Multiple roles for the extracelllular matrix protein Tenascin-X in nerve gut function

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Statement of Originality

I, Rubina Aktar, confirm that the research included within this thesis is my own work or that it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated.

All the studies were performed and analysed by myself except as stated. Mr Max Walmsley obtained IHC images for the KO mouse under my supervision. Images obtained from the DRG and spinal cord were assisted by Dr Eduardo Araujo De Almeida. Dr Jean-Marie Delalande helped obtain IHC images of rectal prolapse. Gastric emptying breath samples were run by Dr Simon Eaton and obtained and analysed by myself. Dr Madusha Peiris wrote the ethics for the gastric emptying tests and assisted with the gastric emptying experiments and analysis.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

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Signature: Date: 30th June 2016

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Abstract

Tenascin X (TNX) is a matricellular protein involved in regulating cellular functions by interacting with other extracellular matrix (ECM) proteins within the cell matrix and has anti-adhesive properties evidenced in tumours and wound healing. TNX is the only member of the tenascin family that is lost in Joint Hypermobility Syndrome (JHS) and exerts a crucial architectural function. Of importance, TNX deficient and JHS patients have gastrointestinal (GI) dysfunction. Despite this association no study has described the role of TNX in the GI tract. Thus, the aim of this thesis was to characterise the expression of TNX in the stomach and colon in mouse and human tissue. Second, we aimed to elucidate the functional role of TNX using TNX knockout (TNX KO) mice.

Expression studies revealed TNX in vagal afferent endings in the mouse, and myenteric cell bodies in human stomach. In colon, TNX strongly associated with cholinergic submucous and myenteric neurons in both species, however, was not found in CGRP positive fibres. Cell bodies in nodose ganglia, dorsal root ganglia, ventral and dorsal horn were also TNX positive. Functional studies in stomach, using single fibre electrophysiology showed TNX KO mice had increased vagal afferent mechanoreceptor sensitivity. Octanoic acid breath test revealed rapid gastric emptying in TNX KO. Colonic manometry showed the amplitude and frequency of colonic contractions were reduced in TNX KO mice, particularly in the distal colon. Ussing chamber studies measuring changes in ion flux (indirect measure of secretion) showed no major difference between TNX KO and wild type (WT) mice.

The specific localisation of TNX with neuronal structures in the gut is shown here for the first time suggesting that TNX is more than just an architectural protein. Indeed, its role in specific GI functions supports this observation and provides a mechanism for GI symptoms in JHS.

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Abbreviations

ACh	Acetylcholine
AH	After Hyperpolarisation
ANOVA	Analysis of variance
ATP	Adenosine Triphosphate
BJHS	Benign joint hypermobility syndrome
CCK	Cholecystokinin
CED	Cambridge Electronic Design
CGRP	Calcitonin Gene Related Peptide
CFTR	Cystic Fibrosis Transmembrane Receptor
ChAT	Choline Acetyl Transferase
CMMC	Colonic Migrating Motor Complex
CNS	Central Nervous System
CPDR	Cumulative Percentage of 13^{C} Recovered
CRF	Corticotrophin Releasing Factor
DMVN	Dorsal Motor Vagal Nucleus
DRG	Dorsal Root Ganglia
DYN	Dynorphin
ECM	Extracellular Matrix
EDS	Ehlers Danlos Syndrome
EGF	Epidermal Growth Factor
EJP	Excitatory Junction Potentials
EMT	Epithelial-Mesenchymal Transition

ENK	Enkephalins
ENS	Enteric Nervous System
EPSP	Excitatory Post Synaptic Potential
FGID	Functional Gastrointestinal Disorder
FNIII	Fibronectin III
GABA	Gamma-amminobutyric Acid
GEC	Gastric Emptying Coefficient
GI	Gastrointestinal
GMC	Giant Migrating Contraction
GRP	Gastrin Releasing Peptide
HAPC	High Amplitude Propagating Contraction
HCL	Hydrochloric Acid
HT	High Threshold
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IBS-C	Irritable Bowel Syndrome - Constipation
IBS-D	Irritable Bowel Syndrome - Diarrhoea
IBS-M	Irritable Bowel Syndrome - Mixed
ICC	Interstitial Cells of Cajal
IGLE	Intraganglionic Laminar Ending
IHC	Immunohistochemistry
IJP	Inhibitory Junction Potentials
ILK	Integrin Linked Kinase
IMA	Intramuscular Array

IMF	Intrinsic Mesenteric Ganglia
IPSP	Inhibitory Post Synaptic Potential
JaCOP	Just Another Colocalisation Plugin
JG	Jugular Ganglia
JHM	Joint Hypermobility
JHS	Joint Hypermobility Syndrome
KO	Knockout
LD-RPCs	Long Duration-Rhythmic Phasic Contractions
LOS	Lower Oesophageal Sphincter
LSN	Lumbar Splanchnic Nerve
MC	Myoelectric Complex
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
MMP	Matrix Metalloprotease
mRNA	Messenger RNA
NA	Nucleus ambiguous
NANC	Non adrenergic non cholinergic
NE	Norepinephrine
NG	Nodose Ganglia
NK	Neurokinins
NKCC	Na/K co-transporter
NMDA	N-methyl d aspartate
NOLA	N^{ω}nitro-L-arginine
NOS	Nitric Oxide Synthase

NPY	Neuropeptide Y
NTS	Nucleus Tract Solitarius
OCT	Optimal Cutting Temperature
OR	Odds Ratio
PBS	Phosphate Buffered Saline
PDB	Pee Dee Belemnite
PFA	Paraformaldehyde
PKA	Protein Kinase A
PKB	Protein Kinase B
PGP	Protein Gene Product
PNN	Peri-neuronal Nets
PoTS	Postural Orthostatic Tachycardia
RLC	Regulatory Light Chain
RNA	Ribonucleic Acid
RPC	Rhythmic Phasic Contraction
RT	Room Temperature
SD-RPCs	Short Duration-Rhythmic Phasic Contractions
SEM	Standard Error of the Mean
SLC	Small Latent Complex
SNP	Single Nucleotide Polymorphism
SP	Substance P
TC	Tonic Contraction
TGF-β	Transforming Growth Factor-β
TNC	Tenascin C

TNR	Tenascin R
TNX	Tenascin X
TNW	Tenascin W
TK	Tachykinin
TTX	Tetrodotoxin
UPC	Ultra Propulsive Contraction
VEGFR-1	Vascular Endothelial Growth Factor Receptor 1
VIP	Vasoactive Intestinal Peptide
VPAC	Vasoactive Intestinal Peptide Receptor
WDR	Wide Dynamic Range
WT	Wild Type
ZIPK	Zipper-Interacting Protein Kinase
5-HT	5 hydroxytryptamine (Serotonin)

1 Literature Review

1.1 Introduction

The term 'functional gastrointestinal disorder' (FGID) classifies several combinations of chronic or recurrent gastrointestinal (GI) symptoms that cannot be explained by structural or biochemical abnormalities seen on endoscopy, x-ray and blood tests (Chang, 2004). FGID affects up to 36% of the general population (Chang, 2004), however, there is no specific geographical distribution (Gwee, 2005) and it is more common in females (67.8%) than males (32.2%) (Chang, 2004), (Schmulson et al., 2010). Although a causal understanding of FGID has not been established, the symptoms associated with FGIDs relate to a combination of several known physiological determinants. These symptoms are common in inflammatory connective tissue disorders such as scleroderma where symptoms are a consequence of immune mediated fibrosis in the neuromuscular layers of the GI tract (Thoua et al., 2012). In contrast little is known about the GI involvement in non-inflammatory connective tissue disorders such as Marfan syndrome, owing to its rare prevalence, and joint hypermobility (JHM) which is more common but under-diagnosed (Remvig et al., 2011). Interestingly, patients who have JHM report more GI symptoms associated with dysmotility in both the upper and lower GI tract (Fikree et al., 2014).

JHM is defined as a condition whereby the joints stretch beyond the normal capacity (Simmonds and Keer, 2007), and when associated with symptoms it is referred to as joint hypermobility syndrome (JHS). Specifically JHS is defined by the presence of symptoms associated with hypermobility in the absence of rheumatologic disease or life threatening complications (Hakim and Grahame, 2004). Recently, a study characterized the range and prevalence of GI symptoms in JHS and found, in patients with GI symptoms, 33% had JHS and of those, 89% experienced bloating, 79% had early satiety and 66% had alternating bowel habits and abdominal pain compared to non JHS (OR: 2.08 CI: 2.08-8.92) (Fikree et al., 2014). This suggests a strong link between GI and JHS. Thus, GI symptoms may arise from an abnormality of the components that make up the connective tissue since JHS is a connective tissue disorder. Importantly, a subset of JHS patients have a deficiency of the extracellular matrix (ECM) protein Tenascin X (TNX) (Bristow

et al., 2005). Case studies in patients with complete TNX deficiency also report a strong GI link, including symptoms of constipation and rectal prolapse (Schalkwijk et al., 2001). What is clear in both upper and lower gut is that there are distinct morphological categories of neuronal structures that underlie their function. However, what we do not know is what extracellular matrix proteins cements them in place relative to each other and or other structures for them to best perform their functions. We also are unaware of how abnormalities in the ECM lead to disease states. Therefore, TNX is an ideal candidate to study the role of the ECM in GI function as its deficiency is associated with GI symptoms.

This chapter will describe the enteric neural control of the stomach and colon based on symptom location and pattern observed in TNX deficient and JHS patients and explain what happens when neural innervation is defected, that give rise to GI symptoms. The second part of this chapter will explore the literature around the role of TNX in the mammalian system.

1.2 Anatomy of the stomach and colon

The stomach, the initial site of digestion consists of the fundus, corpus and antrum (Janssen et al., 2011) and is connected to the oesophagus by the lower oesophageal sphincter. The stomach is connected to the small intestine via the pylorus. When the stomach is empty, the mucosa lies in large folds called rugae (Kang et al., 2013). The muscularis externa contains three muscle layers an outer longitudinal layer, the middle circular layer and an inner oblique layer. The serosal surface enveloping the stomach is composed of simple squamous epithelium. Functionally, between the muscle layers is the myenteric plexus that plays a chief role in gastric motor control.

Lower down the GI tract, the large intestine extends from the small intestine to the rectum and consists of four regions; caecum, colon, rectum and anal canal (Podolsky, 2016). The ileocaecal sphincter at the caecum separates the small and large intestines where the appendix is attached (Podolsky, 2016). The colon is further divided into ascending, transverse and descending colon. The anatomy of the colon wall is described in Fig 1.1 and consists of the same basic layers as the stomach with distinct location dependent differences. The mucosa is composed of columnar epithelium that facilitate water and nutrient absorption, goblet cells that produce mucus

(Jenkins, 2007), (Podolsky, 1989), enteroendocrine cells and enterochromaffin cells that store and sense nutrients (Steinert et al., 2011) (Symonds et al., 2015). Unlike the stomach. The colon consists of the same basic layers as the stomach, except that the colon has a submucous plexus that is involved in secretory function as well as the myenteric plexus (Fig 1.1). The thickness of colonic smooth muscle varies based on the region and species although the arrangement is consistent in most vertebrates (Nilsson and Holmgren, 1983).

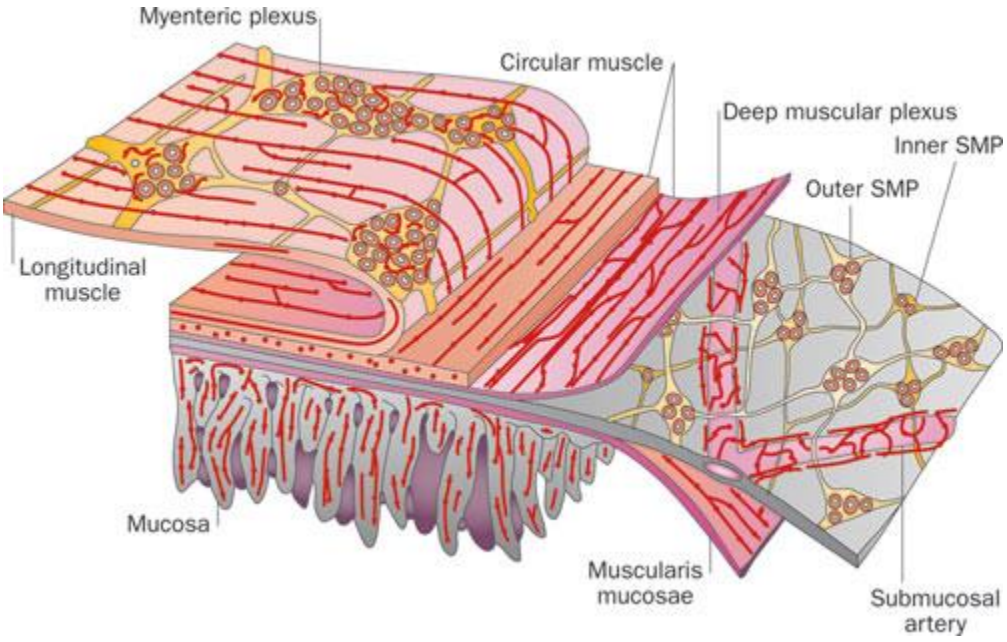


Figure 1.1. Schematic showing organisation of the ENS.

The gut wall consists of the inner mucosa, which is followed by the muscularis mucosa and the submucosa. The submucosa consists of the inner and outer submucosal plexus. The circular and longitudinal muscle follow with the myenteric plexus sandwiched in between, all regions are supplied by extensive neural structures (Furness, 2012).

The same basic structure in the colon extends to the rectum, which is the last portion of the colon. The rectum lies anterior to the sacrum and the rectal terminal is called the anal canal with an opening to the exterior, controlled by the anal sphincter (Jenkins, 2007).

1.3 The enteric nervous system

The human enteric nervous system (ENS) contains around 100 million neurons, which are different in type and distribution depending on the region and species (Hansen, 2003). The ENS consists of sensory, motor and interneurons that form synaptic connections for the flow of information from sensory neurons to interneuronal networks to motor neurons and finally effector neurons (Johnson, 2006). Enteric neurons are bidirectional meaning they communicate to and from the ENS and central nervous system (CNS) (Furness et al., 2014). The ENS forms two major ganglionated plexuses, the myenteric plexus between the longitudinal and circular muscle; and submucous plexus between the circular muscle and mucosa (Johnson, 2006). Both plexuses contain neural cell bodies that are connected to interganglionic nerves projecting to the muscle layers (Johnson, 2006). The myenteric ganglia is found throughout the GI tract while the submucosal ganglia is absent in the stomach and oesophagus but present in the rest of the GI tract (Brehmer et al., 2010). Most motor neurons innervating the circular and longitudinal muscle are found in the myenteric plexus (Costa and Brookes, 1994). The submucous plexus also communicates with the myenteric plexus through axons forming a functionally integrated unit (Costa et al., 2000). The neurons that are found in the myenteric and submucous plexus have been identified in terms of shape, biochemistry and function. Dogiel and La Villa described 6 subtypes of enteric cell bodies and type II is most common (Fig 1.2).

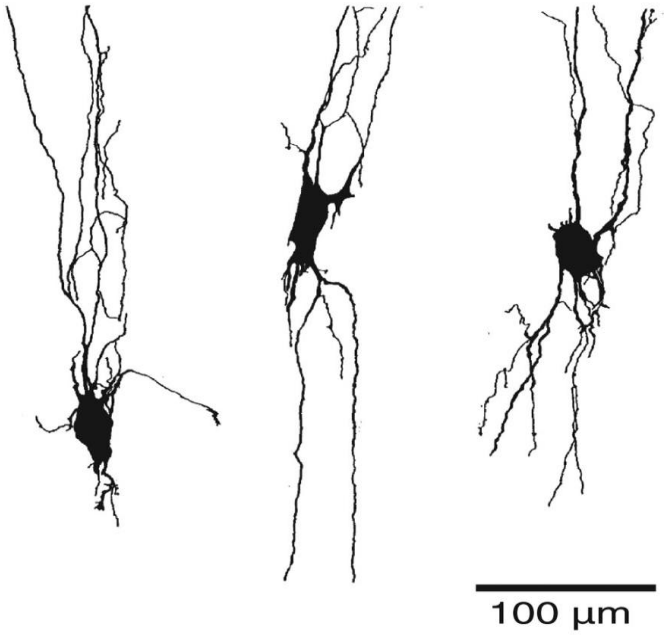


Figure 1.2. Illustration of Dogiel type II neurons

These neurons are easily identified by their rounded cell bodies and long projecting dendrites (Johnson, 2006).

1.4 Neurons defined by function and neurochemistry

The majority of neurochemical and functional studies in the ENS is based on the guinea pig small intestine therefore caution should be applied when extending definitions and classification to all species despite conservation of many neurons in the mammalian system. Recently the rat, mouse and now the human ENS is being characterised with emphasis on regional distribution within the same GI organ.

1.5 Smooth muscle innervation

The smooth muscle layer is supplied by excitatory and inhibitory motor neurons (Brehmer et al., 2010) and pharmacological studies have shown that excitatory neurons contain Acetylcholine (ACh) and tachykinins (TKs) whereas inhibitory motor neurons contain nitric oxide (NO) adenosine triphosphate (ATP) and vasoactive intestinal peptide (VIP) (Sanders et al., 2010).

Accordingly, the presence/absence of these neurotransmitters is used to classify enteric neurons and subsequently their function.

1.6 Inhibitory motor neurons to the circular muscle

The first study to use a neurochemical marker to identify enteric neurons by immunohistochemistry (IHC) used VIP to label circular muscle fibres with cell bodies in the myenteric ganglia of the guinea pig small intestine (Furness and Costa, 1979). In the same region and species, nitric oxide synthase (NOS) immunoreactivity (the enzyme required for NO synthesis) was shown in VIP positive neurons and fibres that extended from the myenteric plexus (Costa et al., 1992). Further studies showed inhibitory motor neurons projecting to circular muscle in both small and large intestine contain VIP and NOS in various species including the mouse (Sang et al., 1997) and human (Wattchow et al., 1997). Retrograde labelling studies have shown NOS neurons to have Dogiel type I morphology (Brookes et al., 1991b). Since the stomach lacks a submucous plexus, nerves originate exclusively from the myenteric plexus. This has been shown in the canine where both inhibitory and excitatory axons degenerated after removal of the myenteric plexus (Furness et al., 1991). Inhibitory neurons project anally whereas excitatory neurons project orally to the muscle (Pfannkuche et al., 1998). NO, NOS inhibitors and scavengers of NO are the main transmitter present in inhibitory neurons innervating both circular and longitudinal muscle (Bult et al., 1990, Li and Rand, 1990, Sanders and Ward, 1992). Human intestinal circular muscle has NOS/VIP positive axons which can contain neuropeptide Y (NPY), this is also true for other species like the rat and mouse (Wattchow et al., 1988).

1.7 Excitatory motor neurons to the circular muscle

Choline acetyl transferase (ChAT) is the enzyme required for the synthesis of ACh and is the primary neurotransmitter to act on muscarinic receptors M_1, M_2 and M_3 (Sander et al., 2006), (Mawe et al., 1986, Wood and Mayer, 1979). ChAT IHC display Dogiel type I morphology in many species including human (Brookes et al., 1991b). Excitatory motor neurons also contain TKs which are thought to mediate the residual component of excitatory muscle function (Holzer and Holzer-Petsche, 1997) (Shimizu et al., 2008). In human circular muscle, excitatory neurons are positive

for both ChAT and TKs (Wattchow et al., 1997), (Brehmer et al., 2006, Wattchow et al., 1997, Porter et al., 1997). These excitatory axons also show immunoreactivity for enkephalins and substance P (SP) which is a TK peptide (Wattchow et al., 1988) that binds to the neurokinin receptor 1 (NK_1) at the neuromuscular junction (Daniel et al., 1995). The proportion of inhibitory and excitatory neurons that innervate the circular muscle is about 30% revealed by retrograde labelling in the human colon (Porter et al., 1997).

1.8 Motor neurons innervating the longitudinal muscle

Similarly, neurons supplying the longitudinal and circular muscle have been defined by immunoreactivity. In guinea pig and mouse, nerve fibres rarely enter the longitudinal muscle itself and instead they adhere to the "tertiary plexus" which is the third component of the myenteric plexus (Furness et al., 2000). In larger mammals this third inner plexus is absent and nerve fibres run parallel to the muscle cells. Cell bodies of motor neurons that innervate longitudinal muscle are smaller than the circular muscle because these neurons have smaller terminal fields (Brookes et al., 1991b, Furness et al., 2000). IHC studies in human, show that some motor neurons can supply both muscle layers, however, the proportion of these axons are unknown (Burleigh, 1990), (Llewellyn-Smith et al., 1984), (Porter et al., 1996). Cell bodies in the longitudinal muscle exhibit Dogiel type I morphology although the diameter is smaller than in the circular muscle (Wattchow et al., 1995).

1.9 Intrinsic sensory neurons

It is widely recognized that the intestine has a remarkable ability to function independently without any extrinsic neural pathways even when dissected and removed from the body (Furness et al., 1998). This phenomenon suggests that there are intestinal neurons that have the ability to sense the physiological state and its environment that in turn produces reflexes. These reflexes could include, contraction and relaxation to aid digestion (tension and length), respond to intraluminal chemical changes as well as to mucosal changes. These autonomous neurons are described as intrinsic primary afferent neurons (IPANs) that can respond either directly or indirectly (Furness et al., 2004b). IPANs are found in and represent around 30% of myenteric

neurons, 14% of submucosal neurons and have a Dogiel type II shape (Costa et al., 1996). IPANs project circumferentially and synapse with other neurons including myenteric ascending/descending interneurons, longitudinal muscle motor neurons, excitatory circular muscle neurons and inhibitory motor neurons (Costa et al., 2000). The function of IPANs has not been conclusively defined as reports suggest an involvement in mechanical stimulation while others have shown this is not entirely true (Spencer and Smith, 2004), (Smith et al., 2007). Other studies suggest a multifunctional role since the mechanosensitive neurons have varied firing rates (Mazzuoli and Schemann, 2009). These 'so called' IPANs are thought to have after hyperpolarising (AH) type of electrophysiological properties, however the neurophysiology of AH neurons does not comply with the definition of sensory neurons and will be further discussed in section 1.14. Dogiel type II neurons contain neurochemicals which differs in region and species, however, in most mammals including the mouse, guinea pig and human neurofilament is present in these type II neurons (Rivera et al., 2009), (Qu et al., 2008), (Brehmer et al., 2002). These sensory neurons primarily contain calcitonin gene related peptide (CGRP) and other excitatory neurotransmitters such as ChAT and TK described earlier. Type II neurons connect with each other forming a functional syncytium whereby activation of one neuron increases the excitability of adjacent neurons (Kunze and Furness, 1999). To initiate a reflex response this large group of connected type II neurons work together and has been described using computer simulation models (Thomas et al., 2004). The transmission in these type II sensory neurons is thought to occur via slow excitatory synaptic potentials with TK influence (Kunze et al., 1993). Type II neurons that innervate the mucosa respond to hormones released by enteroendocrine cells and by peptides such as 5 hydroxytryptamine (5-HT) (Bayguinov et al., 2010) and ATP. There is species variability in the proportion of type II neurons in the myenteric plexus of different species. In the human small intestine, 10% of myenteric neurons are type II and in the mouse these neurons represent 26% (Furness et al., 2004b). In stomach, AH-type neurons are completely absent in corpus (Schemann and Wood, 1989) and very few are seen in the antrum (Tack et al., 1992). The stomach is largely controlled by the vagus nerve providing excitatory function (Schemann and Grundy, 1992) and evidence suggests that the stomach also has intrinsic reflexes (Schubert and Makhlouf, 1993) (Hennig et al., 1997) .

1.10 Interneurons

Interneurons have been studied primarily using lesion experiments, dye filled injection studies and functional studies. These studies describe two types of interneurons; the ascending and descending interneurons (Brookes et al., 1997). Both subtypes have a Dogiel type I morphology in humans (Wattchow et al., 1995). Ascending interneurons project orally to the myenteric plexus and terminate at circular muscle motor neurons through fast nicotinic and non-cholinergic slow synaptic input (Brookes et al., 1997). These interneurons contain ChAT, TKs and opioid peptides (Brookes et al., 1997). Descending interneurons can be subdivided into several classes (Song et al., 1992) but the majority contain ChAT. Other descending interneurons contain NOS and VIP that project aborally and synapse with the myenteric and submucous neurons important for motility and secretion (Wattchow et al., 1995). Studies on interneuron types in the colon (Lomax and Furness, 2000) and stomach (Schemann et al., 2001) of the guinea pig show regional variation. Interneurons are also present in human submucosa but are complex compared to the simpler guinea pig submucosa (Brehmer et al., 2010). 5-HT is found in fibres surrounding the myenteric plexus in the human and can also be used to classify interneurons (Anlauf et al., 2003).

1.11 Secretomotor/vasodilator neurons

Secretomotor neurons innervate secretory glands and periglandular arterioles including goblet cells, crypts of Lieberkühn and Brunner's gland (Johnson and Johnson, 1994). These neurons are important for absorption and secretion of water and electrolytes, and hormone mediated activity (Johnson, 2006). The shape of these neurons are uniaxonal with multiple short dendrites (Johnson, 2006). In the stomach the secretomotor neurons can act on a) parietal cells inducing release of gastric acid and b) chief cells which release pepsinogen (Furness, 2000). Secretomotor neurons are either excitatory that can be either cholinergic or non- cholinergic. Cholinergic neurons release ACh and act on muscarinic receptors on the epithelium, while non-cholinergic neurons contain VIP inducing local reflexes that project to the mucosa and submucosa (Jansson et al., 1970),(Reddix et al., 1994). Some VIP containing neurons also project to the myenteric ganglia, representing integrated control of motility and secretion (Furness, 2000). In guinea pig,

ChAT positive secretomotor neurons contain neuropeptide Y (NPY) and calretinin. The latter is found in the majority of ChAT neurons in many species including human (Beuscher et al., 2014). ACh/calretinin neurons supply the mucosa and submucosal arterioles, while ACh/NPY neurons do not supply the arterioles (Furness, 2000). ACh acts on blood vessels to release NO from the endothelial cells causing an increase in relaxation therefore increasing blood flow enabling stimulated secretion (Vanner and Surprenant, 1996), (Neild et al., 1990). Neurons that cause blood vessel relaxation are called vasodilator neurons (Luo et al., 2010) (Schemann and Mazzuoli, 2010). These neurons can be blocked by tetrodotoxin (TTX) but are unaffected when dennervated from the extrinsic neural supply and myenteric plexus (Vanner and Surprenant, 1991).

Secretomotor/vasodilator neurons respond to non-neural cells for e.g. molecules released from enterochromaffin cells and inflammatory mediators from the immune system. One such molecule is 5-HT released from enterochromaffin cells by mucosal stroking (Vanner et al., 1993).

Axons that reach the myenteric plexus containing VIP extend over several centimetres in an oral/aboral direction (Vanner and Macnaughton, 2004) (Vanner et al., 1993) (Vanner, 2000). These long axons are sensitive to distension of the gut wall. Mucosal responses to stroking is largely attributed by the release of CGRP and SP but to a lesser extent by the neurokinin 3 (NK_3) receptor whereas balloon distension provoked neural reflexes also acts on NK_3 receptors that are independent of CGRP (Patton et al., 2005). The overall effects of secretomotor neurons is to induce secretion of water, sodium chloride and hydrogen carbonate from the intestinal crypts. The vasodilatory effects are important in regulating digestion by increasing blood flow and motility (Reed and Vanner, 2007), (Reed and Vanner, 2003) (Patton et al., 2005).

1.12 Inhibitory/excitatory effects of secretomotor neuron

Secretomotor neurons can be blocked by inhibitory inputs described in electrophysiology, as inhibitory postsynaptic potential (IPSPs). Inhibitory effects are achieved by hyperpolarising the membrane potential reducing the likelihood of firing which suppresses mucosal secretion. In guinea pig, postganglionic neurons from the sympathetic nervous system contribute to inhibitory secretomotor function by releasing norepinephrine which acts on α_2 adrenoceptor (North and

Surprenant, 1985). Moreover inhibiting secretomotor firing prevents excitatory neurotransmitters from being released on the epithelium to suppress secretion. Excitatory receptors on secretomotor neurons include M1 receptors (North et al., 1985), $P2Y_1$ (Hu et al., 2003), NK_1 or NK_3 (Burcher and Bornstein, 1988) (Frieling et al., 1999) (Johnson et al., 1994b) and 5-HT_3 and 5-HT_2 (Frieling et al., 1991) (Camilleri et al., 2001). 5-HT is an important regulator in secretion and increased release causes diarrhoea. Indeed, IBS patients have elevated mast cell and enterochromaffin cells and blocking 5-HT_3 using an antagonist provides a treatment for diarrhoea predominant IBS in women (Camilleri et al., 2000) (Chey et al., 2001, Bose and Farthing, 2001) (O'Sullivan et al., 2000).

1.13 Intestinofugal neurons

The cell bodies of intestinofugal neurons are found in the enteric plexi, with axons that project away from the intestine and synapse at the prevertebral ganglia. These axons then run back to the stomach and intestine bypassing the CNS, this is called the entero-enteric reflex pathway (Furness, 2006). This pathway is involved in regulating gastric function by slowing gastric emptying, acidifying the upper GI tract and slowing colonic transit to allow complete digestion (Furness, 2006). Intestinofugal neurons provide the fast excitatory postsynaptic potentials (EPSPs) in prevertebral ganglia (Furness, 2006). Both IHC studies and pharmacological transmission analysis indicates these neurons are cholinergic (Mann et al., 1995), (Johnson et al., 1994a). Moreover, VIP is also present in these neurons and others contain NOS (Mann et al., 1995), (Lindh et al., 1988).

As well as immunohistochemical classification, enteric neurons have been categorised based on their electrophysiological properties. These were first identified in the guinea pig using intracellular microelectrodes. Two types of enteric neurons have been identified, AH (after hyperpolarising / Dogiel type II) and S (synaptic) /type Dogiel type I neurons (Nishi and North, 1973), (Hirst et al., 1974). S type neurons are uniaxonal and receive fast excitatory postsynaptic potential (fEPSP) whereas AH-type display a prolonged AH post a single somal action potential (Spencer and Smith, 2004).

1.14 AH-type neurons

AH neurons are interconnected to form a circuit whereby stimulation of one neuron excites neighbouring neurons (Bertrand et al., 2000b) The circuit is described as positive feed-forward circuit which enables the simultaneous firing of neurons within the entire circuit along the segment of the bowel. This driver circuit in turn can simultaneously stimulate muscle motor neurons in different areas to form organised propulsion of luminal contents. This circuit can output to the secretomotor neurons to induce mucosal secretion. The communication between the submucosal and myenteric plexus together provide coordinated secretion and contraction allowing efficient peristalsis (Song et al., 1991).

Cell bodies of AH neurons are flat, coin-like and connected by neurites communicating with one another (Johnson and ScienceDirect (Online service), 2006). The axons from the rounded cell bodies project through the circular muscle and innervate the epithelium. The epithelial endings contain $5HT_3$ receptors (Furness et al., 1998) (Bertrand et al., 2000a), (Kunze et al., 1997), ATP and (Bertrand and Bornstein, 2002) HCL (Kunze et al., 1995) (Song et al., 1994). The AH neurons respond to muscle stretch where action potential discharge is observed (Kunze et al., 1998). Therefore myenteric AH neurons are responsive to intrinsic chemical and mechanical stimulation important in both ascending and descending reflexes (Kunze et al., 1999). Gating function of AH-type neural cell bodies are attributed to activation by paracrine mediators and slow excitatory input from adjacent neurons (Johnson and ScienceDirect (Online service), 2006). AH neural gates are fully opened by excitatory synaptic input which results in synchronous firing, whereas when cell soma are inexcitable the gates close. The control of gating regulates the propagation of action potentials in the network of axons that surround the AH neurons (Wood, 2011). The membrane of the AH neurons cell body can exist in different states such as the intermediate, excitable and hyper excitable state. The state of these neurons is affected by various excitatory mediators such as histamine, proteases, and corticotrophin releasing factor (CRF) (Gao et al., 2002), (Liu et al., 2003) (Liu et al., 2010).

AH-type neurons are electrophysiologically distinct from S-type neurons. AH- type neurons have higher resting membrane potential and require lower input resistance than S-type neurons (since

S-type neurons have smaller cell bodies) (Nurgali, 2009). Excitability in the resting state is low since intracellular injection of electrical current does not provoke an action potential (Nurgali, 2009). The action potential shows a prolonged AH followed by a upstroke caused primarily by TTX sensitive sodium (Na+), TTX resistant Na+ and calcium (Ca^{2+}) currents found in Dogiel type II neurons (Nurgali, 2009) and a 'shoulder' in the falling phase which reflects opening of N-type voltage dependent Ca^{2+} channels (Katayama et al., 1979).

Potassium K+ is the primary ion that determines the resting membrane potential which is normally between 20-30mV less negative than the potassium potential of -90mV (Zholos et al., 2002). The resting membrane potential of AH neurons are dependent on free flowing intracellular Ca^{2+} (Akasu and Tokimasa, 1989), (Grafe et al., 1980), (North and Tokimasa, 1987), (Tatsumi et al., 1988). Three types of Ca^{2+} activated K+ channels are generally found in neurons; large/intermediate/small conductance (Johnson, 2006). In AH neurons the intermediate single channel conductance is also called the IK channel which is the primary functional Ca^{2+} activated K+ channel in AH neurons (Vogalis et al., 2002b), (Vogalis et al., 2002a), (Furness et al., 2004a), (Furness et al., 2003). Various neurotransmitters can act to either excite/inhibit the Ca^{2+} activated K+ conductance in AH neurons (Grafe et al., 1980). Inhibitory signals include opioid peptides, galanin and adenosine that increase K+ whereas excitability is induced by SP, 5-HT and histamine by reducing K+ membrane potential and depolarising the membrane (Johnson et al., 1980), (Starodub and Wood, 2000).

1.15 S-type neurons

S-type neurons all exhibit nicotinic fEPSP (<50ms duration) (Nurgali et al., 2004) and are distinct from AH neurons since they have 1) lower resting membrane potentials and higher input resistance (Nurgali, 2009); 2) elevated excitability since microelectrode electrical current injection causes spontaneous discharge of action potentials although the degree of firing depends on how depolarised the neuron is; 3) the degree of membrane depolarisation determines how often neurons repeatedly fire, unlike AH neurons; 4) (Wood, 2011) ; 5) somal spikes are abolished using TTX that block Na+ channels; 6) insensitivity to forskolin stimulated

adenylate cyclase, increases in cyclic adenosine monophosphate (cAMP) and multivalent Ca^{2+} entry blockers.

The rate and amplitude of spikes are depleted in lowering levels of Na+, however, at rest the S-type neurons spontaneously fire during long lasting depolarisation unlike the AH neurons (Spencer and Smith, 2004). S-type neurons are characteristic Dogiel type I uniaxonal neurons that are musculomotor, secretomotor and interneurons (Browning and Lees, 1996).

1.16 Sensory mechanoreceptors

Mechanoreceptors have the ability to control and sense the motile function of the gut in response to distension i.e. stretch and tension and as luminal contents brush against the mucosal surface. It is unclear whether smooth muscle and mucosal mechanoreceptors have cell bodies in the dorsal root ganglia (DRG) and or within the enteric vasculature (Blackshaw et al., 2007) (Wood, 2010).

High threshold mechanoreceptors are unmyelinated C-fibres that respond to balloon distensions at specific thresholds whereas low threshold splanchnic afferents are myelinated α-delta fibres that respond to innocuous distension volumes (Song et al., 2009). It is thought that non painful symptoms such as post prandial fullness and bloating are derived from low threshold afferents (Wood, 2011). High threshold afferents correlate to consciously perceived intense pain exhibited for example in the gall bladder (Cervero and Laird, 1999). Intensity of symptoms is based on the proportion of both high and low mechanoreceptor activation (Cervero and Laird, 1999) (Cervero and Janig, 1992). High threshold nociceptive fibres are indicated in acute visceral pain whilst chronic visceral pain is attributed to mechanoreceptor sensitisation by inflammatory molecules and ischemia in both high and low threshold mechanoreceptors (Wood, 2008).

1.17 Synaptic transmission

Chemical signalling in the ENS is parallel to signalling patterns in the nervous system and neurotransmitters stored in vesicles at synaptic terminals are released by Ca^{2+} triggered exocytosis (Wood, 2011). Opening and release of voltage gated calcium channels is triggered by the depolarisation of action potentials when they arrive at the release terminals (Grundy et al., 2006). Once released they bind to their postsynaptic receptors which evoke either ionotropic (neurotransmitters that are directly coupled to the ion channel) or metabotropic synaptic events (opening and closing of ion channels through a secondary component such as guanosine triphosphate binding proteins and cytoplasmic second messengers such as inositol) (Wood and Kirchgessner, 2004), (Wood, 2011) . Two kinds of synaptic events occur in the ENS, inhibitory IPSPs and fast or slow EPSPs neurons. Initial work by Hirst et al showed that fast EPSPs lasting

less than 50ms were exclusively found in S-type myenteric neurons (Hirst et al., 1974). Further work reported that fast EPSPs can also occur in AH neurons in myenteric and submucous neurons (Wood, 2011). Fast EPSPs are described as the sole mode of transmission between vagal efferents and enteric neurons mediated primarily by ACh acting at post synaptic nicotinic receptors. Other actions behave like fast EPSPs such as the interaction of 5-HT at the 5-HT_3 serotonergic receptor and purine nucleotides at the P2X purinergic receptors. Fast EPSPs can be either serotonergic or purinergic alone or reflect a combination of both input (Galligan et al., 2000), (Ren et al., 2003), (Galligan and North, 2004), (Nurgali et al., 2003a).

Chemicals from neurocrine, paracrine and endocrine systems primarily signal to the enteric cell bodies. Neurocrine signalling (i.e. synaptic transmission) is described as the transmission of chemicals from one neuron to the next. Slow neurocrine signalling can be either in the form of slow EPSPs and IPSP. Paracrine signal arise can arise from enterochromaffin cells, mast cells and macrophages that release substances that reach the neurons by diffusion through non-neuronal cells that are within the vicinity (Wood, 2011). Slow neurocrine transmission can be in the form of IPSPs or slow EPSP. Slow EPSPs are found in cell bodies of the gastric antrum but not the corpus, pancreas or gallbladder (Wood and Mayer, 1978), (Hirst and McKirdy, 1975), (Tack and Wood, 1992a).

1.18 Afferent innervation

1.18.1 Vagal afferents

Cell bodies of vagal afferents reside in the nodose or jugular ganglia (JG) and have central projections that terminate in the nucleus tract solitarius (NTS) located in the brainstem (Fig 1.3) (Berthoud et al., 2004). The nodose ganglia is derived from neural crest cells of the postotic hindbrain whereas the JG arises from the epibranchial placodes (Surdenikova et al., 2012). Vagal afferents project for millimetres in length within the myenteric plexus before leaving the gut through organ specific branches. Vagal afferent fibres extend from the serosa all the way to the mucosa with specialised nerve endings (Wank and Neuhuber, 2001). The morphology of these vagal afferent endings have been established using anterograde labelling studies (Clerc and

Mazzia, 1994), (Neuhuber, 1987), (Berthoud et al., 1992), (Phillips et al., 1997). There are two types of vagal afferent endings in the stomach, intramuscular arrays (IMA) and intraganglionic laminar endings (IGLE) (Phillips and Powley, 2000). IGLEs are lamellar structures that innervate nearly all myenteric neurons in the oesophagus and 50% of the gastric myenteric neurons (Berthoud et al., 1997). In guinea pig stomach, IGLEs associate with connective tissue sheaths surrounding the myenteric ganglia that protect neurons from excessive mechanical stimulation (Zenker and Neuhuber, 1990). The highest density of IGLEs are found in the myenteric ganglia of the stomach (Berthoud and Powley, 1992). Whole mount immunohistochemistry of guinea pig stomach correlated IGLEs with sites of mechanotransduction therefore IGLEs are described as mechanosensory endings that respond to low stretch (Zagorodnyuk and Brookes, 2000), (Zagorodnyuk et al., 2001), (Zagorodnyuk et al., 2003) Although the role of IMAs is less clear they are thought to be tension receptors with axons that extend up to several millimetres parallel to the circular and longitudinal muscle (Phillips and Powley, 2000). Within the muscle layer, IMAs run parallel to the muscle associated with ICCs (Phillips and Powley, 2000), (Berthoud et al., 1990), (Fox et al., 2000), (Berthoud and Powley, 1992), (Zagorodnyuk et al., 2001). Calretinin is a calcium binding protein and a marker for the vagal IGLE/IMA terminals in the gastric myenteric plexus (Castelucci et al., 2003). Vagal mucosal afferent endings in the stomach project to the villi and end at the lamina propria close to endocrine cells, which may release NO (Page et al., 2009). The density of IMAs reduce distally along the GI tract with the highest density of IMAs found in the pyloric sphincter. There are almost no IMAs past the sphincter whereas, the IGLEs continue to extend to the proximal colon only. The reason for such region specificity is unclear, however in neurotrophin-4 KO mice the number of IGLEs were mostly absent in the small intestine whereas IMA distribution was unchanged in the KO vs WT stomach, therefore a lack of neurotrophin-4 causes selective vagal afferent loss dependent on specific gut regions (Fox et al., 2001), suggesting neurotrophin-4 is important in the development of the vagal afferents influencing where they terminate.

1.18.2 Spinal afferents

The cell bodies of spinal afferents are located in the DRG (Fig 1.3) that arise from neural crest cells at the thoracic level (Weston and Thiery, 2015). Spinal afferents have terminal endings that innervate the myenteric plexus, submucosa and mucosa (Grundy, 2002) and are also closely associated with blood vessels within the mesentery and submucosa (Zagorodnyuk et al., 2010). Different regions of the gut are supplied with specific spinal afferents, firstly the gastroesophageal region is innervated by afferents originating from thoracic spinal and greater splanchnic nerves that span the spinal cord from the cervical to the upper lumbar (C1-L2) segment (Berthoud et al., 1995). Secondly the thoracic and lumbar spinal cord innervate the small intestine via the greater splanchnic nerve (Khurana and Petras, 1991), (Clerc, 1983), (Collman et al., 1992), (Brtva et al., 1989). Thirdly colonic innervation is primarily by the splanchnic nerves, thoracolumbar DRG and the rectum via the pelvic nerves and the lumbosacral DRG (Blackshaw et al., 2007). The spinal innervation of the rectum is similar to that of the upper gut in that IGLE like structures are present since they densely innervate the myenteric plexus and have a similar architecture (Lynn et al., 2003).

CGRP is predominantly (90%) expressed in spinal afferents in rodents (Dutsch et al., 1998), (Uddman et al., 1995), (Green and Dockray, 1987) therefore is regarded as a selective marker for the spinal afferents in the oesophagus. CGRP staining using whole mount IHC reveals fine varicose fibres with punctate bead like staining rather than lamellar structures seen with the IGLEs (Green and Dockray, 1987).

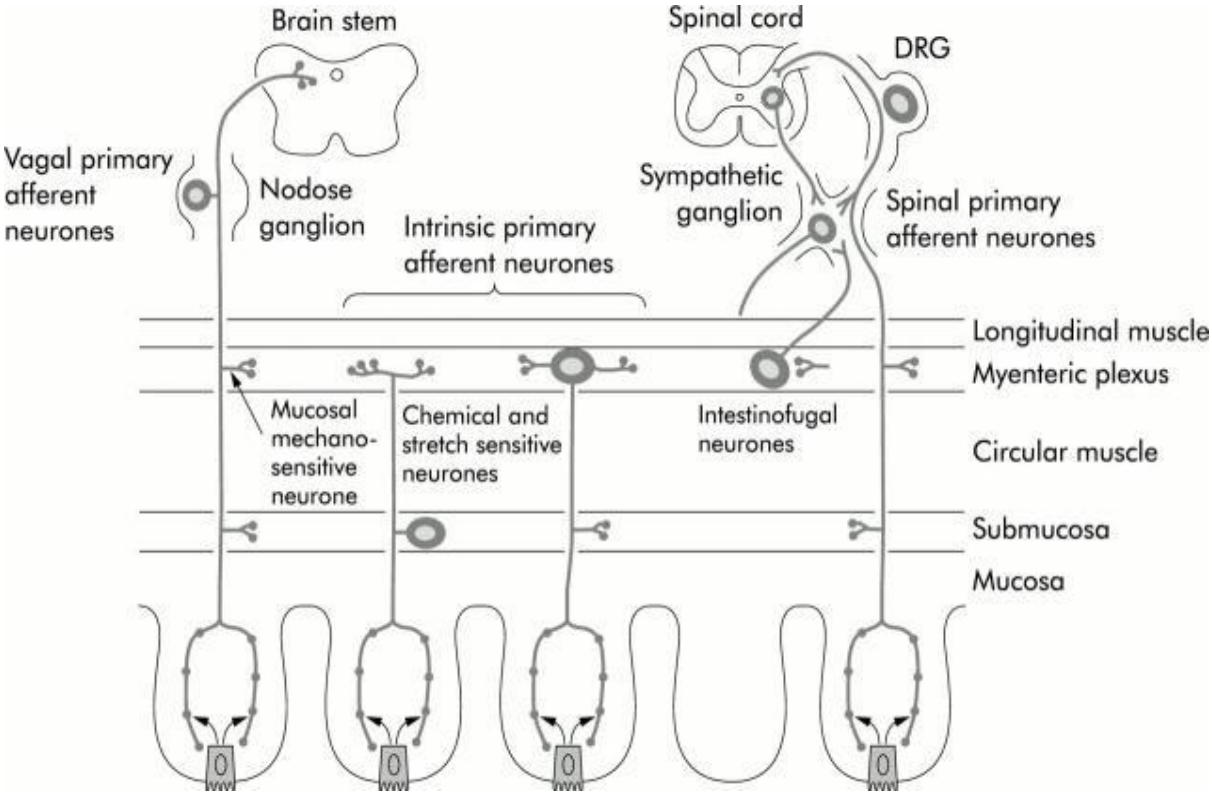


Figure 1.3. Arrangement of the vagal and spinal afferent neurons.

The vagal and spinal neural endings terminate at various layers of the GI tract. Vagal afferents have their cell bodies in the nodose ganglion (NG) whereas the spinal afferents have the cell bodies in the dorsal root ganglion (DRG). Taken from Grundy 2002.

1.19 Structure and functional roles of afferent endings

1.19.1 Mucosal afferent endings

Vagal afferent endings that innervate the oesophagus and gastric mucosa can be repeatedly activated by mucosal stroking. This repeated mucosal stroking in the receptive fields results in a brief burst of action potentials in cat (Clarke and Davison, 1978), (Davison, 1972), ferret (Iggo, 1957a), (Blackshaw and Grundy, 1993b) and mouse (Page et al., 2002). During resting states these afferents are not responsive but are activated post-acute inflammation or mucosal damage likely from the release of 5-HT (Blackshaw and Grundy, 1993a). Mucosal afferents are unresponsive to mechanical stretch and contraction however respond to other stimuli including changes in pH,

osmotic and ionic stimuli (Clarke and Davison, 1978), (Iggo, 1957a), (Page et al., 2002), (Lynn and Blackshaw, 1999). In the stomach and small intestine, mucosal afferents are involved in satiety, nausea and vomiting (Johnson and ScienceDirect (Online service), 2006). Mucosal afferents also send signals to control feedback processes during gastric emptying whereby chyme is not released into the duodenum until trituration of the solid meal is complete (McIntyre et al., 1997). This is important since the stomach does not have abundant intrinsic sensory neurons therefore heavily relies on vagal reflexes (Tack and Wood, 1992b).

In the small intestine, the response of intrinsic sensory neurons to mechanical stimuli suggests a supporting role for enterochromaffin cells to release neurotransmitters, nonetheless, this is unlikely for extrinsic mucosal afferents as responses are observed in calcium free environment and continue to respond without rundown, which would not be the case if epithelial molecules were depleted (Berthoud et al., 2001). In the large intestine few recordings have been made from mucosal afferents of the splanchnic innervation, for example 4% of total fibres were recorded in the mouse (Brierley et al., 2004) and 23% in the rat (Lynn and Blackshaw, 1999) unlike the upper gut where 50% of afferents are mucosal (Page et al., 2002), (Page and Blackshaw, 1998).

Mucosal afferents are found in larger proportion in the pelvic region (distal colon and rectum), for example in the mouse, mucosal afferents constitute 23-38% (Brierley et al., 2004), (Hughes et al., 2009). Consequently, these afferents have a stronger signal to the CNS which is in line with the distal colon and rectum since information regarding stool consistency and transit becomes increasingly important as the materials pass down the colon for expulsion. Thus, pelvic mucosal afferents are important in regulating defecatory processes which allow conscious perception for urgency to defecate. This is also true for mucosal receptors supplying the anal canal shown *in vivo* (Janig and Koltzenburg, 1991).

1.19.2 Tension receptors

Unlike silent mucosal afferents, tension receptors have a resting discharge of action potentials *in vivo* that are mechanosensitive to distension and contraction (Page and Blackshaw, 1998). These receptors slowly adapt in a linear fashion to increases in gut wall tension (Cottrell and Iggo, 1984), (Blackshaw et al., 1987), (Sengupta et al., 1989). Tension receptors relay information to the CNS

about the amplitude and direction of contractions which in turn triggers reflexes for normal GI functions such as motility. As described earlier these tension receptors are possibly IMAs (Phillips and Powley, 2000) and definitively IGLEs that are ideally placed around myenteric neurons which can sense force generated by the smooth muscle from distension or contraction in an 'in-series' manner (Blackshaw et al., 1987). In the ferret corpus, it has been shown that vagal stimulation causes a tonic contraction followed by a long lasting relaxation (Blackshaw et al., 1987). Under isovolumetric conditions afferent discharge increases alongside intraluminal pressure followed by a prolonged decrease, whereas under isobaric conditions the response is unchanged (Blackshaw et al., 1987). This means that the firing rate of tension receptors does not significantly change when a normal meal is ingesting due to accommodation but instead is more responsive to excessive food intake and signalling sensation of satiety and fullness. In the oesophagus, tension receptors have a dual role acting as 1) low threshold mechanoreceptors and 2) nociceptors involved in pain signalling activated by noxious mechanical changes (Kollarik et al., 2007). However, the oesophagus has the ability to reach intraluminal pressures of 200mmHg without any pain sensation (Wang and Powley, 2000).

1.19.3 Muscular afferents

Spinal muscular afferents have large saturation points in terms of firing frequency, well above the maximal range of firing frequency of vagal tension receptors which is 40-50 mmHg maximally (Sengupta et al., 1990). These two populations are described as low threshold (tonic or wide dynamic range; WDR) mechanoreceptors and high threshold (HT; phasic) mechanoreceptors, respectively. They can be discriminated by the shape of the stimulus response function and survival post vagal afferent degeneration (Rong et al., 2004). WDR mechanoreceptors respond to increasing contractions and distension with increasing gut wall tension (Sengupta et al., 1990). Since the response to gastric distension is very high they are likely to be involved in the perception of pain and discomfort, which are common symptoms in IBS (Ozaki and Gebhart, 2001). Both WDR and HT spinal mechanoreceptors are thought to contribute to cramping and colic like symptoms. WDR muscular mechanoreceptors in the pelvic and splanchnic nerves have low thresholds and are more responsive to mechanical stimulation, showing slow adaptation

when distending, therefore supporting the lack of input from the pelvic nerves to nociception. These are a group of receptors that have dual function and are described as both tension and mucosal receptors based on the mechanosensory response (Johnson and Ghishan, 2012).

1.19.4 Serosal and mesenteric afferents

Serosal and mesenteric afferents are determined by their receptive fields (Blumberg et al., 1983), (Bessou and Perl, 1966), (Morrison, 1973). Splanchnic afferents exclusively innervate the mesentery although both splanchnic and pelvic nerves are activated by serosal probing in mouse (Brierley et al., 2004). Serosal afferents are more sensitive to mechanical stimulation of the serosal surface than the mucosal surface (Lynn and Blackshaw, 1999). Both serosal and mesenteric afferent endings have receptive fields in close proximity to or on blood vessels (Janig and Koltzenburg, 1991). These afferents respond to distortion of the colon with small receptive fields but not innocuous circular stretch or mucosal stroking probing (Lynn and Blackshaw, 1999), (Hicks et al., 2002). Serosal afferents are HT afferents that respond to mechanical lateral stretch of 7g and > 45 mmHg pressure (Brierley et al., 2009), (Brierley et al., 2008), (Hughes et al., 2009). Mesenteric afferents are similar to serosal afferents in that they respond to distension in an adaptive manner as do the serosal afferents in high noxious state (Blumberg et al., 1983), (Brierley et al., 2009),(Brierley et al., 2008). Serosal/mesenteric afferents account for 50% and 86% of afferents recorded from lumbar splanchnic nerves (LSN) and 33% from pelvic nerves recorded in electrophysiology experiments. (Brierley et al., 2004), (Hughes et al., 2009), (Lynn and Blackshaw, 1999), (Hicks et al., 2002).

1.20 Neural control: stomach

The stomach is supplied by afferent and efferents, cell bodies of the efferent vagal neurons are located in two nuclei: the dorsomotor vagal nucleus (DMVN) and the nucleus ambiguus (NA) (Grundy and Brookes, 2012). The NA provides motor innervation to the oesophagus and the upper oesophageal sphincter while the DMVN gives rise to branches of the vagus nerve that supply the oesophagus to the proximal colon (Grundy and Brookes, 2012). These afferents relay information to the brainstem about the status of the gut and primarily use glutamate as the

neurotransmitter (Lachamp et al., 2003). Release of glutamate in the NTS stimulates both N-methyl d aspartate (NMDA) and non-NMDA receptors, whereas its release from sensory afferent fibres is subject to modification, for example by CCK (Simasko and Ritter, 2003), hormonal agonists and ATP (Jin et al., 2004). Post-synaptic parasympathetic neurons in the stomach can be either excitatory cholinergic releasing ACh or inhibitory nonadrenergic noncholinergic (NANC). The combined role of the vagal efferents/afferents and the NTS is termed the vago-vagal reflex (Chang et al., 2003). Simply, the sensory vagal afferent pathways initiate the reflex, in turn activating second order neurons within the NTS through the actions of glutamate and NMDA and non-NMDA receptors. The NTS neurons control gastric functions by utilizing several different neurotransmitters completing the vago-vagal loop (Travagli et al., 2006). Cells of the DMVN project to neurons in between the gastric smooth muscle and myenteric plexus that connect with interstitial cells of Cajal (ICC) (Berthoud et al., 2001). The ICCs are described as pacemakers of the gut, where they show distinct regional variations from the fundus to the pylorus (Komuro, 2006). The DMVN neural projections to the fundus have a smaller soma size compared to the corpus, antrum, duodenum and the caecum (Huang et al., 1993). The DMVN gastric projecting neurons can be distinguished from intestinal DMVN neurons by their smaller and faster AH compared to the larger slower AH of the intestinal DMVN (Browning et al., 1999). This suggests that DMVN projecting gastric neurons are more likely to be influenced by changes in the membrane potential from synaptic inputs than intestinal DMVN (Travagli and Gillis, 1994). The myenteric plexus of the stomach therefore is sensitive to slight changes from either the cholinergic excitatory pathway or by NANC inhibitory pathway of the DMVN. Recordings from electrophysiological studies show that the NTS signals to the DMVN through the glutamergic pathway as well as GABAergic (gamma-aminobutyric acid) and catecholaminergic pathways (Rogers et al., 2003). Studies have shown both glutamate and catecholamine agonists applied to the DMVN produce significant effects on motility and tone of the stomach, where glutamate causes gastric excitation and norepinephrine (NE) causes gastric inhibition (Sivarao et al., 1999),(Pagani et al., 1987),(Khachatryan et al., 2010). The majority of DMVN neurons are thought to be cholinergic and thus express ChAT (Armstrong et al., 1990) however some have shown NOS immunoreactivity important in the inhibitory effect of vagal input (Zheng et al., 1999).

Postganglionic excitatory neurotransmission acting on gastric smooth muscle, ICCs and parietal cells heavily rely on ACh acting on muscarinic receptors. When these receptors become activated, smooth muscle and ICC depolarises driving peristalsis (Wood, 1984). Conversely, NOS and VIP are thought to be the main neurotransmitters involved in inhibitory postganglionic transmission with 5-HT having lesser effect (Berthoud et al., 1995).

The sympathetic control of the stomach comes from preganglionic neurons located in the thoracic spinal cord (T6-T9) which project to the postganglionic neurons in the celiac ganglion outside the CNS (Travagli et al., 2006). Spinal sensory neurons provide input at both levels and have cell bodies in DRGs, and peripheral sensory endings found in the gut wall. Sympathetic post ganglionic endings target the myenteric plexus, with sparse innervation of the muscle layers except for the lower oesophageal sphincter, ileocaecal junction and anal sphincter where the innervation of the circular muscle is dense (Elfvin and Lindh, 1982). Although sympathetic effects on gastric motility are inhibitory, this is modest, since cutting the sympathetic nerves does not have a major effect on motor function (Lomax et al., 2010).

The isolated stomach can function independently of the brain and spinal cord and display both inhibitory and excitatory responses to stimulation of the muscle or mucosa (Hennig et al., 1997), (Desai et al., 1991, Paton and Vane, 1963) and unsurprisingly, the stomach contains the main neurotransmitters which mediate and modulate the reflex responses for gastric accommodation (Grundy et al., 1986). Majority of intrinsic gastric motor function is achieved by uniaxonal neurons with cell bodies in the myenteric plexus (Schemann et al., 2001). These myenteric uniaxonal neurons primarily function as motor neurons or interneurons (Schemann et al., 1995). Various markers have been used to functionally classify enteric neurons described in table 1. Calbindin is a calcium binding protein, which stains nerves with long processes important for interneuronal function (Table 1) (Reiche and Schemann, 1999). Based on immunohistochemical characterization studies on guinea pig, NOS (nitrergic) and ChAT (cholinergic) are the two main enzymes found in the myenteric neurons of the stomach that regulate motility (Schemann et al., 1995). In the fundus and corpus there are 40% more cholinergic neurons than nitrergic neurons (Schemann et al., 2008). In all regions of the stomach, ascending pathways predominantly

contain cholinergic motor neurons and descending projections containing nitrergic motor neurons (Pfannkuche et al., 1998). This pattern is also observed in inter-neurons of the stomach (Pfannkuche et al., 1998). Electrophysiological data on the myenteric plexus of the stomach differ from the intestine and colon. The main difference is the absence of AH neurons in the corpus of the stomach (Schemann and Wood, 1989). The behaviour of gastric myenteric neurons is either phasic or largely tonic S (spontaneous firing) termed gastric I and II neurons, respectively (Schemann and Wood, 1989). Synaptic transmission depends on fast EPSPs, be it intrinsic from the myenteric plexus or extrinsic from the vagal afferent fibres. These potentials can be blocked by hexamethonium (Tack and Wood, 1992a).

Extrinsic afferent innervation has been extensively explored using both animal and human extracellular recordings (Page and Blackshaw, 1998). The vagal afferents are important for vago-vagal reflexes that control gastric accommodation, peristalsis of the antrum and gastric inhibition (Andrews et al., 1980) as well as conscious sensations such as post-prandial fullness and nausea (Cervero, 1994). It has been demonstrated that the vagal afferents in the stomach are low threshold fibres and the mechanoreceptor threshold lies outside the nociceptive range (Ozaki et al., 1999). The function of these vagal mechanoreceptors can be divided by region, for example, the antrum is responsive to both stretch and contraction when the stomach is distended while the fundus and the upper part of the corpus mostly responds only to stretch (Takeshima, 1971),(Andrews et al., 1980). Extracellular recordings show that these functions can be attributed to a single class of vagal mechanoreceptors (Andrews et al., 1980), (Blackshaw et al., 1987).

Gastric emptying of a meal into the small intestine can be controlled by various factors including satiety, frequency of eating, and the chemical composition of chyme which affects the tone of the stomach and pylorus. An important factor in controlling gastric emptying is CCK, which activates vagal afferents to evoke reflex relaxation (Olsson and Gibbins, 1999). Other hormones and neurotransmitters such as noradrenaline, ACh, TK, 5-HT, VIP motilin, CGRP and galanin can also affect the rate of gastric emptying (Torsoli and Severi, 1993, Daniel et al., 1994), although it is not clear via which pathway they are involved.

1.21 Neural control: colon

The ENS plays a direct role in regulating colorectal motility by activating enteric plexi and interneurons (Johnson, 2006). The parasympathetic and sympathetic controls have a significant influence on colonic function; however, the ENS through enteric neuronal circuits finally mediates these pathways (Johnson, 2006).

The spinal pathway comprises of extrinsic efferent parasympathetic and sympathetic systems in reflex loops (Brookes et al., 2009). Most of the colon is innervated extrinsically by spinal neurons that travel with sympathetic and parasympathetic sacral nerves from the spinal cord (Sanders and Smith, 1986). The sacral parasympathetic ganglia interact with the enteric neurons in the colon and rectum mostly through nicotinic cholinergic pathways (Olsson et al., 2004).

The spinal afferents reach the colon via splanchnic nerves that travel through the prevertebral sympathetic ganglia and reach the colon via the lumbar colonic nerves (Brookes et al., 2009). Electrophysiology recordings of splanchnic nerves of mouse colon report 86% of these nerves terminate in either the mesentery or the serosa and require harsh probing with Von Frey hairs (Porter et al., 1997). These endings are described as high threshold afferents and can also be activated by strong contractions of colonic muscularis externa. These strong contractions can spasm and therefore induce pain (Brookes et al., 2009).

The sympathetic pathways innervating the colon have various functions including control of blood flow, secretory activity through the submucosal plexus, and colonic motility through the myenteric plexus (Brookes et al., 2009).

The cholinergic excitatory motor neurons that project to the circular and longitudinal muscle express tachykinin peptides such as SP and neurokinins (NK) (Lecci et al., 2006). The NK_1 and NK_2 subtypes are expressed in the circular muscle of the colon and activate contractile activity (Lecci et al., 2006). In mouse colon, TKs acting via NK_1 and NK_2 receptors also play an important role in the formation of colonic migrating motor complexes (CMMC) (Brierley et al., 2001). CMMCs have been described in animal models both *in vitro* (Bywater et al., 1998) (Brierley et al., 2001) (Copel et al., 2013), and *in vivo* (Morita et al., 2012) and in isolated human colon (Zagorodnyuk et al.,

2012). CMMCs function to aid intestinal peristalsis throughout the colon (Brierley et al., 2001), and depend on the ENS entirely, since contractions occur in isolated tissue but do not occur in colonic areas that are aganglionic (Wood and Marsh, 1973). CMMCs give rise to motility patterns in the gut by coordinated contraction and relaxation of the smooth muscle layers (Olsson and Holmgren, 2001). Colonic smooth muscle cells form a functional syncytium interconnected by gap junctions to the ICCs (Johnson, 2006).

Motility of the colon is further regulated by the enteric mechanosensitive viscerofugal neurons which project to the prevertebral ganglia, providing excitatory synaptic drive to increase colonic motility (Hibberd et al., 2012).

Various neurochemical markers label enteric neurons according to their function. The sensory neurons form connections through slow synaptic excitation, ascending and descending interneurons also form connected chains and the muscle motor and secretomotor neurons that innervate the muscle and submucosa (Table 4). Table 4 gives an overview of enteric neural labelling taken from IHC studies in the guinea pig, mouse and human in the stomach, small intestine and colon (Hansen, 2003).

Table 1.1. Functional chemical coding of enteric neurons adapted from Hansen 2003 (Hansen, 2003)

Function	Neurochemical coding
Sensory	ChAT, Calb, CGRP and SP
Interneurons, orally directed	ChAT, calret, ENK, SP
Interneurons, aborally directed	5-HT, DYN, GRP,NOS, somatostatin and VIP
Short excitatory muscle motor neurons	ChAT and SP
Long excitatory muscle motor neurons	ChAT, Calret and SP
Inhibitory muscle motor neurons	DYN, ENK, GRP,NOS and VIP
Secretomotor neurons	ChAT, CCK, CGRP,DYN,NPY, somatostatin and VIP
Abbreviations: ChAT-choline acetyltransferase, NOS- nitric oxide synthetase, Calb-calbindin, Calret-calretinin, DYN-dynorphin, GRP-gastrin releasing peptide, CGRP-calcitonin gene related peptide, SP-SP. ENK-enkephalins, 5-HT-5-hydroxytyptamine, NPY-neuropeptide Y, and VIP-vasointestinal polypeptide.	

1.22 Motility disorders of the stomach

The main disorders that affect motility in the stomach are delayed gastric emptying, rapid gastric emptying and functional dyspepsia.

1.22.1 Delayed gastric emptying (Gastroparesis)

Gastroparesis is a motility disorder where there is a delay in emptying food from the stomach into the duodenum in the absence of a mechanical obstruction (Parkman et al., 2004). The cause is unknown and is present in about 25-55% of patients with insulin dependent diabetes (Kong and Horowitz, 1999). Symptoms include nausea, vomiting and occasionally visceral pain (Soykan et al., 1998). Management of gastroparesis is primarily with prokinetics that increase gastric motility (Parkman et al., 2004) and, ghrelin which improves gastric emptying (Tack et al., 2005). Gastroparesis along with early satiety is common in patients with JHS (Levy, 1993).

1.22.2 Rapid gastric emptying (Dumping syndrome)

Dumping syndrome occurs post gastrectomy where ingested food is rapidly released to the small intestine giving rise to symptoms after a meal (Tack, 2007). Symptoms are specific to either early dumping which occurs straight after a meal or late dumping 1-3 hours post-prandially. Early dumping syndrome causes abdominal pain, bloating, nausea, dizziness, syncope, flushing and late dumping includes transpiration, palpitations, hunger, confusion and diarrhoea (Abell et al., 2006).

1.22.3 Functional Dyspepsia

Dyspepsia is broadly defined as experiencing pain in the epigastric region (Tack and Talley, 2013) and functional dyspepsia (most common GI disorder in clinical practice) is defined by the Rome III criteria as having chronic dyspeptic symptoms that are idiopathic (Talley et al., 2005). Although the exact cause of the disease is unknown, studies have shown that slight structural changes may be important to further investigate such as gastritis post Helicobacter pylori infection (Nesland and Berstad, 1985). Functional dyspepsia and reflux was found to be common in JHS patients attending GI clinics. (Fikree et al., 2014).

1.23 Motility disorders of the colon

The two primary symptoms of motility disorders in the colon are diarrhoea and constipation and additional disorders include rectal evacuatory dysfunction and Hirschsprungs disease.

1.23.1 Diarrhoea

Diarrhoea is characterized by frequent, loose/watery stools with the urge to defecate. It is thought that there is an excess of high amplitude propagating contractions (HPAC) in patients with diarrhoea, enhancing the propulsion of stool in the descending colon (Bazzocchi et al., 1991). Studies have shown that diarrhoea results from central stimuli for example, altered brain-gut interactions arising from stress (Konturek et al., 2011) or peripheral stimuli such as infection (Spiller, 2007), inflammation (Cremon et al., 2009) and immune response (Camilleri et al., 2012). Intestinal ion transport is key in regulating fluid balance in the gut namely though chloride

secretion and sodium absorption. When this balance is affected for example by bacterial infection, intracellular mediators such as cAMP and intracellular calcium activate chloride secretion and alters the sodium/chloride balance causing diarrhoea (Fasano, 2002). Furthermore fermentable products of digestion from bacteria in the gut can cause diarrhoea (Corazziari, 2012).

1.23.2 Constipation

Constipation is defined as experiencing symptoms that include hard and infrequent stools usually less than 3 times a week, straining, incomplete evacuation and prolonged time attempting to defecate (Lembo and Camilleri, 2003). Constipation can be normal transit constipation, slow transit constipation and rectal evacuatory dysfunction (Lembo and Camilleri, 2003). Normal transit constipation is functional, since it is perceived as a difficulty in defecation although transit time and stool frequency is normal. Slow transit constipation is defined as a longer transit time throughout the gut, (Talley et al., 2003). Some constipated patients experience bloating, visceral pain and psychological stress (Ashraf et al., 1996). Rectal evaluation studies show that rectal compliance and rectal sensation is increased in constipated patients (Mertz et al., 1999). Immunohistochemical studies in patients with slow transit constipation show abnormalities in specific neurotransmitters of the ENS including excitatory neurotransmitter SP expressed in myenteric ganglia (Voderholzer et al., 1997), VIP and NOS (Cortesini et al., 1995). IHC studies have also demonstrated a reduction of ICCs which could disrupt coordinated colonic contractions (He et al., 2000)

1.23.3 Rectal evacuatory dysfunction

Unlike functional constipation which is usually defined by symptoms alone, defecatory disorders are defined with the addition of anorectal tests (Bharucha et al., 2006) Defecatory disorders usually overlap with constipation since patients who have functional constipation show abnormal balloon expulsion tests indicating rectal evacuatory disorder (Suttor et al., 2010). Pelvic organ prolapse and collagen disorders are thought to have a common aetiology. A recent study looking at the prevalence of pelvic organ prolapse and previous collagen disorders compared JHM patients (n=100) to a group of controls (n=110) and found that patients with pelvic organ

prolapse had a higher prevalence of JHM and rectal prolapse when compared to controls ($p<0.01$) (Lammers et al., 2012).

1.23.4 Hirschsprungs disease

Hirschsprung's disease is a malformation of the ENS, in which there is a reduction/absence of intrinsic ganglionic cells and a lack of nerves (Kapur, 2006). Calretinin is an essential marker in the diagnosis of Hirschsprung's disease where a loss of calretinin positive cell bodies indicates aganglionosis (Barshack et al., 2004). Intestinal obstruction and megacolon are characteristics of Hirschsprung's caused by a lack of peristalsis in the aganglionic region therefore contents proximal to that region become accumulated (Manber and Gershon, 1979).

1.24 Extracellular matrix

The non-cellular component that is virtually present within all tissues and organs provides essential function. This is called the extracellular matrix (ECM), which not only provides the basic scaffold in organs and tissues but is critical in tissue development, differentiation, signalling, and homeostasis (Frantz et al., 2010). The significance of the ECM is illustrated by genetic abnormalities that give rise to a variety of syndromes (Jarvelainen et al., 2009). The changing microenvironment within tissues and organs determine the unique composition of the ECM. This makes the ECM dynamic that is remodelled serving the needs of that particular microenvironment. Cells adhere with the ECM by ECM receptors, for example integrin's (Harburger and Calderwood, 2009). Adhesive activity regulates cytoskeletal coupling that involves cell migration via the ECM (Schmidt and Friedl, 2010). These interactions determine the mechanical properties of the ECM, such as elasticity and tensile strength as well as maintaining homeostasis and water retention by buffering (Frantz et al., 2010). ECM molecules also communicate with growth factors and interact with cell-surface receptors to initiate signalling mechanisms that regulate cellular functions (Frantz et al., 2010). It is important to note that the components and properties of the ECM varies greatly based on organ type for example the skin and the gut as well as changes within the same organ such as renal cortex and renal medulla and finally in non-diseased and diseased states such as cancer (Frantz et al., 2010).

The ECM is composed of macromolecules that include fibrous proteins and proteoglycans (Jarvelainen et al., 2009), (Schaefer and Schaefer, 2010). Proteoglycans provide structural support and have a significant role in signal transduction mechanisms with regulatory function at various molecular processes (Schaefer and Schaefer, 2010).The main fibrous proteins include the collagens, elastins, fibronectins laminins and the tenascins (Alberts, 2008), (Frantz et al., 2010), (Chiquet-Ehrismann and Tucker, 2004). Collagens are the most abundant fibrous protein that make up 30% of total protein of multicellular animals and have a major role in support, tensile strength, mediating cell adhesion and tissue development (Rozario and DeSimone, 2010). A discussion of the immense number of macromolecules that constitute the ECM is beyond the scope of this thesis, therefore a discussion on the fibrous protein-TNX will be given.

1.25 Tenascin-X (TNX)

TNX was first identified as a partly duplicated opposite strand transcript overlapping the 3' untranslated region of the human 21 hydroxylase gene (CYP21B) (Gitelman et al., 1992), this genetic overlap was found in a case study of a patient who had congenital adrenal hyperplasia as well as flexible joints, velvety skin, easy bruising and poor wound healing (Burch et al., 1997). The phenotype of this patient was further examined from skin biopsies which showed small and sparse collagen fibres, abnormally large elastin bodies, an increase in the ECM around the blood vessels and uneven packing of the myelin sheaths around peripheral nerves (Chiquet-Ehrismann, 2004).

1.25.1 Structure of TNX

TNX forms parts of a large multi-meric tenascin family and is expressed in connective tissue of vertebrates along with tenascin C (TNC), R (TNR) and W (TNW) (Chiquet-Ehrismann, 2004). TNX is predicted to be a 450 kDa glycoprotein with 5 separate domains consisting of a signal domain, 4 heptad hydrophobic domain, 18.5 repeats of epidermal growth factor (EGF), a rich fibronectin type III (FNIII) domain and a globular fibrinogen domain at the C terminus (Valcourt et al., 2015) (Fig 1.4).

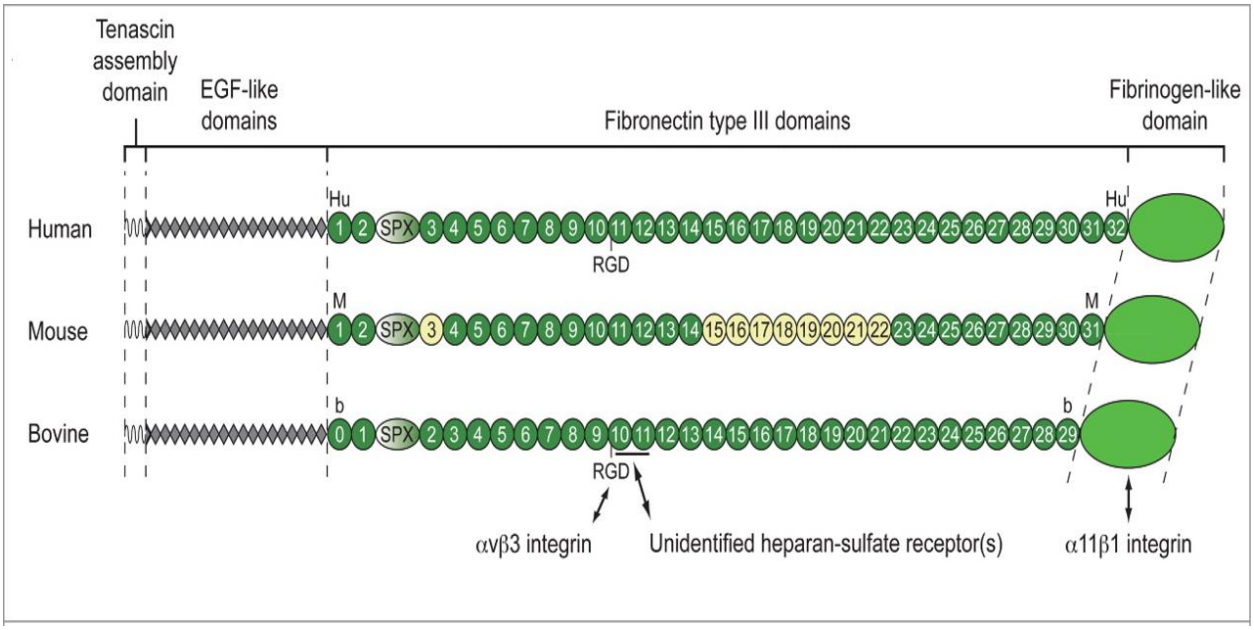


Figure 1.4. Diagrammatic representation of human, mouse and bovine TNX.

The TNX molecule is composed of the N-terminal domain or tenascin assembly domain which is attached to 18.5 EGF-like repeats, followed by 30-32 fibronectin type III repeats and a Fibrinogen (FBG) domain located at the C-terminus. Taken from Valcourt et al 2015.

TNX is a trimer based on its ability to form alpha helices by the hydrophobic rich heptad repeats in the N terminus which also stabilize the trimer forming disulphide bonds with 7 cysteine residues (Kammerer et al., 1998). TNXB gene encodes TNX with variable FNIII repeats in different mammals, for example there are 30 in the bovine (Elefteriou et al., 1997), 31 in the mouse (Ikuta et al., 1998) and 32 in human (Tucker et al., 2006). Mouse TNX has been identified by alternative splicing where in some instances the FNIII repeats is absent, moreover TNX consists of a proline rich sequence followed by the FNIII domain which is unique to TNX within the tenascin family (Valcourt et al., 2015).

1.25.2 Expression of TNX

TNX is only expressed during late stages of foetal development and is preserved in adult tissues (Valcourt et al., 2015). As expression of TNX is later in development, this suggests a role in

organogenesis (Burch et al., 1995). During development, TNX mRNA is expressed in mouse tissues and organs such as the heart, skeletal muscle and limbs (Burch et al., 1995). TNX mRNA is variably expressed in lungs, blood vessels, kidneys, testis, mammary and adrenal glands and interestingly at a higher level in the mouse GI tract including the stomach, small intestine and colon (Matsumoto et al., 1994). In addition, TNX mRNA has been detected in peripheral nerves in mice, pigs (Geffrotin et al., 1995), (Valcourt et al., 2015) and around connective tissue structures such as the epimysium, perimysium, skin dermis, muscularis mucosae and blood vessels (Matsumoto et al., 1994), (Elefteriou et al., 1997). During development and matured adult tissues, the expression of TNX and TNC is distinct. This reciprocal pattern of expression is exemplified by TNX mRNA where low levels are found in pig and mouse spleen, cerebrum and spinal cord whereas TNC is highly expressed in all these tissues (Matsumoto et al., 1994), (Geffrotin et al., 1995). This pattern of TNX/TNC expression is also found in pig and mouse digestive tract, where tissue expression is separate but adjacent (Geffrotin et al., 1995), this is especially true in the mouse oesophagus, stomach and intestines. In oesophagus, TNX is found abundantly in the lamina propria while TNC is found enveloping smooth muscle (Matsumoto et al., 1994). In stomach and intestines, TNX is highly expressed around smooth muscle mesenchyme conversely, TNC is found within the smooth muscle and in dense mesenchyme in close apposition to epithelium at the mRNA level (Matsumoto et al., 1994). This is also true for TNW and TNX in adults although they haven't been compared directly. Moreover, TNR is only expressed in the CNS (Rathjen et al., 1991), (Fuss et al., 1993) while, the expression of TNX has not yet been reported (Valcourt et al., 2015). The opposing expression of TNX and TNC is apparent in processes such as wound healing. TNX is ubiquitously expressed in normal skin dermis associated with the basement membrane, however, in the early stages of wound healing the expression of TNX is negligible in the basement membrane and connective tissue (Egging et al., 2007). During later stages of wound healing TNX is expressed when cells are undergoing matrix assembly and maturation (Egging et al., 2007). On the other hand, TNC expression in the skin is limited and in wound healing it is found below the epidermal-dermal junction enabling migration of keratinocytes (Mackie et al., 1988). Functionally, this suggests TNC enables division of cells and motility whereas, TNX is restrictive and important in skin tissue homeostasis. Similarly in porcine tumour cells TNX expression is

decreased in comparison to the normal dermis while TNC secretion is induced (Geffrotin et al., 2000).

In summary, TNX is expression is highly regulated and mRNA levels have been quantified in a variety of tissues in the developing and mature rat, mouse, pig and cow. The antagonistic expression of TNX/TNC is an interesting phenomenon and suggests that the location of TNX is closely associated with its functional influence within a given tissue and structure ultimately serving a specific role. Importantly, expression of TNX has only been described at the mRNA level in the aforementioned species, and there are no studies describing TNX expression in human tissue specifically in the GI tract.

1.25.3 The role of TNX as a matricellular molecule

Despite expression of TNX in various organs and tissues the function of TNX remains elusive particularly in the GI tract in all species. Studies have suggested a role for TNX as a 'matricellular' protein. Functionally, matricellular proteins do not play a direct role in forming structural elements instead they modulate the function of cells by regulating the interaction between cells and matrices (Bornstein and Sage, 2002). TNR, TNC and TNW are also described as matricellular proteins since they display anti-adhesive properties as well as enabling cells to detach from ECM molecules (Pesheva et al., 1989), (Bristow et al., 1993), (Meloty-Kapella et al., 2006). TNX and members of the tenascin family show overlapping roles, for example in their ability to induce cell rounding (Elefteriou et al., 1997), (Elefteriou et al., 1999) and detachment (Fujie et al., 2009) *in vitro*. Anti-adhesive role of TNX was displayed by a study looking at mouse fibroblast L cells where p38 MAP kinase, known to be important for cell detachment, was knocked down. If TNX is involved in cell detachment higher levels of p38 MAP kinase activity would be expected in TNX expressing cells. Indeed it was found TNX increased p38 MAP kinase activity compared to controls (Fujie et al., 2009) although further studies are necessary to understand the signalling pathway and cell surface receptor attachment associated with TNX. The ability of matricellular proteins to interact with cell-surface receptors and other signalling molecules associated with the membrane has been studied. These interactions can be direct or indirect. Matricellular thrombospondin interacts directly with cell-surface calreticulin which prevents cell-adhesion thus disassembly of

focal adhesions described previously (Goicoechea et al., 2000). Indirect methods involve competing with other ECMs for cell-surface binding sites, this is true for TNC that prevents the HepII site of fibronectin from binding to receptor syndecan by disrupting fibronectin adhesion (Huang et al., 2001). It is unknown whether the anti-adhesive properties of TNX occurs by indirect competitive binding or direct interaction with cell-surface receptors although, in bovine studies it has been reported that regions of the TNX molecule can interact with various cells at adhesion sites (Elefteriou et al., 1999), (Lethias et al., 2001), (Alcaraz et al., 2014). In addition to the anti-adhesive properties of TNX, TNX can also communicate with vascular endothelial growth factor-B (VEGF-B) to stimulate cell proliferation by increasing signals to vascular endothelial growth factor receptor 1 (VEGFR-1) (Ikuta et al., 2000). It has been hypothesized that TNX influences tumour cells and is thought to have a role in tumour suppression. Matrix metalloproteinases (MMP)-2 and MMP-9 are upregulated in TNX KO mice increasing metastasis and tumour invasion (Matsumoto et al., 2001).

1.25.4 TNX and transforming growth factor-β (TGF-β)

The inhibitory cytokine TGF-β is important in fibrosis and tumorigenesis, (Massague, 2008, Wu and Hill, 2009) and initiates epithelial-to-mesenchymal (EMT) transition (Valcourt et al., 2005). TGF-β is a homodimer that is secreted as a latent complex where the prodomain (latency associated peptide-LAP) remains attached (Moustakas and Heldin, 2009). Latent TGF-β is found as a soluble entity called the small latent complex (SLC) and is excreted as a large complex where the SLC is attached to the latent TGF-β (Miyazono and Heldin, 1991). The FBG domain at the C terminus of the TNX molecule interacts with the TGF-β SLC complex.

It has been hypothesized that under certain conditions, TGF-β activation cleaves the TNX FBG domain which interacts with cell surface receptor α11β1 integrin causing a conformational change (Alcaraz et al., 2014). TGF-β then induces further signalling for example, Smad signalling and processes such as EMT transition (Alcaraz et al., 2014). Alcaraz et al also reported that the FNIII domain of TNX was able to oppose EMT response stimulated by the FBG domain, which suggests that these two domains in the TNX molecule are important in both cell plasticity and signalling (Alcaraz et al., 2014).

1.25.5 TNX and role in behaviour

A recent study showed TNX KO mice exhibit behavioural alterations (Kawakami and Matsumoto, 2011). Using the light dark preference test, TNX KO mice displayed anxious behaviour where they spent less time in the light compartment ($p<0.001$) and moved less ($p<0.001$) compared to WT mice (Kawakami and Matsumoto, 2011). Consistent with these findings, TNX KO mice in open field tests showed reduced grooming and rearing (Kawakami and Matsumoto, 2011). Within the tenascin family, TNC is described to act as a guidance molecule by regulating the movement of postnatal granule cell neurons from external to internal cell layer (Husmann et al., 1992). In TNC KO mice, behavioural defects for example hyperlocomotion is apparent due to increases in transmission of dopamine in the CNS (Fukamauchi et al., 1996), (Cifuentes-Diaz et al., 1998 5267). This neural abnormality in the CNS is also evident in TNR KO mice where changes in the ECM is associated with decreased axonal conduction velocities (Weber et al., 1999). This suggests that TNX may be playing a similar role in the CNS that regulates behaviour by increasing

or decreasing neurotransmitters or reduced nerve conduction. Further test are required in TNX KO to unravel the specific mechanism of TNX and behaviour in mice. Of interest, single nucleotide polymorphism (SNP) analysis in humans suggested that a non-synonymous substitution, Glu2578Gly found in exon 23 of TNX, is associated with schizophrenia (Wei and Hemmings, 2004), (Tochigi et al., 2007).

1.25.6 Neuronal function of tenascins

While there are few studies describing the role of TNX in neuronal function, TNR has been explored in the CNS particularly within structures called perineuronal nets (PNNs) (Kwok et al., 2011). PNNs surround the synapses on the neuronal surface and play a direct role in CNS plasticity (Kwok et al., 2011).

TNR is an integral component for the correct assembly and function of PNNs. As shown in Fig 1.5, TNR provides the link between aggrcan molecules and hyaluronan to stabilise the hyaluronic acid-aggrecan complexes and aggregation of these molecules around the dendrites in the CNS (Kwok et al., 2010).

TNR deficient mice lose their capacity to build the barrier for the lateral diffusion of neurotransmitter and neurotrophin (Morawski et al., 2014). Altering the diffusion of these substances in the extracellular space that in turn affects synaptic transmission, efficacy and neuron-glia interactions (Morawski et al., 2014).

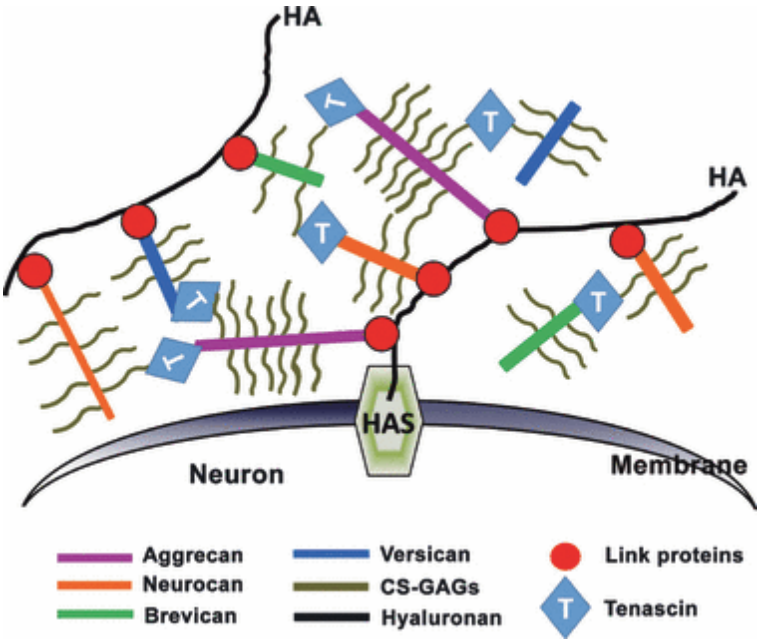


Figure 1.5. Schematic representation of a PNN.

TNR connects CS glycosaminoglycan (GAG) chains to aggrecan, neurocan, brevican and versican that are secured via link proteins to hyaluronan. TNR binds CS- GAGs chains to each other. All PNNs express the transmembrane hyaluroanan synthase which helps to anchor the PNNs to the neuronal membrane to form a complex macromolecule. Image taken from Kwok et al 2010.

1.25.7 Ehlers Danlos Syndrome and TNX

TNX is the only member of the tenascin family, whose deficiency is clearly associated with a pathological disorder in humans (Burch et al., 1997). This disorder is known as Ehlers-Danlos Syndrome (EDS) which is defined as a multi-systemic disorder presenting with variable clinical manifestations affecting primarily the skin, ligaments, joints, blood vessels and internal organs (Klaassens et al., 2012). EDS represents a paradigm collagen disorder among the larger group of heritable connective tissue diseases (Burch et al., 1997). Most forms of EDS recognized to date result from mutations in one of the genes encoding fibrillar collagens or enzymes involved in the biosynthesis of these collagens (De Wandele et al., 2014). The current Villefranche nosology divides EDS into 6 subtypes; the most common is the hypermobility subtype otherwise described as JHS (De Wandele et al., 2014) where the causative gene remains unidentified however, a small subset of patients with JHS have gene mutation in TNX (Bristow et al., 2005).

1.25.8 JHS and TNX

The exact prevalence of JHS in the general population is unknown, but it is estimated to affect 30% of the population, and is more common in females from an Asian background (Grahame, 1999), (Fikree et al., 2013). JHS is thought to be a multi systemic disorder which is taken into account when using the diagnostic criteria for JHS. The gold standard uses the 1998 Brighton criteria (Grahame et al., 2000) recognizing clusters of symptoms (Grahame et al., 2000).

JHS is characterized by velvety skin, easy bruising, musculoskeletal symptoms, skin hyperextensibility, and dislocations (Grahame, 1999). Degenerative joint disease is common and chronic pain, distinct from that associated with dislocations, is a serious complication and can be both physically and psychologically debilitating (Levy, 1993). Other features include rectal and uterine prolapse, hernias, varicose veins, stretch marks, papyraceous scarring, myopia and Marfanoid habitus (Grahame et al., 2000). Another common finding in JHS patients is postural orthostatic tachycardia (PoTs) an autonomic dysfunction (Mathias et al., 2012). JHS is thought to me hereditary through autosomal dominant mode of inheritance (Garcia Campayo et al., 2010).

Hyperextensibility of the skin is a hallmark in JHS patients and is thought to be attributed to the role of TNX in regulating fibrillary spacing and provide elasticity and strength to the skin dermis. The evidence to support this hypothesis, is derived from various studies a) Lethias et al 1996, cloned TNX in cows and showed TNX associated with fibril proteins in the dermal collagen preparations, b) Minamitani et al 2004, suggested that the native form of TNX binds to collagen and increased fibril formation *in vitro*, c) Lethias also showed the ability of TNX to bind to decorin proteoglycan that has a high binding affinity to collagen and modulates fibrillogenesis (Lethias et al., 2001) (Minamitani et al., 2004). A hypothetical schematic describing the role of TNX in regulating fibril space is shown in Fig 1.6.

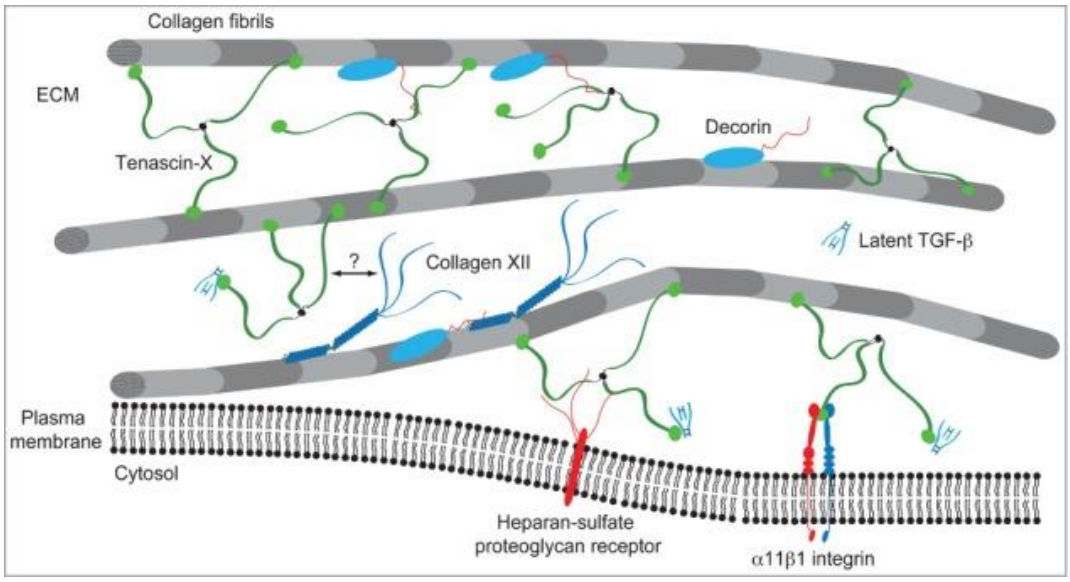


Figure 1.6. Integration of TNX with the collagenous network.

TNX modulates the cohesiveness and spacing between collagen fibrils by direct interaction or secondarily through associations with other ECM molecules like decorin. The N-terminal NC3 domain of collagen type XII is thought to interact with TNX, while collagen fibrils at the cell surface are anchored by TNX by possibly communicating with heparan-sulfate proteoglycan receptor or association with α11β1 integrin. The hypothesised role of TNX in regulating collagen fibrils was supported by immunogold-electron microscopy of human dermis, where inter-fibrillar distance is increased causing the skin to become more loose and flexible leading to hyperextensible skin (Bristow et al., 2005).

1.25.9 TNX deficient humans and mice

Case reports have shown decreased serum TNX in individuals with a phenotype resembling JHS (Schalkwijk et al., 2001). One particular case report showed a 57 year old woman with a complete absence of TNX (homozygote deficiency), who had hyperextensible skin, joint dislocations, displayed hiatus hernia, recurrent rectal prolapse as well as severe longstanding constipation (Schalkwijk et al., 2001). All three of her daughters suffered chronic constipation one of whom also had prolapse (Schalkwijk et al., 2001). This suggests TNX deficient patients may have a defect in motility patterns. Interestingly, heterozygous deficient members of the TNX deficient group

showed TNX levels at 50%, of which half experienced joint hypermobility and were predominantly women (Zweers et al., 2003). Therefore similar phenotypes are presented in homozygous or heterozygous deletions of TNX patients. Another TNX deficient patient study showed generally few complications in pregnancy however the TNX deficient women themselves were born prematurely and have uterine/vaginal prolapse which indicates there is increased laxity in the genito-urinary tracts (Egging et al., 2008). This group also studied TNX KO mice and found rectal prolapse albeit a small percentage (Egging et al., 2008).

Since the discovery of TNX deficiency in patients, the TNX knockout mouse has been generated by two independent groups that have a complete deficiency of the gene (Mao et al., 2002), (Matsumoto et al., 2001). TNX KO mice show similar skin dermal properties as TNX deficient patients where hyperextensibility (Fig 1.7) is a phenotype described above (Mao et al., 2002). Mechanical testing showed reduced tensile strength, collagen content and collagen fibrils (Mao et al., 2002). In addition, TNX KO mouse show TNX has a role in regulating collagen expression for example, TNX KO fibroblasts cells show decreased expression of collagen molecules type I and VI, and collagen fibril molecules type XII, XIV collagen, lumican and fibromodulin (Minamitani et al., 2004). It is unclear whether the lack of TNXB affects expression of various collagens directly or through an adaptive process. However, the study showed that changes in collagen XII expression in mice and human supports an adaptive process, for example collagen XII is reduced in the muscle of TNX KO, however, does not change in human biopsies (Zou et al., 2014). In addition, dermal tissue showed a lower amount of collagen I in both TNX KO mice and TNX deficient patients (Mao et al., 2002), (Zweers et al., 2004b).

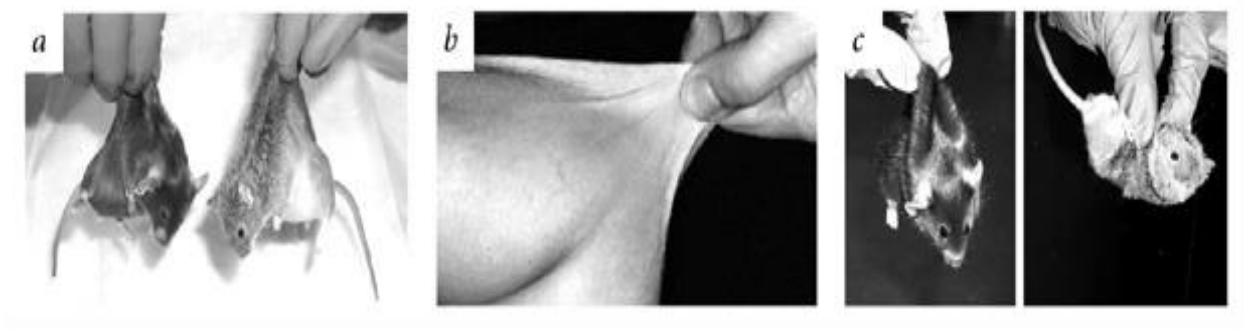


Figure 1.7. Hyperextensibility in TNX KO mice.

(a) WT mouse is darkly coloured and shows normal elasticity when lifted whereas the lighter coloured TNX KO mouse shows hyperextensible skin (b) hyperextensible skin in TNX deficient patient shows parallel hyperextensibility (c) The lighter coloured TNX KO mouse was so hyperextensible that it could turn around and touch the investigators fingers.

In conclusion, TNX can cause hypermobility commonly seen in JHS patients which is recapitulated in TNX KO mouse models. Patients with TNX deficiency have GI symptoms that include constipation and prolapse however, no upper GI symptoms have been reported so far. *In vitro* studies in mouse have described the importance of TNX in cell signalling including its role as a matricellular protein, in regulating TGF-β, its anti-adhesive properties, and its role in tumour suppression. *In vivo* studies show mice lacking TNX have behavioural alterations and a small percentage have rectal prolapse.

Specific examples provide evidence that TNX deficiency and GI symptoms are associated, furthermore it is now recognized that patients who have JHS have a variety of GI symptoms. Although not all JHS patients have been tested for TNX deficiency, similar presentation of GI symptoms suggests TNX may indeed have a role in GI function. Therefore it is important to understand common GI complications in JHS patients that has been widely studied in comparison to TNX deficient group.

1.26 JHS and GI dysfunction

1.26.1 JHS + functional gastrointestinal disorders (FGID)

Over the past few years it is becoming increasingly recognized that JHS is associated with GI symptoms, many gastroenterologists are recognizing this phenotype in patients presenting to GI clinics with unexplained gut symptoms/FGID who do not respond to various drugs and are difficult to manage. The link between JHS and FGID has been described in adults as well as in (Fikree et al., 2014) paediatric clinics (Pacey et al., 2015).

The first large scale study to describe the association of the syndrome (JHS) was from a group of 778 new referrals to a tertiary care GI clinic that were eligible. Of those, around 30% had JHS that were not from rheumatology clinics therefore resembling a large cohort. (Fikree et al., 2014) (Hakim and Grahame, 2004). These patients showed significant (p <0.005) upper GI symptoms including heartburn, waterbrash and postprandial fullness, (Fikree et al., 2014). Other upper GI symptoms such as dysphagia, reflux, bloating and early satiety and lower GI symptoms including alternating bowel habits was higher in the JHS population but was not significant. Separate to GI dysfunction these patients also reported to have PoTS. It is postulated that there is interplay between JHS, GI symptoms and the autonomic system, however the mechanism is not understood (Kanjwal et al., 2010). This study was questionnaire based and patients referred to gastroenterology clinic may be biased in their response since they have underlying GI issues. Based on this criticism the same study was replicated in a group of healthy university students that are unbiased in that they are regarded “healthy” and have not been previously diagnosed with JHS. Interestingly students with JHS had significantly more GI symptoms which included early satiety, post prandial fullness, orthostatic autonomic symptoms, somatic symptoms and particularly dyspeptic symptoms (Fikree et al., 2015a). Other questionnaire based studies also showed 84% of EDS patients in general have FGID and of those 48% had IBS. GI symptoms include epigastric pain (79%), nausea (71%), heartburn (69%), regurgitation (69%), postprandial fullness (68%) and functional constipation (36%) (Zeitoun et al., 2013). The studies mentioned are questionnaire based and few physiology studies exist in patients with JHS. Most recently a published study from the Mayo clinic found that GI symptoms are commoner in EDS

hypermobility type/JHS patients than EDS patients in general. Upper GI symptoms in this group of JHS patients commonly experienced nausea, heartburn, vomiting and dysphagia and lower GI symptoms were most commonly constipation, IBS like symptoms and diarrhoea. Physiology tests were performed on all EDS subtypes and upper physiology tests reveal abnormal gastric emptying which included a mixture of rapid (8.7%) and delayed emptying (19.6 %). Lower physiology tests showed abnormal colonic transit (28.3%) and rectal evacuatory disorder in 60% of patients who underwent anorectal manometry. Since the results from the physiology studies looked at EDS in general therefore the same physiology tests are required in JHS patients. Overall, JHS patients most commonly took proton pump inhibitors, histamine H2 blockers (38.4%) and drugs for constipation (25.8%) (Nelson et al., 2015).

Most of the studies described show a strong association with upper GI symptoms and the Mayo paper highlights lower GI symptoms too. Constipation seems to be common in JHS patients and has also been shown in the paediatric population. Two studies show that slow transit constipation and faecal incontinence is significantly higher in boys that are hypermobile than those that were not (Reilly et al., 2008), (de Kort et al., 2003). In adults a larger study looking at 1000 women who primarily were referred for assessment of lower urinary tract dysfunction and have JHM, showed a strong association with childhood constipation (Manning et al., 2003). A possible explanation for this link could be that of disordered collagen synthesis, since children who have slow transit constipation display hypoplastic desmosis (lack of connective tissue in the muscularis propria) of the colon, and thus disturbed gut mechanics (Meier-Ruge et al., 2001), or they may have a neural dysfunction which alters the motility of the colon. Despite this association, there is no gold standard to diagnose hypermobility in children unlike in adults where the Beighton and Brighton score is used. As well as in paediatric JHM, patients also reported a higher incidence of severe constipation, rectal evacuatory dysfunction (Mohammed et al., 2010) and delayed colonic transit (Zarate et al., 2010).

Interestingly a case study of a 28 year old female patient who had a rare GI manifestation: visceroptosis (prolapse of the internal organs) suffered from bloating and abdominal distension causing significant pain which could only be alleviated by using analgesics. No known cause was

identified until she was diagnosed with JHS (Reinstein et al., 2012). The aetiology of visceroptosis is unknown but is thought to be due to the increased laxity of the ligaments holding the internal organs in place.

1.26.2 JHS is associated with GI pain

Pain experienced in JHS can be subdivided into 3 groups. The pain can be nociceptive pain which includes arthralgia's (Sacheti et al., 1997), dislocations (Voermans et al., 2011) soft tissue injuries (Hudson et al., 1998) and back pain (Simmonds and Keer, 2008). Neuropathic pain includes peripheral neuropathy (Voermans et al., 2011) and fibromyalgia (Ofluoglu et al., 2006) and dysfunctional pain includes dysmenorrhea (Castori et al., 2010a) and functional abdominal visceral pain (Hakim and Grahame, 2004), (Castori et al., 2011). The process leading to abdominal visceral pain in JHS is largely unknown however dolichocolon which is an abnormally long and large intestine is common in JHS which may contribute to the pain (Castori et al., 2012). Patients with JHS may have increased colonic compliance, which are reported to contribute (25%) to gas and pain sensation in a group of healthy subjects (Iturrino et al., 2012). Therefore an increased laxity in the colonic wall of these patients may explain an increase in visceral hypersensitivity. The altered adaptive response seen in FGIDs involves the sensory motor neurons, mechanoreceptors and visceral motor activity, thus it may be likely that an increase in compliance exacerbates the perception to visceral stimuli resulting in lower pain thresholds. A study looking at dysphagic JHS patients showed an increase in oesophageal dysmotility and hypomotility in a cohort of 17 patients (Fikree A, 2011). This study highlights oesophageal dysmotility to be the underlying mechanism giving rise to upper GI symptoms in JHS patients rather than symptoms as a consequence of reflux. Although a significant proportion of JHS patients report unspecific widespread pain, the most potent analgesics such as opiates are not successful and cognitive behavioural therapy is the treatment of choice (Daniel HC. . In: Hakim AJ). Most of these studies assessing compliance are on few patients therefore larger compliance studies are required in the JHS population and TNX population to understand pain processing and compliance in gut regions.

In conclusion, it is clear that JHS patients present with a myriad of GI complications and a small albeit significant number of TNX deficient patients also present with GI symptoms, thus it is plausible to suggest that TNX has a role in functioning of the GI tract. No study to date has explained the role of TNX in the GI tract nor its expression pattern at the protein level despite GI symptoms being present in TNX deficient patients. The lack of data suggests a need to understand the role of TNX in the GI tract which forms the basis of this PhD.

1.27 Hypothesis

TNX is specifically localised and important for normal sensorimotor functioning of the gut.

1.28 Overall aims

To understand the role of TNX in GI function in the stomach and colon. Specifically, to determine where in the normal stomach and colon TNX is found - which layers express TNX (neuronal, muscular, and epithelial). To identify the functional role of TNX using KO mice based on localisation data.

1.28.1 Objectives

1. Characterise the expression pattern of TNX in the enteric layers of the stomach and colon using known neural markers in normal mouse and human tissue.
2. To assess the functional role of TNX in the TNX KO mouse, colon function will be measured by
    a. *In vitro* colonic manometry study that measures number, amplitude and migration of contractions in specific regions of the colon. Drugs will also be used to increase or decrease contractions to assess the change in response if any in WT vs. KO. Therefore assessing overall contractile ability of the colon in each group
    b. Changes in short circuit current using the Ussing chamber will be used to measure changes in active Cl-secretion, which is a reliable method for assessing changes in ion flux and therefore an indirect measure of colonic secretion.

Stomach function will be measured by

a. Vagal afferent electrophysiology which can measure the rate of nerve firing in spontaneous and distended conditions.
b. Gastric emptying studies which measures the rate at which the stomach empties using the octanoic acid breath test.

1.29 General Methods

1.29.1 Animals

The mice used in all studies originated from parent mice donated by Professor Manuel Koch at the University of Cologne, Germany. 8 mice were donated, of which 4 mice were TNX homozygous KO and 4 WT siblings from C57BL/6N background. Within each group of 4, 1 male and 3 females were provided. The mice were born on the 3.8.13 (2 WT and 1 KO) and 10.8.13 (2 WT and 3 KO). The mice were received on the 11.4.14 at 8 months old. TNX KO mice were generated by homologous recombination. This involved inactivating murine TNXB by targeting the 5' end of the gene, replacing the first five coding exons with *lacz* and a neomycin resistance cassette. The correct targeting of TNXB was confirmed by Southern blotting which showed TNX KO mice lacked both TNXB mRNA and the protein. The mice that were used for breeding were previously genotyped at the University of Cologne before being transferred to the UK. The health report of the mice were provided, which stated all mice presented in good condition, with good coverage of body hair and no evidence of faecal soiling or any other physical abnormalities. These animals were provided standard laboratory food (chow) and water and housed in controlled conditions maintained by animal house employees. All 4 animals from each group were housed in the same cage and reproduced litter. A specific home office license for breeding was used (PPL7008598). For gastric emptying experiments another license was obtained (Home office license PPL7007378). Mice used for all other experiments were aged between 8-12 weeks and killed by asphyxiation using carbon dioxide in accordance with the UK Home Office guidelines (Schedule 1, Animals Act 1986). The death of the mice was confirmed by cervical dislocation.

1.29.2 Human Tissue

For IHC experiments full thickness colonic tissue was obtained from patients undergoing colonic resections for cancer. The tissue obtained was 5cm away from the cancer and thus deemed non pathological and healthy. Similarly gastric tissue was obtained from patients undergoing bariatric surgery. Tissue was collected using a pan GI ethics approval (NREC 09/H0704/2) from

Barts and the London NHS trust which comprises the Royal London Hospital, Whipps Cross Hospital and the Homerton hospital.

1.29.3 Sample size and statistics

Sample size in all studies were derived from previously published studies that used the same or similar technique. All data from each chapter was analysed using GraphPad Prism, V.7.02, GraphPad Software, Inc. Data that showed a significant difference was further discussed. Specific details of the type of statistical test used can be found within the methods of each chapter.

2 Anatomical characterisation of TNX

2.1 Introduction

TNX deficient and JHS patients experience a range of GI complications (Castori et al., 2015), however the most common symptoms occur in the colon (TNX deficiency) and stomach and manifest as alternating bowel habits and post-prandial symptoms, respectively (Hendriks et al., 2012), (Fikree et al., 2014). JHS patients frequently experience defecatory problems including incomplete evacuation, constipation (de Weerd et al., 2012) (Chen and Jao, 2007) (Dordoni et al., 2013) (Sardeli et al., 2005) and rectal prolapse also observed in TNX deficient patients (Hendriks et al., 2012) (Chen and Jao, 2007) (Reinstein et al., 2012), that were recurring in some cases (Douglas and Douglas, 1973). On the other hand, upper GI manifestations are present in JHS patients that include dyspepsia, abdominal bloating, distension (Reinstein et al., 2012) and changes in gastric emptying (Nelson et al., 2015). It is now well recognized that there is a strong association between the common connective tissue disorder, JHS and GI symptoms. Despite this comorbidity, the pathogenesis of GI symptoms in this group of patients is largely unknown. One study suggested that dysautonomia may contribute to the reported functional GI complaints (Zarate et al., 2010) (Castori et al., 2013), however, this hypothesis is largely based on descriptive studies and does not explain the pathophysiology. Since JHS is an underlying connective tissue disorder, studies have recognized that the components of connective tissue may be a predisposing factor in gut changes (Timpone et al., 2011). This may include, alterations in elastic fibres, which are found within the gastro-oesophageal junction in patients with reflux and hiatus hernia (Curci et al., 2008). Moreover abnormal connective tissue disposition may increase gut compliance due to large distensions and more specifically alter mechanoreceptors which are embedded within the muscle layers (Grundy and Schemann, 2006). The summative effects of these changes may give rise to gut motility problems such as constipation and abdominal symptoms described in both JHS and those who have TNX deficiency.

To date, the role of connective tissue and its components in the GI tract is not fully characterised. Region-specific GI manifestations are present in JHS patients and those who have a deficiency of

TNX (Schalkwijk et al., 2001). The range of gut symptoms are difficult to explain based on alterations in connective tissue. It may be that TNX is important in other functions such as neural control of the gut, which may underlie symptoms. Therefore it is important to understand the neurochemistry and functional relevance of various neurochemical markers in the extrinsic and intrinsic nervous system in order to understand the role of TNX based on expression pattern.

Neurons in the ENS can be divided into broad categories. First, by morphology for example Dogiel type I-VII; secondly, electrophysiological properties such as S type or AH-type described in detail in Chapter 1 section 1.13; thirdly, chemical classification based on the neurotransmitters and other markers; and finally functional properties, for example categorisation into sensory neurons, interneurons, muscle motor neurons and secretomotor neurons (Hansen, 2003). This chapter will be focusing on the chemical classification to understand the anatomical distribution of TNX relative to the ENS.

The earliest method for staining enteric neurons dates back to the late nineteenth century where methylene blue and silver impregnation revealed distinct morphological characteristics identifying specific enteric neurons (Furness and Costa, 1987). Histochemical localization using a range of neuronal markers has elucidated enteric neuronal pathways, place of origin (using retrograde tracing), and target organ innervation. Morphologically, A.S. Dogiel classified enteric neurons into the subtypes, I, II, III which have now been extended to incorporate types IV-VII (Brehmer, 2006). Moreover, there is a further subtype described as giant neurons, however, types I and II are the most common (Furness, 2000). Dogiel type I display short, flattened dendrites similar to lamella and a single axon whereas Dogiel type II have multiple long processes with a large oval or rounded cell bodies (Brookes et al., 1991a). Type II neurons are also referred to as intrinsic primary afferent neurons and AH neurons based on their electrophysiological properties and encompass two thirds of all enteric neurons in the myenteric plexus (Kustermann et al., 2011).

Neurons in the ENS express various neurotransmitters and the enzymes involved in the production of neurotransmitters. The type of neurotransmitters released in specific neurons is classified and termed the chemical coding system. The chemical code varies according to the

neuronal subtype, species studied as well as the region and deeper layers of the gut (Hansen, 2003). Commonly, fluorescence IHC is used to visualize the chemical code, which allows visualising and semi-quantifying the density of specific neurons in different target areas as well as cell bodies of origin. There are a host of neurotransmitters involved in classifying neurons, however for the purposes of this chapter, focus will be given on the neural markers that were used in this study.

As described previously (Chapter 1 section 1.17), extrinsic sensory neurons are either vagal or spinal. The vagal afferents that originate from the NG supply the mammalian stomach and can be divided into IGLEs and IMAs. IGLEs are described as tension receptors while IMAs detect stretch and length at regions that commonly change in muscle length and tension, for example at the sphincters (Phillips et al., 2000), (Wang and Powley, 2000). In brief, IGLEs are found around myenteric ganglia-muscle sheets which are thought to interact with neural and connective tissue components around the myenteric ganglia (Neuhuber, 1987). The distribution of IGLEs was first identified in the oesophagus and cardia tissue (Nonidez, 1946); (Rodrigo et al., 1975), (Rodrigo et al., 1982) (Neuhuber, 1987) and more recently in the stomach and intestine (Berthoud and Powley, 1992), (Williams et al., 1997), (Phillips et al., 1997), (Wang and Powley, 2000). Detailed IHC data from rat studies show distribution of IGLEs in myenteric plexus in the gut (Wang and Powley, 2000). In contrast, IMAs have long parallel terminal fibres that connect via bridging collaterals to form a network in the smooth muscle layer. IMAS are thought to interact with ICCs as well as fibroblast cells in connective tissue surrounding smooth muscle fibers (Berthoud and Powley, 1992). In stomach, IMAs are concentrated around fundus/forestomach and both lower oesophageal and pyloric sphincters (Kressel et al., 1994), (Wang and Powley, 2000), (Phillips et al., 1997). Using wheat germ agglutinin-horseradish peroxidase or dextran-tetramethylrhodamine conjugated to biotin, Fox et al described the distribution of IGLEs and IMAs in the mouse stomach (Fox et al., 2000). This study measured density of IGLE and IMA innervation based on stomach regions and gave percentages of total ending types in the regions of the mouse stomach. The results showed changes in IMA expression based on region, for example the majority of IMAs (86%) are found in the fundic circular muscle whereas only 12% and 2% are found in the corpus and antrum circular muscle, respectively (Fox et al., 2000). IMAs

were also present in longitudinal muscle of the mouse stomach specifically the fundus (Fox et al., 2000). IGLEs, on the other hand were distributed more evenly in the fundus (36%) and corpus (50%), but decreases in the antrum to 14% (Fox et al., 2000). Finally it was also reported that some endings consisted of both IMA and IGLEs that had diverged from the same parent axon in the fundus. (Fox et al., 2000). This pattern of vagal afferent distribution is similarly shown in rat (Berthoud and Powley, 1992) and guinea pig (Schemann et al., 1995). To identify both populations of vagal afferent endings calretinin was first used in the rat oesophagus as a selective marker (Neuhuber, 1987), (Berthoud and Powley, 1992) that indirectly labels vagal afferents. Although calretinin is routinely used, horseradish peroxidase and biotinamide label vagal afferents very clearly (Patterson et al., 2002). Additionally, retrograde labelling studies using Dil injection allows definitive identification of specific subsets of vagal structures (Berthoud et al., 1990).

The spinal afferent innervation of the rodent stomach uses CGRP as a selective marker. In mouse stomach the majority of CGRP is found around the myenteric plexus but not in myenteric cell bodies (Sharrad et al., 2015), whereas in the circular muscle and mucosa, expression is reduced to 25% and 16%, respectively (Sharrad et al., 2015). Using retrograde tracing studies, mouse colonic afferents contained 81% CGRP (Robinson et al., 2004) and rat spinal cord showed CGRP immunoreactivity in L6-S1 (70%) and L1-L2 (46%) regions (Keast and De Groat, 1992).This suggests CGRP is a selective maker for spinal afferents and thus widely used. Additionally CGRP immunoreactivity is present in NG neurons albeit to a much lesser extent (Hayakawa et al., 2011) than calretinin (Ichikawa et al., 1991). Therefore, extrinsic vagal and spinal afferents can be identified using calretinin and CGRP respectively.

The myenteric plexus largely controls the intrinsic reflexes of the stomach since the stomach lacks a well-developed submucosal plexus (Schemann et al., 2001). These neurons are uniaxonal some of which exhibit various axon collaterals that branch and innervate the layers of the stomach (Schemann and Schaaf, 1995). ACh is the main excitatory neurotransmitter that uses the enzyme ChAT for its synthesis, which is a common marker used to detect cholinergic neurons that are found in the majority of ascending neurons. Conversely, the enzyme NOS, required to produce

NO, is used to label nitrergic neurons that are inhibitory and found in descending neurons (Costa et al., 1986). The distribution of neurons containing ChAT and NOS in the stomach varies across regions and species. For example a large proportion of ChAT neurons are found in mucosal endings (71%) of the guinea pig stomach. In the mouse, the number of ChAT (79.3%) positive neurons outnumber NOS (20.7%) positive neurons in the corpus myenteric plexus, (Pfannkuche et al., 1998). In human fundus the ChAT: NOS ratio is 57.2% and 40.8% (Pimont et al., 2003). This relatively equal distribution of ChAT:NOS neurons is also present in the corpus of the guinea pig smooth muscle with 54% and 46% found in circular muscle and 53% and 47% in the longitudinal muscle, respectively (Pfannkuche et al., 1998). Overall, as ChAT and NOS are the main chemical code in the stomach, they are classically used to identify cholinergic and nitrergic neurons.

In the lower gut, both the aforementioned neural markers are expressed in mouse and human colon, albeit in different densities and locations. In human colon, 48% of ChAT neurons and 43% of NOS neurons are found in the myenteric plexus (Porter et al., 1997). In the human colon ascending interneurons contain 90% of ChAT, and therefore are described as cholinergic whereas NOS is absent in this population. Descending interneurons contain either a combination of ChAT and NOS or ChAT or NOS alone (Porter et al., 2002). For example in humans, 46% of neurons express NOS alone and 20% express ChAT alone in colonic myenteric plexus. (Porter et al., 2002). In the smooth muscle layer, circular muscle motor neurons that project orally contain ChAT and those projecting anally contain NOS. 98% of these neurons have a Dogiel type I morphology that function as inhibitory and excitatory neurons within the circular muscle (Porter et al., 1997). In mouse submucous plexus, ChAT positive neurons are differentially expressed along the mouse colon. This means that the density of ChAT positive neurons increases distally along the colon, however, the overall proportion of cholinergic neurons is small in comparison to VIP containing neurons (Foong et al., 2014). NOS is found abundantly in all layers of the mouse colon with highest expression in the myenteric plexus (Sang and Young, 1996). Unlike the stomach, in mouse colon, calretinin is found in cell bodies and not just nerve fibres, 62% of calretinin neurons are ChAT positive in the myenteric plexus and circular muscle (Sang and Young, 1998). Along with ChAT and NOS, calretinin is also found in the human and mouse colon and colocalised with ChAT but not NOS, therefore is described as a secondary cholinergic marker (Furness, 2000). In human

and mouse colon, calretinin is also found in myenteric and submucosal plexus that co-label with VIP. These neurons have a Dogiel type II morphology, (Furness et al., 2004b), (Beuscher et al., 2014). In the rectum, calretinin is also used to classify cholinergic cell bodies and as such is a marker for Hirschsprungs disease which is characterised by aganglionosis (Kapur et al., 2009).

Sensory fibres are routinely identified primarily using the neuropeptide CGRP and are found in the myenteric plexus (Furness et al., 2004b) and submucosal neurons in the small intestine (Qu et al., 2008). Further studies have also shown CGRP varicosities in the myenteric plexus co-express transient receptor potential cation channel subfamily V member 1 (TRPV1), a key ion channel in pain sensing (Sharrad et al., 2015). Few studies have examined CGRP expression in the human colon, but nevertheless, expression has been observed in colonic mesentery and blood vessels (De Fontgalland et al., 2008) and colonic mucosa (de Jong et al., 2015). In summary, these neuronal markers are established identifiers of sensory and motor neurons in the ENS and are therefore important tools for characterising expression patterns of associated proteins and other molecules, in this case TNX.

There are no studies describing the localisation of TNX in the ENS, however associated ECM proteins are expressed in the gut during ENS development (Goldstein et al., 2013). In the CNS, ECM proteins are studied much more extensively. In the mouse brain, a small proportion of calretinin positive cell bodies are surrounded by PNNs (Karetko-Sysa et al., 2014). In rodent hippocampus, col19a1, the gene encoding collagen XIX, is expressed in subsets of hippocampal neurons that contain immunoreactivity for neuropeptide Y and calbindin, but not calretinin (Su et al., 2010). The majority of studies looking at ECM proteins and neuronal function is based on neuronal growth, plasticity, injury and repair (Meiners and Mercado, 2003), (Dityatev and Schachner, 2003), (Dityatev and Schachner, 2006), (Gaudet and Popovich, 2014), but there is a lack of studies describing the distribution and density of TNX in the CNS and importantly in the ENS.

In order to address this knowledge gap, this chapter will establish the expression pattern of TNX in the enteric layers of the stomach and colon using known neural markers in mouse and human.

2.2 Materials and Methods

2.2.1 Mouse tissue preparation

Surgical scissors (Moria) were used to dissect the mouse; a midline incision through the abdominal wall was made and the entire gut was immersed in Krebs solution. The whole gut was dissected free and transferred to a sylgard petri dish. The fundus and distal colon were then cut free and food products were dislodged by gentle pipetting using Krebs solution. Both tissues were cut open and flattened on the petri dish. Small etymology pins were used to stretch the tissue and keep it in place with the serosal surface facing down. The Krebs solution was discarded and the petri dish was replaced with 4% paraformaldehyde (PFA) (Sigma-Aldrich) and left overnight at 4^{o}C. For NG dissection, the vagus nerve was followed from the oesophagus and into the brain and carefully dissected, while the DRG beginning at position T13 and moving caudally were harvested, both tissues were fixed in 4% PFA for two hours.

All fixed tissues were then washed in phosphate buffered saline (PBS) (Sigma-Aldrich) (3x5 mins). For the fundus and colon, tissue was flattened onto the silicone dish and delaminated with fine forceps (Dumont) so that the muscle is separated from the mucosal layer. The colon and stomach tissues were then washed again (2x5 mins) to remove any excess PFA. The fundus and colon were then cut into smaller pieces and each piece placed in a 12 well plate along with the NG and DRG tissue. Serum protein block (Dako) was applied for 1 hr to prevent non-specific binding. The serum was then removed using a pipette and the primary antibody solution applied using the appropriate optimal concentration. This was left overnight at 4^{o}C agitated on a shaker. After overnight incubation the tissue was washed again (3x10 mins) using PBS on a shaker and the secondary antibody mixture applied for 1hr. The tissue was then washed 3x10 mins and placed on a charged slide (VWR International Superfrost Plus). A drop of mountant (Mowiol) was placed on top of the tissue and cover slipped. The slides were then left to air dry in the dark. Mouse sections followed the same protocol as human sections described below.

2.2.2 Human tissue preparation

Excess fat was cut from the full thickness tissue and fixed in the same way the mouse tissue was fixed using PFA. Fixed tissues were then washed in PBS (3x5 mins) and placed in 30% sucrose: PBS (Sigma) solution at 4°C overnight for cryoprotection. The tissue was then placed in a 50:50 solution of OCT and 30% sucrose PBS at 4°C overnight. Tissue was then rapidly frozen with optimal cutting temperature (OCT) (Tissue-Tek, Sakura) in liquid nitrogen and stored at -80°C until further use. Frozen blocks of tissue were cut into 10µm sections using a cryostat (Leica CM 1850), and air dried at room temperature for 1 hr. Sections were then washed in PBS (3x10 mins) and followed the same procedure as the whole mounts with the serum block, then application of primary antibody and secondary antibodies. The tissue was then mounted using Vectashield hardset without DAPI (Vector labs), with a coverslip (VWR) and left to air dry.

2.2.3 Solutions:

Krebs Buffer: (pH 7.4; 124mM NaCl, 4.8mM KCl, 1.3mM NaH_2PO_4, 1.2mM MgSO4.7H_2O, 2.5mM $CaCl_2$, 11.1mM Glucose, 25.0mM $NaHCO_3$),

0.1M Phosphate Buffered Saline (PBS): pH 7.4: 154mM NaCl, 7.68mM Na_2HPO_4, and 2.67mM NaH_2PO_4),

Antibody diluent (0.1M PBS with 0.2% (v/v) Triton-X100)

Mowiol Mounting Media (6% (w/v) Mowiol 4-88(Calbiochem, UK), 24% (v/v) Glycerin, 0.5% (w/v) DABCO (Sigma, UK) and 6% (v/v) DH2O in PBS).

4% Paraformaldehyde: (0.1M PBS, 4%PFA)

Table 2.1. Primary antibodies used for mouse whole mount immunohistochemistry, dilutions used, host and supplier

Primary Antibody	Dilutions	Host (supplier)
Tenascin-x	1 in 200	Rabbit (Santa Cruz)
Calretinin	1 in 300	Goat (Swant)
CGRP	1 in 200	Goat (Abcam)

Table 2.2. Primary antibodies used for mouse and human section immunohistochemistry, dilutions used, host and supplier

Primary Antibody	Dilutions mouse/human	Host (supplier)
Tenascin-x	1 in 200	Rabbit (Santa Cruz)
Calretinin	1 in 400	Mouse (Abcam)
CGRP	1 in 400	Mouse (Abcam)
ChAT	1 in 500	Sheep (Abcam)
NOS	1 in 500	Goat (Abcam)
PGP	1 in 1000	Mouse (Abcam)

Table 2.3 Secondary antibodies used for mouse and human section immunohistochemistry, dilutions used.

Secondary Antibody	Dilutions	Conjugated
Donkey anti Rabbit	1 in 300	568-red (Invitrogen)
Donkey anti Mouse	1 in 300	488-green (Invitrogen)
Donkey anti Goat	1 in 300	488-green (Invitrogen)
Donkey anti Sheep	1 in 300	488-green (Invitrogen)

All slides were imaged using an epi-fluorescent microscope. (Olympus BX61)

2.2.4 Mouse tissue analysis:

The images acquired from the epifluorescence microscope was used to qualitatively analyse whole mount tissue stained with TNX and other neuronal markers in 5 wild type mice. The cell bodies within the myenteric plexus and submucous plexus were observed to assess if any tissues were positive for TNX and whether positive labelling was associated with other specific neuronal population according to expression of specific markers.

2.2.5 Human tissue analysis:

Frozen human sections were used to analyse the expression of TNX with calretinin, CGRP and NOS as described in the mouse. With human tissue the addition of ChAT was also used as a marker for cholinergic neurons. Full thickness colonic resection tissue from patients undergoing surgery was obtained and only the healthy tissue a distance away from the cancer was used for IHC. Each colonic layer was examined separately including the submucosal plexus, myenteric plexus, circular muscle and longitudinal muscle. From each layer five fields of view were captured using 40x and 10x magnification and assessed for level of co-expression using the same pixel overlap method employed in the mouse. This was done in normal sigmoid colon, and in a preliminary study on human fundic stomach in patients undergoing bariatric surgery.

Image J was used for human and mouse tissue analysis using a JaCOP plugin, where the overlap of one pixel labelled in red (TNX) and the other pixel labelled in green (neural) is measured giving a final value showing the total percentage overlap. Once images were acquired they were converted to black and white images and a threshold was set so that only positive fibres and neurons were black and the background was white. The images were then converted to a binary image in each channel. The positive area was then highlighted using a freehand tool giving a region of interest. This region of interest was applied to the corresponding image in the other channel. The image was then measured for number of pixels. Therefore two values were obtained for each image for example one image showed the number of pixels that were positive in the green channel and the other showed the number of positive pixels in the red within the same region of interest. The overlap of pixels between two images were ascertained by using the

Manders coefficient that measures the percentage positive pixel overlap in one channel and another.

Whole mounts were used in addition to sections because, whole mount preparations gives a clearer picture of the nerve endings, since the ENS system is three-dimensional and enteric nerves are connected at different layers. In total, sections and whole mounts from the fundus (N=5) and distal colon (N=5) were used for IHC and analysed. The tissue was double stained with TNX and calretinin, CGRP, ChAT and NOS. Therefore it is possible to establish relative TNX co-expressed with sub populations of enteric neurons and endings

2.2.6 Confocal analysis

Confocal images of mouse and human tissue were obtained using Zen software on Zeiss LSM 710. These images were not quantified but used for illustrative purposes. Z stacks were obtained with interval of 2.1µm and compressed to obtain a high definition image.

2.2.7 Statistical analysis:

Mean and standard error of the mean (SEM) were calculated using Microsoft excel 2007. Colocalisation values were obtained using the Manders coefficient (measures the overlap of one positive pixel over another) where 1.0= complete overlap and 0= no overlap. Statistical significance was calculated using GraphPad Prism (one way ANOVA). A *p* value of less than 0.05 was considered statistically significant.

2.3 Results

2.3.1 TNX is found in neural structures in the ENS

TNX is a component of the ECM, therefore, we expected to see TNX staining in gut connective tissue, which is abundant in most layers of the gut. Surprisingly, TNX shows an intriguing pattern of expression, almost exclusively associated with neural structures.

2.3.2 Characterisation of TNX in mouse tissue

2.3.2.1 Characterisation of TNX in mouse colon

In the mouse proximal colon, TNX positive cell bodies were found in the myenteric and submucous plexus. TNX positive nerve endings were also observed in the circular muscle and longitudinal muscle (Fig 2.1). Calretinin was co-expressed with TNX, particularly in the submucous plexus (Fig 2.1A). There was little co-localisation between TNX and CGRP, although some submucous plexus cell bodies expressed CGRP and TNX (Fig 2.1B). Moreover CGRP positive nerve fibres surrounded the myenteric ganglia that contained TNX positive cell bodies (Fig 2.2B). TNX and NOS showed co-labelling (Fig 2.1C-yellow) in the MP in the proximal colon, whereas, in the distal colon only a few NOS neurons co expressed TNX (Fig 2.2C). In the distal colon, TNX colocalisation with calretinin and CGRP showed similar patterns of staining as the proximal colon, where the myenteric positive TNX neurons colocalised with calretinin and CGRP fibres were distinct (Fig 2.2B). This is represented graphically, where pixel quantification showed a higher % overlap with calretinin than CGRP in the mouse distal colon (p=0.0104) (Fig 2.3).

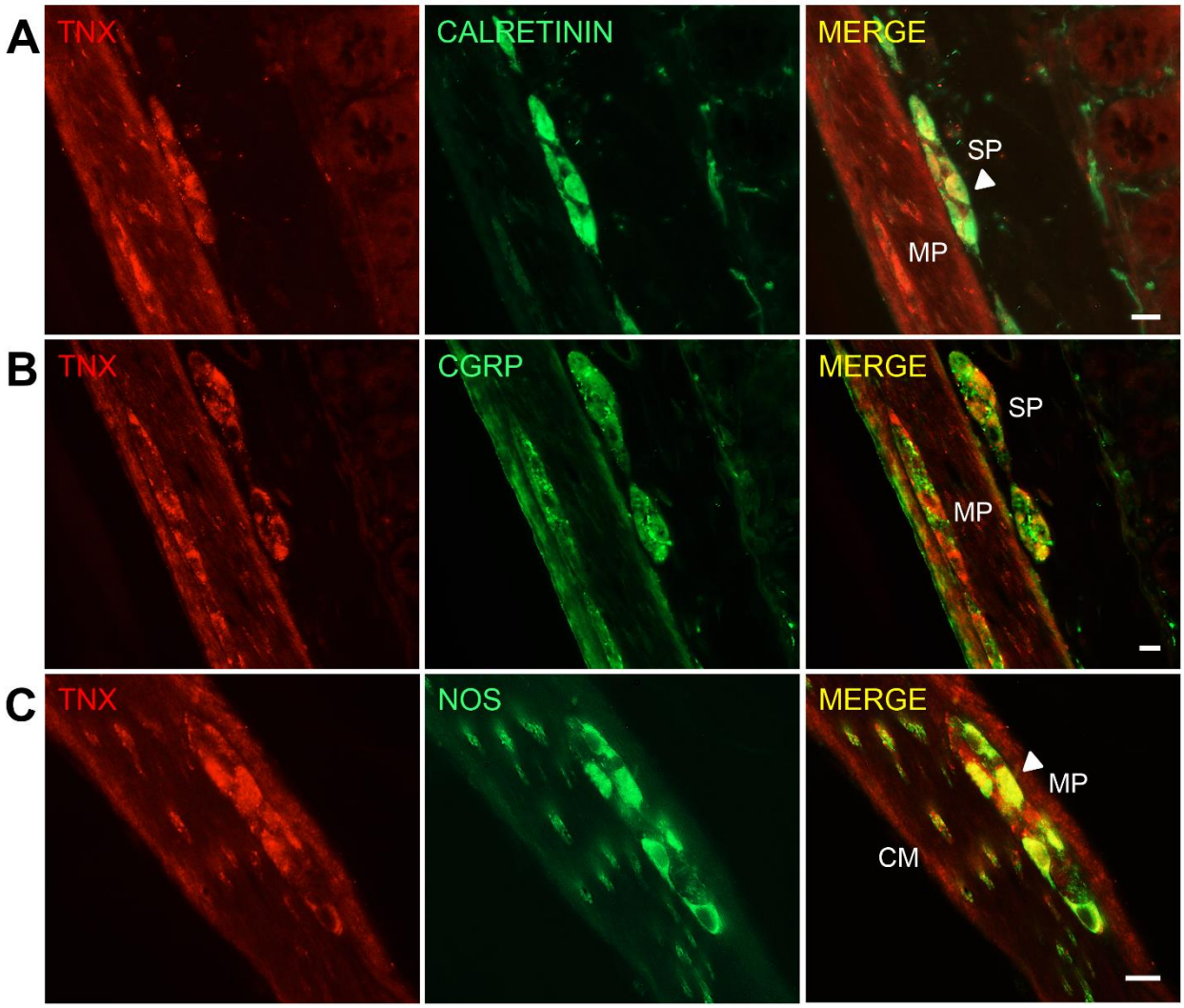


Figure 2.1. TNX associated with neural structures in mouse proximal colon.

(A) TNX (red), labels cell bodies in the MP, SP and smooth muscle layers. Calretinin (green) shows positive cell bodies only in the SP which co-expresses TNX (merge-arrowhead), (B) Positive CGRP is around the MP and SP, which is separate to TNX in the MP (merge). (C) NOS (green) shows co-labelling with TNX in the MP (Arrowhead). MP= Myenteric plexus, M= Mucosa, SP = Submucous plexus, CM= Circular muscle, LM=Longitudinal muscle. Scale bar 25µm.

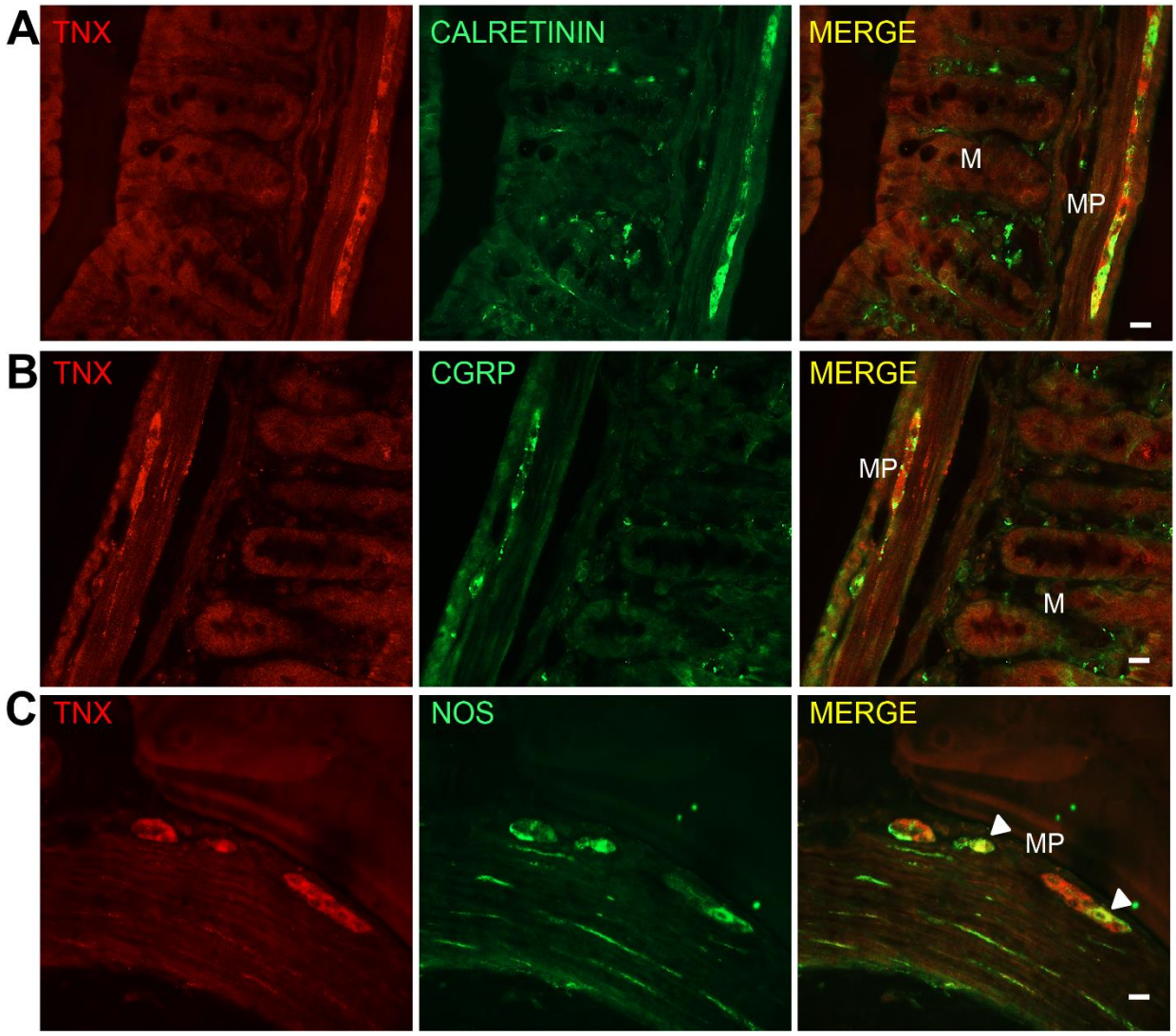


Figure 2.2. TNX associated with neural structures in mouse distal colon.

(A) TNX (red) and calretinin (green) labels cell bodies in the MP. Merge shows co-labelling of TNX and calretinin. (B) TNX (red) MP cell bodies and CGRP (green) surrounding MP that are separate. (C) TNX (red) and NOS (green) cell bodies in the MP. Some cell bodies co-express TNX and NOS (yellow) indicated with white arrowheads. Scale bar 25µm

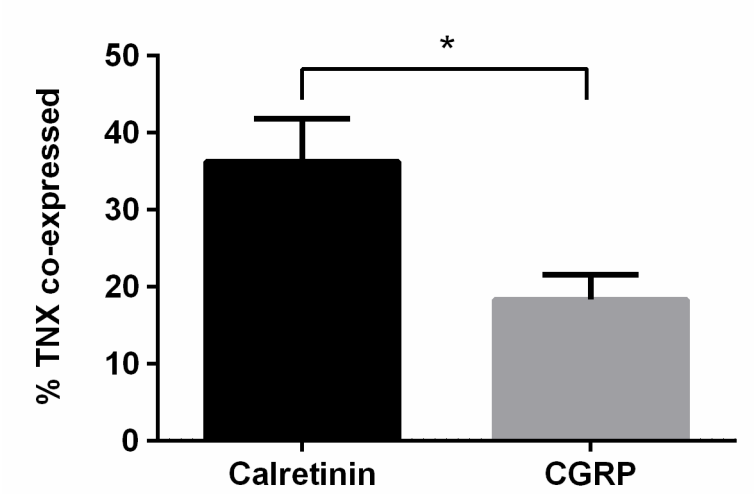


Figure 2.3. TNX is co-expressed with calretinin in the distal colon.

Total % of TNX that was co-expressed with calretinin and CGRP in mouse distal colon (N=5). TNX co-expressed with calretinin (37%) more than CGRP (17%) (p=0.0104). Error bars represent SEM.

It is important to note that the co-expression of CGRP and TNX may be an overrepresentation since although no co-localisation was apparent, CGRP surrounding the cell bodies are found on top of the positive TNX cell body, therefore the JACOP software cannot distinguish. From qualitative analysis CGRP and TNX positive fibres as well as TNX positive cell bodies were separate to CGRP.

In the rectum, TNX shows similar pattern of staining in the MP and SP and smooth muscle layers. TNX colocalised with calretinin in the MP (Fig 2.4A). CGRP and TNX showed distinct patterns of staining (Fig 2.4B), whereas some NOS myenteric neurons expressed TNX albeit few (Fig 2.4C).

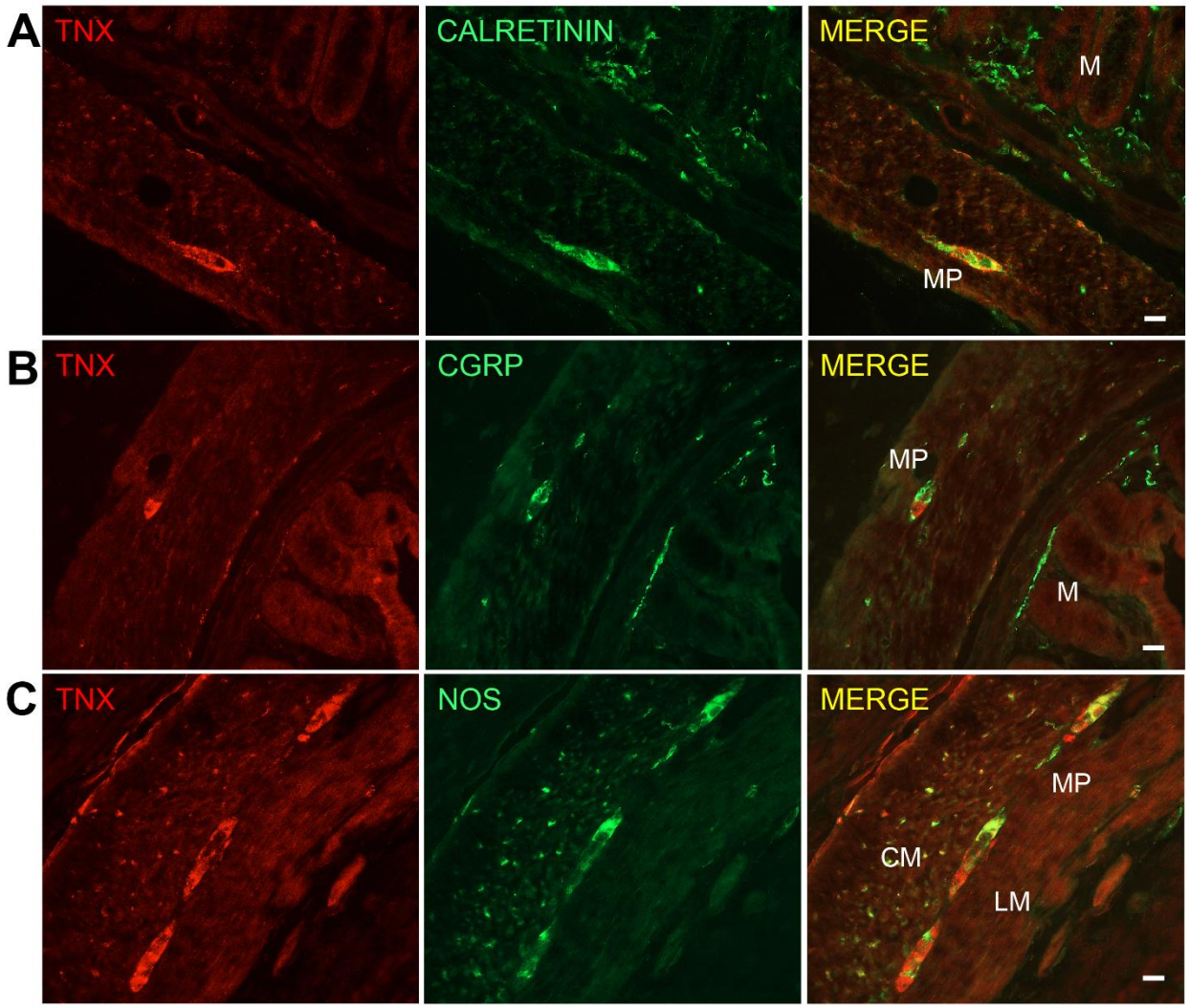


Figure 2.4. TNX in neural structures in mouse rectum.

(A) TNX (red) and calretinin (green) positive ganglia in the MP. Merge shows co-labelling of TNX and calretinin. Calretinin positive fibres found in the mucosa but not TNX. (B) TNX (red) and CGRP (green) positive MP cell bodies that are separate (merge). (C) Shows TNX (red) and NOS (green) cell bodies in the MP and CM. Few cell bodies in the MP and nerve endings in the CM co-express TNX and NOS (yellow). Scale bar 25µm

The pattern of TNX expression is restricted to the deeper regions of the colon however is completely absent in the colonic mucosa (Fig 2.5A)

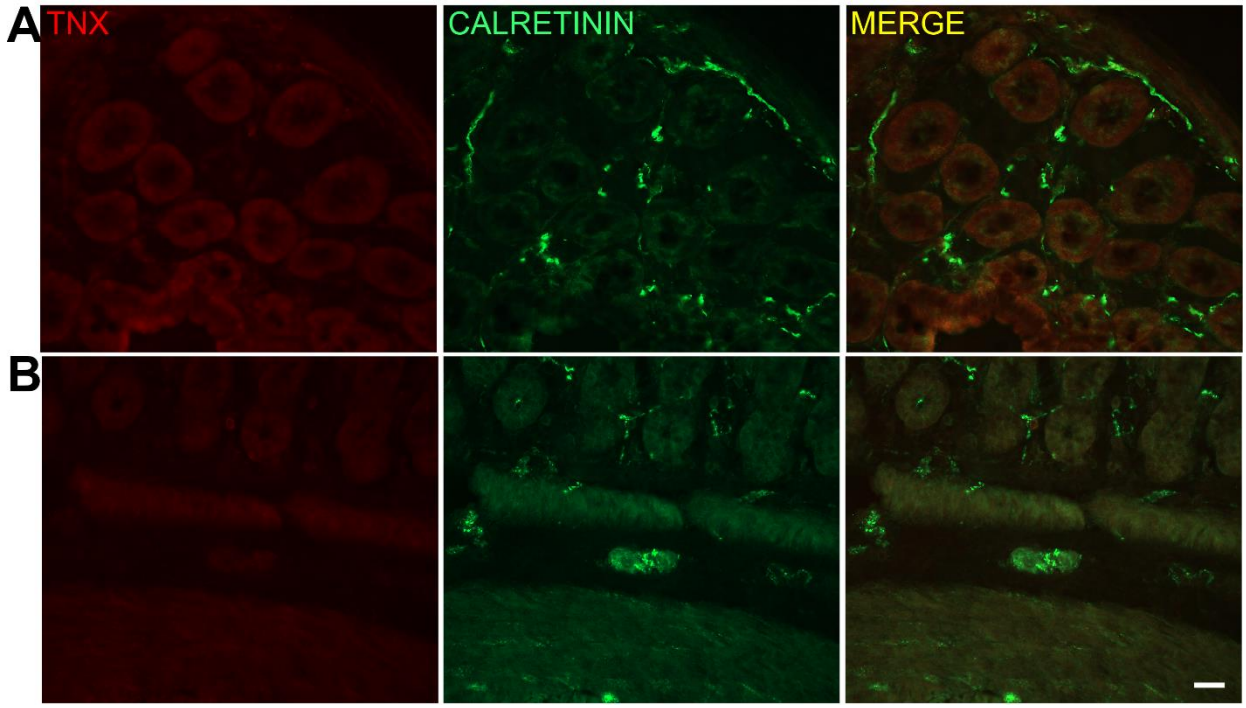


Figure 2.5.TNX absent in mouse colonic and gastric mucosa

(A) Colon. TNX (red) is absent in epithelium. Calretinin (green) positive endings around crypts. (B) Stomach. TNX (red) is absent in gastric epithelium. Calretinin (green) positive endings innervating the mucosa and myenteric plexus. Scale bar 25µm.

Therefore in the mouse colon TNX was co-expressed with calretinin in the proximal/distal colon and the rectum. CGRP shows some overlap but to a much lesser degree than calretinin. NOS and TNX showed differential colocalisation based on region whereby in the proximal colon TNX and NOS positive cell bodies were double-labelled, whereas in the distal colon and rectum a few cell bodies were TNX and NOS positive. TNX was absent in colonic mucosa of the mouse.

2.3.2.2 Characterisation of TNX in mouse stomach

In mouse stomach, TNX and calretinin positive fibres were seen in the circular muscle layer (Fig 2.6A) and longitudinal muscle layer (Fig 2.6B). Some of these fibres were labelled for both TNX and calretinin. TNX did not co label with CGRP and is represented graphically, where pixel quantification showed a significantly higher % overlap with calretinin than CGRP in the mouse fundus ($p<0.0001$) (Fig 2.8).

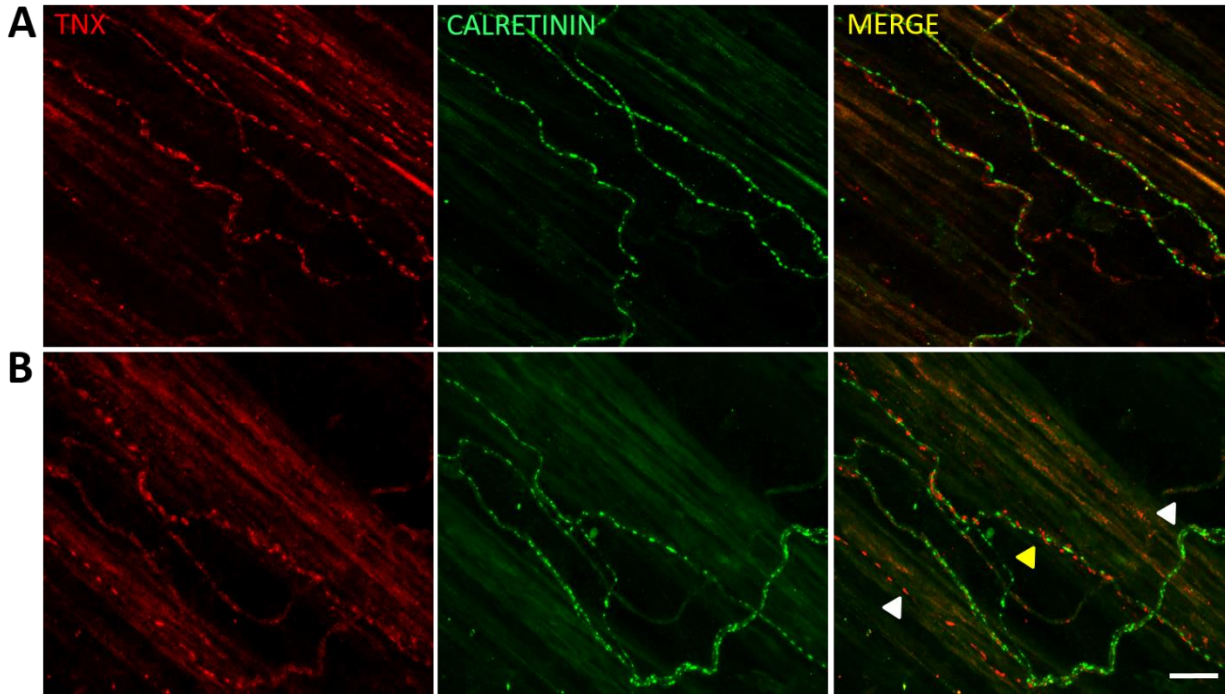


Figure 2.6. TNX found in IMAs in mouse fundus.

Confocal image of mouse fundus whole-mounts. (A) Calretinin labels an intramuscular array in the longitudinal muscle that is TNX positive indicated with yellow arrow, merge shows some fibres are independently stained with TNX and calretinin (white arrows). (B) Calretinin positive fibres in the circular muscle. Scale bar= 25µm

Similarly, TNX was also found around IGLEs that surround myenteric ganglia but not in cell bodies (Fig 2.7A). TNX was not found associated with CGRP positive fibres in the mouse fundus.

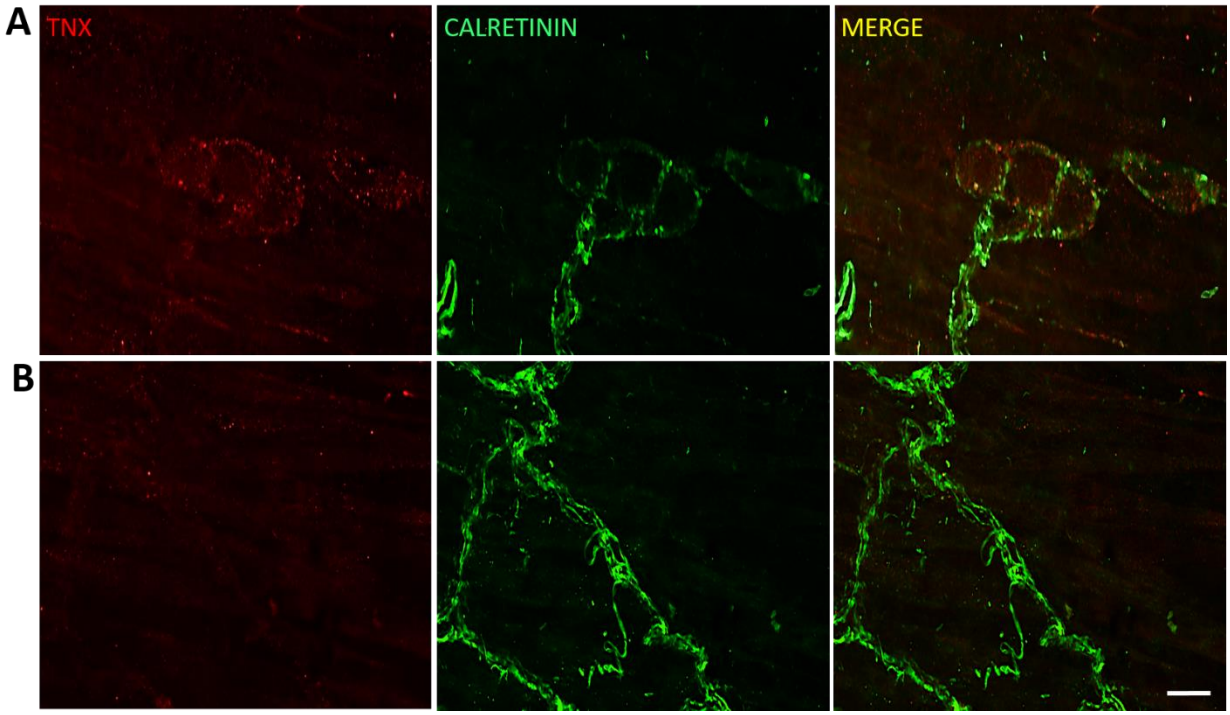


Figure 2.7. TNX found in IGLE nerve endings in mouse fundus but not in cell bodies.

Confocal image of mouse fundus whole-mounts. (A) Calretinin labels intraganglionic laminar endings in muscle layer innervating the myenteric ganglia that is TNX positive. (B) Calretinin positive IGLE fibres innervating the myenteric plexus in the same section, TNX negative cell bodies. Scale bar = 25µm.

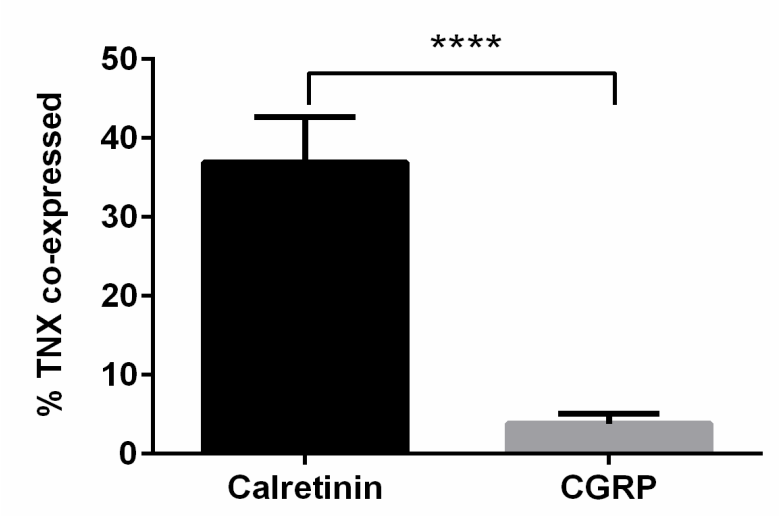


Figure 2.8. TNX is co-expressed with calretinin in the fundus.

Total % of TNX that was co-expressed with calretinin and CGRP in mouse distal colon (N=5). TNX co-expressed with calretinin (37%) more than CGRP (6%) (p=<0.0001). Error bars represent SEM.

2.3.2.3 Characterisation of TNX in mouse CNS

In the CNS, TNX was found in cell bodies, for example in the NG. TNX positive cell bodies were present however were separate to CGRP positive cell bodies (Fig 2.9A). In DRGs, TNX positive cell bodies were also seen which were mostly separate to CGRP with a few that overlap. An example is shown (Fig 2.9B). Similarly in the ventral horn (Fig 2.9C) and dorsal horn (Fig 2.9D), TNX immunoreactive cell bodies were present and co-expressed CGRP.

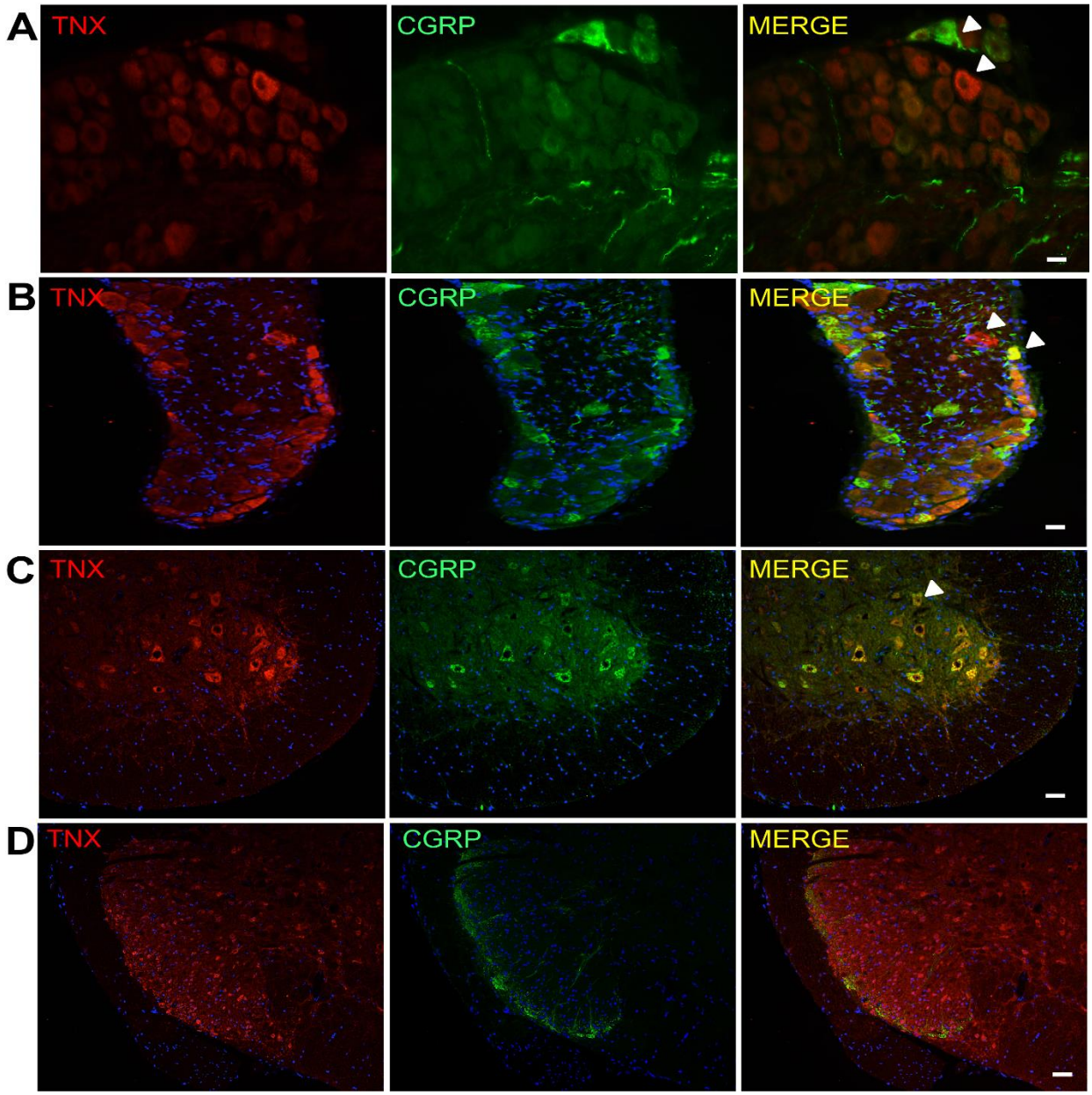


Figure 2.9. TNX found in mouse nodose ganglia, dorsol root ganglia and dorsal and ventral horn.

(A) TNX (red) and CGRP (green) positive cell bodies in the NG are distinct highlighted with white arrows (merge). (B) TNX (red) positive cell bodies and CGRP (green) positive cell bodies in DRGs. Merge shows co-labelling of TNX and CGRP and separate staining (white arrow). (C) TNX (red) positive cell bodies and CGRP (green) positive cell bodies in ventral horn. Merge shows co-labelling

of TNX and CGRP (yellow). (D) TNX positive cell bodies found in the dorsal horn that are separate to CGRP fibres. Scale bar 25µm.

2.3.2.4 TNX KO rectum

In the rectum of the KO mouse, hypertrophy of the smooth muscle layer was apparent. IHC staining with PGP showed positive myenteric plexus in both WT and KO (Fig 2.10 green).

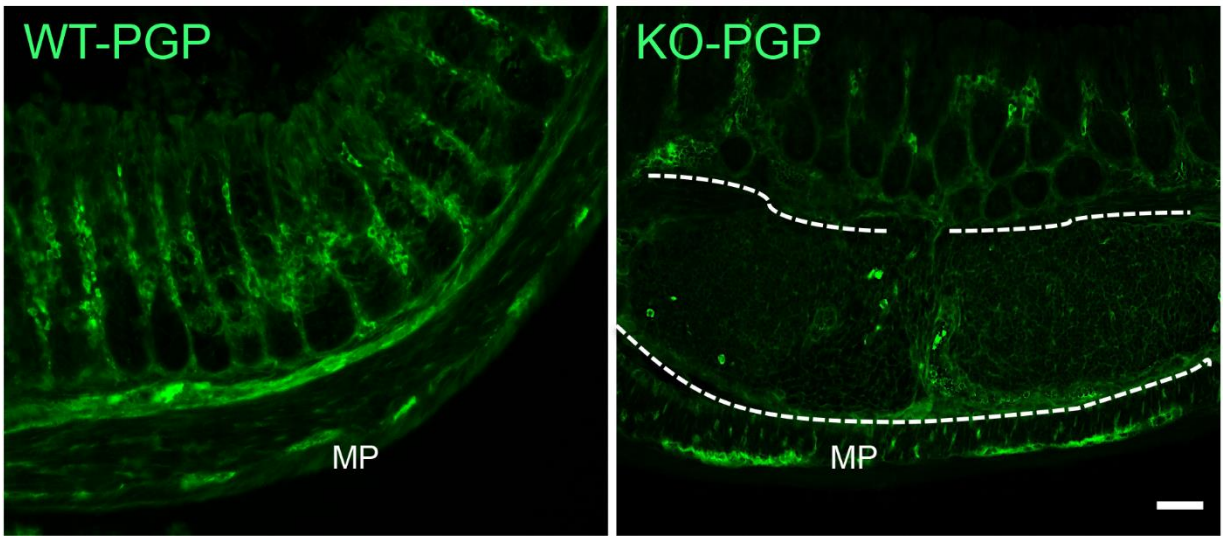


Figure 2.10. TNX KO mouse has hypertrophy of muscle and TNX is absent.

PGP labelling in normal WT mouse, KO mouse shows hypertrophy highlighted with the dotted white lines. Scale bar = 25µm.

In the KO mouse, TNX was used in the stomach and colon to confirm the specificity of the antibody. There was no TNX labelling in the mouse colon or stomach (Fig 2.11 and Fig 2.12). Qualitative analysis of the expression pf CGRP and calretinin in the KO mouse showed no observable changes compared to WT mice. In the stomach calretinin positive fibres surrounding the myenteric ganglia (Fig 2.11A) and CGRP fibres were observed (Fig 2.11B).

Therefore deletion of the TNXB gene did not observably influence the pattern or neurochemistry of innervation of the mouse GI tract.

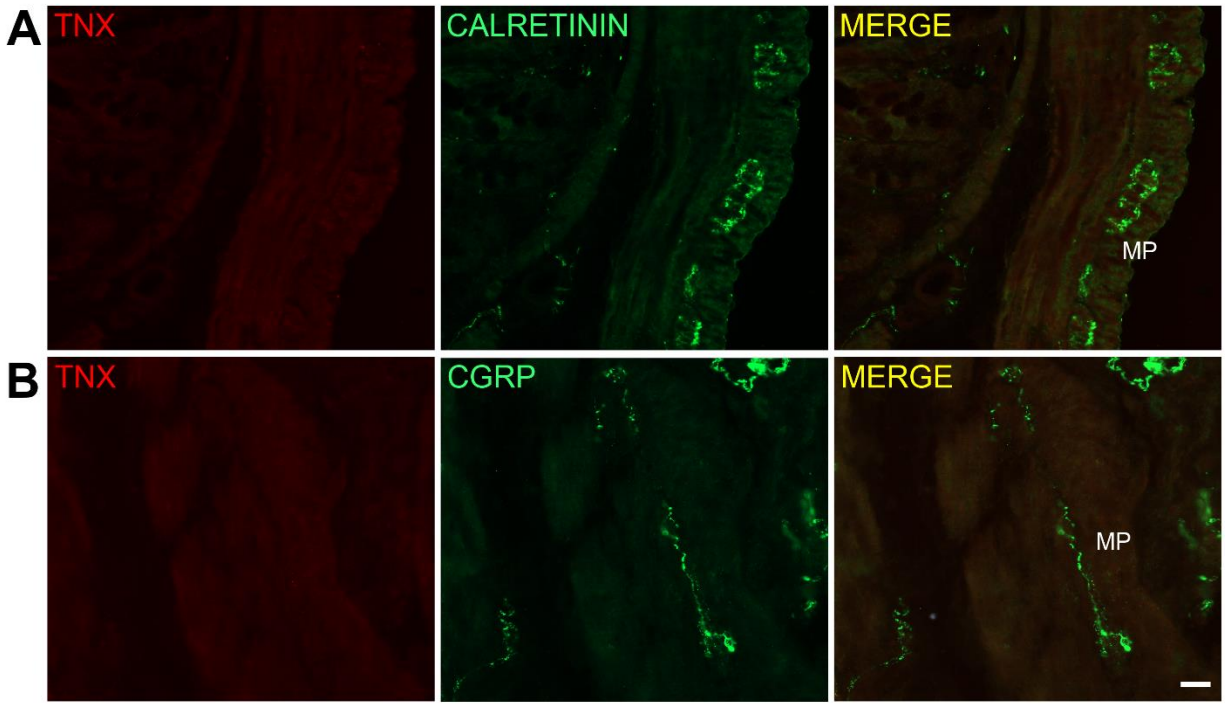


Figure 2.11. TNX KO mouse fundus shows no change in neural expression.

(A) No TNX (red) is found in the TNX KO but calretinin (green) positive fibres innervating the myenteric ganglia are observed. Merge shows labelling of calretinin only in the MP and mucosa. (B) No TNX (red) is found in the TNX KO but CGRP (green) positive fibres innervating the myenteric ganglia and mucosa is observed Merge shows only CGRP staining. Scale bar 25µm

Similarly, in the KO colon calretinin positive myenteric ganglia and fibre tracts in the mucosa were observed (Fig 2.12A). CGRP fibres were also found coursing through the myenteric plexus as well as the mucosa (Fig 2.12B).

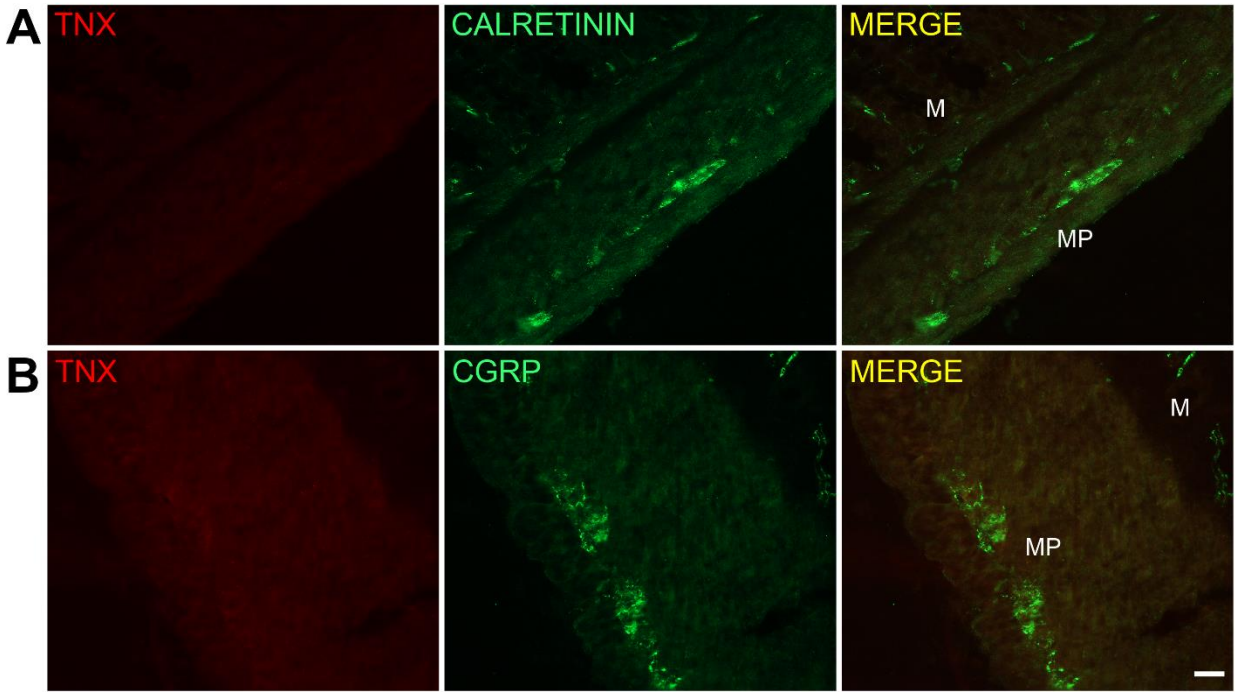


Figure 2.12. TNX KO mouse colon shows no change in neural expression.

(A) No TNX (red) is found in the TNX KO colon but calretinin (green) positive fibres innervating the myenteric ganglia is observed. Merge shows labelling of calretinin only in the MP and mucosa. (B) No TNX (red) is found in the TNX KO but CGRP (green) positive fibres innervating the myenteric ganglia and mucosa is observed. Merge shows only CGRP staining. Scale bar 25µm

2.3.3 Characterisation of TNX in human tissue

2.3.3.1 TNX is expressed in human colonic smooth muscle nerve fibres

Co-expression of TNX with specific nerve markers within the circular muscle (CM) and longitudinal muscle (LM) is shown graphically in Fig 2.13. TNX was found mostly in the circular muscle when compared to the longitudinal muscle (note Y scale on Fig 2.13). In the circular muscle, TNX showed similar levels of co expression with NOS (20.48% ±4.75) and ChAT (19.45% ±4.58). As described in the mouse, TNX was co expressed on calretinin positive fibres (16.23% ±4.08) in the circular muscle followed by CGRP which was less than 15% (Fig 2.13).

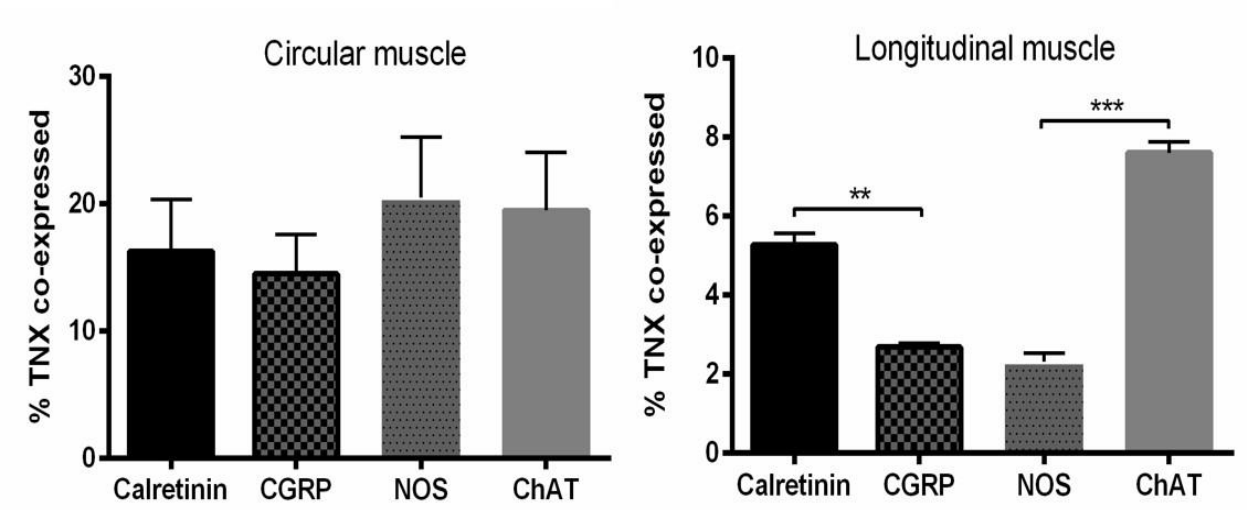


Figure 2.13. TNX found in human colonic nerve fibres smooth muscle

*A comparison of colocalisation of TNX with CGRP, NOS, Calretinin, and ChAT fibres separately in circular muscle and longitudinal muscle in the human colon (n=20). TNX is predominantly found in ChAT and calretinin containing neurons in both plexuses. Error bars represent the SEM. **= p<0.01) ***= p<0.001.*

2.3.3.2 TNX is expressed in human colonic cholinergic myenteric and submucous neurons

In the myenteric plexus, TNX showed most colocalisation with ChAT positive cell bodies (41.43% ±3.92) followed by PGP (42.83% ±4.96). Colocalisation with NOS and calretinin was below 25%, and TNX was rarely expressed with CGRP (8.05 ±1.97) (Fig 2.14). Similarly in the submucosal plexus, TNX colocalised most with ChAT positive neurons (52.56% ±5.34) followed by calretinin (38.52% ±5.30) shown in Fig 2.14.

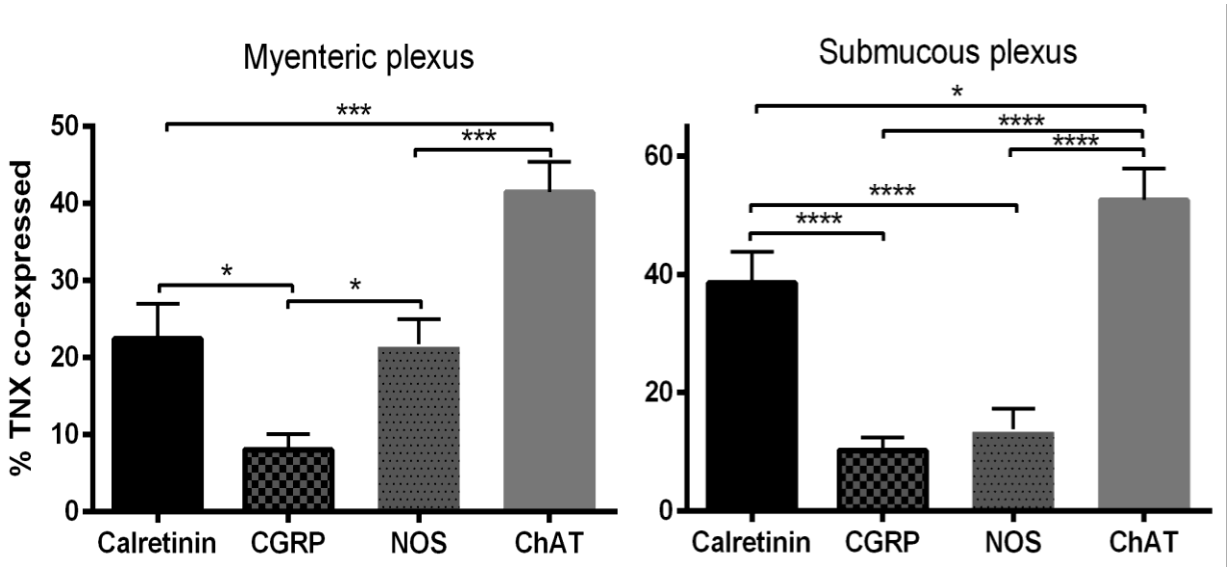


Figure 2.14. TNX found in human cholinergic neuronal cell bodies.

*A comparison of colocalisation with TNX with calretinin, CGRP, NOS and ChAT separately in the MP and SP in human colon (n=20). *= p<0.05, **= p<0.01) ***= p<0.001 and ****= p <0.0001. Error bars represent the SEM. TNX is predominantly found in ChAT and calretinin containing neurons in both plexuses. Error bars represent the SEM.*

IHC data showed the presence of TNX in both myenteric (Fig 2.15B) and submucous plexus (Fig 2.15A). CGRP and TNX were mutually exclusive and did not overlap (Fig 2.15B-merge), however, TNX found in calretinin positive submucous neurons showed complete co-labelling in yellow (Fig 2.15D-merge). NOS immunoreactivity with TNX was less common (Fig 2.15C), although some neurons expressed both (not shown).

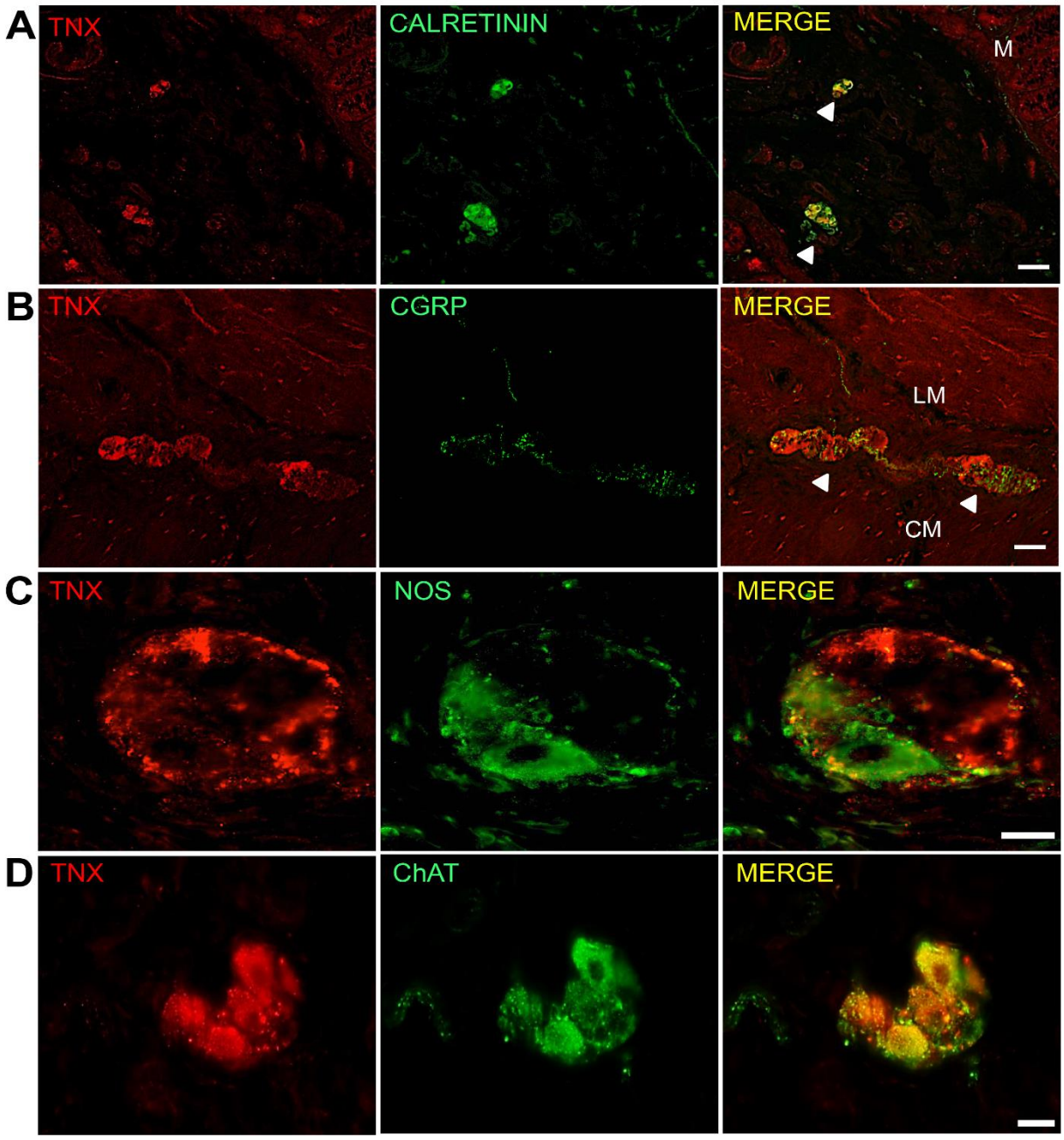


Figure 2.15. TNX found in neural structures in human sigmoid colon.

(A) TNX (red) and calretinin (green) positive cell bodies colocalised in SP (merge). (B) TNX (red) positive cell bodies in MP and smooth muscle layer, CGRP around MP and nerve fibre. Merge shows separate TNX and CGRP labelling. (C) TNX (red) and NOS (green) cell bodies in the MP that

do not co-label (merge). (D) Complete co-labelling of TNX (red) and ChAT (green) in MP (merge). Scale bar in (A) and (B) =50µm (C) =25µm and (D) = 20µm

Unlike the mouse, in the human fundus TNX was found in myenteric cell bodies (Fig 2.16A) where few cell bodies co-labelled calretinin (Fig 2.16A).

2.3.3.3 TNX is expressed in human gastric myenteric neurons

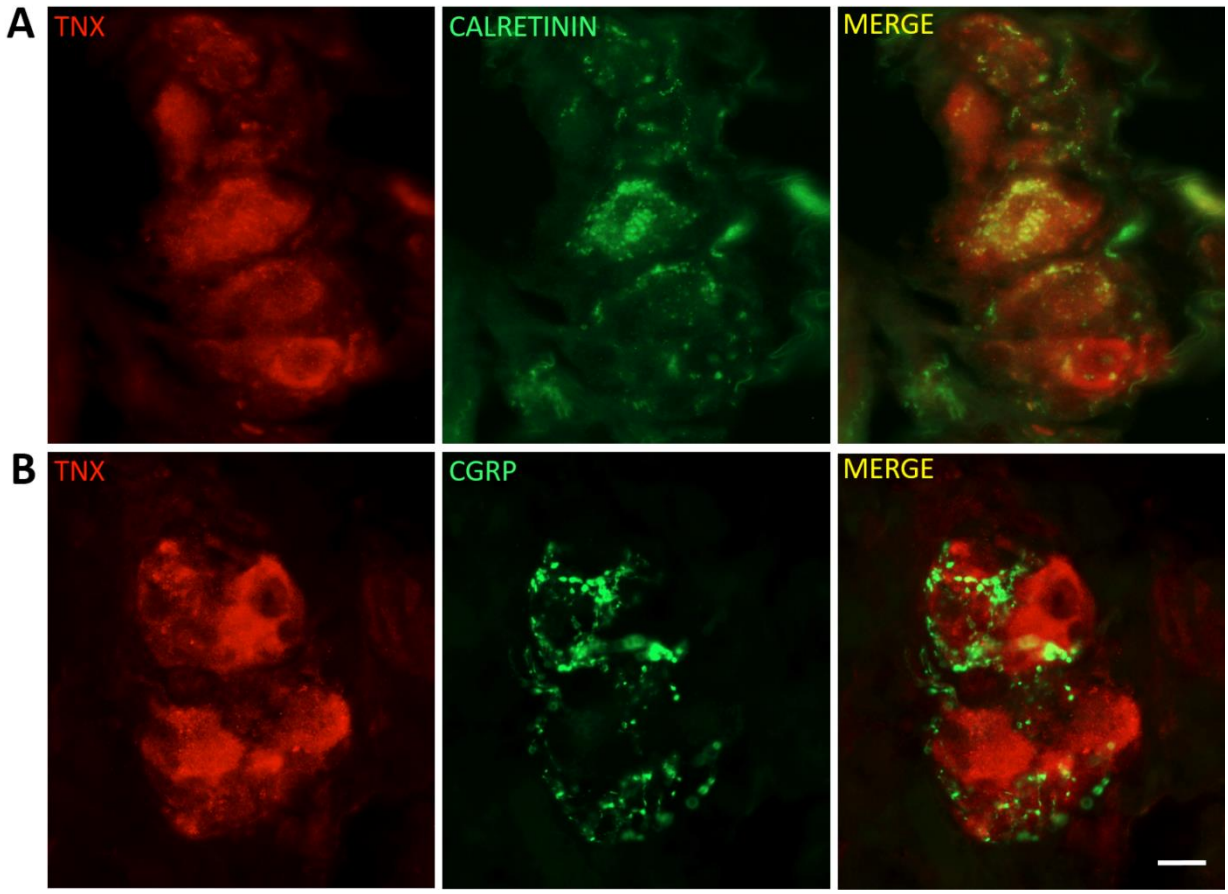


Figure 2.16. TNX found in neural structures in human stomach.

(A) TNX (red) and calretinin (green) positive cell bodies and fibre endings seen colocalised in fundus MP (merge). (B) TNX (red) positive cell bodies in MP and CGRP in MP. Merge shows separate TNX and CGRP labelling. Scale bar 25µm

IHC on tissue without the primary antibody was completed and serves as the control. This confirmed the specificity of the antibodies used. Examples can be found in Appendix 2.1 and 2.2.

2.4 Discussion

In this chapter, fluorescence IHC was used to determine the expression pattern of TNX. The choice of regions was based on origin of symptom observed in patients with JHS and TNX deficiency. Functional markers important in sensory and motor function were used together with TNX to understand the expression pattern of this ECM glycoprotein relative to intrinsic and extrinsic neurons. In mouse and human tissue, IHC shows that TNX is exclusively associated with neural structures and not with epithelial cells or connective tissue, which was unexpected given the critical role of TNX in elasticity of skin (Bristow et al., 2005). With regard to the extrinsic innervation of the gut, TNX shows a lack of association with sensory fibres containing CGRP that are associated with the transmission of noxious stimuli. Instead TNX was found primarily in distinct subpopulations of intrinsic enteric neurons. Vagal afferent endings in the mouse stomach were positive for TNX which are likely to originate from the TNX positive NG cell bodies. In addition, TNX was expressed in the mouse CNS including cell bodies of the NG, DRG, spinal and dorsal horn. Overall, it is clear that in both human and mouse TNX is expressed in specific neural structures such as vagal afferent endings in the stomach and cholinergic neurons in the colon that may serve specific functions. A small number of TNX KO mice had rectal prolapse, where IHC showed hypertrophy of the smooth muscle layer. Qualitative assessment of calretinin and CGRP in the KO colon and fundus showed no obvious changes in neuronal expression. TNX was absent confirming the specificity of the antibody.

In the colon, TNX was observed in all the layers except for the mucosa. Specifically in the mouse and human colon, TNX was found in the cell bodies of both plexuses and in nerve fibres of both muscle layers. In human colon, TNX colocalised predominantly with ChAT and calretinin expressing neurons in the submucous and myenteric plexus. The varying quantity of TNX expression and specific co-labelling with distinct neural markers suggests that TNX has a specific role. One such role could involve providing a supporting structure around the neurons similar to the hypothesis described earlier whereby TNX provides correct positioning of the vagal endings in the stomach. The notion that TNX can provide structural support has been proposed in the skin, where TNX regulates fibrillar spacing (Bristow et al., 2005). A loss of TNX in the skin causes

dysregulation in the spaces between fibrils giving rise to hyperextensibility. Similarly in the gut, TNX may provide spacing between neurons to allow efficient transmission of signals from pre synaptic to post synaptic terminals regulating neural function.

It is known that TNR and TNC are components of PNNs found in the CNS (Kwok et al., 2011). As described in Chapter 1, TNR and TNC from part of the tenascin family, that are found in PNNs which form a lattice like matrix enveloping soma and dendrites in neurons (Celio and Blumcke, 1994). PNNs are thought to play a direct role in the control of CNS plasticity and are composed of various molecules that include TNR and TNC as well as hyaluronan, and chondroitin sulfate proteoglycans (Kwok et al., 2011). The ENS may also contain a PNN-like structure that is similarly composed of various ECM molecules including TNX. This TNX may form part of the 'glue' that holds neuro-neuronal and neuromuscular connections together. This idea warrants further investigation by IHC studies charaterising known ECM molecules found in CNS PNNs in the stomach and colon.

Within the colonic submucosal plexus, TNX colocalised with calretinin positive neurons. Studies show that calretinin positively stains the majority of submucosal neurons that are multi-dendritic (Kustermann et al., 2011), moreover the majority of these neurons are cholinergic and co-stain with ChAT (Beyer et al., 2013). Therefore since ChAT and calretinin colocalised, the finding that TNX is mostly expressed in cholinergic ChAT and calretinin neurons was not unexpected. Abundant TNX in submucosal cholinergic neurons suggests a role in secretion which can be further studied using the Ussing chamber and segments of mouse gut, extensively used by (Cox et al., 2001). In colonic myenteric plexus TNX colocalised with ChAT and calretinin, but to a lesser extent than the submucous plexus, which has been corroborated in a previous study showing that only one tenth of the myenteric neurons are calretinin-positive (Beuscher et al., 2014). ChAT catalyses the synthesis of ACh, which when released from cholinergic neurons, stimulates colonic motility and tone via activation of muscarinic cholinergic receptors found on smooth muscle (Anlauf et al., 2003). Previous studies characterizing the distribution of ChAT, showed a dense network in the myenteric plexus which function as either inter-neurons and/or sensory neurons (Porter et al., 1996). Furthermore these cholinergic neurons were described as ascending and

descending inter-neurons involved in contraction and relaxation of the gut during peristalsis (Grider, 1989). Since TNX colocalised within cholinergic myenteric neurons, TNX may be important for normal peristalsis in the human and mouse colon. Therefore, in KO mice we may expect a loss of TNX to affect the cholinergic pathway resulting in abnormal contractile motility which could reflect symptoms seen in patients with JHS such as alternating bowel habits (Fikree et al., 2013) and constipation in TNX deficiency (Schalkwijk et al., 2001). In human colonic smooth muscle layers, TNX was mostly co-expressed in the circular muscle and a small percentage found in the longitudinal muscle fibres. Nerve fibres found in the circular muscle have a Dogiel type I shape cell body and function together with ICC cells driving muscle activity via the electrical junctions and muscle cells (Sanders et al., 2010). TNX was co expressed with both NOS and ChAT in the circular muscle at similar levels suggesting a role for muscle contractility. This needs to be further explored by electrical field stimulation using muscle strip that directly assesses muscle contraction (Sanger et al., 2013).

Vagal afferent fibres regulate gastric function in two ways. First by communicating with central mechanisms i.e. vago-vagal reflexes and secondly through direct effector systems via axon collaterals (Raybould et al., 1991) Functionally, the stomach can be divided into a) gastric reservoir (fundus and part of the corpus) which is characterised by tonic activity, and b) the gastric pump (distal corpus and antrum) which is characterised by phasic activity based on the different properties of the smooth muscle in each region. In the mouse and human fundus, TNX was expressed in vagal afferent nerve endings in the muscle layer, whereas in the human stomach TNX positive myenteric cell bodies were also observed. This suggests TNX may have a role in vagal afferent mechanoreceptor, functioning specifically as tension/stretch receptors in mouse. (Phillips et al., 2000), (Wang and Powley, 2000). Whereas in the human stomach, an additional role is possible for intrinsic motor control. A possible role for TNX around these endings may be to provide support in terms of anchoring the nerve in the appropriate location. Thus a loss of TNX may affect the response of vagal mechanoreceptors to length and tension induced by distension in turn affecting the rate of firing.

There is a paucity of data showing functional relevance of other ECM proteins in neurons in the gut, however, ECM proteins are found around vagal nerves and are important in migration. For example in mice, laminins are found around vagal sensory nerve endings and TNC which is structurally similar to TNX (Jones and Jones, 2000), actively modifies the microenvironment of vagally derived enteric neural crest cells regulating migration during foetal development (Akbareian et al., 2013). In human stomach TNX positive cell bodies were found in the myenteric plexus, where some co-expressed calretinin. TNX around cell bodies may have a role in forming a network allowing diffusion of neurotransmitters to bind to its receptors at the neuromuscular junction. Studies have shown collagen Q found at the neuromuscular junction anchors acetylcholinesterase. This enzyme regulates the release of ACh levels (Barros et al., 2011) which is the main transmitter important for excitation of contraction in the stomach (Schemann et al., 2001). Therefore TNX may play a similar role in anchoring neurons via similar mechanisms to collagen Q.

Few ECM molecules are associated with the vagus nerve, however, netrins, which are members of the ECM laminin superfamily (Rajasekharan and Kennedy, 2009) are thought to be critical for vagal innervation of the gut during development. Netrins are secreted by intrinsic ganglia and axons that direct the vagal axons expressing the associated receptor to their destination as either IGLE or IMAs (Ratcliffe et al., 2011) similar to the results obtained with TNX found associated with vagal afferents and myenteric ganglia. Furthermore netrins are associated with PGP 9.5 expressing neural structures in the myenteric plexus of the developing mouse stomach (Ratcliffe et al., 2011) which is parallel to TNX, being associated with myenteric neuronal cell bodies. Studies show that the survival of vagal sensory endings such as IGLE/IMA are dependent on intrinsic neurons secreting netrins, since endings degenerate in its absence (Ratcliffe et al., 2006). In the developing mouse gut, similar to netrins, TNX found around vagal afferent endings in the mouse and cell bodies in the human stomach may acts as a stop signal for sprouting of endings or a spacer to maintain the correct width of a synapse. This may also increase or decrease neuronal activation subsequently. Therefore, this concept means that TNX may act as a 'signalling' molecule, which when lost may cause disordered neural regulation leading to GI symptoms, however this requires further exploration. One method of assessing the importance

of TNX in neuronal growth, is by using enteric neural stem cell cultures in the presence and absence of TNX as previously used (Raghavan et al., 2010). This study involved growing gut smooth muscle sheets in the presence of ECM proteins and functionally testing the contractility of each muscle grown under different ECM conditions in response to electrical field stimulation (Raghavan and Bitar, 2014).

There was very little overlap of TNX and CGRP in the stomach and colon of both mouse and human tissue, which suggests that TNX is not involved in nociception. This is surprising since patients with JHS report increased abdominal pain (Nelson et al., 2015), (Fikree et al., 2015b), (Fikree et al., 2014). Another possible explanation is that pain sensitive JHS patients may fall under another subgroup caused by a deficiency or dysregulation in another connective tissue protein that may be associated with pain markers. This is plausible since not all patients who have JHS have TNX deficiency or have been screened for TNX deficiency. Alternatively, pain may result from disordered motility caused by TNX deficiency. Additionally, TNX was not found in the mucosa of gastric and colonic tissue in both species studied, suggesting TNX does not have a role in epithelial function or sensory mechanisms.

The expression of TNX in cell bodies of mouse NG was unsurprising, since one would expect TNX positive vagal afferents to derive from TNX positive cell bodies originating from NG. Similarly the extrinsic fibres in the colon that were positive for TNX may arise from TNX positive cell bodies seen in the DRG, ventral and dorsal horn. TNX may have a similar role in these cell bodies as described in the intrinsic gut.

A small percentage of TNX KO mice showed internal rectal prolapse which when stained showed hypertrophy of the smooth muscle. Similarly fibulin 3 and 5 involved in elastic fiber synthesis are important for maintaining the elasticity of the vaginal wall. An absence of fibulin 5 causes pelvic organ prolapse in 90% of mice and an absence of fibulin 3 causes rectal prolapse in 26.9% of mice (Rahn et al., 2009). TNX may serve a similar function to fibulins in the rectum whereby a deficiency causes irregular elastic fiber deposition and hypertrophy of the muscle is observed. To explore this possibility the expression of elastins around structures of the rectum is important. An alternative explanation could be that disordered motility is present, thus conditions like

prolapse and diverticulosis are likely due to pseudo-obstruction as demonstrated in the TNX deficient patients (Hendriks et al., 2012). Qualitative assessment in the KO mouse showed no obvious changes in neuronal innervation. This is likely since a loss of neurons and nerve fibres results in severe abnormalities, for example a loss of enteric neurons results in Hirschsprungs disease (Kapur, 2006), and no severe GI defects have been reported in TNX deficient patients and TNX mice. Rather, TNX may have a molecular role at the ultrastructural level in the GI tract that gives rise to disordered motility present in TNX deficient patients.

This study is not without limitations, firstly only the human colon was studied in terms of regions with a variety of markers and this should be done in the mouse colon and mouse and human stomach. This will give a more comprehensive understanding of the expression pattern of TNX and localisation in specific neuronal sub-types. Specifically the use of VIP along with ChAT/calretinin will delineate whether TNX containing submucous neurons are indeed implicated in secretion since VIP is a major player in colonic secretion (Reddix et al., 1994). Additionally co-staining of TNX with Hu protein which is a RNA binding protein that selectively labels all enteric nerve cell bodies relative to others (Murphy et al., 2007), will give an understanding of the total population of TNX containing cell bodies. Quantification by two independent observers would increase the validity of the results by removing subjective bias acquired while thresholding images for pixel analysis using JaCOP Image J software. For full characterisation of TNX, other regions such as the small bowel and oesophagus are also needed since reflux is commonly seen in JHS patients, which may be caused by dysfunction of the ENS in the oesophagus. Finally, the next optimal translational study would be to obtain tissue from JHS and or TNX deficient patients and observe if there are changes in TNX or neural expression in comparison to normal patient tissue.

In summary this is the first study to charaterise ECM protein TNX in the GI tract. It is evident that TNX is found in specific neural structures in both mouse and human tissue relative to others. TNX is primarily found in cholinergic neurons in the mouse and human colon suggesting a role for colonic contractions whereas TNX is found in the mouse and human stomach in vagal afferent endings and human myenteric neurons only. This suggests that TNX may have a role in regulating

afferent firing as well as the intrinsic motor control of the stomach. Therefore an absence of the gene may be reflected in changes in gastric emptying which is needed, as well as using electrophysiological recordings of vagal afferents innervating the stomach. In colon, alterations in motility and secretion may be present. To understand the functional role of the TNX expression described here it is important to assess changes in neural control of colonic motility by manometry and the function of submucosal neurons involved in secretion by using Ussing chambers that measure change in ion flux as an indirect measure of secretion. The chapters that follow will present these data.

3 The role of TNX in colonic motility

3.1 Introduction

Research into understanding intestinal motility started as early as 1899 when Bayliss and Starling stated “excitation at any one point of the gut excites contraction above, inhibition below”, which formed the famous law of the intestine (Bayliss and Starling, 1899). Since then, understanding of motility patterns has advanced and it is known that motility of the colon is highly regulated and characterized by contractions of the smooth muscle at single points along the entire length of the colon. This complex coordinated contraction requires input from the intrinsic and extrinsic nervous system and understanding how smooth muscle cells interact with enteric neural circuits is still under investigation. Colonic motility and secretory function are closely integrated, the latter will be described in detail in Chapter 4. Disturbances in colonic motility and sensation gives rise to constipation, diarrhoea and abdominal pain which falls under the umbrella term of IBS. Disturbances to sensorimotor function can also occur in organic inflammatory conditions such as Crohns and colitis.

IHC data in mouse colon shows TNX expression in calretinin positive neurons but not CGRP positive fibres in the myenteric plexus, although TNX positive neurons and CGRP positive fibres are in close proximity (Chapter 2, section 2.3). Calretinin positive neurons are co-expressed with ChAT positive neurons and can therefore act as a secondary marker for excitatory cholinergic function. In human colon similar results are observed where TNX is mostly expressed in ChAT and calretinin positive myenteric neurons (section 2.3.2). Clinical data from TNX deficient patients suggests a role for TNX in constipation since chronic constipation associated with diverticulosis and rectal prolapse was observed (Lindor and Bristow, 2005). Together, the characterization data and anatomical findings imply TNX is involved in either regulating or influencing normal colonic motility particularly the excitatory pathway evidenced by the association of TNX and ChAT and will be further explored in this chapter.

3.2 The structure and role of the colon

The structure of the colon directly reflects its role in absorption and propulsion. The musculature of the colon consists of the circular and longitudinal muscle. The circular muscle consists of thick cell bundles of smooth muscle separated by connective tissue (Hamilton, 1984). Circular muscle contractions partially or fully occlude the lumen to effectively mix/turn and propel during propagation (Sarna, 2010). The longitudinal muscle forms a thin layer below the serosa where contraction shortens the length of the colon and has little effect on mixing/turning and propulsion (Podolsky, 2016). The thickness of longitudinal muscle varies through the length of the colon, for example, longitudinal fibres are scattered in sigmoid colon (Johnson, 2006). In smooth muscle, taenia coli provide elastic support enabling contraction of the circular muscle (Gabella, 1983). The length of taenia coli are shorter than other muscle layers giving a segmented appearance called haustrations that are not fixed (Feldman et al., 2016). The rise of haustra are thought to be neutrally mediated as they appear, disappear and reappear during propulsion (Langer and Takacs, 2004) Haustrations look like pouches thus have less flow than the colonic lumen that is tubular, thus may serve a role for bacterial fermentation as well as absorption of water and ions (Brown et al., 1995), (Lange et al., 1995). Propulsion occurs via the coordinated movement of circular and longitudinal muscle. Motor patterns and force of contractions are determined by neuronal firing, excitability and contractile activity of the smooth muscle via myogenic mechanisms (Johnson and ScienceDirect (Online service), 2006).

The mammalian colon has adapted to perform major functions which include, a) water and electrolytes absorption, b) uptake of short-chain fatty acids and bacterial by-products, c) absorption of carbohydrates and bile salts, d) enabling movement of colonic contents in an aboral direction, e) providing a storage area for waste products until defecation and f) rapidly expelling stored waste products (Scott, 2003).

3.3 Colonic contractions and propulsion of luminal contents

The complexity involved in colonic motility suggests it is not mediated by a single type of contraction that are consistent in amplitude and frequency such as the rhythmic phasic contractions (RPC). In addition, overall colonic propulsion of luminal contents does not solely occur by creating a change in pressure at different regions of the colon (Sarna, 2010). Rather, propulsion of digesta occurs via propagation akin to the peristaltic pump (Sarna, 2010). This propagation and effective mixing/turning over of digesta is relative to the spatiotemporal characteristics (Cook et al., 2000). These characteristics include the direction, speed and distance of propagation which determine if digesta is propelled, mixed/turned over by forward and backward motion or both (Cowles and Sarna, 1990), (Johnson et al., 1997), (Sethi and Sarna, 1995), (Sarna, 1993). Resident smooth muscle cells are responsible for generating independent contractions while communication between these cells via gap junctions and neuronal networks in the plexi co-ordinate spatial contractions to varying degrees based on the colonic region (Sarna, 2010). Contractions this communication generates can either be propagating i.e. contractions that occur sequentially at adjacent regions to other parts of the gut at a distance, or non-propagating where they propagate over short distances or do not propagate at all (Sarna, 2010). These non-propagating contractions commonly occur in the colon producing back and forth movement of digesta that induce intense mixing/turning with slow net propulsion, to allow efficient absorption. The combination of propagation and amplitude of contraction determines the length a single bolus of digesta is propelled in the colon through each propagation (Sarna, 2010).

After ingestion of a meal and inter-digestive state, the colonic smooth muscle can generate three types of distinct contractions; the RPC mentioned earlier, ultrapropulsive contractions (UPCs) and tonic contractions (TCs) (Sarna, 1993).

The RPCs are the primary workhouse in the colon enabling slow net distal movement while mixing and turning over digesta. In humans and dogs, the viscosity of the faeces changes along the colon. In ascending colon contents are fluid and become semi-solid to solid in the sigmoid colon through absorption of water. To enable this change in viscosity, the colon generates two RPCs; short-

duration RPCs (SD-RPCs) that last 2-3 seconds and occur frequently, and long-duration RPCs (LD-RPCs) that last 15-20 seconds (Sarna, 1986), (Sarna et al., 1982), (Bueno et al., 1980). SD-RPCs display little propagation with varying amplitudes while LD-RPCs propagate across short segments (Sarna, 1986). Increased time of LD-RPCs allows luminal content to efficiently turn the semisolid contents to solid. Colonic RPCs are disorganised and change in amplitude and frequency allowing slow propulsion to turn over digesta (Sarna, 2010). In rodent colon, RPCs are small when animals are awake (Li et al., 2002), (Gourcerol et al., 2009), (Hipper and Ehrlein, 2001) Giant migrating UPCs are much larger in amplitude and duration than RPCs and can be divided into GMCs (giant migrating contractions) and retrograde giant contractions (RGCs). The propagation velocity of GMCs is rapid (~1 cm/sec) and in the aboral direction. Both these types of contractions produce mass and rapid movement of digesta giving little time for the luminal contents to contact the mucosal surface as such preventing digestion and absorption during this movement (Sarna, 2010). GMCs are two fold stronger (Sarna, 1987), last longer and propagate over lengthy distances compared to RPCs. In non-rodents and humans GMCs occur spontaneously in both fasting and postprandial state (Karaus and Sarna, 1987), (Otterson and Sarna, 1994), (Bassotti et al., 1999). In rodents, RPCs do not have the strength to effectively propel hard pellets along the colon, therefore contractile activity in rodents are predominantly via GMCs (Li et al., 2002), (Fida et al., 1997), (Bush et al., 2000), (Gourcerol et al., 2009), (Gonzalez and Sarna, 2001b), (Gonzalez and Sarna, 2001a), (Hipper and Ehrlein, 2001), occurring at a frequency of 15-25/hour (Gourcerol et al., 2009). GMCs in rats propagate only over short distances allowing gradual distal movement of pellets (Li et al., 2002). The third group of contractions are termed tonic contractions that are sustained by circular smooth muscle cells found in the lower oeosphageal and internal anal sphincters, the pylorus and ileocecal junction (Sarna, 2010). Sustained tonic contraction allows these areas to be partially or fully close to prevent reflux. The direction of these contractions is anal and regulated by enteric inhibitory neurons that reduce tone (Sarna, 2010). The resting shape, length and diameter of the basal tone and resting shape is maintained by smooth muscle cells (Sarna, 2010). Postprandially, the circular muscle tone increases in the colon, constricting but not occluding the lumen (Coulie et al., 2001), (Ford et al., 1995), (Coffin et al., 1994). Duration and amplitude of tone is dependent on the

volume and calorie content of ingested food (Ford et al., 1995). The tone itself does not cause propulsion, mixing and turning over, instead it narrows the lumen to enhance motile function of RPCs, and this allows the weaker RPCs to sometimes occlude the lumen increasing the effectiveness of mixing/turning and propulsion of digesta (Ford et al., 1995).

The strong lumen-occluding GMCs can propel digesta over long distances without any interruptions, propelling the increasing bolus size along the length of the colon (Karaus and Sarna, 1987). This increase in mass causes the receiving segment to distend by relaxing the tone and inhibiting RPCs to reduce resistance against the propelling bolus. This is mediated by descending inhibition (Karaus and Sarna, 1987), (Bassotti et al., 1999), (Chey et al., 2001) which occurs when GMCs compress the colonic wall. This in turn stimulates descending interneurons that connect inhibitory motor neurons innervating smooth muscle (Otterson and Sarna, 1994), (Sarna, 2007). This inhibition further relaxes the sphincters for ease of passage (Shi et al., 1998). Conversely, RPCs do not induce descending inhibitory signals since they do not induce strong colonic wall compression.

The colon generates all three type of contractions, however, the spatiotemporal characteristics differs between species, indeed GMCs in rodents occur frequently but at irregular intervals (Li et al., 2002), (Fida et al., 1997), (Bush et al., 2000), (Gourcerol et al., 2009), (Gonzalez and Sarna, 2001b), (Gonzalez and Sarna, 2001a), (Hipper and Ehrlein, 2001), hence care must be taken when extrapolating animal studies to human colonic motility in health and disease.

3.4 Colonic motility: regulatory mechanism

Organised occurrence of gut contraction into spatiotemporal patterns such as propagating and non-propagating contractions is autonomous, in other words, extrinsic input from hormones and extrinsic nerves is not needed (Sarna, 2010). Instead the resident smooth muscle cells and the enteric neurons can regulate motility independently. Nonetheless, both parasympathetic and sympathetic components can partly influence enteric neural and myogenic activity in turn altering gut contractile responses to the environment. Autonomic nerves indirectly influence motility function via modulation of the ENS (Sarna, 2010).

3.4.1 Excitation-contraction coupling

Excitation-contraction coupling is a complex process and a detailed description is beyond the scope of this thesis chapter. In brief, smooth muscle contraction is regulated by phosphorylation of myosin chain subunits of myosin (MLC20) dependent on a) calcium/calmodulin action on myosin light chain kinase (MLCK) or b) calcium independent action of various kinases such as Rho-kinase, integrin-linked kinase (ILK), and c) zipper-interacting protein kinase (ZIPK) (Sanders, 2008). Smooth muscle cells contract when the MLCK contracts therefore increasing calcium in the cytoplasm which is the primary driver in initiating contractions. In addition, MLC20 phosphorylation enables actin and myosin to associate and form cross-bridge cycling (Sanders, 2008). Excitation-contraction coupling has two main components that is; electromechanical and pharmacomechanical coupling (Sanders, 2008).

In colon, depolarisation of circular smooth muscle cells results in electromechanical coupling. An individual slow wave has 3 parts; the upstroke, plateau and repolarization (Huizinga et al., 1986). The resting membrane potential is -60 to -80 mV (Huizinga et al., 1986), (Serio et al., 1991). Membrane depolarisation during plateau is less negative at -40 to -60 mV (Huizinga et al., 1986), (Serio et al., 1991), (Sarna, 2010). Ca^{2+} influx by opening of voltage-gated calcium channels is induced by slow wave depolarisation, however this depolarisation is below threshold for RPCs (Liu et al., 2001). Slow wave depolarisation creates small contraction inefficient in propelling and mixing digesta (Liu et al., 2001). In-vitro organ bath studies in circular smooth muscle show small RPCs that associate with RPCs (Lu et al., 1997), (Sato et al., 1994), (Huizinga and Waterfall, 1988), (Sanders, 1983), however, the methodology induces tension which may open cation channels causing influx of Ca^{2+} in turn phosphorylating RLC by MLCK activation (Large, 2002), (Ji et al., 2002), (Kirber et al., 1988). Similar to electromechanical coupling, pharmacomechanical coupling cannot generate strong contractions for propulsion and mixing and turning over contents. However propulsion and mixing/turning over does occur by pharmacomechanical coupling if slow wave depolarisation in conjunction with ACh release and binding to M_3 muscarinic receptor (McFadzean and Gibson, 2002). This coupling enhances contraction amplitude without increasing intracellular Ca^{2+} by a complex process called adjustment of calcium sensitivity (Somlyo and

Somlyo, 2003). The complex signalling pathways are not described here but are crucial in generating all 3 contraction types mentioned earlier particularly GMCs and TCs. In humans, colonic slow waves occur in 3-12 cycles per min and are variable in amplitude and frequency (Sarna et al., 1980). It has been reported that ICCs are important in regulating slow waves (Sanders, 2006), however in the colon propagation of slow waves is disordered thus attributed to communication that occurs in smooth muscle cells via gap junctions to induce colonic propagation (Sarna, 2008). Thus ICCs alone have a limited role in inducing propagation.

3.4.2 Excitation-inhibition coupling

Suppression of excitation-contraction coupling is mediated by signals from the spinal cord, hormones and inflammatory/stress mediator resulting in excitation-inhibition coupling. This primarily occurs by NO and VIP released from inhibitory motor neurons (Furness, 2012). Cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) are also released and together inhibit smooth muscle contraction by sequestering intracellular calcium and reduce MLCP (Somlyo and Somlyo, 2003). cGMP and cAMP communicate with protein kinase A or B (PKA/PKB) respectively (Somlyo and Somlyo, 2003), (Murthy, 2001) and at low concentrations VIP activates PKA while at high concentrations protein kinase G (PKA-G) is activated (Huber et al., 1998). Together the signalling pathways of the aforementioned molecules make specific contribution in colonic motility.

3.4.3 Enteric neuronal regulation of colonic motility

The axons of the Dogiel type I motor neurons branch extensively along the circumference of the colon innervating the myenteric plexus and circular muscle. This branching allows neurotransmitters to act around the circumference of the colon causing ring like contractions (Lomax et al., 1999), (Nurgali et al., 2003b). In the resting state, low levels of both excitatory and inhibitory neurotransmitters are continuously released to maintain a counterbalance achieving the resting tone. When ACh is further released by excitation-contraction coupling as described before, the balance is shifted and the system is overwhelmed inducing contraction. When NO oxide is released, the excitation contraction coupling is blocked and the colon relaxes (excitation inhibition coupling). This has been demonstrated where NOS inhibitors such as L-name increased

the amplitude of RPCs in fed and fasted states (Takahashi et al., 2005), (Takahashi et al., 2005), (Orihata and Sarna, 1994), (Sarna, 1993). The excitation-inhibition coupling is crucial in descending inhibition prior to a GMC (Grider, 2003), (Grider and Makhlouf, 1986 5554), (Sarna, 2003). Some studies show that ICCs act as intermediaries between motor neurons and smooth muscle cells (Beckett et al., 2005), (Horiguchi et al., 2003), (Wang et al., 2003). The idea is that the motor neurons excite ICC cells in the intramuscular layer which then communicate signals to the smooth muscle cells to contract through nerve varicosities in the gap junctions (Sarna, 2008). Although ICCs may influence the coordination of contraction, it is well known that the motor neurons and smooth muscle cells largely generate RPCs (Goyal and Chaudhury, 2010), (Zhang et al., 2010).

Dogiel type I interneurons are important messengers between adjacent ganglia in oral, anal and circumferential directions. Depending on the direction of interneuron projection, they either activate excitatory or inhibitory motor neurons in the circular muscle layer (Furness, 2006). The peristaltic reflex described by Bayliss and Starling state that enteric reflexes can generate contractions above the site of stimulation (i.e. oral end) and simultaneously below it (i.e anal end) (Bayliss and Starling, 1899). The signal in the oral direction releases ACh from excitatory motor neurons in small quantities and SP in larger amounts to cause the initial contraction, whereas the signal in the anal direction initiates release of NO and VIP causing relaxation and lowers muscle tone (Sarna, 1993). The interneurons form a relay system to provide signals from sensory nerve endings to motor effector neurons that stimulate release of their respective neurotransmitters (Lomax et al., 1999) It has been shown that 90% of ascending and 50% of descending interneurons contain ChAT alone or ChAT and NOS (Grider, 1989), (Frantzides et al., 1987), (Sarna et al., 1981). Both types of interneurons synapse with nicotinic receptors that induce ascending and descending contractions and relaxations (Furness, 2000). A small number of descending interneurons are also immunoreactive for VIP, TKs, 5-HT and neuropeptide Y (Wattchow et al., 1997), (Wardell et al., 1994 4013) however, the exact role of these peptides in interneuron function is unclear.

The Dogiel type II/AH intrinsic sensory neurons respond to chemical or mechanical stimuli that transmit signals to a wider field resulting in ring like contractions. In the guinea pig small intestine, mucosal stimulation by stroking causes fast and slow EPSPs on postsynaptic neurons (Pan and Gershon, 2000). This is dependent on neurons since TTX and hexamethonium blocks the fast EPSP. The colon can generate contractions in the absence of any luminal contents which is attributed to Dogiel type I and II neurons that generate fast and slow EPSPs (Sarna, 1992), (Lomax et al., 1999). These neurons release neurotransmitters at effector junctions through motor neurons and contribute to CMMC (Sarna et al., 1981), (Sarna, 1985). Timing of slow waves are independent of EPSP generation and may not occur simultaneously with excitatory and inhibitory transmitter release. Partial/complete overlap of slow waves and fast EPSPs changes the size of action potentials during the plateau phase giving varied contractions in terms of amplitude

The peristaltic reflex does not propel luminal contents autonomously, slow-wave contraction/inhibition coupling is also required to propagate digesta allowing propulsion. Understanding of this reflex since Bayliss and Starling explained intestinal peristalsis has greatly improved particularly through studies in rodents and guinea pigs using a variety of techniques including flat sheet colonic preparations, ex-vivo colonic segments, electrophysiology and IHC (Grider, 1993), (Grider and Jin, 1994), (Grider, 2003), (Grider and Makhlouf, 1986), (Wade and Wood, 1988a), (Wade and Wood, 1988b), (Kadowaki et al., 1996), (Gershon, 2004), (Costa and Furness, 1976), (Grider et al., 1996), (Bian et al., 2004). Based on these seminal studies the following has been established. Principal stimulation of mucosa or circumferential stretch in the muscle initiates orally directed excitatory signals and anally directed inhibitory signals in a length of colon by nicotinic synapses found on interneurons }. Higher intensity stimulation release SP while ACh is released at smaller intensities. These neurotransmitters stimulate the smooth muscle cells in the circular muscle by creating an excitatory junction potential (EJP) and increase circular muscle tone or induce RPCs (Sarna, 2010). Anally mediated signals release NO and VIP in the lower colon inducing inhibitory junction potentials (IJP) to reduce circular muscle tone (Sarna, 2010). In the mucosa, 5-HT is released in response to mucosal stroking which stimulates 5-HT receptors (5-HT_{1P}/5-HT_4) on intrinsic sensory axons in the mucosa that subsequently release CGRP/ACh to stimulate interneurons projecting orally and anally, (Kadowaki et al., 1996), (Grider

et al., 1996), (Gershon, 2004). 5-HT_4 on intrinsic nerve endings may further regulate ACh/CGRP release (Kadowaki et al., 1996).

The arrival of a meal is detected by sensory nerve endings within the mucosa that express chemoreceptors and mechanoreceptors and this information is related to interneurons, this results in a cascade of effector motor neuron activation (Sarna, 2010). The myenteric ganglia is interconnected to other ganglia throughout the whole colon, therefore, the response to luminal contents produces a global response such as colonic contraction (Sarna, 2010). Once signals reach the excitatory and inhibitory motor neurons, they release neurotransmitters that mediate excitation (contraction) and inhibition (relaxation) of motor neurons (Sarna, 2010). Excitatory neurotransmitters are; Ach, tachykinins and SP (Costa et al., 1980) while NO is the primary neurotransmitter that inhibits motor neurons (Furness, 2000), (Grider, 1989), (Grider and Jin, 1994), although VIP is also involved and expressed on inhibitory motor neurons (but VIP antagonist does not block descending inhibition in animals) (Sarna, 2007).

The gut has a complex dual system that allows rhythmic propagation by communication between enteric nerves and the smooth muscle cells.

3.4.4 Colonic migrating motor complex (CMMC)

CMMC is a distinct pattern of electrical and mechanical contraction in the smooth muscle. CMMCs function to expel luminal contents in a propagative manner and are characterized by retrograde contractions i.e. aborally, but anterograde contractions do occur occasionally. The earliest study describing CMMCs was in the dog (Sarna, 1985), however the mouse is now the animal of choice when studying CMMC since the entire colon can be excised and studied *in vitro* (Bush et al., 2000), (Brierley et al., 2001), (Fida et al., 1997) including direct pharmacological intervention. CMMCs in mammals seems to be the equivalent to the HAPC seen in human colonic recordings (Smith et al., 2014). It is important to recognize that CMMCs are a slowly migrating group of contractions whereas GMCs described earlier are large contractions that occur more frequently.

Using extracellular electrodes, spontaneous electrical activity i.e. CMMCs were measured in the isolated mouse colon propagated approximately every 2 mins (Brann and Wood, 1976) and has been described in various mouse strains including C57BL/6 (Bush et al., 2001), (Bush et al., 2000 3912) which is the background of TNX KO mice. Using this technique electrical activity in the circular smooth muscle cells can be recorded in the whole mouse colon and electrical activity is termed the myoelectric complex (MC) which forms an integrated unit with the CMMCs (Lyster et al., 1995), (Bywater et al., 1989), (Spencer et al., 1998b). The frequency of the MC is similar to the CMMC (~ 2-4 min) and propagation persists in the presence of Ca^{2+} channel blocker meaning MC does not require CMMCs to initiate (Bywater et al., 1989). Since CMMCs can be recorded *in vitro* extrinsic neural control may not be required for its generation, indeed MC activity is not modified when lumbar colonic nerves from the inferior mesenteric ganglion (IMG) are severed (Lyster et al., 1995). The MC has two components; fast oscillations in membrane potential at ~2 Hz, and the underlying slow membrane depolarization that is longer and lasts for ~20-60s (Lyster et al., 1995), (Bywater et al., 1989), (Spencer et al., 1998b). The rapid first component of the MC is cholinergic since muscarinic antagonists such as hyoscine and atropine abolish MCs and reduce CMMC amplitude unlike the slow secondary component that was unaffected (Spencer et al., 1998a). Since the rapid component can be abolished using atropine, the onset of MCs are thought to be via release of ACh from circular muscle motor neurons (Bush et al., 2000). MCs and CMMCs are neural in origin since the rapid oscillations as well as the slow depolarisations are abolished with hexamethonium or tetrodotoxin (TTX) (Bush et al., 2000). The application of TTX caused the circular muscle cells to depolarize to a similar level as the secondary slow depolarization in each spontaneous MC therefore in between each MC the circular muscle is under tonic inhibition (-55Mv) by inhibitory motor neurons (Spencer et al., 1998b). The tonic inhibition is caused by the release of NO since inhibitors of NO such as N-nitro-l-arginine (NOLA) increase the frequency of CMMCs (Fig 3.1).

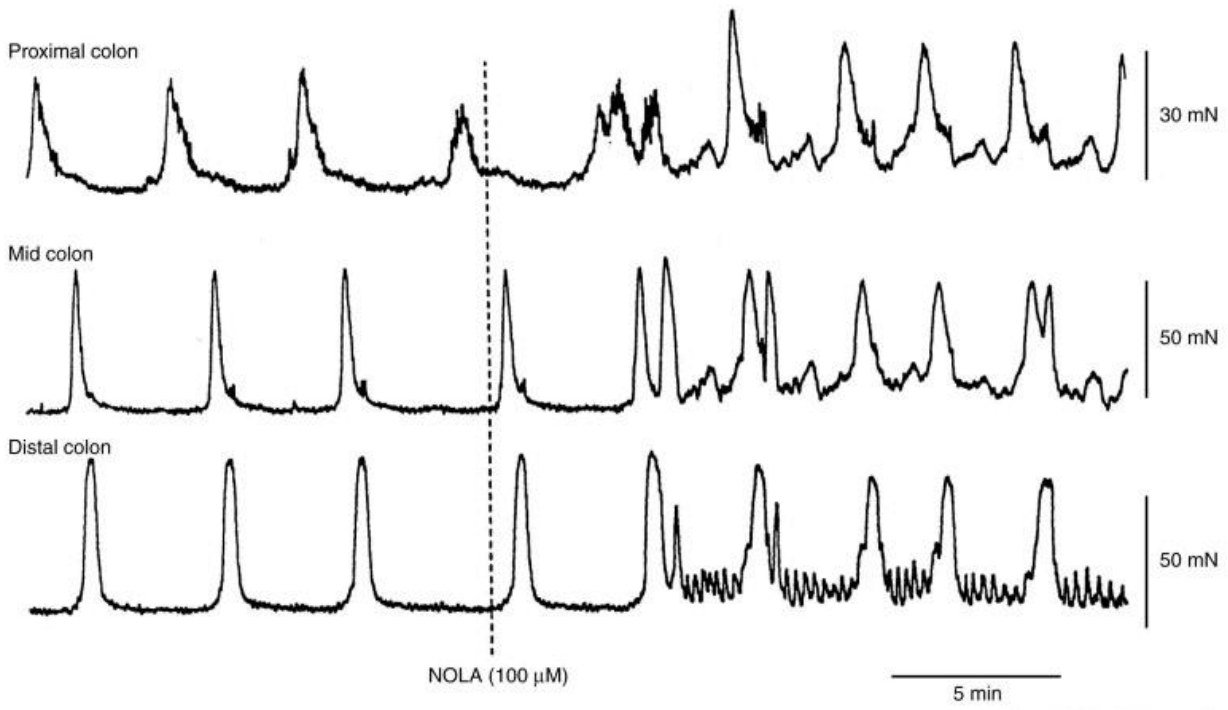


Figure 3.1. Typical mouse CMMC.

The effects of NOLA (NO synthesis inhibitor) on the spontaneous activity of CMMCs in the whole mouse colon, the frequency of CMMCs and the resting tone is increased with the application of NOLA (Fida et al., 1997)

3.5 Disorders of Colonic motility

Disorders of colonic motility and sensation manifest as different forms of constipation, diarrhoea and abdominal symptoms (IBS).

3.5.1 Colonic motility and constipation

Chronic constipation is a common, heterogeneous condition that is associated with unsatisfying defecation, infrequent stool formation and/or difficulty in passing stools (American College of Gastroenterology Chronic Constipation Task, 2005). This condition affects 14% of the population and can have a severe impact on quality of life (Suares and Ford, 2011), (Wald et al., 2007). Patients who have chronic constipation can be divided into 3 groups, 1) normal-transit

constipation, 2) isolated slow transit constipation, and 3) functional defecatory disorders (Sung, 2008). The pathophysiology of chronic constipation is unknown and majority of cases are idiopathic. The cause is thought to be multifactorial including diet, colonic motility and absorption, anorectal disorder and psychological factors (Basilisco and Coletta, 2013). Studies have focused attention on one or another causative factor, however, it is likely that overlapping factors contribute.

It has been long advised that fibre supplementation is useful for colonic function and a diet lacking fibre can give rise to constipation (Muller-Lissner, 1988). Despite this finding, the amount of dietary fibre in constipated vs non constipated subjects showed no disparity (Muller-Lissner et al., 2005). In addition fibre intake is not universally beneficial in constipation, for example, symptoms improve in patients with normal colonic and anorectal transit that consume fibre but is not useful in patients with delayed transit and defecatory disorders irrespective of fibre intake (Preston and Lennard-Jones, 1986), (Voderholzer et al., 1997). Therefore the use of fibre to alleviate constipation and symptoms is regarded as a myth and may in fact worsen symptoms resulting from gas production during fibre digestion (Basilisco and Coletta, 2013).

Delayed colonic transit is correlated with small and hard stools (O'Donnell et al., 1990) that are difficult to expel (Bannister et al., 1987). A longer transit time is correlated with faecal consistency, thus increased transit time favours water absorption in the colon (Basilisco and Coletta, 2013). Transit times can also alter the bacterial population which can influence absorption and secretion (Basilisco and Coletta, 2013). The cause of delayed transit can be attributed either to a direct defect in colonic motor activity (Bassotti et al., 1988), or indirect defect via conscious stool retention (Klauser et al., 1990), defecatory conditions (Nullens et al., 2012) or a lack of nutrition (Nullens et al., 2012). Colectomy studies have reported that defects in motility may arise from abnormalities in ICCs (Lyford et al., 2002) or increased progesterone receptors in circular colonic muscle (Xiao et al., 2005), (Cong et al., 2007).

A sense of incomplete evacuation and reduced frequency in defecation is a common complaint in patients suffering from constipation (Basilisco and Coletta, 2013). The defecatory process involves a complex interplay between rectal motor and sensory functions. Patients with

defecatory disorders have defects in sphincter relaxation during defecation (Preston and Lennard-Jones, 1985), (Rao et al., 1998). Moreover, these patients have hypertonic anal sphincter (Nullens et al., 2012)and reduced rectal propulsion (Rao et al., 1998). Lastly, there is uncoupling of motor activity, they have an urgency to defecate (Dinning et al., 2004) and structural changes are observed such as rectoceles and rectal prolapse (van Dam et al., 2000), (Collinson et al., 2010). These defects may present alone or in combination but may be highly subjective since some assessments are based on questionnaires.

Behavioural factors particularly in children may induce functional constipation based on previous negative experiences associated with defecation (Mugie et al., 2011), leading to prolonged stool retention causing rectal distension. Reports suggest this negative association can progress into adulthood (Bongers et al., 2010) and is observed in hospitalised elderly patients leading to faecal impaction (Read et al., 1995). Psychological factors such as anxiety, depression and somatization are commonly associated with constipation (Rao et al., 2007).

3.5.2 Colonic motility and diarrhoea

Chronic diarrhoea is described as persistently loose and watery stools, urgency to defecate and is a feature of malabsorption (Spiller, 2006). Diarrhoea is characterised by accelerated transit which arises from rapid movement of chyme into the small and large bowel that generates propulsive motor contractions (Spiller, 2006). Diarrhoea is seen in dumping syndrome post gastric surgery where there is malabsorption of simple carbohydrates like fructose (Christopher and Bayless, 1971) and bile salts (Williams et al., 1991). Bile acid malabsorption is easily diagnosed (Niaz et al., 1997), and commonly occurs after gastroenteritis (Sinha et al., 1998) while severe cases are caused by terminal ileal resections which results in bile acids entering the colon displacing the balance between sodium and water absorption thus accelerating transit (Spiller, 2006). Inflammation also disturbs the normal contractile mechanism interfering with ICCs, altering transmission of neurons and sensitising visceral afferents (Spiller, 2006). Inflammation causes a decrease in normal mixing but increases in propulsion indicated by HAPC (Spiller, 2006).

3.5.3 Colonic motility and IBS

Disordered colonic motility gives rise to symptoms of IBS (Sinha et al., 1998). Indeed, almost 30% of FGID patients have disordered motility in a cohort of 286 patients assessed my scintigraphy (Manabe et al., 2010). At 24h 4.2% of IBS-constipation (IBS-C) and 33.3% of IBS-D had accelerated transit, while at 48h 22.9% of IBS-C, 4.5% of IBS-D and 6.7% of IBS-mixed (IBS-C and IBS-D) had delayed transit (Chey et al., 2001). Increases in HAPCs and frequent eating may cause accelerated transit, which in part may explain post-prandial symptoms such as abdominal discomfort and urgency to defecate in IBS-D patients (Chey et al., 2001). Another study using colonic and rectal balloon distension showed 21-65% of IBS patients have visceral hypersensitivity as measured by an increase in perception (Camilleri, 2011), (Mayer et al., 2008).

In comparison to control, IBS-D patients show many fold increases in total transit time measured by radiopaque markers in comparison to control (Chey et al., 2001), (Camilleri et al., 2008), (Bouchoucha et al., 2006). Moreover, increases in both frequency and amplitude of spontaneous GMCs is observed in IBS-D (Chey et al., 2001). These GMCs cause an urgency to defecate and 90% of IBS-D patients had symptoms of intermittent abdominal cramping (Chey et al., 2001), (Thompson et al., 1999). The intensity of RPCs in IBS-D patients is only different in descending colon but not elsewhere compared to healthy controls (Chey et al., 2001). Therefore, symptoms of IBS-D can result from motility defects. Conversely, in severe cases of constipation there is an absence or scarcity of GMCs (Bassotti et al., 1988), (Bassotti et al., 2003 5527), (Bassotti et al., 2005), (Hagger et al., 2003), (Rao et al., 2004), (Dinning et al., 2004). In slow transit constipation, contractile activity is reduced in all colonic regions by 24-hour ambulatory recordings as well as reduced surge in secretion commonly observed when awakening (Rao et al., 2004). Constipation arising from pelvic floor dysfunction do not show changes in frequency or amplitude of colonic GMCs, however GMCs are noticeably reduced when there is urgency to defecate (Dinning et al., 2004). This paradox can be explained by the accumulation of faeces in the distal colon and rectum and thus RPCs occur and thus urgency is perceived. Lastly, IBS-M patients experience both diarrhoea and constipation, therefore, based on the data from individual IBS subtypes, GMCs in this group may fluctuate from one extreme to another.

3.5.4 Colonic motility and IBD

Data on colonic motility in IBD is limited due to the risk of perforation but much information has been obtained from inflammation induced in animal models. Motility disturbances in IBD suppress RPCs and tonic contractions while GMCs are enhanced as observed in IBS-D (Coulie et al., 2001), (Sethi and Sarna, 1991), (Kern et al., 1951), (Loening-Baucke et al., 1989), (Snape, 1991), (Connell, 1962). The contraction frequency, be it RPC or GMCs, correlate to the degree of inflammation and subsequent symptoms (Sethi and Sarna, 1991), (Kern et al., 1951). Regions of intense inflammation in the colon present with highest stimulation of GMCs and inhibition on RPCs. Inflammation in one region can also influence RPCs in another region (Collins, 1996). Therefore, inflammation of the colon can affect the properties of different contractions that arise in the colon.

3.6 Measuring motility

There are various methods of assessing motility in humans which can be divided into 4 components, 1) measuring the underlying myoelectric component using electromyography, 2) phasic or tonic contractile activity using the barostat, 3) intraluminal manometry and intraluminal transit using isotope scintigraphy and 4) radio opaque markers (Scott, 2003). *In vitro* animal studies in mouse have been conducted using a variety of recording techniques. Contractions in this tissue occurs devoid of any luminal contents which has been described in various papers (Bywater et al., 1989, Shimizu et al., 2011), (Bush et al., 2000), (Brierley et al., 2001). Brann et al first described contractions along the length of the colon as “peristaltic waves” with or without luminal contents using external suction electrodes (Brann and Wood, 1976). Since then Taylor and Bywater have measured contractile activity of the colon using intracellular microelectrodes to record electrical activity in the smooth muscle cells from the circular muscle in full length strips of mouse colon (Bywater et al., 1989). This method requires clips be attached to the colon which is then attached to a force transducer which could damage the colon. In the rata multi-lumen manometry catheter is inserted into the lumen after the small intestine is explanted into an organ bath (Frisby et al., 2007). The catheter had 6 side holes which could then record pressure waves every time the small intestine contracted and occluded these side holes (Frisby et al., 2007). The

frequency, amplitude, direction of propagation could be analysed (Frisby et al., 2007). The advantage of this method is that it optimizes the viability of the mucosal and enteric neural tissue throughout the experiment. This method has been adapted and used in the TNX KO mouse in this study.

In summary, mucosal stimulation activates sensory nerves that transmit information relayed by interneurons to effector motor neurons that release neurotransmitters orally (excitatory) and anally (inhibitory). These neurons and their released neurotransmitters work with smooth muscle cells causing rhythmic short and long duration contractions. Postprandial propulsion occurs when the lumen is occluded in the anal direction. The differential release of neurotransmitters causes variation in the amplitude and duration of contractions that allows the slow mixing/turning over of luminal contents. It is important to remember that neuronal regulatory mechanisms associated with colonic motility is variable amongst species therefore interpretation must be applied with caution when comparing. It is obvious that the regulatory mechanisms underlying motility is complex therefore TNX may be involved in a specific signalling pathway in this intricate system.

3.7 Methods

3.7.1 Removing the whole colon

The mouse was pinned out and a mid-line incision was made to expose the colon, Krebs solution (at 4^{o}C) was poured over and further dissected to free the colon from the mouse. The colon was then removed and transferred to another dish and immersed in Krebs solution at room temperature. A small syringe with a pipette tip attached was filled with Krebs solution to flush the colon. Once the colon was completely clean, it was transferred to the organ bath and a multi-lumen catheter was threaded through from the oral end making sure that the colon covered the 4cm of the catheter with the sideholes. The distal region of the colon was always placed 3cm from the end of the catheter so it covered the last sidehole, the rest of the colon covered the other 3 sideholes. In addition the colon was qualitatively assessed for abnormalities in gross structure when excised for manometry experiments

3.7.2 Animals for pellet count

9 KO and 9 WT mice (5 females and 4 males in each group) were used to assess pellet output over a period of 14 days. Each mouse was housed in an individual cage attached to a shoot that had a wire bottom and a metal container to collect the pellets. Animals were housed individually overnight only and placed back with other animals the next day. This alternating method was used for two weeks to get a reading of 6 days of pellet output per mouse. The total number of pellets were counted and weighed each day and an average for each mouse was calculated. All data are presented as mean ± SEM and statistical analysis was performed using a student's (t-test). A $p<0.05$ was taken to be significant (GraphPad Prism, V.7.02, GraphPad Software, Inc).

3.7.3 Multilumen catheter

A multilumen saline-perfused catheter made from silicone rubber (Dentsleeve Pty, Wayville, and South Australlia) was modified for the mouse and used for pressure recordings. The silicon catheter consisted of 4 smaller lumens inside. At one side (3cm from the end) a sidehole was made using a modified flat ended needle (23 gauge), this was done very carefully to avoid piercing the other lumens. 3 more holes were then created in each lumen 1cm apart in the same way. A small piece of plastic was placed inside the individual holes and was pushed back with tweezers to leave the hole exposed to allow the perfusate to exit the sidehole. This was repeated for all 4 sideholes. The silicon tubing was sealed at one end with glue to prevent the perfusate from coming out of the catheter therefore occlusion of the sidehole due to colonic contraction would increase pressure which would be recorded as a peak. On the other end of the catheter, four microloader tips (details) were inserted into each of the lumens connected to a needle attached to the manometry measurement device. Glue was applied around the 4 microloader tips to secure it in place. The catheter was continuously perfused with degassed saline at a rate of 0.04ml/min. For perfusion recording, each separate lumen was coupled to a strain gauge pressure transducer of a capillary perfusion system. A constant perfusion rate was achieved by the use of saline from a reservoir maintained at a known pressure. Once the colon wall occluded the manometric ports, the resistance of perfusate increased which was transmitted as a pressure change. The stronger the occlusion by the colon, the higher the perfusate resistance resulting in a larger pressure represented as a rise in the wave.

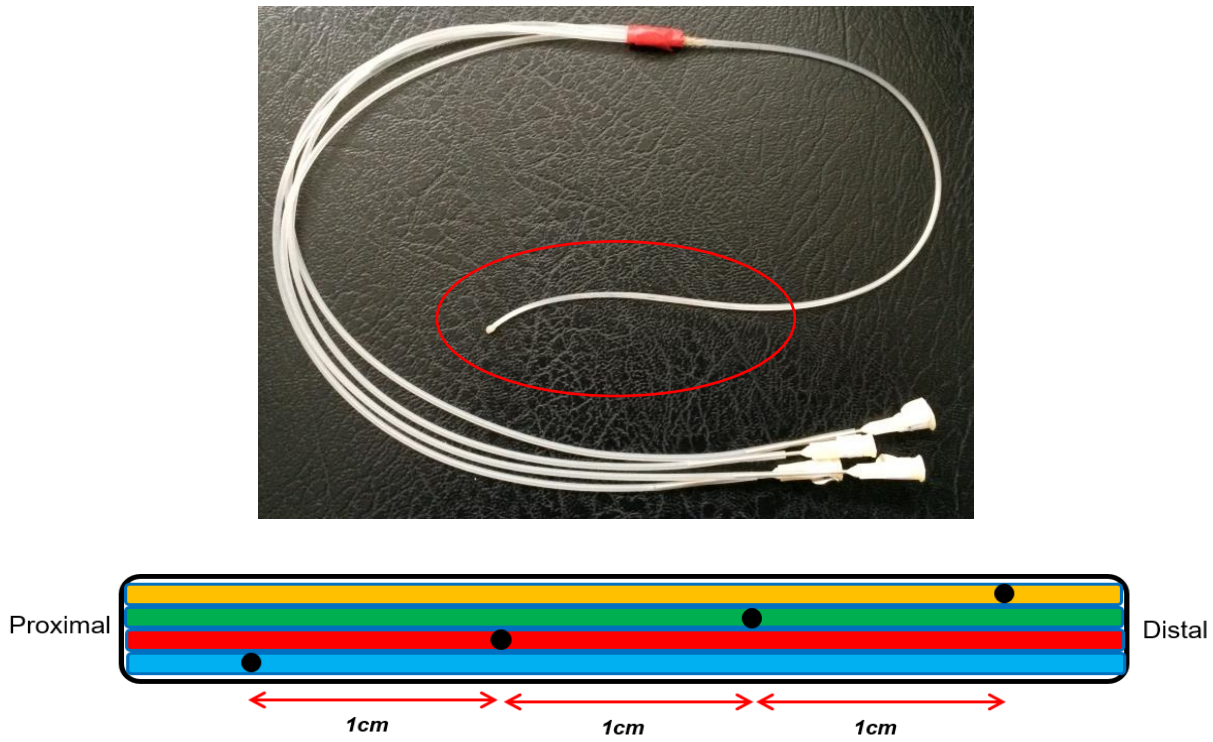


Figure 3.2. Modified catheter designed for mouse colon.

The red circle indicates the region of the four sideholes were made. The schematic below shows four sideholes made in each separate lumen within the catheter represented in blue, red, green and yellow. Sideholes in each lumen were 1 cm apart between each sidehole from the proximal to the distal region.

3.7.4 Solutions

3.7.4.1 0.9% Degassed saline

Degassed saline was made by adding 9g of sodium chloride (Sigma Aldrich) to 1L of distilled water and mixed until dissolved at a concentration of 0.9% w/v. The solution was then placed on a hot plate and boiled until 60OC. Once boiled, the bottle (Scott Duran) was carefully removed with heatproof gloves and the cap was quickly closed. This was left at RT to cool and stored at 4^{o}C

3.7.4.2 Modified Krebs Solution

we used the same Krebs as published colonic motility studies in isolated mouse colon (Lyster et al., 1995, Fraser et al., 1997) with the following recipe: NaCl, 120; KCl, 5.0; $CaCl_2$, 2.5; $MgCl_2$, 1; NaH_2PO_4, 1; $NaHCO_3$, 25; and glucose, 11 mM. The solution was gassed continuously with 95% 0_2 and 5% CO_2.

3.7.5 Interventions

Nitric oxide synthase inhibitor- Nωnitro-L-arginine (NOLA) (Sigma) and secondary bile acid-deoxycholic acid (DCA-(Sigma)) were both used at a concentration of 100µM made up in distilled water.

3.7.6 Data capture and analysis

Manometric data was recorded using a portable manometric device and data analysed using commercial software (Flexisoft III, Oakfield Instruments Ltd) and Medical Measurement System (MMS). Using the Flexisoft III we calculated number and amplitude of contraction and number of single/double/multi peak contractions. Using MMS software number and amplitude of contractions were measured. We were unable to determine whether contractions were propagating or retrograde since the catheter spanning the colon was only 4cm in length using the software, therefore, this was completed manually.

Using the Flexisoft manometry setup, spontaneous contractions were measured over 60 mins. 1ml of NOLA (100µM) was added directly to the organ bath and then perfused for 15 mins at the same concentration. Once all the NOLA had been added, it was allowed to flush out of the bath for 30 min. 1ml bile (100µM) was then directly added to the bath and then continuously perfused with bile (100µM) for 10 mins. We then flushed the colon with Krebs and continued recording over 30 mins before the experiment ended. A total of N=6 WT vs. N=6 KO and it was possible using Flexisoft III software to calculate the number of single/double/multi peaks in the recordings over the spontaneous recording and before/after NOLA and bile addition. The mean amplitude (mmHg) and mean durations (seconds) was also calculated during each time interval.

However, the results we obtained from the method described may not give a true response of the colon to bile since NOLA has already affected the neural component of the colon. We therefore repeated the experiments with NOLA (N=6 WT vs. N=6 KO) and bile (N=5 WT vs. N=5 KO). This was done using the portable MMS system, from which the total number of peaks, duration of peaks, and frequency of peaks, amplitude and area under the curve (AUC) could be calculated. Using this method spontaneous activity was recorded over 30 mins post equilibration. NOLA and bile were then added for 15 min and 10 min in the same way described above but in separate experiments. After the NOLA or bile, spontaneous activity was recorded for 45 mins before the experiment ended. Most data in the results section will be based on the separate studies described here. However spontaneous contraction data from the Flexisoft and MMS system were collated, therefore in total N=11 WT vs. N=11 KO were analysed. Raw values for all the recordings (spontaneous, NOLA and bile) can be obtained in Appendix 3.1-3.6.

Analysis of CMMCs was done manually where the frequency, direction of propagation i.e. retrograde, anterograde or synchronous was noted, as well as the propagation velocity calculated by dividing distance between recording sites by the time measured between the onset of contractions (within 5%) as used previously Fida et al 2000 (Fida et al., 2000). (N=8 WT vs. N=9 KO). Full CMM raw data can be found in Appendix 3.7.

All results are presented as mean ± SEM. Statistical analysis was performed using a two way analysis of variance (ANOVA) using Sidaks multiple comparison test to compare each region of the colon between the two groups. A $p<0.05$ was considered significant (GraphPad Prism, V.7.02, GraphPad Software, Inc). Sample sizes were chosen using previously published studies that used the similar protocol. Data that showed significant differences were further discussed.

3.8 Results

3.8.1 Gross anatomical changes in TNX KO mouse

The first observation made when the colon was excised was that a small percentage (12% of 38 mice) of TNX KO mice had an internal rectal intussusception or internal rectal prolapse (Fig 3.3).

Figure 3.3. Internal rectal prolapse in TNX KO.

The WT colon shows a normal rectum at the anal segment whereas in the KO colon the rectum shows inflammation indicated by blue arrow. This was observed in 12% of 38 TNX KO mice of which a mixture of males and females were studies.

Since the prolapse was observed in both genders in the KO mouse colony, this is indicative of a rectal problem rather than a vaginal or uterine prolapse.

3.8.2 The function of TNX in generating spontaneous contractions

Colonic motility has been extensively studied using *in vitro* preparations in the isolated mouse colon. Using similar techniques we can understand the overall function of the colon in the KO mouse and measure the effects post the addition of drugs. The data described below is based on the studies completed using the MMS and Flexisoft software. In total N=11 WT vs. N=11 KO were analysed for spontaneous colonic activity of which 6 female and 5 males were used in each group. The number of peaks/min is representative of the number of contractions.

In KO the number of contractions is reduced compared to the WT and this was significant in the mid proximal (WT=0.502±0.061 vs. KO=0.300±0.034 peaks/min; p=0.0475) and distal colon (WT=0.296±0.020 vs. KO=0.012±0.004 peaks/min; p=0.0021) (Fig 3.4A). In proximal colon the number of contractions was unchanged in both WT and KO. There was no significant change observed in the mid distal colon.

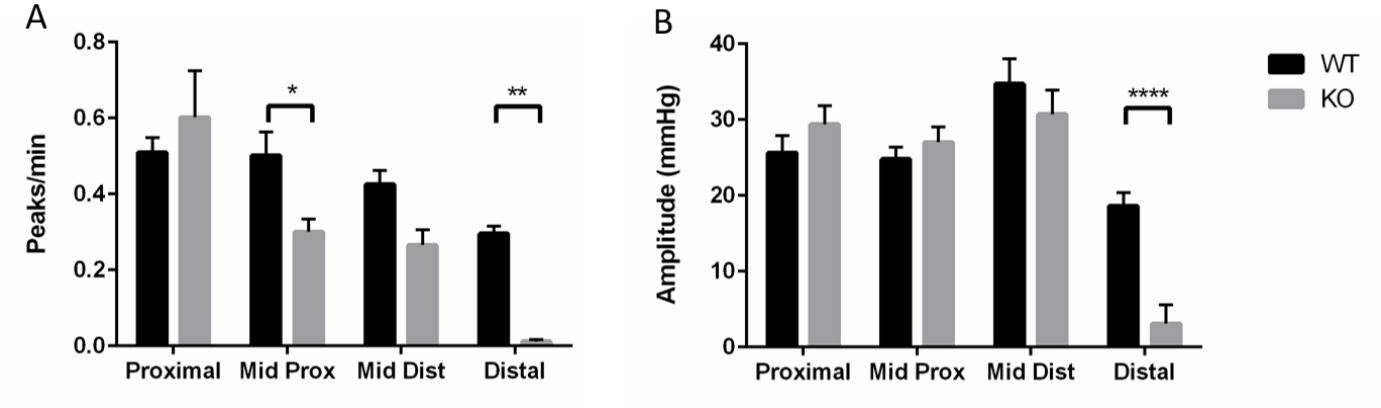


Figure 3.4. Spontaneous colonic contractions and amplitude reduced KO mice.

(A) Spontaneous contractions were significantly reduced in the mid-prox and distal colon in the KO vs. WT. (B) Amplitude of spontaneous contractions was only significantly reduced in the distal region in KO mice. (N=11 WT vs. N=11 KO)

The mean amplitude of spontaneous contractions was only significantly reduced in the distal region (WT=18.60±1.74 vs. KO=5.80±2.51 mmHg; *p*=0.0014), therefore the strength of contractions was weakest in the distal region in the KO mouse compared to WT (Fig 3.4B).

The manometry trace in Fig 3.5 illustrates spontaneous contractions and amplitude in each region of the colon - proximal (blue), mid proximal (green), mid distal (red) and distal (pink). It is clear that the number of contractions and amplitude of contractions is reduced in the KO mice compared to the WT particularly in the distal colon.

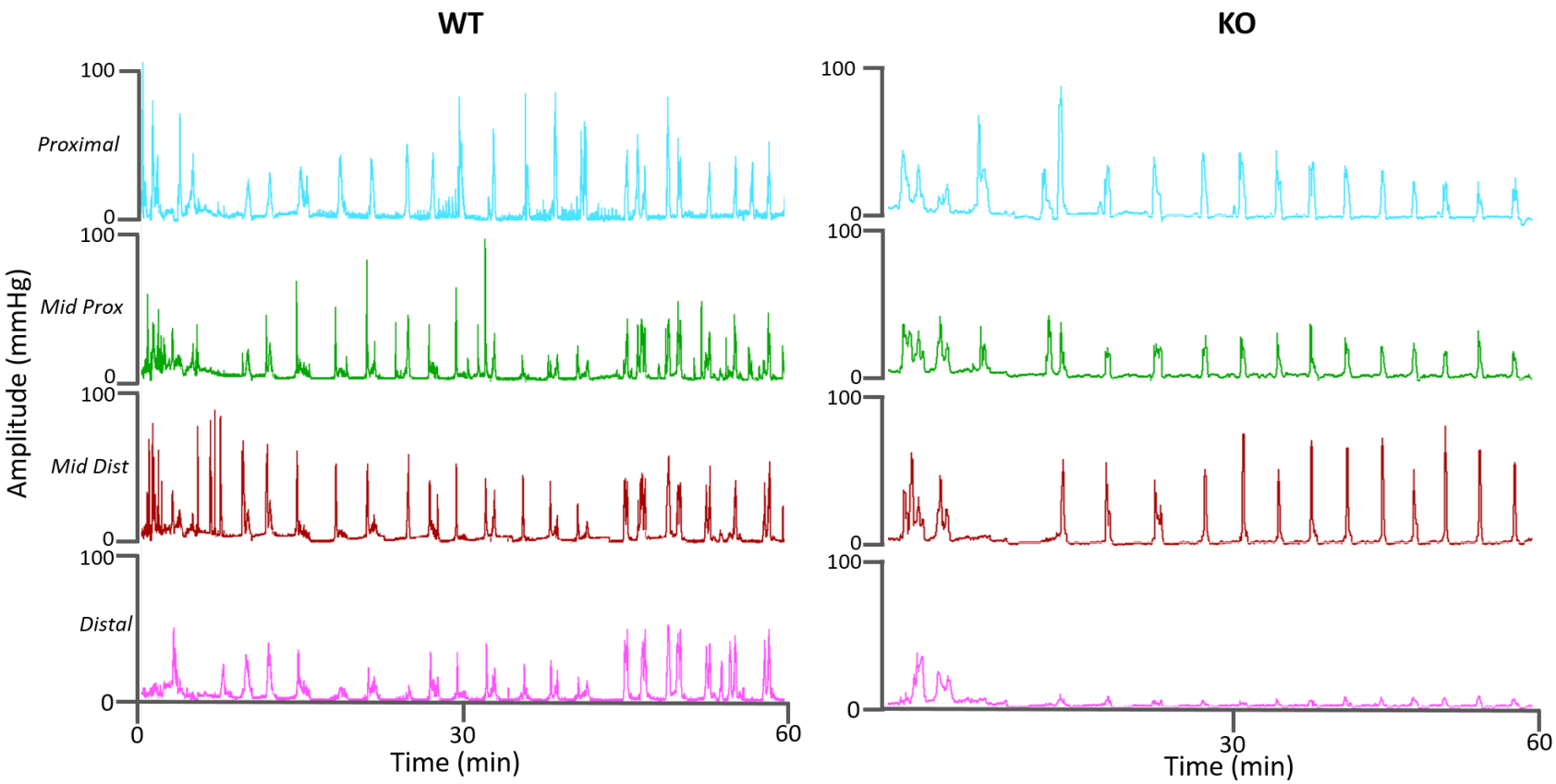


Figure 3.5. Representative motility trace over 60 mins showing spontaneous contractions.

Manometry recordings from different regions of the colon in WT vs KO show frequency of spontaneous contractions is reduced in all regions of the colon. The distal region (pink) in the KO mouse colon shows a significant reduction in both contraction and amplitude.

In addition to looking at overall motility patterns, the number of spontaneous spikes that were single/double or multiphasic were analysed. The multiphasic contractions are suggestive of CMMCs that are well described in the isolated mouse colon. The number of single contractions in the mouse was significantly reduced in all regions of the KO colon except in the proximal colon compared to WT: Proximal (WT=4.2 ±1.7 vs. KO=5.60 ±2.72;), Mid Prox (WT 11.2 ±3.3 vs. KO=3.17 ±0.95; *p*=0.0410), Mid Dist (WT 11.6±1.7 vs. KO=3.80 ±1.35; *p*=0.0495), Distal (WT=11.0 ±2.9 vs. KO=3.20± 1.54; *p*=0.0495) (Fig 3.6).

The number of spontaneous double and multi peaks did not show significant changes in any regions of the colon in WT vs KO, however, there was a general decrease in the number of double and multi peaks in the KO except for proximal colon (Fig 3.6B & 3.6C)

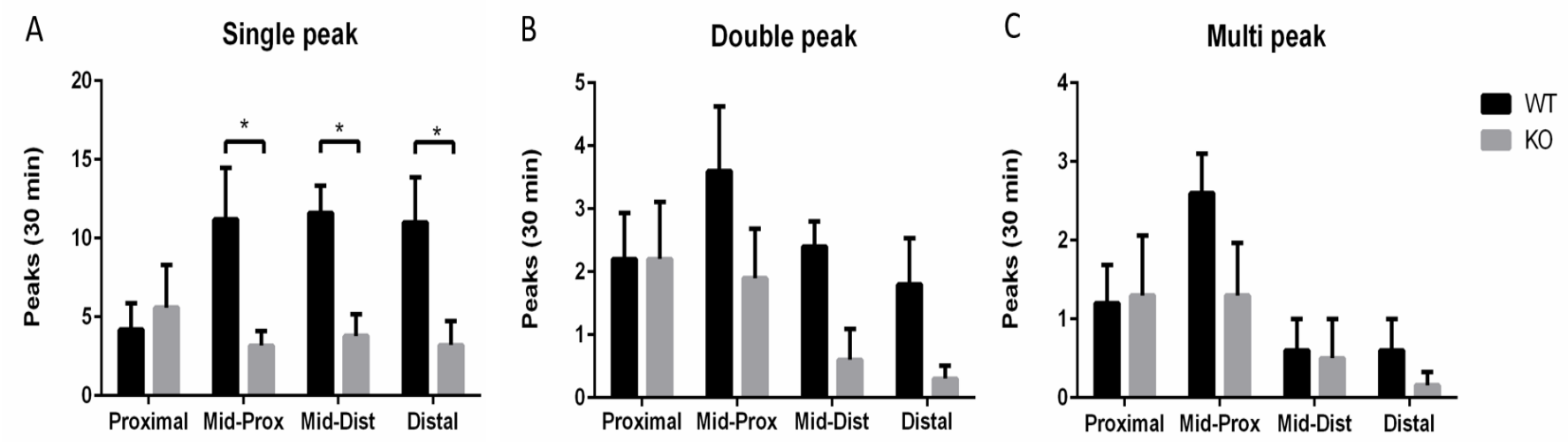


Figure 3.6. The total number of single peaks reduced in the KO.

Graphs showing total number of spontaneous single/double/multi peaks in the WT vs KO colon over 30 mins. (A) Significant reduction in the number of single peaks over 30 mins from mid-prox to distal colon in KO mice compared to WT. (B) No change in number of double and (C) multi peaks in proximal region. Reduction in spontaneous double and multi peak contractions in all other regions of WT vs KO colon, however, this was not significant. (N=6 WT vs. N=6 KO)

In summary, spontaneous recordings show that the number and amplitude of contractions is reduced in the KO mouse colon compared to WT mouse colon. This is particularly true for the distal region.

3.8.3 Pharmacological intervention to study the role of TNX

3.8.3.1 Effects of NOLA

NOS inhibitor NOLA blocks action of NO on inhibitory motor neurons which allows the measurement of the primary effect on cholinergic neurons where TNX is most expressed.

The manometry recording shows the effect of adding NOLA (100µM) in WT and KO colon (Fig 3. 7). In WT and KO mouse, the interval between the contractions i.e frequency is increased from around 3 CMMC complexes in 15 mins to around 5 CMMC's complex in 15 mins. The amplitude of contraction is significantly increased in both WT and KO after the addition of NOLA.

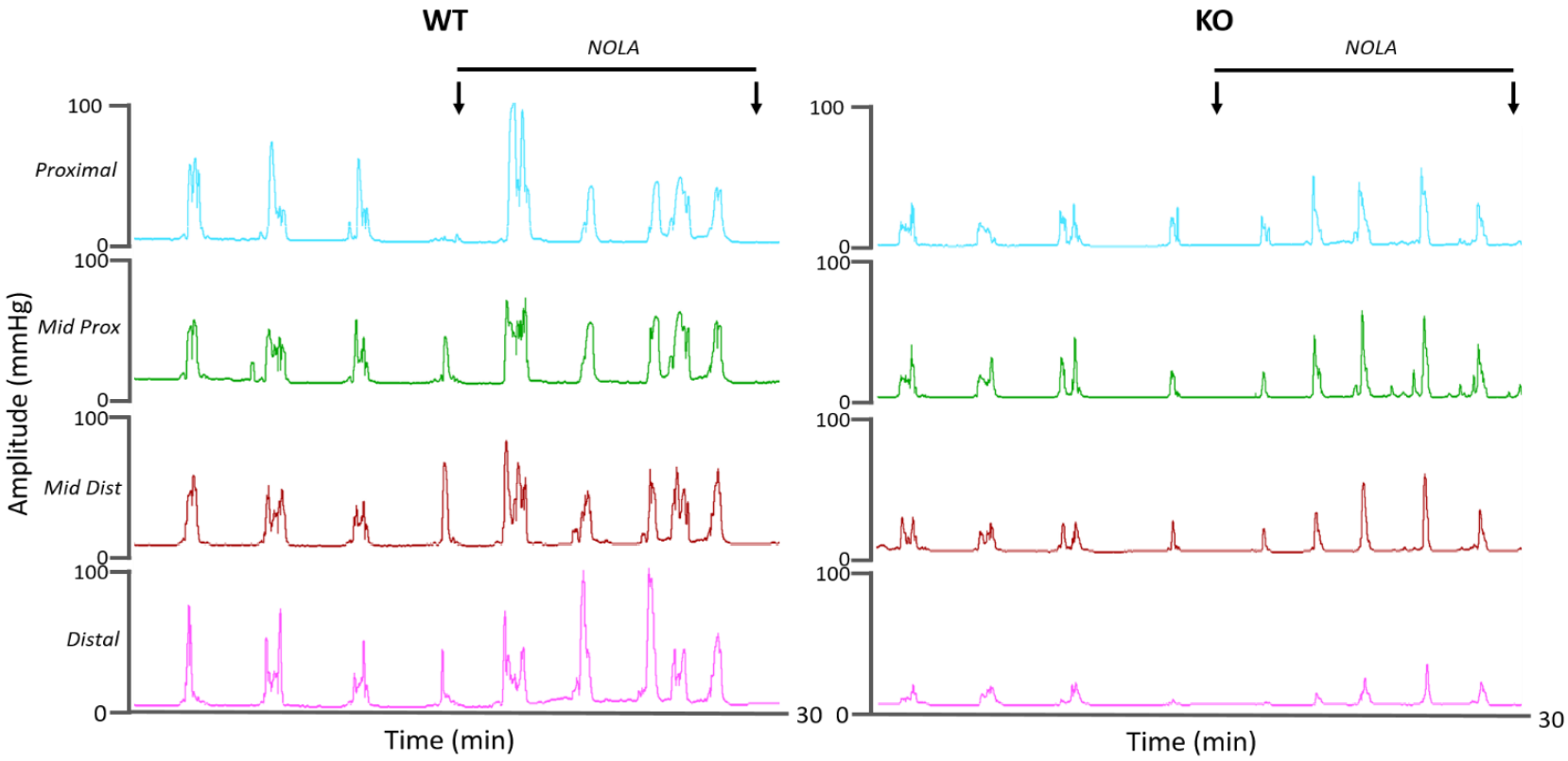


Figure 3.7. Representative spontaneous trace with NOLA.

This trace represents a segment of the experiment where 0 does not indicate 15 mins into the experiment. Spontaneous manometry recording from different regions of the colon in WT vs KO. NOLA (100µM) was added after 30 min (first arrow) (where 0 is 15 mins), for a duration of 15 min (second arrow). In both WT and KO, frequency of CMMCs increases following NOLA addition. The number and amplitude of contractions was reduced in KO colon particularly in the distal region (pink). (N=6 WT vs. N=6 KO)

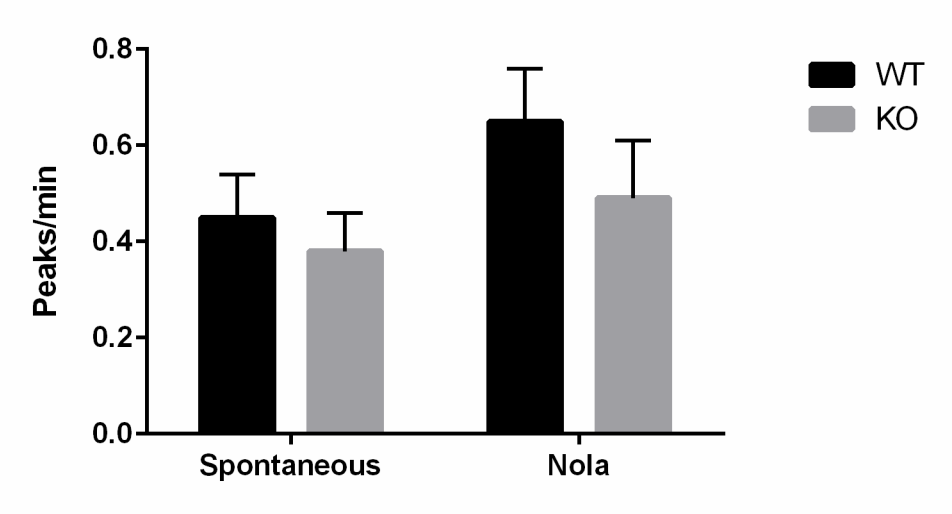


Figure 3.8. The overall effects of NOLA in WT vs KO unchanged.

In the presence of NOLA (100µM) contractions increased similarly in WT and KO compared to spontaneous (prior to NOLA addition), however, this increase was not significant.

As NOLA had no significant effect on the overall number of colonic contractions in either WT or KO, it was important to know if NOLA induced region specific differences so responses to NOLA were analysed per colonic region. In proximal colon, the number of spontaneous contractions (peaks/min) did not change in either WT or KO, or in the presence of NOLA (Fig 3.9A). In mid proximal colon the number of contractions increased after NOLA addition in WT (WT: spontaneous= 0.512 ±0.096 vs. NOLA= 0.808 ±0.065, p=0.023) but was unchanged in KO (Fig3.9B). In mid distal colon, WT mice showed no increase in contractions in response to NOLA, however, there was a significant increase in contractions of KO mice (Fig 3.9C). Finally, in the distal colon the largest effect of NOLA was observed in WT (Fig 3.9D). Distal colon of KO mice showed a slight increase but this was not significant (Fig 3.9D). The effect of NOLA along the length of the colon is variable. In WT colon, the overall increase in peaks/min is observed in mid proximal and distal colon whereas in the KO this increase is small across most regions and only significant in mid distal colon.

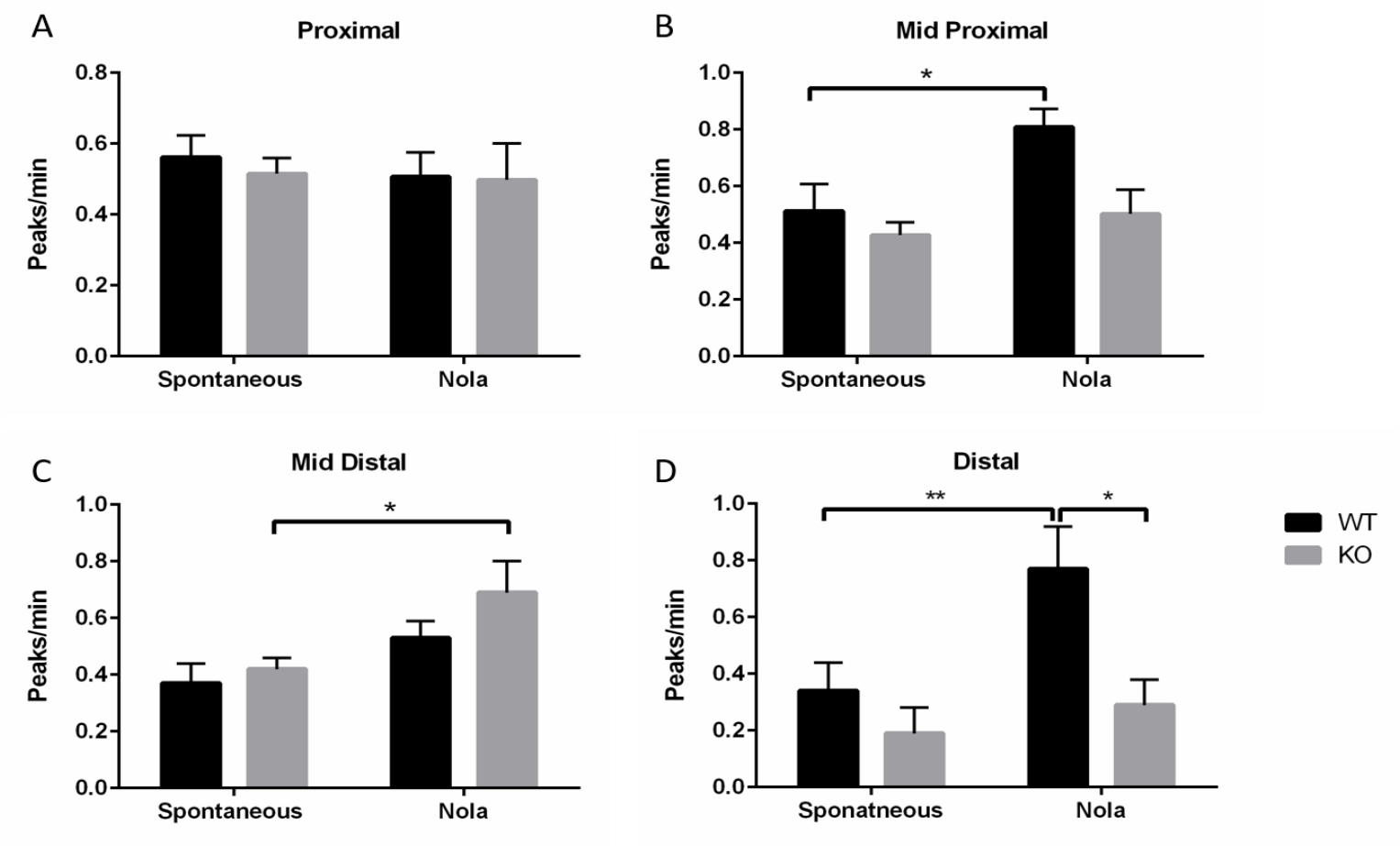


Figure 3.9. Effect of NOLA in WT vs KO was unchanged at each colonic region.

(A) In the proximal region, NOLA (100μM) had no effect on the number of contractions in either WT or KO mice. (B) In mid-proximal colon, NOLA significantly increased contractions only in WT. (C) in mid distal colon, NOLA significantly increased contractions in KO mice only. (D) In the distal region WT mice demonstrated a significant increase in contractions following NOLA addition. There was a significant difference in WT vs KO after NOLA addition. (N=6 WT vs. N=6 KO)

3.8.3.2 Effects of Bile

Secondary bile acid DCA causes relaxation of the longitudinal muscle and therefore reduces contraction via activation of the bile acid receptor TGR5, which is expressed on inhibitory motor neurons that releases NO causing an inhibitory effect (Alemi et al., 2013). Since bile acid affects the nitrergic system, investigating the effect of bile in WT and KO will in part clarify the role of TNX in the cholinergic system as TNX is found in cholinergic neurons in the distal gut of both mouse and human (Chapter 2, section 2.3).

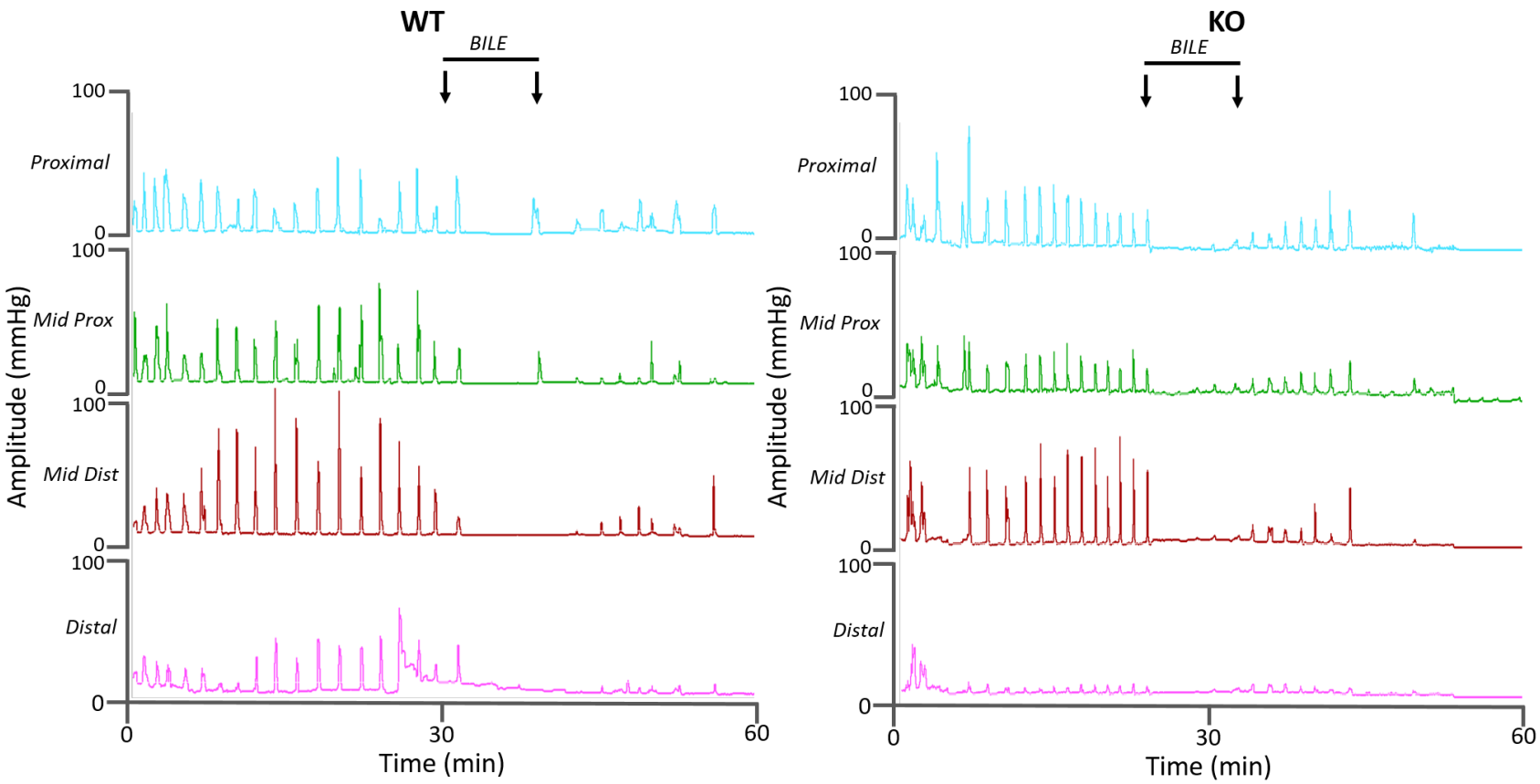


Figure 3.10. Representative spontaneous trace with Bile.

Spontaneous manometry recording from different regions of the colon in WT vs KO. Bile (100µM) was added after 30 min (first arrow) for a duration of 10 min (second arrow). Contractions are abolished in the presence of bile in both WT and KO (N=5 WT vs. N=5 KO).

Representative manometry trace shows the effect of bile on amplitude and thus contractions in the different regions of the colon - proximal (blue), mid proximal (green), mid distal (red) and distal (pink) (Fig 3.10). The trace demonstrates that following the addition of bile, there is an immediate ablation of contractions in all regions of the colon, regardless of TNX phenotype (Fig 3.10).

In order to observe the effects of bile on contractions across WT and KO animals studied, the total number of contractions and contraction amplitude across all regions was analysed. This showed bile significantly reduced the number and amplitude of contractions in WT (Fig 3.11B). In KO mice, there were decreased contractions and amplitude, however not significant, (*p* value = 0.0688, Fig 3.11). In addition, each region was analysed separately and the effect of bile was the same as observed in total contractions and amplitude.

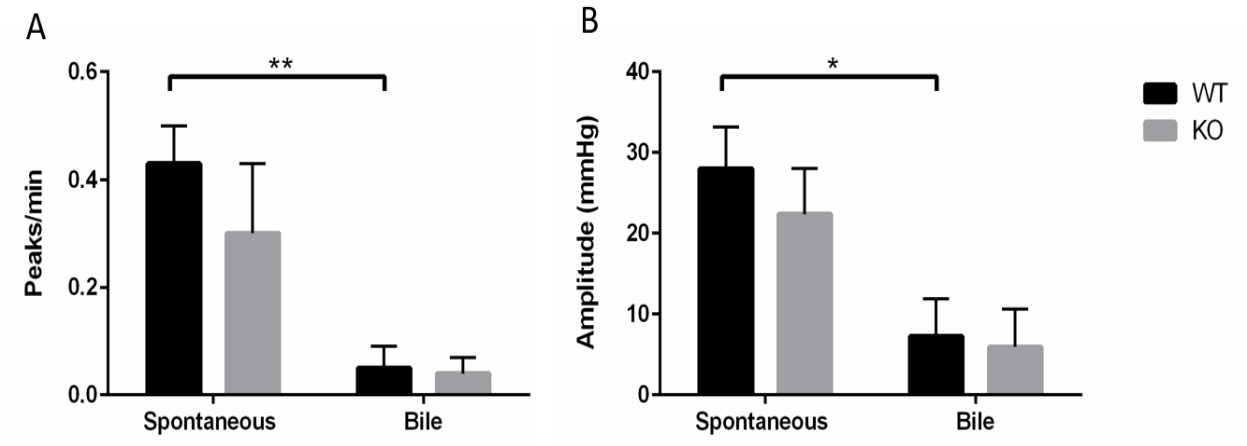


Figure 3.11. The effects of bile on colonic contractions unchanged in WT vs KO

WT mice had a significant decrease in contractions (peaks/min) after addition of bile (100µM) (p=0.0066), while contractions in KO mice also decreased but did not reach significance (p=0.0618). Amplitude of contractions followed the same pattern with bile decreasing amplitude in WT (p=0.0205) and in KO although not significantly (p=0.0688).

In summary, bile decreases colonic contractions and amplitude of these contractions in both WT and KO suggesting both WT and KO colon respond similarly to the addition of bile.

3.8.4 The role of TNX in colonic migrating motor complex (CMMC)

CMMC analysis gives us an understanding of the regulatory process for colonic motility, for example, the direction of contraction and propagation velocity. Specialized software has been developed using the MMS recorder to analyse CMMCs in humans, however, automated analysis does not exist for mouse colon. Due to this reason CMMC analysis was carried out manually based on published methods (Fida et al., 2000). A CMMC was taken as contractions that propagated either anterograde (orally), retrograde (aborally) or remained static (simultaneous) across at least 3 channels which is around half the length of the total colon (Sarna, 1985).

The direction of propagation was calculated by dividing the number of CMMCs going in either the anterograde or retrograde direction or those that remained static, by the total number of CMMCs counted. The direction of propagation was mostly anterograde in both WT (81.7% ± 10.9%) and KO (92.7% ± 3.16%). A smaller number of contractions were retrograde and static in WT (retrograde: 14.1% ± 10.7%, static: 4.21% ± 2.75%) and KO (retrograde: 4.89% ± 3.23%, static: 4.67% ± 3.16%), (Fig 3.12A). The average number of CMMCs calculated as described earlier showed no differences between WT (2.81 ±0.24) and KO (3.14 ±0.35) when measured under spontaneous conditions (Fig 3.12B). Finally, the propagation velocity was unchanged between WT and KO. Therefore, TNX does not contribute to the generation of CMMCs as measured by this methodology.

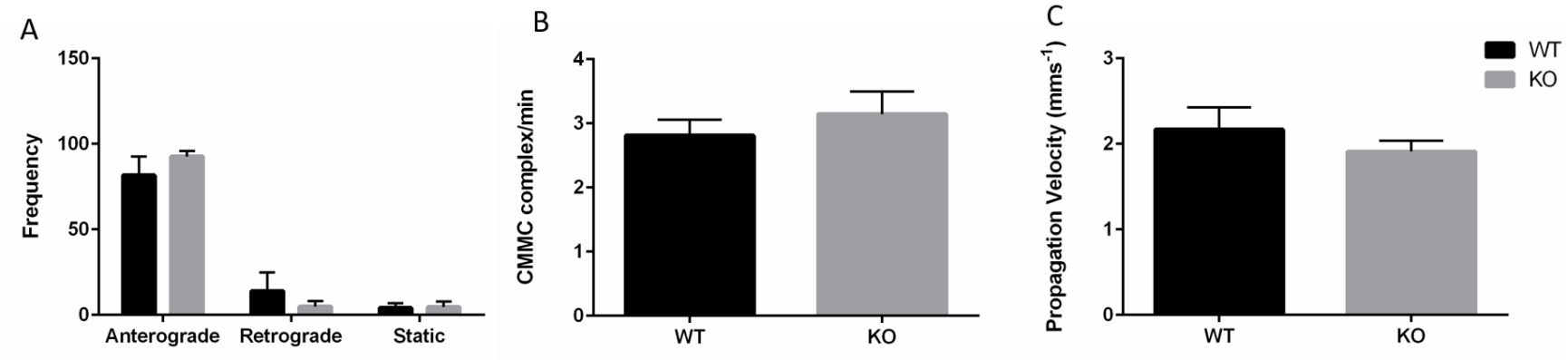


Figure 3.12. No change in CMMC direction, frequency and propagation velocity in WT vs KO.

(A) The lack of TNX gene did not alter the direction of CMMC propagation, (B) frequency and (C) propagation velocity.

3.8.5 TNX effects on stool output

In addition to looking at specific control of colonic motility, the function of the whole colon was investigated using stool output as a global measure of colon function. The number of stools produced over each day showed a reduction in KO compared to WT on day 1 but none of the other days (Fig 3.13A). There was no significant change in the total number of stools in the KO vs WT.

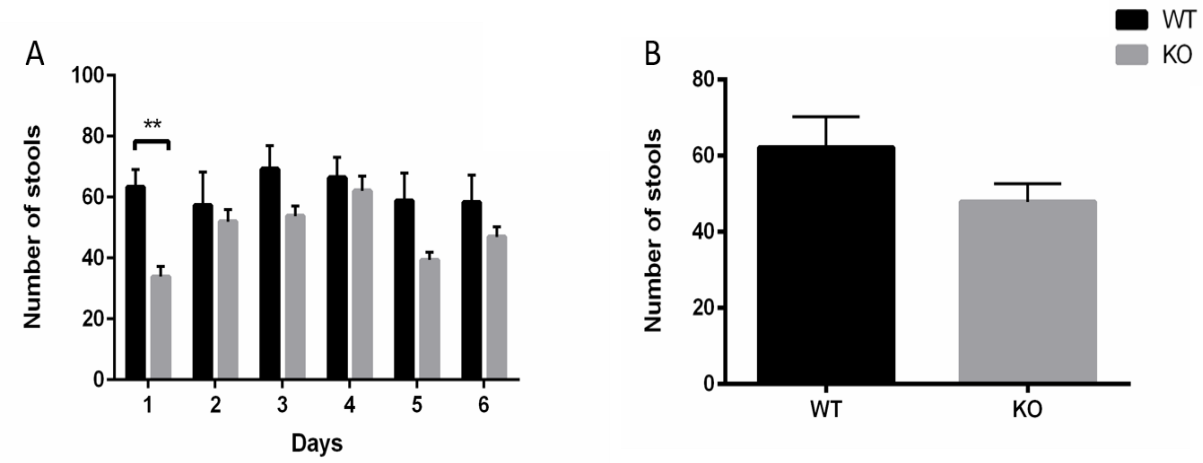


Figure 3.13. No change in stool number over 6 days between WT and KO.

A Small reduction in the number of pellets in the KO (47.9 ± 4.68) vs WT (62.2 ± 8.1) over all 6 days however only significant on day 1 (p=0.0099). The overall mean number of pellets was not significantly different (N=9 WT vs. N=9 KO).

Similarly, weight of pellets produced over each day showed a decreased in KO group only on day 1 (Fig 3.13A) and there was no overall change in the weight of pellets in the KO vs WT.

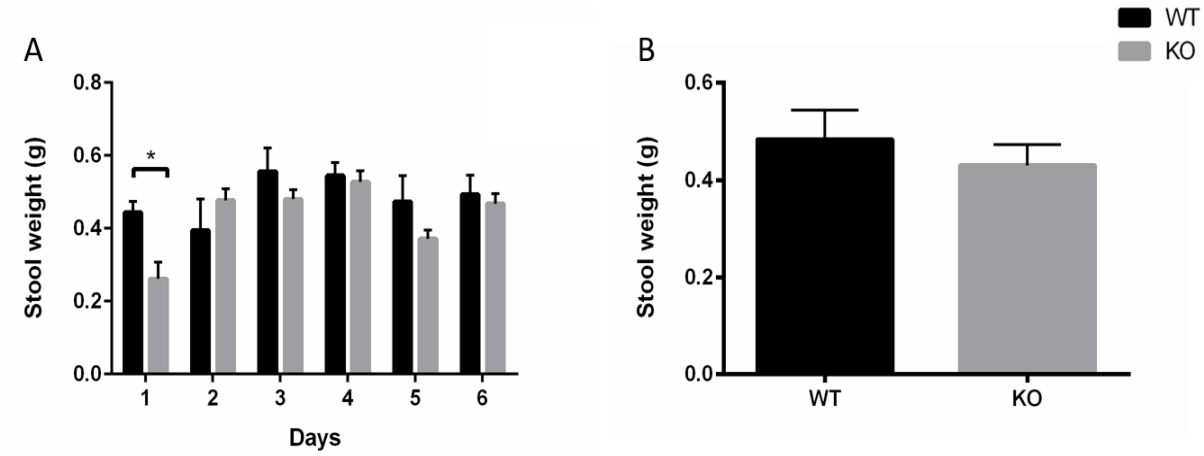


Figure 3.14. No change in pellet weight over 6 days.

(A) Small reduction in the number of pellets on day 1 (p=0.0485), however, the rest of the days weights were similar. (B) Overall mean weight of pellets was not significantly different WT (0.48g ± 0.06g) vs. KO (0.43g ± 0.042g). (N=9 WT vs. N=9 KO)

Thus, the number and weight of faecal output in the TNX KO mouse is normal suggesting that TNX does not contribute to stool production and output.

3.9 Discussion

The main findings from this chapter show TNX may be important in maintaining the number of spontaneous contractions and amplitude particularly in the distal colon. Internal rectal prolapse is present in the TNX KO, however, overall pellet output was unchanged. Intervention analysis using NOS inhibitor did not produce a strong response in increasing the number and amplitude of colonic CMMC in the TNX deficient mice. Furthermore, CMMC analysis showed no change in CMMC propagation or velocity. Lastly, inhibitory effects of secondary bile acids showed similar effects in TNX deficient and WT mice. Therefore, the lack of TNX gene reduces a single type of contraction and amplitude particularly in the distal colon without affecting CMMC.

The first gross observation of the colon showed an internal rectal prolapse present in a small number of TNX deficient mice. This is interesting and has been reported in other extracellular matrix KO mice. The fibulin family consist of 7 extracellular matrix proteins important in varying degrees for assembling elastic fibers. Most of these fibulins bind to tropoelastin and some are critical in elastic fiber integrity and survival, for example fibulin-4 KO mice die at or during birth due to aortic ruptures (McLaughlin et al., 2006). Fibulin-3 is important in maintaining abdominal connective tissue by regulating elastic fiber assembly (Rahn et al., 2009) while fibulin 5 is important in motility, adhesion and elastogenesis (Nakamura et al., 2002). Fibulin 3 and 5 KO mice are phenotypically similar to the TNX KO mice in that they have stretchy skin (Rahn et al., 2009) (Liu et al., 2006), moreover fibulin-3 KO mice have rectal prolapse (34%) (Rahn et al., 2009). As well as fibulins, other extracellular serine proteases such as plasminogen causes rectal dysfunction. Plasminogens are implicated in activating latent growth factors and pro-collagenase, degrading extracellular matrix components and clearing fibrin (Bugge et al., 1996). In plasminogen KO mice, 52% developed rectal ulceration which progressed into rectal prolapse causing euthanasia of the animal due to high levels of distress (Bugge et al., 1996). Importantly, TNX deficiency in patients also results in external rectal prolapse in a case study of a patient with lower GI symptoms (Schalkwijk et al., 2001). The disparity in prevalence of rectal prolapse in humans may be explained by a lack of examination and that internal prolapse is not obvious like the external rectal prolapse. There is a lack of studies showing rectal prolapse in patients with

other extracellular matrix deficiencies, however, a study by Joshi et al, showed a significant decrease in fubulin-5 skin biopsies of male patients that had rectal prolapse compared to males without rectal prolapse. This study is limited for various reasons, first the sample size was small, second they only looked at fibulin-5 expression in skin biopsies and thirdly they did not look at the expression of fibulin-5 in the rectum which may be different than in skin. Therefore based on the studies mentioned above it is sensible to suggest that TNX together with other extracellular matrix molecules causes rectal prolapse by reducing the integrity of the rectal fascia. The level of contribution from TNX in causing rectal prolapse is unknown thus warrants further investigation.

One of the major functions of the colon is to move digesta/pellets from the proximal colon to the distal colon and rectum where the material can be expelled. This process requires the colon to produce waves of muscle contraction which involves various enteric neural and myogenic regulatory mechanisms. As discussed in chapter 2, TNX is selectively expressed in cholinergic neurons in the myenteric plexus which is the neural centre involved in generating colonic contractions. TNX was also expressed in nerve fibres within the muscle that communicate with neurons in the plexi to generate contractions through the release of neurotransmitters. TNX was found on inhibitory nitrergic neurons but to a lesser extent than the excitatory cholinergic neurons. Therefore TNX may have a functional role in generating colonic contractions owing to the cholinergic pathway rather than being involved in relaxation attributed to the nitrergic pathway. To test this hypothesis, colonic motility patterns were analysed using the TNX KO mouse and compared to controls. The first major finding showed that a lack of TNX causes a significant reduction in the number of spontaneous contractions in all regions except for the proximal colon. This reduction was most apparent in the distal colon where the amplitude was also significantly decreased. Since TNX was in neurons it is likely that the decrease in amplitude and frequency of contractions is a neurogenic phenomenon. Although the overall number of contractions was reduced the coordination of contractions exemplified by CMMC analysis showed no change. First it is necessary to confirm that the nature of contractions and CMMC's observed in the WT mouse was parallel to published data. Fida et al described the migration pattern of CMMC's in the isolated mouse colon using isometric force transducers that showed 90% of all CMMCs propagated in the aboral direction (Fida et al., 1997). The data presented in

this chapter also show similar propagation patterns with CMMC's in both WT and KO mice propagating aborally 82% and 92%, respectively. Moreover this data showed 14% of orally propagating CMMC's in the WT, which is the same as CMMC's reported in another study that showed 14% oral propagation in control tissue (Fida et al., 2000). Similarly the migration velocity observed in the WT (2.17 ±0.26 min) correlated with published data in control mice (2.9 ±0.4/min) (Brierley et al., 2001). Lastly Brierley et al measured the interval between each CMMC in the mouse colon which showed an average CMMC frequency of 2.8 ±0.2/min which was identical to this study where CMMC interval in the WT was 2.81 ±0.24/min. In this chapter a novel methodology was adapted for the colon which has only been used in the small intestine before (Fraser et al., 1997), however, the data presented here is analogous to published results from different laboratories using different methods on isolated mouse colon. Therefore, the method described here is suitable for assessing mouse colonic motility patterns.

In the proximal colon the number of contractions and amplitude of contractions was unaltered in the TNX KO mouse. Specific peak type analysis also showed no differences in the proximal region when separating single, double and multi-peaks. This may be due to differential expression of TNX along the colon, whereby there is a greater expression in the distal colon rather than proximal colon. Thus explaining why colonic motility is unaffected in this region. However, to confirm this, quantifying TNX expression in different colonic regions needs to be completed in future experiments. Another explanation for this lack of change could be due to the innervation pattern of extrinsic neurons in different colonic regions. Vagal afferents terminating in the proximal colon are IGLEs with no IMA structures present (Phillips and Powley, 2000). TNX was expressed in gastric smooth muscle nerve endings in structures that resembled both IMAs and IGLEs. Since there are no IMAs in the proximal colon the effect of TNX that may be around IGLEs in the proximal colon is not significant enough to affect proximal colonic contractions. Therefore in the KO model no significant changes in contractile activity was observed in the proximal colon.

As described previously, TNX was expressed mainly on cholinergic neurons, therefore if it promotes function of these neurons, the TNX KO would lack preferentially cholinergic function rather than nitrergic function, which would give rise to a nett reduction of contractile activity. More specifically, if TNX is on excitatory myenteric neurons, the effect would more likely manifest in reduced contraction amplitude. If TNX is on interneurons, the effect would be uncoordinated contractions, probably resulting in lack of organisation and propagation of CMMC. Therefore the former is probably the case, since we observed only changes in number and amplitude of contraction, and no sign of changes in their organisation into distinct propagating CMMC. However, in the TNX KO there was a reduction in the occurrence of CMMC with a single pressure oscillation, whereas those with obvious separate pressure waves within them were unaffected. This may indicate fewer prolonged contractions (which appear as a single pressure wave in manometry) and more short duration contractions. This may in turn reflect changes in the organisation of neural signals prior to the final signal to smooth muscle being generated in excitatory motor neurons, but presumably at a less complex level than the organisation of CMMC, which requires communication throughout the whole colon to achieve migration of contractile complexes from one end to the other and repetition at given intervals. Of course myenteric neurons can act as interneurons (Reiche et al., 2000), (Porter et al., 2002) so these difference does not imply necessarily that TNX is affecting specific subpopulation of neurons.

The largest decrease in the number and amplitude of contractions between WT and KO was observed in the distal colon. This could be in part explained by a higher expression of TNX in the distal colon, therefore it is likely that they will be in cholinergic neurons thus altering the effects of ACh. In TNX KO mice, the number and amplitude of contractions is decreased particularly in distal colon suggesting that TNX influences specifically the function of ACh containing neurons. This is also true in a study showing the number of large contractions decrease particularly in the distal colon in muscarinic receptor KO mice compared to WT controls (Kondo et al., 2011).

When spontaneous parameters were analysed two differences were observed. First, there was a significant difference observed in the single type of contractions from mid distal through distal segments. Second, the amplitude of contractions in the distal colon in the KO mouse was

significantly reduced compared to WT. In order to elucidate whether TNX influences overall contractile function owing to its expression in cholinergic neurons, the mouse colon was challenged with NOS inhibitor with the aim of revealing the contribution of TNX in cholinergic function. The NOS inhibitor NOLA has been extensively used to study CMMC patterns in isolated mouse colon (Brierley et al., 2001), (Powell et al., 2003), (Spencer et al., 1998b). NOLA blocks the release of NOS from inhibitory neurons that otherwise induce relaxation important in the peristaltic reflex. In this arm of the study the effects of NOLA increasing the number of contractions was mostly observed in WT mid proximal and distal colon and in the KO mid distal colon. Therefore the overall response of NOLA in the KO mouse colon was minimal suggesting TNX does not play a critical role in excitatory contractions but affects a single type of contraction.

The role of TNX was further explored by using secondary bile acid - DCA. The role of bile acids in intestinal motility is controversial, where *in vivo* studies report bile acids to be stimulatory or have no effect in human ileum (Penagini et al., 1988), (Penagini et al., 1989), (Van Ooteghem et al., 2002). *In vitro* studies in rabbit ileum (Armstrong et al., 1993) and guinea pig ileum (Romero et al., 1993) show inhibitory effects on motility. Specifically in the mouse colon, bile acids are shown to produce an inhibitory effect by activating TGR5 receptors that in turn release NO and inhibit contractility (Alemi et al., 2013). Even though only a smaller proportion of NOS containing neurons co expressed TNX, it was important to know whether TNX had a role in the function of inhibitory neurons. Adding bile acid had an inhibitory effect on contractility in both WT and KO mouse colon when bile acid was applied to the serosa. Specifically, the number and amplitude of spontaneous contractions was significantly reduced in WT but in the KO significance was not reached (p=0.0618 peaks/min, amplitude p=0.0688). Bile acid may have similar effects on KO as in WT mice and further studies to increase N numbers could elucidate this. Comparison between WT and KO showed no difference, collectively indicating TNX does not have a role on inhibitory neurons which was expected since IHC data showed co-expression of TNX with nitrergic NOS to be infrequent.

The aim of this chapter was to identify the functional role of TNX in colonic motility by neural mechanisms. The data described shows that the role of TNX in colonic motility is specific and

does not affect coordinated motility or stool output. To further understand motility patterns it is important to look at total gut transit time perhaps using radio opaque markers (Ghoshal et al., 2007) used previously in mouse colon as well as *in vivo* studies that gives an indication of colonic motility in a truer natural state.

4 The role of TNX in secretory function of the colon

4.1 Introduction

In a case study, absence of the TNX gene resulted in chronic constipation and rectal prolapse (Schalkwijk et al., 2001). Moreover, a large scale study performed at the Mayo clinic showed that patients with JHS most commonly had symptoms associated with constipation and diarrhoea (Nelson et al., 2015). Physiology tests in these patients showed abnormal colonic transit (28.3%) and they commonly took drugs for constipation (25.8%) (Nelson et al., 2015). The pathophysiology of constipation is unknown and likely to be multifactorial including dysregulation of colonic motility and secretion (Lembo and Camilleri, 2003). Secretory function of the colon is largely controlled by the submucous plexus, which maintains fluid homeostasis. This balance is regulated, in part by the action of secretomotor and vasodilatory neurons which control water and electrolyte secretion, and blood flow (Vanner and Surprenant, 1996), (Vanner and Macnaughton, 2004). Furthermore defects in the submucous plexus, predominantly at the level of neuronal excitability causes bowel disorders such as diarrhoea and constipation (Foong et al., 2014).

4.2 Reflexes in submucous neurons

The presence of neuronal reflexes in the colon that modulate ion secretion and blood flow are well recognized and a variety of studies have tried to establish the organization of these reflexes (Vanner and Macnaughton, 2004). Specifically *in vitro* studies in the guinea pig ileum have shown mucosal stroking activates the submucosal reflexes controlling secretion and vasodilation (Vanner et al., 1993), (Cooke et al., 1997b), (Kirchgessner et al., 1996), (Pan and Gershon, 2000). In order to reveal the autonomy of the submucous plexus and mucosa in regulating secretion the myenteric plexus was removed in the aforementioned studies. These studies were able to conclude that the submucosal reflex consists of afferent and efferent elements confined to the submucous plexus and mucosa. (Vanner and Macnaughton, 2004, Furness et al., 1998). Stimulating the mucosa by mechanical distortions activates enteroendocrine cells that release 5-HT which act on nerve terminals found on the submucous neurons that are in close proximity to

the epithelial cells (Wapnir and Teichberg, 2002). Specifically 5-HT acts on 5-HT_{1P} receptors found on submucosal primary afferent neurons whereas second order neurons respond to cholinergic/CGRP containing primary afferents with nicotinic fast EPSPs and CGRP mediated slow EPSPs which are necessary for excitation to spread across the submucosal plexus (Pan and Gershon, 2000). In addition to CGRP, SP is also known to mediate slow EPSPs in second order neurons (Cooke, 1998), (Pan and Gershon, 2000). SP containing nerves have axon collaterals that release SP which act directly on enterocytes in the guinea pig distal colon (Cooke et al., 1997a). The submucous reflex has been shown to have an oral-anal polarity in distance, number and type of neurons in the colon which can amplify the mucosal signal and thus secretion (Neunlist et al., 1998), (Neunlist and Schemann, 1998). This has been demonstrated by simultaneously activating adjacent submucous neurons using intracellular recording techniques. This study demonstrated that cholinergic neurons provide diverging and converging inputs to VIP containing neurons to enhance secretomotor effects, the former attributed to AH neurons (Reed and Vanner, 2003). Despite this ability to induce effects on neighbouring submucous neurons, the overall reflex is confined to distances of 1-2 mm and no more than 5mm (Moore and Vanner, 1998). The myenteric projections that reach the submucous plexus were first identified using IHC studies combined with electrophysiology suggesting functional synaptic connections exist (Bornstein et al., 1987). Specifically *in vitro* studies in submucosal and myenteric preparations showed direct evidence of the connection between these two plexuses and surrounding nerves (Moore and Vanner, 2000). Neuronal input to submucous neurons from the myenteric plexus are S-type and receive both fast and slow EPSPs. Video microscopy has shown that axons connecting both submucous and myenteric neurons can functionally cause submucous blood vessels to dilate (Vanner, 2000). Therefore it is unsurprising that mucosal or muscle distension can affect the myenteric submucosal vasodilator pathway (Reed and Vanner, 2003). In summary the submucosal reflex is driven by a variety of neurotransmitters that plays a key role in normal secretory function. Constipation and diarrhoea can occur when there is dysfunction at any level of these pathways.

4.3 Neurotransmitter control of ion secretion

The epithelium acts as a barrier that controls the movement of ions across the lumen. Secretion of ions is maintained by coordinated mechanisms to allow for normal secretion and absorption, mucus fluidity and removal harmful agents (Cooke, 2000). One of the most important aspects of colonic secretion modulated by neurotransmitters is the movement of chloride into the lumen. The Cystic Fibrosis Transmembrane Regulator (CFTR) is expressed on the apical membrane of colonocytes and is important for the movement of Cl ions in order to maintain an osmotic gradient (Strong et al., 1994). Opening of CFTR is controlled by increasing intracellular cAMP which regulates active movement of Cl- into the lumen (Tabcharani et al., 1991). This occurs when VIP binds to its receptor found on the epithelium, which is a G protein-linked activator of adenylyl cyclase that in turn causes phosphorylation of protein kinase A (PKA) and an increase of cAMP by using ATP (Field, 2003). This process actively secretes chloride into the lumen through the CFTR channel (Banks et al., 2005). Secondly the sodium/potassium/chloride (co-transporter (NKCC) is found on the basolateral membrane which mediates active chloride uptake where two Cl- and two cations (K+ and Na+) are transported in an electroneutral manner, therefore unaffected by the membrane voltage (Barrett and Keely, 2000). The NKCC1 isoform is most prominently expressed in the colon and has 10-30 fold higher expression on secretory epithelial cells compared to other cell types (Payne and Forbush, 1995). In essence the NKCC1 transporter provides a mechanism where the loss of Cl- by CFTR across the apical membrane is matched to the rate of Cl- entry enabling sustained chloride secretion. This communication between the apical and basolateral membranes by CFTR and NKCC1 reflects a controlled integrated process of the colonic epithelium (Barrett and Keely, 2000). Lastly there are two types of potassium channels found at the basolateral membrane involved in Cl- secretion (McRoberts et al., 1985), (Lomax et al., 1996), (Greger et al., 1997). One of these channels is thought to be activated by increases in cytosolic calcium (Barrett and Keely, 2000), moreover data suggests that carbachol which is a calcium dependent agonist activates this channel by a G-protein and increased sensitivity to intracellular calcium (Devor and Duffey, 1992). ACh is the other major secretomotor transmitter and acts via M_3 receptors found on enterocytes that releases intracellular calcium (Hirota and McKay, 2006). This causes Cl- secretion and is owed to the opening of calcium gated

K+ channels (Hirota and McKay, 2006). VIP is the predominant secretomotor transmitter while ACh plays a lesser transient role contributing to short term changes in secretion. VIP and ACh are thought to be contained in separate neuron populations in guinea pig and mouse studies, whereas in humans they are described as co-transmitters found in the same neurons (Anlauf et al., 2003), (Harrington et al., 2010). Both VIP and ACh are found in nerve terminals of the colonic mucosa suggesting secretion is under direct neural control. VIP is found in 50% of submucosal neurons in the guinea pig ileum while the other 50% expresses ChAT (the enzyme responsible for ACh synthesis) (Keast, 1987).

To summarise, the presence of neuronal reflexes in the colon is important for normal ion secretion through the release of neurotransmitters both from the mucosa and, nerve terminals found in the submucous and myenteric plexus. At the level of the epithelial cell, secretion is controlled by activation of transporters found on the apical and basolateral membrane that maintain an electrochemical gradient. Cl- is taken up by epithelial cells by the basolateral Na+/K+/Cl- co- transporter mechanism and exits the cell by cAMP activated CFTR, or by Ca^{2+} activated Cl- channels (Ji et al., 1998), (Cartwright et al., 1984).

Interestingly as described in chapter 2, TNX was most abundantly co-expressed in cholinergic neurons containing ChAT and calretinin in the submucous plexus. Specifically, in mouse tissue, calretinin which is co-expressed with ChAT (a marker for cholinergic neurons), was found abundantly with TNX in the submucous plexus of the mouse colon (Chapter 2, section 2.3.2). In human colon, ChAT showed the highest level of co expression with TNX followed by calretinin in the submucous neurons (Chapter 2 section 2.3.3). Collectively, these data demonstrate the expression of TNX in cholinergic submucous neurons in both human and mouse, suggesting TNX may possibly influence secretory mechanisms in the colon. Due to the co-expression of TNX preferentially in cholinergic submucous neurons, TNX may modulate secretory pathways by affecting the release of neurotransmitters. Changes in short circuit current, which can be measured by observing changes in active Cl-secretion, is a reliable method used to assess changes in ion fluxes and therefore an indirect measure of secretion in the mouse intestine (Clarke, 2009).

Therefore, this chapter will study WT and KO mice to observe the effects of TNX on secretion in response to neural stimulation.

4.4 Methods

4.4.1 Tissue dissection

The mouse was then pinned out and the abdomen was cut open carefully to prevent damage to the colon. Once the colon was exposed, cold Krebs solution at 4^{o}C was poured over and further dissected to free the colon from the mouse. The colon was then removed and transferred to another dish that contained damp tissue on a sylgard. The colon was carefully cut along the mesenteric border. The pellets were then gently removed using a damp tissue without touching the mucosa. The whole colon was then turned over so that the mucosa was facing down and the serosa was on top. Using fine forceps (Dumont) the muscle layer was carefully peeled from the distal to proximal colon leaving the mucosa and submucosa intact. Using scissors (Moria) the colon was then cut into 4 equal pieces 1cm x 1cm pieces, 1cm above the rectum and 1cm below caecum. Each piece was defined by location for example the first 1cm piece was in the proximal colon, then subsequent 1cm pieces followed and denoted as mid proximal, mid distal and lastly distal colon (Fig 4.1). This was done quickly to avoid tissue from drying up and placed in the Ussing chamber setup.

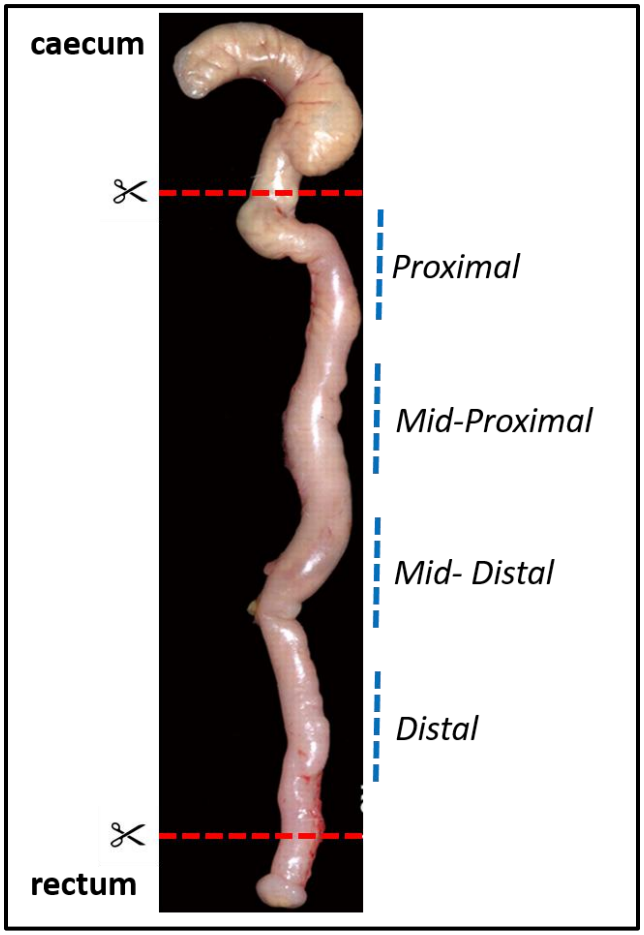


Figure 4.1. Dissected mouse colon.

Colonic regions used for secretion experiment are highlighted with dashed blue lines. (Adapted from Toivola et al 2004)

4.4.2 Intervention

Veratridine is a naturally occurring alkaloid that is a sodium channel activator. (Tikhonov and Zhorov, 2005). This activation stimulates neurons to release ACh and VIP, which are two prominent transmitters involved in Cl- secretion (Barrett and Keely, 2000). On the epithelial cells, VIP binds to its receptors that is associated with G coupled proteins. This interaction activates adenyl cyclase, in turn increasing intracellular calcium and activating the CFTR resulting in sustained Cl- secretion in the continued presence of the agonist (Barrett and Keely, 2000). Conversely, ACh binds to muscarinic M_3 receptors that is associated with another G protein

(Dharmsathaphorn and Pandol, 1986), (Vajanaphanich et al., 1995). Activation of phospholipase C and phosphatidylinositol hydrolysis yields the calcium mobilizing messenger (Yamada, 1995), (Sidhu and Cooke, 1995), in turn intracellular calcium. Which activates potassium channels and chloride channels to induce Cl- secretion (Barrett and Keely, 2000). The combined effects of VIP and ACh are synergistic evidenced by using different signalling events (Barrett and Keely, 2000). Therefore, through this mechanism veratridine is a non-selective potent stimulator of secretion. Veratridine was added to the serosal hemi-chamber at a concentration of 30µM after 20 mins of stable basal secretion was recorded.

4.4.3 Ussing chamber setup

The method used has been described in detail by Hyland and Cox 2005. In short, preparations were mounted on Ussing chambers (exposed area 0.14 cm2) and bathed continuously with 5ml carbogenated Krebs maintained at 37^{o}C. The mucosae were voltage clamped at 0 mV using a voltage clamp (DVC 1000, World Precision Instruments, Stevenage U.K.) and the resulting short-circuit current Isc was recorded using voltage and current electrodes EKV and EKC respectively (World Precision Instruments), which gave a measure of secretory function. Once a stable basal Isc was reached (20 mins), veratridine which causes epithelial anion secretion was added to each serosal hemi chamber at 30µM and Isc was recorded until the end of the experiment. Peak changes were recorded as change in amplitude (µA).

4.4.4 Agar filled electrode cartridges

Electrode cartridges were filled with agar gel (99% purity-Life Technologies). This was made by dissolving 2-4% (by weight) agar in 3M KCl solution at 80°C. Once the agar cartridges were attached to the electrodes they were stored in the dark with the cartridge tips in 3M KCl to prevent the gel from drying out.

4.4.5 Modified Krebs solution

Based on published secretion studies in mouse colon (Hyland et al., 2003) the same Krebs Heinseleit buffer was used studies, which includes NaCl, 117; KCl, 4.7; $CaCl_2H_2O$, 2.5; $MgSO_4$.7

H_2O, 2.5; NaH_2PO_4, 1; $NaHCO_3$, 24.8; and D-glucose, 11.1 mM. The solution was gassed continuously with 95% 0_2 and 5% CO_2.

4.4.6 Data acquisition

Data was acquired using the 1401 and Spike 2 software (Cambridge Electronic Design, Cambridge UK). All results are presented as mean ± SEM. Statistical analysis was performed using a two way analysis of variance (ANOVA) using Sidaks multiple comparison test to compare the two groups and a $p<0.05$ was considered significant (GraphPad Prism, V.7.02, GraphPad Software, Inc). All raw traces are presented in Appendix 4.1-4.3 for WT mice and 4.4-4.6 for KO mice.

4.5 Results

To understand the role of TNX in submucous neurons colonic secretion was measured by changes in electrogenic short circuit current (Isc) after the addition of veratridine (induces secretion) in WT (N=11) vs. KO (N=9).

There were no significant changes in basal Isc across all regions of the colon. There was an increase in secretion in the mid-distal and distal colon of the KO mouse, however, not significant (Fig 4.2).

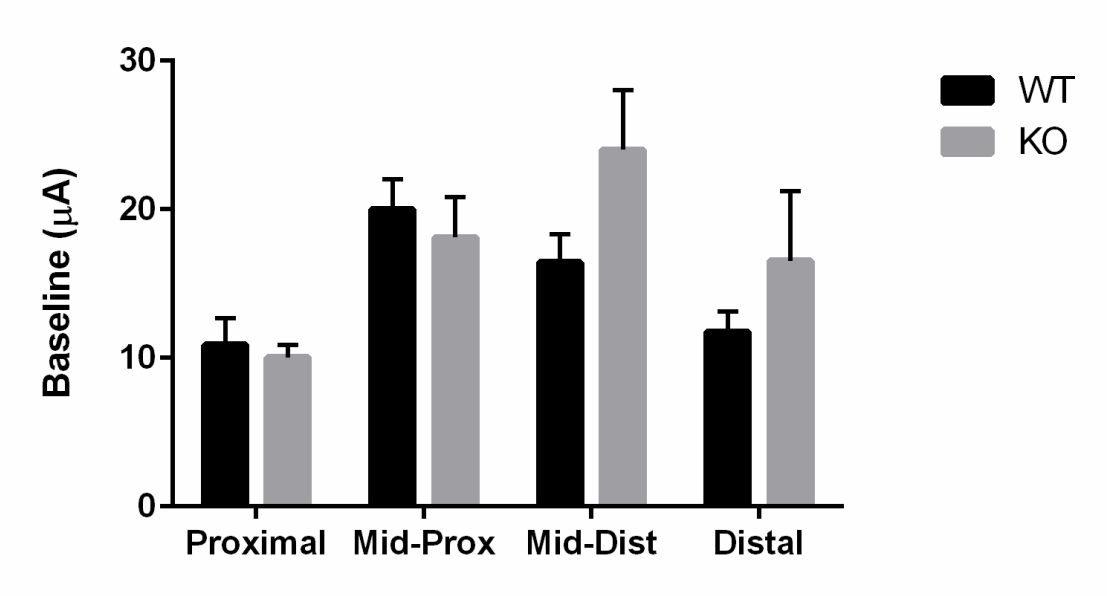


Figure 4.2. Spontaneous basal secretion unchanged in TNX KO.

No significant changes were observed in basal secretion levels in WT vs KO in proximal (WT=10.90 ±1.77 vs. KO=10.04 ±0.82), mid-prox (WT 20.00 ±2.00 vs. KO=18.11 ±0.82), mid-distal (WT 16.43±1.92 vs. KO=24.00 ±4.00), and distal (WT=11.77 ±1.36 vs. KO=16.54± 4.67) regions.

This lack of change is evident in the raw trace (Fig 4.3) which showed no changes in basal secretion. Furthermore in all colonic regions the response to veratridine was similar in WT and KO (Fig 4.3). The shape of the response in each region of the colon were similar in WT and KO.

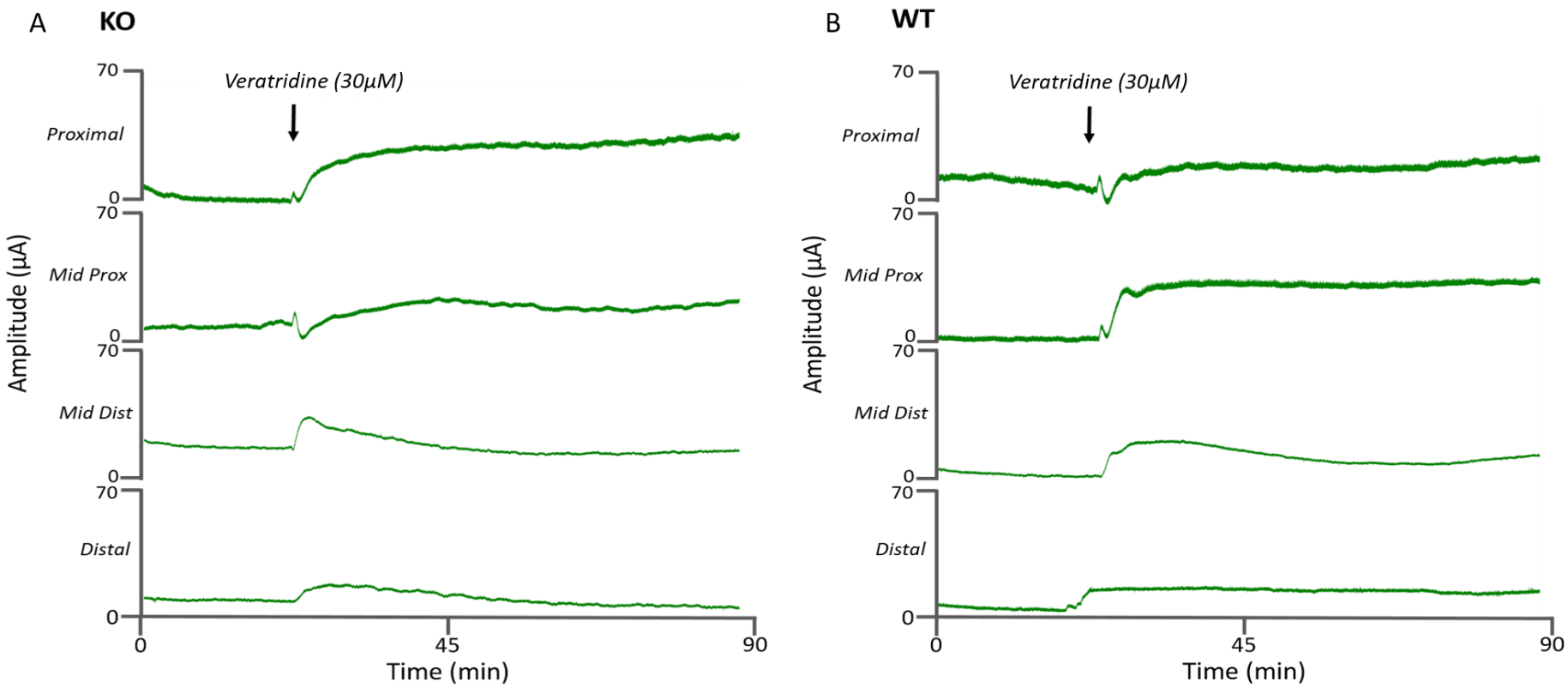


Figure 4.3. Representative trace in WT and KO over 90 mins.

Basal secretion recording from different regions of the colon in WT (A) vs KO (B). At 20 min Veratridine was added and Isc response was recorded until the end of the 90 min. Veratridine showed an increase in Isc in both WT and KO across all regions.

As described previously, veratridine causes an increase in Isc and was observed in both WT and KO (Fig 4.3). The WT and KO mouse show similar levels of secretion in the presence of veratridine across all regions of the colon (Fig 4.4A). In addition primary, secondary and tertiary outcomes were considered. The initial response to veratridine in terms of peak shape was analysed. A triphasic response refers to an initial increase above base line, followed by a decrease below baseline, finishing with the overall maximum peak. There were no triphasic responses in the WT distal colon and KO proximal colon. The number of triphasic responses showed an increase in the mid distal (KO=6 vs WT=2) and distal regions (KO=5 vs WT=2) of the KO compared to WT colon (Fig 4.4C) but this was not significant. Based on these triphasic responses three parameters were considered, the primary outcome which measures the initial peak within the first min, the secondary outcome which measures the decrease after the initial peak within 2 mins and lastly the tertiary outcome which measures the peak within the first 10 mins. There were no significant changes in all three parameters in WT vs KO (Fig 4.4B)

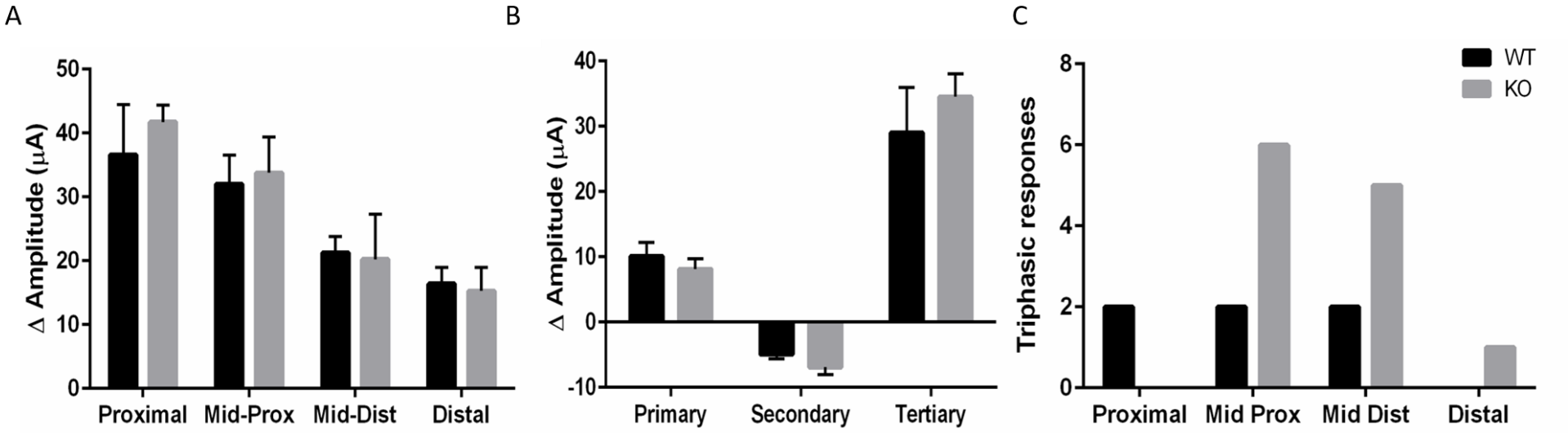


Figure 4.4. No change by veratridine induced Isc in WT vs KO.

A: No change observed by the addition of veratridine in WT vs. KO, as measured by change in amplitude of short circuit current. B: Overall colonic response to veratridine shows no change in response to drug at initial peak (over 1 min, i.e. primary), second dip (within 2 min, i.e. secondary) or tertiary peak (10 min). C: The number of mice with triphasic responses to veratridine was increased in Mid Prox, Mid Dist and Distal regions of KO colon compared to WT (N=11) vs. KO (N=9).

Finally, after the addition of veratridine, the time taken to reach a peak was calculated, which showed no significant changes in latency to peak.

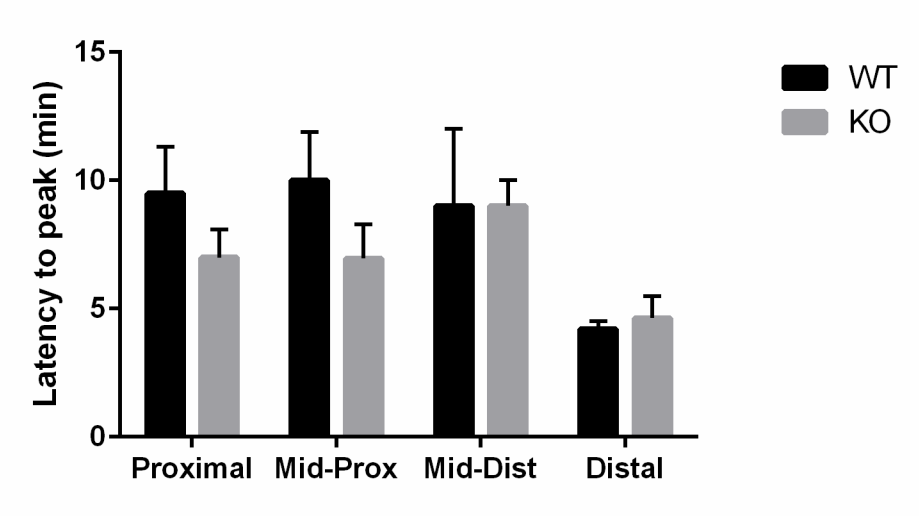


Figure 4.5. No change in latency to peak in TNX KO.

Latency to peak in each region of the WT and KO colon showed no significant changes. WT (N=11) vs. KO (N=9).

In summary, TNX deficient mice show no changes in basal secretion compared to WT mice. Intervention analysis using veratridine also showed no changes in any region of the KO colon except that the number of complex responses i.e. triphasic response was increased in the KO.

4.6 Discussion

The results obtained from this chapter suggest TNX does not have a major role in overall secretory function of the mouse colon. In terms of basal secretion no changes were observed between the KO and WT mouse nor did the KO colon respond differently to veratridine. Specifically, the time taken to reach a peak and the change measured from basal level to peak level post veratridine addition showed no significant change. Lastly, the number and shape of triphasic responses was measured which gives an indication of the true complexity of veratridine-induced responses. The number of triphasic responses was increased in the mid distal to distal colon post veratridine, however, when considering the individual elements of the triphasic responses for example primary, secondary and tertiary responses there were no significant differences in both groups.

Activation of sodium channels by veratridine activates VIP and ACh containing neurons which trigger signalling events releasing chloride. This indirect mechanism results in an increase in secretion which can be electrically measured. The lack of change in basal secretion between WT and KO in distal colon may be explained by the density and distribution of cholinergic submucous neurons involved in secretion, as well as the proportion of ChAT containing neurons expressing TNX in the mouse colon. It has been reported that the number of ChAT positive neurons in the submucous plexus increases distally whereas the number of VIP positive neurons decreases (Neunlist and Schemann, 1998). However, VIP containing submucous neurons account for 80% of all submucosal neurons in the mouse colon (Foong et al., 2014), therefore the proportion of ChAT positive neurons influencing colonic secretion in the distal region is still relatively low. This means that overall secretion in TNX deficient mice is unlikely to be affected particularly since ACh has a secondary effect in secretion. Furthermore, it is plausible that VIP neurons compensate for any TNX related effects on cholinergic submucous neurons.

Due to the importance of VIP in mediating secretion, and from the data obtained in this chapter, it is clear that investigating the expression pattern of TNX in and around VIP containing neurons is an important next step. In addition, it is necessary to establish whether there are region based differences in TNX expression within the colon. Finally, it is important to account for species

diversity in neurotransmitter expression in submucous neurons. For example in the colon, separate populations of cholinergic (ChAT) and non-cholinergic (VIP) positive neurons exist in the mouse (Foong et al., 2014) and other mammals (Keast et al., 1985), (Hubel et al., 1987), (Kuwahara et al., 1989), (Biagi et al., 1990). However studies in human and pig tissues show an overlap between VIP and ChAT or vesicular acetylcholine transporter which is an alternative cholinergic marker (Poonyachoti et al., 2002), (Bornstein, 2012). IHC data in both mouse and human submucous plexus showed overlap with cholinergic neurons, thus TNX may have a larger role in secretion in human tissue since ChAT and VIP are co-expressed unlike the mouse colon where the number of ChAT/VIP containing submucosal neurons are small. Thus it is pertinent to obtain human colonic tissue from both normal and TNX deficient patients to measure any changes in colonic secretion that may have been missed in the mouse model.

Veratridine was used for intervention studies and has been previously shown to increase Isc associated with net increases in Cl- secretion in mouse jejunum (Sheldon et al., 1990). In myenteric neurons of guinea pigs, veratridine and veratrine (mixture of veratridine alkaloids and veratridine) both stimulate release of SP, VIP (Belai and Burnstock, 1988), and ACh (Yau et al., 1986), as well as VIP release from myenteric nerve terminals (Allescher et al., 1996). Additionally, veratridine non-selectively stimulates submucosal neurons increasing release of VIP and ACh to produce prolonged increases in Isc. In this study, the overall response to veratridine was unchanged in all parameters for example, latency to peak, change in secretion measured by change in amplitude and the nature of triphasic responses. This may be explained by Isc studies in the mouse colon and jejunum that show in the presence of muscarinic and nicotinic antagonists, veratridine induced responses are unaltered (Foong et al., 2014) (Sheldon et al., 1989). Therefore the observed veratridine induced increase in secretion (Isc) is occurring via non-cholinergic VIP pathway rather than the cholinergic ChAT pathway. This may explain the lack of veratridine induced responses in WT and KO mouse, since TNX is predominantly found in cholinergic neurons and therefore the effects of the main secretagogue, VIP is unaffected. However, this suggestion can only be confirmed once characterization of VIP and TNX in the submucous plexus is established as suggested previously. In addition, veratridine induced triphasic responses were analysed. Triphasic responses reflect complex response patterns post

veratridine and can indicate such mechanisms as initial excitability of the VIP/ACh containing neurons as depicted by the initial peak (primary response) resulting from Cl- release. The secondary fall in secretion (secondary response) may represent the gap between Isc induced by non-synaptic neural activation and Isc induced by output from sustained activation of the submucous plexus network. Finally, the tertiary response is the overall effect of ACh/VIP in increasing chloride secretion thus increase in Isc is observed. No significant change was observed in triphasic response of the colon to secretion in WT vs. TNX KO. Further pharmacological intervention studies that selectively stimulate cholinergic neurons in the presence of non-cholinergic VIP blocker, for example VPAC receptor antagonist is required to fully delineate the role of TNX in cholinergic submucous neurons in the mouse colon.

Together, the data from this chapter suggests TNX does not have a major role in secretory function in the mouse colon. No changes in basal secretion or veratridine induced changes could be observed and further pharmacological intervention studies are needed to understand the nature of triphasic responses. Importantly, the distribution of TNX in different regions of the colon is necessary along with VIP co-expression. Lastly, secretion studies using normal and TNX deficient colonic tissue will address whether secretory function is conserved amongst species as well as shed light on the cause of symptoms seen in patients. Although this is ideal, such tissue is rare and very hard to identify before surgery. Nonetheless, based on the mouse data so far, tailored drug treatment for TNX deficient patients may be necessary as it is evident from the data thus far that secretion is less involved in generating symptoms. Therefore, the use of a prokinetic drug such as prucalopride rather than a pro-secretory drug such as linaclotide used for constipation may be more useful for TNX deficient patients.

5 The role of TNX in vagal afferents: electrophysiology study

5.1 Introduction

Upper GI complaints are common in patients with JHS (Fikree et al., 2015a); these include bloating, heartburn, nausea, early satiety, regurgitation and pain (Kovacic et al., 2014), (Fikree et al., 2015a), (Zeitoun et al., 2013), (Nelson et al., 2015). Functional dyspepsia and reflux are also found to be common in JHS patients attending GI clinics. (Fikree et al., 2014). Interestingly, TNX-deficient patients have upper GI symptoms that include indigestion, reflux and abdominal pain (Aktar & Fikree, unpublished data). These symptoms are likely to be caused by multiple factors and it is known that vagal afferents play a major role in normal gastric function by communication between the ENS and CNS. Therefore in these patients there may be a possible dysregulation in vagal afferents which is an important treatment target (Andrews and Sanger, 2002). As described in Chapter 1, section 1.17 vagal afferents can be divided into two types of nerve endings based on morphology and innervation patterns, the IMAs and IGLEs. IHC data in Chapter 2 section 2.3.2, showed whole mounts of TNX around IMAs in muscular layers of mouse stomach and around IGLEs in the myenteric plexus. It is known that IGLEs are mechanosensory and respond to stretch (Zagorodnyuk and Brookes, 2000) whereas the specific role of IMAs is yet to be established. Nevertheless, IMAs are thought to respond to tension and stretch that extend up to several millimetres parallel to the circular and longitudinal muscle in the stomach (Phillips and Powley, 2000). Calretinin, a marker for vagal IGLE/IMA terminals in the stomach (Castelucci et al., 2003), was co expressed with TNX in the mouse stomach. Moreover, TNX containing cell bodies were present in mouse nodose ganglia therefore indicating a vagal origin (Berthoud et al., 2004). Collectively, anatomical mouse data and upper GI symptoms present in TNX deficient patients points towards a possible role for TNX in modulating gastric vagal afferents.

Extrinsic innervation of the stomach is by vagal and spinal afferents and their cell bodies that reside in the NG/JG and DRG, respectively (Berthoud et al., 2004). The morphology of IGLE/IMA vagal afferent endings has been established using anterograde labelling studies (Clerc and Mazzia, 1994), (Neuhuber, 1987), (Berthoud and Powley, 1992),(Phillips et al., 1997), described in further detail in Chapter 1 section 1.17. Although morphological data suggests two types of afferent endings differing in terms of a) target tissue, b) structure of

nerve endings and c) regional distribution, early electrophysiology studies describe a single population of tension receptors that are mechanoreceptors (Iggo, 1955). These mechanoreceptors are described as “in-series” to the smooth muscle that monitor smooth muscle contraction, motility, propulsion of contents and stretch of the gastric wall based on intra-gastric changes in volume (Phillips and Powley, 2000). Based on these properties, confusion exists as to whether IMAs and IGLEs are one and the same in terms of function.

The vagal afferent fibre response to distension can be divided into three parts: Firstly; the dynamic response secondly; the non-adapting phase and lastly, the silent period. When distension is initiated the vagal afferents produce an immediate burst of action potentials which then acclimatise to a steady discharge in concordance with the amount of gastric fill (Davison and Grundy, 1978). The dynamic response is thought to reflect the initial tension undergone by the endings due to active resistance by smooth muscle cells through induction of muscular stretch (Grundy and Brookes, 2012). Once distension reaches a plateau, so does the firing frequency to a static level maintained during the length of distension. This ongoing firing suggests that vagal afferents are slow adapting receptors (Roberts et al., 1986), (Paintal, 1954). The decreased firing is caused by accommodation or receptive relaxation of the gastric muscle wall decreasing intra-gastric pressure and muscle tension (Iggo, 1986). Lastly, when the distension period is over, the firing rate falls back to spontaneous levels and in some instances even below pre-distension levels, which is parallel to the drop in gastric muscle tension and pressure. Occasionally there is a silent period in firing which is correlated to the intensity of distension and speed of gastric relaxation (Davison and Clarke, 1988). In addition to distension, vagal afferents also respond to isometric contraction resulting in an increase in discharge irrespective of whether the innervating organ is distended or flaccid (Blackshaw et al., 1987), (Iggo, 1957b), (Iggo, 1955), moreover, afferent firing is greatly increased when contractions are superimposed on distensions compared to individual effects on firing (Iggo, 1957b), (Iggo, 1955). As well as the nature of firing patterns, the vagal afferents innervating different regions of the stomach have distinct roles, for example, the fundus and corpus serve as storage compartments and the antrum grinds and pumps food into the duodenum (Andrews et al., 1980).

Not only are vagal afferent endings found in the muscle layer, they are also found in mucosa where they have distinct properties. The slow adapting vagal mechanoreceptors are found in

the muscle wall and not in the mucosa, this was demonstrated when mucosal and serosal stroking did not induce a response (Paintal, 1954), moreover rendering the mucosa inactive by use of chemical anaesthetics, showed no changes in vagal afferent activity (Clerc and Mei, 1983) nor did scraping the mucosa (Bitar et al., 1975), (Sengupta et al., 1989). Vagal afferents that innervate the mucosa are thought as rapidly adapting and responded to the aforementioned stimuli that cause mucosal afferent firing. TNX was found around vagal mechanoreceptors and not around endings innervating the mucosa, therefore, mucosal activation is unlikely to show differences in WT and KO mouse.

Alterations in afferent function would most likely give rise to a variety of symptoms associated with the gastrointestinal tract. Symptoms associated with FGIDs are likely to include nausea, early satiety, and bloating and epigastric discomfort mediated by altered vagal afferent function (Andrews and Sanger, 2002). Patients with dysmotility and functional dyspepsia have post-prandial symptoms including bloating and altered gastric accommodation due to incraesed distension (Troncon et al., 1995). These symptoms are parallel to those found in a group of post-vagotomy patients suggesting an underlying vagal mediated pathway (Troncon et al., 1995). Similarly the role of the vagal neurocircuits are of critical importance that provide targets to reduce symptoms such as vomiting that is not caused by food poisoning but by exaggerated relaxation of the diaphragm most likely due to an vagal afferent dysfunction. Furthermore a high density of vagal afferent innervation is found in the lower oesophageal sphincter, which is another area that may be disrupted. Over relaxation of the sphincter causes dysregulation in gastric emptying and can give rise to reflux and nausea (Andrews and Sanger, 2002). It is evident that disordered vagal input or central processing of vagal afferent signals gives rise to a host of GI symptoms.

Single unit electrophysiological recordings of vagal afferents have provided a wealth of information including specificity of activation thresholds and receptive fields. In the mouse single fibre recordings of vagal afferents using a flat sheet preparation has been extensively used (Page et al., 2002). In this study we used an adapted form of the *in vitro* mouse preparation keeping the stomach whole and recording afferent firing in response to filling of the stomach. In this way the stomach is less prone to damage and reflects a more natural state of the stomach as if it were *in vivo* in comparison to a flat sheet.

In conclusion, as IHC data has shown TNX co-expressed with calretinin positive vagal afferent endings, and the symptoms present in TNX deficient patients may indeed be vagally mediated. The hypothesis of this chapter is that TNX is important for normal sensory function of vagal afferents in the stomach. The aims of this chapter were to assess spontaneous firing and firing in response to distension in WT and KO mice using whole nerve vagal afferent nerve recordings.

5.2 Methods

KO and WT mice, either male or female were assessed for electrophysiology studies. The death of the mice was confirmed by snipping the heart instead of cervical dislocation to prevent damaged to the vagus nerve.

5.2.1 Tissue dissection

The mouse was pinned out and a mid-line incision was made, the skin was then pinned and once the internal organs were exposed cold Krebs solution (4^{o}C) was poured over. The whole stomach and oesophagus with attached vagal nerves were carefully dissected and transferred to a sylgard (Dow Corning) dish containing ice cold carbogenated Krebs. The vagus nerve was carefully peeled from the side of the oesophagus using fine scissors (Moria) and forceps (Dumont) until 1cm above the stomach. Keeping the stomach whole with attached oesophagus and vagus nerve, the proximal duodenum was cut and stomach contents were gently flushed out with a syringe. Once clear a small rubber tubing filled and attached to a Krebs filled syringe was inserted into the stomach and the duodenum was tied securely with a black silk tie. The whole stomach was then secured and a pin was placed through the oesophagus in one side of the organ bath the bath was perfused with carbogenated Krebs buffer at 34^{o}C. A sliding wall with a small hole for the vagus nerve to pass through was positioned so that the vagus nerve can extend into the second chamber onto a glass plate bathed in paraffin oil. The edge of the sliding wall was sealed with paraffin wax to prevent the paraffin oil mixing with the Krebs solution in the adjacent chamber. Under a dissection microscope (6x: Wild Heerbrugg M5A, Germany), using fine forceps (Dumont) the sheath was peeled and the vagus nerve was dissected into 6-10 bundles. One nerve bundle was placed on a platinum recording electrode. A reference electrode rested on the glass plate with a pool of Krebs surrounding it away from the recording electrode. Once a nerve fibre was placed on the recording electrode, any spontaneous discharge was observed. Fibres that had spontaneous discharge often responded to distension than those that were silent. Thus recordings from spontaneously active fibres in spite of firing rate was tested. There was a resting period of 10 mins before any distension was applied. Once 10 mins was over the stomach was distended using the syringe filled Krebs, if there was no response to distension, the next fibre was placed on the recording electrode. This was repeated on all dissected fibres

to delineate single fibres that responded to distension. Once a distension sensitive fibre was identified, 1ml of Krebs was carefully inserted in the stomach and distension lasted approximately 1min before removing the tip of the syringe and the stomach naturally deflated. The same fibre was distended again in the same way after a rest period of 5 mins. Repeated distensions were obtained 3 times for all fibres that responded to distension.

5.2.2 Data capture and analysis

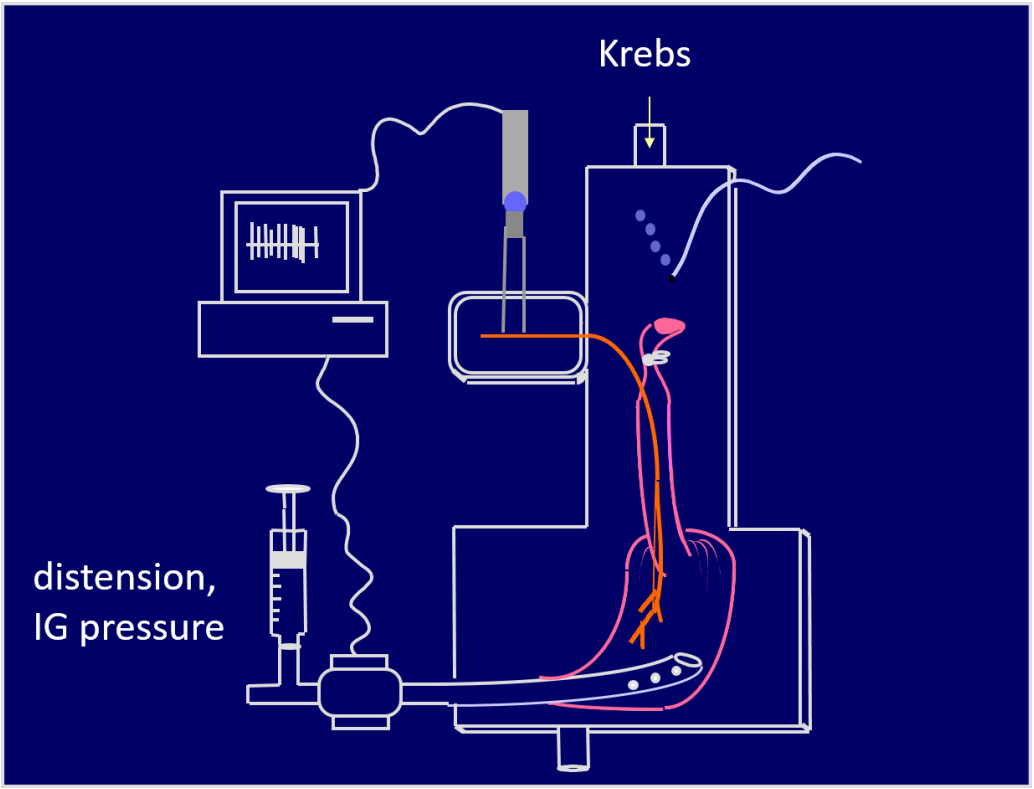


Figure 5.1. *In vitro* setup of mouse whole stomach.

The whole stomach is placed in a Krebs filled bath outlined in pink, a flexible tube is inserted stomach and tightly sealed with silk. The other end of the flexible tubing is attached to a 5ml syringe filled with Krebs. The dissected afferent nerves resting on the platinum electrode was continuously recorded using Spike 2 software.

5.2.3 Recording protocol

A platinum electrode head stage (World Precision Instruments) was used to record nerve activity (NL100). This was amplified using a preamp (A.C. Preamp NL104; Gain 5K) and filtered (Filters NL125; Neurolog System Digitimer Ltd, UK). This signal was then digitised (CED Micro 1401 MkII; CED, UK). 50/60Hz electrical noise was removed (Humbug 50/60Hz Noise Eliminator; Quest Scientific, Canada). The recording was then high-pass filtered at 10,000Hz and low-pass filtered at 1000Hz to remove background noise. An IBM-compatible PC using Spike 2 was used to record the raw traces. Spikes were counted offline using Spike 2 software (CED, UK)

Spike units were discriminated using wavemark analysis, histograms and discharge traces. Data is presented as mean ± SEM. Differences in spontaneous afferent activity and response to distension were evaluated using two tailed unpaired *t-test* to compare groups N=4 WT vs. N=5 KO (GraphPad Prism, V.7.02, GraphPad Software, Inc).

5.2.4 Modified Krebs solution:

Based on published studies using a similar *in vitro* preparation, the same Krebs composition was used for our studies, which includes NaCl, 117.9; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4(H_2O)_7$, 1; NaH_2PO_4, 1.2; $NaHCO_3$, 25; and D-Glucose, 11.1 mM. The solution was gassed continuously with 95% 0_2 and 5% CO_2.

5.3 Results

Using a novel whole stomach *in vitro* preparation, it was possible to record the rate of vagal afferent firing under spontaneous and distended conditions. Overall 42 preparations were made from the WT and KO mice combined. Of those 42, few recordings were obtained at the onset, however after practice afferent activity was observed and recorded. 14 and 18 single units were recorded from the WT and KO mice respectively.

Overall the KO mouse showed higher spontaneous afferent activity as well as increased response to distension (Fig 5.2). This response was reproducible (Fig 5.2). In Fig 5.2 the green trace represents the raw trace that measures afferent firing rate denoted as Spikes (s), the blue histogram represents the rate of afferent discharge.

The average number of spontaneous spikes in the WT mouse was 0.237 ±0.067 per second whereas in the KO this was increased (0.6911 ±0.252) however missed significance (Fig 5.3A). The response to distension was significantly increased in the KO compared to the WT mouse (WT: 2.37 ±0.66 vs. KO: 9.67 ±2.15, p=0.0364), (Fig 5.3B). Repeated responses to distension showed similar results in WT vs KO mice, where the response to distension was twice as high in the KO than in the WT (Fig 5.4), for example the rate of firing was around 18.2 in the WT and 38.3 in the WT during distension (Fig 5.2- blue histogram).

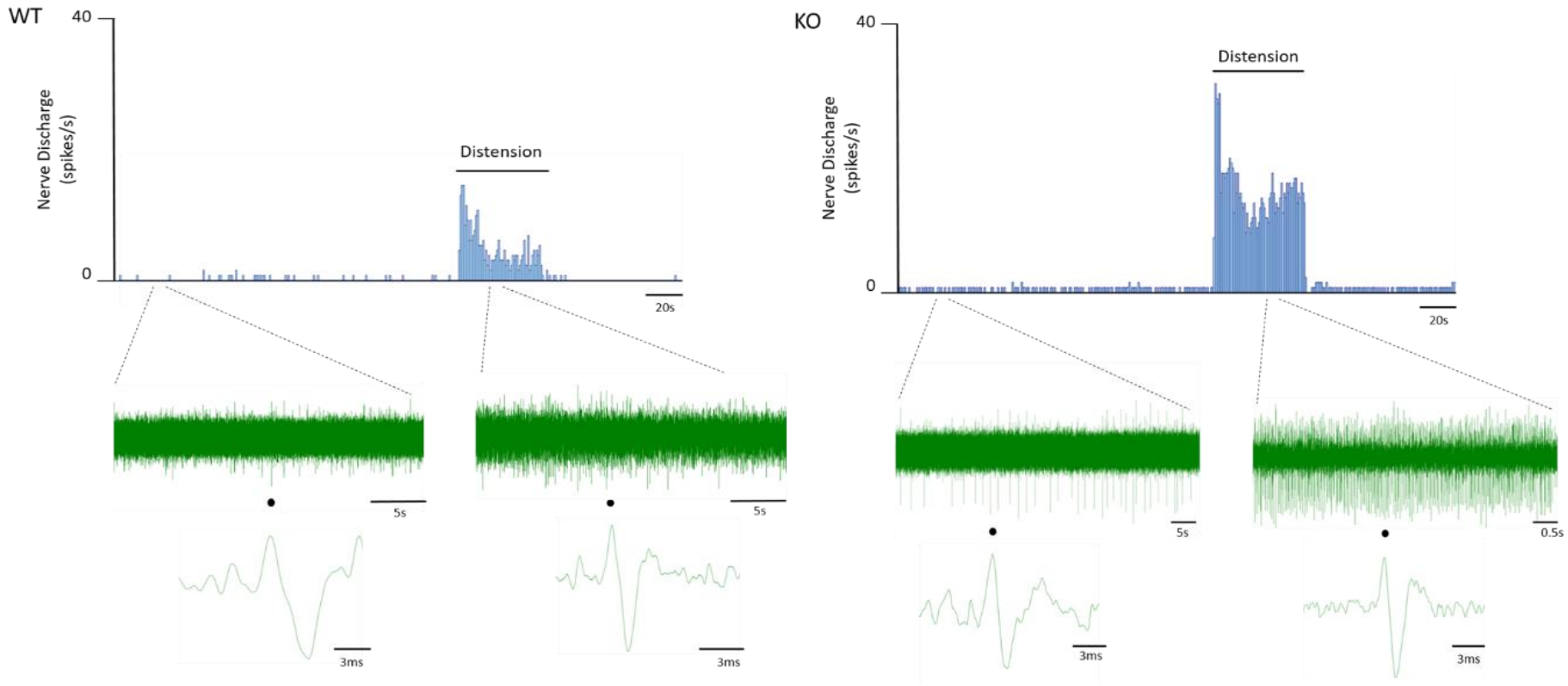


Figure 5.2. Responses to distension from single vagal afferent units innervating the stomach.

Comparison of WT and KO raw traces and histogram illustrates the increase in nerve activity spontaneously as well as during distension. Upper trace shows the nerve discharge rate is two-fold higher in KO vs. WT in response to distension. Middle left trace shows the raw recordings before and after distension in the WT, and in the KO (middle right). Clearly the number of spikes before and after distension are increased in the KO (middle panel) compared to WT. Lower panel shows a single action potential; lower left panel shows the WT and lower right panel shows the KO (n=14 WT vs. n=18 KO).

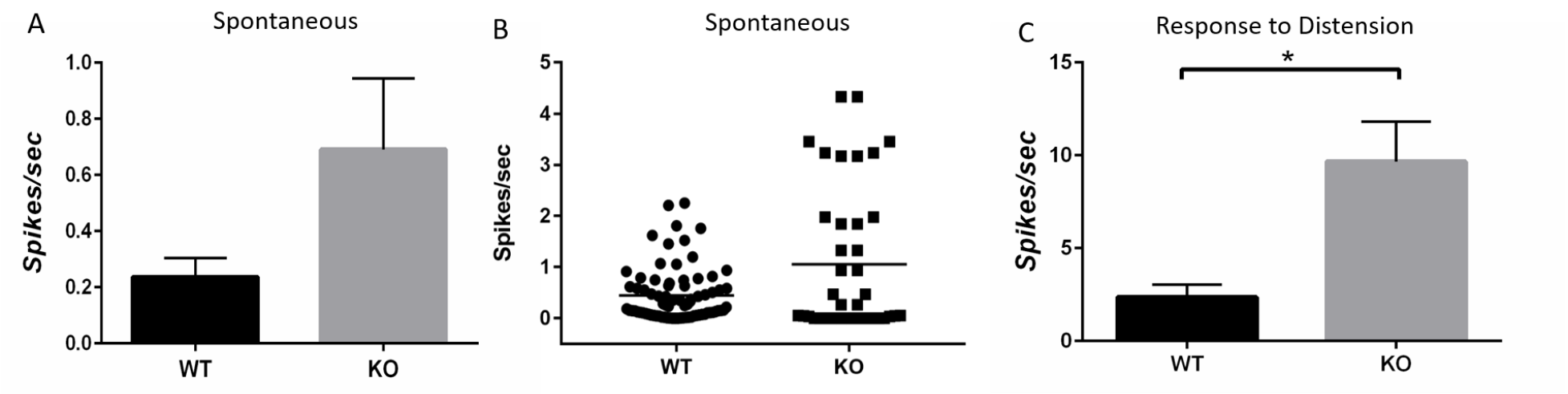


Figure 5.3. Graphical representation of the raw traces.

(A) Mean spontaneous vagal afferent nerve firing is increased in the KO compared to the WT (not significant). (B) Scatter plot of data A, showing individual values (C) Response to distension was significantly increased in the KO compared to WT.

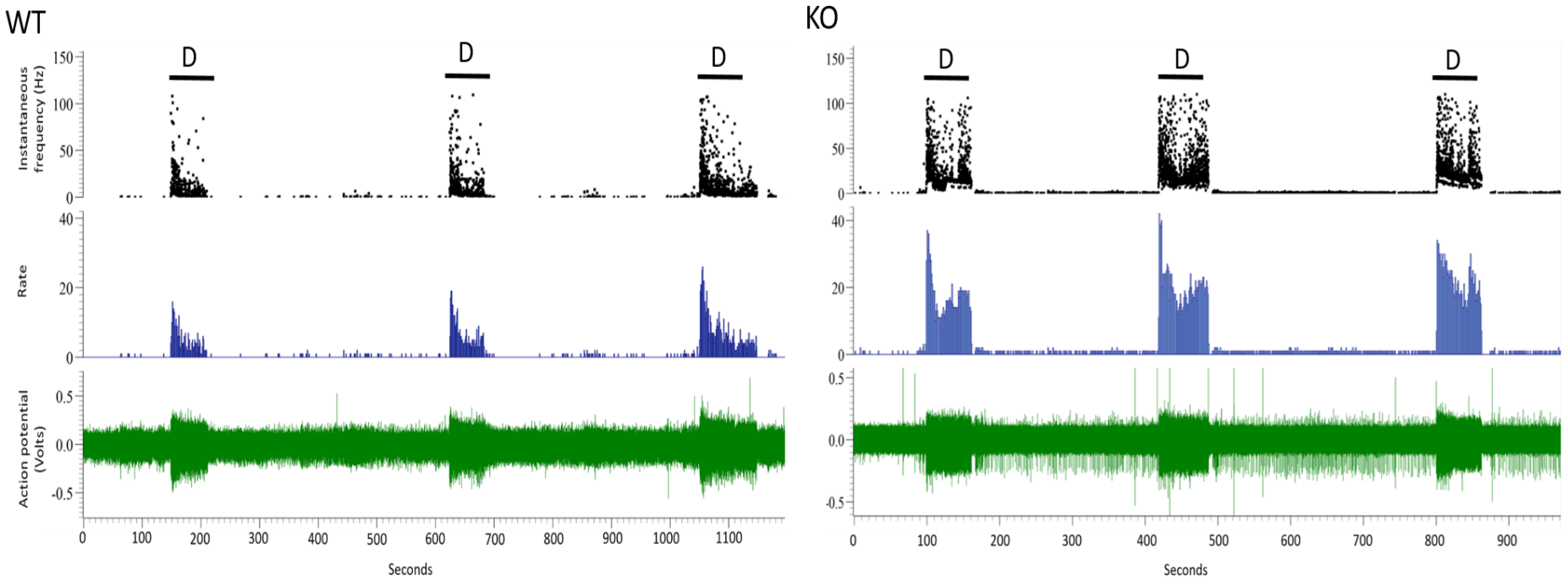


Figure 5.4. Raw traces showing repeated responses to distension in WT and KO.

Green trace shows the raw action potential of the nerve fibre, blue histogram describes the rate of afferent nerve firing and black dots illustrate the instantaneous frequency. Evidently in the traces, KO showed higher afferent nerve firing rate in spontaneous and repeated distended conditions. (n=14 WT vs. n=18 KO).

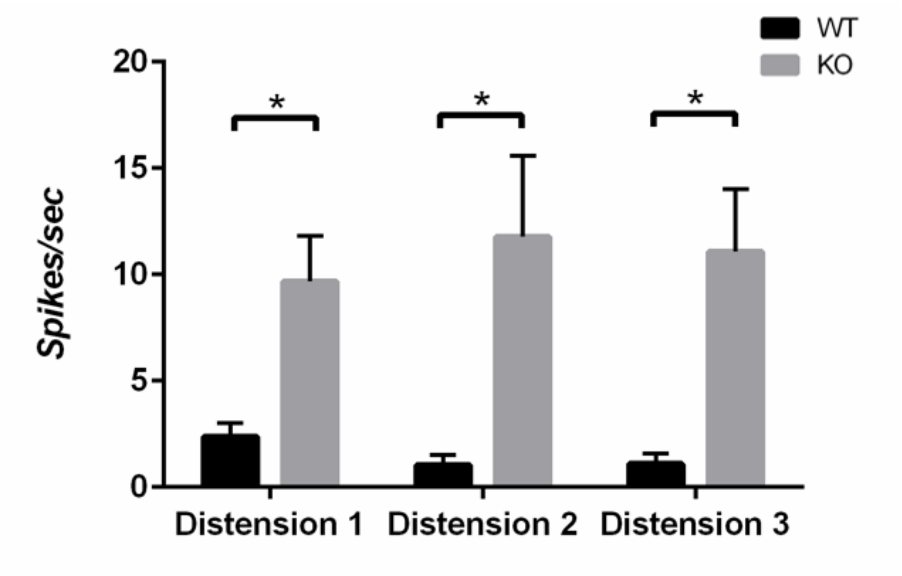


Figure 5.5. Repeated responses to distension.

Significant increases in afferent nerve firing in the KO compared to WT in all distensions. Distension 1 (WT: 2.37 ±0.66 vs. KO: 9.67 ±2.15, p=0.0364), Distension 2 (WT: 1.04 ±0.49 vs. KO: 11.78 ±3.80, p=0.0104), Distension 3 (WT: 1.08 ±0.49 vs. KO: 11.07 ±2.93, p=0.0192).

5.4 Discussion

In this chapter a novel *in vitro* mouse-vagus preparation was used to study the properties of extrinsic vagal afferent endings in the stomach. One of the interesting findings from this study was an important role for TNX in maintaining normal afferent firing frequency. TNX KO mouse showed higher levels of spontaneous afferent discharge and the response to distension was on average twice as high compared to its WT counterpart. The reasons for increased vagal activity in the TNX KO mouse will be discussed.

The purpose of using a whole stomach was to prevent tissue damage as well as mimic an *in vivo* state of the stomach. Spontaneous activity in mouse vagal afferents using the flat sheet preparation yielded a median discharge of 2.5 impulses/s (Page et al., 2002), while in the anaesthetised ferret whole stomach, the mean discharge was around 1 impulse/sec (Blackshaw et al., 1987). Moreover, ferret baseline afferent discharge differed based on the region of the stomach, for example in the forestomach/corpus, afferent discharge was 0.35-7.7 Hz (Andrews et al., 1980) and 5.6 impulses/s (Blackshaw et al., 1987)}. In the ferret corpus alone afferent discharge increased to 10 ±2.3 impulses/s whereas, in the antrum a lower value of 3.19 ±0.6 impulses/s was reported (Blackshaw and Grundy, 1990). In TNX WT mice mean spontaneous discharge was lower than reported data. The difference may be attributed to differences in methodology. The flat sheet preparation cuts the stomach along the greater curvature, which may result in increased spontaneous afferent firing due to muscle responses to the trauma. Moreover, the mouse tissue was pinned out which may have increased tension at the points of insertion resulting in greater baseline activity compared to the whole stomach recording which was performed here. Other studies recording from vagal afferents in rat stomach showed variable spontaneous firing based on recording (1.5-2.5 impulses/s) (Davison and Clarke, 1988) and (0.01-2.1 spikes/s) (Wang et al., 1997). Although there is large variability in reported spontaneous afferent firing, the afferent firing rate reported in this chapter is within the same range as previous studies suggesting that recording from whole stomach is a comparable method. In addition, the WT spontaneous activity is the baseline to which KO afferent discharge rates is compared. Therefore any changes observed in the TNX KO mouse was relative to the WT controls.

An explanation for increased firing during distension in TNX KO mice may be due to changes in neuronal plasticity. The term neuronal plasticity encompasses a host of changes in the neuronal structure in response to changes in input (Giaroni et al., 1999), for example trains of pre synaptic action potentials induce post-tetanic potentiation which in effect increases the release of specific neurotransmitters (Dityatev and Schachner, 2003). It is well known that changes in neuronal plasticity have important physiological and clinical implications. In the CNS, there is strong evidence to suggest a link between changes in neuronal plasticity and chronic pain (Zhang et al., 2014). Interestingly, in the ENS enteric neuropathy may present the primary cause of disease for instance in Hirschsprungs disease (Kenny et al., 2010) or secondarily in the case of Crohn's or ulcerative colitis (Geboes and Collins, 1998). Additionally, changes in neural plasticity and gut symptoms in neurological diseases such as Parkinson's is being studied further (Singaram et al., 1995). Therefore various studies have focused on neural plasticity to elucidate the pathophysiology of such diseases. ECM proteins such as laminin, reelin, TNR, TNC, brevican and neurocans are reported to be involved in synaptic plasticity (Dityatev and Schachner, 2003). Mice deficient in ECM molecules show abnormal levels of neural plasticity without any obvious developmental changes (Dityatev and Schachner, 2003). Specifically, the ECM molecule agrin is thought to be important in forming neuromuscular junctions and maintaining synapses between cholinergic preganglionic axons and sympathetic neurons (Gingras et al., 2002). Mice lacking a part of the agrin molecule show marked reduction in acetylcholine receptor which indicates this molecule is crucial in normal synapse function in the CNS (Gingras et al., 2002), (Gautam et al., 1996 4716). Moreover, agrin increases nicotinic transmission at the synapse by modulating the space between the gap-junction-mediated electrical coupling (Martin et al., 2005). Similar to agrins, TNX may have a role in modulating the vagal afferent microenvironment by regulating the intercellular spaces between the vagal afferent terminals and smooth muscle or ICC. An extensive review describing ECM molecules and neural plasticity showed ECM molecules play a role in neural strength by both biochemical and morphological changes using electrophysiological recordings (Dityatev and Schachner, 2003). Therefore, it is likely that TNX plays a similar role to other ECM proteins in modulating ENS neural connectivity. Perhaps TNX is important in maintaining the shape of vagal afferent endings, therefore the absence of TNX may transduce mechanical forces more efficiently. The morphology of vagal afferent endings in the KO model

has not been carefully characterised and may be altered therefore this needs to be further explored.

An alternative explanation of increased vagal discharge could be attributed to changes in ion channels and biochemistry within nerve endings. There are a variety of ion channels expressed on gastric vagal afferents, one example is the acid-sensing ion channels (ASICs) which are involved in detecting pH levels and mechanical stimuli by the influx of sodium ions (Page et al., 2005b). In particular deletion of ASIC1a increases the mechanical sensitivity of gastro-oesophageal vagal afferents (Page et al., 2005a), (Page et al., 2004). In TNX deficiency, ion channels that detect mechanical sensitivity may be altered, thus affecting the movement of sodium influx initiating the action potential. This change could then result in increased afferent firing observed in the KO. Interestingly, TNR is shown to modulate the activity of sodium channels (Weber et al., 1999), in particular cells containing β1 and β2 subunits (Xiao et al., 1999). TNR deficient mice show no change in the distribution of sodium channels however action potentials recorded from optic nerves showed a significant decrease in conduction velocity (Weber et al., 1999). Therefore since TNX and TNR are within the same family, the functional importance of voltage gated sodium channels that are involved in action potential generation may also be affected.

The method of recording vagal afferents from whole mouse stomach was first used in this study and comes with limitations. Firstly, flushing the contents of the stomach prior to recording may induce stretch of the stomach wall thus activating mechanoreceptors through this action. *In vivo* measurements of the fasted animal as described in anesthetised models earlier, would eliminate mechanical stimulation while also retaining the complete anatomical environment and thus giving a truer reflection of the role of TNX in afferent discharge. In addition, an increase in N and n numbers would increase the validity of the observed results and may show significant increase in spontaneous discharge in the KO. The only intervention in my studies was whole stomach distension, therefore changes in circumferential and longitudinal stretch were not considered. Using the cantilever claw system described by Page *et al* could delineate the effect of stretch and the type (e.g. circumferential vs. longitudinal) on KO afferent firing. Finally, when recording vagal afferents it is not possible to distinguish whether endings are IGLEs or IMAs, although whether they are indeed two functionally separate population's remains to be seen.

In summary, TNX has an important role in mediating vagal afferent firing since its absence causes hypersensitivity in both spontaneous and distended conditions. Based on the importance of ECM proteins in neuronal plasticity, it is reasonable to suggest that TNX may be playing a similar role in the vagal afferents. The underlying mechanism in the tissue microenvironment is currently unknown and opens up a novel field of research. Understanding the role of TNX at this level will help elucidate whether increased firing is due to changes in ion channels and biochemistry, changes in action potential generation or changes in morphology. This will in effect shed light on what induces GI symptoms in patients such as dyspepsia that is thought, in part to be contributed by altered vagal activity (Tougas, 1999), (Holtmann et al., 1998)

6 The role of TNX in gastric emptying

6.1 Introduction:

In stomach, TNX was found around vagal afferent nerve endings in mouse smooth muscle that resembled IMAs and IGLEs around the myenteric plexus in section 2.3.2. Additionally, in humans TNX was found in myenteric cell bodies 2.3.3. It is known that IMAs are varicose nerve fibres branching and running for millimetres parallel to the smooth muscle fibres (Neuhuber, 1987). Based on morphological structure, IMAs are assumed to be in-series tension receptors (Berthoud and Powley, 1992). IMAs are almost exclusively found in the smooth muscle layers of the stomach whereas IGLEs are also found in the myenteric plexus ganglia (Berthoud and Neuhuber, 2000). The presence of TNX around these vagal afferent endings suggests a possible function. In addition to understanding the electrophysiological properties of vagal afferent nerve endings, stomach function can also be tested *in vivo* using gastric emptying tests. This method assess overall efficiency of the stomach to empty and may highlight the role of TNX association around vagal afferent terminals in meal emptying.

6.1.1 Functional anatomy of the stomach in relation to gastric emptying

The stomach is divided into the proximal part, including the cardia, fundus and a portion of the corpus, and the distal region that includes part of the corpus and the antrum (Schemann and Grundy, 1992). Each region has a specific function, for example, the proximal stomach provides a reservoir for the meal, whereas the distal stomach grinds solid food in preparation

for gastric emptying and trituration (Lee et al., 2004). Motor activity is a specific function of the distal stomach. The smooth muscle cells found in the circular and longitudinal layer generate electrical slow waves that are closely associated with ICC cells that regulate contraction (Sanders, 1996). During gastric emptying when food is passed through the pylorus, motor activity is stimulated in the duodenum (Wang et al., 2005).

6.1.2 Efferent and afferent integration of gastric function

The duodenum and colon can act independently to control their functions, however, the stomach requires more sophisticated neural input from the brainstem for min to min coordinated control of gastric ingestion and emptying. The vagal efferent neurons receive input from the DMVN that is then communicated to the ENS (Schemann and Grundy, 1992). The vagal efferent fibres project extensively within the myenteric plexus communicating with excitatory and inhibitory motor neurons innervating the stomach (Zheng and Berthoud, 2000). These vagal efferent fibres release ACh that act on postsynaptic myenteric neurons that stimulate nicotinic excitatory receptors (Wood, 2011). Contraction of the stomach is mediated by the cholinergic system and vagally evoked relaxation occurs through release of NO, VIP and ATP from the inhibitory neurons (Chang et al., 2003, Kuo et al., 2009). Efferent nerve fibres connect to both excitatory and inhibitory systems, for example, activation of cholinergic system alongside simultaneous inhibition of the inhibitory system is required for gastric contraction (Wood, 2011). This reciprocal nature seems to be inherent in the brainstem that controls this complex excitatory and inhibitory synaptic transmission (Hornby, 2001). Predominantly vagal afferent fibres project to second order neurons in the NTS that control sensory information of the stomach but some connect monosynaptically to DMVN cell bodies (Kalia and Sullivan, 1982).

6.1.3 Gastric action potential and the antral pump

The gastric pacemaker located in the corpus, initiates gastric action potentials that determine strength, duration and direction of antral phasic contractions. The action potential generated crosses the circumference of the gastric musculature in a ring like fashion, these contraction ends in the gastroduodenal junction. The action potentials travel from one muscle fibre to the next via the gap junctions and electrical syncytial properties of the gastric musculature (Daniel and Irwin, 1971). The gastric action potential has a rising depolarisation phase, a plateau

phase and a repolarisation falling phase (Daniel and Irwin, 1971). The contraction generated by the action potential is called the leading contraction and is produced by the rising phase followed by the trailing (Morgan et al., 1981). The leading contraction has a minimum amplitude that remains constant as the contraction travels to the pylorus (rising phase) (Morgan et al., 1981). Once the contractions reach the pylorus, the gastroduodenal sphincter closes (Morgan et al., 1981). The trailing contraction then follows and reaches the pylorus forcing the stomach contents into the antrum ref. This action is repeated allowing particles to be ground down by trituration until the contents are ready to be expelled into the duodenum (Johnson, 2006). Neurotransmitters released from vagal afferents determine the amplitude of the plateau phase, whereby ACh increases amplitude and NO has the opposite effects (Johnson, 2006).

The concentration of neurotransmitter and receptor density on the gastric musculature determine the magnitude of inhibitory/excitatory response (Johnson, 2006). Firing of musculomotor neurons determine the actions on the plateau phase and with sufficient release of neurotransmitters, the plateau phase exceeds the threshold for contraction to occur (Johnson and ScienceDirect (Online service), 2006). A dysregulation of electrical and contractile behaviour can alter the gastric emptying rates (Johnson and ScienceDirect (Online service), 2006).

6.1.4 Gastric reservoir- focus on neural control

A functions of the gastric reservoir is to accommodate the arrival of a meal without creating large changes in gastric pressure. Failure to accommodate can give rise to symptoms that include bloating, epigastric pain and nausea (Johnson and ScienceDirect (Online service), 2006). Another function is to maintain a constant force enabling antral motor activity that cycles the food. Failure of this mechanism by pharmacological interventions such as insulin injections can suppress gastric emptying (Loavenbruck et al., 2015). The gastric reservoir is richly supplied with enteric excitatory and inhibitory motor neurons; collectively the vagal efferent and enteric afferent neural networks that control the rate of neural firing (Schemann et al., 2001). The rate of firing from motor neurons adjusts the volume and intra gastric pressure needed for ingestion and emptying of a meal (Johnson and ScienceDirect (Online service), 2006). When firing rate is increased in the excitatory pathway and firing in the

inhibitory pathway is simultaneously decreased, a decrease in volume and increase in pressure a contraction occurs in the reservoir (Johnson and ScienceDirect (Online service), 2006). The opposite is true when there is an increase in the firing rate of inhibitory neurons causing relaxation i.e. an increase in volume and decrease in pressure ref. Three types of relaxations are recognised; receptive relaxation which is triggered by swallowing, adaptive relaxation by the distension of the reservoir, and feedback relaxation by nutrients in the duodenum (Johnson and ScienceDirect (Online service), 2006). Adaptive relaxation involves mechanosensitive stretch receptors that send information through the vagal afferents into the DMVN to activate the efferent outflow to inhibitory motor neurons ref. A vagotomy can cause the motor firing pattern to increase in response to distension because there is an increase in gastric tone thereby decreasing compliance which causes the stretch receptors to become more sensitive (Faxen et al., 1979).

6.1.5 Gastric emptying

When a meal is consumed, peristaltic waves move from the mid corpus into the pylorus and the antrum. The antrum mixes food contents by retropulsion and breaks it down with gastric juices so it can pass into the duodenum. Pressure within the distal stomach is increased by the increase in tone in the proximal stomach, which aids propulsion of the meal. The release of food into the duodenum is dependent on various factors including the strength of antral contraction, the pyloric sphincter muscle relaxation and duodenal resistance. The duodenum slows down gastric emptying when it is full since the intragastric volume is limited in comparison to the stomach. This is called the “intestinal brake” which is primarily thought to occur because of an increase in CCK and gastrin (Liddle et al., 1986).

6.1.6 Physiology of gastric emptying:

Gastric emptying is a complex physiological process that is influenced by multiple factors, such as nervous stimulation, the interaction of hormones together with pH, volume, consistency of food and lifestyle habits such as smoking, alcohol and stress (Hellmig et al., 2006) (Hellmig et al., 2006). Both the proximal and distal stomach are neurally regulated by extrinsic and intrinsic nerves (Olsson and Holmgren, 2001), and hormones such as ghrelin to name a few (Tack et al., 2005). The rate of gastric emptying from the stomach into the duodenum is such that the function of the intestine to secrete and absorb nutrients is not exceeded. The rate of

gastric emptying is affected by neurotransmitters such as NO, Ach, 5HT, tachykinins, VIP, motilin, CGRP and galanin (Daniel et al., 1994) (Torsoli and Severi, 1993). Many of these neurotransmitters act on vagal afferent endings relaying signals to the CNS and back. It has been shown that the rate of gastric emptying is delayed by the inhibition of NOS by an increased tone in the pyloric region and a loss of co-ordination between the regions of the stomach (Orihata and Sarna, 1994).

6.1.7 Gastric motility disorders

Gastric motility disorders arise when one or a combination of stomach processes are not regulated and can include inter-digestive ability, gastric reservoir/accommodation and gastric emptying (Tack, 2007). Patients with gastric motility disorders have a range of symptoms that include post prandial fullness, early satiety, epigastric pain, nausea and vomiting depending on the type of disorder (Tack, 2007). There is no conclusive evidence to show a link between delayed gastric emptying (gastroparesis) and symptom pattern, however, a study showed an association between rapid gastric emptying and post-prandial fullness (Delgado-Aros et al., 2004). Gastroparesis is a delay in emptying food from the stomach into the duodenum in the absence of a mechanical obstruction (Parkman et al., 2004). The cause is unknown and is present in about 25-55% of patients with insulin dependent diabetes (Kong and Horowitz, 1999). Symptoms include nausea, vomiting and occasionally visceral pain (Soykan et al., 1998). Management of gastroparesis is predominantly with prokinetics that increase gastric motility (Delgado-Aros et al., 2004) such as domperidone (Tack, 2007). Conversely rapid gastric emptying termed dumping syndrome generally occurs after a gastrectomy where ingested food is rapidly emptied (Tack, 2007). Symptoms are specific to either early dumping which occurs straight after a meal or late dumping 1-3 h post-prandially. Early dumping syndrome causes abdominal pain, bloating, nausea, dizziness, syncope, flushing while late dumping includes transpiration, palpitations, hunger and confusion (Abell et al., 2006).

Another major gastric motility disorder is dyspepsia, which is broadly defined as experiencing pain in the epigastric region (Tack and Talley, 2013) (Avau et al., 2013). Functional dyspepsia (most common GI disorder in clinical practice) is defined by the Rome III criteria as having chronic dyspeptic symptoms that are idiopathic and heterogeneous (Talley et al., 2005). Although the exact cause of the disease is unknown, studies have shown that slight structural

changes may be important such as those caused by gastritis post Helicobacter pylori infection (Nesland and Berstad, 1985). It is estimated that up to 40% of patients with functional dyspepsia have impaired accommodation in the corpus or proximal colon (Tack et al., 2004). Similar to gastroparesis, prokinetics are used to treat functional dyspepsia that show motility dysfunction (Moayyedi et al., 2006), however, no targeted treatment is available for impaired accommodation. Some pharmacotherapies have been proposed for including 5-HT_4 agonists (Moayyedi et al., 2006).

The IHC data obtained in Chapter 2 demonstrating the close correlation between TNX positive staining and vagal and intrinsic neurons in the stomach suggests that TNX may have a role in gastric function. Since gastric emptying is largely controlled by the vagal afferent and intrinsic motor control of the stomach it is an obvious choice to measure gastric emptying rates in the TNX KO mouse model.

6.2 Methods

6.2.1 Fasting mice

KO and WT mice, male or female in equal numbers, aged between 10-14 weeks were assessed for gastric emptying. The day prior to the test, mice were fasted overnight for 12 h with free access to water. Once the test was complete, the animals were killed as described before by asphyxiation.

6.2.2 ^{13}C Octanoic acid breath test

The gold standard to measure gastric emptying is the non-invasive gastric scintigraphy test that provides a quantitative measure of gastric emptying time (Bruno et al., 2013). However this technique is limited as it requires use of radioactive substances, it is expensive, requires trained staff and not recommended for repeated assessments in children and pregnant women (Choi et al., 1997). An array of new techniques are now available to measure the rate of gastric emptying including the ^{13}C octanoic acid breath test. The ^{13}C octanoic acid breath test is a validated method in humans that is well correlated with the scintigraphy technique (Ghoos et al., 1993). The test has also been validated with high reproducibility in small animals such as mice (Symonds et al., 2000). This test is most attractive for such animals because the method of interval breath testing used does not require handling of the animal nor does the animal need be killed to perform the test (Enck and Wienbeck, 1989). Octanoic acid is a medium chain fatty acid that consists of 8 carbon atoms and is present in the fat of foods such as the egg yolk. Octanoic acid is efficiently absorbed into the duodenum and transported to the liver where it is oxidised to $^{13}CO_2$. ^{13}CO2 then mixes with the pool of plasma bicarbonate and is exhaled in the breath (Perri et al., 2005). Octanoic acid is not at risk from digestion and post gastric emptying modifications, moreover it is not affected by the liver, kidney or lung disease (van de Casteele et al., 2003) (Keller et al., 2009). Therefore it is a good substrate for the measurement of stomach emptying by quantifying $^{13}CO_2$ in the breath.

6.2.3 Test meal preparation

To prepare the solid egg yolk meal, the yolk was separated from the whole egg and weighed in a beaker. For every gram of egg yolk, 1µL of [^{13}C]-octanoic acid (99% enrichment)

(Cambridge Isotopes, USA) was added. The egg yolk was then mixed evenly and baked at 60°C until it was set. The cooked egg yolk was then cut into 0.1g pieces for each mouse.

6.2.4 Breath testing

Each mouse was individually placed in a 100ml glass container (Schott Duran, Germany) and left to acclimatise for 10 mins. A wire mesh was then placed on the top to allow fresh air to ventilate the container. The mouse was then given 0.1g of the solid egg yolk which was consumed within 1 min. The lid of the glass container was modified to fit a two-way valve with a 10ml syringe inlet. Once the egg yolk meal was consumed, the wire mesh was removed and replaced with a modified lid attached securely to a 10ml syringe. The breath was then accumulated in the glass container for 120 s and the syringe was drawn and injected in an evacuated helium flushed 10mL tube (Labco, Exetainer Ltd). The lid was then removed and the wire mesh placed on top. The breath samples were collected at 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, 120, 135, 150 mins by repeating the same process. Once all the breath samples were collected, the mouse was sacrificed by asphyxiation.

6.2.5 Data analysis:

Breath CO_2 was analysed for $^{13}CO_2/^{12}CO_2$ enrichment by gas chromatography using a Gasbench (Thermo Finnigan,Bremen, Germany) containing a CP-Poraplot-Q column (Varian Inc., UK) followed by isotope-ratio mass spectrometry on a Thermo Finnigan Delta-XP (Thermo Finnigan, Germany). Sample $^{13}CO_2/^{12}CO_2$ enrichment was standardized against a CO_2 cylinder (5.0 grade, BOC Special Gases, UK), which was calibrated against the international standard Pee Dee Belemnite (PDB) (Iso-Analytical, Sandbach, UK). The results are expressed as the relative difference (δ‰) between the sample and the international limestone standard PDB using the formula below.

$$^{13}C = [(R_S/R_{PDB)}-1] * 1000$$

$R_S = {}^{13}C/{}^{12}C$ in the sample

$R_{PDB} = {}^{13}C/{}^{12}C$ in the sample in PDB (=0.0112372)

The isotope mass spectrometer gives δ values that are then converted to percentage of ^{13}C per hour of the initial dose given (%dose/h) and as cumulative percentage of administered dose of ^{13}C over time (CPDR). The calculations are described below.

$$\%dose/h = \frac{\text{mmol } ^{13}C \text{ excess in breath}}{\text{mmol } ^{13}C \text{ excess dose}} \times 100$$

Before calculating the mmol of ^{13}C excess in breath, the concentration of ^{13}C at the interval time ($\%^{13}C_t$) post ingestion of the egg yolk meal and the concentration of ^{13}C at time zero ($\%^{13}C_0$) was calculated.

$$\%^{13}C_t = \frac{(\delta_t/1000+1) \times R_{PDB}}{((\delta_t/1000+1) \times R_{PDB} + 1}$$

$$\%^{13}C_{t0} = \frac{(\delta_t/1000+1) \times R_{PDB}}{((\delta_t/1000+1) \times R_{PDB} + 1}$$

δ = δ value at interval time

$R_{PDB} = {}^{13}C/{}^{12}C$ in the sample in PDB (=0.0112372)

Both of these equations were then used to calculate the mmol of ^{13}C excess in breath described below.

$$\text{mmol } ^{13}C \text{ excess in breath} = \frac{\%^{13}C_t - \%^{13}C_0}{100} \times CO_2 \text{ production}$$

The rate of CO_2 production was assumed to be 40ml/kg/min; this is based on normal values for resting metabolic parameters measured in mice with C57BL/6 background (Symonds et al., 2000).

The mmol of ^{13}C excess dose was calculated using the equation below.

$$\text{mmol excess } ^{13}\text{C dose} = \left\{ \frac{\%^{13}C_s - \%^{13}C_{t0}}{100} \right\} \times \frac{m}{M} \times n$$

Where the percentage of $^{13}C_s$ is the concentration of ^{13}C in administered octanoic acid, M is the molar mass of the octanoic acid (145.21), m is the amount of octanoic acid in mg (0.091mg-calculated using the concentration 1µl/1mg with molar mass) and n is the number of ^{13}C-labelled atoms in the octanoic acid (99-since it is enriched with 99% ^{13}C).

The % ^{13}C in the cumulative dose is derived from the $\%^{13}C$ dose/hr data calculated by the following equation:

$$\%^{13}C_{\text{umalat dose ti+1}} = \%\ ^{13}C_{\text{umalat dose ti}} + \left\{ \frac{\%^{13}\text{Cdose}_{ti} - \%^{13}\text{Cdose}_{ti+1}}{2} \right\} \times 1/n$$

Where n is the number of samples taken per hour, therefore samples taken at 0, 5, 10, 15, 25 and 30 mins means the n value is 12 and samples taken from 45, 60, 75, 90, 105, 120, 135 and 150 mins means the n value is 4. ti represents the time.

The %dose/h curve gives an indication of the rate at which the stomach empties, highlighting delays, accelerations or any other abnormalities. After plotting the %dose/hr curve against time, the typical curve starts with an ascending curve, peak excretion and then a descending curve. Rapid gastric emptying shifts the curve to the left and delayed gastric emptying shifts the curve to the right as shown in Fig 6.1.

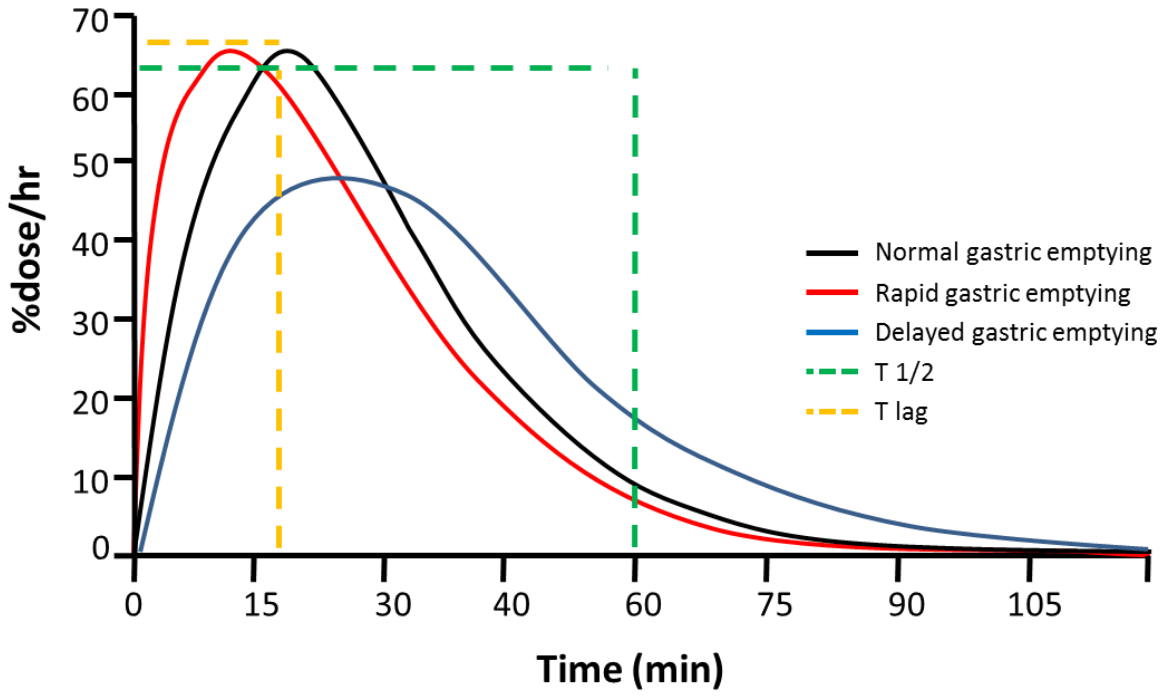


Figure 6.1. Theoretical representation of gastric emptying.

Black curve shows normal gastric emptying, blue curve shows delayed gastric emptying and shifts to the right, and red curve shows rapid gastric emptying and shifts the curve to the left. Dashed yellow line indicates T-lag and dashed green line represents the T ½. Adapted from Symonds et al 2000 (Symonds et al., 2000).

The $^{13}CO_2$ curve was mathematically analysed to give two formulas using non-linear regression analysis on the Solver program in Excel (Microsoft Corporation, Redmound, WA, USA). The first formula is purely mathematical to derive the *abc* fit describing the characteristic shape of the $^{13}CO_2$ curve shown below (Symonds et al., 2000)

$$y = at^{b}e^{-ct}$$

Where y is the percentage of $^{13}CO_2$ excreted in the breath per hour, t is the time in hours and a, b, c are regression estimated constants derived from the solver function in Excel.

The second formula describes the cumulative $\%^{13}C$ excretion curve owing to the fact that the cumulative dose in function of time is inversely analogue to the scintigraphy curve of gastric emptying.

$$y = mk\beta e^{-kt}\ (1-e^{-kt})^{\beta-1}$$

Where y is the percentage of $^{13}CO_2$ excreted in the breath per hour, t is the time in hours and m, k, β are regression estimated constants derived from the solver function in Excel. The m describes the total amount of $^{13}CO_2$ when time is infinite; β describes the initial phase of emptying and k describes the decline of $^{13}CO_2$ excretion curve.

Both mathematically derived formulas can be statistically compared using a Pearson's product moment correlation to make sure that the curves fit the measured %dose/h curve. A correlation coefficient of r ≥0.95 was taken as an acceptable mathematical fitting.

Based on these two mathematically derived formulas, four main parametres of gastric emptying can be obtained. The first is the half life (T ½) which measure the time taken for half of the stomach contents to empty (Ghoos et al., 1993). The T ½ also informs of the post gastric process in terms of metabolism and absorption of the solid egg yolk meal as well as the excretion of $^{13}CO_2$. The following equation is used to obtain the T ½ (min).

$$T\ ½ = (-1/k)\ln(1-2^{-1/\beta})$$

Any fraction of gastric emtpying can be analysed using the general equation:

$$T_{1/n}\ (-1/k)\ln(1-n^{-1/\beta})$$

Where the n value is the fraction of gastric emptying time that is being calculated.

The mathematical index describing the overall gastric emtying rate is defined as the gastric emptying coefficient (GEC), this can be influenced by the rate of appearance and disappearance of the octanoic acid in the egg yolk meal.

$$GEC = \ln(a)$$

T_{LAG} represents the initial delay in gastric emptying giving an indication of the time required for the stomach to breakdown the solid egg yolk meal so that it is fine enough to pass into the pylorus.

$$T_{lag} = (-1/k)\ln(1-20^{-1/\beta})$$

T_{MAX} corresponds to the time at which the $^{13}CO_2$ excretion was at a maximum in the the %dose/hr curve.

$$T_{MAX} = (\ln\beta)/k$$

It is important to note that correction factors have not been included when calculating the various parameters. In humans, correction factors have been developed to adjust the gastric emptying rate, used for radioscintigraphy, which is thought to be the gold standard (Smout and Mundt, 2009). The correction factors account for the post gastric processes in humans based on a 250kcal ingested meal labelled with ^{14}C. The post gastric process is variable and dependent on which substrate is adminstered and has not been developed for the mouse.

All the parameters were calculated for both WT and KO mice and statistically analysed. Mean and standard error of the mean (SEM) were calculated using Microsoft excel 2007. Statistical significance was calculated using GraphPad Prism (Students *t-test*). A *p* value of less than 0.05 ($p < 0.05$) was considered statistically significant. All calculated values are presented in Appendix 6.1 and 6.2

6.3 Results

Octanoic acid breath technique is an indirect method of measuring gastric emptying rates because it is the rate limiting step. Therefore the rate of gastric emptying and production of $^{13}CO_2$ will be defined as a measure of stomach function in terms of gastric emptying. In total WT (N=15) and KO (N=17) mice were breath tested with all mice weighing between 24-25g. WT (N=9) and KO (N=15) mice were used for analysis. 6 WT mice were excluded, since 4 WT mice did not fulfil the Pearson product correlation of r ≥0.95 and 2 WT mice did not show normal pattern of $^{13}CO_2$ excretion with multi-peaks. It was important to exclude data that did not fit the Pearson product correlation in order to ensure that the measured $^{13}CO_2$ production fitted the theory curve (curve of best fit) for gastric emptying, measured values were correlated with values mathematically fitted to the theory curve. A correlation coefficient of $r \geq 0.95$ was taken as indicating acceptable mathematical fitting. Multi peak data was excluded from the analysis since the curves produced did not fit the theory equation for the $^{13}CO_2$ excretion curve but most likely represent a rare, but nevertheless real, biphasic gastric emptying curve for which a mathematical expression has yet to be determined. Since this mathematical expression is yet to be determined this set of data were excluded.

The mathematically derived calculations on T ½, T Max, T Lag and GEC were compared in WT vs KO. It is important to note that these calculations are based on the rate of octanoic acid excretion as an indirect predictor of gastric emptying rate.

The excretion of $^{13}CO_2$ in the WT mouse showed a gradual increase with a peak at 50 mins whereas in the KO there was a rapid increase and the peak was seen at 30mins (Fig 6.2). The amplitude showing excretion of $^{13}CO_2$ in KO mice was much higher than in the WT mice. There was a gradual decline around 100 mins in the WT mice unlike the KO, showing a rapid decline earlier at 45 mins. Before applying the mathematical calculations it is clear from the graphs there is faster gastric emptying in the KO mice compared to the WT (Fig 6.2).

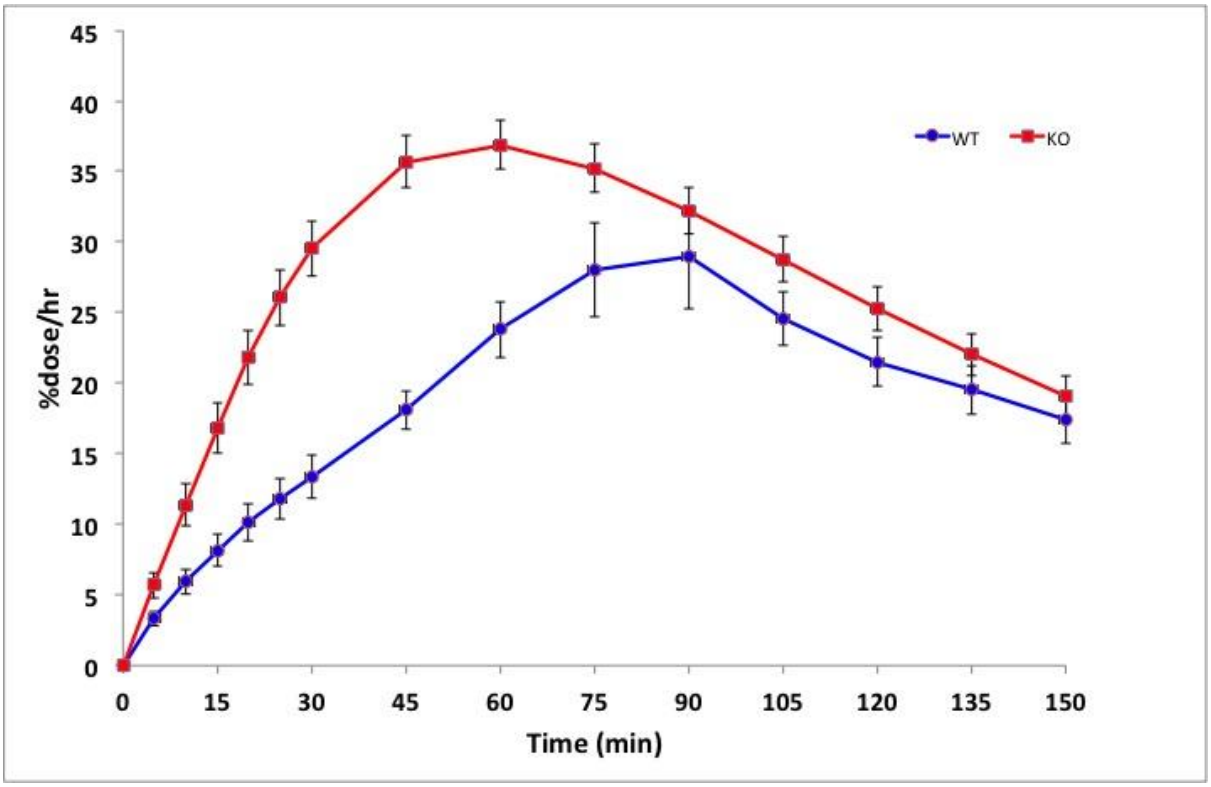


Figure 6.2. Rapid gastric emptying in the TNX KO mouse.

Mean results showing $^{13}CO_2$ excretion curve in WT vs. KO. WT curve (blue) shows increase in gastric emptying up to 74-90 mins, then declines. Gastric empting rates in the KO (red) mice is rapid, peaking at 45 mins and declining after 60 mins.

6.3.1 Gastric emptying: WT vs KO mice

The gastric emptying half-life in KO and WT mice is illustrated in Fig 6.3. The time taken for half of the stomach contents to empty in the KO mice was significantly faster compared to WT mice (WT=158±21.8 min vs. KO=104±10.5 min; *p*=0.0188).

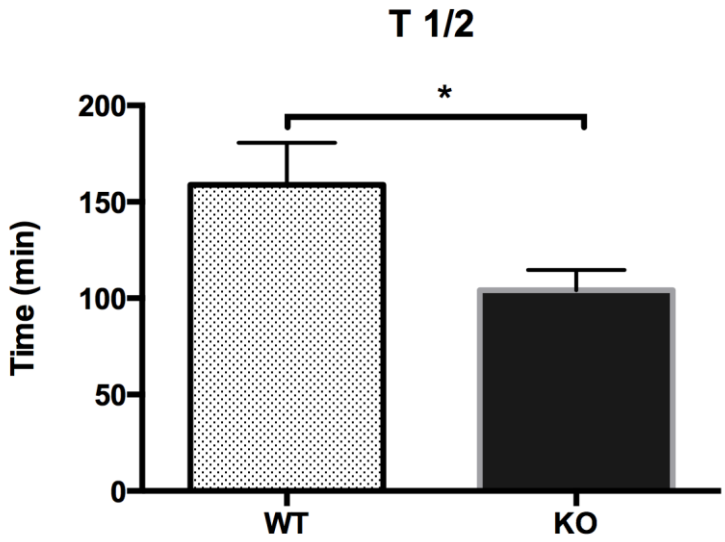


Figure 6.3. Gastric emptying half-life (T½) is reduced in TNX KO.

The half-life in WT is 158±21.8 which is significantly reduced in the KO (103±12.9 min) p=0.0277.

6.3.2 Gastric emptying T-Lag in WT vs KO mice

T Lag results shows KO mice take a significantly longer time to digest the solid egg yolk meal into small enough particles so that it can pass into the pylorus (WT=38.93±5.724 min vs. KO=23.91±2.642 min; *p*=0.0188).

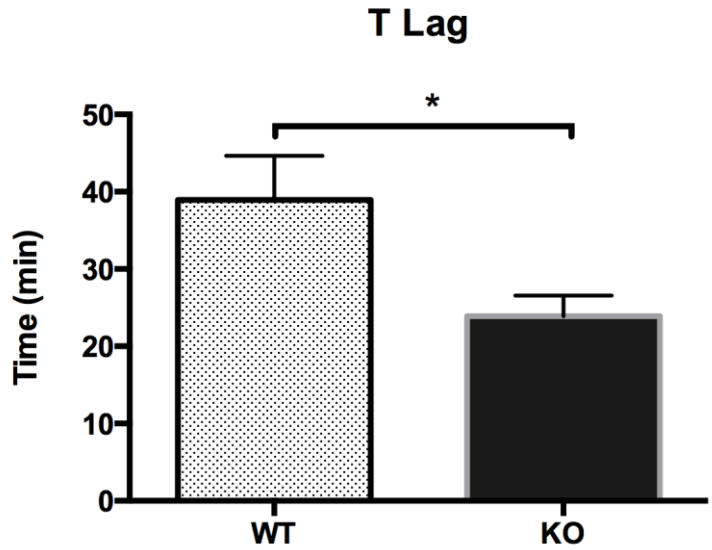


Figure 6.4. T lag is reduced in the TNX KO.

*T-lag in WT (*38.93±5.724*) and is significantly reduced in KO* (23.91±2.642).

T_{MAX} , which corresponds to the time at which the $^{13}CO_2$ excretion was at a maximum in the %dose/h curve, was significantly lower in the KO compared to WT. This suggests that it takes a shorter time to reach the maximum amount of $^{13}CO_2$ produced in the KO compared to the WT, again indicating rapid emptying

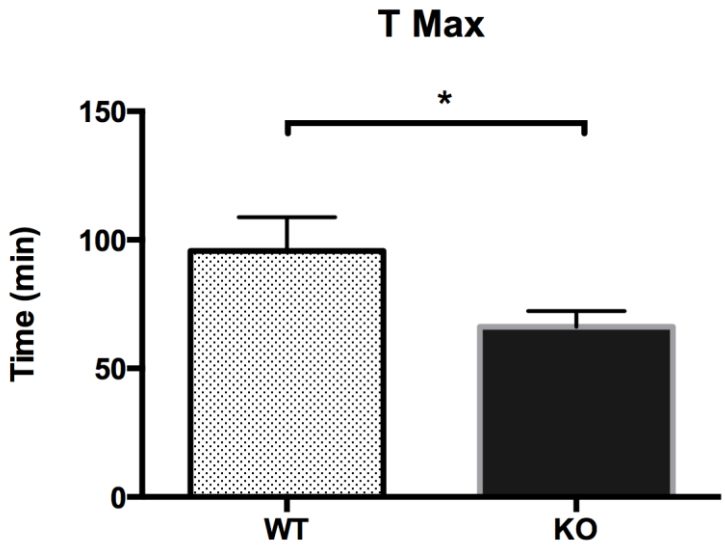


Figure 6.5. T Max reduced in TNX KO.

Similarly the T Max was also reduced in the KO mouse (WT=95.68±13.11 and KO=66.15±6.200 min; p=0.0315)

The gastric emptying coefficient describes the overall effect including the appearance and disappearance of octanoic acid (Deane et al., 2010). There were no significant differences in global gastric emptying.

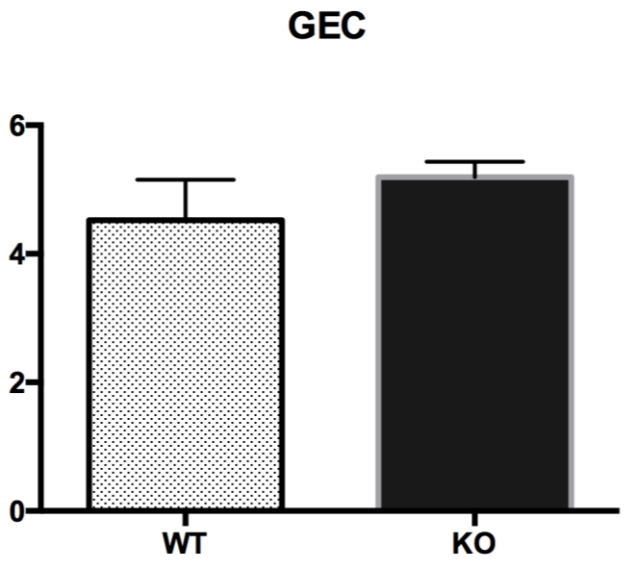


Figure 6.6. No change in GEC.

The overall effect of gastric emptying however shows no differences in both groups, WT=4.520±0.6320 and KO=5.193±0.2398 min; p=0.2542.

6.4 Discussion:

Collectively, the results from the breath tests show that mice lacking TNX exhibit faster gastric emptying rates compared to wild type. This has been illustrated by calculating the gastric emptying parameters, that show KO mice take a shorter time for 50% of the contents to empty (T ½) and a shorter time to digest the solid food into small enough particles that can be released into the pylorus (T Lag). Therefore, it is clear there is a physiological change causing gastric rapid emptying when TNX is absent.

The GEC which gives an index of overall gastric emptying did not show a difference, this may be because this mathematically derived index is based on all the time points in the octanoic acid excretion curve. As evident in the graph (Fig 6.2), the initial gastric emptying time points show a significant difference between the two groups (time point 15-90min), however, from time point 105 to 150min there is no significant difference. Since GEC accounts for all data points, the mathematical index cancels out the initial difference and the lack of difference towards the end of the graph. This explains why there is no overall difference observed in GEC. Therefore, GEC may not be a sensitive measure of gastric emptying but rather a global index which doesn't account for changes at certain time points. The mean results showing the dose/hr curve of excreted octanoic acid in breath is the best measure of assessing if there are changes to the gastric emptying rate by considering curve shifts. Based on the excretion curve graph and other mathematical calculations such as T ½, T-Lag and T-Max it is clear that TNX KO mice have significantly rapid gastric emptying compared to WT counterparts during the initial stages of emptying.

The reasons for this will be further explored in this section, highlighting changes in gastric emptying in mouse models. Furthermore, the clinical relevance in terms of symptoms arising from rapid gastric emptying will be discussed.

To date, there are few studies looking at the gastric emptying rate in patients with JHS, however, two recent abstracts showed 56% of patients diagnosed with functional dyspepsia have JHS (Fikree et al., 2015a) and another study showed that gastric emptying was faster following a meal (Fikree A, 2011).

In the mouse stomach, TNX was localised around vagal afferent nerve endings in the smooth muscle layer described in detail in Chapter 2. The architecture and location of these TNX positive endings are characteristic of IMA mechanoreceptors in the smooth muscle and IGLEs enveloping the myenteric plexus. IMAs are primarily found in the circular/longitudinal muscle and lower oesophageal and duodenal sphincters, whereas IGLEs are found innervating the myenteric neurons (Berthoud and Powley, 1992), (Kressel et al., 1994), (Wang and Powley, 2000), (Fox et al., 2000), (Fox et al., 2001). In the last few decades, studies have focused on IGLE vagal mechanoreceptors and IMAs were deemed functionally unimportant at least by electrophysiological techniques where no recordings could be made (Brookes et al., 2013). However, Powley and Phillips rebutted this in a comprehensive analysis of IMAs and showed they act as stretch receptors (Powley et al., 2016). In light of this and previous studies, in this thesis IMAs are regarded as important vagal mechanoreceptors thus the distribution of TNX in IMAs and IGLEs allowed to hypothesise an important role for TNX in vagal afferent function. Contrary to reports in the literature that describe gastroparesis in JHS patients (Levy, 1993), an increase in gastric emptying was observed in TNX KO mice in line with increased vagal afferent discharge described in Chapter 5 in both spontaneous and distended conditions. The rate of gastric emptying is dependent on factors including the relaxation of the gastric reservoir which is the upper portion of the stomach, the depth of constriction of the lower antral waves, the rate of pyloric sphincter opening and the receptive relaxation and contraction of the duodenum (Schemann et al., 2001). It is important to note gastric emptying of a solid and liquid meal are different in terms of region and their function. In TNX KO mice, when the solid meal is ingested there is tonic contraction of the fundus and upper corpus with a deep antral peristalsis which accelerates the gastric emptying rate. This enhanced activity of the fundus is largely influenced by increased vagal mechanoreceptor activity observed in the electrophysiology study of the KO mouse. When a meal is ingested, the stomach becomes distended and afferent fibres produce a burst of action potentials which reverts to a steady discharge correlating to the volume of ingested meal (Schemann and Grundy, 1992). The increase in gastric emptying rates may be because TNX provides anchoring of vagal afferent endings, therefore, a loss may cause the vagal afferents to have increased sensitivity to intrinsic changes in the stomach under tonic conditions. This suggests that slight changes in the stomach caused by spontaneous secretion, muscular tone or motility causes increased spontaneous firing, which then sends signals to the CNS, in turn stimulating efferent neurons

projecting to inhibitory and excitatory axons innervating the stomach. This vago-vagal reflex causes gastric relaxation of the stomach before any stimulation by distension. In the TNX KO when a solid meal is ingested the stomach may be in a 'pre prepared' state caused by an increase in spontaneous discharge explained previously. Thus, when already activated vagal mechanoreceptors are subsequently activated *in vitro* or by eating a meal, the distension causes the afferent to fire at an even higher rate. This increase in firing post distension enhances the afferent messages to reach the DMVN which uses glutamate and sends signals via efferents to the stomach that can cause contraction of the corpus via the release of ACh and the relaxation of the antrum by the release of NO, VIP and ATP (Grundy and Brookes, 2012). This possibly enhanced integrated control of the vago-vagal loop likely allows the fast passage of food into the duodenum resulting in rapid emptying observed in the KO mouse.

There are various studies looking into mouse studies showing delayed gastric emptying with few studies describing rapid gastric emptying. However, exploring the opposite effect in terms of pathophysiology may shed light on why the TNX KO mouse displays rapid gastric emptying. Neuronal NO and the enzyme responsible for its synthesis NOS are of most interest in understanding delayed gastric emptying since fundic accommodation and pyloric relaxation predominantly rely on inhibitory nitrergic nerves (Takahashi and Owyang, 1997) (Shah et al., 2004). The first study looking into the role of inhibitory NOS in gastroparesis was through a knockout model that lacked the NOS producing gene NOS 1. These mice showed delayed gastric emptying and gastric dilation (Vittal et al., 2007). Therefore a loss of NOS affects synaptic neurotransmission in these sites and in turn a loss of inhibitory control. Receptive relaxation and gastric accommodation is impaired and may give rise to symptoms of early satiety and rapid gastric emptying of liquids (Takahashi and Owyang, 1997), (Desai et al., 1991). It is plausible to suggest that a loss of TNX surrounding these vagal afferent nerve endings may indirectly disrupt sensory neurotransmission thus give rapid gastric emptying rates.

Another mechanism for rapid emptying may be due to the elastic properties of the stomach. IMAs are highly concentrated in the pyloric sphincter and are thought to be important in sphincter function. A loss of TNX around these vagal afferent IMAs may cause changes in elasticity meaning the sphincter may not open and close in a regulated manner. This defect in sphincter function may allow the passage of food into the duodenum before the grinding

of the food is completed causing symptoms observed in JHS patients. It has been demonstrated that rapid gastric emptying is caused by lower pyloric resistance (Abell et al., 2008) and may be also true in the TNX deficient mice due to the change in elasticity. This needs to be further studied by using compliance tests on stomach tissue.

In terms of clinical data, it has been shown that 40% of patients with dyspepsia have impaired gastric accommodation (Mihara et al., 2013). It has also been described that 66.7% (n=21) of patients who have JHS also have dyspeptic symptoms (Castori et al., 2010b). Around 20-30% of patients with functional dyspepsia are thought to have delayed gastric emptying (Feinle-Bisset et al., 2004), but it is becoming recognised that symptoms of dyspepsia are associated with rapid gastric emptying (Bharucha et al., 2011). For example, functional dyspeptic patients who have post prandial symptoms display low fasting gastric volumes and rapid gastric emptying compared to controls (Delgado-Aros et al., 2004). The symptoms associated with both rapid and delayed gastric emptying overlap, so it is difficult to distinguish whether rapid vs. delayed gastric emptying is present in patients with functional dyspepsia. Results from this chapter suggest patients who have functional dyspepsia may be divided into two groups; those that have rapid emptying and those that have delayed emptying. The group with rapid gastric emptying may have a higher prevalence of JHS and TNX deficiency compared to the group with delayed gastric emptying and functional dyspepsia, although this needs to be further explored.

JHS patients also commonly experience autonomic dysfunction (78%) (Gazit et al., 2003) particularly postural orthostatic tachycardia syndrome (POTS) (Kanjwal et al., 2010). POTS is defined as the symptoms associated with orthostatic intolerance causing an increase in heart rate that occurs from suddenly standing up. The cause of POTS is thought to be the inability of the peripheral vasculature to maintain resistance from the orthostatic stress, thus causing pooling of blood (Rowe et al., 1999) (Gazit et al., 2003). It is thought that increased laxity in JHS patients means a greater degree of vascular dispensability leading to an exaggerated amount of blood pooling resulting in tachycardia (Streeten et al., 1988). The prevalence of POTs in TNX deficiency is unknown, however, may also be present since laxity is also present in these patients. Patients who have POTS commonly experience GI symptoms including nausea (40%), bloating (24%) , diarrhoea (18%) and constipation (15%) and abdominal pain (Thieben et al., 2007). Interestingly, two thirds of patients who have POTS and GI symptoms

have abnormal gastric emptying and rapid emptying is the most frequently observed (Park et al., 2013). In light of this, the prevalence of POTS in TNX deficient patients is needed.

This study has limitations in that only solid gastric emptying was measured. Liquid gastric emptying is also needed since the mechanism of solid and liquid emptying is different thus measuring both in the TNX deficient mice will help highlight the specific pathway(s) that are affected. Additionally, it is important to characterise liquid emptying of the stomach as it is primarily controlled by the proximal stomach where TNX expression was observed (Collins et al., 1991). It is also important to measure how compliant the stomach is to clarify whether increased emptying is primarily due to increased compliance or increased synaptic transmission as hypothesised.

In summary, since it has been demonstrated that gastric emptying rates are faster in mice lacking TNX, this may be caused by a lack of coordinated motor control by vagal afferent nerves or by changes in elastic properties that disallow sphincters to relax and close tightly. It is important to confirm the rate of gastric emptying in TNX deficient patients to ascertain whether the results from TNX KO mice are translational.

7 Main Discussion

7.1 Summary of data

TNX was characterised in the locations where GI symptoms are most commonly present i.e. the stomach and colon. To clarify the functionality of this ECM protein, the expression of TNX was studied in human and mouse GI tissue and was colocalised with well characterised neuronal markers. Data from these studies led to formulate specific functional experiments in WT and TNX KO mice, allowing direct correlation of GI consequence based on TNX gene expression. Specifically, the role of TNX in the stomach was evidenced by an increase in vagal afferent firing and gastric emptying. As may be expected anatomical data showed TNX was not only expressed in extrinsic and intrinsic neural structures innervating the stomach but also in cell bodies. TNX plays an important role in colonic motility particularly in the distal region where the amplitude was significantly reduced in TNX KO mice, moreover some of these mice had rectal intussusception. Similarly, it is well documented that patients with long term chronic constipation can secondarily develop rectal prolapse as a consequence of prolonged dysmotility (Fox et al., 2014). TNX does not play a significant role in overall secretion however TNX may have a role in complex secretory responses evidenced by using drugs that stimulate secretion. Taken together, the expression of TNX associated with neuronal structures and the functional changes observed, we provide evidence that TNX has an important role in GI function. The literature documents other tenascins and ECM molecules play a critical role in neuronal development (Chiquet-Ehrismann and Tucker, 2004), neuronal plasticity (Dityatev and Schachner, 2003) and as a matrix molecule providing architectural support (Pihlajaniemi, 2013). These concepts in relation to TNX and its role in the ENS will be further discussed.

7.2 TNX expressed in enteric neurons

The first and most interesting finding was the location of TNX in neural structures in both species and locations studied. As an ECM protein, TNX was expected to be expressed around connective tissue, however, this study found TNX localised in specific neural structures. The expression of TNX protein in neurons was conserved amongst both species studied indicating that it serves a particular function related to neurons. It is possible that TNX is expressed

within neurons and outside neural structures. The localisation of TNX showed cytoplasmic expression in neuronal cell bodies thus described as intracellular, whereas, TNX positive fibres found in muscle may be extracellular and serve a motor or sensory role. Epi fluorescent and confocal imaging did not clearly identify the precise location of TNX and thus electron microscopy may reveal the exact location of TNX in relation to neural structures. Although the exact molecular location remains open the expression of TNX within or outside neural structures is novel. This finding may not be as surprising as initially thought, since other tenascins are found associated with neural structures in the CNS (Kwok et al., 2011).

7.3 Functional differences of TNX based on microenvironment

From the functional studies, it was apparent that TNX serves different roles in each region of the GI tract. In the TNX KO mice activity in the stomach was increased, evidenced by rapid gastric emptying and hypersensitivity of vagal afferents, whereas in the colon, colonic contractions were reduced and there was no change in secretion in comparison to the WT mice. This distinct role of TNX based on region may be attributed to differences in the microenvironment or different interactions between other molecules.

It has been demonstrated ECM composition and quantity is variable in different tissues and within tissues that can give rise to changes in ECM function (Hynes, 2009), (Baker and Chen, 2012), (Watt and Fujiwara, 2011). For example, TNC is described as pro-migratory and adhesive in one cell type and in another, TNC was inhibitory and anti-adhesive (Jones and Jones, 2000). Additionally, in cell culture studies, when DRG explants were plated on ECM substrates, sensory neurites grew past laminin and the TNC border (Wehrle-Haller and Chiquet, 1993) suggesting a pro-migratory role. On the other hand, satellite cells in the presence of TNC stopped migrating, failed to spread and lined up bordering TNC rather than growing past (Wehrle-Haller and Chiquet, 1993) suggesting an inhibitory role. In the gut the composition and environment of the stomach and colon are varied and have evolved to serve different roles. Within the stomach or colon itself there are variations in structure composition and function, therefore it is probable, that the function of TNX is different depending on the cellular microenvironment and the molecules it interacts within these regions.

7.4 TNX acts like a signaling molecule

Of all four tenascins, TNR and TNC have been most widely studied and have a significant role in neural development. Tenascins have a similar structure whereby specific regions are implicated in different functions, for example the EGFL domain found in all tenascins. This domain has a counter-adhesive role in neuronal guidance in both CNS and PNS. The ability of tenascins to inhibit attachment to neuronal receptors on neurons and glial cells has been well documented (Prieto et al., 1992), (Lochter and Schachner, 1993 5012), (Crossin, 1994). In particular, regions within TNR and TNC can bind to various cell adhesion molecules (CAMs) that can influence neurite extension and repulsion (Jones and Jones, 2000). Moreover, tenascins have the ability to switch binding preferences for CAMs from hetrophilic binding to homophilic binding (Volkmer et al., 1998) (Jones and Jones, 2000). This binding preference is thought to influence migration of neurons, for example axons migrate contralaterally (Dodd et al., 1988) or when axons from the thalamocortical regions project to their targets (Gotz et al., 1997, Gotz et al., 1996). Based on the structural similarities between tenascin molecules and the characterisation of TNX in neural structures, it is indicative that TNX may play a role as a signalling molecule for neurons and fibres in the developing ENS. In support of this theory, other ECM molecules such as netrins have shown to co-express neuronal PGP 9.5 in myenteric plexus, which directs the growth of vagal mechanoreceptors to their endings. Netrins have the ability to direct vagal afferents by being released from intrinsic ganglia and nerve fibres in the ENS (Ratcliffe et al., 2011). When intrinsic neurons are deleted in receptor tyrosine kinase (Ret) KO mice netrin is secondarily absent. Thus without this guidance molecule vagal afferents terminate before the aganglionic segments of the bowel (Ratcliffe et al., 2011). Netrins are similar to TNX since they are also associated with neural structures therefore, it is sensible to suggest that TNX may also guide the vagal afferent IGLEs/IMAs to terminate in the appropriate region of the gut, thus in the TNX KO the vagal afferent IMAs and IGLEs may have altered termination points due to the inability of TNX to exert its “counteradhesive” role. Thus in the absence of TNX as the proposed guidance molecule for vagal afferents the function may be altered based on whether the ending terminated in close range or at a distance to the associated neural structure the ending communicates with.

7.5 TNX and synaptic transmission

Heightened nerve activity has been reported in TNR deficient mice whereby basal synaptic transmission (Saghatelyan et al., 2001) and hippocampal activity is increased (Brenneke et al., 2004). Increased hippocampal activity in TNR KO was attributed to increased number of spikes in the hippocampal cornu ammonis (CA) region of the brain compared to the WT. The study went on to use a GABA(A) receptor blocker picrotoxin that only partially reduced the observed increase suggesting that TNR has a role in GABAergic inhibition and increased excitability of CA region 1 (CA1) pyramidal cells (Brenneke et al., 2004). TNX KO may also have specific roles in increasing excitability of vagal afferent fibres innervating the stomach observed in the electrophysiology study in Chapter 5. Interestingly, IHC data from the aforementioned study showed a low number of calretinin positive interneurons in the CA1 and CA3 region (Brenneke et al., 2004). This suggests that TNR has a role in determining the density of calretinin in the brain regions described. It is important therefore to quantitatively assess the number of calretinin neural fibres and neurons in stomach and colon in the TNX KO. This will help delineate if the loss of TNX affects the number of calretinin expressing neurons.

PNNs, composed of lecticans, hyaluronan, TNR and other glycoproteins, have a putative role in synaptic transmission (Kwok et al., 2010). PNNs connect neuronal surfaces found in the brain that form networks described in detail in Chapter 1 (Kwok et al., 2010). TNR, an integral component of PNNs, has a critical role in mediating synaptic function, since TNR KO have reduced synaptic plasticity (Carulli et al., 2010). Specifically, it is proposed that molecules including TNR within PNNs insulate synaptic contacts (space between synapses), preventing the released neurotransmitters from spilling over to adjacent synapses or extra synaptic neurotransmitter receptors to maintain ion homeostasis and diffusion properties (Dityatev et al., 2006). PNNs are found around neural structures in the CNS (Kwok et al., 2010) and TNX is expressed around enteric neurons. TNX around cell bodies may be part of a larger sheath around neurons similar to TNR in PNNs and may provide similar roles in synaptic insulation and neurotransmitter homeostasis. Thus when TNX is absent, the neurotransmitters may become more diffuse and may not bind to the appropriate receptors in an organised and timely manner. For example, delayed binding of ACh to muscarinic receptors in the colon. A PNN like structure that consists of TNX is only speculation and needs further study. The

structure of a PNN in the CNS surrounds neuronal soma in a honeycomb like net that wrap around the proximal dendrites but not the distal dendrites. The holes in the net are occupied by synaptic boutons from afferent axons (de Winter et al., 2016). To confirm if the ENS also has a PNN like structure, neurons from the myenteric/submucous neurons and nodose ganglia can be grown in culture to see if a honeycomb like structure exists.

7.6 TNX role in the shape of neural structures

An alternative explanation for reduced neural activity may be attributed to the unique ability of tenascins to shape cells within tissues. This 'shaping' would alter transduction pathways by modifying the amplitude and duration of intracellular signalling by mediators such as focal adhesion kinase (FAK) and extracellular signal regulated kinase (ERK) (Jones and Jones, 2000). It is known that TNX can influence cell rounding of bovine osteosarcoma *in vitro* (Elefteriou et al., 1997), (Elefteriou et al., 1999 5063). Cells from the MG63 osteosarcoma cell line are normally oval to spindle shaped, without any branching processes (Pautke et al., 2004), however, when treated with TNX these cells display a round morphology (Elefteriou et al., 1997). Based on these studies it is possible to suggest a similar role for TNX in the ENS where TNX may be involved in shaping neural structures by rounding neuronal cell bodies found in the GI tract. It has been shown neural guidance is strongly dependent on geometry of ECM proteins, in other words laminin needs to be organised in order to guide neurite growth (Clark et al., 1993). Therefore ECM proteins are key in guiding neurons to grow and develop in an ordered fashion so as to carry out their intended function. Loss of TNX may similarly create disorder resulting in ENS structures that are not correctly shaped and therefore may not function correctly. Electron microscopy will reveal if there are any differences at the ultrastructural level.

7.7 TNX functions as a structural molecule

An emerging role for tenascins is regulation of biomechanical properties and their role in providing support to anchoring of structures. In particular TNX provides architectural support for other structural complexes such as collagens, fibrils and elastic fibres (Valcourt et al., 2015). Ultrastructural dermal analysis of skin in both TNX deficient mice and patients showed increase distance between fibrils (Bristow et al., 2005), (Mao et al., 2002). Based on these

studies and further *in vitro* studies, a model for the interaction between TNX and collagen fibrils was proposed whereby TNX bridges collagen fibrils to regulate the collagenous network either directly by fibrillary collagen interaction and/or indirectly via interaction with other ECM components like other collagens (XII and XIV) or decorin (Bristow et al., 2005), (Veit et al., 2006). In addition, TNX increases collagen content in 3-dimensional gel matrices which reinforces the gel (Margaron et al., 2010). Loss of TNX can change this interaction with collagens weakening the matrix and give rise to hyperextensibility of the skin (Zweers et al., 2004a).

7.8 Secondary role of TNX

The expression of TNX based in specific gut regions and even regions within tissues may provide different functional roles. IHC studies in submucous neurons showed strong TNX expression, however, functional studies looking at secretion showed no overall change. This could be because TNX that labelled ChAT neurons in the submucosal plexus projects to the circular muscle rather than the mucosa involved in secretion. Indeed 97% of submucosal neurons labelled from the circular muscle were found in the intermediate layer of the human submucosal plexus (Porter et al., 1999). Therefore, TNX containing submucous neurons may be important for motility rather than secretion. VIP containing neurons are important in regulating secretion therefore to rule out a role for TNX in secretion requires TNX and VIP co-staining.

7.9 Specific hypothesised role of TNX in the stomach and colon

Based on the proposed roles of TNX described previously it is possible to suggest specific roles for TNX in the stomach and colon. TNX found around vagal afferent IGLE and IMAs in the stomach may be used to maintain the space (Fig 6.7) between the tension receptors and the smooth muscle cells that it communicates with. Specifically TNX around these mechanoreceptors may allow efficient spontaneous activity of these low threshold endings. TNX around these endings may act to set the threshold for optimum nerve firing, thus in its absence the afferent fibre may become hypersensitive due to increased contact resulting in increased firing as observed in chapter 5.

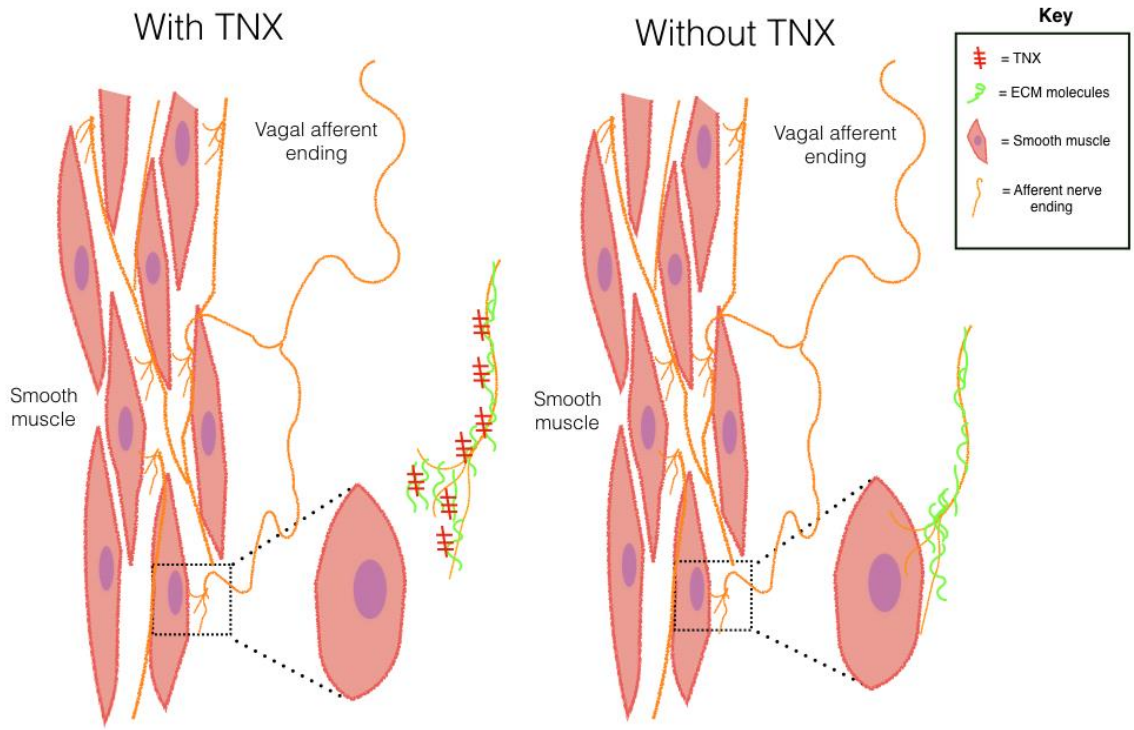


Figure 6.7. Possible role of TNX around vagal afferents in stomach. *TNX (red) is found around the nerve endings along with other ECM molecules (green). TNX may provide an anchor between the afferent ending and smooth muscle. Specifically, TNX around these mechanoreceptors may allow efficient spontaneous activity of these low threshold endings. TNX around these endings may act to set the threshold for optimum nerve firing, thus in its absence the afferent fibre may become hypersensitive due to reduced spacing between the nerve ending and smooth muscle.*

In the colon, TNX may act as a spacer and thus regulate the distance between the cholinergic neurons, for example TNX may allow the efficient transmission of neurotransmitters specifically ACh from cholinergic neurons released in the synaptic cleft allowing the neurotransmitter to bind to the appropriate receptor (Fig 6.8). In the absence of TNX the neurotransmitter may not bind to the receptor easily altering transmission of signals. This may lead to reduced cholinergic transmission to smooth muscle and therefore hypomotility of the distal colon as described in chapter 3.

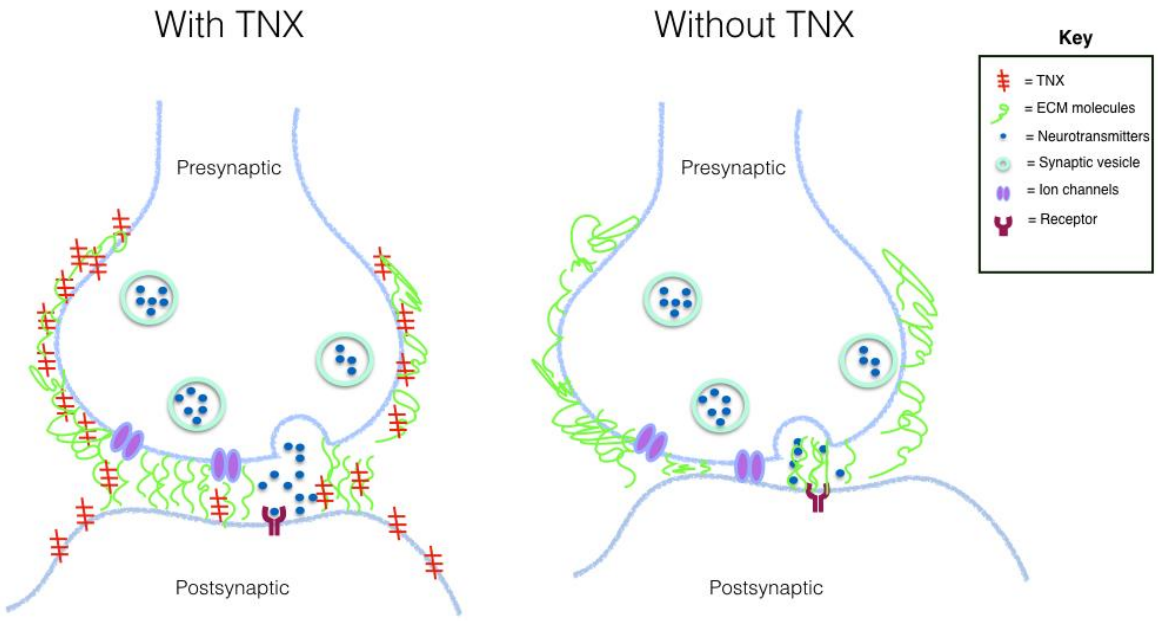


Figure 6.8. Possible role of TNX in colonic cholinergic neuron. *TNX (red) is found around the neurons along with other ECM molecules (green) exerting an anti-adhesive role. This maintains the correct position between the postsynaptic and presynaptic neuron and thus may be described as a spacer. In the absence of TNX this specific distance is altered which could result in neurotransmitters not appropriately binding to the respective receptor dysregulating the neural signal.*

7.10 Limitations and future work

Modifications of known experimental techniques used in this thesis is novel and directly served the purposes of our aims. Nonetheless, as with any research project, there is always room for improvement. In IHC studies, further exploration of TNX and quantitative assessment of neural expression with a range of neural markers in the KO model is needed to understand if there is indeed a change in distribution of neurons. Regional distribution of TNX is required within an organ as well as in different gut regions such as the oesophagus to fully characterise TNX in the gut. Ideally, tissue from TNX deficient patients and parallel physiology tests are required to compare whether the results are translational. In addition electron microscopy may reveal the precise location of TNX to definitively confirm its location within neural structures. Finally in situ hybridisation and polymerase chain reaction (PCR) in specific gut tissue will clarify if the gene is expressed as well as the observed protein expression.

Regarding the functional experiments, all studies except for gastric emptying were performed *in vitro.* The difficulty with *in vitro* studies lies in not being able to reflect the natural process that requires communication between different systems in the intact animal. However, motility and secretion studies are difficult to perform *in vivo* while single fibre vagal afferent recordings in the mouse would prove even more difficult. Nonetheless, colonic motility studies can be improved by additionally measuring pellet propulsion with spatiotemporal maps. This would enable visually identifying the muscle region that may be defected in the TNX KO as well as give an indication of overall transit time in the colon. Secretion studies can also be improved by using a broad range of pharmacological interventions that may clearly reveal the difference we started to observe in the complex response post the addition of veratridine. A limitation of the electrophysiology study was not controlling for compliance, however, this will be performed in the future. Using whole mouse stomach to perform afferent recording is advantageous since it reflects the natural state of the stomach in comparison to using a flat sheet preparation that can damage the endings when pinning down.

To address the importance of TNX, further work is needed to measure the growth of neurons in the presence and absence of TNX which will exemplify its importance in neural development. This may be done using tissue engineered innervated muscle sheets from

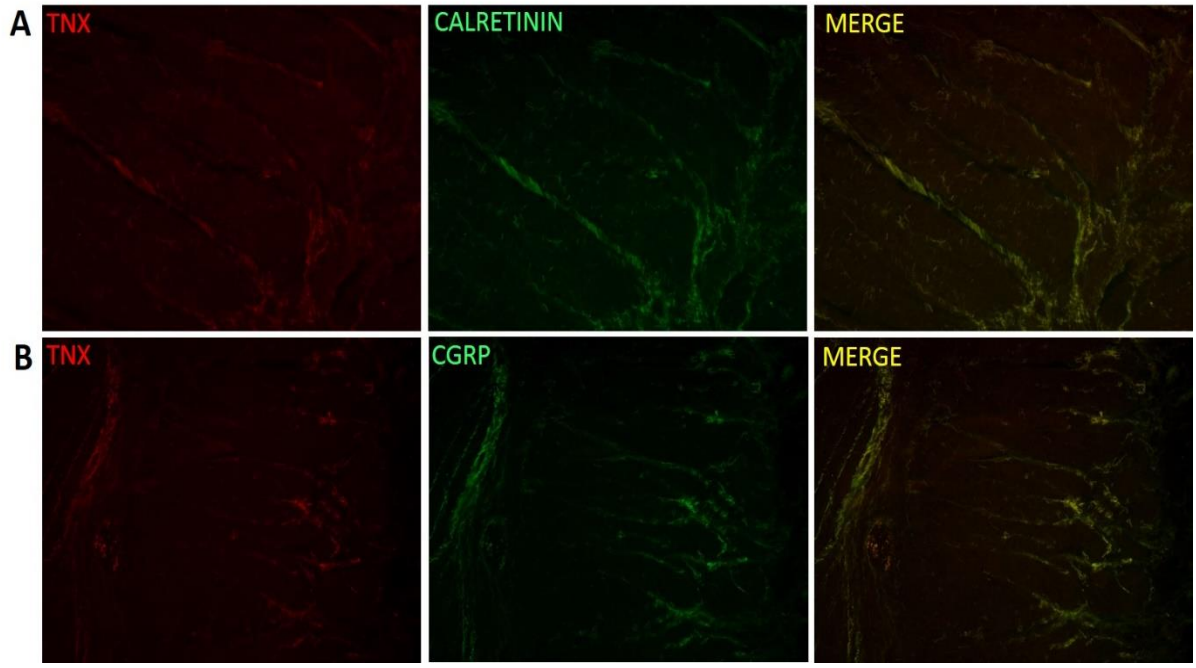
enteric neural stem cells grown in medium containing TNX and without (Raghavan and Bitar, 2014). This technique has shown that the smooth muscle sheets maintain contractile activity which can be tested *in vitro* (Raghavan et al., 2010). The functional efficiency of the muscle can be tested to see if TNX promotes contractile activity. Furthermore, a battery of intervention studies using drugs can be applied if there is a defect in smooth muscle sheets that were not exposed to TNX. Perhaps a specific drug will reverse the defected contractile activity more than another which could implicate tailored treatments in patients.

7.11 Conclusions

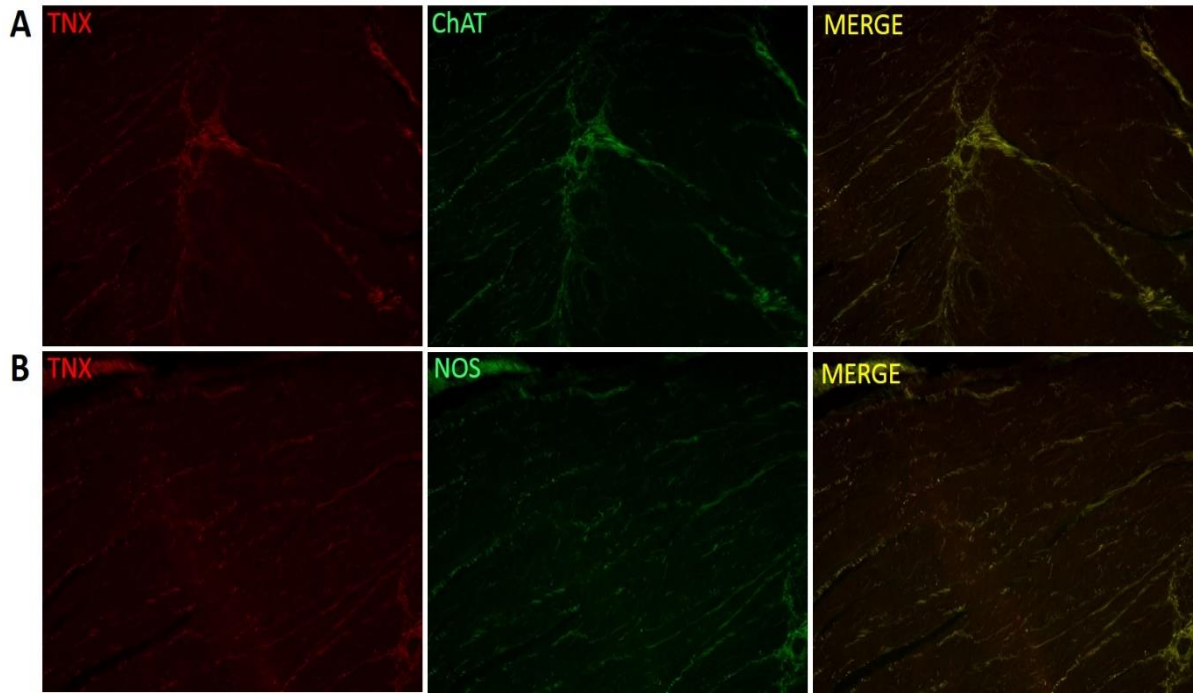
In summary, the precise role of TNX requires further investigation, however, it is clear that TNX may have a role in regulating the space between neural connections by exerting an anti-adhesive role, thus can act as a spacer and an anchor between neurons. It may also be important in neuronal development acting as a signalling molecule for axonal guidance and migration. TNX may also be critical in synaptic plasticity in the ENS, as exemplified by a host of other ECM molecules allowing the efficient communication of signals between synapses. Finally TNX may have a role in mechanical support through its interaction with other matrix molecules such as collagens. In light of the characterisation and functional studies presented in this thesis, it is probable to suggest a critical role for TNX in the complex function of the neurons. Further studies looking at the role of TNX at a molecular levels is now needed to further understand the refined role of this intricate protein.

Tenascins were once labelled ‘talented proteins in search of function” (Hsia and Schwarzbauer, 2005), since then research has shown multiple roles for various tenascins particularly in the CNS. However, the expression pattern and the specific role of TNX in the ENS remained a mystery. This body of work has identified and highlighted the localisation of TNX and its importance in the gastrointestinal tract, so we can relabel this protein as a talented protein with multiple functions, at least in the ENS. We have demonstrated the possible complexity of TNX in regulating gut function suggesting structural proteins have a true functional role. This opens a novel area of research to examine the underlying mechanism for TNX dependent modulation of neurons and their function in the ENS.

8 Appendix



Appendix 2.1. No primary control. A. TNX (red), Calretinin (green) and Merge (Yellow) shows no staining for both antibodies. B. TNX (red), CGRP (green) and Merge (Yellow) shows no staining for both antibodies. Magnification 40x



Appendix 2.2. No primary control. A. TNX (red), ChAT (green) and Merge (Yellow) shows no staining for both antibodies. B. TNX (red), NOS (green) and Merge (Yellow) shows no staining for both antibodies. Magnification 40x

WT	DURATION (MIN)				PEAKS				PEAKS/MIN				AMPLITUDE (mmHg))			
MOUSE	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal
1	60	60	60	60	17	28	24	30	0.28	0.46	0.39	0.49	45	32	32	50
2	53	53	53	53	32	19	15	11	0.6	0.36	0.28	0.21	28	29	23	24
3	42	42	42	42	31	41	28	23	0.73	0.96	0.66	0.54	36	36	36	29
4	44	44	44	44	23	23	19	29	0.53	0.51	0.42	0.65	27	39	23	35
5	60	60	60	60	43	35	21	8	0.6	0.49	0.29	0.11	24	26	31	23
6	60	60	60	60	42	19	13	14	0.63	0.29	0.2	0.21	30	29	23	23
7	41	41	41	41	30	32	19	12	0.72	0.77	0.46	0.29	23	27	48	9
8	40	40	40	40	19	15	23	16	0.47	0.37	0.57	0.39	26	17	39	19
9	53	53	53	53	23	13	13	11	0.43	0.24	0.24	0.21	38	24	56	19
10	40	40	40	40	22	23	17	11	0.54	0.57	0.42	0.27	24	31	43	22
11	40	40	40	40	16	23	18	13	0.39	0.56	0.44	0.32	17	25	28	24
MEAN	**42.8**	**42.8**	**42.8**	**42.8**	**22**	**21.2**	**18**	**12.6**	**0.51**	**0.502**	**0.426**	**0.296**	**25.6**	**24.8**	**42.8**	**18.6**
SEM	**1.72**	**1.72**	**1.72**	**1.72**	**1.58**	**2.28**	**1.08**	**0.62**	**0.03**	**0.06**	**0.03**	**0.01**	**2.32**	**1.54**	**3.14**	**1.73**

Appendix 3.1: WT spontaneous raw data. The duration, number of peaks, number of peaks/min and amplitude was measured in each region of the colon (proximal, mid-proximal, mid-distal and distal). The mean and standard error of the mean (SEM) was calculated. A total of 11 mice in the WT group were analysed.

KO	DURATION (MIN)				PEAKS				PEAKS/MIN				AMPLITUDE (mmHg)			
MOUSE	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal
1	49	49	49	49	24	20	17	19	0.61	0.3	0.22	0.08	24	20	17	19
2	60	60	60	60	31	18	28	15	0.5	0.29	0.45	0.24	27	22	33	19
3	45	45	45	45	30	22	21	2	0.66	0.48	0.46	0.04	40	34	37	18
4	60	60	60	60	31	32	27	34	0.52	0.54	0.45	0.57	27	4	47	32
5	51	51	51	51	18	28	25	1	0.35	0.54	0.48	0.02	20	26	30	82
6	46	46	46	46	21	19	21	3	0.45	0.41	0.45	0.06	29	27	38	33
7	42	42	42	42	21	9	14	0	0.49	0.21	0.33	0	26	19	43	0
8	30	30	30	30	27	14	14	1	0.89	0.46	0.46	0.03	37	22	32	11
9	45	45	45	45	12	13	6	0	0.27	0.29	0.13	0	22	36	24	0
10	38	38	38	38	8	7	7	0	0.21	0.18	0.18	0	23	27	19	0
11	30	30	30	30	35	11	7	1	1.15	0.36	0.23	0.03	39	31	18	18
MEAN	**37**	**37**	**37**	**37**	**20.6**	**10.8**	**9.6**	**0.4**	**0.602**	**0.3**	**0.266**	**0.012**	**29.4**	**27**	**27.2**	**5.8**
SEM	**2.06**	**2.06**	**2.06**	**2.06**	**3.30**	**0.86**	**1.21**	**0.16**	**0.12**	**0.031**	**0.03**	**0.00**	**2.41**	**2.05**	**3.14**	**2.508**

Appendix 3.2: KO spontaneous raw data. The duration, number of peaks, number of peaks/min and amplitude was measured in each region of the colon (proximal, mid-proximal, mid-distal and distal). The mean and standard error of the mean (SEM) was calculated. A total of 11 mice in the KO group were analysed.

WT-NOLA	DURATION (MIN)				PEAKS				PEAKS/MIN				AMPLITUDE (mmHg)			
	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal
BEFORE NOLA																
1	60.00	60.00	60.00	60.00	17.00	28.00	24.00	30.00	0.28	0.46	0.39	0.49	45.00	32.00	32.00	50.00
2	53.00	53.00	53.00	53.00	32.00	19.00	15.00	11.00	0.60	0.36	0.28	0.21	28.00	29.00	23.00	24.00
3	42.00	42.00	42.00	42.00	31.00	41.00	28.00	23.00	0.73	0.96	0.66	0.54	36.00	36.00	36.00	29.00
4	44.00	44.00	44.00	44.00	23.00	23.00	19.00	29.00	0.53	0.51	0.42	0.65	27.00	39.00	23.00	35.00
5	60.00	60.00	60.00	60.00	43.00	35.00	21.00	8.00	0.60	0.49	0.29	0.11	24.00	26.00	31.00	23.00
6	60.00	60.00	60.00	60.00	42.00	19.00	13.00	14.00	0.63	0.29	0.20	0.21	30.00	29.00	23.00	23.00
MEAN	**51.80**	**51.80**	**51.80**	**51.80**	**34.20**	**27.40**	**19.20**	**17.00**	**0.62**	**0.52**	**0.37**	**0.34**	**29.00**	**31.80**	**27.20**	**26.80**
SEM	**3.49**	**3.49**	**3.49**	**3.49**	**3.41**	**4.10**	**2.39**	**3.57**	**0.03**	**0.11**	**0.07**	**0.10**	**1.83**	**2.22**	**2.46**	**2.13**
DURING NOLA																
1	13.00	13.00	13.00	13.00	7.00	12.00	7.00	8.00	0.54	0.93	0.54	0.62	35.00	34.00	41.00	42.00
2	14.00	14.00	14.00	14.00	10.00	13.00	6.00	14.00	0.70	0.91	0.42	0.98	38.00	43.00	19.00	32.00
3	15.00	15.00	15.00	15.00	5.00	15.00	10.00	9.00	0.33	0.99	0.66	0.66	25.00	28.00	25.00	27.00
4	13.00	13.00	13.00	13.00	6.00	10.00	8.00	8.00	0.46	0.77	0.62	0.62	37.00	25.00	23.00	29.00
5	13.00	13.00	13.00	13.00	9.00	8.00	4.00	4.00	0.69	0.62	0.31	0.31	27.00	28.00	37.00	21.00
6	12.00	12.00	12.00	12.00	4.00	8.00	8.00	16.00	0.32	0.63	0.63	1.27	21.00	33.00	28.00	37.00

MEAN	**13.40**	**13.40**	**13.40**	**13.40**	**6.80**	**10.80**	**7.20**	**10.20**	**0.50**	**0.78**	**0.53**	**0.77**	**29.60**	**31.40**	**26.40**	**29.20**
SEM	**0.47**	**0.47**	**0.47**	**0.47**	**1.06**	**1.27**	**0.93**	**1.97**	**0.08**	**0.07**	**0.06**	**0.15**	**3.08**	**2.90**	**2.76**	**2.42**
POST NOLA																
1	51.00	51.00	51.00	51.00	7.00	28.00	33.00	26.00	0.14	0.55	0.64	0.51	15.00	24.00	31.00	34.00
2	39.00	39.00	39.00	39.00	26.00	24.00	14.00	14.00	0.65	0.60	0.35	0.35	29.00	33.00	21.00	24.00
3	59.00	59.00	59.00	59.00	51.00	40.00	41.00	33.00	0.86	0.68	0.69	0.56	32.00	28.00	40.00	28.00
4	40.00	40.00	40.00	40.00	13.00	24.00	28.00	21.00	0.33	0.61	0.72	0.54	25.00	31.00	23.00	22.00
5	59.00	59.00	59.00	59.00	24.00	33.00	39.00	13.00	0.40	0.55	0.65	0.22	27.00	26.00	26.00	19.00
6	38.00	38.00	38.00	38.00	11.00	15.00	6.00	7.00	0.28	0.39	0.15	0.18	25.00	35.00	20.00	17.00
MEAN	**47.00**	**47.00**	**47.00**	**47.00**	**25.00**	**27.20**	**25.60**	**17.60**	**0.50**	**0.57**	**0.51**	**0.37**	**27.60**	**30.60**	**26.00**	**22.00**
SEM	**4.48**	**4.48**	**4.48**	**4.48**	**6.51**	**3.91**	**6.26**	**4.06**	**0.10**	**0.04**	**0.10**	**0.07**	**1.21**	**1.49**	**3.33**	**1.76**

Appendix 3.3: WT raw data with NOLA. The duration, number of peaks, number of peaks/min and amplitude was measured in each region of the colon (proximal, mid-proximal, mid-distal and distal) before, during and after NOLA. The mean and standard error of the mean (SEM) was calculated. A total of 6 mice in the WT group were analysed.

KO-NOLA	DURATION (MIN)				PEAKS				PEAKS/MIN				AMPLITUDE (mmHg)			
	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal
BEFORE NOLA																
1	49	49	49	49	24	20	17	19	0.61	0.3	0.22	0.08	24	20	17	19
2	60	60	60	60	31	18	28	15	0.5	0.29	0.45	0.24	27	22	33	19
3	45	45	45	45	30	22	21	2	0.66	0.48	0.46	0.04	40	34	37	18
4	60	60	60	60	31	32	27	34	0.52	0.54	0.45	0.57	27	4	47	32
5	51	51	51	51	18	28	25	1	0.35	0.54	0.48	0.02	20	26	30	82
6	46	46	46	46	21	19	21	3	0.45	0.41	0.45	0.06	29	27	38	33
MEAN	**52.4**	**52.4**	**52.4**	**52.4**	**26.2**	**23.8**	**24.4**	**11**	**0.496**	**0.452**	**0.46**	**0.19**	**28.6**	**22.6**	**37**	**36.8**
SEM	**2.98**	**2.98**	**2.98**	**2.98**	**2.54**	**2.456**	**1.34**	**5.74**	**0.046**	**0.043**	**0.01**	**0.09**	**2.952**	**4.597**	**2.63**	**10.7**
DURING NOLA																
1	10	10	10	10	6	7	4	2	0.56	0.65	0.37	0.19	34	32	33	15
2	14	14	14	14	6	5	13	4	0.4	0.34	0.88	0.27	26	30	29	16
3	13	13	13	13	12	6	8	2	0.92	0.46	0.62	0.15	25	25	41	14
4	13	13	13	13	2	11	9	9	0.15	0.81	0.67	0.67	17	42	48	35
5	13	13	13	13	7	3	6	1	0.53	0.23	0.46	0.08	17	14	19	16
6	11	11	11	11	5	6	13	3	0.43	0.52	1.13	0.26	21	25	56	18

MEAN	**12.8**	**12.8**	**12.8**	**12.8**	**6.4**	**6.2**	**9.8**	**3.8**	**0.486**	**0.472**	**0.75**	**0.29**	**21.2**	**27.2**	**38.6**	**19.8**
SEM	**0.45**	**0.447**	**0.45**	**0.45**	**1.49**	**1.204**	**1.27**	**1.27**	**0.114**	**0.09**	**0.11**	**0.09**	**1.742**	**4.137**	**6.03**	**3.52**
POST NOLA																
1	60	60	60	60	26	30	19	1	0.38	0.44	0.28	0.01	22	31	16	11
2	60	60	60	60	18	18	29	4	0.28	0.28	0.45	0.06	17	20	27	19
3	45	45	45	45	29	27	18	7	0.64	0.59	0.39	0.15	35	32	17	15
4	80	80	80	80	2	17	33	21	0.02	0.21	0.41	0.26	13	31	46	33
5	54	54	54	54	8	3	13	16	0.15	0.05	0.24	0.29	14	15	23	15
6	59	59	59	59	26	25	40	9	0.44	0.42	0.68	0.15	24	26	34	13
MEAN	**59.6**	**59.6**	**59.6**	**59.6**	**16.6**	**18**	**26.6**	**11.4**	**0.306**	**0.31**	**0.43**	**0.18**	**20.6**	**24.8**	**29.4**	**19**
SEM	**5.25**	**5.249**	**5.25**	**5.25**	**4.7**	**3.851**	**4.5**	**2.84**	**0.099**	**0.084**	**0.06**	**0.04**	**3.726**	**2.964**	**4.55**	**3.32**

Appendix 3.4: KO raw data with NOLA. The duration, number of peaks, number of peaks/min and amplitude was measured in each region of the colon (proximal, mid-proximal, mid-distal and distal) before, during and after NOLA. The mean and standard error of the mean (SEM) was calculated. A total of 6 mice in the KO group were analysed.

WT-BILE	DURATION (MIN)				PEAKS				PEAKS/MIN				AMPLITUDE (mmHg)			
	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal
BEFORE BILE																
1	41	41	41	41	30	32	19	12	0.72	0.77	0.46	0.29	23	27	48	9
2	40	40	40	40	19	15	23	16	0.47	0.37	0.57	0.39	26	17	39	19
3	53	53	53	53	23	13	13	11	0.43	0.24	0.24	0.21	38	24	56	19
4	40	40	40	40	22	23	17	11	0.54	0.57	0.42	0.27	24	31	43	22
5	40	40	40	40	16	23	18	13	0.39	0.56	0.44	0.32	17	25	28	24
MEAN	**42.8**	**42.8**	**42.8**	**42.8**	**22**	**21.2**	**18**	**12.6**	**0.51**	**0.502**	**0.43**	**0.3**	**25.6**	**24.8**	**42.8**	**18.6**
SEM	**2.56**	**2.557**	**2.56**	**2.56**	**2.35**	**3.382**	**1.61**	**0.93**	**0.058**	**0.091**	**0.05**	**0.03**	**3.444**	**2.289**	**4.66**	**2.58**
DURING BILE																
1	9	9	9	9	0	0	0	0	0	0	0	0	0	0	0	0
2	9	9	9	9	1	0	0	1	0.11	0	0	0	22	0	0	0
3	10	10	10	10	0	0	0	0	0	0	0	0	0	0	0	0
4	9	9	9	9	2	1	0	1	0.21	0.11	0	0.11	26	21	0	23
5	9	9	9	9	2	0	2	1	0.22	0	0.22	0.11	22	0	18	13
MEAN	**9.2**	**9.2**	**9.2**	**9.2**	**1**	**0.2**	**0.4**	**0.6**	**0.108**	**0.022**	**0.04**	**0.04**	**14**	**4.2**	**3.6**	**7.2**
SEM	**0.2**	**0.2**	**0.2**	**0.2**	**0.45**	**0.2**	**0.4**	**0.24**	**0.048**	**0.022**	**0.04**	**0.03**	**5.762**	**4.2**	**3.6**	**4.68**

POST BILE																
	1	38	38	38	38	14	1	6	5	0.36	0.03	0.15	0.13	16	10	22	13
	2	30	30	30	30	8	3	2	1	0.27	0.1	0.07	0.03	15	19	24	19
	3	36	36	36	36	5	4	4	3	0.14	0.11	0.11	0.08	15	23	53	20
	4	39	39	39	39	10	4	3	0	0.25	0.1	0.08	0	16	25	19	0
	5	35	35	35	35	8	4	3	0	0.22	0.11	0.08	0	18	18	19	0
MEAN		**35.6**	**35.6**	**35.6**	**35.6**	**9**	**3.2**	**3.6**	**1.8**	**0.248**	**0.09**	**0.1**	**0.05**	**16**	**19**	**27.4**	**10.4**
SEM		**1.57**	**1.568**	**1.57**	**1.57**	**1.48**	**0.583**	**0.68**	**0.97**	**0.036**	**0.015**	**0.01**	**0.03**	**0.548**	**2.588**	**6.47**	**4.41**

Appendix 3.5: WT raw data with BILE. The duration, number of peaks, number of peaks/min and amplitude was measured in each region of the colon (proximal, mid-proximal, mid-distal and distal) before, during and after BILE. The mean and standard error of the mean (SEM) was calculated. A total of 5 mice in the WT group were analysed.

KO-BILE	DURATION (MIN)				PEAKS				PEAKS/MIN				AMPLITUDE (mmHg)			
	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal	Proximal	Mid-Prox	Mid-Dist	Distal
BEFORE BILE																
1	42	42	42	42	21	9	14	0	0.49	0.21	0.33	0	26	19	43	0
2	30	30	30	30	27	14	14	1	0.89	0.46	0.46	0.03	37	22	32	11
3	45	45	45	45	12	13	6	0	0.27	0.29	0.13	0	22	36	24	0
4	38	38	38	38	8	7	7	0	0.21	0.18	0.18	0	23	27	19	0
5	30	30	30	30	35	11	7	1	1.15	0.36	0.23	0.03	39	31	18	18
MEAN	**37**	**37**	**37**	**37**	**20.6**	**10.8**	**9.6**	**0.4**	**0.602**	**0.3**	**0.27**	**0.012**	**29.4**	**27**	**27.2**	**5.8**
SEM	**3.07**	**3.066**	**3.07**	**3.07**	**4.91**	**1.281**	**1.81**	**0.24**	**0.182**	**0.051**	**0.06**	**0.0073**	**3.586**	**3.05**	**4.66**	**3.72**
DURING BILE																
1	11	11	11	11	0	0	0	0	0	0	0	0	0	0	0	0
2	10	10	10	10	8	1	1	1	0.23	0.1	0.1	0.1	38	14	18	12
3	10	10	10	10	0	0	0	0	0	0	0	0	0	0	0	0
4	11	11	11	11	2	2	0	0	0.17	0.17	0	0	22	15	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MEAN	**8.4**	**8.4**	**8.4**	**8.4**	**2**	**0.6**	**0.2**	**0.2**	**0.08**	**0.054**	**0.02**	**0.02**	**12**	**5.8**	**3.6**	**2.4**
SEM	**2.11**	**2.112**	**2.11**	**2.11**	**1.55**	**0.4**	**0.2**	**0.2**	**0.05**	**0.035**	**0.02**	**0.02**	**7.772**	**3.555**	**3.6**	**2.4**

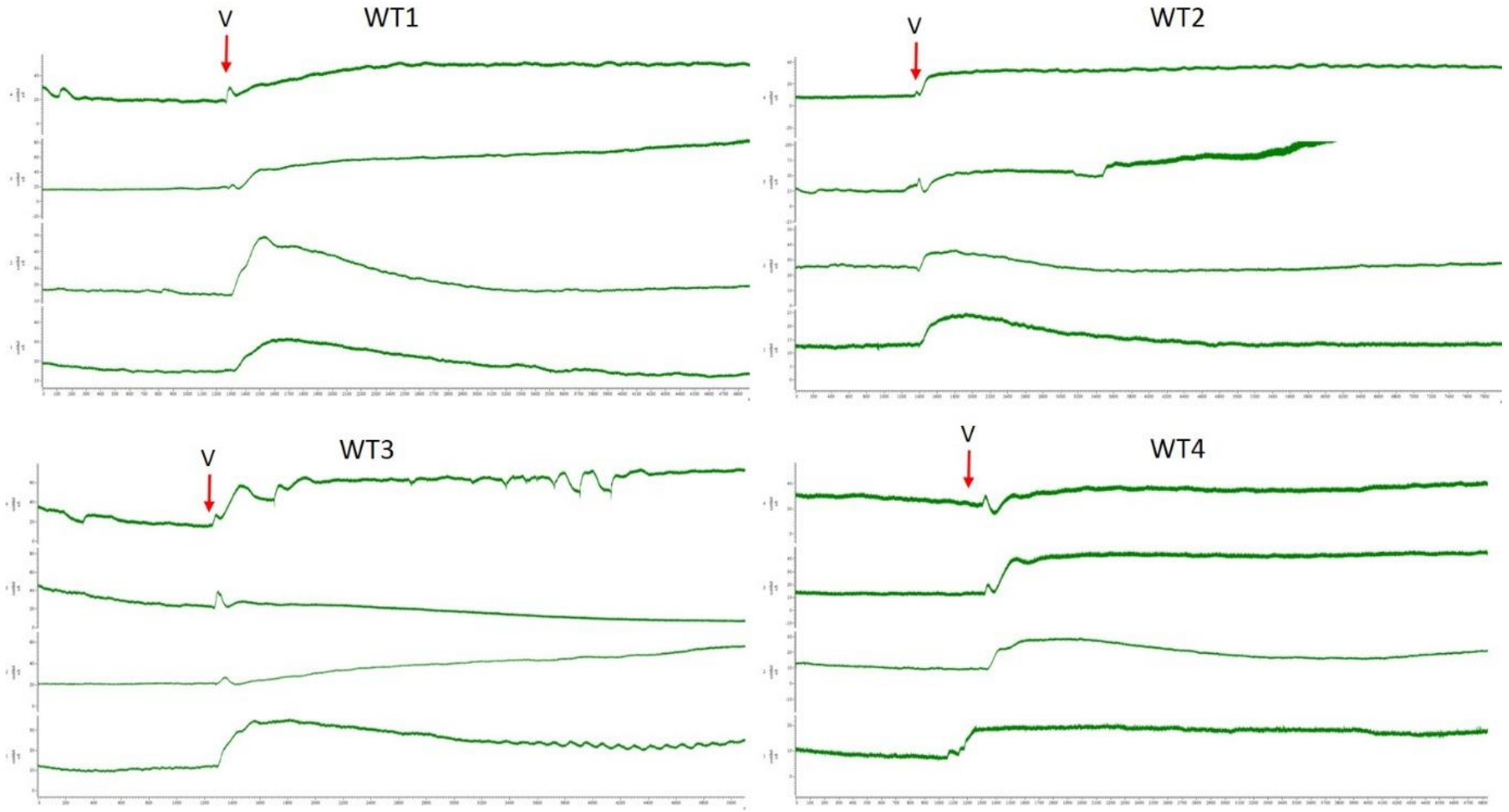
POST BILE																
	1	31	31	31	31	5	3	3	0	0.16	0.09	0.09	0	20	14	20	0
	2	39	39	39	39	9	2	16	1	0.23	0.05	0.4	0.03	22	15	19	11
	3	35	35	35	35	5	1	2	0	0.14	0.03	0.06	0	16	11	17	0
	4	27	27	27	27	2	1	3	1	0.07	0.04	0.11	1	14	18	29	12
	5	37	37	37	37	11	8	16	8	0.29	0.21	0.43	0.21	27	17	27	16
MEAN		**33.8**	**33.8**	**33.8**	**33.8**	**6.4**	**3**	**8**	**2**	**0.178**	**0.084**	**0.22**	**0.248**	**19.8**	**15**	**22.4**	**7.8**
SEM		**2.15**	**2.154**	**2.15**	**2.15**	**1.6**	**1.304**	**3.27**	**1.52**	**0.038**	**0.033**	**0.08**	**0.192**	**2.289**	**1.225**	**2.36**	**3.29**

Appendix 3.6: KO raw data with BILE. The duration, number of peaks, number of peaks/min and amplitude was measured in each region of the colon (proximal, mid-proximal, mid-distal and distal) before, during and after BILE. The mean and standard error of the mean (SEM) was calculated. A total of 5 mice in the KO group were analysed.

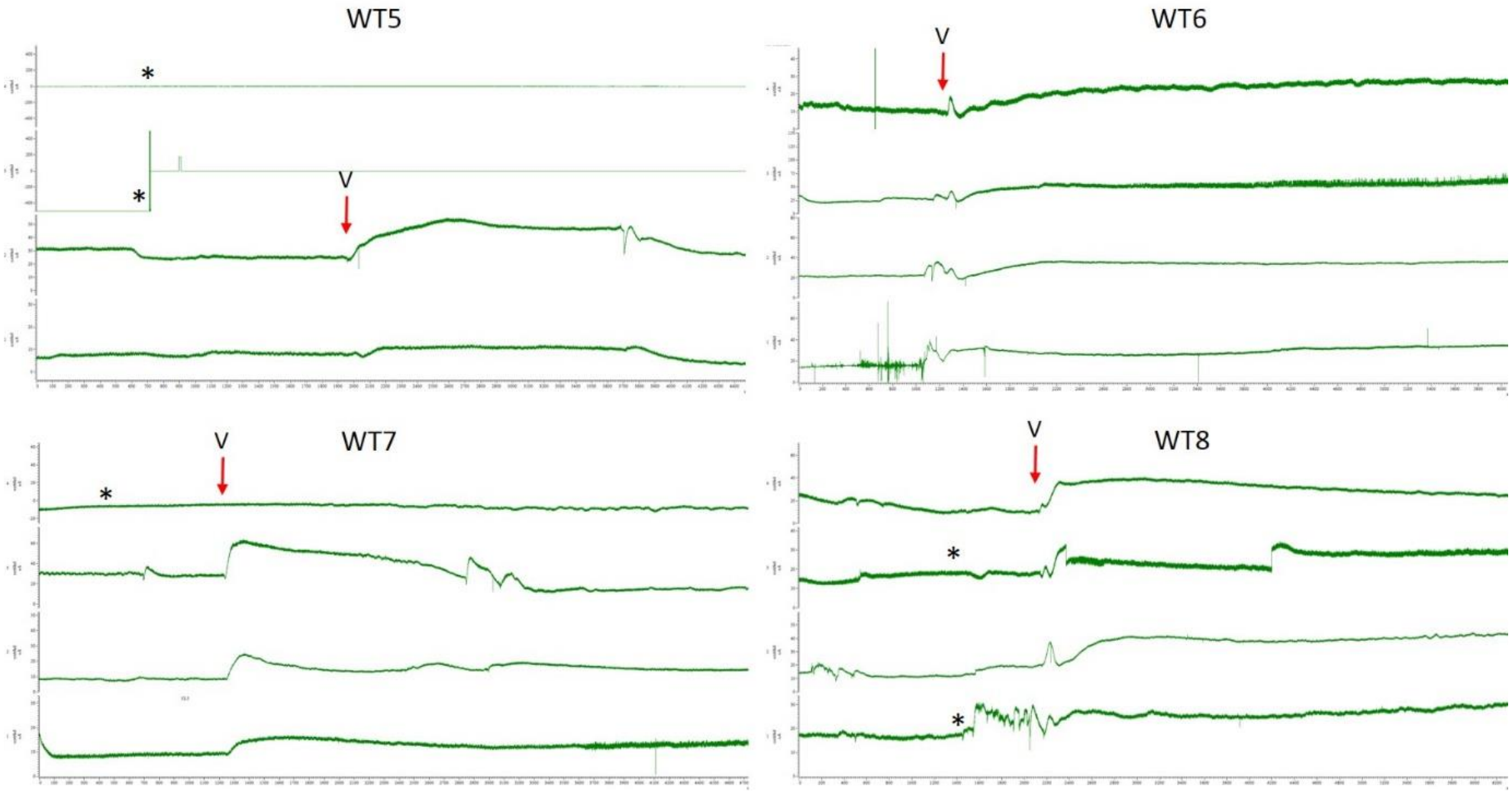
WT	FREQUENCY	TIME MIN	FREQUENCY COMPLEX/MIN	PROPAGATION VELOCITY (S)	ANTEROGRADE (%)	RETROGRADE (%)	STATIC (%)
1	12	26	2.166667	3.45	75	8.3	16.7
2	10	22	2.2	1.79	100	0	0
3	5	14	2.8	2.37	100	0	0
4	6	24	4	2.15	100	0	0
5	6	16	2.666667	1.47	66	17	17
6	8	30	3.75	1.06	12.5	87.5	0
7	4	10	2.5	2.44	100	0	0
8	5	12	2.4	2.62	100	0	0
MEAN	**7**	**19.25**	**2.810417**	**2.16875**	**81.6875**	**14.1**	**4.2125**
SEM	**0.981981**	**2.561738**	**0.245332**	**0.261202**	**10.98251**	**10.71019**	**2.757874**

KO	FREQUENCY	TIME MIN	FREQUENCY COMPLEX/MIN	PROPAGATION VELOCITY (S)	ANTEROGRADE (%)	RETROGRADE (%)	STATIC (%)
1	11	30	2.727272727	1.76	100	0	0
2	18	30	1.666666667	1.98	88	22	0
3	7	30	4.285714286	1.51	100	0	0
4	6	30	5	1.34	100	0	0
5	9	22	2.444444444	2.33	100	0	0
6	9	32	3.555555556	2.13	88	22	0
7	11	27	2.454545455	1.62	100	0	0
8	6	22	3.666666667	2.09	83	0	17
9	4	10	2.5	2.45	75	0	25
MEAN	**9**	**25.8889**	**3.144540645**	**1.912222222**	**92.66666667**	**4.888888889**	**4.66667**
SEM	**1.37437**	**2.32406**	**0.352105576**	**0.126114782**	**3.157882554**	**3.233696047**	**3.15788**

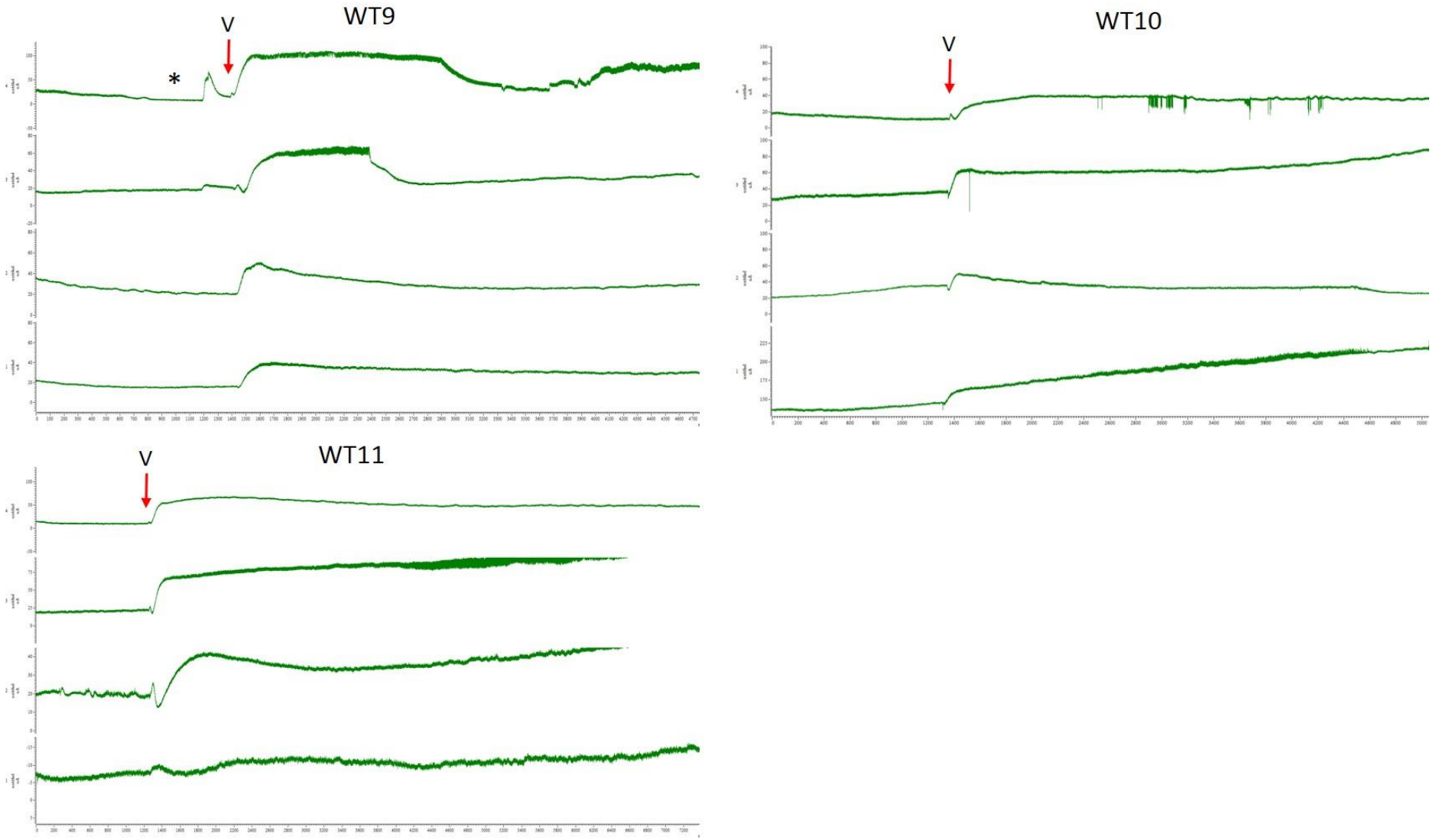
Appendix 3.7: WT and KO CMMC analysis. The frequency, given time CMMC were measured across, frequency of CMMC complex/min was measured. The direction of CMMC (anterograde, retrograde or static) were also counted. The mean and standard error of the mean (SEM) was calculated. A total of 8 mice in the WT and 9 mice in the KO group were compared.



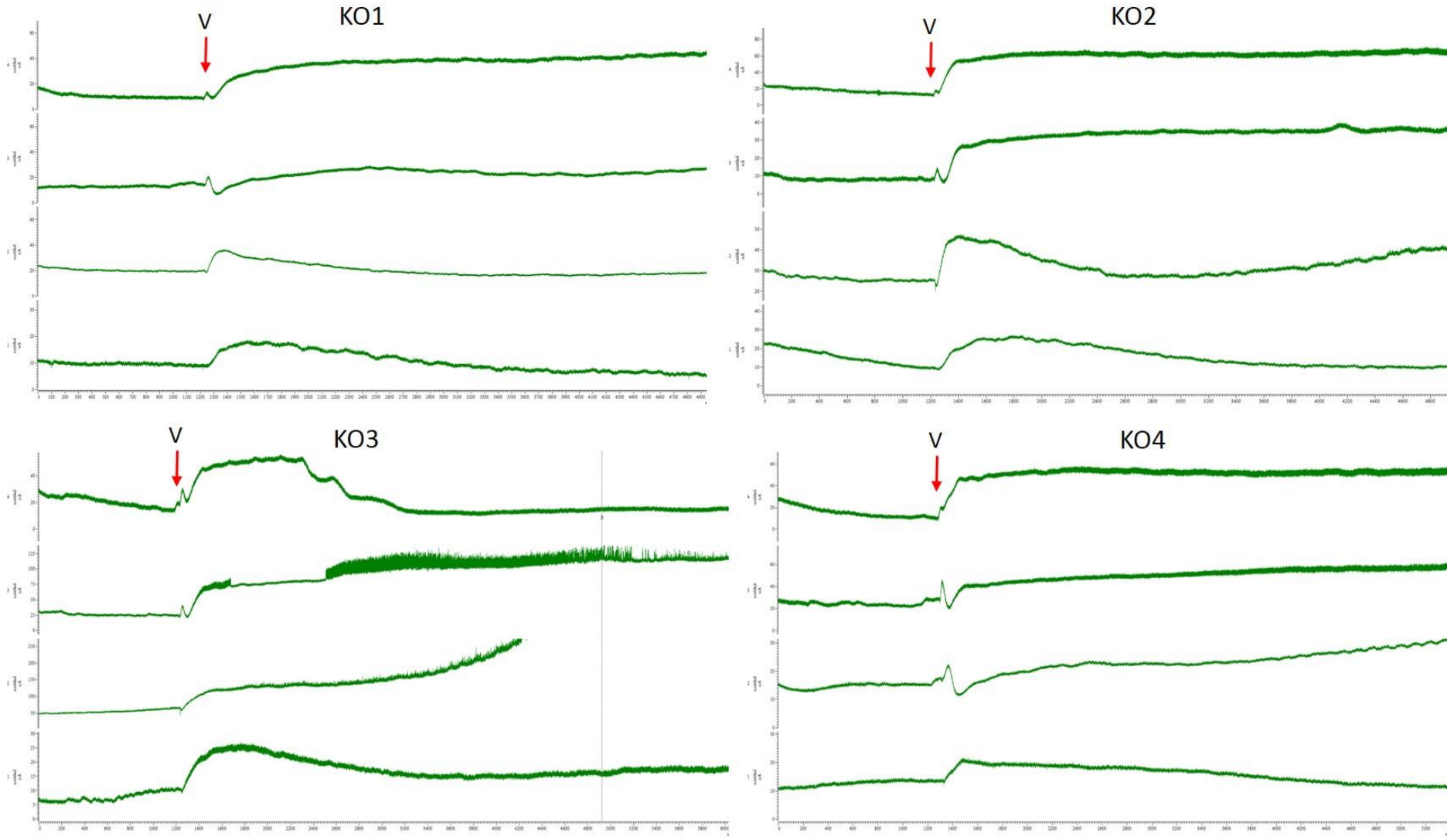
Appendix 4.1: WT1-WT4 raw traces. Basal secretion was measured in each colonic region, the topmost trace is proximal, followed by mid-proximal and mid-distal and lowest trace is the distal colon. V denotes the time at which veratridine was added at a concentration of 30µM.



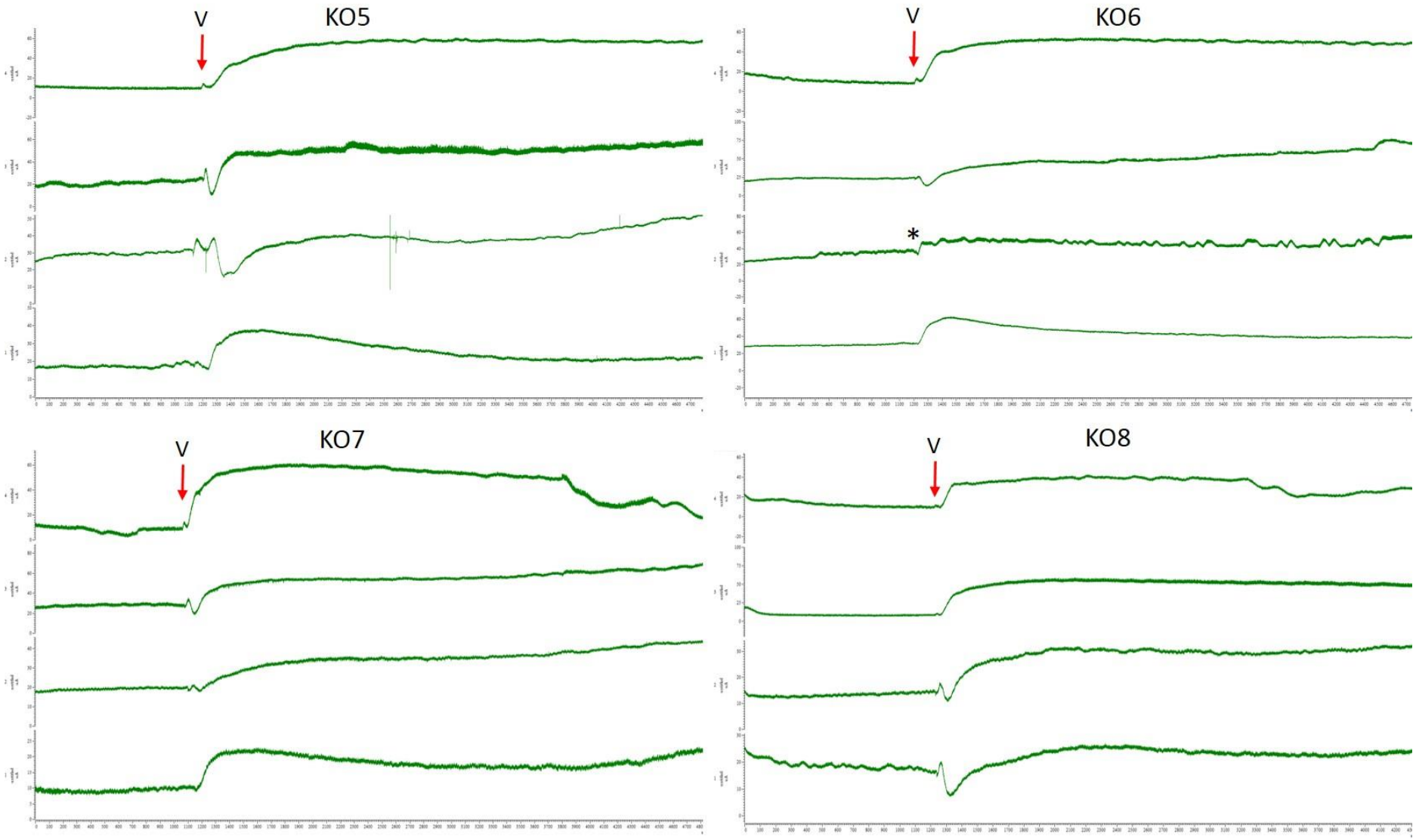
Appendix 4.2: WT5-WT8 raw traces. Basal secretion was measured in each colonic region, the topmost trace is proximal, followed by mid-proximal and mid-distal and lowest trace is the distal colon. V denotes the time at which veratridine was added at a concentration of 30µM. * Represents trace that was not analysed



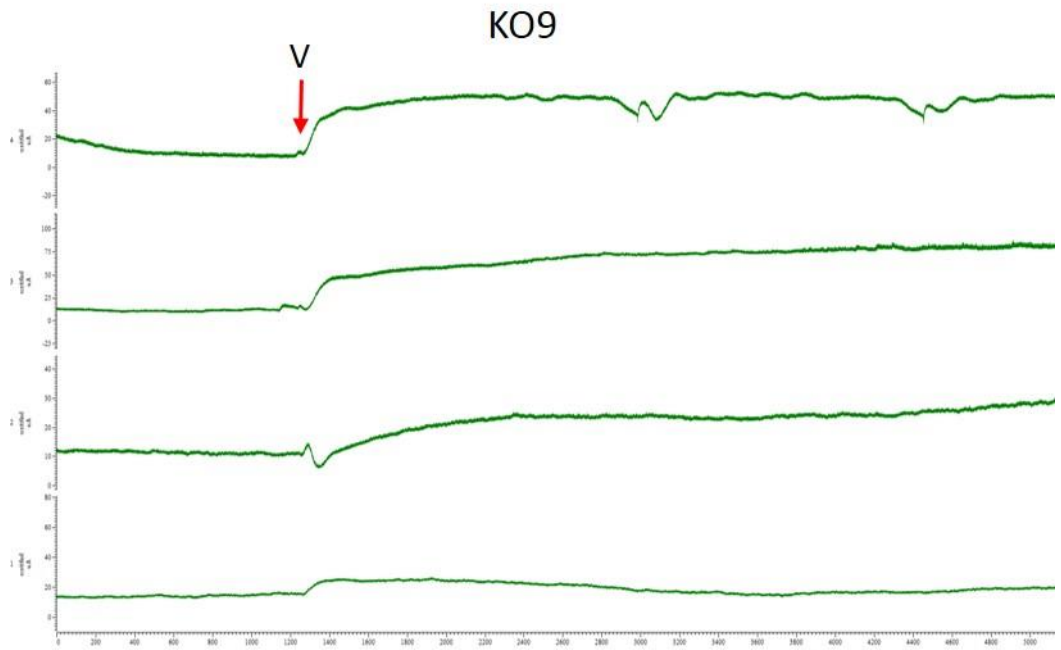
Appendix 4.3: WT9-WT11 raw traces. Basal secretion was measured in each colonic region, the topmost trace is proximal, followed by mid-proximal and mid-distal and lowest trace is the distal colon. V denotes the time at which veratridine was added at a concentration of 30µM. * Represents trace that was not analysed



Appendix 4.4: KO1-KO4 raw traces. Basal secretion was measured in each colonic region, the topmost trace is proximal, followed by mid-proximal and mid-distal and lowest trace is the distal colon. V denotes the time at which veratridine was added at a concentration of 30µM.



Appendix 4.5: KO5-KO8 raw traces. Basal secretion was measured in each colonic region, the topmost trace is proximal, followed by mid-proximal and mid-distal and lowest trace is the distal colon. V denotes the time at which veratridine was added at a concentration of 30µM. * Represents trace that was analysed



Appendix 4.6: KO9 raw trace. Basal secretion was measured in each colonic region, the topmost trace is proximal, followed by mid-proximal and mid-distal and lowest trace is the distal colon. V denotes the time at which veratridine was added at a concentration of 30µM.

WT1				WT2				WT3			
Time (min)	**D13C**	**%13C**	**%dose/hr**	**Time (min)**	**D13C**	**%13C**	**%dose/hr**	**Time (min)**	**D13C**	**%13C**	**%dose/hr**
0	-20.777	1.088	0.000	**0**	-20.247	1.089	0.000	**0**	-20.586	1.089	0.000
5	-20.196	1.089	2.469	**5**	-19.622	1.090	2.433	**5**	-19.747	1.090	2.852
10	-20.109	1.089	2.838	**10**	-19.061	1.090	4.615	**10**	-19.317	1.090	4.312
15	-19.835	1.089	4.003	**15**	-18.292	1.091	7.607	**15**	-18.309	1.091	7.735
20	-19.576	1.090	5.102	**20**	-17.899	1.092	9.135	**20**	-17.523	1.092	10.406
25	-19.703	1.090	4.562	**25**	-18.152	1.091	8.151	**25**	-17.241	1.092	11.364
30	-19.585	1.090	5.064	**30**	-17.905	1.092	9.115	**30**	-15.893	1.094	15.941
45	-19.444	1.090	5.664	**45**	-17.972	1.091	8.854	**45**	-15.346	1.094	17.799
60	-19.453	1.090	5.625	**60**	-16.890	1.093	13.064	**60**	-15.930	1.094	15.817
75	-19.302	1.090	6.266	**75**	-15.131	1.095	19.909	**75**	-17.415	1.092	10.772
90	-19.038	1.090	7.391	**90**	-15.442	1.094	18.699	**90**	-15.900	1.094	15.919
105	-18.766	1.091	8.545	**105**	-17.924	1.092	9.038	**105**	-14.831	1.095	19.551
120	-19.318	1.090	6.198	**120**	-18.519	1.091	6.724	**120**	-15.748	1.094	16.434
135	-18.954	1.090	7.746	**135**	-18.896	1.090	5.258	**135**	-17.191	1.092	11.534
150	-18.704	1.091	8.807	**150**	-20.334	1.089	-0.339	**150**	-17.890	1.092	9.157

WT4				WT5				WT6			
Time (min)	**D13C**	**%13C**	**%dose/hr**	**Time (min)**	**D13C**	**%13C**	**%dose/hr**	**Time (min)**	**D13C**	**%13C**	**%dose/hr**
0	-20.525	1.089	0.000	**0**	-19.959	1.089	0.000	**0**	-20.360	1.089	0.000
5	-19.731	1.090	2.762	**5**	-17.775	1.092	6.455	**5**	-17.517	1.092	8.660
10	-19.753	1.090	2.684	**10**	-17.124	1.092	8.381	**10**	-14.784	1.095	16.981
15	-19.845	1.089	2.363	**15**	-17.454	1.092	7.404	**15**	-13.937	1.096	19.561
20	-20.028	1.089	1.729	**20**	-17.063	1.092	8.559	**20**	-12.813	1.097	22.984
25	-20.038	1.089	1.694	**25**	-17.074	1.092	8.529	**25**	-12.074	1.098	25.236
30	-19.945	1.089	2.016	**30**	-16.949	1.093	8.898	**30**	-12.942	1.097	22.592
45	-19.440	1.090	3.773	**45**	-15.469	1.094	13.273	**45**	-13.209	1.097	21.779
60	-19.548	1.090	3.396	**60**	-15.148	1.095	14.222	**60**	-11.453	1.099	27.127
75	-18.531	1.091	6.932	**75**	-15.353	1.094	13.616	**75**	-12.631	1.097	23.539
90	-18.340	1.091	7.596	**90**	-13.564	1.096	18.904	**90**	-12.714	1.097	23.286
105	-17.541	1.092	10.374	**105**	-13.426	1.096	19.312	**105**	-13.303	1.097	21.493
120	-17.497	1.092	10.527	**120**	-15.088	1.095	14.399	**120**	-16.431	1.093	11.967
135	-18.037	1.091	8.649	**135**	-15.871	1.094	12.083	**135**	-18.841	1.091	4.628
150	-17.855	1.092	9.280	**150**	-15.515	1.094	13.137	**150**	-19.989	1.089	1.129

	WT7				WT8				WT9		
Time (min)	D13C	%13C	%dose/hr	Time (min)	D13C	%13C	%dose/hr	Time (min)	D13C	%13C	%dose/hr
0	-20.546	1.089	0.000	**0**	-20.541	1.089	0.000	**0**	-20.154	1.089	0.000
5	-19.374	1.090	3.763	**5**	-19.596	1.090	3.197	**5**	-18.865	1.090	3.990
10	-19.129	1.090	4.547	**10**	-18.913	1.090	5.508	**10**	-17.772	1.092	7.373
15	-17.997	1.091	8.184	**15**	-18.574	1.091	6.655	**15**	-16.684	1.093	10.743
20	-17.610	1.092	9.427	**20**	-18.195	1.091	7.937	**20**	-15.840	1.094	13.355
25	-17.070	1.092	11.160	**25**	-18.916	1.090	5.497	**25**	-15.061	1.095	15.766
30	-16.945	1.093	11.562	**30**	-18.686	1.091	6.276	**30**	-14.283	1.096	18.174
45	-16.331	1.093	13.531	**45**	-18.322	1.091	7.508	**45**	-13.395	1.097	20.924
60	-16.426	1.093	13.226	**60**	-17.449	1.092	10.461	**60**	-7.378	1.103	39.550
75	-14.319	1.095	19.991	**75**	-17.530	1.092	10.188	**75**	3.596	1.115	73.511
90	-14.586	1.095	19.135	**90**	-16.871	1.093	12.417	**90**	7.615	1.120	85.946
105	-13.705	1.096	21.962	**105**	-15.248	1.094	17.904	**105**	no value	no value	no value
120	-14.741	1.095	18.637	**120**	-14.656	1.095	19.906	**120**	no value	no value	no value
135	-14.304	1.096	20.040	**135**	-15.268	1.094	17.838	**135**	-8.108	1.102	37.291
150	-16.389	1.093	13.347	**150**	-15.558	1.094	16.856	**150**	-14.816	1.095	16.525

	WT10				WT11				WT12		
Time (min)	D13C	%13C	%dose/hr	Time (min)	D13C	%13C	%dose/hr	Time (min)	D13C	%13C	%dose/hr
0	-20.472	1.089	0.000	**0**	-19.428	1.090	0.000	**0**	-19.589	1.090	0.000
5	-20.027	1.089	2.719	**5**	-18.959	1.090	2.842	**5**	-18.976	1.090	3.671
10	-18.670	1.091	11.014	**10**	-18.631	1.091	4.825	**10**	-18.796	1.091	4.750
15	-17.851	1.092	16.019	**15**	-18.064	1.091	8.262	**15**	-18.793	1.091	4.769
20	-16.856	1.093	22.106	**20**	-18.008	1.091	8.602	**20**	-18.787	1.091	4.808
25	-15.522	1.094	30.259	**25**	-17.171	1.092	13.670	**25**	-18.676	1.091	5.469
30	-15.102	1.095	32.825	**30**	-15.426	1.094	24.238	**30**	-18.677	1.091	5.468
45	-15.035	1.095	33.237	**45**	-14.695	1.095	28.660	**45**	-16.731	1.093	17.129
60	-15.641	1.094	29.530	**60**	-14.900	1.095	27.421	**60**	-13.302	1.097	37.676
75	-15.891	1.094	28.003	**75**	-15.830	1.094	21.791	**75**	-13.863	1.096	34.315
90	-16.689	1.093	29.000	**90**	-15.142	1.095	25.957	**90**	-12.614	1.097	41.797
105	-15.679	1.094	29.300	**105**	-14.396	1.095	30.475	**105**	-13.141	1.097	38.643
120	-14.219	1.096	38.225	**120**	-11.567	1.099	47.605	**120**	-13.910	1.096	34.031
135	-15.653	1.094	29.454	**135**	-13.531	1.096	35.708	**135**	-12.822	1.097	40.550
150	-17.972	1.091	15.280	**150**	-15.109	1.095	26.156	**150**	-12.902	1.097	40.074

	WT13				WT14				WT15		
Time (min)	D13C	%13C	%dose/hr	Time (min)	D13C	%13C	%dose/hr	Time (min)	D13C	%13C	%dose/hr
0	-19.548	1.090	0.000	**0**	-20.589	1.089	0.000	**0**	-20.449	1.089	0.000
5	-18.554	1.091	5.956	**5**	-19.194	1.090	7.397	**5**	-19.132	1.090	6.385
10	-18.162	1.091	8.308	**10**	-17.566	1.092	16.028	**10**	-18.848	1.091	7.763
15	-17.370	1.092	13.052	**15**	-16.098	1.094	23.811	**15**	-18.438	1.091	9.754
20	-17.010	1.093	15.210	**20**	-15.529	1.094	26.827	**20**	-18.357	1.091	10.147
25	-15.875	1.094	22.016	**25**	-15.240	1.094	28.360	**25**	-18.079	1.091	11.495
30	-14.985	1.095	27.349	**30**	-16.054	1.094	24.041	**30**	-17.970	1.091	12.023
45	-15.058	1.095	26.909	**45**	-17.580	1.092	15.953	**45**	-17.140	1.092	16.046
60	-16.402	1.093	18.852	**60**	-18.214	1.091	12.594	**60**	-17.129	1.092	16.101
75	-15.157	1.095	26.317	**75**	-17.725	1.092	15.183	**75**	-16.793	1.093	17.730
90	-14.321	1.095	31.323	**90**	-17.052	1.092	18.749	**90**	-15.595	1.094	23.543
105	-12.838	1.097	40.210	**105**	-16.860	1.093	19.771	**105**	-14.853	1.095	27.141
120	-12.788	1.097	40.511	**120**	-16.192	1.093	23.308	**120**	-13.535	1.096	33.531
135	-12.273	1.098	43.599	**135**	-15.486	1.094	27.054	**135**	-13.159	1.097	35.352
150	-11.929	1.098	45.662	**150**	-16.149	1.093	23.539	**150**	-15.875	1.094	22.183

Appendix 6.1: Raw values for WT mice. At each time point a breath sample was taken. D13C is the raw δ value which is then converted to percentage of ^{13}C per hour (%13C) of the initial dose given (%dose/h). The calculation for each time point is described in detail Chapter 6, see methods.

KO1				KO2				KO3			
Time (min)	D13C	%13C	%dose/hr	Time (min)	D13C	%13C	%dose/hr	Time (min)	D13C	%13C	%dose/hr
0	-20.586	1.089	0.000	0	-20.326	1.089	0.000	0	-20.751	1.088	0.000
5	-19.747	1.090	3.843	5	-19.672	1.090	3.291	5	-18.732	1.091	11.524
10	-19.317	1.090	5.811	10	-19.349	1.090	4.911	10	-17.872	1.092	16.432
15	-18.309	1.091	10.424	15	-18.852	1.091	7.412	15	-16.695	1.093	23.154
20	-17.523	1.092	14.022	20	-17.966	1.091	11.864	20	-15.164	1.095	31.890
25	-17.241	1.092	15.313	25	-17.068	1.092	16.379	25	-15.376	1.094	30.679
30	-15.893	1.094	21.482	30	-17.158	1.092	15.929	30	-13.889	1.096	39.168
45	-15.346	1.094	23.986	45	-15.727	1.094	23.121	45	-13.736	1.096	40.045
60	-15.930	1.094	21.315	60	-14.281	1.096	30.390	60	-14.009	1.096	38.482
75	-17.415	1.092	14.516	75	-12.545	1.097	39.117	75	-12.762	1.097	45.600
90	-15.900	1.094	21.452	90	-12.295	1.098	40.374	90	-11.959	1.098	50.183
105	-14.831	1.095	26.346	105	-13.964	1.096	31.987	105	-15.101	1.095	32.254
120	-15.748	1.094	22.146	120	-13.393	1.097	34.853	120	-16.050	1.094	26.836
135	-17.191	1.092	15.544	135	-13.249	1.097	35.578	135	-18.401	1.091	13.414
150	-17.890	1.092	12.340	150	-14.110	1.096	31.249	150	-19.697	1.090	6.014

KO4				KO5				KO6			
Time (min)	D13C	%13C	%dose/hr	Time (min)	D13C	%13C	%dose/hr	Time (min)	D13C	%13C	%dose/hr
0	-20.623	1.089	0.000	0	-21.122	1.088	0.000	0	-19.674	1.090	0.000
5	-20.110	1.089	2.306	5	-20.454	1.089	2.685	5	-18.814	1.091	2.479
10	-19.911	1.089	3.195	10	-20.079	1.089	4.191	10	-17.263	1.092	6.951
15	-19.502	1.090	5.033	15	-19.460	1.090	6.677	15	-14.330	1.095	15.404
20	-19.359	1.090	5.674	20	-19.127	1.090	8.012	20	-11.563	1.099	23.379
25	-19.021	1.090	7.191	25	-19.238	1.090	7.567	25	-9.395	1.101	29.629
30	-18.523	1.091	9.425	30	-19.319	1.090	7.243	30	-7.985	1.102	33.694
45	-16.314	1.093	19.339	45	-18.864	1.090	9.069	45	-6.992	1.104	36.553
60	-14.764	1.095	26.297	60	-18.432	1.091	10.806	60	-6.543	1.104	37.849
75	-14.280	1.096	28.468	75	-17.459	1.092	14.715	75	-5.691	1.105	40.304
90	-13.045	1.097	34.012	90	-14.050	1.096	28.407	90	-11.054	1.099	24.847
105	-13.494	1.096	31.994	105	-17.441	1.092	14.787	105	-18.036	1.091	4.722
120	-14.164	1.096	28.988	120	-16.702	1.093	17.754	120	-21.061	1.088	-3.998
135	-14.611	1.095	26.982	135	-16.945	1.093	16.777	135	-20.933	1.088	-3.629
150	-17.843	1.092	12.477	150	-18.598	1.091	10.141	150	-21.569	1.088	-5.464

KO7				KO8				KO9			
Time (min)	**D13C**	**%13C**	**%dose/hr**	**Time (min)**	**D13C**	**%13C**	**%dose/hr**	**Time (min)**	**D13C**	**%13C**	**%dose/hr**
0	-20.365	1.089	0.000	**0**	-20.909	1.088	0.000	**0**	-20.487	1.089	0.000
5	-18.970	1.090	4.609	**5**	-20.054	1.089	4.130	**5**	-19.945	1.089	1.863
10	-17.917	1.092	8.089	**10**	-19.963	1.089	4.571	**10**	-19.261	1.090	4.214
15	-16.913	1.093	11.406	**15**	-19.669	1.090	5.989	**15**	-17.657	1.092	9.725
20	-14.797	1.095	18.394	**20**	-19.290	1.090	7.822	**20**	-16.119	1.094	15.008
25	-14.040	1.096	20.895	**25**	-19.189	1.090	8.311	**25**	-14.853	1.095	19.361
30	-13.482	1.096	22.739	**30**	-19.179	1.090	8.360	**30**	-14.201	1.096	21.600
45	-13.373	1.097	23.100	**45**	-17.368	1.092	17.106	**45**	-10.063	1.100	35.816
60	-15.121	1.095	17.326	**60**	-16.520	1.093	21.201	**60**	-11.197	1.099	31.923
75	-14.577	1.095	19.123	**75**	-16.600	1.093	20.816	**75**	-11.814	1.098	29.800
90	-16.216	1.093	13.706	**90**	-16.931	1.093	19.215	**90**	-9.711	1.101	37.027
105	-18.580	1.091	5.897	**105**	-17.039	1.093	18.693	**105**	-8.963	1.101	39.597
120	-20.166	1.089	0.656	**120**	-16.463	1.093	21.475	**120**	-9.625	1.101	37.324
135	-21.580	1.088	-4.015	**135**	-17.322	1.092	17.328	**135**	-8.128	1.102	42.467
150	-21.283	1.088	-3.033	**150**	-17.641	1.092	15.787	**150**	-9.814	1.100	36.675

KO10				KO11				KO12			
Time (min)	**D13C**	**%13C**	**%dose/hr**	**Time (min)**	**D13C**	**%13C**	**%dose/hr**	**Time (min)**	**D13C**	**%13C**	**%dose/hr**
0	-20.602	1.089	0.000	**0**	-20.703	1.088	0.000	**0**	-20.536	1.089	0.000
5	-19.450	1.090	4.170	**5**	-20.126	1.089	2.757	**5**	-17.165	1.092	15.650
10	-18.689	1.091	6.924	**10**	-19.759	1.090	4.509	**10**	-14.116	1.096	29.803
15	-17.207	1.092	12.285	**15**	-18.169	1.091	12.110	**15**	-12.476	1.098	37.417
20	-16.131	1.094	16.180	**20**	-15.711	1.094	23.852	**20**	-12.816	1.097	35.838
25	-15.206	1.095	19.527	**25**	-14.863	1.095	27.906	**25**	-12.947	1.097	35.229
30	-14.930	1.095	20.527	**30**	-11.960	1.098	41.776	**30**	-12.669	1.097	36.519
45	-14.066	1.096	23.652	**45**	-7.447	1.103	63.336	**45**	-11.282	1.099	42.957
60	-13.660	1.096	25.124	**60**	-7.903	1.103	61.158	**60**	-11.407	1.099	42.380
75	-11.484	1.099	32.998	**75**	-9.879	1.100	51.715	**75**	-11.921	1.098	39.991
90	-12.506	1.097	29.297	**90**	-8.212	1.102	59.683	**90**	-12.873	1.097	35.571
105	-12.663	1.097	28.731	**105**	-8.812	1.102	56.813	**105**	-17.270	1.092	15.165
120	-12.640	1.097	28.815	**120**	-11.511	1.099	43.922	**120**	-19.796	1.089	3.435
135	-16.080	1.094	16.364	**135**	-14.391	1.095	30.162	**135**	-20.800	1.088	-1.222
150	-16.533	1.093	14.725	**150**	-17.115	1.092	17.147	**150**	-20.675	1.089	-0.642

KO13				KO14				KO15			
Time (min)	**D13C**	**%13C**	**%dose/hr**	**Time (min)**	**D13C**	**%13C**	**%dose/hr**	**Time (min)**	**D13C**	**%13C**	**%dose/hr**
0	-20.334	1.089	0.000	**0**	-20.816	1.088	0.000	**0**	-19.137	1.090	0.000
5	-18.325	1.091	9.628	**5**	-19.385	1.090	8.131	**5**	-14.173	1.096	25.768
10	-18.045	1.091	10.973	**10**	-18.786	1.091	11.530	**10**	-13.488	1.096	29.322
15	-17.816	1.092	12.066	**15**	-18.070	1.091	15.597	**15**	-13.896	1.096	27.206
20	-17.275	1.092	14.661	**20**	-17.548	1.092	18.564	**20**	-13.652	1.096	28.471
25	-16.993	1.093	16.011	**25**	-17.289	1.092	20.033	**25**	-13.600	1.096	28.740
30	-16.142	1.093	20.089	**30**	-17.311	1.092	19.910	**30**	-13.441	1.096	29.564
45	-16.931	1.093	16.308	**45**	-16.724	1.093	23.242	**45**	-13.189	1.097	30.876
60	-14.965	1.095	25.732	**60**	-15.747	1.094	28.790	**60**	-11.995	1.098	37.067
75	-11.952	1.098	40.172	**75**	-12.963	1.097	44.602	**75**	-9.624	1.101	49.372
90	-13.428	1.096	33.098	**90**	-12.172	1.098	49.093	**90**	-8.437	1.102	55.535
105	-12.338	1.098	38.322	**105**	-13.036	1.097	44.190	**105**	-9.593	1.101	49.535
120	-12.604	1.097	37.045	**120**	-13.115	1.097	43.739	**120**	-13.382	1.097	29.873
135	-13.048	1.097	34.917	**135**	-15.262	1.094	31.547	**135**	-16.523	1.093	13.570
150	-15.357	1.094	23.853	**150**	-16.042	1.094	27.116	**150**	-18.395	1.091	3.855

KO16				KO17			
Time (min)	**D13C**	**%13C**	**%dose/hr**	**Time (min)**	**D13C**	**%13C**	**%dose/hr**
0	-20.270	1.089	0.000	**0**	-20.655	1.089	0.000
5	-14.169	1.096	28.712	**5**	-20.223	1.089	2.801
10	-9.115	1.101	52.498	**10**	-19.962	1.089	4.490
15	-8.896	1.101	53.528	**15**	-19.552	1.090	7.150
20	-7.633	1.103	59.472	**20**	-18.048	1.091	16.895
25	-7.693	1.103	59.188	**25**	-16.191	1.093	28.920
30	-7.172	1.103	61.637	**30**	-15.002	1.095	36.627
45	-9.401	1.101	51.149	**45**	-12.884	1.097	50.347
60	-9.348	1.101	51.399	**60**	-12.027	1.098	55.898
75	-12.450	1.098	36.801	**75**	-10.641	1.100	64.877
90	-13.918	1.096	29.894	**90**	-9.396	1.101	72.941
105	-16.991	1.093	15.429	**105**	-11.650	1.098	58.339
120	-18.461	1.091	8.513	**120**	-14.223	1.096	41.673
135	-19.966	1.089	1.430	**135**	-14.956	1.095	36.920
150	-20.479	1.089	-0.986	**150**	-17.558	1.092	20.069

Appendix 6.2: Raw values for KO mice. At each time point a breath sample was taken. D13C is the raw δ value which is then converted to percentage of ^{13}C per hour (%13C) of the initial dose given (%dose/h). The calculation for each time point is described in detail Chapter 6, see methods.

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