Modulation of enteric neural activity and its influence on brain function and behaviour

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ABSTRACT

The gut brain axis (GBA) facilitates bidirectional communication between the enteric nervous system (ENS) of the gastrointestinal (GI) tract and the central nervous system (CNS). The location of the ENS along the GI tract enables it to serve as a relay station along the GBA. A key regulator of the GBA is the diverse population of microbial communities inhabiting the GI tract, also called the gut microbiota. Given the proximity of the gut microbiota to the ENS, gut microbes are considered to be an important contributory factor in influencing not only ENS functions, such as gut motility, but also brain function and behaviour. A diverse and healthy gut microbiota is key to normal GI physiology and good mental health. Exploring the physiological host factors that influence and control the gut microbiota is essential for understanding its variability in health and in states of dysbiosis. Movement of the luminal content along the GI tract, primarily driven by the rhythmic contractions of the GI smooth muscles, is one factor that has been shown to affect gut microbiota growth and population dynamics. In this thesis, a series of *ex vivo* and behavioural experiments were conducted in rodents to better understand control of gut motility by the ENS and determine its impact on anxiety related behaviour.

Initially, the effect of a specific pharmacological agent was evaluated on colonic motility patterns using *ex vivo* techniques (Chapter Two). Observations from this study provided fundamental insights into the mechanisms underlying ENS function and its regulation of colonic motility. Moreover, this study laid the foundation for further studies exploring the impact of altered colonic motility via ENS manipulation on gut microbiota composition (Chapter Three) and anxiety related behaviour (Chapter Four). In my second study (Chapter Three), I aimed to investigate whether the pharmacological modulation of the ENS, that resulted in reduced colonic motility patterns, as shown in Chapter Two, had any downstream effects on the gut microbiota. The results of this study revealed significant changes in gut microbiota composition following reduced colonic motility. Specifically, there was a decrease in the relative abundance of certain bacterial species, as well as alterations in overall community structure.

The third and final study of my PhD research aimed to provide a comprehensive understanding of the relationship between ENS manipulation and brain function and behaviour by inducing

alterations in gut motility. To evaluate anxiety related behaviour in rats I used open field and elevated plus maze tests. I evaluated anxiety related behaviour in rats that had been exposed to a pharmacological agent previously shown to slow colonic motility via activation of specific receptors in the ENS. To understand if any behaviour changes from ENS modulation involved modification of specific neural pathways, brain gene expression was also studied in key regions. To gain insight into possible GBA communication pathways, I also assessed whether there might be a relationship between the gut microbiota and brain function and behaviour. I investigated whether ENS modulation (and behavioural effects) were associated with changes in gene expression and microbiota profile in the large intestine. The findings from this study indicated that modulation of ENS function altered anxiety related behaviour in a sex specific manner with female rats showing anxiogenic effects with corresponding changes in brain and proximal colon gene expression compared to their male counterparts. This study not only shed light on the sexually dimorphic communication between the gut and brain but also suggested that multiple gene/pathways may be involved in influencing anxiety related behaviour in females.

This comprehensive exploration through three interrelated studies has provided new knowledge spanning the regional specificity of ENS receptors in relation to regulation of colonic motility (Chapter Two), the consequence of slowed gut transit on microbiota composition (Chapter Three), through to the overarching physiological consequences of ENS modulation on brain function and anxiety with associated sex differences (Chapter Four). A combined analysis of these new findings (Chapter 5) discusses their implications for our understanding of the ENS as a key player in regulating the gut brain axis.

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As I reach this significant milestone in my career, I am filled with overwhelming gratitude and a deep sense of humility. Completing this doctoral journey would not have been possible without the encouragement and well-wishes of numerous individuals who have significantly contributed to shaping my academic and personal growth.

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To my long-suffering wife, your infinite love, patience, and support have been my pillar of strength during the highs and lows of this journey. Your unwavering confidence in my capabilities and the sacrifices you made are beyond measure, and I feel truly fortunate to have you as my life partner.

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Peer-reviewed Articles

Parkar N, Dalziel JE, Spencer NJ, Janssen P, McNabb WC, Young W. Slowed gastrointestinal transit is associated with an altered caecal microbiota in an aged rat model. Frontiers in Cellular and Infection Microbiology. 2023;13. https://doi.org/10.3389/fcimb.2023.1139152

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Parkar N, Young W, Spencer N, Janssen P, McNabb W, Dalziel J. Sa1885 activation of μ opioid receptors in the gut influences anxiety related behaviour in female rats. Gastroenterology. 2023 May 1;164(6):S-475. Available from: https://doi.org/10.1016/s0016-5085(23)02099-1

Conference Proceedings

Parkar N, Young W, Spencer N, Janssen P, McNabb W, Dalziel J. Activation of μ opioid receptors in the gut influences anxiety related behaviour in female rats. Poster session presented at: Digestive Disease Week; 2023 May 6-9; Chicago, USA.

Parkar N, Dalziel J, Spencer N, Janssen P, McNabb W, Young W. Microbial composition is altered in a pharmacological model of slowed GI transit in aged rats. ePoster session presented at: Asian Pacific Digestive Week; 2021 Aug 19-22; Kuala Lumpur, Malaysia.

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4EPS	4-Ethylphenyl sulfate
5-HT	Serotonin
ACh	Acetylcholine
ACTH	Adrenocorticotropin hormone
ANCOM	Analysis of composition of microbiomes
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
ATP/ βNAD	Adenosine triphosphate/β-nicotinamide adenine dinucleotide
BBB	Blood brain barrier
CMC	Colonic motor complex
CNS	Central nervous system
CRH	Corticotropin releasing hormone
CRHR	Corticotropin releasing hormone receptor
CRS	Chronic restraint stress
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EEC	Enteroendocrine cell
EGC	Enteric glial cell
ENS	Enteric nervous system
EPM	Elevated plus maze
FDR	False discovery rate
FGID	Functional gastrointestinal disorder
GABA	Gamma aminobutyric acid
GB	Gut barrier
GBA	Gut brain axis
GF	Germ free
GI	Gastrointestinal tract
GSEA	Gene set enrichment analysis
HPA	Hypothalamic pituitary adrenal
IBS	Irritable bowel syndrome
ICC	Interstitial cells of Cajal
IFN	Interferons
IPAN	Intrinsic primary afferent neuron
ITS	Internal transcribed spacer
KEGG	Kyoto encyclopedia of genes and genomes
LPS	Lipopolysaccharide
MAG	Metagenome assembled genome
MyD88	Myeloid differentiation primary response 88
NF- κB	Nuclear factor kappa B
NO	Nitric oxide
NOD	Nucleotide binding oligomerisation domain
NTS	Nucleus tractus solitarius
OF	Open field
OTU	Operational taxonomic unit

PAMP	Pathogen associated microbial pattern
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PFC	Prefrontal cortex
PGN	Peptidoglycan
PRR	Pattern recognition receptor
PVN	Paraventricular nucleus
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SCFA	Short chain fatty acid
SEM	Standard error of mean
SIBO	Small intestinal bacterial overgrowth
SPF	Specific pathogen free
ST	Spatiotemporal
TAK-242	Resatorvid
TBI	Traumatic brain injury
TGR5	G protein coupled bile acid receptor
TLR	Toll like receptor
TRPA1	Transient receptor potential ankyrin A1
VIP	Vasoactive intestinal peptide
WGS	Whole genome shotgun

Units of measurement

^{o}C	Degree Celsius
μM	Micromolar
cm	Centimetre
g	Gram
h	Hour
mg/kg	Milligrams per kilogram
mm	Millimetre
mm/s	Millimetres per second
min	Minute
nM	Nanomolar
s	Second

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

In the nineteenth century, an emerging interest to understand the connections between the central nervous system (CNS) and the gastrointestinal (GI) tract was sparked by studies which showed that disturbances in emotional state altered GI functions.[1] One such study was by Cannon, who through his newly discovered technique for studying GI motility showed that cats confronted by growling dogs had changes in the flow of gut content, implying stress induced changes in intestinal motility.[2] Like Cannon, other prominent physiologists and psychologists have critically evaluated the influences of emotional state on GI function.[3-5] It was in the 1980s that the bidirectionality of brain to gut signalling became appreciated largely because of advances in neuroimaging technology. In these studies, it was shown that alterations in the GI tract (e.g., distension) led to key pathways being activated in the brain, implying gut to brain signalling.[6] This two way signalling linking the gut and brain is referred to as the gut brain axis (GBA).[7] The GBA monitors and regulates GI physiology and influences higher cognitive functions.

The gut has a functionally independent nervous system, the enteric nervous system (ENS). The discovery of the ENS in the mid nineteenth century led to a greater understanding of how the GI tract interacts with the brain.[8-10] The ENS lies within the walls of the GI tract and has been referred to as the 'brain in the gut' not only due to its similarities to the brain in terms of aspects such as neuronal components and neurotransmitters,[11,12] but also due to its ability to orchestrate intestinal behaviour independent of the CNS.[13] The significance of the ENS is highlighted by the life-threatening consequences that can occur if a component or segment of the ENS is missing, such as

that which occurs in Hirschsprung's Disease.[13] Similarly, the absence of ENS components in several other species such as rats and mice can also be lethal.[14]

The GI tract constantly monitors the dynamic microenvironment of the gut wall and its lumen.[15] This constant surveillance allows the GI tract to adapt and respond to a wide range of stimuli, including variations in nutrient availability, presence of harmful pathogens, and disturbances in the microbiota composition. The mammalian GI tract is colonised by a diverse population of microbial communities known as the gut microbiota.[16] Research in the past 15 – 20 years has established a crucial role for the gut microbiota in regulating gut and brain function and as a result the term GBA has been expanded into microbiota gut brain axis. The gut microbiome has been demonstrated to influence the development and functioning of both the ENS and CNS.[16] Alterations in ENS neurochemistry and function by the gut microbiome have been highlighted in numerous studies using various animal models. For example, in germ free (GF) animals (specially-raised animals devoid of all microorganisms) a reduced number of enteric neurons were observed that corelated to abnormal GI motility.[17,18] In addition, changes in microbiota composition or dysbiosis have been shown to influence cognitive function, mood and behaviour.[19,20] Altering the microbiota composition through diet and pharmacological agents is therefore becoming sought after as a potential strategy to manage GI and mental health. However, a mechanistic understanding of how gut microbiota influences both the ENS and CNS is still lacking. Nevertheless, efforts are being made to identify potential signalling pathways between microbiota, gut and brain.

Studies focussed on the gut microbiome are a starting point in answering the question of how gut derived neural signals can affect brain function. The location of the ENS along the GI tract enables it to serve as a relay station along the GBA. Communication between the ENS and CNS provides opportunities for gut luminal factors (e.g., diet, microbial metabolites) to influence not only local

gut function but also the CNS. Therefore, understanding these communication networks along the GBA is important.

This chapter summarises the current knowledge regarding our understanding of the underlying mechanisms of GI motility and stress within the context of gut brain axis research. The role of microbiota in influencing GI motility and stress will also be discussed.

1.2. Gut-Brain axis (GBA)

The GBA is a term that refers to the bidirectional crosstalk between the GI tract and brain. This bidirectional signalling network that links the ENS and CNS not only regulates normal GI functions but also plays a key role in shaping higher cognitive functions via hormonal, immunological and neural routes of communication.[21,22]

Section 1.2 will discuss the concept of GBA, highlighting the structure of the ENS and its role in GI motility. The second half of this section focusses on the role of stress within the context of gut-brain interactions.

1.2.1. Enteric nervous system (ENS): Structure

The gut wall is organised in layers consisting of the lumen, mucosal layer, submucosal layer, muscularis and serosa (***Fig. 1***). The muscularis comprises of an inner circular muscle layer and an outer longitudinal muscle layer. The ENS consists of two major nerve networks (also referred to as plexus): myenteric (or Auerbach's) and submucosal (or Meissner's) plexus.[23] These plexi are characterised by many bundles of nerve processes called ganglia, each containing neuronal cell bodies and glial cells.[24] The gut wall is innervated by enteric nerve fibres, with inputs to and from different intestinal layers including the mucosa.[25] The submucosal plexus is positioned within the

submucosal layer and plays a key role in regulating GI blood flow and controlling epithelial cell function and secretion.[26] The myenteric plexus sits between the longitudinal and circular muscle layers and mainly regulates the contraction and relaxation of these two muscle layers. Both plexi communicate with each other through axons to form a functionally coordinated unit.[27]

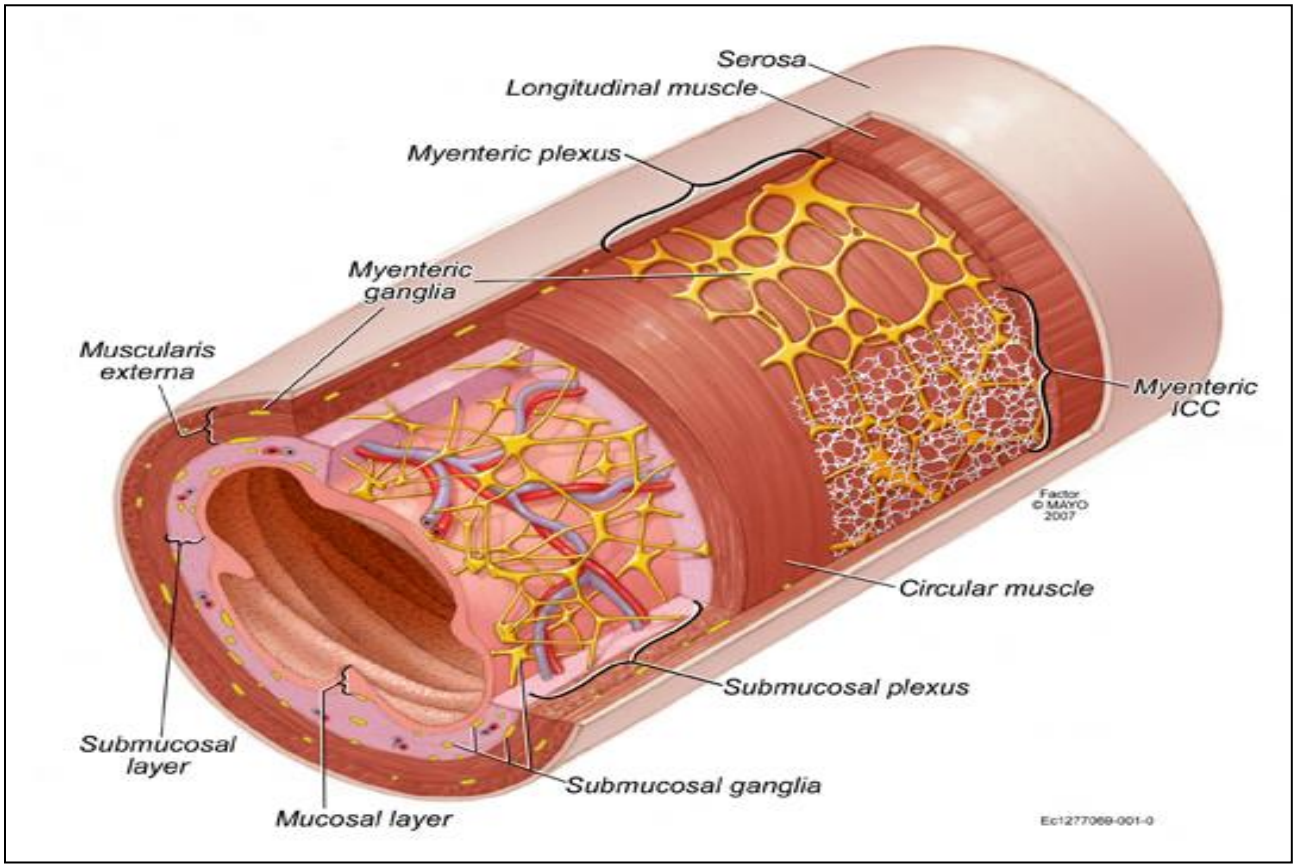


Figure 1: *Structural framework of the gut wall encompassing the various elements of the enteric nervous system.*[28]

1.2.1.1 ENS composition and function

The ENS mediates important GI functions such as propulsion of food content, regulation of fluid exchange and immunological defence.[8] Although the ENS receives regulatory signals from the CNS, it can function independently because of its expansive network of neuronal cell bodies (***Fig. 2***).[23] The ENS contains distinct types of neurons namely sensory neurons, interneurons and motor

neurons.[29] These neurons are supported by glial cells. Enteric neurons contain and release various chemical messenger substances such as neurotransmitters.[30] These neurons express various receptors to these chemical messenger substances which, upon activation contribute to neurotransmission. For example, nicotinic acetylcholine receptors localised in myenteric neurons mediate synaptic excitation upon activation by acetylcholine.

Sensory neurons such as intrinsic primary afferent neurons (IPANs), mostly positioned in the myenteric and submucosal plexi, sense the intestinal physical state. They also extend projections to mucosal epithelium. IPANs are mostly polymodal, i.e., they respond to both chemical and mechanical stimulation of the mucosa.[31] IPANs contribute to the intrinsic reflex circuitry that regulates intestinal blood flow, water and electrolyte secretion, and motility.

Motor neurons provide innervation to the longitudinal and circular muscle layers. Excitatory motor neurons use acetylcholine to induce contraction of smooth muscle. On the other hand, inhibitory motor neurons use neurotransmitters such as vasoactive intestinal peptide (VIP) and nitric oxide (NO) to evoke muscle relaxation. The collective work of these motor neurons enables rhythmic circular and longitudinal muscle layer activity or peristalsis that aids in moving contents along the GI tract.

A diverse group of specialised cells referred to as the interstitial cells of Cajal (ICC) have been identified as the pacemaker cells of the GI tract because of their ability to initiate electrical rhythmic slow waves within the muscle layers of the ENS.[32] ICCs represent another important control system mediating initiation of neurotransmission from intestinal motor neurons towards the

intestinal muscle cells and regulating coordination of contractile activity of intestinal smooth muscle.[33]

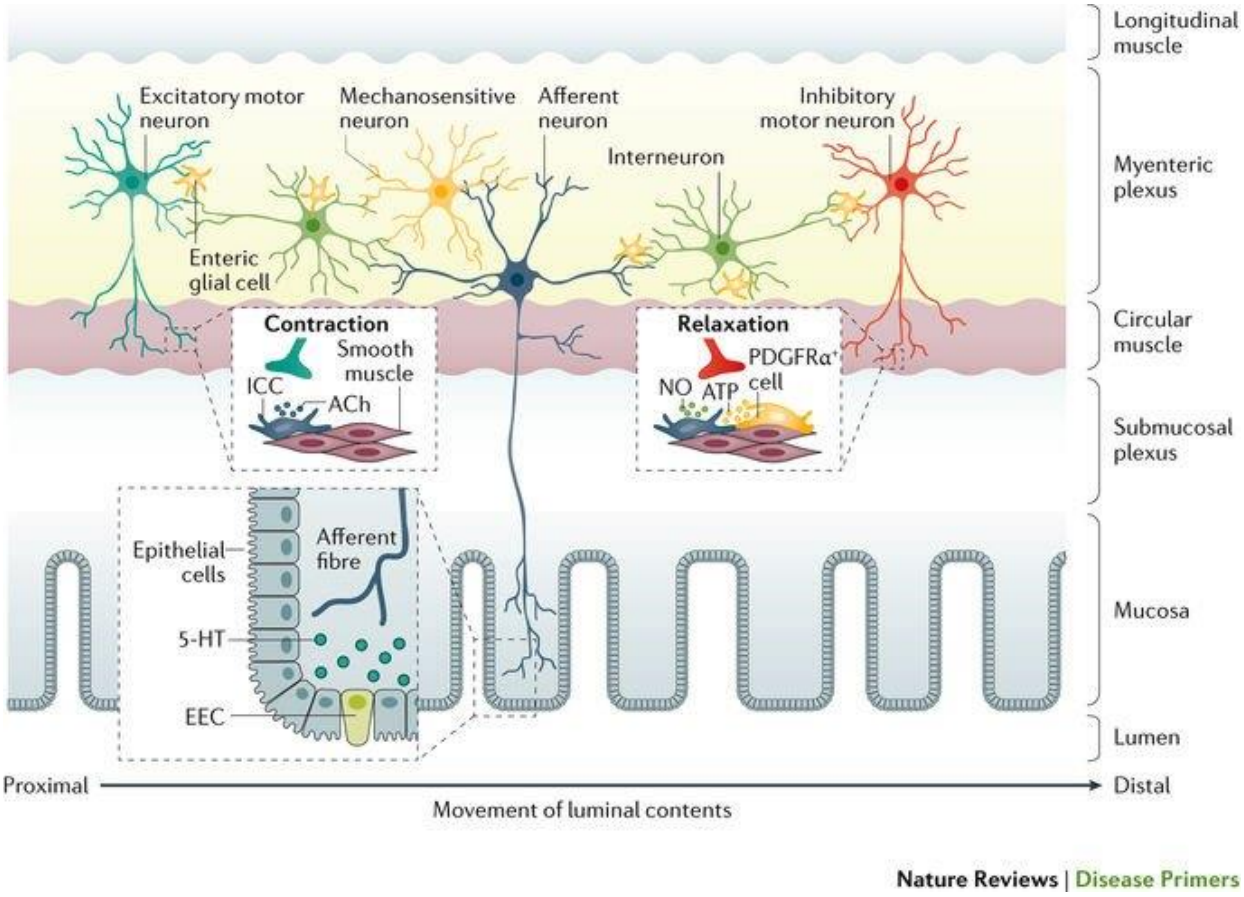


Figure 2: *Neuronal components of the ENS.[34] EEC – enteroendocrine cells; EGCs – enteric glial cells; IPANs – intrinsic primary afferent neurons; ICCs – interstitial cells of Cajal; ACh – acetylcholine; NO – nitric oxide.*

1.2.1.2. Intestinal motility patterns

An important aspect of digestion is the controlled progression of luminal contents along the GI tract which is coordinated by various patterns of intestinal muscle movement that occur as a result of the interplay between the ENS and ICC.[35-37] The two fundamental mechanisms underlying controlled intestinal muscle movements include myogenic mechanisms, which involve ICCs and smooth muscle cells, and neurogenic mechanisms, which involve the enteric neural circuits embedded

within the gut wall.[38] The ENS's unique characteristic of regulating intestinal movements independently of the CNS is not fully understood and the role of enteric neuronal networks in facilitating motility in isolated segments of intestine requires further investigation.[39] From studies investigating the cellular basis of intestinal motility, it has been strongly suggested that the fundamental mechanisms of motility patterns are preserved across humans and other mammalian species.[38] The mouse colon has served as an excellent model species for investigating intrinsic mechanisms underlying intestinal motility.[40,41] Moreover, its small size makes it feasible to remove the entire intestine or segments of the intestine enabling testing of interventions and recording data in a highly controlled lab setting.[38]

In the small intestine, two fundamental motor patterns have been described: peristalsis or propagating contractile complexes, which aid in the propulsion of luminal contents in the aboral direction, and segmentation which causes mixing to facilitate nutrient and water absorption.[42,43] Both motor patterns have been shown to be orchestrated by enteric neural circuits.[42,44] However, a study by Huizinga and colleagues using mouse small intestine demonstrated segmentation to occur even after exposure to the nerve toxin, tetrodotoxin, implying myogenic control involves two ICC networks in coordinating segmentation motor activity.[37] Although there have been some inconsistencies between study findings with regards to the mechanisms underlying coordinated intestinal movements there is a certain agreement that the neurogenic and myogenic systems operate together to coordinate intestinal motility.

The large intestine exhibits a rich variety of motor patterns, especially the proximal colon where the densities and neurochemical profiles of myenteric neurons are found to be higher compared to the distal colon.[45] One pattern that has been studied extensively in various experimental animals is the

colonic motor complex (CMC).[46-49] For example, it has been shown that CMCs are the predominant mechanical motor pattern of the mouse colon.[50] CMCs are neurally mediated spontaneous propagating contraction of the colonic smooth muscles that propagate over longer distances and aid in the propulsion of luminal contents.[51] When exposed to tetrodotoxin, or an antagonist of nicotinic fast synaptic transmission in the ENS, such as hexamethonium, their activity stops immediately, implying neural origin.[49,52]

The characteristics of CMCs are influenced by the presence of content in the large intestine and the extent of circumferential stretching exerted on the colon wall.[53] For example. Barnes and colleagues demonstrated that increasing the circumferential stretch of the mouse colon increases the frequency of CMCs.[53] The mechanism by which initiation of content dependent neural peristalsis and of CMCs have also been investigated in studies using mouse colon *in vitro*. Keating and Spencer through their experiments showed that the enteric neuronal networks responsible for CMC generation are located in the myenteric plexus and muscularis externa.[54]

Myogenic motor activity generated by ICCs have also been shown to play a role in coordinating colonic motility. Studies by Chen and colleagues[55], and Huizinga and colleagues[56] suggested that the myogenic system may be an important component facilitating peristaltic contractions in the rat colon. Costa and colleagues confirmed these findings in the rat and rabbit colon; however, their study did not detect such myogenic mechanisms in mouse and guinea pig colon.[57] It was concluded that in some species, myogenic activity is capable of being propulsive and it is likely that both neurogenic and myogenic activities work in tandem to create colonic motility patterns. Although the ability of myogenic activity in propelling luminal contents has been detected in some species, in

general under healthy conditions, myogenic mechanisms are not sufficient (nor required) for significant propulsion of colonic contents and it is evident that the ENS is essential for survival.[58]

1.2.1.2.1 Techniques to quantify and analyse intestinal motility patterns

The distinctive feature of the ENS in controlling intestinal movements autonomously means that this ability is retained when the intestine is excised and maintained in a tissue bath. This enables the study of enteric neuronal circuits and their role in regulating motility in isolated segments of the intestine. Analysing the movements of the intestinal wall and luminal content in muscle mechanics presents a considerable challenge due to the spontaneous nature of the movements throughout the entire gut. Nonetheless, advancements in video recording and spatiotemporal (ST) mapping techniques have made it feasible to visually represent, describe, and study intricate motility patterns in *ex vivo* whole segments of the small intestine and colon. High definition video imaging of the intestines allows for the measurement of changes in intestinal diameter. It has become possible to create maps (Diameter maps - DMaps) that show changes in diameter across the length of the isolated intestinal segments in real time (***Fig. 3***)[59,60] These maps offer a straightforward, easily interpretable visual representation of motility over time ranging from a few seconds to several minutes. The dynamic nature of GI motility patterns and the use of video imaging techniques has not only facilitated detailed descriptions of motor patterns on isolated intestinal preparations of several experimental mammalian species but has also enabled the analysis of functional alterations in response to pharmacological agents.[41,57,61,62]

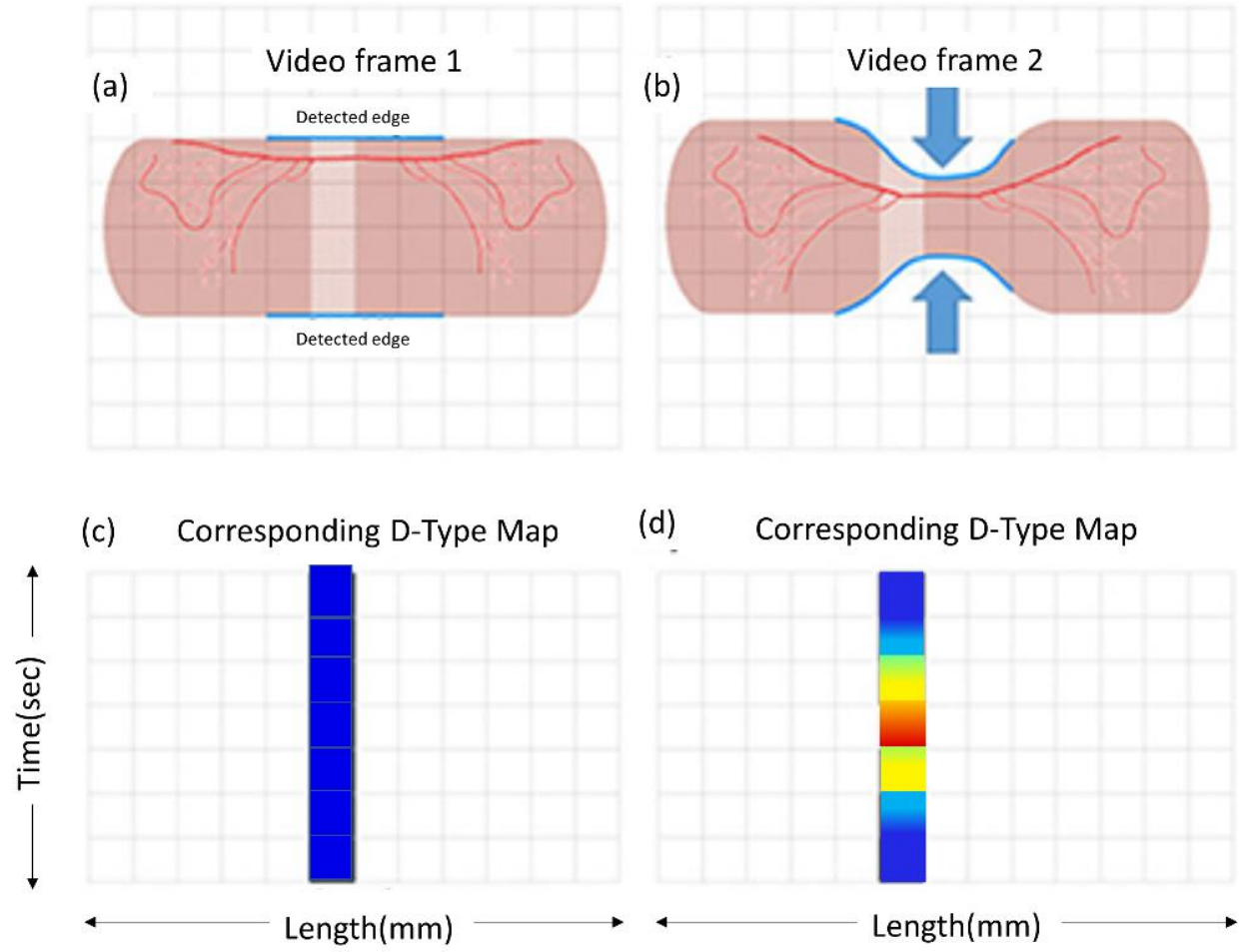


Figure 3: *Fundamental approach employed in diameter (D type) spatiotemporal maps. (Image adapted; licensed under CC BY 4.0)*[63] *(a) The boundaries of an intestinal segment (pink) are identified (blue line) and the number of pixels is quantified between the edges of consecutive columns within the shaded region (pale area). (b) In frame 2, a circumferential contraction causing a reduction in the diametric profile is shown. (c) The quantified pixels are assigned specific colours to correspond with each column in every frame. Frame 1 of the time series of this DMap displays blue squares indicating relaxation state. (d) Red square indicates maximum contraction in the time series of the DMap.*

1.2.2. Stress-Gut-Brain axis communication network

According to Hans Selye, stress is defined as 'any threat to the homeostasis of an organism, be it real (physical) or perceived (psychological), which may be posed by events in the outside world or

from within'.[64] Stress can trigger anxiety and depression related behaviours.[65,66] The hypothalamic-pituitary-adrenal (HPA) axis that consists of the hypothalamus, pituitary gland and the adrenal gland is considered a crucial endocrine mediator in brain to gut interactions that control adaptive responses to stress.[67] In contrast, the amygdala, hippocampus and the prefrontal cortex (PFC) are the main brain regions that regulate the HPA stress response.[68] Activation of neural circuits in these regions during stress stimulates the hypothalamus to induce an endocrine stress response.[69] The median paraventricular nucleus (PVN) of the hypothalamus synthesises corticotropin releasing hormone (CRH) that is delivered to the pituitary. Stimulation of the pituitary releases adrenocorticotropin hormone (ACTH) into the bloodstream which then acts on the adrenal cortex, where the secretion of glucocorticoids (cortisol in humans and corticosterone in rodents) occurs. Cortisol has a range of systemic effects which can be beneficial during situations of acute stress.[70] In addition to the hypothalamus, the central nucleus of the amygdala also synthesises CRH in response to psychological stress.[71]

Animal studies suggest that stress can modulate GI function via the ENS.[72,73] For example, in a stress induced rat model (chronic restraint stress), enhanced intestinal propulsive activity and increased intestinal mucosal secretion was observed.[72] Upon further analysis it was demonstrated that these changes induced by stress were because of alterations in the secretomotor and musculomotor neurons, found in the submucosal plexus and myenteric plexus of the ENS, respectively. This study shows that changes in the ENS reflect abnormal GI function in response to stress. Another study has shown that the impact of acute stress on GI physiology is mediated by CRH via activation of CRH receptors in the brain.[74]

In order to focus on how gut derived signals influence brain function via the ENS, the role of the vagus nerve is often studied in rodent models.[75] For example, a study by Klarer and colleagues demonstrated that disruption of vagal afferent signalling from the gut to the CNS via surgical disconnection of vagal afferents (subdiaphragmatic vagal deafferentation; SDA) reduced anxiety-like behaviour and enhanced cognitive flexibility in rats.[76] In addition, a growing body of experimental data from animal studies focussed on the enteric microbiome provide further support that vagus nerve integrity is important for mediating anxiolytic effects.[77,78] A more detailed discussion on the role of microbiota in anxiety and stress responsivity highlighting gut to brain signalling is discussed in ***section 1.3.2.4.***

1.2.2.1 Sex differences in stress responses

Understanding the development of stress related behaviour requires careful consideration of the role played by sex differences in the GBA. HPA response variability may explain some of the sexually dimorphic changes in behavioural response to stress.[79] For instance, female rats respond more strongly to both physical and psychological stressors in terms of their endocrine system function, and their average glucocorticoid concentration is higher than that of male rats.[80] Gonadal steroid hormones are thought to function in various parts of the brain to regulate HPA negative feedback.[79,81] Gaskin and colleagues in their study showed that prepubertally castrated males exposed to ether stress had a much higher level of circulating glucocorticoids than control (intact) males.[82] However, when exogenous testosterone was given, glucocorticoid levels returned to levels of control males. Handa and colleagues went a step further and showed that testosterone not only diminished the release of glucocorticoids into the bloodstream during stress but also lowered the levels of circulating ACTH, potentially indicating impaired receptor mediated negative feedback within the hypothalamus.[79] Interestingly, the PVN of the hypothalamus expresses both androgen

and estrogen receptors, further suggesting that sex hormones may function at the level of the hypothalamus to control the production of glucocorticoids.[83] Moreover, studies have shown CRH receptors to be co-localised with estrogen receptors in the hypothalamus, raising the possibility of a neuroendocrine interaction between CRH signalling pathways and estrogens.[84]

1.2.2.2 Behavioural assays to study anxiety in rodents

Charles Darwin's early discovery that animals and humans have comparable traits in the display of emotions raises the prospect of researching the aetiology of stress related disorders in other mammals.[85] Several animal models and behavioural assays have been developed in an attempt to simulate human pathological anxiety. For example, experimental models using neonatal maternal separation, acute restraint stress and water avoidance stress as stressors have been developed to replicate irritable bowel syndrome (IBS) symptoms in animals to investigate cellular mechanisms responsible for gut brain axis dysfunction.[86] Behavioural based tests using rodents are widely used to understand the mechanisms by which a stressful stimuli triggers anxiety related behaviour.[87] The notion that fundamental physiological mechanisms responsible for generating fear and anxiety in rodents may be compared to those similar in humans gives these behavioural tests a degree of apparent validity.[88] It is important to keep in mind that many of these behavioural tests have been developed and refined to maximise sensitivity to anti-depressive and anxiolytic pharmacological treatments rather than to gain insights to the signs and symptoms of anxiety in humans. As a result, they are more appropriately thought of as assays of drug effects than as models of anxiety and depression. Moreover, the use of rodents in behavioural research gives researchers the ability to explore the neurochemistry and neuroanatomy associated with stress related behaviour that may also provide valuable insights into potential targets for the treatment of anxiety.[89]

To determine the level of anxiety or depression induced by various stress-inducing protocols, a variety of behavioural tests have been developed that pose an acute stress challenge. Anxiety related behaviours recorded from behavioural tests can be defined as behavioural and physiological measures evoked by aversive stimuli that could be of short duration.[90] Several of these behavioural assays incorporate an approach-avoidance conflict aimed to hinder an ongoing animal behaviour.[90] These approach-avoidance conflict paradigms allow the assessment of anxiety related behaviour in experimental animals through the examination of the conflict between motivations to approach and responses of avoidance or fear. The list of these behavioural tests is extensive and is beyond the scope of this thesis. Listed below is a brief overview of the two commonly used rodent behavioural assays.

Open field (OF) test

The OF test is the most commonly used behavioural test which places a rodent in a walled arena 90 cm in diameter. It is designed to exploit conflict between the normal tendency of rodents to engage in exploratory activity versus avoiding the unprotected centre of the arena.[87,91] It consists of an enclosed circular space which is brightly illuminated (***Fig. 4***). Experimental animals will normally spend more time exploring the area close to the wall (thigmotaxis), than the exposed centre area. Animals that spend more time exploring the exposed centre region exhibit reduced anxiety like behaviour. Overall, the OF test is a valuable tool for evaluating locomotor activity, exploration of novel environments, and serves as an initial screening method to assess anxiety related behaviours in experimental animals.[91]

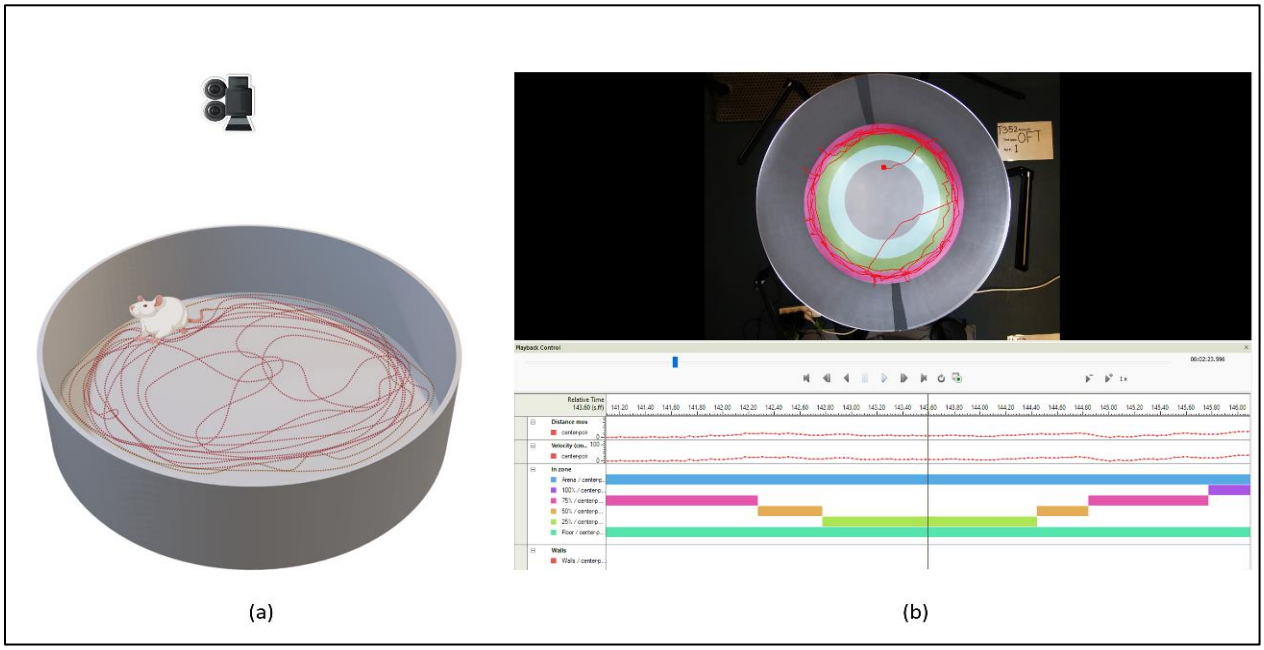
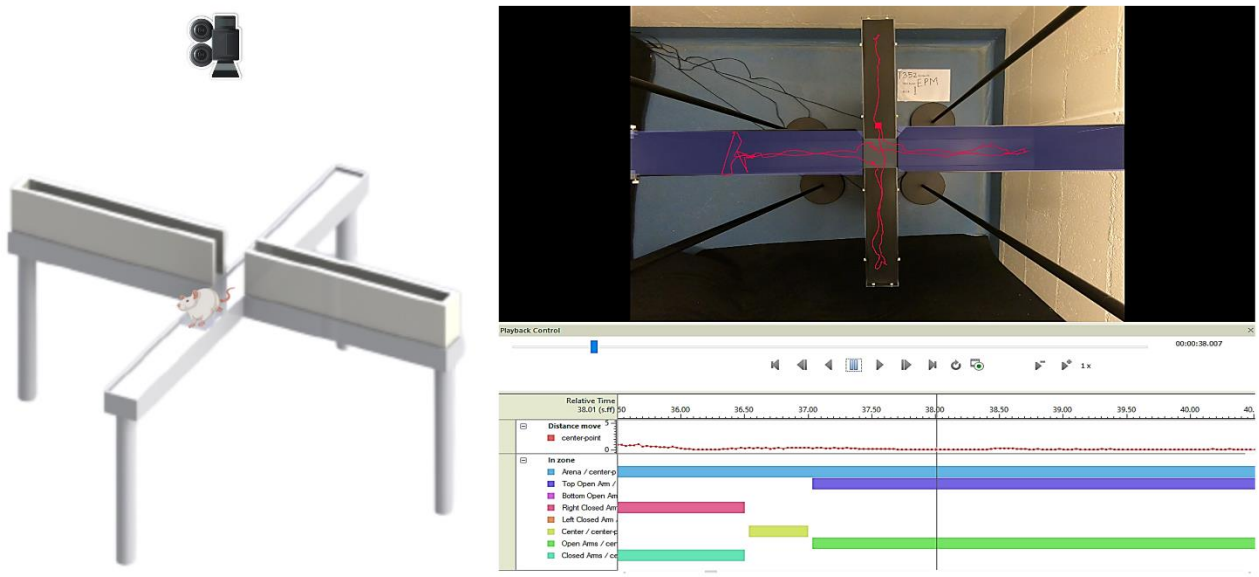


Figure 4: *Representative diagram of the open field test showing (a) Tracked movement (red lines) of experimental animal in the open field apparatus. (b) Screenshot of the automated video tracking software that is showing the path travelled by the experimental animal (above). The video tracking software detects the centre point (small red square) of the experimental animal and automatically scores anxiety-like parameters (below).*

Elevated plus maze (EPM)

The EPM is another well used approach-avoidant behavioural test in which environmental stimuli may be considered as threatening by the experimental animal.[92,93] The EPM consists of a plus shaped maze, with two opposing open arms and two opposing enclosed arms, elevated from the ground (***Fig. 5***). The test takes advantage of the natural preferences of the experimental animal to explore dark/enclosed spaces as opposed to the open, brightly lit elevated areas. This approach-avoidance conflict leads to actions that have been linked to increased levels of physiological stress markers. Parameters analysed such as increased frequency and time spent in the open arms are interpreted as the experimental animal showing lower anxiety levels.



(a) (b)

Figure 5: *Representative diagram of the elevated plus maze (EPM) test showing (a) Experimental animal in the open arm of the EPM apparatus. (b) Screenshot of the automated video tracking software that is showing the path travelled (red lines) by the experimental animal. The blue highlighted (above) area shows the enclosed arms of the EPM. The video tracking software detects the centre point (small red square) of the experimental animal and automatically scores anxiety-like parameters (below).*

1.3. Gut microbiota

The mammalian GI tract is colonised by a diverse population of microbial communities consisting of bacteria, but also viruses, archaea, helminth parasites, yeasts, and protozoa.[94] The diversity and abundance of these microbial communities is influenced by the nutritional, immunological and chemical profile along the GI tract. Each species has evolved to execute specialised roles in designated GI tract environmental niches. The importance of these microbial communities in maintaining physiologic homoeostasis and in modulating disease processes has become a topic of widespread interest over the past several decades. In order to understand the gut microbiota's role as a causative or correlative factor in human illnesses, small animal models have been developed.[95-

[97] For example, specially raised mice devoid of all microorganisms known as germ free (GF) mice have been used to investigate how manipulation of the microbiota affects the GBA.[97] Another approach is through the use of antibiotics which can deplete the prevalence of specific bacteria or the whole gut bacterial microbiome.[98] Given their extensive similarities to humans in terms of physiology, genetics and anatomy, and the feasibility of microbiota manipulation, murine models are utilised more often in gut microbiota research to investigate the function and mechanisms of the gut microbiota and its association with human pathologies.[99]

The mammalian gut microbial community consists of five major phyla which include Proteobacteria, Verrucomicrobia, Actinobacteria, Bacteroidetes and Firmicutes.[100] However, inter-species differences among mammals in terms of relative abundance and diversity on species level have been observed.[100] In general, Bacteroidetes and Firmicutes are the two major bacterial phyla that dominate the gut microbiota of humans and mice.[101,102] It has also been shown that humanised rat models have a similar Firmicutes:Bacteroidetes ratio to human donors.[103] The Bacteroidetes phylum includes the key genera *Prevotella*, *Bacteroides*, and *Xylanibacter*, which are recognised to be efficient degraders of dietary fibre.[104] Firmicutes members include the butyrate producers *Roseburia, Faecalibacterium* and *Eubacterium,* as well as the genera *Ruminococcus, Lactobacillus* and *Clostridium*.[104] A study by Nguyen and colleagues[99] identified about 79 genera that are shared between humans and mice, very similar to the 80 genera identified in a study by Krych and colleagues.[105] The authors observed that *Mucispirillum* belonging to the phylum *Deferribacteres* was the only genus found to be exclusive to mice, whereas multiple genera namely *Megasphera, Paraprevotella, Mitsuokellla, Succinivibio, Phascolarctobacterium, Dialister, Faecalibacterium*, *Sutterella* and *Asteroleplasma* were found to be present exclusively in humans.[105] However, *Faecalibacterium* was detected in the mouse gut microbiota in several other studies.[106,107] Likely

explanations for these observed inconsistencies include differences in analysis methods, databases used, sequencing depth, and constant updates on microbial phylogeny and nomenclature. Although absolute comparisons between murine models and human gut microbiota may be challenging, murine models are useful for investigating the processes that cause microbiota variation and alterations in response to perturbation, which may then be approximated to humans.

1.3.1. Techniques to measure the microbiome

Investigation of the microbiome regularly involves the use of bioinformatic and sequencing approaches. Our knowledge of the diversity, abundance and functional properties of the microorganisms constituting the microbiome has been greatly improved by the use of next-generation sequencing, which offers a snapshot of the microbial DNA and RNA present in a community. Bioinformatics, which refers to the application of analytic and computational tools to store and interpret complex biological data, has played an integral role within the microbiome research space as it enables the efficient processing of massive amounts of microbiome data.[108] Bioinformatics combined with sequencing technology is central to the analysis of microbiome data. Two sequencing techniques namely whole genome shotgun sequencing (WGS) and amplicon sequencing have been developed which have enabled researchers to gain a deeper understanding of the gut microbiome.[109,110] Amplicon sequencing of specific genetic regions, e.g., the 16S rRNA gene (16S) in bacteria or the nuclear ribosomal internal transcribed spacer (ITS) region in fungi, provide a reasonably affordable method to assess the relative abundance of microbial communities in a sample. This method is based on the notion of amplifying a genetic sequence that is present across all bacterial members of the microbiota, but which varies according to taxonomy, using polymerase chain reaction (PCR). In reality though, no single genetic region is able to resolve all differences between taxa. The relative abundance of these amplified sequences, or amplicons, in a

sample may then be estimated by grouping them according to how closely they are related genetically. In contrast to amplicon sequencing, WGS is more costly and resource intensive, however, this method can provide information on microbiota abundance and functional capability, and in some circumstances can provide strain-level identifications.[111] Using WGS, all of the DNA in a sample is analysed as opposed to only analysing specific marker genes. The sequences are then aligned to reference databases for taxonomic and functional identifications. Furthermore, sequences can also be assembled to create whole genomes, commonly referred to as "metagenome assembled genomes" (MAGs).

1.3.1.1 Metrics for microbiome data description

When analysing microbiome data, it is important to acknowledge that both amplicon sequencing and whole-genome sequencing yield compositional data, which represent quantitative information about the proportions between different components of the whole, providing relative information.[112] Comparing relative abundance is usually the most straightforward way to compare the presence of specific microbes in two samples.

Microbiome diversity is used primarily to describe the degree of variability within a sample or to quantify how different two samples are from one another. There are two types of diversity measurements usually used to describe a microbial community.[113] Alpha diversity represents the distribution of species abundances within a sample, summarized into a single value that is dependent on species richness and evenness. On the other hand, beta diversity quantifies similarity or dissimilarity between communities (samples).

In order to visualise high dimensional data such as microbiome data, which may consist of hundreds of different species or tens of thousands of genes, a multidimensional scaling method such as principal coordinates analysis (PCoA) is commonly used.[114] In PCoA, variables are transformed into a similarity matrix and dimensions that account for the maximum distances between samples are extracted as coordinates. The obtained coordinates can be utilised to graphically represent each sample as a point in either two-dimensional or three-dimensional space, employing the principal coordinates as the axes. In the resulting plot, samples that are in close proximity to each other indicate a higher degree of compositional similarity.

1.3.2. Microbiota-Gut-Brain axis

Data from clinical and pre-clinical studies show that the gut microbiota has a significant influence on the GBA, interacting not only with the ENS and ICCs but also with the CNS via immune, neuronal and endocrine pathways. The importance of gut microbiota in regulating the GBA stems from the fact that bacterial colonisation in the gut is critical for the development and maturation of both the ENS and CNS.[115,116] Moreover, numerous experimental approaches using animal models such as GF animals, antibiotics, probiotics and infection studies have been used to investigate how manipulation of the gut microbiota affects the GBA including the ENS.[97,117] For example, studies using GF animals have shown a wide range of physiological functions to be altered ranging from gut sensory motor functions to higher cognitive functions.

This section provides an overview of the expanding literature characterising the role of gut microbiota in the GBA.

1.3.2.1 Influence of microbiota on ENS components and function

Studies using GF animals have shown that lack of bacterial colonisation is linked to alteration of ENS components and functions such as slowed GI transit.[118,119] Moreover, studies have shown that development of ENS can be influenced by the gut microbiota.[120,121] In a study by Collins and colleagues, it has been shown that the development of ENS in early life is influenced by the gut microbiota. Using immunohistochemistry techniques, the authors showed that GF mice on postnatal day 3 had a structurally abnormal myenteric plexus in the jejunum and ileum of the small intestine compared to dams conventionalised with a simplified microbiota or to offspring born in a specific pathogen free (SPF) environment. Moreover, the authors showed that these abnormal myenteric plexi in GF mice were characterised by decreased neuronal density, reduced number of neurons per ganglion, and an increased proportional expression of myenteric nitrergic neurons, corresponding to abnormal intestinal motility patterns.[120] Whilst this study did not extend its analysis further into adulthood or postnatal stages of development, in another study it was shown that 4 week old GF mice exhibited decreased expression of myenteric nitrergic neurons.[121] Husebye and colleagues demonstrated using GF rats that lack of microbial colonisation significantly decreased contractile activity of the small intestine and slowed GI transit, which was partially reversed when these rats were colonised with *Lactobacillus acidophilus* together with *Bifidobacterium bifidum*.[118] In another study it was shown that conventionalisation of GF mice with the bacterium *Bacteroides thetaiotamicron* restored the expression of excitatory and inhibitory motor neuron subpopulations.[122] The role of gut microbiota in regulating gut motility also comes from studies that have used antibiotics as a tool to deplete the gut microbiota. A study by Obata and colleagues showed that mice treated with antibiotics showed a significant increase in intestinal transit time.[123] Similarly, Ge and colleagues showed antibiotics-treated mice to have decreased intestinal motility, with prolonged GI and colonic transit, and inhibition of contraction of the proximal colon

longitudinal muscles.[124] Collectively, these studies provide invaluable information on the importance of gut microbiota in control of intestinal motility.

As mentioned in ***section 1.2.1.2***, ICCs have shown to play an important role in coordinating intestinal motility. The gut microbiota has been shown to influence ICCs. For example, a study by Zhang and colleagues who used a traumatic brain injury (TBI) mouse model demonstrated that administration of a probiotic mixture which consisted of four different lactic acid bacteria (*Pediococcus pentosaceus 16:1, Lactobacillus casei ssp. paracasei 19, Leuconostoc raffinolactis 23~77:1 and Lactobacillus plantarum 2362)* improved the structure and density of ICCs improving contractile activity of the small intestine.[125] In a TBI mouse model, the functionality of the intestinal smooth muscle contractility is compromised, resulting in decreased defecation and body weight. In another study, Sui and colleagues showed that the probiotic strain *Clostridium butyricum* promoted gastrointestinal motility by regulating ICC proliferation.[126] Although, studies identifying the role of gut microbiota in influencing ICC structure and function are still in their infancy, there is some evidence that gut microbiota may impact ICCs controlling intestinal motility.

1.3.2.2 Role of Toll-like receptors (TLRs) in gut motility

The role of gut microbiota in modulating GI motility is supported by several studies focussed on TLRs located in the GI tract. These transmembrane receptors have an important role in the innate immune response by recognising pathogen associated microbial patterns (PAMPs) consisting of microbial cell wall derived components.[127] For this reason, TLRs are referred to as pattern recognition receptors (PRRs). TLRs recognise a variety of PAMPs that include bacterial cell wall components such as lipopolysaccharide (LPS), peptidoglycan (PGN), and bacterial DNA and viral double-stranded RNA.[127] The TLRs are a diverse family of receptors that are specific to different

components. For example, TLR4 detects lipopolysaccharides (LPS), a cell wall component of gram-negative bacteria and binding to TLR4 leads to downstream activation of immune pathways via nuclear factor – kappa B (NF- κB).[127] While in humans only ten have been discovered (TLR1 to TLR10), twelve have been identified in mice (TLR1 to TLR9, TLR11, TLR12 and TLR13). TLRs are primarily expressed in various intestinal epithelial and immune cells. Experiments have shown that they are also expressed in neurons of the ENS.[128] Most reports examining the roles of TLR in motility have studied TLR2 and TLR4.

Functionally, mice deficient in TLR4 exhibited delayed intestinal motility characterised by reduced colonic relaxation which was found to be associated with a significant decrease in ENS nitrergic inhibitory neurons compared to controls.[121] These changes were also seen in both antibiotic treated and GF mice in the same study.[121] Furthermore, similar changes were also observed in mice deficient in ENS specific MYD88, which is an adaptor molecule important for downstream TLR4 signalling,[121] Overall, this study has highlighted the relationship between bacteria and TLR4 signalling pathways in influencing intestinal motility via the ENS.

1.3.2.3 Role of gut microbial metabolites on gut motility

The production of metabolites or other end products of bacterial fermentation is another way by which the gut microbiota can affect gut motility (***Table 1***). Short chain fatty acids (SCFAs) and tryptophan metabolites are two major categories of bacterial products that have been extensively researched in connection to altered gut motility.

SCFAs can alter gut contractility as evidenced in an *in vitro* study where propionate, butyrate, and valerate initiated phasic contractions in the mid and distal rat colon.[129] Similarly, in a study by Soret

and colleagues, the rat enteric nervous system was shown to be affected by a resistant starch diet, intracaecal butyrate infusion, and butyrate application to cultured myenteric ganglia, which resulted in an increase in the proportion of cholinergic neurones that led to an increase in colonic circular muscle contractility and shorter colonic transit time.[130] In another study, Fukumoto and colleagues highlighted the role of SCFAs in inducing intestinal propulsive movement in rats.[131] In contrast, a study by Cherbut and colleagues showed that SCFA infused into the rat colon *in vivo* reduced colonic motility thereby increasing intestinal transit time.[132] These discrepancies between studies could be because of differences in experimental methods, animal models and the nature and form of SCFA used.

Table 1: *Examples of published literature evidence of gut microbial metabolites shown to impact gut motility.*

Authors	**Bacterial metabolic product**	**Impact on intestinal motility**	**Experimental technique**	**Model**
Hurst et al.[133]	SCFAs (Butyrate, propionate, acetate)	Full length propagating contractions in the proximal colon increased upon butyrate administration, while propionate inhibited them, and acetate reduced the frequency of propagations.	Measurements of colonic contractility were conducted using an *ex vivo* organ bath model.	Pigs
Yano et al.[134]	SCFAs (Butyrate, propionate)	Increased serotonin (5-HT) release from enterochromaffin cells (corresponding to reduction in intestinal transit time).	Measurements of 5-HT release.	RIN14B cell line *in vitro*
Bhattarai et al.[135]	Tryptophan metabolites (Tryptamine)	Accelerated GI transit induced by activation of serotonergic	Epithelial ionic flux assessment conducted using Ussing chamber	Mice

		receptors on the epithelium of proximal colon.	technique, and *in vivo* GI transit assay employing carmine red for measuring gut motility.	
Ye et al.[136]	(Indole and indole-3-carboxaldehyde)	Promote intestinal motility induced by activation of transient receptor potential ankyrin A1 (TRPA1) in enteroendocrine cells, inducing production of 5-HT.	*In vivo* real time monitoring of enteroendocrine cells, specifically investigating the activation TRPA1 receptors and its impact on gut motility. Additionally, amperometry was employed to measure the release of 5-HT.	Zebrafish
Alemi et al.[137]	Bile acids	Promote gastrointestinal motility through the activation TGR5 receptors present on enterochromaffin cells	Measurement of colonic motility using an *ex vivo* organ bath model. GI transit was evaluated by employing Evans blue dye and bead expulsion test.	Mice
Zuo et al.[138]	Lipopolysaccharides	Activation of ICCs through TLR4 signalling led to production of NO and simultaneous inhibition of pacemaker currents responsible for gut contractility.	Electrophysiologi-cal properties and membrane currents of cultured ICCs were assessed using whole-cell patch clamp technique. Additionally, RT-PCR analysis was conducted on cultured ICCs derived from the small intestine to examine gene expression profiles.	Mice

1.3.2.4 Influence of microbiota on stress related behaviour

Evidence mainly from animal studies has shown that the microbiota is a key mediator in regulating stress related alterations in physiology, brain function and behaviour (***Table 2***). Sudo and colleagues were the first to show that mice devoid of gut microbiota had a heightened response to acute restraint stress as shown by elevated corticosterone levels.[139] The observation that GF mice display HPA axis hyperresponsivity led researchers to ask whether the gut microbiota could also influence stress related behaviour.

Neufeld and colleagues using female germ-free mice found that anxiety like behaviour in the EPM was reduced in animals without conventional microbiota.[140] In contrast, studies have also demonstrated that GF animals exhibit increased stress reactivity and anxiety-like behaviour, which is associated with an increased stress response with elevated ACTH and cortisol.[141,142] It is interesting to note that reduced anxiety like behaviour as a result of absent microbiota seems to be a species-specific effect. GF rats with an absent microbiota display the opposite phenotype from mice and are characterised by increased anxiety like behaviour.[142] Regardless of these differences in the direction of shift, these findings show that microbiota influence stress related behaviour. Furthermore, a direct role for microbiota composition in influencing behaviour was shown in a study where the transfer of caecal microbiota from stress prone BALB/c to GF Swiss Webster (SW) mice demonstrated increased levels of anxiety compared to normal SW mice. On the other hand, transfer of caecal microbiota from SW to GF BALB/c mice reduced anxious behaviour in comparison to normal BALB/c mice.[143] This provides compelling evidence for microbiota-initiated influence on the GBA in mice. In another experiment, increased locomotor activity was observed in GF mice moncolonised with *Lactobacillus plantarum* PS128 in contrast to control GF mice. This change in behaviour was linked to elevated levels of serotonin, dopamine and their metabolites in

the striatum.[144] To further support the role of microbiota in influencing behaviour, administration of antibiotics in adolescent mice altered the diversity and composition of the gut microbiota with a concomitant reduction in anxiety like behaviour,[145] in line with that for GF adults.

The influence of microbiota on brain function and behaviour also comes from studies involving gut microbial metabolites such as SCFAs. For example, a study by Marcel van de Wouw and colleagues showed that oral administration of a cocktail of three SCFAs of acetate, propionate and butyrate in mice induced a reduction in depressive like behaviour as shown in the forced swim test and decreased anxiety like behaviour as shown in the open field test.[146] Moreover, the authors showed that the gene expression of CRH receptor 1 related to stress signalling was found to be decreased in the hippocampus, hypothalamus and colon.

Table 2: *Examples of published literature evidence of influence of microbiota on stress related behaviour using different approaches.*

Authors	**Approach**	**Behavioural test**	**Findings**	**Conclusion**	**Experi-mental animal**
Hao et al.[147]	Antibiotics	OF and EPM test	Reduction in total distance travelled in centre of open field arena in antibiotic treated mice. Decrease in time spent in open arms of the EPM.	Increased anxiety related behaviour associated with gut microbiota depletion.	Mice (male)
Grant et al.[148]	Antibiotics	OF test	Less time spent in centre of the OF arena in mice treated with antibiotics.	Increased anxiety related behaviour exhibited associated with gut microbiota depletion.	Mice (female)
Crumeyroll e-Arias et al.[142]	Germ free	OF test	GF rats exhibited an increase in the time spent and latency to explore the corners of the open field arena, along with an	The lack of gut microbiota amplifies the neuroen-docrine and	F344 Rats (male)

			increase in the frequency of defecations. Increase in serum corticosterone concentration, elevated CRF mRNA expression in the hypothalamus was observed. A lower turnover rate of dopamine was observed in the hippocampus, striatum, and frontal cortex.	behavioral response to an acute stressor.	
Needham et al.[149]	Gut microbial metabolite: 4-Ethylphenyl sulfate (4EPS)	EPM, OF test and light/dark box	A decrease in the time spent in the centre of the open field arena and the open arms of the EPM was observed in mice administered with 4EPS. In contrast, an increase in the time spent in the dark area of the light/dark box test was observed.	Gut microbial metabolite 4EPS induces anxiogenic effects	Mice
Jaglin et al.[150]	Gut microbial metabolite: Indole	EPM, OF test, novelty test, tail suspension	Decrease in time spent and entries into the open arms of the EPM, as well as decrease in the distance travelled and the number of rearings in the OF test were observed in GF rats colonized with *Escherichia coli.* Moreover, a longer latency time to visit/explore novel object in the novelty test and an extended immobility time in the tail suspension test was observed.	GF rats colonized with *Escherichia coli*, a bacterial species known for producing indole, exhibited heightened anxiety like and helplessness behaviours in comparison to the control group.	F344 rats (male)
Lyte et al[151]	Infectious agent: *Citrobacter rodentium*	OF test/hole-board apparatus	Mice treated with *C. rodentium* avoided the centre zone of the OF/hole board test, as evidenced by a shorter distance travelled and fewer entries into centre zone compared to the control group. The expression of c-Fos protein positive neurons,	Mice treated with *C. rodentium* displayed heightened anxiety like behaviour likely influenced by activation of	CF-1 mice (male)

			indicating vagal afferents activation, was significantly higher in the *C. rodentium* treated group compared to the control group.	vagal sensory neurons, indicating a neural pathway for communicatio n between the gut and brain.	

1.3.2.5 Modulation of microbiota-GBA to manage GI and mental health

Dysfunction of the ENS can cause abnormal GI motility[152] leading to altered gut microbial composition.[141] This may cause disturbances in microbiota-gut-brain signalling pathways affecting brain function and behaviour.[141] Dietary interventions such as probiotics that alter the microbiota composition or function are therefore becoming sought after as potential strategies to manage GI and mental health.

Studies have shown that manipulation of gut microbiota with probiotics has therapeutic potential for treating conditions of dysmotility.[153] For example, the probiotic strain *Lactobacillus reuteri* enhanced the excitability of myenteric neurons and decreased colonic motility in rats.[154] Another probiotic strain *Lactobacillus rhamnosus GG* expanded the number of myenteric neurons in mice and accelerated GI motility.[155] Overall, these studies suggest that effects of probiotics on GI motility are mediated via the ENS.

Probiotic preparations have also been studied to investigate their therapeutic potential in improving mental health. A study by Bravo and colleagues demonstrated that male BALB/c mice fed with *L. rhamnosus* showed increased entries and time spent in the centre of the OF test and increased entries into the open arms of the EPM, indicating anxiolytic effects.[156] Depression related behaviour

was also assessed using the forced swim test where *L. rhamnosus* fed mice spent less time immobile compared with control mice, indicating reduced depressive like behaviour. In another study, *Lactobacillus reuteri*, that is known to modulate the ENS,[154] showed anxiolytic effects as measured on the elevated plus maze. This probiotic strain also reduced stress-induced increases in corticosterone levels in mice.[157] In both studies, vagotomy in mice prevented the anxiolytic and antidepressant effects of the probiotic strains, implying that vagal integrity is important for modulating behavioural responses.

It is evident that specific probiotic strains can modulate various aspects of the microbiome-gut-brain axis and have potential in managing GI and mental health as shown in some studies. However, we are yet to identify the magnitude of the effects as well as the biological sites of action and pathways within the GBA that are responsible for these alterations. More studies are needed to find out how signals originating at the ENS level can impact brain function and behaviour.

1.3.2.6. Pathways of the microbiota-GBA (bottom-up signalling)

We have not yet fully unravelled the processes by which the gut microbiota affects brain function and behaviour, however a large body of experimental data from animal studies has indicated that there are three interacting routes through which gut microbes communicate with the CNS which include neuronal, endocrine, and immune signalling pathways. These pathways rely on efficient communication through specialised physiological barriers, namely the gut barrier (GB) and the blood brain barrier (BBB). These barriers serve as crucial defences that safeguard the body against external disruptions while facilitating communication between distinct anatomical regions. The coordination and interaction between the GB and BBB are essential for maintaining normal

physiological functions, achieved through the continuous exchange of peripheral signals in response to environmental needs.

1.3.2.6.1 Afferent vagal signalling pathway

Perhaps the most direct and fastest route for the gut microbiota to influence the brain is via the vagus nerve, which is the best studied neural pathway for bidirectional signalling between gut, microbiota and brain.[78] This dual nerve consists of vagal efferent fibres (10-20%) that send signals "down" from brain to gut and the vagal afferent fibres (80-90%) that convey sensory information "up" from the gut to the brain. Vagal afferent fibres are distributed across all layers of the gut wall but do not pass beyond the epithelial layer.[158] These fibres can detect microbial signals indirectly, through the diffusion of bacterial factors such as SCFAs, or through specialised epithelial cells such as enteroendocrine cells (EECs) that relay luminal signals.[159] For example, Lal and colleagues showed that sodium butyrate, when luminally perfused in the small intestine of anaesthetised male rats, stimulated vagal afferent nerve discharge. Furthermore, the authors went on to show that by damaging the integrity of the vagus nerve (vagotomy) these effects were abolished.[160] A likely explanation for the stimulation of vagal afferents could be the absorption of these metabolites across the mucosal epithelium to the nerve terminal in the lamina propria, an area that has shown arborisation of vagal afferent fibres. A diverse array of sensory receptors are also expressed on vagal afferent fibres that can respond to a variety of gut signals for example, vagal afferent fibres express TLR4 that can detect bacterial products such as LPS.[161,162]

Change in anxiety related behaviour through vagus nerve signalling can be induced by specific bacterial strains. Mice orally challenged with the diarrhoea causing pathogen *Campylobacter jejuni*, showed increased anxiety related behaviour, as indicated by increased avoidance of the centre

regions in the hole board test, compared to saline-treated controls. Furthermore, these mice also showed increased c-Fos (a neuronal activation marker utilised for mapping brain pathway) immunoreactivity in cell bodies of vagal afferents and the nucleus tractus solitarius (NTS), which is the main hub of gut-related vagal afferents in the brain.[163] In another study, the therapeutic effects of *Lactobacillus rhamnosus JB1* on anxiety and depressive like behaviour was blocked following vagotomy.[156] Similarly, the anxiolytic effect of *Bifidobacterium longum* was abrogated when the vagus nerve was disconnected in a mice model of chronic colitis associated with anxiety related behaviour.[164] The authors went on to show that *Bifidobacterium longum* decreased the excitability of enteric neurons of the myenteric plexus *in vitro*. The ability of *Bifidobacterium longum* to modulate the enteric neurons suggests that its anxiolytic effects could be mediated via vagal pathways originating at the ENS level.

1.3.2.6.2 Enteroendocrine signalling pathway

Another crucial pathway which enabled gut microbiota and their metabolites to communicate with the CNS involves the specialised EECs distributed between epithelial cells of the gut throughout the length of the GI tract.[165] These specialised cells produce a vast array of neuropeptides which have a wide range of functions that are not only associated with regulating gut secretion and motility but also brain function.[166,167] EECs are known to be stimulated by microbiota metabolites such as SCFA (e.g., butyrate, acetate and propionate) and amino acid derived metabolites (e.g., from tryptophan).[166] Upon stimulation, EECs secrete neuropetides that can diffuse through the lamina propria, en route to the blood circulation or act on ENS neurons or vagal afferents, carrying signals to the brain.[168,169] Another signalling pathway involving EECs was demonstrated by Bohorquez and colleagues who showed direct paracrine signalling between neurons innervating the small intestine

and colon and EECs, serving as a sensory interface for facilitating luminal gut microbiota signals to the CNS via the ENS.[170]

1.3.2.6.3 Immune signalling pathway

The immune system serves as a vital link in the dynamic balance that occurs between the gut and the brain.[171] The immune system is in constant interaction with the ENS and CNS including the HPA axis.[172,173] The gut is a critical immune organ as it harbours the highest concentration of immune cells that serve as a physical barrier to limit the contact of intestinal microbiota with the visceral tissue and also to identify harmful pathogens.[174] Immune cells include chemosensory cells, enterocytes, secretory cells, and gut-associated lymphoid tissue.[175] The gut-associated lymphoid tissue makes use of lymphocytes to generate a more targeted immune response, whereas enterocytes contain innate immune receptors and have the ability to produce cytokines and chemokines. Cytokines are chemical messengers that are categorised as pro inflammatory or anti-inflammatory and are able to promote or inhibit inflammatory processes, respectively.[176] They can infiltrate the lymphatic and blood circulatory systems, as well as affect neural signals carried by vagal and spinal afferent neurones, allowing constant communication with the brain. The role of the immune system in gut-brain crosstalk has been demonstrated in animal studies that used LPS to induce an immune response (mimicking a bacterial infection), triggering an increase in proinflammatory cytokines such as IL-1β, IL-2, IL-6, TNF-α, and interferons (IFNs) that act on the brain and induce depressive and anxiety like behaviour.[177-179] For example, a study by Fields and colleagues showed that gut derived LPS increased anxiety-like behaviours in male mice, as measured by reduced time spent in the centre zone of the OF test.[179] Furthermore, LPS increased IL-6 expression levels in the gut. The authors concluded that the effects of LPS on behaviour were mediated by the TLR4 signalling pathway. Their study showed microbiota-induced gut inflammation resulting from gut microbiota

dysbiosis can induce behavioural alterations via immune signalling pathways. The balance of the gut microbial ecosystem is critical to maintain immune balance.

1.4 Rationale for study

As discussed in the previous sections, the gut microbiota has gained recognition for its impact on the GBA. The ENS, positioned along the GI tract, acts as a crucial intermediary within the GBA. Close proximity between the gut microbiota and the ENS highlights the substantial role of the gut microbes in not only regulating local gut functions but also influencing brain function and behaviour. Although how the gut microbiota impacts the ENS has been studied extensively, understanding the feedback or reverse regulation of ENS and its function on the microbiota is required to fully understand the microbiota-ENS interplay. There have been relatively few studies that have demonstrated the ENS influence on the gut microbiome.[180] A study by Rolig and colleagues showed that zebrafish devoid of an ENS, resulting from a mutation in *sox10* transcription factor gene (a gene associated with Hirschsprung disease), developed a pro-inflammatory microbiota profile following dysbiosis. However, these effects were reversed by transplanting wild-type ENS precursors into *sox10* mutant zebrafish or introducing anti-inflammatory bacterial strains.[180] Although this study indicates that changes in enteric neural activity may precede alterations in the gut microbiota, it is still unclear whether these changes in the gut microbiota are caused directly by the ENS circuits or if they are simply a consequence of abnormal gut motility.

When diseases, such as functional gastrointestinal disorders (FGIDs), have a demonstrated link with altered microbiome composition, it becomes increasingly important to assess the underlying cause of microbiome variation. There is an obvious gap in knowledge about the underlying physiological

factors shaping the gut microbiota. Movement of the luminal content is one factor that has shown to affect gut microbiota growth and population dynamics. Cremer and colleagues emphasised the significance of flow and mixing in shaping colonic microbiota.[181] Through mathematical modelling and *in vitro* experiments, they illustrated that regulated contractions of the colon have a substantial impact on the density and composition of the microbiota.[181] The role of gut motility in influencing the gut microbiota also comes from studies that involve a commonly used marker of gut motility: gut transit time.[51] Studies have shown that changes in gut transit time, which refers to the movement of luminal contents along the GI tract, are associated with gut microbiome variation, suggesting it to be a major driver in shaping the gut microbial ecosystem.[182,183]

Diet has been shown to be a major factor in influencing the gut microbial composition.[184,185] Moreover, studies have also shown diet to influence psychological health via changes associated with gut microbiota.[186,187] However, the effect of diet on gut motility or gut transit time is often overlooked. Modulation of gut motility via ENS manipulation, which in turn may affect the gut microbiome and subsequently higher cognitive functions is one underlying 'gut to brain' mechanism that hasn't been fully explored.

Understanding the impact of gut motility on higher cognitive functions such as anxiety requires an animal model in which the ENS can be independently manipulated. One useful approach to studying the ENS would be using pharmacological agents that target specific receptors in the ENS. For example, loperamide, an opioid agonist has been shown to act on mu opioid receptors in the ENS decreasing the activity of the myenteric plexus within the ENS which in turn reduces the tone of the longitudinal and circular smooth muscles of the intestine inhibiting peristaltic activity.[188-190] Moreover, loperamide has shown to inhibit acetylcholine release, the primary excitatory

neurotransmitter released by myenteric neurons to induce muscle contractions, by interacting with opiate receptor sites in the myenteric plexus.[191] As the thesis focuses on unravelling the intricate gut to brain mechanism, it is essential to employ a pharmacological tool that allows for precise manipulation of the ENS without any interference from the CNS. Loperamide serves as an advantageous pharmacological tool due to its limited ability to cross the BBB,[192] which allows for the investigation of localised effects on ENS, shedding light on the intricate interplay between the gut and the brain.

1.5 Scientific aims and hypotheses

The overarching goal of my thesis was to explore the impact of ENS manipulation on brain function and behaviour via the GBA. I chose to use a modulator that activates the ENS mu opioid receptor present in the large intestine which is chemically and anatomically connected to the brain. Since ENS mu opioid receptor activation also decreases gut motility, this receptor target was also useful to investigate the relationship between gut motility and the microbiota.

To achieve this objective, the following research questions were posed, and hypotheses formulated:

1. How can the ENS in isolation induce colonic motility alterations?

   Hypothesis: Administration of an opioid agonist will alter colonic motility patterns throughout the colon.

   The aim of this study was to gain a fundamental understanding of the effect of an opioid agonist on colonic motility. This would serve as a representative model of ENS manipulation. The use of an *ex vivo* assay for motility enables observations and measurements of the spontaneous contractions of the colon in the absence of input from the CNS.

2. Does altering colonic motility affect the gut microbiota composition?

   Hypothesis: Changes in colonic motility induced by an opioid agonist will lead to microbiota variation.

   This research question aims to explore whether altering colonic motility using an opioid agonist can lead to changes in the composition and abundance of gut microbiota.

3. Does modulating the ENS (and subsequently) inhibiting colonic motility affect anxiety related behaviour?

   Hypothesis: Altered gut motility will impact brain function and behaviour, leading to increased anxiety related behaviour.

   The aim of this study was to gain a comprehensive understanding of the relationship between slowed gut motility and anxiety related behavior.

1.6 Structure of thesis

Review	**Chapter 1**	**Introduction** **Literature Review**
	Section 1	Introduction
	Section 2	Overview of the enteric nervous system (ENS) structure and function
	Section 3	Introduction to gut microbiota and review of its influence on gastrointestinal motility (GI), and stress related behaviour
	Section 4	Rationale for study
	Section 5	Research aims and hypotheses
Research chapters	**Chapter 2**	**Impact of ENS modulation on colonic motility parameters**
	Purpose of study	Exploring the role of ENS mu opioid receptors in regulating colonic motility
	Technique	Spatiotemporal (Diameter map) mapping of motility in *ex vivo* preparations of mouse colon
	Chapter 3	**Slowed GI transit and its impact on caecal microbiota composition**
	Purpose of study	Understanding the impact of slowed GI transit on caecal microbiota composition
	Technique	16S rRNA gene amplicon sequencing technique for identification of bacterial communities and diversity
	Chapter 4	**Impact of ENS modulation on anxiety related behaviour**
	Purpose of study	Examining the broader physiological consequences of ENS modulation on brain function and anxiety, with a specific emphasis on exploring potential sex differences
	Technique	▪ Behavioural tests (open field and elevated plus maze) using rats ▪ Gene expression in the proximal colon, prefrontal cortex, hippocampus, and amygdala was assessed (RNAseq) ▪ Caecal microbiota composition determined (shotgun metagenomic sequencing)
	Chapter 5	**Integrated discussion, limitations and potential areas for future research**

CHAPTER 2: IMPACT OF ENS MODULATION ON COLONIC MOTILITY PARAMETERS

This chapter is currently under review for publication in Frontiers in Neuroscience journal.

Effects of a peripherally acting mu opioid agonist on the characteristics of colonic motor complexes in the isolated mouse colon.

2.1 Introduction

Embedded within the gut wall lies a complex neural circuitry containing millions of neurons that are critical for the modulation of GI functions including motility and control of fluid movement. This autonomous neural system that has the unique ability to coordinate GI functions independent of the CNS is referred to as the ENS.[26,193] Control of GI functions is dependent on the activity of diverse neurochemical classes of enteric neurons that are contained within interconnected micro-ganglia that form two distinct plexi, known as the submucosal and myenteric plexi.[52] The myenteric plexus lies between the circular and longitudinal muscle layers of the gut wall and includes excitatory and inhibitory motor neurons, descending and ascending interneurons and a unique population of intrinsic sensory neurons which are responsible for coordinating GI motor activity enabling propulsive movement along the intestine.[194,195] The ENS's unique characteristic of regulating intestinal movements independently of the CNS allows for the exploration of enteric neuronal networks and their role in facilitating motility in isolated segments of intestine.[39]

An important aspect of digestion is the controlled progression of luminal contents along the GI tract, which is coordinated by various patterns of intestinal movements that occur as a result of the

interplay between enteric neural circuits, Interstitial cells of Cajal (ICC) and spontaneous intestinal smooth muscle activity.[35-37] Colonic motor complexes (CMCs) are neurally mediated spontaneous propagating contraction of the colonic smooth muscle that propagate over longer distances and aid in the propulsion of luminal contents.[51] They can readily be recorded and analysed from isolated intact colon preparations as they are known to occur in the absence of input from the CNS. The mouse colon has served as an excellent model species for recording CMCs because of the regular and rhythmic occurrence of these events in the isolated colon.[40,41,49] They are the predominant mechanical motor pattern of the mouse colon and are visualised as rhythmic contractions that propagate along an axial distance that is at least half the total length of the colon.[50]

Our knowledge of intestinal motility has improved as a result of technological advancements. Analysing the movements of the intestinal wall and luminal content in muscle mechanics presents a considerable challenge due to the spontaneous nature of the movements throughout the entire gut. Nonetheless, advancements in video recording and spatiotemporal (ST) mapping techniques have made it feasible to visually represent, describe, and study intricate motility patterns in *ex vivo* whole segments of the colon and intestine. High-definition video imaging of the intestines allows for the measurement of changes in intestinal diameter. It has become possible to create maps (Diameter maps - DMaps) that show changes in diameter across the length of the isolated intestinal segments in real time.[59,60] These maps offer a straightforward, easily interpretable visual representation of motility over time ranging from a few seconds to several minutes. The spontaneity of GI motility patterns and the use of video-imaging techniques has not only facilitated detailed descriptions of motor patterns on isolated colon preparations of several experimental animals but has also enabled the analysis of functional alterations in response to pharmacological and infectious agents.[41,57,61,62,196]

Opioids have been used for centuries as anti-diarrheal agents that work by inhibiting enteric neuronal activity, reducing propulsive colonic peristaltic contractions, and delaying GI transit.[197] Several studies have reported exogenous opioids to alter intestinal motility both *in vivo and in vitro* settings.[198-201] The impact of opioids on GI motility is ascribed to the activation of opioid receptors (μ/mu, κ/kappa, and δ/delta), with the mu opioid receptor playing a significant role in mediating anti-peristaltic effects specifically in the large intestine.[202] Loperamide is one such opioid agonist that acts on mu opioid receptors decreasing the activity of the myenteric plexus within the ENS which in turn reduces the tone of the longitudinal and circular smooth muscles of the intestine inhibiting peristaltic activity.[188,189] In the guinea pig ileum, loperamide has been shown to act via mu opioid receptors to inhibit excitatory motor neurons of the ENS.[190] Moreover, loperamide has shown to inhibit acetylcholine release, the primary excitatory neurotransmitter released by myenteric neurons to induce muscle contractions, by interacting with opiate receptor sites in the myenteric plexus.[191] Although there is detailed knowledge about the pharmacological activity of loperamide, little is known of its effects on the characteristics of colonic motility patterns in *ex vivo* colon. This is surprising and is an obvious gap in knowledge, despite it being a commonly used drug to treat diarrhoea. The current study assays the effect of loperamide on colonic motor complexes in isolated colon of mice using DMaps. We hypothesised that the opioid agonist, loperamide, will inhibit colonic motor complexes.

2.2. Materials and methods

2.2.1 Animals

The research described in this chapter was carried out in compliance with the Animal Welfare Act, 1999 (NZ) and after obtaining ethical approval from the AgResearch Grasslands Animal Ethics

Committee (Palmerston North, New Zealand) under application AE 15540. C57BL/6 mice were bred at The University of Otago breeding unit (Dunedin, New Zealand) and were raised in groups with their littermates. Mice were kept in controlled conditions, with a consistent temperature of 21 °C and a regular light/dark cycle. They were housed in cages lined with sawdust, either made of plastic or stainless steel, and had unrestricted access to food and water. The mice's weight, food intake, and general health were monitored daily using a scoring system ranging from 1 to 5, following the NZ Animal Health Care Standard.

2.2.2 Drugs and Solutions

The composition of Krebs solution used was (in mM): NaCl. 118; KCl. 4.7; $NaHPO_4.2H_2O$. 1.0; $NaHCO_3$. 25; $MgCl.6H_2O$. 1.2; D-Glucose. 11; $CaCl_2.2H_2O$. 2.5. The pH of Krebs solution was maintained at 7.3-7.4 with constant aeration of 95% O_2 and 5% CO_2 at 35 ± 0.5ºC.

The drugs used in these experiments included loperamide and naloxone that were purchased from Selleck Chemicals (Houston, TX, USA). Both Loperamide and naloxone were prepared as stock solutions in, Dimethyl sulfoxide (DMSO) and MilliQ water (based on solubility), respectively. Final concentrations of the drugs were made using Krebs solution (the final concentration of the drugs was calculated based on the organ bath tank volume).

2.2.3 Dissection protocol and colonic tissue preparation

Male mice aged between 7 and 14 weeks were humanely euthanised by isoflurane overdose inhalation followed by cervical dislocation, following approved protocols from the AgResearch Grasslands Animal Ethics Committee. The mouse was securely fixed to a dissection board by pinning its four paws, enabling the exposure of its ventral side. Using dissecting forceps and scissors, an incision was made through the epidermis, revealing the lower abdominal muscle layers.

The abdominal cavity was opened along the midline towards the sternum. To prevent tissue dehydration during the dissection, Krebs solution at room temperature, aerated with carbogen gas (95% O_2 and 5% CO_2), was poured onto the abdominal contents at regular intervals (every 30-60 seconds). The surrounding tissues, such as the urinary bladder and testes, were carefully removed. Two vertical incisions, approximately 0.5 cm from the midline, were made to cut the pelvic bone on each side of the colon. The colon, still attached to the caecum and rectum, was isolated. Using forceps, the caecum was held while the proximal end of the colon was separated from it. Next, the entire length of the colon was placed in a glass tray filled with warm Krebs solution (30-35°C), which was continuously bubbled with carbogen. Fine dissection scissors were used to trim the mesentery attached to the tissue. Care was taken not to stretch the gastrointestinal tissue while trimming the adjoining mesentery. To empty the intestinal contents and faecal pellets, the isolated colon was kept in Krebs solution and constantly bubbled with carbogen for 30 min. Any remaining contents were flushed out by gently applying pressure at the oral end using a 5 ml syringe attached to a blunt needle filled with Krebs solution. A stainless-steel rod (1.9mm thickness) was cautiously inserted into the colon lumen. The proximal and distal ends of the colon were secured onto barbed tubing connectors and secured with surgical knots. The tubing connectors were anchored to a base plate, which could be easily transferred into an organ bath. The setup, including the isolated colon segment with the stainless-steel rod, was then placed into an organ bath containing warm Krebs solution (maintained at 35 ± 0.5°C) and continuously aerated with carbogen (***Fig. 6***). The lumen of the colon was perfused with warm Krebs solution at a rate of 0.13 mL/min from the proximal end using a peristaltic pump. This constant flow provided the necessary pressure for recording consistent propagating contractions. The distal end of the colon, attached to the tubing connector, was cannulated to an outlet tube. Intraluminal pressure was measured by determining the difference

in height between the end of the outlet tube and the meniscus of the Krebs solution within the organ bath, maintained at a constant level of 40-50 mm.

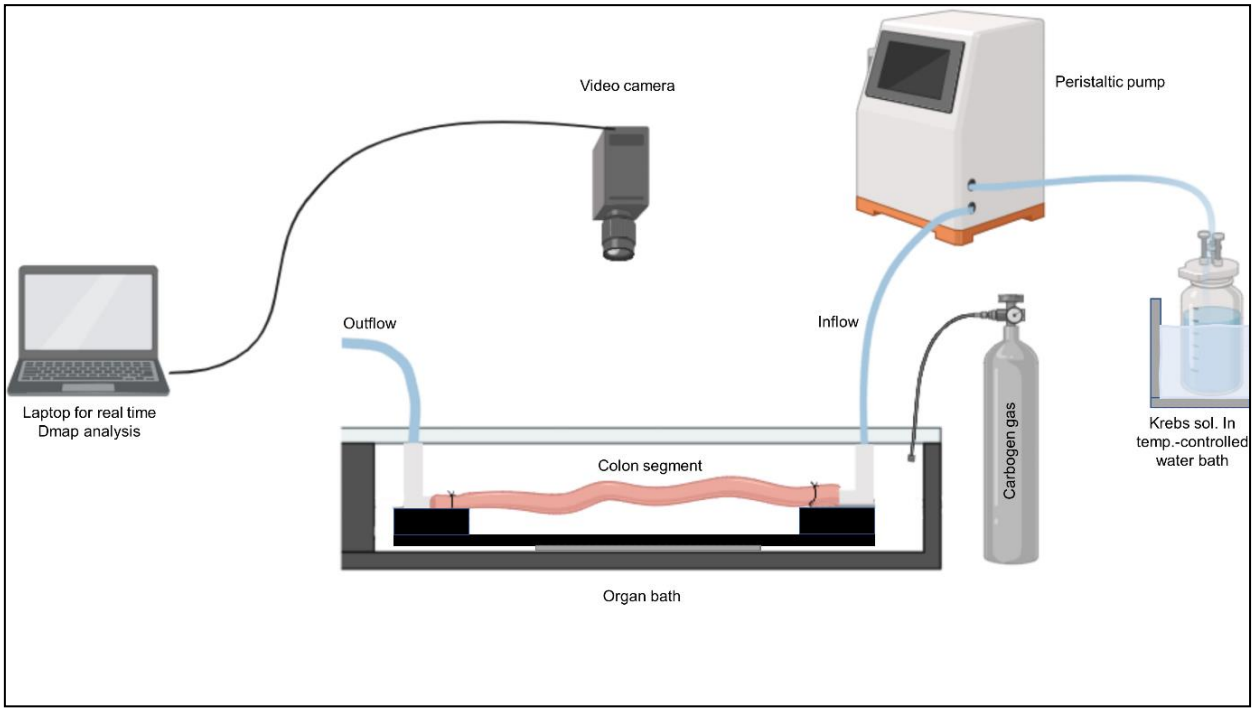


Figure 6: *Schematic representation of the ex vivo motility experiment set up. (Created using BioRender)*

2.2.4 Study design

Motility experiments began with a 30 min equilibration period, followed by a 10 min control video recording. In specific experiments, loperamide was applied to the organ bath in isolation from any antagonists or combined with the opioid antagonist naloxone. In other experiments, naloxone was added on its own. In each experiment where the tissue was exposed to pharmacological agents, motility was recorded for 10 min. Each tissue preparation served as its own control prior to drug application. Pharmacological agents were applied to the serosal side of each preparation.

2.2.5 Video Imaging and analysis

Colonic contractions or motility patterns were recorded *ex vivo* using a camera (Logitech HD Pro C920; JB Hi-Fi, New Zealand) mounted directly above the organ bath. QuickTime (Apple Inc.) software was used to record videos. The captured video segments (10 min duration) were analysed using MATLAB (MathWorks, Natick, MA, USA) to create spatiotemporal maps (ST) where the diameter of the colon is mapped (displayed as a heat map) along the length of the segment as a function of time (***Fig. 8***). The x axis represents increasing time (s), and the y axis represents length of colon (in mm). A colour bar indicated luminal diameter where relaxed tissue is represented by blue–green pixels on the ST maps while yellow–red pixels represented constricted region: Four parameters of colonic motor activity were examined. CMCs which originated at the oral end and propagated aborally at least half the length of the colon were analysed and from these CMC propagating velocity, CMC interval and CMC propagation distance were assessed. The frequency of CMCs was quantified from ST maps while the velocity of CMC propagation, interval between CMCs and CMC propagation distance were measured using MATLAB. The CMC propagation velocity was determined using the scale produced with each map (showing distance and time) to determine over what distance of the colon the contraction occurred and the time it took for the full contraction to take place. Interval between CMCs was calculated as the average time between contractions at a point that was selected as a consistent reference location for comparing different preparations.

2.2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 9.5.1, GraphPad software Inc., USA). Differences in CMC parameters between multiple treatment groups were assessed using Kruskal-Wallis ANOVA with Dunn's multiple comparison test. Results are

expressed as mean ± standard error of mean (SEM). P value less than 0.05 was considered statistically significant.

2.3. Results

2.3.1 General observations

After euthanasia and inspection of the abdominal cavity, it was established that each of the 20 colonic preparations had an average of 3 faecal pellets within the lumen (range: 1 - 5). After placing each preparation containing pellets into the glass tray containing warm Krebs (30-35°C; aerated with carbogen gas), it was found that 7 of these 20 preparations naturally expelled all pellets within 30 min. Four colon preparations did not expel any pellets. Each colon preparation was gently flushed with approximately 5 ml of warm Krebs solution.

After an initial equilibrium period (30 min), CMC typically became temporally coordinated between the proximal and distal ends of the isolated colon.

2.3.2 Effect of loperamide on CMC parameters

To assess the effect of mu opioid receptor agonist loperamide on CMC activity, we examined CMC parameters of frequency, velocity, interval between CMCs and CMC propagating distance, and compared them to control conditions. It was found that loperamide 10 nM did not significantly alter any of the CMC parameters (***Fig. 7 and Fig. 8c***). The effect of increasing loperamide concentration on CMC parameters was also analysed. Loperamide at 1 μM potently inhibited CMCs (***Fig. 8d***) to within the recording noise. Loperamide at 100 nM significantly reduced CMC frequency compared to the control period (control: 0.69 ± 0.04 per min; lop. 100 nM: 0.36 ± 0.03 per min; n=12; P=0.0001) (***Fig. 7a***). Moreover, this concentration significantly reduced CMC velocity by 46%

compared to the control period (control: 2.39 ± 0.27 mm/s; lop. 100 nM: 1.28 ± 0.21 mm/s; n=12; P= 0.0103) (***Fig. 7b, Fig. 8a and 8b***). Similarly, the extent of CMC propagation was significantly reduced when 100 nM loperamide was added to the bath (control: 38.60 ± 1.42 mm; lop. 100 nM: 29.70 ± 0.84 mm; n=12; P=0.0002) (***Fig. 7c***). Also, the interval between CMCs increased by 40% when 100 nM loperamide was applied (control: 67.12 ± 5.06 s; lop. 100 nM: 93.97 ± 8.36 s; n=12; P=0.0299) (***Fig. 7d).***

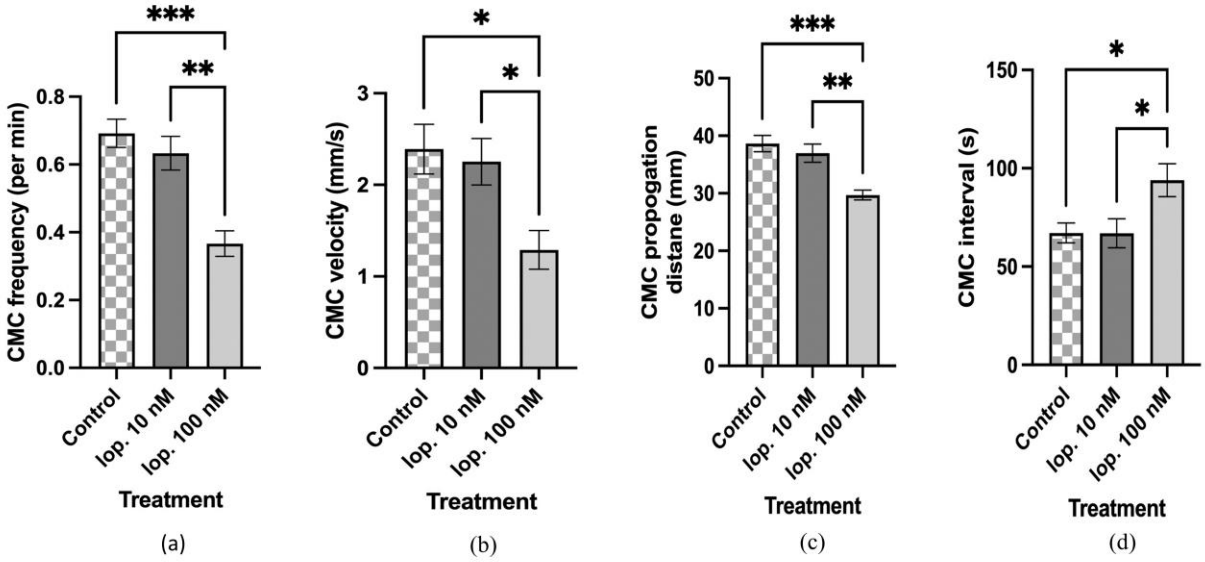


Figure 7: *Summary graphs of different treatment effects on* ***(a)*** *CMC frequency per min* ***(b)*** *CMC velocity (mm/s)* ***(c)*** *CMC propagation distance (mm) and* ***(d)*** *CMC interval (s). Loperamide at 10 nM (Lop. 10 nM) concentration was without significant effect on CMC parameters. Loperamide at an increased concentration of 100 nM (Lop. 100 nM) significantly reduced CMC frequency, CMC velocity and CMC propagation distance compared to controls. CMC intervals increased with lop. 100 nM compared to controls. Asterisks indicate statistical significance (*P<0.0332; **P<0.0021; ***P<0.0002; ****P<0.0001). Data shown as mean with error bars indicating SEM. CMC: colon motor complex; lop: loperamide.*

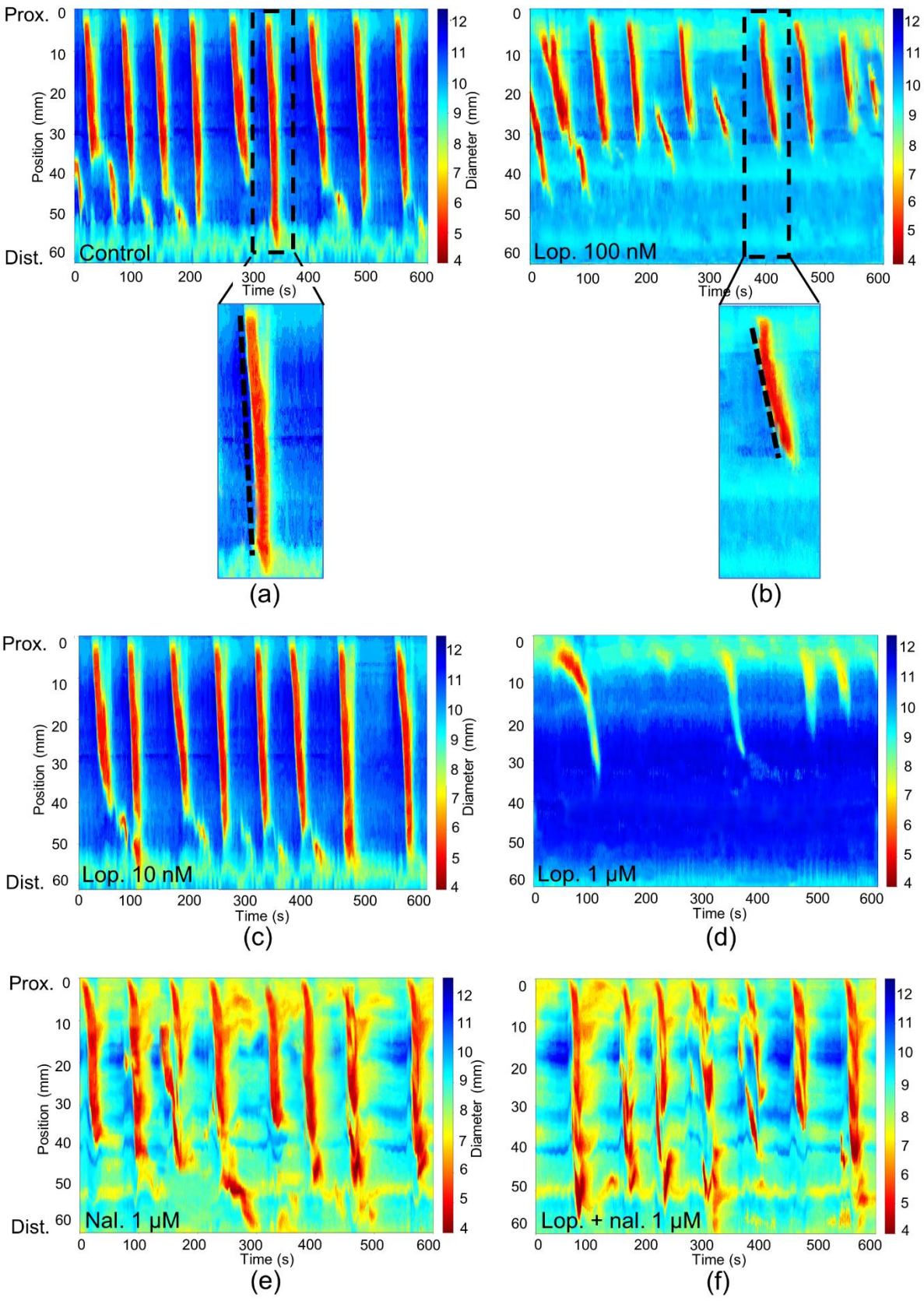

Prox.
Dist.
Position (mm)
Time (s)
Diameter (mm)
Control
(a)
Lop. 100 nM
(b)
Lop. 10 nM
(c)
Lop. 1 µM
(d)
Nal. 1 µM
(e)
Lop. + nal. 1 µM
(f)

Figure 8: *Spatiotemporal heat maps (DMaps) showing CMCs in* ***(a)*** *control* ***(b)*** *loperamide at 100 nM concentration* ***(c)*** *loperamide at 10 nM concentration* ***(d)*** *loperamide at 1 μM concentration* ***(e)*** *naloxone at 1 μM concentration* ***(f)*** *naloxone at 1 μM added to the bath containing preparations which were exposed to loperamide at 100 nM concentration. The x-axis represents increasing time in seconds and the y-axis represents length of colon in millimetres from the proximal to distal end. The colour bar on the right of each map indicates the width of the colon for each captured frame during the 10 min video recording. Red-yellow regions show constricted areas whereas blue-green regions show relaxed tissue. The broken lines within the enlarged images of the spatiotemporal maps in* ***(a)*** *and* ***(b)*** *show the velocity of the CMCs. The slower the speed, the steeper the broken lines/slope. Lop: loperamide; nal: naloxone.*

2.3.3 Effect of naloxone in the presence of loperamide

Using the opioid receptor antagonist naloxone (nal), we examined whether the inhibitory effect of loperamide could be prevented. Naloxone at 1 μM was added to the bath containing preparations which were exposed to loperamide at 100 nM concentration (***Fig. 8f***). Loperamide significantly reduced CMC frequency compared to control activity, and this effect was prevented by naloxone when co-applied with loperamide (lop. 100 nM: 0.35 ± 0.02 per min; lop.+nal. 1 μM: 0.56 ± 0.02 per min; n=6; P=0.0377), which was not significantly different from controls (***Fig. 9a***). Similarly, the reduced velocity of CMCs after loperamide application was restored by naloxone (lop. 100 nM: 1.16 ± 0.16 mm/s; lop.+nal. 1 μM: 2.01 ± 0.16 mm/s; n=6; P=0.0174), to similar levels as controls (***Fig. 9b***). CMC propagation distance which was significantly reduced with 100 nM loperamide was also restored by naloxone to distances similar to controls (lop. 100 nM: 30.67 ± 0.97 mm; lop.+nal. 1 μM: 39.98 ± 1.99 mm; n=6; P=0.0061) (***Fig. 9c***). The interval between CMCs increased significantly in the presence of loperamide, however, this effect was not prevented by naloxone (lop. 100 nM: 84.71 ± 3.17 s; lop.+nal. 1 μM: 74.89 ± 4.52 s; n=6; P=0.4323) (***Fig. 9d***).

To confirm that there was no intrinsic effect caused by naloxone or the presence of endogenous opioids, naloxone was added to the organ bath by itself in the absence of loperamide. Naloxone at 1 μM alone did not affect CMC parameters as compared to the control period (***Fig. 8e***).

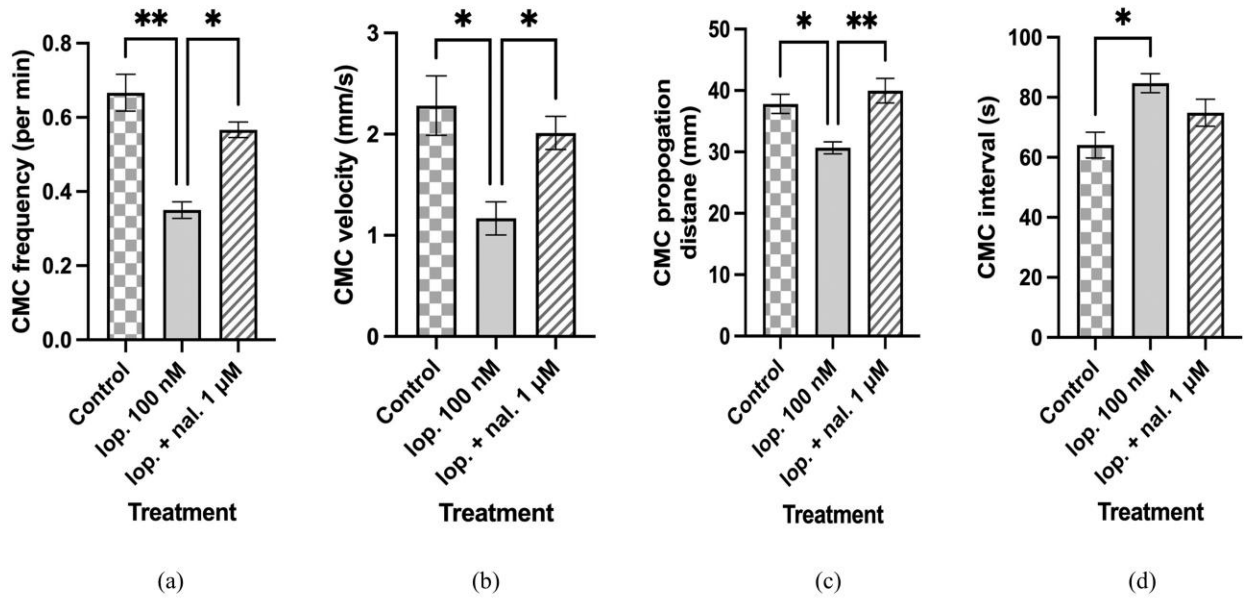


Figure 9: *Summary graphs of different treatment effects on* ***(a)*** *CMC frequency per min* ***(b)*** *CMC velocity (mm/s)* ***(c)*** *CMC propagation distance (mm) and* ***(d)*** *CMC interval (s). Loperamide at 100 nM (lop. 100 nM) significantly reduced CMC frequency, CMC velocity and CMC propagation distance compared to controls. CMC intervals increased with lop. 100 nM compared to controls. Naloxone at 1 µM prevented the inhibitory effects of loperamide, except for CMC intervals, when applied to preparations that were exposed to 100 nM loperamide (lop. + nal. 1 µM). Asterisks indicate statistical significance (*P<0.0332; **P<0.0021; ***P<0.0002; ****P<0.0001). Data shown as mean with error bars indicating SEM. CMC: colonic motor complex; lop: loperamide; nal: naloxone.*

2.4 Discussion

In this study, we characterised the effects of loperamide on CMC characteristics utilising custom written spatiotemporal D-mapping software created from high resolution video recordings of isolated intact mouse colon preparations. Our results revealed that loperamide at 100 nM reduced the frequency, velocity, and propagating distance of CMCs compared to control conditions. Moreover, the interval between CMCs was prolonged in loperamide treated colon preparations. When loperamide was decreased to 10 nM, this concentration was too low to be effective. The attenuation of inhibitory effects of loperamide on CMCs by naloxone confirmed the involvement of

opioid receptor signalling. The findings confirm the potent inhibitory effects of mu opioid agonists in retarding colonic transit. Perhaps the most startling effects of the action of loperamide was the regional inhibition of CMCs to the mid and distal colon. That is, CMCs were not blocked in the proximal colon. This has not been reported in the literature to date.

As previously classified, CMC contractions that migrated at least half the length of the colon were termed as CMCs.[51] At baseline or control conditions, CMCs occurred at a frequency of 0.69 per min which is similar to previous mouse studies, where CMCs in control conditions have been shown to occur at a frequency of 0.5-3 per min.[49,58,203,204] However, the techniques used to stimulate colonic preparations to record CMC activity in these studies were different from the current study. With regards to the velocity of propagation of CMCs, the findings of this study were found to be similar to a study by Balasuriya and colleagues[196] who used a similar technique to record CMCs. That is, maintained colonic distension via infusion of intraluminal fluid that acted as stimuli to enable video recordings of CMC activity. CMCs require gut wall distension in mouse colon and are infrequent in the absence of an applied mechanical stimulus.[53] A study by Barnes and colleagues showed that CMCs occurred frequently when stretch or mechanical stimulus was applied to isolated mouse colon but were absent or occurred rarely when the colon was devoid of endogenous faecal pellets.[53] The mechanism by which initiation of content dependent neural peristalsis and of CMCs have been investigated in studies using mouse colon *in vitro*. Keating and Spencer showed that the neural circuits responsible for CMC generation lie in the myenteric plexus and/or muscularis externa.[54] Moreover, they went on to show that colon preparations devoid of mucosa did not affect propagating velocities of CMCs compared to dissected colon preparations devoid of mucosa and submucosal plexus.[54] These findings suggest that the submucosal plexus and mucosa are not required for either the initiation or propagation of these colonic motor patterns. In another study,

Zagorodnyuk and Spencer demonstrated that stretch applied on the luminal wall increases CMC frequency which remains unaffected even after removal of mucosa and submucosa.[205] Overall, this implies that, at least in rodents, all the mechanosensory components and neuronal circuits driving CMCs and neural peristaltic contractions must be in the myenteric plexus and/or muscularis externa.

Suppression of GI motor activity is a well-known effect of opioids based on studies involving animals and humans.[198.199.206,207] In the present study, loperamide at a relatively higher concentration suppressed CMC parameters compared to controls. This is similar to a study by Beckett and colleagues who in their experiments showed that morphine, another mu opioid receptor agonist, reduced the amplitude and frequency of CMCs in isolated mouse colon compared to control conditions.[208] The inhibition of GI motor activity by exogenous opioids such as loperamide and morphine is largely due to the existence of a widely distributed opioid system in the gut.[209] Opioid receptors of the mu, kapa and delta-subtype are expressed by myenteric and secretomotor neurons of the ENS in rodents, guinea pig and humans, but with a varied distribution across GI region and species.[210-214] Gut motility is controlled by myenteric neurons via the release of neurotransmitters acetylcholine (ACh) and substance P which induce muscle contraction (excitatory) and adenosine triphosphate/β-nicotinamide adenine dinucleotide (ATP/βNAD), nitric oxide (NO), vasoactive intestinal peptide (VIP) to cause muscle relaxation (inhibitory).[215] The inhibitory effect of mu opioid agonists have been shown to arise primarily from interruption of both excitatory and inhibitory enteric neural inputs controlling muscle activity.[190,191,216,217] A study by Yagasaki and colleagues using isolated guinea pig ileum demonstrated that exposure to loperamide results in suppression of ACh and substance P release.[191] Since the predominant input to longitudinal muscle is excitatory, the absence of ACh and substance P result in inhibition of

longitudinal muscle contraction. In another study Iwata and colleagues using electrophysiological techniques elucidated that contraction in circular muscle of the isolated mouse ileum induced by morphine administration was strongly inhibited by NG-nitro-L-arginine and tetrodotoxin, suggesting that morphine's contractile effects on circular muscle may be associated with the inhibition of NO release from inhibitory nerves.[217] Unlike the longitudinal muscle, the circular muscle layer primarily receives inhibitory input. In a state of rest, the continuous firing of inhibitory motor neurons leads to the relaxation of the circular muscle layer. Consequently, this relaxation of the bowel aids in accommodating advancing intraluminal contents. However, when exposed to an opioid agonist, the suppression of NO, as demonstrated by Iwata et al., triggers heightened contractile activity within this muscle layer, inhibiting the descending relaxation necessary for peristalsis. Thus, the combined absence of longitudinal muscle contraction and circular muscle relaxation would explain loperamide's ability to suppress propulsive motility patterns.

A major finding of this study was that loperamide reduced CMC propagation distance. This occurred via a suppression of CMC contraction propagation from proximal to mid and distal regions of colon. In control conditions CMCs propagated longer distances, frequently reaching the distal end of the colon. However, when loperamide was applied, this effect was potently reduced. A possible explanation for these findings is that opiate receptors activated by loperamide are either more densely distributed on interneurons and motor neurons in the mid to distal colon, or that a similar opiate receptor density exists throughout the ENS, but the receptors have a greater sensitivity to opiate agonists between the mid to distal regions. This finding could certainly help explain why constipation is common in people who frequently consume opiates.
Indeed, a difference in the relative distribution of mu opioid receptors compared with ACh receptors might disproportionately influence the effect of their involvement in CMCs. Moreover, Li

and colleagues have shown that the proportion of nitrergic neurons was found to be slightly higher in the myenteric plexus of the distal compared to the proximal colon.[218] The findings from previous studies that have shown inhibitory effect of mu opioid agonists to arise mainly from interruption of both excitatory and inhibitory neurotransmitters controlling muscle activity could be a likely explanation for the suppressed contractile activity of loperamide in the distal colon.

The spatiotemporal DMap protocol is primarily designed to detect changes in luminal diameter. However, motility patterns that minimally impact the luminal diameter such as myogenic motor patterns are not easily visualised. This problem can be overcome by coupling this technique to other functional measurements such as intraluminal pressure and muscle tension (e.g., combining DMap analysis and measurements of muscle contractions captured by force transducers). This integrated method enables a more detailed functional analysis of DMaps produced from the video recordings.

2.5 Conclusion

The spatiotemporal DMap technique enables a collective assessment of effects on the ENS by measuring an overall change in colonic motility patterns. Using this method, we showed that loperamide inhibited colonic peristaltic contractions in a dose dependent manner and preferentially between the mid and distal colon. The findings from this study may help us better understand the impact of GI opioid pharmacodynamics on the biomechanics of colonic motility.

CHAPTER 3: SLOWED GASTROINTESTINAL TRANSIT IS ASSOCIATED WITH AN ALTERED CAECAL MICROBIOTA COMPOSITION.

This chapter has been published: Parkar N, Dalziel JE, Spencer NJ, Janssen P, McNabb WC, Young W. Slowed gastrointestinal transit is associated with an altered caecal microbiota in an aged rat model. Frontiers in Cellular and Infection Microbiology. 2023;13.

https://doi.org/10.3389/fcimb.2023.1139152

3.1 Introduction

Gastrointestinal (GI) motility is an integral part of digestive function. The enteric nervous system (ENS) plays a major role in control of GI motility.[52] Gut transit time, which refers to the transit of luminal content along the GI tract, is commonly used as a marker of gut motility and function.[51] Measurement of gut transit time is relevant when addressing GI motility disorders such as irritable bowel syndrome (IBS) and constipation.[219]

The mammalian GI tract is colonized by a diverse population of microbial communities. These gut microbes are vital in maintaining host health.[220] Numerous studies have pointed out that an association exists between the gut microbiota and GI motility, and that this relationship is likely to be bidirectional.[221,222] Experiments using germ free (GF) animal models have demonstrated that a lack of microbial colonization correlates with altered ENS functions such as delayed gastric emptying and slowed GI transit.[117] Conversely, alterations in GI motility can modify the resident microbial population as seen in the case of Small Intestinal Bacterial Overgrowth (SIBO), a clinical syndrome often associated with altered GI motility.[223] Environmental factors have also been shown to influence gut microbiota composition. In this context, it can be considered that changes in gut

motility likely led to changes in microbiota composition and function. For example, some microbial taxa benefit from increases in GI motility, relative to other species adapted to conditions associated with slower motility.[224] This concept is consistent with ecological principles of r/K selection in response to environmental disturbance.[224] As GI transit time decreases, such as with diarrhea, species better adapted to grow rapidly during reduced competition (r-selected) will dominate the gut. In contrast, prolonged colonic transit may facilitate the amplification and colonization of slow-growing species, better adapted to persist in competitive environments (K-selected); these species include metabolically economical taxa. The direct effects of gut motility on specific microbial communities could cascade into broad ecosystem changes as the community is interconnected metabolically. The question of identifying which microbial species are impacted by motility is important because shifts from normal microbiota composition can lead to metabolic changes with possible physiological consequences.

Animal models mimicking symptoms of constipation have been developed by way of pharmacological manipulations. Loperamide is an opioid receptor agonist that works by activating the mu opioid receptors located in the myenteric plexus of the ENS [188,189] and does not cross the blood brain barrier.[192] Upon binding to the opioid receptors, loperamide decreases the activity of the myenteric plexus, which subsequently reduces the tone of the circular and longitudinal smooth muscles of the gut wall. This in turn reduces propulsion and extends the total stay time of luminal contents.[225] Through our experiments we have previously shown that loperamide works by inhibiting enteric neuronal activity, delays GI transit, inducing constipation in aged rats.[199] Similarly other studies have reported that loperamide inhibits colonic peristalsis and intestinal water secretion causing delayed GI transit time.[206,226] Loperamide-induced slowed transit is therefore considered to be a model of spastic constipation due to increased colonic contractions and inhibition of stool

frequency.[227] Although the dose and time for loperamide administration has been described to vary among rodent studies, loperamide has shown to be effective in inducing constipation when administered subcutaneously[228-230], orally[206] or intra-peritoneally[231] at doses ranging from 0.2 to 5 mg/kg body weight for 3 to 7 days. Although longer transit times have been associated with increased relative abundances of taxa such as *Akkermansia, Bacteroides* and *Alistipes*,[232] little is known about the changes in gut microbiota profile resulting specifically from pharmacologically induced slowed GI motility in rats. Moreover, the relationship between gut microbiota and altered intestinal motility are based on studies using faecal samples, which are easier to obtain but do not accurately reflect the intestinal microbial community.[233] The caecum serves as a bacterial reservoir that populates the large intestine.[234] The aim of this study was to examine how slowed GI transit due to opioid receptor agonism in the ENS affects the caecal microbiota. We hypothesised that a prolonged GI transit induced by the opioid agonist, loperamide, will lead to microbiota variation.

3.2. Materials and methods

3.2.1 Animals

Male Sprague Dawley rats were bred at the AgResearch Ruakura Small Animal Unit (Hamilton, New Zealand) and raised in group housing with littermates to 18 months of age (804 ± 13 g).[235] The rats were maintained under a 12-hour light/dark cycle with water and food provided ad libitum. Rats were fed a nutritionally balanced diet (OpenStandard Rodent Diet, Research Diets, Inc., New Brunswick, NJ, USA) as previously described.[235] They were monitored three times weekly for General Health Score (1–5; NZ Animal Health Care Standard), weight and food intake. The experiment was performed in accordance with the Animal Welfare Act, 1999 (NZ). The protocol was approved by the AgResearch Grasslands Animal Ethics Committee (Ethics approval No.: AE12933).

3.2.2 Study design and pharmacological treatment

This study included a loperamide-treated group and a control group with age and weight balanced amongst treatment groups. Loperamide hydrochloride (S2480) was purchased from Selleck Chemicals (Houston, TX, USA). Rats were administered 1 mg/kg/day loperamide (in 100% Dimethyl sulfoxide (DMSO) or DMSO Vehicle only (control) for seven days. The drug dose has been previously determined to be effective over seven days.[199] The route of administration was via a subcutaneous 2 mL capacity slow-release osmotic mini pump (Durect Corporation, Alzet Osmotic Pumps, Cupertino, CA, USA) as previously described.[199] The control group received DMSO vehicle only via the same delivery method. The control group consisted of 13 rats and the loperamide-treated group had 11. Some rats died before the end of the study from age related issues.[235]

3.2.3 Caecal microbiota

Caecal content samples were collected rapidly after euthanasia, using carbon dioxide inhalation overdose, and snap frozen in liquid nitrogen and stored at -80 °C before use. Metagenomic DNA was extracted using the NucleoSpin Soil kit (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's instructions, using SL2 lysis buffer and SC enhancer, with the addition of bead beating for four minutes using a BioSpec Mini Beadbeater 96 (Bartlesville, OK, USA) set to maximum speed.

DNA samples were then analysed by 16S rRNA gene amplicon sequencing using the Illumina MiSeq platform with 2 × 250 bp paired-end sequencing with PCR primers targeting the V3 and V4 region:[236]

Forward Primer: 5'-

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Reverse Primer: 5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

PCR thermal cycler conditions were used as specified in the Illumina library preparation protocol (95 °C for 3 minutes; 25 cycles of [95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds]; 72 °C for 5 minutes; Hold at 4 °C).[236] Sequence reads were quality trimmed using the following parameters in QIIME 2:[237] Adapter sequences were removed using the cutadapt function, paired reads joined using vsearch with a minimum overlap of 20 bp, reads were quality trimmed with a 25 q-score cut off, remaining reads denoised and chimera checked using the deblur algorithm. Single nucleotide variants were classified by aligning against the Silva 132 small subunit ribosomal RNA database. Alpha diversity was assessed using the Faith's Phylogenetic Diversity and Chao1 index. Beta diversity was compared using Principal Coordinate Analysis (PCoA) of weighted unifrac phylogenetic distances. The sampling depth used for alpha and beta diversity analysis was 32000 reads. Differences in taxa were analysed using ANCOM-BC[238] with $q<0.05$ considered significant. Differences in overall community profiles were analysed by permutation multivariate analysis of variation (PERMANOVA) using the anosim function in the 'vegan' package for R. R version 1.4.1103 was used for all statistical analyses.[239] Data is presented as mean percentage +/- SEM.

Sequence reads can be downloaded from the NCBI Sequence Read Archive (SRA) under accession PRJNA819534.

3.3. Results

3.3.1 Taxonomic composition at the phylum level

A total of 10 bacterial phyla were detected in both loperamide-treated and control groups, which included four frequently detected phyla: *Bacteroidetes, Actinobacteria, Firmicutes* and *Proteobacteria,* and six minor phyla *Tenericutes, Deferribacteres, Patescibacteria, Cyanobacteria, Elusimicrobia* and *Verrucomicrobia* (***Fig. 10***). All 24 samples contained the four frequently found phyla. No more than six samples contained each of the six minor phyla. No clear differences between treatment were observed at the phylum level ($q>0.05$).

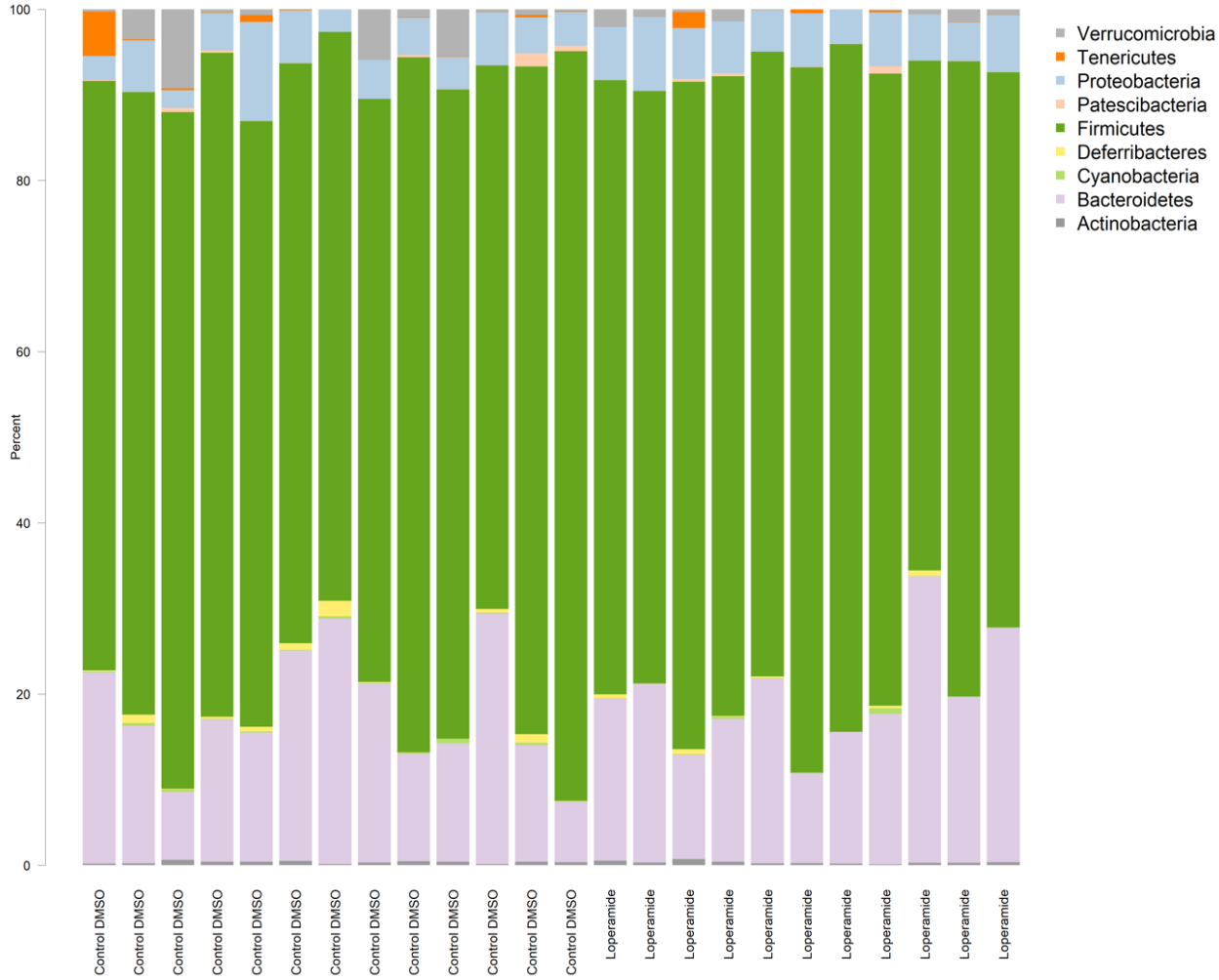


Figure 10: *Distribution of the gut microbiota at the Phylum level. The x axis represents treatment, and the y axis represents relative abundance in percent.*

3.3.2 Microbiota at the family and genus levels

Analysis of the microbiota at the family level showed significant differences ($q<0.05$) between the loperamide-treated and control groups in eleven families (***Table 3***). Of these, approximately half were from the Firmicutes phylum; *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Streptococcaeae*, *Defluviitaleaceae* and *Lachnospiraceae*, which were the most abundant of the significantly different families (loperamide 25.746% ± 1.94; control 31.69% ± 2.141).

Extensive differences at the genus level were also observed with 29 genera significantly different between treatments (***Table 4***). Of the most abundant taxa, *Roseburia* (loperamide 1.527% ± 0.695; control 4.489% ± 0.844) and Unclassified *Lachnospiraceae* (loperamide 2.655% ± 0.649; control 7.575% ± 1.168) were significantly lower in loperamide-treated rats. Genera that were significantly more abundant in the loperamide-treated group and that had a mean relative abundance greater than 1% included *Bacteroides, Phascolarctobacterium, Ruminococcaceae* UCG-005, *Lactobacillus, Blautia, Christensenellaceae* R-7 group and the *Ruminococcaceae* NK4A214 group.

Table 3: *Families with significantly different relative abundances between loperamide- treated and control rats. Data represented as mean percent ± standard error of mean (SEM).*

Phylum	Family	Control	Loperamide	q-value
Bacteroidetes	Marinifilaceae	0.127 ± 0.022	0.337 ± 0.041	0.0009
Proteobacteria	Unclassified Rhodospirillales	0.008 ± 0.003	0.042 ± 0.012	0.03
Proteobacteria	Pseudomonadaceae	0.013 ± 0.002	0.008 ± 0.002	0.01
Actinobacteria	Corynebacteriaceae	0.010 ± 0.003	0.003 ± 0.001	0.03
Cyanobacteria	Unclassified Gastranaerophilales	0.187 ± 0.040	0.111 ± 0.056	0.02
Firmicutes	Aerococcaceae	0.025 ± 0.005	0.010 ± 0.003	0.005
Firmicutes	Carnobacteriaceae	0.187 ± 0.018	0.125 ± 0.017	0.001
Firmicutes	Enterococcaceae	0.066 ± 0.011	0.036 ± 0.005	0.005
Firmicutes	Streptococcaceae	0.329 ± 0.043	0.217 ± 0.030	0.01
Firmicutes	Defluviitaleaceae	0.043 ± 0.007	0.022 ± 0.004	0.01
Firmicutes	Lachnospiraceae	31.69 ± 2.141	25.746 ± 1.94	0.02

Table 4: *Genera with significantly different relative abundances between loperamide- treated and control rats. Data represented as mean percent ± standard error of mean (SEM).*

Family	Genus	Control	Loperamide	q-value
Bifidobacteriaceae	Bifidobacterium	0.007 ± 0.002	0.041 ± 0.017	0.036
Bacteroidaceae	Bacteroides	4.964 ± 0.709	7.731 ± 1.113	0.013
Ruminococcaceae	Candidatus Soleaferrea	0.029 ± 0.006	0.037 ± 0.005	0.014
Barnesiellaceae	Barnesiella	0.027 ± 0.010	0.052 ± 0.012	0.013
Ruminococcaceae	Ruminococcaceae NK4A214 group	0.912 ± 0.108	1.427 ± 0.163	<0.001
Ruminococcaceae	Ruminococcaceae UCG-005	3.789 ± 0.751	7.616 ± 1.177	0.003
Ruminococcaceae	Ruminococcaceae UCG-010	0.221 ± 0.036	0.072 ± 0.016	0.003
Marinifilaceae	Butyricimonas	0.096 ± 0.016	0.235 ± 0.033	<0.001
Marinifilaceae	Odoribacter	0.031 ± 0.007	0.102 ± 0.015	<0.001
Erysipelotrichaceae	Erysipelotrichaceae UCG-003	0.220 ± 0.072	0.623 ± 0.135	0.013
Erysipelotrichaceae	Faecalibaculum	0.068 ± 0.045	0.155 ± 0.040	0.023
Erysipelotrichaceae	Unclassified Erysipelotrichaceae	0.536 ± 0.159	0.054 ± 0.041	0.007
Acidaminococcaceae	Phascolarctobacterium	4.701 ± 1.213	8.507 ± 1.172	0.025
Unclassified Rhodospirillales	Unclassified Rhodospirillales	0.008 ± 0.003	0.042 ± 0.012	0.003
Burkholderiaceae	Parasutterella	0.327 ± 0.074	0.535 ± 0.069	0.003
Enterobacteriaceae	Kluyvera	0.001 ± 0.0003	0.002 ± 0.001	0.015
Carnobacteriaceae	Granulicatella	0.002 ± 0.001	0	0
Lactobacillaceae	Lactobacillus	3.469 ± 0.642	7.088 ± 0.809	0.001
Christensenellaceae	Christensenellaceae R-7 group	0.940 ± 0.096	1.688 ± 0.259	0.003
Atopobiaceae	Unclassified Atopobiaceae	0.004 ± 0.001	0.012 ± 0.003	0.014

Family XIII	Anaerovorax	0.047 ± 0.010	0.056 ± 0.004	0.021
Lachnospiraceae	ASF356	0.180 ± 0.1	0	0
Lachnospiraceae	Blautia	3.251 ± 1.132	5.246 ± 0.874	0.025
Eggerthellaceae	Enterorhabdus	0.001 ± 0.001	0	0
Lachnospiraceae	GCA-900066575	0.259 ± 0.049	0.083 ± 0.028	0.042
Eggerthellaceae	Gordonibacter	0.009 ± 0.001	0.013 ± 0.002	0.013
Lachnospiraceae	Marvinbryantia	0.243 ± 0.069	0.912 ± 0.213	<0.001
Lachnospiraceae	Roseburia	4.489 ± 0.844	1.527 ± 0.695	0.013
Lachnospiraceae	Unclassified Lachnospiraceae	7.575 ± 1.168	2.655 ± 0.649	0.014

3.3.3 Microbiota diversity

Principal coordinate analysis of Unifrac phylogenetic distances showed strong separation overall between caecal microbiotas from control and loperamide-treated rats (***Fig. 11***). Permutation multivariate analysis of variance (PERMANOVA) confirmed that the overall differences in communities were significant (P=0.008). The microbiotas of control rats were also significantly more diverse with more observed operational taxonomic units (OTUs) (P=0.017), and a higher Faith's phylogenetic distance (P=0.06) compared to loperamide-treated rats (***Fig. 12***).

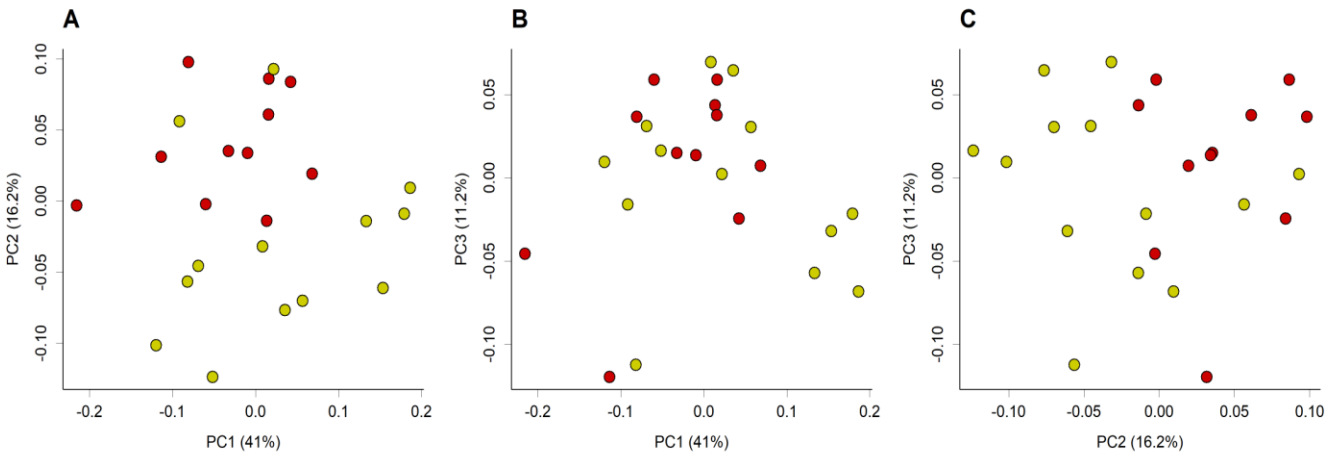


Figure 11: *Principal coordinate analysis (PCoA) plot of weighted unifrac phylogenetic distances of caecal microbiotas from control (yellow) or loperamide (red) groups. Plots show (A) PC1 vs PC2, (B) PC1 vs PC3, and (C) PC2 vs PC3. Percentages on axes indicate the proportion of variation explained by each dimension. Communities between groups were significantly different (PERMANOVA P=0.008).*

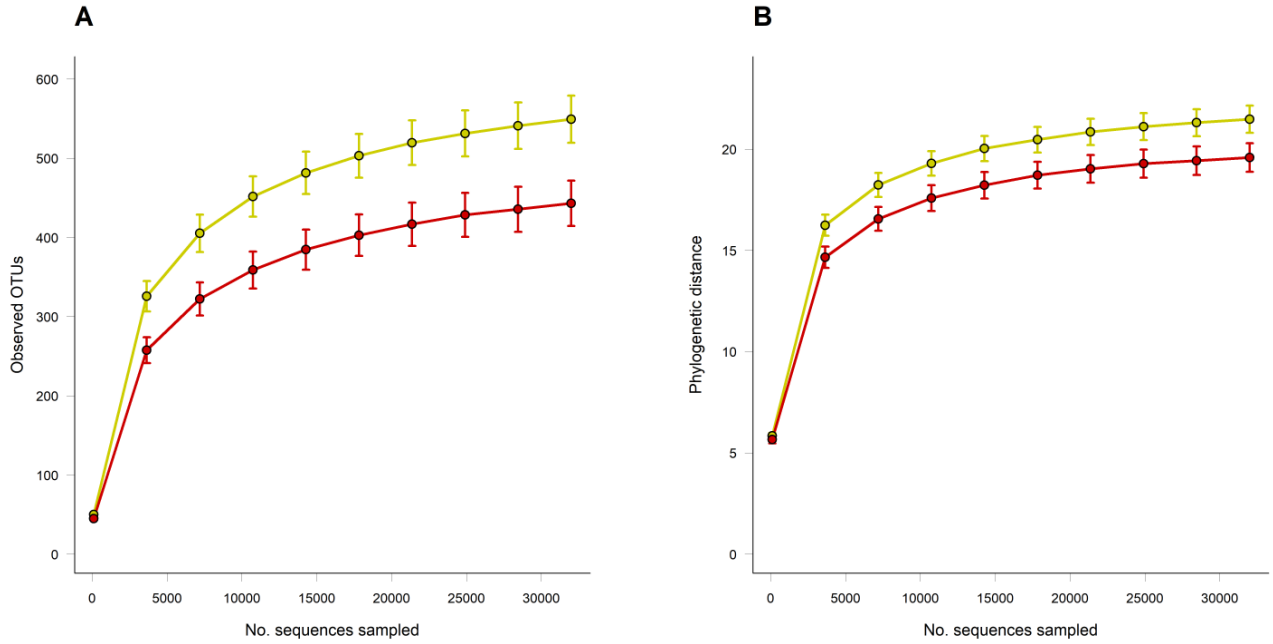


Figure 12: *Rarefaction curves of (A) observed OTUs and (B) Faith's phylogenetic distance (PD) between caecal microbiotas from control (yellow) or loperamide-treated (red) groups, showing reduced diversity in the loperamide group. Error bars show SEM of 10 iterations per sampling depth. Observed OTUs P=0.017, Faith's PD P=0.06. OTU: Operational taxonomic unit.*

3.4 Discussion

It is important to understand the association between GI transit time and the gut microbiota due to the potential impact of the gut microbiota on host physiology and the transition between healthy and diseased states. Dalziel and colleagues have previously reported that the pharmacological drug loperamide delayed GI transit in rats compared to un-treated.[235] Delayed GI transit time, as seen in people with constipation, affects the microbiota composition by decreasing beneficial bacteria and increasing harmful bacteria.[240] Interestingly, *Bacteroides*, the predominant genus in the human gut and a beneficial symbiont/commensal,[241] in this study was found to be more relatively abundant in loperamide-treated rat caecal samples compared to controls. This finding is consistent with a previous study in which *Bacteroides* were shown to be significantly increased in constipated women compared to controls.[242] The loperamide-induced prolonged transit time[235] might have facilitated the increase in relative abundance of *Bacteroides*, indicating that they can adapt well in a slow and competitive environment (Based on r/K selection theory of microbial ecology).[224] Increased relative abundance of *Bacteroides* may be associated with alteration of gut microbiota homeostasis. Given their large genome bank, *Bacteroides* have the ability to turn on certain genes to shift from friendly commensal to harmful bacteria.[241] In contrast, loperamide-induced slowed transit caused a decrease in the relative abundance of the phylum *Actinobacteria* and selected genus in the phylum *Firmicutes* (*Roseburia*) that are associated with faster colonic transit.[243] These findings suggest that taxa of these phyla do not adapt well in a slowed transit luminal environment. Overall, these findings suggest that normal gut motility is key in maintaining a balanced gut ecosystem and gut homeostasis.

In this study, we investigated if changes in microbiota is associated with changes in gut transit time (using data from a previous study where loperamide was effective at inhibiting GI transit compared

to controls).[235] We found that the ecological diversity and richness in the caecal microbiota differed significantly between loperamide-treated rats and controls. Alpha-diversity analysis showed that the richness and diversity of the bacterial communities was significantly lower in the loperamide-induced slowed transit group compared to the control group. This is in line with a study by Ren et al., who showed that the control group exhibited higher bacterial diversity and richness than the constipation group, concluding that higher microbial diversity may correspond to healthier ecosystems.[244] In contrast, several studies have reported the diversity and richness of bacterial communities to be higher in the constipation group compared to controls.[245,246] Furthermore, these studies went on to show that increased alpha diversity was significantly associated with longer colonic passage, the explanation being diversification as an adaption to a perturbed ecosystem (i.e., depletion of nutrients, switch from microbial saccharolytic to proteolytic fermentation, microbial competition and decreased water availability).[246] These contrasting findings indicate that microbial diversity should be interpreted within the physiological context and reduced microbial alpha diversity should not necessarily be represented as reduced microbiota stability.

Numerous studies have documented the role of gut microbiota-derived molecules in regulating gut motility.[132,247,248,249] Production of short chain fatty acids (SCFA), especially butyrate, by the gut microbiome was shown to influence GI motility.[249] In our study, the butyrate producing genus *Roseburia* was found to be significantly reduced in loperamide-induced constipated rats. This is similar to a study by Chassard et al. in which butyrate-producing *Roseburia - E. rectale* group was found to be lower in the IBS-with-constipation group compared to controls. The authors concluded that a reduced relative abundance of butyrate producers makes colonic transit slower.[250] Experiments by Soret et al., and Reigstad et al., showed that butyrate producing bacteria may increase colonic motility by inducing the release of serotonin or promoting cholinergic

pathways.[130,251] Conversely, studies have reported butyrate producing genera to be associated with constipation.[252] Butyrate has been shown to impact various colonic effects; such as inhibition of smooth muscle contractions in the colon, reduction of stool volume through stimulation of colonic electrolyte and water absorption, predisposing to constipation.[252] These inconsistencies in the literature can be addressed by carrying out further research to identify the mechanisms and involvement of butyrate producers in prolonged colonic transit.

We speculate that a slowed gut transit might modify the spatial organization and proportion of the microbiota by creating a luminal microenvironment for the growth of specific bacterial taxa, or by affecting bacterial colonization. Moreover, the influence of luminal microenvironments might be relevant in regions where key motility patterns are initiated such as the proximal colon. In our study, a slowed GI transit time induced by loperamide led to increased relative abundance of families *Bacteroidaceae* and *Marinifilaceae,* belonging to the phylum Bacteroidetes. Several studies have proposed Bacteroidetes to be the dominant gram-negative bacteria in the GI tract.[101] Alterations in distribution of gram-negative bacteria is associated with elevated levels of lipopolysaccharides (LPS), a cell wall component of gram-negative bacteria.[253] LPS is thought to be an important mediator of the microbiome's influence on host physiology. Several studies have pointed out an inhibitory role of LPS on GI motility. Mikawa et al., through their experiments showed that LPS-induced nitric oxide synthase produced nitric oxide, which in turn inhibited GI motility.[254] It would therefore follow that changes in the composition of gram-negative bacteria might further cause GI motility disturbances. We did not measure LPS levels in this study; future studies might shed more light on the possible specific association of gram-negative bacteria and LPS on GI motility.

In the present study, we used 16S rRNA gene amplicon sequencing to analyse DNA samples. Although there is valuable information gained from 16S sequencing, there are also some limitations. The sequencing depth may not be sufficient for short amplicon sequencing to capture novel or low abundance microbial species. Moreover, this method does not directly provide information about the functional capacities of the organisms. In contrast, whole genome sequencing would have revealed a strain level resolution of both microbiota abundance and functional capacity and would have given a comprehensive understanding regarding the association between varied gut transit time and dysbiosis. This could be the subject of further studies.

3.5 Conclusion

Our findings indicate that the loperamide-induced alterations in gut transit time affected the diversity and relative abundance of caecal microbial communities. We speculate that slowed colonic transit facilitates the amplification and colonization of select genera such as *Bacteroides* that adapt well in slow and competitive environments (corresponding to prolonged transit and limited resources). The relationship between gut motility and microbiota is relevant in experimental models used to study several functional GI disorders associated with the gut microbial composition, such as IBS and constipation, where GI transit is also altered. Understanding the link between specific microbial species and varying transit times is crucial to design microbiota-based interventions to treat intestinal motility disorder.

CHAPTER 4: MODULATING THE ENS (AND SUBSEQUENTLY) INHIBITING COLONIC MOTILITY INFLUENCES ANXIETY RELATED BEHAVIOUR

This chapter, after adapting it to a journal manuscript format, is planned for submission to the British Journal of Pharmacology. Findings have been published in abstract form in Gastroenterology journal.

Activation of mu opioid receptors in the gut influences anxiety related behaviour in a sex specific manner.

4.1 Introduction

It has long been known that the brain exerts an influence on gut function but there is increasing evidence that the brain and gut communicate both ways.[7,255] This complex bidirectional communication system that not only ensures proper maintenance and coordination of GI functions, but also permits signalling from the gut to influence mood, higher cognitive functions and mental health is enclosed in the denomination of GBA. The two main components of the GBA are the CNS and ENS.[6] The gut wall is embedded with an intrinsic nervous system, referred to as the ENS which, along with its diverse population of enteric neurons and glial cells orchestrates gastrointestinal functions such as intestinal motility and secretion.[13] The ENS has been referred to as the 'brain in the gut' due to its similarities to the brain in terms of various aspects such as neuronal components, neurotransmitters, and functional independence.[8,13] The ENS communicates with the brain not only via complex extrinsic neural circuitry but also chemical signalling pathways,

that provide opportunities for gut luminal factors to influence not only gut function but also the brain.

Increasing evidence suggests that the enteric microbiome greatly impacts on gut-brain communication and experiments investigating the impact of the gut microbiota on brain and behaviour are gaining recognition.[77] As a result the term GBA has been expanded into microbiota gut brain axis. The gut microbiome has been shown to impact the development and function of both the ENS and CNS.[16] Alterations in ENS neurochemistry and function by the gut microbiome have been highlighted in numerous studies using various animal models. For example, in germ free (GF) mice (specially raised animals devoid of all microorganisms) reduced number of enteric neurons were observed that corelated to abnormal GI motility.[17,18] In addition, changes in microbiota composition or dysbiosis has been shown to influence cognitive function, mood and behaviour.[19,20] With the help of behavioural tests such as the open field (OF) and elevated plus maze (EPM) using rodents, these studies also confirm that microbiota affects the anxiety and stress system by influencing brain neurochemistry.[90] For example, Neufeld and colleagues using female germ-free mice demonstrated that the absence of a conventional microbiota resulted in a reduction in anxiety behaviour in the EPM, a well validated model of anxiolytic tendencies.[140] Given the proximity of the gut microbiome to the ENS, the gut microbiome has emerged as a major player to influence the ENS with subsequent effects on GI physiology and potentially gut to brain signalling. However, due to the dynamic and complex interactions between ENS, CNS and gut microbes, understanding the mechanisms responsible for these effects is a difficult task.

The endogenous opioid system regulates pain relief, emotion, stress, feeding motivation and eating behaviour.[256] It consists of central and peripheral divisions which consist of mu, delta, and kappa

receptors that are widely distributed in the brain and GI tract. These receptors can be activated endogenously by endorphins, dynorphins, enkephalins and endomorphin as well as exogenously by opioid agonists. Endogenous opioid peptides, including enkephalins, dynorphins and endorphins, regulate gastrointestinal function by slowing motility and secretomotor activity.[257] Moreover, as described previously, opioid agonists such as morphine, mediate their effects on the gut through activation of the mu opioid receptors expressed by enteric neurons, as demonstrated by mechanistic studies using selective mu opioid receptor targeting drugs and *oprm1* knockout mice.[190,258] Recent studies have shown that mu opioid receptors expressed in the gut can regulate the gut to brain neural circuitry involved in satiety.[259,260] A study by Kaneko and colleagues showed that orally ingested soymorphin (soy derived mu opioid peptide) suppressed food intake via activation of gut mu opioid receptors.[260] Moreover, Sudokov and colleagues showed that mu opioid agonist administered via oral gavage reduced anxiety like behaviour in rats as shown in the EPM where loperamide treated rats spent more time in the open arms and exhibited increase in the number of entries into the open arms compared to controls.[261] Targeting the peripheral opioid receptors presents a viable approach to understanding how gut derived signals can impact brain function. Furthermore, the presence of sex differences in opioid receptor expression, which results in significant variations in opioid receptor mediated effects between males and females,[262,263] represents a signalling pathway that can potentially enhance our understanding of the physiological and behaviour disparities in the gut-brain research space.

The aim of this study was to find out whether altering ENS activity has any effects on behavioural responses and to understand underlying mechanisms of gut to brain signalling. I used an ENS modulating drug – loperamide to investigate its effect on brain neurochemistry, and anxiety related behaviour using behavioural tests. Loperamide is an antimotility drug that acts on the ENS to

decrease gut motility. It works by activating ENS opioid receptors primarily of the mu type in the myenteric plexus of the large intestine, decreasing the activity of the myenteric plexus which decreases the tone of the longitudinal and circular smooth muscles of the intestine causing slowed GI transit.[189] It does not cross the blood brain barrier[192] and therefore it was used in this study as an effective tool to investigate how modulating gut ENS activity impacts anxiety related behaviour. This involved observing and measuring various behavioural parameters related to anxiety using the OF and EPM tests. Furthermore, the study aimed to uncover changes in gene expression within specific brain regions that play critical roles in stress responses, namely the amygdala, hippocampus, and prefrontal cortex. To further elucidate the possible influence of ENS mu opioid receptor activation on gut to brain signalling, gut (specifically the proximal colon) gene expression, and caecal microbiome were also investigated.

4.2. Materials and methods

4.2.1 Animals

Adult male Sprague Dawley rats (weight 200–300 g; 6-8 weeks of age; AgResearch Ruakura, New Zealand) were used in this study. The experiment was performed in accordance with the Animal Welfare Act, 1999 (NZ). The ethics application was approved by the AgResearch Grasslands Animal Ethics Committee (Ethics approval No.: AE15077). Every possible measure was taken to minimize distress and employ the minimum number of animals required to obtain dependable scientific data. Initially, all rats were pair-housed in individual cages, maintaining standard laboratory conditions with a room temperature of 22°C and 55% humidity. They were subjected to a 12-hour light/dark cycle (with lights on from 8 am to 8 pm) and provided unrestricted access to rat chow and tap water. Upon their arrival, the rats underwent a one-week acclimation period to adjust to their new living environment.

4.2.2 Study design

This study involved the use of 32 Sprague Dawley rats (16 male and 16 females), approximately 6-8 weeks old at the time of behavioural tests. The experiment was run in 4 blocks. Each block consisted of 8 rats; 4 rats from each treatment group (control or loperamide; 2 males and 2 females per treatment group). Rats arrived from the breeding centre and were acclimatised to the new living environment for 1 week after which they were handled by an experimenter for another week (***Fig. 13a***). On day 15, rats were administered subcutaneously with DMSO (Control) or loperamide two hours prior to the first behaviour test which was the OF test (***Fig. 13b***). On day 16 rats were re-administered with control or loperamide two hours prior to sampling (***Fig. 13c***).

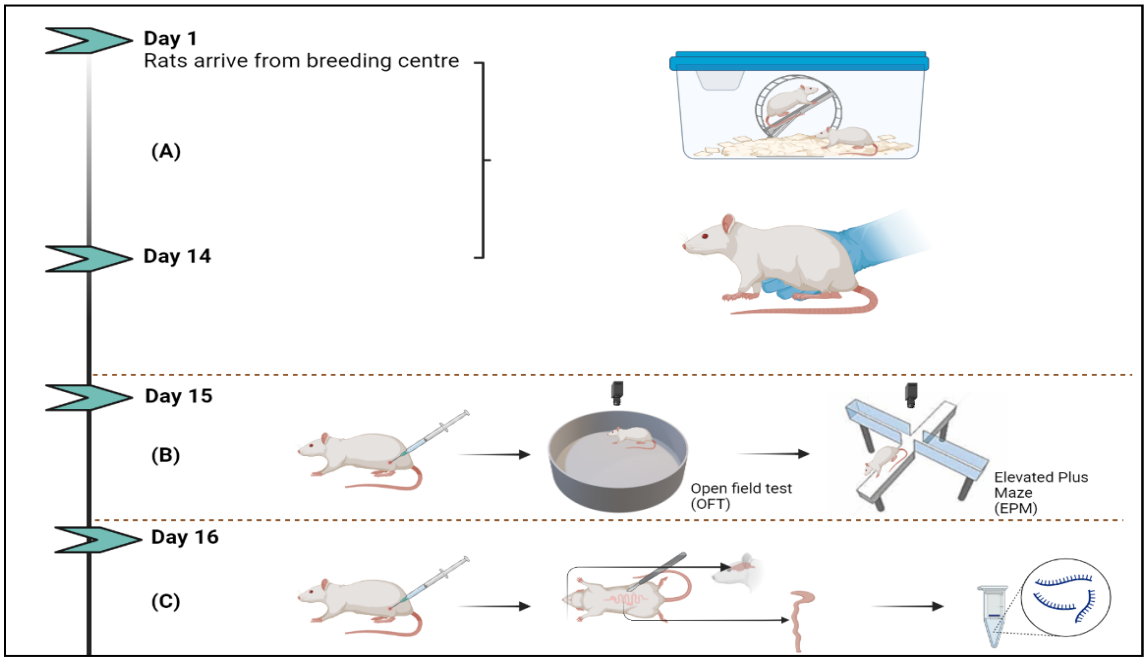


Figure 13: *Study design. (a) Mice were acclimatised to the new living environment for one week after which they were handled for one week. (b) On the day of the behaviour tests rats were administered with loperamide or control 2 hours prior to the first behaviour test (OF test). (c) Rats were re-administered with control or loperamide on the next day 2 hours prior to sampling.*

4.2.3 Behavioural tests

On the day of behaviour testing, animals were first left to acclimatise for at least 30 min in the same experimental room where they later underwent the open field test and elevated plus maze test. To minimise stress during experiments, mice were handled for 5 min every day for one week prior to behavioural tests.

The OF test was used to assess anxiety like exploratory and locomotor behaviours. The testing arenas consisted of an enclosed circular area with a diameter of 90 cm and a 50 cm high wall. The experiments were performed during the light phase of the light/dark cycle in a bright lit environment level (800-900 lux) adjacent to the housing room. Each animal was placed in the testing arena and allowed to move freely for 10 min while being recorded by a video camera (HERO 7, GOPro, USA) positioned 130 cm above the testing arena. Surfaces were cleaned with 70% ethanol between animals.

The EPM was used to test anxiolytic/anxiogenic effects of the drug. The EPM consisted of four arms (10 cm wide x 100 cm deep; elevation from floor: 65 cm) arranged around a small centre platform in a plus shape. Two opposite arms were enclosed with high walls (35 cm high) and the other two were open arms. This task utilises the natural preference rodents have for enclosed areas to test for anxiety. The rodent was placed in the centre facing one of the open arms and was allowed to explore the maze for 5 min while being recorded by an overhead camera. Anxiety-like metrics assessed in the EPM were total distance moved (cm), velocity (cm/s), percentage number of open and closed arm entries (scored when the centre point of the body crossed into the respective zone), and percentage time spent in open and closed arms of the EPM.

The recorded videos were analysed using EthoVision XT 10 (Noldus, Wageningen, The Netherlands). The video tracking software tracked the centre-point of the rat as the reference point to record variables for the OF and EPM tests. The experimenter conducting the analysis was blinded to the treatment groups. EthoVison software automatically recorded the following variables: mean velocity (cm/s), total distance moved during the test (cm), the frequency and duration (s) spent in each of the zones of interest [OF: 4 concentric zones (***Fig. 14d***), EPM: Closed arms, open arms, and centre zone (***Fig. 15***)] and the number of rearings.

4.2.4 Sample collection

Brain, gut tissue, and caecal content were collected immediately after euthanasia. The brain regions of interest were the hippocampus, prefrontal cortex, and the amygdala. Gut tissue samples were dissected from proximal colon. Samples from these regions were stored in RNAlater (Ambion, Life Technologies, Carlsbad, CA) for 24 h and then transferred to -20^{o}C for later analysis of gene expression. Caecal content that was collected for microbiome analysis was snap frozen in liquid nitrogen and stored at -80^{o}C for later analyses.

4.2.5. Gene expression

4.2.5.1 RNA isolation

Gene expression profiles in proximal colon tissue and different brain regions were analysed by RNA-seq. Isolation of RNA from brain tissue was done using QIAzol Lysis Reagent (Qiagen, Valencia, CA) followed by clean up using a RNeasy Lipid Tissue Mini Kit (Cat. No. QLAG74804, Qiagen, Valencia, CA. Tissues samples were added to the QIAzol lysis buffer and homogenised for 30 s using a rotor homogeniser (TissueRuptor, Qiagen, Netherlands), followed by incubation at room temperature for 5 min. Chloroform (200 ml) was then added, vortexed for 15 s and incubated

at room temperature for 2-3 min. The mixture was then centrifuged at 12,000 g for 15 min. at 4. Approximately 600 ml of the upper aqueous phase was placed into a new tube which was added with one volume of 70% ethanol and vortexed for 15 s. 700 ml was then transferred to a RNeasy spin column for RNA clean up following the manufacturer's instructions. The RNA was eluted with 30 ml RNAse free water and stored at -80^{o}C for subsequent sequencing.

4.2.5.2 RNA analysis

To prepare strand-specific cDNA libraries, NEBNext® Ultra Directional RNA Library Prep Kits for Illumina® (New England Biolab, Ipswich, MA, USA) were utilized. The libraries were size-selected to include fragments of 250-300 bp and were sequenced using an Illumina Novaseq 6000, generating 150 bp paired-end sequences. Trimmomatic 0.36 was employed for quality trimming of the reads, and the remaining read pairs that passed the quality trimming were aligned to the Rattus norvegicus genome (Rnor 6.0 release 102) using STAR. Uniquely mapped read pairs were then aggregated for each gene and subjected to analysis using a likelihood ratio generalized linear model in the EdgeR package for R. Genes exhibiting a > 1.5-fold difference (i.e., |log fold change| > 0.58) with a false discovery rate (FDR) < 0.05 were considered differentially expressed. Additionally, gene expression was assessed through gene set enrichment analysis (GSEA) using the mroast function from limma, with Reactome pathways serving as the gene sets. In GSEA, the collective expression of groups of genes is examined as a single unit rather than analysing individual genes independently.[264]

4.2.6 Caecal microbiota

Metagenomic DNA extraction was performed using the NucleoSpin Soil kit (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's instructions, with the inclusion of bead

beating for 4 minutes using a BioSpec Mini Beadbeater 96 (Bartlesville, OK, USA) at maximum speed.

Indexed sequencing libraries were created using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (New England Biolab, Ipswich, MA, USA) following the manufacturer's guidelines. In summary, DNA samples were sonicated to fragment sizes of 300 bp, followed by end-polishing, A-tailing, and ligation with full-length adaptors for sequencing on an Illumina Novoseq 6000 instrument, generating 150-bp paired-end reads. Trimmomatic v.0.36 was used for quality trimming of the reads, and PEAR version 0.9.6 was employed to merge the read pairs. Removal of host reads was performed using the bbduk.sh function from the BBMAP package version 38.22-0, using the Rattus norvegicus genome (Rnor 6.0 release 102) as a reference. The "blastx" function of DIAMOND version 0.9.22 was utilized to align the reads against the "nr" NCBI database. To assign putative functions to the DIAMOND alignment files against the KEGG database,[265] MEGAN6 Ultimate Edition was utilized. Differences in the relative abundances of individual taxa and gene functions were analyzed using the MaAsLin2 package in R.[266]

4.2.7 General statistical analysis

Animal metric data and behavioural analysis was performed using GraphPad Prism. Animal metric data was analysed using a repeated measure two-way ANOVA (sphericity not assumed; Greenhouse-Geisser correction applied). Significant ANOVA results were further analysed using Sidak's test for multiple comparisons as post hoc analyses.

Behavioural data was analysed using two-way ANOVA (sphericity not assumed; Greenhouse-Geisser correction applied) with significant ANOVA results further analysed using Tukey's

multiple comparison test as post hoc analyses. Statistical significance was set at an alpha level of less than 0.05. Data are presented as mean ± standard error of mean (SEM).

4.3. Results

4.3.1. Animal metrics

4.3.1.1 Food intake, body weight and faecal output

Table 5 shows data on bodyweight, food intake and faecal output. Food intake was significantly reduced 24 h post loperamide treatment by 38% in male rats and by 44% in female rats compared to pretreatment controls. Similarly, loperamide treatment significantly reduced faecal output by 31% in male rats and by 38% in female rats compared to pretreatment controls. No changes were observed in bodyweights in any of the treatment groups.

Table 5: *Effect of loperamide on rat body weight, food intake, and faecal output. Significant differences in food intake and faecal output observed posttreatment compared with pretreatment in both male and female rats, **P<0.01; ***P<0.001. Mean values for each treatment group ± SEM. Pretreatment measurement taken on day 15, and posttreatment on day 16.*

Gender	Treatment	Bodyweight (g)		Food intake (g)		Faecal output (g)		Rat
		Pre	Post	Pre	Post	Pre	Post	n
Male	Control/ DMSO	481.62 ± 14.30	480.75 ± 13.82	32.12 ± 1.79	31.37 ± 1.08	9.61 ± 0.46	9.18 ± 0.61	8
	loperamide	480.87 ± 16.20	474.87 ± 17.54	33.37 ± 1.20	20.75 ± 2.01***	9.62 ± 0.44	6.68 ± 0.53***	8
Female	Control/ DMSO	266.87 ± 8.64	265.12 ± 8.22	21.87 ± 1.07	19.12 ± 0.76	5.93 ± 0.24	5.71 ± 0.22	8
	loperamide	278.85 ± 8.44	275.42 ± 8.65	22.85 ± 0.67	12.85 ± 1.71**	6.08 ± 0.16	3.80 ± 0.45**	7

4.3.2. Behavioural Test

4.3.2.1 Open Field Test

In terms of total distance moved, loperamide treated female rats moved less compared to controls (Control: 6176.97 ± 428.18 cm, n=8; loperamide trt.: 4322.57 ± 647.11 cm, n=7; P<0.04) **(*Fig. 14a***). Similarly, loperamide treated female rats moved slower compared to controls (Control: 10.29 ± 0.71 cm/s, n=8; loperamide trt.: 7.20 ± 1.07 cm/s; n=7; P<0.04) (***Fig. 14b***). In contrast, total distance moved, and velocity did not significantly differ between loperamide treated males and male controls (n=16; P>0.05). Loperamide treated female rats reared less against the walls compared to female controls (Control: 48.25 ± 5.50, n=8; loperamide trt.: 29.24 ± 6.79, n=7; P<0.03) (***Fig. 14c***). On the other hand, rearing behaviour did not differ significantly between loperamide treated male rats and male controls (n=16; P>0.05). No differences were observed between treatment groups in both male and female rats with respect to the time spent in different zones of the open field arena (***Fig. 14e and f***)

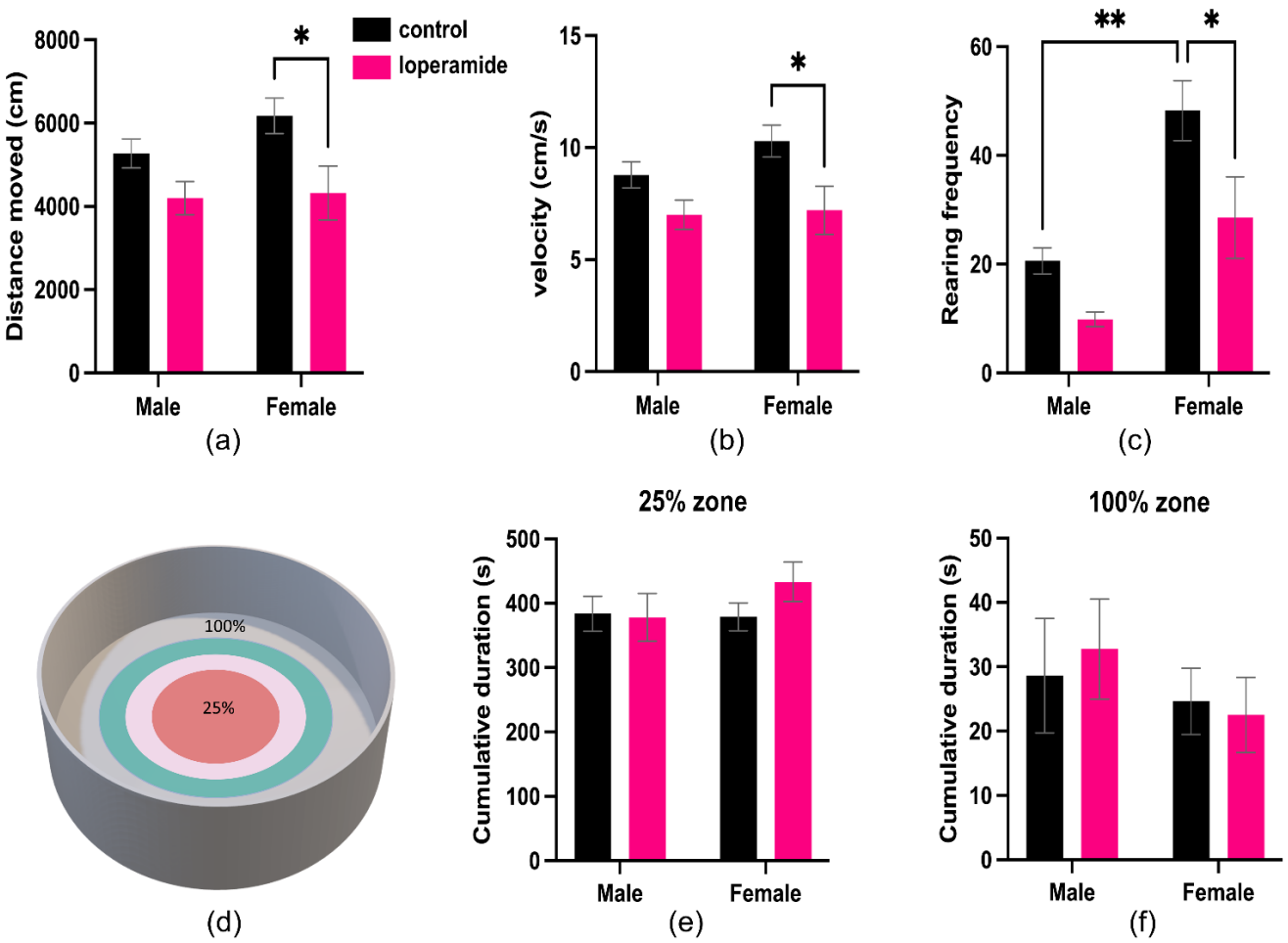


Figure 14: *Open field test (a) Distance moved by male and female rats in the OF test. (b) Velocity of tracked movement. (c) Rearing frequency. (d) The coloured concentric circles are representative of different zones in the arena (red represents centre or 25% zone; grey represents periphery or 100% zone). (e) Time spent in 25% or centre zone of OF arena. (f) Time spent in the 100% zone or periphery of the OF arena. Asterisks indicate statistical significance (*P<0.05; **P<0.01). Data shown as mean with error bars indicating SEM.*

4.3.2.2 Elevated plus maze

Loperamide treatment in females affected their performance in the EPM. Loperamide treated female rats moved less compared to female controls (Control: 2478.31 ± 83.71 cm, n=8; loperamide trt.: 1959.46 ± 116.62 cm, n=7; P<0.005) (***Fig. 15a***). Moreover, the velocity of loperamide treated female rats was slower compared to female controls (Control: 8.26 ± 0.27 cm/s, n=8; loperamide trt.: 6.53 ± 0.38 cm/s, n=7; P<0.005) (***Fig. 15b***). In contrast, total distance moved, and velocity did not significantly differ between loperamide treated male rats and male controls (n=16; P>0.05). A significant effect of sex on total distance moved and velocity was observed in the EPM with control female rats moving more and quicker than control male rats (***Fig. 15a and b***). In terms of percentage time spent and percentage entries to different arms of the EPM, loperamide treated female rats showed a significantly lower percentage of time spent in the open arms of the EPM compared to female controls (Control: 44.49 ± 2.39 %, n=8; loperamide trt.: 33.95 ± 3.09 %, n=7; P<0.03) (***Fig. 15d***). However, no significant differences were observed in terms of percentage entries to open arms between loperamide treated female rats and female controls (n=15; P>0.05) (***Fig. 15c***). Similarly, percentage time spent and percentage entries to closed arms did not differ significantly between loperamide treated female rats and female controls (n=15; P>0.05). Loperamide administration in male rats did not affect their performance in any of the EPM parameters when compared to controls (n=16; P>0.05). No significant differences were observed in terms of percentage time spent in centre zone of the EPM between loperamide treated male and female rats and their control counterparts (P>0.05).

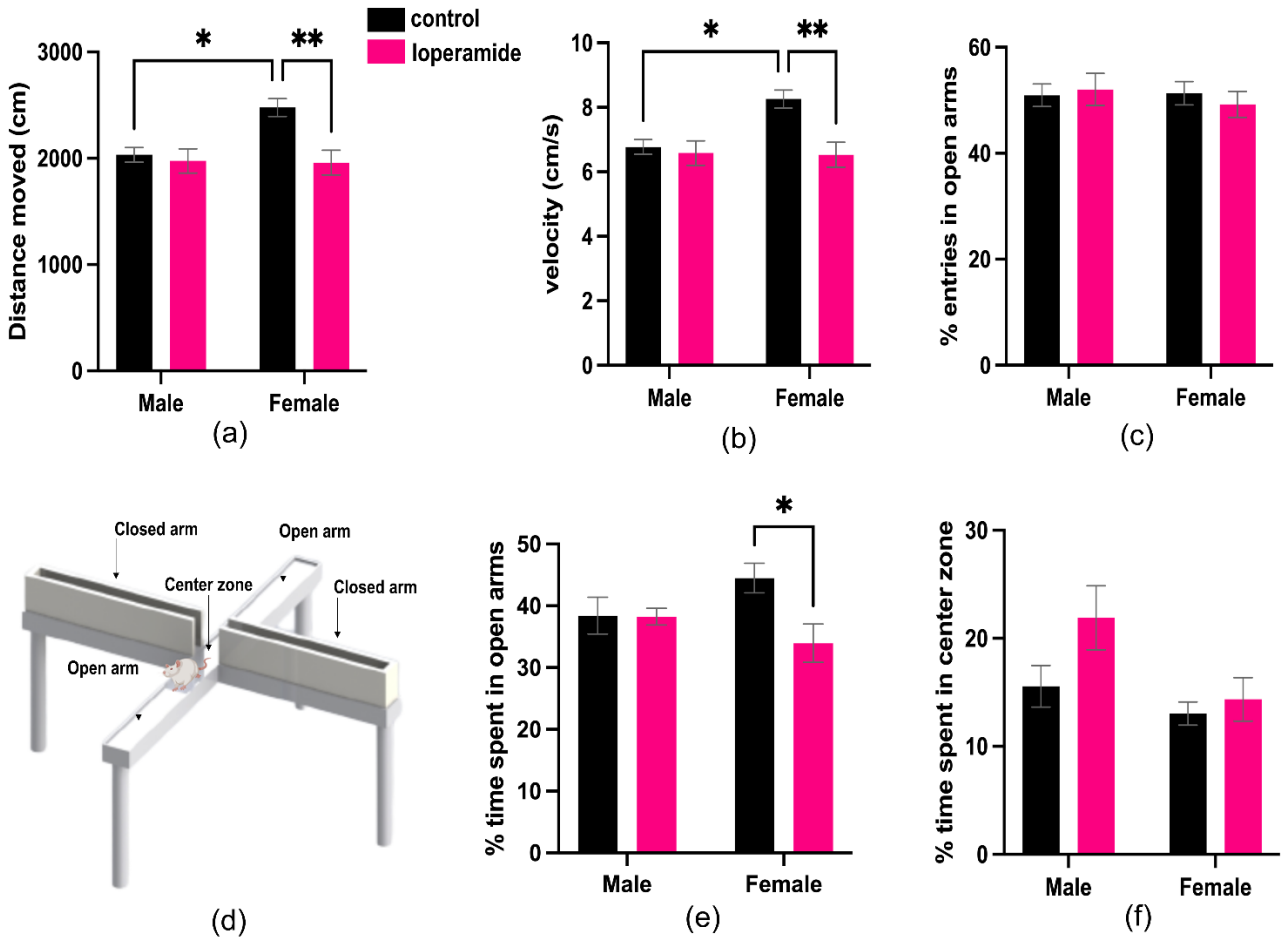


Figure 15: *Elevated plus maze (a) Graph showing total distance moved in EPM. (b) Graph showing velocity (c) Graph showing % entries in open arms of the EPM. (d) Graph showing % time spent in open arms of the EPM. (e) Graph showing % time spent in centre zone of the EPM. Asterisks indicate statistical significance (*P<0.05; **P<0.01). Data shown as mean with error bars indicating SEM.*

4.3.3. Brain and gut tissue transcriptome

4.3.3.1 Amygdala, prefrontal cortex, and hippocampus

In male rats, there were no significant differences in gene expression between treatment groups at the individual transcript level in any of the brain regions examined, namely amygdala, prefrontal cortex, and hippocampus. There were no significant differences in gene expression at the individual transcript level in the amygdala and prefrontal cortex in female rats. However, in female rats, 9 genes in the hippocampus were found to be differentially expressed between control and loperamide groups (***Figure 16***).

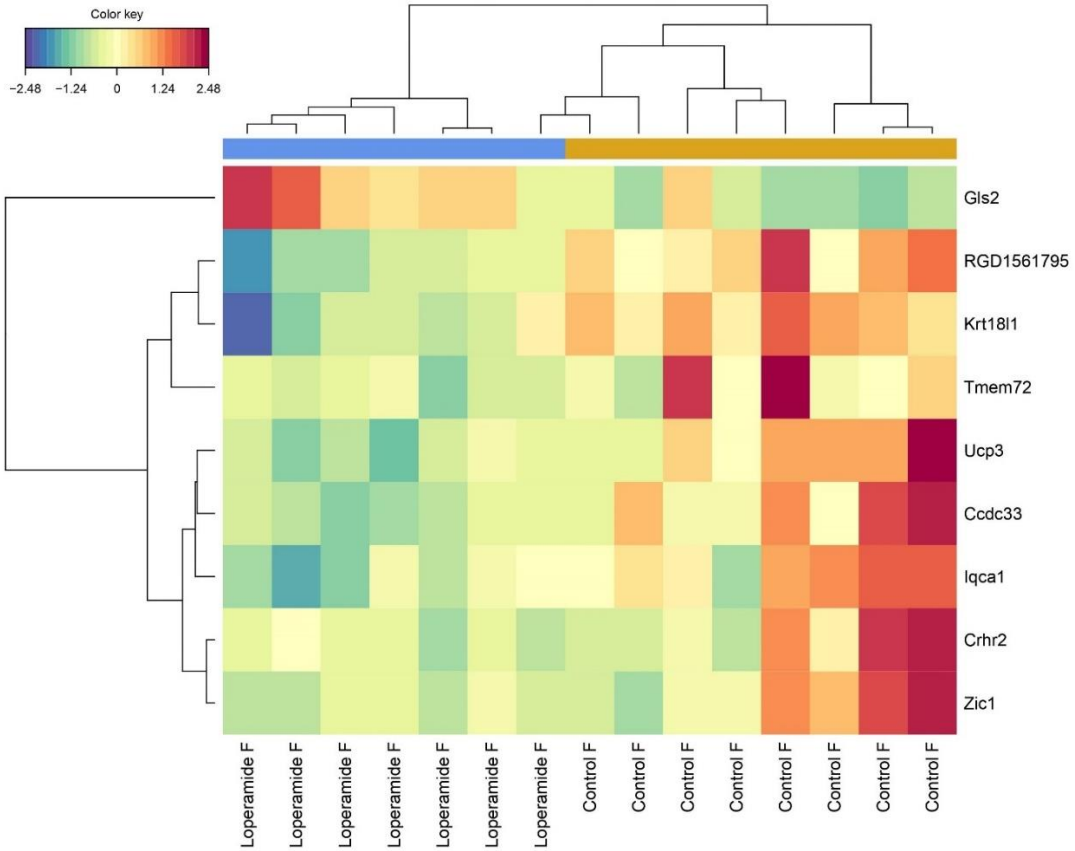


Figure 16*: Heatmap showing differentially expressed genes in the hippocampus of loperamide treated and control female rats. The red and blue colour scale represents expression, with red being higher and blue being lower. The values are scaled by row, which means the actual expression (counts) have been converted to standard deviations above and below the median which is set at zero.*

4.3.3.2 Proximal colon

The RNA expression profiles of the proximal colon tissue of rats in the control and loperamide group were determined using RNAseq. Genes with an FDR < 0.05 and log fold change |logFC| > 0.585 were considered differentially expressed. Overall, more than 200 genes of the proximal colon were found to be differentially expressed between control and loperamide group (***Fig. 17***). 95 genes were more highly expressed in the loperamide group compared to controls. In terms of sex differences, 145 genes were differentially expressed between control and loperamide groups in male rats (***Appendix A***), whereas 60 genes were differentially expressed between control and loperamide groups in female rats (***Appendix B***). The differentially expressed genes across treatment groups in both males and females, were mainly associated with biological processes such as response to corticosterone, cellular response to glucocorticoid stimulus, and various signal related pathways such as lipopolysaccharide mediated signalling pathway, phospholipase C-activating G protein-coupled receptor signalling pathway and peroxisome proliferator activated receptors (PPAR) signalling pathway.

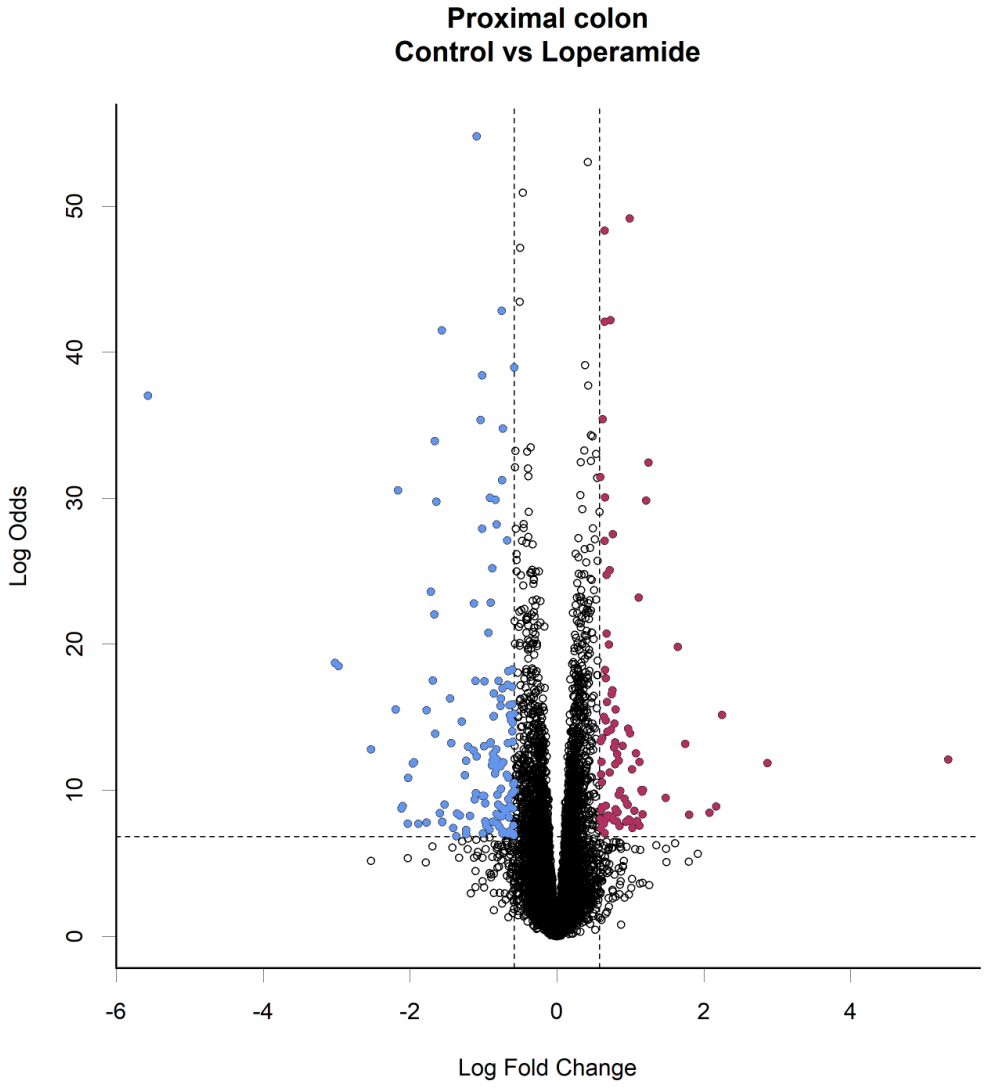


Figure 17: *Volcano plot showing differences in gene expression for control and treatment groups. The volcano plots show logFC vs LogOdds, where log odds represent significance. The horizontal dotted line represents FDR=0.05, with FDR values less than 0.05 above the line. Vertical dotted lines represent +1.5 and -1.5 fold change. Any genes that are in the upper left and right quadrants are considered differentially expressed and have been coloured accordingly.*

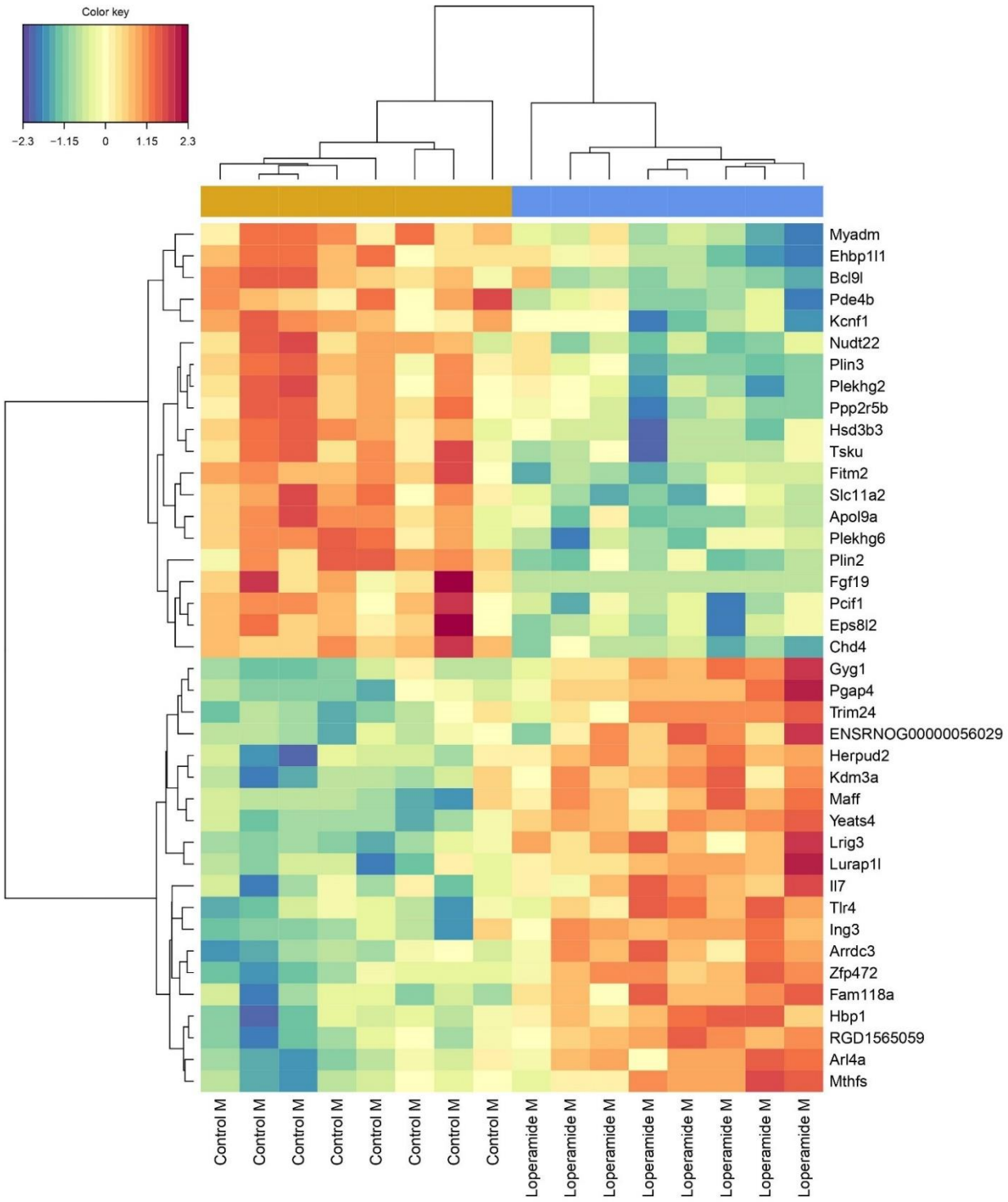


Figure 18: *Heatmap showing top 40 differentially expressed genes in the proximal colon of loperamide treated and control male rats. The red and blue colour scale represents expression, with red being higher and blue being lower. The values are scaled by row, which means the actual expression (counts) have been converted to standard deviations above and below the median which is set at zero.*

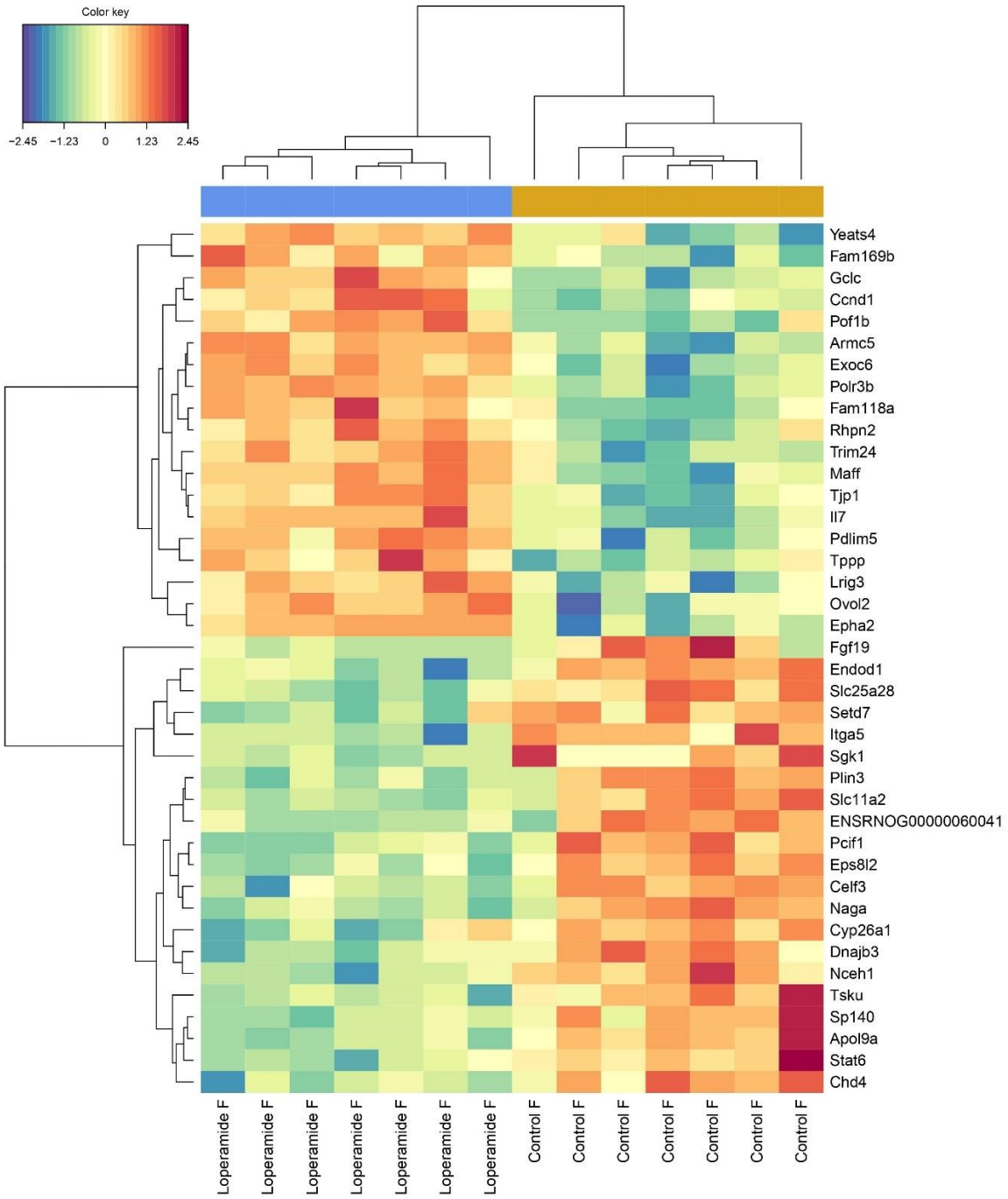


Figure 19: *Heatmap showing top 40 differentially expressed genes in the proximal colon of female rats. The red and blue colour scale represents expression, with red being higher and blue being lower. The values are scaled by row, which means the actual expression (counts) have been converted to standard deviations above and below the median which is set at zero.*

4.3.4 Gene set enrichment analysis - Brain

While the analysis of individual genes showed significant differences only in expression levels in the hippocampus of female rats, GSEA showed that the collective expression patterns of genes within numerous reactome pathways also differed in the amygdala and prefrontal cortex between groups (***Fig. 20***). In the amygdala, pathways related to ERK and NOD1/2 showed increased expression in loperamide treated female rats. The greatest number of differentially expressed pathways were seen in the hippocampus (33 pathways) which included several immune signalling related pathways involving TAK1, NOD1/2 and CD28, showing higher expression in loperamide treated rats, while pathways related to GABA synthesis, release, reuptake, and degradation showed a significant overall decrease in expression. In the prefrontal cortex, 7 pathways were differentially expressed, with most showing higher expression in the loperamide treated female group. Two pathways showed lower expression in loperamide treated female rats compared to controls that included MAP kinase activation and interleukin 17 signalling.

Figure 20: *Reactome pathways differentially expressed by GSEA (P<0.05) in amygdala, hippocampus, and prefrontal cortex of female rats. Red circles indicate overall significantly higher expression in Loperamide rats compared to Controls and blue circles indicate overall significantly lower expression compared to Controls. Grey circles indicate pathway not differentially expressed (P>0.05). Size of circle proportional to the number of genes up or down regulated.*

4.3.5 Caecal microbiome

4.3.5.1 Microbiota at the family and genus level

Having determined that the faecal output was significantly reduced in both male and female rats after loperamide administration, we evaluated the effect of loperamide on caecal microbiota composition. Microbiota profiles differed significantly between control and loperamide-treated groups in both male and female rats. However, there were no significant differences between male and female rats, nor was there a significant interaction between gender and treatment **(*Table 6*)**. Analysis of the microbiotas at the family level showed significant differences ($q<0.05$) between the control and loperamide-treated groups in ten families (***Fig. 21***). Of these, the majority were from the Firmicutes phylum: *Lactobacillaceae, Clostridiaceae, Eubacteriaceae, Lachnospiraceae, Ruminococcaceae.* Except for *lactobacillaceae,* all other families from the Firmicutes phylum were less abundant in the loperamide-treated group. Four families from the Bacteroidetes phylum: *Bacteroidaceae, Barneseillaceae, Muribaculoceae and Tannerellaceae* were more abundant in the loperamide-treated group.

At the genus level, 20 genera differed significantly between treatments. Genera that were significantly more abundant in the loperamide-treated group and that had a mean relative abundance greater than 1% included *Bacteroides, Lactobacillus, Butyricicoccus, Clostridium, Colidextribacter, Eubacterium, Acetatifactor, Blautia, Dorea, Enterocloster, Roseburia, Schaedlerella, Flavonifractor and Ruminococcus*. The largest effect of treatment on the microbiota composition was seen in *Bacteroides* and *Lactobacillus*. Loperamide treatment increased the relative abundance of *Bacteroides* by 75% in male rats and 32% in female rats compared to controls (***Fig. 22***). Similarly, the abundance of *Lactobacillus* increased in loperamide treated male and female rats by 171% and 100% respectively, compared to control.

Table 6: *Taxa with significantly different relative abundance between control and loperamide-treated male and female rats. Data represented as mean percent ± standard error of mean (SEM).*

Phylum	**Family**	**Genus**	**Male**		**Female**		**q-value**	
			Control	Lop.	Control	Lop.	Treatment	Gender
Bacteroidetes	Bacteroidaceae	Bacteroides	3.83 ± 0.25	6.72 ± 0.62	5.33 ± 0.50	7.01 ± 0.95	0.004	0.180
Bacteroidetes	Barnesiellaceae	Barnesiella	0	0.42 ± 0.12	0.14 ± 0.09	0.44 ± 0.12	0.004	0.415
Bacteroidetes	Muribaculaceae	Duncaniella	0	0.58 ± 0.09	0.33 ± 0.13	0.49 ± 0.13	0.009	0.303
Bacteroidetes	Muribaculaceae	Muribaculum	0.77 ± 0.12	1.31 ± 0.11	1.19 ± 0.13	1.35 ± 0.16	0.041	0.143
Bacteroidetes	Tannerellaceae	Parabacteroides	0	0.73 ± 0.13	0.21 ± 0.14	0.71 ± 0.13	<0.001	0.416
Firmicutes	Lactobacillaceae	Lactobacillus	3.66 ± 1.01	9.93 ± 0.91	4.38 ± 1.09	8.78 ± 1.03	0.001	0.971
Firmicutes	Lactobacillaceae	Limosilactobacillus	0.21 ± 0.14	0.44 ± 0.14	0.32 ± 0.16	1.11 ± 0.11	0.016	0.076
Firmicutes	Clostridiaceae	Butyricicoccus	2.16 ± 0.13	1.54 ± 0.20	2.34 ± 0.24	1.76 ± 0.25	0.016	0.429
Firmicutes	Clostridiaceae	Clostridium	5.82 ± 0.38	4.15 ± 0.68	7.05 ± 0 .68	3.77 ± 0.72	0.003	0.767
Firmicutes		Colidextribacter	2.51 ± 0.06	1.74 ± 0.16	1.80 ± 0.14	1.65 ± 0.23	0.026	0.066
Firmicutes	Eubacteriaceae	Eubacterium	4.47 ± 0.39	2.98 ± 0 .43	6.56 ± 0.81	4.55 ± 1.44	0.026	0.176
Firmicutes	Lachnospiraceae	Acetatifactor	2.82 ± 0.23	1.35 ± 0.19	2.34 ± 0.23	1.19 ± 0.20	<0.001	0.339
Firmicutes	Lachnospiraceae	Blautia	1.61 ± 0.05	1.05 ± 0.12	1.74 ± 0.13	1.02 ± 0.16	<0.001	0.930

Firmicutes	Lachnospiraceae	Dorea	1.61 ± 0.16	1.06 ± 0.14	1.95 ± 0.15	1.12 ± 0.11	<0.001	0.278
Firmicutes	Lachnospiraceae	Enterocloster	1.38 ± 0.08	0.92 ± 0.09	1.42 ± 0.09	0.90 ± 0.11	<0.001	0.951
Firmicutes	Lachnospiraceae	Roseburia	5.22 ± 0.63	3.36 ± 0.54	4.56 ± 0.51	2.71 ± 0.61	0.011	0.347
Firmicutes	Lachnospiraceae	Schaedlerella	2.59 ± 0.30	1.51 ± 0.19	3.69 ± 0.40	1.97 ± 0.28	<0.001	0.066
Firmicutes	Ruminococcaceae	Flavonifractor	1.32 ± 0.02	0.94 ± 0.08	0.98 ± 0.08	0.92 ± 0.13	0.041	0.111
Firmicutes	Ruminococcaceae	Ruminococcus	6.14 ± 0.20	5.04 ± 0.49	5.39 ± 0.27	4.17 ± 0.28	0.008	0.105
Proteobacteria	Enterobacteriaceae	Klebsiella	0.67 ± 0.03	0.15 ± 0.10	0.40 ± 0.12	0.23 ± 0.11	0.008	0.411

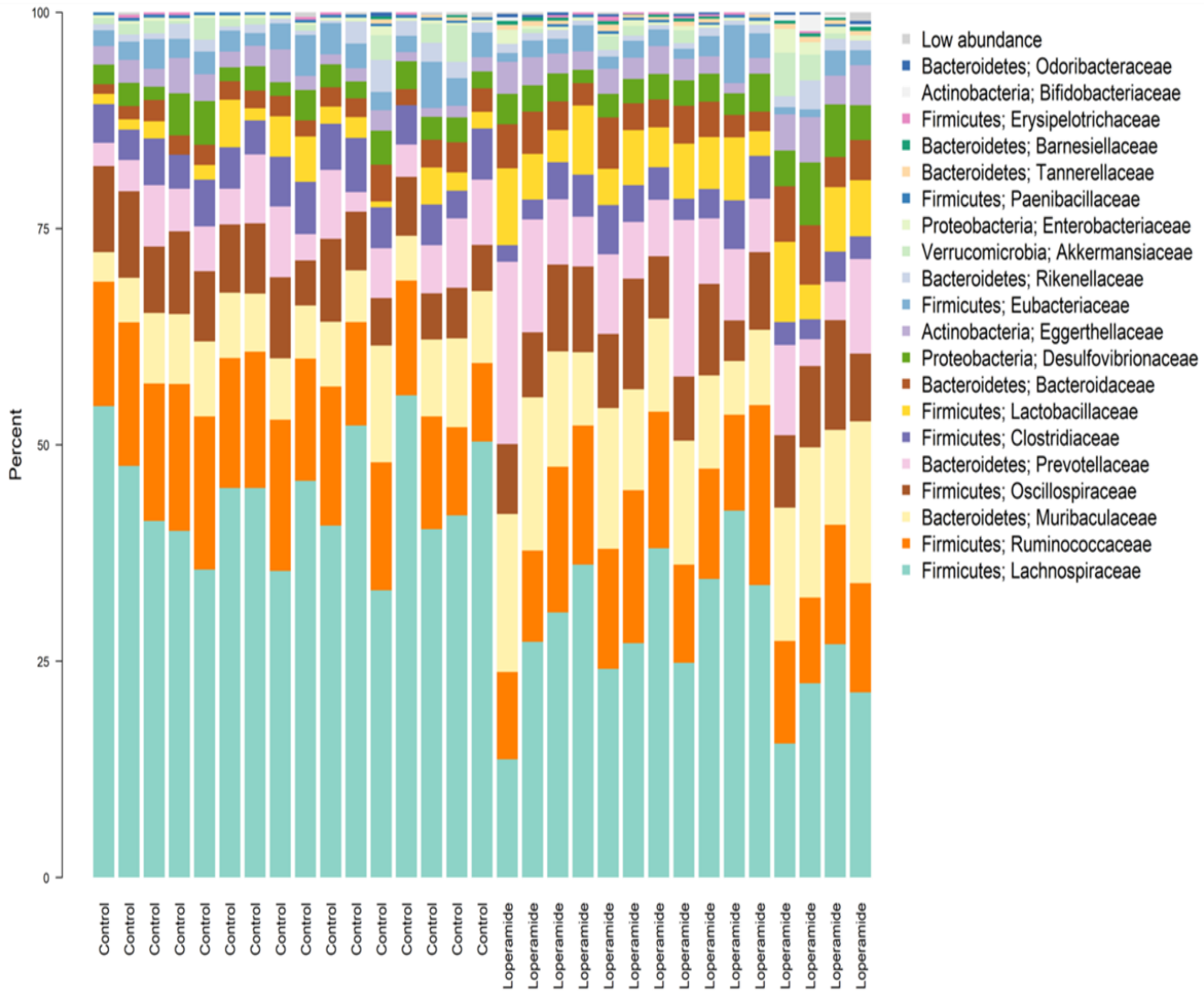


Figure 21: *Taxonomic composition of the caecal microbiota at the family level. The x axis represents treatment, and the y axis represents relative abundance in percent.*

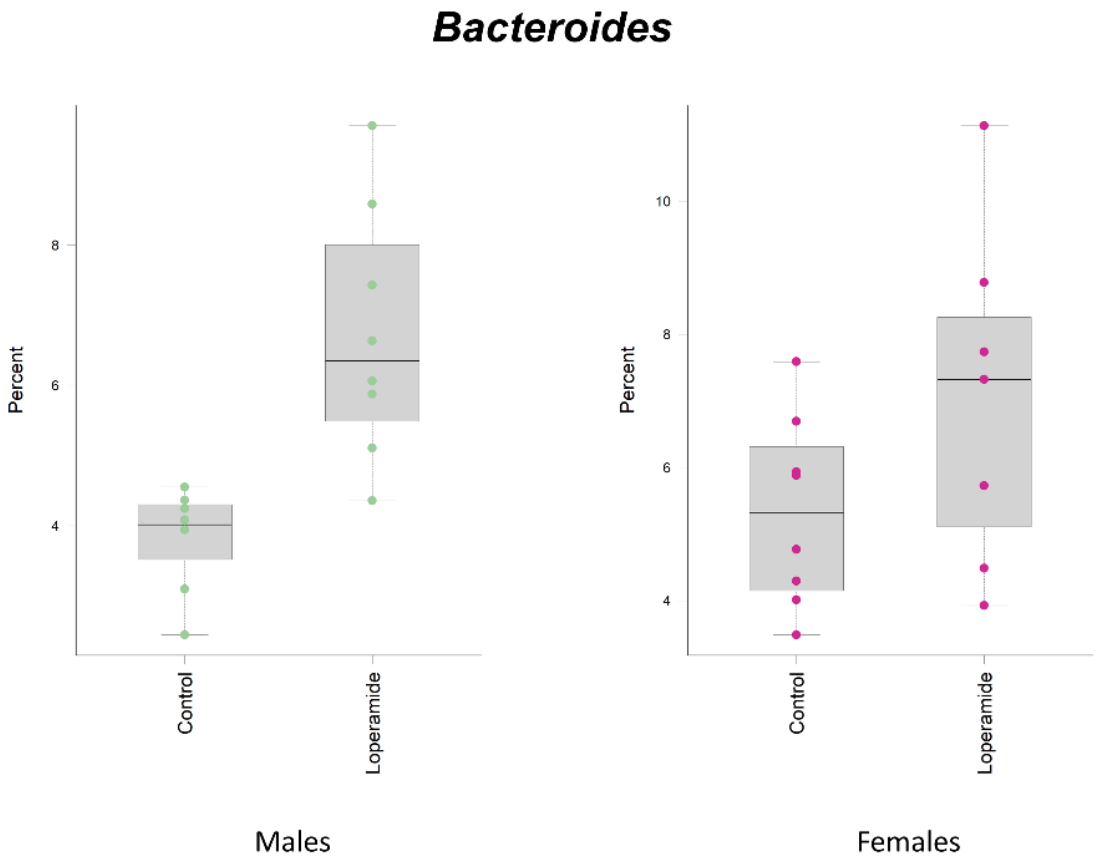


***Figure 22*:** Box plots showing Bacteroides to be more abundant in loperamide treated male and female rats compared to controls.

4.3.5.2 Microbiota diversity

Principal coordinate analysis of Unifrac phylogenetic distances showed strong separation overall between caecal microbiotas from control and loperamide-treated male and female rats (***Fig. 23***).

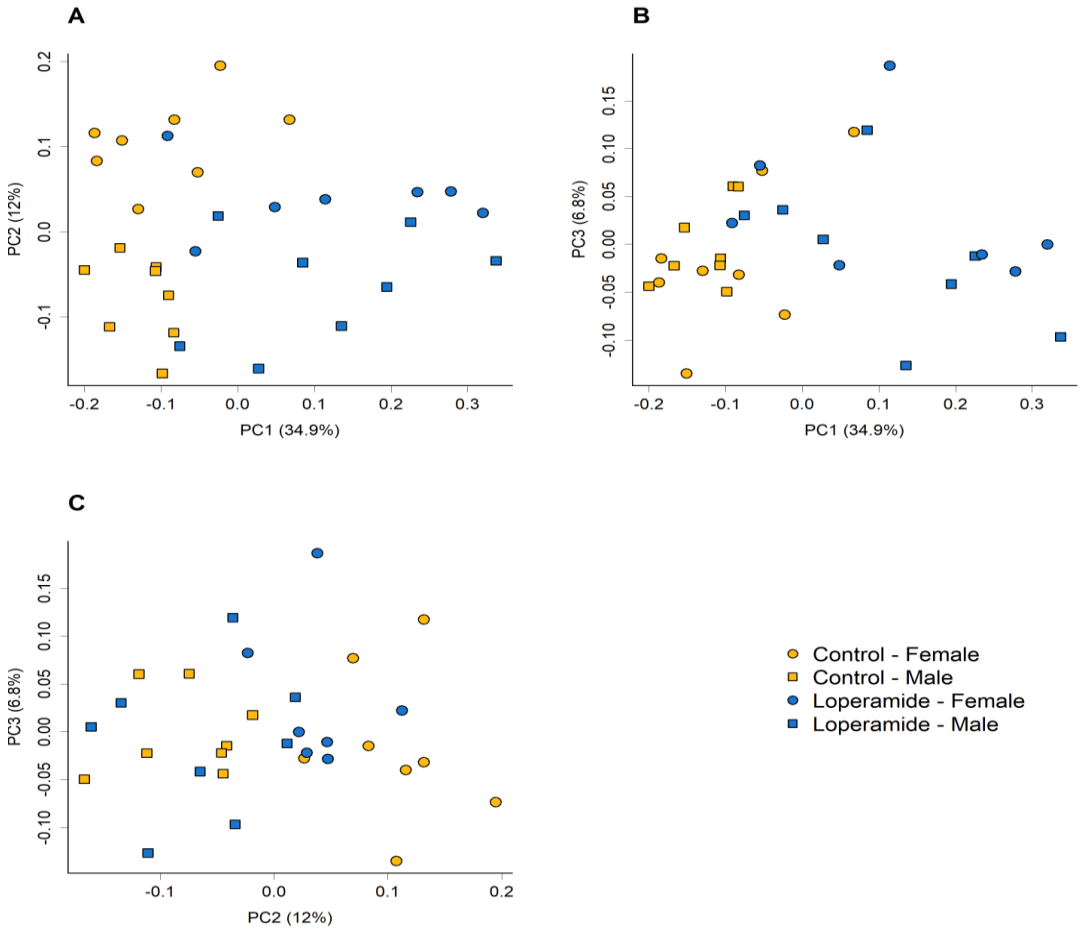


Figure 23: *Principal coordinate analysis (PCoA) plot of weighted unifrac phylogenetic distances of caecal microbiotas from control (yellow) or loperamide (blue) groups. Plots show (A) PC1 vs PC2, (B) PC1 vs PC3, and (C) PC2 vs PC3. Percentages on axes indicate the proportion of variation explained by each dimension. Permutation analysis of variance indicated a significant difference between loperamide and control communities (ANOSIM P value = 0.001, R statistic = 0.449)*

4.3.5.3 Metagenome community function

In addition to characterising changes in the caecal microbiota composition, the functional characterisation of the caecal microbiome among different treatment groups were identified. Functional annotations were carried out for the updated gene catalogue using KEGG database. In male rats, at the third classification level, the top 5 KEGG pathways based on significance ($q<0.05$) that showed significant changes in abundance when comparing the control and loperamide groups

were K1000240 Pyrimidine metabolism, K1000230 Purine metabolism, K1000010 Glycolysis / Gluconeogenesis, K1002020 Two-component system, and K1000300 Lysine biosynthesis (***Table 7***). These reference pathways were associated with nucleotide metabolism, carbohydrate metabolism, signal transduction and amino acid metabolism. In female rats, the top 5 KEGG pathways based on significance ($q<0.05$) that showed significant changes in abundance when comparing the control and loperamide groups included K1000361 Chlorocyclohexane and chlorobenzene degradation, K1000230 Purine metabolism, K1000040 Pentose and glucuronate interconversions, K1000040 Pentose and glucuronate interconversions, K1000480 Glutathione metabolism and K1000052 Galactose metabolism (***Table 8***). These reference pathways were associated with xenobiotics biodegradation and metabolism, nucleotide metabolism, carbohydrate metabolism and metabolism of other amino acids.

Table 7: *Relative abundance (percentage) of top 5 caecal microbiome function pathways (based on significance; q<0.05) in male rats (KEGG database functional gene annotation at classification level 3). Lop.: loperamide.*

Level 1	Level 2	Level 3	Control	Lop.	q-value
Metabolism	Nucleotide Metabolism	K1000240 Pyrimidine metabolism	2.615 ± 0.015	2.849 ± 0.028	<0.001
Metabolism	Nucleotide Metabolism	K1000230 Purine metabolism	6.904 ± 0.054	7.293 ± 0.054	0.010
Metabolism	Carbohydrate Metabolism	K1000010 Glycolysis / Gluconeogenesis	2.780 ± 0.020	2.950 ± 0.025	0.010
Environmental Information Processing	Signal Transduction	K1002020 Two-component system	6.910 ± 0.136	5.664 ± 0.195	0.010
Metabolism	Amino Acid Metabolism	K1000300 Lysine biosynthesis	1.346 ± 0.014	1.433 ± 0.011	0.011

Table 8: *Relative abundance (percentage) of top 5 caecal microbiome function pathways (based on significance; q<0.05) in* female rats *(KEGG database functional gene annotation at classification level 3). Lop.: loperamide.*

Level 1	Level 2	Level 3	Control	Lop.	q value
Metabolism	Xenobiotics Biodegradation and Metabolism	K1000361 Chlorocyclohexane and chlorobenzene degradation	0.037 ± 0.001	0.051 ± 0.001	0.015
Metabolism	Nucleotide Metabolism	K1000230 Purine metabolism	6.758 ± 0.070	7.219 ± 0.090	0.049
Metabolism	Carbohydrate Metabolism	K1000040 Pentose and glucuronate interconversions	1.598 ± 0.028	1.429 ± 0.031	0.049
Metabolism	Metabolism of Other Amino Acids	K1000480 Glutathione metabolism	0.051 ± 0.004	0.076 ± 0.004	0.049
Metabolism	Carbohydrate Metabolism	K1000052 Galactose metabolism	3.208 ± 0.069	2.838 ± 0.060	0.049

4.4 Discussion

The premise for this work was that acute dosing of animals using a blood brain barrier impermeable ENS modulator would allow us to examine possible gut to brain signalling pathways. The main findings from this study were that ENS opioid receptor activation altered anxiety related behaviour in a manner that indicates that gut to brain signalling occurred. The behavioural changes were most prominent in females and were associated with changes in hippocampal gene expression and corresponded with changes in caecal microbiome and colonic gene expression. That changes occurred in the caecal microbiome in a relatively short timeframe, just 48 hours after acute dosing,

shows rapid shifts in community composition are possible following slowed colonic transit, as determined from the reduced faecal output.

The OF and EPM behavioural tests conducted in this study provided useful information on differences in anxiety levels. Particularly noteworthy is the emergence of a sexually dimorphic influence in several parameters of these behavioural tests. Notably, the percentage of time spent in open arms of the EPM emerged as a significant parameter, highlighting sex specific distinctions (occurred only in females) in response to modulation of the ENS by loperamide. The open arms of the EPM elicit a stronger fear response and higher levels of avoidance behaviour compared to enclosed arms.[267] In the present study loperamide treated female rats exhibited a lower percentage of time spent in open arms, implying heightened anxiety when compared to their female control counterparts. Conversely, no differences were observed in loperamide treated males compared to control males. These findings are in contrast to the results reported by Sudakov and colleagues, who demonstrated that intragastrical administration of loperamide (5 mg/kg) in male rats led to increased time spent and entries in the open arms of the EPM, indicating an anxiolytic effect of loperamide.[261] Although the observed discrepancies in the results of our study and that of Sudakov and colleagues may stem from differing administration routes and rat strains utilised, a reasonable explanation for these differences could be due the dosage of loperamide applied. It has been shown that peripheral administration of morphine, another mu opioid agonist, induces biphasic effects on behavioural parameters in a dose dependent manner.[268] Thus, future studies must be conducted to explore the impact of varied loperamide dosages on behavioural parameters.

In terms of the OF behavioural parameters assessed, locomotor activity was altered whereas exploratory behaviour in the OF test was not (no difference among OF test zones). Rats with high

levels of anxiety typically exhibit decreased locomotion and rearing. In the present study, the parameters used to analyse locomotor activity, such as total distance moved and speed, revealed that subcutaneous administration of loperamide suppressed locomotor activity in female rats and not male rats after acute dosing (in both the OF and EPM behaviour tests). The decreased locomotor activity in loperamide treated female rats may be attributable to increased anxiety. We are not aware of prior studies that have investigated the effects of loperamide on rodents in the open field test; however, the effects of other opioids have been investigated.[268,269] Peripheral administration of morphine has been shown to induce dose dependent biphasic effects on locomotor activity.[268] A study by Paul and colleagues showed that a low dose of morphine reduced distance moved in the open field test in male rats indicating suppressed locomotor activity. In contrast, increased dose of morphine induced hyperlocomotion in the open field test in the same study.[268] Furthermore, sex differences in behavioural responses on morphine exposure have also been reported.[270] In a study by Zhan and colleagues, morphine increased locomotor activity in female mice compared to controls, whereas no differences were observed in male mice, which indicates that male and female mice respond differently to morphine exposure.[270] These findings are contradictory to the results from our study where, locomotor activity was found to be decreased in female rats compared to controls. This discrepancy may be due to species differences which emphasize the need to exercise caution when comparing behavioural data across species. Moreover, although both loperamide and morphine belong to the same class of drugs, comparing them is complex due to the different pharmacological profiles of these drugs. Morphine crosses the blood brain barrier[271] unlike loperamide[192], and it is well documented that the effect of morphine is centrally mediated involving dopaminergic systems.[272] On the other hand, loperamide induced activation of peripheral opioid receptor influencing behavioural response in rats is indicative of a gut to brain signalling.

Merely assessing basic locomotion cannot encompass all the parameters of the OF behavioural test comprehensively. A more promising approach involves combining it with additional behavioural tendencies. In this study, we assessed rearing behaviour, which consists of rats standing on both hind paws in a vertical upright position.[273] It can be classified into unsupported and supported rears.[274] Rearing behaviour is indicative of exploratory behaviour in rodents, associated with information gathering and cognition. Moreover, it has been utilised as an additional measure of anxiety in the OF test.[273] The neurotransmission of gamma-aminobutyric acid (GABA) controlled by the $GABA_A$ receptor in the hippocampus is closely linked to rearing behaviour.[275] Notably, the Gene Set Enrichment Analysis unveiled a significant downregulation of the GABA synthesis, release, reuptake, and degradation pathway in the hippocampus of loperamide treated female rats compared to the control group. The down regulation of many GABA pathway genes might be expected to lead to a reduction in basal inhibition of excitability in the hippocampus and contribute to increased excitability across neural pathways. The hippocampus responds to stress as a conflict resolution and inhibitory control center.[276] It inhibits ongoing behaviour during confrontational situations and promotes engagement in exploratory behaviour when additional information is required to resolve the conflict, as described by Gray and McNaughton.[276] It is thought that the frequency of rearing behaviour increases during ambiguous situations, where the drive to explore the environment is balanced against perceived danger. Conversely, exploratory behaviour is expected to decrease in situations of extremes in relation to safe or aversive environments. In our study, noteworthy sex differences were observed, with females displaying a considerably higher frequency of supported rearing compared to males at baseline. These findings are in line with a study by Zhan and colleagues where female mice (controls) exhibited higher rearing frequency compared to their male counterparts.[270] This indicates that females may have higher levels of

exploratory tendencies compared to males. While sex differences in response to stress have been extensively studied in rodents,[277,278] sex differences in terms of rearing behaviour in relation to stress are reported much less frequently. Although the hormonal status of female rats in this study was not obtained, one cannot rule out the possibility of the observed sex differences mediated due to organizational effects of sex hormones. Ovarian hormones elicit clear effects on hippocampal function and morphology,[279] and impact hippocampus dependent behaviors.[280,281] In contrast to the increased rearing frequency in control females, our findings revealed that loperamide administration significantly reduced rearing frequency in females, but no differences were observed in males. Although there are no studies showing loperamide affecting rearing behaviour in rodents, our results are in accordance with the study of Patti and colleagues, who reported that the opioid agonist morphine produced a decrease in rearing activity in female mice.[269] The authors suggested that morphine introduces aversive behavioural reactions. Considering these findings, it can be said that activation of opioid receptors in females might be an anxiety provoking factor. Moreover, the observed significant reduction in rearing frequency across treatment in female rats might indicate a possible interaction between the peripheral opioid system and ovarian hormones. There is evidence which shows interaction between the hippocampal opioid system and estrogen, suggesting potential involvement of ovarian hormones in drug addiction processes.[282] However, no studies have investigated the effects involving the peripheral opioid system and ovarian hormones on behavioural parameters. Nevertheless, these investigations show the importance of routinely incorporating both males and females in fundamental research, as well as employing multiple behavioural tests to ensure robustness in drawing conclusions regarding the generalisation of observed effects.

The fact that brain gene expression was only altered in female rats supports these changes as contributing to the behavioural changes. Our study demonstrated that loperamide administration led to a reduction in CRHR2 (corticotropin releasing hormone receptor 2) gene expression in the hippocampus of female rats compared to controls. CRHR2 is one of the receptors through which corticotropin releasing hormone (CRH) acts to regulate stress responses.[283] Studies have shown that alterations in CRH signalling play an important role in the development of anxiety and depression.[284] Moreover, both CRHR1 and CRHR2 have crucial roles in regulating stress sensitivity.[284] Alterations in the expression of these receptors are linked to behavioural disorders. For example, Bale and colleagues showed that CRHR2 knock out male and female mice exhibit increased anxiety as shown in the EPM where mice spent less time and entered the open arms less frequently.[285] Based on these studies it appears that CRHR2 plays a role in reducing anxiety like behaviour. In this context it can be said that the downregulation of CRHR2 gene in loperamide treated female rats may contribute to the anxiogenic effects seen in the behavioural tests. Furthermore, our study also points to a possible role of the peripheral opioid system in modulating the expression of CRHR2. Mechanistic studies are needed to further explore how the peripheral opioid system regulates CRHR2 expression.

Gene expression profiles in proximal colon tissue for this study showed that TLR4 (Toll like receptor 4) was increased in loperamide treated male rats. TLRs are critical pattern recognition molecules of the innate immune system that play a crucial role in maintaining homeostatic functions.[121] Our finding that TLR4 was upregulated in loperamide treated male rats is in line with other studies showing that opioids (morphine) activate Toll-like receptor 4 (TLR4).[286,287] Farzi and colleagues showed that TLR4 is involved in the anti-peristaltic effects of opioids in the GI tract, particularly in the colon.[287] In their study they used a TLR4 blocker TAK-42 (4 mg/kg) that

attenuated the anti-peristaltic effect of morphine (3 mg/kg) in the mice colon but not in the small intestine *in vivo*.[287] These findings raise the question on whether the contribution of TLR4 to the inhibitory effect of the opioid system on colonic motility is sex specific, as the data in the aforementioned study and our study was shown in males (rather than only in females). That TLR4 expression was not altered in loperamide treated females points to a TLR4 independent mechanism related to the opioid induced anti peristaltic effect in the colon as we determined from the reduction in faecal output post loperamide treatment in females.

The reduction in food intake by loperamide treatment together with decreased faecal output indicates that loperamide dose was effective at altering ENS function. Because this was altered in both male and female rats, this suggests that despite the lack of behavioural effect of loperamide on male rats the dose was sufficient. The involvement of opioid receptors has been shown in the regulation of food intake[256] and was expected. Activation of mu opioid receptors in a number of brain structures leads to activation of eating behaviour in rodents.[288] In contrast, activation of peripheral, predominantly gastric opioid receptors leads to suppression of food intake in rats.[258] The fact that we observed reduced food intake is consistent with a peripheral origin of action for loperamide as we would have expected. This is in line with a study by Sudakov and colleagues who showed that vagal afferentation had a role in the anorexigenic effect of loperamide administration.[289]

The caecal microbiota composition between the control and loperamide treated groups showed significant differences in the abundance of Bacteroidetes. The prolonged transit time induced by loperamide could have contributed to the rise in the relative abundance of Bacteroidetes, implying their adaptability in a slow and competitive environment, as per the r/K selection theory of

microbial ecology. Conversely, the decelerated transit caused by loperamide resulted in a decrease in the relative abundance of the genus *Roseburia,* which is typically associated with faster colonic transit. These outcomes indicate that *Roseburia* may not thrive well in a slowed transit luminal environment. Collectively, these findings suggest that maintaining normal gut motility is crucial for preserving a balanced gut ecosystem and gut homeostasis. Because the changes in microbiota were detected only 48h after acute dosing, this raises the question as to whether this may also have indirectly contributed to the gut to brain signalling that mediated the behaviour changes. However, the sex differences observed suggest such an effect was likely minor.

The functional characterisation of the caecal microbiome using the KEGG database revealed gender specific effects of loperamide treatment. In males, the administration of loperamide led to a decrease in the relative abundance of genes involved in the two-component system, which is an integral part of the bacterial quorum sensing mechanism.[290] This suggests that loperamide has a disruptive effect on the communication and interaction between microbial communities specifically in males, while no significant impact was observed in females. On the other hand, in females, loperamide induced an increase in the abundance of genes related to xenobiotic and glutathione metabolism. These pathways play a crucial role in the detoxification of harmful chemicals and substances, providing a defence mechanism for the microbes.[291] The observed upregulation of these detoxification pathways suggests the activation of defence mechanisms in females to counteract potential threats and maintain microbial equilibrium. These results highlight the gender-specific effects of loperamide on the microbiome function and shed light on the differential responses and protective mechanisms employed by males and females in the face of loperamide treatment.

While the study provides valuable insights into the influence of ENS on anxiety related behaviour, it does have some limitations. Notably, the data indicates an anxiogenic phenotype in female rats, but the study did not account for the stage of estrous cycle. Ovarian hormone fluctuations during the rodent estrous cycle significantly affect both gut and brain physiology. Determining the estrous stage of the female rats would have allowed for a more comprehensive understanding of the observed anxiety related behavior.

4.5 Conclusion

The findings from this study indicate that modulation of ENS function via mu opioid receptor activation increased anxiety related behaviour in female, but not male, rats with corresponding changes in proximal colon and brain tissue gene expression. Our findings show that the anxiogenic gut to brain signalling effect via ENS modulation is associated with stress-related genes and pathways in the hippocampus, involving CRH, and GABA synthesis, release, reuptake, and degradation pathway. With similar changes observed in the microbiota profile following loperamide administration in both males and female rats, it is improbable that the microbiota factor alone is responsible for the behavioural effect observed in females.

In addition to recognizing the role of ENS in influencing higher cognitive function, this study also sheds light on the sexually dimorphic communication between the gut and the brain. Our data underscores the need to investigate these sex differences further. Using a targeted approach, we can delve deeper into the gene/pathways identified in our study, expanding our understanding of their specific roles in gut-brain signalling.

CHAPTER 5: GENERAL DISCUSSION

This thesis encompasses three studies that have allowed for a thorough investigation and the generation of new insights on the involvement of the ENS in modulating the GBA. In chapter Two, the focus was on exploring the role of ENS receptors (opioid receptors) in regulating colonic motility. The results obtained from this study shed light on the significant inhibitory effects observed on colonic peristaltic contractions in response to mu opioid receptor activation in the ENS. Notably, this inhibitory effect was region specific, being prominently evident in the mid to distal colon compared with the proximal region, suggesting the presence of unique regulatory pathways or receptor distributions in these regions. Moving forward to chapter Three, the investigation shifted towards understanding the impact of slowed gut transit on microbiota composition, uncovering the intricate relationship between gut motility and microbial balance. Slowed gut transit induced by mu opioid receptor activation altered the microbiota composition and reduced the richness and diversity of bacterial communities. Finally, in chapter Four, the emphasis was on examining the broader physiological consequences of ENS modulation on brain function and anxiety, with a specific emphasis on exploring potential sex differences. The results obtained from this study revealed that the modulation of ENS activity through mu opioid receptor activation had distinct effects on anxiety related behaviour in female and male rats. Specifically, the activation of mu opioid receptors led to an increase in anxiety related behaviour in female rats, while no significant behavioural changes were observed in male rats. Furthermore, these behavioural differences were accompanied by specific alterations in gene expression patterns within the brain tissue, which were evident in females only. Additionally, variation in caecal microbial communities and changes in proximal colon gene expression were observed in both male and females.

This final chapter offers a holistic perspective that integrates the findings from each study to present a coherent and unified understanding of the thesis main objective. To streamline the discussion and facilitate clarity, this chapter has been divided into two distinct subsections. The first subsection focusses on the findings from chapters two, three and four, offering new knowledge on the effects of ENS mu opioid receptor activation on the biomechanics of colonic motility, and its subsequent impact on the gut microbiota. The second subsection focusses on the impact of ENS modulation, specifically through the activation of mu opioid receptor, on anxiety related behaviour. By addressing these two distinct aspects, this thesis provides significant insights into the ENS's pivotal role in the GBA at the level of receptor and tissue function in a region-specific GI tract environment, as well as at the physiological whole system level.

Impact of ENS mu opioid receptor activation on the biomechanics of colonic motility: A driving force for changes in microbial composition

While there has been extensive research on the factors influencing the gut microbiota, the specific impact of intestinal contractile activity on the gut microbiota has remained relatively unexplored. My research findings, that activation of mu opioid receptors slowed colonic motility by inhibiting propagations with a predominant distal region effect *ex vivo*, together with reducing caecal microbial diversity *in vivo*, show the importance of the large intestine in providing an ecological niche for microbial colonisation due to its anatomical and physiological characteristics.[292] It is important to understand the motility aspect of the colon in particular due to its specialised functions and the significant role it plays not only in facilitating efficient digestion but also ensuring a balanced gut ecosystem. The movement of gut bacteria, their byproducts, and the nutrients they depend on is facilitated by the luminal flow within the gut.[293] This flow, which is generated by gut motility, plays a crucial role in determining the stability of the bacterial population.[181] Through the

utilisation of mathematical modelling and *in vitro* experiments, Cremer and colleagues effectively showcased how regulated contractions within the colon exert a substantial influence on both the density and composition of the microbiota.[181]

Luminal flow can be characterised into radial and longitudinal flows. Radial flows influence the mixing of metabolites, their availability for absorption across the gut wall, and their distribution across the gut.[294] On the other hand, longitudinal flows impact how solutes disperse longitudinally and the time it takes for them to move through the gut.[294] Different types of motility patterns have distinct effects on both radial and longitudinal flows. For example, peristalsis predominantly generates a strong net forward flow that aids in cleansing.[295,296] In contrast, segmentation slows down the movement of contents, allowing for increased absorption.[37,297] These motility patterns result in significant forward and backward longitudinal flows, which increase dispersion but contribute to a lower net forward movement.[294] By altering its motility, the gut can modify radial flows, mixing, and the overall net forward flow, thereby influencing the gut transit time.

My findings (chapter two) that opioid receptor activation decreased frequency, velocity, and propagating distance of CMCs together with the observation that the interval between CMCs was significantly prolonged, strongly indicate a reduction in the movement and coordination of contractions within the colon. Consequently, it can be inferred that opioid receptor activation may contribute to a decrease in net forward flow and hinder the mixing characteristics of the contents within the colon, thereby prolonging the colonic transit time and influencing microbiota density and composition. The findings from chapters three and four demonstrated that altered ENS activity through mu opioid receptor modulation significantly influenced the microbiota composition and diversity. One plausible explanation for these observed changes lies in the reduced motility induced

by altered ENS activity, which limits the luminal flow carrying nutrients. Nutrient inflow exerts a profound influence on microbiota composition through its impact on luminal pH.[297] As a consequence of the slower flow of luminal contents, there is a decrease in nutrient availability, leading to reduced bacterial density engaged in fermentation.[297] Consequently, lower concentrations of short-chain fatty acids (SCFAs) are produced.[297] This reduction in SCFA concentration results in a moderate drop in pH. Changes in pH values, caused by the altered nutrient levels affecting acidification and fermentation products in the colon, have been shown to impact bacterial growth.[298,299] Notably, previous research by Cremer and colleagues has demonstrated that in mildly acidic environments, Bacteroidetes exhibit faster growth compared to Firmicutes, leading to a higher relative abundance of Bacteroidetes.[297] Conversely, at normal or higher nutrient inflow rates, increased fermentation occurs, resulting in elevated SCFA production and a more significant drop in pH, creating a stronger acidic luminal environment.[297] This creates a growth advantage for Firmicutes.[297,300] Drawing upon these compelling lines of evidence, it can be said that the increased relative abundance of Bacteroidetes and decreased abundance of Firmicutes observed in rats subjected to loperamide treatment, as shown in chapters three and four, might stem from reduced motility induced by opioid receptor activations that would alter the luminal pH making the luminal environment less acidic and more favourable for Bacteroidetes to grow or adapt to. These findings are consistent with a previous study by Walker and colleagues, who also concluded that a lower pH resulting from substrate fermentation in the colon may limit the growth of *Bacteroides*.[301] Although direct SCFA measurements were not conducted in this research, the observed decrease in SCFA producing bacteria such as butyrate producing genera *Roseburia, Clostridium, Ruminococcus* and *Eubacterium* suggests that prolonged colonic transit induced by mu opioid receptor activation may have implications on SCFA production. Furthermore, the downregulation of G protein coupled receptor 4 (GPR4), a pH sensing receptor known to be activated by acidic pH,[302] in the loperamide

treated group (***Appendix A and B***) further supports the notion of a less acidic environment when the colonic transit time is prolonged. Overall, these observations support the hypothesis that a slow colonic transit caused by reduced ENS activity may lead to a less acidic luminal environment due to depletion of SCFAs, subsequently leading to alterations in the microbial composition.

An alternative explanation for the influence of reduced motility on microbiota composition and diversity centres around substrate availability and utilisation. Colonic transit time can be influenced by various factors such as diet, drugs and other variables.[303] This transit time, in turn, has been shown to impact digestive efficiency, consequently affecting the substrates that are available for the gut microbiota to consume.[304] Certain gut microbes possess the capability to adapt their substrate utilisation efficiency according to the availability of resources.[241,305] For example, *Bacteroides* have the ability to adjust their metabolism and metabolic products through a metabolic switch.[241] This adaptability grants them a competitive advantage, enabling them to thrive and outcompete other bacteria particularly during periods of reduced substrate availability. The findings presented in chapters three and four, where the abundance of *Bacteroides* increases with prolonged transit time, corroborate the significance of this metabolic adaptability.

In conclusion, modulating the ENS appears to have a significant impact on the complex interplay of hydrodynamic and mechanical forces within the GI tract. These alterations subsequently lead to significant changes in the luminal chemical environment and the availability of substrates, ultimately influencing the composition of the gut microbiota.

Modulating the ENS via mu opioid receptor activation influences anxiety related behaviour in a sex specific manner: Involvement of neural pathways and sex hormones

When examining anxiety related behaviour in chapter four, the percentage of time spent in open arms of the elevated plus maze emerged as a significant parameter that revealed sex differences (occurred only in females) in response to ENS modulation. Regarded as anxiety provoking, the open arms of the EPM elicit a stronger fear response and higher levels of avoidance behaviour compared to enclosed arms.[267] It is linked to GABA neurotransmission, regulated by the $GABA_A$ receptor in the hippocampus, a key target area in the stress response.[306] Notably, the Gene Set Enrichment Analysis conducted in chapter four unveiled a significant downregulation of the GABA synthesis, release, reuptake, and degradation pathway in the hippocampus of loperamide treated female rats compared to the control group. GABA serves as the principal inhibitory neurotransmitter in the CNS and has long been recognised as a pivotal regulator of anxiety like behaviour in mammals.[306,307] Activation of brain mu opioid receptor has been shown to inhibit GABA release in the brain, thereby disrupting the delicate balance of inhibitory neurotransmission, and potentially contributing to anxiety related behaviour.[308] The observed downregulation of the GABA synthesis, release, reuptake, and degradation pathway in the hippocampus of loperamide treated female rats, as shown in chapter four, aligns with these findings and may underlie the decreased percentage of time spent in open arms of the EPM by loperamide treated females, further supporting the involvement of GABAergic neurotransmission in anxiety related behaviours. While previous research has established that the activation of mu opioid receptors inhibits GABA release in the brain, this study provides novel insights into the impact of peripheral mu opioid receptor activation on the GABAergic system in the brain implying a gut to brain signalling pathway (Note that loperamide does not cross the blood brain barrier so only a peripheral site of action is considered). As discussed in the literature review, three interacting routes linking the ENS with the

CNS have been identified through experimental data from animal studies which include neural, endocrine, and immune signalling pathways. Regarding the neural pathways involved in gut and brain communication, it has been observed that vagal afferent fibres, traditionally believed to be distributed throughout the upper GI tract, also extend their innervation to the distal colon in rats and mice.[309,310] My findings (chapter two) that mu opioid receptors activation inhibited colonic propagations with a predominant distal region effect *ex vivo*, may suggest a motility GBA effect involving the vagus nerve, potentially influencing anxiety related behaviour in females. However, this speculation needs further investigation as the distal region of the colon is also innervated by spinal afferents.[310] Furthermore, given that loperamide exerts its effects throughout the GI tract (has also been shown to slow gastric emptying),[199] the observed GBA effect could involve another vagally innervated GI region. Considering the complex and dynamic interplay between the gut and brain, characterised by multifaceted communication pathways, limiting the focus solely to a neural pathway would be an oversimplification. Nonetheless, this observation represents an important advancement in understanding the influence of neural signals originating in the gut on anxiety related behaviour.

The reduced percentage of time spent in the open arms of the EPM along with downregulation of the GABA pathway observed in females but not males could potentially be attributed to the complex interaction between ENS opioid system, female sex hormones and GABAergic system. Although the hormonal status of female rats in this study was not assessed, it is plausible that the observed sex differences may be mediated through the organizational effects of sex hormones. Ovarian hormones have well documented effects on the GABAergic system significantly influencing behaviour.[311] Moreover, there is evidence that indicates an interaction between the hippocampal opioid system and ovarian hormones.[282] However, there is a lack of research

investigating the effects of the interplay between the peripheral opioid system, female sex hormones and the GABAergic system on behavioural parameters. Further investigations are warranted to unravel the complex interplay among these systems, and their impact on behavioural outcome.

The results from chapter four of this thesis demonstrating a shift in caecal microbiota profile by ENS opioid receptor activation in both sexes occurred within a relatively short timeframe (less than 48 hours), indicate rapid shifts in community composition following slowed colonic transit. It is tempting to speculate that this rapid alteration in microbiota composition following ENS mu opioid receptor mediated prolonged colonic transit may have played a significant role in influencing anxiety related behaviour specifically in female rats. Although similar changes in microbiota profile upon loperamide administration were observed in males too, no behavioural changes were observed. As a result, it is unlikely that microbiota factor alone is responsible for the behavioural effect observed in females but not males. However, it is worth noting that although the behavioural differences in male rats did not reach statistical significance, there was a notable trend towards increased anxiety. This observation suggests that the contributory effect of microbiota on behavioural outcomes should not be ruled out.

Limitations and potential areas for future research

A major finding of this study was the sex specific difference in anxiety related behaviour upon ENS modulation. While anxiety and depression are reported to be more common in women,[312,313] the integration of sex as a biological variable in fundamental neuroscience research remains limited. In animal studies, females are often disregarded due to concerns about potential behavioural variations resulting from the female reproductive cycle and their impact on experimental outcomes.[314] However, meta-analyses have revealed that naturally cycling female rodents do not exhibit

significantly greater variability in neural and behavioural measures compared to males in broad categories.[315,316] This is consistent with my behavioural study data too. Nevertheless, a comprehensive investigation into anxiety and depressive like behaviours in rodents, considering the influence of estrous cycle stages, has been inadequately explored, impeding a thorough understanding of the role of cycle stages in previously observed sex differences in emotional behaviour. In theory, this could be measured and factored into statistical analysis. Additionally, the direction and magnitude of these differences, influenced by sex or cycle stage, seem to rely on various factors, such as stress exposure, gonadectomy, testing paradigms, and data measurement and quantification methods. Consequently, discrepancies in findings have arisen. Therefore, it is crucial to examine the responses of males and naturally cycling females in mildly anxiety-inducing situations to establish foundational information that can guide future investigations focusing on the specific contribution of estrous cycle stages to sex differences in anxiety and depression. This bears particular significance in determining whether data can be aggregated across sexes or cycle stages when exploring the underlying neurobiological mechanisms of anxiety and depression.

While the results from my study indicate that the acute change in composition of the microbiota (in both male and females) with loperamide may not correlate with behavioural changes, the fact that the males trended toward increased anxiety (in measures of rearing, total distance travelled and velocity) might suggest that the microbiota is able to have some influence on behaviour.
To gain a better understanding of the role of the microbiota in this context, further research could be done using antibiotics. By methodically designing a study that incorporates the usage of antibiotics, it would be possible to assess the impact of loperamide in the absence of the microbiome, thus providing clearer insights into its specific effects. It is important to consider the ability of the chosen antibiotic to cross the blood brain barrier, as the thesis focuses on the signalling from the gut

to brain. Some antibiotics, such as metronidazole and minocycline, can potentially enter the CNS and can have direct action on brain and behaviour,[317] complicating interpretation of results. One approach would be to use non absorbable antibiotics that do not enter systemic circulation, thereby avoiding any potential systemic and even CNS effects.[318]

The use of a mu opioid agonist as a research tool to investigate ENS function can provide insights into how specific dietary ingredients might affect the functioning of the gut and brain by modulating the peripheral opioid system. Certain foods contain substances with opioid activity, such as casomorphin derived from milk casein, exorphins and gliadorphin from gluten, and soymorphin from soybeans. These compounds can activate peripheral opioid receptors located in the GI tract. While previous studies have explored their effects on gut motility,[235,260] their potential impact on higher cognitive functions and the underlying molecular mechanisms remain largely unexplored. It would also be interesting to explore whether they exhibit sex specific effects through mu opioid receptor agonism in the ENS.

Rodents are commonly used models in research, but it is important to acknowledge that translation of findings from animal models to humans may encounter challenges due to inherent differences in anatomy, physiology, and genetic makeup. While animal models can provide valuable insights, they may not fully replicate the complexity and interactions seen in the human GBA. Therefore, care must always be taken when extrapolating results from animal studies to humans. Nonetheless, animal models remain an invaluable tool under the right circumstances as they can provide mechanistic insights that could not be obtained through cell models or even human intervention studies.

Clinical relevance

The thesis findings that loperamide induces anxiety-related behaviour in female rats suggest concerns may arise about its impact on brain function in humans if these findings in rats translate to humans. Despite the effectiveness of loperamide in alleviating GI symptoms in humans, these findings suggest that clinical investigations to assess its use in female patients are necessary to gain deeper insights on the gut-brain axis effect of loperamide in humans in relation to anxiety.

The findings of this thesis highlight the necessity to explore the fundamental processes that regulate sexual dimorphism in gut brain interactions. Understanding these mechanisms will enable the development of sex-specific prevention and treatment strategies, moving us closer to personalised medicine.

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APPENDICES

Appendix A: differentially expressed genes in the proximal colon of male rats (FDR < 0.05 and |logFC| > 0.585).

Gene	logFC	FDR	PValue	Expression
RGD1565059	0.809159363	5.28E-09	5.32E-13	Increased in Loperamide
Fam118a	0.690889485	7.58E-07	3.06E-10	Increased in Loperamide
Apol9a	-1.093673469	8.08E-07	4.08E-10	Decreased in Loperamide
Bcl9l	-0.589863545	1.17E-06	8.27E-10	Decreased in Loperamide
Fitm2	-0.852574492	1.17E-06	7.70E-10	Decreased in Loperamide
Ppp2r5b	-0.682169222	1.23E-06	9.91E-10	Decreased in Loperamide
Plekhg6	-1.208234377	1.31E-06	1.19E-09	Decreased in Loperamide
Ing3	0.742990975	2.58E-06	2.61E-09	Increased in Loperamide
Maff	1.024391959	3.58E-06	3.97E-09	Increased in Loperamide
Mthfs	0.693058674	5.22E-06	6.32E-09	Increased in Loperamide
Arrdc3	0.597170866	1.65E-05	2.50E-08	Increased in Loperamide
Plin3	-0.590688557	2.33E-05	4.22E-08	Decreased in Loperamide
Tsku	-0.790842687	2.72E-05	5.50E-08	Decreased in Loperamide
Il7	0.725549886	3.52E-05	7.45E-08	Increased in Loperamide
Tlr4	0.804038911	5.17E-05	1.38E-07	Increased in Loperamide
Kcnf1	-2.397073047	5.17E-05	1.34E-07	Decreased in Loperamide
Fgf19	-6.139562686	5.17E-05	1.45E-07	Decreased in Loperamide

Plekhg2	-0.618643848	6.46E-05	2.41E-07	Decreased in Loperamide
Bcar3	0.6752945	6.83E-05	2.96E-07	Increased in Loperamide
Nudt22	-0.618959778	6.83E-05	2.67E-07	Decreased in Loperamide
Bcorl1	-0.674063619	6.83E-05	2.93E-07	Decreased in Loperamide
Mat2a	-0.819385067	6.83E-05	2.84E-07	Decreased in Loperamide
Hsd3b3	-1.120783613	6.83E-05	2.83E-07	Decreased in Loperamide
Bcl2l11	0.62687217	7.74E-05	3.75E-07	Increased in Loperamide
LOC108348086	-0.95159035	0.000113611	5.96E-07	Decreased in Loperamide
Gpr160	0.698650908	0.000123501	6.60E-07	Increased in Loperamide
Klf11	0.630509997	0.000132053	7.75E-07	Increased in Loperamide
Ankrd34a	-1.399574137	0.000263074	1.75E-06	Decreased in Loperamide
Gvin1	0.835861198	0.000282593	2.02E-06	Increased in Loperamide
Itga5	-0.801576194	0.000337613	2.62E-06	Decreased in Loperamide
Fdxacb1	0.615982598	0.000406655	3.32E-06	Increased in Loperamide
Wnt11	-0.77579504	0.000446391	3.78E-06	Decreased in Loperamide
Dhrs9	0.661211214	0.000513641	4.60E-06	Increased in Loperamide
Cyp26a1	-1.627249281	0.000513641	4.61E-06	Decreased in Loperamide
Cyp8b1	1.28156516	0.000589248	5.76E-06	Increased in Loperamide
Gpr4	-0.886586827	0.000589248	5.77E-06	Decreased in Loperamide
Tceanc	0.884451202	0.00085146	9.11E-06	Increased in Loperamide

Fam169b	1.174567611	0.000879755	9.59E-06	Increased in Loperamide
Alas1	-0.590557937	0.000922436	1.02E-05	Decreased in Loperamide
Scd2	-0.659805939	0.000922665	1.04E-05	Decreased in Loperamide
Kansl1l	0.741785739	0.000995052	1.15E-05	Increased in Loperamide
Fst	-0.781257878	0.001135204	1.41E-05	Decreased in Loperamide
Sgk1	-1.227161528	0.001385494	1.80E-05	Decreased in Loperamide
Mmp10	0.701045036	0.001585529	2.17E-05	Increased in Loperamide
Mab21l4	0.684918488	0.001585529	2.18E-05	Increased in Loperamide
Zc3h6	0.675460475	0.001651615	2.32E-05	Increased in Loperamide
Gja5	-1.739620379	0.00165931	2.34E-05	Decreased in Loperamide
Nr0b2	-1.590324595	0.001673308	2.38E-05	Decreased in Loperamide
Vxn	-1.100867215	0.002021797	2.98E-05	Decreased in Loperamide
Exoc3l2	-0.829031927	0.002060763	3.06E-05	Decreased in Loperamide
Clec2e	0.817154202	0.002217326	3.46E-05	Increased in Loperamide
Rhod	-0.976318537	0.002217326	3.48E-05	Decreased in Loperamide
Atp2b2	-1.038450544	0.002217326	3.48E-05	Decreased in Loperamide
B4galt1	-1.087202795	0.002638172	4.38E-05	Decreased in Loperamide
C2cd4b	-1.716534027	0.002638551	4.44E-05	Decreased in Loperamide
Akap3	-1.269216327	0.003357366	6.33E-05	Decreased in Loperamide
Foxs1	-1.216561031	0.003393214	6.45E-05	Decreased in Loperamide

Aqp8	-3.463459917	0.003873757	8.01E-05	Decreased in Loperamide
Tac1	-0.779442135	0.004003297	8.32E-05	Decreased in Loperamide
Nos3	-0.693686363	0.004065903	8.53E-05	Decreased in Loperamide
Spsb4	-0.607767944	0.004329188	9.13E-05	Decreased in Loperamide
Efcc1	-0.910624618	0.005324148	0.000124629	Decreased in Loperamide
Acer2	0.592576286	0.005978648	0.00014537	Increased in Loperamide
LOC103690878	-0.982330805	0.006622963	0.000173075	Decreased in Loperamide
Prap1	-1.74149968	0.007187955	0.000195818	Decreased in Loperamide
Cox6a2	-1.153746637	0.007201827	0.000197043	Decreased in Loperamide
Ifit1bl	0.695877856	0.008034198	0.000234273	Increased in Loperamide
Ptger4	0.650774308	0.008410059	0.000249476	Increased in Loperamide
Slc6a14	0.946544266	0.009071826	0.000276429	Increased in Loperamide
RGD1308544	0.691483126	0.009361333	0.0002907	Increased in Loperamide
Fut9	0.664539063	0.009361333	0.000291762	Increased in Loperamide
Dnajb3	-0.609060884	0.009361333	0.000291863	Decreased in Loperamide
Hmx3	-1.268627823	0.00941334	0.000294811	Decreased in Loperamide
Gbp3	1.180530119	0.009758575	0.000315079	Increased in Loperamide
Adra1d	-0.933807938	0.009758575	0.000314798	Decreased in Loperamide
Krt77	1.828489869	0.009809082	0.000318689	Increased in Loperamide
Sln	-1.084123559	0.010470193	0.000347315	Decreased in Loperamide

Slc5a12	2.737536203	0.010636242	0.000356294	Increased in Loperamide
Lrrc4	-1.413254417	0.010643276	0.000357604	Decreased in Loperamide
Lratd1	0.664582658	0.011349525	0.000387059	Increased in Loperamide
Abca12	0.597644201	0.011368684	0.000390006	Increased in Loperamide
Pax8	0.628823771	0.011372366	0.000391954	Increased in Loperamide
LOC103690302	-0.842695782	0.012481118	0.000448318	Decreased in Loperamide
Cyp2d4	-0.743491872	0.012667653	0.000464366	Decreased in Loperamide
Crb2	1.49582598	0.013912871	0.000529225	Increased in Loperamide
Caps2	0.807381716	0.013949385	0.000532022	Increased in Loperamide
C2cd4a	-1.26745886	0.014304512	0.000557112	Decreased in Loperamide
Slc37a2	-1.985938138	0.014349536	0.000560873	Decreased in Loperamide
RGD1561916	-0.85777589	0.014512713	0.000569614	Decreased in Loperamide
Duoxa2	-1.363829404	0.014650974	0.000576519	Decreased in Loperamide
Ccdc30	0.588889022	0.014926329	0.000591872	Increased in Loperamide
Depp1	1.228995445	0.015322689	0.000622661	Increased in Loperamide
Cgas	-0.611355698	0.015556881	0.00064042	Decreased in Loperamide
Nrarp	-0.692729513	0.015899149	0.000658316	Decreased in Loperamide
Macc1	0.86539678	0.017388912	0.000761456	Increased in Loperamide
Lrrc69	0.791720543	0.017644222	0.000777976	Increased in Loperamide
Tmem151b	-1.150599206	0.017699037	0.000785588	Decreased in Loperamide

Adra2a	-0.631024642	0.018480291	0.000853998	Decreased in Loperamide
Nr4a1	-1.496890892	0.018880039	0.000874376	Decreased in Loperamide
Rdh12	1.792876845	0.019948638	0.000946629	Increased in Loperamide
Angptl3	1.299446404	0.019948638	0.000954057	Increased in Loperamide
Rasl12	-0.667434481	0.019948638	0.000951724	Decreased in Loperamide
Gpr150	0.682192966	0.020230222	0.000983317	Increased in Loperamide
F2	0.655570627	0.020230222	0.000985239	Increased in Loperamide
Lgi1	-0.743865317	0.020230222	0.000985062	Decreased in Loperamide
Acsbg2	0.924900594	0.021106619	0.001047771	Increased in Loperamide
Slc16a14	-0.908612327	0.022075648	0.001122604	Decreased in Loperamide
Erich2	0.923854483	0.024416595	0.001313091	Increased in Loperamide
Tubb4a	-0.662105069	0.024504407	0.001322758	Decreased in Loperamide
Ceacam1	-0.957711415	0.025581316	0.001422188	Decreased in Loperamide
Ms4a4c	1.287448658	0.025748829	0.001436697	Increased in Loperamide
Samd5	-1.610948816	0.027094464	0.001552924	Decreased in Loperamide
Klhl38	0.646502057	0.027783729	0.001626363	Increased in Loperamide
Hspa12a	-0.587487977	0.027795064	0.00163301	Decreased in Loperamide
Lgsn	1.276840639	0.028553822	0.001731495	Increased in Loperamide
Amn	-1.124320863	0.02922413	0.001811813	Decreased in Loperamide
Nek11	0.643425106	0.02991818	0.001877622	Increased in Loperamide

Zfp786	0.697054024	0.030018035	0.001896003	Increased in Loperamide
Baiap2l2	-2.209016801	0.033011222	0.002214959	Decreased in Loperamide
Atp6v1c2	0.841004115	0.033357628	0.002255031	Increased in Loperamide
Chst8	-0.927286719	0.034979482	0.002430791	Decreased in Loperamide
Exd1	0.846765272	0.035071337	0.002445192	Increased in Loperamide
Ciart	1.469340205	0.035293444	0.002480143	Increased in Loperamide
Gja4	-0.712991317	0.035798677	0.002532022	Decreased in Loperamide
Pou2f3	-1.282318997	0.036048014	0.002568571	Decreased in Loperamide
Lrrn1	-0.722593579	0.037267196	0.002711094	Decreased in Loperamide
Fosb	-2.183886973	0.037494692	0.002755519	Decreased in Loperamide
Armcx1	-0.596605481	0.038931146	0.002934272	Decreased in Loperamide
Slc7a11	0.906770113	0.039005108	0.002946377	Increased in Loperamide
Six2	-2.300769603	0.039005108	0.002951564	Decreased in Loperamide
Tifa	0.587479526	0.039815688	0.003061888	Increased in Loperamide
Tmem35a	-0.649177853	0.039998146	0.003095407	Decreased in Loperamide
Has1	-1.198324666	0.040691245	0.003169591	Decreased in Loperamide
Trpv5	1.372152604	0.040858189	0.003190822	Increased in Loperamide
Bsnd	-2.513371923	0.042826943	0.003392105	Decreased in Loperamide
Cyp24a1	-2.248153061	0.043109945	0.00341887	Decreased in Loperamide
Plg	1.08748823	0.043552219	0.003484705	Increased in Loperamide

Cntn4	1.196522713	0.043630043	0.003505033	Increased in Loperamide
LOC100910656	-1.134830569	0.043630043	0.003526149	Decreased in Loperamide
Ifit3	0.670639907	0.047577657	0.004085217	Increased in Loperamide
P2ry4	0.696761254	0.048220962	0.004162569	Increased in Loperamide
Arhgef33	0.598121804	0.048313105	0.00421584	Increased in Loperamide
Fgf22	-1.000990445	0.048498408	0.004286611	Decreased in Loperamide
Tmprss12	0.96540252	0.048535691	0.004304598	Increased in Loperamide
Plppr5	-0.699784703	0.049088433	0.004363526	Decreased in Loperamide

Appendix B: *Differentially expressed genes in the proximal colon of female rats (FDR < 0.05 and |logFC| > 0.585).*

Gene	logFC	FDR	PValue	Expression
Sgk1	-1.943497391	3.46E-06	4.83E-10	Decreased in Loperamide
Dnajb3	-1.121197457	3.46E-06	6.97E-10	Decreased in Loperamide
Apol9a	-1.090214877	2.00E-05	6.04E-09	Decreased in Loperamide
Fam118a	0.609000077	0.000369411	1.86E-07	Increased in Loperamide
Maff	0.95230172	0.000443141	2.94E-07	Increased in Loperamide
Il7	0.728824099	0.000443141	3.42E-07	Increased in Loperamide
AABR07002677.2	-1.176075439	0.000443141	3.82E-07	Decreased in Loperamide
Itga5	-0.886794726	0.000830182	1.30E-06	Decreased in Loperamide
Celf3	-0.958745089	0.000830182	1.35E-06	Decreased in Loperamide
Nceh1	-0.67817898	0.001165931	2.24E-06	Decreased in Loperamide
Fgf19	-5.290924354	0.001233225	2.65E-06	Decreased in Loperamide
Epha2	0.656988671	0.001923533	4.66E-06	Increased in Loperamide
Fam169b	1.332752607	0.001984979	5.01E-06	Increased in Loperamide
Cyp26a1	-1.717416087	0.003290278	1.03E-05	Decreased in Loperamide
Tsku	-0.665893413	0.004602289	1.85E-05	Decreased in Loperamide
Gp2	-2.322349114	0.004602289	2.00E-05	Decreased in Loperamide
Fitm2	-0.629353529	0.004905769	2.18E-05	Decreased in Loperamide
Lrrc32	-0.735788632	0.004946857	2.25E-05	Decreased in Loperamide
Zhx2	-0.728298959	0.004964717	2.35E-05	Decreased in Loperamide

Nr0b2	-1.696588812	0.005045521	2.44E-05	Decreased in Loperamide
LOC108348086	-0.857892362	0.005157375	2.55E-05	Decreased in Loperamide
Dhrs9	0.640418674	0.006229083	3.27E-05	Increased in Loperamide
Slc6a20	0.665261263	0.007561634	4.45E-05	Increased in Loperamide
Nhsl2	-0.749900449	0.008584005	5.46E-05	Decreased in Loperamide
Wnt11	-0.7205387	0.009526039	6.44E-05	Decreased in Loperamide
Gpr4	-0.882076358	0.010203728	7.10E-05	Decreased in Loperamide
Usp2	-0.677496784	0.012675118	9.46E-05	Decreased in Loperamide
Mab21l4	0.665220484	0.012965927	0.000100734	Increased in Loperamide
Kansl1l	0.693333544	0.01326845	0.000105762	Increased in Loperamide
Emp3	-0.763871641	0.013486473	0.00010977	Decreased in Loperamide
Hoxc6	1.295314762	0.013486473	0.000110221	Increased in Loperamide
C2cd4b	-1.72434346	0.014613742	0.000129756	Decreased in Loperamide
Nell1	-0.959829835	0.016152374	0.000148779	Decreased in Loperamide
Plekhg6	-0.79721918	0.016684796	0.000158245	Decreased in Loperamide
AABR07062068.1	1.117094849	0.017147452	0.000164364	Increased in Loperamide
Bsnd	-3.26849119	0.018346516	0.000194368	Decreased in Loperamide
Hsd3b3	-0.869921827	0.018571773	0.000200502	Decreased in Loperamide
Mmp10	0.649163583	0.019882556	0.000228697	Increased in Loperamide
Pygm	-0.690560007	0.020032766	0.000234467	Decreased in Loperamide
Pla2g2d	-1.21235774	0.020228494	0.000243904	Decreased in Loperamide

Cd40	-0.853212721	0.020844586	0.000255237	Decreased in Loperamide
Gvin1	0.684860698	0.021140796	0.000262367	Increased in Loperamide
RGD1563354	-0.89406471	0.021401626	0.000287198	Decreased in Loperamide
Kcnf1	-1.748640531	0.023293995	0.000321993	Decreased in Loperamide
Ifit1bl	0.717593309	0.024349619	0.000340348	Increased in Loperamide
Syt17	-0.593910392	0.025282253	0.000369885	Decreased in Loperamide
AABR07009161.1	1.024900347	0.026226226	0.00039428	Increased in Loperamide
Tacstd2	0.743055198	0.026666566	0.000404945	Increased in Loperamide
AABR07049792.1	-1.053156606	0.029350284	0.00047086	Decreased in Loperamide
Neu2	-0.620308112	0.030061663	0.000485324	Decreased in Loperamide
Unknown	3.510097889	0.030061663	0.00049311	Increased in Loperamide
AABR07065895.1	-3.055681647	0.030061663	0.000504257	Decreased in Loperamide
LOC689065	0.835360711	0.030061663	0.000511622	Increased in Loperamide
Gja5	-1.533857581	0.030061663	0.000512604	Decreased in Loperamide
Klrd1	0.718548547	0.033687182	0.000627045	Increased in Loperamide
Sln	-1.150846006	0.035910199	0.000677551	Decreased in Loperamide
Fut9	0.663072388	0.03628577	0.00069196	Increased in Loperamide
Ifit3	0.858222442	0.036582267	0.000712378	Increased in Loperamide
Cyp2b1	-2.057577401	0.037569274	0.000740505	Decreased in Loperamide
Slc25a34	-0.594136764	0.038544406	0.000773922	Decreased in Loperamide
Lypd6	-0.860306148	0.038950097	0.000804508	Decreased in Loperamide

Echdc2	-0.597668644	0.038950097	0.000805647	Decreased in Loperamide
Il12a	-0.963611288	0.041081242	0.000874598	Decreased in Loperamide
Peg12	-1.022016352	0.0429799	0.000923693	Decreased in Loperamide
Armcx2	-0.672038141	0.045752775	0.001017049	Decreased in Loperamide
Numbl	-0.677634943	0.04847417	0.001110245	Decreased in Loperamide
Tmem121	-1.16595644	0.04946576	0.001162902	Decreased in Loperamide

Gene	logFC	FDR	PValue	Expression
RGD1565059	0.809159363	5.28E-09	5.32E-13	Increased in Loperamide
Fam118a	0.690889485	7.58E-07	3.06E-10	Increased in Loperamide
Apol9a	-1.093673469	8.08E-07	4.08E-10	Decreased in Loperamide
Bcl9l	-0.589863545	1.17E-06	8.27E-10	Decreased in Loperamide
Fitm2	-0.852574492	1.17E-06	7.70E-10	Decreased in Loperamide
Ppp2r5b	-0.682169222	1.23E-06	9.91E-10	Decreased in Loperamide
Plekhg6	-1.208234377	1.31E-06	1.19E-09	Decreased in Loperamide
Ing3	0.742990975	2.58E-06	2.61E-09	Increased in Loperamide
Maff	1.024391959	3.58E-06	3.97E-09	Increased in Loperamide
Mthfs	0.693058674	5.22E-06	6.32E-09	Increased in Loperamide
Arrdc3	0.597170866	1.65E-05	2.50E-08	Increased in Loperamide

Plin3	-0.590688557	2.33E-05	4.22E-08	Decreased in Loperamide
Tsku	-0.790842687	2.72E-05	5.50E-08	Decreased in Loperamide
Il7	0.725549886	3.52E-05	7.45E-08	Increased in Loperamide
Tlr4	0.804038911	5.17E-05	1.38E-07	Increased in Loperamide
Kcnf1	-2.397073047	5.17E-05	1.34E-07	Decreased in Loperamide
Fgf19	-6.139562686	5.17E-05	1.45E-07	Decreased in Loperamide
Plekhg2	-0.618643848	6.46E-05	2.41E-07	Decreased in Loperamide
Bcar3	0.6752945	6.83E-05	2.96E-07	Increased in Loperamide
Nudt22	-0.618959778	6.83E-05	2.67E-07	Decreased in Loperamide

Gene	**logFC**	**FDR**	**PValue**	**Expression**
Sgk1	-1.943497391	3.46E-06	4.83E-10	Decreased in Loperamide
Dnajb3	-1.121197457	3.46E-06	6.97E-10	Decreased in Loperamide
Apol9a	-1.090214877	2.00E-05	6.04E-09	Decreased in Loperamide
Fam118a	0.609000077	0.000369411	1.86E-07	Increased in Loperamide
Maff	0.95230172	0.000443141	2.94E-07	Increased in Loperamide
Il7	0.728824099	0.000443141	3.42E-07	Increased in Loperamide
AABR07002677.2	-1.176075439	0.000443141	3.82E-07	Decreased in Loperamide
Itga5	-0.886794726	0.000830182	1.30E-06	Decreased in Loperamide

Celf3	-0.958745089	0.000830182	1.35E-06	Decreased in Loperamide
Nceh1	-0.67817898	0.001165931	2.24E-06	Decreased in Loperamide
Fgf19	-5.290924354	0.001233225	2.65E-06	Decreased in Loperamide
Epha2	0.656988671	0.001923533	4.66E-06	Increased in Loperamide
Fam169b	1.332752607	0.001984979	5.01E-06	Increased in Loperamide
Cyp26a1	-1.717416087	0.003290278	1.03E-05	Decreased in Loperamide
Tsku	-0.665893413	0.004602289	1.85E-05	Decreased in Loperamide
Gp2	-2.322349114	0.004602289	2.00E-05	Decreased in Loperamide
Fitm2	-0.629353529	0.004905769	2.18E-05	Decreased in Loperamide
Lrrc32	-0.735788632	0.004946857	2.25E-05	Decreased in Loperamide
Zhx2	-0.728298959	0.004964717	2.35E-05	Decreased in Loperamide
Nr0b2	-1.696588812	0.005045521	2.44E-05	Decreased in Loperamide

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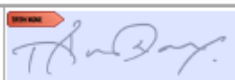
Student's signature:		Main supervisor's signature:	Warren McNabb Digitally signed by Warren McNabb Date: 2024.06.13 09:30:29 +12'00'

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