible implications of this on normal gut function, immune response and in disease susceptibility.

1.2 The nervous system and the gastro intestinal tract-

1.2.1 Organisation and anatomy

The nervous system consists of the central nervous system (CNS) - brain, spinal cord, retina and cranial nerves, and the peripheral nervous system (PNS) - nerves and ganglia outside of the spinal cord communicating with the CNS (Figure 1.1).

The somatic division of the PNS regulates movement whilst the autonomic division of the PNS, the autonomic nervous system (ANS) can be subdivided into the

sympathetic, parasympathetic and enteric systems; this controls involuntary, automated processes such as heart rate and blood pressure (Wood et al., 1999). The sympathetic branch of the ANS is associated with the 'fight or flight' response and utilises the noradrenergic pathway (Ulrich-Lai and Herman, 2009). Postganglionic nerve fibres release epinephrine and norepinephrine; this binds with adrenoreceptors and can result in increased heart rate, vasodilatation in muscles and vasoconstriction in the gut (Elenkov et al., 2000). Sympathetic nerve fibres have been identified in several areas of the gastro intestinal (GI) tract, notably much of the colon (Mackner et al., 2011). The parasympathetic branch of the ANS controls biological functions such as return of heart rate to normal following activity, hormone secretion, digestion and gut peristalsis, also known as 'rest and digest' activities (van der zanden et al., 2009) via the vagus nerve following release of acetylcholine and activation of the cholinergic pathway (Felten et al., 1988; Borovikova et al., 2000). Parasympathetic nerve fibres have been identified in the GI tract including the stomach, small intestine and proximal colon (van der zanden et al., 2009). The enteric nervous system (ENS) is sometimes known as the gut minibrain (Wood, 1994); it possesses sensory and motor neurons capable of controlling reflex actions such as bloodflow, secretion and GI motility (Blackshaw et al., 2007) independently of the CNS (Schemann et al., 2001).

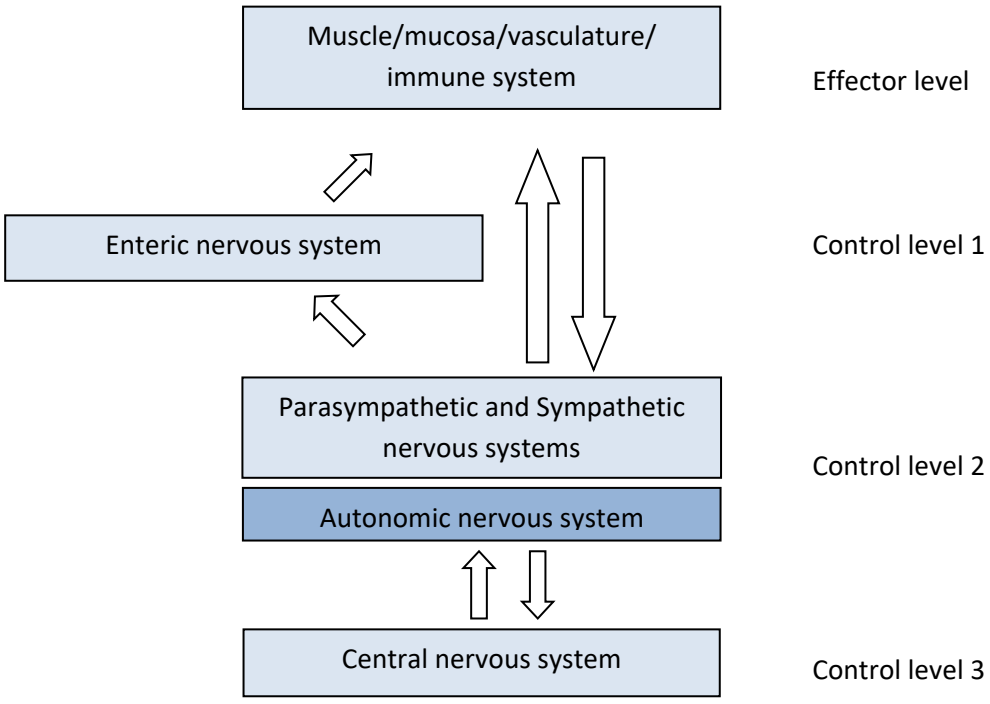


Figure 1.1 Hierarchy of neural control in the gut. The first level of control is the enteric nervous system capable of operating independently and considered a division of the ANS. The parasympathetic and sympathetic nervous systems are the second level of control conveying information to and from the CNS. Higher brain centres of the CNS coordinate integrative functions (adapted from Straub et al., 2006).

The gastrointestinal (GI) tract is the site of digestion, responsible for absorption of nutrients, water and electrolytes and removal of waste; it is a muscular tube lined with a mucous membrane roughly 9 metres in length in humans that passes from the mouth to the anus. It consists of the mouth, pharynx, oesophagus, stomach, the small intestine (duodenum, jejunum and ileum) and large intestine (caecum, colon, rectum, and anus) (Gray, 1918). The microscopic anatomy of the GI tract varies slightly from region to region; for the purposes of this report we will focus on gut anatomy within the small and large intestine. The outer layer of the gut, the serosa, consists of connective tissue. Underneath this lies the muscularis consisting of longitudinal and circular muscle. We then find the submucosa consisting of

connective tissue, lymph and blood vessels. The final layer of the gut wall prior to the lumen is the mucosa and includes the muscularis mucosae, lamina propria and epithelium, the barrier between the gut contents and host. In the small intestine, the epithelial surface is folded into finger like projections known as villi; this maximises gut surface area for nutrient absorption (Gelberg, 2014). Enteric ganglionated nerve plexuses can be found within the myenteric plexus (located between the circular and longitudinal muscle) and submucosal plexus (located between the mucosa and circular muscle) with axonal projections directed towards the epithelial surface (Figure 1.2). As with any mucosal membranous surface, the gut is lined with a layer of mucous which forms a physical barrier over the delicate epithelial surface protecting against potential damage from luminal contents and microbial invasion; within this layer the symbiotic commensal gut bacteria that comprise the microbiome can be found (Lennon et al., 2014).

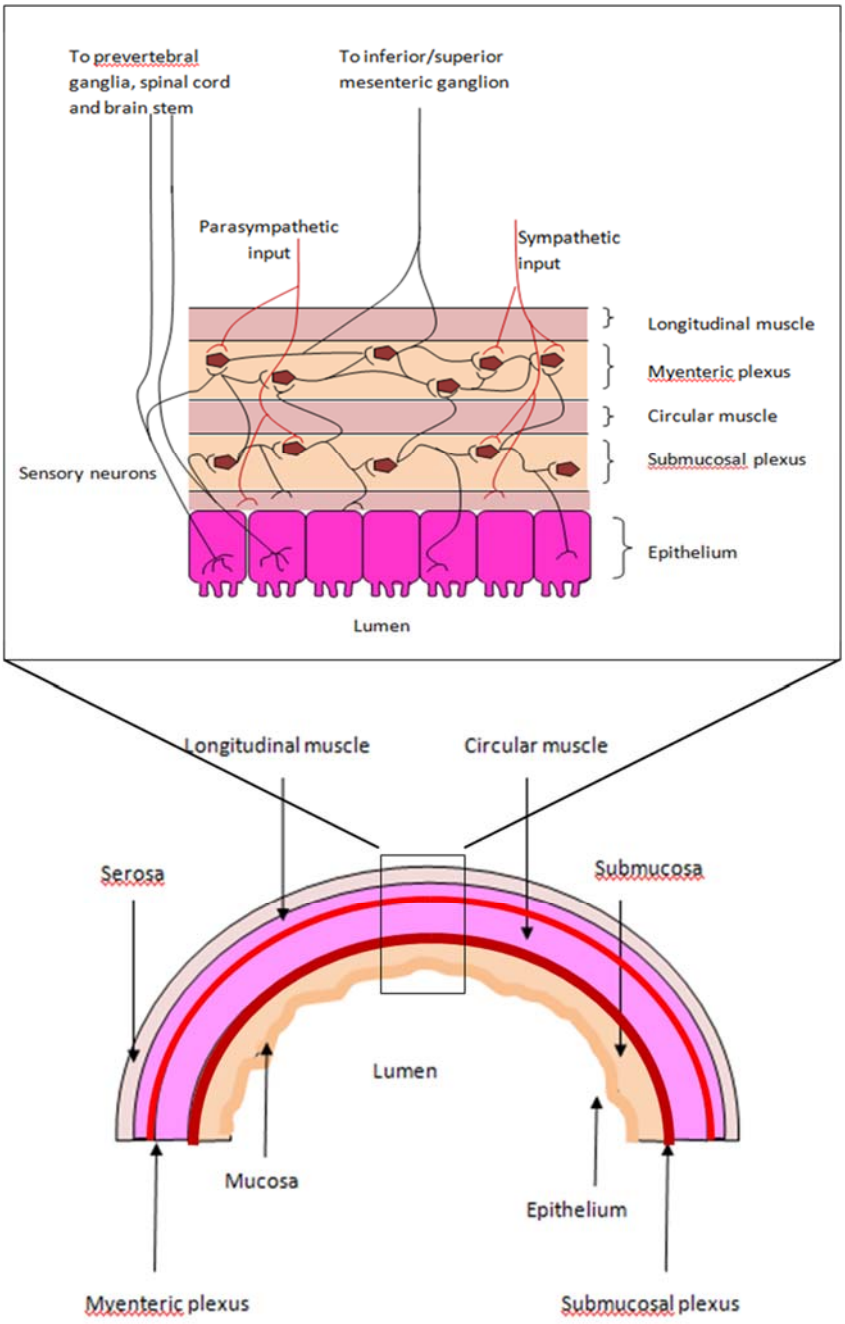


Figure 1.2 Cross section of the gut wall. The outer layer of the gut is the serosa, followed by the muscularis, submucosa and mucosa before reaching the epithelium, the barrier between the gut contents and host. Nerve cell bodies can be found within the myenteric plexus that lies between the longitudinal and circular muscle, and the submucosal plexus found beneath the mucosa, with axonal projections branching towards the epithelial surface.

1.2.2 Enteric neuronal subsets

Neurons innervating the gut have been divided into several subsets; within these subsets, there are distinctions based on morphology and polarisation. For instance, the majority of neurons are described as Dogiel type I or type II; the former are uniaxonal whereas the latter have many processes (Metzger, 2010). Neurons have also been divided into S (synaptic) and AH (after hyperpolarisation) categories (Hirst et al., 1974). S neurons have high synaptic output with a short after hyperpolarisation potential, are usually of the Dogiel type I morphology and have been identified within the myenteric plexus (Hirst et al., 1974; Smith et al., 1992; Smith et al., 1999). AH neurons have one large action potential with a long after hyperpolarisation potential, are usually of the Dogiel type II morphology and respond to changes in chemistry and contractility (Hirst et al., 1974; Neunlist et al., 1999; Kunze et al., 1999).

1.2.2.1 Extrinsic and intrinsic innervation

Extrinsic innervation, subdivided into sympathetic and parasympathetic, has been found throughout the gut and is vital in maintenance of normal function (Chen et al., 2015). Sympathetic efferent stimulation downregulates gut function affecting motility and absorption allowing blood flow to be redirected from the gut, possibly to support a 'fight or flight' situation (Lomax et al., 2010). Preganglionic sympathetic neurons project from the intermediolateral column of the spinal cord and form synapses with postganglionic sympathetic neurons within the prevertebral and paravertebral ganglia (Jänig, 1988); these neurons then project to the myenteric and submucosal plexuses of the intestine where they influence the action of intrinsic enteric neurons (Lomax et al., 2010). Parasympathetic afferent innervation conveys information regarding satiety, defaecation, chemical or noxious stimuli and pain from the gut to the CNS (Moynes et al., 2014; Brookes et al., 2016). Parasympathetic cell bodies located within either the nodose or jugular ganglions project to the gastrointestinal system via the vagus nerve, innervating mainly the upper GI tract, with little presence in the colon (Moynes et al., 2014). However, parasympathetic afferent innervation has been identified in both the distal colon and rectum from cell bodies located within pelvic ganglia (Chen et al., 2015). Intrinsic innervation regulates blood flow, secretion and motility, is primarily cholinergic and has been subdivided into several subsets including intestinofugal afferent neurons (IFANs) (Lomax and Furness, 2000), intrinsic primary afferent neurons (IPANs) (Kunze et al., 1999), rapidly adapting mechanosensitive enteric neurons (RAMENs) (Mazzuoli and Schemann, 2009) and colospinal or rectospinal afferent neurons (Suckow and

Caudle, 2008). IPANS, located in both the myenteric and submucosal plexuses with projections to the villi, respond to changes in chemistry or motility to regulate enteric reflexes by co-ordinating with interneurons and motor neurons (Moynes et al., 2014; Costa et al., 2000).

1.2.2.2 Nitrergic and cholinergic neurons

Neurons located in the myenteric plexus are thought to be involved in reflex mechanosensory regulation of GI muscles whereas neurons within the submucosal plexus are believed to control mucosal vaso/secretomotor processes and smooth muscle contractility (Lomax and Furness, 2000; Phillips and Powley, 2007). Neurons within the myenteric plexus have been categorised as either cholinergic (producing acetylcholine) or nitrergic (producing nitric oxide) (Mann et al., 1999); however, some colocalisation between nadph-d and acetylcholinesterase in enteric neurons of chicks has been identified that appeared to decrease with age, suggesting these two subsets of neurons are not consistently mutually exclusive (Cserni et al., 2007). In relation to gastrointestinal motility, nitric oxide (NO) is inhibitory and acetylcholine is excitatory (Nishiyama et al., 2014; Fornai et al., 2016). The neurotransmitter acetylcholine and lachykinin Substance P are associated with increased intestinal motility whilst NO regulates non cholinergic, non adrenergic relaxation; incubation with a nitric oxide synthase (NOS) inhibitor resulted in increase in gut tension in human and rat studies (Maggi et al., 1991; Calignano et al., 1992).

Both acetylcholine and nitric oxide have been associated with inflammation during an immune response. Acetylcholine is the natural ligand of the alpha seven nicotinic acetylcholine receptor; stimulation of this receptor, present on neurons and many types of immune cell, disrupts translocation of nuclear factor κ beta (NFκB) to the nucleus during an inflammatory response, reducing production of pro-inflammatory cytokines, such as IFNγ and TNFα. This is known as the cholinergic anti-inflammatory pathway (Tracey, 2002). Nitric oxide is involved in the pathophysiology of several conditions including autoimmunity, degenerative diseases and infection and it is known that several cells of the immune system both produce and respond to NO; however, its involvement in the immune response is still poorly understood (Tripathi, 2007; Bogdan, 2001). We will further examine the involvement of the sympathetic and parasympathetic nervous systems and associated neurotransmitters in the immune response later within this chapter.

1.2.2.3 Neuropeptide expression

Several neuropeptides have been identified and associated with enteric neuronal subsets including Substance P (SP), vasoactive intestinal peptide (VIP) and calcitonin gene related peptide (CGRP) (Hens et al., 2001; Brookes, 2001); there is known plasticity in expression of neuropeptides within the enteric nervous system (Ekblad and Bauer, 2004) and these neuropeptides are capable of influencing the immune response (Parkman et al., 1993; Ma and Szurszewski, 1996)

In the guinea pig intestine receptors for SP have been identified on epithelial cells and on VIP expressing submucosal neurons (Keast et al., 1985). SP was found to increase VIP levels in the small intestine in a feline model; this effect was mitigated by administration of the nicotinic receptor agonist hexamethonium or fast sodium channel blocker tetrodotoxin suggesting SP may act via cholinergic nerves or ion channels (Brunsson et al., 1995). However in the feline colon hexamethonium did not affect the contraction and vasodilatation caused by SP suggesting a noncholinergic mechanism of action in this area (Hellström et al., 1991). SP is involved in nociception (Mello et al., 2007) and has been linked with psychiatric conditions following identification of increased levels found in patients suffering from anxiety and stress (Geracioti et al., 2006). In the GI tract SP is implicated in vasodilation, secretion of electrolytes and water into the lumen (Kuwahara and Cooke, 1990), excitation and contraction (Barthó et al., 1994).

VIP is associated with secretion, relaxation and vasodilation; increased VIP levels and subsequent vasodilation was found in the rectum following stimulation of the pelvic nerve (Andersson et al., 1983). VIP has been found to be neuroprotective in the ENS; mice with trinitrobenzenesulfonic-acid (TNBS) induced colitis treated with VIP showed reduced pathology attributed to reduction in inflammation caused by downregulation of inflammatory cytokines such as TNFα and IL-6 and increased IL-10 levels (Abad et al., 2003). VIP also induces release of growth factors (e.g. neurotrophin 3) and inhibits neuronal cell death (Sandgren et al., 2003). Perhaps as a result of this, overexpression of VIP and VIP receptors has been found in many cancer cell lines including carcinomas, breast cancer tissue samples and metastases; VIP was able to further increase its own secretion when added to human breast cancer cells (Valdehita et al., 2010).

CGRP is commonly found alongside SP or acetylcholine (Rosenfeld et al., 1983; Wimalawansa, 2001). It is important in secretion, nociception and vasodilation (Arulmani et al., 2004; Straub et al., 2006). CGRP is released following stimulation

of transient receptor potential vanilloid 1 (TRPV1), which responds to capsaicin, heat (>43°C), acidity, lipids and is considered important in nociception (Assas et al., 2014); TRPV1 knockout mice were less sensitive to colonic distension than wild type mice (Jones et al., 2005). In the CNS, CGRP has been shown to increase release of SP and modulate release of and response to acetylcholine via upregulation of acetylcholine receptors (Oku et al., 1987; Rossi et al., 2003). The role of CGRP in the immune response has yet to be fully elucidated, however, it is known that many immune cells, including T cells, B cells, monocytes and macrophages, can both produce and respond to CGRP (Engel et al., 2011; Assas et al., 2014); exposure of murine endothelial cells to CGRP in culture skewed T cells towards a Th17 lineage and decreased interferon γ (IFNγ) production (Ding et al., 2016) and CGRP increased tumour necrosis factor α (TNFα) production by mast cells (Niizeki et al., 1997). Administration of CGRP was found to be protective in a TNBS model of colitis in rats; interestingly, this effect was abolished by vagotomy (Mazelin et al., 1999). Some research has been unable to find any difference in CGRP levels between patients with ulcerative colitis and a control group (Renzi et al., 1998); however, more recently, reduced CGRP levels were identified as a potential marker for ulcerative colitis (Li et al., 2013).

It is clear that these neuropeptides fulfil a role in normal gut function; however, current evidence suggests each peptide also has a role, though currently poorly understood, in the neuro-immune link. Therefore, any variation in these factors could influence susceptibility and response to disease.

1.2.3 Enteric glial cells

Enteric neuronal cell bodies in ganglia, interganglionic processes and synapses are surrounded by enteric glial cells; the function of these cells is not yet clearly understood, however they are up to four times higher in number than neurons and have been compared with astrocytes in the CNS (Bassotti et al., 2007). Enteric glial cells are important in functional support and maintenance of enteric neurons, but can also influence neuromediator expression, cytokine secretion and gut wall integrity (Neunlist et al., 2014). The majority of enteric glial cells, particularly those within the myenteric plexus, express the astrocyte markers S100β and glial fibrillary acidic protein (GFAP), though these are not always co-localised (Aubé et al., 2006; Ochoa-Cortes et al., 2016; Boesmans et al., 2015).

The neuronal-glial-epthelial unit is integral in maintaining a healthy intestinal epithelial barrier, therefore disruption of this unit is likely to be related to gut pathology (Neunlist et al., 2013). Ablation of enteric glia resulted in severe gut inflammation, increased mucosal permeability and haemorrhagic necrosis resulting in death (Bush et al., 1998). Following co culture of human, rat and mouse intestinal epithelial cells with enteric glial cells, intestinal epithelial cell number was reduced but cell size increased and intestinal epithelial permeability was reduced (Soret et al., 2013). A significant reduction in enteric glial cell number was found in patients with severe constipation and idiopathic megacolon (Bassotti et al., 2007) and ulcerative colitis (Celikbilek et al., 2014a; Celikbilek et al., 2014b) suggesting a certain link between enteric glial cells and pathology. However, variation in enteric glial cell markers have also been linked with disruption in homeostasis within the gut. Increased levels of S100β were associated with upregulated macrophage recruitment and toll-like receptor activation leading to increased inflammation, and inhibition of S100β mediated glial cell activation has been suggested as an anti-inflammatory therapy (Esposito et al., 2014). Co-culture of rat colon with pro-inflammatory cytokines resulted in upregulated GFAP expression (von Boyen et al., 2004). However, upregulation of GFAP following inflammation was reduced in patients with ulcerative colitis and Crohn's disease compared with a control group; reduced GFAP and S100β levels were also seen in these patients in a steady state (Cornet et al., 2001) suggesting upregulation of GFAP and S100β may be part of a regulatory mechanism during inflammation. These factors suggest a definite role for enteric glial cells in gut pathology; however, whether changes in dynamic factors associated with these cells are the cause, or result of, pathology remains to be seen.

Natural variation in enteric glial cell numbers in different strains of healthy mice may provide an answer to the question of susceptibility, or may certainly contribute towards this answer. If these cells are able to modulate the enteric neural map in health or following a pathogenic challenge and the enteric nervous system affects the immune system, they may influence changes in susceptibility throughout life.

1.2.4 Absorption and motility

The gut is required to digest food, absorb nutrients, electrolytes and water and eliminate waste; this requires co-ordination between neuronal subsets and smooth muscle.

The small intestine, primarily the jejunum, is the site of nutrient absorption via diffusion through columnar epithelial cells of the villi and microvilli. Water absorption occurs within the colon; 1-2 litres of effluent passes through the ileo-caecal valve each day, and the colon absorbs up to 90% of this (Phillips and Giller, 1973). Water absorption is directly related to sodium absorption and the capacity for these processes varies segmentally, for instance, the proximal colon has a greater capacity for sodium and water absorption than the descending distal colon (Levitan et al., 1962). Absorption within both the small and large intestine is affected by nervous input; a reduction in fluid absorption and increased secretion via cholinergic nerves has been reported (Hubel, 1985), and stimulation of the sympathetic nervous system increased absorption within the jejunum of rats (Sjövall, 1984). Inflammation affects absorption both within the small and large intestine. Within the small intestine, inflammation affects villus and crypt size, permeability via tight junctions and metabolic activity (Peuhkuri et al., 2010). In patients suffering from IBD, absorption of sodium and chlorine is decreased, reducing the amount of water absorbed from the colon and leading to diarrhoea (Sandle et al., 1990).

Movement of the gut, known as peristalsis, is phasic and occurs throughout the GI system in response to size or chemical nature of luminal contents (Bitar, 2003). Peristalsis, consisting of orad contraction and caudad relaxation of the circular muscle, occurs following contraction of longitudinal muscle causing shortening of the gut and contents (Hennig et al., 1999). This requires interaction between the neural system, smooth muscle and interstitial cells of Cajal, which act as a pacemaker (Sanders, 1996). There are several small areas of gut, such as the oesophageal, pyloric and anal sphincters, where movement is tonic; these areas remain closed until neuronal input and luminal stretching facilitate opening, ensuring unidirectional movement of contents (Bitar, 2003).

Activation of the ascending excitatory and descending inhibitory pathways by stretch sensitive intrinsic mechanosensory neurons, possibly interneurons, initiates peristalsis (Spencer et al., 2002) and within the colon, it has been suggested that extrinsic primary spinal afferent neural input is vital (Costa et al., 2000). Contraction of smooth muscle is initiated by calcium sensitisation; intracellular calcium binds with calmodulin, which in turn activates myosin light chain kinase (MLCK). This then phosphorylates myosin regulatory light chain (MLC_{20}) which is responsible for smooth muscle contraction (Harnett and Biancani, 2003). This calcium may be intracellular, released by the sarcoplasmic reticulum, or influx into the cell via a calcium channel (Bolton et al., 1999). Binding of acetylcholine with nicotinic or

muscarinic acetylcholine receptors on smooth muscle of the GI tract initiates smooth muscle contractility via calcium influx into the cell (LePard and Galligan, 1999; Ehlert et al., 1999). Conversely, this calcium influx is also responsible for initiation of smooth muscle relaxation by nitric oxide (NO); calcium that binds to calmodulin then associates with endothelial nitric oxide synthase (eNOS) leading to electron flux and NO production (Zhao et al., 2015; Van Hove et al., 2009). Thus we can see that both acetylcholine and NO are essential in control of normal gut motility.

1.3 The role of genetics in the ENS

Evolution and the genetic variation that accompanies this is the basis for the infinite multitude of species inhabiting the planet. Darwinism suggests that within each of these species exists a mixture of individual organisms; those genetically primed to increase chances of survival until sexual maturity will reproduce ensuring passage of their DNA to the next generation and survival of the species. Within these species themselves lie infinite possibilities for genetic variation, some of which may not prove advantageous for survival. It is well documented that many pathologies are caused or influenced by predetermined specific genetic factors. Some of these can be pinpointed; for instance, inherited mutations in the BRCA1 tumour suppressor gene are associated with an 82% lifetime risk of developing breast cancer (King et al., 2003). However, when considering the gut and inflammatory bowel disease (IBD) specifically, over 163 associated gene loci have been identified in genome wide association studies (GWAS), with some new loci added thought to be heterogenous between populations (Liu et al., 2015). Therefore, GWAS have shown that IBD is not the result of a single genetic trait, but a polygenic disease.
If we look more broadly at the issue of health and disease susceptibility, the well documented link between the nervous system and immune system (Kelley & McCusker, 2014) may lead us to suggest that genetic variations between individuals that result in differences in nerve function and the neural map may impact the immune response, both in the gut and throughout the body. Given the vast amount of nerves within the gastro-intestinal system (Gershon & Erde, 1981) this is likely to be a major contributing factor in disease development. Could this be a reason why some individuals are affected by gut disease and others are not?

Evidence for genetic variation in the peripheral nervous system has been found within different strains of the same species in experimental mouse models, thus

allowing us to remove the effects of any environmental influence or experience that cannot be accounted for in a human sample. Mogil et al. (1999) found that in 12 different tests of nociception on 11 different mouse strains, geneology significantly affected response to stimulus. In further research, it was also found that each strain produced a varied response to a different category of noxious stimuli; this suggests that different neuronal subsets are responsible for response to different stimuli, and that the concentration or function of these subsets is affected by genetic input (Lariviere et al., 2002; Mogil et al., 1999b). The largest divergence in response to pain was seen between the AKR and C57BL/6 strains; on further examination this difference was attributed to expression of and response to CGRP (Mogil et al., 2005). It seems fair to suggest that these variations in the peripheral nervous system could extend to the enteric nervous system. Given that CGRP has a direct role in the neuro-immune link (Assas et al., 2014), the variation in CGRP between these two strains may be responsible for the varied response to infection with *Trichuris muris*, i.e., chronic infection in AKR mice and resistance in C57BL/6 mice (Blackwell and Else, 2002; Cliffe et al., 2007). This perhaps also suggests that genetic differences between individuals may influence susceptibility to and recovery from gut disease as a result of variation in enteric neuronal subsets.

1.4 Innervation and disease

Activation of both the sympathetic and parasympathetic extrinsic enteric neural pathways has been described as anti-inflammatory during disease processes. The sympathetic branch of the nervous system is thought to reduce inflammatory cytokines TNFα and IFNγ and associated pathology in the gut via β adrenergic mediated pathways (Straub et al., 2006). Activation of nicotinic acetylcholine receptors on parasympathetic nerves has also facilitated reduction in levels of inflammatory cytokines, such as IFNγ and TNFα, via the 'cholinergic anti-inflammatory pathway' (Borovikova et al., 2000; Tracey, 2002). This suggests a definite role for neuroimmunomodulation in recovery from disease. Interestingly, neuropathy is frequently associated with a variety of pathologies; however, whether some kind of neuropathy precedes and possibly contributes towards development of pathology remains to be seen. This would lead us to look more closely at development and maintenance of normal innervation and any factor that may disrupt this.

1.4.1 Neurogenesis

The pattern for innervation of the adult ENS begins in the embryo. Innervation of the GI tract is derived from multipotent stem cells that migrate from the dorsal neural tube following neurulation in the embryo; the Ret/GFRa1/GDNF pathway is important in facilitating a normal rostro-caudal pattern of neural crest cell colonisation of the gut, although this is not yet totally understood (Metzger, 2010). Mutations in the Ret and GDNF gene have been found in up to 50% of cases of Hirschsprung's disease, a condition characterised by congenital intestinal aganglionosis; however, as yet, no genetic cause can be found for up to 50% of cases (Tomuschat and Puri, 2015; Butler Tjaden and Trainor, 2013). Glial cell-line derived neurotrophic factor (GDNF) binds with the Ret receptor to facilitate proliferation of ENS cell precursors (Iwashita et al., 2003). Transgenic mice (GFAP-*Gdnf*) expressing increased GDNF levels in the CNS and enteric glia prenatally from E18 demonstrated an increase in submucosal neuron density of 19% in the small intestine and 70% in the colon; myenteric neurons increased by 28% in the colon but remained unaltered in the small intestine in comparison to wild type mice. This would suggest that changes in volume and timing of pre natal GDNF expression can affect the ratio of myenteric to submucosal neurons in the ENS. Once mature, GFAP-*Gdnf* mice with increased GDNF expression showed no change in size or distribution of most enteric neuronal subsets compared with wild type mice; however, increased cell size and fibre density of nadph-d+ VIP expressing neurons in both the myenteric and submucosal plexus was found, suggesting the importance of GDNF in maintenance and support of this subset of mature enteric neurons. Thirty day old mice injected with GDNF presented a 57% increase in NADPH-d neuron density; the authors speculate that this may not be solely due to precursor cell proliferation and that GDNF may be capable of affecting gene expression and changing cell fate (Wang et al., 2010).

It is known that in many species there is lifelong active neurogenesis in the CNS (Kuhn et al., 1996); however, in the ENS, evidence for development of new neurons has been confined to small areas of local damage despite the presence of neuronal stem cells (Joseph et al., 2011; Kruger et al., 2002). Studies demonstrated that following neural insult, administration of a 5-hydroxytryptamine 4 receptor agonist, mosapride, was able to re-establish neuronal connections, with evidence of new cell and neurofilament growth along with restoration of the defaecation reflex (Matsuyoshi et al., 2010; Takaki et al., 2014). Very recently it has been suggested that although neural number and subset types are thought to remain fairly constant,

neurogenesis occurs constantly within the ENS with a turnover of 5% per day in the small intestine of healthy adult mice (Kulkarni et al., 2017); however, this evidence is yet to be corroborated by other groups.

Factors that affect neurogenesis in the CNS throughout life with as yet poorly understood effects on the ENS include species, age, gender, stress, learning, exercise, sexual experience and the microbiome (Miller and Szurszewski, 1997; Schoenfeld and Gould, 2012; Kuhn et al., 1996; Ait-Belgnaoui et al., 2014). It could be that these factors may be capable of changing the enteric neural map and associated immune influencing soluble factors, therefore modulating susceptibility to disease throughout life, in a way that we do not yet understand. Stress associated reduced hippocampal neurogenesis in rats attributed to increased glucocorticoid levels was reversed by environmental enrichment, exercise and probiotics suggesting the possibility for reversal of stress associated effects on neurogenesis (Veena et al., 2009; Fabel et al., 2003; Ait-Belgnaoui et al., 2014). This alludes to an interesting possibility for a restorative solution for disrupted neurogenesis; however, any possible involvement of the ENS and the mechanisms behind these processes remains largely unknown.

1.4.2 Factors affecting innervation

1.4.2.1 Stress

If neuropathy is associated with many pathologies, factors that affect existing innervation will play a role in initiating change in neuronal number or subset and therefore may be important in creating disease susceptibility. One of these factors is stress. Stress is defined as a potential physical or psychological threat that results in activation of the sympathetic pathways to ready the organism for a 'flight or fight' response (Selye, 1998; Cannon, 1915). This leads to activation of a compensatory negative feedback in order to restore homeostasis; however, recurrent stress can result in the organism consistently failing to reach normal levels of neural functioning which leads to pathological changes (Chrousos, 2009). Within the CNS, chronic stress induced functional and structural changes to pyramidal neurons in the prefrontal cortex in an animal model; these changes were reversible following removal of stress (Goldwater et al., 2009; Radley et al., 2005). However, prolonged stress in monkeys resulted in extensive neuronal damage including reduced hippocampal size and loss of neurons within the dentate gyrus, due to

increased levels of neurotoxic gluco-corticoids (Uno et al., 1989). The ENS is also a target for pathology induced by stress; early life adversity has been associated with increased incidence of IBD, suggesting long term impact of stress on the gut nervous system (Bradford et al., 2012; Talley et al., 1994). In a porcine model, early life adversity increased the number choline acetyltransferase (ChAT) positive neurons and increased cholinergic activity within the ileum (Medland et al., 2016). In IBD patients, stress has been associated with exacerbation of symptoms, and increased sympathetic output was linked with increased nociception and mechanical hypersensitivity (Spaziani et al., 2008; Azpiroz, 2002). Therefore, it is possible that stress may affect gut function and neuroimmune balance via disruption of cholinergic homeostasis both in early life and adulthood.

1.4.2.2 Ageing

The enteric neural map is also affected by ageing. Many studies have investigated age related change throughout the GI tract, with most focussing on the myenteric plexus (Saffrey, 2013), and information about natural neurodegeneration within the submucosal plexus or mucosa is sparse. Saffrey (2013) also points out the great variety of protocols employed between different studies, such as differences in counting methods, distension of samples during fixation and use of a variety of 'pan neuronal' markers, must be accounted for when comparing results. Age associated changes in size and shape of myenteric ganglia have been found in the myenteric plexus of the guinea pig ileum (Abalo et al., 2005), and human ileum and colon (Hanani et al., 2004). Studies of total neuronal population within the myenteric plexus have found a reduction in neuronal number as ageing occurs, with cholinergic neurons the phenotype more susceptible to death in animal models and a human population (Phillips et al., 2003; Belai et al., 1995; Bernard et al., 2009); this reduction in the excitatory neurotransmitter acetylcholine is likely to be the cause of increased transit time associated with old age (Phillips and Powley, 2007). Although these studies focussed on the myenteric plexus and did not investigate neuronal subsets within the submucosal plexus, age related degeneration of the entire neuronal population has been identified within the submucosal plexus (Phillips et al., 2007). It would be reasonable to speculate that this loss within the submucosal plexus is also likely to be of a cholinergic phenotype. Although the reason for survival of one subset and loss of another is, as yet, poorly understood, it could be related to the inherent neurotoxicity of NO. NO has been associated with N-Methyl-D-Aspartate (NMDA) induced hypoxic neurotoxicity (Dawson et al., 1996) as it reacts with superoxide to produce the highly neurotoxic oxidant peroxynitrite.

Superoxide dismutase competes for superoxide with nitric oxide is therefore protective against NO induced neurotoxicity (Beckman and Koppenol, 1996), thus it may be possible that the NO positive neurons are less susceptible to death due to the presence of superoxide dismutase, which is protective against the potentially devastating effects of overproduction of highly toxic peroxynitrite.

It is well known that the immune system reaches a phase of reduced responsiveness during old age termed immune senescence; this is, as yet, a poorly understood process. It could be that the normal loss of neurons associated with ageing is directly linked with this immune phenomenon (Bellinger et al., 1992). This remains to be elucidated.

1.4.2.3 The microbiome

Commensal symbiotic bacteria found within the mucosal layer of the gut constitute the microbiome. It is now understood that the microbiome, consisting of trillions of bacteria and varying greatly between individuals, can impact both physical and mental health, and is susceptible to change based on environmental and host factors (Cresci and Bawden, 2015). The nervous system of the GI tract is important in recognition and tolerance of the host to naturally occurring bacteria in the gut and neurotransmitters such as norepinephrine are able to affect the composition of the microbiome (Collins and Bercik, 2009). Just as the nervous system is able to change and influence the metabolic activity and composition of the microbiome, conversely, the microbiome is known to exert change in neural function in the CNS; this is known as the gut-brain axis (Mayer et al., 2014). During development the gut microbiome can affect neural gene expression; germ free mice demonstrated higher tryptophan concentration, increased anxiety and increased activity in comparison with specific pathogen free mice. This effect could be mitigated by introducing commensal bacteria to the germ free strain; the direct impact of this on the ENS was not investigated (Diaz Heijtz et al., 2011; Clarke et al., 2013). However, the microbiome does have known effects on development of the ENS. In the postnatal period when the ENS is still developing and highly plastic, the microbiome is essential for healthy innervations patterns; germ free mice demonstrated significantly reduced neuronal excitability and motility in the small intestine (Collins et al., 2014; McVey Neufeld et al., 2013). A reduction in the number of calbindin positive neurons was also found in the jejunum of germ free mice in comparison with specific pathogen free (SPF) mice; interestingly, germ free mice recolonised with intestinal bacteria had increased gut motility and significantly more calbindin

positive neurons than SPF mice. The mechanisms behind the variations in the ENS of these animals is not currently known (McVey Neufeld et al., 2015) but does stress the importance of the microbiome in modulating gut innervation.

In adulthood, variations within the microbiome can cause change within the CNS affecting mood and memory and are implicated in several pathological conditions such as fibromyalgia and chronic fatigue syndrome (Galland, 2014). Effects such as reduced caution and vigilance in germ free mice could be replicated in SPF mice following administration of antibiotics for seven days, confirming the influence of commensal bacterial subsets on behavioural traits (Bercik et al., 2011). The microbiome is also increasingly linked to anxiety and depression. It is interesting to consider that the link between the microbiome, development of the ENS and the CNS may be key. For instance, patients with depression showed alleviation of symptoms when fructose was removed from the diet; malabsorption of fructose affects bacterial fermentation, bowel motility and tryptophan metabolism, possibly via variations in commensal bacteria (Ledochowski et al., 2000). Autism has been associated with changes in commensal bacteria including increase in clostridial species and greater variety in comparison to controls (Song et al., 2004; Finegold et al., 2010). Spontaneous relapsing-remitting experimental autoimmune encephalitis does not develop in germ free mice until they are given commensal bacteria; this is thought to be associated with a defective Th17 inflammatory response (Ochoa-Repáraz et al., 2011; Ochoa-Repáraz et al., 2010). Although the role of nerve development has never been studied, disorders of the CNS frequently present alongside immunological abnormalities such as inflammation and dysregulation of T cells (Qureshi and Mehler, 2013). Expression of toll-like receptors (TLR) 2 and 4 was reduced in germ free mice; polymorphisms in the TLR2 gene are associated with inflammatory gut pathologies and TLR2 knockout mice demonstrate enteric functional and structural abnormalities similar to germ free mice (Brun et al., 2013). However, during recolonisation TLR2 expression was upregulated, suggesting the importance of the microbiome in this process (Lundin et al., 2008). Surely it must be recognised that the interplay between the CNS, ENS and microbiome is likely to be vitally important in both gut innervation and maintenance of health.

1.4.2.4 Obesity

Obesity is associated with onset of several pathologies linked with inflammation and change in neural subsets or function, such as arthritis, diabetes and coronary heart disease. In a traumatic model of sciatic nerve injury, obese mice displayed

significantly thinner myelin sheath and axon diameter than a control group (Bekar et al., 2014). In a diet induced obesity murine model, intra epidermal nerve fibre number and nerve conduction velocity was significantly reduced (Yorek et al., 2015) and mice were hypertensive due to disrupted sympathetic neural pathways in the kidney that affected the cardiopulmonary baroreflex (Khan et al., 2015). This impairment of baroreflex control in obese mice has been attributed to the presence of inflammatory cytokines TNFα and IL-6, suggesting a link between obesity and inflammation as a causal factor for autonomic dysregulation (Khan et al., 2017). Vasoconstriction due to increased sympathetic stimulation in diet induced obesity has also been linked with increased adrenergic and purinergic neurotransmission (Haddock and Hill, 2011). The ability of cholecystokinin (CCK) to induce plasticity in vagal afferent neurons is limited in mice with diet induced obesity. The normal anorexigenic phenotype, associated with decreased cannabinoid 1 receptor and increased neuropeptide Y (NPY) Y2 receptor expression on vagal afferent nerves, that follows feeding in response to increase in circulating CCK does not occur, upregulating appetite and exacerbating the issue (Browning et al., 2017). However, vagotomy has been linked with loss of diet induced weight gain and reduced fat deposits, and TRPV1 stimulation with capsaicin also reduced fat deposits by preventing adipogenesis and stimulating lipolysis (Leung, 2014) suggesting a possible solution for weight loss induction. It is clear that there is much to learn about the link between obesity and neural function.

1.4.2.5 Inflammation

Differences in innervation between infected and control groups have been investigated in several pathologies. In a rat model, osteoarthritis has been linked with an increase in sympathetic nerve fibres, both surrounding the joint and within adjacent skin; increased pain sensation in these animals could be ablated by administration of guanethidine (Longo et al., 2013). Autonomic neuropathy associated with GI symptoms including altered visceral sensation was found in diabetic patients (Brock et al., 2013). Abnormalities of the sympathetic branch of the autonomic nervous system were identified in children suffering from abdominal pain, possibly a result of unmodulated peristalsis (Chelimsky et al., 2001). In a study of eight patients with GI disease, seven patients displayed hypomotility and uncoordinated contractions in the small intestine and every patient demonstrated sympathetic denervation suggesting involvement of the nervous system in GI disorders (Camilleri and Fealey, 1990). Interestingly, the Th2 mediated IBD ulcerative colitis is associated with increase in adrenergic nerve fibres (Kyösola et

al., 1977). β-adrenergic signalling via norepinephrine was found to induce immunoglobulin production and Th2 cytokine secretion suggestive of increased pathology in ulcerative colitis (Elenkov et al., 2000; Kohm and Sanders, 2000). However, loss of sympathetic neurons was identified in patients with Th1 mediated Crohn's disease along with increased VIP expression (Belai et al., 1997). Despite findings that VIP can reduce inflammatory cytokines (Abad et al., 2003), in a study of patients with IBS, VIP was significantly increased compared to a control group; patients with IBS in an anxiety depressive state demonstrated significantly higher levels of VIP than IBS patients in a normal emotional state, suggesting VIP is involved in changes in GI function and motility associated with IBS and these changes can be exacerbated by stress (Han, 2013). SP and CGRP have been linked with gut inflammation (Figini et al., 1997; Engel et al., 2011). Increased levels can cause extravasation of plasma into tissue and infiltration of immune cells. SP binds to NK1 receptors; blocking the NK1 receptor was found to be protective against inflammation in a murine model of chronic colitis in SCID mice (Gad et al., 2009). NK1 receptors are upregulated in patients with Crohn's disease and ulcerative colitis suggesting a role for SP in IBD related inflammation (Mantyh et al., 1988).

Interestingly, a functioning immune system was deemed crucial in normal regeneration of neurons in the adult brain. T lymphocytes were found to be essential in hippocampal neurogenesis, spatial learning, memory and expression of brain derived neurotrophic factor (BDNF) in the mouse dentate gyrus; mice with severe combined immunodeficiency syndrome (SCID) demonstrated reduced hippocampal neurogenesis that was not improved following environmental enrichment but was recovered following administration of T cells with CNS specific antigen (Ziv et al., 2006).

Extensive research suggests a definite link between pathology and disruption of normal neural pathways or neuropeptide functioning; however, results between pathologies, even the two variants of IBD (ulcerative colitis and Crohn's disease), are disparate. In order to try to understand this, we must look more closely at the link between the nervous system and the immune system, the way that these two systems integrate and mechanisms behind immune mediated loss of neurons.

1.5 The neuro-immune link

The immune response is initiated following damage to or invasion of the host. Inflammation is associated with local heat, swelling and redness accompanied by influx of immune mediating factors such as immune cells and cytokines. Pathogen associated molecular patterns (PAMPs) on an invading pathogen or damage associated molecular patterns (DAMPs) expressed during trauma are recognised by specific receptors (e.g. Toll or NOD like receptors) on circulating cells of the innate immune system, such as macrophages (Tang et al., 2012). These cells can phagocytose invading pathogens, activate complement receptors and release inflammatory mediators, such as chemokines and cytokines, resulting in an influx of immune cells such as monocytes and neutrophils. Both macrophages and dendritic cells act as antigen presenting cells, contributing towards maintaining an innate immune response whilst also activating T cells and initiating an adaptive immune response. T cells may then go on to activate the humoral adaptive response by presenting antigen to B cells within lymphoid tissue (Janeway et al., 2001). How then does this immune process link with and become influenced by the nervous system?

1.5.1 Effects of the immune response on the nervous system

During infection, the immune system is activated but also the CNS must regulate factors such as sleep, pain and appetite to ensure the best recovery and survival of the host. This alludes to a necessary cross talk. It has long been known that the immune response and in particular, cytokines, are able to exert some influence over the nervous system during infection (Rosas-Ballina et al., 2008). Despite the inability of most pathogens to cross the blood brain barrier, increased inflammatory cytokines, such as IL-6 and TNF, were found alongside cognitive impairment and reduced nerve growth factor (NGF) within the brain during infection with the influenza virus, suggesting that activation of the immune system peripherally results in changes within the CNS (Jurgens et al., 2012). Cytokines IL- 1β, IL-6 and TNFα cross the blood brain barrier to induce fever via direct communication with hypothalamic neurons (Blatteis, 2000). In both human and animal models, the proinflammatory cytokine IL-1 activates the hypothalamic-pituitary-adrenal axis, causing release of adrenocorticotrophic hormone and subsequent glucocorticoid hormone production in a neuroendocrine stress pathway (Berkenbosch et al., 1987;

Sapolsky et al., 1987). Therefore, there are several mechanism via which the immune system impacts the nervous system.

1.5.2 Effects of the nervous system on the immune response

1.5.2.1 The sympathetic nervous system

It has been more recently understood that, conversely, the nervous system is able to exert some control over the immune system, contributing to both initiation and resolution of a response. Following initiation of an immune response by skin contact sensitivity, increase in areas of brain activity could be seen within twenty minutes on blood oxygen level dependent functional magnetic resonance imaging scans; this effect was ablated following administration of capsaicin for one week. This evidence supports a direct and almost immediate link between pathogen recognition and the central nervous system (Thinschmidt et al., 2015). This is further supported by extensive evidence that the thymus, spleen, lymph nodes, bone marrow and gut associated lymphoid tissue are directly innervated by peptidergic and noradrenergic sympathetic neurons. These nerve terminals are in direct contact with T cells and macrophages within white and red pulp; beta adrenoreceptors and receptors for neurotransmitters have been found on many cells of the immune system including macrophages and lymphocytes (Felten et al., 1987; Felten et al., 1988; Bellinger et al., 1987). A role for sympathetic noradrenergic input has been suggested during development of the immune system as innervation of lymphoid tissue is evident prior to compartmentalisation of the immune system (Bellinger et al., 1992). Stimulation of the sympathetic nervous system was found to increase levels of circulating IL-6 and TNF serum levels (Bernstein et al., 2009; Pöyhönen-Alho et al., 2008); paradoxically, sympathetic nervous stimulation has also been associated with reduced inflammatory cytokine production (Straub et al., 2005) and increased bacterial infections related to immunosuppression in a rat model of stroke could be alleviated by sympathectomy (Prass et al., 2003). Toll like receptors 3, 4 and 7 have been found within dorsal root ganglia, myenteric and submucosal plexuses within murine and human small and large intestine (Barajon et al., 2009). Straub et al. (2005) found that the impact of the sympathetic nervous system on efficacy of the immune response was dependent on the bacteria being gram positive or gram negative; norepinephrine decreased production of TNF in macrophages but increased IL-4 production by peripheral mononuclear cells. Therefore sympathetic stimulation was considered

beneficial in infection with gram positive bacteria (e.g. *Staphylococcus aureus*) and negative in terms of bacterial load during gram negative infection (e.g. *E.coli*). This evidence suggests that a definite role for the sympathetic nervous system during immune stimulation, though it would appear that this role may not always be beneficial.

1.5.2.2 The parasympathetic nervous system

The parasympathetic nervous system also has a role to play during infection. Inflammation has been associated with modulation of vagal function, including increased mechanosensitivity, changes in ion channel expression and adenosine tri phosphate signalling and upregulation of TRPV1 receptors (Liu et al., 2007; Bielefeldt et al., 2002; Schwartz et al., 2011). Vagal afferent nerves have receptors for IL-1 (Hansen et al., 2001), and TNF and IL-1β reduce gastric motility via inhibiton of vagal motorneurons in the dorsal motor nucleus of the vagus (Mo et al., 1996). It has been proved that the parasympathetic nervous system can exert an anti inflammatory influence during infection via production of principal neurotransmitter and neuroimmunomodulator, acetylcholine, by the vagus nerve (van der zanden et al., 2009). Acetylcholine is present within blood and mononuclear leukocytes and is predominantly produced by T cells (Fujii et al., 1999); it binds with muscarinic or nicotinic acetylcholine receptors, present throughout the CNS and PNS, and is important in stimulation of normal gut motility (LePard and Galligan, 1999; Ehlert et al., 1999). However, Tracey et al. (2002) found that incubation of macrophages with acetylcholine or nicotine reduced translocation of NFκB to the nucleus of the cells and subsequently reduced production of inflammatory cytokines including TNF, IL-1, IL-6 and IL-18. The alpha 7 nicotinic acetylcholine receptor was found to be necessary for initiation of this pathway, termed the 'cholinergic anti inflammatory pathway' and that stimulation of this receptor with its natural ligand acetylcholine or with nicotine reduced the risk of a potentially fatally unresolved immune response by neurally inducing reduction of inflammation (Wang et al., 2003). This receptor has been identified within the CNS and PNS, but also on many cells involved in the immune response including macrophages, T cells, B cells, neutrophils, mast cells and dendritic cells (de Jonge and Ulloa, 2007). The cholinergic anti inflammatory pathway is thought to occur predominantly in the spleen; however, the spleen does not have any known vagal innervation (Gautron et al., 2013). Despite this, vagal stimulation reduced inflammation within the spleen; in rats with splenic denervation, this effect was not seen. Catecholaminergic nerve fibres infiltrating the spleen arise from the celiac and superior mesenteric ganglia, where vagal efferent fibres

terminate; depletion of catecholamine stores in rats ablated the anti inflammatory effect of vagal stimulation. This suggests that the cholinergic anti inflammatory effect within the spleen is mediated by a preganglionic vagal neuron originating from the dorsal motor nucleus synapsing with a postganglionic adrenergic catecholaminergic nerve in the celiac or superior mesenteric ganglia that terminates within the spleen and facilitates acetylcholine synthesis in adjacent choline acetyltransferase positive T cells (Rosas-Ballina et al., 2008).

Effects of vagal stimulation may be dependent on the immune environment and cytokine milieu. In an experimental model used in mice to investigate multiple sclerosis, experimental autoimmune encephalitis (EAE), studies revealed a 50% reduction in the Th1 transcription factor T-bet and a 350% increase in the Th2 transcription factor GATA-3 following administration of nicotine leading to the conclusion that stimulation of the α7nAChR skews an immune response from a Th1 to Th2 phenotype (Nizri et al., 2009). Interestingly, in CD4+CD62L+ T cells cultured with a cytokine traditionally associated with a Th1 response, IL-12, expression of the α7nAChR mRNA was downregulated, whereas incubation with the Th2 associated cytokine IL-4 resulted in upregulation of α7nAChR mRNA (Galitovskiy et al., 2011). This may be a homeostatic mechanism to avoid loss of necessary inflammation and immune cell infiltration required to ensure eradication of a pathogen. However, smoking is known to exacerbate the Th1 mediated gut autoimmune condition Crohn's disease, whereas the Th2 mediated gut autoimmune disease ulcerative colitis is known as a disease of non smokers (Lawrance et al., 2013; Lunney and Leong, 2012). This could be due to the ability of acetylcholine to inhibit epithelial apoptosis, which would alleviate symptoms associated with widespread epithelial ulceration found in ulcerative colitis but exacerbate areas of T cell infiltrated chronic inflammation that are characteristic of Crohn's disease (Itoh et al., 2001; Iwamoto et al., 1996).

Therefore, evidence identifies a role for parasympathetic innervation in the immune response, often in a regulatory capacity; however perhaps more research is needed to clearly identify the role of vagal stimulation in different immune profiles.

1.6 Mechanisms of neuropathy

Due to the involvement of the nervous system in the immune response, changes in neuronal number are likely to affect not only normal physiological functions such as

motility and absorption within the gut, but also response to disease. Although Cashman and Hoke (2015) suggest that neuronal loss occurs as a secondary result of a predisposing condition, peripheral neuropathy is evident in 15% of the population of the United States of America over the age of forty (Gregg et al., 2004) suggesting that neuropathy may precede and contribute to development of pathology. However, disruption of homeostasis by disease can result in neuronal death.

The biggest predictor of mortality in diabetes, autonomic neuropathy, is a serious and common complication associated with a host of co-morbidities that affects the cardiovascular, genitourinary, neurovascular and gastrointestinal systems (Vinik et al., 2013). This has been linked with accumulation of advanced glycation end products (AGE) associated with hyperglycaemia; AGE is capable of modification of laminin, a key component in the basement membrane of neurons, resulting in reduced neurite outgrowth (Federoff et al., 1993). Increase in AGE and in particular the receptor for these products, RAGE, has also been associated with increased inflammation (Schmidt et al., 2001); RAGE knockout mice demonstrated delayed development of neuropathy (Toth et al., 2008). Although constant low grade inflammation is associated with diabetes and AGE-RAGE signalling has been associated with changes in intracellular signalling due to upregulation of IL-6 and TNF, autonomic dysfunction and changes in neuronal number have been found prior to signs of inflammation (Lieb et al., 2012) suggesting that although inflammation contributes to neuronal death, it is likely that a level of neuropathy was evident prior to, and possibly contributed to, development of the pathology. Loss of heart rate variability associated with autonomic neuropathy was linked with increased levels of the inflammatory marker C-reactive protein; it was thought that this may be a result of loss of parasympathetic neurons and associated reduction in activation of the cholinergic anti inflammatory pathway (Thayer and Fischer, 2009) suggesting that a change in the nervous system was the cause of inflammation. Inhibition of G6PD during hyperglycaemia results in accumulation of sorbitol and associated polyols within neural tissue impairing regeneration and myelination (Zhang et al., 2010; Gabbay et al., 1966). Increased apoptosis and decreased proliferation are also evident, and as conversion of glucose into sorbitol requires the antioxidant NADPH, intracellular levels are depleted resulting in increased ROS levels (Zhang et al., 2010). Impairment of function or damage to mitochondria, linked with influx of oxygen and electrons, or endoplasmic reticulum, linked with

protein synthesis, has been linked with neuropathy as these organelles are the likely site of ROS synthesis (Cashman and Höke, 2015).

NO is important in maintenance of normal gut function, regulating vasodilation and promoting relaxation of smooth muscle. NO is synthesised by nitric oxide synthases, which convert L-arginine to L-citrulline, NO and water in the presence of dioxygen and NADPH. Calmodulin is also involved in NOS activation but the impact of this is variable dependent on the isoform of NOS utilised. NOS occurs in three isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Whilst levels of nNOS and eNOS remain relatively static, iNOS is produced during infection when levels rise dramatically upregulating NO production (Vallance and Leiper, 2002). NO has anti inflammatory properties and was found to reduce production of pro inflammatory cytokines TNFα, IL-1β and macrophage inflammatory protein- 1α in human alveolar macrophages (Thomassen et al., 1997). NO also increased lymphocyte apoptosis during infection (Moulian et al., 2001). However, as the process of NO formation involves oxygen and water, due to the redox active capacity of NO, it is rapidly converted into a nitrogen oxide species (Tennyson and Lippard, 2011; Koppenol et al., 1992). Superoxide production is also upregulated during inflammation, produced by activated polymorphonuclear leukocytes; this increase results in inequity between levels of superoxide and its neutralising factor superoxide dismutase, resulting in interaction between NO and superoxide to produce peroxynitrite alongside loss of normal NO function (McCord and Omar, 1993). Peroxynitrite, a reactive oxygen species (ROS), is highly toxic both to the cell within which it is produced and the surrounding environment and has been associated with reduced T and B lymphocyte generation and cytotoxic neuronal cell death following brain injury (Ames et al., 1993; Beckman et al., 1990). Some markers of peroxynitrite damage include lipid peroxidation (Radi et al., 1991b), DNA breakage (Salgo et al., 1995), oxidative damage to protein, induction of protein carbonyls (Ischiropoulos and al-Mehdi, 1995), oxidation of thiols (Radi et al., 1991a) and hydroxylation of phenolics (Beckman et al., 1990). Therefore the presence of NO, particularly during infection, can be a double edged sword.

Acetylcholine does not have the potentially damaging properties associated with nitric oxide; indeed, loss of cholinergic neurons and targeting increased cholinergic stimulation has been the focus of therapies for several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and schizophrenia (Holzgrabe et al., 2007; Posadas et al., 2013). However, alongside anti-inflammatory effects, upregulation of acetylcholine has been linked with reduced apoptosis; stimulation of

the α7nAChR increased expression of the anti-apoptotic protein Bcl-2 (Mai et al., 2003) and caused mutations within the tumour suppressor gene p53 (Gibbons et al., 2014). Nicotine, a ligand of the α7nAChR, upregulated cell proliferation and increased expression of growth factors and growth factor receptors, such as VEGF, TGFβ and EGFR (Egleton et al., 2008). Acetylcholine receptors have been associated with pathogenesis of cancer and resistance to chemotherapeutic agents (Dang et al., 2016); small cell lung carcinomas upregulate nAChRs and acetylcholine production, facilitating survival and increased cell proliferation, thus enhancing tumour progression (Egleton et al., 2008). This highlights a sinister side to acetycholine upregulation and emphasises the importance of neural regulation in the host in maintenance of health.

1.7 *Trichuris muris* as a model of gut inflammation

Trichuris muris (*T.muris*) is a laboratory model of *Trichuris trichiura*, a human whipworm. Following oral gavage, parasite eggs travel to the caecum and proximal colon where they hatch after ninety minutes. The first larvae then burrow through the epithelium where they undergo molts at approximately days 9-11, 17 and 22 post infection. By day 32 post infection, the adult worms have moved from the epithelium to the gut lumen, and eggs are expelled within the faecal matter of the host (Klementowicz et al., 2012; Dixon et al., 2010; Antignano et al., 2011). *T.muris* provides a robust model of Th2 driven inflammation, requiring a strong Th2 immune profile and associated cytokines such as IL-4, IL-5 and IL-9 to eradicate the pathogen; this response requires infection with 200 to 300 eggs. However, reduction of antigen load (less than 40 eggs) resulted in loss of the Th2 immune mediated phenotype with skew towards an uncharacteristic Th1 mediated immune response demonstrating increased IFNγ levels and resulting in chronic infection. However, this is useful as it enables the study of both Th1 and Th2 mediated inflammation within the same area of the gut. Some strains of mice, interestingly including the AKR, are unable to produce an effective Th2 response to *T.muris* infection resulting in a Th1 phenotype and chronic infection (Bancroft et al., 1994).

1.8 In summary

It is evident from research that there is a cross talk between the immune system and the nervous system and vice versa. This plays a pivotal role in establishing and

maintaining an appropriate response to invading pathogens, and immune regulation to re-establish homeostasis following pathogen eradication. Perhaps it should come as no surprise that these two highly organised systems integrate in order to best serve the host during immune invasion; the nervous system is widespread, constantly monitoring conditions within the body with the capacity to invoke an immediate response. In this sense, it is far more efficient than the immune system, which relies on circulating cells and cytokines and takes days to establish an adaptive response (Janeway et al., 2001).

Although inflammation can cause neuropathy, this is frequently investigated as a consequence of long term chronic pathology, and long term effects following resolution of common infection are unclear. Some studies demonstrate evidence of neuropathy prior to development of pathology associated inflammation. Perhaps this is a reflection of genetic factors or influence of environmental factors, possibly previous infection and inflammation, which may be capable of impacting neuronal number and subsets. The impact of genetic background and resolved inflammation from different immune profiles on enteric neuronal function and number has not been greatly examined. Genetic variation within the enteric nervous system and any long term effects of resolved infection on the enteric nervous system are likely to be reflected in changes in gut physiology. The importance of the neuro-immune link would also suggest that these changes would affect the host's response to enteric pathology, and therefore influence susceptibility to disease.

Hypothesis

1. Host, developmental and environmental factors influence enteric nerve distribution and function throughout life. We hypothesize therefore that different strains of mice have different density of neuronal subsets in the gut at adulthood, primarily due to host genetic factors.
2. We hypothesize that density and function of enteric neuronal subsets can be affected differently by infection with different immune profiles, and that this change in neuronal density will remain following resolution of infection.
3. We hypothesize that differences in density of enteric neuronal subsets due to genetic factors or resolved infection will affect gut physiology.
4. Lastly, we hypothesize that variations in type and density of enteric neuronal subsets may ultimately influence susceptibility to enteric disease via the neuro-immune link. This means that natural genetic variation or previous infection will affect the enteric neural map in a human population and therefore this will be reflected in differences in gut physiology, response to, and susceptibility to, disease.

Aims

1. To investigate and compare impact of host genetic factors on enteric nervous system function by comparing gut physiology in two different strains of mice with known differences in the peripheral nervous system, the AKR and the C57BL/6. We will investigate gross and microscopic anatomical factors, such as gut length and muscle thickness, to provide information about any strain specific differences. We will also examine faecal pellet parameters such as wet/dry weight, length, number and transit time to identify differences in gut absorption and motility. Finally, we will measure differences in colon motility using neuroreceptor agonists to identify any differences in expression between strains that may impact motility. As neuroreceptors can affect gut motility but are also involved in the neuro-immune link, strain specific differences may explain different responses to infection between strains and this could be echoed in a human population.
2. To investigate and compare impact of host genetic factors on the enteric nervous system by comparing enteric neuronal density between two different strains of mice with known differences in the peripheral nervous system, the AKR and the C57BL/6. This will require identification of a suitable pan neuronal marker via immunofluorescence or histochemical protocols. We

will trial markers commonly and historically used in enteric neuronal study and aim to identify a technique that is pan neuronal. This will enable us to elucidate and strain specific differences in the enteric neural map that may impact gut physiology or the neuro-immune link and therefore susceptibility to, and recovery from, disease that could be echoed in a genetically varied human population.

3. To investigate and compare impact of resolved infection with Th1 or Th2 immune profile on enteric nervous system function by comparing gut physiology in mice infected with a high or low dose of *Trichuris muris* and a control naive group. We will examine gross and microscopical anatomical factors, faecal pellet parameters and neuroreceptor expression, as previously described, to examine any differences in gut physiology that may remain post infection. This will identify if infection has an impact on gut physiology following resolution, and clarify if this difference varies dependent on the immune profile of the infection. This may mean that infection with a different immune profile may have a different long term impact on gut function in a human population.
4. To investigate and compare impact of resolved infection with Th1 or Th2 immune profile on enteric nervous system function by comparing enteric neuronal density in mice infected with a high or low dose of *Trichuris muris* and a control naive group. We will use previously identified pan neuronal markers to identify if the immune profile impacts the enteric neural map both during and following resolution of infection. We will also examine enteric glial cell number and expression, using S100β and GFAP, to identify any immune profile specific differences both during and following infection that may affect the enteric nervous system number and function. We will also examine NOS levels as increased NOS is associated with increased cell death. As NOS, in particular iNOS, is known to play a role in the immune response, differences in NOS expression between different immune profiles may be identified. This in turn may impact the immune response and amount of neuronal death, which would also impact susceptibility to and recovery from disease due to the neuro-immune link.

Chapter 2
Methods and Materials

2.1 Animals

Male AKR/J mice were obtained from Harlan Olac at 4-6 weeks old or bred in the University of Manchester Biological Services Facility. Male C57BL/6N and SCID mice were bred in the University of Manchester Biological Services Facility. C57BL/6N animals were infected at 6-8 weeks of age (weight approximately 30g). All animals were housed in the Biological Services Unit under 12:12 hour light dark cycles with free access to food and water for the duration of the experiment. All experimental procedures were performed under the regulations of the Home Office Scientific Procedures Act of 1986 (amended Jan 2013).

2.2 Parasites

Trichuris muris eggs were prepared as described previously (Wakelin, 1967). In brief, SCID mice were infected with 300 *T.muris* eggs. At day 35 post infection mice were culled, the caecum and proximal colon removed, cut open longitudinally, washed and adult worms pulled out gently using curved forceps. Worms were then cultured overnight at 37°C in RPMI (Sigma Aldrich) with 100U penicillin and 100µg/ml streptomycin (Sigma Aldrich). Released eggs were isolated, washed by centrifugation (1000g, 15 minutes) and stored for 6 weeks in the dark at room temperature in Milli-Q water to embryonate.

C57BL/6 mice were infected at 6 weeks with oral administration of either 300 (high dose group) or 20 (low dose group) *T.muris* eggs. Mice from each group were culled on day 21 and caecum and proximal colon removed, placed in PBS and sectioned longitudinally to assess worm burden. Adult worms were removed and counted using light microscopy (Leica MZ7.5, 10xs mag).

2.3 Antihelminthic Treatment

The antihelminthic treatment mebendazole (Ovex 100mgs/5ml) was administered orally, resuspended in Milli-Q water at a dose of 50mg/kg, to ensure expulsion of parasites and resolution of chronic infection in the low dose group. Treatment was given to animals in the high dose and naive control groups aswell to control for effect of mebendazole.

2.4 Measurement of gut physiology

2.4.1 Weight measurement

Mice were weighed weekly for 6 weeks from age 9 weeks in naive groups or from day 21 post infection for animals in the infection experiment. Mean average was calculated for each group.

2.4.2 Food and water intake

Food and water intake was measured weekly for 6 weeks from age 9 weeks in naive groups or from day 21 post infection for animals in the infection experiment. Intake was calculated as absolute values per cage and mean average intake per mouse.

2.4.3 Transit time

At day 55 post infection or on the eve of the cull at week 16 in uninfected groups, each mouse was orally gavaged with 200µl of Miili-Q (Micropore) water containing 5% Evans Blue (Sigma T0195) and 5% gum arabic (Sigma G8752). Mice were then housed separately with access to food and water *ad libitum* and observed. Time taken for a blue faecal pellet to be produced was noted for each animal and average time calculated per group.

2.4.4 Faecal pellet number, length and water content

At day 55 post infection or on the eve of the cull at week 16 in uninfected groups, each mouse was housed separately with access to food and water *ad libitum* and number of faecal pellets produced per animal in a four hour period counted. The length of the first 5 faecal pellets produced by each animal was measured and the mean average per animal calculated. Pellets were weighed, dried in an oven at 90°C for 48 hours and reweighed. Water content as a percentage per group was calculated using the following equation;

$$\text{Water content \%} = \frac{(\text{wet weight} - \text{dry weight})}{\text{wet weight x } 100}$$

Mean average values were calculated per group for all parameters measured.

2.4.5 Measurement of gut length

At day 21, 45 and 56 post infection or week 16 in uninfected groups, animals were culled using carbon dioxide inhalation; small and large intestine was removed and length measured. Mean average length per group was calculated.

2.4.6 Measurement of gut contractility

Sections of proximal colon approximately 2cms long were taken from each animal, mounted on a tissue hook and placed under tension calibrated to 1g in individual 50ml tissue baths filled with oxygenated Kreb's solution (see recipes) maintained at 37°C. Following 30 minutes of rest to allow equilibration, spontaneous contractility of tissue was measured for a 3 minute period using a transducer attached to a bridge pod and PowerLab (26T) data acquisition system analogue to digital converter; data were recorded using LabChart 7 software (all AD instruments). Average number and strength of contractions for each animal was measured and a mean average number for each group calculated. Increasing concentrations of carbachol (0.1μM, 1μM and 10μM, Sigma PHR1511), capsaicin (0.1μM, 1μM and 10μM, Sigma 360376) and PNU 282987 (0.1μM, 1μM and 10μM, Tocris T0195) was added to organ baths containing tissue from naive AKR/J and C57BL/6N animals. Increasing concentrations of PNU 282987 (0.1μM, 1μM, 10μM and 20μM, Tocris T0195) was added to organ baths containing tissue from infected groups and the associated control group. Tissue was flushed with oxygenated Kreb's solution three times and rested for 5 minutes between additions of each treatment. Average number and strength of contractions for each animal during a three minute period was measured and mean average per group calculated.

2.5 Gut samples- tissue preparation

Small and large intestine was removed from animals, snips of tissue were taken from the jejunum and proximal colon, flushed with PBS to remove any faecal matter, stored in histology cassettes and immersed in 4% PFA for 12 hours at room temperature or snap frozen in liquid nitrogen and stored at -80°C. Whilst transverse sections demonstrated a cross section of neural infiltration from the submucosal plexus into the lamina propria towards the epithelium, a large amount of enteric neurons sit within the myenteric plexus (see figure 2.1); this lies at 90 degrees to the epithelium. We did not want to use a whole mount technique that employed microdissection due to possible damage to tissue that could affect validity of results; however, our attempts to visualise this area using whole mount techniques with the

gut intact were not successful. To counteract this problem a new technique was employed to visualise the myenteric plexus; gut was cut along the longitudinal axis (figure 2.1), opened out and placed flat between sponges in histology cassettes. Samples fixed in 4% PFA were processed through 70% and 100% alcohol, followed by xylene (Shandon Citadel tissue processor, Citadel 2000, RHYS International Ltd.) before being embedded in molten wax. Once set, 10µm transverse and longitudinal sections were cut (microtome, Microm HM 325) and collected on superfrost slides (Fisher Scientific, Leicestershire). Samples snap frozen and stored at -80°c were embedded in optimal cutting temperature (OCT) compound, sectioned at a thickness of 10µm (cryostat,Leica CM3050) and collected on superfrost slides.

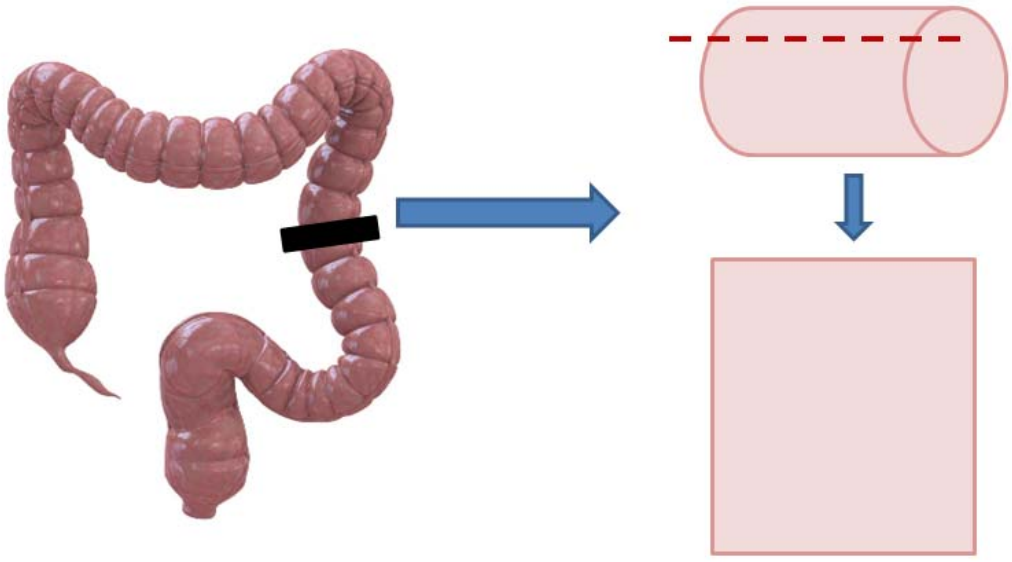


Figure 2.1 Longitudinal sectioning of gut snips. In order to visualise neurons in the myenteric plexus that cannot be visualised on transverse sections of gut tissue, snips of colon were cut along the longitudinal axis, opened out and processed flat in between sponge in histology cassettes. This tissue was then embedded in paraffin flat and sectioned on a microtome to visualise the myenteric plexus.

2.6 Histology

2.6.1 Paraffin embedded tissue

Prior to histological staining, tissue embedded in paraffin and sectioned at a thickness of 10 µm was dewaxed in xylene (H.D. Supplies, Buckinghamshire; 10

minutes) and rehydrated by passing through graded Industrial Methylated Spirit (IMS) (Fisher Scientific, Leicestershire) of 100% (twice), 90% and 70% respectively for 2 minutes each. Slides were then washed in distilled water two times for 3 minutes and stained as follows:

2.6.1.1 Haemotoxylin and Eosin staining

To identify muscle, crypt and villus depth, slides were then placed in Harris haemotoxylin (Sigma-Aldrich) for 10 minutes. Slides were then rinsed in running tap water for 5 minutes prior to immersion in acid alcohol (5ml concentrated HCl plus 495ml 90% ethanol) for 10 seconds, rinsed again in running tap water for 5 minutes and counter stained with 1% aqueous yellowish eosin (Sigma-Aldrich) for 4 minutes before further washing in running tap water for 5 minutes.

2.6.1.2 Sevier-Munger silver stain

For identification of neurons using the Sevier-Munger silver stain technique, deparaffinised slides were washed in Milli-Q (Micropore) deionised water for 1 hour, placed in a coplin jar containing pre warmed 20% silver nitrate solution and placed in an oven at 65°C for 15 minutes. Slides were then removed from the oven, rinsed in Milli-Q water and placed in a coplin jar filled with ammoniacal silver nitrate solution (see recipes) on a shaker as a photographic development step for 15 minutes. Slides were then rinsed again in Milli-Q water.

2.6.1.3 Luxol fast blue stain

In order to identify myelinated neurons, deparaffinised slides were placed in Luxol fast blue solution (see recipes) overnight in an oven at 56°C. Slides were immersed in 95% ethyl alcohol rinse off excess stain, rinsed in distilled water then placed in lithium carbonate for 30 seconds and 70% ethyl alcohol for 30 seconds to differentiate before rinsing in distilled water. Tissue was then counter stained with cresyl violet solution (see recipes) for 30 seconds before rinsing in distilled water again and further differentiation in 95% ethyl alcohol for 5 minutes.

2.6.1.4 Picrosirius red stain

For identification of collagen fibres, slides were immersed in Picrosirius red solution (see recipes) for one hour. Slides were then blotted and rinsed in distilled water.

Following staining outlined in sections **2.6.1.1**, **2.6.1.2**, **2.6.1.3** and **2.6.1.4**, slides were dehydrated by passing through increasing gradients of IMS (70%, 90% and 100% respectively) for 2 minutes each and placed in xylene (10 minutes). They

were then mounted with DPX mounting medium (Lamb Lab Supplies, U.K.), covered with a coverslip and left overnight to dry before being examined.

2.7 Immunofluorescence

Paraffin embedded tissue sections were dewaxed in citroclear (H.D. Supplies, Buckinghamshire; 10 minutes) and rehydrated by passing through graded Industrial Methylated Spirit (IMS) (Fisher Scientific, Leicestershire) (100%, 90%, 70% and 50% respectively) for 2 minutes each. For antigen retrieval slides were then immersed in warmed citrate buffer (0.1M pH 6.0, see recipes) and placed into a microwave (SHARP 800W) at p60 for 3 minutes. Slides were then allowed to cool for 5 minutes before being microwaved again at p60, then cooled for 3 minutes and moved to distilled water for 3 minutes. Slides were then placed in a Sequenza slide rack (Thermo-Fisher), covered with a blocking serum (10% goat serum, 0.3%Triton X in PBS) and incubated at room temperature for 30 minutes. Following this slides were washed in PBS twice for 2 minutes each. Tissue was either single stained with Neuro-Chrom pan neuronal marker (raised in mouse, clone MAB2300, 1:500, EMD Millipore), double stained with primary antibodies PGP9.5 (raised in rabbit, clone 7863-0504, 1:200, Life Technologies) and β tubulin (raised in mouse, clone ab78078, 1mg/ml 1:200, Abcam), or triple stained with primary antibodies S100β (raised in mouse, clone 15E2E2, 1mg/ml 1:200, Millipore), GFAP (raised in rat, clone 2.2B10, 0.5mg/ml 1:50, Invitrogen) and NOS (raised in rabbit, clone 15203, 0.2mg/ml 1:200, Abcam). 100µl was added to each slide. A control slide with primary antibody omitted was used in each experiment. Slides were stored at 4°C overnight. Slides were then rinsed in PBS (two times for two minutes) and secondary antibodies were applied for 1 hour at room temperature (goat anti rabbit IgG Texas Red, 1:100; goat anti mouse IgG AlexaFluor 488, 1:400, goat anti rat IgG AlexaFluor 350, 1:400: all Life Technologies). After being rinsed in PBS (two times for two minutes) slides were mounted in vectashield aqueous mounting medium (Vectorlabs, Peterborough, UK).

2.8 Recipes

Ammoniacal silver nitrate solution: Concentrated ammonium hydroxide solution (30%) was added drop by drop to 50mls 10% silver nitrate solution (w/v) until precipitate disappeared. 0.5mls of sodium carbonate (8g dissolved in 30mls deionised water) was added and shaken well. A further 25 drops of concentrated

ammonium hydroxide was added during vigorous shaking. Immediately prior to use 10 drops of 0.8% formaldehyde was added.

Citrate buffer (0.1M pH6.0): 1.92g citric acid was added to 1 litre of distilled water. Sodium hydroxide was added until the pH was 6.

Cresyl fast violet solution (0.1%): 0.1gm cresyl fast violet in 100ml distilled water. Prior to use add 10 drops glacial acetic acid and filter.

Krebs solution: 117mM NaCl, 4.75mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$ $7H_20$, 3.8mM $CaCl_2$ $2H_20$. Bubble solution with 95% O_2, 5% CO_2 for 20 minutes. Add 11mM glucose solid and 25mM $NaHCO_3$.

Luxol fast blue solution (0.1%): 0.1gm luxol fast blue powder, 0.5ml glacial acetic acid, 100ml ethyl alcohol (95%).

Lithium carbonate solution (0.05%): 0.05gm lithium carbonate, 100ml distilled water.

4% paraformaldehyde: 500mls of deionised water was heated to 60°C. 40g paraformaldehyde was added. This was stirred for several minutes and 2.5ml 1M sodium hydroxide added until the solution cleared. The solution was then filtered and a further 500mls 0.2M PBS added.

Picrosirius red solution: 500ml saturated aqueous solution picric acid, 0.5g Siruis red.

2.9 Microscopy

Slides treated with silver, haemotoxylin and eosin, luxol fast blue, and nadph-d stains or PGP9.5, βtubulin, soxβ100, GFAP, NOS and Pan neuronal marker antibodies were examined using the Pannoramic 250 Flash II slide scanner (3D Histech, Hungary). Slides were viewed on Panoramic Viewer software 1.15.4 (RTM 3DHistech). Crypt depth, submucosal depth, muscle thickness, staining and immunofluorescence was quantified at 20xs magnification using Histoquant (3D Histech). Slides treated with picrosirius red stain were examined using a Zeiss Imager M2 with an Axiocam MRc camera running ZEN software and qualitatively compared.

2.10 Statistics

Data were analysed using GraphPad prism 7.0. Data sets were analysed using two way ANOVA with posthoc Tukey analysis. Where stated, Student's t-test were also performed using Microsoft excel 2010. Results were considered significant where $P<0.05$. Data expressed as mean ± SEM.

Chapter 3

There are differences in gut physiology between the AKR and C57BL/6 mouse strains that are likely to reflect variation within the enteric nervous system

3.1 Introduction

In order to investigate the impact of genetics on the enteric nervous system map and therefore the role this may play in susceptibility to gastro-intestinal disease in humans, it would be pertinent to consider an animal model in which genetic variation can be controlled and the impact of environmental influence minimised. We decided to use two strains of mice that have been previously studied and are known to be neurally different; C57BL/6 and AKR. It has been established that different strains of mice produce different heritable responses to pain including thermal, mechanical and chemical nociception; of the 11 strains studied, the greatest variation was found to be between the C57BL/6 and AKR strains (Mogil et al., 1999a; Lariviere et al., 2002). AKR mice produced a much delayed response to heat induced pain and reduced heat induced CGRP release in the hindpaw in comparison with C57BL/6 mice, though this strain specific response was not ablated by administration of CGRP (Mogil et al., 2005). Coincidentally these two strains of mice also elicit very different responses to parasitic infection. The C57BL/6 mouse strain is characterised by resistance to high dose whipworm infection producing a characteristic T Helper 2 type response found in humans and animals necessary for successful elimination of parasites (Cliffe et al., 2007). The AKR mouse is susceptible to parasitic infection producing a delayed Th1 response leading to chronic infection (Blackwell and Else, 2002). If the reason for the variation in response to parasites is linked with the nervous system, and given that the parasites used in the aforementioned studies are those which enter the host via the gastro-intestinal tract, then this neural difference is likely to be in the gut.

It would, perhaps, be acceptable to surmise that variation in the gut nervous system between two strains of mice would manifest itself primarily in a variation in normal gut physiology in the absence of pathology. Therefore, this study was designed to examine the normal gut function of these two strains of mice in order to ascertain if any noticeable differences arose that may warrant further investigation of the enteric nervous system map.

Hypothesis

There are known differences in the peripheral nervous system between the AKR and C57BL/6 mouse strains that may be reflected in the enteric nervous system. As the enteric nervous system controls gut physiology, this genetic difference will therefore be reflected in differences in gut function between the two strains.

Differences in the enteric nervous system will ultimately impact susceptibility to and recovery from disease via the neuro-immune link; this genetic influence is likely to be reflected in a human population and therefore may be of importance in understanding susceptibility to gastro-intestinal disease in a human population.

Aim

To assess any differences in gut physiology that will reflect differences in the enteric nervous system in two different inbred strains of mice (C57BL/6 and AKR) with known peripheral nervous system differences.

Assessments of variation in physical and functional parameters that will be used include comparison between strains of

- Weight gain
- Length of small and large intestines
- Total and longitudinal muscle thickness
- Food and water intake
- Faecal pellet number, length and water content
- Faecal transit time
- Spontaneous motility of the proximal colon, including number and tension of contractions
- Initial response of the proximal colon to an acetylcholine receptor agonist at 3 concentrations
- Average response of the proximal colon, including number and tension of contractions, to an acetylcholine receptor agonist at 3 concentrations in a 3 minute period
- Average response of the proximal colon, including number and tension of contractions, to a TRPV1 receptor agonist at 3 concentrations in a 3 minute period

Results will highlight strain specific differences in physical size, function and neuroreceptor expression that may impact gut function and reflect a varied enteric nervous system. This may be echoed in a genetically varied human population and impact human gut function and ultimately disease susceptibility via the neuro-immune link.

3.2 Weight gain and gut length

3.2.1 Strain of mouse affected weight gain

AKR (n=3) and C57BL/6 (n=3) mice were weighed every 7 days for 5 weeks from the age of 9 weeks to 13 weeks and the mean average per group calculated for each week (Figure 3.1, A). Average weight of AKR mice was consistently significantly heavier (two way anova $p<0.0001$) than the average weight of the C57BL/6 mouse (West et al., 1992).

3.2.2 Strain of mouse did not affect length of the small or large intestine

Length of small intestine and large intestine was measured in AKR (n=3) and C57BL/6 (n=3) mice at age 13 weeks and mean average length per group was calculated. Average length of small intestine and large intestine was not statistically significantly different between strains (Student's t-test) (Figure 3.1, B, C).

3.3 Total muscle and longitudinal muscle depth

3.3.1 Significant differences in total muscle depth in the jejunum and in longitudinal muscle depth in the jejunum and colon between strains

Total muscle thickness and depth of longitudinal muscle was measured in the jejunum and colon of AKR (n=5) and C57BL/6 (n=5) mice. In the jejunum, the C57BL/6 mice had significantly thicker total muscle (Student's t-test $p<0.00005$) and longitudinal muscle ($p<0.001$) than the AKR mice. In the colon, the C57BL/6 mice had significantly thicker ($p<0.05$) longitudinal muscle than the AKR mice; however, there was no significant difference in total muscle thickness in the colon between strains (Figure 3.2).

3.4 Food and water intake, faecal pellet number and length

3.4.1 Effect of mouse strain on food and water intake

Food and water intake per cage (1 cage of AKR mice (n=3), 1 cage of C57BL/6 mice (n=3)) was measured over a 5 week period and calculated as a mean average value per mouse. AKR mice appeared to eat more food (Figure 3.3, A) and drink less water (Figure 3.3, B) than the C57BL/6 mice. Data was measured as absolute values per cage.

3.4.2 Mouse strain had an inconsistent effect on faecal pellet number

Faecal pellet number produced per animal in a 4 hour time period was measured in AKR (n=3) and C57BL/6 (n=3) mice. AKR mice produced significantly fewer faecal pellets (Student's t-test $p<0.05$) than the C57BL/6 strain (Figure 3.2, C.i). However, this was not found to be statistically significant at retrial (AKR n=6, C57BL/6 n=5) (Figure 3.3, C.ii).

3.4.3 Mouse strain affected faecal pellet length

The first five faecal pellets produced by 3 AKR and 3 C57BL/6 mice were collected and measured to give a mean average faecal pellet length per animal. AKR mice had significantly longer faecal pellets (Student's t-test $p<0.05$) than the C57BL/6 mouse (Figure 3.3, D.i); this difference was consistent at retrial and found to be more highly significant (Student's t test $p<0.002$, AKR n=6, C57BL/6 n=5) (Figure 3.3, D.ii).

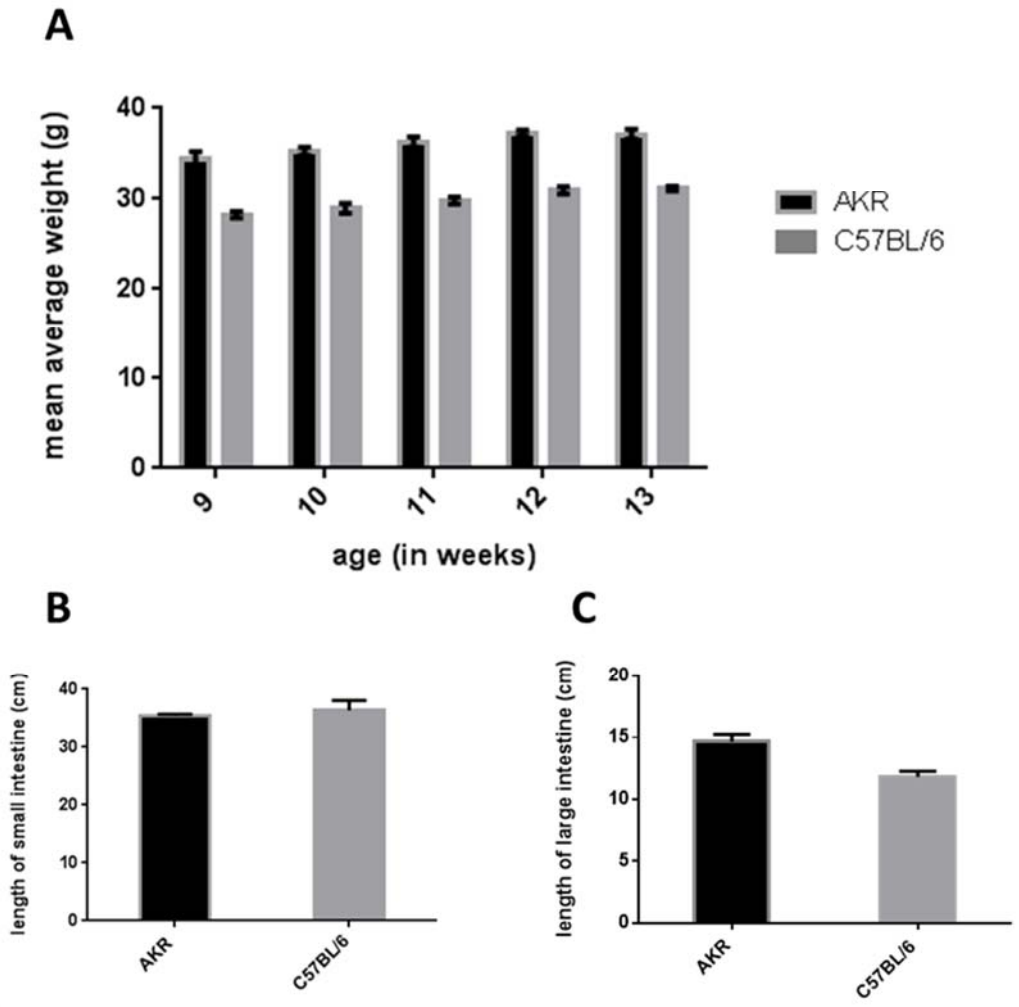


Figure 3.1 Comparison of weight and gut length between the AKR and C57BL/6 strains. **A:** weekly weight of AKR and C57BL/6 mice from age 9 to 13 weeks. Two way ANOVA $p<0.0001$. **B&C:** Average length of small intestine (**B**) and large intestine (**C**) for AKR and C57BL/6 mice. Data are shown as a mean value ± SEM. N=3 per group.

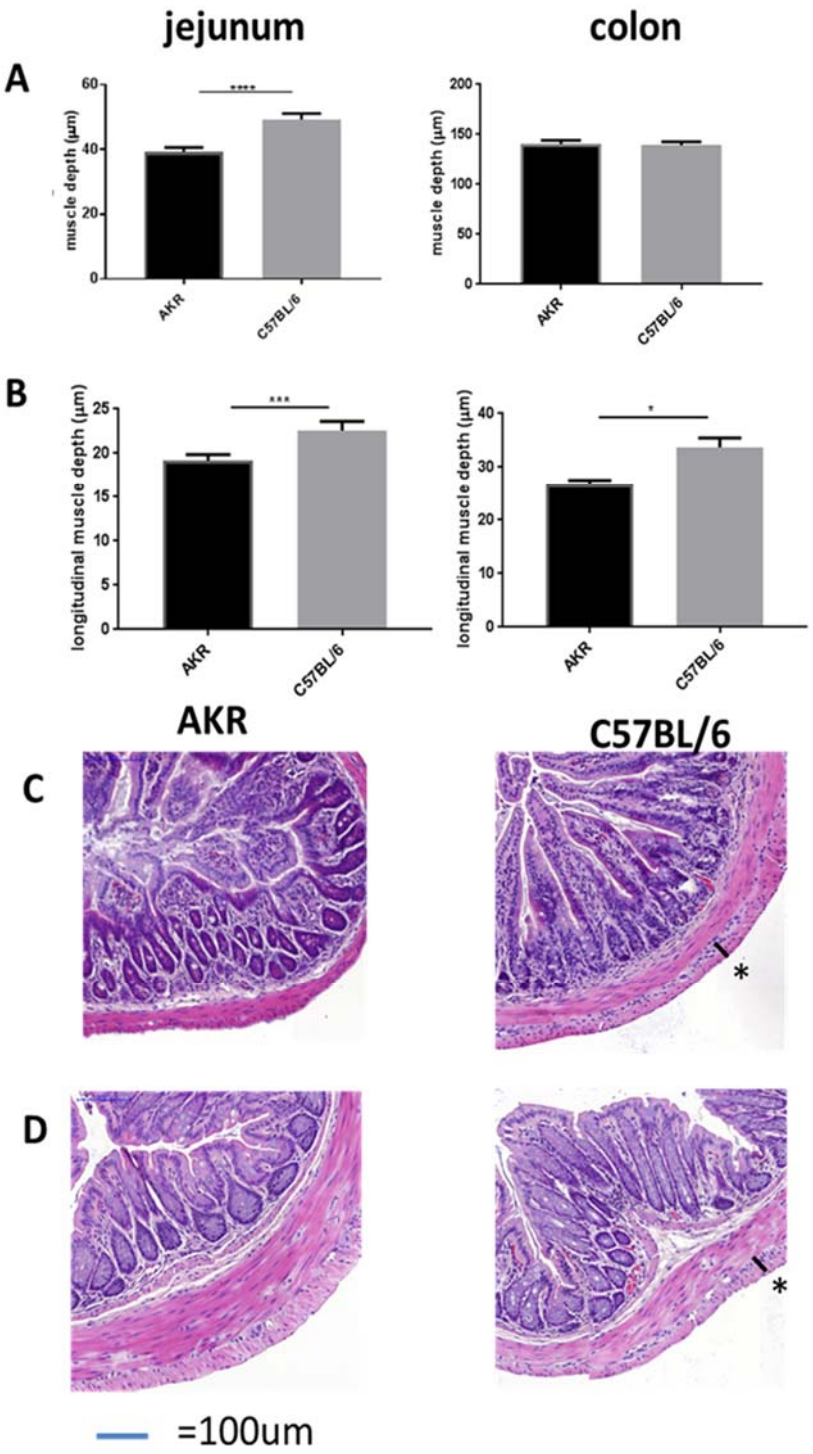


Figure 3.2 Significantly thicker total muscle in the jejunum and longitudinal muscle in the colon in the C57BL/6 mouse strain. A&B: Total muscle depth (**A**) and longitudinal muscle depth (**B**) in the jejunum and colon of AKR and C57BL/6 mice. **C&D:** Representative images of muscle thickness in the jejunum (**C**) and colon (**D**) for AKR and C57BL/6 mice (longitudinal muscle marked with line, see asterisk). Images shown at 200xs magnification. Images were viewed using panoramic viewer. N=5 per group, 4 sections per animal. Student's t-test, * $p<0.05$, *** $p<0.001$, **** $p<0.0001$.

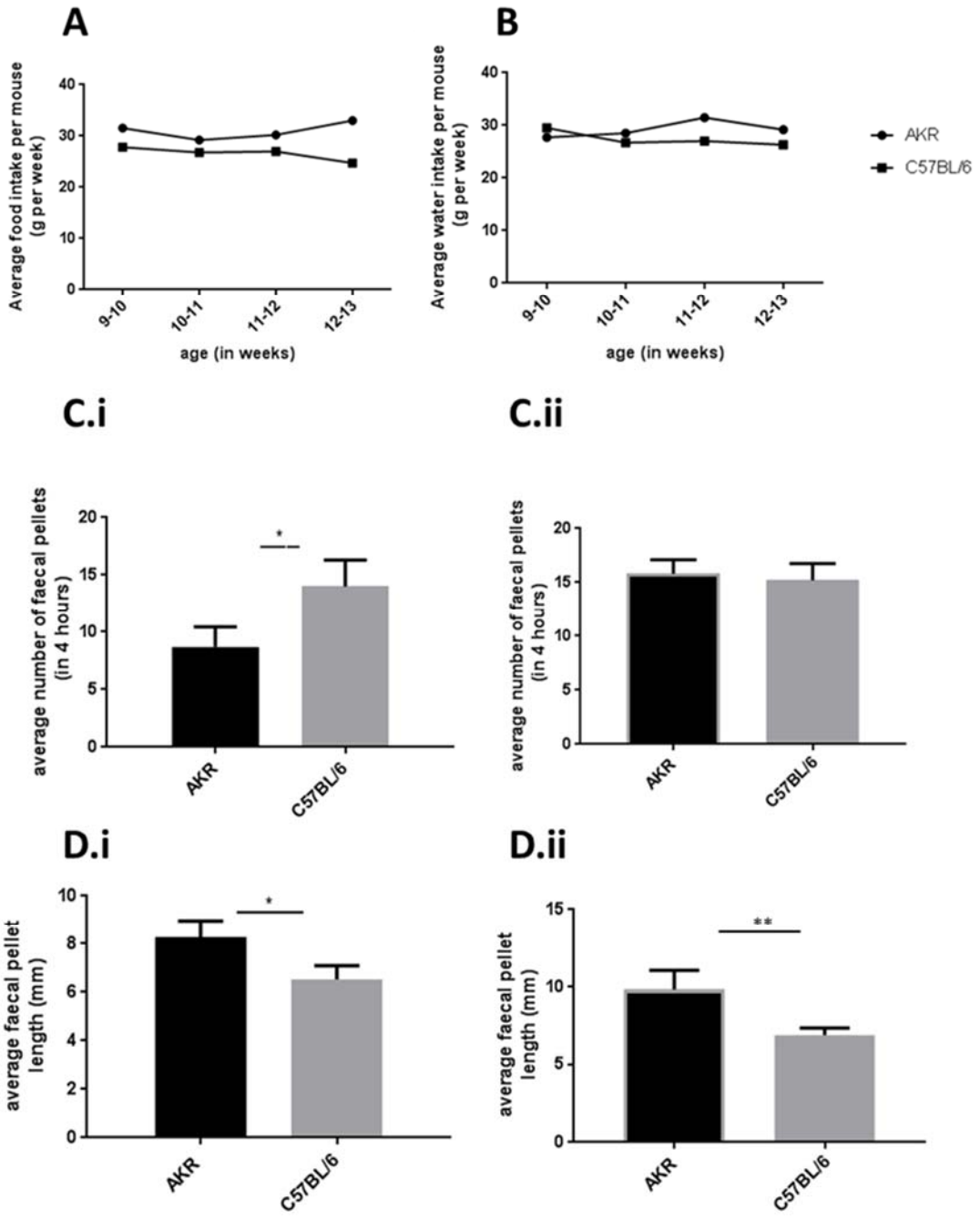


Figure 3.3 Measurement of food and water intake, faecal pellet number and length. A&B: Food (**A**) and water (**B**) intake for AKR mice and C57BL/6 mice was measured per cage for a 4 week period. Data are shown as absolute values per cage. **C:** Average faecal pellet number produced per animal in a 4 hour time period by AKR and C57BL/6 mice (**C.i**) and at retrial (**C.ii**). **D:** Average faecal pellet length (mm) in the AKR and C57BL/6 mice (**D.i**) and at retrial (**D.ii**). Data are shown as a mean value ± SEM. N=3-6 per group. Student's t-test, *p<0.05, **p<0.01.

3.5 Faecal water content

3.5.1 Mouse strain did not have any effect on faecal water content

Faecal pellets from AKR and C57BL/6 mice were collected over a 4 hour time period and water content calculated. There was no significant difference in faecal water content between strains on two separate occasions (AKR n=3, C57BL/6 n=3; AKR n=6, C57BL/6 n=5) (Figure 3.4). Actual values for percentage water content can be seen in table 3.1.

These data suggest that the AKR generates more faecal matter over time as pellets are longer but not increased in number.

Table 3.1. Average faecal water content.

Mouse	Average faecal water content per group
AKR (n=3)	29
C57BL/6(n=3)	26
Retrial	
AKR (n=6)	51
C57BL/6 (n=5)	50

3.6 Comparison of transit time

3.6.1 Mouse strain affected transit time

AKR and C57BL/6 mice were gavaged with 5% Evans blue and 5% gum Arabic in MQ water and the time taken for a blue faecal pellet to be passed was monitored. On two separate occasions AKR mice (n=3, n=6) had a significantly longer transit time (Student's t-test $p<0.01$, $p<0.05$) than C57BL/6 mice (n=3, n=5) (Figure 3.5, A.i and A.ii).

3.7 No link between transit time and water content.

Though the significant difference in transit time between the AKR and C57BL/6 mouse strains remained consistent, surprisingly there appeared to be no link between transit time and water content, this remaining similar for each strain but varying greatly between two separate occasions (Figure 3.6).

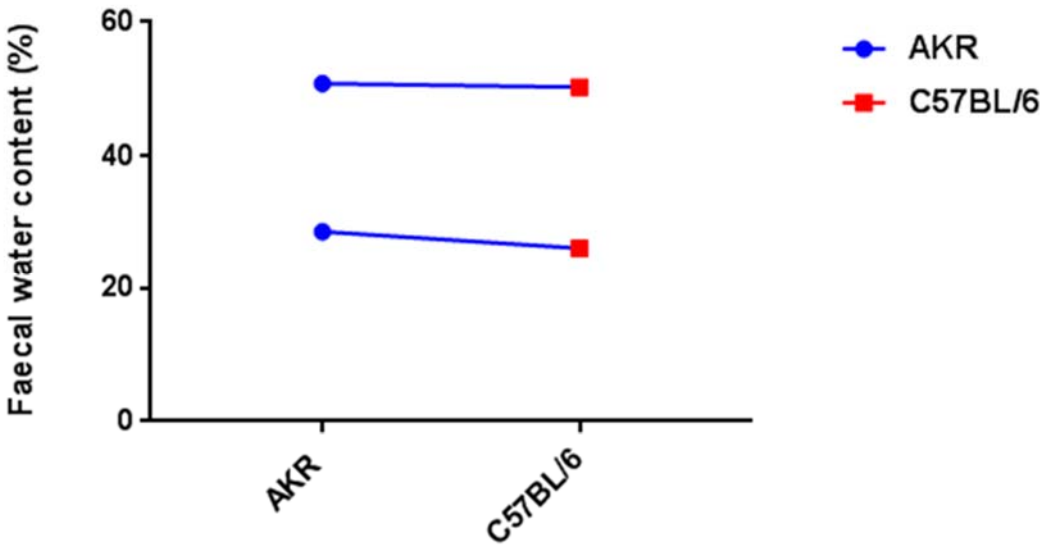


Figure 3.4 Average faecal water content. Faecal pellets were collected from AKR and C57BL/6 mice and water content per group calculated as a percentage. Data are expressed as mean average per group of animals (actual values can be seen in Table 3.1). N=3-6.

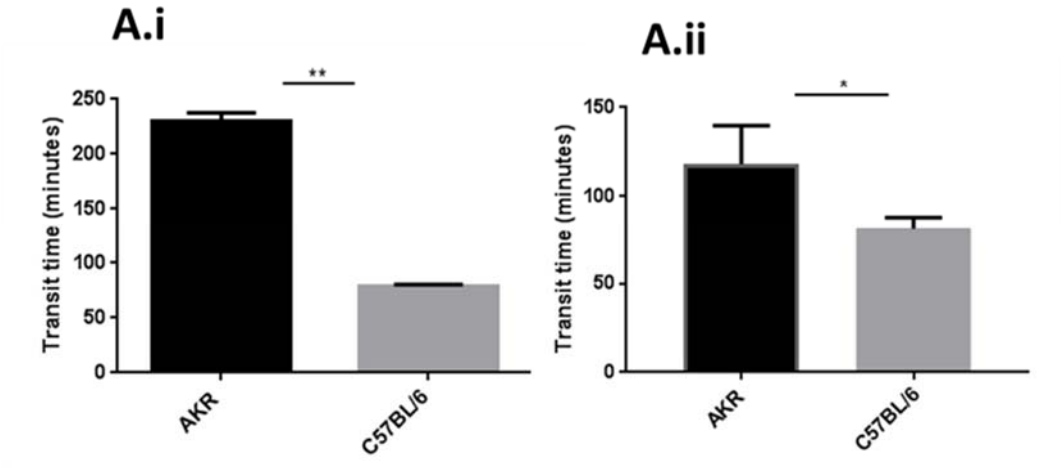


Figure 3.5 Significantly longer transit time in AKR mice than C57BL/6 mice. A: Faecal transit time in minutes measured for AKR and C57BL/6 mice (**A.i**) and at retrial (**A.ii**). Data are shown as a mean value ± SEM. N=3-6 per group. Student's t-test, *p<0.05, **p<0.01.

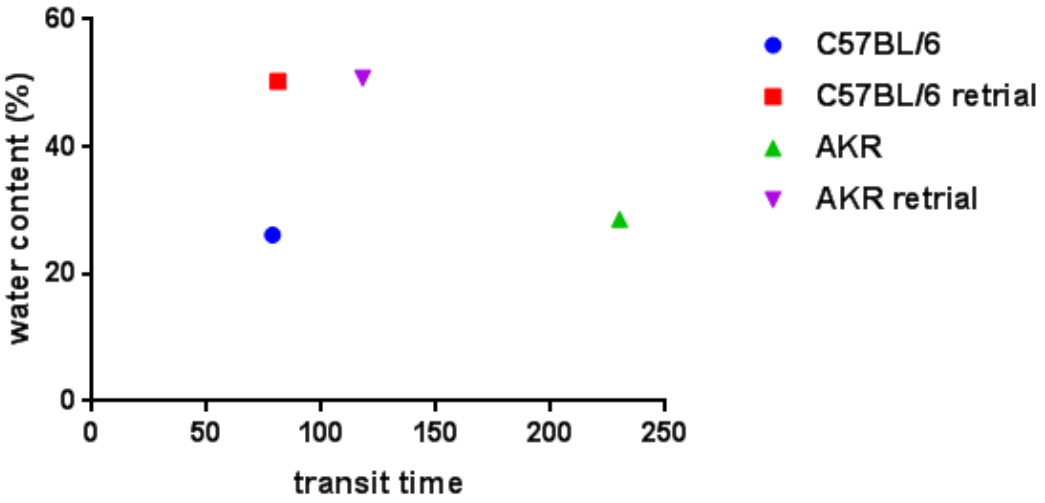


Figure 3.6 Variations in water content and transit time. There appeared to be variation in faecal water content in animals of the same strain on two different occasions. AKR mice appeared to have a slower transit time but similar water content to the corresponding C57BL/6 group.

3.8 Motility of the colon

3.8.1 No strain specific significant differences in spontaneous gut motility was found

To examine any strain specific differences in gut movement, a small section of proximal colon was taken from both the AKR (n=3) and C57BL/6 (n=3) strain of mice and placed in an organ bath containing oxygenated Kreb's solution. After 30 minutes of resting to ensure tissue equilibrium the number of gut contractions (number of events) and average strength of contractions over a three minute period was measured and the two strains compared. Although the difference between number of events and tension per event in a three minute period was not statistically significant (Student's t-test), the AKR strain appeared to demonstrate greater tension per contraction in comparison to the C57BL/6 strain. It was thought that this difference remained insignificant due to one animal per group that displayed results incongruent with this trend (AKR mouse 3, C57BL/6 mouse 2) (Figure 3.7).

3.9 Dose response to acetylcholine receptor agonist concentration

3.9.1 Strain specific significant difference in initial increase in gut tension following administration of carbachol 1μM

There was an immediate increase in tension in gut tissue from all animals following initial administration of carbachol at concentrations of 1μM and 10μM (it was thought that this initial increase was not evident at a carbachol concentration of 0.1μM due to this being below the threshold for causing any effect). This difference in tension was measured and strains compared. At a concentration of 1μM, carbachol caused a significantly greater increase in tension (Student's t-test $p<0.05$) in AKR mice (n=3) than in C57BL/6 mice (n=3). Although the immediate increase in tension in gut tissue was seen following administration of carbachol at a concentration of 10μM in all tissue, there was no significant difference between the two strains (Figure 3.8).

3.10 Acetylcholine receptor agonism and gut motility; carbachol.

3.10.1 No strain specific significant difference in gut motility following administration of carbachol (0.1μM)

In order to investigate any strain specific differences in acetylcholine receptor expression that may affect gut function, a muscarinic and nicotinic acetylcholine receptor agonist, carbachol, was added at increasing concentrations in accordance with protocols for experiments in the jejunum (Grasa et al., 2015) to the oxygenated Kreb's solution in the organ bath and effect on frequency and strength of gut contractions was measured. There was no significant difference between strains in number or strength of gut contractions during a three minute period following administration of carbachol at a concentration of 0.1μM (Student's t-test) (Figure 3.9).

3.10.2 No strain specific significant difference in gut motility following administration of carbachol (1μM)

There was no significant difference between the AKR (n=3) and C57BL/6 (n=3) mice in number or strength of gut contractions during a three minute period following administration of carbachol at a concentration of 1μM (Student's t-test) (Figure 3.10).

3.10.3 Strain specific significant difference in average tension of gut contraction following administration of carbachol (10μM)

Average tension per gut contraction, or event, in response to carbachol at a concentration of 10μM for a three minute period was significantly greater (Student's t-test $p<0.05$) in AKR mice (n=3) than in C57BL/6 mice (n=3) (Figure 3.11), suggesting a strain specific difference in muscarinic or nicotinic receptor expression or function. There was no significant difference between strains in number of gut contractions during a three minute period following administration of carbachol at a concentration of 10μM.

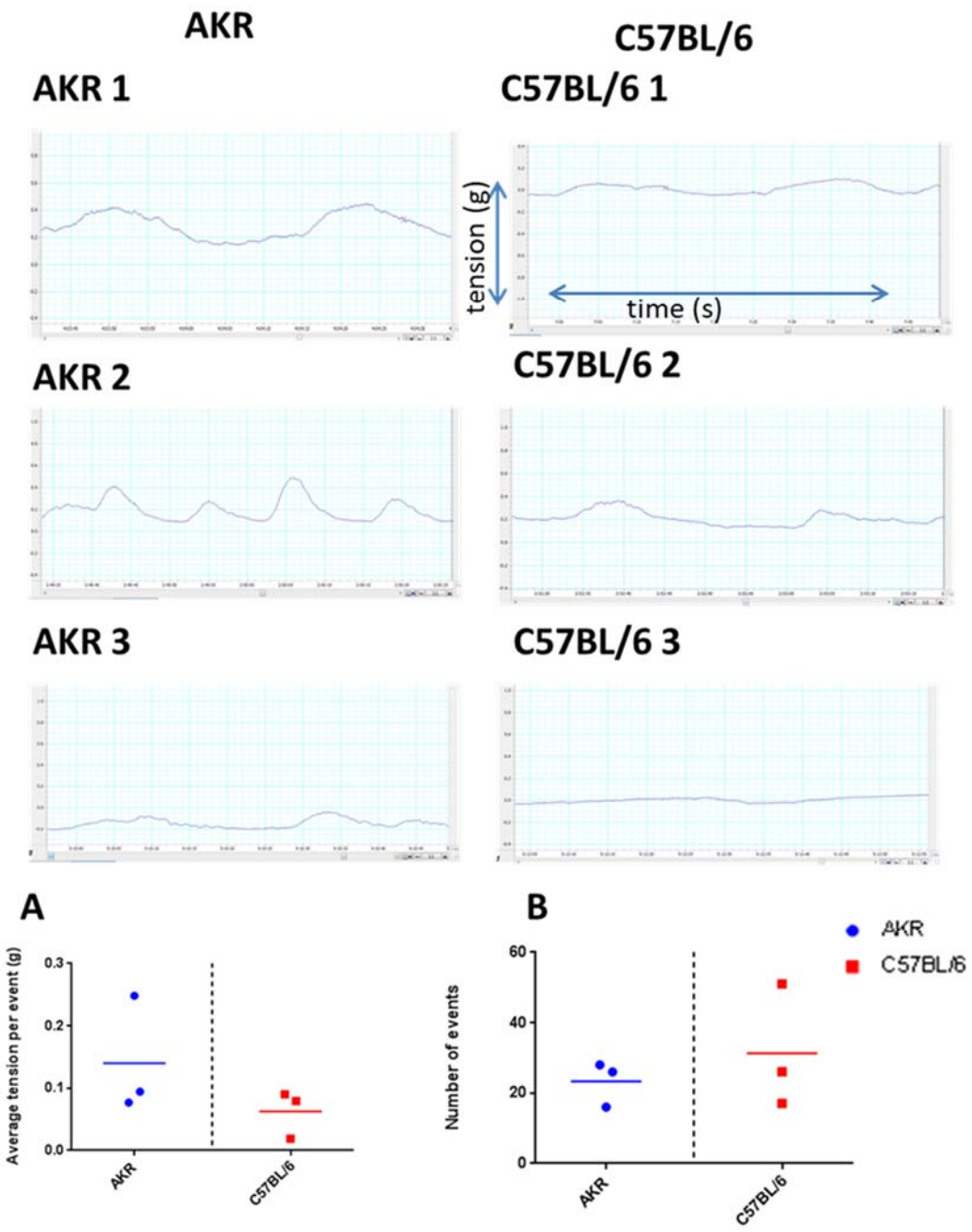


Figure 3.7 No significant difference between AKR and C57BL/6 mice in spontaneous motility of the proximal colon. A&B: Average tension per event (**A**) and average number of events (**B**) in the proximal colon was compared between AKR and C57BL/6 mice over a 3 minute period. Actual traces for each animal and mean average values per animal can be seen above (**A&B**). N=3 per group.

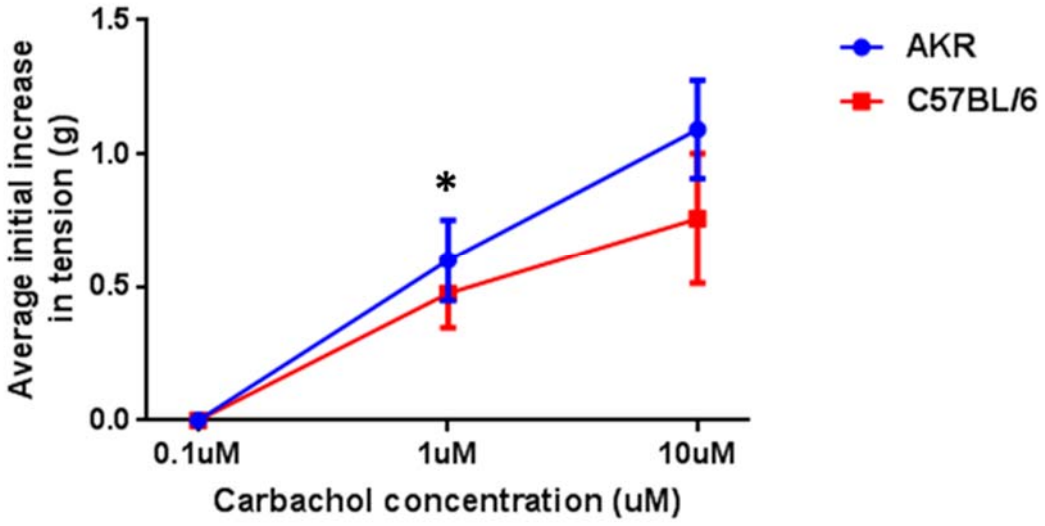


Figure 3.8 Significantly greater effect of carbachol administration (1μM) on tension of the colon in the AKR mouse strain than the C57BL/6 mouse strain. A dose response curve showing the average initial increase in tension (g) in the proximal colon in AKR mice and C57BL/6 mice following immersion in carbachol at a concentration of 0.1μM, 1μM and 10μM. Data are shown as mean average per group ± SEM. N=3. Student's t-test, *$p<0.05$.

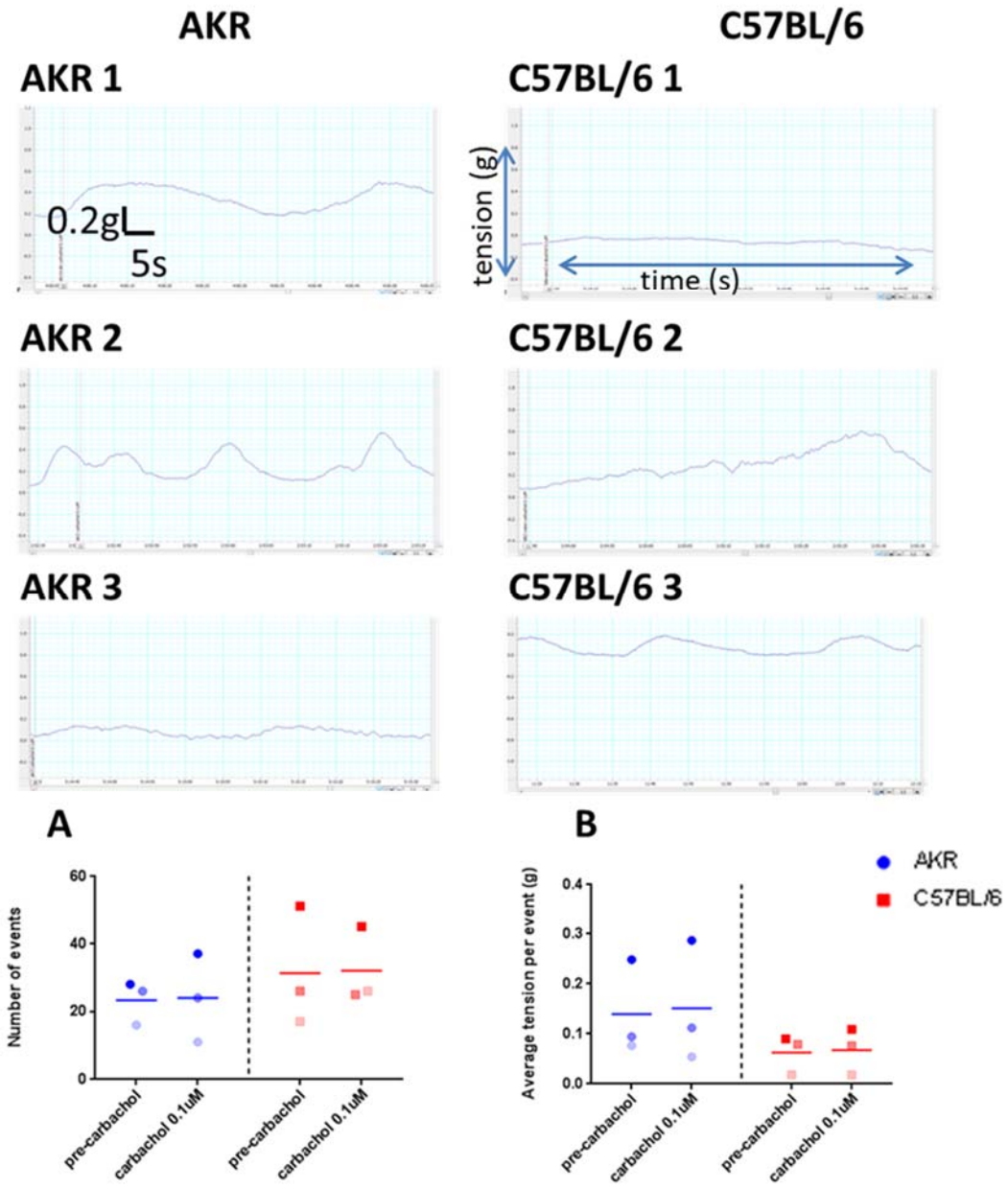


Figure 3.9 No significant difference between strains following administration of carbachol 0.1μM. A&B: Carbachol 0.1μM was administered to proximal colon in AKR and C57BL/6 mice and average number of events (**A**) and average tension per event (**B**) per animal was measured over a 3 minute period. Individual traces for each animal can be seen above. N=3 per group.

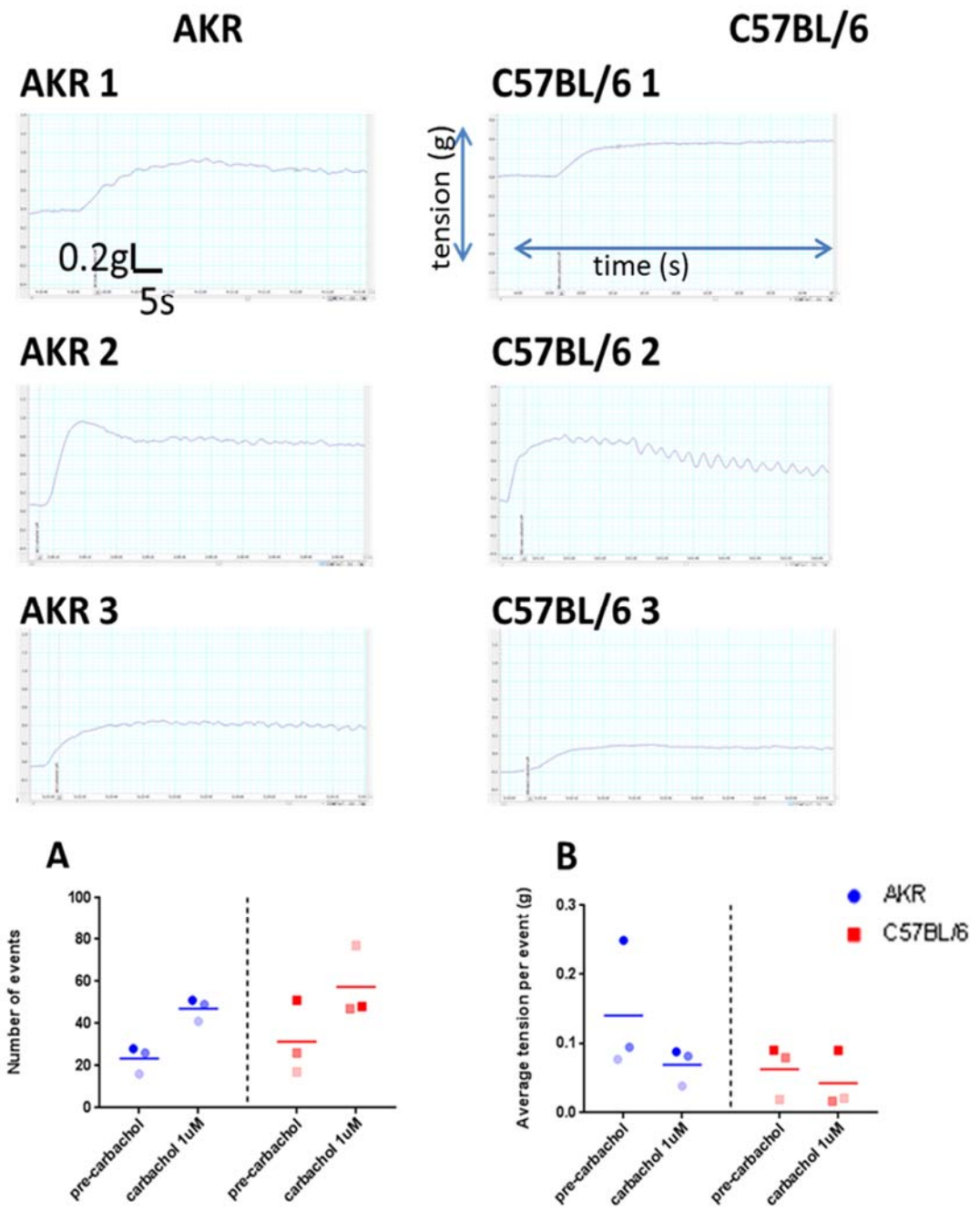


Figure 3.10 No significant difference between strains following administration of carbachol 1µM. A&B: Carbachol 1µM was administered to proximal colon in AKR and C57BL/6 mice and average number of events (**A**) and average tension per event (**B**) per animal was measured over a 3 minute period. Individual traces for each animal can be seen above. N=3 per group.

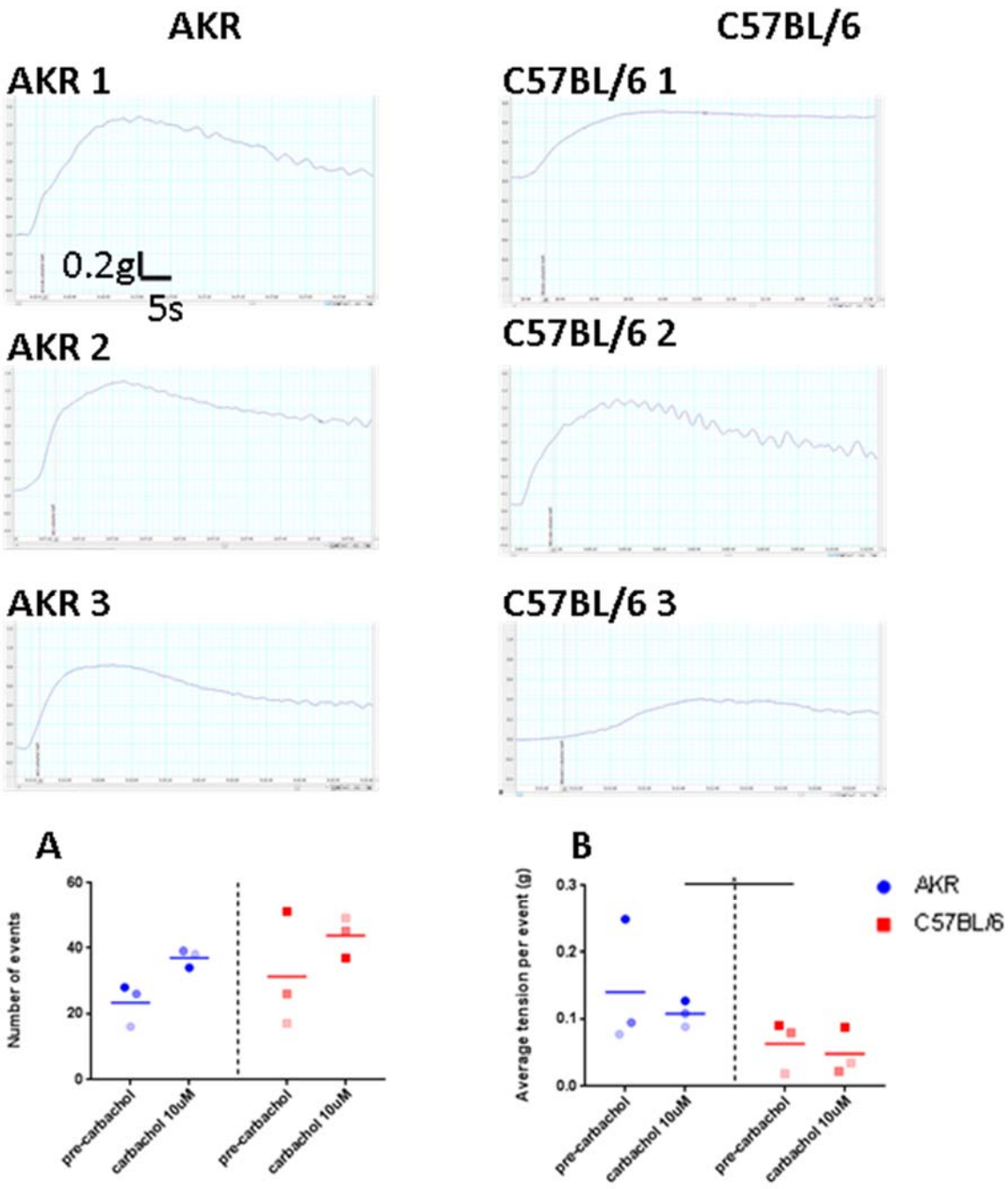


Figure 3.11 Significant increased average tension per event in the AKR mouse strain following administration of carbachol 10μM. A&B: Carbachol 10μM was administered to proximal colon in AKR and C57BL/6 mice and average number of events (**A**) and average tension per event (**B**) per animal was measured over a 3 minute period. Individual traces for each animal can be seen above. N=3 per group. Student's t-test, *p<0.05.

3.11 TRPV1 receptor agonism and gut motility

3.11.1 No strain specific significant difference in gut motility following administration of capsaicin (0.1μM, 1μM and 10μM)

The effect of TRPV1 agonism on gut motility and any possible strain specific differences in TRPV1 expression or function was investigated using the TRPV1 specific agonist capsaicin. Capsaicin was added at increasing concentrations to the oxygenated Kreb's solution in the organ bath and effect on frequency and strength of gut contractions was measured. There was no significant difference between strains in number or strength of gut contractions during a three minute period following administration of capsaicin at concentrations of 0.1μM, 1μM and 10μM (Student's t-test) (Figure 3.12). However, capsaicin appeared to consistently reduce the number of events, or gut contractions, in the C57BL/6 strain at 0.1μM and 10μM.

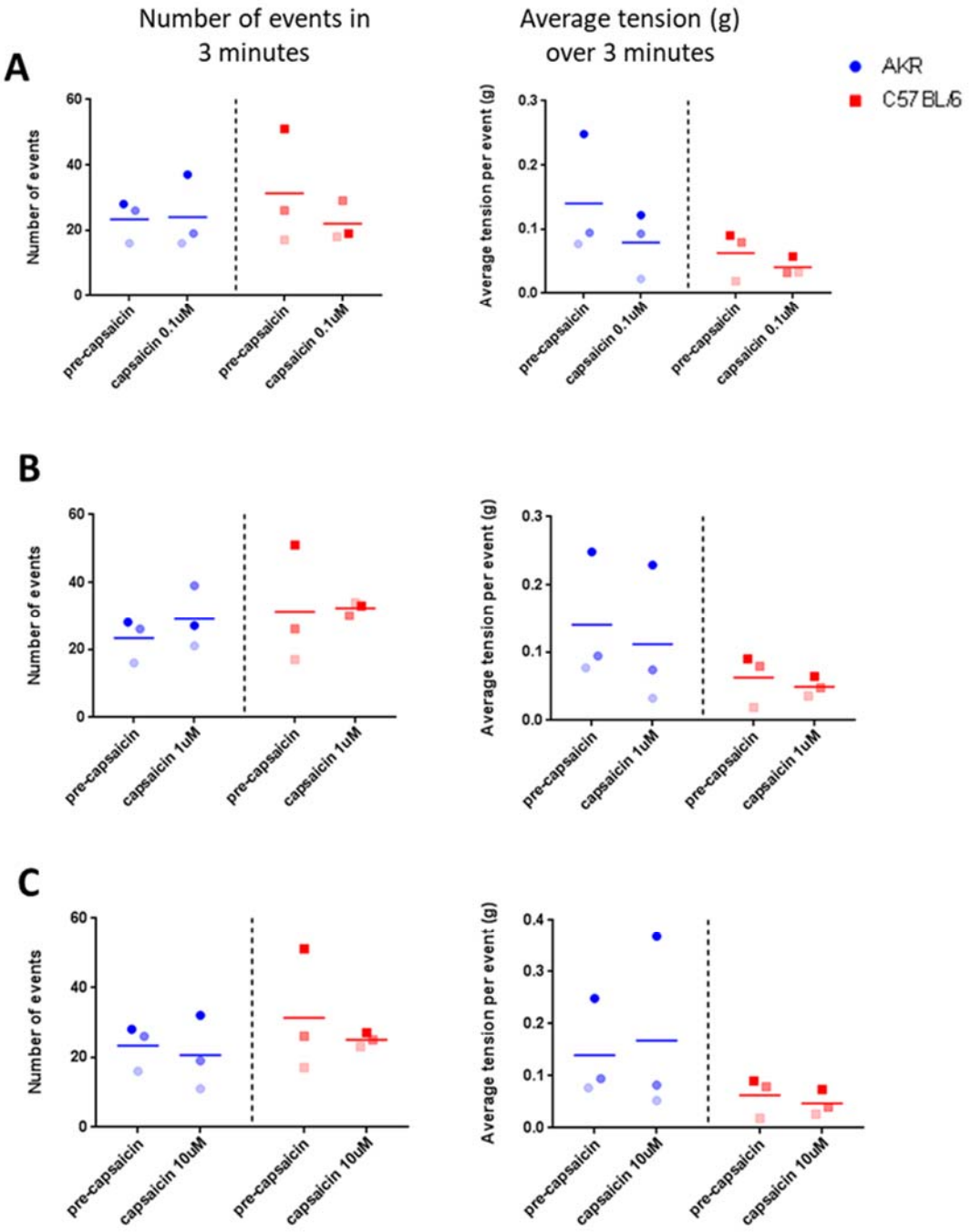


Figure 3.12 No difference between strains following administration of capsaicin (0.1μM, 1μM and 10μM). Capsaicin was administered to proximal colon in AKR and C57BL/6 mice at concentrations of 0.1μM (A), 1μM (B) and 10μM (C) and average number of events and average tension per event per animal was measured over a 3 minute period. N=3 per group.

3.12 Discussion

It has been documented that there is a clear link between the immune system and the nervous system and so normal variations in the enteric neural map due to genetic background may influence susceptibility to gastro-intestinal disease in both humans and animals. Despite this, there is little information comparing different nervous system maps and the possible effect of this on function between different strains of the same species. Much research on gut physiology has focussed on manipulation of normal conditions or effects of drugs rather than comparing baseline function in different strains of the same species (Bove, 2015; Choi and Chang, 2015; Grasa et al., 2015). To investigate if genetic variation leads to differences in gut function, possibly via a varied enteric nervous system, this study investigated potential differences in gross anatomy and physiological function of the gut between two different strains of mice (AKR and C57BL/6) by monitoring and measuring a number of physical parameters. Several previously unidentified variations in normal gut anatomy between the two strains were found.

3.12.1 Genetic influence has some consistent effects on gut anatomy and physiology

Along with impact on the enteric neural network, genetic variation could influence normal gut physiology in several ways. We found that although both strains of mice gained weight steadily each week, during a five week period AKR mice were consistently heavier than C57BL/6 mice, ate slightly more food and drank slightly less water, despite having the same access to food, water and exercise. West et al., (1992) found that AKR mice are not significantly different in weight from the C57BL/6 strain at age five weeks but are more sensitive to dietary obesity on a high fat diet. It was thought that this may be linked with a quantitative trait locus controlling mesenteric fat deposit size and variation in tissue adiposity on chromosome 15 (York et al., 1996) and may prove advantageous in the wild by facilitating survival through periods of famine (West et al., 1992). However, more recent studies have shown links between obesity and the microbiome (Ley, 2010) which may be impacted by factors such as genetics, transfer of microbes from the vaginal canal during birth and stress (Jiminez et al., 2015); variation in the microbiome has also been shown to affect neuronal function (McVey Neufeld et al., 2013) and therefore may be having an impact on enteric nervous system physiology.

In this study, we found C57BL/6 mice to have significantly thicker total and longitudinal muscle thickness in the jejunum and thicker longitudinal muscle in the colon than AKR mice. As the longitudinal muscle is believed to shorten the gastro-intestinal tract, this could explain our other findings of a trend for AKR mice to have a longer large intestine than the C57BL/6; conversely, there is a trend for the C57BL/6 mice to have a longer small intestine than the AKR mice, suggesting the thickness of longitudinal muscle has an inconsistent effect on gut length. However, we did find significant differences in transit time between strains; the shorter transit time of the C57BL/6 strain could be linked to thicker muscle found in the jejunum and colon producing stronger contractions and propelling faecal contents through the gastro-intestinal tract at a comparatively faster pace than the AKR strain. It is perhaps surprising to note that we found no difference in faecal water content between the strains despite the variations in transit time, suggesting that the speed at which the faecal pellet passes through the gut has no effect on the amount of water absorbed.

We also found significant differences in pellet length between the two strains; the AKR mice produced significantly longer faecal pellets than the C57BL/6 mice on two separate occasions ($p<0.05$, $p<0.005$). Consequentially it was surprising that the faecal pellet number was inconsistent across experiments. This suggests that there is no link between muscle thickness, transit time and faecal pellet number.

3.12.2 Genetic influence affects muscle contractility within the proximal colon

Much previous published work investigating muscle contractility has focussed on manipulation of normal conditions, such as effects of drug administration and changes in microbiome constitution. Our results present a novel finding in the understanding of basic gut physiology; we found that stimulation of acetylcholine and TRPV1 receptors in the proximal colon with a chemical agonist produced some differences in muscle contractility between the AKR and C57BL/6 strains. This suggests variation in expression of neuroreceptor expression, function, or possibly both which would result in variation in normal parasympathetic nervous function between strains. This difference could be a result of variation in muscle thickness; however, it is interesting to note that the AKR strain was found to have thinner longitudinal muscle in the colon but produced the greater tension during contraction in response to stimulation of nicotinic and muscarinic acetylcholine receptors. This suggests that number and function of these neuroreceptors is not linked to the

volume of muscle present but is controlled by another factor. Interestingly, the alpha 7 nicotinic acetylcholine receptor has been linked directly with the inflammatory response, in macrophages in particular (Tracey, 2002).

Administration of a TRPV1 receptor agonist appeared to reduce the number of gut contractions in the C57BL/6 strain but had no effect on the AKR mice, suggesting some strain specific impairment in function of or response to TRPV1 stimulation in the AKR. Whilst previous studies have reported similar findings in the hindpaw (Mogil et al., 2005), this is the first known study to highlight a similar strain specific difference in the gut related to gut function. As stimulation of this receptor leads to CGRP production (Meng et al., 2009), known to be a factor in the establishment of a Th2 response (Mikami et al., 2011), this could also directly contribute to immune response variation to parasite invasion.

In this study we investigated the effect of genetic variation via two different strains of mice (C57BL/6 and AKR) on normal gut anatomy and physiology. We found several differences between the two strains, some of which were inconsistent. There was also some variation within the strains. This difference in gut function suggests that known differences in the peripheral nervous systems of these two inbred mouse strains are reflected within the enteric nervous system. It was then decided to investigate the enteric nervous system within these animals to see if any differences could be identified.

Summary of key findings

Significant differences in-
Weight gain- AKR consistently heavier than C57BL/6
Total muscle depth- thicker in C57BL/6 in jejunum than AKR
Longitudinal muscle depth- thicker in C57BL/6 jejunum and colon than AKR
Faecal pellet length- AKR has longer pellets than C57BL/6
Faecal transit time- AKR has longer transit time than C57BL/6
Initial response to carbachol (1μM)- AKR greater increase in tension than C57BL/6
Average tension gut contraction in response to carbachol (10μM)- AKR greater tension (3 minute period)

No significant differences in-
Length of small or large intestine
Faecal pellet number
Water content
Spontaneous gut motility

Initial response to carbachol (0.1μM and 10μM)
Average tension gut contraction in response to carbachol (0.1μM and 1μM) in a 3 minute period
Average number of gut contractions in response to carbachol (0.1μM and 1μM) in a 3 minute period
Initial response to capsaicin
Average tension or number of gut contractions in response to capsaicin (0.1μM, 1μM and 10μM) in a 3 minute period

Chapter 4

Immunofluorescence and histochemical investigation revealed some differences in the enteric nervous system of the colon between the AKR and C57BL/6 mouse strains

4.1 Introduction

Previous results identified some differences in gut physiology, such as variation in transit time and faecal pellet length, between two inbred strains of mice (AKR and C57BL/6); it is likely that these differences are a result of variation in the enteric nervous system that reflect known differences in the peripheral nervous system of these mice. Many studies have investigated the anatomy of the enteric nervous system in various species including the pig (Brown and Timmermans, 2004), guinea pig (Hu et al., 2002), rat (Sundler et al., 1989) and mouse (Sang and Young, 1996). However, information regarding comparison of different strains of animal from the same species, and therefore the impact of genetic variation, is sparse. This information may be useful in understanding the effect of genetic variation on the enteric nervous system and the effect of this in human physiology and disease susceptibility. To investigate this, jejunum and colon was taken from AKR and C57BL/6 mice and evaluated with histochemistry and immunofluorescence techniques to quantify and compare enteric neurons.

Many studies have mapped the enteric nervous system in a variety of species with a focus on quantifying and categorising neurons within the myenteric plexus, the larger plexus of the two, utilising a whole mount technique with microdissection to separate the submucosal plexus and lamina propria from the myenteric plexus (Galvis et al., 1987; Yu et al., 2009). However, if we consider the neuro-immune link important and the differing response of these two strains to parasitic gut infection, then any nerves present in close proximity with the epithelium and within the lamina propria would be of particular interest as these are the first to encounter the parasite. For this reason, a whole mount approach was trialled without microdissection. A pan neuronal marker was required to identify any differences in overall neural expression between the two strains. Much published work examining the peripheral nervous system and the gut in particular utilised beta III tubulin or PGP9.5 immunofluorescent antibodies (Eisenman et al., 2013; Harrington et al., 2010; He et al., 2016; Betolli et al., 2008). Beta III tubulin is a component of the microtubule structure of all neurons believed to be exclusive to the nervous system (Caccamo et al., 1989), and PGP 9.5 is a ubiqutination hydrolase product thought to be a sensitive marker of neural cells and neural fibres (Campbell et al., 2003). These antibodies highlight cell bodies along with axonal projections, therefore it was decided that these would be acceptable markers to trial. Despite numerous attempts and a variety of protocols, the whole mount technique remained

unsuccessful, perhaps due to inability of antibodies to penetrate tissue of such thickness or issues with depth of tissue that the confocal microscope could illuminate. Therefore, tissue was sectioned along two axes at a thickness of 10μm, transversely and longitudinally along the length of the gut, with transverse sections demonstrating primarily nerves in the submucosal plexus and lamina propria, and longitudinal sections demonstrating the myenteric plexus.

As the accuracy and reliability of antibody markers has been questioned (Baker, 2015), an older and less frequently used histochemical technique for identification of nerves was also employed, the Sevier-Munger silver stain technique. Despite some issues with sensitivity and specificity, silver stains have provided a useful tool for neuropathologists (Uchihara, 2007). The Sevier-Munger stain, modified for paraffin embedded tissue from the Bielchowsky method, is an argyrophilic technique that highlights neurons and neurofibres (Sotelo et al., 1980). It was hoped that a combination of these old and new techniques would visualise the enteric nervous system in its entirety and allow comparisons of quantification to be made. Any difference in the enteric nervous system may explain the functional differences in physiology, and therefore maybe immunity, between the two strains.

Hypothesis

There are known differences in the peripheral nervous system between the AKR and C57BL/6 mouse strains that may be reflected in the enteric nervous system. As we identified differences in gut function between the two strains it is likely that these differences extend to the enteric neural map. These differences will ultimately impact susceptibility to and recovery from disease via the neuro-immune link; this genetic influence is likely to be reflected in a human population and therefore may be of importance in understanding susceptibility to and recovery from gastro-intestinal disease.

Aim

To investigate and compare anatomy of the enteric nervous system in two different inbred strains of mice (C57BL/6 and AKR). Various immunofluorescence and histochemical studies will be utilised in order to identify a ‘pan-neuronal’ marker for the enteric neural map in the myenteric plexus and submucosal plexus and lamina propria of the jejunum and colon. These markers include immunofluorescence antibodies commonly used for identification of gut nerves, such as beta III tubulin and PGP 9.5, alongside a newer antibody cocktail containing beta III tubulin,

neurofilament medium (NEFM), Neuronal Nuclei (NeuN) and microtubule associated protein 2 (MAP2) and described as ‘pan neuronal’. Histochemical techniques including Sevier-Munger silver stain, Luxol Fast Blue and Picrosirius red stain will also be utilised to identify all nerves, myelinated nerves and collagen, respectively. Amount of fluorescence/ density of staining within a section will be measured and compared to elucidate any strain specific differences. Genetic strain specific differences in the enteric neural map are likely to reflect previous strain specific differences in gut physiology and therefore are likely to influence susceptibility to and recovery from gastro-intestinal disease via the neuro-immune link. This is likely to be reflected in a human population.

4.2 Immunofluorescence in the jejunum and colon

4.2.1 Variation in neurons identified within the gut with different 'pan neuronal' markers

Antibodies to pan neuronal components beta III tubulin and PGP9.5 were trialled on a C57BL/6 mouse using a double staining method and compared; despite some overlap of fluorescence being evident, subsets of neurons could be seen adjacent to each other that were specific for each of these individual markers but not both. Beta III tubulin appeared to show more nerves than PGP9.5 (Figure 4.1).

4.3 Beta III tubulin immunofluorescence in the submucosal plexus and lamina propria

4.3.1 No significant difference in beta III tubulin immunofluorescence in submucosal plexus and lamina propria in the jejunum or colon between strains

Jejunum and proximal colon was taken from AKR and C57BL/6 mice, embedded in paraffin, sectioned transversely at a thickness of 10μm and incubated with beta III tubulin antibody. Immunofluorescence in the submucosal plexus and lamina propria was quantified and despite a trend for higher level of fluorescence in the C57BL/6 mice, no significant difference was found between strains (Student's t-test) in the jejunum (Figure 4.2) and colon (Figure 4.3).

4.4 Beta III tubulin immunofluorescence in the myenteric plexus

4.4.1 No significant difference in beta III tubulin immunofluorescence in the myenteric plexus in the jejunum or colon between strains

Jejunum and proximal colon was taken from AKR and C57BL/6 mice and cut longitudinally, flattened and embedded in paraffin, sectioned at a thickness of 10μm and incubated with beta III tubulin antibody. Immunofluorescence in the myenteric plexus was quantified and no significant difference was found between strains (Student's t-test) in the jejunum (Figure 4.4) and colon (Figure 4.5); variation in immunofluorescence levels could be seen within the groups in both the jejunum and

colon with a trend for increased beta III tubulin immunofluorescence in the C57BL/6 in the colon.

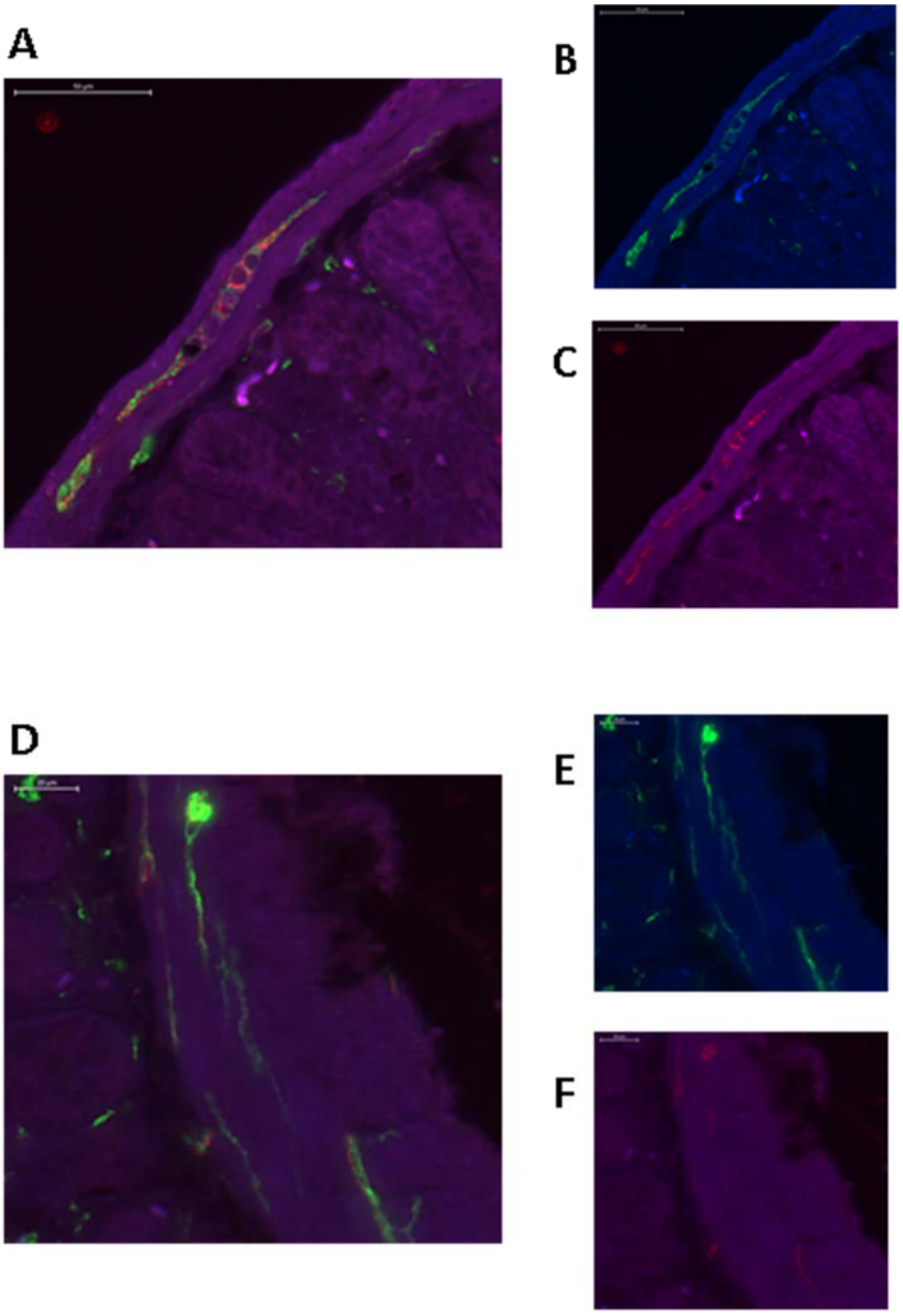


Figure 4.1. Variation in nerves in the intestine demonstrated with pan markers via immunofluorescence. A cross section of jejunum (A, B, C- 600xs mag, D, E, F- 700xs mag) with beta III tubulin (green) and PGP9.5 (red) immunofluorescence staining, both considered pan neuronal markers, clearly showing some neurons are double stained (A, D- yellow) and a subset are single stained with either beta III tubulin (B, E- green) or PGP9.5 (C, F- red). Images B and E show beta III tubulin immunofluorescence, C and F show PGP9.5 immunofluorescence, A and D show merged images.

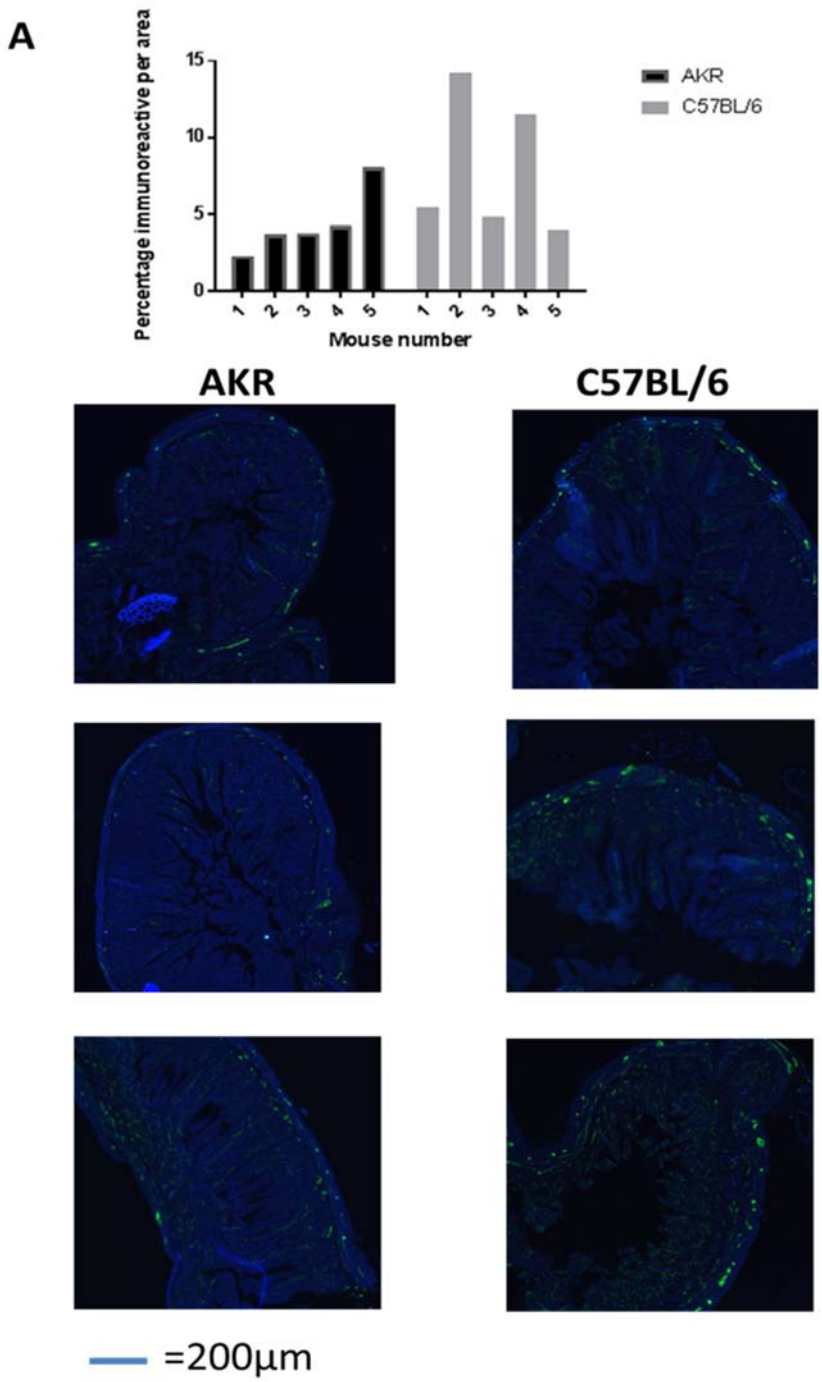


Figure 4.2. No strain specific significant difference in beta III tubulin immunofluorescence in the submucosal plexus and lamina propria of the jejunum. Quantification of beta III tubulin expression (**A**) in the jejunum of AKR and C57BL/6 mice by immunohistochemistry showing data for each animal. Representative images of beta III tubulin (green) expression in the jejunum shown at 100xs magnification. Images were viewed using panoramic viewer. N=5 per group, 3-4 sections per animal.

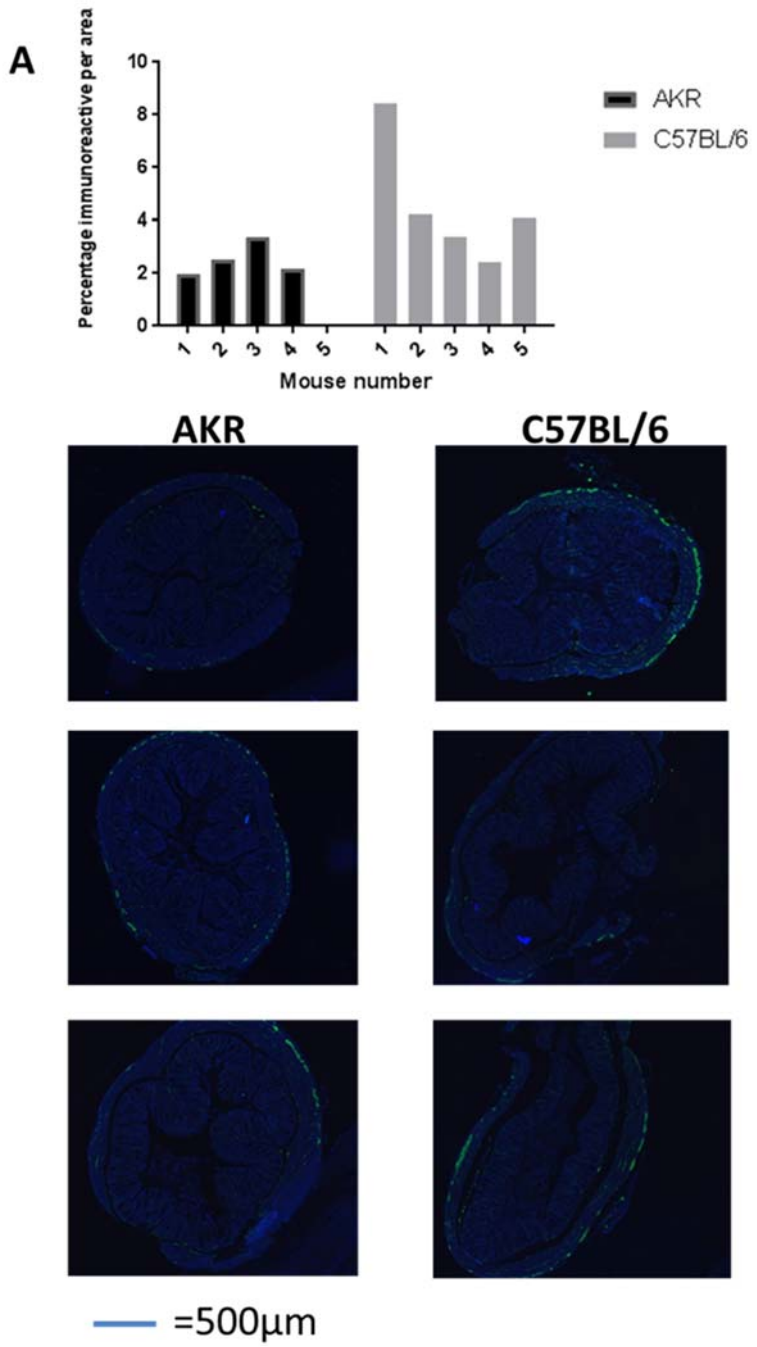


Figure 4.3. No strain specific significant difference in beta III tubulin immunofluorescence in the submucosal plexus and lamina propria of the colon. Quantification of beta III tubulin expression (**A**) in the colon of AKR and C57BL/6 mice by immunohistochemistry showing data for each animal. Representative images of beta III tubulin (green) expression in the colon (images shown at 50xs magnification). Images were viewed using panoramic viewer. N=4-5 per group, 3- 4 sections per animal.

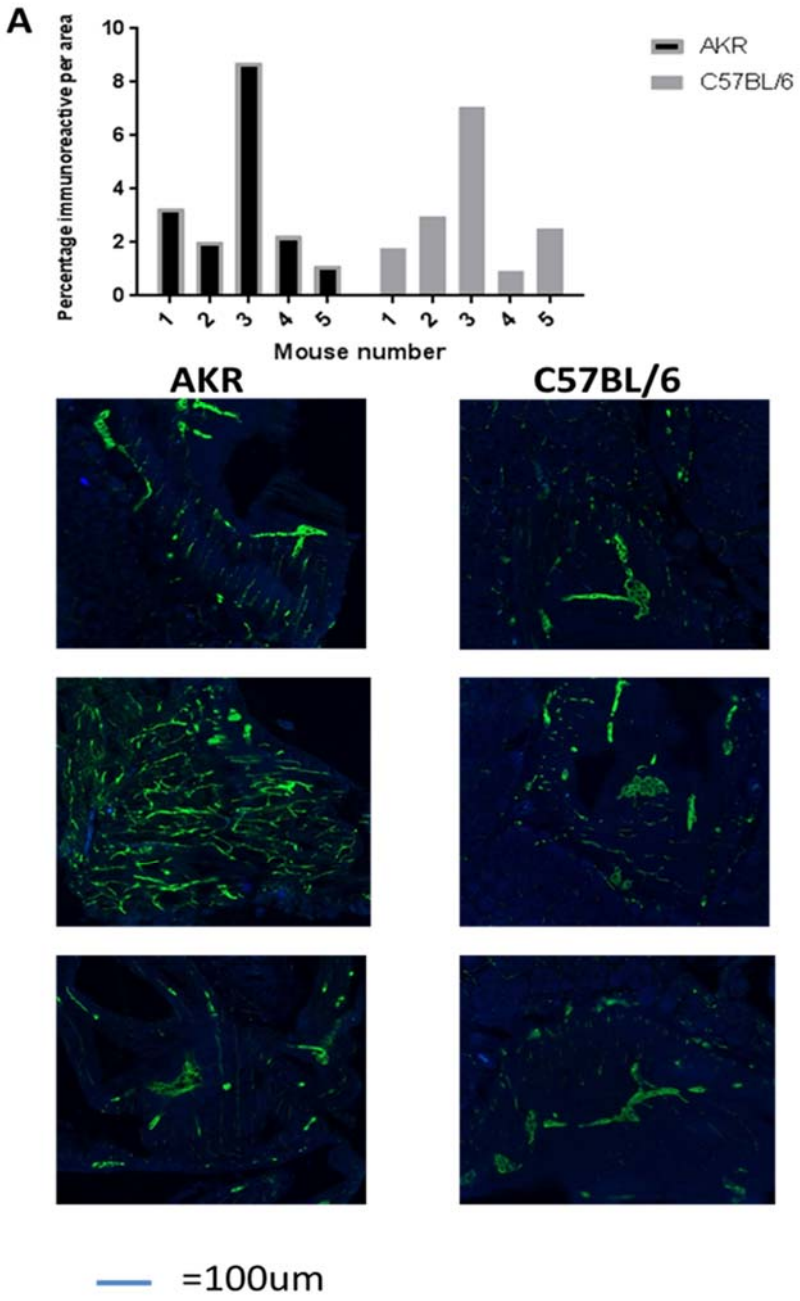


Figure 4.4 No strain specific significant difference in beta III tubulin immunofluorescence in the myenteric plexus of the jejunum. Quantification of beta III tubulin expression (**A**) in the jejunum of AKR and C57BL/6 mice by immunohistochemistry showing data for each animal. Representative images of beta III tubulin (green) expression in the jejunum (images shown at 200xs magnification). Images were viewed using panoramic viewer. N=5 per group, 3- 4 sections per animal.

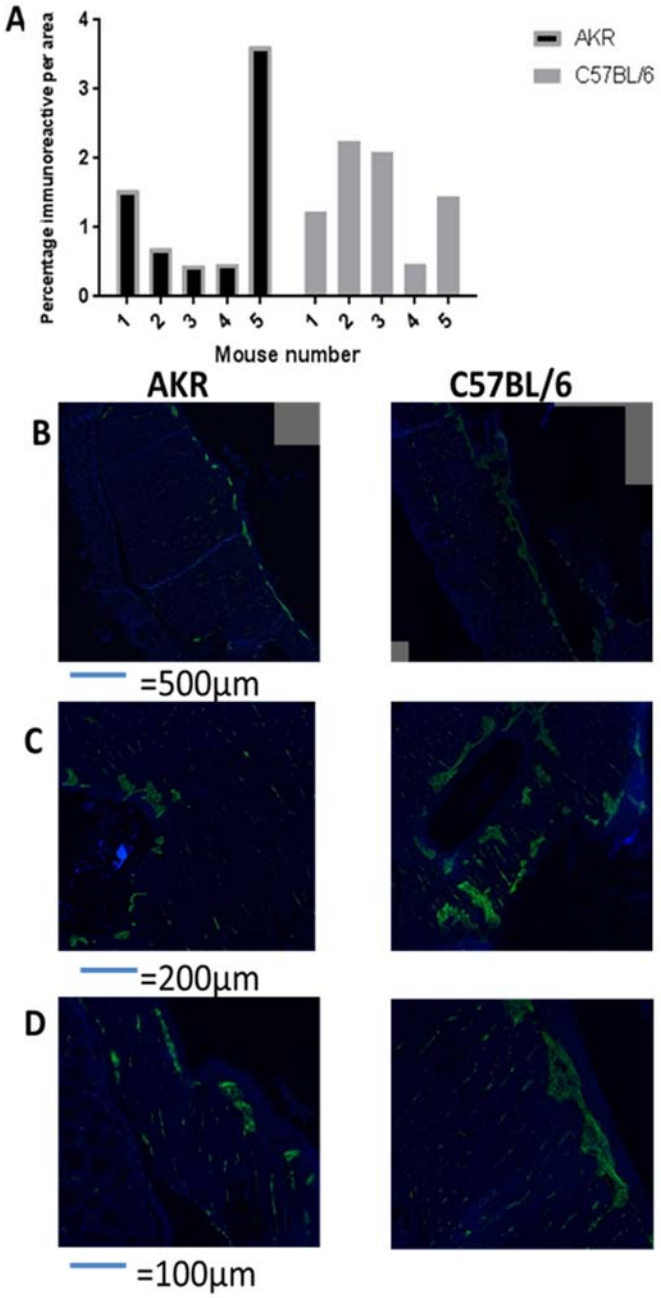


Figure 4.5. No strain specific significant difference in beta III tubulin immunofluorescence in the myenteric plexus of the colon. Quantification of beta III tubulin expression (**A**) in the colon of AKR and C57BL/6 mice by immunohistochemistry showing data for each animal. Representative images of beta III tubulin (green) expression in the colon (images shown at B- 50xs, C-100xs and D-200xs magnification). Images were viewed using panoramic viewer. N=5 per group, 3- 4 sections per animal.

4.5 'Pan neuronal' antibody immunofluorescence in the submucosal plexus and lamina propria

4.5.1 No significant difference in pan neuronal antibody immunofluorescence in the submucosal plexus and lamina propria in the jejunum or colon between strains

Jejunum and proximal colon was taken from AKR and C57BL/6 mice, embedded in paraffin, sectioned transversely at a thickness of 10μm and incubated with an antibody described as 'pan neuronal', containing epitopes for beta III tubulin, neurofilament medium (NEFM), Neuronal Nuclei (NeuN) and microtubule associated protein 2 (MAP2). Immunofluorescence in the submucosal plexus and lamina propria was quantified and despite one AKR mouse showing very high levels of fluorescence in the colon, no significant difference was found between strains (Student's t-test) in the jejunum (Figure 4.6) and colon (Figure 4.7). Interestingly, although this antibody contained a beta III tubulin epitope along with NEFM, NeuN and MAP2, these sections appeared to show a lower level of immunofluorescence than a previous incubation with a different beta III tubulin antibody alone (Figure 4.2, Figure 4.3).

4.6 Sevier-Munger technique silver stain in the submucosal plexus and lamina propria

4.6.1 No significant difference in Sevier-Munger technique silver stain in submucosal plexus and lamina propria in the jejunum between strains

Jejunum was taken from AKR and C57BL/6 mice, embedded in paraffin, sectioned transversely at a thickness of 10μm and Sevier-Munger technique silver stain applied. Stain per section in the submucosal plexus and lamina propria was quantified and no significant difference was found between strains in the jejunum (Student's t-test) (Figure 4.8). There was noticeable variation in stain between animals in the AKR group.

4.6.2 Strain specific difference in Sevier-Munger technique silver stain in submucosal plexus and lamina propria in the colon

Proximal colon was taken from AKR and C57BL/6 mice, embedded in paraffin, sectioned transversely at a thickness of 10μm and Sevier-Munger technique silver

stain applied. Stain per section in the submucosal plexus and lamina propria was quantified and the AKR strain were found to have a significantly reduced amount of stain per percentage area (Student's t-test, $p<0.05$) compared with the C57BL/6 strain in the colon (Figure 4.9). This difference between strains can be clearly seen at a higher magnification (Figure 4.10). It is also interesting to note that there is noticeable variation in stain in the same section of the same animal (Figure 4.11) suggesting possible differences in gut innervation within a very small area.

4.7 Comparison of beta III tubulin immunofluorescence and Sevier-Munger silver stain technique

4.7.1 Variation in nerves identified with beta III tubulin immunofluorescence and Sevier-Munger silver stain technique in serial sections of the colon

Transverse serial sections of proximal colon from a C57BL/6 mouse were taken and stained using the Sevier-Munger silver stain technique and incubated with beta III tubulin immunofluorescence labelled antibodies to compare the neurons demonstrated with each. Figure 4.12 clearly shows that although beta III tubulin is thought to be contained within all neurons (Betolli et al., 2008) there are many shown with Sevier-Munger silver stain technique in the lamina propria that are not visualised with beta III tubulin antibody. There is also a subset of nerves demonstrated clearly within the myenteric plexus with beta III tubulin that are not shown with the Sevier-Munger silver stain technique. A trial of a neurofilament triplet light antibody did not yield results similar to the silver stain (results not shown).

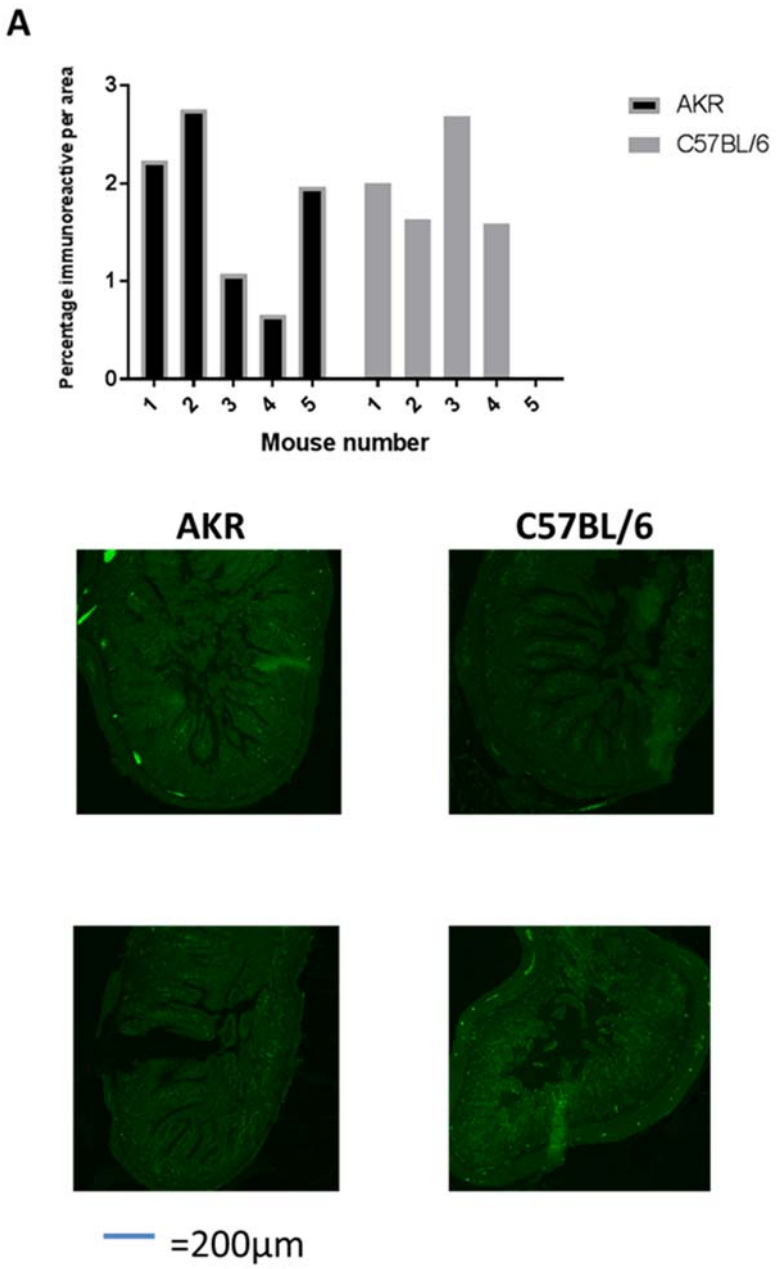


Figure 4.6. No strain specific significant difference in 'Pan neuronal' antibody (beta III tubulin, NEFM, NeuN, MAP2) immunofluorescence in the submucosal plexus and lamina propria of the jejunum. Quantification of 'pan neuronal' antibody expression (**A**) in the jejunum of AKR and C57BL/6 mice by immunohistochemistry showing data for each animal. Representative images of pan neuronal antibody (green) expression in the jejunum (images shown at 100xs magnification). Images were viewed using panoramic viewer. N=5 per group, 3- 4 sections per animal.

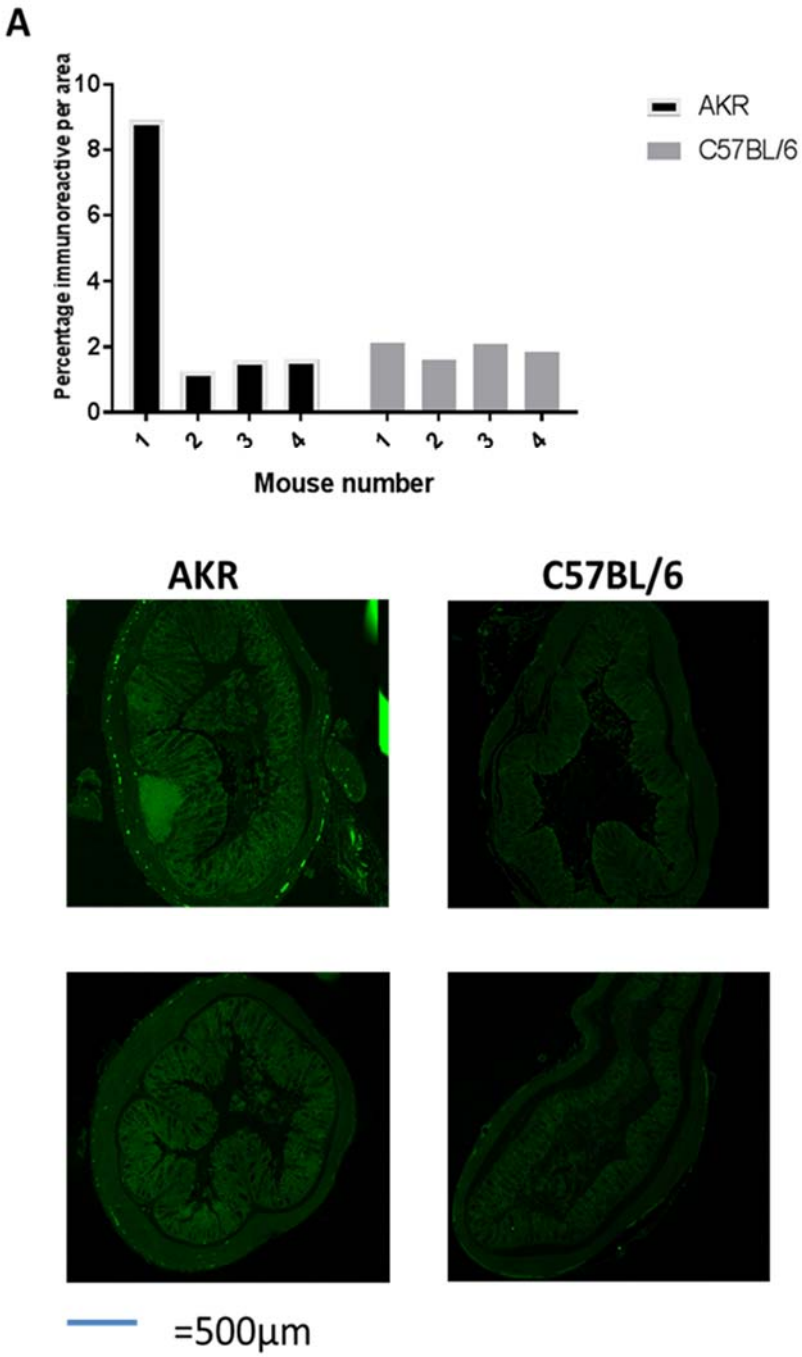


Figure 4.7. No strain specific significant difference in 'Pan neuronal' antibody (beta III tubulin, NEFM, NeuN, MAP2) immunofluorescence in the submucosal plexus and lamina propria of the colon. Quantification of 'pan neuronal' antibody expression (**A**) in the colon of AKR and C57BL/6 mice by immunohistochemistry showing data for each animal. Representative images of pan neuronal antibody (green) expression in the colon (images shown at 50xs magnification). Images were viewed using panoramic viewer. N=5 per group, 3- 4 sections per animal.

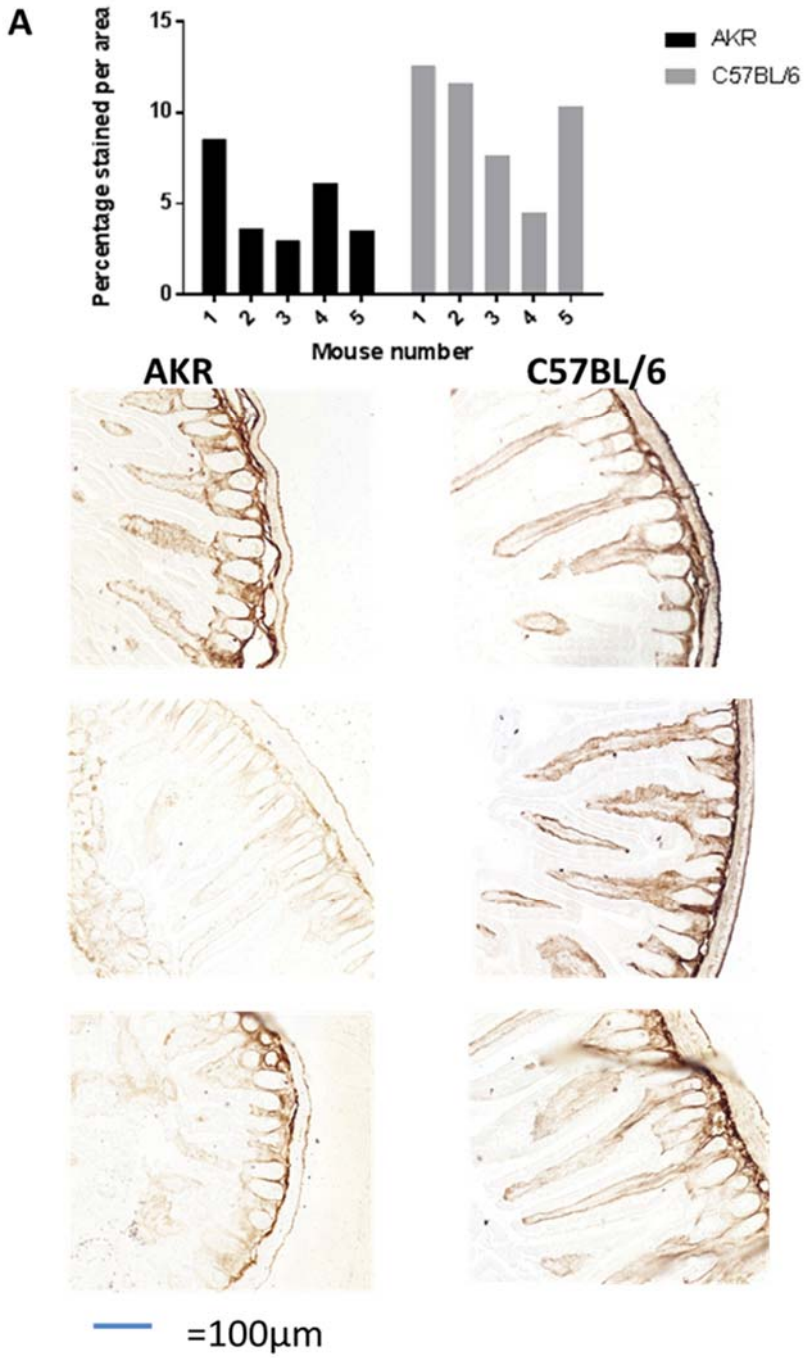


Figure 4.8. No strain specific significant difference in Sevier-Munger technique silver stain in the submucosal plexus and lamina propria of the jejunum. Quantification of Sevier-Munger technique silver stain (**A**) in the jejunum of AKR and C57BL/6 mice showing data for each animal. Representative images of Sevier-Munger technique silver stain in the jejunum (images shown at 200xs magnification). Images were viewed using panoramic viewer. N=5 per group, 3-4 sections per animal.

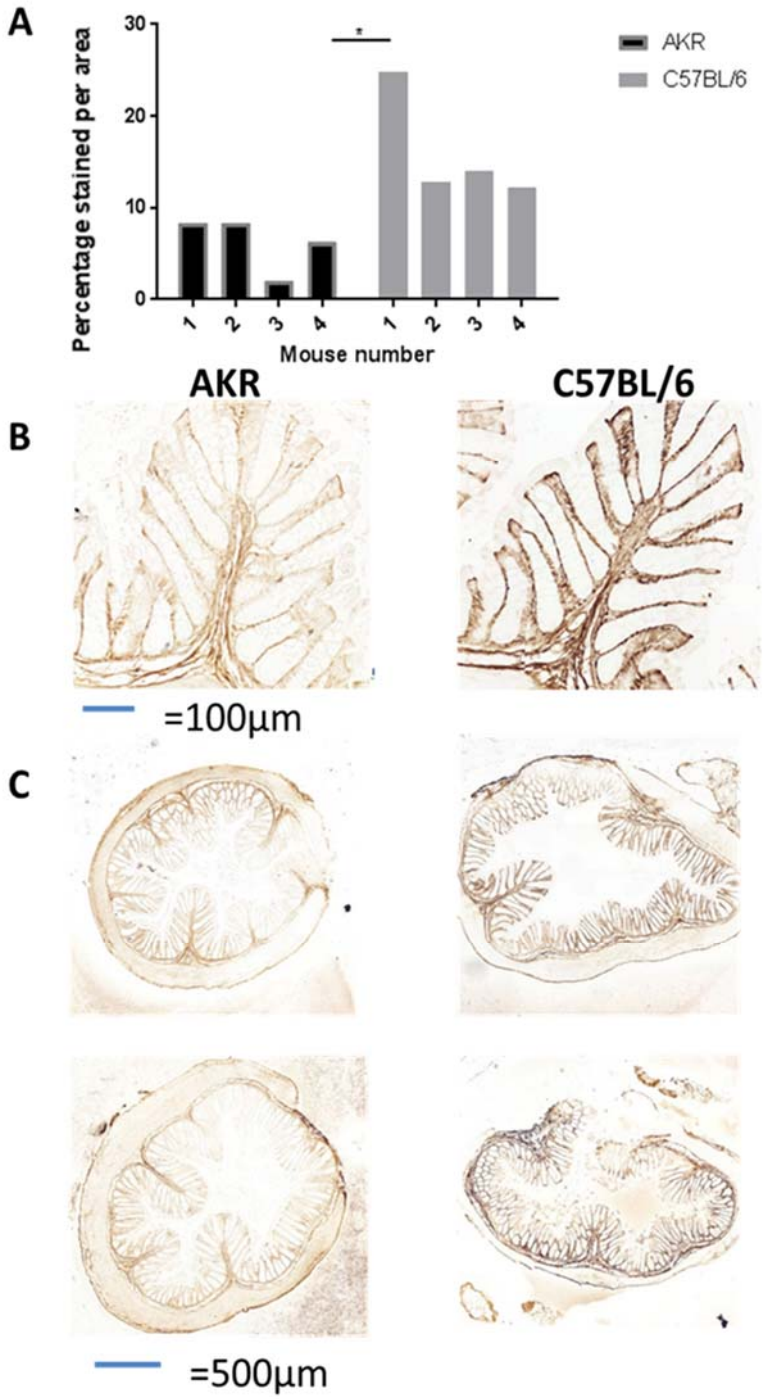


Figure 4.9 Significantly increased staining in C57BL/6 mice using the Sevier-Munger technique silver stain in the submucosal plexus and lamina propria of the colon. Quantification of Sevier-Munger technique silver stain (**A**) in the colon of AKR and C57BL/6 mice showing data for each animal. Representative images of Sevier-Munger technique silver stain in the colon (images shown at B- 200xs and C- 50xs magnification). Images were viewed using panoramic viewer. N=4 per group, 3-4 sections per animal. Student's t-test, *p<0.05.

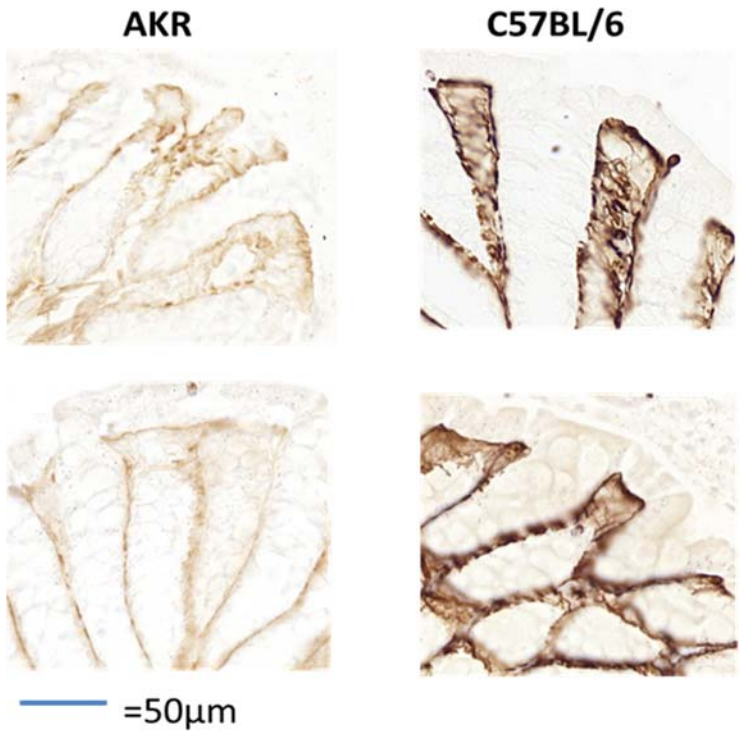


Figure 4.10 Strain specific differences in Sevier-Munger technique silver stain in the submucosal plexus and lamina propria of the colon. Images at higher magnification clearly demonstrate reduced stain per percentage area in AKR mice in comparison with C57BL/6 mice (all 600xs magnification).

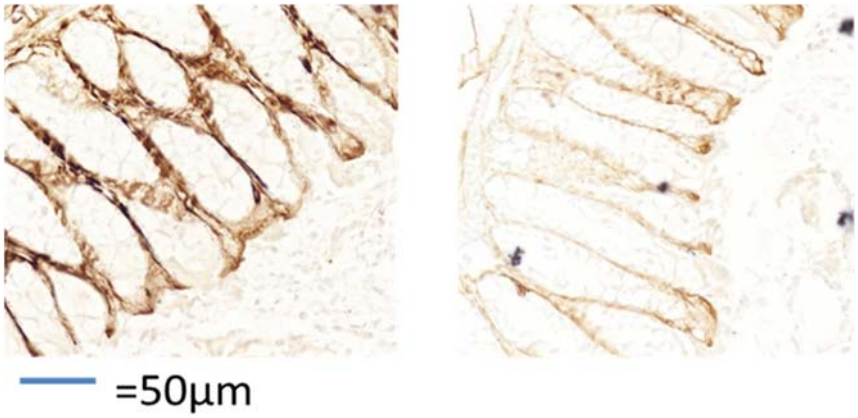


Figure 4.11 Variation in Sevier-Munger technique silver stain within the same section in the submucosal plexus and lamina propria of the colon. Two images taken from different areas of the same 10µm thick section of the colon of a C57BL/6 mouse (400xs mag) demonstrating variation in stain suggestive of protocol issues or nervous system variation within the same small area of colon.

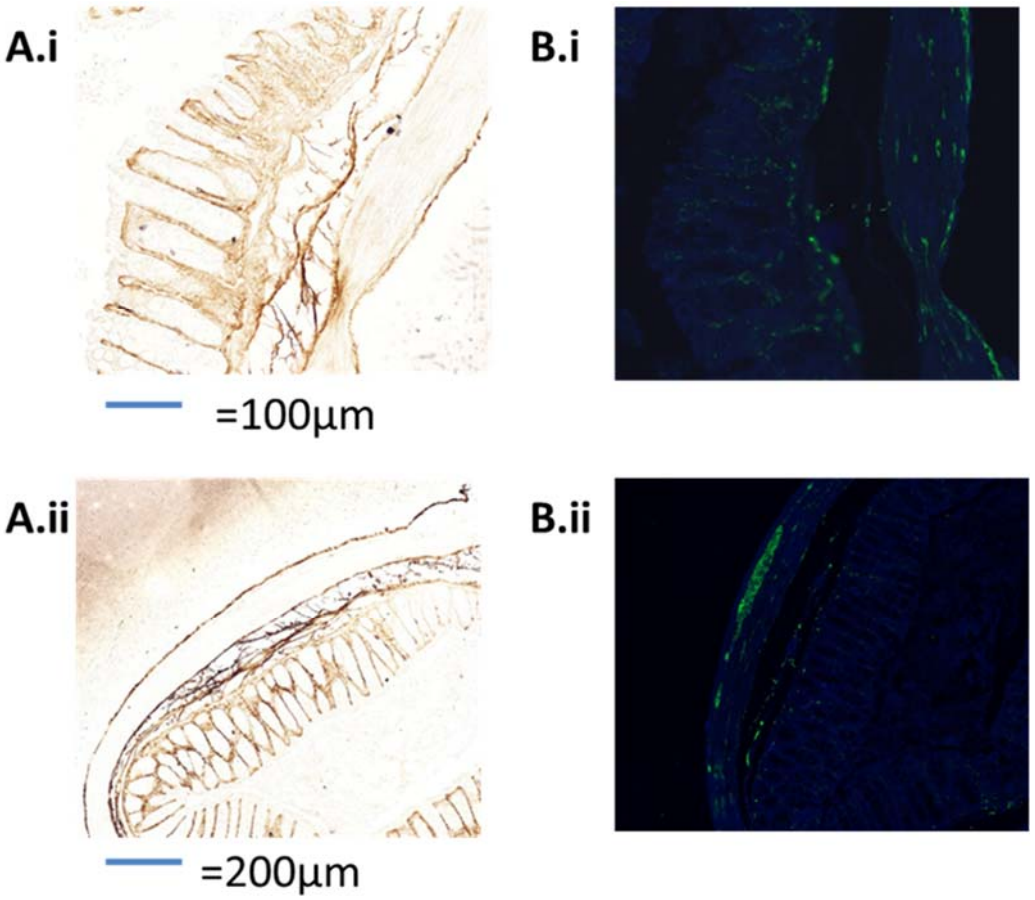


Figure 4.12 Variation in nerves identified with the Sevier-Munger silver stain technique and beta III tubulin antibodies in serial sections of the submucosal plexus and lamina propria of the colon. Two images taken from 10µm thick serial sections of the colon of a C57BL/6 mouse (**A**.i, **B**.i, 200xsmag, **A**.ii, **B**.ii, 100xs mag) using the Sevier-Munger silver stain technique (**A**) and beta III tubulin immunofluorescence labelled antibodies (green) (**B**) demonstrating variation in neurons identified with each, despite the attributed pan neuronal identification qualities of beta III tubulin.

4.8 Luxol Fast Blue stain in the colon

4.8.1 Luxol Fast Blue did not visualise neurons in the enteric nervous system

In order to try and visualise myelinated enteric neurons, the Luxol Fast Blue histological stain was trialled in the colon. Myelinated nerve fibres were clearly visible in the rat brain and spinal cord but no neurons could be clearly seen within mouse colon (Figure 4.13) suggesting that this stain is not useful for visualisation of enteric neurons. This may be due to complications in identification of neurons amongst the complex morphology of the lamina propria of the gut or it may be that the number of myelinated neurons within the lamina propria is limited.

4.9 Picrosirius red stain in the colon

4.9.1 No apparent difference in collagen fibres identified with Picrosirius red collagen stain between strains in the colon

The extracellular matrix is known to be important in facilitating neurite outgrowth via secretion of neurotrophic factors (Madl and Heilshorn, 2015) and cultured neural cells trapped in collagen developed normal polarity and growth patterns in comparison with those in agarose gel which were unable to survive (O'Connor et al., 2001). In order to investigate if variation in collagen fibres within the gut may reflect and influence variation in enteric neurons between strains, a Picrosirius red stain was performed. There was no visible difference between the AKR and the C57BL/6 mice in appearance or amount of collagen fibres in the colon (Figure 4.14). However, as some studies have found several extracellular matrix functions and abnormalities to be linked with the process of myelination (Mehta and Piao, 2017; Röhr et al., 2017), the similarity in collagen between strains may suggest that any genetic variation in enteric nerves may be limited to a subsection of non-myelinated neurons such as C fibres (Gebhart, 2000).

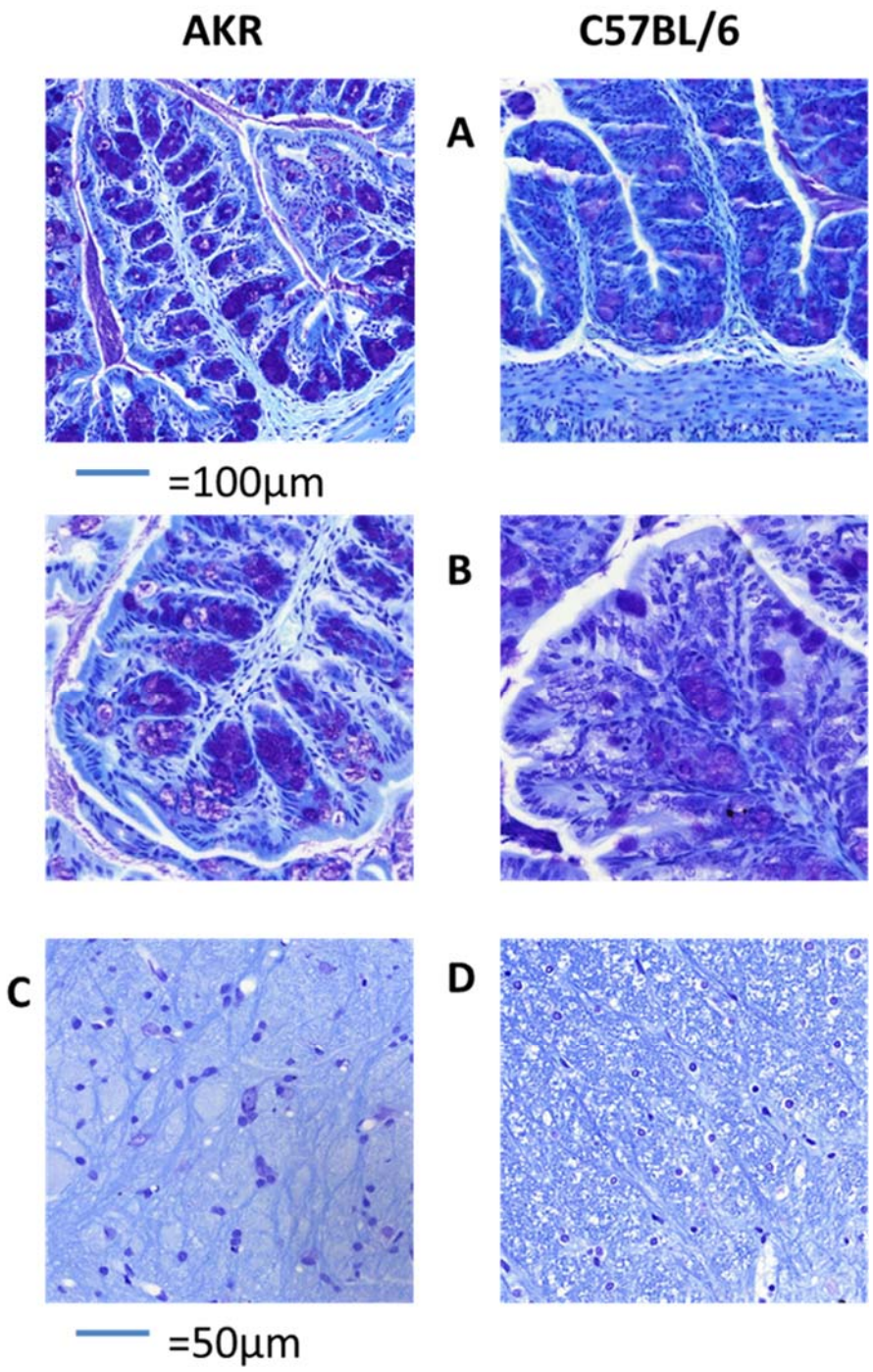


Figure 4.13 Luxol Fast Blue stain in the colon. Images of colon from mouse strains AKR and C57BL/6 stained with Luxol Fast Blue demonstrating the apparent lack of or difficulty in visualising myelinated nerve fibres (**A**-200xs, **B**-400xs magnification). Myelinated nerve fibres were clearly visible in the rat brain (**C**) and spinal cord (**D**) (both images at 400xs magnification).

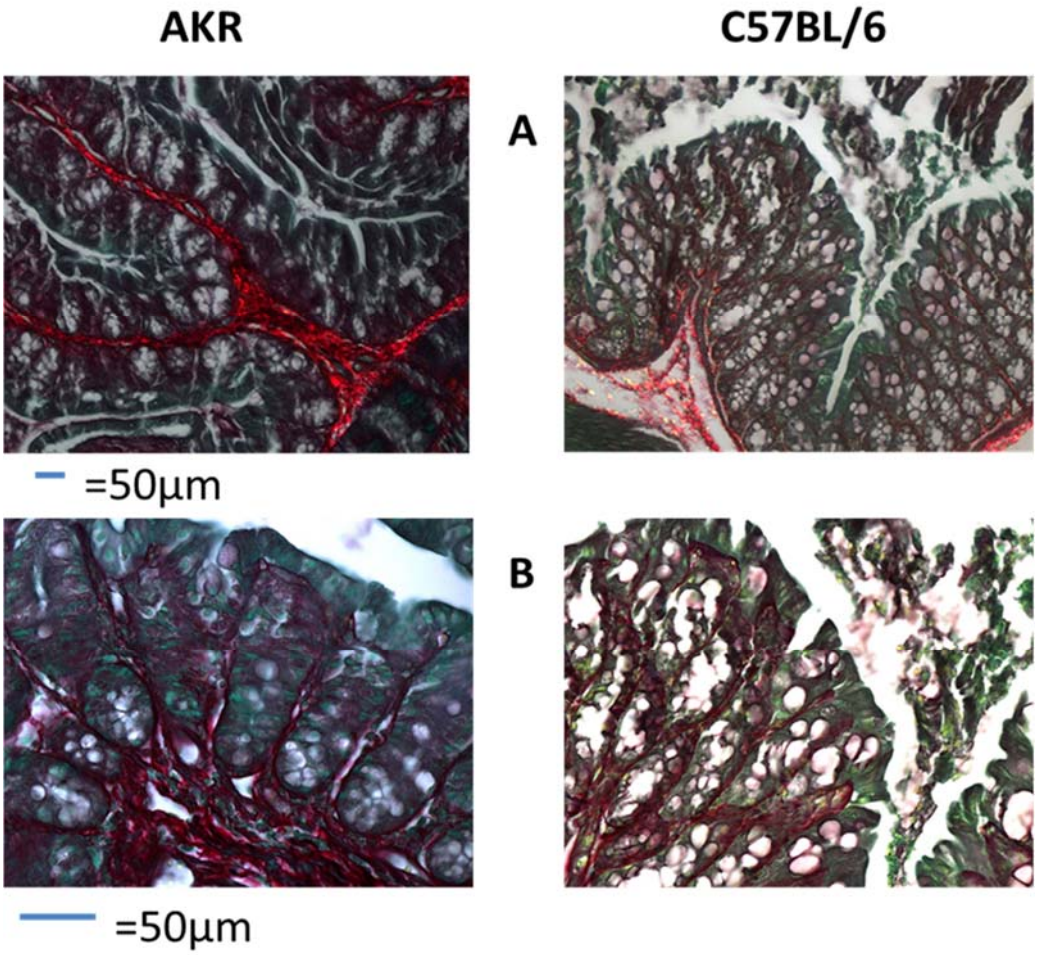


Figure 4.14 Picrosirius red stain in the colon. Images of colon from mouse strains AKR and C57BL/6 stained with Picrosirius red demonstrating similar appearance of collagen fibres between strains (**A**-200xs magnification, **B**-400xs magnification).

4.10 Discussion

Our previous findings identified several variations in gut physiology between the AKR and C57BL/6 strains of mice. Despite the known variation in the peripheral nervous system between these two strains of mice (Mogil et al., 2005), the enteric nervous systems have not been mapped and compared. We used immunofluorescence and histochemistry techniques to visualise and compare the enteric nervous system in AKR and C57BL/6 mice and found some interesting and novel results.

4.10.1 Difficulties in establishing a reliable pan neuronal marker within the gut

The alpha/beta tubulin heterodimer is an essential component in the eukaryotic cytoskeleton (Dutcher, 2001) and different beta tubulin isotypes have been found to be ubiquitously expressed or tissue specific (Leandro-García et al., 2010); we chose to investigate beta III tubulin, an accepted tubulin isotype associated with commitment to neuronal lineage (Qu et al., 2013; Marei et al., 2017; Caccamo et al., 1989). Another marker specific to neuronal fibres is ubiqutination hydrolase product PGP9.5 (Campbell et al., 2003). For this reason, beta III tubulin and PGP9.5 have been considered reliable pan neuronal markers widely used in research of the peripheral and enteric nervous system (Betolli et al., 2008; Harrington et al., 2010; He et al., 2016; Eisenman et al., 2013). However, a trial using a double stain immunofluorescence technique for both of these antibodies presented interesting results; whilst some nerves were clearly double labelled, a definite subset was found to be labelled with only one marker (Figure 4.1) suggesting that caution must be exercised in attributing a label of pan neuronal to either of these antibodies within the colon or small intestine.

Interestingly, in many papers beta III tubulin immunohistochemical protocol within the gut is limited to the longitudinal muscle myenteric plexus (LMMP) whole mount protocol as this is considered the main site of nerve ganglia (Su et al., 2016; Harrington et al., 2010). However, in this study, it was felt that possible strain specific variation in neurons in the lamina propria and submucosa important to investigate for several reasons: nerves closest to the epithelium would be first to encounter and react to sensory input from the gut lumen including general changes in environment, microbiome variation and parasitic infection. Thus, differences within the enteric nervous system close to the epithelium may be responsible for the differences in gut physiology we identified and known variation in response to

parasitic infection between strains. It has been suggested that the microbiome can play an important role in gut function and impact immune response (Round and Mazmanian, 2009). As inter-species variation in the human gut microbiome is an accepted phenomena (Arumugam et al., 2011), it is likely that these different strains of mice will have different microbiomes and although genetic background is influential it may be that interaction between gut bacteria and the nervous system exerts some control in the impact of this difference.

There are few papers examining beta III tubulin antibody stain in the submucosa of the colon. A similar pattern of beta III tubulin immunofluorescence to our study was found in both the mouse (Khalil et al., 2016) and human colon (Badizadegan et al., 2014). Despite a trend for higher quantities of immunofluorescence in the C57BL/6 mice, there was some variation within the groups and no strain specific significant difference was found in beta III tubulin antibody immunofluorescence in our work. However, serial sections of colon treated with the Sevier-Munger silver stain technique appeared to demonstrate a higher number of nerves than those treated with beta III tubulin antibody. Interestingly, the Sevier-Munger silver stain technique used on transverse sections of gut found AKR mice to have significantly reduced ($p<0.05$) levels of stain in the proximal colon in comparison with the C57BL/6 mice; at a higher magnification it appeared that these animals had fewer nerves (Figure 4.10). Variation in staining was also found within individual sections for all animals, suggesting a possible issue with staining protocol. However, this could also represent variation in the enteric nervous system throughout cross sectional areas of the gut (Figure 4.11). This must be considered relevant when some studies have used a small area from a cross section of guinea pig colonic mucosa to investigate innervation with beta III tubulin antibody (Robinson et al., 2017); the current study would suggest validity of results may be compromised if stain pattern from across the entire section is not considered.

Also intriguing was the fact that in our study, comparison of serial sections of small and large intestine appeared to demonstrate beta III tubulin immunofluorescence and Sevier-Munger silver stain in different areas; beta III tubulin immunofluorescence clearly shows many more nerves within the myenteric plexus but less nerves within the lamina propria than the Sevier-Munger silver stain in the colon (Figure 4.12). It cannot be ignored that despite the appearance that these experiments were successful, there may be some as yet unidentified issue with this particular antibody and stain technique protocol that may be affecting results; this was compounded by the fact that the Sevier-Munger silver stain technique was

unsuccessful on a number of following occasions despite repeated attempts, and known issues with reproducibility have been highlighted (Uchihara, 2007). However, as literature suggests that beta III tubulin is present as a structural component of almost all neurons (Caccamo et al., 1989), the possibility that we may have uncovered a subset of neurons within the gut that are beta III tubulin negative represents an interesting and novel finding. It has been reported that some interneurons within the retina known as amacrine cells do not contain always contain beta III tubulin, and it is interesting to note that the projections of these cells are dendritic rather than axonal (Balasubramanian and Gan, 2014); it may be that our results suggest a similar as yet undiscovered subset of neurons within the gut. A Luxol Fast Blue stain was not able to clearly highlight any myelinated submucosal nerves suggesting that this as yet unidentified subset of nerves is unmyelinated such as C fibres (Gebhart, 2000). As the sensitivity and tissue specificity of argyrophiIlic silver staining is dependent on the method utilised (Uchihara, 2007), we performed a Picrosirius red stain to confirm that the Sevier-Munger silver stain technique had indeed highlighted nerves rather than collagen fibres; collagen fibres were different in appearance to nerve fibres and no strain specific difference was identified.

In the hope of highlighting any nerves that may not be beta III tubulin specific, a second antibody was also used described as ‘pan neuronal’ and suitable for the peripheral nervous system containing markers for beta III tubulin but also other common biomarkers thought to be specific to neurons including the neuronal nuclei marker NeuN, microtubule associated protein 2 (MAP2), and neurofilament medium. No strain specific significant difference in immunofluorescence was found and images did not appear to be markedly different from those demonstrated with beta III tubulin and certainly did not appear to highlight the same subset of sub-epithelial nerves as the Sevier-Munger silver stain technique.

4.10.2 Evidence of influence of genetic background on enteric nervous system

Mogil et al. (2005) identified a difference in thermal nociception in the hindpaw of the AKR and C57BL/6 strains; it appeared that the AKR demonstrated a reduced response to pain that could be rectified with administration of CGRP. CGRP release is triggered by stimulation of the TRPV1 receptor (Meng et al., 2009), which is located on thinly myelinated alphaδ fibres and unmyelinated sensory C fibres (Michael and Priestley, 1999) suggesting a reduction in function or amount of these

peripheral sensory nerves in the AKR mouse. Our work demonstrates that this known peripheral difference in sensory innervation in the AKR may be reflected in the enteric nervous system, as a reduced infiltration of nerves in the lamina propria and submucosa was identified by the Sevier-Munger silver stain technique in the AKR strain in comparison with the C57BL/6 strain. This has never been previously documented to our knowledge; however, a defect in nerves in this area of the colon in the AKR strain may explain the inability of these animals to produce a Th2 immune response to parasitic infection given that CGRP release is associated with Th2 immune profile induction (Mikami et al., 2011).

In this study we investigated and compared the enteric nervous system in two different inbred strains of mice (AKR and C57BL/6). We were unable to identify a reliable pan neuronal marker but did find a possible variation in amount of nerves in the submucosal plexus and lamina propria between strains. Although this difference between strains was only demonstrated in one histochemical stain, it may be the reason for the variation in function previously found between the two strains.

Summary of key findings

Significant differences in-
Sevier-Munger silver stain technique- AKR significantly less stain per percentage area than C57BL/6

No significant differences in-
Beta III tubulin immunofluroescence, jejunum and colon, myenteric plexus or submucosal plexus/lamina propria
'Pan neuronal' antibody immunofluorescence, jejunum and colon, submucosal plexus/lamina propria
Sevier-Munger silver stain technique, jejunum, submucosal plexus/lamina propria
Spontaneous gut motility
Initial response to carbachol (0.1μM and 10μM)
Average tension gut contraction in response to carbachol (0.1μM and 1μM) in a 3 minute period
Average number of gut contractions in response to carbachol (0.1μM and 1μM) in a 3 minute period
Initial response to capsaicin
Average tension or number of gut contractions in response to capsaicin (0.1μM, 1μM and 10μM) in a 3 minute period

Chapter 5

There are differences in gut physiology in C57BL/6 mice on return to health following resolution of high/low dose *Trichuris muris* infection that are likely to reflect differences within the enteric nervous system post infection

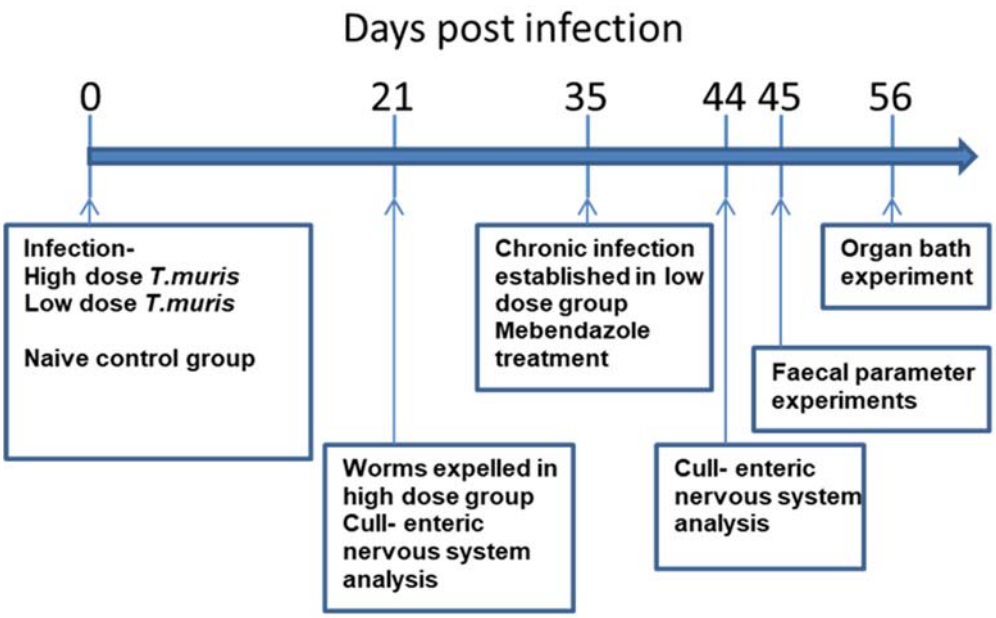
5.1 Introduction

The nervous system is vital in the control of physiological functions within the gut regulating movement, absorption and defaecation (Gray, 1918). Invasion by a pathogen can activate a cascade of events that results in the death of cells, including neurons. During infection, NO produced by the nervous system contributes to formation of ROS and cell death (Tennyson and Lippard, 2011; McCord and Omar, 1993) and activated macrophages release nerve repellent factors specific to sympathetic nerve fibres and reduce NGF production that may lead to a reduced overall number of sympathetic nerve fibres (Straub et al., 2006; Straub et al., 2008). Therefore, it is likely that inflammation affects neuronal number and as a result of this, physiology; the long term effect of these possible changes on ENS neuronal number and gut function is not yet fully understood.

It is not yet clear if an initial minor neuropathy or varied neural map prior to diagnosis may contribute to disease onset. Peripheral autonomic neuropathy resulting from has change in neuronal number has been associated with many pathological conditions involving chronic inflammation and inappropriate immune activation such as hypertension and diabetes (Gregory et al., 2012; Hosseini and Abdollahi, 2013). This is increasingly linked with complications and leads to comorbidities; for instance, in diabetic patients, cardiovascular disease that develops secondary to the condition itself it a leading cause of mortality (Vinik et al., 2013). Some chronic pathologies such as diabetes and the CNS neurological disorders Alzheimers disease and Parkinsons disease, are associated with GI disturbances, suggesting the ENS is affected (Brock et al., 2013; Holzgrabe et al., 2007). In diabetic patients, some alteration in heart rate variation indicative of autonomic neuropathy could be identified prior to onset of any inflammatory signals, suggesting that some element of neuropathy, perhaps minor, may precede or perhaps contribute to, development of the pathology (Posadas et al., 2013). In healthy adults, Thayer and Fischer (2009) found an inverse correlation between levels of C-reactive protein in plasma and vagally modulated heart rate variability; this increase in inflammatory marker C-reactive protein was attributed to reduced activation of the cholinergic anti-inflammatory pathway caused by loss of cholinergic nerves in these individuals which could therefore potentially lead to chronic low level inflammation. This would exacerbate any underlying neuropathy and may lead to development of pathology.

As individuals we are all different genetically, but we also encounter environmental influences that could impact our gut nerves; if a prior neuropathy is indicative of or associated with pathology, these factors may contribute to disease initiation and progression. We encounter a variety of pathogens every day and most people, if not everyone, will at some point succumb to a gut bacteria or virus that triggers the immune system. This will usually lead to a Th1 profile immune response leading to successful eradication of the pathogen and return to health; if we become infected by a worm or parasite, a Th2 immune response will usually be triggered (Romagnani, 1999). Whilst we know that there is a link between oxidative stress, inflammation and neuropathy (Reuter et al., 2010), the long term effect of resolved Th1 or Th2 immune profile infection on the gut nervous system of healthy individuals is not known. Interestingly, patients with Crohn's disease, a condition with an elevated Th1/Th17 immune profile, were found to have progressive sympathetic neuropathy (Ohlsson et al., 2007) whilst patients with the Th2 driven ulcerative colitis demonstrated a lower heart reaction to deep breathing ratio (E/I ratio) and dysfunctional vagal cardiac control suggestive of vagal neuropathy (Coruzzi et al., 2007). Perhaps we could hypothesize that infection activating different immune profiles will affect the nervous system differently, and this will be reflected by variations in enteric neuronal number and gut physiology. This will therefore affect the quality of life of the individual and may affect susceptibility to future disease.

We decided to investigate the impact on gut physiology of resolved infection with the parasite *Trichuris muris*; C57BL/6 mice were used as they are known to be resistant to parasitic infection whereas the AKR mice have a known irregularity in neural phenotype (Mogil et al., 2005) that may be linked with the susceptibility of this strain to parasitic infection (Artis et al., 1999). We used two different doses to elicit two different immune profiles, a high dose of parasites to initiate a Th2 response and a low dose to initiate a Th1 response (Bancroft et al., 2001; Bancroft et al., 1994); as the Th1 response is not sufficient to eliminate *T.muris* (Bancroft et al., 2001), an anti parasitic drug used commonly in humans, mebendazole, was given to mice to resolve chronic infection. Mebendazole was given to animals in the high and low dose groups and a naive control group to ensure any significant differences between groups could not be attributed solely to the action of the drug. It would be expected that any lasting impact of resolved infection from either immune profile on the enteric nervous system would be reflected in changes in gut physiology and may warrant further investigation of gut nerves.



Hypothesis

Resolved Th1 or Th2 immune response to infection in the caecum and proximal colon will lead to long term changes in the enteric nervous system. This will result in differences in normal gut physiology between animals and may impact future susceptibility and recovery from gastro-intestinal disease via the neuro-immune link. These post infection changes may be echoed in a human population and thus resolved infection may be a factor in influencing human susceptibility to disease.

Aim

To investigate gut physiology that will reflect differences in the enteric nervous system in C57BL/6 mice infected with a high or low dose of *T.muris*, and compare to an age matched uninfected control group.

Assessments of variation in physical and functional parameters that will be used include comparison between groups of

- Weight gain
- Length of small and large intestines
- Muscle thickness, crypt size and submucosal depth
- Food and water intake
- Faecal pellet number, length and water content
- Faecal transit time

- Spontaneous motility of the proximal colon, including number and tension of contractions
- Average response of the proximal colon, including number and tension of contractions, to an α7 nicotinic acetylcholine receptor agonist at 3 concentrations

Results will highlight differences in physical size, function and neuroreceptor expression specific to each infection group that may impact gut function following resolution of infection. These post infection changes may be echoed in a human population and impact human gut function and ultimately disease susceptibility via the neuro-immune link.

5.2 Weight gain and gut length

5.2.1 Infection affected weight gain at day 35 post infection

Mice from each group (high dose infection with *Trichuris muris*, low dose infection with *T.muris* and a control group) were weighed every 7 days for 5 weeks and mean average weight of animals per group calculated each week. Naive mice (n=5) weighed significantly more than those infected with a high dose ($p<0.0001$, n=5) and a low dose ($p<0.0001$, n=5) infection of *T.muris* at day 35 post infection. There was no significant difference between high and low dose groups at day 35 post infection and no significant difference in weight at any other time point between any of the groups (two way ANOVA) (Figure 5.1).

5.2.2 Dose of infection did not consistently affect length of the small or large intestine

Length of small intestine and large intestine was measured in each group at day 21 post infection and post treatment with mebendazole at day 45 and 56 post infection; mean average length per group for each time point was calculated. There was no significant difference in length of small intestine or large intestine between groups at day 21 post infection and post treatment with mebendazole at day 45 or 56 post infection (two way ANOVA) (Figure 5.2).

5.3 Muscle, submucosa and crypt measurements and infection

5.3.1 Infection affected crypt depth, submucosal depth and muscle thickness in the colon

To confirm that any change found in gut physiology following resolution of infection was not purely a result of physical changes, muscle thickness, submucosal depth and crypt depth were measured at day 21 post infection; mice infected with a low dose of *T.muris* (n=5) had significantly longer crypts ($p<0.001$) and thicker submucosa ($p<0.05$) in the colon than naive mice (n=5). Mice infected with a high dose of *T.muris* had significantly thicker muscle depth than mice in the low dose group ($p<0.01$) and naïve mice ($p<0.005$) (two way ANOVA) (Figure 5.3).

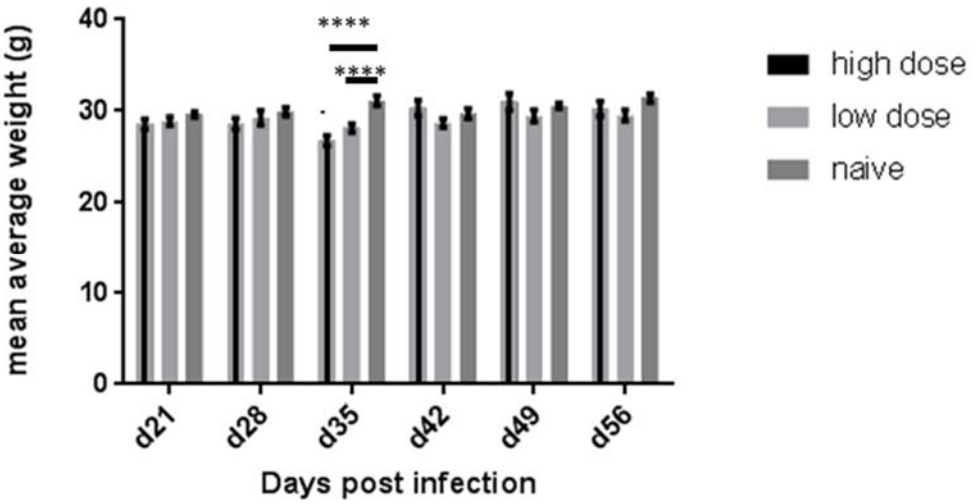


Figure 5.1 Weight of C57BL/6 mice infected with high or low dose *Trichuris muris* and a naive control group. Mice from each group were weighed every 7 days for 5 weeks from day 21 post infection. Data are shown as a mean value ± SEM. N=5. Two way ANOVA $p<0.0001$.

5.4 Food and water intake, faecal pellet number and length

5.4.1 Effect of infection on food and water intake

Food and water intake per group per cage was measured (1 cage of mice infected with a high dose of *T.muris* (n=5), 1 cage of mice infected with a low dose of *T.muris* (n=5) and 1 cage of uninfected control mice (n=5)) over a 5 week period and calculated as a mean average value per mouse. At day 21-28 post infection, animals infected with a low dose of *T.muris* demonstrated reduced food intake in comparison to animals infected with a high dose of *T.muris* and a control group. At day 28-35 post infection, both infected groups demonstrated reduced food intake in comparison to a control group. Food intake in infected groups returned to similar levels as the control group between days 35-42 post infection and remained stable for the remainder of the experiment (Figure 5.4 A). Water intake was stable for all groups throughout the experiment other than the high infection group, which demonstrated reduced water intake between days 28-35 post infection returning to normal between days 35-42 post infection (Figure 5.4 B).

5.4.2 Effect of resolved infection on faecal pellet number

Faecal pellet number produced per group in a 4 hour time period was measured for all groups (n=5), alongside a fourth group, naive control animals that had not been treated with mebendazole (n=3) at day 56 post infection. No significant difference was found between groups in the number of faecal pellets produced in a 4 hour time period (two way ANOVA) (Figure 5.4 C).

5.4.3 Mebendazole treatment affected faecal pellet length in naive animals

The first five faecal pellets produced by all groups including naive animals not treated with mebendazole were collected and measured to give a mean average faecal pellet length per animal at day 56 post infection. Average faecal pellet length (mm) in naive C57BL/6 mice treated with mebendazole (50mgs/kg, n=5) was significantly longer than naive animals ($p<0.02$, n=3), animals in the high dose group treated with mebendazole ($p<0.02$, n=5) and animals in the low dose group treated with mebendazole ($p<0.02$, n=5). No significant difference was found in average faecal pellet length between naive mice and the high and low dose groups post treatment (two way ANOVA) (Figure 5.4 D).

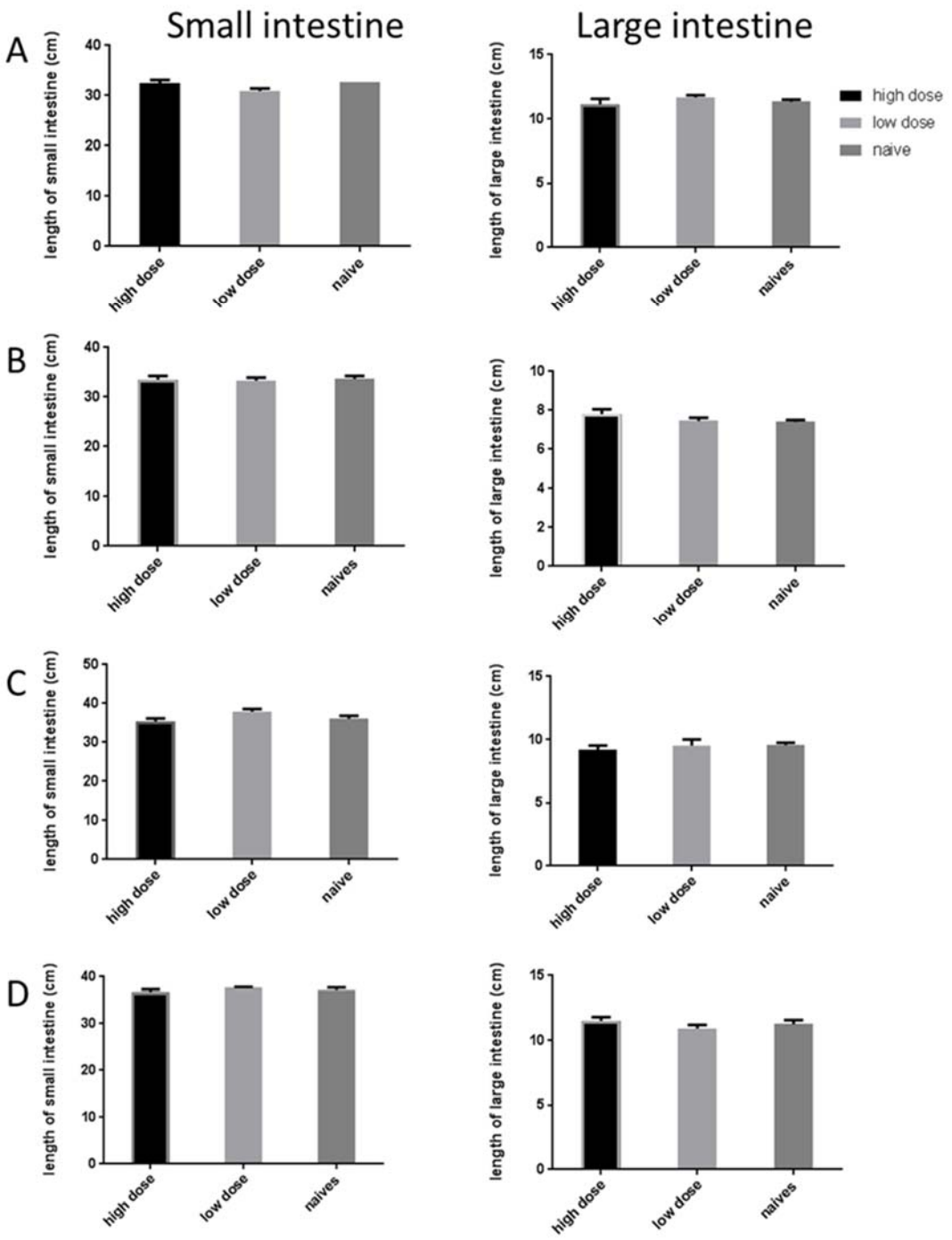


Figure 5.2 Small and large intestinal length shown for C57BL/6 mice infected with *Trichuris muris* at a high dose, low dose and a naive control group. A&B: Small and large intestinal length day 21 post infection for two independent experiments. **C&D:** Small and large intestinal length for day 45 (C) and day 56 (D) post infection. Data are shown as a mean value ± SEM. N=3-5.

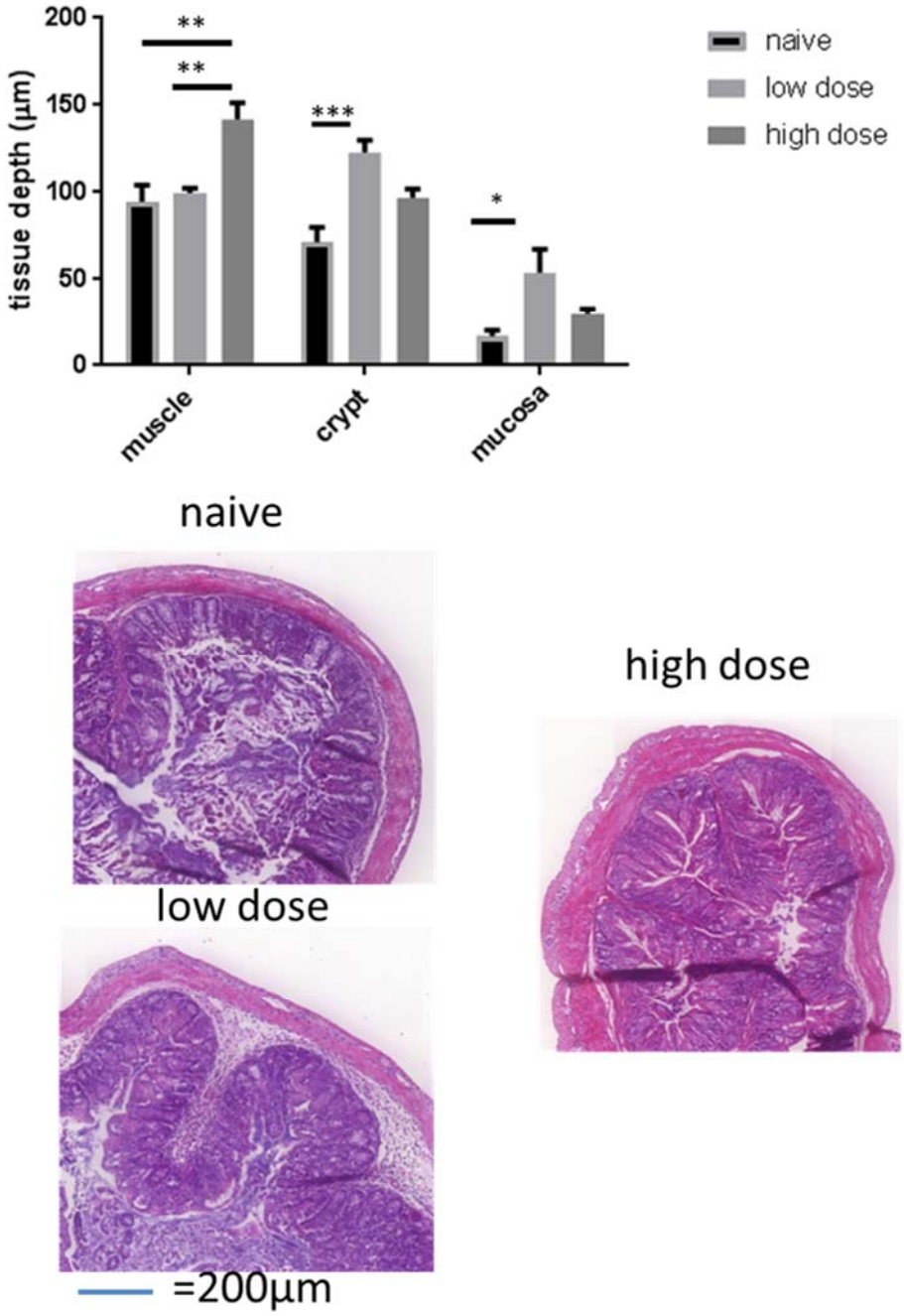


Figure 5.3 Significant difference in pathology in the colon of C57BL/6 mice infected with *Trichuris muris* at a high dose, low dose and a naive control group. **A:** Measurement of crypt depth, submucosal depth and muscle thickness in C57BL/6 mice infected with *T.muris*. Data are shown as a mean value ± SEM. **B:** Representative H&E staining of proximal colon in uninfected (naïve) and infected (high or low dose) C57BL/6 mice. N=5, 3-4 sections per individual. Two way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

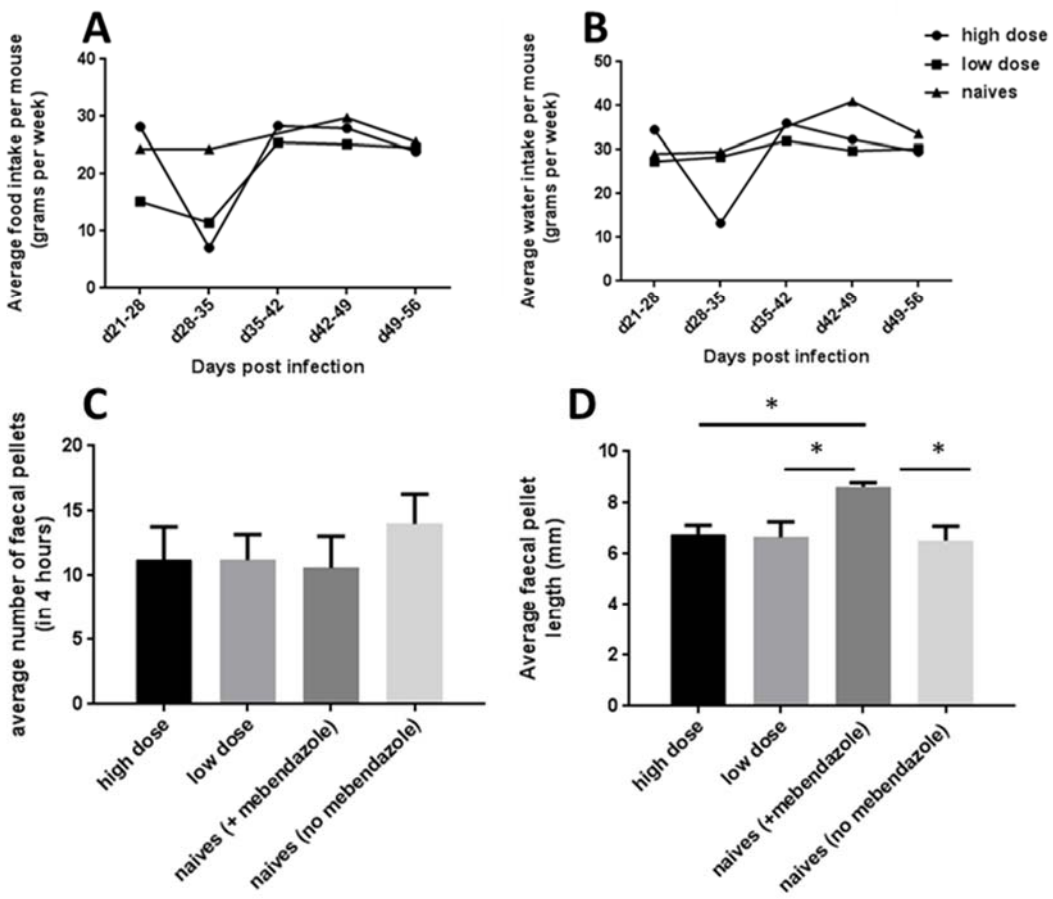


Figure 5.4 Measurement of food and water intake, faecal pellet number and length in C57BL/6 mice infected with *Trichuris muris* at a high dose, low dose and a naive control group. A&B: Food and water intake was measured for a 5 week period from day 21 post infection. **C**: Average faecal pellet number produced per animal in a 4 hour time period. **D:** Average faecal pellet length per animal (mm). Data are shown as a mean value ± SEM (**C, D**). N=5. Two way ANOVA, *p<0.05.

5.5 Faecal water content

5.5.1 Resolved infection did not have any effect on faecal water content

At day 56 post infection, faecal pellets were collected from each group of mice and water content per group calculated as a percentage. Naive animals not treated with mebendazole had a lower faecal water content than all other groups followed by naive mice treated with mebendazole and animals in the high dose group. Animals in the low dose group had the highest faecal water content (Figure 5.5). Actual values for percentage water content can be seen below in table 5.1.

Table 5.1 Average faecal water content.

	Faecal water content (mean average per group)
high dose (n=5)	41
low dose (n=5)	43
naïve (+ mebendazole) (n=5)	39
naïve (no mebendazole) (n=3)	26

5.6 Comparison of transit time

5.6.1 Resolved infection and mebendazole treatment affected transit time

At day 56 post infection, mice from each group were gavaged with 5% Evans blue and 5% gum Arabic in MQ water and the time taken to pass a blue faecal pellet was monitored. Transit time for naive mice was significantly shorter than naive mice treated with mebendazole (50mgs/kg, $p<0.025$), animals in the high dose group ($p<0.01$) and animals in the low dose group ($p<0.0001$). Animals in the low dose group had a significantly longer transit time than naive control animals treated with mebendazole ($p<0.0029$) and animals in the high dose group ($p<0.0063$). There was no significant different in transit time between naive animals treated with mebendazole and animals in the high dose group (two way ANOVA) (Figure 5.6).

5.7 Link between transit time and water content.

There appeared to be a correlation between water content and transit time; in all groups, water content increased as transit time increased. Naive mice had the fastest transit time and lowest water content. Animals treated with mebendazole and animals treated with a high dose of *T.muris* had similar higher levels of water content and slower transit time in comparison with the naive group, whilst animals in the low dose group had both the slowest transit time and highest water content on day 56 post infection (Figure 5.7).

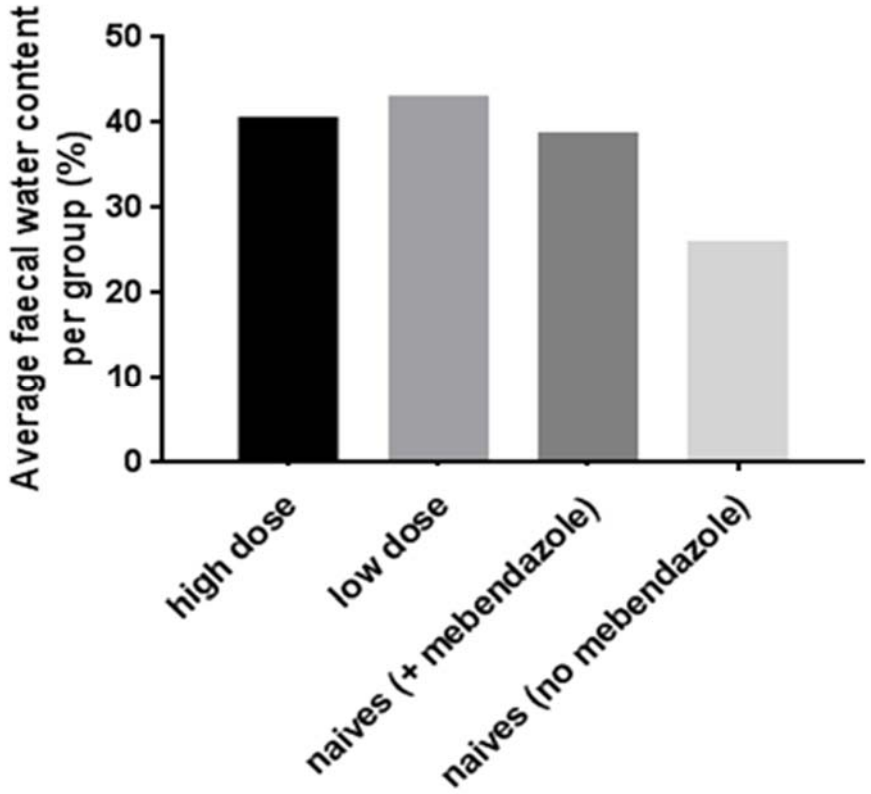


Figure 5.5 Average faecal water content for C57BL/6 mice infected with *Trichuris muris* at a high dose, low dose and two naive control groups. Faecal pellets were collected from each group of mice and water content per group calculated as a percentage (actual values can be seen in Table 5.1). N=3-5.

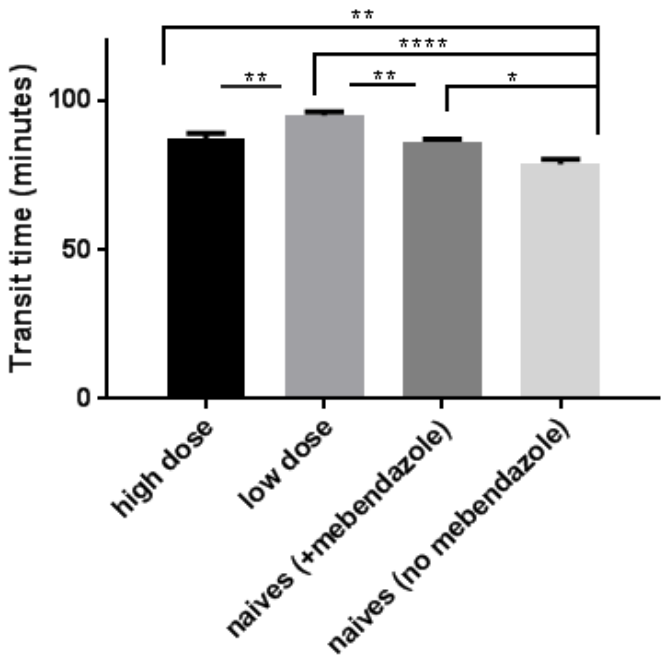


Figure 5.6 Resolved infection and treatment with mebendazole affected transit time in C57BL/6 mice infected with *Trichuris muris* at a high dose, low dose and two naive control groups at day 56 post infection. Data are shown as a mean value ± SEM. N=3-5 per group. Two way ANOVA, *p<0.05, **p<0.01, ****p<0.0001.

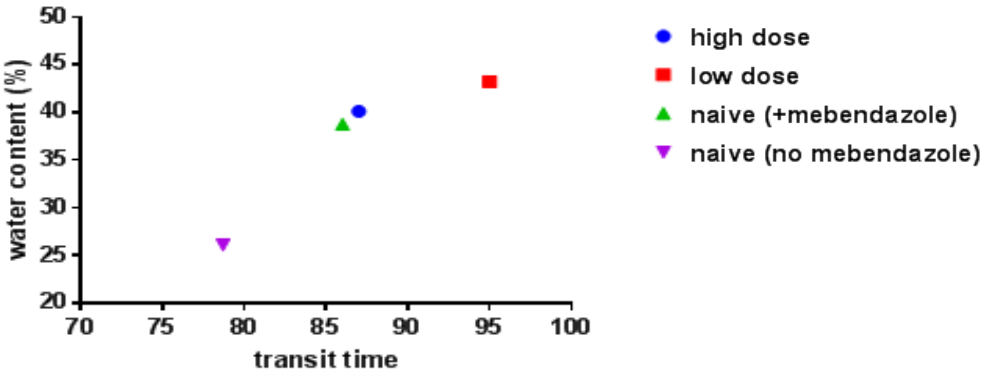


Figure 5.7 Increased transit time associated with increased water content. There was a positive correlation between water content and transit time in C57BL/6 mice infected with *Trichuris muris* at a high dose, low dose and two naive control groups at day 56 post infection. N=3-5.

5.8 Motility of the colon

5.8.1 No significant differences in spontaneous gut motility related to resolution of infection was found

To examine any lasting effect of resolved infection on gut movement, a small section of proximal colon was taken from all animals and suspended in an organ bath containing oxygenated Kreb's solution. After resting for 30 minutes to ensure tissue equilibrium, the number of gut contractions (number of events) and average strength of contractions over a three minute period was measured and animals in each group compared. Although the difference between number of events and tension per event in a three minute period was not statistically significant (two way ANOVA), the naive group not treated with mebendazole and the low dose group appeared to demonstrate greater tension per event in comparison with the other two groups (Figure 5.8); this would suggest that colon from all groups demonstrated a similar number of contractions but that these contractions were stronger in the naive group not treated with mebendazole and the low dose group, also treated with mebendazole. However, results were not consistent within the groups and this difference may have been coincidental; individual traces demonstrate time and strength of contractions can be seen in figure 5.9.

5.9 Alpha 7 acetylcholine receptor agonism and gut motility

Acetycholine receptors have many functions throughout the body and are vital in autonomic synaptic function (De Biasi, 2002). The nicotinic acetylcholine receptor antagonist succinylcholine is used as a neuromuscular blocking agent during endotracheal intubation (Langeron et al., 2009) highlighting the importance of nicotinic acetylcholine receptors in normal muscular motility. Reduced neuromuscular function in myasthenia gravis and resultant paralysis has been associated with reduced acetylcholine receptor expression (Binks et al., 2016; Fambrough et al., 1973). The alpha 7 nicotinic acetylcholine receptor is involved in the neuro-immune link, having an anti-inflammatory effect (Tracey, 2002); the expression or function of this receptor has been shown to vary in different immune profiles (Galitovskiy et al., 2011). It could be that the variation in transit time identified between groups is due to variation in expression of the α7nAChr as a result of the profile of immune response. We decided to investigate if there was a long term effect on α7nAChr expression following resolution of infection that may impact gut physiology.

5.9.1 No significant difference in gut motility related to resolution of infection following administration of PNU 282987 (0.1μM, 1μM, 10μM, 20μM)

In order to investigate any differences in alpha 7 acetylcholine receptor expression that may affect gut function between groups, an alpha 7 nicotinic acetylcholine receptor agonist, PNU 282987, was added to oxygenated Kreb's solution in the organ bath at increasing concentrations to judge the optimum response level, in accordance with protocols for experiments in the jejunum; gut was rested and washed with fresh Kreb's solution prior to and following addition of the agonist, and effect on frequency and strength of colon contractions during a three minute period was measured. Although there appeared to be a trend towards reduced tension per event in the naive group in response to PNU 282987 (0.1μM), variation within groups meant that differences were not significant either in number or strength of gut contractions during a three minute period (two way ANOVA) (Figure 5.10). Individual traces can be seen in Figure 5.11.

There was no significant difference in number of events (gut contractions) or tension per event between groups following administration of PNU 282987 at a concentration of 1μM (two way ANOVA). The naive group appeared to display increased number of contractions and reduced tension, however there was variation within the group (Figure 5.10). Individual traces can be seen in Figure 5.12.

There was no significant difference in number or tension of gut contractions between groups following administration of PNU 282987 at a concentration of 10μM (tow way ANOVA). The naive group appeared to display increased number of events and reduced tension per event, a trend which was echoed to a lesser extent in the high dose group. Interestingly, the low dose group appeared to demonstrate increased tension per event following administration of PNU 282987 at a concentration of 10μM (Figure 5.10). Again there was variation within groups. Individual traces can be seen in Figure 5.13.

There was no significant difference in number or tension of gut contractions between groups following administration of PNU 282987 at a concentration of 20μM (two way ANOVA). The naive group appeared to display increased number of events and reduced tension per event, a trend which was echoed to a lesser extent in the high dose group, and in the low dose group a slight increase in tension could be seen; again there was variation between groups (Figure 5.10). Individual traces can be seen in Figure 5.14.

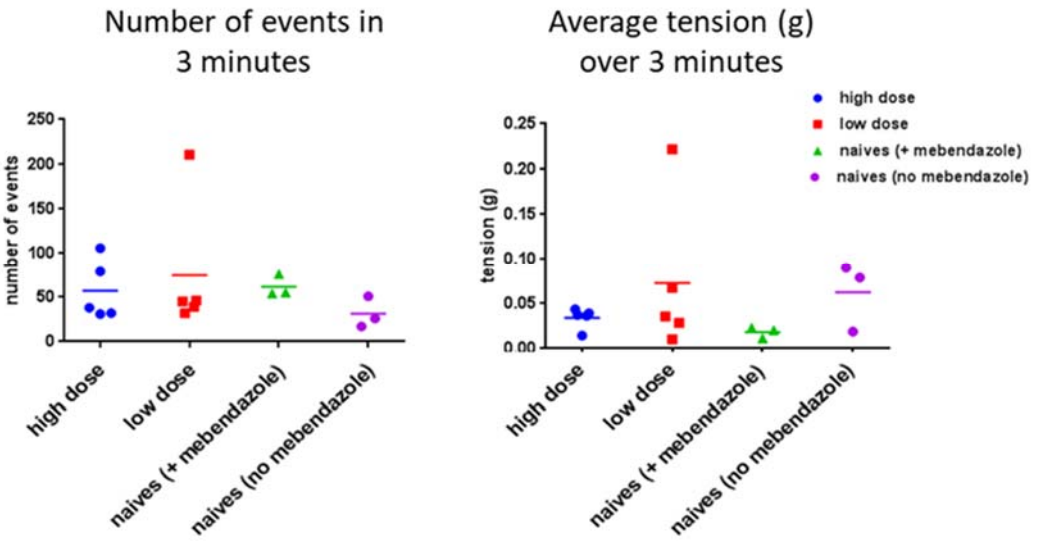


Figure 5.8 Spontaneous motility of the colon. Proximal colon from C57BL/6 mice at day 56 post infection and post treatment with mebendazole was taken and placed in an organ bath containing oxygenated Kreb's solution and spontaneous motility compared. Individual traces can be seen in Figure 5.9. N=3-5.

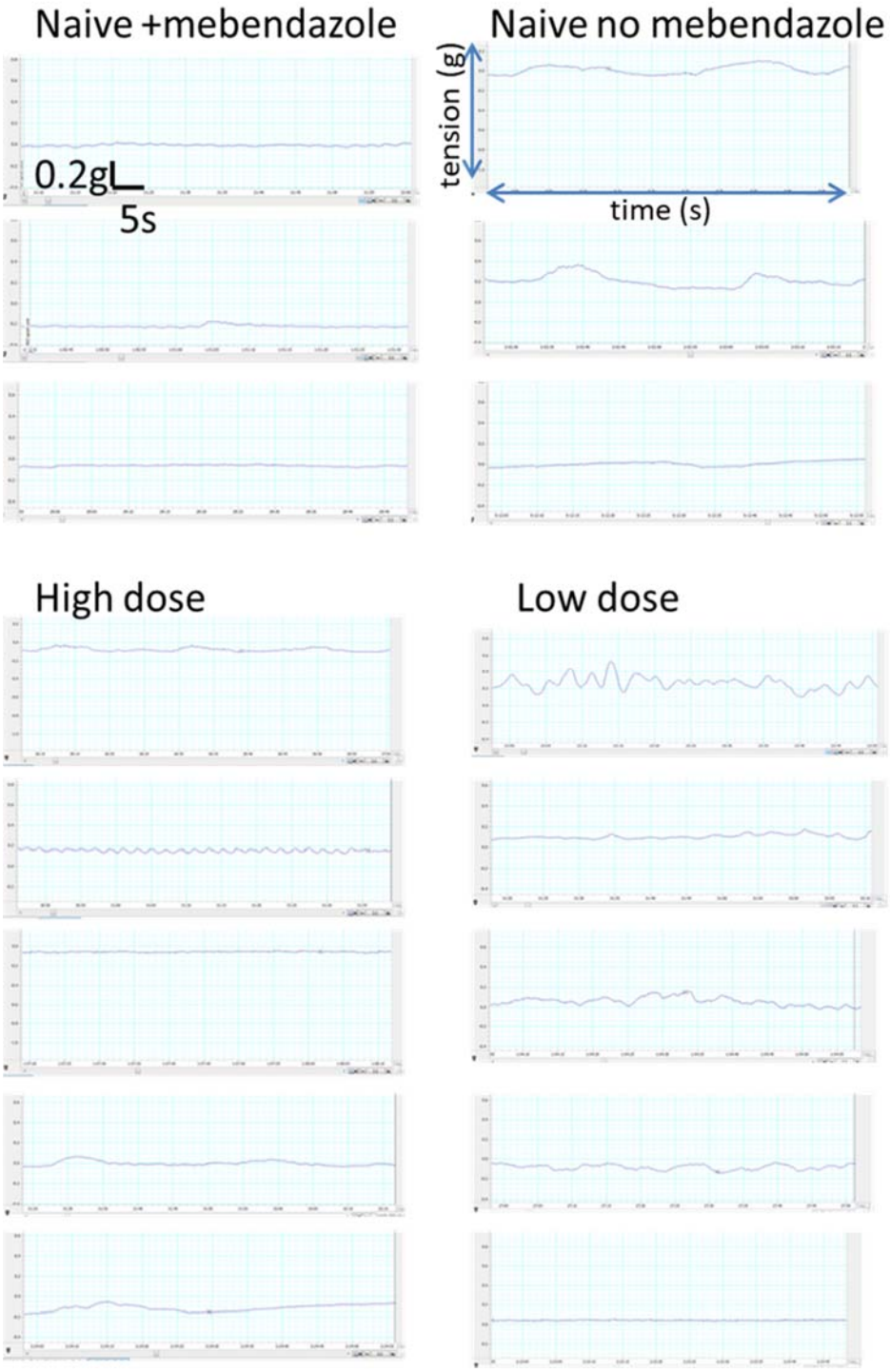


Figure 5.9 Spontaneous motility of the colon. Proximal colon from C57BL/6 mice at day 56 post infection with *Trichuris muris* at a high dose, low dose, a naive control group (all treated with mebendazole) and a naive control group not treated with mebendazole was taken and placed in an organ bath containing oxygenated Kreb's solution and spontaneous motility compared. Individual traces can be seen above. N=3-5.

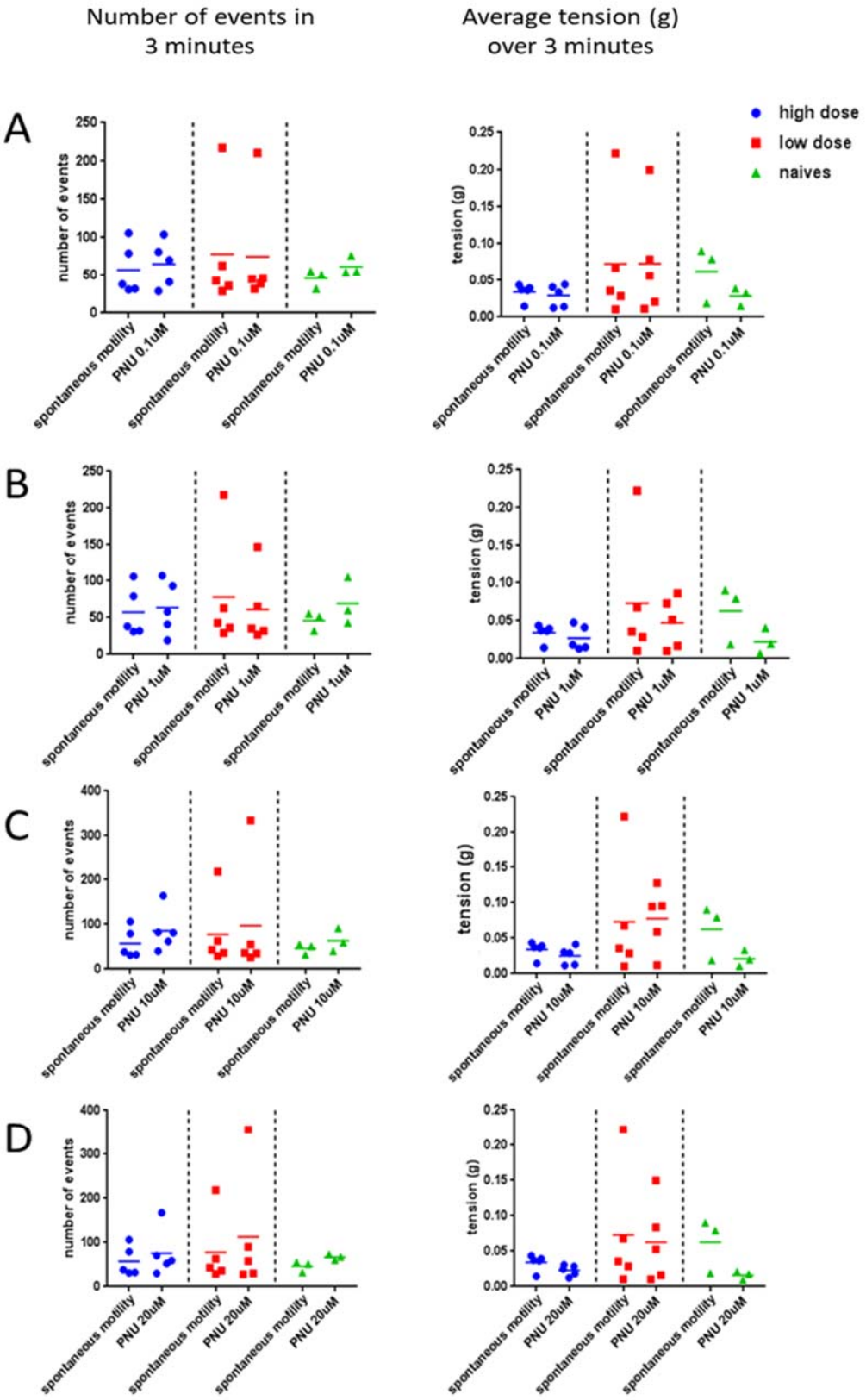


Figure 5.10 Comparison of spontaneous motility and administration of PNU 282987 (0.1μM, 1μM, 10μM and 20μM) on tension of the colon in C57BL/6 mice infected with *Trichuris muris* at a high dose, low dose and a naive control group at day 56 post infection. **A:** 0.1μM, **B:** 1μM, **C:** 10μM and **D:** 20μM. N=3-5.

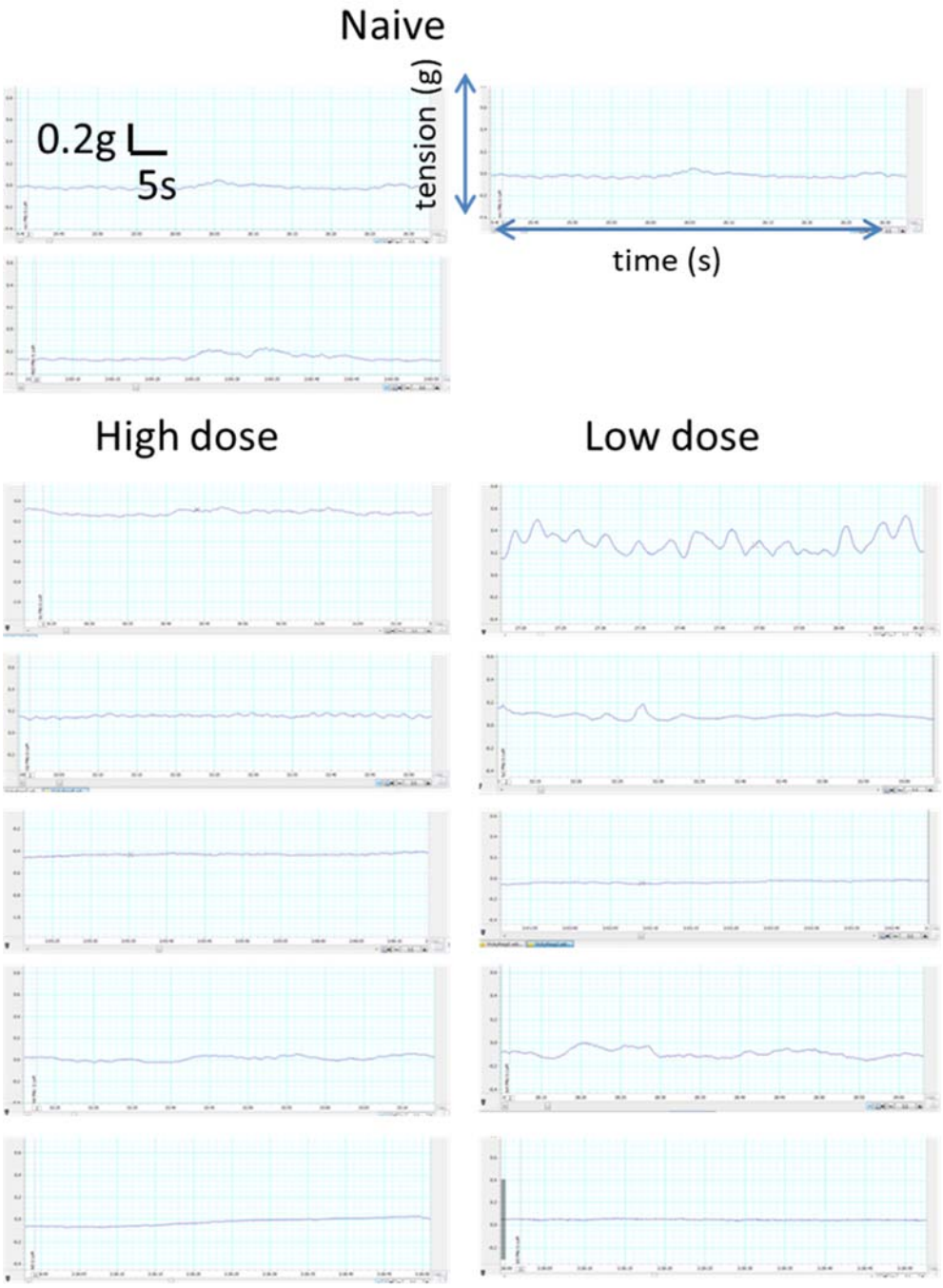


Figure 5.11 Effect of alpha 7 nicotinic acetylcholine receptor agonism on the proximal colon. PNU 282987 (0.1μM) was administered to proximal colon from C57BL/6 mice at day 56 post infection with *Trichuris muris* at a high dose, low dose and a naive control group (all treated with mebendazole. Individual traces for each animal following PNU 282987 treatment can be seen above. N=3-5.

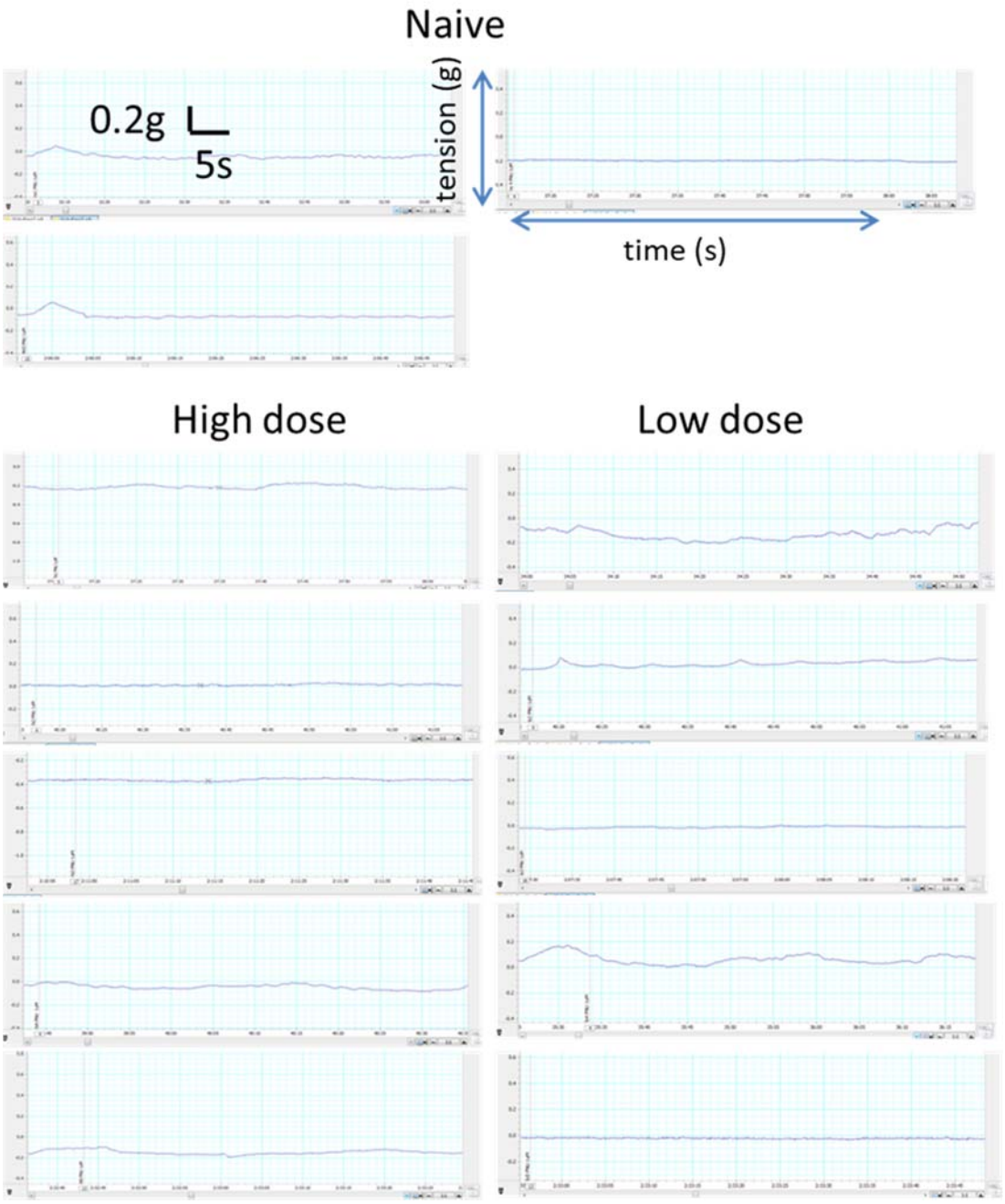


Figure 5.12 Effect of alpha 7 nicotinic acetylcholine receptor agonism on the proximal colon. PNU 282987 (1μM) was administered to proximal colon from C57BL/6 mice at day 56 post infection with *Trichuris muris* at a high dose, low dose and a naive control group (all treated with mebendazole). Individual traces for each animal following PNU 282987 treatment can be seen above. N=3-5.

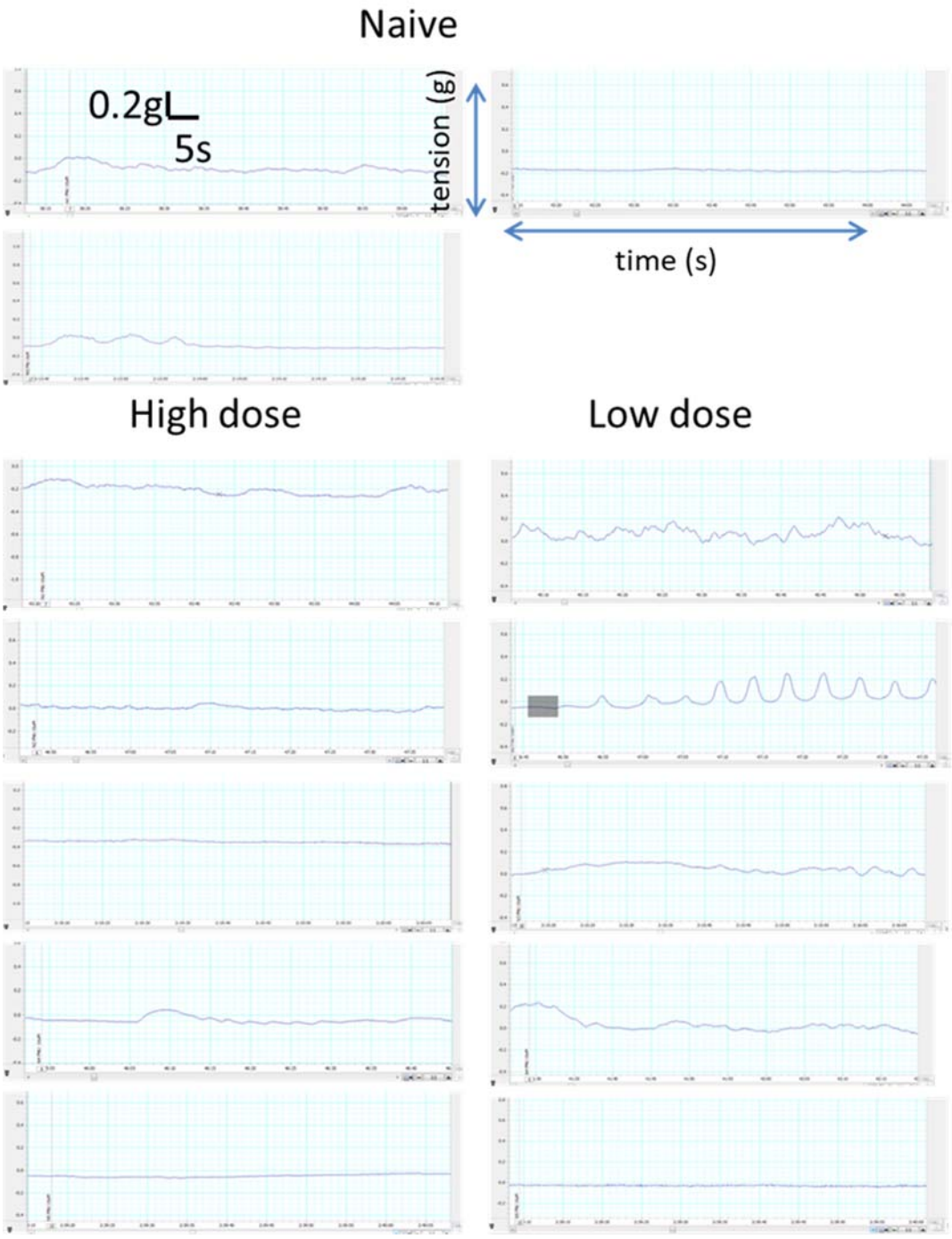


Figure 5.13 Effect of alpha 7 nicotinic acetylcholine receptor agonism on the proximal colon. PNU 282987 (10μM) was administered to proximal colon from C57BL/6 mice at day 56 post infection with *Trichuris muris* at a high dose, low dose and a naive control group (all treated with mebendazole). Individual traces for each animal following PNU 282987 treatment can be seen above. N=3-5.

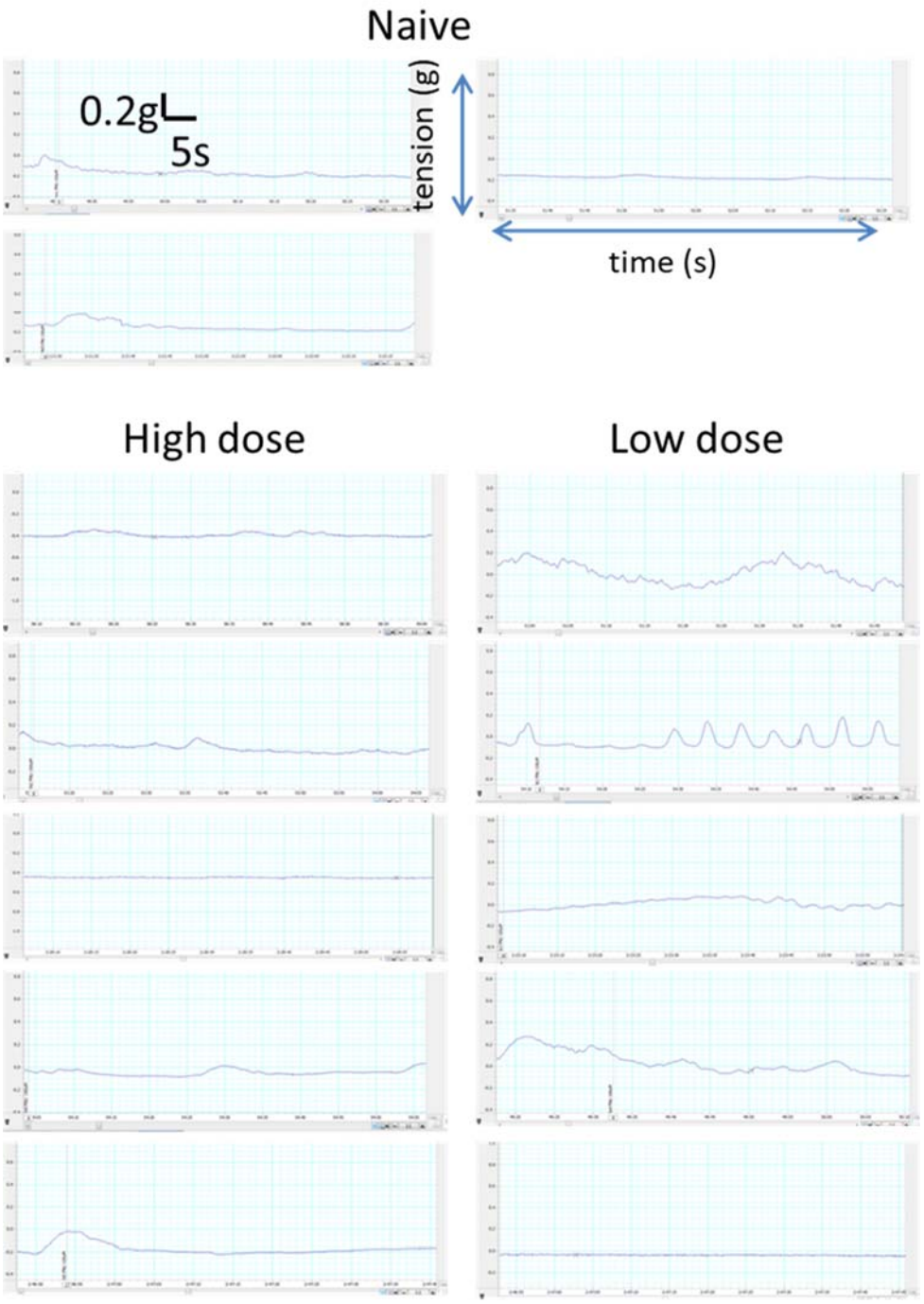


Figure 5.14 Effect of alpha 7 nicotinic acetylcholine receptor agonism on the proximal colon. PNU 282987 (20μM) was administered to proximal colon from C57BL/6 mice at day 56 post infection with *Trichuris muris* at a high dose, low dose and a naive control group (all treated with mebendazole). Individual traces for each animal following PNU 282987 treatment can be seen above. N=3-5.

5.10 Discussion

Gastroenteritis caused by infection with *Shigella*, *Campylobacter Jejuni*, *Escherichia Coli* and *Salmonella* is known in many cases to lead to post infection Irritable Bowel Syndrome (PI-IBS) (Ji et al., 2005; Spiller et al., 2000; Okhuysen et al., 2004; Mearin et al., 2005); repeated infection with *Campylobacter Jejuni* and the parasite *Trichinella Spiralis* has been used to induce PI-IBS in animal models (Jin et al., 2017; Spiller et al., 2000; Sung et al., 2013). PI-IBS is associated with increased intestinal permeability, T-lymphocyte invasion, altered cytokine levels, visceral hypersensitivity and dysbiosis in gut flora. Jin et al. (2017) found that the antibiotic rifaximin had beneficial effects in PI-IBS in a mouse model but had little effect on the microbiome; therapeutic effect was deemed to be a result of altered cytokine and tight junction protein levels. None of these studies have investigated the possibility of neural involvement in post infection IBS despite the known integration between the nervous and immune systems (Kelley and McCusker, 2014) and known neuropathy associated with many conditions such as Crohn's disease, Ulcerative Colitis, hypertension and diabetes (Coruzzi et al., 2007; Gregory et al., 2012; Hosseini & Abdollahi, 2013). Much research has focussed on the effect of disease progression on nerves, and it is known that inflammation during pathology can lead to neuropathy via production of reactive oxygen species (ROS) (Erbas et al., 2017). However, there is little information examining impact on gut nerves of inflammation that occurs during a gut immune response and if any changes are sustained following return to health. To investigate if infection eliciting a Th1 or Th2 immune profile leads to variations in gut function following resolution of infection, this study measured a variety of physical parameters to identify potential variations in gut physiology between groups of C57BL/6 mice infected with different doses of *T.muris* and a control group. We identified some differences in normal gut function between groups.

5.10.1 There are some consistent effects on gut physiology following resolution of infection

We found no long term significant effect on gross anatomy of the gut and any impact of infection on weight and food and water intake had resolved by post treatment day 42 post infection. Although there was no significant difference in faecal pellet number between groups, a control group that had not been treated with mebendazole appeared to produce more faecal pellets and had lower faecal water content than the infected groups and the naive group treated with mebendazole. Interestingly, faecal pellet length was significantly longer in the naive animals

treated with mebendazole than in the other three groups, suggesting mebendazole may be having an as yet unknown effect on gut physiology. There were also significant differences in transit time between groups, with naive animals displaying a significantly faster transit time than the other groups. The low dose infection group had a significantly longer transit time than other groups, whilst there was no significant difference found between the high dose group and naive animals treated with mebendazole. This suggests that following resolution of infection, gut physiology is affected and transit time is increased, and the effect was greater in a resolved Th1 immune profile infection (low dose infection group) rather than a resolved Th2 profile infection (high dose infection group). There also appeared to be a link between transit time and faecal water content suggesting that resolved infection reduces the amount of water absorbed from the faecal pellet. This is an interesting finding and may be relevant in etiology of IBS and associated symptoms such as altered gut function commonly associated with diarrhoea or loose stools (Sayuk et al., 2017). *Trichuris* infection was shown to cause change in the host microbiome in pigs (Li et al., 2012) and mice (Houlden et al., 2015a); in humans this has been associated with changes in faecal pellet consistency and transit time (Vandeputte et al., 2016). Interestingly, a human study found no difference in microbiome phenotype between individuals infected with *Trichuris trichuria* and a control group (Cooper et al., 2013). Importantly, mebendazole had the greatest impact on water content suggesting that this may be the reason for the variation found between groups rather than resolved infection with *T.muris*.

A very interesting coincidental finding was that mebendazole appeared to affect gut physiology increasing faecal pellet length and affecting transit time and water content in a way that appeared similar to resolved Th2 infection in the high dose infection group. This is a particularly important finding when we consider that large scale deworming initiatives employing the use of mebendazole, amongst other anti-parasitic drugs, are employed in many countries where worms are endemic (Levecke et al., 2014). Mebendazole prevents parasite growth via disruption of beta tubulin function in the parasite gut (Bai et al., 2011); as 5-10% of drug administered is absorbed by the human host (Drugbank, 2017), it may be that there is some impact on beta tubulin in the gut of the host along with that of the parasite. If we consider that mebendazole has been shown to inhibit some cell cancer lines due to disruption of mitotic spindle formation, then the possibility of some effect on the gut following oral administration is feasible (Martarelli et al., 2008); gut physiology tests were performed 3 weeks after administration of treatment with mebendazole

suggesting that effects are long term if we consider the limited life span of the mouse and the possible equivalent time span in human terms. However, it may be that effects are most dramatic immediately following administration and reduce over time; this could be evaluated by adding in a later time point. Also, given that mebendazole increases faecal water content there is a possibility that it may have some as yet unknown application in the treatment of constipation; faecal water content has been linked with variation in the microbiome (Vandeputte et al., 2016) suggesting that the impact of mebendazole may be caused by effects on gut flora.

5.10.2 Resolution of infection has inconsistent effects on gut motility and expression of a neuro-immune receptor

There was a trend for animals in the low dose group and naive animals treated with mebendazole to have increased strength of gut contractions compared to animals in the high dose group and naive animals; however, this difference was not significant in the small group sizes tested here. As the alpha 7 acetylcholine receptor is commonly accepted as important in the neuro-immune link and has been previously described as varying in expression in different immune profiles (Galitovskiy et al., 2011), we decided to investigate expression and function of this receptor following resolution of infection. Any lasting impact on expression of this receptor could be considered particularly important as upregulated acetycholine production and receptor expression has been associated with reduced apoptosis and some tumour profiles (Paleari et al., 2008; Song et al., 2003; Catassi et al., 2008). Although there was no significant difference in frequency or strength of gut contractions between groups at any concentrations of PNU 282987 incubation, there was a trend for reduced strength and increased number of contractions in naive animals treated with mebendazole. Interestingly, at a concentration of 10μM and 20μM, PNU 282987 appeared to have a similar effect on animals in the high dose group whilst increasing the strength of contractions in the low dose group. This suggests that there may be differences in expression or function of the α7nAChR that affect gut motility, and we could speculate that as the naive and high dose groups also displayed similar results in transit time and water content, this receptor may be important in regulation of gut physiology.

In this study we investigated the effect of resolved infection with a high or low dose of *T.muris* on normal gut anatomy and physiology of C57BL/6 mice. We found several differences between groups, some of which were inconsistent, and some variation within groups. Incidentally, we found that treatment with the anti-parasitic

drug mebendazole resulted in differences in gut physiology in naive animals. These differences suggest that the impact of infection on gut physiology is not limited to the time of infection but persists to some extent following resolution of infection. This would suggest long term differences in the enteric nervous system that may impact the immune response and influence susceptibility to and recovery from future disease. It was then decided to investigate the enteric nervous system within these animals to see if any differences between groups could be identified.

Summary of key findings

Significant differences in-
Weight gain- lower weight in both infection groups than a naive group (day 35 post infection)
Crypt length- longer crypts in low dose infection group than a naive group (day 21 post infection)
Submucosal depth- thicker submucosa in low dose infection group than a naive group (day 21 post infection)
Muscle thickness- thicker muscle in high dose group than a low dose group and naive group (day 21 post infection)
Faecal pellet length- longer faecal pellets in naive animals treated with mebendazole than a naive untreated group and high or low dose infection groups treated with mebendazole (day 56 post infection)
Faecal transit time- shorter transit time in naive animals than naive, high or low dose infection groups treated with mebendazole. Shorter transit time in naive animals treated with mebendazole and a high dose infection group than a low dose infection group (day 56 post infection)

No significant differences in-
Length of small or large intestine
Crypt length between high dose and a low dose infection group or naive group
Submucosal depth between high dose and a low dose infection group or naive group
Muscle thickness between a low dose infection group and a naive group
Faecal pellet number between all groups
Spontaneous gut motility between all groups
Average tension or number of gut contractions in response to PNU 282987 (0.1μM, 1μM and 1μM) in a 3 minute period

Chapter 6

Immunofluorescence and histochemical investigation revealed some differences in the enteric nervous system of the colon in C57BL/6 mice during and post infection with high/low dose *Trichuris muris* infection

6.1 Introduction

Previous results found physiological differences in gut function between groups of mice following resolution of infection with a high or low dose of *Trichuris muris* and a control group, including differences in transit time and water content. These variations may be a result of post infection changes in the gut environment similar to those found following gastroenteritis, including changes in cytokine milieu, gut wall permeability and the microbiome, that can lead to post infection irritable bowel syndrome (Jin et al., 2017; Sung et al., 2013). Much previous research has focussed on these aspects without investigating the influence of or impact on the enteric nervous system of infection, despite the known link between the immune system and the nervous system (Kelley and McCusker, 2014), and the known link between inflammation and neuropathy (Zhou and Zhou, 2014). We investigated the enteric nervous system in mice following resolution of infection with a high or low dose of *Trichuris muris* and a control group using immunofluorescence and histochemistry techniques.

We had discovered previously (see chapter 4) that markers in the gut considered to be pan neuronal appeared to highlight different neural subsets and that identification of a truly pan neuronal marker was difficult. In our earlier work we had used a Sevier-Munger silver stain technique; despite this not being pan neuronal, it highlighted many nerves within the lamina propria that were not shown with beta III tubulin. Unfortunately, we were unable to successfully repeat the protocol despite many attempts. We chose to use beta III tubulin and PGP 9.5 as neuronal markers; despite our previous work suggesting that these markers are not pan neuronal, but highlight different subsets of nerves with some cross over (see figure 4.), these markers have been used extensively during investigation of the peripheral nervous system in much published research (Harrington et al., 2010; Bettolli et al., 2012; Eisenman et al., 2013; He et al., 2016). It was decided to investigate the gut nerves both during infection and following resolution of infection, to see if any differences highlighted were transient or long lasting.

The enteric nervous system is maintained and supported by cells known as enteric glial cells that sit around and on nerve cell bodies and cell projections, akin to astrocytes within the CNS; however more recent research has suggested an expanded role for these cells including cytokine secretion, neuromediator expression and maintenance of gut wall integrity during infection (Neunlist et al., 2014). This suggests that enteric glial cells are important in regulation and

maintenance of a healthy gut nervous system both during infection and on return to health. Typical markers for enteric glial cells are similar to those found in astrocytes in the CNS, namely S100β and glial fibrillary acidic protein (GFAP) (Ochoa-Cortes et al., 2016). Some studies have suggested that these markers are not always co-expressed and increased GFAP proportions are associated with inflammation and nerve regeneration in the dorsal root ganglion and colon (Siemionow et al., 2009; da Silveira et al., 2009). Increased levels of both S100β and GFAP was found in the hippocampus of a streptozotocin rat model of diabetes (Baydas et al., 2003); however, a study of human diabetic subjects found no detectable GFAP and reduced S100β levels in serum in comparison with controls (Celikbilek et al., 2014a). Increased GFAP has been associated with chagasic megacolon and was considered to have a protective effect (da Silveira et al., 2009). Culture of isolated human primary enteric glial cells incubated with LPS or IFNγ showed signs of becoming highly activated with increased expression of S100β, GFAP, iNOS and subsequent nitric oxide production (Cirillo et al., 2011). As the levels of enteric glial cell markers have been associated with inflammation and have been clearly identified within the gut in several studies (da Silveira et al., 2009; von Boyen et al., 2004; Liu et al., 2010), we decided it relevant to investigate enteric glial cells, in particular the markers S100β and GFAP.

We had observed some effects on muscle contractility following administration of an α7nAChR agonist in the colon (a trend towards increased number of contractions in the naive and high dose group and increased strength of contractions in the low dose group) that may reflect differences in transit time across groups. In relation to gastrointestinal motility, the natural ligand of the α7nAChR, acetylcholine, is thought to have an excitatory effect, whereas nitric oxide has been found to have an inhibitory effect (Olsson and Holmgren, 2011). Activated enteric glial cells have increased expression of iNOS and therefore increased NO production. Nitric oxide synthase (NOS) is essential in modulation of vascular tone and exists in three forms; neuronal, endothelial and inducible (Knowles and Moncada, 1994). Inducible NOS (iNOS) is greatly upregulated during infection and expressed by inflammatory phagocytic cells leading to upregulated nitric oxide production and the resultant anti bacterial and anti-viral effects assist in pathogen clearance (Neves-Souza et al., 2005). During infection with *Schistosoma mansoni* in mice, iNOS and subsequent elevated NO levels have been associated with increased IL-12 production and a resultant skew from a Th2 to Th1 cytokine profile, suggesting a role for NO in immune modulation (Hesse et al., 2000). Interestingly, NOS has also been

associated with α7nAChR function; NO is released following stimulation of the α7nAChR in the hippocampus, and inhibition of NOS blocked α7nAChR activation in the rat hippocampus (Adams et al., 2000). Therefore, variation during infection of NOS levels on the α7nAChR and also nitric oxide production is likely to be responsible for changes in transit time found between groups, so we decided to investigate NOS levels.

Hypothesis

Differences in gut physiology between groups of mice infected with a high or low dose of *Trichuris muris* and a control group may be a result of variation in the enteric nervous system map, enteric glial cell population or nitric oxide synthase levels both during and post infection.

Aim

To investigate and compare the enteric nervous system, enteric glial cell population and nitric oxide synthase levels between groups of mice infected with a high or low dose of *Trichuris muris* and a control group both during and following resolution of infection. Immunofluorescence and histochemical studies utilised in chapter 4 and commonly used for identification of gut nerves, such as beta III tubulin and PGP 9.5, will be used to identify the enteric neural map in the submucosal plexus and lamina propria of the jejunum and colon, to identify any group specific differences. Histochemical techniques including Sevier-Munger silver stain and Luxol Fast Blue will also be utilised to identify all nerves and myelinated nerves, respectively. Enteric glial cell markers S100β and GFAP will be used to identify any group specific differences in glial cell number and expression both during and following resolution of infection. NOS levels will be quantified in groups both during and following resolution of infection as increased NOS is associated with cell death; if NOS levels vary between different immune profiles then this may mean that neuronal cell survival and the enteric neuronal map will differ post infection dependent on the immune profile of the pathology. Amount of fluorescence/ density of staining within a section will be measured and compared to elucidate any group specific differences. Differences in the enteric neural map between groups are likely to reflect differences found in gut physiology and therefore are likely to influence susceptibility to and recovery from gastro-intestinal disease via the neuro-immune link. This is likely to be reflected in a human population following resolution of infection with differing immune profiles.

6.2 Beta III tubulin immunofluorescence in the submucosal plexus and lamina propria; day 21 post infection

6.2.1 No significant difference in beta III tubulin immunofluorescence in submucosal plexus and lamina propria in the jejunum or colon between groups

Jejunum and proximal colon was taken from mice infected with a high dose or low dose of *T.muris* and a group of naive control animals. Tissue was embedded in paraffin, sectioned transversely at a thickness of 10μm and incubated with beta III tubulin antibody. Immunofluorescence in the submucosal plexus and lamina propria was quantified and despite a trend for higher levels of fluorescence in the high dose group in the colon, no significant difference was found between groups in the jejunum and colon at day 21 post infection (two way ANOVA) (Figure 6.1).

6.3 PGP 9.5 immunofluorescence in the submucosal plexus and lamina propria; day 21 post infection

6.3.1 Significant upregulation in PGP 9.5 immunofluorescence in the high dose group in the submucosal plexus and lamina propria of the colon

Immunofluorescence in the submucosal plexus and lamina propria was quantified and animals in the high dose group (n=5) had significantly higher levels of PGP 9.5 immunofluorescence than the low dose group (two way ANOVA, $p<0.05$, n=4) and naive animals ($p<0.05$, n=3) in the colon at day 21 post infection. No significant difference was found between groups in the jejunum at day 21 post infection (Figure 6.2).

6.4 Beta III tubulin immunofluorescence in the submucosal plexus and lamina propria; day 45 post infection

6.4.1 Significant downregulation of beta III tubulin immunofluorescence in the high dose group in the submucosal plexus and lamina propria of the colon

Proximal colon was taken from mice infected with a high or low dose of *T.muris*, and a group of naive control animals, embedded in paraffin, sectioned transversely at a thickness of 10μm and incubated with beta III tubulin antibodies. Immunofluorescence in the submucosal plexus and lamina propria was quantified and animals in the high dose group (n=4) had significantly lower levels of beta III tubulin than animals in the naive group (two way ANOVA, $p<0.05$, n=3) in the colon at day 45 post infection. There was no difference between the low dose group (n=5) and any other group (Figure 6.3).

6.5 PGP 9.5 immunofluorescence in the submucosal plexus and lamina propria; day 45 post infection

6.5.1 No significant difference in PGP 9.5 immunofluorescence in the submucosal plexus and lamina propria in the colon between groups

Immunofluorescence of PGP9.5 in the submucosal plexus and lamina propria was quantified; there was variation within the groups but no significant difference was found between groups in the colon at day 45 post infection (two way ANOVA) (Figure 6.3).

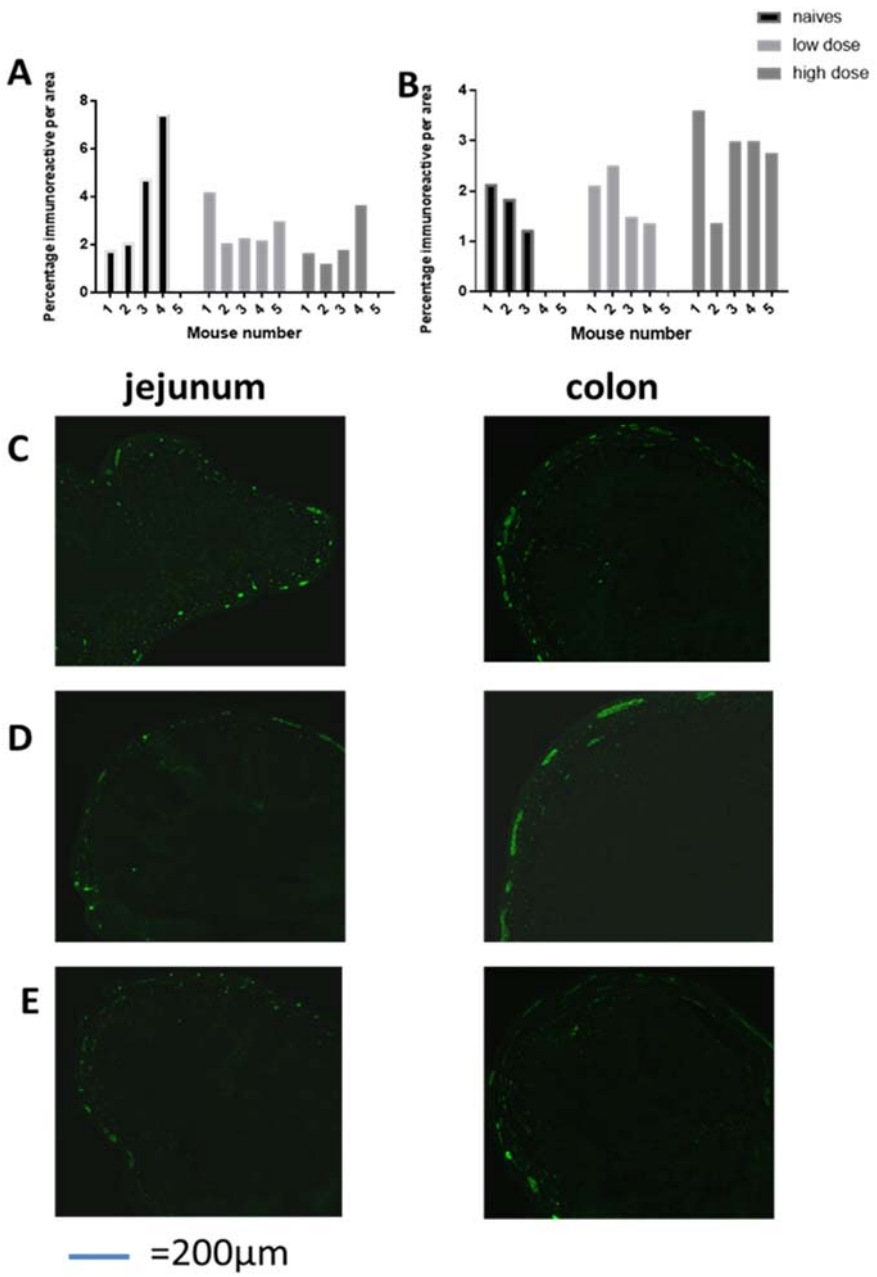


Figure 6.1 No significant difference in beta III tubulin immunofluorescence in the submucosal plexus and lamina propria of the jejunum and colon in C57BL/6 mice infected with *Trichuris muris* at a high dose, low dose and a naive control group at day 21 post infection. A: beta III tubulin immunofluorescence in the submucosal plexus and lamina propria of the jejunum. **B:** beta III tubulin immunofluorescence in the submucosal plexus and lamina propria of the colon between the naive (**C**), low dose (**D**) and high dose (**E**) groups at day 21 post infection (all 100xs mag). Graph shows individual data per animal. N=3-5, 3-4 sections per animal.

.

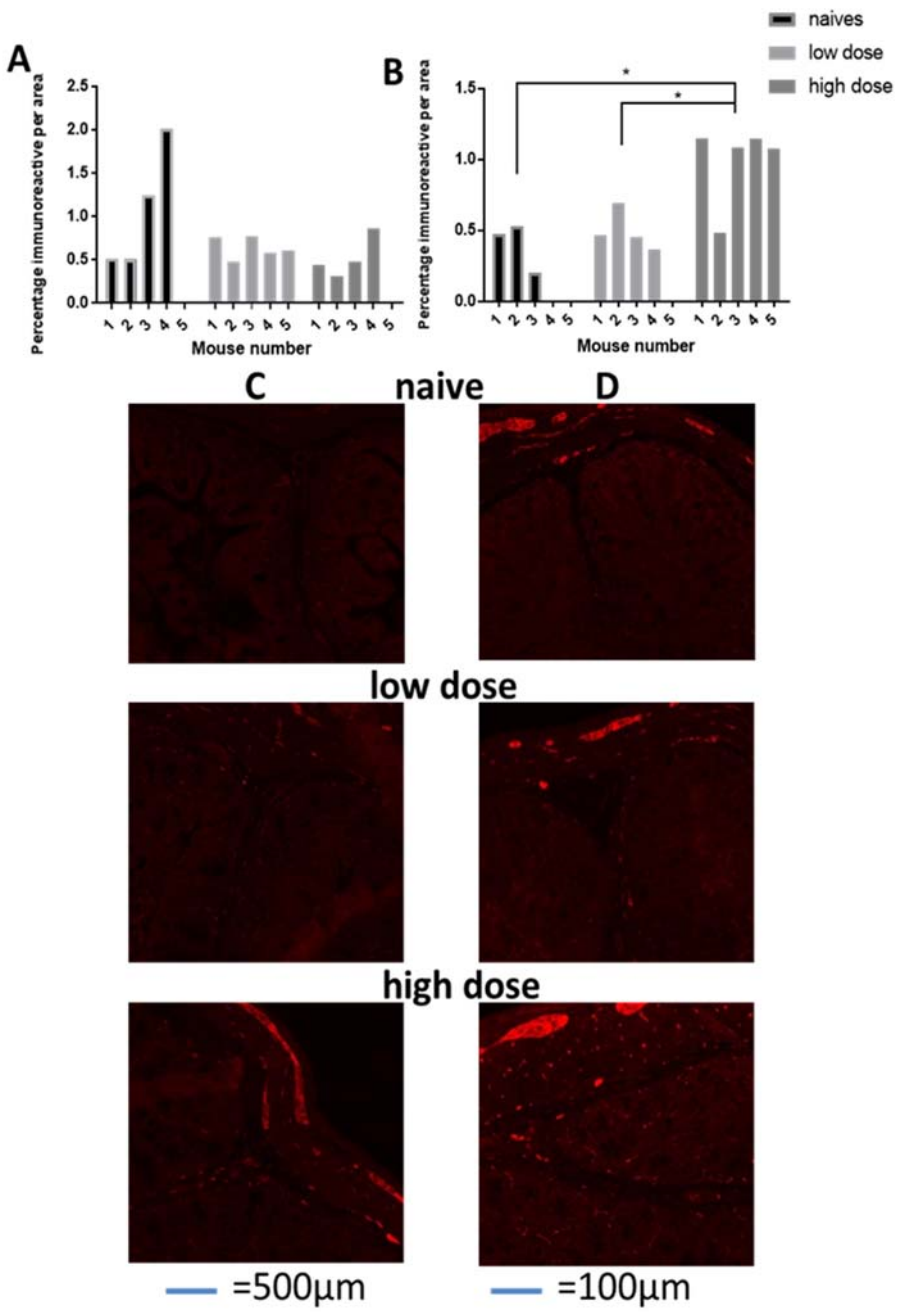


Figure 6.2 Significant upregulation of PGP 9.5 immunofluorescence in the high dose group in submucosal plexus and lamina propria of the colon. **A:** PGP 9.5 immunofluorescence in the submucosal plexus and lamina propria in the jejunum. **B:** PGP 9.5 immunofluorescence in the submucosal plexus and lamina propria in the colon. **C&D:** Representative images of beta III tubulin and PGP 9.5 expression in the colon for naïve, low and high dose *T. muris* infected mice at 50xs magnification (**C**) and 200xs magnification (**D**). Graph shows individual data per animal. N=3-5, 3-4 sections per animal. Two way ANOVA, *p<0.05.

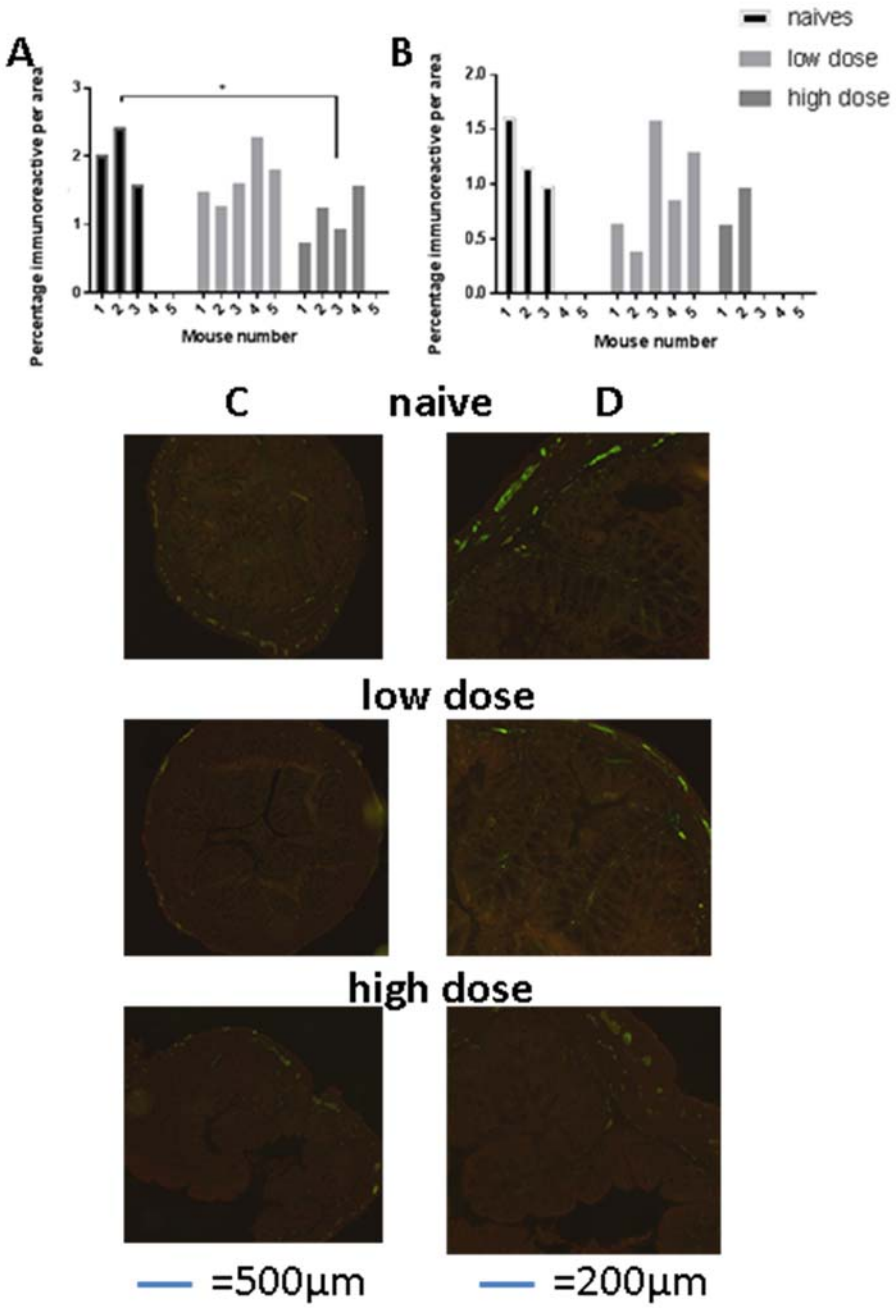


Figure 6.3 Significant downregulation of beta III tubulin immunofluorescence in the high dose group in submucosal plexus and lamina propria of the colon at day 45 post infection. **A:** beta III tubulin immunofluorescence in the submucosal plexus and lamina propria of the colon. **B:** PGP 9.5 immunofluorescence in the submucosal plexus and lamina propria of the colon. **C&D:** Representative images of beta III tubulin and PGP 9.5 expression in the colon for naïve, low and high dose *T. muris* infected mice at 50xs magnification (**C**) and 100xs magnification (**D**). Graph shows individual data per animal. N=3-5, 3-4 sections per animal. Two way ANOVA, *p<0.05.

6.6 GFAP and S100β immunofluorescence in the submucosal plexus and lamina propria; day 21 post infection

Jejunum and proximal colon was taken from mice infected with a high dose of *T.muris*, a low dose of *T.muris*, and a group of naive control animals, embedded in paraffin, sectioned transversely at a thickness of 10μm and triple stained with enteric glial markers GFAP and S100β and NOS antibodies.

6.6.1 No significant difference in GFAP immunofluorescence in the submucosal plexus and lamina propria in the jejunum and colon between groups

Immunofluorescence with GFAP antibodies was quantified in the submucosal plexus and lamina propria in the jejunum and colon day 21 post infection. There was variation within the groups in the jejunum, and levels were very low in the colon. There was no significant difference between groups in GFAP immunofluorescence in the jejunum or colon at day 21 post infection (two way ANOVA) (Figure 6.4).

6.6.2 No significant difference in S100β in the submucosal plexus and lamina propria in the jejunum and colon between groups

Immunofluorescence with s100β antibodies was quantified in the submucosal plexus and lamina propria in the jejunum and colon day 21 post infection. There was variation within the groups and despite naive animal showing a trend for higher levels of S100β, there was no significant difference in S100β immunofluorescence in the jejunum or colon at day 21 post infection (two way ANOVA) (Figure 6.4).

6.7 NOS immunofluorescence in the submucosal plexus and lamina propria; day 21 post infection

6.7.1 Significant differences in NOS immunofluorescence in the submucosal plexus and lamina propria in the colon between groups

Immunofluorescence with NOS antibodies was quantified in the submucosal plexus and lamina propria in the jejunum and colon day 21 post infection. There was a clear distinction between groups, with animals in the high dose group (n=4) presenting significantly greater levels of NOS immunofluorescence than animals in the low dose group (two way ANOVA, $p<0.005$, n=4) and animals in the naive group

($p<0.0001$, $n=4$) at day 21 post infection. Animals in the low dose group demonstrated significantly greater levels of NOS immunofluorescence than animals in the naive group ($p<0.05$). There was no significant difference between groups in NOS immunofluorescence in the jejunum at day 21 post infection. (Figure 6.5)

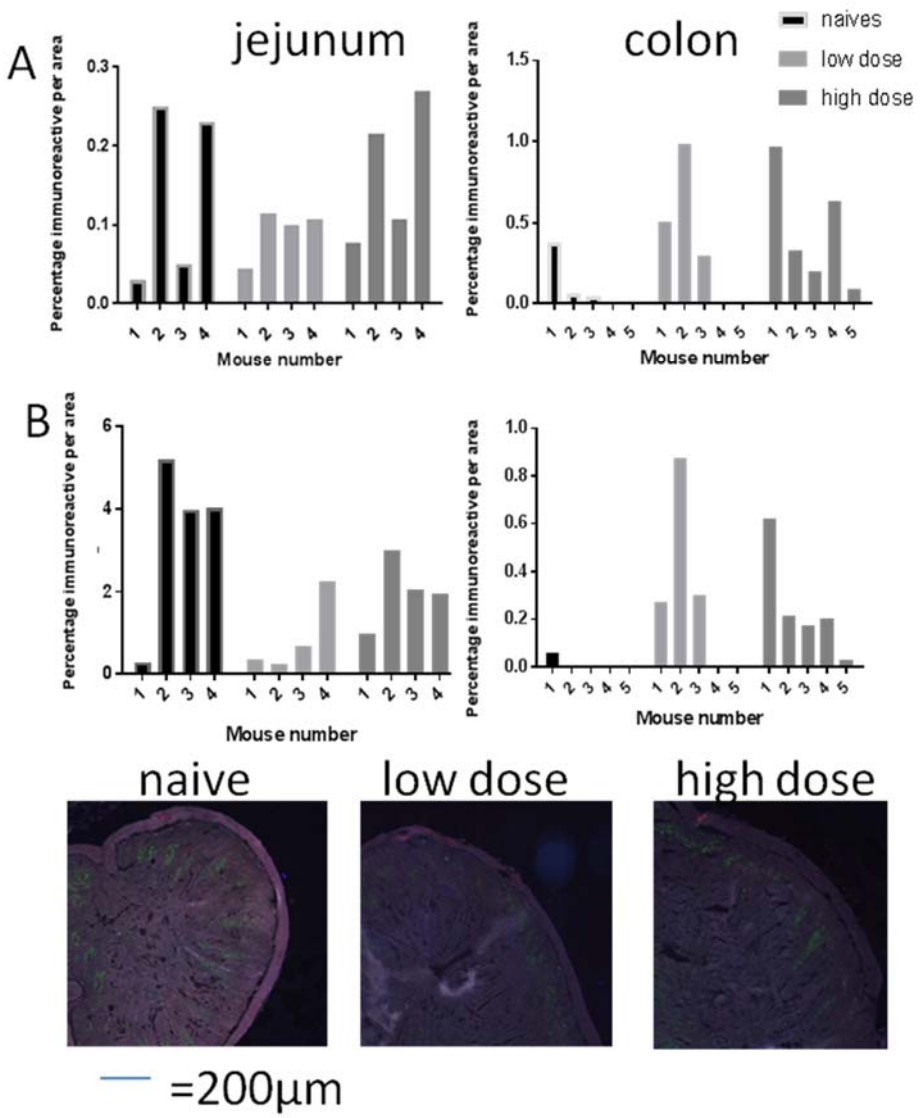


Figure 6.4 No significant difference between groups in GFAP and S100β immunofluorescence in the submucosal plexus and lamina propria of the jejunum and colon at day 21 post infection. A: GFAP immunofluorescence (red) in the submucosal plexus and lamina propria of the jejunum and colon. **B:** S100β immunofluorescence (green) in the submucosal plexus and lamina propria of the jejunum and colon. Representative images of GFAP and S100β expression in the jejunum for naïve, low and high dose *T. muris* infected mice (all 100xs mag). Graph shows individual data per animal. N=3-5, 3-4 sections per animal.

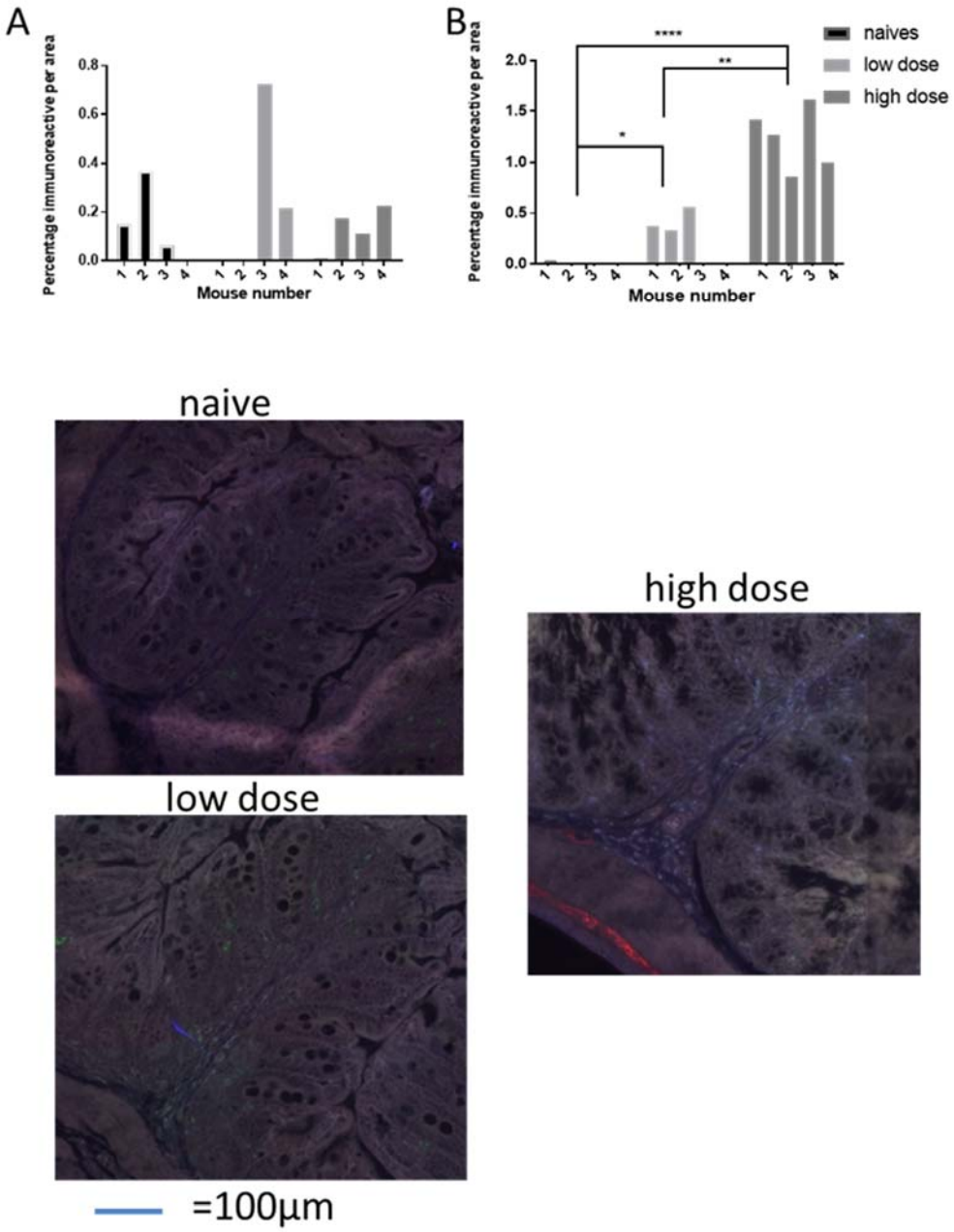


Figure 6.5 Significant differences in NOS immunofluorescence in the submucosal plexus and lamina propria of the colon. A&B: Quantification of NOS expression (ultra violet) in jejunum **(A)** and colon **(B)** of C57BL/6 mice by immunohistochemistry. **C:** Representative images of NOS expression in the colon for naïve, low and high dose *T. muris* infected mice. Images shown at 200xs magnification. Images were viewed using panoramic viewer. N=5 per group, 3-4 sections per animal. Two way ANOVA, *p<0.05, **p<0.01, ****p<0.0001.

6.8 GFAP and S100β immunofluorescence in the submucosal plexus and lamina propria; day 45 post infection

6.8.1 No significant difference in GFAP immunofluorescence in the submucosal plexus and lamina propria in the jejunum and colon between groups

Jejunum and proximal colon was taken from mice infected with a high dose of *T.muris*, a low dose of *T.muris*, and a group of naive control animals, embedded in paraffin, sectioned transversely at a thickness of 10μm and incubated with GFAP antibodies. There was variation within groups but no significant difference was found between groups in GFAP immunofluorescence in the jejunum or colon at day 45 post infection (two way ANOVA) (Figure 6.6).

6.8.2 No significant difference in S100β in the submucosal plexus and lamina propria in the jejunum and colon between groups

Jejunum and proximal colon was taken from mice infected with a high dose of *T.muris*, a low dose of *T.muris*, and a group of naive control animals, embedded in paraffin, sectioned transversely at a thickness of 10μm and incubated with S100β antibodies. There was variation within the groups and naive animals showed a trend for higher levels of S100β; interestingly this was also seen in naive animals at day 21 post infection. However there was no significant difference between groups in S100β immunofluorescence in the jejunum or colon at day 45 post infection (two way ANOVA) (Figure 6.6).

6.9 NOS immunofluorescence in the submucosal plexus and lamina propria; day 45 post infection

6.9.1 No significant difference in NOS immunofluorescence in the submucosal plexus and lamina propria in the colon between groups

Jejunum and proximal colon was taken from mice infected with a high dose of *T.muris*, a low dose of *T.muris*, and a group of naive control animals, embedded in paraffin, sectioned transversely at a thickness of 10μm and incubated with NOS antibodies. No evidence of NOS antibody immunofluorescence could be seen in any animal in any of the groups (data not shown).

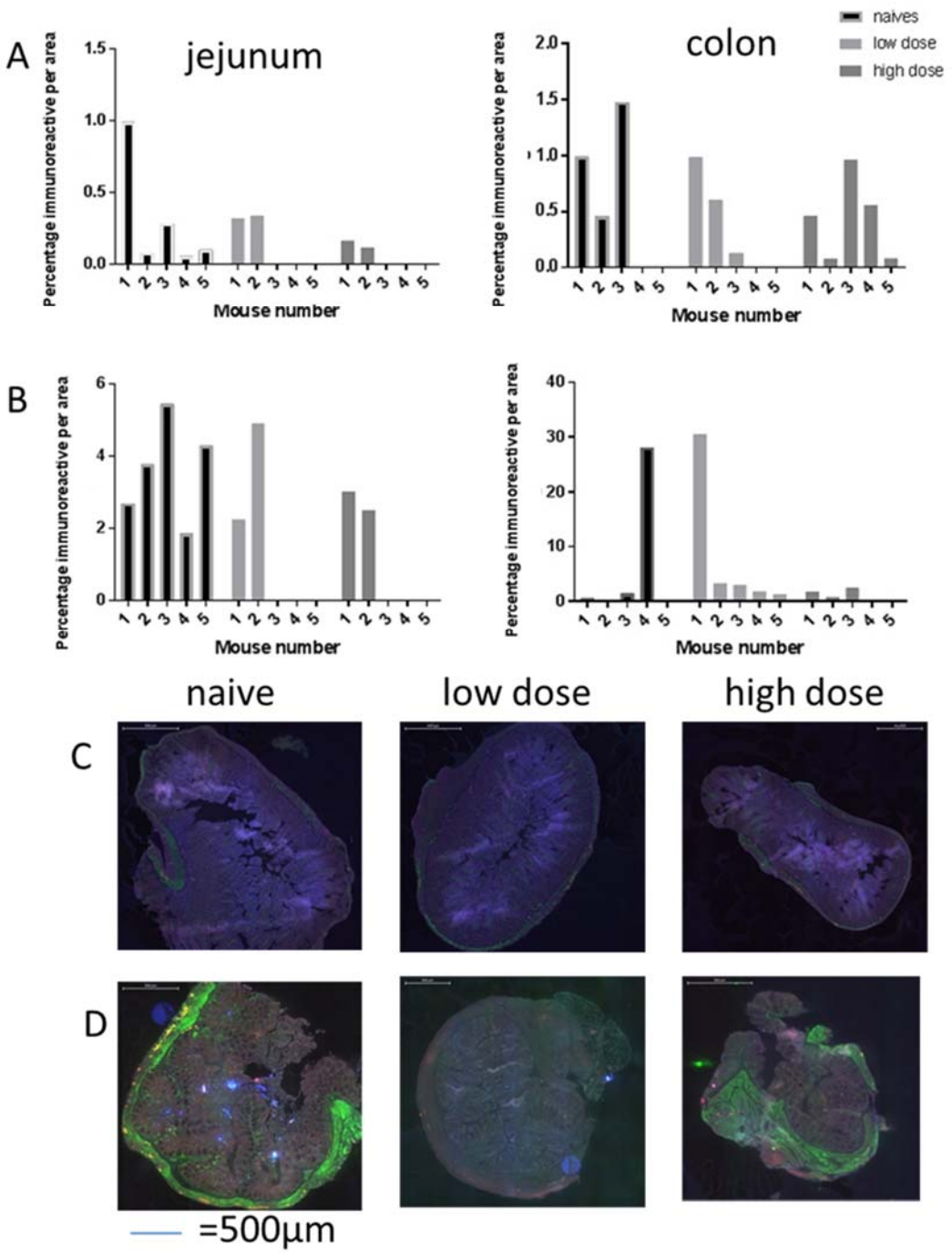


Figure 6.6 No significant differences in NOS immunofluorescence in the submucosal plexus and lamina propria of the colon. A&B: Quantification of GFAP (**A**) and S100β expression (**B**) in jejunum and colon of C57BL/6 mice by immunohistochemistry. **C&D:** Representative images of GFAP (red) and S100β (green) expression in the jejunum (**C**) colon (**D**) for naïve, low and high dose *T. muris* infected mice. Images shown at 50xs magnification. Images were viewed using panoramic viewer. N=5 per group, 3-4 sections per animal.

6.10 Luxol Fast Blue stain of colonic enteric neurons

6.10.1 Luxol Fast Blue stain did not clearly visualise any quantifiable difference in neurons in the enteric nervous system between groups

The Luxol Fast Blue histological stain was previously trialled in the colon and though myelinated nerve fibres within the rat brain and spinal cord could be clearly seen it was more difficult to identify neurons within the complex morphology of the large intestine. Colon embedded in paraffin and sectioned at a thickness of 10μm from mice infected with a high dose of *T.muris*, a low dose of *T.muris*, and a group of naive control animals was stained with Luxol Fast Blue solution to investigate if a quantifiable difference in myelinated nerve fibres could be seen between groups. Identification of fibres was again complex and it was not possible to measure quantifiable difference between groups (Figure 6.7).

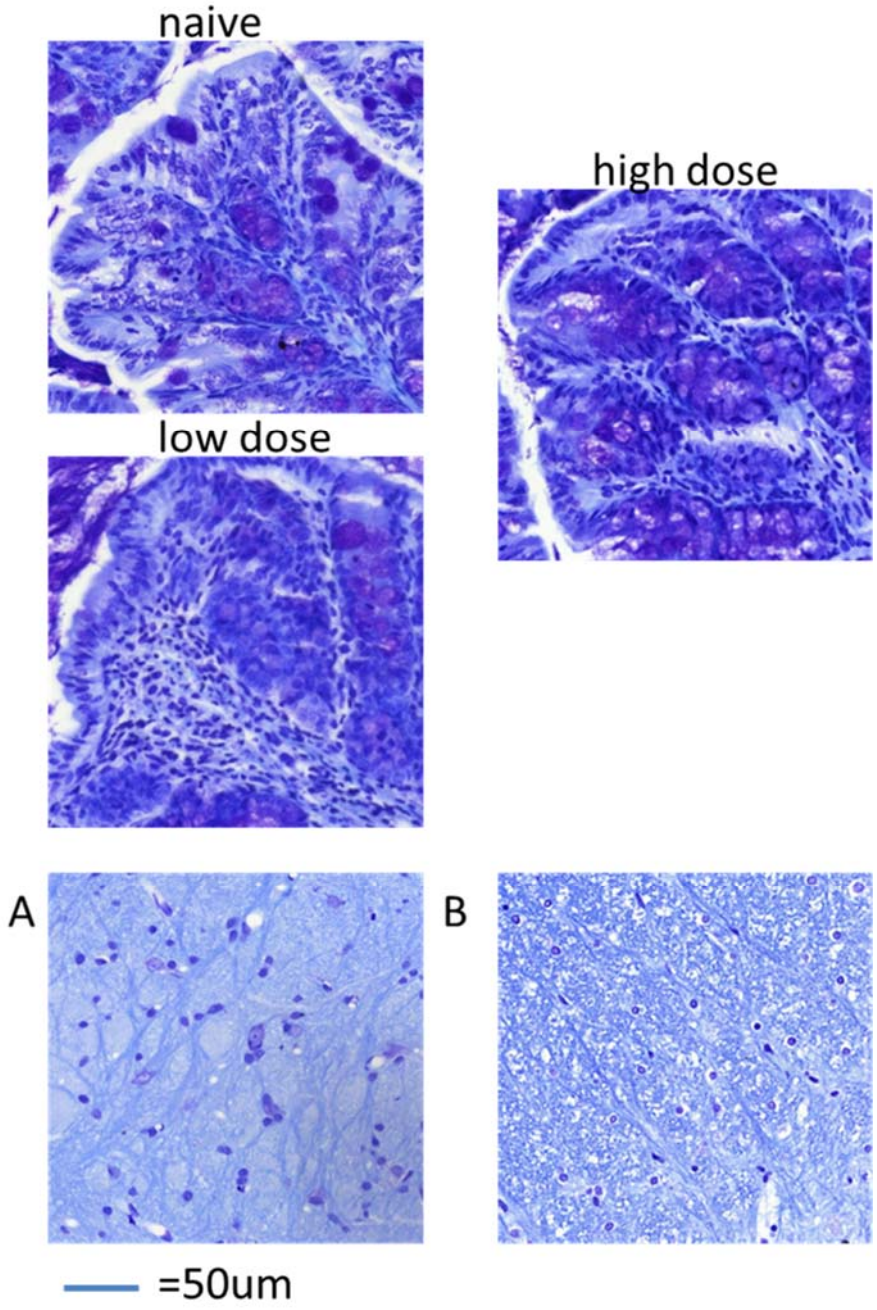


Figure 6.7 Luxol Fast Blue stain in the colon. Images of colon stained with Luxol Fast Blue in C57BL/6 mice infected with a high or low dose of *T.muris* and a naive control group at day 45 post infection demonstrating the apparent lack of or difficulty in visualising myelinated nerve fibres. Myelinated nerve fibres were clearly visible in the rat brain (**A**) and spinal cord (**B**) (all images 400xs mag).

6.11 Discussion

Previous findings highlighted some differences in gut physiology in mice following resolution of infection with a high or low dose of *Trichuris muris* and a control group. This suggests that there has been some lasting impact on the gut nerves. Despite the known link between the neural and immune systems (Kelley and McCusker, 2014) and previous research suggesting that resolved infection with *Shigella*, *Campylobacter Jejuni*, *Escherichia Coli* and *Salmonella* can affect the gut in a long term manner leading to symptoms of IBS (Ji et al., 2005; Spiller et al., 2000; Okhuysen et al., 2004; Mearin et al., 2005), there has been little research investigating the role of the enteric nervous system following resolution of infection. We investigated the enteric nervous system in mice during and following resolution of infection with a high or low dose of *Trichuris muris* and a control group using immunofluorescence and immunohistochemical techniques in order to highlight any differences between groups.

6.11.1 Differences in immunofluorescence between groups

No significant difference was found at any time point in any immunofluorescence in the jejunum. At day 21 post infection the level of PGP 9.5 immunofluorescence in the colon of mice infected with a high dose of *T.muris* was significantly higher than mice in the low dose and naive group; however this difference was not maintained and post treatment at day 45 post infection there was no significant difference in PGP 9.5 levels between groups. Interestingly, there was no significant difference in beta III tubulin levels at day 21 post infection, but at post treatment day 45 post infection, animals in the naive group showed significantly higher levels of beta III tubulin immunofluorescence than those in the high dose group. The variation in findings at different time points confirmed previous findings that these two markers considered ‘pan neuronal’ are not always co-expressed or pan neuronal.

Tests of gut function between groups found differences in transit time and water content; however, the most significant differences identified were between the low dose group and the naive group. At day 56 post infection, following administration of α7nAChR agonist PNU28987, there was a trend seen towards increased number of contractions in the high dose and naive group whereas the low dose group showed a trend towards increased tension of contractions. These physiological findings suggest that any difference in the enteric nervous system should be seen between the low dose and naive group and therefore does not correlate with immunofluorescence findings which demonstrated significant differences in PGP 9.5

and beta III tubulin between the high dose and the naive group at different timepoints in the colon. It may be that the subsection of nerves that are responsible for physiological changes evident in these groups following resolution of infection are not shown by beta III tubulin or PGP 9.5. Previous findings comparing two different strains of mice, the AKR and C57BL/6, also demonstrated significant differences in transit time but no significant differences in beta III tubulin immunofluroescence; the significant difference found between the AKR and C57BL/6 mice was seen using the Sevier-Munger silver stain technique, which appeared to show a different subsection of nerves to beta III tubulin immunofluorescence (see figure 4.12). Unfortunately we were unable to replicate this technique despite a number of attempts. However, this combination of results may lead us to speculate that the subset of nerves demonstrated with the Sevier-Munger technique, that we were not able to show in this experiment, could be involved in changes affecting motility. This is an intriguing finding given that acetylcholine is linked with gut motility and we have found a trend for differences in contractility with an α7nAChR agonist suggesting the nerves visualised with the Sevier-Munger technique may be cholinergic. However, previous research considered beta III tubulin pan neuronal and suggested it to be the best method for visualisation of the entire enteric nervous system (Matsuda et al., 2006) and immunostaining for a high affinity choline re-uptake receptor, CHT1, present in cholinergic nerve fibres, was found to be co-localised with beta III tubulin (Harrington et al., 2010). Despite this, images of the colon show a similar level of staining to our beta III tubulin images, suggesting that there is a definite subset of nerves that are not being seen. Regardless of this, we can say that resolution of infection with a high dose of *T.muris*, and potentially therefore any Th2 infection, results in a reduction of beta III tubulin positive nerves in the submucosal plexus and lamina propria of the gut.

6.11.2 Differences in NOS expression between groups during infection

Given the importance of enteric glial cells in maintenance and regulation of the enteric nervous system environment, we studied immunofluorescence levels of common glial cell markers, S100β and GFAP. Despite literature suggesting changes in levels of these markers during inflammation (da Silveira et al., 2009; Liu et al., 2010), we found no significant differences both during and following resolution of infection suggesting that there is no effect on glial cell markers during infection with *T.muris* regardless of the cytokine milieu. Given the suggested role of enteric glial cells in survival, differentiation and generation of peripheral neurons (Ochoa-

Cortes et al., 2016), the lack of change in enteric glial cell markers is likely to influence homeostasis of the ENS during infection with *T.muris* in an as yet unknown way. We also decided to investigate levels of NOS as production of iNOS is linked with response to infection (Hesse et al., 2000) and NO has been associated with activation of the acetylcholine receptor α7nAChR (Adams et al., 2000). We found NOS immunofluorescence levels to be significantly higher in the high dose group than the low dose group, and significantly higher in the low dose group than the naive group during infection. This is interesting as the high dose group experience a Th2 immune response (Bancroft et al., 1994) and upregulation of iNOS has traditionally been associated with a skew from Th2 to a Th1 profile (Hesse et al., 2000). It could be that during the immune response in the high dose group, increased NOS levels and associated Th1 cytokine milieu may prevent upregulation of the resistant response. Also, increased NO and resultant oxidative stress has been associated with creating an inhospitable environment during parasitic infection and thus is beneficial for pathogen clearance; increased iNOS levels in eosinophil ablated mice infected with *Trichinella spiralis* resulted in enhanced parasitic clearance by NO. However, this effect was ablated following increased eosinophil production, which resulted in downregulation of iNOS and upregulation of Th2 immune cells (Gebreselassie et al., 2012). Increased NO production was found to be protective in hepatocytes following infection with Schistosoma mansoni; prolonged NO production in IL-4 knockout mice suggested that the Th2 response is involved in regulation of NO production (Brunet et al., 1999). This significant difference in NOS levels was not evident at day 45 post infection. This suggests that on return to health, levels of iNOS and NO return to pre-infection levels. However, the increase on NO during infection may have resulted in some longer term loss of enteric neurons, as demonstrated via changes in gut physiology and in differences in beta III tubulin immunofluorescence, that may prevail despite a return to health. Undiagnosed autonomic neuropathy has been identified in healthy adults and neuropathy has been associated with initiation of chronic inflammation via the reduced activation of the cholinergic anti-inflammatory pathway (Thayer and Fischer, 2009). Therefore we could speculate that changes within the ENS that remain following resolution of infection may underlie and contribute to development of inflammatory pathological conditions such as IBD. As Crohn's disease and ulcerative colitis are associated with loss of different neuronal subsets (Ohlsson et al., 2007; Coruzzi et al., 2007), it could be that predisposition to a particular inflammatory condition is dependent on the balance of neuronal subsets

within the gut, and that this balance could have been influenced by the immune profile of any previous infection.

In this study we investigated the enteric nervous system with immunofluorescence techniques and found differences in PGP 9.5 and beta III tubulin expression during and following infection respectively, in mice infected with a high dose of *T.muris* and a naive group. This was in contrast with physiological examinations which concluded that the greatest difference in function was between mice infected with a low dose of *T.muris* and a naive group. Work showing strain specific differences in function between the AKR and C57BL/6 mice highlighted strain specific differences in neuronal subsets shown with the Sevier-Munger silver stain technique; unfortunately, this could not be repeated on the infection groups due to protocol issues. Differences in NOS expression between groups were seen during infection but resolved following infection clearance. This work suggests that infection has an impact on the enteric nervous system that can be seen following return to health.

Sunmmary of key findings

Significant differences in-
PGP 9.5- higher levels of immunofluorescence in the colon in a high dose infection group compared with low dose infection and a naive group (day 21 post infection)
Beta III tubulin- lower levels of immunofluorescence in the colon in a high dose infection group compared with low dose infection and a naive group (day 45 post infection)
NOS- lower levels of immunofluorescence in a naive group than both infection groups. Lower levels in a low dose than a high dose group (both day 45 post infection).

No significant differences in-
PGP 9.5 immunofluorescence in submucosal plexus/lamina propria of jejunum between any groups (day 21 post infection)
PGP 9.5 immunofluorescence in submucosal plexus/lamina propria of colon between low dose infection and naive group (day 21 post infection)
Beta III tubulin immunofluorescence in submucosal plexus/lamina propria of jejunum and colon (day 21 post infection)
Beta III tubulin immunofluorescence in the colon between a low dose infection group and a naive group (day 45 post infection)
GFAP and S100β immunofluorescence between any groups in the submucosal plexus/lamina propria of jejunum and colon (day 21 and day 45 post infection)
NOS immunofluorescence between groups in the jejunum and colon(day 21 post post infection) or jejunum at day 45 post infection

Chapter 7
Discussion

7.1 Discussion

Neural input and function within the GI tract is important in maintenance of normal gut physiology; however, increased understanding of the neuro-immune link means that we must also appreciate the value of the enteric nervous system in responding to pathogen invasion and re-establishment of homeostasis following resolution of infection. This means that anything that affects the enteric nervous system has the potential to impact not only gut physiology, but susceptibility and response to gut challenge. Despite this, little is known about the impact of host genetics or resolving inflammation on the enteric nervous system (ENS). In this study, we aimed to elucidate the influence of host genetics and resolving inflammation on gut physiology and enteric neuronal density.

To investigate the impact of genetic background on the ENS, we compared gut physiology and enteric neuronal density between AKR and C57BL/6 mice, two strains with known differences in peripheral innervation in the skin (Mogil et al., 1999a) and response to gut inflammation (Blackwell and Else, 2002). As the physiology of the gut is controlled by the ENS, any differences between groups within the ENS should be reflected in gut function.

We chose to investigate any variation in gut physiology first to identify if there were any differences between groups that would warrant immunofluorescent investigation. We found that genetic background had some significant effects. The AKR mouse had significantly increased weight and longer faecal pellets, with slower intestinal transit time and reduced total gut thickness in the jejunum and longitudinal muscle thickness in the colon in comparison to the C57BL/6 mouse. There was a trend towards increased tension during colonic contraction in the AKR.

As we identified several differences, we went on to investigate neuronal numbers with immunofluorescent and histological techniques. We found that the AKR mouse demonstrated a significantly reduced number of enteric nerves in the submucosal plexus and lamina propria highlighted by the Sevier-Munger silver stain technique when compared with the C57BL/6 mouse. However, there was no difference between strains in length of small or large intestine, faecal pellet number, water content and amount of beta III tubulin positive nerves identified in the myenteric plexus or submucosal plexus of the jejunum and colon.

To investigate the impact of resolved infection on the ENS, we compared gut physiology and enteric neuronal number between mice with infected with a high or

low dose of *T.muris*, to elicit a Th2 and Th1 response respectively (Bancroft et al., 1994) and a naive control group, following resolution of infection. Animals in the low dose infection group required treatment with an anti helminthic, mebendazole, to eradicate chronic infection; to account for any effects of this drug, animals in the high dose group and a naive control group were also given mebendazole. We found animals within the high, low and naive groups treated with mebendazole had significantly longer transit time, higher faecal water content and produced significantly fewer faecal pellets than a naive group that were not treated with mebendazole. Animals in the high dose group and naive animals treated with mebendazole displayed similar results with a faster transit time, lower water content and lower tension in gut contractility than the low dose group. During infection, there was a significant increase in NOS expression seen with immunofluorescence in the high dose group and low dose group respectively, and an increase in PGP9.5 positive nerves in the high dose group; this difference had resolved and returned to the same level as a naive control group by day 45 post infection post treatment with mebendazole. However, at day 45 post infection, number of beta III tubulin positive nerves was reduced in the high dose group, with no significant differences between the low dose and naive group treated with mebendazole. There was no difference in weight gain, food intake, thickness or length of small or large intestine or faecal pellet number between groups. There was also no difference between groups in glial cell markers S100β or GFAP expression at day 21 or day 45 post infection

In our study we found that both genetic background and resolved infection affected gut physiology and enteric neuronal number. However, we did find variation within groups that we were unable to account for; this could have been caused by one or several factors, including variation in the microbiome from birth, position relative to the placenta in utero, or stress caused by aggressive behaviour of mice within the cage. Despite this, the differences found suggest that genetics and resolved inflammation influence neural function or survival within the ENS; understanding this may help us to explain differences in disease susceptibility between different individuals and perhaps in the future, this understanding could be utilised for therapeutic potential.

7.2 Measuring differences between groups within the ENS-physiology

7.2.1 Genetic background, but not resolved infection, influenced weight gain in mice

Our results found differences in gut physiology between strains of mice with known variation in peripheral innervation. We found that the AKR strain ate more food and were consistently significantly heavier than the C57BL/6 strain suggesting a genetic link with weight gain in this strain. This has been identified in other research; in a study of the effect of high fat diet on nine mouse strains, the AKR strain gained the most weight and ingested the most food suggesting a propensity towards an obese phenotype (West et al., 1992). This was linked with a quantitative trait locus on chromosome 15 that affects tissue adiposity and mesenteric fat deposit size that has been associated with resistance to obesity in a high fat diet in the CAST/Ei and SWR/J and susceptibility to obesity following administration of a high fat diet in the AKR and C57BL/6 strains (York et al., 1996; West et al., 1994). However, if there is any genetic link between neuronal subsets and obesity, this is very relevant, given that the number of people classified as obese is over 1.1 billion and on the increase, leading to premature death and a range of health problems including diabetes, arthritis, cardiovascular disease and cancer (Haslam and James, 2005). This places a great strain on provision of medical services. Despite the impact of increased prevalence of sedentary lifestyles and increased intake of calorific foods, our research corroborates that of others suggesting an underlying genetic predisposition to weight gain that may exert itself according to environmental changes (Xia and Grant, 2013; West et al., 1992); this may be useful in understanding the aetiology and physiology of obesity. Some risk factors associated with gut function or the neural network have been identified. Mutation in the pro-hormone convertase 1 gene caused absorptional dysfunction within the small intestine and diarrhoea despite obesity development and no sign of retarded growth (Jackson et al., 2003). Mutation in the gene SIM1, a transcription factor involved in midline neurogenesis, is associated with obesity; SIM1, expressed within the nervous system and essential in neurogenesis, is also thought to be important in regulation of food intake due to its role in melanocortin 4 receptor expression within the paraventricular nucleus of the hypothalamus (Holder et al., 2000). Lipolytic functions of catecholamines, including epinephrine and norepinephrine, are mediated by the beta 2 adrenoreceptor (Meirhaeghe et al., 2001); mutations in both

the beta 2 and beta 3 adrenoreceptor genes resulting in decreased receptor activity have also been associated with development of obesity (Clément et al., 1995; Meirhaeghe et al., 2001). This suggests that the nervous system, via catecholamine production, is fundamental in regulation of obesity, and any factor that affects the nervous system, such as genetic background, may impact susceptibility to obesity.

Interestingly, a mutation involving the immune system has been implicated in development of obesity; the TNFα gene leading to increased TNFα expression has been linked with increased body mass index (BMI) and insulin resistance (Hotamisligil, 1999). This is interesting as it suggests a direct role for this inflammatory cytokine in development of obesity and diabetes; as TNFα is considered an inflammatory cytokine, and inflammation is associated with neuropathy, this suggests that changes in TNFα during inflammation may precede development of a pathology. Increased expression of this cytokine may be caused by infection or by an underlying autonomic neuropathy that results in downregulation of the cholinergic anti-inflammatory pathway (Thayer and Fischer, 2009). Interestingly, despite research proving that TNFα is upregulated during *T.muris* infection and enhances the immune response (Hayes et al., 2007), there was no long term effect on weight in animals following resolution of infection with a high or low dose of T.muris. This would suggest that normal upregulation of TNFα during infection followed by return to homeostasis leaves no lasting effect on weight in mice.

Increased weight gain has been associated with changes in metabolism and adipocity due to differences in the gut microbiome; however, studies investigating composition of the gut microbiome associated with obesity have yielded inconsistent and diverse results (Sanmiguel et al., 2015). Genetic background has been associated with microbiome composition in both mouse models and human studies (Zhang et al., 2014), and GWAS studies have identified several loci linked with obesity in adolescents and adults (Graff et al., 2013). However, there is still some question as to whether differences within the microbiome associated with obesity are a result of obesity itself; the answer is difficult to extricate due to the complex relationships between the microbiome and diet, obesity and the immune response (Sanmiguel et al., 2015). As we did not assess the microbiome during this experiment, any existing differences between strains and the influence of this on weight gain cannot be accounted for. Interestingly, during infection with *T.muris* in C57BL/6 mice, changes within the microbiome were identified that persisted for 91

days following infection before returning to those of a naive animal (Houlden et al., 2015b). As we saw no differences in weight gain between our infected groups and a naive control group, we can confirm that during our experiment, any change caused by *T.muris* to the microbiome of C57BL/6 mice during and following infection did not result in weight gain.

7.2.2 Genetic background and resolved infection did not affect length of the small or large intestine

No significant difference was found between any groups in length of the small or large intestine, despite a trend for C57BL/6 animals to have a longer small intestine and shorter colon than AKR mice. Literature suggests that the small intestine has a length that is surplus to dietary requirement; this facilitates maintenance of absorptive function during infection when rapid transit is initiated, or during a pathology such as Coeliac disease or Crohn's disease with associated enteropathy (Weaver et al., 1991). Therefore it would appear that any possible impact on small intestinal function is accounted for biologically prior to any infection. This would explain why no change in small intestinal length was seen within the animals infected with a high or low dose of *T.muris*. Wide variation in small intestinal length was found within a human population (Weaver et al., 1991); during pregnancy and lactation, increased nutrient requirement facilitates hypertrophy and lengthening of the small intestine; however, starvation can cause atrophy (Lo and Walker, 1989). Human studies have also attributed variation in the length of the large intestine to differences in length of the rectosigmoid colon (Phillips et al., 2015). As none of these factors are relevant to this study, and we did not measure the length of the rectosigmoid colon in animals, we are unable to account for the trend we saw for a lengthier small intestine and shorter large intestine in the C57BL/6 mice compared with the AKR mice. Interestingly, contraction of longitudinal muscle has been associated with shortening of the GI tract (Mackenna and McKirdy, 1972); however, it has been shown that radial stretching of the gut resulted in passive shortening of the GI tract that persisted in the absence of longitudinal muscle and innervation (Wood and Perkins, 1970). Therefore any genetic or infection associated variation in gut length may be associated with changes in ratio of longitudinal and circular smooth muscle subsets. Although this may influence gut physiology in a live animal, it is unlikely to be relevant in measurement of gut length ex-vivo due to loss of muscle tone (Weaver et al., 1991).

7.2.3 Genetic background and resolved infection affected muscle thickness in the gut in mice

We found significantly thicker total muscle in the colon of animals infected with a high dose of *T.muris* compared with a low dose and naive group. Interestingly, inflammatory cytokines IL-1β, TNFα and IL-17 alongside platelet derived growth factor (PDGF) increased proliferation in cultured rat circular smooth muscle cells via upregulation of the PDGF-Rβ receptor (Nair et al., 2014). These cytokines are traditionally associated with a Th1 immune profile, and therefore it was surprising to see that our results found no difference in muscle thickness in animals infected with a low dose of *T.muris* and a naive group, given that this level of infection is associated with a Th1 cytokine milieu (Bancroft et al., 1994). However, it has been shown that TNFα driven IL-13 is essential for elimination of *T.muris* in C57BL/6 mice, therefore this cytokine is likely to be present during high dose infection and could contribute to the increased thickness of muscle found within this group (Hurst and Else, 2013). Also interestingly, Th1 immune profile in low level *T.muris* infection has been confirmed by levels of IFNγ, and levels of TNFα have not been investigated (Bancroft et al., 1994; Bancroft et al., 2001).

We also found that genetic background affected muscle thickness between strains. AKR mice had a significantly thinner total muscle depth within the jejunum, and thinner longitudinal muscle depth but the same total muscle thickness within the proximal colon in comparison with the C57BL/6 mice. Interestingly, an investigation of muscle thickness in the sigmoid colon in a human sample found that whilst the total thickness of muscle remained the same, depth of circular muscle increased and longitudinal muscle decreased up to the age of 30; this was accompanied by loss of NO positive neurons that was attributed to loss of longitudinal muscle mass and was associated with growth (Southwell et al., 2010). However, this does not explain our findings as our mice were age and gender matched adults. As NO is important if facilitating gut relaxation via an inhibitory descending sympathetic pathway, loss of NO positive neurons within the longitudinal muscle may be reflected in changes in relaxation of the small and large intestine; however, NO positive neurons have also been found in the circular smooth muscle layer of the colon, which may act in a compensatory manner (Nishiyama et al., 2014).

During peristalsis, activation of cholinergic ascending excitatory and non-cholinergic descending inhibitory nervous pathways stimulate oral contraction and anal

relaxation of circular and longitudinal muscles, facilitating propulsion of contents through the gut lumen (Smith and Robertson, 1998). Whilst it was demonstrated as early as the 1900's that contraction of circular muscle propels intraluminal contents in an oral to anal direction (Bayliss and Starling, 1900), the contribution of the longitudinal muscle was less clearly understood. Despite studies demonstrating that these two muscles contract together, innervation of the two muscle layers is separate, and longitudinal muscle moves in slow waves with frequent action potential in contrast with the more sporadic movement of the circular muscle (Mackenna and McKirdy, 1972). The contraction of longitudinal smooth muscle is inhibited by the muscarinic acetylcholine receptor antagonist atropine but not by a nicotinic acetylcholine receptor antagonist hexamethonium, suggesting that muscarinic acetycholine receptors are integral to contraction of the longitudinal muscle (Kosterlitz and Lees, 1964). Inhibition of circular muscle contraction could be induced by administration of atropine or hexamethonium, suggesting both muscarinic and nicotinic acetylcholine receptors are important in contraction of circular muscle (Kosterlitz and Robinson, 1959). As the ratio of these muscles is affected by genetic variation in the AKR and C57BL/6 mice, and these muscles are directly responsible for gut peristalsis, we could hypothesize that this may result in differences between strains in gut transit time (see section 3.6). Perhaps it is reasonable to suggest that genetic variation in a human population may result in replication of this observation. Consequentially, as different effects of muscarinic and nicotinic acetylcholine receptor antagonism have been shown between longitudinal and circular muscle, the presence of any ligand specific to these individual receptors, such as nicotine, is likely to have a different effect on gut contractility in the genetically different individuals.

7.2.4 Genetic background and resolved infection affected faecal transit time

We found that genetic background can influence faecal transit time, with AKR mice demonstrating significantly longer transit time than the C57BL/6 mice. We also found that resolved infection and mebendazole affected faecal transit time; naive mice not treated with mebendazole had the fastest transit time, followed by a naive uninfected control group and high dose group (no significant difference between these two groups), with the low dose group having the longest transit time. In groups within the infection experiment, increased faecal transit time was directly

correlated with increased faecal water content; however, this was not the case in the genetic variation group, within which the AKR consistently had a longer transit time but similar faecal water content to the C57BL/6 mice. Increased transit time associated with increasing age has been attributed to loss of neurons and neurotransmitters; therefore it could be that a variation in the ENS caused by genetics or resolved infection is influencing transit time. However, slower transit time has consistently correlated with reduced faecal water content in studies of ageing and constipation, directly contradicting our findings and suggesting a complex relationship between faecal water content and transit time (Britton and McLaughlin, 2013; Xu et al., 2011).

Movement of the faecal pellet through the colon has been linked with stool size and consistency. Speed of pellet movement increased with pellet diameter; pellet movement was halted by administration of tetradotoxin but only partially abrogated by hexamethonium, suggesting the origin of this movement is neural in nature but not reliant on nicotinic acetylcholine receptors (Costa et al., 2015). However, in our study, we found that the AKR strain demonstrated longer transit time but larger faecal pellets than the C57BL/6 group; this pattern was replicated in our infection experiment, where the naive group treated with mebendazole had a slower transit time and longer faecal pellet than the naive control group not treated with mebendazole. It could be that the AKR has a longer transit time as it has significantly thinner total muscle in the jejunum that could affect strength of peristaltic propulsion; it also has thinner longitudinal muscle but thicker circular muscle within the proximal colon, suggesting that the more frequent action potentials in the longitudinal muscle are important in regulating transit time. However, in the infection group, the low dose group have a longer transit time but the same muscle thickness as naive animals; the high dose group had significantly thicker muscle than both the low dose and naive animals, but had the same transit time as the naive animals. Again this suggests that the relationship between these physiological factors is not straightforward. Increased oxytocin and oxytocin receptor levels have been linked with reduced GI motility, reduced stool mass and water content and reduced inflammation (Welch et al., 2014) adding another level of complexity to results that cannot be accounted for.

7.2.5 Genetic background and resolved infection had inconsistent effects on gut contractility

We found no significant differences in the strength or number of spontaneous contractions within the proximal colon between two different strains of mice, the AKR and C57BL/6 mice, and groups of mice given a high or low dose infection with *T.muris* and a naive control group. This was surprising as we had found significant differences in transit time between most groups. This would suggest that the proximal colon does not play a large role in influencing transit time, or could be a result of tissue being ex-vivo. It must also be noted that there was variation within the groups; there appeared to be a trend towards increased tension of contraction in AKR mice compared to C57BL/6 mice, however, one animal in each group did not fit with this trend. Interestingly, a trend for lower tension in the high dose group and naive animals treated with mebendazole in the infection experiment echoed similar results found between these two groups in transit time and water content. However, animals in the low dose group demonstrated a trend for comparatively higher tension per contraction that was replicated by the naive control animals not treated with mebendazole. This is surprising as the low dose group had the slowest transit time and naive animals treated with mebendazole had the fastest transit time out of all four groups. Perhaps this suggests that the tension of gut contraction cannot be consistently associated with transit time.

Following administration of carbachol, a muscarinic and nicotinic acetylcholine receptor antagonist, AKR mice displayed a greater initial increase in tension within the proximal colon, this being significant at 1μM. This suggests that the AKR mouse strain has either increased expression or function of acetylcholine receptors. This was corroborated by a significant increase in tension of contractions in the AKR mouse treated for a period of three minutes with carbachol (10μM). This is a confusing finding as acetylcholine is known to have excitatory functions within the gut and loss of cholinergic neurons has been associated with reduced transit time in the elderly (Nishiyama et al., 2014; Phillips et al., 2003), but the AKR has slower transit time than the C57BL/6 mouse. We could speculate that the finite number of cholinergic neurons in the AKR may be comparatively lower than the C57BL/6 and that upregulation of acetycholine receptor expression or function is a compensatory mechanism in an attempt to maintain homeostasis. However this currently remains as speculation.

Findings that the AKR has a peripheral variation in innervation that reduces nociceptive sensitivity via impairment in function of or response to TRPV1 stimulation were echoed to an extent in our study within the ENS (Mogil et al., 1999a; Mogil et al., 2005). Administration of a TRPV1 receptor ligand capsaicin (0.1μM, 1μM, 10μM) induced a trend for reduced tension of contractions in the C57BL/6 mice; however there was no effect on the AKR strain.

Due to the role of the α7nAChR in the cholinergic anti inflammatory pathway (Tracey, 2002), we decided to investigate the effects of an α7nAChR agonist (PNU 282987) on gut contractility following resolution of infection with a high or low dose of *T.muris*. As acetylcholine is excitatory, we would expect stimulation of this receptor to increase gut contractility; as we are only stimulating one acetycholine receptor and the gut has both nicotinic and muscarinic acetylcholine recptors, the effect seen may be limited. However, if this expression of this receptor is affected by different immune profiles (Galitovskiy et al., 2011) and any differences persist following return to health, this should be reflected in differences in gut contractility between our high and low dose *T.muris* infection groups. Again no significant differences were identified, but as we may have expected, a trend for an increased number of contractions associated with reduced tension of contractions was seen following administration of PNU 282987 in naive animals treated with mebendazole. Interestingly, at a higher concentration this trend was echoed in the high dose group, whereas the low dose group appeared to show sign of increased tension of contractions. This echoes functional results for transit time in which there was correlation between animals in the high dose group and naive control animals treated with mebendazole, with significantly longer transit time in the low dose group and suggests there is a trend towards a persistent difference in expression or function of the α7nAChR which is immune profile dependent. Interestingly, the trend towards increased tension contraction was echoed in the AKR group following administration of carbachol, suggesting some link between acetylcholine receptor expression or function, increased tension of gut contraction and longer transit time.

7.2.6 Genetic background, but not resolved infection affected faecal pellet length in mice

There was no significant difference in faecal pellet number between groups infected with a high or low dose of *T.muris* and a control group and despite the higher food intake in AKR mice compared with C57BL/6 mice, there was no consistent

significant difference in faecal pellet number between strains. Results of one experiment that found the AKR mouse produced significantly fewer faecal pellets in a four hour period compared with the C57BL/6 strain but these were not replicated during a repeat experiment. However, significantly longer faecal pellets were found in the AKR mouse strain in comparison with the C57BL/6 mice. In a naive control group treated with mebendazole faecal pellets were significantly longer than groups infected with a high or low dose of *T.muris* treated with mebendazole and a naive control group not treated with mebendazole, suggesting that mebendazole had an effect on pellet length that was mitigated by high or low dose infection with *T.muris*.

Changes in faecal pellet number and increased pellet size have been correlated with increasing age in many animal studies (Lee et al., 2016); this has been used successfully as a non invasive method of monitoring animal population. However, studies of the ageing gut have not addressed this issue in a human population but instead have focussed on factors that may impact quality of life (Britton and McLaughlin, 2013). Research has identified age associated changes in structure and density of nerves within the enteric nervous system and changes in gut physiology such as increased transit time (Saffrey, 2013); these age associated changes are likely to be responsible for the difference in faecal pellet length and transit time found in these animals (Phillips et al., 2003; Phillips and Powley, 2007). It may be that the variation we identified within the gut of the AKR is homologous to age related change. Whether this change is present from birth or represents a premature ageing remains to be seen. We could speculate that anything that affects gut physiology or the ENS in a similar way to ageing may also affect faecal pellet size. Interestingly, mice treated with antibiotics for a four week period produced significantly fewer but lengthier faecal pellets than a naive control group, suggesting a role for the microbiome in modulation of faecal pellet size and number (Ge et al., 2017a). Transfer of microbiota from human patients with slow transit constipation into pseudo-germ free mice resulted in reduced number of faecal pellets produced, but this was accompanied by reduction in faecal pellet size (Ge et al., 2017b). This highlights a complex role for the microbiome in influencing faecal pellet length that is likely to be affected by an as yet unspecified composition of bacteria and could play a role in strain specific differences.

As previously mentioned, the microbiome is known to be affected by infection with *T.muris* (Houlden et al., 2015b), but we found no significant differences in pellet length between high and low dose infection groups and a naive control group in either strain. This could highlight the importance of bacterial species affected within

the microbiome in influencing potential changes in gut function. Low dose infection with *T.muris* results in a Th1 cytokine milieu and chronic infection (Blackwell and Else, 2002); to eradicate this and ensure the infected animals returned to health for the purpose of the experiment, an anti parasitic medication called mebendazole was administered. In order to account for any possible effects of this drug, it was given to animals in both the high and low dose groups, and a naive control group; however, the only significant difference in pellet length following mebendazole treatment was seen in the uninfected group.

Mebendazole is one of only four drugs recommended by the WHO for treatment of common soil transmitted helminths; despite limited efficacy, these treatments have been used for over thirty years in equatorial countries where helminths are pandemic and affect millions per year (Keiser and Utzinger, 2008). Treatment has been associated with some mild side effects including stomach cramps, headache and feeling bloated (Steinmann et al., 2011). Mebendazole inhibits helminth growth by irreversibly inhibiting uptake of glucose (Seah, 1976); reduced glycogen, free glucose and soluble protein in *in vivo* and *in vitro* treated *T.Spiralis* larvae (Criado Fornelio et al., 1987). It causes transient increase in ROS and reduction in antioxidants (Locatelli et al., 2004) and also interferes with tubulin polymerisation causing mitotic arrest (Sasaki et al., 2002). For this reason, cytotoxic and antiproliferative effects have been attributed to the anti-parasitic drugs mebendazole and albendazole; due to comparatively low levels of ROS generation associated with mebendazole in comparison to albendazole, therapeutic potential of mebendazole has been under investigation in the treatment of several types of cancer including lung and colon (Nygren et al., 2013; Sasaki et al., 2002). As 5-10% of the drug is absorbed by the body, it is feasible to suggest that administration is likely to have some effect on the host, particularly in the area of absorption, in this case the gut (Drugbank, 2017). This suggests there is an as yet unconsidered physiological consequence following administration of mebendazole that must be considered when administering the drug as an anti-helminthic treatment to a human population. In our study, one of these effects was increased faecal pellet length; unfortunately the effect of mebendazole on the microbiome is not currently known and therefore cannot be accounted for.

7.2.7 Resolved infection but not genetic background affected faecal water content

We did not find any differences between the AKR and C57BL/6 mice in faecal water content, suggesting that genetic background was not a factor in control of reabsorption of water throughout the GI tract during digestion in our experiment. However, we did find differences in water content in animals in the infection experiment; the lowest water content of 28% was found in a naive control group not treated with mebendazole. This was followed by a naive group treated with mebendazole which demonstrated water content levels over 10% higher than that of the untreated naive group. A further increase was seen in the high dose group and the low dose group respectively; however the difference between the groups treated with mebendazole was a maximum of 3%. This suggests that mebendazole, either via a direct action such as tubulin disruption and glucose uptake in the host, or via an indirect method such as impact on bacteria in the gut microbiome, affects water reabsorption within the GI tract. Our faecal parameter tests were performed 21 days after treatment with mebendazole, suggesting any physiological effect caused persists for a degree of time following treatment; we are unable to conclude whether these effects are permanent or reversible as there were no later timepoints examined within our study. Our results suggest that resolved infection also affects water reabsorption, as animals infected with a low dose of T.muris resulting in a Th1 cytokine profile produced faecal pellets with a higher water content than those in the high dose group eliciting a Th2 infection, and both were higher than the control group. As all of these groups had been treated with mebendazole, any persisting differences identified in water content must be attributed to resolved infection, and differences between infected groups suggest that these differences are immune profile dependent.

Membrane bound proteins regulate absorption of water throughout the gut in response to changes in osmotic gradients (Leiper, 1998). The peptide transporter protein PEPT1 is expressed at high levels in the small intestine and low levels within the colon and has been associated with changes in water absorption; PEPT1 knockout mice produced faecal pellets with a significantly higher water content than wild type mice (Wuensch et al., 2013; Wuensch et al., 2014). Upregulation of PEPT1 within the colon has been associated with IBD, and increased levels of proinflammatory cytokines TNFα and IFNγ upregulated PEPT1 expression in a human colon carcinoma cell line and in mouse colon, but not small intestine; cytokines IL-1β, IL-2, IL-8 and IL-10 had no effect on PEPT1 expression (Vavricka

et al., 2006). However, Wuensch et al. (2014) found that PEPT1 expression was not upregulated in murine models of DSS and TNBS induced colitis or a human cohort with cute IBD. This suggests a complex relationship between PEPT1 expression and local cytokine milieu, but does suggest that there is likely to be some relationship between the two that will influence faecal water content; exactly how this works is not yet fully understood.

7.3 Influence of genetic background and resolved infection on the ENS; histological and immunofluorescence studies

We encountered significant difficulty in establishing a satisfactory pan neuronal marker that was able to demonstrate both cell bodies and surrounding neuropil within the complex morphology of the ENS. Despite many trials with several antibodies, we were unable to replicate the results found with the Sevier-Munger silver stain technique. No significant difference between the AKR and C57BL/6 strains was seen in beta III tubulin expression, and despite a successful protocol with high levels of immunofluorescence in the myenteric plexus, demonstration of nerves within the submucosa and lamina propria was poor with this antibody. The number of studies investigating the ENS within the submucosa and lamina propria is limited with many studies focussing on nerves within the myenteric plexus, and the submucosal and lamina propria nerve infiltration is considered much reduced in rodents compared to larger mammals (Timmermans et al., 2001). However, the Sevier-Munger silver stain technique demonstrated a rich infiltration of nerves throughout the lamina propria with significantly more nerves in the submucosa and lamina propria of the colon in C57BL/6 mice compared to the AKR. This difference in enteric neuronal number could represent an enteric reflection of the known differences in peripheral innervation between these two strains. It could also be the reason behind the differences in gut function and neuroreceptor expression or function that we found between the strains.

Unfortunately we were unable to replicate the Sevier-Munger silver stain technique for animals in the infection experiment, despite numerous attempts. However we did see significant increase in expression of PGP9.5 at day 21 post infection and significant decrease in beta III tubulin expression at day 45 post infection in the high dose group. This again confirms that these markers are not pan neuronal within the ENS. It does suggest that infection with a Th2 immune profile, but not a Th1

immune profile, results in a loss of beta III tubulin neurons that persists following resolution of infection. However, it is difficult to link this result to differences in gut physiology between groups, as function of the high dose group closely resembled that of the naive group. Despite the impact of genetic background on gut physiology, no significant difference was seen between the AKR and C57BL/6 mouse strains in beta III tubulin expression. Therefore we are unable to currently elucidate the meaning of these results in beta III tubulin expression within the high dose group.

7.3.1 Upregulation of NOS during infection is immune profile dependent

Under normal physiological conditions, NO is a potent neurotransmitter with effects on vasodilation and is considered inhibitory in terms of gut movement (Nishiyama et al., 2014). During infection, massive upregulation of iNOS results in increased synthesis of NO that contributes to the elimination of an invading pathogen via ROS production and resultant cell death (Vallance and Leiper, 2002; Salgo et al., 1995). We found that during infection, NOS levels were higher within animals infected with a high dose of *T.muris* eliciting a Th2 immune profile than within animals with a low dose infection and resultant Th1 based response; this effect did not persist and NOS staining was minimal in both groups on resolution of infection. This was an interesting observation as increased iNOS levels have been associated with a Th1 immune profile; it was demonstrated that during metabolism of arginine in macrophages, Th1 cytokines induced formation of iNOS, whereas Th2 cytokines resulted in arginase production (Munder et al., 1999). Also, TNFα and iNOS producing dendritic cells were shown to increase proliferation of IFNγ producing CD4 T cells thus upregulating a Th1 immune profile (Chong et al., 2011). However, increase in NO levels have also been associated with reduced expression of pro inflammatory cytokines TNFα and IL-1β in macrophages (Thomassen et al., 1997). Several studies have shown the importance of iNOS and NO in immunity to parasitic infection. Resistance to the parasite *Toxoplasma gondii* and reduction of intracellular parasite replication was dependent on NO production in macrophages; mice treated with an iNOS inhibitor L-NAME became susceptible to infection (Woods et al., 2013). Activation of the P13K/Akt pathway during infection with the parasite *Leishmania amazonensis* reduced iNOS production; inhibition of this pathway was associated with reduced parasite replication (Calegari-Silva et al., 2015), and inhibition of iNOS during infection with *Trypanosoma cruzi* resulted in

increased parasitic growth (Talvani et al., 2002). Interestingly, during early stages of *Schistosoma mansoni* infection, NO mediated protective effects; however, IL-4 knockout mice expressed higher levels of NO over a prolonged period that resulted in deleterious effects on hepatic cells. This highlights the influence of IL-4 in mediating NO production (Brunet et al., 1999) and suggests a link between increased iNOS and a skew from a Th2 to Th1 profile as a means of mediating cytotoxic damage (Hesse et al., 2000). Along with our results, this suggests that increased levels of iNOS are required for elimination of some parasitic infections, and these levels are controlled by the Th2 cytokine IL-4. The transient increase in ROS generation associated with high levels of iNOS during *T.muris* infection in the high dose group appears to leave little influence on gut physiology, but could be the reason for reduced beta III tubulin positive nerves following resolution of infection. However, further research is needed.

7.3.2 No difference in S100β or GFAP positive enteric glial cells during or following infection with a high or low dose of *T.muris*

Despite much research highlighting the importance of enteric glial cells in survival and generation of peripheral neurons, and regulation of enteric glial cell markers S100β or GFAP in inflammation, we found no difference in expression of these markers during or following high or low dose infection with *T.muris* (da Silveira et al., 2009; Ochoa-Cortes et al., 2016). This could mean that the relevant time frame for examination of these markers was missed during our study, or that enteric glial cell markers S100β and GFAP are not affected during infection with *T.muris* in mice.

7.4 Identification of neurons within the gut- problems and pitfalls

Valid, reliable and repeatable methods for measurement of gut physiology were established without considerable difficulty; despite not being used to compare effect of genetic background or resolved infection with different immune profiles on the ENS, these methods had been employed by many studies to investigate the effect of pathology and drugs on ENS function (Marks et al., 2013; Bove, 2015; Ge et al., 2017a). However, there were several issues to take into account when investigating number of neurons within an area of the gut (Kapur, 2013).

Many studies investigating the ENS with immunofluroescence techniques focus on the neural network within the myenteric plexus using whole mount techniques with microdissection to remove the submucosal plexus. However, we felt it important to investigate neurons within the submucosal plexus and lamina propria; this is because infection with the parasite *T.muris* involves penetration of the parasite through the gut epithelium, therefore any nerves in this area would be likely to be affected in the infected groups. We were interested in nerves in this area in the AKR mice as these mice are known to have differences in peripheral nociception (Mogil et al., 1999a) and are also unable to produce a normal Th2 immune response to parasitic invasion with *T.muris*, succumbing instead to chronic infection and a Th1 based cytokine milieu as opposed to the highly resistant C57BL/6 strain (Blackwell and Else, 2002). It could be that variation in nerves between strains within the lamina propria are responsible for this variation in response to parasitic infection along with differences in gut physiology identified. We were unable to effectively utilise a whole mount technique without employing microdissection due to issues with antibody penetration and microscopic visualisation. Therefore we established a histological method that permitted investigation of both the myenteric plexus and submucosal plexus without using microdissection. This also eliminated any problems associated with tissue stretch and shrinkage ex vivo in whole mount techniques (Kapur, 2013).

There is a vast quantity of markers available attributed with pan neuronal qualities. When considering what reliable pan neuronal marker to use to establish neuronal number, we focussed on those able to identify both the cell body and surrounding neuropil that other studies of the ENS had used, namely PGP9.5 and beta III tubulin (Eisenman et al., 2013; Harrington et al., 2010; He et al., 2016; Betolli et al., 2008). PGP9.5 is a ubiquitination hydrolase product and beta III tubulin is a component of the nerve cell wall; both of these markers have been described as neuron specific and pan neuronal (Campbell et al., 2003; Caccamo et al., 1989). We used antibodies for these markers previously used successfully by our lab. However, initial double staining trials clearly identified that these markers were not consistently co-expressed and could be seen adjacent to each other in mutually exclusive nerves, and that comparatively, levels of beta III tubulin expression were higher than PGP9.5. It could be that this is an issue with antibody binding or states of phosphorylation. However, this is a novel and important finding suggesting that caution must be used when considering these markers pan neuronal.

Antibody techniques are utilised during many experimental protocols such as immunofluorescence, enzyme linked immunosorbent assays (ELISA) and fluorescence activated cell sorting (FACS) techniques. Despite this, issues with reliability of antibodies leading to questions over validity of results has been highlighted (Baker, 2015). We decided then also to utilise the Sevier-Munger silver stain, a histological technique adapted from the Bielchowsky method for use in paraffin embedded tissue (Sotelo et al., 1980). This is an argyrophillic technique that highlights nerves and nerve fibres and has been described as a useful tool for neurologists (Uchihara, 2007). We found that the Sevier-Munger silver stain identified a subset of nerves within the villi of the jejunum and lamina propria of the colon that could not be seen with beta III tubulin immunofluorescence in serial sections. Interestingly, a subset of neurons within the myenteric plexus that was clearly demonstrated with beta III tubulin immunofluorescence was not visible in a serial section with the Sevier-Munger silver stain technique. This suggests previously unidentified complications in attributing a pan neuronal marker within the gut that researchers must be aware of and suggests that all enteric neurons may not contain all of the markers previously considered pan neuronal.

Issues with size of area studied has also been considered problematic when comparing results of one study with another (Kapur, 2013). We were able to account for this by quantifying neurons within a complete histological cross section of the gut; interestingly, we found variation in expression of markers in an animal within the same section. This does indeed highlight a possible issue in validity of results in studies that quantify only a small area of a histological cross section rather than the section in its entirety.

7.5 Future work

This study has yielded several interesting results; it is clear that variation within the ENS can be a result of both genetic background and resolved infection, and these differences were reflected within gut physiology. However, due to difficulty establishing a reliable pan neuronal marker and protocol issues, immunofluorescent and histological examination of the ENS was not extensive. Further examination of enteric neuronal number with a truly pan neuronal marker would be useful; however, this marker is yet to be identified. It would be interesting to identify different enteric neuronal subsets affected by genetic variation or infection; quantification of nitrergic and cholinergic neurons may help to elucidate the cause of variations between

groups in gut functions such as transit time and contractility. Investigation of neuropeptides such as CGRP, VIP and SP would also be interesting, particularly CGRP within the AKR mice. This strain has a peripheral dysfunction with TRPV1 response or function associated with disrupted CGRP expression (Mogil et al., 2005); as CGRP has been associated with downregulation of a Th1 cytokine immune profile (Ding et al., 2008; Ding et al., 2016), issues with expression of this neuropeptide peripherally echoed within the ENS may be the reason why AKR mice are susceptible to parasitic infection with *T.muris*, producing a chronic Th1 response (Bancroft et al., 1994).

We identified a difference between the AKR and C57BL/6 strains in expression or function of acetylcholine receptors following administration of carbachol. As expression of the α7nAChR has been associated with the immune response, it may be interesting to investigate the amount or function of these receptors to see if this may be a factor in the different immune response to *T.muris* infection between these strains. Further investigation of gut contractility using a higher number of animals may clarify possible significance within trends that were seen; however, we did find variation within genetically identical animals in the same groups during both physiological and immunofluorescence examinations. This may be the case regardless of sample size.

It would also be interesting to investigate the impact of the microbiome in variation within the ENS as a result of both genetic background and resolved infection, as changes within the microbiome have been associated with variation in both (Houlden et al., 2015a; Goodrich et al., 2014). These commensal bacteria may influence gut function and therefore the ENS.

Examination of animals at later timepoints may enable us to ascertain if post infection changes found in gut physiology and the enteric nervous system can eventually resolve. Houlden et al. (2015a) found that changes in intestinal microbiota identified during *T.muris* infection in a murine model lasted for 91 days following infection clearance before returning to the profile of an uninfected animal, suggesting that infection related changes within the microbiome at least are not permanent. Perhaps restoration of homeostasis may be able to correct post infection changes in the enteric nervous system in time; however, if disease susceptibility is affected prior to this, further studies to confirm this phenomenon and elucidate relevant timespan required for resolution would be useful. It may also be that factors affecting neurogenesis such as age, stress and the microbiome are

influential in restoration of pre infection function and further examination of manipulation of these factors post infection may prove interesting.

7.6 Wider implications

Our study found that genetic background and resolved infection affect some aspects of gut physiology and enteric neuronal number, suggesting that both of these factors have an influence on the ENS. If we consider the extensive role of the neuro immune link (Kelley and McCusker, 2014), this means that differences within the ENS between different people will affect susceptibility and response to disease. Patients with Th1/Th17 autoimmune mediated Crohn's disease demonstrated sympathetic neuropathy whereas those with Th2 mediated ulcerative colitis present with a vagal neuropathy, suggesting that activation of different immune profiles affects the ENS differently (Ohlsson et al., 2007; Coruzzi et al., 2007); our results confirmed this. Ulcerative colitis is known as a disease of non smokers, which highlights the role of the nervous system, in particular stimulation of nicotinic acetylcholine receptors, in disease susceptibility (Bastida and Beltrán, 2011). Neuropathy is commonly associated with inflammatory pathologies (Gregory et al., 2012; Hosseini and Abdollahi, 2013); autonomic neuropathy has also been identified in asymptomatic adults and neuropathy preceded signs of inflammation in diabetic patients (Thayer and Fischer, 2009; Posadas et al., 2013; Vinik et al., 2013). Understanding the loss of neurons, in particular the subset lost prior to disease development, may help us to further understand susceptibility; to take this a step further, if we were able to somehow arrest this neuronal loss or stimulate neuroregeneration, this has the therapeutic potential to prevent or arrest pathology development.

We identified an increase in acetylcholine receptor expression in the AKR mouse compared with the C57BL/6 mouse; this could be associated with the reduced quantity of neurons within the lamina propria in this strain. During ageing, numbers of cholinergic neurons fall and this is associated with increased transit time; this may be similar to our findings in the AKR. Increased expression of acetylcholine receptors in this strain may represent the body's attempt to maintain homeostasis. The α7nAChR is widely expressed throughout the nervous system, particularly within the gut and CNS, and is also present on many immune cells (Bencherif et al., 2011); although increased expression of the α7nAChR has been associated with an anti inflammatory immune response, there are some more negative consequences.

Stimulation of this receptor by nicotine increased the anti apoptotic Bcl-2 and facilitated cell growth in cancer cells (Kihara et al., 2001; Shin et al., 2008). The link between acetylcholine and increased cell survival in hypoxic conditions associated with cancer further corroborates this (Kim et al., 2008). This is particularly relevant following the advent of e-cigs and the proclamation by Cancer Research that nicotine does not cause cancer (McEwen, 2016). Therefore, upregulation of acetylcholine receptors may precede cancer development, and it would be beneficial if factors upregulating receptor expression could be understood and accounted for. However, the therapeutic potential of upregulation of acetylcholine receptors has been investigated in pathogenesis of CNS disorders Alzheimer's disease and Parkinsons disease, both of which are associated with loss of cholinergic neurons and receptors (Kihara and Shimohama, 2004; Fujita et al., 2006). Interestingly, these conditions are also associated with gastrointestinal disturbances, suggesting that differences within the ENS may reflect pathological differences in systemic neural function. In Parkinson's disease, it is thought that disrupted gut function caused by chronic inflammation associated with disease onset is evident prior to CNS associated symptoms (Houser and Tansey, 2017). Gastrointestinal disturbances have also been associated with several other disorders of the CNS including schizophrenia, depression and anxiety, and autism (de Magistris et al., 2010; Severance et al., 2016; Shah et al., 2014). These conditions are also associated with disturbances in normal commensal bacteria (Houser and Tansey, 2017) and disrupted acetylcholine receptor function (Martin and Freedman, 2007; Deutsch et al., 2010; Philip et al., 2010). Perhaps the gut and assessment of functional parameters represents an as yet unutilised diagnostic tool capable of representing not only potential gut disease, but also systemic changes in nervous function prior to establishment of any inflammatory pathology.

References

Abad, C., Martinez, C., Juarranz, M. G., Arranz, A., Leceta, J., Delgado, M. & Gomariz, R. P. (2003). Therapeutic effects of vasoactive intestinal peptide in the trinitrobenzene sulfonic acid mice model of Crohn's disease. *Gastroenterology,* 124(4)**,** 961-71.

Abalo, R., José Rivera, A., Vera, G. & Isabel Martín, M. (2005). Ileal myenteric plexus in aged guinea-pigs: loss of structure and calretinin-immunoreactive neurones. *Neurogastroenterol Motil,* 17(1)**,** 123-32.

Adams, C. E., Stevens, K. E., Kem, W. R. & Freedman, R. (2000). Inhibition of nitric oxide synthase prevents alpha 7 nicotinic receptor-mediated restoration of inhibitory auditory gating in rat hippocampus. *Brain Res,* 877(2)**,** 235-44.

Ait-Belgnaoui, A., Colom, A., Braniste, V., Ramalho, L., Marrot, A., Cartier, C., Houdeau, E., Theodorou, V. & Tompkins, T. (2014). Probiotic gut effect prevents the chronic psychological stress-induced brain activity abnormality in mice. *Neurogastroenterol Motil,* 26(4)**,** 510-20.

Ames, B. N., Shigenaga, M. K. & Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci U S A,* 90(17)**,** 7915-22.

Andersson, P. O., Bloom, S. R., Edwards, A. V., Järhult, J. & Mellander, S. (1983). Neural vasodilator control in the rectum of the cat and its possible mediation by vasoactive intestinal polypeptide. *J Physiol,* 344**,** 49-67.

Antignano, F., Mullaly, S. C., Burrows, K. & Zaph, C. (2011). Trichuris muris infection: a model of type 2 immunity and inflammation in the gut. *J Vis Exp,* (51).

Artis, D., Potten, C. S., Else, K. J., Finkelman, F. D. & Grencis, R. K. (1999). Trichuris muris: host intestinal epithelial cell hyperproliferation during chronic infection is regulated by interferon-gamma. Exp Parasitol.

Arulmani, U., Maassenvandenbrink, A., Villalón, C. M. & Saxena, P. R. (2004). Calcitonin gene-related peptide and its role in migraine pathophysiology. *Eur J Pharmacol,* 500(1-3)**,** 315-30.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., Batto, J. M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Kurokawa, K., Leclerc, M., Levenez, F., Manichanh, C., Nielsen, H. B., Nielsen, T., Pons, N., Poulain, J., Qin, J., Sicheritz-Ponten, T., Tims, S., Torrents, D., Ugarte, E., Zoetendal, E. G., Wang, J., Guarner, F., Pedersen, O., de Vos, W. M., Brunak, S., Doré, J., Antolín, M., Artiguenave, F., Blottiere, H. M., Almeida, M., Brechot, C., Cara, C., Chervaux, C., Cultrone, A., Delorme, C., Denariaz, G., Dervyn, R., Foerstner, K. U., Friss, C., van de Guchte, M., Guedon, E., Haimet, F., Huber, W., van Hylckama-Vlieg, J., Jamet, A., Juste, C., Kaci, G., Knol, J., Lakhdari, O., Layec, S., Le Roux, K., Maguin, E., Mérieux, A., Melo Minardi, R., M'rini, C., Muller, J., Oozeer, R., Parkhill, J., Renault, P., Rescigno, M., Sanchez, N., Sunagawa, S., Torrejon, A., Turner, K., Vandemeulebrouck, G., Varela, E., Winogradsky, Y., Zeller, G., Weissenbach, J., Ehrlich, S. D., Bork, P. & Consortium, M. (2011). Enterotypes of the human gut microbiome. *Nature,* 473(7346)**,** 174-80.

Assas, B. M., Pennock, J. I. & Miyan, J. A. (2014). Calcitonin gene-related peptide is a key neurotransmitter in the neuro-immune axis. *Front Neurosci,* 8**,** 23.

Aubé, A. C., Cabarrocas, J., Bauer, J., Philippe, D., Aubert, P., Doulay, F., Liblau, R., Galmiche, J. P. & Neunlist, M. (2006). Changes in enteric neurone phenotype and intestinal functions in a transgenic mouse model of enteric glia disruption. *Gut,* 55(5)**,** 630-7.

Azpiroz, F. (2002). Gastrointestinal perception: pathophysiological implications. *Neurogastroenterol Motil,* 14(3)**,** 229-39.

Badizadegan, K., Thomas, A. R., Nagy, N., Ndishabandi, D., Miller, S. A., Alessandrini, A., Belkind-Gerson, J. & Goldstein, A. M. (2014). Presence of intramucosal neuroglial cells in normal and aganglionic human colon. *Am J Physiol Gastrointest Liver Physiol,* 307(10)**,** G1002-12.

Bai, R. Y., Staedtke, V., Aprhys, C. M., Gallia, G. L. & Riggins, G. J. (2011). Antiparasitic mebendazole shows survival benefit in 2 preclinical models of glioblastoma multiforme. *Neuro Oncol,* 13(9)**,** 974-82.

Baker, M. (2015). Reproducibility crisis: Blame it on the antibodies. *Nature,* 521(7552)**,** 274-6.

Balasubramanian, R. & Gan, L. (2014). Development of Retinal Amacrine Cells and Their Dendritic Stratification. *Curr Ophthalmol Rep,* 2(3)**,** 100-106.

Bancroft, A. J., Else, K. J. & Grencis, R. K. (1994). Low-level infection with Trichuris muris significantly affects the polarization of the CD4 response. *Eur J Immunol,* 24(12)**,** 3113-8.

Bancroft, A. J., Else, K. J., Humphreys, N. E. & Grencis, R. K. (2001). The effect of challenge and trickle Trichuris muris infections on the polarisation of the immune response. *Int J Parasitol,* 31(14)**,** 1627-37.

Barajon, I., Serrao, G., Arnaboldi, F., Opizzi, E., Ripamonti, G., Balsari, A. & Rumio, C. (2009). Toll-like receptors 3, 4 and 7 are expressed in the enteric nervous system and dorsal root ganglia. *J Histochem Cytochem*, 57(11), 1013-1023.

Barthó, L., Maggi, C. A., Wilhelm, M. & Patacchini, R. (1994). Tachykinin NK1 and NK2 receptors mediate atropine-resistant ileal circular muscle contractions evoked by capsaicin. *Eur J Pharmacol,* 259(2)**,** 187-93.

Bassotti, G., Villanacci, V., Fisogni, S., Rossi, E., Baronio, P., Clerici, C., Maurer, C. A., Cathomas, G. & Antonelli, E. (2007). Enteric glial cells and their role in gastrointestinal motor abnormalities: introducing the neuro-gliopathies. *World J Gastroenterol,* 13(30)**,** 4035-41.

Bastida, G. & Beltrán, B. (2011). Ulcerative colitis in smokers, non-smokers and ex-smokers. *World J Gastroenterol,* 17(22)**,** 2740-7.

Baydas, G., Nedzvetskii, V. S., Tuzcu, M., Yasar, A. & Kirichenko, S. V. (2003). Increase of glial fibrillary acidic protein and S-100B in hippocampus and cortex of diabetic rats: effects of vitamin E. European Journal of Pharmacology.

Bayliss, W. M. & Starling, E. H. (1900). The movements and the innervation of the large intestine. *J Physiol,* 26(1-2)**,** 107-18.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. & Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A,* 87(4)**,** 1620-4.

Beckman, J. S. & Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol,* 271(5 Pt 1)**,** C1424-37.

Bekar, E., Altunkaynak, B. Z., Balcı, K., Aslan, G., Ayyıldız, M. & Kaplan, S. (2014). Effects of high fat diet induced obesity on peripheral nerve regeneration and levels of GAP 43 and TGF-β in rats. *Biotech Histochem,* 89(6)**,** 446-56.

Belai, A., Boulos, P. B., Robson, T. & Burnstock, G. (1997). Neurochemical coding in the small intestine of patients with Crohn's disease. *Gut,* 40(6)**,** 767-74.

Belai, A., Cooper, S. & Burnstock, G. (1995). Effect of age on NADPH-diaphorase-containing myenteric neurones of rat ileum and proximal colon. *Cell Tissue Res,* 279(2)**,** 379-83.

Bellinger, D. L., Felten, S. Y., Collier, T. J. & Felten, D. L. (1987). Noradrenergic sympathetic innervation of the spleen: IV. Morphometric analysis in adult and aged F344 rats. *J Neurosci Res,* 18(1)**,** 55-63, 126-9.

Bellinger, D. L., Lorton, D., Felten, S. Y. & Felten, D. L. (1992). Innervation of lymphoid organs and implications in development, aging, and autoimmunity. *Int J Immunopharmacol,* 14(3)**,** 329-44.

Bencherif, M., Lippiello, P. M., Lucas, R. & Marrero, M. B. (2011). Alpha7 nicotinic receptors as novel therapeutic targets for inflammation-based diseases. *Cell Mol Life Sci,* 68(6)**,** 931-49.

Bercik, P., Park, A. J., Sinclair, D., Khoshdel, A., Lu, J., Huang, X., Deng, Y., Blennerhassett, P. A., Fahnestock, M., Moine, D., Berger, B., Huizinga, J. D., Kunze, W., McLean, P. G., Bergonzelli, G. E., Collins, S. M. & Verdu, E. F. (2011). The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. *Neurogastroenterol Motil,* 23(12)**,** 1132-9.

Berkenbosch, F., van Oers, J., del Rey, A., Tilders, F. & Besedovsky, H. (1987). Corticotropin-releasing factor-producing neurons in the rat activated by interleukin-1. *Science,* 238(4826)**,** 524-6.

Bernard, C. E., Gibbons, S. J., Gomez-Pinilla, P. J., Lurken, M. S., Schmalz, P. F., Roeder, J. L., Linden, D., Cima, R. R., Dozois, E. J., Larson, D. W., Camilleri, M., Zinsmeister, A. R., Pozo, M. J., Hicks, G. A. & Farrugia, G. (2009). Effect of age on the enteric nervous system of the human colon. *Neurogastroenterol Motil,* 21(7)**,** 746-e46.

Bernstein, I. M., Damron, D., Schonberg, A. L., Sallam, R. M. & Shapiro, R. (2009). The relationship of plasma volume, sympathetic tone, and proinflammatory cytokines in young healthy nonpregnant women. *Reprod Sci,* 16(10)**,** 980-5.

Betolli, M., De Carli, C., Jolin-Dahel, K., Bailey, K., Khan, H. F., Sweeney, B., Krantis, A., Staines, W. A. & Rubin, S. (2008). Colonic dysmotility in postsurgical patients with Hirschsprung's disease. Potential significance of abnormalities in the interstitial cells of Cajal and the enteric nervous system. J Pediatr Surg.

Bettolli, M., De Carli, C., Cornejo-Palma, D., Jolin-Dahel, K., Wang, X. Y., Huizanga, J., Krantis, A., Rubin, S. & Staines, W. A. (2012). Interstitial cell of Cajal loss correlates with the degree of inflammation in the human appendix and reverses after inflammation. J Pediatr Surg.

Bielefeldt, K., Ozaki, N. & Gebhart, G. F. (2002). Experimental ulcers alter voltage-sensitive sodium currents in rat gastric sensory neurons. *Gastroenterology,* 122(2)**,** 394-405.

Binks, S., Vincent, A. & Palace, J. (2016). Myasthenia gravis: a clinical-immunological update. *J Neurol,* 263(4)**,** 826-34.

Bitar, K. N. (2003). Function of gastrointestinal smooth muscle: from signaling to contractile proteins. *Am J Med,* 115 Suppl 3A**,** 15S-23S.

Blackshaw, l. a., brookes, s. j. h., grundy, d. & schemann, m. (2007). Sensory transmission in the gastrointestinal tract. *Neurogastroenterology & Motility,* 19(s1)**,** 1-19.

Blackwell, N. M. & Else, K. J. (2002). A comparison of local and peripheral parasite-specific antibody production in different strains of mice infected with Trichuris muris. *Parasite Immunol,* 24(4)**,** 203-11.

Blatteis, C. M. (2000). The afferent signalling of fever. *J Physiol,* 526 Pt 3**,** 470.

Boesmans, W., Lasrado, R., Vanden Berghe, P. & Pachnis, V. (2015). Heterogeneity and phenotypic plasticity of glial cells in the mammalian enteric nervous system. *Glia,* 63(2)**,** 229-41.

Bogdan, C. (2001). Nitric oxide and the immune response. *Nat Immunol,* 2(10)**,** 907-16.

Bolton, T. B., Prestwich, S. A., Zholos, A. V. & Gordienko, D. V. (1999). Excitation-contraction coupling in gastrointestinal and other smooth muscles. *Annu Rev Physiol,* 61**,** 85-115.

Borovikova, L. V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G. I., Watkins, L. R., Wang, H., Abumrad, N., Eaton, J. W. & Tracey, K. J. (2000). Vagus nerve stimulation

attenuates the systemic inflammatory response to endotoxin. *Nature,* 405(6785)**,** 458-62.

Bouchard, C., Tremblay, A., Després, J. P., Nadeau, A., Lupien, P. J., Thériault, G., Dussault, J., Moorjani, S., Pinault, S. & Fournier, G. (1990). The response to long-term overfeeding in identical twins. *N Engl J Med,* 322(21)**,** 1477-82.

Bove, G. M. (2015). A non-invasive method to evaluate gastrointestinal transit behavior in rat. *J Pharmacol Toxicol Methods,* 74**,** 1-6.

Bradford, K., Shih, W., Videlock, E. J., Presson, A. P., Naliboff, B. D., Mayer, E. A. & Chang, L. (2012). Association between early adverse life events and irritable bowel syndrome. *Clin Gastroenterol Hepatol,* 10(4)**,** 385-90.e1-3.

Britton, E. & McLaughlin, J. T. (2013). Ageing and the gut. *Proc Nutr Soc,* 72(1)**,** 173-7.

Brock, C., Søfteland, E., Gunterberg, V., Frøkjær, J. B., Lelic, D., Brock, B., Dimcevski, G., Gregersen, H., Simrén, M. & Drewes, A. M. (2013). Diabetic autonomic neuropathy affects symptom generation and brain-gut axis. *Diabetes Care,* 36(11)**,** 3698-705.

Brookes, S. (2001). Retrograde tracing of enteric neuronal pathways. *Neurogastroenterol Motil,* 13(1)**,** 1-18.

Brookes, S., Chen, N., Humenick, A., Spencer, N. J. & Costa, M. (2016). Extrinsic Sensory Innervation of the Gut: Structure and Function. *Adv Exp Med Biol,* 891**,** 63-9.

Brown, D. R. & Timmermans, J. P. (2004). Lessons from the porcine enteric nervous system. *Neurogastroenterol Motil,* 16 Suppl 1**,** 50-4.

Browning, K. N., Verheijden, S. & Boeckxstaens, G. E. (2017). The Vagus Nerve in Appetite Regulation, Mood, and Intestinal Inflammation. *Gastroenterology,* 152(4)**,** 730-744.

Brun, P., Giron, M. C., Qesari, M., Porzionato, A., Caputi, V., Zoppellaro, C., Banzato, S., Grillo, A. R., Spagnol, L., De Caro, R., Pizzuti, D., Barbieri, V., Rosato, A., Sturniolo, G. C., Martines, D., Zaninotto, G., Palù, G. & Castagliuolo, I. (2013). Toll-like receptor 2 regulates intestinal inflammation by controlling integrity of the enteric nervous system. *Gastroenterology,* 145(6)**,** 1323-33.

Brunet, L. R., Beall, M., Dunne, D. W. & Pearce, E. J. (1999). Nitric oxide and the Th2 response combine to prevent severe hepatic damage during Schistosoma mansoni infection. *J Immunol,* 163(9)**,** 4976-84.

Brunsson, I., Fahrenkrug, J., Jodal, M., Sjöqvist, A. & Lundgren, O. (1995). Substance P effects on blood flow, fluid transport and vasoactive intestinal polypeptide release in the feline small intestine. *J Physiol,* 483 (Pt 3)**,** 727-34.

Bush, T. G., Savidge, T. C., Freeman, T. C., Cox, H. J., Campbell, E. A., Mucke, L., Johnson, M. H. & Sofroniew, M. V. (1998). Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. *Cell,* 93(2)**,** 189-201.

Butler Tjaden, N. E. & Trainor, P. A. (2013). The developmental etiology and pathogenesis of Hirschsprung disease. *Transl Res,* 162(1)**,** 1-15.

Caccamo, D. V., Herman, M. M., Frankfurter, A., Katsetos, C. D., Collins, V. P. & Rubinstein, L. J. (1989). An immunohistochemical study of neuropeptides and neuronal cytoskeletal proteins in the neuroepithelial component of a spontaneous murine ovarian teratoma. Primitive neuroepithelium displays immunoreactivity for neuropeptides and neuron-associated beta-tubulin isotype. *Am J Pathol,* 135(5)**,** 801-13.

Calegari-Silva, T. C., Vivarini, Á., Miqueline, M., Dos Santos, G. R., Teixeira, K. L., Saliba, A. M., Nunes de Carvalho, S., de Carvalho, L. & Lopes, U. G. (2015). The human parasite Leishmania amazonensis downregulates iNOS expression via NF-κB p50/p50 homodimer: role of the PI3K/Akt pathway. *Open Biol,* 5(9)**,** 150118.

Calignano, A., Whittle, B. J., Di Rosa, M. & Moncada, S. (1992). Involvement of endogenous nitric oxide in the regulation of rat intestinal motility in vivo. *Eur J Pharmacol,* 229(2-3)**,** 273-6.

Camilleri, M. & Fealey, R. D. (1990). Idiopathic autonomic denervation in eight patients presenting with functional gastrointestinal disease. A causal association? *Dig Dis Sci,* 35(5)**,** 609-16.

Campbell, L. K., Thomas, J. R., Lamps, L. W., Smoller, B. R. & Folpe, A. L. (2003). Protein gene product 9.5 (PGP 9.5) is not a specific marker of neural and nerve sheath tumors: an immunohistochemical study of 95 mesenchymal neoplasms. Mod Pathol.

Cannon, W. B. (1915). Bodily Changes in Pain, Hunger, Fear and Rage: An Account of Recent Researches into the Function of Emotional Excitement. D.Appleton and company; New York and London.

Cashman, C. R. & Höke, A. (2015). Mechanisms of distal axonal degeneration in peripheral neuropathies. *Neurosci Lett,* 596**,** 33-50.

Catassi, A., Servent, D., Paleari, L., Cesario, A. & Russo, P. (2008). Multiple roles of nicotine on cell proliferation and inhibition of apoptosis: implications on lung carcinogenesis. *Mutat Res,* 659(3)**,** 221-31.

Celikbilek, A., Akyol, L., Sabah, S., Tanik, N., Adam, M., Celikbilek, M., Korkmaz, M. & Yilmaz, N. (2014a). S100B as a glial cell marker in diabetic peripheral neuropathy. *Neurosci Lett,* 558**,** 53-7.

Celikbilek, A., Celikbilek, M., Sabah, S., Tanık, N., Borekci, E., Dogan, S., Akin, Y., Baldane, S., Deniz, K., Yilmaz, N., Ozbakir, O. & Yucesoy, M. (2014b). The Serum S100B Level as a Biomarker of Enteroglial Activation in Patients with Ulcerative Colitis. *Int J Inflam,* 2014**,** 986525.

Chelimsky, G., Boyle, J. T., Tusing, L. & Chelimsky, T. C. (2001). Autonomic abnormalities in children with functional abdominal pain: coincidence or etiology? *J Pediatr Gastroenterol Nutr,* 33(1)**,** 47-53.

Chen, B. N., Sharrad, D. F., Hibberd, T. J., Zagorodnyuk, V. P., Costa, M. & Brookes, S. J. (2015). Neurochemical characterization of extrinsic nerves in myenteric ganglia of the guinea pig distal colon. *J Comp Neurol,* 523(5)**,** 742-56.

Choi, C. H. & Chang, S. K. (2015). Alteration of gut microbiota and efficacy of probiotics in functional constipation. *J Neurogastroenterol Motil,* 21(1)**,** 4-7.

Chong, S. Z., Wong, K. L., Lin, G., Yang, C. M., Wong, S. C., Angeli, V., Macary, P. A. & Kemeny, D. M. (2011). Human $CD8^+$ T cells drive Th1 responses through the differentiation of TNF/iNOS-producing dendritic cells. *Eur J Immunol,* 41(6)**,** 1639-51.

Chrousos, G. P. (2009). Stress and disorders of the stress system. *Nat Rev Endocrinol,* 5(7)**,** 374-81.

Cirillo, C., Sarnelli, G., Turco, F., Mango, A., Grosso, M., Aprea, G., Masone, S. & Cuomo, R. (2011). Proinflammatory stimuli activates human-derived enteroglial cells and induces autocrine nitric oxide production. *Neurogastroenterol Motil,* 23(9)**,** e372-82.

Clarke, G., Grenham, S., Scully, P., Fitzgerald, P., Moloney, R. D., Shanahan, F., Dinan, T. G. & Cryan, J. F. (2013). The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol Psychiatry,* 18(6)**,** 666-73.

Cliffe, L. J., Potten, C. S., Booth, C. E. & Grencis, R. K. (2007). An increase in epithelial cell apoptosis is associated with chronic intestinal nematode infection. *Infect Immun,* 75(4)**,** 1556-64.

Clément, K., Vaisse, C., Manning, B. S., Basdevant, A., Guy-Grand, B., Ruiz, J., Silver, K. D., Shuldiner, A. R., Froguel, P. & Strosberg, A. D. (1995). Genetic variation in the beta 3-adrenergic receptor and an increased capacity to gain weight in patients with morbid obesity. *N Engl J Med,* 333(6)**,** 352-4.

Collins, J., Borojevic, R., Verdu, E. F., Huizinga, J. D. & Ratcliffe, E. M. (2014). Intestinal microbiota influence the early postnatal development of the enteric nervous system. *Neurogastroenterol Motil,* 26(1)**,** 98-107.

Collins, S. M. & Bercik, P. (2009). The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. *Gastroenterology,* 136(6)**,** 2003-14.

Cooper, P., Walker, A. W., Reyes, J., Chico, M., Salter, S. J., Vaca, M. & Parkhill, J. (2013). Patent Human Infections with the whipworm, Trichuris trichiura, Are Not Associated with Alterations in the Faecal Microbiota. PLoS One.

Cornet, A., Savidge, T. C., Cabarrocas, J., Deng, W. L., Colombel, J. F., Lassmann, H., Desreumaux, P. & Liblau, R. S. (2001). Enterocolitis induced by autoimmune targeting of enteric glial cells: a possible mechanism in Crohn's disease? *Proc Natl Acad Sci U S A,* 98(23)**,** 13306-11.

Coruzzi, P., Castiglioni, P., Parati, G., Brambilla, V., Brambilla, L., Gualerzi, M., Cademartiri, F., Franze, A., De Angelis, G., Di Rienzo, M. & Di Mario, F. (2007). Autonomic cardiovascular regulation in quiescent ulcerative colitis and Crohn's disease. Eur J Clin Invest.

Costa, M., Brookes, S. J. & Hennig, G. W. (2000). Anatomy and physiology of the enteric nervous system. *Gut,* 47 Suppl 4**,** iv15-9; discussion iv26.

Costa, M., Wiklendt, L., Simpson, P., Spencer, N. J., Brookes, S. J. & Dinning, P. G. (2015). Neuromechanical factors involved in the formation and propulsion of fecal pellets in the guinea-pig colon. *Neurogastroenterol Motil,* 27(10)**,** 1466-77.

Cresci, G. A. & Bawden, E. (2015). Gut Microbiome: What We Do and Don't Know. *Nutr Clin Pract,* 30(6)**,** 734-46.

Criado Fornelio, A., Rodriguez Caabeiro, F. & Jimenez Gonzalez, A. (1987). The mode of action of some benzimidazole drugs on Trichinella spiralis. *Parasitology,* 95 (Pt 1)**,** 61-70.

Cserni, T., Paran, S. & Puri, P. (2007). The effect of age on colocalization of acetylcholinesterase and nicotinamide adenine dinucleotide phosphate diaphorase staining in enteric neurons in an experimental model. *J Pediatr Surg,* 42(2)**,** 300-4.

da Silveira, A. B., Freitas, M. A., de Oliveira, E. C., Neto, S. G., Luquetti, A. O., Furness, J. B., Correa-Oliveira, R. & Reis, D. (2009). Glial fibrillary acidic protein and S-100 colocalization in the enteroglial cells in dilated and nondilated portions of colon from chagasic patients. *Hum Pathol,* 40(2)**,** 244-51.

Dang, N., Meng, X. & Song, H. (2016). Nicotinic acetylcholine receptors and cancer. *Biomed Rep,* 4(5)**,** 515-518.

Dawson, V. L., Kizushi, V. M., Huang, P. L., Snyder, S. H. & Dawson, T. M. (1996). Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. *J Neurosci,* 16(8)**,** 2479-87.

De Biasi, M. (2002). Nicotinic mechanisms in the autonomic control of organ systems. *J Neurobiol,* 53(4)**,** 568-79.

de Jonge, W. J. & Ulloa, L. (2007). The alpha7 nicotinic acetylcholine receptor as a pharmacological target for inflammation. *Br J Pharmacol,* 151(7)**,** 915-29.

de Magistris, L., Familiari, V., Pascotto, A., Sapone, A., Frolli, A., Iardino, P., Carteni, M., De Rosa, M., Francavilla, R., Riegler, G., Militerni, R. & Bravaccio, C. (2010). Alterations of the intestinal barrier in patients with autism spectrum disorders and in their first-degree relatives. *J Pediatr Gastroenterol Nutr,* 51(4)**,** 418-24.

Deutsch, S. I., Urbano, M. R., Neumann, S. A., Burket, J. A. & Katz, E. (2010). Cholinergic abnormalities in autism: is there a rationale for selective nicotinic agonist interventions? *Clin Neuropharmacol,* 33(3)**,** 114-20.

Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., Hibberd, M. L., Forssberg, H. & Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci U S A,* 108(7)**,** 3047-52.

Ding, W., Stohl, L. L., Wagner, J. A. & Granstein, R. D. (2008). Calcitonin gene-related peptide biases Langerhans cells toward Th2-type immunity. *J Immunol,* 181(9)**,** 6020-6.

Ding, W., Stohl, L. L., Xu, L., Zhou, X. K., Manni, M., Wagner, J. A. & Granstein, R. D. (2016). Calcitonin Gene-Related Peptide-Exposed Endothelial Cells Bias Antigen Presentation to CD4+ T Cells toward a Th17 Response. *J Immunol,* 196(5)**,** 2181-94.

Dixon, H., Little, M. C. & Else, K. J. (2010). Characterisation of the protective immune response following subcutaneous vaccination of susceptible mice against Trichuris muris. *Int J Parasitol,* 40(6)**,** 683-93.

Drugbank (2017). Mebendazole. Available at :https://www.drugbank.ca/drugs/DB00643:, accessed (21.01.2017)

Dutcher, S. K. (2001). The tubulin fraternity: alpha to eta. Current Opinion in Cell Biology.

Egleton, R. D., Brown, K. C. & Dasgupta, P. (2008). Nicotinic acetylcholine receptors in cancer: multiple roles in proliferation and inhibition of apoptosis. *Trends Pharmacol Sci,* 29(3)**,** 151-8.

Ehlert, F. J., Sawyer, G. W. & Esqueda, E. E. (1999). Contractile role of M2 and M3 muscarinic receptors in gastrointestinal smooth muscle. *Life Sci,* 64(6-7)**,** 387-94.

Eisenman, S. T., Gibbons, S. J., Singh, R. D., Bernard, C. E., Wu, J., Sarr, M. G., Kendrick, M. L., Larson, D. W., Dozois, E. J., Shen, K. R. & Farrugia, G. (2013). Distribution of TMEM100 in the mouse and human gastrointestinal tract--a novel marker of enteric nerves. *Neuroscience,* 240**,** 117-28.

Ekblad, E. & Bauer, A. J. (2004). Role of vasoactive intestinal peptide and inflammatory mediators in enteric neuronal plasticity. *Neurogastroenterol Motil,* 16 Suppl 1**,** 123-8.

Elenkov, I. J., Wilder, R. L., Chrousos, G. P. & Vizi, E. S. (2000). The sympathetic nerve--an integrative interface between two supersystems: the brain and the immune system. *Pharmacol Rev,* 52(4)**,** 595-638.

Engel, M. A., Becker, C., Reeh, P. W. & Neurath, M. F. (2011). Role of sensory neurons in colitis: increasing evidence for a neuroimmune link in the gut. *Inflamm Bowel Dis,* 17(4)**,** 1030-3.

Erbas, O., Taşkıran, D., Oltulu, F., Yavaşoğlu, A., Bora, S., Bilge, O., Çınar, B. P. & Peker, G. (2017). Oxytocin provides protection against diabetic polyneuropathy in rats. *Neurol Res,* 39(1)**,** 45-53.

Esposito, G., Capoccia, E., Turco, F., Palumbo, I., Lu, J., Steardo, A., Cuomo, R., Sarnelli, G. & Steardo, L. (2014). Palmitoylethanolamide improves colon inflammation through an enteric glia/toll like receptor 4-dependent PPAR-α activation. *Gut,* 63(8)**,** 1300-12.

Fabel, K., Tam, B., Kaufer, D., Baiker, A., Simmons, N., Kuo, C. J. & Palmer, T. D. (2003). VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *Eur J Neurosci,* 18(10)**,** 2803-12.

Fambrough, D. M., Drachman, D. B. & Satyamurti, S. (1973). Neuromuscular Junction in myasthenia gravis: decreased acetylcholine receptors. Science.

Federoff, H. J., Lawrence, D. & Brownlee, M. (1993). Nonenzymatic glycosylation of laminin and the laminin peptide CIKVAVS inhibits neurite outgrowth. *Diabetes,* 42(4)**,** 509-13.

Felten, D. L., Felten, S. Y., Bellinger, D. L., Carlson, S. L., Ackerman, K. D., Madden, K. S., Olschowki, J. A. & Livnat, S. (1987). Noradrenergic sympathetic neural interactions with the immune system: structure and function. *Immunol Rev,* 100**,** 225-60.

Felten, S. Y., Felten, D. L., Bellinger, D. L., Carlson, S. L., Ackerman, K. D., Madden, K. S., Olschowka, J. A. & Livnat, S. (1988). Noradrenergic sympathetic innervation of lymphoid organs. *Prog Allergy,* 43**,** 14-36.

Figini, M., Emanueli, C., Grady, E. F., Kirkwood, K., Payan, D. G., Ansel, J., Gerard, C., Geppetti, P. & Bunnett, N. (1997). Substance P and bradykinin stimulate plasma extravasation in the mouse gastrointestinal tract and pancreas. *Am J Physiol,* 272(4 Pt 1)**,** G785-93.

Finegold, S. M., Dowd, S. E., Gontcharova, V., Liu, C., Henley, K. E., Wolcott, R. D., Youn, E., Summanen, P. H., Granpeesheh, D., Dixon, D., Liu, M., Molitoris, D. R. & Green, J. A. (2010). Pyrosequencing study of fecal microflora of autistic and control children. *Anaerobe,* 16(4)**,** 444-53.

Fornai, M., Pellegrini, C., Antonioli, L., Segnani, C., Ippolito, C., Barocelli, E., Ballabeni, V., Vegezzi, G., Al Harraq, Z., Blandini, F., Levandis, G., Cerri, S., Blandizzi, C., Bernardini, N. & Colucci, R. (2016). Enteric Dysfunctions in Experimental Parkinson's Disease: Alterations of Excitatory Cholinergic Neurotransmission Regulating Colonic Motility in Rats. *J Pharmacol Exp Ther,* 356(2)**,** 434-44.

Fujii, T., Tajima, S., Yamada, S., Watanabe, Y., Sato, K. Z., Matsui, M., Misawa, H., Kasahara, T. & Kawashima, K. (1999). Constitutive expression of mRNA for the same choline acetyltransferase as that in the nervous system, an acetylcholine-synthesizing enzyme, in human leukemic T-cell lines. *Neurosci Lett,* 259(2)**,** 71-4.

Fujita, M., Ichise, M., Zoghbi, S. S., Liow, J. S., Ghose, S., Vines, D. C., Sangare, J., Lu, J. Q., Cropley, V. L., Iida, H., Kim, K. M., Cohen, R. M., Bara-Jimenez, W., Ravina, B. & Innis, R. B. (2006). Widespread decrease of nicotinic acetylcholine receptors in Parkinson's disease. *Ann Neurol,* 59(1)**,** 174-7.

Gabbay, K. H., Merola, L. O. & Field, R. A. (1966). Sorbitol pathway: presence in nerve and cord with substrate accumulation in diabetes. *Science,* 151(3707)**,** 209-10.

Gad, M., Pedersen, A. E., Kristensen, N. N., Fernandez, C. e. F. & Claesson, M. H. (2009). Blockage of the neurokinin 1 receptor and capsaicin-induced ablation of the enteric afferent nerves protect SCID mice against T-cell-induced chronic colitis. *Inflamm Bowel Dis,* 15(8)**,** 1174-82.

Galitovskiy, V., Qian, J., Chernyavsky, A. I., Marchenko, S., Gindi, V., Edwards, R. A. & Grando, S. A. (2011). Cytokine-induced alterations of α7 nicotinic receptor in colonic CD4 T cells mediate dichotomous response to nicotine in murine models of Th1/Th17- versus Th2-mediated colitis. *J Immunol,* 187(5)**,** 2677-87.

Galland, L. (2014). The gut microbiome and the brain. *J Med Food,* 17(12)**,** 1261-72.

Galvis, D. A., Nakazato, Y., Wells, T. R. & Landing, B. H. (1987). Demonstration of myenteric plexus abnormalities in genetic diseases by a microdissection technique: preliminary studies. *Am J Med Genet Suppl,* 3**,** 329-42.

Gautron, L., Rutkowski, J. M., Burton, M. D., Wei, W., Wan, Y. & Elmquist, J. K. (2013). Neuronal and nonneuronal cholinergic structures in the mouse gastrointestinal tract and spleen. *J Comp Neurol,* 521(16)**,** 3741-67.

Ge, X., Ding, C., Zhao, W., Xu, L., Tian, H., Gong, J., Zhu, M., Li, J. & Li, N. (2017a). Antibiotics-induced depletion of mice microbiota induces changes in host serotonin biosynthesis and intestinal motility. *J Transl Med,* 15(1)**,** 13.

Ge, X., Zhao, W., Ding, C., Tian, H., Xu, L., Wang, H., Ni, L., Jiang, J., Gong, J., Zhu, W., Zhu, M. & Li, N. (2017b). Potential role of fecal microbiota from patients with slow transit constipation in the regulation of gastrointestinal motility. *Sci Rep,* 7(1)**,** 441.

Gebhart, G. F. (2000). Visceral Pain- Peripheral Sensitisation. Gut.

Gebreselassie, N. G., Moorhead, A. R., Fabre, V., Gagliardo, L. F., Lee, N. A., Lee, J. J. & Appleton, J. A. (2012). Eosinophils preserve parasitic nematode larvae by regulating local immunity. *J Immunol,* 188(1)**,** 417-25.

Gelberg, H. B. (2014). Comparative anatomy, physiology, and mechanisms of disease production of the esophagus, stomach, and small intestine. *Toxicol Pathol,* 42(1)**,** 54-66.

Geracioti, T. D., Carpenter, L. L., Owens, M. J., Baker, D. G., Ekhator, N. N., Horn, P. S., Strawn, J. R., Sanacora, G., Kinkead, B., Price, L. H. & Nemeroff, C. B. (2006). Elevated cerebrospinal fluid substance p concentrations in posttraumatic stress disorder and major depression. *Am J Psychiatry,* 163(4)**,** 637-43.

Gibbons, D. L., Byers, L. A. & Kurie, J. M. (2014). Smoking, p53 mutation, and lung cancer. *Mol Cancer Res,* 12(1)**,** 3-13.

Goldwater, D. S., Pavlides, C., Hunter, R. G., Bloss, E. B., Hof, P. R., McEwen, B. S. & Morrison, J. H. (2009). Structural and functional alterations to rat medial prefrontal cortex following chronic restraint stress and recovery. *Neuroscience,* 164(2)**,** 798-808.

Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J. T., Spector, T. D., Clark, A. G. & Ley, R. E. (2014). Human genetics shape the gut microbiome. *Cell,* 159(4)**,** 789-99.

Graff, M., Ngwa, J. S., Workalemahu, T., Homuth, G., Schipf, S., Teumer, A., Völzke, H., Wallaschofski, H., Abecasis, G. R., Edward, L., Francesco, C., Sanna, S., Scheet, P., Schlessinger, D., Sidore, C., Xiao, X., Wang, Z., Chanock, S. J., Jacobs, K. B., Hayes, R. B., Hu, F., Van Dam, R. M., Crout, R. J., Marazita, M. L., Shaffer, J. R., Atwood, L. D., Fox, C. S., Heard-Costa, N. L., White, C., Choh, A. C., Czerwinski, S. A., Demerath, E. W., Dyer, T. D., Towne, B., Amin, N., Oostra, B. A., Van Duijn, C. M., Zillikens, M. C., Esko, T., Nelis, M., Nikopensius, T., Metspalu, A., Strachan, D. P., Monda, K., Qi, L., North, K. E., Cupples, L. A., Gordon-Larsen, P., Berndt, S. I. & Consortium, G. (2013). Genome-wide analysis of BMI in adolescents and young adults reveals additional insight into the effects of genetic loci over the life course. *Hum Mol Genet,* 22(17)**,** 3597-607.

Grasa, L., Abecia, L., Forcén, R., Castro, M., de Jalón, J. A., Latorre, E., Alcalde, A. I. & Murillo, M. D. (2015). Antibiotic-Induced Depletion of Murine Microbiota Induces Mild Inflammation and Changes in Toll-Like Receptor Patterns and Intestinal Motility. *Microb Ecol,* 70(3)**,** 835-48.

Gray, H. (1918). Anatomy of the Human Body. Philadelphia: Lea and Febiger.

Gregg, E. W., Sorlie, P., Paulose-Ram, R., Gu, Q., Eberhardt, M. S., Wolz, M., Burt, V., Curtin, L., Engelgau, M., Geiss, L. & survey, -. n. h. a. n. e. (2004). Prevalence of lower-extremity disease in the US adult population >=40 years of age with and without diabetes: 1999-2000 national health and nutrition examination survey. *Diabetes Care,* 27(7)**,** 1591-7.

Gregory, J. A., Jolivalt, C. G., Goor, J., Mizisin, A. P. & Calcutt, N. A. (2012). Hypertension-induced peripheral neuropathy and the combined effects of hypertension and diabetes on nerve structure and function in rats. *Acta Neuropathol,* 124(4)**,** 561-73.

Haddock, R. E. & Hill, C. E. (2011). Sympathetic overdrive in obesity involves purinergic hyperactivity in the resistance vasculature. *J Physiol,* 589(Pt 13)**,** 3289-307.

Han, B. (2013). Correlation between gastrointestinal hormones and anxiety-depressive states in irritable bowel syndrome. *Exp Ther Med,* 6(3)**,** 715-720.

Hanani, M., Fellig, Y., Udassin, R. & Freund, H. R. (2004). Age-related changes in the morphology of the myenteric plexus of the human colon. *Auton Neurosci,* 113(1-2)**,** 71-8.

Hansen, M. K., O'Connor, K. A., Goehler, L. E., Watkins, L. R. & Maier, S. F. (2001). The contribution of the vagus nerve in interleukin-1beta-induced fever is dependent on dose. *Am J Physiol Regul Integr Comp Physiol,* 280(4)**,** R929-34.

Harnett, K. M. & Biancani, P. (2003). Calcium-dependent and calcium-independent contractions in smooth muscles. *Am J Med,* 115 Suppl 3A**,** 24S-30S.

Harrington, A. M., Lee, M., Ong, S. Y., Yong, E., Farmer, P., Peck, C. J., Chow, C. W., Hutson, J. M. & Southwell, B. R. (2010). Immunoreactivity for high-affinity choline transporter colocalises with VAChT in human enteric nervous system. *Cell Tissue Res,* 341(1)**,** 33-48.

Haslam, D. W. & James, W. P. (2005). Obesity. *Lancet,* 366(9492)**,** 1197-209.

Hayes, K. S., Bancroft, A. J. & Grencis, R. K. (2007). The role of TNF-alpha in Trichuris muris infection II: global enhancement of ongoing Th1 or Th2 responses. *Parasite Immunol,* 29(11)**,** 583-94.

He, D., Manzoni, A., Florentin, D., Fisher, W., Ding, Y., Lee, M. & Ayala, G. (2016). Biologic effect of neurogenesis in pancreatic cancer. *Hum Pathol,* 52**,** 182-9.

Hellström, P. M., Söder, O. & Theodorsson, E. (1991). Occurrence, release, and effects of multiple tachykinins in cat colonic tissues and nerves. *Gastroenterology,* 100(2)**,** 431-40.

Hennig, G. W., Costa, M., Chen, B. N. & Brookes, S. J. (1999). Quantitative analysis of peristalsis in the guinea-pig small intestine using spatio-temporal maps. *J Physiol,* 517 (Pt 2)**,** 575-90.

Hens, J., Vanderwinden, J. M., De Laet, M. H., Scheuermann, D. W. & Timmermans, J. P. (2001). Morphological and neurochemical identification of enteric neurones with mucosal projections in the human small intestine. *J Neurochem,* 76(2)**,** 464-71.

Hesse, M., Cheever, A. W., Jankovic, D. & Wynn, T. A. (2000). NOS-2 mediates the protective anti-inflammatory and antifibrotic effects of the Th1-inducing adjuvant, IL-12, in a Th2 model of granulomatous disease. *Am J Pathol,* 157(3)**,** 945-55.

Hirst, G. D., Holman, M. E. & Spence, I. (1974). Two types of neurones in the myenteric plexus of duodenum in the guinea-pig. *J Physiol,* 236(2)**,** 303-26.

Holder, J. L., Butte, N. F. & Zinn, A. R. (2000). Profound obesity associated with a balanced translocation that disrupts the SIM1 gene. *Hum Mol Genet,* 9(1)**,** 101-8.

Holzgrabe, U., Kapková, P., Alptüzün, V., Scheiber, J. & Kugelmann, E. (2007). Targeting acetylcholinesterase to treat neurodegeneration. *Expert Opin Ther Targets,* 11(2)**,** 161-79.

Hosseini, A. & Abdollahi, M. (2013). Diabetic Neuropathy and Oxidative Stress: Therapeutic Perspectives. Oxidative Medicine and Cellular Longevity.

Hotamisligil, G. S. (1999). The role of TNFalpha and TNF receptors in obesity and insulin resistance. *J Intern Med,* 245(6)**,** 621-5.

Houlden, A., Hayes, K. S., Bancroft, A. J., Worthington, J. J., Wang, P., Grencis, R. K. & Roberts, I. S. (2015a). Chronic Trichuris muris Infection in C57BL/6 Mice Causes Significant Changes in Host Microbiota and Metabolome: Effects Reversed by Pathogen Clearance. PLoS One.

Houlden, A., Hayes, K. S., Bancroft, A. J., Worthington, J. J., Wang, P., Grencis, R. K. & Roberts, I. S. (2015b). Chronic Trichuris muris Infection in C57BL/6 Mice Causes Significant Changes in Host Microbiota and Metabolome: Effects Reversed by Pathogen Clearance. *PLoS One,* 10(5)**,** e0125945.

Houser, M. C. & Tansey, M. G. (2017). The gut-brain axis: is intestinal inflammation a silent driver of Parkinson's disease pathogenesis? *NPJ Parkinsons Dis,* 3**,** 3.

Hu, H. Z., Gao, N., Lin, Z., Gao, C., Liu, S., Ren, J., Xia, Y. & Wood, J. D. (2002). Chemical coding and electrophysiology of enteric neurons expressing neurofilament 145 in guinea pig gastrointestinal tract. *J Comp Neurol,* 442(3)**,** 189-203.

Hubel, K. A. (1985). Intestinal nerves and ion transport: stimuli, reflexes, and responses. *Am J Physiol,* 248(3 Pt 1)**,** G261-71.

Hurst, R. J. & Else, K. J. (2013). Trichuris muris research revisited: a journey through time. Parasitology.
Ichihara, S. & Yamada, Y. (2008). Genetic factors for human obesity. *Cell Mol Life Sci,* 65(7-8)**,** 1086-98.
Ischiropoulos, H. & al-Mehdi, A. B. (1995). Peroxynitrite-mediated oxidative protein modifications. *FEBS Lett,* 364(3)**,** 279-82.
Itoh, J., de La Motte, C., Strong, S. A., Levine, A. D. & Fiocchi, C. (2001). Decreased Bax expression by mucosal T cells favours resistance to apoptosis in Crohn's disease. *Gut,* 49(1)**,** 35-41.
Iwamoto, M., Koji, T., Makiyama, K., Kobayashi, N. & Nakane, P. K. (1996). Apoptosis of crypt epithelial cells in ulcerative colitis. *J Pathol,* 180(2)**,** 152-9.
Iwashita, T., Kruger, G. M., Pardal, R., Kiel, M. J. & Morrison, S. J. (2003). Hirschsprung disease is linked to defects in neural crest stem cell function. *Science,* 301(5635)**,** 972-6.
Jackson, R. S., Creemers, J. W., Farooqi, I. S., Raffin-Sanson, M. L., Varro, A., Dockray, G. J., Holst, J. J., Brubaker, P. L., Corvol, P., Polonsky, K. S., Ostrega, D., Becker, K. L., Bertagna, X., Hutton, J. C., White, A., Dattani, M. T., Hussain, K., Middleton, S. J., Nicole, T. M., Milla, P. J., Lindley, K. J. & O'Rahilly, S. (2003). Small-intestinal dysfunction accompanies the complex endocrinopathy of human proprotein convertase 1 deficiency. *J Clin Invest,* 112(10)**,** 1550-60.
Janeway, C. A., Travers, P., Walport, M. & *al., e.* (2001). Immunobiology: the immune system in health and disease. 5th Ed.: Garland Science, New York.
Ji, S., Park, H., Lee, D., Song, Y. K., Choi, J. P. & Lee, S. I. (2005). Post-infectious irritable bowel syndrome in patients with Shigella infection. *J Gastroenterol Hepatol,* 20(3)**,** 381-6.
Jiminez, J. A., Uwiera, T. C., Douglas Inglis, G. & Uwiera, R. R. (2015). Animal models to study acute and chronic intestinal inflammation in mammals. *Gut Pathog,* 7**,** 29.
Jin, Y., Ren, X., Li, G., Li, Y., Zhang, L., Wang, H., Qian, W. & Hou, X. (2017). Rifaximin exerts beneficial effects in PI-IBS mouse model beyond gut microbiota. *J Gastroenterol Hepatol*.
Jones, R. C., Xu, L. & Gebhart, G. F. (2005). The mechanosensitivity of mouse colon afferent fibers and their sensitization by inflammatory mediators require transient receptor potential vanilloid 1 and acid-sensing ion channel 3. *J Neurosci,* 25(47)**,** 10981-9.
Joseph, N. M., He, S., Quintana, E., Kim, Y. G., Núñez, G. & Morrison, S. J. (2011). Enteric glia are multipotent in culture but primarily form glia in the adult rodent gut. *J Clin Invest,* 121(9)**,** 3398-411.
Jurgens, H. A., Amancherla, K. & Johnson, R. W. (2012). Influenza infection induces neuroinflammation, alters hippocampal neuron morphology, and impairs cognition in adult mice. *J Neurosci,* 32(12)**,** 3958-68.
Jänig, W. (1988). Integration of gut function by sympathetic reflexes. *Baillieres Clin Gastroenterol,* 2(1)**,** 45-62.
Kapur, R. P. (2013). Counting neurons is not as easy as 'one-two, three'. *Neurogastroenterol Motil,* 25(7)**,** 549-53.
Keast, J. R., Furness, J. B. & Costa, M. (1985). Different substance P receptors are found on mucosal epithelial cells and submucous neurons of the guinea-pig small intestine. *Naunyn Schmiedebergs Arch Pharmacol,* 329(4)**,** 382-7.
Keiser, J. & Utzinger, J. (2008). Efficacy of current drugs against soil-transmitted helminth infections: systematic review and meta-analysis. *JAMA,* 299(16)**,** 1937-48.
Kelley, K. W. & McCusker, R. H. (2014). Getting nervous about immunity. *Semin Immunol,* 26(5)**,** 389-93.

Khalil, H. A., Kobayashi, M., Rana, P., Wagner, J. P., Scott, A., Yoo, J. & Dunn, J. C. (2016). Mouse model of endoscopically ablated enteric nervous system. *J Surg Res,* 200(1)**,** 117-21.

Khan, S. A., Sattar, M. Z., Abdullah, N. A., Rathore, H. A., Abdulla, M. H., Ahmad, A. & Johns, E. J. (2015). Obesity depresses baroreflex control of renal sympathetic nerve activity and heart rate in Sprague Dawley rats: role of the renal innervation. *Acta Physiol (Oxf),* 214(3)**,** 390-401.

Khan, S. A., Sattar, M. Z. A., Abdullah, N. A., Rathore, H. A., Ahmad, A., Abdulla, M. H. & Johns, E. J. (2017). Improvement in baroreflex control of renal sympathetic nerve activity in obese Sprague Dawley rats following immunosuppression. *Acta Physiol (Oxf)*.

Kihara, T. & Shimohama, S. (2004). Alzheimer's disease and acetylcholine receptors. *Acta Neurobiol Exp (Wars),* 64(1)**,** 99-105.

Kihara, T., Shimohama, S., Sawada, H., Honda, K., Nakamizo, T., Shibasaki, H., Kume, T. & Akaike, A. (2001). alpha 7 nicotinic receptor transduces signals to phosphatidylinositol 3-kinase to block A beta-amyloid-induced neurotoxicity. *J Biol Chem,* 276(17)**,** 13541-6.

Kim, M. H., Kim, M. O., Heo, J. S., Kim, J. S. & Han, H. J. (2008). Acetylcholine inhibits long-term hypoxia-induced apoptosis by suppressing the oxidative stress-mediated MAPKs activation as well as regulation of Bcl-2, c-IAPs, and caspase-3 in mouse embryonic stem cells. *Apoptosis,* 13(2)**,** 295-304.

King, M. C., Marks, J. H., Mandell, J. B. & Group, N. Y. B. C. S. (2003). Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. *Science,* 302(5645)**,** 643-6.

Klementowicz, J. E., Travis, M. A. & Grencis, R. K. (2012). Trichuris muris: a model of gastrointestinal parasite infection. *Semin Immunopathol,* 34(6)**,** 815-28.

Knowles, R. G. & Moncada, S. (1994). Nitric oxide synthases in mammals. *Biochem J,* 298 (Pt 2)**,** 249-58.

Kohm, A. P. & Sanders, V. M. (2000). Norepinephrine: a messenger from the brain to the immune system. *Immunol Today,* 21(11)**,** 539-42.

Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H. & Beckman, J. S. (1992). Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem Res Toxicol,* 5(6)**,** 834-42.

Kosterlitz, H. W. & Lees, G. M. (1964). Pharmacological analysis of intrinsic intestinal reflexes. *Pharmacol Rev,* 16**,** 301-39.

Kosterlitz, H. W. & Robinson, J. A. (1959). Reflex contractions of the longitudinal muscle coat of the isolated guinea-pig ileum. *J Physiol,* 146(2)**,** 369-79.

Kruger, G. M., Mosher, J. T., Bixby, S., Joseph, N., Iwashita, T. & Morrison, S. J. (2002). Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. *Neuron,* 35(4)**,** 657-69.

Kuhn, H. G., Dickinson-Anson, H. & Gage, F. H. (1996). Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci,* 16(6)**,** 2027-33.

Kulkarni, S., Micci, M. A., Leser, J., Shin, C., Tang, S. C., Fu, Y. Y., Liu, L., Li, Q., Saha, M., Li, C., Enikolopov, G., Becker, L., Rakhilin, N., Anderson, M., Shen, X., Dong, X., Butte, M. J., Song, H., Southard-Smith, E. M., Kapur, R. P., Bogunovic, M. & Pasricha, P. J. (2017). Adult enteric nervous system in health is maintained by a dynamic balance between neuronal apoptosis and neurogenesis. *Proc Natl Acad Sci U S A,* 114(18)**,** E3709-E3718.

Kunze, W. A., Clerc, N., Bertrand, P. P. & Furness, J. B. (1999). Contractile activity in intestinal muscle evokes action potential discharge in guinea-pig myenteric neurons. *J Physiol,* 517 (Pt 2)**,** 547-61.

Kuwahara, A. & Cooke, H. J. (1990). Tachykinin-induced anion secretion in guinea pig distal colon: role of neural and inflammatory mediators. *J Pharmacol Exp Ther,* 252(1)**,** 1-7.

Kyösola, K., Penttilä, O. & Salaspuro, M. (1977). Rectal mucosal adrenergic innervation and enterochromaffin cells in ulcerative colitis and irritable colon. *Scand J Gastroenterol,* 12(3)**,** 363-7.

Langeron, O., Birenbaum, A. & Amour, J. (2009). Airway Management in Trauma. Minerva Anestesiol.

Lariviere, W. R., Wilson, S. G., Laughlin, T. M., Kokayeff, A., West, E. E., Adhikari, S. M., Wan, Y. & Mogil, J. S. (2002). Heritability of nociception. III. Genetic relationships among commonly used assays of nociception and hypersensitivity. *Pain,* 97(1-2)**,** 75-86.

Lawrance, I. C., Murray, K., Batman, B., Gearry, R. B., Grafton, R., Krishnaprasad, K., Andrews, J. M., Prosser, R., Bampton, P. A., Cooke, S. E., Mahy, G., Radford-Smith, G., Croft, A. & Hanigan, K. (2013). Crohn's disease and smoking: is it ever too late to quit? *J Crohns Colitis,* 7(12)**,** e665-71.

Leandro-García, L. J., Leskelä, S., Landa, I., Montero-Conde, C., López-Jiménez, E., Letón, R., Cascón, A., Robledo, M. & Rodríguez-Antona, C. (2010). Tumoral and tissue-specific expression of the major human beta-tubulin isotypes. *Cytoskeleton (Hoboken),* 67(4)**,** 214-23.

Ledochowski, M., Widner, B., Bair, H., Probst, T. & Fuchs, D. (2000). Fructose- and sorbitol-reduced diet improves mood and gastrointestinal disturbances in fructose malabsorbers. *Scand J Gastroenterol,* 35(10)**,** 1048-52.

Lee, Y., Alam, M. M., Noh, M., Rönnegård, L. & Skarin, A. (2016). Spatial modeling of data with excessive zeros applied to reindeer pellet-group counts. *Ecol Evol,* 6(19)**,** 7047-7056.

Leiper, J. B. (1998). Intestinal water absorption--implications for the formulation of rehydration solutions. *Int J Sports Med,* 19 Suppl 2**,** S129-32.

Lennon, G., Balfe, A., Earley, H., Devane, L. A., Lavelle, A., Winter, D. C., Coffey, J. C. & O'Connell, P. R. (2014). Influences of the colonic microbiome on the mucous gel layer in ulcerative colitis. *Gut Microbes,* 5(3)**,** 277-85.

LePard, K. J. & Galligan, J. J. (1999). Analysis of fast synaptic pathways in myenteric plexus of guinea pig ileum. *Am J Physiol,* 276(2 Pt 1)**,** G529-38.

Leung, F. W. (2014). Capsaicin as an anti-obesity drug. *Prog Drug Res,* 68**,** 171-9.

Levecke, B., Montresor, A., Albonico, M., Ame, S. M., Behnke, J. M., Bethony, J. M., … & Vercruysse, J. (2014). Assessment of Anthelmintic Efficacy of Mebendazole in School Children in Six Countries Where Soil-Transmitted Helminths Are Endemic. PLoS Neglected Tropical Diseases.

Levitan, R., Fordtran, J. S., Burrows, B. A. & Ingelfinger, F. J. (1962). Water and salt absorption in the human colon. *J Clin Invest,* 41**,** 1754-9.

Ley, R. E. (2010). Obesity and the human microbiome. *Curr Opin Gastroenterol,* 26(1)**,** 5-11.

Li, F. J., Zou, Y. Y., Cui, Y., Yin, Y., Guo, G. & Lu, F. G. (2013). Calcitonin gene-related peptide is a promising marker in ulcerative colitis. *Dig Dis Sci,* 58(3)**,** 686-93.

Li, R. W., Wu, S., Li, W., Navarro, K., Couch, R. D., Hill, D. & Urban, J. F. (2012). Alterations in the porcine colon microbiota induced by the gastrointestinal nematode Trichuris suis. *Infect Immun,* 80(6)**,** 2150-7.

Lieb, D. C., Parson, H. K., Mamikunian, G. & Vinik, A. I. (2012). Cardiac autonomic imbalance in newly diagnosed and established diabetes is associated with markers of adipose tissue inflammation. *Exp Diabetes Res,* 2012**,** 878760.

Liu, C. Y., Mueller, M. H., Grundy, D. & Kreis, M. E. (2007). Vagal modulation of intestinal afferent sensitivity to systemic LPS in the rat. *Am J Physiol Gastrointest Liver Physiol,* 292(5)**,** G1213-20.

Liu, J. Z., van Sommeren, S., Huang, H., Ng, S. C., Alberts, R., Takahashi, A., Ripke, S., Lee, J. C., Jostins, L., Shah, T., Abedian, S., Cheon, J. H., Cho, J., Daryani, N. E., Franke, L., Fuyuno, Y., Hart, A., Juyal, R. C., Juyal, G., Kim, W. H., Morris, A. P., Poustchi, H., Newman, W. G., Midha, V., Orchard, T. R., Vahedi, H., Sood, A., Sung, J. J., Malekzadeh, R., Westra, H. J., Yamazaki, K., Yang, S. K., Barrett, J. C., Franke, A., Alizadeh, B. Z., Parkes, M., B K, T., Daly, M. J., Kubo, M., Anderson, C. A., Weersma, R. K., Consortium, I. M. S. G. & Consortium, I. I. G. (2015). Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet,* 47(9)**,** 979-86.

Liu, W., Yue, W. & Wu, R. (2010). Effects of diabetes on expression of glial fibrillary acidic protein and neurotrophins in rat colon. *Auton Neurosci,* 154(1-2)**,** 79-83.

Lo, C. W. & Walker, W. A. (1989). Changes in the gastrointestinal tract during enteral or parenteral feeding. *Nutr Rev,* 47(7)**,** 193-8.

Locatelli, C., Pedrosa, R. C., De Bem, A. F., Creczynski-Pasa, T. B., Cordova, C. A. & Wilhelm-Filho, D. (2004). A comparative study of albendazole and mebendazole-induced, time-dependent oxidative stress. *Redox Rep,* 9(2)**,** 89-95.

Lomax, A. E. & Furness, J. B. (2000). Neurochemical classification of enteric neurons in the guinea-pig distal colon. *Cell and Tissue Research,* 302(1)**,** 59-72.

Lomax, A. E., Sharkey, K. A. & Furness, J. B. (2010). The participation of the sympathetic innervation of the gastrointestinal tract in disease states. *Neurogastroenterol Motil,* 22(1)**,** 7-18.

Longo, G., Osikowicz, M. & Ribeiro-da-Silva, A. (2013). Sympathetic fiber sprouting in inflamed joints and adjacent skin contributes to pain-related behavior in arthritis. *J Neurosci,* 33(24)**,** 10066-74.

Lundin, A., Bok, C. M., Aronsson, L., Björkholm, B., Gustafsson, J. A., Pott, S., Arulampalam, V., Hibberd, M., Rafter, J. & Pettersson, S. (2008). Gut flora, Toll-like receptors and nuclear receptors: a tripartite communication that tunes innate immunity in large intestine. *Cell Microbiol,* 10(5)**,** 1093-103.

Lunney, P. C. & Leong, R. W. (2012). Review article: Ulcerative colitis, smoking and nicotine therapy. *Aliment Pharmacol Ther,* 36(11-12)**,** 997-1008.

Ma, R. C. & Szurszewski, J. H. (1996). Release of calcitonin gene-related peptide in guinea pig inferior mesenteric ganglion. *Peptides,* 17(1)**,** 161-70.

Mackenna, B. R. & McKirdy, H. C. (1972). Peristalsis in the rabbit distal colon. *J Physiol,* 220(1)**,** 33-54.

Mackner, L. M., Clough-Paabo, E., Pajer, K., Lourie, A. & Crandall, W. V. (2011). Psychoneuroimmunologic factors in inflammatory bowel disease. *Inflammatory Bowel Diseases,* 17(3)**,** 849-857.

Madl, C. M. & Heilshorn, S. C. (2015). Matrix interactions modulate neurotrophin-mediated neurite outgrowth and pathfinding. *Neural Regen Res,* 10(4)**,** 514-7.

Maggi, C. A., Barbanti, G., Turini, D. & Giuliani, S. (1991). Effect of NG-monomethyl L-arginine (L-NMMA) and NG-nitro L-arginine (L-NOARG) on non-adrenergic non-cholinergic relaxation in the circular muscle of the human ileum. *Br J Pharmacol,* 103(4)**,** 1970-2.

Mai, H., May, W. S., Gao, F., Jin, Z. & Deng, X. (2003). A functional role for nicotine in Bcl2 phosphorylation and suppression of apoptosis. *J Biol Chem,* 278(3)**,** 1886-91.

Mann, P. T., Furness, J. B. & Southwell, B. R. (1999). Choline acetyltransferase immunoreactivity of putative intrinsic primary afferent neurons in the rat ileum. *Cell Tissue Res,* 297(2)**,** 241-8.

Mantyh, C. R., Gates, T. S., Zimmerman, R. P., Welton, M. L., Passaro, E. P., Vigna, S. R., Maggio, J. E., Kruger, L. & Mantyh, P. W. (1988). Receptor binding sites for substance P, but not substance K or neuromedin K, are expressed in high concentrations by arterioles, venules, and lymph nodules in surgical specimens obtained from patients with ulcerative colitis and Crohn disease. *Proc Natl Acad Sci U S A,* 85(9)**,** 3235-9.

Marei, H. E., El-Gamal, A., Althani, A., Afifi, N., Abd-Elmaksoud, A., Farag, A., Cenciarelli, C., Caceci, T. & Hasan, A. (2017). Cholinergic and Dopaminergic Neuronal Differentiation of Human Adipose Tissue Derived Mesenchymal Stem Cells. *J Cell Physiol*.

Marks, L., Beard, E., Cobey, D., Moore, N., Motyer, V., Valentin, J. P. & Ewart, L. (2013). An evaluation of the non-invasive faecal pellet assessment method as an early drug discovery screen for gastrointestinal liability. *J Pharmacol Toxicol Methods,* 68(1)**,** 123-36.

Martarelli, D., Pompei, P., Baldi, C. & Mazzoni, G. (2008). Mebendazole inhibits growth of human adrenocortical carcinoma cell lines implanted in nude mice. *Cancer Chemother Pharmacol,* 61(5)**,** 809-17.

Martin, L. F. & Freedman, R. (2007). Schizophrenia and the alpha7 nicotinic acetylcholine receptor. *Int Rev Neurobiol,* 78**,** 225-46.

Matsuda, H., Hirato, J., Kuroiwa, M. & Nakazato, Y. (2006). Histopathological and immunohistochemical study of the enteric innervations among various types of aganglionoses including isolated and syndromic Hirschsprung disease. *Neuropathology,* 26(1)**,** 8-23.

Matsuyoshi, H., Kuniyasu, H., Okumura, M., Misawa, H., Katsui, R., Zhang, G. X., Obata, K. & Takaki, M. (2010). A 5-HT(4)-receptor activation-induced neural plasticity enhances in vivo reconstructs of enteric nerve circuit insult. *Neurogastroenterol Motil,* 22(7)**,** 806-13, e226.

Mayer, E. A., Knight, R., Mazmanian, S. K., Cryan, J. F. & Tillisch, K. (2014). Gut microbes and the brain: paradigm shift in neuroscience. *J Neurosci,* 34(46)**,** 15490-6.

Mazelin, L., Theodorou, V., Fioramonti, J. & Bueno, L. (1999). Vagally dependent protective action of calcitonin gene-related peptide on colitis. *Peptides,* 20(11)**,** 1367-74.

Mazzuoli, G. & Schemann, M. (2009). Multifunctional rapidly adapting mechanosensitive enteric neurons (RAMEN) in the myenteric plexus of the guinea pig ileum. *J Physiol,* 587(Pt 19)**,** 4681-94.

McCord, J. M. & Omar, B. A. (1993). Sources of free radicals. *Toxicol Ind Health,* 9(1-2)**,** 23-37.

McEwen, A. (2016). 10 common questions about e-cigarettes answered, Cancer Research UK. Available at :http://scienceblog.cancerresearchuk.org/2016/05/17/10-common-questions-about-e-cigarettes-answered/:, accessed (03.08.2017).

McVey Neufeld, K. A., Mao, Y. K., Bienenstock, J., Foster, J. A. & Kunze, W. A. (2013). The microbiome is essential for normal gut intrinsic primary afferent neuron excitability in the mouse. *Neurogastroenterol Motil,* 25(2)**,** 183-e88.

McVey Neufeld, K. A., Perez-Burgos, A., Mao, Y. K., Bienenstock, J. & Kunze, W. A. (2015). The gut microbiome restores intrinsic and extrinsic nerve function in germ-free mice accompanied by changes in calbindin. *Neurogastroenterol Motil,* 27(5)**,** 627-36.

Mearin, F., Pérez-Oliveras, M., Perelló, A., Vinyet, J., Ibañez, A., Coderch, J. & Perona, M. (2005). Dyspepsia and irritable bowel syndrome after a Salmonella gastroenteritis outbreak: one-year follow-up cohort study. *Gastroenterology,* 129(1)**,** 98-104.

Medland, J. E., Pohl, C. S., Edwards, L. L., Frandsen, S., Bagley, K., Li, Y. & Moeser, A. J. (2016). Early life adversity in piglets induces long-term upregulation of the enteric

cholinergic nervous system and heightened, sex-specific secretomotor neuron responses. *Neurogastroenterol Motil,* 28(9)**,** 1317-29.

Mehta, P. & Piao, X. (2017). Adhesion G-protein coupled receptors and extracellular matrix proteins: Roles in myelination and glial cell development. Developmental Dynamics.

Meirhaeghe, A., Luan, J., Selberg-Franks, P., Hennings, S., Mitchell, J., Halsall, D., O'Rahilly, S. & Wareham, N. J. (2001). The effect of the Gly16Arg polymorphism of the beta(2)-adrenergic receptor gene on plasma free fatty acid levels is modulated by physical activity. *J Clin Endocrinol Metab,* 86(12)**,** 5881-7.

Mello, D. M., Marcinichen, D. R., Madruga, D., Branco, R., Paschoalini, M. A. & De Lima, T. C. (2007). Involvement of NK1 receptors in metabolic stress markers after the central administration of substance P. *Behav Brain Res,* 181(2)**,** 232-8.

Meng, J., Ovsepian, S. V., Wang, J., Pickering, M., Sasse, A., Aoki, K. R., Lawrence, G. W. & Dolly, J. O. (2009). Activation of TRPV1 mediates calcitonin gene-related peptide release, which excites trigeminal sensory neurons and is attenuated by a retargeted botulinum toxin with anti-nociceptive potential. *J Neurosci,* 29(15)**,** 4981-92.

Metzger, M. (2010). Neurogenesis in the enteric nervous system. *Arch Ital Biol,* 148(2)**,** 73-83.

Michael, G. J. & Priestley, J. V. (1999). Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. *J Neurosci,* 19(5)**,** 1844-54.

Mikami, N., Matsushita, H., Kato, T., Kawasaki, R., Sawazaki, T., Kishimoto, T., Ogitani, Y., Watanabe, K., Miyagi, Y., Sueda, K., Fukada, S., Yamamoto, H. & Tsujikawa, K. (2011). Calcitonin gene-related peptide is an important regulator of cutaneous immunity: effect on dendritic cell and T cell functions. *J Immunol,* 186(12)**,** 6886-93.

Miller, S. M. & Szurszewski, J. H. (1997). Colonic mechanosensory afferent input to neurons in the mouse superior mesenteric ganglion. *Am J Physiol,* 272(2 Pt 1)**,** G357-66.

Mo, Z. L., Katafuchi, T. & Hori, T. (1996). Effects of IL-1 beta on neuronal activities in the dorsal motor nucleus of the vagus in rat brain slices. *Brain Res Bull,* 41(4)**,** 249-55.

Mogil, J. S., Miermeister, F., Seifert, F., Strasburg, K., Zimmermann, K., Reinold, H., Austin, J. S., Bernardini, N., Chesler, E. J., Hofmann, H. A., Hordo, C., Messlinger, K., Nemmani, K. V., Rankin, A. L., Ritchie, J., Siegling, A., Smith, S. B., Sotocinal, S., Vater, A., Lehto, S. G., Klussmann, S., Quirion, R., Michaelis, M., Devor, M. & Reeh, P. W. (2005). Variable sensitivity to noxious heat is mediated by differential expression of the CGRP gene. *Proc Natl Acad Sci U S A,* 102(36)**,** 12938-43.

Mogil, J. S., Wilson, S. G., Bon, K., Lee, S. E., Chung, K., Raber, P., Pieper, J. O., Hain, H. S., Belknap, J. K., Hubert, L., Elmer, G. I., Chung, J. M. & Devor, M. (1999a). Heritability of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception. *Pain,* 80(1-2)**,** 67-82.

Mogil, J. S., Wilson, S. G., Bon, K., Lee, S. E., Chung, K., Raber, P., Pieper, J. O., Hain, H. S., Belknap, J. K., Hubert, L., Elmer, G. I., Chung, J. M. & Devor, M. (1999b). Heritability of nociception II. 'Types' of nociception revealed by genetic correlation analysis. *Pain,* 80(1-2)**,** 83-93.

Moulian, N., Truffault, F., Gaudry-Talarmain, Y. M., Serraf, A. & Berrih-Aknin, S. (2001). In vivo and in vitro apoptosis of human thymocytes are associated with nitrotyrosine formation. *Blood,* 97(11)**,** 3521-30.

Moynes, D. M., Lucas, G. H., Beyak, M. J. & Lomax, A. E. (2014). Effects of inflammation on the innervation of the colon. *Toxicol Pathol,* 42(1)**,** 111-7.

Munder, M., Eichmann, K., Morán, J. M., Centeno, F., Soler, G. & Modolell, M. (1999). Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J Immunol,* 163(7)**,** 3771-7.

Nair, D. G., Miller, K. G., Lourenssen, S. R. & Blennerhassett, M. G. (2014). Inflammatory cytokines promote growth of intestinal smooth muscle cells by induced expression of PDGF-Rβ. *J Cell Mol Med,* 18(3)**,** 444-54.

Neunlist, M., Dobreva, G. & Schemann, M. (1999). Characteristics of mucosally projecting myenteric neurones in the guinea-pig proximal colon. *J Physiol,* 517 (Pt 2)**,** 533-46.

Neunlist, M., Rolli-Derkinderen, M., Latorre, R., Van Landeghem, L., Coron, E., Derkinderen, P. & De Giorgio, R. (2014). Enteric glial cells: recent developments and future directions. *Gastroenterology,* 147(6)**,** 1230-7.

Neunlist, M., Van Landeghem, L., Mahé, M. M., Derkinderen, P., des Varannes, S. B. & Rolli-Derkinderen, M. (2013). The digestive neuronal-glial-epithelial unit: a new actor in gut health and disease. *Nat Rev Gastroenterol Hepatol,* 10(2)**,** 90-100.

Neves-Souza, P. C., Azeredo, E. L., Zagne, S. M., Valls-de-Souza, R., Reis, S. R., Cerqueira, D. I., Noqueira, R. M. & Kubelka, C. F. (2005). Inducible nitric oxide synthase (iNOS) expression in monocytes during acute Dengue Fever in patients and during in vitro infection. BMC Infect Dis.

Niizeki, H., Alard, P. & Streilein, J. W. (1997). Calcitonin gene-related peptide is necessary for ultraviolet B-impaired induction of contact hypersensitivity. *J Immunol,* 159(11)**,** 5183-6.

Nishiyama, K., Azuma, Y. T., Shintaku, K., Yoshida, N., Nakajima, H. & Takeuchi, T. (2014). Evidence that nitric oxide is a non-adrenergic non-cholinergic inhibitory neurotransmitter in the circular muscle of the mouse distal colon: a study on the mechanism of nitric oxide-induced relaxation. *Pharmacology,* 94(3-4)**,** 99-108.

Nizri, E., Irony-Tur-Sinai, M., Lory, O., Orr-Urtreger, A., Lavi, E. & Brenner, T. (2009). Activation of the cholinergic anti-inflammatory system by nicotine attenuates neuroinflammation via suppression of Th1 and Th17 responses. *J Immunol,* 183(10)**,** 6681-8.

Nygren, P., Fryknäs, M., Agerup, B. & Larsson, R. (2013). Repositioning of the anthelmintic drug mebendazole for the treatment for colon cancer. *J Cancer Res Clin Oncol,* 139(12)**,** 2133-40.

O'Connor, S. M., Stenger, D. A., Shaffer, K. M. & Ma, W. (2001). Survival and neurite outgrowth of rat cortical neurons in three-dimensional agarose and collagen gel matrices. *Neurosci Lett,* 304(3)**,** 189-93.

Ochoa-Cortes, F., Turco, F., Linan-Rico, A., Soghomonyan, S., Whitaker, E., Wehner, S., Cuomo, R. & Christofi, F. L. (2016). Enteric Glial Cells: A New Frontier in Neurogastroenterology and Clinical Target for Inflammatory Bowel Diseases. Inflamm Bowel Dis.

Ochoa-Repáraz, J., Mielcarz, D. W., Begum-Haque, S. & Kasper, L. H. (2011). Gut, bugs, and brain: role of commensal bacteria in the control of central nervous system disease. *Ann Neurol,* 69(2)**,** 240-7.

Ochoa-Repáraz, J., Mielcarz, D. W., Wang, Y., Begum-Haque, S., Dasgupta, S., Kasper, D. L. & Kasper, L. H. (2010). A polysaccharide from the human commensal Bacteroides fragilis protects against CNS demyelinating disease. *Mucosal Immunol,* 3(5)**,** 487-95.

Ohlsson, B., Sundkvist, G. & Lindgren, S. (2007). Subclinical sympathetic neuropathy appears early in the course of Crohn's disease. BMC Gastroenterology.

Okhuysen, P. C., Jiang, Z. D., Carlin, L., Forbes, C. & DuPont, H. L. (2004). Post-diarrhea chronic intestinal symptoms and irritable bowel syndrome in North American travelers to Mexico. *Am J Gastroenterol,* 99(9)**,** 1774-8.

Oku, R., Satoh, M., Fujii, N., Otaka, A., Yajima, H. & Takagi, H. (1987). Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rats. *Brain Res,* 403(2)**,** 350-4.

Olsson, C. & Holmgren, S. (2011). Autonomic control of gut motility: A comparative view. Autonomic Neuroscience.

Paleari, L., Catassi, A., Ciarlo, M., Cavalieri, Z., Bruzzo, C., Servent, D., Cesario, A., Chessa, L., Cilli, M., Piccardi, F., Granone, P. & Russo, P. (2008). Role of alpha7-nicotinic acetylcholine receptor in human non-small cell lung cancer proliferation. *Cell Prolif,* 41(6)**,** 936-59.

Parkman, H. P., Ma, R. C., Stapelfeldt, W. H. & Szurszewski, J. H. (1993). Direct and indirect mechanosensory pathways from the colon to the inferior mesenteric ganglion. *Am J Physiol,* 265(3 Pt 1)**,** G499-505.

Peuhkuri, K., Vapaatalo, H. & Korpela, R. (2010). Even low-grade inflammation impacts on small intestinal function. *World J Gastroenterol,* 16(9)**,** 1057-62.

Philip, N. S., Carpenter, L. L., Tyrka, A. R. & Price, L. H. (2010). Nicotinic acetylcholine receptors and depression: a review of the preclinical and clinical literature. *Psychopharmacology (Berl),* 212(1)**,** 1-12.

Phillips, M., Patel, A., Meredith, P., Will, O. & Brassett, C. (2015). Segmental colonic length and mobility. *Ann R Coll Surg Engl,* 97(6)**,** 439-44.

Phillips, R. J., Kieffer, E. J. & Powley, T. L. (2003). Aging of the myenteric plexus: neuronal loss is specific to cholinergic neurons. *Auton Neurosci,* 106(2)**,** 69-83.

Phillips, R. J., Pairitz, J. C. & Powley, T. L. (2007). Age-related neuronal loss in the submucosal plexus of the colon of Fischer 344 rats. *Neurobiol Aging,* 28(7)**,** 1124-37.

Phillips, R. J. & Powley, T. L. (2007). Innervation of the gastrointestinal tract: patterns of aging. *Auton Neurosci,* 136(1-2)**,** 1-19.

Phillips, S. F. & Giller, J. (1973). The contribution of the colon to electrolyte and water conservation in man. *J Lab Clin Med,* 81(5)**,** 733-46.

Posadas, I., López-Hernández, B. & Ceña, V. (2013). Nicotinic receptors in neurodegeneration. *Curr Neuropharmacol,* 11(3)**,** 298-314.

Prass, K., Meisel, C., Höflich, C., Braun, J., Halle, E., Wolf, T., Ruscher, K., Victorov, I. V., Priller, J., Dirnagl, U., Volk, H. D. & Meisel, A. (2003). Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. *J Exp Med,* 198(5)**,** 725-36.

Pöyhönen-Alho, M. K., Manhem, K., Katzman, P., Kibarskis, A., Antikainen, R. L., Erkkola, R. U., Tuomilehto, J. O., Ebeling, P. E. & Kaaja, R. J. (2008). Central sympatholytic therapy has anti-inflammatory properties in hypertensive postmenopausal women. *J Hypertens,* 26(12)**,** 2445-9.

Qu, C., Dwyer, T., Shao, Q., Yang, T., Huang, H. & Liu, G. (2013). Direct binding of TUBB3 with DCC couples netrin-1 signaling to intracellular microtubule dynamics in axon outgrowth and guidance. *J Cell Sci,* 126(Pt 14)**,** 3070-81.

Qureshi, I. A. & Mehler, M. F. (2013). Towards a 'systems'-level understanding of the nervous system and its disorders. *Trends Neurosci,* 36(11)**,** 674-84.

Radi, R., Beckman, J. S., Bush, K. M. & Freeman, B. A. (1991a). Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem,* 266(7)**,** 4244-50.

Radi, R., Beckman, J. S., Bush, K. M. & Freeman, B. A. (1991b). Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys,* 288(2)**,** 481-7.

Radley, J. J., Rocher, A. B., Janssen, W. G., Hof, P. R., McEwen, B. S. & Morrison, J. H. (2005). Reversibility of apical dendritic retraction in the rat medial prefrontal cortex following repeated stress. *Exp Neurol,* 196(1)**,** 199-203.

Renzi, D., Mantellini, P., Calabrò, A., Panerai, C., Amorosi, A., Paladini, I., Salvadori, G., Garcea, M. R. & Surrenti, C. (1998). Substance P and vasoactive intestinal polypeptide but not calcitonin gene-related peptide concentrations are reduced in patients with moderate and severe ulcerative colitis. *Ital J Gastroenterol Hepatol,* 30(1)**,** 62-70.

Reuter, S., Gupta, S. C., Chatervedi, M. M. & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med.

Robinson, A. M., Rahman, A. A., Miller, S., Stavely, R., Sakkal, S. & Nurgali, K. (2017). The neuroprotective effects of human bone marrow mesenchymal stem cells are dose-dependent in TNBS colitis. *Stem Cell Res Ther,* 8(1)**,** 87.

Romagnani, S. (1999). Th1/Th2 cells. *Inflamm Bowel Dis,* 5(4)**,** 285-94.

Rosas-Ballina, M., Ochani, M., Parrish, W. R., Ochani, K., Harris, Y. T., Huston, J. M., Chavan, S. & Tracey, K. J. (2008). Splenic nerve is required for cholinergic antiinflammatory pathway control of TNF in endotoxemia. *Proc Natl Acad Sci U S A,* 105(31)**,** 11008-13.

Rosenfeld, M. G., Mermod, J. J., Amara, S. G., Swanson, L. W., Sawchenko, P. E., Rivier, J., Vale, W. W. & Evans, R. M. (1983). Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature,* 304(5922)**,** 129-35.

Rossi, S. G., Dickerson, I. M. & Rotundo, R. L. (2003). Localization of the calcitonin gene-related peptide receptor complex at the vertebrate neuromuscular junction and its role in regulating acetylcholinesterase expression. *J Biol Chem,* 278(27)**,** 24994-5000.

Round, J. L. & Mazmanian, S. K. (2009). The gut microbiome shapes intestinal immune responses during health and disease. Nature Reviews Immunology.

Röhr, D., Halfter, H., Schulz, J. B., Young, P. & Gess, B. (2017). Sodium-dependent Vitamin C transporter 2 deficiency impairs myelination and remyelination after injury: Roles of collagen and demethylation. *Glia*.

Saffrey, M. J. (2013). Cellular changes in the enteric nervous system during ageing. *Dev Biol,* 382(1)**,** 344-55.

Salgo, M. G., Stone, K., Squadrito, G. L., Battista, J. R. & Pryor, W. A. (1995). Peroxynitrite causes DNA nicks in plasmid pBR322. *Biochem Biophys Res Commun,* 210(3)**,** 1025-30.

Sanders, K. M. (1996). A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterology,* 111(2)**,** 492-515.

Sandgren, K., Lin, Z., Fex Svenningsen, A. & Ekblad, E. (2003). Vasoactive intestinal peptide and nitric oxide promote survival of adult rat myenteric neurons in culture. *J Neurosci Res,* 72(5)**,** 595-602.

Sandle, G. I., Higgs, N., Crowe, P., Marsh, M. N., Venkatesan, S. & Peters, T. J. (1990). Cellular basis for defective electrolyte transport in inflamed human colon. *Gastroenterology,* 99(1)**,** 97-105.

Sang, Q. & Young, H. M. (1996). Chemical coding of neurons in the myenteric plexus and external muscle of the small and large intestine of the mouse. *Cell Tissue Res,* 284(1)**,** 39-53.

Sanmiguel, C., Gupta, A. & Mayer, E. A. (2015). Gut Microbiome and Obesity: A Plausible Explanation for Obesity. *Curr Obes Rep,* 4(2)**,** 250-61.

Sapolsky, R., Rivier, C., Yamamoto, G., Plotsky, P. & Vale, W. (1987). Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor. *Science,* 238(4826)**,** 522-4.

Sasaki, J., Ramesh, R., Chada, S., Gomyo, Y., Roth, J. A. & Mukhopadhyay, T. (2002). The anthelmintic drug mebendazole induces mitotic arrest and apoptosis by depolymerizing tubulin in non-small cell lung cancer cells. *Mol Cancer Ther,* 1(13)**,** 1201-9.

Sayuk, G. S., Wolf, R. & Chang, L. (2017). Comparison of symptoms, healthcare utilisation and treatment in diagnosed and undiagnosed individuals with diarrhea-predominant irritable bowel syndrome. Am J Gastroenterology.

Schemann, M., Reiche, D. & Michel, K. (2001). Enteric pathways in the stomach. *The Anatomical Record,* 262(1)**,** 47-57.

Schmidt, A. M., Yan, S. D., Yan, S. F. & Stern, D. M. (2001). The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J Clin Invest,* 108(7)**,** 949-55.

Schoenfeld, T. J. & Gould, E. (2012). Stress, stress hormones, and adult neurogenesis. *Exp Neurol,* 233(1)**,** 12-21.

Schwartz, E. S., Christianson, J. A., Chen, X., La, J. H., Davis, B. M., Albers, K. M. & Gebhart, G. F. (2011). Synergistic role of TRPV1 and TRPA1 in pancreatic pain and inflammation. *Gastroenterology,* 140(4)**,** 1283-1291.e1-2.

Seah, S. K. (1976). Mebendazole in the treatment of helminthiasis. *Can Med Assoc J,* 115(8)**,** 777-9.

Selye, H. (1998). A syndrome produced by diverse nocuous agents. 1936. *J Neuropsychiatry Clin Neurosci,* 10(2)**,** 230-1.

Severance, E. G., Yolken, R. H. & Eaton, W. W. (2016). Autoimmune diseases, gastrointestinal disorders and the microbiome in schizophrenia: more than a gut feeling. *Schizophr Res,* 176(1)**,** 23-35.

Shah, E., Rezaie, A., Riddle, M. & Pimentel, M. (2014). Psychological disorders in gastrointestinal disease: epiphenomenon, cause or consequence? *Ann Gastroenterol,* 27(3)**,** 224-230.

Shin, V. Y., Jin, H. C., Ng, E. K., Yu, J., Leung, W. K., Cho, C. H. & Sung, J. J. (2008). Nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induce cyclooxygenase-2 activity in human gastric cancer cells: Involvement of nicotinic acetylcholine receptor (nAChR) and beta-adrenergic receptor signaling pathways. *Toxicol Appl Pharmacol,* 233(2)**,** 254-61.

Siemionow, K., Klimczak, A., Brzezicki, G., Siemionow, M. & McLain, R. F. (2009). The effects of inflammation on glial fibrillary acidic protein expression in satellite cells of the dorsal root ganglion. *Spine (Phila Pa 1976),* 34(16)**,** 1631-7.

Sjövall, H. (1984). Sympathetic control of jejunal fluid and electrolyte transport. An experimental study in cats and rats. *Acta Physiol Scand Suppl,* 535**,** 1-63.

Smith, T. K., Bornstein, J. C. & Furness, J. B. (1992). Convergence of reflex pathways excited by distension and mechanical stimulation of the mucosa onto the same myenteric neurons of the guinea pig small intestine. *J Neurosci,* 12(4)**,** 1502-10.

Smith, T. K., Burke, E. P. & Shuttleworth, C. W. (1999). Topographical and electrophysiological characteristics of highly excitable S neurones in the myenteric plexus of the guinea-pig ileum. *J Physiol,* 517 (Pt 3)**,** 817-30.

Smith, T. K. & Robertson, W. J. (1998). Synchronous movements of the longitudinal and circular muscle during peristalsis in the isolated guinea-pig distal colon. *J Physiol,* 506 (Pt 2)**,** 563-77.

Song, P., Sekhon, H. S., Jia, Y., Keller, J. A., Blusztajn, J. K., Mark, G. P. & Spindel, E. R. (2003). Acetylcholine is synthesized by and acts as an autocrine growth factor for small cell lung carcinoma. *Cancer Res,* 63(1)**,** 214-21.

Song, Y., Liu, C. & Finegold, S. M. (2004). Real-time PCR quantitation of clostridia in feces of autistic children. *Appl Environ Microbiol,* 70(11)**,** 6459-65.

Soret, R., Coquenlorge, S., Cossais, F., Meurette, G., Rolli-Derkinderen, M. & Neunlist, M. (2013). Characterization of human, mouse, and rat cultures of enteric glial cells and their effect on intestinal epithelial cells. *Neurogastroenterol Motil,* 25(11)**,** e755-64.

Sotelo, J., Gibbs, C. J., Gajdusek, D. C., Toh, B. H. & Wurth, M. (1980). Method for preparing cultures of central neurons: cytochemical and immunochemical studies. *Proc Natl Acad Sci U S A,* 77(1)**,** 653-7.

Southwell, B. R., Koh, T. L., Wong, S. Q., King, S. K., Ong, S. Y., Lee, M., Farmer, P. J., Peck, C. J., Sutcliffe, J. R., Stanton, M. P., Keck, J., Cook, D. J., Chow, C. W. & Hutson, J. M. (2010). Decrease in nerve fibre density in human sigmoid colon circular muscle occurs with growth but not aging. *Neurogastroenterol Motil,* 22(4)**,** 439-45, e106.

Spaziani, R., Bayati, A., Redmond, K., Bajaj, H., Mazzadi, S., Bienenstock, J., Collins, S. M. & Kamath, M. V. (2008). Vagal dysfunction in irritable bowel syndrome assessed by rectal distension and baroreceptor sensitivity. *Neurogastroenterol Motil,* 20(4)**,** 336-42.

Spencer, N. J., Hennig, G. W. & Smith, T. K. (2002). A rhythmic motor pattern activated by circumferential stretch in guinea-pig distal colon. *J Physiol,* 545(Pt 2)**,** 629-48.

Spiller, R. C., Jenkins, D., Thornley, J. P., Hebden, J. M., Wright, T., Skinner, M. & Neal, K. R. (2000). Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute Campylobacter enteritis and in post-dysenteric irritable bowel syndrome. *Gut,* 47(6)**,** 804-11.

Steinmann, P., Utzinger, J., Du, Z. W., Jiang, J. Y., Chen, J. X., Hattendorf, J., Zhou, H. & Zhou, X. N. (2011). Efficacy of single-dose and triple-dose albendazole and mebendazole against soil-transmitted helminths and Taenia spp.: a randomized controlled trial. *PLoS One,* 6(9)**,** e25003.

Straub, R. H., Grum, F., Strauch, U., Capellino, S., Bataille, F., Bleich, A., Falk, W., Schölmerich, J. & Obermeier, F. (2008). Anti-inflammatory role of sympathetic nerves in chronic intestinal inflammation. *Gut,* 57(7)**,** 911-21.

Straub, R. H., Pongratz, G., Weidler, C., Linde, H. J., Kirschning, C. J., Glück, T., Schölmerich, J. & Falk, W. (2005). Ablation of the sympathetic nervous system decreases gram-negative and increases gram-positive bacterial dissemination: key roles for tumor necrosis factor/phagocytes and interleukin-4/lymphocytes. *J Infect Dis,* 192(4)**,** 560-72.

Straub, R. H., Wiest, R., Strauch, U. G., Harle, P. & Scholmerich, J. (2006). The role of the sympathetic nervous system in intestinal inflammation. *Gut,* 55(11)**,** 1640-1649.

Stunkard, A. J., Harris, J. R., Pedersen, N. L. & McClearn, G. E. (1990). The body-mass index of twins who have been reared apart. *N Engl J Med,* 322(21)**,** 1483-7.

Su, L., Zhang, Z., Gan, L., Jiang, Q., Xiao, P., Zou, J., Li, Q. & Jiang, H. (2016). Deregulation of the planar cell polarity genes CELSR3 and FZD3 in Hirschsprung disease. *Exp Mol Pathol,* 101(2)**,** 241-248.

Suckow, S. K. & Caudle, R. M. (2008). Identification and immunohistochemical characterization of colospinal afferent neurons in the rat. *Neuroscience,* 153(3)**,** 803-13.

Sundler, F., Ekblad, E. & Håkanson, R. (1989). Projections of enteric peptide-containing neurons in the rat. *Arch Histol Cytol,* 52 Suppl**,** 181-9.

Sung, J., Morales, W., Kim, G., Pokkunuri, V., Weitsman, S., Rooks, E., Marsh, Z., Barlow, G. M., Chang, C. & Pimentel, M. (2013). Effect of repeated Campylobacter jejuni infection on gut flora and mucosal defense in a rat model of post infectious functional and microbial bowel changes. *Neurogastroenterol Motil,* 25(6)**,** 529-37.

Takaki, M., Goto, K. & Kawahara, I. (2014). The 5-hydroxytryptamine 4 Receptor Agonist-induced Actions and Enteric Neurogenesis in the Gut. *J Neurogastroenterol Motil,* 20(1)**,** 17-30.

Talley, N. J., Fett, S. L., Zinsmeister, A. R. & Melton, L. J. (1994). Gastrointestinal tract symptoms and self-reported abuse: a population-based study. *Gastroenterology,* 107(4)**,** 1040-9.

Talvani, A., Machado, F. S., Santana, G. C., Klein, A., Barcelos, L., Silva, J. S. & Teixeira, M. M. (2002). Leukotriene B(4) induces nitric oxide synthesis in Trypanosoma cruzi-infected murine macrophages and mediates resistance to infection. *Infect Immun,* 70(8)**,** 4247-53.

Tang, D., Kang, R., Coyne, C. B., Zeh, H. J. & Lotze, M. T. (2012). PAMPs and DAMPs: Signal 0s that Spur Autophagy and Immunity. Immunol Rev.

Tennyson, A. G. & Lippard, S. J. (2011). Generation, translocation, and action of nitric oxide in living systems. *Chem Biol,* 18(10)**,** 1211-20.

Thayer, J. F. & Fischer, J. E. (2009). Heart rate variability, overnight urinary norepinephrine and C-reactive protein: evidence for the cholinergic anti-inflammatory pathway in healthy human adults. *J Intern Med,* 265(4)**,** 439-47.

Thinschmidt, J. S., King, M. A., Korah, M., Perez, P. D., Febo, M., Miyan, J. & Grant, M. B. (2015). Central neural activation following contact sensitivity peripheral immune challenge: evidence of brain-immune regulation through C fibres. *Immunology,* 146(2)**,** 206-16.

Thomassen, M. J., Buhrow, L. T., Connors, M. J., Kaneko, F. T., Erzurum, S. C. & Kavuru, M. S. (1997). Nitric oxide inhibits inflammatory cytokine production by human alveolar macrophages. *Am J Respir Cell Mol Biol,* 17(3)**,** 279-83.

Timmermans, J. P., Hens, J. & Adriaensen, D. (2001). Outer submucous plexus: an intrinsic nerve network involved in both secretory and motility processes in the intestine of large mammals and humans. *Anat Rec,* 262(1)**,** 71-8.

Tomuschat, C. & Puri, P. (2015). RET gene is a major risk factor for Hirschsprung's disease: a meta-analysis. *Pediatr Surg Int,* 31(8)**,** 701-10.

Toth, C., Rong, L. L., Yang, C., Martinez, J., Song, F., Ramji, N., Brussee, V., Liu, W., Durand, J., Nguyen, M. D., Schmidt, A. M. & Zochodne, D. W. (2008). Receptor for advanced glycation end products (RAGEs) and experimental diabetic neuropathy. *Diabetes,* 57(4)**,** 1002-17.

Tracey, K. J. (2002). The inflammatory reflex. *Nature,* 420(6917)**,** 853-9.

Tripathi, P. (2007). Nitric oxide and immune response. *Indian J Biochem Biophys,* 44(5)**,** 310-9.

Uchihara, T. (2007). Silver diagnosis in neuropathology: principles, practice and revised interpretation. Acta Neuropathol.

Ulrich-Lai, Y. M. & Herman, J. P. (2009). Neural regulation of endocrine and autonomic stress responses. *Nature Reviews Neuroscience,* 10(6)**,** 397-409.

Uno, H., Tarara, R., Else, J. G., Suleman, M. A. & Sapolsky, R. M. (1989). Hippocampal damage associated with prolonged and fatal stress in primates. *J Neurosci,* 9(5)**,** 1705-11.

Valdehita, A., Bajo, A. M., Fernández-Martínez, A. B., Arenas, M. I., Vacas, E., Valenzuela, P., Ruíz-Villaespesa, A., Prieto, J. C. & Carmena, M. J. (2010). Nuclear localization of vasoactive intestinal peptide (VIP) receptors in human breast cancer. *Peptides,* 31(11)**,** 2035-45.

Vallance, P. & Leiper, J. (2002). Blocking NO synthesis: how, where and why? *Nat Rev Drug Discov,* 1(12)**,** 939-50.

van der zanden, e. p., boeckxstaens, g. e. & de jonge, w. j. (2009). The vagus nerve as a modulator of intestinal inflammation. *Neurogastroenterology & Motility,* 21(1)**,** 6-17.

Van Hove, C. E., Van der Donckt, C., Herman, A. G., Bult, H. & Fransen, P. (2009). Vasodilator efficacy of nitric oxide depends on mechanisms of intracellular calcium mobilization in mouse aortic smooth muscle cells. *Br J Pharmacol,* 158(3)**,** 920-30.

Vandeputte, D., Falony, G., Vieira-Silva, S., Tito, R. Y., Joossens, M. & Raes, J. (2016). Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. *Gut,* 65(1)**,** 57-62.

Vavricka, S. R., Musch, M. W., Fujiya, M., Kles, K., Chang, L., Eloranta, J. J., Kullak-Ublick, G. A., Drabik, K., Merlin, D. & Chang, E. B. (2006). Tumor necrosis factor-alpha and interferon-gamma increase PepT1 expression and activity in the human colon carcinoma cell line Caco-2/bbe and in mouse intestine. *Pflugers Arch,* 452(1)**,** 71-80.

Veena, J., Srikumar, B. N., Raju, T. R. & Shankaranarayana Rao, B. S. (2009). Exposure to enriched environment restores the survival and differentiation of new born cells in the hippocampus and ameliorates depressive symptoms in chronically stressed rats. *Neurosci Lett,* 455(3)**,** 178-82.

Vinik, A. I., Erbas, T. & Casellini, C. M. (2013). Diabetic cardiac autonomic neuropathy, inflammation and cardiovascular disease. *J Diabetes Investig,* 4(1)**,** 4-18.

von Boyen, G. B., Steinkamp, M., Reinshagen, M., Schäfer, K. H., Adler, G. & Kirsch, J. (2004). Proinflammatory cytokines increase glial fibrillary acidic protein expression in enteric glia. *Gut,* 53(2)**,** 222-8.

Wakelin, D. (1967). Acquired immunity to Trichuris muris in the albino laboratory mouse. *Parasitology,* 57(3)**,** 515-24.

Wang, H., Hughes, I., Planer, W., Parsadanian, A., Grider, J. R., Vohra, B. P., Keller-Peck, C. & Heuckeroth, R. O. (2010). The timing and location of GDNF expression determines enteric nervous system structure and function. Journal of Neuroscience.

Wang, H., Yu, M., Ochani, M., Amella, C. A., Tanovic, M., Susarla, S., Li, J. H., Yang, H., Ulloa, L., Al-Abed, Y., Czura, C. J. & Tracey, K. J. (2003). Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature,* 421(6921)**,** 384-8.

Weaver, L. T., Austin, S. & Cole, T. J. (1991). Small intestinal length: a factor essential for gut adaptation. *Gut,* 32(11)**,** 1321-3.

Welch, M. G., Margolis, K. G., Li, Z. & Gershon, M. D. (2014). Oxytocin regulates gastrointestinal motility, inflammation, macromolecular permeability, and mucosal maintenance in mice. *Am J Physiol Gastrointest Liver Physiol,* 307(8)**,** G848-62.

West, D. B., Boozer, C. N., Moody, D. L. & Atkinson, R. L. (1992). Dietary obesity in nine inbred mouse strains. *Am J Physiol,* 262(6 Pt 2)**,** R1025-32.

West, D. B., Goudey-Lefevre, J., York, B. & Truett, G. E. (1994). Dietary obesity linked to genetic loci on chromosomes 9 and 15 in a polygenic mouse model. *J Clin Invest,* 94(4)**,** 1410-6.

Wimalawansa, S. J. (2001). Blood pressure and cardiovascular tone: role of CGRP family of peptides. Scientific World.

Wood, J. D. (1994). Physiology of the enteric nervous system. *In:* Johnson, L. R., Alpers, D. H., Christensen, J., Jacobsen, E. D., Walsh, J. H. & (eds) (eds.) *Physiology of the gastrointestinal tract.*: Raven Press, New York.

Wood, J. D., Alpers, D. H. & Andrews, P. L. R. (1999). Fundamentals of neurogastroenterology. *Gut,* 45(Supplement 2)**,** ii6-ii16.

Wood, J. D. & Perkins, W. E. (1970). Mechanical interaction between longitudinal and circular axes of the small intestine. *Am J Physiol,* 218(3)**,** 762-8.

Woods, S., Schroeder, J., McGachy, H. A., Plevin, R., Roberts, C. W. & Alexander, J. (2013). MAP kinase phosphatase-2 plays a key role in the control of infection with Toxoplasma gondii by modulating iNOS and arginase-1 activities in mice. *PLoS Pathog,* 9(8)**,** e1003535.

Wuensch, T., Schulz, S., Ullrich, S., Lill, N., Stelzl, T., Rubio-Aliaga, I., Loh, G., Chamaillard, M., Haller, D. & Daniel, H. (2013). The peptide transporter PEPT1 is expressed in distal colon in rodents and humans and contributes to water absorption. *Am J Physiol Gastrointest Liver Physiol,* 305(1)**,** G66-73.

Wuensch, T., Ullrich, S., Schulz, S., Chamaillard, M., Schaltenberg, N., Rath, E., Goebel, U., Sartor, R. B., Prager, M., Büning, C., Bugert, P., Witt, H., Haller, D. & Daniel, H. (2014). Colonic expression of the peptide transporter PEPT1 is downregulated during intestinal inflammation and is not required for NOD2-dependent immune activation. *Inflamm Bowel Dis,* 20(4)**,** 671-84.

Xia, Q. & Grant, S. F. (2013). The genetics of human obesity. *Ann N Y Acad Sci,* 1281**,** 178-90.

Xu, H. M., Han, J. G., Na, Y., Zhao, B., Ma, H. C. & Wang, Z. J. (2011). Colonic transit time in patient with slow-transit constipation: comparison of radiopaque markers and barium suspension method. *Eur J Radiol,* 79(2)**,** 211-3.

Yorek, M. S., Obrosov, A., Shevalye, H., Holmes, A., Harper, M. M., Kardon, R. H. & Yorek, M. A. (2015). Effect of diet-induced obesity or type 1 or type 2 diabetes on corneal nerves and peripheral neuropathy in C57Bl/6J mice. *J Peripher Nerv Syst,* 20(1)**,** 24-31.

York, B., Lei, K. & West, D. B. (1996). Sensitivity to dietary obesity linked to a locus on chromosome 15 in a CAST/Ei x C57BL/6J F2 intercross. *Mamm Genome,* 7(9)**,** 677-81.

Yu, B., Han, J., He, Y. T., Guo, S., Li, S. F. & Mei, F. (2009). Immunohistochemical study of CD44 immunopositive cells in the muscular layers of the gastrointestinal tract in adult guinea pigs and mice. *Acta Histochem,* 111(4)**,** 382-90.

Zhang, X., Zeng, B., Liu, Z., Liao, Z., Li, W., Wei, H. & Fang, X. (2014). Comparative diversity analysis of gut microbiota in two different human flora-associated mouse strains. *Curr Microbiol,* 69(3)**,** 365-73.

Zhang, Z., Liew, C. W., Handy, D. E., Zhang, Y., Leopold, J. A., Hu, J., Guo, L., Kulkarni, R. N., Loscalzo, J. & Stanton, R. C. (2010). High glucose inhibits glucose-6-phosphate dehydrogenase, leading to increased oxidative stress and beta-cell apoptosis. *FASEB J,* 24(5)**,** 1497-505.

Zhao, Y., Vanhoutte, P. M. & Leung, S. W. (2015). Vascular nitric oxide: Beyond eNOS. *J Pharmacol Sci,* 129(2)**,** 83-94.

Zhou, J. & Zhou, S. (2014). Inflammation: therapeutic targets for diabetic neuropathy. *Mol Neurobiol,* 49(1)**,** 536-46.

Ziv, Y., Ron, N., Butovsky, O., Landa, G., Sudai, E., Greenberg, N., Cohen, H., Kipnis, J. & Schwartz, M. (2006). Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nat Neurosci,* 9(2)**,** 268-75.