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Novel Insights into Early Life Stress-Induced Dysfunction of the Gut-Brain Axis

Thesis presented by

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents, I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Author contributions

All of the work conducted in this thesis was performed independently by the author with the following exceptions.

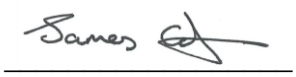
Chapter 2: Siobhain O'Mahony, Niall Hyland, Gerard Clarke, Marcela Julio-Pieper, and Patrick Fitzgerald carried out the *in vivo* and *ex-vivo* aspects of the study.

Chapter 3: Valentina Caputi carried out the *in vivo* aspects of the study.

Chapter 4: Mónica Tramullas and Patrick Fitzgerald carried out the *in vivo* aspects of the study.

Chapter 6: James Keane carried out immunoassays and statistical analysis of results.

Signed



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Publications and presentations

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Tramullas M*, **Collins JM***, Fitzgerald P, Dinan TG, O' Mahony SM, Cryan JF (2021) Estrous cycle and ovariectomy-induced changes in visceral pain are microbiota-dependent. iScience 24:102850.

Wilmes L*, **Collins JM***, O'Riordan KJ, O'Mahony SM, Cryan JF, Clarke G (2021) Of bowels, brain and behavior: A role for the gut microbiota in psychiatric comorbidities in irritable bowel syndrome. Neurogastroenterology and motility: the official journal of the European Gastrointestinal Motility Society 33:e14095.

Keane JM, Khashan AS, McCarthy FP, Kenny LC, **Collins JM**, O'Donovan S, Brown J, Cryan JF, Dinan TG, Clarke G, O'Mahony SM (2021) Identifying a biological signature of prenatal maternal stress. JCI Insight 6.

Conference posters

Collins JM, Caputi V, Wilmes L, Cryan JF, O'Mahony SM. Gut Microbiota Depletion in Early Life Reduces Visceral Sensitivity and Anxiety-Like Behaviour in Adulthood. European Behavioural Pharmacology Society biennial meeting, Online, 13th-6th July 2021.

Collins JM, Caputi V, Manurung S, Gross G, Dinan TG, Cryan JF, O'Mahony SM: Modulation of Dietary Lipids Reverses Early Life Stress-Induced Alterations of Gut-Brain Axis and Behaviour in the Rat. Irish Pain Society Annual Scientific Meeting, Dublin, Ireland, 19th October 2019.

Collins JM, Caputi V, Manurung S, Gross G, Dinan TG, Cryan JF, O'Mahony SM: Modulation of Dietary Lipids Reverses Early Life Stress-Induced Alterations of Gut-Brain Axis and Behaviour in the Rat. European Behavioural Pharmacology Society biennial meeting, Braga, Portugal, 28th -31st August 2019.

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Manuscripts in preparation/submitted

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Collins JM, Wilmes L, Caputi V, Cryan JF, O'Mahony SM. Gut microbiota in early life shapes adult anxiety-like behaviour and pain perception. *Manuscript in preparation.*

Lannon AS, Brocka M, **Collins JM**, Fitzgerald P, Cryan JF, O'Mahony SM, Moloney RD. A novel model for understanding secondary traumatic stress and visceral pain. *Manuscript in preparation.*

Caputi V, Wilmes L, **Collins JM**, Bastiaanssen TF, Clarke G, Cryan JF, O'Mahony SM. Decoding early life stress-induced comorbidities: susceptibility to different domains of altered microbiota-gut-brain axis signalling. *Manuscript in preparation.*

Abbreviations list

5-HT - Serotonin

ACTH - Adrenocorticotropic hormone

AMU - IgA median units

AR - Adrenoceptor

AVP - Arginine vasopressin

BBB - Blood-brain barrier

CC - Conventionally colonised

CNS - Central nervous system

CORT - Corticosterone

CRD - Colorectal distension

CRF - Corticotropin-releasing factor

CRP - C-reactive protein

DAPI - 4′,6-diamidino-2-phenylindole

DHA - Docosahexaenoic acid

DNA - Deoxyribonucleic acid

DRG - Dorsal root ganglia

E2 - 17β-oestradiol

ECC - Enterochromaffin cells

ECL - Electrochemiluminescence

ELS - Early life stress

ENS - Enteric nervous system

EPDS - Edinburgh postnatal depression scale

ER - Oestrogen receptor

FFAR - Free fatty acid receptor

FITC - Fluorescein isothiocyanate

FMT - Faecal microbiota transplantation

FODMAP – Fermentable oligosaccharide, disaccharide, monosaccharide, and polyol

FOS - Fructooligosaccharide

GABA - Gamma-aminobutyric acid

GF - Germ-free

GI - Gastrointestinal

GMU - IgG median units

GOS - Galactooligosaccharide

HMO - Human milk oligosaccharides

HPA - Hypothalamic-pituitary-adrenal

HPLC - High-performance liquid chromatography

IBS - Irritable bowel syndrome

IFABP - Intestinal fatty acid binding protein

IFN - Interferon

IL - Interleukin

I_{SC} - Short circuit current

KC/GRO - Keratinocyte chemoattractant/growth-related oncogene

Kyn - Kynurenine

LBP - Lipopolysaccharide binding protein

LMMP - Longitudinal muscle-myenteric plexus

LPS - Lipopolysaccharide

MFGM - Milk fat globule membrane

mRNA - Messenger ribonucleic acid

MS - Maternal separation

MWM - Morris water maze

NA - Noradrenaline

nNOS - Neuronal nitric oxide synthase

NS - Non-separated

OCT - Optimal cutting temperature

OVX - Ovariectomy

PBS – Phosphate-buffered saline

PBS-T – phosphate-buffered saline-Triton x100

PFA - Paraformaldehyde

PND - Postnatal day

PO - Perorally

PRR - Pattern recognition receptors

PSD - Postsynaptic density protein

PSS - Perceived stress scale

PUFA - Polyunsaturated fatty acid

S.C - Subcutaneous

SCFA - Short-chain fatty acids

sCD14 - Soluble CD14

SCOPE - Screening for pregnancy endpoints

SEM - Standard error of the mean

SHRP - Stress hyporesponsive period

SPF - Specific pathogen free

STAI - State-trait anxiety inventory

TBS - Tris-buffered saline

TLR - Toll-like receptor

TNF - Tumour necrosis factor

Trp - Tryptophan

TRPV1 - Transient receptor potential cation channel subfamily V member 1

VH - Visceral hypersensitivity

VMR - Visceromotor response

Abstract

Visceral pain, a debilitating hallmark of disorders of gut-brain axis interactions such as irritable bowel syndrome, has a major impact on quality of life. Given the increasing prevalence of irritable bowel syndrome over the past number of years, as well as a lack of effective treatments for disorders of visceral pain, new strategies need to be undertaken to develop successful interventions. The use of both dietary and pharmacological interventions to reduce visceral pain has yielded some promising results, however, these require further investigation. There is also a pressing need to unravel the mechanisms behind the aetiology of these disorders.

In this thesis, we focused on prenatal and postnatal stress-induced dysfunction of the gut-brain axis and provide novel insights into the factors that modulate the visceral pain response.

Firstly, the potential of CL-316243, a pharmacological intervention, and milk fat globule membrane (MFGM), a dietary intervention, as potential novel strategies to ameliorate visceral hypersensitivity resultant from exposure to stress in the early postnatal period were assessed. Specifically, using Sprague Dawley rats exposed to maternal separation (MS) for 3 hours per day from postnatal day 2-12, a well-established rodent model of early life stress, we administered either CL-316243 via the oral route or MFGM in the diet to assess their efficacy in ameliorating MS-induced visceral hypersensitivity. Here, we report that both interventions were successful in reducing MS-induced visceral hypersensitivity and this occurred independently of changes at the level of central serotonergic signalling and secretomotor activity (CL-316243), or the enteric nervous system and intestinal permeability (MFGM).

Next, we investigated the role of female sex hormones and the gut microbiota as modulators of visceral sensitivity using female germ-free mice. Here, we observed that the oestrous cycle modulated the visceral pain response in a microbiota-dependent manner and ovariectomy resulted in visceral hypersensitivity in conventional animals only.

We then assessed alterations in the immune profiles of pre-adolescent rats and the consequent impact of MS. Here, we reported modest pre-adolescent changes in the plasma immune profile and spleen weight in male rats, with no changes seen in the gut immune profile at this same timepoint.

Finally, we propose the use of several biological markers of systemic inflammation and gastrointestinal permeability as indicators of prenatal maternal stress during the second trimester of healthy pregnancies. The utilisation of these biomarkers could help to negate or prevent the deleterious impacts of early life stress both on foetal development and maternal health.

Overall, the results of this thesis provide novel insights into early life stress-induced dysfunction of the gut-brain axis as well as potential therapeutic strategies.

Chapter 1

General introduction

1. Historical and functional perspectives of the gut-brain axis

The concept of complex crosstalk between the gut and the brain is not new, but rather spans over the past 2,000 years beginning with philosophers including Hippocrates, Plato, and Aristotle suggesting that the mind and body are fully integrated and inseparable from each other. However, the nineteenth century saw a period of accelerated investigation into the communication pathways that exist between the gut and the brain in both health and disease, which resulted in advances in treatment strategies for disease in the field of neurogastroenterology. Overviews of the historical investigations leading to the discovery of the gut-brain axis have been provided in several reviews (Cryan et al., 2019; Margolis et al., 2021; Mayer, 2011), which detail the extensive reach and function of these gut-brain communication pathways.

In more recent times, the bidirectional communication between the gastrointestinal (GI) system and the brain has been termed the "the gut-brain axis". This axis is key to both the maintenance of homeostasis of the entire body via the extensive communication pathways and contributions of multiple systems and is also reciprocally influenced by the systems of the body. This idea was uncovered in the 1840's by William Beaumont who showed that varying emotional states affect the rate of digestion, thus suggesting that the brain and the gut are communicating reciprocally. These bidirectional communication pathways allow for the GI tract to impact on nervous system-related functions such as mood, emotion, pain, stress reactivity, and neurochemistry (Foster et al., 2017; Gao et al., 2019; Huang and Wu, 2021; Huang et al., 2019; Mayer et al., 2015a; Mayer et al., 2014), while in turn exerting local effects

in the GI tract by affecting motility, secretion, and permeability (Covasa et al., 2019; Moyat et al., 2022; Waclawiková et al., 2022).

Most recently, the communication networks of the gut-brain axis have been expanded to reflect the role of the gut microbiota, the hundreds of trillions of microorganisms that reside in the gut, and is now referred to as the "microbiota-gut-brain axis" (Cryan et al., 2019; Morais et al., 2021; Sherwin et al., 2016). The number of microbial cells in the body outnumbers the number of human cells to the order of 1.3:1 (Sender et al., 2016), so it is not unimaginable that this community of microorganisms has widespread effects on physiology not just restricted to the GI tract (Contijoch et al., 2019; Cryan and Dinan, 2012; Dinan and Cryan, 2017). While other distinct microbial communities exist both within and on the surface of organisms, including the oral microbiota (Kilian et al., 2016), pulmonary microbiota (Lynch, 2016), and skin microbiota (Grice and Segre, 2011), the most extensive microbiota population is that of the GI tract (Cryan et al., 2019). These bacteria, eukarya, and archaea have evolved with their hosts over millennia to form a complex and mutually beneficial relationship (Backhed et al., 2005; Neish, 2009). Some of the physiologically beneficial effects that the microbiota confers to the host include reinforcement of the gut barrier against pathogens (Baumler and Sperandio, 2016; Natividad and Verdu, 2013), regulation of host immunity (Belkaid and Hand, 2014), and energy balance and metabolism (den Besten et al., 2013). The gut microbiota is also pivotal in the communication between the gut and the brain and implicated in disorders of both (Bastiaanssen et al., 2018; Mayer et al., 2015b; Morais et al., 2021; Wilmes et al., 2021), details of which will be discussed later in this review of the literature.

2. Early life development of the microbiota-gut-brain axis

The individual components of the microbiota-gut-brain axis undergo extensive development in early life to allow these complex interactions to be carried out appropriately. A brief overview of the function, and an in-depth summary of the role of the gut microbiota in the development of these components is provided in the below sections.

2.1. Development of the gut microbiota

The term "microbiota" is the hypernym used to describe the 10-100 trillion microbial cells living within and on the surface of multicellular organisms (Blaser, 2014; Gilbert et al., 2018). A concentration gradient is evident from the upper intestine, with a population of 10^2 to 10^4 cells per gram in the duodenum to 10^7 to 10^9 cells per gram in the ileum, to the colon which is home to 10^{11} to 10^{12} cells per gram (Derrien and van Hylckama Vlieg, 2015; Kovatcheva-Datchary et al., 2013; O'Hara and Shanahan, 2006) **(Figure 1)**. The different bacterial communities as well as their relative abundances found along the human GI tract are also outlined in **Figure 1**. The bacterial species that comprise the gut microbiota are extremely important not only for normal GI function, but also for gut development.

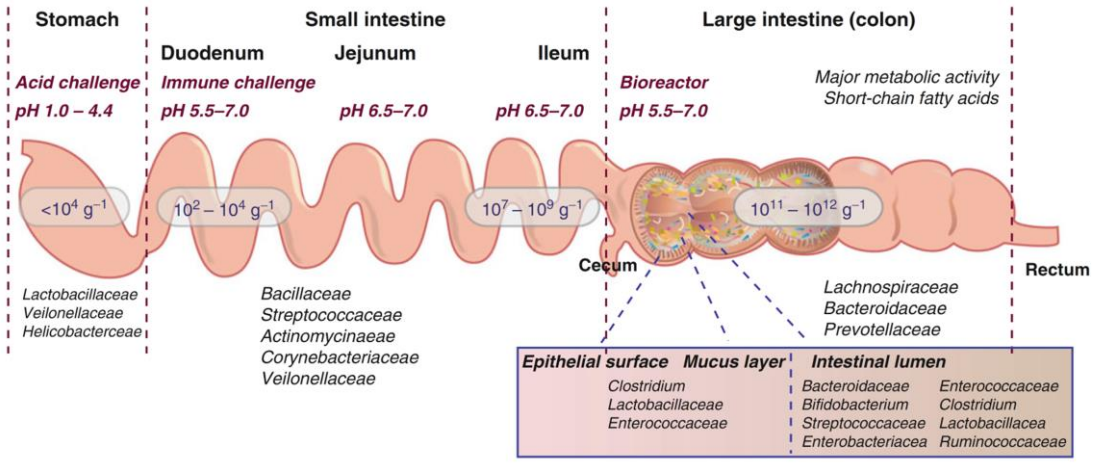


Figure 1. Representation of gut microbiota composition and abundance along the GI tract. *Major features that shape the gut microbiota into different anatomical regions of the gut are indicated. A concentration gradient of the gut microbiota from the small to the large intestine is also seen. From (Kovatcheva-Datchary et al., 2013).*

The gut microbiota is essential in early life development. Colonisation of the GI tract with microbes is a dynamic process and for the first number of years of life, the composition and diversity of the gut microbiota fluctuates until the establishment of a more consistent "adult microbiota" which occurs at approximately age 3 in humans (Nylund et al., 2014; Rodríguez et al., 2015). Several factors are involved in early colonisation and development of the neonatal gut microbiota such as mode of delivery (Martin et al., 2016; Rouge et al., 2010), gestational duration (Barrett et al., 2013), early life antibiotic exposure (Mbakwa et al., 2016), mode of feeding (Madan et al., 2016), and epigenetics (Gacesa et al., 2022) and examples and each of these have been reviewed extensively previously (Tamburini et al., 2016; Yee et al., 2020).

2.1.1. Prenatal influences on the microbiota

Prenatal factors including microbial seeding from the mother, maternal antibiotic use, maternal diet, and maternal stress exposure all impact upon foetal development and infant microbiota establishment. In early life, the diversity of the infant microbiota is

initially low prior to the observed expansion and diversification. Maternal antibiotic use has been shown to alter infant gut microbiota composition at 3 and 12 months of age in humans versus non-exposed infants (Zhang et al., 2019b), as well as decrease microbiota diversity (Dierikx et al., 2020). Similarly, maternal diet during pregnancy has been shown to heavily impact upon infant gut microbiota composition (Chu et al., 2016; Lundgren et al., 2018; Mirpuri, 2021). Maternal stress results in shifts in the maternal vaginal and gut microbiota, which then impacts foetal development and infant microbiota composition (Jašarević et al., 2017).

A current topic of intense discussion in research is the question of the sterility of the uterus. This also sparks the debate of whether the infant gut is sterile *in utero* and the infant microbiota is acquired both from the mother during and after birth as well as from the environment after birth, the so-called "sterile womb hypothesis" (Funkhouser and Bordenstein, 2013), or is there a distinct uterine microbiota colonising the foetal gut *in utero*, the "*in utero* colonisation hypothesis" (Collado et al., 2016). Since the early 20th century, it has been thought that the uterus is sterile due to the work of Henry Tissier (Tissier, 1900). Evidence exists to both support and disprove the notion of a sterile womb, however the current consensus is that there is no microbiome in the *in utero* environment and any bacteria found in the uterine cavity, meconium, placenta, umbilical cord, or indeed the neonate are likely invaders and may be reflective of a pathogenic state or due to contamination of samples (Baker et al., 2018; Bushman, 2019; de Goffau et al., 2019; Kennedy et al., 2021; Khan et al., 2015; Lauder et al., 2016; Perez-Muñoz et al., 2017; Rehbinder et al., 2018; Theis et al., 2019).

Mode of delivery, whether it be via caesarean section or vaginal delivery, has profound effects on early colonisation of the gut. A study has shown that neonates born via caesarean section have a different microbiota composition compared to vaginally-born

neonates, with those born by caesarean section initially being exposed to the skin microbiota with a higher representation of *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. versus infants delivered vaginally with a high representation of *Lactobacillus*, *Prevotella*, and *Sneathia* spp. (Dominguez-Bello et al., 2010). Another study also reported a lesser abundance of *Bifidobacterium* spp. in caesarean section-born mice, which may impair the ability of these infants to break down the human milk oligosaccharides (HMO) in breastmilk resulting in an increased prevalence of atopic diseases (Fujimura et al., 2016) and metabolic disorders (Stuivenberg et al., 2022), further highlighting the major role of mode of delivery on early GI colonisation (Morais et al., 2020). Interestingly, exposure of neonates born by caesarean section to their mother's vaginal microbiota resulted in a shift in microbiota composition towards that of vaginally-born infants during the first month of life (Dominguez-Bello et al., 2016). This same narrowing of the difference in microbiota composition between caesarean section and vaginally-born infants has been reported in another study using orally-delivered maternal faecal microbiota (Korpela et al., 2020). However, the efficacy of this mother-to-infant microbiota seeding method is somewhat disputed, with evidence suggesting that oral administration of maternal vaginal microbiota did not affect caesarean section-born offspring's microbiota composition (Wilson et al., 2021). In summary, the mode of delivery is responsible for major shifts in gut microbiota composition, however, further studies are needed to determine the benefits of mother-to-infant microbiota seeding in caesarean section-born infants directly after birth.

2.1.2. Postnatal influences on the microbiota

Aside from prenatal input, postnatal factors such as method of feeding also play a major role in early life microbiota colonisation. Breastfeeding has been marked as an important source of nutrition for neonates (Lyons et al., 2020). The microbiota composition of preterm infants has been seen to be lacking in two major genera, *Bifidobacterium* and *Lactobacillus,* versus full-term infants (Barrett et al., 2013). Breastfeeding provides bifidogenic factors and HMOs to aid the growth of beneficial bacteria in the gut and help to develop a more stable microbiome (Dai and Walker, 1999) and has been shown to compensate for the missing genera seen in both preterm and caesarean section-delivered infants (Costello et al., 2012). Not surprisingly, it has been seen that there is greater *Bifidobacterium* population diversity in breastfed versus formula-fed infants (Roger et al., 2010). Interestingly, it has been shown that breastfed infants harbour a less diverse microbiota (lower α-diversity) versus formula-fed infants, which is thought to be the result of a higher capacity to degrade HMOs in breastfed infants, which has shown importance in the development of the immune system (Ma et al., 2020). The different methods of transfer and modulation of neonatal microbiota both prenatally and postnatally are summarised in **Figure 2**. It has also been suggested that the development of the microbiota, in tandem with multiple other systems including the brain, immune system, and hypothalamic-pituitary-adrenal (HPA) axis reflects a critical window in the development of the gut-brain axis (Cowan et al., 2020). As outlined above, several factors may directly affect the gut microbiota. One such factor is stress and associated HPA axis activation. It is thought that the early postnatal stage is critical for the development of a healthy microbiome as it is the most dynamic phase of microbiota development (O'Mahony et al., 2014). This will be discussed in more detail below in the relevant sections.

Other postnatal factors that impact upon the infant gut microbiota include exposure to stress in early life (D'Agata et al., 2019; Vogel et al., 2020) and postnatal antibiotic exposure which results in an increase abundance of pathogenic bacteria including *Klebsiella* and *Enterococcus* spp. (Reyman et al., 2022).

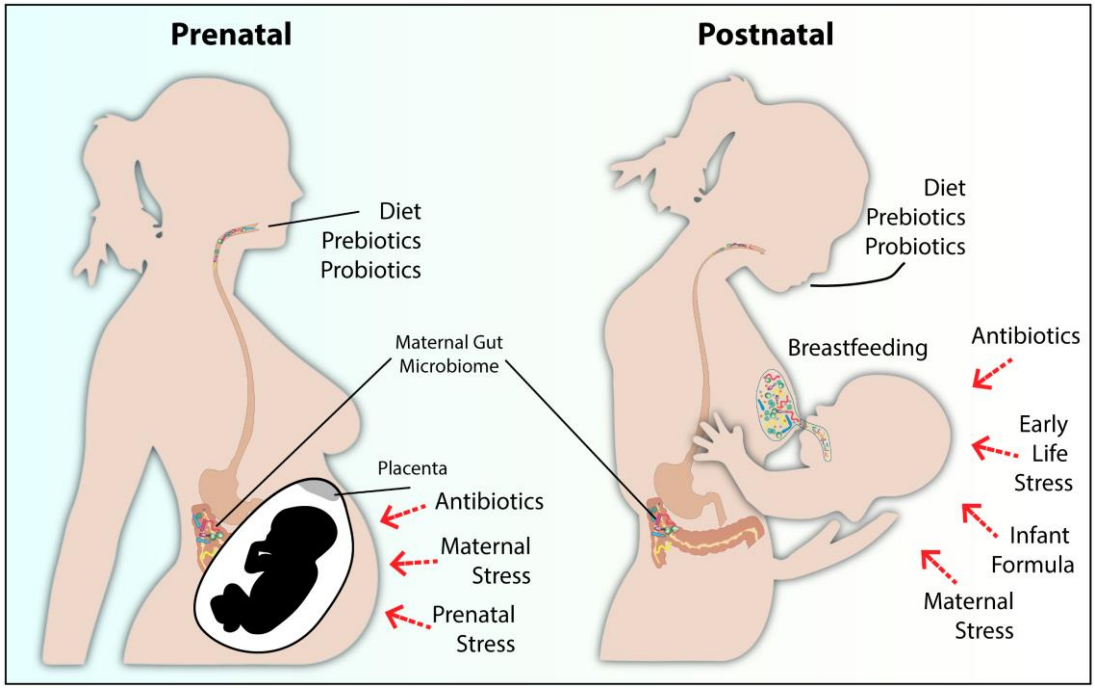


Figure 2. Pre- and postnatal factors influencing neonatal gut microbiota including mode of feeding and maternal diet, antibiotic use, and exposure to stress. *Prenatal factors that influence infant gut microbiota colonisation include maternal diet, which shapes the maternal microbiota which the infant is exposed to at birth. In the postpartum period and early life, postnatal factors including maternal diet, mode of feeding as well as neonatal antibiotic treatment and early life stress all play major modulatory roles in early life shaping of the gut microbiota. Red arrows indicate influencing factors.*

2.2. Functional anatomy of the gastrointestinal tract

The GI tract is the series of hollow organs beginning at the mouth and terminating at the anus and is responsible for the digestion and absorption of nutrients from food, control of motility, secretion, and also performs a role in immunity.

Digestion begins in the mouth both mechanically and chemically. Here, the salivary glands produce saliva containing amylase which mixes with the ingested food and begins digesting starch. Mucins contained within saliva also aid in the formation of a bolus which is then transferred down the oesophagus to the stomach via peristaltic contractions (the muscular contraction of the GI tract). In the stomach, the partially digested food is mixed with enzymes and hydrochloric acid secreted by gastric cells to aid digestion. The stomach is anatomically divided into the fundus, body, and pylorus which enables accumulation, and mechanical digestion respectively. Next, each segment of the remaining GI tract performs specific processes related to digestion and absorption of food. For instance, the small intestine, usually between 3-6 meters long, is comprised of the duodenum (20-25cm in length), jejunum (~2.5 metres in length), and ileum (~3 metres in length), and is mainly involved in the absorption of nutrients.

Chemical digestion of foodstuffs is carried out in the duodenum as it receives pancreatic juices and bile from the gall bladder, with the jejunum and ileum being involved in more absorptive roles. As there is no clear juncture between the jejunum and ileum, the jejunum may be recognised by the absence of Peyer's patches, groupings of lymphoid tissue which are part of gut-associated lymphoid tissues which are seen in the ileum (Kobayashi et al., 2019), and the absence of Brunner's glands, which secrete mucous to neutralise chyme from the stomach, present in the duodenum.

The jejunum also has circular folds, villi, and microvilli, all of which increase the available surface area for absorption of nutrients. The jejunum may be differentiated from the ileum by its' greater thickness, increased vasculature, and more separated circular folds (Mahadevan, 2020).

The small intestine is composed of 4 layers. The serosa, the outermost layer, is comprised of mesothelium **(Figure 3 below)**. The muscularis houses two smooth muscle layers, one being the longitudinal muscle responsible for elongation or shortening of the gut, and the other being the circular muscle, which constricts the gut. This layer is also home to neurons organised into myenteric plexi. The submucosa contains blood vessels, lymphatics, and other plexi of neurons called submucosal plexi which are responsible for the regulation of motility and secretion. The mucosa is the innermost layer and is optimised for absorption by virtue of the presence of numerous villi which increase total surface area. Finger-like projections of the mucosa, the villi, increase surface area and are present throughout the small intestine and are longest in the duodenum and shortest in the distal ileum. Specialised epithelial cells, including crypt cells which control mitosis and secretion of fluids, divide and give rise to other specialised cells including goblet cells located between enterocytes which coat the villi in mucin to protect the wall of the GI tract and aid in movement of the chyme, enteroendocrine cells which produce hormones necessary for regulation of digestion through alterations in motility and secretion, Paneth cells which play a role in immunity via secretion of anti-microbial molecules, or enterocytes (Campbell et al., 2019).

Peristalsis results in the movement of chyme along the ileum to the large intestine though the ileo-caecal junction and into the caecum where any remaining water and required salts are absorbed. The colon is approximately 1.5 metres in length. In

summary, the journey of the remaining chyme begins in the ascending colon, which becomes the transverse colon, then the descending colon and the final part of the colon, the sigmoid colon. From the sigmoid colon, the contents of the gut pass into the rectum, and finally to the anus where it is expelled from the body. The main functions of the large intestine are absorption of water and electrolytes, formation of faeces, and digestion by gut bacteria (Nigam, 2019). The musculature of the wall of the colon is comprised of the outer longitudinal and inner circular layers. In the colon, the musculature, enteric nervous system (ENS), and interstitial cells of Cajal modulate contraction. The interstitial cells of Cajal extend connections to the myenteric and submucosal plexi as well as the circular muscle layer to regulate absorption. The interstitial cells of Cajal also play a modulatory role in motility via electrical propagation along smooth muscle cells, while also being involved in sensitivity to mechanical stimuli. Mechanoreceptors located in the mucosa have been shown to respond to touch, whereas those expressed in the serosa respond to distension (Bharucha and Camilleri, 2019).

Located within the monolayer of epithelial cells, a host of immune cells including dendritic cells, B and T cells, as well as macrophages maintain intestinal homeostasis. Dendritic cells are located throughout the lamina propria, gut associated lymphoid tissue, and in discrete lymphoid aggregates and are among the most potent antigen presenting cells and are capable of initiating the adaptive immune response (Iwasaki and Medzhitov, 2015). Under pathological conditions, dendritic cells recognise microbial-borne antigens and mount an immune response, however, given the vital role of the microbiota in digestion and gut to brain signalling, intestinal dendritic cells have adapted to tolerate oral antigens and maintain a symbiotic relationship between the host and the microbiota (Chieppa and Eri, 2013).

2.3. The role of the gut microbiota in the development of the enteric nervous system

The ENS is a division of the autonomic nervous system and is comprised of networks of neuronal and glial cells embedded in the gut wall along the entire length of the intestine. It exerts control over normal physiological function of the GI tract and is comprised of two major ganglionated plexi; the myenteric plexus and submucosal plexus **(Figure 3)**. Although the ENS is capable of functioning independently of the central nervous system (CNS) to control GI secretomotor activity including peristalsis, gastric secretion, and nutrient and water absorption, there is constant bidirectional communication between the ENS and CNS via intestinofugal neurons which synapse onto sympathetic ganglia, where sensory information travels along spinal and vagal afferent routes to the CNS (Furness, 2012), and from the CNS to ENS preganglionic neurons of vagal efferents from the dorsal motor nucleus of the vagus nerve which innervate the muscular and mucosal aspects of the gut (Breit et al., 2018). The ENS has also been shown to play a major role in gut-brain axis communication (Carabotti et al., 2015) and responds either directly or indirectly to the luminal contents of the gut, including microbial metabolites and mediators. Intrinsic primary afferent neurons of the ENS, neurons with their cell bodies, processes, and connections located in the gut wall detect the luminal environment including microbial metabolites and mechanical manipulations and are involved in chemosensing and initiation of secretomotor and vasomotor reflexes. The extrinsic primary afferent neurons are neurons with differing locations depending on function; those with their cell bodies located in the nodose and jugular ganglia are vagal afferents, whereas for spinal afferents these are located in the dorsal root ganglia (DRG). The function of these extrinsic primary afferent neurons is to relay sensory information such as nociceptive

signals to the CNS and are hypothesised to exert effects on myenteric neurons of the ENS, resulting in smooth muscle contraction (Smith-Edwards et al., 2019). Even before being established in the foetus, the microbiota plays a major role in gut development as products produced by the maternal gut microbiota begin exerting indirect effects on gut physiology and development *in utero* (Heiss and Olofsson, 2019). The influence of the gut microbiota on ENS development has been discussed at length previously (Hyland and Cryan, 2016; Obata and Pachnis, 2016).

It is seen that the major developmental processes associated with ENS formation begin early in gestation during the formation of the neural tube (Goldstein et al., 2013). In humans, the colonisation process of the rudimentary gut by neural crest cells begins at approximately gestational week 4 (Nagy and Goldstein, 2017), with the myenteric plexus developing from week 4 until shortly before birth, and submucosal plexus development lagging behind by 3 weeks with continued development postnatally (Fu et al., 2004; Wallace and Burns, 2005). This same enteric neural crest cell colonisation process may be seen in mice with the myenteric plexus forming from embryonic day 8.5-9.5 to 13.5-14, with some semblance of a submucosal plexus forming from embryonic day 14.5-16.5 (Rao and Gershon, 2018).

Shortly following birth, the GI tract is rapidly colonised by microorganisms as indicated above. The role of the microbiota in ENS development may be seen in that germ-free (GF) mice have a lower number, as well as density of myenteric neurons, indicating a potentially reduced functional capacity compared to relative controls (Collins et al., 2014). The gut microbiota has been identified as one of the main modulators of ENS development (Foong et al., 2020) as in rodents, both the ENS and gut microbiota undergo substantial parallel development postnatally. This begs the question of whether these developmental processes are linked and is supported by the

observation that in GF mice, improper development and functionality of the ENS is seen (Kabouridis and Pachnis, 2015). Caputi and colleagues demonstrated this in C57BL/6 mice by depleting the gut microbiota via administration of broad-spectrum antibiotics and observed the abnormal development of glial networks and a reduction in the number of neurons in the myenteric plexi (Caputi et al., 2017b). To further support the link between the development of the ENS and the gut microbiota, colonisation of GF mice with conventional microbiota rescues the function of enteric neurons altered as a result of a lack of microbiota (McVey Neufeld et al., 2015; Vadder et al., 2018).

Antibiotic depletion of the gut microbiota has also been shown to decrease GI transit (Ge et al., 2017; Lendrum et al., 2016). Mechanisms underlying this effect are thought to be related to decreased inhibitory neuronal nitric oxide synthase (nNOS) nitrergic signalling as antibiotic-induced dysbiosis reduced nNOS signalling, leading to altered inhibitory input to the gut, resulting in dysmotility (Caputi et al., 2017b). This is reinforced in a study by Anitha et al who treated mice with broad-spectrum antibiotics, resulting in a reduction in bacterial load, a decrease in the number of $nNOS^{+}$ neurons, and delayed motility (Anitha et al., 2012). The microbiota is required for normal excitability of gut neurons, in particular, myenteric intrinsic primary afferent neurons as seen in a study in GF mice where colonisation of GF mice rescued the excitability lost as a result of the GF condition, highlighting the importance of the gut microbiota in appropriate gut function (McVey Neufeld et al., 2013). The expression of the aryl hydrocarbon receptor on enteric neurons of both the proximal and distal GI tract enables a response to luminal content, including to microbial metabolites (Obata et al., 2020). These findings together demonstrate the necessity of the gut microbiota in normal development of the gut and the ENS.

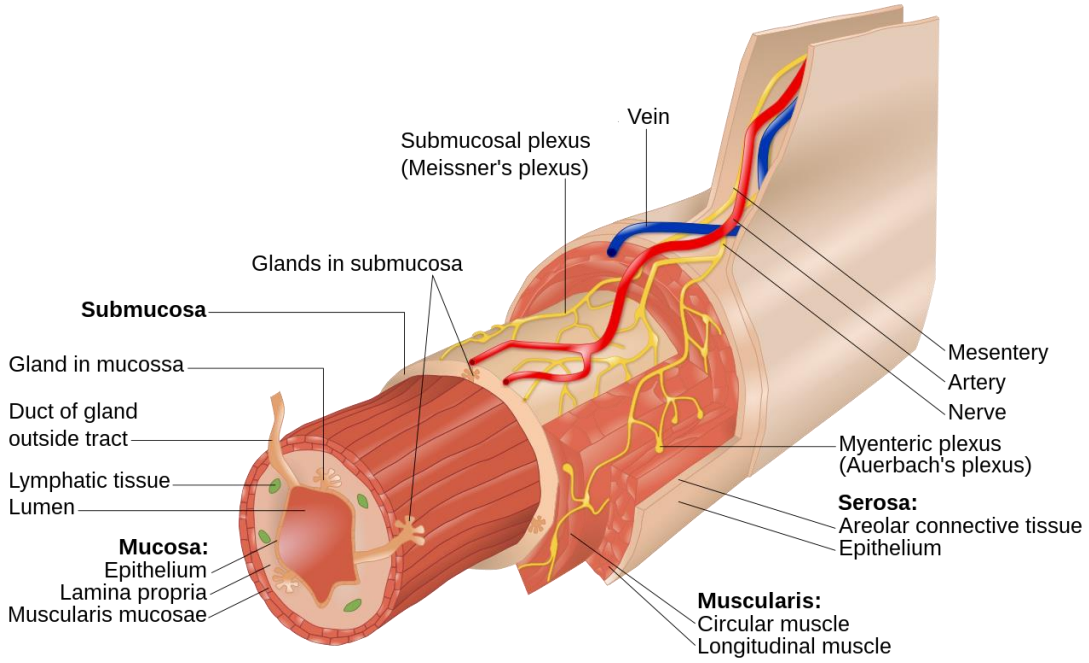


Figure 3. Structure of gastrointestinal tract including the enteric nervous system. *The schematic illustrates the laminar organization of the bowel in three dimensions from the mesentery to the lumen. The two major plexuses of the ENS are the myenteric plexus, located between the circular and longitudinal muscle layers in the muscularis externa, and the submucosal plexus, located in the submucosa. Image obtained from Wikimedia and reproduced under a creative commons attribution-share alike 4,0 international license.*

2.4. The role of the gut microbiota in the development of the gut barrier

At the interface between the enteric microbiota, luminal contents of the GI tract, and the host lies the gut barrier. This barrier is comprised of two main layers; (i) a physical barrier to regulate diffusion between cells and host tissue and (ii) a functional barrier to allow immune tolerance and nutrient uptake. During the prenatal period, the GI tract is already capable of the following; (i) digestion of food (ii) defence against pathogens (iii) hormone and signalling molecule secretion (iv) osmoregulation (v) detoxification of metabolites resultant of metabolism (Buddington and Sangild, 2011), indicating that the infant GI tract is primed to respond to postnatal microbial colonisation.

There are three main methods by which molecules may pass through the epithelial barrier; (i) passive diffusion across the cell membranes (ii) passive diffusion through spaces between neighbouring cells (iii) carrier/receptor-mediated transport (Chelakkot et al., 2018). The innermost layer of the gut barrier is the mucous layer which serves as a buffer between the luminal contents of the GI tract and the gut epithelial cells. This layer is formed following secretion of mucins such as MUC2, a highly glycosylated mucin which prevents bacterial adherence to the epithelial layer, by goblet cells and is comprised of 2 layers; the inner and outer mucous layer. The inner layer is approximately 100µm thick, contains concentrated antimicrobials, and has a low density of bacteria whereas the outer layer contains more diluted antimicrobials and allows some bacterial penetration (Natividad and Verdu, 2013). Mucous structure also varies depending on location in the gut as well as microbiota composition. The next layer, a single layer of epithelial cells comprised of endocrine cells, goblet cells, Paneth cells, enterocytes, and M cells, forms a physical barrier between luminal contents and visceral tissues and organs. This epithelial layer is fortified by tight junctions formed by the interaction of tight junction proteins including zonula occludens ZO-1, ZO-2, occludin, and claudin.

A critical regulator of gut barrier development is the gut microbiota as it is seen that gut barrier function is impaired in GF animals (Smith et al., 2007). A study in piglets found that at birth, physiological and molecular parameters of the gut mucosal barrier of the ileum and colon, including fluorescein isothiocyanate (FITC)-dextran flux, crypt depth, and gene expression levels of toll-like receptors (TLR), are already distinct with little variation occurring in these parameters in the colon, while some variation was noted in the ileum until postnatal day (PND)28 (Arnaud et al., 2020). Further studies have reinforced the role of the gut microbiota in gut barrier

development whereby the outer mucous layer of GF mice was similar to that of conventional animals, but the inner mucous layer was more permeable to bacteria-sized beads, an effect that was rescued by colonisation of the GI tract with microbiota (Johansson et al., 2015). The role of the gut microbiota in development of the gut barrier has been discussed at length previously (Takiishi et al., 2017).

The gut barrier and immune system develop in tandem, with the epithelial barrier playing a major role in early life priming of the immune system, and the mechanism by which the gut microbiota exerts its effects on gut barrier development and function has been suggested to be mediated by the immune system.

2.5. The role of the gut microbiota in the development of the immune system

The immune system undergoes extensive development *in utero* as well as in the early postnatal period, with this evolutionary capacity persisting throughout the lifespan as the host encounters various immune challenges (Simon et al., 2015). The immune system may be broadly split into two subsystems, the innate and adaptive immune system, which function in synchrony to mount a defence against pathogens and other foreign particles. The innate immune system provides a non-specific front line of defence against potentially infectious agents and includes both physical and chemical barriers such as the skin and mucous membranes, myeloid-derived immune cells, and soluble mediators (Pettengill et al., 2014; Williams, 2011).

The adaptive immune system is more specific in its response, utilising immune cells of lymphoid lineage to mount a pathogen-specific response (Simon et al., 2015). The adaptive immune system also retains an “immunological memory” of previously

encountered pathogens, and thus is more effective should the host be exposed to that same pathogen again (Flajnik and Kasahara, 2010).

It is also seen that optimal colonisation of the GI tract in neonates is central for appropriate immune development in the gut (Langhendries, 2006). Further evidence for the vital role of the gut microbiota in early life immune system development is found in studies reporting that a lack of *Bifidobacteria* and depletion of genes associated with HMO utilisation was found to result in immune dysregulation and systemic inflammation in early life (Henrick et al., 2021). For quite some time it has been proposed that a decreased exposure of infants to microbes as a result of increased cleanliness in the developed world, the hygiene hypothesis (Garn et al., 2021), negatively impacts upon immune system development. It has been suggested that exposure to this increasingly sterile environment compromises immune system development and function, leading to a rise in the incidence of atopic diseases, namely asthma and eczema, in childhood (Strachan, 1989). Evidence to support this theory includes the observation that the incidence of atopic diseases during early childhood in those that are exposed to pets, other children at home (siblings), or a farm environment is far lower than those who grew up without experiencing these same environments (Ball et al., 2000; Benn et al., 2004). The immune system of the gut is very well developed with a vast array of immune cells and mechanisms capable of mounting appropriate immune defences against pathogens and other harmful foreign particles. One such mechanism includes the litany of receptor molecules present in the intestinal epithelial cells for recognition of bacterial cell surface ligands such as capsular polysaccharides, peptidoglycan, and lipopolysaccharide (LPS) (Platt and Mowat, 2008).

At the centre of how immune system development may be affected by the gut microbiota lie the TLRs. It is seen that both pro- and anti-inflammatory cytokines are released following the activation of TLRs (Takeda and Akira, 2005). TLRs are activated by bacterial products such as LPS and therefore development of the immune system is closely regulated by the gut microbiota. It is seen in human children that early life perturbations in the gut microbiota may lead to the development of immune diseases such as Crohn's disease and inflammatory bowel disease (Matsuoka and Kanai, 2015).

The role of the gut microbiota in the development of the immune system is further bolstered by the observation that GF animals display a dysregulated and ineffective immune system (Round and Mazmanian, 2009). It may also be noted that through communication between the enteric microbiota and the host at the gut epithelial barrier, the microbiota is involved in early life priming of the immune system. This is accomplished via recognition of self-versus non-self-antigens by pattern recognition receptors (PRR), such as the TLRs in the gut, which aid in tolerance to the host microbiota by the immune system (Chu and Mazmanian, 2013; Fasano and Shea-Donohue, 2005; Francino, 2014). Further, short-chain fatty acids (SCFA) such as butyrate have been shown to reinforce the mucosal barrier through stimulation of mucous production (Jung et al., 2015; Willemsen et al., 2003). Through the evidence above, it is no surprise that any microbiota-targeted insult in early life would have detrimental effects on proper immune system development and later function.

2.6. The role of the gut microbiota in the development of the brain

The gut microbiota also plays a pivotal role in CNS development as it is seen that in the absence of the maternal gut microbiota in GF mice, the microglia which play a major role in brain circuitry exhibit alterations in gene expression levels (Thion et al., 2018). Early neural tube development has been shown to be susceptible to both internal and external factors prenatally such as maternal diet, stress, and infection. In a study by Diaz Heijtz and colleagues, it was noted that the gut microbiota may alter neurodevelopment by altering levels of synaptophysin and postsynaptic density protein (PSD)-95 (markers of synaptic vesicle maturation as an indirect marker of synaptogenesis and maturation of excitatory synapses respectively) in the striatum in a critical window of development. This may lead to alterations in synaptogenesis via long-term regulation of synaptogenesis or by direct modulation of neurotransmitters such as serotonin (5-HT), melatonin and gamma-aminobutyric acid (GABA) (Heijtz et al., 2011). It has also been reported that maternal microbiota disruption alters uterine environmental conditions, leading to abnormalities in the foetal brain which are lessened by the addition of microbial metabolites to foetal thalamic explants (Vuong et al., 2020). Interestingly, in GF mice it is seen that there is increased neurogenesis, or birth of new neurons, in the dorsal hippocampus versus conventional controls which was not reversed by recolonisation with microbiota (Ogbonnaya et al., 2015). Further, hippocampal and amygdalar volumes are shown to be increased in GF mice (Luczynski et al., 2016b), and CNS serotonergic signalling is altered in GF mice which is not rescued by postnatal recolonisation with microbiota (Clarke et al., 2013). These findings highlight the prenatal or very early postnatal modulatory role of the gut microbiota on neuronal processes in the brain which occur during critical windows of development (Vuong et al., 2020).

The behavioural correlate of the developmental alterations in these microbiota-mediated CNS structures includes decreased anxiety (Neufeld et al., 2011), non-spatial memory deficits (Gareau et al., 2011), as well as exaggerated stress responsivity (Clarke et al., 2013). The modulatory role of the gut microbiota on brain and behaviour has been extensively reviewed previously (Luczynski et al., 2016a).

It is known that the gut microbiota may affect blood-brain barrier (BBB) permeability either directly or by action of released metabolites as well as via the release of cytokines (Logsdon et al., 2018). Gut microbes, or their metabolites have also been shown to cross the BBB and exert effects on both BBB permeability and brain function (Parker et al., 2020). Evidence to support the effect of the gut microbiota on neurodevelopmental processes lies in the concept that the permeability of the BBB at the hippocampus, frontal cortex, and striatum is thought to be controlled by the microbiota. Interestingly, increased BBB permeability has also been seen in GF versus conventional mice (Braniste et al., 2014).

The gut microbiota is capable of synthesis of many neuromodulatory molecules including GABA, noradrenaline (NA), 5-HT, as well as SCFAs and tryptophan, and as such may affect gut-brain signalling (Bistoletti et al., 2020; Bosi et al., 2020). It has also been shown that specific bacteria may modulate neurotransmission within the CNS via alteration of precursors for example, *Bifidobacterium infantis* has been seen to increase peripheral tryptophan levels and possibly decrease 5-HT degradation in the brain (Desbonnet et al., 2008).

Interestingly, the neural circuitry required for perception of pain are already developed by the end of the second trimester. It has been shown that the nerve tracts involved in the spinothalamic tract are completely myelinated to the level of the thalamus by

gestational week 30, and the neurons from the thalamus to higher brain regions in the cortex are myelinated by week 37 (Anand and Carr, 1989). The late gestational and early postnatal period is considered a critical window for neuronal development as functional connections are still being formed and myelinated. Thus, any insult either prenatally or in the early postnatal period may impact upon the nociceptive system, resulting in sensitisation including in perception of visceral pain.

2.7. The role of the gut microbiota in HPA axis development

The HPA axis undergoes major development both *in utero* and in the postnatal period. Development of the pituitary gland and activity of adrenocorticotropic hormone (ACTH) has been detected as early as 9 to 10 weeks gestational age (Pavlova et al., 1968) as well as development of the foetal hypothalamus (Koutcherov et al., 2002). By week 12, corticotropic releasing factor (CRF) is detected in the foetal hypothalamus (Wood and Walker, 2015). The adrenal cortices of the foetus are active from early gestation and undergo substantial development both prenatally and postnatally (Ishimoto and Jaffe, 2011).

A role for the gut microbiota in HPA axis development and function has also been observed. It is seen that chronically high levels of cortisol, the stress hormone in humans, can impact upon the gut microbiota and intestinal epithelial barrier permeability in a reciprocal manner (for review see (Kelly et al., 2015)). Stress responsivity and anxiety-like behaviour are noted to be elevated in GF versus conventional mice (Clarke et al., 2013; Sudo et al., 2004), indicating that early life microbiota products and potentially the systems they affect are capable of influencing stress system development and function.

Irritable bowel syndrome (IBS) is a disorder of gut-brain axis interactions that is associated with both an altered gut microbiota as well as an exaggerated cortisol response to CRF stimulation (Dinan et al., 2006). This association was investigated in GF mice whereby decreased anxiety-like behaviour was observed versus specific pathogen free (SPF) mice, mice whose exact gut composition is known and controlled, on the elevated plus maze (Neufeld et al., 2011). This same decreased anxiety-like behaviour was seen by Clarke and colleagues using the light-dark box (Clarke et al., 2013). However, only this reduction in anxiety-like behaviour, but not the CNS neurochemical alterations, was normalised following reconstitution of the microbiota post-weaning, highlighting a temporal critical window of development (Clarke et al., 2013).

Stress, particularly in early life exerts effects on HPA axis function, resulting in alterations in cortisol at baseline or response to a subsequent stressor (Butler et al., 2017; Ouellet-Morin et al., 2019). Mounting evidence exists to support the idea that the gut microbiota is required for an appropriate stress response. Sudo and colleagues noted that the stress response, as measured by plasma ACTH and corticosterone (CORT), was elevated in GF versus SPF mice in response to a restraint stress (Sudo et al., 2004). Further justification for the necessity of the gut microbiota in the mounting of an appropriate stress response is seen where colonisation of GF mice with *Bifidobacterium infantis* ameliorated the exaggerated stress response observed (Sudo et al., 2004). Colonisation of naturally timid GF BALB/c mice with the microbiota of SPF NIH Swiss mice resulted in an increase in exploratory behaviour whereas the converse is true if GF NIH Swiss mice are colonised with microbiota from BALB/c mice (Bercik et al., 2011a), further supporting the role of gut microbiota in gut-brain axis signalling.

3. Pathways of communication of the microbiota-gut-brain axis

The main pathways of communication between the GI tract and the CNS including the role of the gut microbiota and its' metabolites, the ENS, the gut epithelial barrier, immune signalling, HPA axis, and the vagus nerve are summarised in **Figure 4**. Here, the different routes of communication will be discussed.

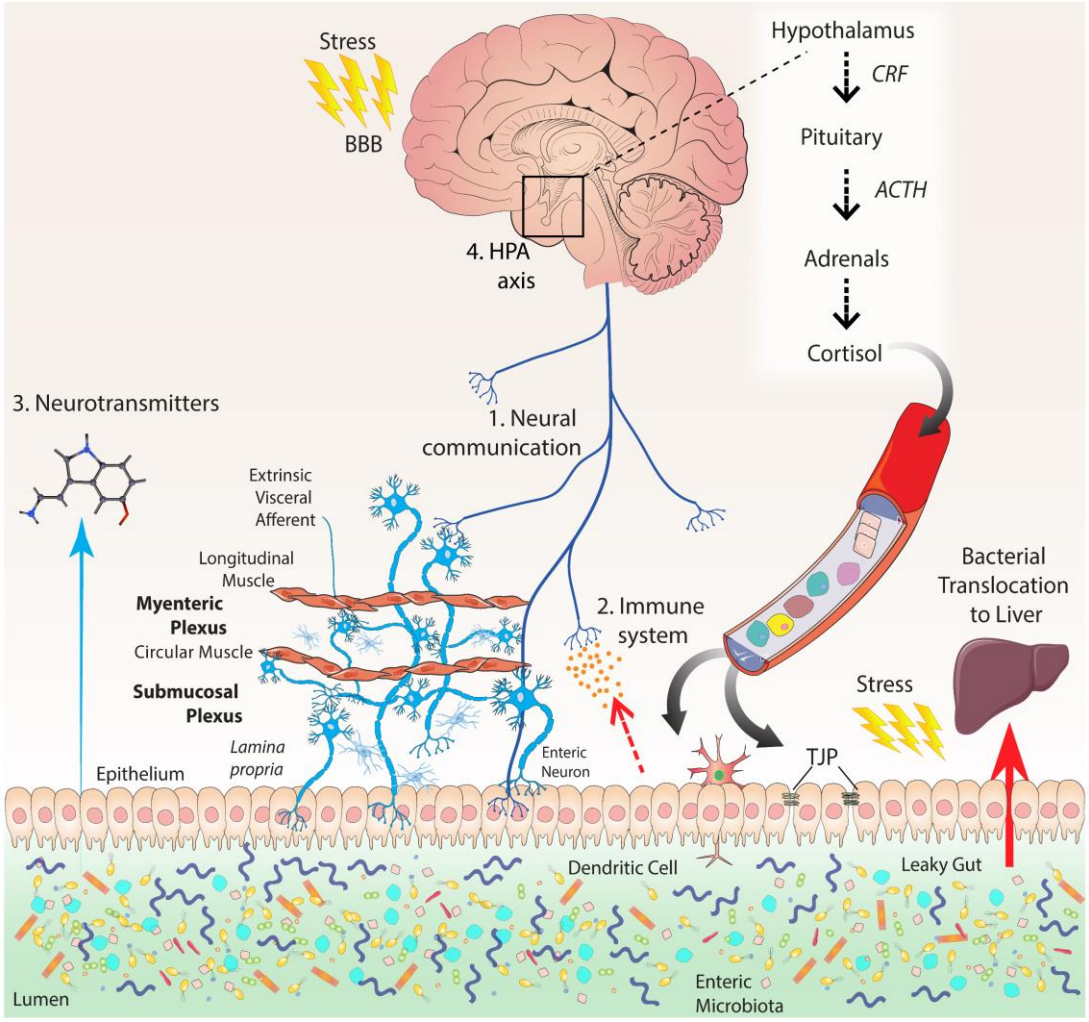


Figure 4. Routes of communication between the gut microbiota and the central nervous system including proposed humoural and neural routes through 1. Neural routes 2. Immune signalling 3. Release of neurotransmitters 4. HPA axis modulation. *Factors that affect gut barrier permeability are also outlined including stress and circulating corticosteroids, which may affect tight junction protein expression and function. The influence of the enteric nervous system on control of gastrointestinal function is also shown. ACTH–Adrenocorticotropic Hormone, BBB – Blood Brain Barrier, CRF–Corticotropin Releasing factor, HPA – Hypothalamic-Pituitary-Adrenal, TJP – Tight Junction Protein.*

3.1. Neural communications

One of the primary routes of communication between the gut and the brain is via the vagus nerve of the parasympathetic nervous system **(Figure 4 point 1)**. The vagus nerve is a mixed nerve composed of 80% afferent (sensory) and 20% efferent (motor) fibers. Afferent fibers of the vagus nerve innervate both the mucosal and smooth muscle layers of the intestine from the duodenum to the distal colon (Wang and Powley, 2000; Wang and Powley, 2007). The vagus nerve, through its afferent connections, can sense the local environment in the gut and send this information to the CNS where it may be integrated into the autonomic network before a response is generated (Bonaz et al., 2018). These local changes include detection of microbial metabolites produced by the gut microbiota and in this way, the microbiota exert influence over gut-brain axis signalling (Bonaz et al., 2018). The vagus nerve may be directly activated by SCFAs produced by enteric bacteria such as butyric acid (Breit et al., 2018). Evidence to support the major role of the vagus nerve in gut-brain communication includes that vagotomy prevents the beneficial *in vivo* effects of the bacteria *Lactobacillus rhamnosus* JB-1 seen in mice where a reduction in the CORT response to a stressor and a reduction in stress-induced depressive and anxiety-like behaviours is seen in normal mice treated with this bacteria (Bravo et al., 2011). Other studies have also supported the role of the vagus nerve in gut-brain axis signalling showing that the *in vivo* anxiolytic effects of *Lactobacillus rhamnosus JB-1* (Liu et al., 2021), and *Bifidobacterium longum* subsp. *longum* NCC3001 in a mouse model of colitis were absent in vagotomised mice, implicating the vagus nerve in gut-brain axis signalling (Bercik et al., 2011b).

The sympathetic nervous system is also involved in gut-brain communication. The majority of the viscera receive both parasympathetic and sympathetic innervation via

the vagus nerve and splanchnic nerves respectively each of which exert opposing effects. For example, parasympathetic innervation results in vasodilation and increased GI motility and secretion, whereas sympathetic innervation results in vasoconstriction and decreased GI motility and secretion (Breit et al., 2018).

It is generally accepted that pain signals are transmitted via spinal mechanisms including via the spinoreticular, spinomesencephalic, spinohypothalamic, and spinothalamic tracts (Millan, 2002). The spinoreticular tract projects to the dorsal reticular nucleus to regulate the affective component of pain, while the spinomesencephalic tract projects to the periaqueductal gray, with information regarding the intensity and location of the pain. Conversely, the spinohypothalamic tract links to the hypothalamus to coordinate affective and behavioural responses to pain. In pain perception, the thalamus acts as the gatekeeper for the many nociceptive and anti-nociceptive signals entering and leaving the CNS before being relayed to higher brain areas including the anterior cingulate cortex and somatosensory cortex (Cryan et al., 2019).

3.2. Immune modulation

Another key mediator in the modulation of gut to brain signalling is the immune system **(Figure 4 point 2)**. The distribution of immune cells in the gut is by far the densest in the body (Cryan et al., 2019), with these immune cells in continuous contact with the gut microbiota either by direct contact or via sensing released molecules. To prevent the mounting of an immune response, the mucous layer lining the GI tract limits contact between the enteric microbiota and the internal organs. It is at this mucosal barrier that the crosstalk between the immune system and the gut microbiota occurs. Epithelial PRRs monitor intestinal homeostasis and bacterial infiltration by

sensing microbe-associated molecular patterns, which once activated, mount an intestinal immune response (Duerkop et al., 2009).

The autonomic nervous system, with integration from the ENS and CNS, is responsible for the maintenance of physiological homeostasis. The autonomic nervous system, in concert with endocrine signalling of the HPA axis, may induce local alterations in the gut such as changes in motility, mucous production, permeability of the intestine, and the mucosal immune response (Mayer et al., 2015a).

The ENS lies at the interface between the microbiota along with the luminal content of the gut and the host and is primed to communicate with the microbiota either directly or indirectly **(Figure 4)**. One mechanism by which the microbiota effect change in GI physiology is via activation of PRRs such as TLRs. Several TLRs are expressed throughout the ENS and are activated by products and components of the microbiota (Hyland and Cryan, 2016). One such receptor is TLR4, which is expressed in myenteric plexi (Anitha et al., 2012). TLR4 has been shown to have importance in normal functioning of the gut and has been linked to proper development of the ileum, specifically in the development of normal ileal morphology (Caputi et al., 2017a). Interestingly, TLR4 is expressed in the nodose ganglion of the vagus nerve which can detect LPS, a component of gram-negative bacteria, further supporting the influence of the gut microbiota in gut to brain signalling (Hosoi et al., 2005). LPS activation of TLR4 and NF-κB has also been linked to increased enteric neuronal survival (Anitha et al., 2012). TLR4 is involved in colonic motility as seen by TLR4 knockdown or knockout which resulted in delayed motility (Anitha et al., 2012). This is also seen by blocking TLR4 signalling by means of introduction of a spontaneous mutation $TLR4^{lps\text{-}d}$ which mimics GF and antibiotic-induced colonic dysmotility by removing the sensitivity of the receptor to its ligand, LPS (Caputi et al., 2017a). The important

role of TLRs in the modulation of physiological processes is further reinforced in that TLR4$^{-/-}$ mice display decreased inhibitory signalling-mediated relaxation of the colon, delayed colonic motility, and decreased faecal pellet output associated with decreased inhibitory nNOS signalling (Anitha et al., 2012). Knockout of TLR2 also results in a decrease in the number of nNOS^{+} neurons in the myenteric ganglia (Brun et al., 2013). TLR4 has even been suggested to play a regulatory role in the communication between glia and neurons in the small intestine (Caputi et al., 2017a) and may thus be involved in gut signalling and normal gut function. Concluding from evidence above, it is clear that both immune-mediated TLR signalling and the gut microbiota are required for normal physiological function of the gut.

3.3. Enterochromaffin cells

Enterochromaffin cells (ECCs) play a vital role in communication between the gut and the brain and are of pivotal importance in the neuroendocrine system within the gut. ECCs are responsible for the regulation of secretion, motility, and absorption (O'Mahony et al., 2011) via production and release of neurotransmitters, namely 5-HT **(Figure 4 point 3)**. ECCs are innervated by vagal afferents and via this route, may effect change in both local enteric and central nervous systems (Lutgendorff et al., 2008) and may possibly play a role in control of the immune response (Yang and Lackner, 2004). ECCs produce 5-HT and along with the neurons of the ENS, store ~95% of this neurotransmitter (Kim and Camilleri, 2000) with all 5-HT in the blood being gut-borne (Toh, 1954). 5-HT may affect host physiology as it is central to the control of motility and secretion in the gut, and modulation of levels of 5-HT in the gut by the gut microbiota also affects host GI function (Yano et al., 2015).

Depending on the 5-HT receptor activated, the response to 5-HT differs. 5-HT is known to play a crucial role in control of gut motility (Martin et al., 2022). Treatments for constipation centre around agonism of the 5-HT_4 receptor whereas treatments for diarrhoea revolve around antagonism of the 5-HT_3 receptor, highlighting the major regulatory role of 5-HT in motility (Kendig and Grider, 2015). The gut microbiota plays an important role in the production of 5-HT in the gut (De Vadder et al., 2018). This is supported by the fact that GF mice, that have not been colonised by bacterial species and are sterile in microbiological terms, show a large decrease in the amount of 5-HT present in the plasma versus their conventional counterparts (Clarke et al., 2013; Hata et al., 2017; Wikoff et al., 2009). As the host is unable to synthesise tryptophan, the precursor of 5-HT, itself, the gut microbiota plays a vital role in the metabolism of tryptophan into bioactive molecules, including via the kynurenine pathway (for review see (Gheorghe et al., 2019)). Further, GF animals also show a reduction in tryptophan metabolism along the kynurenine pathway that is normalised following colonisation of the GI tract (Clarke et al., 2013). It is seen that following microbiota depletion using antibiotics, circulating 5-HT levels drop (De Vadder et al., 2018), thus reinforcing the role of the gut microbiota in tryptophan metabolism.

3.4. Stress reactions of the axis

The HPA axis, also referred to as the stress axis, is another main factor that influences gut-brain communication **(Figure 4, point 4)**. Under conditions of stress, CRF and arginine vasopressin (AVP) are released from the paraventricular nucleus of the hypothalamus which stimulates production and systemic release of ACTH from the anterior pituitary, resulting in glucocorticoid synthesis and release from the adrenal

glands, namely cortisol in humans, CORT in rodents. This cascade results in a physiological stress response and may be activated by the experience of stress. This endocrine signalling pathway is controlled at brain level by negative feedback of cortisol against CRF, AVP, and ACTH by acting on the glucocorticoid receptor when concentrations of cortisol are high. Studies in GF animals have outlined the regulatory role of the gut microbiota on HPA axis reactivity with studies reporting stress hyperreactivity in response to restraint stress in GF animals versus conventional controls (Clarke et al., 2013).

3.5. Mediators

Neurotransmitters are key signalling molecules in the gut-brain axis. Here, a brief overview of the neurotransmitters and SCFAs known to be involved in gut to brain communication are discussed.

5-HT is produced from its precursor tryptophan in the ECCs in the gut. 5-HT may affect GI motility, behaviour, secretomotor activity, as well as peristalsis (Huang and Wu, 2021). GF animals have also been shown to harbour less 5-HT in the colonic lumen and caecum compared to conventional animals, which was reversed following colonisation (Hata et al., 2017). Further, dysfunction of serotonergic signalling is associated with stress-related psychiatric disorders and IBS (Wilmes et al., 2021). The extensive function of 5-HT signalling in the gut-brain axis is described in section 3.3 above.

GABA is the major inhibitory neurotransmitter of the nervous system. GABA exerts effects on GI motility as well as enteric immunomodulation (Auteri et al., 2015). It has been shown that microbes including *Escherichia* spp. and *Lactobacillus* spp. can

synthesise GABA (Richard and Foster, 2003; Siragusa et al., 2007). To further support the role of GABA in gut-brain axis communication, GF animals have been shown to have less luminal GABA versus their conventional controls (Matsumoto et al., 2012).

Catecholaminergic neurotransmitters such as NA were some of the first neurotransmitter systems to be demonstrated to be involved in gut-brain communication. Interestingly, GF animals display higher levels of NA compared to their conventional controls (Kingsley et al., 1991). Further, NA and adrenaline have been shown to promote pathogenesis and growth of bacteria (Lyte et al., 2016).

Other major contributors to gut-brain communication are microbial metabolites such as SCFAs, the most abundant of which are butyrate, acetate, and propionate (Silva et al., 2020). SCFA's are produced following the fermentation of dietary fibers and are either absorbed by enterocytes by non-ionic diffusion or via transporter proteins such as the monocarboxylate transporter-1 (Tamai et al., 1995) and sodium-coupled monocarboxylate transporter-1 (Miyauchi et al., 2004) and are broken down along the Kreb's cycle, resulting in adenosine triphosphate as an energy source (Schönfeld and Wojtczak, 2016). SCFAs may act locally in the GI tract and in the periphery via activation of several G-protein-coupled receptors, the most studied being the free fatty acid receptors (FFAR) 2 and 3 (Dalile et al., 2019; Schlatterer et al., 2021). Interestingly, it has been reported that butyrate may affect ENS physiology with respect to cholinergic neuronal representation in myenteric neurons, suggesting a role for SCFA's in normal gut physiological function (Soret et al., 2010). SCFA's have also been shown to activate FFAR3 on the vagus nerve, resulting in increased activity in several brain areas (De Vadder et al., 2014).

4. Adverse early life events

Over eighty years ago, Hans Selye defined stress as "the non-specific response of the body to any demand for change". An individual's experience of stress differs hugely, so much so that one may be considered more resilient or susceptible to the effects of stress (Liu et al., 2018). While the individual response to stress exposure may vary, it is well-known that stress activates the HPA axis and results in the release of the stress hormone cortisol in humans (Dickerson and Kemeny, 2004) and CORT in rodents (Smith and Vale, 2006), which then goes on to effect behaviour.

Stress during the early stages of life, ranging from the *in utero* to early postnatal period may have drastic effects on both gut and brain development and later life function. For the purpose of this thesis, the impact of stress during both the prenatal and early postnatal periods on later life gut-brain communication will be investigated. Stress exists in numerous forms ranging from physical, inflammatory, and psychological stressors, to less often considered experiences such as mode of delivery and method of feeding. The range of factors that influence gut to brain communication from the prenatal period into adulthood is summarised in **Figure 2**.

4.1. Stress in the prenatal period

4.1.1. Effect of stress in the prenatal period on mothers

The prenatal period encompasses the time from conception until birth. The result of exposure to stress in the prenatal period is two-fold in that not only is the foetus experiencing this stress, but so too is the mother. Prenatal maternal stress may manifest as depression, anxiety, perceived stress, or any combination (Painter et al., 2012). Prenatal stress has been associated with several unfavourable outcomes, including

increased risk of preterm birth (Garcia-Flores et al., 2020; Lilliecreutz et al., 2016), delivering an infant with low birth weight (Fan et al., 2018; Khashan et al., 2014; Nkansah-Amankra et al., 2010), and preeclampsia (Maher et al., 2017), a complication of pregnancy characterised by heightened blood pressure. Throughout the course of pregnancy, maternal cortisol rises to up to 3-fold basal levels by the third trimester (Jung et al., 2011) and while glucocorticoids play a major role in foetal development, overexposure may have devastating effects on foetal development (Reynolds, 2013).

The wide-ranging impacts of stress on the mothers includes the development of stress-related psychiatric disorders such as depression and anxiety either during pregnancy or in the early postpartum period. It has also been suggested that a depressed mood may be one of the most common complications associated with childbirth, with an incidence of 10-20% in the first year following parturition (Reid and Taylor, 2015). Postpartum depression has also been shown to dysregulate the HPA axis (Zorn et al., 2017) and increase cortisol levels in the mothers in the postpartum period (Syam et al., 2021). Further, the levels of prolactin, a hormone involved in lactation, generally increase towards the end of pregnancy before dropping off to within normal range in the first 3 weeks postpartum. Prolactin also plays a role in the regulation of mood and maternal behaviour, with studies reporting that the elevated levels of prolactin during pregnancy reduced anxiety in the postpartum period in the mothers (Larsen and Grattan, 2012). Further to this, women experiencing prenatal depressive symptoms and possible pregnancy-related anxiety were less likely to plan to breastfeed their child, however this tendency did not translate to a lower incidence of breastfeeding (Fairlie et al., 2009). More recent studies have shown that breastfeeding rate is negatively affected by prenatal depression (Figueiredo et al., 2014), and that mothers who breastfeed have a lower incidence of postnatal depression (Pope and Mazmanian,

2016). However, the directionality of the changes in intention and initiation of breastfeeding is still under investigation.

Maternal experience of depression has also been reported to exert negative effects on maternal physical and mental health together with a lower overall quality of life. Further, these women also experienced difficulties with social relationships as well as a perceived lower social support and higher suicidal ideations (for review see (Slomian et al., 2019)). Prenatal stress also negatively impacts the maternal gut-brain axis and leads to alterations in the maternal gut microbiota and increased maternal GI permeability, which may lead to inflammation and alterations in foetal development (Jašarević et al., 2017). These disorders associated with stress in the perinatal period may then lead to negative outcomes in the offspring as described below.

4.1.2. Effect of stress in the prenatal period on offspring

The effects of prenatal stress on the foetus have also been investigated extensively. Heightened maternal levels of the steroid hormones glucocorticoids such as cortisol in humans may impact foetal development by crossing the placental barrier and impacting neurodevelopment and affecting foetal programming (Moisiadis and Matthews, 2014). Furthermore, decreased expression of 11β-hydroxysteroid dehydrogenase type-2, the glucocorticoid-inactivating enzyme expressed in the placenta, negatively impacts foetal development, resulting in foetal growth restriction (Cottrell et al., 2014). There is also preclinical evidence for altered development of the HPA axis in the offspring (Glover et al., 2010; Thayer et al., 2018). This same altered programming is also seen in humans whereby infants exposed to high levels of maternal cortisol displayed an exaggerated cortisol response to heel-prick (Davis et

al., 2011). It may also be seen that infants who were exposed to prenatal stress display behavioural problems and lower cognitive skills (Bergman et al., 2010; Berthelon et al., 2021; Lin et al., 2017). Prenatal maternal anxiety has also been shown to impact on neurodevelopment whereby those exposed to prenatal maternal stress during gestation display a decrease in grey matter density in the brain (Buss et al., 2010), as well as postnatal growth of the hippocampus (Qiu et al., 2013). Interestingly, long-term cortisol levels measured in maternal hair correlated with infant amygdala development in a sex-specific manner whereby structural changes were restricted to males, and connectivity changes were found in females (Stoye et al., 2020).

Under normal conditions, the placenta serves as a barrier to protect the foetus from high circulating levels of maternal glucocorticoids, however, this barrier has been shown to be compromised following reports of anxiety in the mother via reduction of 11β-hydroxysteroid dehydrogenase type-2 (O'Donnell et al., 2012), resulting in increased exposure of the foetus to high maternal glucocorticoids. Interestingly, glucocorticoid treatment may also be used to reduce the likelihood and severity of respiratory distress in neonates where there is a high risk of preterm birth (Agnew et al., 2018), highlighting the importance of temporality of glucocorticoid exposure on developmental outcomes.

4.2. Stress in the postnatal period

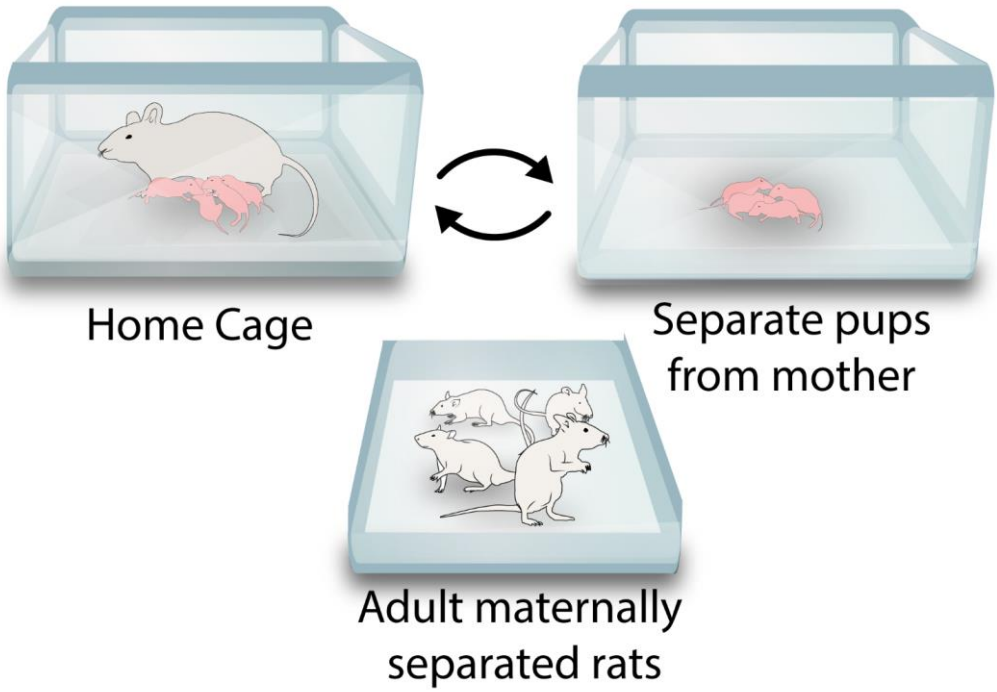
Microbes rapidly colonise the GI tract directly after birth and for the first year of life there is major maturation of both the gut and the bacterial species colonising it (Backhed et al., 2015). In this way, exposure to early life stress (ELS) during this developmental window may be particularly detrimental for the establishment of the various systems of the gut-brain axis as outlined below.

4.2.1. Maternal separation

4.2.1.1. Impacts on the gut microbiota

A well-established animal model of ELS is maternal separation (MS) and involves the separation of the pups (either singly or together) from their mother for a certain period in early life. MS has been shown to cause alterations in the gut microbiota (Cryan and O'Mahony, 2011; O'Mahony et al., 2011), as well as alter gut-brain axis signalling (O'Mahony et al., 2009) **(Figure 5)**. Alterations in faecal microbiota have also been seen following exposure to MS (O'Mahony et al., 2009; O'Mahony et al., 2020) as well as alterations in microbial diversity with an increased representation of *Prevotella* and *Flexibacter*, which have been reported to be associated with colitis (Pusceddu et al., 2015a). Interestingly, there appears to be a sex-specific effect of MS on microbiota composition as it is seen that in males, MS reduced the relative abundance of staphylococcus, yet increased that of streptococcus, *Gracilibacter*, and *Alkalibaculum*, whereas in females a decrease in *Barnesiella*, *Anaerovorax*, and *Mucispirillum* was noted (Park et al., 2021). *Mucispirillum* has been associated with inflammation in the GI tract and has been reported to be increased in colitis (Rooks et al., 2014) and this decrease in females may suggest that an inflammatory response in the gut is not induced. MS has also been shown to increase *Bacteroides* and decrease *Lachnospiraceae* (Park et al., 2021*)*. Further, another study in mice reported decreases in *Lachnospiraceae* and *Porphyromonadaceae*, while increases in *Bacteroides*, *Lactobacillus*, *Porphyromonas*, *Alloprevotella*, and *Firmicutes* were seen in males, only a decrease in *Mucispirillum* and *Lactobacillus* was noted in females (Rincel et al., 2019b). Other studies in rats have reported a decrease in *Fusicatenibacter* (Fukui et al., 2018*)*, which has also been shown to be decreased in patients with ulcerative colitis (Takeshita et al., 2016). Studies have also reported a decrease in beneficial

bacteria including *Lactobacillus* following MS in primates (Bailey and Coe, 1999) and rats (Murakami et al., 2017). However, the functional output of these specific changes in the gut microbiota induced by MS is as yet unclear, and only correlative conclusions may be drawn.


Home Cage
Separate pups from mother
Adult maternally separated rats

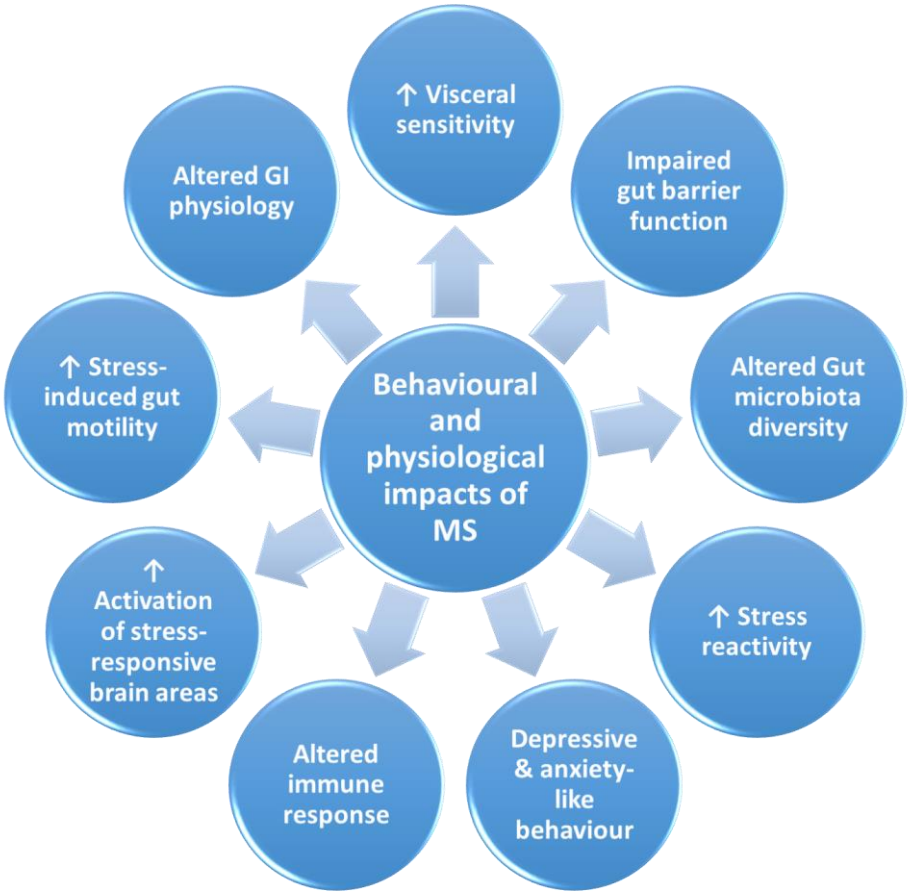

↑ Visceral sensitivity
Impaired gut barrier function
Altered Gut microbiota diversity
↑ Stress reactivity
Depressive & anxiety-like behaviour
Altered immune response
↑ Activation of stress-responsive brain areas
↑ Stress-induced gut motility
Altered GI physiology
Behavioural and physiological impacts of MS

Figure 5. Behavioural and physiological impacts of maternal separation. *These changes are resultant from a 3-hour separation from postnatal day 2-12 and include the induction of an anxio-depressive phenotype, alterations in stress reactivity, increased visceral sensitivity and activation of stress-responsive brain areas, and alterations in the immune response. This phenotype is evident through adulthood. GI = Gastrointestinal; MS = Maternal separation; ↑ indicates an increase; ↓ indicates a decrease.*

4.2.1.2. Impacts on physiology and behaviour

The effects of MS on offspring behaviour are quite well characterised (for review see (Rincel and Darnaudéry, 2020; Wang et al., 2020)) in rats, however, the effects of MS in mice are not clear as studies consistently report opposite findings, or no effect of MS in their mouse model (Savignac et al., 2011; Tractenberg et al., 2016). For the purpose of this review of the literature, the existing MS literature has been broadly split into brief (≤15 mins) and prolonged (>1 hr) separation paradigms, and the latter is discussed in detail here.

The effects of prolonged MS are generally agreed upon in the literature **(see Figure 5)**. MS (3 hrs per day from PND2 to 12) resulted in decreased locomotor activity and induced deficits in spatial learning as measured by the Morris water maze (Berg et al., 2015). This same paradigm has also been seen to induce an exaggerated stress (CORT) response and increased anxiety-like behaviour as measured by the elevated plus maze in rats (Huot et al., 2001). Prolonged MS (6 hours per day from PND 2 to 15) led to increased serum levels of CORT and also immunological alterations along with depressive and anxiety-like behaviours into adulthood (Roque et al., 2014). Another study in rats using a 6-hour separation reported increased anxiety-like behaviour with accompanying activation of neural circuits relevant to anxiety (Troakes and Ingram, 2009). SPF mice that underwent prolonged MS (3 hours per day from PND1-21) had significantly higher plasma CORT levels when compared to control mice (De Palma

et al., 2015). PND 9 pups that had undergone prolonged MS (3 hours per day from PND2-8) also displayed an exaggerated stress response in response to a restraint stress (Kuhn and Schanberg, 1998). 3-hour MS has also been shown to increase the activation of stress-responsive brain regions including the paraventricular nucleus, medial amygdaloid nucleus, and supraoptic nucleus (Fóscolo et al., 2022). However, studies have also shown no change or decreased anxiety and depressive-like behaviours in C57BL/6 mice following prolonged MS (3 hours per day from PND1-14) (Savignac et al., 2011), further supporting that MS in mice is not as reproducible as in rats.

Another parameter that is altered following MS is pain sensitivity. Sensitivity to pain is increased in adulthood following early life exposure to prolonged MS (3hrs per day from PND2-15) (Vilela et al., 2017). MS for 3 hours per day from PND2-14 resulted in visceral hypersensitivity from the post-weaning period, which persisted into adulthood (Yi et al., 2017). Visceral hypersensitivity resulting from a 3 hour per day separation from PND2-12 has also been noted (Collins et al., 2022; O'Mahony et al., 2009; O'Mahony et al., 2020). It may therefore be posited that exposure to ELS, such as MS, may sensitise specific areas of the CNS such as the amygdala, cingulate cortex, and basal ganglia which have been identified as having altered activation patterns in IBS patients (Bonaz et al., 2002) and may be implicated in the pathophysiology of visceral hypersensitivity (O'Mahony et al., 2009). MS also exerts effects on nociceptive circuitry. MS results in increased excitability of afferent neurons in the DRG and increased activity of the superficial and deeper layers of the spinal cord as well as altering the activity of the rostroventral medulla, periaqueductal gray, and hypothalamus which are involved in descending control of pain (Melchior et al., 2021). Interestingly, ELS in the form of limited nesting material affects the activation

of specific brain regions involved in pain perception and processing including the somatosensory, insular, cingulate and prefrontal cortices, periaqueductal gray, and thalamus (Holschneider et al., 2016), suggesting that other forms of ELS such as MS may also alter this pain circuit.

Exposure to ELS also has detrimental effects on the CNS. It is seen that Wistar rats that underwent MS for 3 hours per day from PND1-10 display higher activation of the amygdala following contextual fear conditioning in adulthood (Diehl et al., 2014). MS (1 hour per day from PND2-9) has also been shown to have an impact upon hippocampal neuroplasticity whereby induction and length of hippocampal long term potentiation was increased (Kehoe and Bronzino, 1999), which is the neural correlate of learning and memory (Bliss and Collingridge, 1993). Several studies have shown that MS results in deficits in cognition and different types of memory (Hulshof et al., 2011; McVey Neufeld et al., 2020). Sprague Dawley rats separated for 3 hours per day for the first two weeks of life were also found to have decreased hippocampal levels of 5-HT (Lee et al., 2007). From the above studies, it is clear that prolonged MS has deleterious effects on neurochemistry possibly relating to the increased anxiety and depressive-like behaviours seen following MS.

MS has also been shown to result in various dysfunctions of the GI system (Pohl et al., 2015). It has been reported that MS results in alterations in GI motility including delayed gastric emptying and increased colonic motility at baseline as well as in response to a subsequent stressor (Babygirija et al., 2012; Bülbül et al., 2012; O'Mahony et al., 2009). MS has also been shown to result in an increase in the number of enteric cholinergic neurons (Gareau et al., 2007a) as well as an increase in gut epithelial barrier permeability (Barreau et al., 2007; Moussaoui et al., 2017; O'Mahony et al., 2011). Interestingly, an increase in the number of ECCs as well as

in circulating and colonic 5-HT is seen in MS animals (Bian et al., 2010), which is also seen in patients with IBS. A decrease in the number of Paneth cell as well as endocrine cell hyperplasia is also seen following MS (Estienne et al., 2010). Alterations in gut motility are also seen following MS with alternating bouts of diarrhoea and constipation seen from pre-adolescence to adulthood (Yi et al., 2017).

Not only are deficits seen in rodent pups exposed to MS, but deficits are also seen in human children. In a population of Romanian children from the 1980's to 1990's who were exposed to separation from their parents and psychosocial deprivation by being raised in substandard orphanages, several social and cognitive deficits were observed such as quasi-autism, cognitive impairment, and inattention or hyperactivity (Gunnar, 2010; Rutter et al., 2007). Another form of ELS is childhood sexual abuse which has an occurrence rate of 20% in females and 5-10% in males (Runyan et al., 2002) which results in the development of psychiatric disorders such as post-traumatic stress disorder, anxiety and panic, and poorer physical health including abdominal pain and GI complaints (Leserman, 2005). This type of ELS also has further long-lasting psychological outcomes such as increased suicidal tendencies, self-harm, and cognitive impairment (Runyan et al., 2002). Childhood sexual abuse also increases HPA axis activity (Charmandari et al., 2003). Reasoning behind the cognitive deficits induced by ELS may lie in the fact that early life exposure to a sustained stressor, such as childhood sexual abuse or MS, results in hyperactivation of the HPA axis leading to hyperfunction of the fear centre of the brain, the amygdala, accompanied by decreased activation of the hippocampus which is responsible for learning, cognition, and negative feedback of glucocorticoids. Suppression of growth may also be seen due to suppression of growth hormone by the HPA axis (Charmandari et al., 2003).

Considering all the evidence on the effect of MS on offspring, is it clear that there are several factors that must be considered when elucidating the effect of MS on gut-brain communication and physiology such as genetic strain, length of separation, and life stage at time of separation. Evidence above suggests that prolonged MS is sufficient to induce a lasting depressive and anxiety-like phenotype into adulthood with an exaggerated CORT response to stressors.

4.2.1.2.1. Impact of maternal care in early postnatal life

The mother-child dyad is of paramount importance, in particular, in early life. Rodent pups are dependent upon their mother as paternal input is minimal and pups are therefore heavily affected by changes in quality of maternal care (Franklin et al., 2012). Of course, there are inter-individual differences in the quality of maternal care seen in both rodents and humans whereby good maternal care, including provision of a safe environment and reliability of care, has been linked with a resilience to exposure to stress (Champagne et al., 2003; Jaffee, 2007). Conversely, poor maternal care, including neglect and abuse, exacerbates susceptibility to stress in later life (Henningsen et al., 2012) and predisposes to development of mood disorders and behavioural problems in later life as well as a disruption in cognitive development including language abilities at 18 months (Jaffee, 2007). Disruption of this relationship between mother and child can lead to alterations in behaviour and even lead to the manifestation of stress-related psychiatric disorders later in life. Interestingly, the cortisol and pain responses of infants undergoing the heel prick test was reduced by exposure of the infant to the odour of their own mother's milk (Nishitani et al., 2009), further highlighting the importance of the mother-child dyad. Several studies have shown the importance of this early life association between mother and child both in

rodents and in humans. Interestingly, the stress hyporesponsive period (SHRP) seen in rodents between PND4 and 14 whereby a decreased response to stressors and downregulated HPA axis activity is observed, is dependent upon the presence of the mother as separation from the mother for 24 hours elicited an increase in CORT levels in the pups, even when housed with their littermates (Cirulli et al., 1992). This is of importance for the use of the MS model, as one of the most often used paradigms involves daily separation from PND2-12, during the SHRP. It is also observed that maternal input such as licking and grooming is required for the upkeep of the SHRP (Levine, 2002). However, there is no correlate of the SHRP seen in mammals which may question the translatability of investigating this factor in a murine model.

In terms of further relevance to MS, maternal behaviour has been shown to modulate the ultrasonic vocalisations of pups exposed to a single isolation in both control and MS animals. Of interest, higher maternal care was associated with lower frequency calls in control pups and higher frequency calls in MS animals which may be associated with distress (Kaidbey et al., 2019). It has also been shown that MS impacts heavily on maternal care with studies reporting an increase in licking and grooming as well as arched-back nursing (for review see (Orso et al., 2019)). Overall, MS impacts on maternal care, generally resulting in increased care using the prolonged MS paradigm.

4.2.2. Weaning – a critical time period

Weaning is a critical timepoint that sees a change in diet from the mother's milk to solid food, and in animals, a change in housing conditions whereby they are permanently separated from their mother. This period of change is usually also accompanied by a decrease in voluntary food take as the animal adapts to the new

solid diet. The age at which weaning occurs has long lasting effects on physiology. Appropriate weaning has been shown to increase gut barrier permeability which is gradually reduced over the next two weeks post-weaning (Moeser et al., 2007). The GI immune system is also activated due to weaning in response to the vast expansion of the gut microbiota (Al Nabhani et al., 2019). Weaning has also been associated with an increase in the intestinal expression of pro-inflammatory cytokines (Pié et al., 2004).

It has been shown that precocious weaning results in a litany of physiological alterations (Campbell et al., 2013). Mice that were weaned early displayed a predisposition to stress-related psychiatric disorders such as anxiety and a dysregulated CORT response to a stressor in later life (Kikusui et al., 2019). Early weaning stress has also been shown to impact heavily upon GI physiology (Zheng et al., 2021), resulting in impaired mucosal barrier function in pigs (Smith et al., 2009), deeper crypts, lower villus/crypt ratio, and smaller villus area which is normalised before adulthood in rats (Crispel et al., 2019), leading to an impaired capability for absorption. Further, early weaning is associated with impaired gut barrier function (Hu et al., 2013a). Precocious weaning also exerts effects on the ENS whereby enteric neuronal numbers did not decline with age as was seen with late weaned controls and was associated with increased cholinergic activity (Medland et al., 2016). Alterations in cholinergic activity have been linked to immune responsivity (Dhawan et al., 2012), epithelial barrier function, and secretory diarrhoea (Hirota and McKay, 2006). Thus, weaning is an important sensitive period which has long lasting ramifications on host physiology and health.

5. Disorders of early life stress

The idea that perturbations in the gut microbiota may be a causative factor in the development of acute and chronic diseases both in the brain and the gut has gathered momentum in recent years. Several studies have implicated the gut microbiota in functional and psychiatric disorders (Foster and McVey Neufeld, 2013; Hyland and Cryan, 2016). Here, ELS-related disorders will be discussed.

5.1. Irritable bowel syndrome

IBS is one of the most prevalent disorders of gut-brain axis interactions and now accounts for 20-50% of the GI workload worldwide (Sperber et al., 2021) and has an estimated worldwide prevalence of 20% (Lovell and Ford, 2012a). IBS displays a female predominance with symptom onset occurring as early as age 35 (Maxwell et al., 1997) and is characterised by abdominal pain and/or bloating, altered bowel habits, and abdominal distension (Mearin et al., 2016). IBS is currently diagnosed using the Rome IV criteria (Palsson et al., 2016) and although several factors including (epi)genetic predisposition, immunological involvement, alterations in gut microbiota, inflammatory agents, stress, and neuropsychiatric disorders have been suggested to play a role in the onset of this disorder of gut-brain axis interactions, the aetiology of IBS is not clearly understood (Bellini et al., 2014). IBS is thought to reflect altered gut-brain axis homeostasis, and as such may be classified as a microbiota-gut-brain axis disorder (Mayer et al., 2015b; O'Mahony et al., 2017; Wilmes et al., 2021).

Exposure to stress, particularly in early life, has been shown to induce gut dysbiosis and has also been flagged as a risk factor for the development of IBS in later life (Drossman, 2011; O'Mahony et al., 2017). In patients with IBS who have a history of

ELS, it was found that the cortisol response to sigmoidoscopy was higher than that of patients without IBS, and that a faster speed of return to baseline cortisol correlated with a lower symptom severity and higher quality of life (Videlock et al., 2009). Patients with IBS have also been shown to display altered cognition (Kennedy et al., 2015; Kennedy et al., 2014c) and heightened response to an acute stressor (Kennedy et al., 2014b). Numerous classifications of IBS exist based on bowel movement type including IBS-diarrhoea predominant, IBS-constipation predominant, IBS-mixed stool pattern, and IBS-unclassified (Mearin et al., 2016). Another subset of IBS patients include those who present with psychiatric comorbidities, namely anxiety or depression which includes 44% and 25% of IBS patients respectively (Midenfjord et al., 2019).

5.2. Stress-related psychiatric disorders

It has been observed that up to 60% of patients presenting with depression or anxiety also report GI issues (Liu and Zhu, 2018). Although the precise cause of IBS is still unclear, a role for the gut microbiota in the apparition of this disorder is emerging.

The gut microbiota also appears to play a role in the manifestation of stress-related psychiatric disorders such as depression as a depressive phenotype may be conferred by use of faecal microbiota transplantation (FMT) from a depressed patient to a rodent (Kelly et al., 2016b). Several theories exist attempting to elucidate the method by which alterations in the gut microbiota may be related to the manifestation of disease. One such school of thought is the leaky gut hypothesis. This hypothesis identifies a breakdown in gut barrier function leading to bacterial translocation **(see Figure 6)** causing inflammation and is thought to contribute to the apparition of some psychiatric

disorders (Kelly et al., 2015). Not only has impaired gut barrier function been implicated as a causative factor, but due to translocation of bacteria from the gut, LPS may enter the brain due to increased BBB permeability, leading to neuroinflammation (Kelly et al., 2015; Smythies and Smythies, 2014). **Figure 6** depicts the effect of stress on gut and brain function.

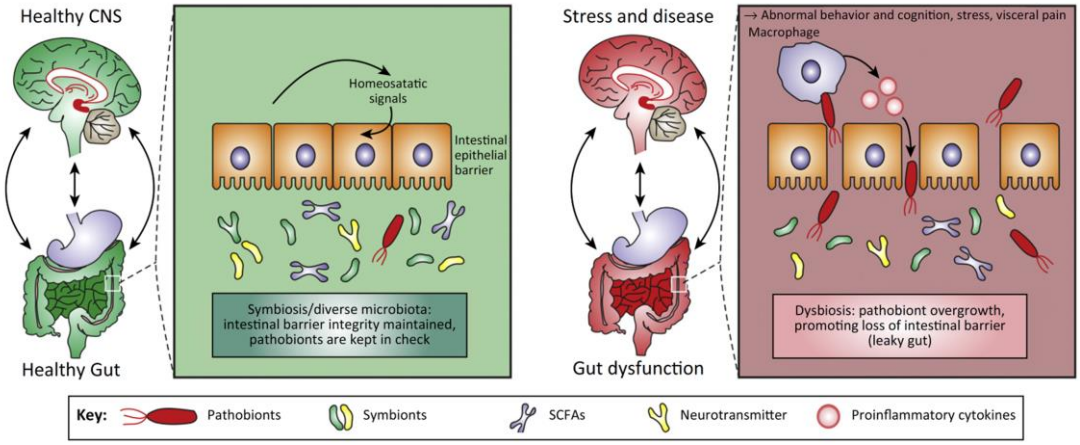


Figure 6. Gut-brain axis crosstalk under normal and diseased conditions. *On the left, normal gut barrier function and symbiosis is seen whereas on the right a "leaky" gut is seen following exposure to stress or disease which may trigger an immune response along with intestinal dysbiosis resulting in inflammation. CNS–Central Nervous System, SCFA–Short chain fatty acids. From (Borre et al., 2014).*

5.2.1. Biomarkers for stress-related psychiatric disorders

Stress-related psychiatric disorders including depression and anxiety disorders affect approximately 10% of the global population each year (Craske et al., 2017; Otte et al., 2016). There is a clinical need to identify biomarkers for these disorders to aid in earlier diagnosis and treatment for patients. In recent years, these potential biomarkers have been classified into diagnostic, monitoring, pharmacodynamic, predictive, prognostic, and safety depending on their application (Califf, 2018). Biomarkers identified for stress include salivary cortisol (Pearlmutter et al., 2020), catecholamines, interleukin (IL)-6 and 8, and c-reactive protein (CRP) (reviewed by (Noushad et al., 2021)).

5.2.1.1. Biomarkers for anxiety disorders

Some biomarkers for anxiety-related disorders have been identified. As anxiety disorders can affect the immune system, some immune biomarkers have been proposed. These include immunoglobulins such as IgA, where a strong association between perceived stress and anxiety and low salivary IgA has been observed (Engeland et al., 2016). CRP, a marker of inflammation has also been shown to be higher in the blood of males with anxiety disorders (Vogelzangs et al., 2013). Genetic biomarkers for anxiety have received less research attention, however the gene for monoamine oxidase A has been suggested to play a role in the aetiology of generalised anxiety disorder (Tadic et al., 2003). There is also some evidence to support a link between a polymorphism in the gene for brain-derived neurotrophic factor and generalised anxiety disorder (Moreira et al., 2015), however, whether these genetic alterations may be useful as biomarkers is still a topic of debate in research, with some studies reporting no association (Wang et al., 2015). Although, currently the most well-known biomarker for anxiety is the brain-derived neurotrophic factor gene, which is linked to alterations in deoxyribonucleic acid (DNA) methylation (Doherty et al., 2016; Miao et al., 2020). Some research into epigenetic biomarkers for anxiety disorders has also reported promising results. A study by Cerveira de Baumont and colleagues reported that telomere length in adolescents with persistent anxiety did not change over time, suggesting a delay in neuronal development (Cerveira de Baumont et al., 2021). Epigenetic mechanisms related to anxiety disorders include DNA methylation and histone modifications (Lin and Tsai, 2020).

5.2.1.2. Biomarkers for depression

Suggested biomarkers for depression again highlight an immune role in stress-related psychiatric disorders. CRP and IL-6 have been reported to be heightened in depression (Haapakoski et al., 2015). Not surprisingly, those with depression also display hypercortisolaemia (Stetler and Miller, 2011). Genetic biomarkers for depression include a noted decrease in 5-HT_{1A} messenger ribonucleic acid (mRNA) levels in the hippocampus and prefrontal cortex of patients with major depressive disorder (López-Figueroa et al., 2004). Epigenetic factors, that is the interplay between environment and genes, including histone modification and DNA methylation resulting in a decrease in brain-derived neurotrophic factor in the brain have also been suggested as biomarkers for depression (Roth et al., 2009; Tsankova et al., 2006). It has also been suggested that epigenetic modulation by early life adversity in patients with depression resulting in hypermethylation of the 5-HT transporter gene may also prove a useful biomarker for depression (Kang et al., 2013). Depression has also been linked with alterations in the gut microbiota. Surprisingly, patients with major depressive disorder have been shown to have a greater α-diversity versus healthy control patients (Jiang et al., 2015). Conversely, a decreased species richness and α-diversity in depressed patients has also been reported (Kelly et al., 2016b). A decrease in the amount of *Bifidobacterium* and *Lactobacillus*, thought to have a beneficial effect against stress and depressive disorders, in patients with major depressive disorder was found by Aizawa and colleagues (Aizawa et al., 2016). Patients with bipolar disorder present with a significantly altered gut microbiota composition, namely a decreased representation of *Faecalibacterium*, versus healthy controls (Evans et al., 2017). Interestingly, in the same study, Evans et al. correlated the relative amount of *Faecalibacterium* with better health as scored using several self-reporting

questionnaires. Conversely, no significant differences in species richness between depressed patients and control subjects were found by another study (Naseribafrouei et al., 2014). A variation in the microbiome between depressed patients and healthy controls is also seen and most notably, in the absence of gut microbiota, i.e. in a GF condition, depressive symptomatology develops (Zheng et al., 2016).

5.3. Irritable bowel syndrome with psychiatric comorbidities

IBS is a double-edged sword in that not only may one experience the functional GI disturbances associated with the manifestation of IBS symptoms, but a comorbidity with psychiatric disorders such as depression and anxiety may also be seen (Foster et al., 2017; Wilmes et al., 2021). A vicious cycle then ensues as mood disorders such as depression and anxiety may worsen the severity of IBS, which may in turn exacerbate the intensity of the comorbid mood disorders. Importantly, the severity of GI symptoms has been associated with the presence of a comorbid psychiatric disorder, highlighting the inextricable link between psychiatric disorders and IBS symptom severity (Stasi et al., 2019). The global incidence of anxiety and depressive disorders ranges from 7 to 10% (Baxter et al., 2013; Craske, 1999; Otte et al., 2016) and these disorders are still diagnosed based on symptoms which, when comorbid with IBS, make clinical management difficult given the suboptimal treatment options (Clarke, 2020). In recent years, a role for the gut-brain axis in neuropsychiatric disorders has gained traction. A causal role for the gut microbiota in mood disorders has also been suggested whereby FMT from patients with depression to rodents conferred depressive-like symptoms (Kelly et al., 2016b; Knudsen et al., 2021). Given the impact of IBS on quality of life, there is an apparent need for more effective treatments.

5.4. Visceral pain as a hallmark of IBS

Abdominal pain is the cardinal symptom of IBS. Heightened sensitivity to pain in the abdominal region is termed visceral hypersensitivity and may be broken down into 2 constituent elements: (i) allodynia, the perception of a non-noxious stimulus as painful and (ii) hyperalgesia, an augmented response to pain. There is a significant relationship between female sex and development of IBS symptomatology, including visceral hypersensitivity (Adeyemo et al., 2010; Lovell and Ford, 2012b). As a hallmark of functional GI disorders (Enck et al., 2016), the presence of visceral hypersensitivity is used as a major diagnostic criterion for IBS as epidemiological reports show that between 30 and 90% of those with IBS display visceral hypersensitivity (Bouin et al., 2002; Ludidi et al., 2012; Posserud et al., 2007; van der Veek et al., 2008). A recent review has discussed the pathophysiology of visceral pain and potential treatments in great detail (Lucarini et al., 2020a).

5.4.1. Ascending and descending pathways of visceral pain

Perception of visceral pain beings at extrinsic nociceptors with cell bodies located in the DRG and nerve endings throughout the wall of the GI tract. These nociceptors are capable of sensing and responding to pH, stretch or distension, microbial metabolites, inflammation, and neurotransmitters released by the ENS (Sengupta, 2009). Receptors such as the transient receptor potential cation channel subfamily V member 1 (TRPV1) expressed on these neurons detect signals such as histamine, substance P, acetylcholine, as well as changes in pH. The nociceptive signal travels to the dorsal horn of the spinal cord and via the release of molecules including substance P, glutamate, vasoactive intestinal peptide, and somatostatin, the signal is transmitted to the contralateral side of the spinal cord. From here, depending on where the cell body

is located, the nociceptive signal travels along either the ascending spinothalamic (originating in the deep dorsal horn) or spinoparabrachial (originating in the superficial dorsal horn) tract (involved in sensory and affective aspects of pain respectively) to reach the brain (Hunt and Mantyh, 2001). Once this signal reaches the brain, specifically the thalamus for the spinothalamic tract, it is sent to cortical and limbic areas including the somatosensory cortex and anterior cingulate cortex for localisation and intensity grading. For the spinoparabrachial tract, this signal reaches the parabrachial nuclei and is sent to the amygdala and hypothalamus for affective processing. Once processed in these structures, an inhibitory or facilitatory response will be engaged. The descending inhibitory pathway is then activated either resulting in the release of inhibitory neurotransmitters in the dorsal horn of the spinal cord, resulting in inhibition of these nociceptive signals, or the signal is facilitated. The regulation of this response is mediated by the periaqueductal gray in connection with the dorsal horn of the spinal cord via the rostroventral medulla and receives input from higher brain structures including the anterior cingulate cortex, nucleus tractus solitarius, and the hypothalamus and mainly involves the release of 5-HT, opioids, cannabinoids, or NA. Repeated activation of these nociceptors may lead to chronic visceral pain via central sensitisation, involving increased excitability of spinal cord and higher order neurons, or peripheral sensitisation of the visceral nociceptors (Moloney et al., 2016a; Moshiree et al., 2006; Sikandar and Dickenson, 2012). The ascending and descending pathways of visceral pain modulation are summarised in **Figure 7**.

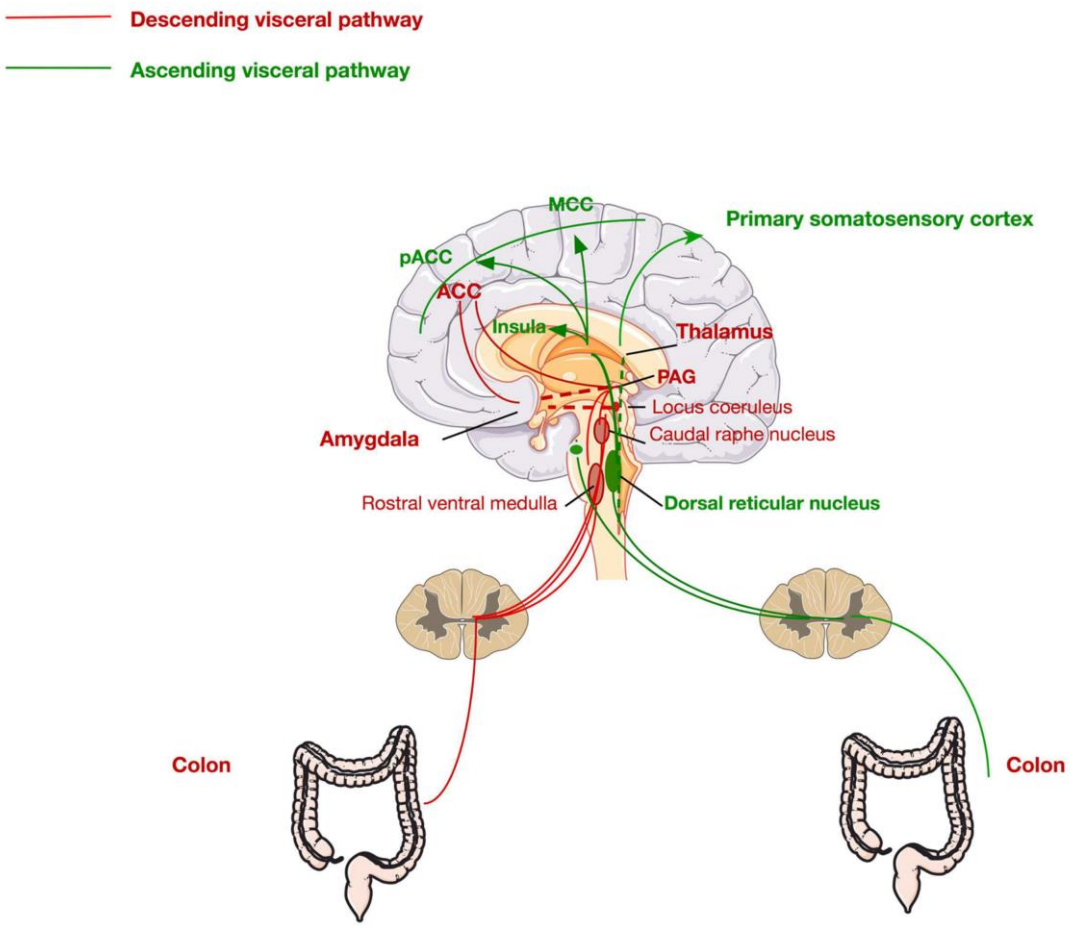


Figure 7. Ascending and descending pathways of visceral pain modulation. *The ascending pathway for visceral pain perception from the periphery through the dorsal root ganglia via the dorsal reticular nucleus to the primary somatosensory cortex, insula, pregenual anterior cingulate cortex (pACC), and the midcingulate cortex (MCC). The descending pathway is mediated via signals from the ACC, thalamus, and amygdala to the periaqueductal gray (PAG), locus coeruleus, and raphe nucleus, returning via the rostral ventral medulla to the colon. From (Moloney et al., 2015a).*

5.4.2. Assessment of visceral sensitivity

In a laboratory setting, visceral sensitivity may be measured by quantifying the response to colorectal distension (CRD) which involves the controlled inflation of a balloon in the colorectal region to a given pressure and is commonly used both clinically (Camilleri, 2002; J. van der Schaar, 1999) and preclinically (O'Mahony et al., 2012) for visceral sensitivity assessment. This can be achieved in several ways including measurement of the threshold pressure, cumulative number of pain behaviours, or electromyographic recordings in response to the distension.

5.4.2.1. Electromyography

Electromyography is considered a more objective measure of visceral sensitivity as it is used to quantify the abdominal contractions in response to a noxious colonic stimulus such as CRD via electrodes implanted into the muscles of the abdominal wall and is not subject to observer bias or unclear pain behaviours. This technique has been used extensively in the literature as a readout of visceral pain (Kogure et al., 2020; Lucarini et al., 2020b; Parisio et al., 2019).

5.4.2.2. Pain threshold and cumulative number of pain behaviours

Another often-used method of quantification of visceral sensitivity involves recording the threshold pressure, or the pressure at which the first pain behaviour is displayed. It has been shown experimentally that a lower pain threshold to colonic distention is indicative of visceral hypersensitivity. Similarly, the cumulative number of pain behaviours displayed across the distension protocol may be used to assess visceral sensitivity whereby a higher total numbers of pain behaviours is indicative of visceral hypersensitivity. The number of pain behaviours may also be represented per pressure gradient, whereby it is seen that as distension pressure increases, so too does the number of pain behaviours (Yang et al., 2006).

5.4.2.3. Verbal reporting

In human patients with IBS, verbal reporting of pain in response to distension of the colorectal region has proven to be an effective and reliable measure of visceral sensitivity (Keszthelyi et al., 2012). However, this technique in humans also has its pitfalls as it is vulnerable to psychological factors including anxiety and heightened

vigilance seen in IBS patients, which may lead to reports of pain, rather than true visceral hypersensitivity (Dorn et al., 2007). Verbal reporting is not suitable for use preclinically for reasons that are readily apparent.

5.4.3. Factors modulating the visceral pain response

Given its' complex nature, several factors have been suggested to play a role in the pathophysiology of visceral hypersensitivity including: (i) gut microbiota (ii) sex hormones and (iii) neurotransmitters. Similarly, many of the factors that feed into the pathophysiology of visceral pain may be seen as modulators of the visceral pain response.

5.4.3.1. The gut microbiota

The gut microbiota has been shown to be a major modulator of the visceral pain response. In a study by Luczynski et al, it was seen that male mice devoid of any microbiota (GF) are spontaneously viscerally hypersensitive, and this was reversed by colonisation by conventional microbiota (Luczynski et al., 2017). This observation was sex specific as it is seen that female GF mice do not display visceral hypersensitivity (Tramullas et al., 2021). It has also been seen that FMT from a viscerally hypersensitive patient to a mouse resulted in visceral hypersensitivity, highlighting that the gut microbiota exerts a major role in visceral hypersensitivity (Crouzet et al., 2013). Further, visceral pain has also been reported following antibiotic depletion of the gut microbiota (O'Mahony et al., 2014). The increasing emphasis of the gut microbiota in visceral sensitivity has been reported extensively in the literature (Defaye et al., 2020; Rea et al., 2019; Theodorou et al., 2014).

5.4.3.2. Sex hormones

Female sex hormones, namely oestrogen, have been shown to exert modulatory effects on visceral pain perception. The oestrous cycle in rodents mirrors the menstrual cycle in humans, but only last between 4-5 days and consists of four distinct phases; (i) proestrus (ii) estrus (iii) metestrus (iv) diestrus (Byers et al., 2012b). Levels of oestrogen fluctuate across the oestrous cycle, with highest levels present during proestrus and lower levels seen during diestrus (Hong and Choi, 2018). It has been shown that visceral sensitivity varies across the oestrous cycle (Moloney et al., 2016b). However, there is currently no consensus as to the specific changes induced by oestrogen on visceral pain perception. Intriguingly, female GF mice do not display baseline visceral hypersensitivity seen in male GF mice (Luczynski et al., 2017) and are insensitive to ovariectomy-induced visceral pain. Further, alterations in visceral sensitivity across the oestrous cycle were seen in their conventional counterparts, and ovariectomy-induced visceral pain was reversed by exogenous oestradiol administration (Tramullas et al., 2021), highlighting the role of female sex hormones on visceral pain perception. This is also of relevance given the female predominance of IBS. The dichotomy in results of the anti- versus pro-nociceptive properties of oestrogen has been reviewed extensively (Sun et al., 2019).

Clinically, it has been seen that women experience heightened pain, and sensitivity to experimentally-induced pain versus men (Paller et al., 2009). This topic has been extensively reviewed in the literature (Mogil, 2018; Sorge and Strath, 2018; Templeton, 2020), however, it should also be known that there is a sex bias in the reporting of studies showing a male predominance (Mogil, 2020), leading to a gap in knowledge surrounding pain management in females. Similarly, the amplitude of the visceromotor response revealed that female maternally separated rats display

heightened visceral sensitivity versus males (Yi et al., 2017). Interestingly, intrathecal administration of antisense oligodeoxynucleotides designed to decrease expression of glucocorticoid receptors alleviated Paclitaxel (a chemotherapy drug)-induced mechanical hyperalgesia in males, but not females. Moreover, intrathecal administration of antisense oligodeoxynucleotides designed to reduce expression of β2-adrenoceptor (AR) on nociceptors attenuated mechanical nociception to a greater degree in females (Ferrari et al., 2020). Some of these sex differences in perception of pain may be attributed to circulating female sex hormones, which has been discussed above. Further, sex differences in central regulation of pain also exist. For instance, neurons of the ventrolateral periaqueductal gray-dorsal raphe differentially and sex-dependently regulate pain behaviours via connections to the bed nucleus of the stria terminalis in mice (Yu et al., 2021a). Brain imaging studies have also reported differential sex-dependent activation in brain regions known to play a role in pain processing including the anterior cingulate, insular, and medial prefrontal cortices (Traub and Ji, 2013). Specifically in response to visceral stimuli, higher activation of the ventromedial prefrontal cortex, right anterior cingulate cortex, and left amygdala was noted in females with IBS, while in males with IBS, the right dorsolateral prefrontal cortex, insula, and dorsal periaqueductal gray displayed greater activation (Naliboff et al., 2003). Interestingly, mechanisms of pain and pain transmission have been shown to be different in males versus females. It has been suggested that in males, chronic pain is driven by the innate immune system via neutrophil recruitment to the vasculature of the spine, as well as infiltration of monocytes and activation of microglia-neuron crosstalk in the CNS, whereas in females this is accomplished by the adaptive immune system via central or peripheral activation and infiltration and activation of T-lymphocytes (Gregus et al., 2021).

5.4.3.3. Neurotransmitters

The catecholaminergic neurotransmitter NA plays an important role in visceral pain perception. NA acts at α- and β-ARs and is involved in descending inhibitory control of pain. Postganglionic sympathetic nerve fibers provide the main source of catecholamines in the periphery, and it has been shown that all three subtypes of the α1-AR are expressed in the DRG of the lumbar region, supporting their role in the transmission of pain (Nicholson et al., 2005). While the ascending NA pathways in the brain have been shown to facilitate nociception, descending NA inhibition originating from the locus coeruleus has been suggested to be anti-nociceptive (Hickey et al., 2014). While the majority of studies investigating the role of NA in pain perception have focused primarily on α-AR, the β-ARs have received increasingly more attention as a therapeutic target for disorders of pain. β-AR are G protein-coupled receptors that are generally coupled to a Gs protein (and in some cases Gi) which results in activation of adenylate cyclase, resulting in increased cAMP activity (Schena and Caplan, 2019). There are 3 subtypes of β-AR; β1-AR, β2-AR, and β3-AR which are widely expressed throughout the body. Antagonism of the β2-AR has been shown previously to alleviate stress-induced visceral hypersensitivity (Winston et al., 2010; Zhang et al., 2014). The β3-AR is expressed on cholinergic neurons of both the myenteric and submucosal plexi of the ENS in rodents and humans (Cellek et al., 2007; Nasser et al., 2006), frontal cortex and hippocampus in humans (Rodriguez et al., 1995), hippocampus, cerebral cortex, hypothalamus, striatum, and brainstem in rats (Summers et al., 1995). The β3-AR is also expressed in the urinary system (Michel and Vrydag, 2006), white and brown adipose tissue (Nahmias et al., 1991), and cardiac tissue (De Matteis et al., 2002). Furthermore, agonism of this receptor has been shown to result in visceral analgesia (Cellek et al., 2007) whilst also decreasing the

excitability of submucosal enteric neurons in a somatostatin-dependent manner (Schemann et al., 2010). Clinically, the role of NA signalling has been exploited by utilising β3-AR agonists as a treatment option for overactive bladder (Keam, 2018).

5-HT also plays a major role in pain. All 5-HT receptors are G protein-coupled receptors, with the exception of 5-HT_3 which is a ligand-gated ion channel (McCorvy and Roth, 2015). There is disagreement as to whether 5-HT signalling is pro- or anti-nociceptive, however alterations in 5-HT signalling have been reported in patients with IBS (Crowell, 2004). Acute tryptophan depletion, resulting in decreased 5-HT, led to increased pain in patients with IBS, which then resulted in heightened pain and urge scoring (Kilkens et al., 2004). In contrast, chronic exposure to 5-HT in mice increased visceral pain (Feng et al., 2014). Agonists of the $5HT_4$ receptor have shown anti-nociceptive properties (Hoffman et al., 2012; Sabaté et al., 2008), whereas antagonists of the 5-HT_3 receptor inhibited spinal dorsal horn neuronal activation in response to CRD (Kozlowski et al., 2000) and increased colonic compliance (Delvaux et al., 1998). Intraduodenal administration of 5-HT has also been shown to decrease the visceromotor response to CRD (Feng et al., 2014). All of these studies report a role for 5-HT in modulation of the visceral pain response and highlight the further need for research into its mechanism of action.

5.4.3.4. The immune system

The immune system not only plays a vital role in defence of the host, but also in the perception of pain. For instance, the immune system may lead to direct or indirect activation of nociceptive neurons. Following pathogen recognition by the immune system, a cascade of inflammatory cytokines may be released which leads to activation of nociceptive neurons, resulting in perception of pain. Nociceptors on neurons may

also be activated directly following detection of microbial metabolites and this is mediated through either PRRs or independent of this pathway (Chiu et al., 2013; van Thiel et al., 2020). Not surprisingly, during inflammation the action potential threshold for nociceptive neurons to fire is significantly reduced, resulting in heightened sensitivity. Mast cells, cells that regulate innate and adaptive immune responses among other functions (Krystel-Whittemore et al., 2016), have been shown to be increased in the gut of patients with IBS (Singh et al., 2020) and have been shown to play a strong mediating role in pain perception, particularly from the viscera (Héron and Dubayle, 2013; Zhang et al., 2016a). When activated, mast cell degranulation results in the release of inflammatory cytokines, which act on nociceptors to sensitise pain (Aich et al., 2015; Chatterjea and Martinov, 2015). This sensitisation and crosstalk is thought to be a key mechanism by which the immune system influences pain perception (Pinho-Ribeiro et al., 2017).

5.4.3.5. Interaction of stress and pain

As is evidenced above, stressors may induce visceral pain, therefore the two are inextricably linked. It has been seen that ELS-induced visceral hypersensitivity in females was accompanied by an increase in glucocorticoid receptor and CRF expression in the central amygdala, and knockdown of the glucocorticoid receptor or CRF induced visceral hypersensitivity (Prusator and Greenwood-Van Meerveld, 2017). Furthermore, adrenalectomised rats were not sensitive to Paclitaxel-induced mechanical hyperalgesia (Ferrari et al., 2020), further reinforcing the modulatory role of the stress axis on pain. However, the effect of stress on pain perception is bidirectional as it has been shown that exposure to acute stressors results in temporary

pain suppression (Butler and Finn, 2009). This stress-induced analgesia is thought to have developed as part of the body's response to stressors to enable the fight or flight response or coping and is mediated by the opioid system (Amit and Galina, 1986). Conversely, chronic exposure to stress has been shown to result in stress-induced hyperalgesia in both healthy subjects and patients with disorders of chronic pain (Crettaz et al., 2013), thus highlighting the divergent effects of stress on pain perception. The neurological basis for stress-induced hyperalgesia has been suggested to involve the anterior cingulate cortex, amygdala, periaqueductal gray, rostral ventromedial medulla, spinal cord, and the HPA axis (Jennings et al., 2014; Olango and Finn, 2014).

5.5. Preclinical models of visceral pain

There are many preclinical models of visceral pain that may be used. These have been recently extensively reviewed by (West and McVey Neufeld, 2021). Here, the most commonly used models will be discussed and are listed in T**able 1**.

Table 1. List of the preclinical models of visceral pain discussed.

Preclinical model of visceral pain	Induction method	Reference
Wistar-Kyoto Rats	Genetically predisposed to stress	(Gunter et al., 2000; O'Mahony et al., 2013)
Antibiotic Depletion of the Gut Microbiota	Microbiota disruption	(Aguilera et al., 2015; Hoban et al., 2017; O'Mahony et al., 2014; Verdú et al., 2006)
Maternal Separation	Early life stress induced	(Botschuijver et al., 2019; Moloney et al., 2015b; O'Mahony et al., 2009)
Faecal Microbiota Transplantation	Microbiota disruption	(Crouzet et al., 2013)
Water Avoidance Stress	Psychological stress	(Nozu et al., 2017)

5.5.1. Genetically susceptible stress models

The concept of a genetic susceptibility to many disorders is not new. It has been seen that stress-induced visceral hypersensitivity may be modulated by epigenetic changes in gene expression. These changes include alterations in DNA-methylation and histone-acetylation patterns in the brain of rodents (Tran et al., 2013). Another study reported baseline visceral hypersensitivity in Wistar-Kyoto versus Sprague Dawley (O'Mahony et al., 2013) and Fisher-344 rats (Gunter et al., 2000).

5.5.2. Gut microbiota depletion

To further support the role of the gut microbiota in stress-induced pain, depletion of the gut microbiota with an antibiotic cocktail in early life results in visceral hyperalgesia in adulthood in male rats, highlighting the importance of the gut microbiota in the regulation of visceral pain responses (O'Mahony et al., 2014). Verdu and colleagues also showed that administration of antibiotics induces visceral hypersensitivity (Verdú et al., 2006) in mice. In contrast, the administration of an antibiotic cocktail to adult rats reversed MS-induced visceral hypersensitivity (Hoban et al., 2017) and capsaicin-induced visceral hypersensitivity was attenuated by antibiotic administration in mice (Aguilera et al., 2015). Other studies have also shown that the gut microbiota is required for appropriate sensation of visceral stimuli (Luczynski et al., 2017; Tramullas et al., 2021), however the efficacy of gut microbiota depletion using antibiotics as a model of visceral pain remains unclear. It may be suggested that antibiotic administration alone results in visceral hypersensitivity, but when used following the induction of visceral pain via another model as mentioned above, may have beneficial effects.

5.5.3. Early life adversity and neonatal maternal separation

MS is a widely used animal model of ELS, which involves repeatedly separating the mother from her pups in early life and elicits visceral hypersensitivity and increased HPA axis activity (O'Mahony et al., 2009). MS has reproducibly resulted in heightened visceral sensitivity in rats and is therefore an appropriate model of visceral hypersensitivity induction (Botschuijver et al., 2019; Collins et al., 2022; Moloney et al., 2015a; Moloney et al., 2015b; Yi et al., 2017). However, although MS does not reproducibly result in a robust behavioural phenotype in mice, MS in mice does induce visceral hypersensitivity (Riba et al., 2017; Riba et al., 2018). The plethora of behavioural and physiological effects of MS on both mother and offspring has been extensively discussed in a previous section. As the gut microbiota plays a role in the management of the stress response, so too is it involved in stress-induced pain. Early life, both in humans and rodents, is a critical timepoint at which neural pain circuitry is programmed and thus early life is a time at which pain circuitry, and therefore pain perception, may be most susceptible to alteration from external factors such as early life exposure to adverse events such as ELS (Prusator et al., 2016) or internal factors such as ELS-induced alterations in HPA-axis activity (Videlock et al., 2009). Research to support this is evident in cases of IBS where stress is a known causative factor for the development of this disorder associated with visceral pain (Farzaei et al., 2016). Interestingly, in a human study, those who reported that they had experienced early life adversity presented with higher perceived pain than those who did not experience this same adversity in early life (Sachs-Ericsson et al., 2007). In another study, it was found that the severity of IBS symptoms was worse in those who had experienced early life adversity (Kanuri et al., 2016). Moreover, studies in humans have shown that differences in abundance of several genera are seen in patients with IBS versus healthy

controls, further supporting the role of the microbiota in visceral pain (Noor et al., 2010).

5.5.4. Faecal microbiota transplantation

FMT involves the transfer of faecal material from one individual to another and has been classically used as a treatment for recurrent *Clostridium difficile* infection (Bakken et al., 2011; Kelly et al., 2016a; Smillie et al., 2018). This technique is subject to a lot of variation in administration (Gheorghe et al., 2021), but is still a proven effective method of effecting change in the recipient. Interestingly, the use of FMT has not only highlighted the benefits of microbiota-targeted interventions but has also aided in the elucidation of mechanisms behind visceral pain. For instance, a study which inoculated GF rats with faecal microbiota from patients with IBS presenting with visceral hypersensitivity resulted in the transfer of visceral hypersensitivity (Crouzet et al., 2013). Similarly, FMT from rats with DNBS-induced colitis induced visceral hypersensitivity in control recipient rats (Lucarini et al., 2021).

5.5.5. Water avoidance stress

Another preclinical model of visceral pain is water avoidance stress. Water avoidance stress involved placing the rodent on an escape platform surrounded by water such that it cannot escape either for a single exposure, or 1 hour daily for up to 10 days (Hong et al., 2009; Luo et al., 2020; Tran et al., 2013). This model has been shown to reproducibly result in heightened visceral sensitivity (Nozu et al., 2017). Potential mechanisms behind water avoidance stress-induced visceral hypersensitivity have been suggested to include corticotropin-releasing hormone receptors, TRPV1 (Nash

et al., 2012), neurokinin-1 receptors (Schwetz et al., 2004b), and 5-HT receptors (Bradesi et al., 2007; Gilet et al., 2014).

6. Interventions for disorders of the gut-brain axis

6.1. Microbiota-targeted interventions for disorders of the gut-brain axis

It is now known that diet can impact heavily upon gut microbiota composition and therefore gut function (Wu et al., 2011). In recent times, prebiotics, probiotics, and psychobiotics have come to the fore as main modulators of the gut microbiome (Bastiaanssen et al., 2018) and have even been used to treat disorders such as IBS (Moloney et al., 2014) and mood disorders (Dinan et al., 2013). Several reviews on the efficacy of pre- and probiotics have been published, highlighting the need for future studies into their use as treatment strategies for disorders of the gut-brain axis such as IBS (Asha and Khalil, 2020; de Souza et al., 2022; Ford et al., 2018).

6.1.1. Prebiotics

Prebiotics are defined as a substrate (non-digestible fiber) that is selectively utilised by host microorganisms conferring a health benefit (Gibson et al., 2017). Prebiotic use to benefit host wellbeing received a large amount of interest in the 1990's where fructooligosaccharide (FOS) and galactooligosaccharide (GOS) were the main prebiotics being investigated (Gibson and Roberfroid, 1995) and even today, FOS and GOS are front-runners in prebiotic studies as they have been shown to confer a health benefit in both preclinical models (Burokas et al., 2017) and human infants (Rao et al., 2009). These prebiotics aim to confer health benefit by supporting the growth of beneficial microbes (Bastiaanssen et al., 2018) and may be used as modulators of the microbiota (Vulevic et al., 2013) and immune system (van Vlies et al., 2012). Prebiotics have been used in the treatment of IBS and comorbid psychiatric conditions including depression and anxiety, however, mixed results on the effects of prebiotics

on these symptoms has resulted in a lack of consensus on the benefit of their use (Wilson et al., 2019). Studies using prebiotics such as oligofructose (Hunter et al., 1999) and FOS (Olesen and Gudmand-Høyer, 2000) reported no beneficial effects on symptoms. Another study utilising a trans-galactooligosaccharide mixture produced from lactose by *Bifidobacterium bifidum* NCIMB 41171 found that abdominal pain and discomfort were reduced, and stool pattern normalised (Silk et al., 2009). Prebiotics have been shown in several preclinical studies to ameliorate stress-induced changes in behaviour and alter host biochemistry. The reduction in locomotor activity induced in rats by MS is reversed by polydextrose and GOS administration, and deficits in spatial working memory are reversed with the addition of *Lactobacillus rhamnosus* GG to the prebiotic formulation (Berg et al., 2015). The use of polyphenols as a microbiota-targeted intervention against the effects of ELS has also proven effective whereby polyphenol intake in rats reversed ELS-induced deficits in depressive- and anxiety-like behaviour (Donoso et al., 2020). Chronic administration of both FOS and GOS together displayed both anxiolytic and anti-depressive effects in mice as well as reducing stress-induced CORT levels and normalising the gut microbiota following stress (Burokas et al., 2017). The exact mechanisms of action of FOS and GOS are not readily apparent, however it is known that FOS and GOS tightly regulate the gut microbiota (Burokas et al., 2017). Not only do prebiotics act at a local level in the gut, but they may also have effects on the CNS. GOS has been shown to increase hippocampal brain-derived neurotrophic factor levels (Savignac et al., 2013). Prebiotics have also been shown to increase 5-HT levels in the prefrontal cortex which may suggest that the behavioural changes observed with respect to depressive and anxiety-like behaviours following prebiotic administration may be 5-HT-mediated as

it has been shown that an increase in 5-HT levels has an anti-depressive-like effect (Cryan et al., 2002). Another family of prebiotics, HMOs will be discussed below.

6.1.2. Probiotics

Probiotics, on the other hand, are defined as live bacteria that have a positive effect on host health when ingested in adequate quantities (Bastiaanssen et al., 2018). This method of dietary intervention exploits the use of introducing previously identified beneficial bacteria to confer health benefits to the host. Similarly to prebiotics, probiotics have been shown to ameliorate the stress-induced behavioural changes in mice such as anxiety (Messaoudi et al., 2011), reduce stress-induced serum CORT levels (Bravo et al., 2011), as well as rescue memory deficits (Savignac et al., 2015). Interestingly, the increased GI permeability seen in major depressive disorder and IBS is reduced by pre-treatment with a probiotic as is the associated increased HPA axis activation (Ait-Belgnaoui et al., 2012).

Probiotic use in the treatment of IBS has yielded more benefit that prebiotics, however, studies also report a lack of efficacy of *Lactobacillus plantarum* 299V (Sen et al., 2002), and *Lactobacillus reuteri* (Amirimani et al., 2013) against the symptoms of IBS including stool quality, abdominal pain, and urge for defecation. Studies using probiotic strains *Lactobacillus delbruekii*, *Lactobacillus fermentum*, and other *Lactobacillus* and *Bifidobacteria* have reported benefits on overall IBS severity scoring scale and quality of life (Fawzy et al., 2021; Zhang et al., 2016b). More recent studies have shown a higher efficacy of multi-strain probiotics against symptoms of IBS. These include the multi-strain probiotic BioKult comprised of 14 different bacterial strains, namely *Bifidobacteria* and *Lactobacillus*) (Ishaque et al., 2018), Bifico (containing 3 strains; *Bifidobacterium*, *Lactobacillus*, *Enterococcus)* (Zhang et

al., 2019a), NordBiotic (four *Bifidobacteria*, five *Lactobacillus*, and one *Streptococcus*) (Skrzydło-Radomańska et al., 2021), and VSL#3 (eight strains of *Bifidobacteria*, *Lactobacillus*, and *Streptococcus*) (Guandalini et al., 2010) which reduced abdominal pain, bloating, and overall symptom severity measured by the IBS severity scoring scale.

Psychobiotics were originally discovered as a new class of probiotic that act against the symptoms of anxiety and depression (Dinan et al., 2013), however more recently the definition of psychobiotics has received a lot of attention with calls to broaden the definition to include prebiotics (Sarkar et al., 2016). One such potential psychobiotic is *Bifidobacerium infantis* which has been shown to be effective in increasing peripheral tryptophan levels in Sprague Dawley rats suggesting that this strain may have potential as an anti-depressant even though classic anti-depressant activity in the forced swim test was not observed (Desbonnet et al., 2008). Psychobiotics have also been seen to be effective in ameliorating the MS-induced depressive-like symptomatology whereby chronic treatment with *Bifidobacterium infantis* reversed this behavioural deficit (Desbonnet et al., 2010). Psychobiotics have also been shown to be effective in reducing anxiety in BALB/c mice which were pre-treated with *Mycobacterium vaccae* (Matthews and Jenks, 2013), and in rats treated with *Lactobacillus rhamnosus* GG in combination with prebiotics (McVey Neufeld et al., 2019). Interestingly, psychobiotics have even been suggested to have the potential to act on memory as following infection with *Citrobacter rodentium*, which resulted in memory dysfunction when coupled with acute stress in C57BL/6 mice, pre-treatment with a combination of *Lactobacillus rhamnosus* (R0011) and *Lactobacillus helveticus* (R0052) countered this memory dysfunction (Gareau et al., 2011). Interestingly, it is seen that GF mice display impaired memory, suggesting a role for

the gut microbiota (Cryan and O'Mahony, 2011), further reinforcing the role that psychobiotics may play in memory. Not only have psychobiotics been shown to be beneficial in murine models of stress but they have also been shown to be efficacious in human studies where anti-depressant activity was seen by a reduction in awakening cortisol levels following administration of Bimuno GOS (Schmidt et al., 2015) as well as a reduction in depressed mood following treatment with a probiotic blend comprised of *Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus salivarius*, and *Lactococcus lactis* (Steenbergen et al., 2015).

6.1.3. Postbiotics

Postbiotics are defined as a preparation of inanimate microorganisms and/or their components that confers a health benefit to the host (Salminen et al., 2021). Postbiotics include heat-killed bacteria as well as their metabolites and have the added benefit of circumvention of colonisation efficacy and viability at high doses as they are non-living. Their use as a microbiota-targeted therapy for disorders include the reported efficacy of heat-killed *Lactobacillus acidophilus* LB with its' culture medium when added to an oral rehydration solution for the treatment of diarrhoea in humans (Liévin-Le Moal et al., 2007). Postbiotics have also been shown to affect gut physiology whereby lambs which were administered *Lactobacillus plantarum* RG14 showed improved gut barrier function via the upregulation of tight junction protein mRNA expression and alterations in pro-inflammatory cytokine mRNA expression (Izuddin et al., 2019). However, the benefit and efficacy of postbiotics as a treatment method for stress and non-stress related disorders is not fully clear, and as such, further studies into their uses should be conducted.

6.1.4. Faecal microbiota transplantation

As previously mentioned, the use of FMT as a model of visceral pain has yielded promising results. Not only may FMT be utilised as a model to induce visceral pain-associated disorders, but it may also be used as a treatment approach. The use of FMT from healthy controls in treating disorders of the gut-brain axis has proven to be effective against the symptoms of IBS in humans (Cui et al., 2021; El-Salhy and Mazzawi, 2018; El-Salhy et al., 2020; Johnsen et al., 2018). Some studies have reported no effect of FMT against the symptoms of IBS (Halkjær et al., 2018), however, the majority of studies support the safety and efficacy of FMT as a treatment for IBS. Similarly, evidence exists to support the use of FMT in the treatment of stress-related psychiatric disorders such as depression and anxiety (Chinna Meyyappan et al., 2020; Kurokawa et al., 2018). Further large-scale studies are needed to fully investigate the efficacy of FMT as a treatment strategy for disorders of the gut-brain axis.

6.1.5. Diet

Manipulation of the gut microbiota through diet is an effective method of effecting change in host physiology and behaviour as it is well known that diet modulates gut microbiota composition (Leeming et al., 2019). Similarly, the gut microbiota may also influence host nutrition either directly or indirectly (for review see (Ezra-Nevo et al., 2020)). This concept led to the introduction of the field of nutritional psychiatry as diet can positively impact on brain function. There is an inextricable link between diet and stress-related psychiatric disorders such as depression as it is seen that by use of dietary interventions including omega-3 fatty acids, the symptoms of depression are reduced (Ekong and Iniodu, 2021; Firth et al., 2019; Liao et al., 2019; Marx et al.,

2019; Owens et al., 2020). It has been shown that omega-3 fatty acids upregulate adult neurogenesis, or birth of new neurons, and brain-derived neurotrophic factor expression (Beltz et al., 2007; Paduchová et al., 2021), while also preventing cortisol-induced reductions in neurogenesis (Borsini et al., 2020). The Mediterranean diet, a plant-based diet high in omega-3 fatty acids, has also been shown to reduce the symptoms of depression (Dinan et al., 2019) (McMillan et al., 2011). Similarly, dietary supplementation with vitamin D has been shown to be effective in the treatment of depression (Parker et al., 2017).

Given that diet majorly impacts on the symptoms of IBS, the exploitation of diet as a treatment strategy has received increasingly more attention in recent years. For example, a diet low in fermentable oligosaccharide, disaccharide, monosaccharide, and polyol (FODMAP) has been shown to lead to vast improvements in IBS symptomatology including abdominal pain, motility issues, and abdominal bloating (Black et al., 2021). Other dietary supplements that have shown promising results against IBS symptoms include peppermint oil (Alammar et al., 2019), however its efficacy has been subjected to scrutiny with calls for further investigation (Black et al., 2020b; Cash, 2020).

6.1.6. Infant formula supplementation

As mentioned previously in this review, one of the main methods of microbiota seeding in early life is through early life nutrition, whether it be from breastfeeding, or formula-feeding. When breastfeeding is not an option, infant formula may be used to supplement the infant with critical nutrients. Producers of infant formula are constantly attempting to better reflect the nutritional value of breastmilk in their formulas (Ahern et al., 2019), with the majority of the protein component being

derived from bovine milk (Lönnerdal, 2014). For example, supplementation with bovine milk fat globule membrane (MFGM), the membrane surrounding milk fat droplets, has been shown to reduce the impact of ELS on visceral pain and memory in rodents (Collins et al., 2022; O'Mahony et al., 2020). Further studies have reported higher cognitive scores as measured by the Bayley scales of infant development at 12 months of age in infants fed an MFGM-supplemented diet (Timby et al., 2014), or MFGM and lactoferrin-supplemented diet (Li et al., 2019). MFGM has also been shown to have beneficial effects on GI physiology in pigs whereby measures of intestinal health including villus height, crypt depth, and expression of tight junctions were positively altered (Zhang et al., 2020). The addition of other bioactive fractions of bovine milk to increase the nutritional value of infant formula has also proven effective. These protein components include α-lactalbumin and lactoferrin (Skolnick et al., 2020). However, the main notable difference between breastmilk and infant formula is the lack of a diverse microbial community, fuelling the production of infant formula with prebiotics to better recapitulate the beneficial microbes found in breastmilk.

6.1.7. Breastmilk

Human breastmilk is the gold standard for infant feeding as it provides the necessary nutrients and bioactive compound which together promote growth and immune system competency. Breastmilk in itself possesses a distinct microbiome, which also plays a major role in infant gut microbiota development (Rodríguez, 2014). Breastmilk is produced in the presence of prolactin (Gargiulo, 2017), and the necessary nutrients are brought via the blood and lymph system and secreted into the breastmilk through mammary epithelial cells. The composition of breastmilk varies depending on the

developmental stage of the infant as various other nutrients are required at different developmental stages ranging from preterm birth, to weaning off the mother's milk (Bauer and Gerss, 2011). Casein, lactoferrin, lysozyme, secretory IgA, and serum albumin are among the most abundant proteins found in breastmilk (Lyons et al., 2020). HMOs are non-digestible glycans grouped into fucosylated, sialylated, and non-fucosylated neutral and are found in human breastmilk (Garwolińska et al., 2018). HMOs have been shown to modulate gut-brain axis activity through promotion of bacterial species in the gut such as *Bifidobacteria* (Kirmiz et al., 2018), leading to the generation of metabolites such as SCFAs (Šuligoj et al., 2020) and limiting the growth of pathogenic bacteria (Triantis et al., 2018). The HMOs 3'Sialyllactose and 6'Sialyllactose have been shown to result in a decrease in anxiety-like behaviour in mice (Tarr et al., 2015), as well as positively impact on memory in rats (Oliveros et al., 2018). Importantly, the HMOs 2'-*O*-fucosyllactose and 3'Sialyllactose have been shown to reduce GI barrier permeability (Chleilat et al., 2020), which may be of use for treatment of disorders of the gut-brain axis. Interestingly, a blend of six HMOs reduced stress-induced visceral hypersensitivity in mice, an effect that was not seen in mice with a depleted microbiota, suggesting that HMOs act via the gut microbiota (Ferrier et al., 2022). 2'-*O*-fucosyllactose and lacto-N-neotetraose together improved IBS symptoms and overall quality of life as well as increasing the abundance of *Bifidobacterium* spp., further supporting the use of HMOs in the treatment of IBS (Iribarren et al., 2020; Iribarren et al., 2021; Palsson et al., 2020).

6.2. Pharmacological interventions for disorders of the gut-brain axis

There are currently no approved pharmacological treatments specifically to treat visceral pain given its multifaceted aetiology, however, there are a number of pharmacological interventions that are of use against visceral pain-associated disorders. The interventions that have shown most efficacy clinically for the disorder of gut-brain interactions, IBS are summarised in **Table 2**.

Table 2. Overview of current pharmacological interventions for disorders of gut-brain communication.

Intervention	Patient pool	Result	Proposed mechanism	Reference
Microbiota-targeted				
Rifaximin	Non-constipated IBS, IBS-D	Global IBS symptom improvement (visceral pain, bloating, altered bowel habits).	Rifaximin alters the gut microbiota and reduces the immune response in the gut, likely a multifactorial mechanism.	(Lembo et al., 2020; Menees et al., 2012; Pimentel et al., 2011; Schey and Rao, 2011)
Antidepressants				
Tricyclic antidepressants Amitriptyline, imipramine, desipramine	IBS-D	Decreased visceral hypersensitivity.	Can act via central modulation of visceral afferents.	(Ford et al., 2019; Rahimi et al., 2009; Thoua et al., 2009)
Receptor-specific				
Gabapentin	IBS-M, IBS-C, IBS-D	Reduction of bloating, discomfort, and abdominal pain.	Binds to α-2 delta subunits of voltage-gated Ca^{2+} channels and prevents membrane expression of Ca^{2+} channels, preventing glutamate, substance P release from primary afferents.	(Houghton et al., 2007; Lee et al., 2005)
Eluxadoline	IBS-D	Improvement in bowel movement urgency and frequency, quality of life.	Acts as a μ and κ-opioid receptor agonist, and a δ-opioid receptor antagonist which delays intestinal transit and alters secretion and sensation respectively.	(Dove et al., 2013; Lembo et al., 2016)
Ebastine	IBS	Reduced visceral hypersensitivity, reduced abdominal pain.	Histamine receptor 1 antagonist which blocks histamine-induced sensitisation of TRPV1.	(Wouters et al., 2016)

Ibodutant	IBS-D	Relief of IBS symptoms and abdominal pain.	Neurokinin-2 receptor antagonist, possible inhibition of neurokinin-induced visceral sensory nerve activation.	(Tack et al., 2017)
Loperamide	IBS-D	Reduced abdominal pain, improved stool consistency.	Gastrointestinal tract μ-opioid receptor agonist, decreases smooth muscle contraction in the intestinal wall.	(Efskind et al., 1996)
Alosetron	IBS-D	Reduced abdominal pain, discomfort, and urgency.	5-HT_3 receptor antagonism.	(Moore et al., 2013)
Antispasmodics				
Otilonium bromide	IBS	Reduction in abdominal pain and bloating severity	Targets L- and T-type Ca^{2+} channels and tachykinin neurokinin-2 receptors.	(Clavé and Tack, 2017; Triantafillidis and Malgarinos, 2014)

7. Summary and conclusion

The gut microbiota is heavily involved in all aspects of the development of the gut-brain axis and its component parts. Stress in early life, whether it be in the prenatal, perinatal, or postnatal period, exerts deleterious effects on the function of the gut-brain axis and results in a wide range of disorders of gut-brain axis interactions and alterations in behaviour as outlined above. Pharmacological and dietary interventions aimed at restoration of proper function of the gut-brain axis have proven effective in some cases with future research into potential mechanisms of action required. Further, early detection of stress during pregnancy and early life may aid in the treatment of these disorders and reduce potential negative outcomes associated with stress in early life.

This review of the literature highlights the need for novel investigations into treatments of disorders of gut-brain axis interactions such as IBS and associated hallmark visceral pain, as well as a better understanding of the factors that modulate these changes. Further studies are required to provide alternative and more effective treatments for early life stress-induced disorders of the gut-brain axis, and more reliable biomarkers for stress-related disorders should be investigated.

8. Aims of the thesis

The overarching aim of this thesis is to lead a novel investigation into dysfunction of the gut-brain axis with a focus on visceral pain and explore dietary and pharmacological interventions that may ameliorate these deleterious effects.

8.1. Aim 1: Can dietary or pharmacological interventions ameliorate early life stress-induced dysfunction of the gut-brain axis?

Firstly, we characterised the effect of early life stress on behavioural and physiological measures in the rat. These studies were carried out with a focus on visceral sensitivity and the enteric nervous system (Chapters 2 and 3) and the effects of novel treatment strategies including the β3-adrenoceptor agonist CL-316243 and milk fat globule membrane were investigated.

8.2. Aim 2: Is there a role for the gut microbiota and female sex hormones in perception of visceral pain?

To establish whether the gut microbiota and female sex hormones impact on visceral pain perception, we used germ-free and conventional mice that were ovariectomised to cease the main production of female sex hormones and plotted the visceral pain response across the oestrous cycle (Chapter 4).

8.3. Aim 3: What is the impact of early life stress on pre-adolescent changes in circulating and gut immune profiles?

Given that the immune system is an integral part of the gut-brain axis, we sought to investigate the impact of early life stress on the changes that occur during the pre-adolescent period (Chapter 5).

8.4. Aim 4: Can biological markers related to the immune system and intestinal permeability be utilised as biomarkers for prenatal maternal stress?

Given that prenatal stress has been linked to a dysfunctional brain-gut axis in animal models we wanted to investigate potential biomarkers of this stress pregnant women with and without IBS (Chapter 6).

Chapter 2

Beta 3-Adrenoceptor Agonism Ameliorates Early Life Stress-Induced Visceral Hypersensitivity

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Abstract

Background: Visceral hypersensitivity, a hallmark of disorders of the gut-brain axis, is associated with early life stress (ELS). Agonism of neuronal β3-adrenoceptors (AR) has been shown to alter central and peripheral levels of tryptophan and reduce visceral hypersensitivity. Here, we aimed to determine the potential of β3-AR agonism in reducing ELS-induced visceral hypersensitivity and possible underlying mechanisms.

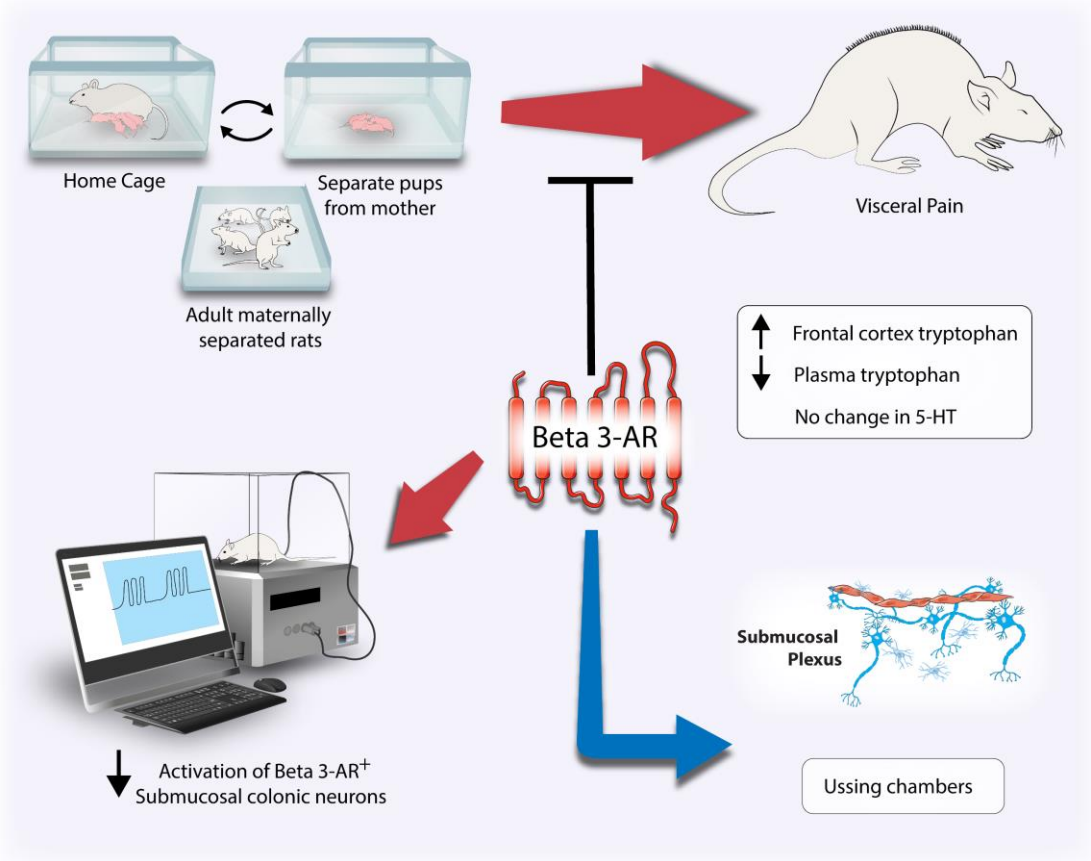
Methods: ELS was induced using the maternal separation (MS) model, where Sprague Dawley rat pups were separated from their mother (postnatal day 2-12). Visceral hypersensitivity was confirmed in adult offspring using colorectal distension (CRD). CL-316243, a β3-AR agonist, was administered to determine anti-nociceptive effects to CRD. Distension-induced enteric neuronal activation was assessed by quantifying c-Fos positive neurons as well as β3-AR positive neurons in the colonic submucosa. Tryptophan metabolism was assessed both centrally and peripherally as well as colonic secretomotor function.

Key Results: Here we show that CL-316243 significantly ameliorated MS-induced visceral hypersensitivity. While no impact of MS was evident in the enteric nervous system, CL-316243 did lead to a significant decrease in the distension-induced activation of β3-AR^{+} colonic submucosal neurons. Furthermore, MS altered plasma tryptophan metabolism and colonic adrenergic tone while CL-316243 reduced both central and peripheral levels of tryptophan and affected secretomotor activity in the presence of tetrodotoxin.

Conclusions and Inferences: This study supports a beneficial role of CL-316243 in reducing ELS-induced visceral hypersensitivity and suggests that targeting the β3-AR can significantly influence gut-brain axis activity through modulation of enteric

neuronal activation, tryptophan metabolism, and colonic secretomotor activity which may synergistically contribute to offsetting the effects of ELS.

Graphical abstract



Introduction

Stressful experiences, particularly in early life may have detrimental effects on host development and physiology. A well-established model of early-life stress (ELS) in rodents is maternal separation (MS), which involves separating pups from their dams for a specified amount of time, usually during the stress hyporesponsive period. One of the most often used paradigms involves a 3-hour daily separation from postnatal day 2-12 which results in robust effects on behaviour and physiology (O'Mahony et al., 2009; O'Mahony et al., 2020). These changes include alterations in central and peripheral nervous systems (De Palma et al., 2015), hypothalamic-pituitary-adrenal axis function (Juruena et al., 2006; O'Mahony et al., 2009), as well as response to pain (O'Mahony et al., 2009; O'Mahony et al., 2020; O'Mahony et al., 2011; Vilela et al., 2017; Yi et al., 2017). MS has also reproducibly been shown to elicit an anxio-depressive phenotype in rodents (Cui et al., 2020; McVey Neufeld et al., 2019).

Previous studies in rodents have reported increased visceral sensitivity following exposure to MS (O'Mahony et al., 2020; Yi et al., 2017). Visceral hypersensitivity, characterised by a diffuse sensation of pain arising from the midline of the body (Sikandar and Dickenson, 2012), is a hallmark of disorders of gut-brain axis interactions such as irritable bowel syndrome (IBS), a condition for which ELS is a known risk factor (Wilmes et al., 2021) and for which there is currently no satisfactory treatment. Although the exact aetiology behind the manifestation of ELS-induced visceral hypersensitivity is unclear, several factors are known to play a major modulatory role in perception and processing of pain. These factors include the gut microbiota (Luczynski et al., 2017), female sex hormones (Tramullas et al., 2021), and

neurotransmitters such as serotonin (5-HT) and noradrenaline (NA) (O'Mahony et al., 2006).

Noradrenaline is a catecholaminergic neurotransmitter known to play a role in pain perception which acts via α or β-adrenoceptors (AR). β-ARs are G protein-coupled receptors and are generally coupled to a Gs protein which increases cAMP when activated by NA and adrenaline (Schena and Caplan, 2019). In terms of its role in visceral pain perception, NA release in the spinal cord from descending pathways inhibits pain via α-ARs (Pertovaara, 2006). Modulation of β-ARs has also proven useful in the alleviation of visceral pain with antagonism of β2-AR reversing stress-induced visceral hypersensitivity, whereas administration of NA resulted in visceral hypersensitivity in control rats (Zhang et al., 2014). The β3-AR is expressed on cholinergic neurons of both the myenteric and submucosal plexi of the enteric nervous system (Nasser et al., 2006), and agonism of this receptor has been shown to elicit visceral analgesia (Cellek et al., 2007) whilst also decreasing excitability of submucosal enteric neurons in a somatostatin-dependent manner (Schemann et al., 2010). β3-AR expression has also been shown both in the frontal cortex and hippocampus of the human brain (Rodriguez et al., 1995) and in the rat brain, specifically in the hippocampus, cerebral cortex, hypothalamus, brainstem, and striatum (Summers et al., 1995). It has been shown previously that MS alters the levels of β3-AR in adipose tissue (Miki et al., 2013) and decreases NA levels in the brain (Arborelius and Eklund, 2007), which could suggest a role for the β3-AR in MS-induced dysfunction.

CL-316243, a highly selective agonist for the rat β3-AR (Baker, 2005), has been shown to increase central tryptophan (Lenard et al., 2003) and 5-HT (Conley et al., 2006), which when coupled with its anti-nociceptive properties highlights a potential

role for central serotonergic signalling in its mechanism of action against visceral pain. Alterations in both central and peripheral serotonergic signalling have also been reported in patients with IBS (Faure et al., 2010; Stasi et al., 2014). Studies using 5-HT_4 receptor agonists in humans and rodents report anti-nociceptive activity (Hoffman et al., 2012; Sabaté et al., 2008), whereas antagonism of the 5-HT_3 receptor in rodents inhibits spinal dorsal horn neuronal activation in response to colorectal distension (CRD) (Kozlowski et al., 2000) and increased colonic compliance in humans (Delvaux et al., 1998).

Tryptophan, the precursor of 5-HT, has gained increasingly more attention for its potential role in neuropsychiatric disorders and disorders of gut-brain axis interactions such as IBS. Tryptophan may be metabolised to form 5-HT or melatonin, or be broken down into several neuroactive metabolites via the kynurenine pathway which accounts for ~95% of tryptophan metabolism (Peters, 1991). Acute tryptophan depletion, resulting in decreased 5-HT, led to increased pain in patients with IBS, further supporting the role of 5-HT in visceral pain processing (Kilkens et al., 2004). As the precursor of 5-HT, it would stand to reason that alterations in tryptophan availability would disturb 5-HT concentrations both centrally and peripherally, with this altered serotonergic signalling modifying pain perception.

Colonic secretomotor function is closely linked to disorders of gut-brain axis interactions such as IBS. Both visceral hypersensitivity and alterations in gut secretomotor function are seen in cases of IBS (Camilleri, 2015) where hyperactivity of secretomotor neurons results in diarrhoea and hypoactivity results in constipation (Nezami and Srinivasan, 2010). Secretomotor neurons are located both in the myenteric ganglia of the enteric nervous system, where they project to the mucosa, and in the submucosal ganglia which project to the myenteric ganglia and have been

suggested to serve as the link between secretion and motility in the gut (Costa et al., 2000). Distension of colonic tissue from guinea-pigs *ex vivo* has been shown to result in alterations in secretomotor activity, specifically resulting in increased secretion (Weber et al., 2001). Interestingly, MS has been shown to alter baseline short circuit current (I_{SC}) in colonic tissue (Gareau et al., 2006), suggesting that alterations in secretomotor activity may play a role in MS-induced dysfunction.

To date, few studies have investigated the role of the β3-AR in ELS-induced visceral pain, therefore, the aim of this study was to assess the potential of β3-AR agonism against ELS-induced visceral hypersensitivity and investigate possible serotonergic or colonic secretomotor activity-dependent mechanisms.

Methods

Animals

Male and female Sprague Dawley rats (approximately 8 weeks of age) were purchased from Envigo, UK and were mated in the biological services unit, Doughcloyne, and subsequent offspring were used in this study. The day of birth was designated as postnatal day (PND0). All dams and littermates were housed in large plastic cages (15 x 22 x 9cm) in a humidity- (55% ± 10%) and temperature- (21°C ± 1°C) controlled room. The holding room was maintained at a 12-hour light/dark cycle (lights on 7am). All experiments were conducted in accordance with the guidelines of European Directive 86/609/EEC and the Recommendations 2007/526/65/EC and were approved by the Animal Experimentation Ethics Committee of University College Cork.

Maternal separation

Maternal separation was carried out as previously described (O'Mahony et al., 2009). Briefly, at PND0 litters were randomly assigned to maternally separated (MS) or non-separated (NS) groups. At PND2, MS litters were taken from the main colony room to an adjacent room maintained at the same lighting and temperature conditions. The dam was carefully removed from the home cage and placed into a smaller holding cage, following which the pups (entire litters) were gently transferred together to a small cage where they remained for 3 hours. Cages containing the pups were placed on heating pads maintained at between 30°C and 33°C and were filled with 3cm of bedding so the pups could thermoregulate as needed. The dam was returned to the home cage and placed back in the main colony room without the pups for this time period to avoid communication via ultrasonic vocalisation or scent. Following the 3-

hour separation, dams were again brought to the adjacent room and all pups were returned to their original home cages. NS litters were also transported to and from the adjacent room as the MS litters to avoid the confound of transportation stress but were left otherwise undisturbed in their home cages with the exception of weekly cage cleaning. This procedure was carried out daily from PND2 to PND12 inclusive. The period of the separation was carried out at the same time each day (9am-12pm). At PND21, offspring were sexed and weaned, and male offspring were used for the remainder of the study.

In vivo studies

The selective β3-AR agonist CL-316243 (product number C5976, Sigma-Aldrich, Dublin, Ireland) was administered perorally via gavage 1 hour prior to CRD as shown in **Figure 1**. Two different doses were used based on a previous study in the literature – 0.1mg kg^{-1} and 1mg kg^{-1} (Cellek et al., 2007). A vehicle control group with 0.9% saline was used. CL-316243 was not administered to NS animals for the visceral sensitivity assessment as there was no viscerally hypersensitive phenotype to rescue with CL-316243. The volume of the agonist or saline administered was 1ml kg^{-1}. For CRD-induced neuronal activation and tryptophan metabolism, the effect of CL-316243 in NS animals was investigated to assess if this agonist affects the neuronal response to CRD and tryptophan metabolism under control conditions.

Experimental design

Separate cohorts were used for the following assessments:

A. Visceral hypersensitivity assessment: For investigation into the effect of β3-AR agonism on visceral pain perception; NS-Vehicle, MS-Vehicle, MS-CL-316243 (0.1mg kg^{-1} and 1mg kg^{-1}) groups were used during colorectal distension. *n* = 9-16 per group; Colonic submucosal neuronal activation in response to CRD: NS-Vehicle, NS-CL-316243 (0.1mg kg^{-1}), MS-Vehicle, MS-CL-316243 (0.1mg kg^{-1}). *n* = 5-10 per group.

B. β3-AR expression in colonic tissue assessment: For investigating the expression of β3-AR in colonic submucosal plexi; NS-Control, MS-Control. *n* = 4-5 per group; Ussing chamber experiments; NS and MS control. *n* = 9-13 per group.

C. Tryptophan metabolism assessment (HPLC): NS-Vehicle, NS-CL-316243 (0.1mg kg^{-1} and 1mg kg^{-1}), MS-Vehicle, MS- β3-CL-316243 (0.1mg kg^{-1} and 1mg kg^{-1}). *n* = 6-8 per group.

Sample collection details: All rats were euthanised by decapitation for tissue collection. Colonic samples were collected (A) 1 hour following CRD (B) Immediately after decapitation. (C) Whole brain was removed immediately following decapitation and dissected into regions and stored at -80°C until later analysis.

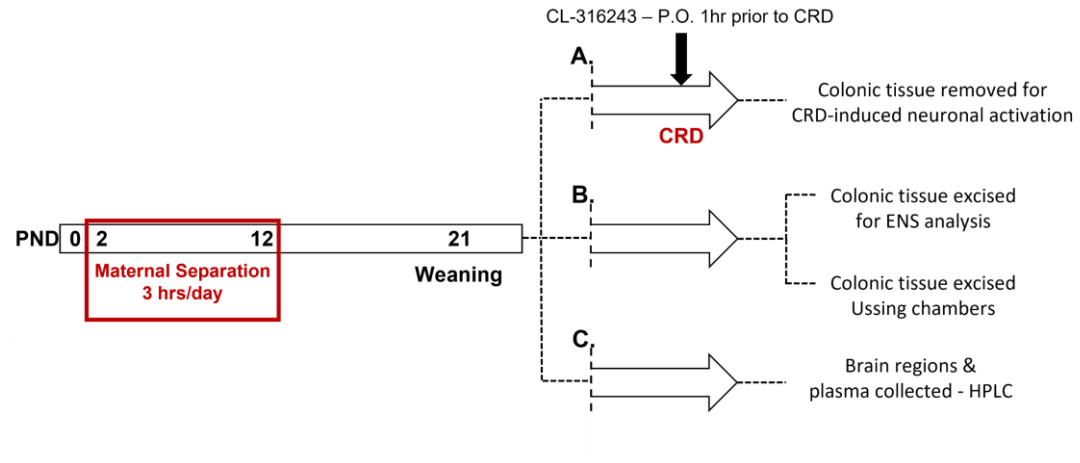


Figure 1. Experimental design. (A) *Animals received CL-316243/saline 1 hour prior to CRD. Colonic tissues were excised for analysis of CRD-induced neuronal activation and the number of CRD-induced pain behaviours was scored.* ***(B)*** *Animals underwent maternal separation and colonic tissue was collected for analysis of β3-AR expression in the enteric nervous system and Ussing chamber experiments.* ***(C)*** *Animals received CL-316243, and brain regions and plasma collected for HPLC analysis. All animals from A, B, C above underwent the same conditions, with any differences shown. CRD; Colorectal distension, ENS; Enteric nervous system, P.O.; Perorally.*

Colorectal distension

The colorectal distension (CRD) protocol was carried out as previously described (O'Mahony et al., 2009) at PND77. Animals were fasted for 16 hours prior to the start of the procedure. Animals were lightly anaesthetised with isoflurane and a 6cm-long polyethylene balloon with a connecting catheter was inserted into the colon, 1cm proximal to the anus. The catheter was secured to the tail of the animal with surgical tape to prevent displacement. Animals were allowed to recover from the anaesthesia for 10 minutes prior to the start of the procedure. The CRD paradigm used was an ascending phasic distension from 0 to 80mmHg over an 8-minute period. Air inflation and pressure were monitored during the procedure using a customised barostat (Distender Series II, G and J Electronics, Toronto, ON, Canada). Pain behaviours were identified as abdominal retraction, withdrawal and stretching (O'Mahony et al., 2012). A trained observer, blinded to the experimental groups, scored each animal for the

threshold pressure, when the first pain behaviour was observed, as well as the number of pain behaviours displayed across all pressure ranges by each animal.

Colonic submucosal neuronal activation following colorectal distension

Given the high level of expression of the β3-AR on submucosal neurons and the role of these neurons in secretomotor activity relevant to IBS, the percentage of colonic submucosal neurons activated in response to CRD was assessed. Animals received CL-316243 via oral gavage 1 hour prior to CRD. Based on the efficacy of the 0.1mg/kg dose of CL-316243 in the visceral sensitivity assessment, this dose only was used here. 1 hour after the CRD had been carried out, colonic tissue was gently excised, rinsed, opened along the mesenteric line, and pinned flat on a Sylgard-coated (SYLGARD™ 184 Silicone Elastomer, Dow, CA, USA) dish where it was incubated for 10 minutes with 1μM nifedipine to induce muscle fiber relaxation. The tissue was then further stretched and fixed in Zamboni's fixative overnight for immunohistochemistry. Preparations of the submucosal plexus were prepared by microdissecting away the mucosa and separating the submucosa containing the submucosal plexus from the underlying circular and longitudinal muscle layers. The submucosal plexus preparations underwent three 15-minute washes in the washing solution (PBS with 0.1% Triton X-100) under gentle agitation. Following the washes, tissues were incubated with a sheep anti-c-Fos primary antibody (Chemicon, cat. no. AB1584) diluted in the washing solution at a concentration of 1:300 for 40 hours at 4°C. Following the incubation with the primary antibody, three 15-minute washes with the washing solution under gentle agitation were carried out. Tissues were then incubated for 2 hours at room temperature under gentle agitation with the secondary antibody

anti-sheep conjugated with CY3 (Jackson Immunoresearch cat. no. 713-165-003) diluted in the washing solution at a concentration of 1:100. A further three 15-minute washes with the washing solution were then carried out. For co-staining with the β3-AR, tissues were incubated with a goat anti-β3-AR primary antibody (Santa Cruz, cat. no. SC1473) diluted in the blocking solution (5% donkey serum in the wash solution) at a concentration of 1:100 for 40 hours at 4°C. Three washes of 15-minutes were then carried out under gentle agitation and the tissues were incubated with an anti-goat conjugated with FITC secondary antibody (Chemicon, cat. no. AP180F) diluted in the blocking solution at a concentration of 1:100 for 3 hours under gentle agitation at room temperature. Following the incubation period, tissues were again washed (three times x 15 minutes each) under gentle agitation before being mounted and coverslipped for imaging. Sections were imaged using an Olympus BX51 fluorescent microscope equipped with an oil immersion 100x objective lens. The number of neurons with nuclear c-Fos staining expressed as a percentage of total c-Fos^{+ve} neurons activated in response to CRD was analysed in 20 randomly selected submucosal ganglia, and an average value per animal was used. The number of neurons with nuclear c-Fos^{+ve} staining expressed as a percentage of β3-AR^{+ve} neurons was assessed using the same method to investigate the involvement of the β3-AR in neuronal response to visceral stimulus.

β3-AR expression in colonic submucosal preparations

To investigate the expression of the β3-AR in colonic submucosal plexi, colonic submucosal plexus preparations were prepared and immunohistochemically stained for the β3-AR. Briefly, colonic tissue was gently excised, rinsed, opened along the

mesenteric line, and pinned flat in a Sylgard-coated (SYLGARD™ 184 Silicone Elastomer, Dow, CA, USA) dish where it was incubated for 10 minutes with 1μM nifedipine to induce muscle fiber relaxation. The tissue was then further stretched and fixed in Zamboni's fixative overnight for immunohistochemistry. Preparations of the submucosal plexus were prepared by microdissecting away the mucosa and separating the submucosa containing the submucosal plexus from the underlying circular and longitudinal muscle layers. The submucosal plexus preparations underwent three 15-minute washes in the washing solution (PBS-0.1% Triton X-100) under gentle agitation before being incubated with the blocking solution (5% donkey serum in the washing solution) for 1 hour at room temperature. Tissues were then incubated for 40 hours at 4°C with a goat anti-β3-AR primary antibody (Santa Cruz, cat. no. SC1473) diluted in the blocking solution at a concentration of 1:100. Following this, three 15-minute washes with the washing solution were carried out under gentle agitation and tissues were then incubated with an anti-goat conjugated with FITC secondary antibody (Chemicon, cat. no. AP180F) diluted in the blocking solution at a concentration of 1:100 for 2 hours under gentle agitation at room temperature. Three 15-minute washes were carried out under gentle agitation, and tissues were incubated with a mouse anti-PGP9.5 primary antibody (UltraClone limited) diluted in blocking solution at a concentration of 1:400 overnight at 4°C. The next morning, three 15-minute washes with the washing solution were carried out under gentle agitation and tissues were incubated with a goat anti-mouse conjugated with rhodamine red secondary antibody (Jackson Immunoresearch, cat. no. 115-295-166) diluted in washing solution at a concentration of 1:400 for 2 hours at room temperature. Three further washes of 15 minutes each were carried out before tissues were mounted and coverslipped for imaging. Tissues were imaged using an Olympus BX51 fluorescent

microscope equipped with an oil immersion 100x objective lens. The number of β3-AR-expressing neurons in 10 randomly selected colonic submucosal ganglia was counted and expressed as a percentage of total PGP9.5^{+ve} neurons and an average value per animal used.

High-performance liquid chromatography (HPLC)

Determination of plasma tryptophan and kynurenine

Given that alterations in peripheral levels of tryptophan may affect central 5-HT and therefore serotonergic signalling, levels of tryptophan and kynurenine in plasma samples were determined as previously described (Fitzgerald et al., 2008). Briefly, 2µl of internal standard (3-Nitro-L-Tryosine) was added to 198µl of plasma prior to being deproteinised with 20µL of 4M perchloric acid. Samples were then centrifuged at 20,000g for 15 minutes at 4°C. 100µl of supernatant was transferred to an HPLC vial for analysis via a 20ul injection volume. Stock solutions of each standard were prepared in HPLC-grade water, and working solutions were prepared, aliquoted, and stored at -80°C until analysis, at which point 20µl of 4M perchloric acid was added and vortexed. 20µl of standards and sample supernatants were vortexed and injected into the HPLC system which consisted of a Waters 510 pump (Waters Ireland, Dublin, Ireland), 717plus cooled autosampler, a 996 PDA detector, a Hewlett Packard 1046A Fluorescent Detector (Waters Ireland, Dublin, Ireland), a waters bus SAT/IN module and a croco-cil column oven. System components were used in conjunction with Waters Empower software (Waters Ireland, Dublin, Ireland). HPLC grade Acetonitrile, acetic acid and perchloric acid were obtained from Alkem/Reagecon (Cork, Ireland). All samples were injected into a reverse phase Luna 3µm C18 100Å

size LC column 150 × 2mm (Phenomenex), which was protected by Krudkatcher disposable pre-column filters (Phenomenex) and SecurityGuard cartridges (Phenomenex). The mobile phase consisted of 50mM acetic acid and 100mM zinc acetate with 3% (v/v) acetonitrile and was filtered through MilliporeSigma 0.45µm HV Durapore membrane filters (AGB) and vacuum degassed prior to use. Compounds were then eluted isocratically over a 30-minute period at a flow rate of 0.3ml min-1 after injection. The column temperature was set to 30°C, and samples/standards were maintained at 4°C in the cooled autoinjector prior to injection. The fluorescence detector was set to an excitation wavelength of 254nm and 404nm emission wavelength. The UV detector was set to 330nm. Tryptophan and its metabolites were identified based on their characteristic retention times compared with injection standards, which were run at regular intervals during the sample analysis. The chromatograms obtained were analysed using the LabSolutions software (Shimadzu) and concentrations were determined using analyte/internal standard peak height ratios. Results are expressed as ng ml-1 of supernatant respectively.

Determination of CNS serotonin and tryptophan

Levels of tryptophan in the brainstem and frontal cortex were measured as the brainstem projects serotonergic processes to many areas of the brain, and the frontal cortex plays a major role in behaviour. Measurement of serotonin was performed as described previously (Browne et al., 2011, 2012). Briefly, brain tissue was sonicated in 1000µl of chilled HPLC grade water spiked with an internal standard 4 ng/20 µl of N-methyl 5-HT (Sigma Chemical Co., UK) as internal standard. The homogenates were centrifuge at 20,000g at 4 °C for 15 min using a MIKRO 22 R refrigerated

centrifuge. 250µl of the resulting sample supernatant was then added to 250µl of HPLC mobile phase prior to injection. For tryptophan analysis, brain tissue samples were homogenised as described for monoamine analysis and 20 µl of 4M perchloric acid was added to 200µl of sample supernatant which were then centrifuged at 20,000g at 4 °C for 15 min using a MIKRO 22 R refrigerated centrifuge. Samples were subsequently analysed as described above for plasma tryptophan The mobile phase for 5-HT analysis consisted of 0.1M citric acid, 0.1M sodium dihydrogen phosphate, 0.01mM EDTA (Alkem/Reagecon, Cork), 5.6mM octane-1-sulphonic acid (Sigma) and 9% (v/v) methanol (Alkem/Reagecon, Cork) and pH was adjusted to pH 2.8 using 4N sodium hydroxide (Alkem/Reagecon, Cork). Homogenates were then centrifuged at 14,000rpm for 15 minutes at 4°C following which 20µl of the supernatant was injected into the HPLC system consisting of an SCL 10-Avp system controller, LC-10AS pump, SIL-10A autoinjector (with sample cooler maintained at 40 °C), CTO-10A oven, LECD 6A electrochemical detector (Shimadzu) and an online Gastorr Degasser (ISS, UK). A reverse-phase column (Synergi 4u C18 250 × 4.6mm, Phenomenex) maintained at 30 °C was employed in the separation (flow rate 2 ml min-1), the glassy carbon working electrode combined with an Ag/AgCL reference electrode (Shimdazu) was operated at +0.8V and the chromatograms generated were analysed using Class-VP 5 software (Shimadzu). Serotonin and its metabolites were determined based on their characteristic retention times as determined by the injection standards which were run at regular intervals during the sample analysis. Concentrations were determined using analyte/internal standard peak heigh ratios and expressed as ng g-1 of tissue.

Ussing chamber studies

Animals naïve to CRD were euthanised by decapitation and distal colonic segments were gently excised and placed in chilled Krebs solution (1.2mM NaH2PO4, 116mM NaCl, 4.8mM KCl, 1.2mM MgCl2, 25mM NaHCO3, 2.5mM CaCl2 and 10mM D-glucose) for Ussing chamber experiments as previously described (Carroll et al., 2013; Hyland et al., 2015) to investigate potential involvement of secretomotor neurons in MS-induced visceral hypersensitivity and potential mechanism of action of CL-316243. Seromuscular stripping was performed by blunt dissection under a stereomicroscope, and both longitudinal and circular muscle layers were removed. The resultant mucosal-submucosal segments were mounted onto vertical NaviCyte diffusion chambers (Harvard apparatus, Kent, UK) and were maintained at 37°C in Krebs solution and were constantly supplied with carbogen (95% O2 and 5% CO2). Tissues were voltage clamped to 0mV using an automatic voltage clamp (DVC-1000/EVC-4000, World Precision Instruments, Saratosa, Florida, USA). Once a stable baseline had been obtained, tissues were treated basolaterally with CL-316243 (1µM) and changes in short circuit current (I_{SC}) were recoded. The effects of the neurotoxin tetrodotoxin (300nM) on the CL-316243 response was also measured as well as the effect of CL-316243 on Ca^{2+}-evoked ion transport. The effect of CL-316243 on neuronal- and cAMP-evoked secretomotor activity using veratridine (30µM) or the pro-secretory agent forskolin (10µM) was also assessed. All measurements were recorded continuously on a computer using the LabTrax data acquisition hardware and analysed using DataTrax software (World Precision Instruments, Saratosa, Florida, USA).

Statistical analysis

Data were analysed using the statistical software package SPSS 28.0 (IBM) and were expressed as mean ± SEM. Data were assessed for normality using the Shapiro-Wilk test and Levene's test for equality of variances. Any statistical outliers were removed prior to analysis using Grubb's test. Normally distributed data were analysed using one-way ANOVA, two-way ANOVA, independent samples t-test, and Tukey's HSD post hoc where appropriate. A p-value of 0.05 was set as the threshold of statistical significance. "*n*" indicates the number of animals per experimental group.

Results

β3-AR agonism reverses maternal separation-induced visceral hypersensitivity

To assess the potential of β3-AR agonism on visceral pain perception, the total number of pain behaviours as well as the threshold pressure to CRD were analysed. Firstly, to investigate the effect of MS on the total number of pain behaviours, independent samples t-test revealed a significant difference between NS-Vehicle and MS-Vehicle groups whereby MS animals displayed a higher total number of pain behaviours in response to CRD (NS-Vehicle; 10.75 ± 1.49, MS-Vehicle; 27.56 ± 2.81, $t(23) = -5.818$, $p < 0.001$) **(Figure 2A)**. Next, to investigate the effect of β3-AR agonism on the total number of pain behaviours in MS animals, one-way ANOVA revealed a significant main effect of pharmacological intervention ($F(2,28) = 7.382$, $p = 0.003$) with Tukey's post hoc revealing specific differences between MS-Vehicle and both MS-0.1mg kg^{-1} ($p = 0.003$) and MS-1mg kg^{-1} ($p = 0.02$) groups whereby the total number of pain behaviours was decreased in both pharmacological intervention groups versus vehicle **(Figure 2A)**.

Similarly, independent samples t-test revealed a significant difference between NS-Vehicle and MS-Vehicle groups whereby MS animals displayed a lower threshold pressure to CRD than their NS counterparts (NS-Vehicle; 50.91 ± 2.74, MS-Vehicle; 29.67 ± 1.58, $t(22.051) = 6.71$, $p < 0.001$) **(Figure 2B)**. When the effect of β3-AR agonism in MS animals was analysed, one-way ANOVA revealed a significant main effect of pharmacological intervention ($F(2,29) = 6.404$, $p = 0.005$) on threshold pressure to CRD. Further analysis using Tukey's post hoc revealed significant differences between MS-Vehicle and MS-0.1mg kg^{-1} ($p = 0.003$) groups whereby the

threshold in the pharmacological intervention group was increased versus vehicle **(Figure 2B)**.

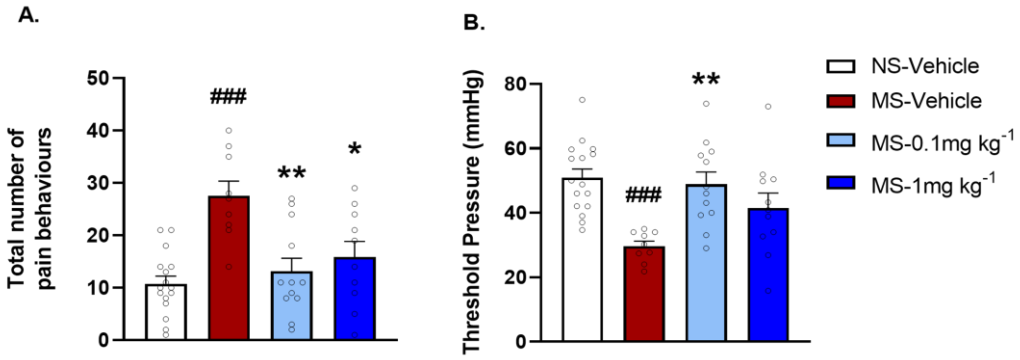


Figure 2. Maternal separation (A) Increases the total number of pain behaviours and (B) Decreases threshold pressure in response to CRD. *###Independent samples t-test NS-Vehicle versus MS-Vehicle, $p \leq 0.001$.* ***β3-AR agonism (A) Decreases the total number of pain behaviours at both doses of CL-316243*** *and* ***(B) Restores the threshold pressure to near control levels at the 0.1mg kg^{-1} dose of CL-316243.*** **$p \leq 0.05$, **$p \leq 0.01$ versus MS-Vehicle group. Data presented as Mean ± SEM. n = 9-16 per group. NS=Non-separated; MS=Maternally separated.*

β3-AR expression in colonic submucosal plexus

To examine the expression of β3-AR in colonic submucosal plexi, the number of β3-AR^{+ve} neurons expressed as a percentage of PGP9.5^{+ve} neurons was analysed. Independent samples t-test showed no difference between the two groups (NS-Control; 86.25 ± 2.76, MS-Control 85.85 ±0.77, t(7) = 0.124, p = 0.904) **(Figure 3A&B)**.

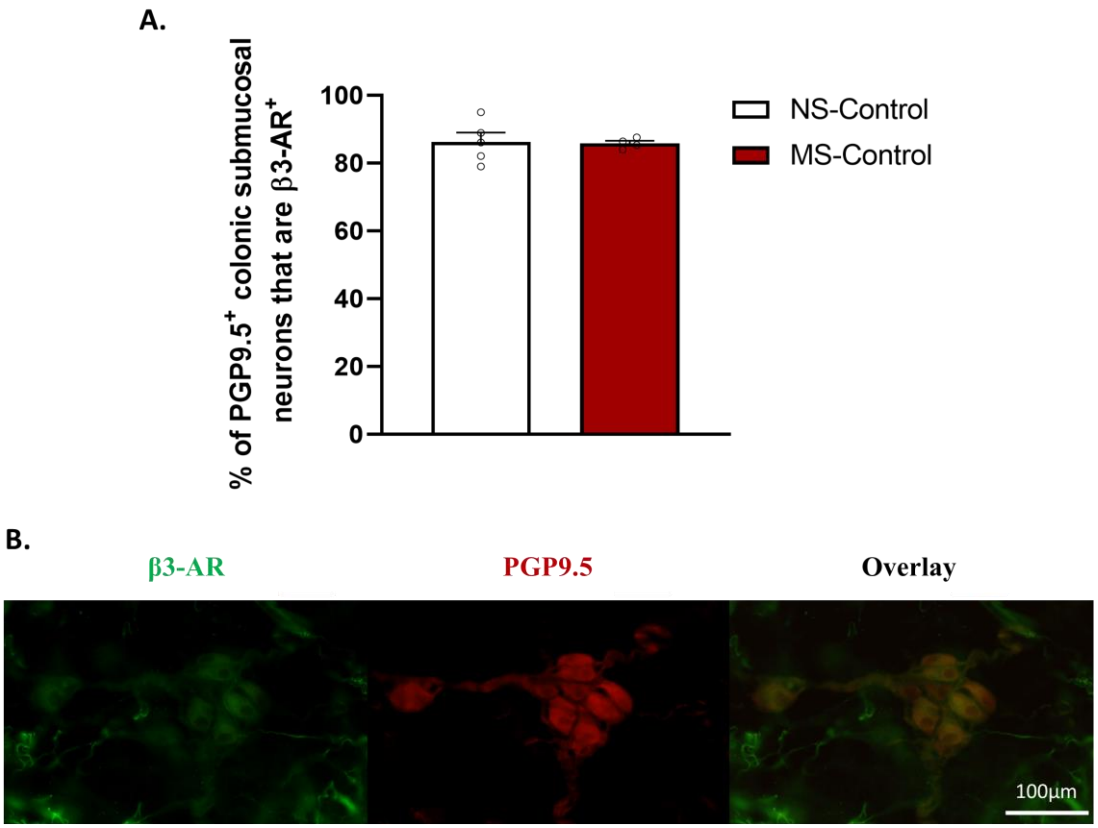


Figure 3. (A) Number of β3-AR^{+ve} neurons expressed as a percentage of PGP9.5^{+ve} neurons in the colonic submucosal plexus. (B) Representative photomicrographs of the expression of β3-AR on PGP9.5^{+ve} neurons. *β3-AR staining in green, PGP9.5 staining in red. Scale bar=100µm. Data presented as Mean ± SEM. n = 4-5 per group. AR=Adrenoceptor; NS=Non-separated; MS=Maternally separated.*

Activation of colonic submucosal neurons following colorectal distension

To assess the activation of colonic submucosal neurons in response to CRD, the percentage of neurons with nuclear c-Fos staining was analysed. Two-way ANOVA revealed a significant main effect of pharmacological intervention ($F(1,27) = 12.016$, $p = 0.002$), but not of ELS ($F(1,27) = 2.387$, $p = 0.134$), nor of an ELS*pharmacological intervention interaction ($F(1,27) = 1.339$, $p = 0.257$) on the percentage of neurons with nuclear c-Fos^{+ve} staining following CRD **(Figure 4A)**. Further analysis using Tukey's post hoc revealed a significant difference between NS-Vehicle and NS-0.1mg kg^{-1} groups whereby the 0.1mg kg^{-1} dose of CL-316243

significantly decreased the percentage of neurons activated following CRD (p = 0.02) **(Figure 4A)**.

To further investigate the involvement of the β3-AR in this neuronal response to the noxious stimulus of CRD, the number of c-Fos^{+ve} neurons was expressed as a percentage of β3-AR^{+ve} neurons. Interestingly, two-way ANOVA revealed a significant main effect of pharmacological intervention ($F(1,27) = 12.62$, $p = 0.001$), but not of ELS ($F(1,27) = 1.863$, $p = 0.184$), nor of an ELS*pharmacological intervention interaction ($F(1,27) = 0.886$, $p = 0.355$) on the percentage of β3-AR^{+ve} neurons activated in response to CRD **(Figure 4B)**. Post hoc analysis using Tukey's post hoc revealed a significant decrease in percentage of β3-AR^{+ve} neurons activated in NS-0.1mg kg^{-1} versus NS-Vehicle animals (p = 0.024) **(Figure 4B)**.

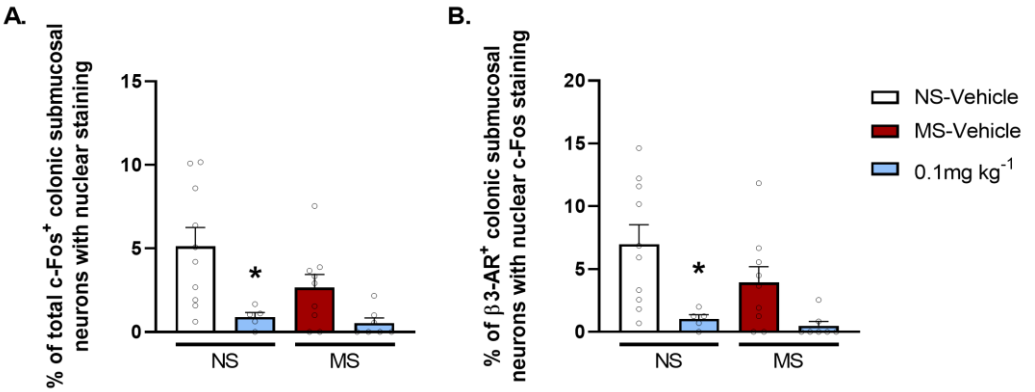


Figure 4. Percentage of (A) Total c-Fos^{+ve} neurons (B) β3-AR^{+ve} neurons activated in response to CRD as noted by nuclear c-Fos staining. **p ≤ 0.05 versus NS-Vehicle group. Data presented as mean ± SEM. n = 5-10 per group. AR=Adrenoceptor; NS=Non-separated; MS=Maternally separated.*

β3-AR agonism alters central and peripheral levels of tryptophan

To investigate the potential involvement of the central serotonergic system in the mechanism of action of β3-AR agonism against visceral hypersensitivity, first plasma levels of tryptophan were analysed. Two-way ANOVA revealed a main effect of

pharmacological intervention ($F(2,39) = 30.128$, $p < 0.001$), but not of ELS ($F(1,39) = 0.262$, $p = 0.611$), nor of an ELS*pharmacological intervention interaction ($F(2,39) = 0.341$, $p = 0.713$) on plasma levels of tryptophan. Further analysis using Tukey's post hoc revealed significant differences between NS-Control and NS-1mg kg^{-1} groups ($p < 0.001$) as well as between NS-0.1mg kg^{-1} and NS-1mg kg^{-1} groups ($p = 0.022$) whereby the 1mg kg^{-1} dose of CL-316243 reduced plasma tryptophan versus both NS groups. Similarly, in MS animals the 0.1mg kg^{-1} and the 1mg kg^{-1} dose of CL-316243 decreased plasma tryptophan versus control ($p = 0.033$ and $p < 0.001$ respectively) **(Figure 5A)**. Two-way ANOVA also revealed a significant main effect of ELS ($F(1,37) = 51.469$, $p < 0.001$), of pharmacological intervention ($F(2,37) = 21.269$, $p < 0.001$), and of an ELS*pharmacological intervention interaction ($F(2,37) = 3.285$, $p = 0.049$) on the plasma kynurenine:tryptophan ratio. Tukey's post hoc revealed that the 1mg kg^{-1} dose of CL-316243 increased the plasma kynurenine:tryptophan ratio versus NS-Control ($p < 0.001$) and versus the 0.1mg kg^{-1} group ($p = 0.004$) **(Figure 5B)**. Interestingly, the plasma kynurenine:tryptophan ratio was lower in MS-Control versus NS-Control animals ($p = 0.047$) as revealed by Tukey's post hoc. Similarly, the 1mg kg^{-1} dose of CL31623 increased the plasma kynurenine:tryptophan ratio in MS animals ($p = 0.05$) **(Figure 5B)**. This change in kynurenine:tryptophan ratio was independent of alterations in levels of kynurenine (data not shown).

Central levels of tryptophan and 5-HT were also measured. Two-way ANOVA revealed a significant main effect of ELS ($F(1,42) = 16.992$, $p < 0.001$), and of pharmacological intervention ($F(2,42) = 8.034$, $p = 0.001$), but not of an ELS*pharmacological intervention interaction ($F(2,42) = 0.791$, $p = 0.46$) on brainstem levels of tryptophan. Post hoc analysis using Tukey's did not reveal any significant differences between groups **(Figure 5C)**. No significant main effect of ELS

($F(1,39) = 3.058$, $p = 0.088$), nor of pharmacological intervention ($F(2,39) = 0.571$, $p = 0.57$), nor of an ELS*pharmacological intervention interaction ($F(2,39) = 1.824$, $p = 0.175$) on brainstem 5-HT levels was found using two-way ANOVA **(Figure 5D)**. Significant main effects of ELS ($F(1,41) = 14.593$, $p < 0.001$), and of pharmacological intervention ($F(2,41) = 7.572$, $p = 0.002$) and of an ELS*pharmacological intervention interaction ($F(2,41) = 3.466$, $p = 0.041$), were noted on frontal cortex tryptophan levels. Tukey's post hoc revealed that the 1mg kg^{-1} dose increased frontal cortex tryptophan levels in NS animals ($p = 0.041$), whereas the 0.1mg kg^{-1} dose increased frontal cortex tryptophan in MS animals ($p = 0.05$) **(Figure 5E)**. Finally, two-way ANOVA revealed a significant main effect of ELS ($F(1,42) = 6.473$, $p = 0.015$), but not of pharmacological intervention ($F(2,42) = 1.583$, $p = 0.217$), nor of an ELS*pharmacological intervention interaction ($F(2,42) = 1.743$, $p = 0.187$) on frontal cortex levels of 5-HT and no significant differences between groups were noted **(Figure 5F)**.

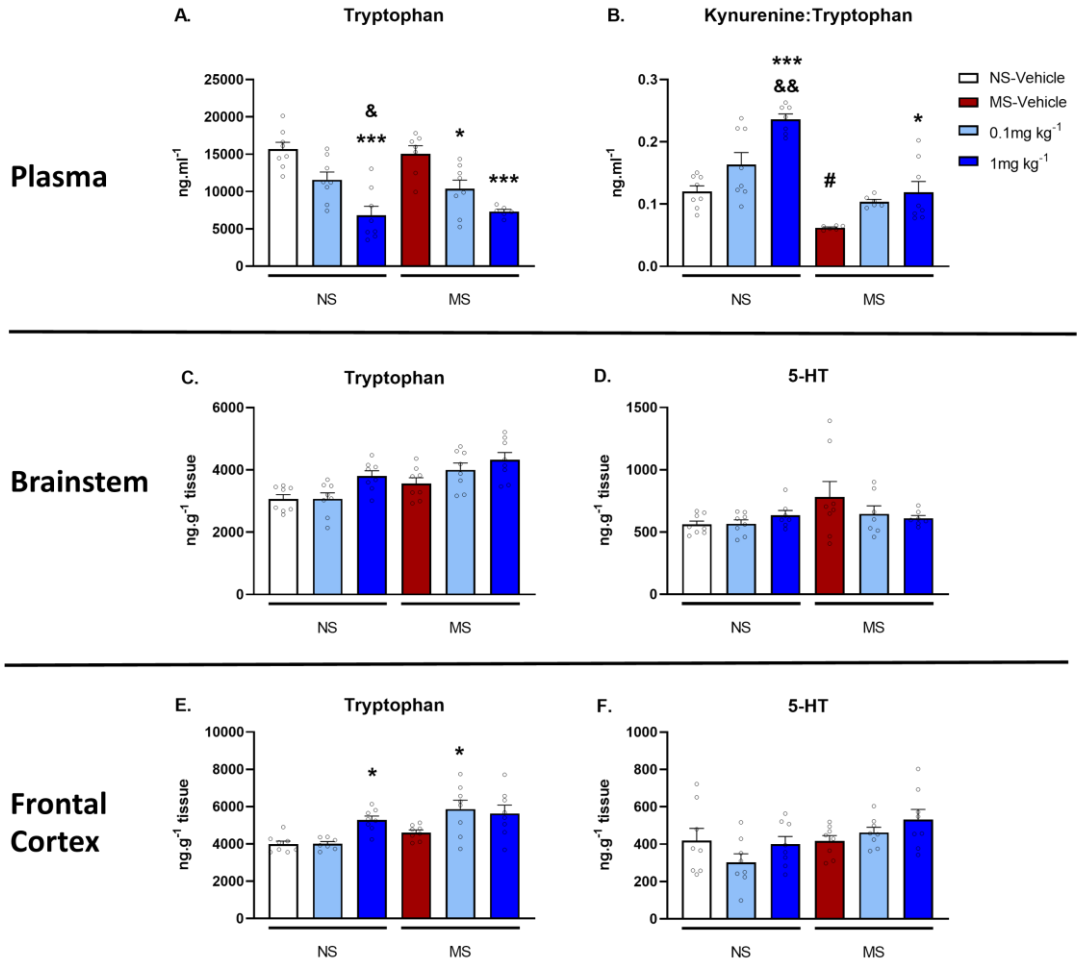


Figure 5. β3-AR agonism alters central and peripheral tryptophan levels. Effect of CL-316243 on (A) Plasma tryptophan (B) Plasma kynurenine:tryptophan ratio (C) Brainstem tryptophan (D) Brainstem serotonin (E) Frontal cortex tryptophan (F) Frontal cortex serotonin. **p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 relative to respective control group, &p ≤ 0.05, &&p ≤ 0.01 versus respective 0.1 mg kg^{-1} CL-316243 group, #p ≤ 0.05 MS-Vehicle versus NS-Vehicle. Data presented as mean ± SEM. n = 6-8 per group. 5-HT=Serotonin; NS=Non-separated; MS=Maternally separated.*

Characterisation of the effects of β3-AR modulation on electrophysiological measures of the colon

To characterise the effects of modulation of the β3-AR on secretomotor activity of the colon both at baseline and in response to tissue challenge, the β3-AR agonist CL-316243 was added to colonic tissue in the Ussing chamber.

Firstly, when the effect of the β3-AR agonist CL-316243 on baseline I_{SC} was analysed, two-way ANOVA between the vehicle-treated and CL-316243-treated groups did not reveal a significant main effect of ELS ($F(1,39) = 0.006$, $p = 0.936$), nor of pharmacological intervention ($F(1,39) = 0.648$, $p = 0.426$), nor of an ELS*pharmacological intervention interaction ($F(1,39) = 0.002$, $p = 0.969$) **(Table 1A)**. When the effect of neural blockade with tetrodotoxin on the CL-316243 response was assessed, two-way ANOVA between the CL-316243-treated and CL-316243 + TTX-treated groups revealed a significant main effect of pharmacological intervention ($F(1,40) = 13.21$, $p < 0.001$), but not of ELS ($F(1,40) = 1.9$, $p = 0.176$), nor of an ELS*pharmacological intervention interaction ($F(1,40) = 1.998$, $p = 0.165$). Further analysis using Tukey's post hoc revealed a significant difference between the NS-CL-316243 and NS-CL-316243 + TTX group whereby short circuit current was higher in the CL-316243 + TTX-treated group ($p = 0.005$) **(Table 1A)**.

When the effect of CL-316243 on Ca^{2+}-evoked secretory responses in colonic tissue was investigated, two-way ANOVA did not reveal a significant main effect of ELS ($F(1,42) = 0.48$, $p = 0.492$), nor of pharmacological intervention ($F(1,42) = 0.105$, $p = 0.747$), nor of an ELS*pharmacological intervention interaction ($F(1,42) = 0.648$, $p = 0.425$) on the second peak of bethanechol stimulation was noted by two-way ANOVA **(Table 1B)**.

Next, when the effect of CL-316243 on cAMP-evoked secretory response was assessed, two-way ANOVA revealed no significant main effect of ELS ($F(1,43) = 1.843$, $p = 0.182$), nor of pharmacological intervention ($F(1,43) = 3.311$, $p = 0.076$), nor of an ELS*pharmacological intervention interaction ($F(1,43) < 0.001$, $p = 0.995$) on short circuit current **(Table 1C)**.

Lastly, when the effect of CL-316243 on neurally-evoked secretory response was investigated, two-way ANOVA revealed a significant main effect of pharmacological intervention ($F(1,36) = 6.467$, $p = 0.015$), but not of ELS ($F(1,36) = 0.846$, $p = 0.364$), nor of an ELS*pharmacological intervention interaction ($F(1,36) = 0.018$, $p = 0.895$) on short circuit current **(Table 1D)**.

Table 1. (A) Effects of neuronal blockade on the β3-AR agonist response. (B) Effect of β3-AR agonist on Ca^{2+}-evoked secretory response after 45 minutes of incubation with bethanechol. (C) Effect of β3-AR agonist on cAMP-evoked secretory response. (D) Effects of β3-AR agonist on neuronally-evoked secretomotor response. *Data presented as Mean ± SEM. n = 9-13 per group. TTX=Tetrodotoxin. *Significantly higher versus NS-CL-316243 alone, p = 0.005.*

Group	Short circuit current (Mean ± SEM (μA.cm^{-2}))	
	Non-separated	Maternally separated
A. Effect of neural blockade with tetrodotoxin of the CL-316243 response		
Vehicle-treated	0.433 ± 0.978	0.648 ± 1.019
CL-316243-treated	1.946 ± 2.372	2.019 ± 1.602
CL-316243 + TTX	**12.509 ± 2.439*	6.667 ± 1.667
B. Effect of β3-AR agonist on bethanechol (Ca^{2+}) -evoked secretory response		
Vehicle-treated	92.847 ± 12.791	76.833 ± 11.656
CL-316243-treated	80.769 ± 10.361	81.97 ± 6.117
C. Effect of β3-AR agonist on forskolin (cAMP)-evoked secretory response		
Vehicle-treated	92.273 ± 11.973	110.5 ± 17.553
CL-316243-treated	116.731 ± 14.233	135.128 ± 10.017
D. Effects of β3-AR agonist on veratridine (neurally)-evoked secretomotor response		
Vehicle-treated	53.889 ± 5.312	67.037 ± 8.256
CL-316243-treated	87.307 ± 15.16	97.13 ± 12.881

Discussion

The current study aimed to assess the therapeutic potential of the β3-AR agonist CL-316243 against ELS-induced visceral hypersensitivity and investigate possible serotonergic or colonic secretomotor activity-dependent mechanisms. We demonstrate that agonism of the β3-AR ameliorated ELS-induced visceral hypersensitivity and decreased the percentage of colonic submucosal neurons activated in response to the noxious stimulus of CRD. We also noted that CL-316243 altered central and peripheral levels of tryptophan in the frontal cortex and plasma respectively without any change in 5-HT levels. Finally, we show that modulation of the β3-AR affects secretomotor activity in colonic tissue *ex-vivo*. Overall, these results support the use of CL-316243 against disorders of visceral pain and provide novel insights into the possible mechanisms of action of CL-316243 via central and peripheral tryptophan and secretomotor, fluid and electrolyte homeostasis.

In agreement with the literature, we demonstrate that MS results in visceral hypersensitivity to CRD (O'Mahony et al., 2009; O'Mahony et al., 2020; O'Mahony et al., 2011; Yi et al., 2017). Visceral hyperalgesia was noted both by an increase in the total number of pain behaviours as well as a decrease in the threshold pressure before the first pain behaviour was displayed in the MS-Vehicle group versus non-separated controls. Interestingly, we show that the 0.1mg kg^{-1} dose of CL-316243 was effective in both reducing the total number of pain behaviours as well as restoring the threshold pressure to control levels, while the 1mg kg^{-1} dose reduced the total number of pain behaviours only. This beneficial effect of CL-316243 has also been noted in another model using mustard oil to induce visceral pain (Cellek et al., 2007), however,

this is the first study to report the beneficial effect of CL-316243 against MS-induced visceral hypersensitivity.

Next, we report that the abundance of β3-AR^{+ve} neurons in colonic submucosal plexi is not significantly different between NS and MS animals. The expression of the β3-AR in cholinergic neurons of the myenteric and submucosal plexi of the enteric nervous system has been noted previously (Cellek et al., 2007), however, we show here for the first time that there is no significant difference in the abundance of β3-AR^{+ve} neurons in colonic submucosal plexi between NS and MS animals. Given that in the visceral sensitivity assessment no dose-dependent difference was noted, the 0.1mg kg^{-1} dose was used to assess CRD-induced enteric neuronal activation. Despite this equal abundance of β3-AR^{+ve} neurons in NS and MS animals, we show that there appears to be fewer β3-AR^{+ve} neurons activated in response to CRD in MS animals. Although this observation did not meet the threshold of statistical significance, it could suggest that this reduced activation of β3-AR^{+ve} neurons may play a role in MS-induced visceral hypersensitivity by decreasing inhibitory signalling mechanisms. The activation of these neurons in response to the noxious stimulus of CRD likely includes the mucosal afferents which are responsive to low-intensity tactile stimulation, but are unresponsive to stretch (Brierley and Gastroenterology, 2004), and the muscular afferents which are distension and stretch receptive (Brookes et al., 2013). Paradoxically, the 0.1mg kg^{-1} dose of CL-316243 further reduced the percentage of both total and β3-AR^{+ve} neurons activated in response to CRD in NS animals only. This may be explained by the observation that GW427353, a human-selective β3-AR agonist decreased the excitability of submucosal enteric neurons in humans (Schemann et al., 2010).

When the effects of β3-AR agonism on tryptophan metabolism were assessed, we found that the 1mg kg^{-1} dose of CL-316243 significantly decreased plasma tryptophan in both NS and MS animals, whereas the 0.1mg kg^{-1} dose of CL-316243 reduced plasma tryptophan levels in both NS and MS animals, although only in the MS animals did this reach the threshold of statistical significance. This decrease in peripheral levels of tryptophan drove changes in the kynurenine:tryptophan ratio whereby the ratio was increased by the 1mg kg^{-1} dose of CL-316243 in both NS and MS animals with no change in plasma kynurenine being seen (data not shown). No changes in brainstem tryptophan or 5-HT were noted, however, an increase in frontal cortex tryptophan induced by the 1mg kg^{-1} dose of CL-316243 in NS animals and the 0.1mg kg^{-1} dose of CL-316243 in MS animals was seen. CL-316243 has been shown previously to increase brain tryptophan in mice (Lenard et al., 2003), as well as hypothalamic 5-HT (Conley et al., 2006). Importantly, these alterations in central and peripheral tryptophan levels do not translate into increased central 5-HT, suggesting that central serotonergic signalling does not play a role in the mechanism of action of CL-316243.

Finally, CL-316243 alone had no effect on baseline I_{SC} in colonic tissue. Interestingly, when the neurotoxin tetrodotoxin was added, the response to CL-316243 increased in both NS and MS tissues, although only significantly in the NS group. This may suggest that neural blockade with tetrodotoxin may be relieving inhibitory enteric neuronal input on secretomotor activity, which when relieved allows for alterations in secretomotor activity induced by CL-316243. This may also suggest a non-neuronal site of action for the β3-AR agonist as once inhibitory enteric nervous system input is relieved by tetrodotoxin, an increase in short circuit current is seen, suggesting that CL-316243 may be acting at a non-neuronal site. Alternatively, it has been shown that different classes of colonic submucosal neurons thought to be related to secretomotor

activity are tetrodotoxin-insensitive (Lomax et al., 2001), which suggests that these neurons that are insensitive to tetrodotoxin may be affecting secretomotor activity when neuronally-mediated inhibitory input is alleviated. Broadly, these secretomotor neurons are classified as cholinergic and non-cholinergic and have been further characterised in the rodent submucosal plexus as vasoactive intestinal peptide/neuropeptide Y/tyrosine hydroxylase/calretin-expressing non-cholinergic and vasoactive intestinal peptide/neuropeptide Y/tyrosine hydroxylase/calretin-expressing cholinergic neurons (Mongardi Fantaguzzi et al., 2009). Given the observation that short circuit current is non-significantly lower in MS tissue even when potential enteric nervous system-mediated inhibitory input is relieved, this may suggest that MS animals display altered secretomotor activity versus control animals. Previously, it has been shown that MS results in an increase in baseline I_{SC} in pre-adolescent rats which decreased to the level of NS animals by PND30 (Gareau et al., 2006). This finding of decreased short circuit current in MS animals, albeit non-significant, suggests either a pro-absorptive or pro-secretory state that may result in a constipation or diarrhoeal phenotype. Although more evidence exists to support increased gastrointestinal motility following MS (Moloney et al., 2015b; O'Mahony et al., 2009), it has been shown previously that exposure to MS results in a constipation phenotype in adolescence but not in adulthood where an increased faecal pellet output is seen (Yi et al., 2017). Further, a study reported that an inherently viscerally hypersensitivity strain of rats, the Wistar-Kyoto, also display decrease baseline I_{SC} versus Sprague Dawley controls, suggesting a possible link between secretomotor function and visceral sensitivity, however, when these Wistar-Kyoto rats were subjected to MS, they displayed an increase in cholinergic-induced ion transport (Hyland et al., 2015). A previous study in guinea pigs using another β3-AR agonist, SR58611A, reported that

its effects on colonic muscle relaxation were resistant to tetrodotoxin (De Ponti et al., 1995), exhibiting potential differential mechanisms of action dependent upon the agonist used.

Next, when the effect of the cholinomimetic bethanechol (Ca^{2+})-mediated secretomotor activity was investigated, we found no difference between the vehicle-treated and CL-316243-treated tissues following 45 minutes of incubation. These data suggest that CL-316243 acts independently of Ca^{2+}-evoked mechanisms to affect secretomotor activity.

The effect of β3-AR agonism on neuronally and cAMP-evoked secretory response was also investigated. Here, CL-316243 augmented the tissue response to challenge with both forskolin and veratridine, although not significantly, suggesting that β3-AR agonism enhances neuronal responsivity in colonic tissue. It has been shown previously through removal of the myenteric ganglia that there are complete secretomotor circuits in the submucosal plexus of the rat colon (Christofi et al., 2004). Based on our results showing that β3-AR agonism reduces the number of colonic submucosal neurons activated in response to CRD, this may suggest that the activity of the β3-AR may differ between pharmacological and physical neuronal stimulation.

The limitations of this study include that we did not further classify the type of neurons being activated in response to CRD. However, previous studies have shown a high level of expression of the β3-AR on cholinergic neurons (Coelho et al., 2017), and given the inhibitory role of cholinergic afferents in spinal pain mechanisms it would be reasonable to surmise that these neurons may play a role in the dampening down of the visceral pain response.

Overall, we demonstrate that CL-316243 ameliorates MS-induced visceral hypersensitivity and decreases the percentage of colonic submucosal neurons activated in response to the noxious stimulus of CRD. We also report changes in peripheral and frontal cortex tryptophan induced by CL-316243, however, these alterations do not translate into altered central 5-HT. Finally, we show that agonism of the β3-AR *ex vivo* results in increases in short circuit current in the presence of tetrodotoxin, supporting an ENS-mediated inhibitory input on β3-AR modulation of short circuit current.

Conclusion

The present study supports the β3-AR as a promising therapeutic target for visceral pain-associated disorders, the effects of which appear to act independently of central serotonergic signalling and secretomotor activity. Further studies are needed to elucidate precise mechanisms of action of β3-AR agonism against visceral hypersensitivity.

Chapter 3

Supplementation with Milk Fat Globule Membrane from Early Life Reduces Maternal Separation-Induced Visceral Pain Independent of Enteric Nervous System or Intestinal Permeability Changes in the Rat

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Abstract

Nutritional approaches have emerged over the past number of years as suitable interventions to ameliorate the enduring effects of early life stress. Maternal separation (MS) is a rodent model of early life stress which induces widespread changes across the microbiota-gut-brain axis. Milk fat globule membrane (MFGM) is a neuroactive membrane structure that surrounds milk fat globules in breast milk and has been shown to have positive health effects in infants, yet mechanisms behind this are not fully known. Here, we investigated the effects of MFGM supplementation from birth on a variety of gut-brain signalling pathways in MS and non-separated control animals across the lifespan. Specifically, visceral sensitivity as well as spatial and recognition memory were assessed in adulthood, while gut barrier permeability, enteric nervous system and glial network structure were evaluated in both early life and adulthood. MS resulted in visceral hypersensitivity, which was ameliorated to a greater extent by supplementation with MFGM from birth. Modest effects of both MS and dietary supplementation were noted on spatial memory. No effects of MS were observed on enteric neuronal or glial networks in early life or adulthood, however an increase in the immunoreactivity of βIII-tubulin in adult colonic myenteric ganglia was noted in the MFGM intervention non-separated group. In conclusion, dietary supplementation with MFGM from birth is sufficient to block MS-induced visceral hypersensitivity, highlighting its potential value in visceral pain-associated disorders, but future studies are required to fully elucidate the mechanistic role of this supplementation on MS-induced visceral pain.

Introduction

Stressful events during the early postnatal period have been shown to have particularly detrimental effects on host development and physiology by leading to long-lasting perturbations in several systems including the gastrointestinal (GI), endocrine (Heim et al., 2002; O'Mahony et al., 2009; Rincel et al., 2019a), peripheral and central nervous systems (Juruena et al., 2020; O'Mahony et al., 2008; Osadchiy et al., 2019). Early life stress (ELS) has been previously shown to result in heightened visceral sensitivity (Coutinho et al., 2002; O'Mahony et al., 2009; O'Mahony et al., 2020; Videlock et al., 2009) characterised by a diffuse sensation of pain centred around the midline of the body and upper abdomen (Sikandar and Dickenson, 2012). The diffuse nature of the sensation of visceral pain results in poor localisation to the site of pain and is due to a paucity of visceral sensory innervation (Sikandar and Dickenson, 2012). ELS is also a known risk factor for the development of both stress-related psychiatric disorders such as depression and anxiety in humans (Cougle et al., 2010; Scott et al., 2010) as well as functional GI disorders such as irritable bowel syndrome (Bradford et al., 2012). Dysfunctional communication between the gut and the brain via the microbiota-gut-brain axis is critical in the manifestation of these disorders, with studies showing a causal relationship between ELS, mood disorders, and gut dysfunction (Bradford et al., 2012) (for review see (Cryan et al., 2019; O'Mahony et al., 2011; Sánchez et al., 2001; Wilmes et al., 2021). Maternal separation (MS) is a well-established rat model of ELS and gut-brain axis dysfunction (O'Mahony et al., 2011). In rats, MS has been shown to induce depressive and anxiety-like behaviours, increase gut epithelial barrier permeability and visceral sensitivity, and lead to stress hyper-responsivity (De Palma et al., 2015; Holschneider et al., 2016; McVey Neufeld et al., 2019; Moussaoui et al., 2017; O'Mahony et al., 2009; O'Mahony et al., 2011).

The enteric nervous system (ENS) is a network of neurons and glial cells embedded in the gut wall that is crucial for normal physiological GI function (Nezami and Srinivasan, 2010) and plays a key role in gut-brain axis signalling (Carabotti et al., 2015). MS has been shown to negatively impact on the ENS by increasing colonic cholinergic activity resulting in alterations in cholinergic regulation of epithelial permeability (Gareau et al., 2007a). Epithelial barrier integrity has also been shown to be compromised following MS, leading to bacterial translocation (Moussaoui et al., 2014). It has also been suggested that the ENS may play a role in the pathophysiology of visceral pain (Vergnolle, 2003). The ENS, spinal sensory afferent nerves, and enteric mast cells have been posited to play a role in visceral pain, however, mechanisms behind this are unclear (Wood, 2011).

Dietary interventions as strategies to ameliorate MS-induced psychopathology have proven effective with studies in rats using probiotics reporting a reversal of MS-induced depressive (Desbonnet et al., 2010) and anxiety-like behaviours (McVey Neufeld et al., 2019), as well as MS-induced gut barrier dysfunction (Gareau et al., 2007b). Modulation of diet for the symptomatic relief of GI disorders has also proven effective with probiotics having been shown to reduce abdominal pain in children with irritable bowel syndrome (Guandalini et al., 2010), as well as reduce the frequency and intensity of abdominal pain occurrences in school-aged children with functional GI disorders (Newlove-Delgado et al., 2017). Other studies have also reported reduced abdominal pain following administration of different probiotics (Ducrotté et al., 2012; Gawrońska et al., 2007; Whorwell et al., 2006).

In recent years, dietary interventions aimed at restoration of proper gut to brain communication have come to the fore as strategies for the management of symptoms of many mood disorders. For example, the Mediterranean diet has been shown to have

positive effects by reducing the symptoms of depression in humans (Dinan et al., 2019; McMillan et al., 2011; Opie et al., 2018), while a diet high in polyunsaturated fatty acids (PUFAs) has been shown to reduce the symptoms of anxiety in a cohort of undergraduate college students (Yehuda et al., 2005). A combination of eicosapentaenoic acid and docosahexaenoic acid (DHA), two PUFAs, has also been shown to reduce anxiety-like behaviour in rats (Pusceddu et al., 2015a) and reverse selective effects of ELS (Pusceddu et al., 2015b).

Milk fat globule membrane (MFGM) is a triple layer membrane structure that surrounds milk fat globules secreted by mammary epithelial cells during lactation and has been shown to potentially confer health benefits and may be responsible for some of the benefits of breastfeeding (Bourlieu and Michalski, 2015; Brink and Lönnerdal, 2020). Breastfeeding has been shown to confer several beneficial effects on health including on cognitive scores (Quigley et al., 2012) and lowering the risk of obesity later in life (Owen et al., 2005). Not only this, but exclusive breastfeeding for a longer duration has been shown to reduce the incidence of GI tract infections in infants (Kramer et al., 2001). It has been proposed that MFGM exerts effects on neurodevelopment and cognitive function with preclinical evidence showing changes in brain development in piglets following dietary supplementation with prebiotics, MFGM, and lactoferrin (Mudd et al., 2016). Furthermore, studies in humans have reported higher cognitive scores in the Bayley scales of infant development at 12 months of age in those whose diets were supplemented with MFGM (Timby et al., 2014). MFGM has also been shown to have beneficial effects on gut physiology whereby MFGM altered intestinal epithelial architecture. Specifically, it was observed that MFGM increased ileal and jejunal villus length, indicators of intestinal health, in a dose-dependent manner (Bhinder et al., 2017).

We have previously shown that post-weaning administration of MFGM ameliorated MS-induced visceral hypersensitivity and lead to an improvement in spatial learning and memory (O'Mahony et al., 2020). We also noted that administration of MFGM from weaning facilitated a faster return of stress-induced corticosterone levels to baseline, whilst also exerting effects on the gut microbiota at the family and genus level. However, it is unclear if interventions would induce greater effects if they began at an earlier timeframe. Thus, based on existent evidence as mentioned above, this current study aimed to lead a novel investigation into the effect of supplementation with MFGM from birth on MS-induced behavioural and GI physiological changes in early life and adulthood, with a particular focus on visceral sensitivity.

Methods

Animals and housing

Male and female Sprague Dawley rats (approximately 8 weeks of age) were purchased from Envigo, UK and were mated in the Biological Services Unit, Western Gateway Building, University College Cork, and subsequent offspring were used in this study. The day of birth was designated as postnatal day 0 (PND0). Dams and littermates were housed in large plastic breeding cages (15 x 22 x 9cm) in a humidity- and temperature-controlled room set to 21°C ± 1°C. The light/dark cycle was set to 12 hours (light phase 7am-7pm). All experiments were conducted in accordance with European Directive 2010/63/EEC, the requirements of S.I No 543 of 2012 and approved by the Animal Experimentation Ethics Committee of University College Cork.

Maternal separation model

Maternal separation was carried out as described previously (O'Mahony et al., 2009). Briefly, at PND0 litters were randomly assigned to maternally separated (MS) or non-separated (NS) groups. At PND2, the litters assigned to MS were moved from the main colony room to an adjacent room maintained at the same temperature (21 ± 2°C) and lighting conditions. The dam was first removed from the home cage and placed into a smaller holding cage, following which, the pups (entire litters) were gently transferred together into a small cage where they remained for 3 hours. Cages containing the pups were placed on heating pads set to 30–33°C and were filled with 3cm of bedding so the pups could thermoregulate as needed. The dam was returned to the home cage and transferred back to the main colony room for this time period to avoid communication between the dam and the pups. After the 3-hour separation, dams were again brought

into the adjacent room and pups were returned to their original home cages. NS litters were also transported to the same room as the MS groups to avoid the confound of transportation stress but were otherwise left undisturbed in their home cages with their dams with the exception of weekly cage cleaning. This procedure was repeated daily from PND2 to PND12 inclusive. The period of separation was carried out at the same time each day (9am–12pm). At PND21, offspring were sexed and weaned, and male offspring were used for the remainder of the study.

Dietary interventions and experimental design

Diets

Two custom rodent diets (control diet and MFGM-enriched diet) were used in this study and were formulated by Mead Johnson Nutrition based on AIN-93G specifications. The composition of the two diets is listed in **Supplementary Table 1**. Both the control and the MFGM-containing diets contained DHA/ARA oil 5.3 g/kg, however, the MFGM diet differs from the control diet by the inclusion of whey protein concentrate MFGM-10 15.9 g/kg. Food pellets containing both the control diet and MFGM-enriched diet were provided to pregnant dams from two days prior to the birth of the pups. Dietary supplementation continued throughout the lifespan of all experimental animals including during behavioural testing. All experimenters were blinded as to the type of diet administered. Upon completion of the experiments and subsequent data analysis, experimenters were informed of the contents of the diets.

Experimental design

Male offspring were divided into four experimental groups: NS-Control, MS-Control, NS-MFGM and MS-MFGM **(**see **Figure 1)** and were tested at two different life stages; PND21 and PND100. At PND21, offspring from each experimental group were culled by decapitation (*n* = 8-9 per group) and ileum and colon segments were gently excised and used for *ex vivo* intestinal permeability and immunohistochemistry experiments. At weaning, the remaining offspring were randomly group-housed (2-3 per cage) and underwent behavioural tests and visceral sensitivity assessment in adulthood **(**see **Figure 1)**. Following the assessment of visceral sensitivity, adult offspring (PND100) were culled by decapitation (*n* = 11-12 per group) and ileum and colon segments were removed and used for *ex vivo* intestinal permeability and immunohistochemistry experiments. From both PND21 and PND100 animals, the entire GI tract (from stomach to anus) was removed, and the length of the small intestine and colon was measured. The weight of the caecum including its contents was also measured. Body weight was recorded at PND21 (prior to culls in early life), at PND60 (prior to behavioural assessment) and at PND100 (prior to culls in adulthood). Experimental design and timeline are shown in **Figure 1**.

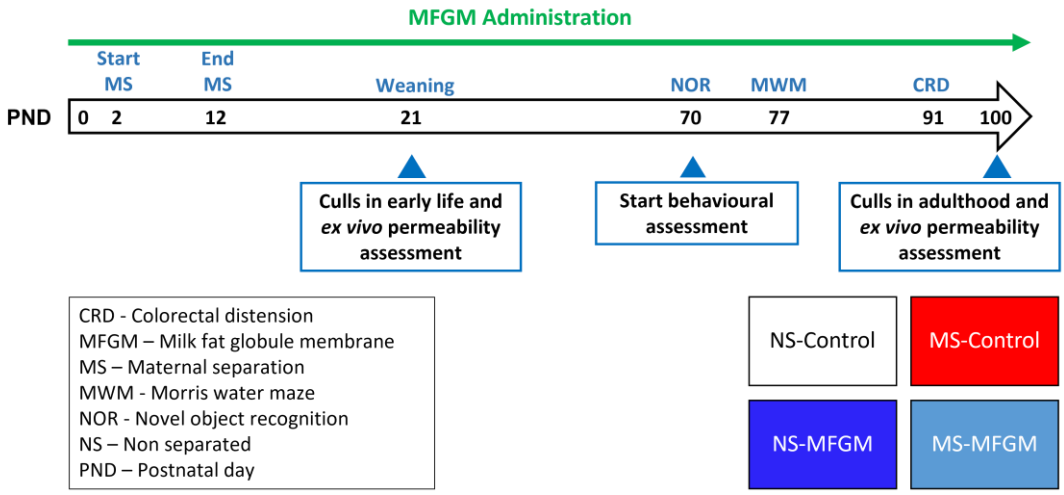


Figure 1: Overview of experimental groups and procedures.

Behavioural procedures

Behavioural assessment began at 10 weeks of age (PND70) in male offspring from each of the experimental groups. Between each test, animals were given a minimum of 1 week of a washout period to reduce the impact of the behavioural battery on subsequent behavioural tests as much as possible.

Novel object recognition

The novel object recognition test was carried out on PND70 and provides a readout of recognition memory and exploratory behaviour. The protocol used was adapted from Bevins and Besheer (Bevins and Besheer, 2006). The test was carried out over 2 days. On day 1, animals were allowed to freely explore the testing apparatus, a rectangular arena equipped with an overhead camera, for 10 minutes (habituation phase). No bedding was used, and the container was wiped with 70% ethanol in between each animal to remove odour cues. On day 2, two identical objects were placed in adjacent corners approximately 5cm from the wall of the arena, and animals were allowed a 10-minute exploration period (acquisition training period). Following the acquisition training period, animals were removed from the arena for a period of 1 hour. After this time, animals were returned to the arena, which this time contained one "familiar" object (the same used in the acquisition training period) and one "novel" object that the animals had not encountered previously. Interactions between the animal and the two objects were recorded by the overhead camera for 5 minutes for later analysis. The objects were cleaned before each trial with 70% ethanol to remove odour cues. The time spent exploring each of the objects was scored blinded using EthoVision (Noldus, UK). Exploratory behaviour was defined as orienting the nose towards the

object at a distance of less than 2cm, or direct contact with the object. A discrimination index was calculated according to the following formula: (t[novel] − t[familiar])/(t[novel] + t[familiar]). As rats are naturally explorative, a decrease in the discrimination index compared with controls indicates a deficit in recognition memory.

Morris water maze

The Morris Water Maze (MWM) was performed at PND77, using a protocol adapted from (O'Mahony et al., 2014). The MWM is used to assess spatial learning and reference memory of rodents, and it relies on distal cues to navigate from different start locations around the perimeter of an open swimming arena to find a submerged escape platform. Spatial learning is assessed across repeated trials, and spatial and reference memory is determined by preference for the platform area when the platform is absent (O'Mahony et al., 2014). The maze used was a circular pool 180cm in diameter and was filled with water (21°C ± 1°C) to a depth of 31cm. A transparent platform with a diameter of 10cm was placed in the middle of one of the quadrants so that it was submerged up to 3cm below the level of the water and was not visible from the surface. Four spatial cues were arranged around the maze to provide landmarks that would aid navigation to the platform. The test was conducted over 5 days. The animals received 4 days of training that consisted of 4 trials per day (acquisition training). At the beginning of each trial, animals were placed in 1 of 4 starting positions facing the wall of the tank and allowed to swim for 120 seconds or until it located the escape platform. A different starting position selected in a semi-randomised pattern was used for each of the 4 trials on a given day. If the animal was unable to locate the

platform within the allocated 120 seconds, the researcher gently guided the animal to the platform and detained it there for 30 seconds. On the fifth day of the test (probe trial), the platform was removed, and the animals were placed in a novel starting position and allowed to explore the arena for 60 seconds. The amount of time spent in the quadrant where the platform was situated previously was recorded using EthoVision (Noldus, UK) and was scored by an observer blinded to the experimental groups. Increased latency to find the platform and a decrease in the time spent in the quadrant where the platform was previously with respect to control animals is indicative of spatial memory deficits.

Visceral sensitivity assessment through colorectal distension

The colorectal distension (CRD) protocol was carried out as previously described (O'Mahony et al., 2009) on PND91. Animals were fasted for 16 hours prior to the start of the procedure. Animals were lightly anaesthetised with isoflurane and a 6-cm long polyethylene balloon with a connecting catheter was inserted into the colon, 1cm proximal to the anus. The catheter was secured to the tail of the animal with surgical tape to prevent displacement. Animals were allowed to recover from the anaesthesia for 10 minutes prior to the start of the procedure. The CRD paradigm used was an ascending phasic distension from 0 to 80mmHg over an 8-minute period. Air inflation and pressure were monitored during the procedure using a customised barostat (Distender Series II, G and J Electronics, Toronto, ON, Canada). Pain behaviours were identified as abdominal retraction, withdrawal and stretching (O'Mahony et al., 2012). A trained observer, blinded to the experimental groups, scored each animal for the

threshold pressure, when the first pain behaviour was observed, as well as the total number of pain behaviours displayed across all pressure ranges by each animal.

Ex-vivo permeability assessment

Intestinal permeability was assessed *ex vivo*. Distal ileum (a 1.5cm segment taken 2cm proximally to the caecum) and middle colon (a 1.5cm segment) specimens of PND21 and PND100 animals were mounted into vertical NaviCyte diffusion chambers with a 4mm round aperture (0.126cm^2 exposed tissue area). No seromuscular stripping was performed. 4mls of Krebs buffer (1.2mM NaH_2PO_4, 116mM NaCl, 4.8mM KCl, 1.2mM $MgCl_2$, 25mM $NaHCO_3$, 2.5mM $CaCl_2$ and 10mM D-glucose) was added to both the mucosal and serosal chambers for colonic specimens. For ileum specimens, 10mM mannitol was added to the mucosal chamber to avoid activation of sodium/glucose co-transporters in the epithelial cells of the small intestine and the associated increase in tight junction permeability (Turner et al., 1997). Chambers were continuously supplied with carbogen (95% O_2 and 5% CO_2). Tissues were not clamped, and electrophysiological measures were not recorded. Transepithelial permeability was investigated by measuring mucosal to serosal flux of 4kDa Fluorescein isothiocyanate (FITC)-dextran (FD4, Sigma-Aldrich, Ireland) using a sampling method as described previously (Golubeva et al., 2017). Briefly, FITC was added to the mucosal chamber to a final concentration of 2.5mg/ml and 200µl samples were taken from the serosal chamber every 30 minutes for the following 180 minutes. Samples taken were measured at 485nm excitation/535nm emission wavelengths. FITC mucosal to serosal flux was presented in µg/hour/cm^2.

Immunohistochemistry of colonic frozen sections

Colonic segments (1.5cm specimens adjacent to those used in the *ex vivo* permeability assessment) were collected from PND21 and PND100 animals from each of the experimental groups. Colonic segments were flushed with a 10mM Phosphate-buffered saline (PBS) and 10mM glucose solution and fixed in 4% paraformaldehyde (PFA) at 4°C for 4 hours. Following the fixation period, segments were washed in 10mM PBS and any excess PFA was dabbed off. Colonic segments were then placed in a cryoprotective solution comprised of 30% sucrose in 10mM PBS and 0.02% NaN_3 for 72 hours at 4°C to prevent microbial growth. Colonic samples were then washed in 10mM PBS and embedded in optimal cutting temperature (OCT) medium (VWR chemicals, Dublin, Ireland). Colonic samples were frozen at -20°C and then placed at -80°C until sectioning. Colonic samples were sectioned at 16µm thickness using a cryostat (Leica CM1900, Germany) and sections were mounted on Superfrost Plus slides for immunohistochemistry as previously described (Caputi et al., 2017b). Briefly, colonic frozen sections were thawed at room temperature for 15 minutes and then rinsed with three washes of 5 minutes with Tris-buffered saline (TBS). Sections were dried and incubated with 0.05M NH_4Cl for 20 minutes at room temperature. After three 5-minute washes with TBS, colonic sections were blocked and permeabilised with blocking solution A (4% goat serum and 0.3% Triton X-100 in TBS) or blocking solution B (3% horse Serum and 0.3% Triton X-100 in TBS) for 90 minutes at room temperature. Colonic sections were incubated with chicken anti-βIII-tubulin (1:100, ab41489, abcam, UK) and mouse biotin-conjugated HuC/D (1:50, Thermo Fisher Scientific, cat no A-21272, UK) diluted in 0.5% goat serum in TBS, or with rabbit monoclonal [EP1576Y] anti-S100β (1:200, ab52642 abcam, UK) diluted in 0.5% horse serum in TBS overnight in a humidity chamber at 4°C. After three 10-

minute washes with TBS, sections were incubated with the following secondary antibodies:- goat anti-chicken IgG Alexa Fluor 568-conjugated (1:500, Thermo Fisher Scientific, cat no A-11041) and streptavidin Alexa Fluor 488-conjugated (1:500, Thermo Fisher Scientific, cat no S32354) diluted in TBS and 0.5% goat serum, or donkey anti-rabbit IgG Alexa Fluor 488-conjugate (1:500, Thermo Fisher Scientific, cat no A-21206) diluted in TBS and 0.5% horse serum. Sections were incubated with secondary antibodies for 2 hours at room temperature. Following three 10-minute washes with TBS, colonic sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000, Thermo Fisher Scientific) diluted in TBS and 0.5% goat serum or 0.5% horse serum for 30 minutes at room temperature. After three 10-minute washes with TBS, sections were dried and mounted with Polyvinyl Alcohol DABCO mounting medium and stored in the dark at -20°C until analysis.

Immunohistochemistry of colonic whole mount preparations

Freshly isolated middle-proximal colon segments (a 3-cm long segment from PND21 animals and a 5-cm long segment from PND100 animals of each of the experimental groups) were gently flushed with a 10mM PBS and 10mM glucose solution to remove any luminal content. Colonic segments were then tied with string at one end and filled with 4% PFA (in 10mM PBS) before being tied off at the other end. Segments were then placed in fixative solution (4% PFA in 10mM PBS) for 2 hours (PND21) or 4 hours (PND100) at room temperature to ensure fixation from the inside and outside of the tissue. After two 30-minute washes in 10mM PBS, colonic segments were placed in a solution of 10mM PBS and 0.02% NaN_3 to prevent microbial growth and were stored at 4°C until analysis. For immunohistochemistry experiments, colonic segments

were divided into 0.5cm segments, opened along the mesenteric border, and placed as a flat sheet onto Sylgard-coated dishes with the mucosal side down. Using a dissecting microscope, tissues were separated into two layers: the outer musculature with adhering serosa, and the submucosa/mucosa. The circular muscle was removed to yield whole mount sections of longitudinal muscle with the myenteric plexus attached (LMMP) as previously performed (Brun et al., 2013). LMMP preparations were gently stretched and pinned down on the bottom of Sylgard-coated dishes and washed in PBS-T (PBS with 1% Triton X-100) for 45 minutes with gentle agitation. After blocking nonspecific binding sites with PBS-T containing 4% goat serum for 1.5 hours at room temperature, LMMPs were incubated overnight at room temperature with mouse biotin-conjugated HuC/D (1:100, Thermo Fisher Scientific, cat no A-21272, UK) diluted in PBS-T and 4% goat serum. Three 15-minute washes with PBS-T were carried out following the incubation period and LMMPs were incubated with streptavidin Alexa Fluor 488-conjugate (1:500, Thermo Fisher Scientific, cat no S32354) diluted in PBS-T and 4% goat serum for 2 hours at room temperature. Following this, three 10-minute washes with PBS-T were carried out. LMMPs were mounted on glass slides using Polyvinyl Alcohol DABCO mounting medium and stored in the dark at -20°C until analysis.

Confocal image acquisition and analysis

Images of colonic frozen sections and LMMP preparations were acquired using an Olympus FV1000 confocal laser scanning microscope equipped with an oil immersion 60x objective lens for colonic frozen sections and 40x, 20x and 10x objective lenses for LMMP preparations. The immunoreactivity of βIII-tubulin, HuC/D, and S100β in

colonic frozen sections was determined as previously described (Stenkamp-Strahm et al., 2013). Four full-thickness images per animal were taken using the 60x objective lens and were processed as maximum intensity projections. A tracing tool was used to define the muscularis externa or the myenteric ganglia in order to estimate tissue area. βIII-tubulin, HuC/D, and S100β staining in the muscularis externa and/or the myenteric ganglia were then thresholded in a blinded fashion to allow Fiji Image J software (version 1.52e) to estimate stained areas within muscularis externa or myenteric ganglia. Density index calculations (stained area/area of muscularis externa or myenteric ganglia) were generated. In colonic LMMP preparations, changes in the number of HuC/D$^+$ neurons were assessed in PND21 and PND100 animals from each of the experimental groups. For cell quantification, five to ten visual fields were blindly chosen within the areas where the myenteric plexus was intact and acquired using a 20x objective (for anti-HuC/D staining in colonic specimen at PND21; 636 x 636μm/visual field) and a 40x objective (for anti-HuC/D staining in colonic specimen at PND100; 318 x 318μm/visual field). HuC/D$^+$ myenteric neurons were blindly quantified and expressed as neuronal count per visual field.

Statistical analysis

Data were analysed using the statistical software package SPSS 24.0 (IBM) and were expressed as mean ± SEM. Differences between the experimental groups were assessed using: a two-way ANOVA, repeated measures two-way ANOVA, mixed design ANOVA, and LSD Fisher post-hoc test where appropriate. A p-value of 0.05 was set as the threshold of statistical significance. "*n*" indicates the number of animals per experimental group.

Results

Perinatal coadministration of DHA and MFGM does not alter rat body weight or gastrointestinal anatomy

The coadministration of DHA and MFGM from birth was well tolerated by the animals and there was no change in body weight observed throughout the lifespan of the animals from each of the experimental groups (data not shown). Food intake was also not affected between experimental groups. No changes in either small intestine or colon length, or caecum or spleen weight were observed at PND21 or PND100 in animals exposed to MS or the dietary intervention (data not shown).

Coadministration of DHA and MFGM reduces maternal separation-induced visceral hypersensitivity

Two-way ANOVA revealed a significant main effect of early life stress ($F(1,39) = 14.785$, $p < 0.001$) and of diet ($F(1,39) = 5.213$, $p = 0.028$) on threshold pressure in response to CRD. No diet*early life stress interaction was observed ($F(1,39) = 0.272$, $p = 0.61$). LSD Fisher post hoc test revealed a significant decrease in threshold pressure in MS-Control animals compared to NS-Control animals ($p = 0.003$) and MFGM administration significantly restored threshold pressure to near control levels in MS-MFGM compared to MS-Control animals ($p = 0.05$) **(Figure 2A)**. Two-way ANOVA revealed a significant main effect of early life stress ($F(1,35) = 25.124$, $p < 0.001$) as well as of diet ($F(1,35) = 11.484$, $p = 0.002$) on the total number of pain behaviours displayed in response to CRD. No diet*early life stress interaction was noted ($F(1,35) = 2.553$, $p = 0.12$). LSD Fisher post hoc test revealed a significant increase in the total number of pain behaviours displayed by MS-Control animals

compared to the NS-Control group ($p < 0.001$) and that the total number of pain behaviours displayed by MS-MFGM animals was significantly less than that of the MS-Control group ($p = 0.001$) **(Figure 2B)**.

Perinatal coadministration of DHA and MFGM improves spatial learning without affecting reference memory in the Morris water maze

Animals successfully learned the location of the platform over the four training days as shown by a repeated-measures two-way ANOVA which revealed a significant main effect of time ($F(3,105) = 104.522$, $p < 0.001$) and of diet with respect to time ($F(3,105) = 2.713$, $p = 0.049$). However, during the acquisition training, no effect of early life stress with respect to time was found ($F(3,105) = 0.563$, $p = 0.64$), nor was there a diet*early life stress interaction with respect to time ($F(3,105) = 0.229$, $p = 0.88$) **(Figure 2C)**. Further investigation using a mixed design ANOVA with trial day as the repeated measures factor and diet as the independent factor revealed that diet approached the threshold of statistical significance as a main effect between NS-Control and NS-MFGM on day one only ($F(1,22) = 3.866$, $p = 0.06$) **(Figure 2D)**. A significant effect of diet was also noted by mixed design ANOVA with trial day as the repeated measures factor and diet as the independent factor between MS-Control and MS-MFGM on day one ($F(1,21) = 4.337$, $p = 0.05$) and day four only ($F(1,21) = 4.704$, $p = 0.04$) **(Figure 2E)**. Mixed design ANOVA with trial day as the repeated measures factor and ELS as the independent factor also revealed an effect of early life stress between NS-Control and MS-Control on day 4 only ($F(1,21) = 5.158$, $p = 0.034$) **(Figure 2F)**. No differences were observed in the percentage of time spent in the platform quadrant during the probe trial between animals from each experimental

group, suggesting that reference memory was not affected by MFGM administration or MS (data not shown).

Maternal separation and MFGM do not affect recognition memory in the novel object recognition test

A two-way ANOVA revealed no effect of diet ($F(1,41) = 1.018$, $p = 0.32$), nor of a diet*early life stress interaction effect ($F(1,41) = 0.098$, $p = 0.76$) on the discrimination index in the novel object recognition test **(Figure 2G)**.

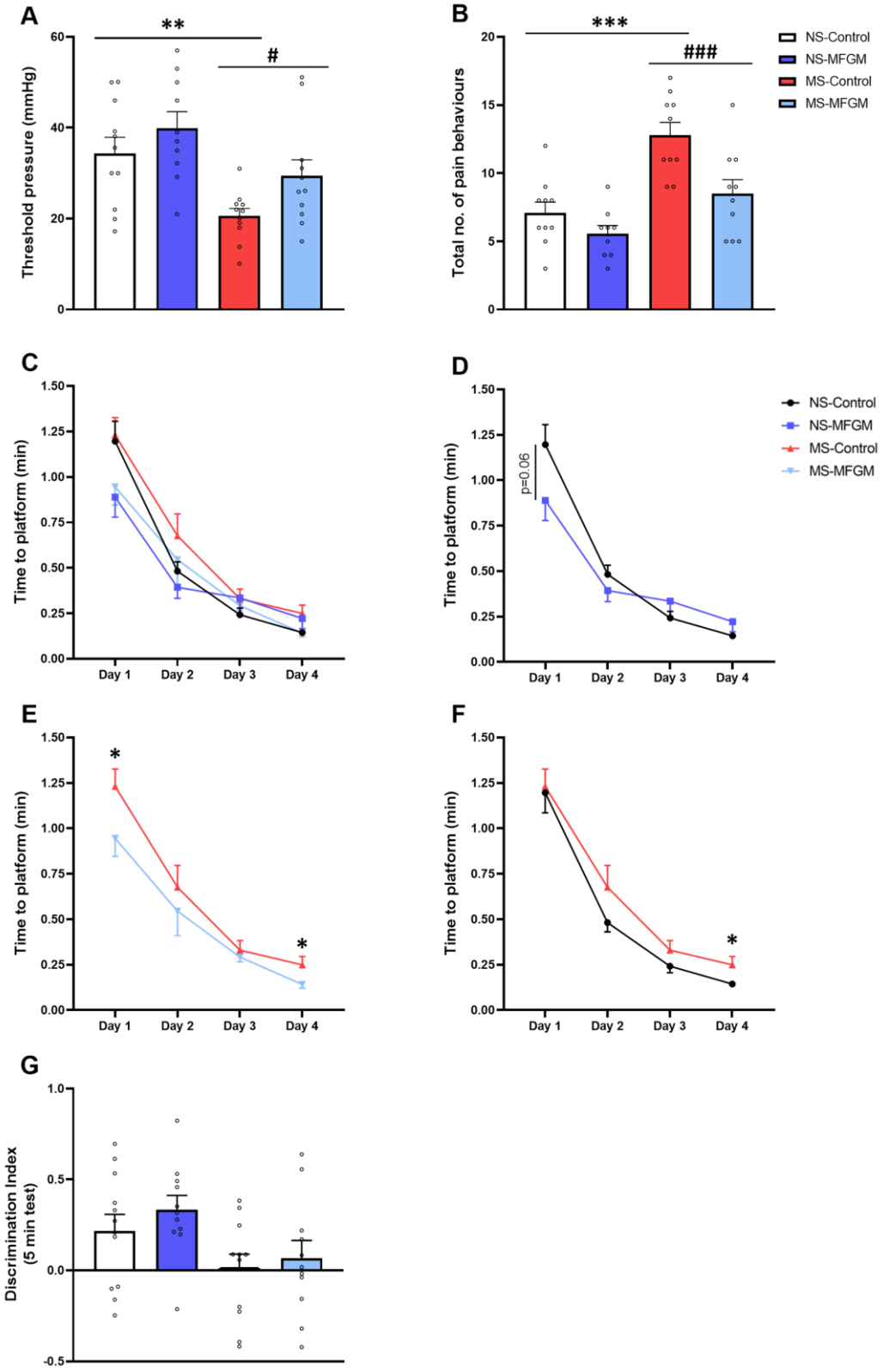


Figure 2. (A&B) Perinatal MFGM administration reduced maternal separation-induced visceral hypersensitivity. *Maternal separation resulted in visceral hypersensitivity as marked by* ***(A)*** *the significantly lower threshold pressure observed in MS-Control animals compared to the NS-Control group. **p ≤ 0.01 MS Control vs NS-Control.* ***(B)*** *the significantly higher number of pain behaviours in MS-Control*

*animals when compared to NS-Control. ***p ≤ 0.001 MS-Control vs NS-Control. MFGM administration was able to significantly* ***(A)*** *increase the threshold pressure to near control levels in MS-MFGM animals. #p ≤ 0.05 MS-MFGM vs MS-Control* ***(B)*** *reduce the number of pain behaviours exhibited by MS-MFGM animals. ###p ≤ 0.001 MS-MFGM vs MS-Control. Data is presented as mean ± SEM. n = 10-11 per group.* ***(C-F) Spatial memory is influenced by MFGM intervention in the MWM.*** ***(C)*** *A main effect of diet and time but not of early life stress was observed on spatial learning.* ***(D)*** *A trend towards a decrease in the time taken to reach the platform was observed on day 1 of the acquisition training in NS-MFGM animals when compared with NS-Control animals. p = 0.06 NS-MFGM vs NS-Control.* ***(E)*** *Administration of MFGM reduced the time taken by MS-MFGM animals to locate the platform on day 1 and 4 of acquisition training. *p ≤ 0.05 MS-MFGM vs MS-Control.* ***(F)*** *Maternally separated animals fed the control diet took longer to locate the platform on day 4 of the acquisition training when compared to NS-Control animals. *p ≤ 0.05 MS-Control vs NS-Control. Data is presented as mean ± SEM. n = 11-12 per group.* ***(G) Maternal separation and MFGM do not affect recognition memory in the novel object recognition test.*** *Data presented as mean ± SEM. n = 11-12 per group.*

Intestinal permeability in early life or adulthood was not affected by maternal separation or dietary interventions

A two-way ANOVA revealed no effect of early life stress on transepithelial permeability of the ileum to 4kDa FITC at PND21 ($F(1,29) = 0.16$, $p = 0.69$) **(Figure 3A)** or PND100 ($F(1,40) = 0.021$, $p = 0.88$) **(Figure 3B)**. Likewise, no effect of diet on transepithelial permeability of the ileum to FITC was noted at PND21 ($F(1,29) <0.001$, $p = 0.99$) **(Figure 3A)** or PND100 ($F(1,40) = 0.014$, $p = 0.91$) **(Figure 3B)**. No diet*early life stress interaction on transepithelial permeability of the ileum to FITC was noted at PND21 ($F(1,29) = 1.529$, $p = 0.23$) **(Figure 3A)** or PND100 ($F(1,40) = 3.411$, $p = 0.07$) **(Figure 3B)**. With respect to the colon, a two-way ANOVA revealed no effect of early life stress on transepithelial permeability of colonic segments to 4kDa FITC at PND21 ($F(1,32) = 0.037$, $p = 0.85$) **(Figure 3C)** or PND100 ($F(1,39) = 0.216$, $p = 0.65$) **(Figure 3D)**. Similarly, no effect of diet on transepithelial permeability of the colon to FITC was noted at PND21 ($F(1,32) = 0.162$, $p = 0.69$) **(Figure 3C)** or PND100 ($F(1,39) = 0.032$, $p = 0.86$) **(Figure 3D)**.

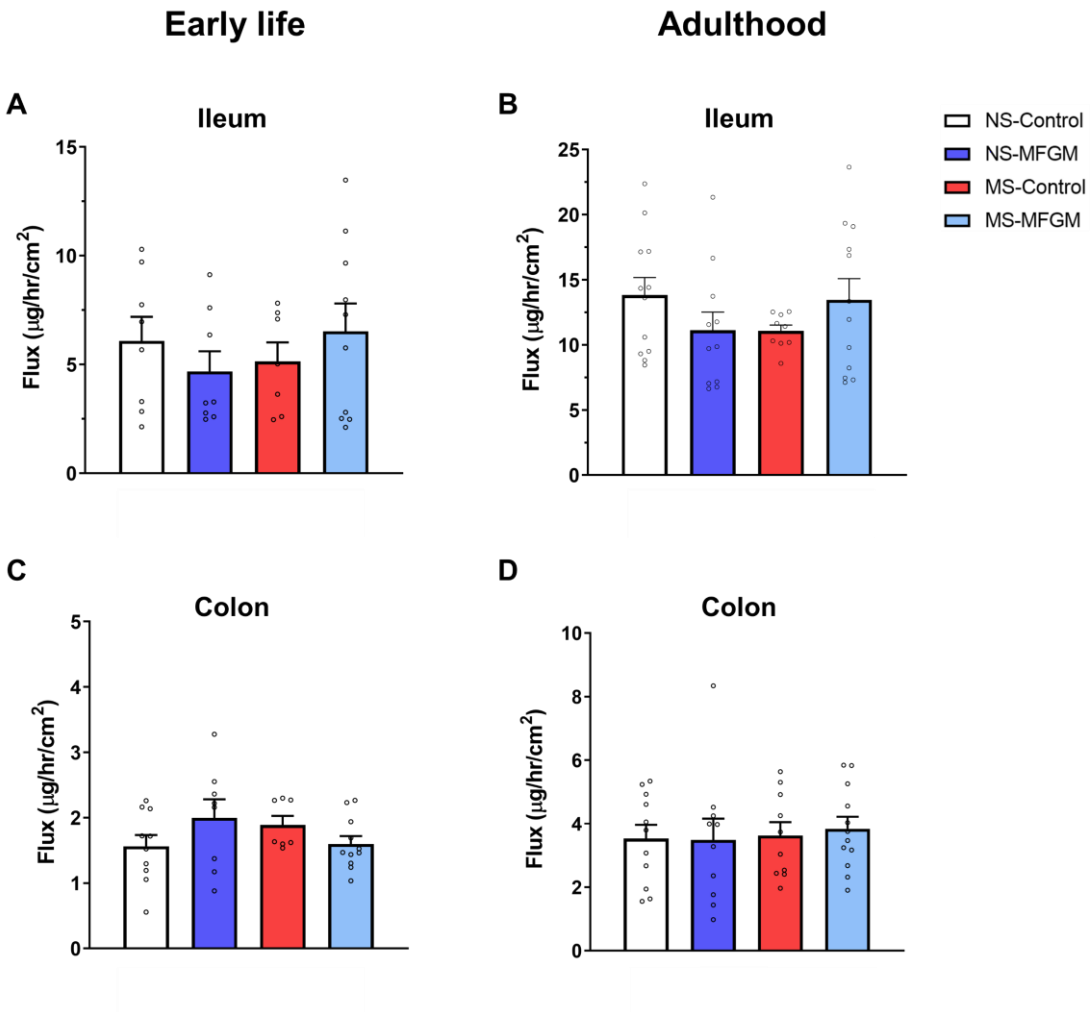


Figure 3. No effect of maternal separation or perinatal coadministration of MFGM and DHA on small and large intestinal permeability in early life or adulthood. *Transepithelial flux of FITC-dextran 4kDa, detected in the serosal chambers after 180 minutes of incubation in the Ussing chamber apparatus, of ileal segments from animals at* ***(A)*** *PND21 or* ***(B)*** *PND100 and of colonic segments from animals at* ***(C)*** *PND21 or* ***(D)*** *PND100. Data presented as mean ± SEM. n = 7-11 per group for PND21 and n = 9-12 per group for PND100.*

No effect of maternal separation or perinatal dietary intervention on neuronal and glial architecture of the enteric nervous system in early life

The immunoreactivity of βIII-Tubulin, a neuronal marker present in the nerve fibres of all subsets of neurons in the ENS, was evaluated in the muscle layer of colonic frozen section from PND21 animals. A two-way ANOVA showed no effect of early life stress ($F(1,31) = 2.168$, $p = 0.15$), nor of diet ($F(1,31) = 1.777$, $p = 0.19$), nor of a diet*early life stress interaction ($F(1,31) = 0.717$, $p = 0.40$) on βIII-Tubulin immunofluorescence in the colonic muscle layer of PND21 animals **(Figure 4A** and **4C)**. The density index of βIII-Tubulin in the colonic myenteric ganglia was also assessed, and no differences were induced by early life stress ($F(1,30) = 3.315$, $p = 0.08$), nor by diet ($F(1,30) = 0.045$, $p = 0.83$), nor by a diet*early life stress interaction ($F(1,30) = 0.007$, $p = 0.93$) **(Figure 4A** and **4D)**. Colonic frozen sections were also stained for HuC/D, a pan-neuronal cell body marker in the ENS, and no effect of early life stress ($F(1,31) = 0.001$, $p = 0.98$), nor of diet ($F(1,31) = 0.187$, $p = 0.67$), nor of a diet*early life stress interaction ($F(1,31) = 1.952$, $p = 0.17$) on HuC/D immunoreactivity was observed in colonic myenteric ganglia from PND21 animals. **(Figure 4A** and **4E)**. Enteric glial networks were also investigated in early life. A two-way ANOVA revealed no effect of early life stress ($F(1,29) < 0.001$, $p = 0.99$), nor of diet ($F(1,29) = 0.001$ $p = 0.98$), nor of a diet*early life stress interaction ($F(1,29) = 2.071$, $p = 0.16$) on density index of the enteric glial marker S100β in myenteric ganglia of PND21 animals. **(Figure 4B** and **4F)**.

Dietary intervention but not maternal separation affects enteric neuronal processes in adulthood in colonic myenteric ganglia

In colonic frozen sections of PND100 animals, a two-way ANOVA showed no effect of early life stress ($F(1,27) = 0.099$, $p = 0.76$), nor of diet ($F(1,27) = 1.749$, $p = 0.2$), nor of a diet*early life stress interaction ($F(1,27) = 2.634$, $p = 0.12$) on the immunoreactivity of βIII-Tubulin in the colonic muscle layer **(Figure 4G** and **4I)**. The immunofluorescence of βIII-Tubulin in the colonic myenteric ganglia was also assessed, and no differences were induced by early life stress ($F(1,26) = 0.03$, $p = 0.86$), however, there was a significant effect of diet ($F(1,26) = 6.772$, $p = 0.015$), and a diet*early life stress interaction ($F(1,26) = 5.98$, $p = 0.022$). LSD Fisher post hoc test revealed a significant increase in βIII-Tubulin immunoreactivity in the myenteric ganglia of NS-MFGM animals when compared to the NS-Control group ($p = 0.001$) **(Figure 4G** and **4J)**. Colonic frozen sections were also stained for HuC/D, however no effect of early life stress ($F(1,27) = 0.066$, $p = 0.8$), nor of diet ($F(1,27) = 3.314$, $p = 0.08$), nor of a diet*early life stress interaction ($F(1,27) = 0.381$, $p = 0.54$) on HuC/D immunoreactivity was noted **(Figure 4G** and **4K)**. The immunoreactivity of the enteric glial marker S100β was also investigated at PND100. A two-way ANOVA revealed no effect of early life stress ($F(1,27) = 0.199$, $p = 0.66$), nor of diet ($F(1,27) = 1.216$, $p = 0.28$), nor of a diet*early life stress interaction ($F(1,27) = 1.079$, $p = 0.31$) on density index of S100β in myenteric ganglia of PND100 animals **(Figure 4H** and **4L)**.

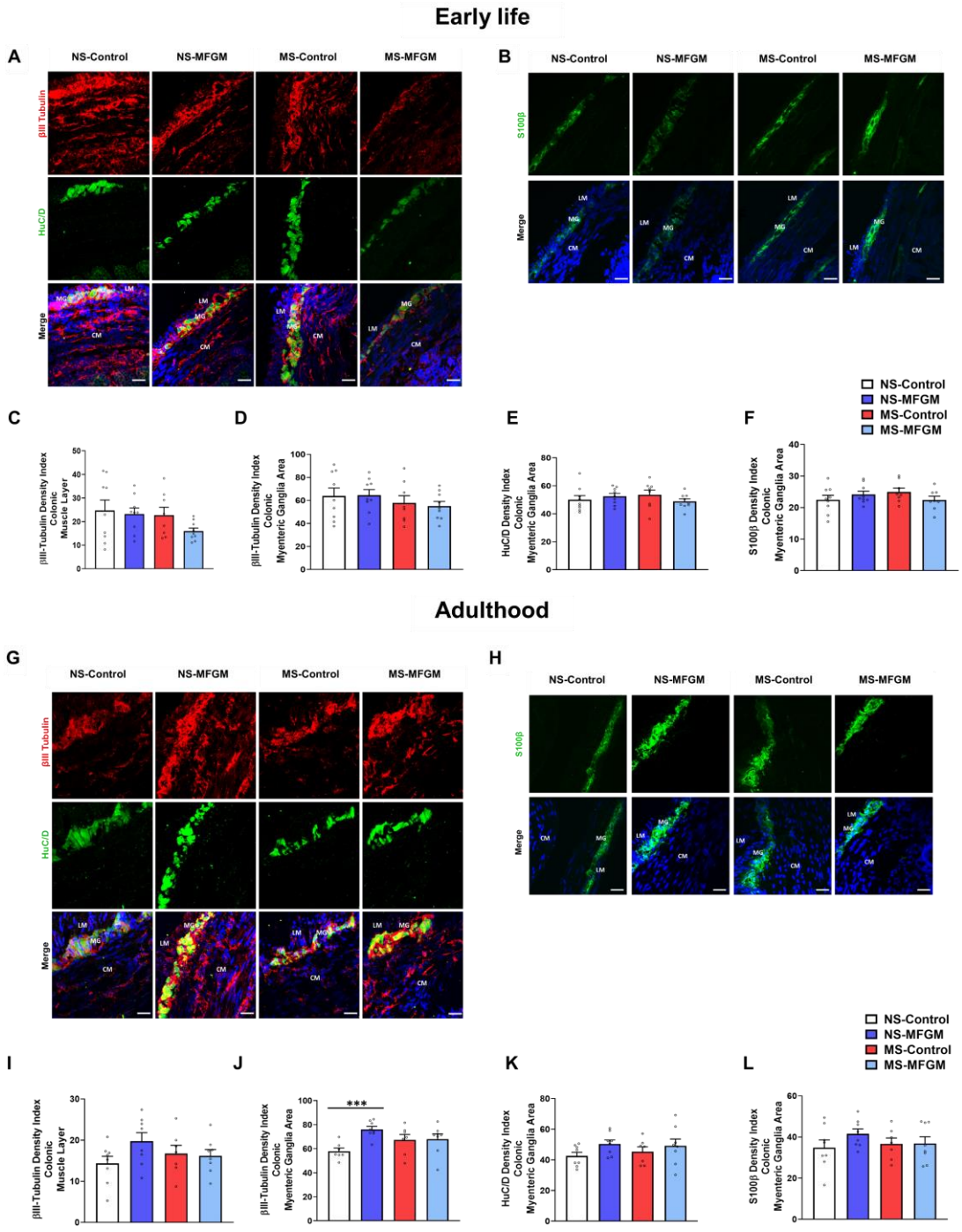


Figure 4. No effect of maternal separation or dietary intervention on enteric neuronal or glial networks in early life. (A) *Representative confocal photomicrographs showing the distribution of βIII-Tubulin (red), HuC/D (green) and of S100β (green,* ***B****) in colonic frozen sections of PND21 animals from each of the experimental groups. Analysis of immunofluorescence of βIII-tubulin in* ***(C)*** *colonic muscle layer or* ***(D)*** *myenteric ganglia and of* ***(E)*** *HuC/D or* ***(F)*** *S100β in colonic myenteric ganglia of PND21 animals from each experimental group. Cell nuclei were stained with DAPI (blue). Scale bars = 30 µm. CM = Circular Muscle; LM = Longitudinal Muscle; MG = Myenteric Ganglia.* ***Diet but not maternal separation affects enteric neuronal representation in adulthood. (G)*** *Representative confocal photomicrographs showing the distribution of βIII-Tubulin, (red), HuC/D (green) and*

*of S100β (green, **H**) in colonic frozen sections of PND100 animals from each experimental group. Analysis of density index of βIII-tubulin in **(I)** colonic muscle layer or **(J)** myenteric ganglia and of **(K)** HuC/D or **(L)** S100β in colonic myenteric ganglia of PND100 animals from each experimental group. Cell nuclei were stained with DAPI (blue). Scale bars = 30 µm. ***p ≤ 0.001 NS-MFGM vs NS-Control. Data presented as mean ± SEM. n = 7-8 per group.*

No effect of maternal separation or diet on enteric nervous system in early life: whole-mount colonic preparations

Two-way ANOVA showed no effect of early life stress ($F(1,26) = 2.754$, $p = 0.11$), nor of diet ($F(1,26) = 0.003$, $p = 0.95$), nor of a diet*early life stress interaction ($F(1,26) = 0.008$, $p = 0.93$) on the number of HuC/D-stained cells in colonic whole mount preparations in early life **(Supplementary Figure 1A** and **1C)**. Similarly, no differences in HuC/D-stained cells in colonic whole mount preparations at PND100 were induced by early life stress ($F(1,29) = 1.087$, $p = 0.31$), nor by diet ($F(1,29) = 1.228$, $p = 0.28$), nor by a diet*early life stress interaction ($F(1,29) = 0.093$, $p = 0.76$) **(Supplementary Figure 1B** and **1D)**.

Discussion

We have previously shown that post-weaning administration of MFGM attenuated the effects of ELS in rats (O'Mahony et al., 2020). The mechanisms underpinning such effects were unknown but may involve changes in ENS function and intestinal permeability (Bhinder et al., 2017; Ortega-Anaya and Jiménez-Flores, 2019; Yu et al., 2021b). In this study, the diet of the dams was supplemented with MFGM from two days prior to birth of the pups and continued throughout the lifespan of the offspring to assess its potential in reversing the effects of ELS. MFGM was selected as the candidate intervention as it has been shown to have beneficial health effects both preclinically (Mudd et al., 2016) and clinically on neurodevelopment (Hernell et al., 2016), and narrow the gap in cognitive performance between formula-fed and breastfed infants. MFGM has also been shown to reduce the incidence of infections (Hernell et al., 2016). However, the mechanistic potential of MFGM in reduction of ELS-induced deficits has not yet been extensively explored.

This study is to our knowledge the first to investigate the effect of dietary supplementation with MFGM from birth on rat ENS and behaviour following exposure to ELS. The MS model is a robust model of ELS and gut-brain axis dysfunction in rats (Botschuijver et al., 2019; Cowan et al., 2019; O'Mahony et al., 2009; O'Mahony et al., 2011; Rincel et al., 2019b).

Visceral sensation from the gut has been shown previously to be heavily influenced by ELS with exposure to stress during the stress hyporesponsive period in early life resulting in heightened sensitivity of the GI tract (Felice et al., 2015; O'Mahony et al., 2012). In line with this, we found that MS resulted in visceral hypersensitivity in response to the noxious stimulus of colorectal distension. This was noted both by an

increased total number of pain behaviours observed in response to distension of the colorectal region and by a decreased threshold to this stimulus. Supplementation with MFGM reduced this, normalising the number of pain behaviours and threshold pressure to near control levels. This is in agreement with our previous study showing that supplementation with MFGM after weaning ameliorated MS-induced visceral hypersensitivity (O'Mahony et al., 2020), supporting the potential value of MFGM against visceral pain-associated disorders.

We noted that animals exposed to ELS displayed a deficit in spatial memory in the Morris water maze on the final day of the acquisition training, suggesting that initially the MS group learned the location of the platform as fast as the NS group, with this effect having tapered off by day 4. This impaired cognitive performance has been previously noted following a longer MS paradigm where the authors suggest that the effects of MS on spatial memory are related to changes in the postnatal development of the hippocampus (Cao et al., 2014). Interestingly, we noted that MS MFGM-treated animals displayed significantly better cognitive performance on day 1 and day 4 of the acquisition training compared to the MS control diet animals, highlighting the potential benefit of MFGM on brain function, particularly on learning. Interestingly, a subtle deficit in visuospatial memory was noted in a cohort of patients with irritable bowel syndrome (Kennedy et al., 2014c), strengthening the link between impaired cognition and visceral pain-associated disorders such as irritable bowel syndrome. Interestingly, the MS-induced deficits in our current study were much more modest than what we have previously shown (Felice et al., 2014; McVey Neufeld et al., 2019; O'Mahony et al., 2009; O'Mahony et al., 2020). This may be due to the fact that the control diet used in the present study was enriched with DHA, a polyunsaturated fatty

acid that has been shown to have pro-cognitive effects (Lauritzen et al., 2016; Mulder et al., 2018).

MS has previously been shown to result in deficits in recognition memory (O'Mahony et al., 2020), yet here we do not observe this deficit potentially due to the addition of DHA in the control diet masking any ELS-induced memory deficits.

The gut barrier serves as a physical barrier between the luminal content, enteric microbiota and the host and serves to protect the host from invading pathogens (Odenwald and Turner, 2017). The gut microbiota plays an important role in proper sensation of pain, and it may be seen that germ-free mice display a blunted inflammatory-induced pain response (Amaral et al., 2008). Importantly, it has been shown that the gut microbiota plays an important role in visceral sensation, with male germ-free mice showing baseline visceral hypersensitivity (Luczynski et al., 2017), while female germ-free mice do not, but instead display microbiota-dependent modulation of visceral pain across the oestrous cycle (Tramullas et al., 2021). Similarly, early life antibiotic-induced depletion of the gut microbiota results in altered sensation of visceral pain in adulthood (O'Mahony et al., 2014). MS has been shown previously to affect transepithelial barrier permeability as noted by an increased flux of Horseradish peroxidase in maternally separated rats (Gareau et al., 2007a; Gareau et al., 2006). Other studies have also reported MS-induced increases in transepithelial permeability (Barreau et al., 2004; Oines et al., 2012; Söderholm et al., 2002). However, a study using a combination of pre- and probiotics reported normalisation of intestinal barrier function following MS (García-Ródenas et al., 2006) while a probiotic, VSL#3, was successful in preventing MS-induced visceral pain, and also tightened the gut epithelial barrier (Dai et al., 2012). This increase in transepithelial permeability may lead to bacterial translocation from the gut, feeding into the

manifestation of visceral pain. Therefore, we hypothesised that epithelial barrier permeability plays a role in MS-induced visceral hypersensitivity. However, no effect of either MS or dietary intervention was noted at either life stage. This is in agreement with a previous study, which found no effect of MFGM on transepithelial barrier permeability to FITC-dextran (Bhinder et al., 2017). Possible reasons behind the protection of epithelial barrier permeability against the effects of MS include the presence of DHA in the control diet. DHA has been shown to be effective against increased permeability as a result of the addition of interleukin-4 to a monolayer of human colon-derived carcinoma cells (Willemsen et al., 2008).

Previous studies have shown that ELS affects the morphology and functionality of the ENS. Piglets exposed to early life adversity (early weaning stress) displayed a higher veratridine (activates voltage-gated sodium channels resulting in neuron depolarisation)-induced short circuit current in the ileum than late weaning controls (Medland et al., 2016). Moreover, early weaning increased the number of ileal submucosal neurons in adulthood compared to late weaning controls. However, how ELS impacts upon development of the ENS is not yet known. Here we show that both the immunoreactivity of HuC/D and βIII-Tubulin in both the colonic myenteric ganglia and the colonic muscle layer at PND21 was unaffected by ELS. However, an increase in the density index of βIII-Tubulin in colonic myenteric ganglia was noted at PND100 in the NS MFGM-treated group only. Similarly, no effect of either ELS or dietary intervention on enteric glial structure was noted at either PND21 or PND100. The same may be said for the colonic whole mount preparations where no effect of ELS or MFGM supplementation was noted at PND21 or PND100 **(Supplementary Figure 1)**.

Despite a clear mechanism of action, MFGM supports immune function in vitro by decreasing cytokine production following splenocyte stimulation (Zanabria et al., 2014). It was observed that the effects of MFGM did not occur under non-stimulatory conditions, therefore potentially suggesting MFGM may act in conditions that, in part, simulate a stressed condition. Immune changes may be one of the driving factors of ELS-induced visceral hypersensitivity as MS can cause immune dysregulation including upregulation of cytokines and growth factors which can sensitise peripheral nociceptors and thus amplify visceral pain perception (Fuentes and Christianson, 2018). It is therefore reasonable to suggest that the effects on the immune system induced by MFGM are likely to reduce the impact of ELS on visceral pain perception.

As was previously shown, the microbiome of MFGM-enriched formula fed rat pups more closely resembles that of rat pups consuming their dam's milk (Bhinder et al., 2017), and we have shown previously that MFGM can alter gut microbiota composition (O'Mahony et al., 2020), possibly suggesting a mechanistic link between MFGM, the gut microbiota, and the immune system.

In summary, MS resulted in visceral hypersensitivity and a deficit in spatial memory – effects that were buffered by the addition of MFGM to the DHA-enriched diet. The potential mechanisms of action of MFGM on memory-dependent tasks may lie in the lipid content of MFGM. It has been suggested that the complex lipids comprising MFGM form the integral basis for neonatal brain development and is evidenced by studies reporting enhanced cognitive development in infants following dietary supplementation with gangliosides, a complex lipid component of MFGM (Gurnida et al., 2012). Another constituent part of MFGM, sphingolipids play a major role in lipid membrane function and dynamics, and thus may alter brain composition and

function (Posse de Chaves and Sipione, 2010) and induce behavioural changes (Mühle et al., 2013; Schverer et al., 2020).

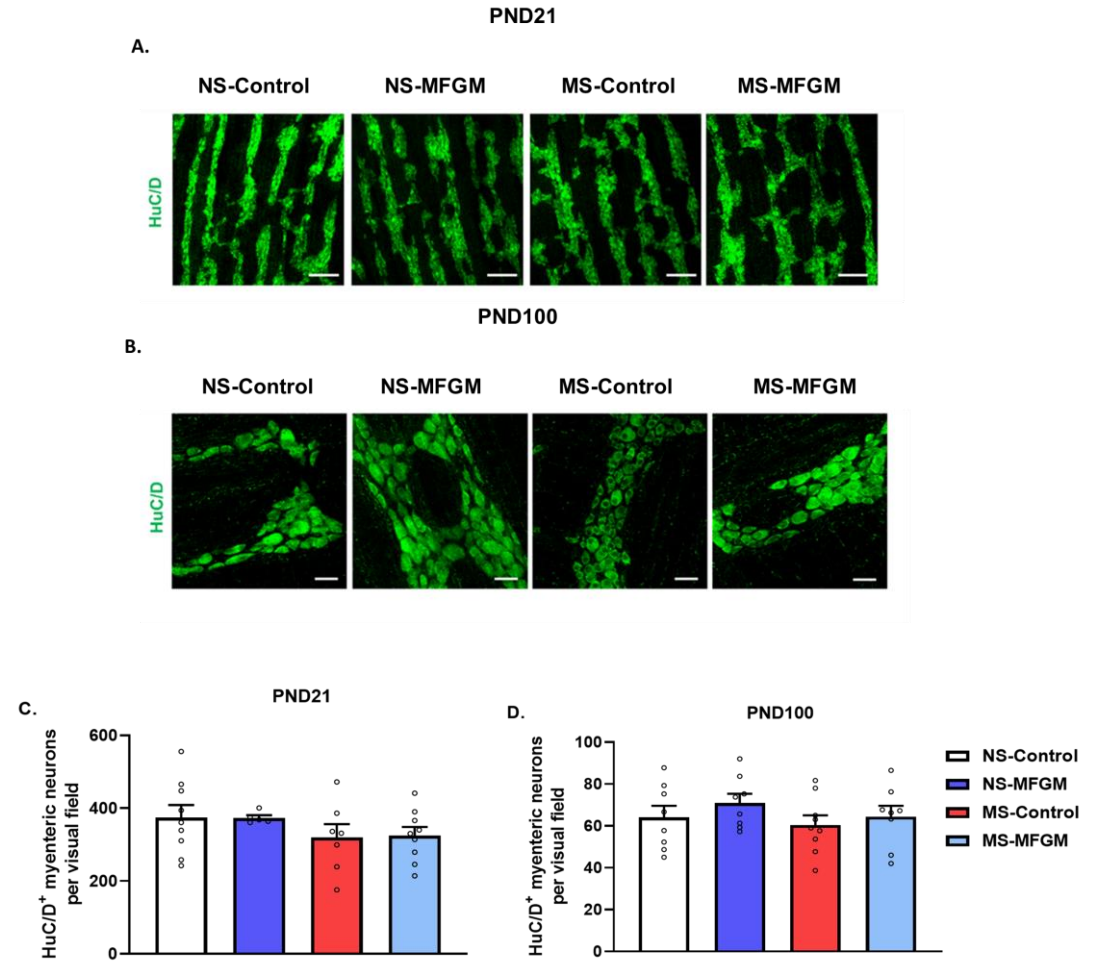
Conclusions

Overall, we add further evidence for the beneficial effects of MFGM in the reduction of ELS-induced visceral hypersensitivity. This occurs independent of changes at the level of the enteric nervous system and intestinal permeability. This has clinical relevance in terms of a potential nutritional intervention for irritable bowel syndrome, for which visceral hypersensitivity is a hallmark.

Supplementary material

***Supplementary table 1*. Breakdown of dietary components of both control and MFGM-enriched diets.** *DHA = docosahexaenoic acid; ARA = Arachidonic acid; MFGM = Milk fat globule membrane.*

Ingredient	Control diet (g/kg)	MFGM diet (g/kg)
Casein	200	187.04
L-cysteine	3	3
Corn starch	392.372	392.464
Maltodextrin	132	132
Sucrose	100	100
Lactose, monohydrate	7.5	7.5
Soybean oil	64.7	61.52
DHA/ARA oil	5.3	5.3
Cellulose	50	50
Mineral mix	13.4	13.4
Calcium phosphate, dibasic	7.2	6.95
Vitamin mix	15	15
Choline Bitartrate	2.5	2.5
Thiamine HCL	0.01	0.01
Vitamin K1	0.002	0.002
TBHQ antioxidant	0.014	0.014
Whey protein concentrate MFGM-10 enriched	-	15.9



Supplementary Figure 1. No effect of either maternal separation or diet on the number of HuC/D positive cells in colonic whole mount preparations at PND21 or PND100. ***(A)*** *Representative confocal photomicrograph of colonic whole mount preparation at PND21 showing pan neuronal marker HuC/D (green).* ***(B)*** *Representative confocal photomicrograph of colonic whole mount preparation at PND100 showing pan neuronal marker HuC/D (green).* ***(C)*** *No effect of diet or maternal separation on the number of HuC/D positive cells at PND21.* ***(D)*** *No effect of diet or maternal separation on the number of HuC/D positive cells at PND100. Scale bar =* ***(A)*** *100µm or* ***(B)*** *40µm. Data presented as Mean ± SEM. n = 5-9 for PND21 and n = 8-9 per group for PND100.*

Chapter 4

Oestrous cycle and ovariectomy-induced changes in visceral pain are microbiota-dependent

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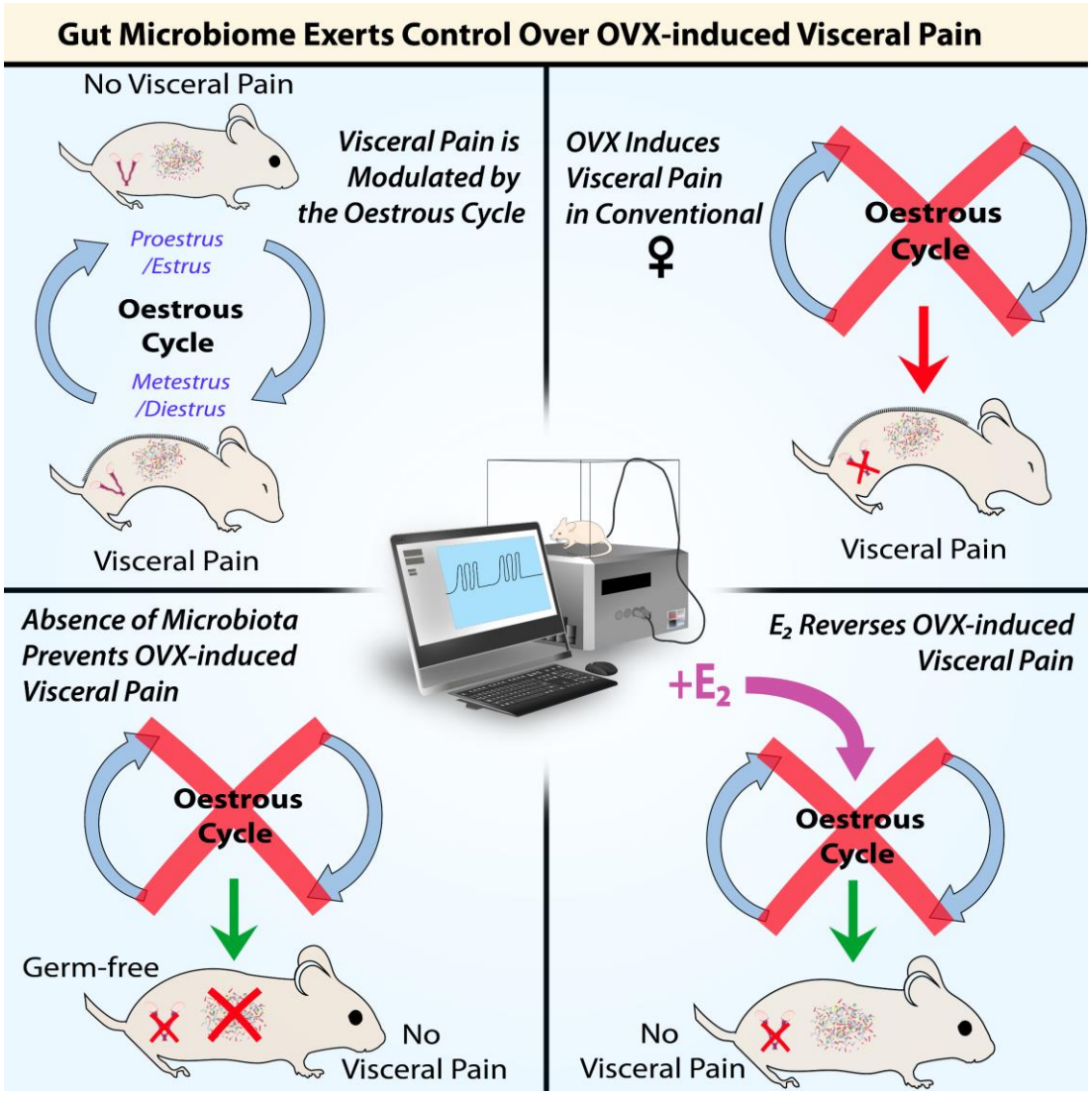
Abstract

Visceral hypersensitivity (VH) is a hallmark of many functional gastrointestinal disorders including irritable bowel syndrome and is categorised by a dull, diffuse sensation of abdominal pain. Recently, the gut microbiota has been implicated in VH in male mice, but the effects in females have yet to be explored fully. To this end, we now show that somewhat surprisingly, female germ-free mice have similar visceral pain responses to colorectal distension (CRD) as their conventional controls. However, we show that although sensitivity to CRD is oestrous cycle stage-dependent in conventional mice, it is not in germ-free mice. Further, ovariectomy (OVX) induced VH in conventional but not germ-free mice, and induced weight gain regardless of microbiota status. Finally, we show that oestrogen-replacement ameliorated OVX-induced VH. Taken together, this study provides evidence for a major role of female sex hormones and the gut microbiota in sensation of visceral pain in females.

Graphical abstract



Introduction

Visceral pain is a common and complex occurrence categorised by a diffuse, often dull sensation of pain centred around the midline and upper abdomen originating from some, but not all internal organs (Cervero and Laird, 1999; Sikandar and Dickenson, 2012). It has been reported that 25% of adults experience intermittent abdominal pain during their lifetime, highlighting the need for a better understanding of the pathophysiology of this disorder (Collett, 2013; Drewes et al., 2020). Visceral pain-associated disorders such as irritable bowel syndrome (IBS) are more commonly presented in women; however, to date the majority of preclinical studies on visceral pain are carried out exclusively in males, with their results being generalised to include females in terms of treatment of these disorders (Lee et al., 2018b). This raises the issue of sex differences in treatment strategies and supports the notion that these treatments for disorders should not follow a singular approach across sexes.

The oestrous cycle is the term used to describe the female reproductive cycle in rodents and is similar to the menstrual cycle in humans. In rodents, this cycle has four phases: (i) proestrus, (ii) estrus, (iii) metestrus, and (iv) diestrus, and lasts between 4 and 5 days (Byers et al., 2012a). During this time, circulating gonadal hormone levels fluctuate. For example, levels of oestrogen are highest during proestrus and lower in diestrus (Hong and Choi, 2018). Interestingly, it has been shown that the visceral pain response varies across the oestrous cycle (Moloney et al., 2016b). However, not all studies agree on the specific changes in visceral pain perception across the oestrous cycle, with some reporting heightened visceral sensation when in proestrus versus metestrus/diestrus, or the converse (Giamberardino et al., 1997; Ji et al., 2008).

Ovariectomy (OVX) in rodents is used to cease the main production of female gonadal hormones (Lemini et al., 2015) and is a useful experimental model to investigate the specific effects of sex hormones on physiological parameters. Moreover, the effects of OVX on visceral pain processing are unclear as it has been shown that OVX results in a reduction in the visceromotor response (VMR) to colorectal distension (CRD) (Ji et al., 2003), or has no effect on visceral pain perception in rats (Traub et al., 2014). Here, we investigate the impact of OVX in female mice.

The gastrointestinal (GI) tract is home to between 10 and 100 trillion microbial cells which comprise the gut microbiota (Blaser, 2014; Gilbert et al., 2018; Lynch and Pedersen, 2016). The gut microbiota forms an essential component of the bidirectional communication between the gut and the nervous system, the microbiota-gut-brain axis, which has received increasingly more attention in recent years for its apparent role in the pathophysiology of many disorders of the gut-brain axis including IBS, a functional GI disorder characterised by visceral pain (Bhattarai et al., 2016; Collins, 2014; Margolis et al., 2021; Mayer et al., 2015b; Wilmes et al., 2021).

There is also an increasing realisation that signalling across the microbiota-gut brain axis is sex dependent (Jaggar et al., 2020; Jašarević et al., 2016). There is substantial evidence for the influence of gonadal hormones on the gut microbiota and vice versa (Tetel et al., 2018), with differences being observed between male and female rodents, as well as effects of gonadectomy and hormone replacement on the microbiota (Jašarević et al., 2016; Org et al., 2016). Altered levels of steroid hormones seen in germ-free (GF) mice, which are devoid of a microbiota and are sterile in microbiological terms (Spichak et al., 2018), further validates the interaction between the microbiota and gonadal hormones (Kamimura et al., 2019).

There is an increasing emphasis on the role of the microbiota in pain responses, particularly in visceral pain (Defaye et al., 2020; Luczynski et al., 2017; O' Mahony et al., 2017; Rea et al., 2017; Rea et al., 2019; Theodorou et al., 2014). We have previously shown that the gut microbiota plays a role in the mediation of visceral pain, whereby male GF mice displayed visceral hypersensitivity (VH) to CRD which was reduced following microbial colonisation of the GI tract (Luczynski et al., 2017). Furthermore, it has also been shown that modulation of the gut microbiota in rodents by use of antibiotics in early life induces VH (O'Mahony et al., 2014). Moreover, manipulation of the gut microbiota using probiotic bacterial species including strains of *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* (Dai et al., 2012; McKernan et al., 2010; Miquel et al., 2016; Verdú et al., 2006), as well as their soluble mediators (McVey Neufeld et al., 2020) has been shown to reduce the visceral pain response.

The underlying sex-dependent effects on visceral pain processing remain largely unexplored, particularly in the context of the gut microbiota. In this study, we investigated the role of the gut microbiota in the visceral pain response in female mice across the oestrus cycle and in response to OVX.

Methods

Experimental model and subject details

For this study, naïve Swiss Webster germ-free and conventional females aged between 6 and 10 weeks old were used. All animals were group housed and littermates were randomly assigned to each experimental group. All experiments were conducted in accordance with the guidelines of European Directive 86/609/ EEC and the Recommendations 2007/526/65/EC and were approved by the Animal Experimentation Ethics Committee of University College Cork. All efforts were made to reduce the number of animals used for the study and minimise animal suffering.

Method details

Animals

Swiss Webster breeding pairs for germ-free (GF) and conventionally colonised (CC) groups were supplied by Taconic (Germantown, New York, USA) and first-generation female offspring were used for all experiments. GF mice were group housed in flexible film gnotobiotic isolators maintained at 21 ± 1°C with 55-60% relative humidity under a 12-hr light/dark cycle in the University College Cork GF Unit. CC mice were group housed in the standard animal facility and maintained under the same temperature, relative humidity, and light/dark cycle as the GF unit. Both GF and CC mice were age matched and fed the same autoclaved pelleted diet (Special Diets Services, product code 801010). Surgery, testing, or euthanasia occurred between 6 and 10 weeks of age. All experimenters were blinded as to the experimental groups until scoring was completed.

Experimental timeline

This study was performed to address two questions as set out in the objectives and the experiments were designed as follows:

(i) Visceral sensitivity assessment in CC and GF mice following ovariectomy with sham control.

(ii) Visceral sensitivity assessment in ovariectomised CC E2 pellet-implanted mice with placebo control.

Ovariectomy

Ovariectomy (OVX) was carried out as previously described (O'Leary et al., 2009) in 6-week-old animals under germ-free conditions. Ketamine (90mg/kg) and Xylazine (10mg/kg) (both Abbeyville Veterinary, Ireland) anaesthetic mix was administered intraperitoneally to induce anaesthesia for ovariectomy surgeries. An area of the dorsal surface of the animal was shaved and sterilised with 70% ethanol and a small 2cm incision was made in the centre of this area. The fat pad of the left ovary was located laterally to this incision and a 0.5cm incision made through the abdominal wall to access the ovary. The ovary was gently taken out through the incision by pulling the accompanying fat and the ovary was then removed and the uterus gently replaced into the abdominal cavity. The incision site was then sutured, and the same procedure was carried out on the other side to remove the right ovary. The initial incision was then also sutured closed and cleaned with sterile saline. To control for the effect of the surgery on the study outcomes, the surgery was performed in the same manner, with the exception of the removal of the ovaries (i.e., the ovaries were gently pulled through the incision site but were immediately returned) to form the sham-OVX group. All

animals received the anti-inflammatory drug carprofen (5mg/kg, s.c., Carprofen, Norbrook) 1 day after the surgery.

17β-oestradiol pellet implantation

17β-Oestradiol (E2) pellet implantation was carried out as previously described (Ingberg et al., 2012). The pellets, 0.1mg E2/pellet (21-day release, IRA, FL, USA cat. No. E-121) or placebo (0.1mg/pellet, 21-day release, IRA, FL, USA cat. No. C-111) were implanted 1 week after the ovariectomy procedure when the animals were 7 weeks old. Animals were anaesthetised with isoflurane (1.5-2% for induction) and the pellet was then inserted subcutaneously into the dorsal aspect of the neck using a stainless-steel reusable precision trocar with regular medical point needle (IRA, FL. USA, cat. No. MP-182). The animal was then monitored for up to 20 min following pellet insertion. The same procedure was carried out for the placebo group with a placebo pellet being implanted in place of the E2 pellet to control for the effects of the surgery.

Colorectal distension

Animals were removed from the germ-free facility to undergo colorectal distension (CRD) as previously described (Luczynski et al., 2017; Tramullas et al., 2012) in 9-10-week-old animals. The CRD apparatus used consisted of a barostat (Distender Series II, G and J Electronics, Toronto, ON, Canada) and a transducer amplifier (LabTrax 4, World Precision Instruments, Sarasota, FL). A custom-made polyurethane balloon (2cm length x 1cm inflated diameter) was tied over a PE60 catheter with silk 4.0. Prior to securing the balloon to the catheter, several holes were

punched in the distal 20mm of the tubing with a 27-gauge needle to allow for inflation of the balloon. Mice were lightly anaesthetised with isoflurane (2% vapor in oxygen, IsoFlo, Abbot, UK) and the lubricated balloon along with a connecting catheter were inserted into the colon, 0.5cm proximal to the anus. The catheter was secured to the base of the tail with tape to prevent removal or displacement. Animals were allowed a recovery time of 10 min prior to the commencement of the CRD procedure. The balloon was connected to the barostat system and an ascending phasic distension protocol from 10 to 80mmHg consisting of three 20s pulses at each pressure with a 5-minute inter-pulse interval was used. The visceromotor response (VMR) to CRD was quantified by the pressure changes observed within the colonic distending balloon during the procedure and the average of the three consecutive pulses for each pressure were used. For each animal, pain threshold was designated as the pressure which exceeded the mean baseline activity plus three times the standard deviation. Following the visceral sensitivity assessment, the balloon was carefully removed, and the animals were sacrificed within 4 hours.

Identification of stages of oestrous

To investigate the effect of the different stages of the oestrous cycle on visceral pain perception, vaginal cytology was used. A vaginal swab using a saline-soaked cotton swab (saline was used to help preserve the morphology of cells taken as water may disturb the structure of the cells, potentially confounding the interpretation of the stage of oestrous (Cora et al., 2015)) was collected by gentle insertion of the cotton swab into the vaginal canal of the restrained animals. The cotton swab was slowly and gently rotated against the vaginal wall and then removed. Cells collected during the swab

were transferred to a dry glass slide and imaged under a light microscope. The stage of the oestrous cycle was determined based on the type and shape of the cells present (Caligioni, 2009). For the purpose of this study, the stages of oestrous were grouped as follows; proestrus/estrus and metestrus/diestrus due to previous reports of heightened visceral sensitivity to tactile stimulation when in metestrus/diestrus versus proestrus/estrus (Giamberardino et al., 1997; Gonzalez and Carrasquillo, 2019).

Quantification and statistical analysis

All data were assessed for normality using the Shapiro-Wilk test and Levene's test for equality of variances. Normally distributed data were analysed using repeated measures two-way ANOVA or mixed deign ANOVA followed by post hoc testing using Bonferroni correction. A p-value of 0.05 was set as the threshold of statistical significance and all data are presented as Mean ± SEM. Significant outliers identified in the analysis were excluded. All statistical testing was performed using SPSS version 27 (IBM Statistics). Detailed statistical reporting is listed throughout the results section under the relevant subheadings and in the figure legends. The number of animals used per experiment are as follows with n representing the individual animal:

- To investigate the role of the microbiota in the visceral pain response, 2 groups were generated: CC and GF (n = 10 per group).
- To analyse the impact of the oestrous cycle on the visceral pain response, 4 groups were used: CC (P/E), CC (M/D), GF (P/E), GF (M/D) (n = 8, 14, 9, 10 per group respectively).
- For investigation into the effects of female sex hormones on visceral pain perception, 2 experiments were run: Firstly using 4 groups to observe the effect

of cessation of production of oestrogen: CC-Sham, CC-OVX, GF-Sham, GF-OVX (n = 12, 10, 10, 12 per group respectively) and secondly to investigate hormone replacement with E2: CC-OVX-Placebo, CC-OVX-E2 (n = 13, 14 per group respectively).

Results

Absence of microbiota does not affect the visceral pain response to colorectal distension in female mice

To assess the role of the microbiota in the visceral pain response, the response to CRD in GF and conventionally colonised (CC) animals was investigated (see methods). To investigate the effect of cessation of production of circulating sex hormones, 6-week-old animals were ovariectomised or underwent sham surgery (see methods). These animals underwent CRD at 9 weeks of age.

Interestingly, unlike what we have previously shown in male mice (Luczynski et al., 2017), a lack of microbiota did not affect the VMR to CRD in female mice. A mixed design ANOVA with pressure as the repeated measures factor and microbiota status as the independent factor showed a main effect of pressure ($F(4,52) = 33.3$, $p < 0.001$) but not of microbiota status with respect to pressure ($F(4,52) = 0.7$, $p = 0.62$) nor of microbiota status alone ($F(1,13) = 0.8$, $p = 0.402$) **(Figure 1)** when oestrous cycle stage was not taken into account.

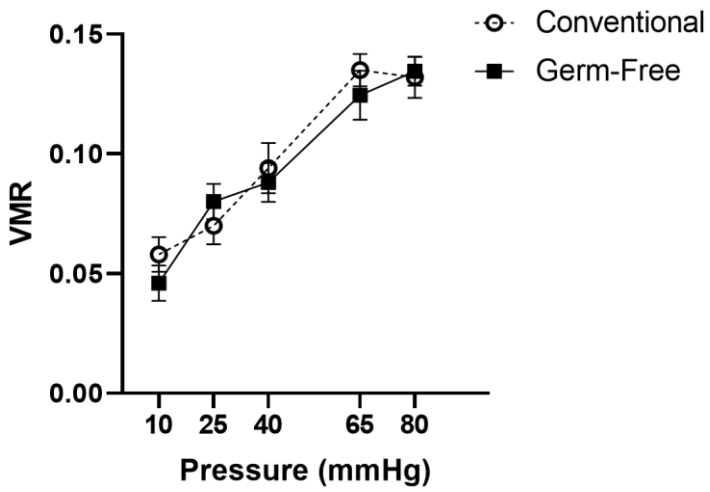


Figure 1. Absence of microbiota does not affect the visceral pain response to colorectal distension in female mice. *No differences observed in the visceromotor response (VMR) in response to colorectal distension between conventional and germ-free animals. Data presented as Mean ± SEM. n = 10 per group.*

Ovariectomy induces an increase in body weight in both conventional and germ-free mice

Here, we observed that OVX induced an increase in body weight in both conventional and GF mice as reported previously (Ding et al., 2017) as found by a repeated measures 2-way ANOVA by a main effect of time ($F(5,175) = 235.8$, $p < 0.001$), of OVX with respect to time ($F(5,175) = 21.5$, $p < 0.001$), of microbiota status with respect to time ($F(5,175) = 9.9$, $p < 0.001$) but not of microbiota status*OVX with respect to time ($F(5,175) = 0.6$, $p = 0.7$) **(Figure 2A)**. Significant main effects of OVX ($F(1,35) = 10.6$, $p = 0.003$) and of microbiota status ($F(1,35) = 7.1$, $p = 0.012$) but not of a microbiota status*OVX interaction ($F(1,35) = 0.01$, $p = 0.921$) on body weight gain were also noted **(Figure 2A)**.

Further analysis using Bonferroni post hoc revealed specific differences in body weight between CC-Sham and CC-OVX groups on day 15 ($p = 0.043$), and day 19 ($p = 0.023$), and between GF-Sham and GF-OVX groups on day 12 ($p = 0.047$), day 15

($p < 0.001$), and day 19 ($p = 0.004$), whereby the OVX groups weighed more than sham controls.

Oestrous cycle modulation of visceral pain is driven by microbiota-dependent mechanisms

In conventional females, a mixed design ANOVA with pressure as the repeated measures factor and oestrous cycle stage as the independent factor revealed a main effect of pressure ($F(4,80) = 21$, $p < 0.001$) but not of oestrous stage with respect to pressure ($F(4,80) = 1.6$, $p = 0.171$). A main effect of oestrous stage alone was also noted ($F(1,20) = 12.4$, $p = 0.002$) **(Figure 2B)**. Post hoc analysis using Bonferroni correction revealed significant differences between CC animals in proestrus/estrus versus when in metestrus/diestrus at pressures of 65mmHg ($p = 0.004$), and 80mmHg ($p = 0.037$), whereby the VMR to CRD was lower in conventional animals during proestrus/estrus versus when in metestrus/diestrus **(Figure 2B)**.

No significant differences in VMR across the oestrous cycle were noted in germ-free animals using a mixed design ANOVA with pressure as the repeated measures factor and oestrous cycle stage as the independent factor reporting a main effect of pressure ($F(4,64) = 22.5$, $p < 0.001$) but not of oestrous stage with respect to pressure ($F(4,64) = 0.4$, $p = 0.819$) nor an effect of oestrous stage alone ($F(1,16) = 2$, $p = 0.176$) **(Figure 2B)**.

When data were grouped by (1) stage of oestrous and (2) microbiota status, repeated measures 2-way ANOVA also revealed a main effect of pressure ($F(4,144) = 43.5$, $p < 0.001$) but not of group with respect to pressure ($F(12,144) = 0.8$, $p = 0.6$). A main effect of group was also noted ($F(3,36) = 5.9$, $p = 0.002$). Further analysis using

Bonferroni post hoc revealed significant differences between conventional animals in proestrus/estrus versus when in metestrus/diestrus at pressures of 65mmHg ($p = 0.041$), whereby conventional females in proestrus/estrus displayed lower VMR to CRD versus conventional females in metestrus/diestrus **(Figure 2B)**.

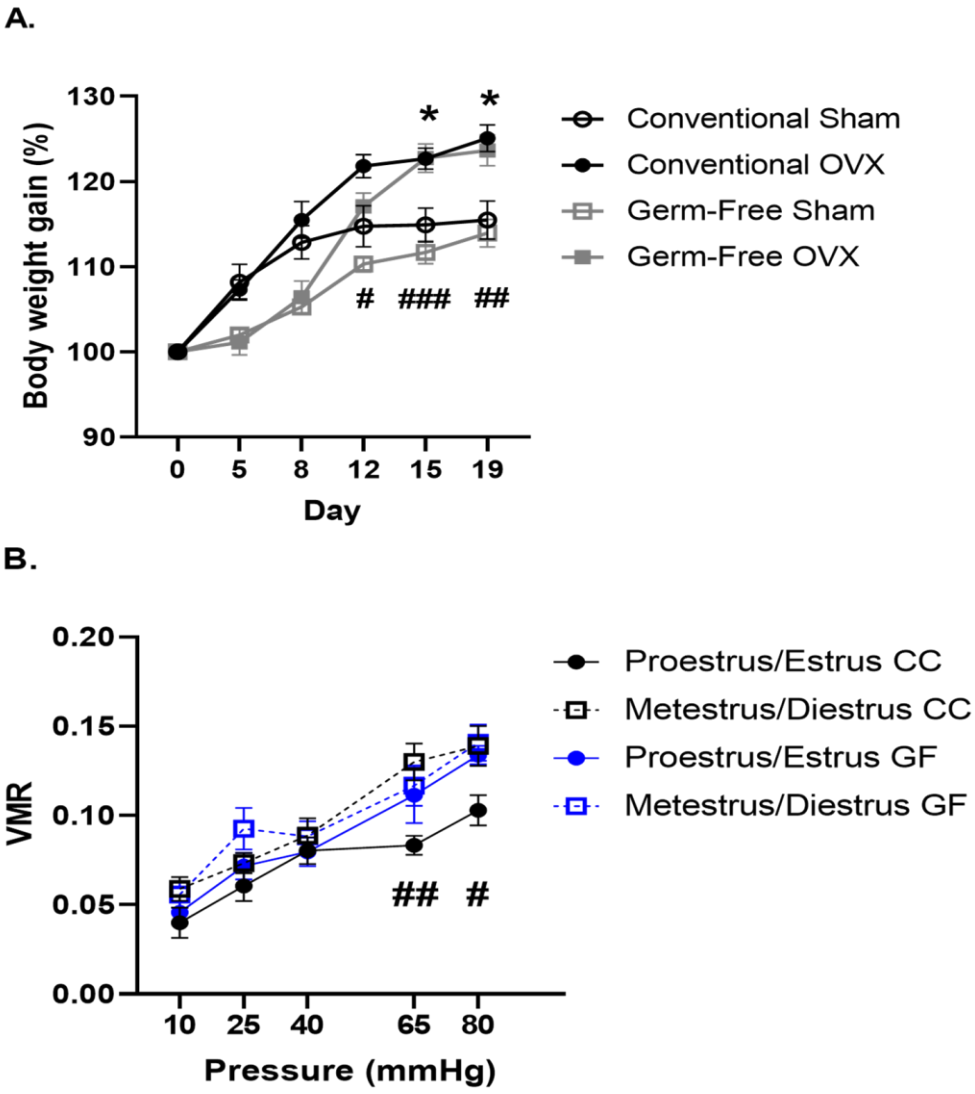


Figure 2. Ovariectomy induced an increase in body weight in both conventional and germ-free mice and oestrous cycle modulation of visceral pain was driven by microbiota-dependent mechanisms (A) *Ovariectomy (OVX) induced an increase in body weight in female mice regardless of microbiota status. *p ≤ 0.05 for conventionally colonised ovariectomised versus sham group, #p ≤ 0.05, ##p ≤ 0.01, ###p ≤ 0.001 for germ-free ovariectomised versus sham group. n = 7–12 per group. Data presented as Mean ± SEM.* ***(B)*** *Oestrous Cycle modulates visceral pain perception in conventional animals only. #p ≤ 0.05, ##p ≤ 0.01 conventionally colonised proestrus/estrus versus metestrus/diestrus group. n = 8–14 per group. Data*

presented as Mean ± SEM. VMR, visceromotor response; GF, germ-free; CC, conventionally colonised.

Ovariectomy induces visceral hypersensitivity in a microbiota-dependent manner

A repeated measures two-way ANOVA revealed a main effect of pressure ($F(4,132) = 61.3$, $p < 0.001$) but not of microbiota status with respect to pressure ($F(4,132) = 0.6$, $p = 0.696$) nor of OVX with respect to pressure ($F(4,132) = 1.1$, $p = 0.347$) or of microbiota status*OVX with respect to pressure ($F(4,132) = 0.5$, $p = 0.75$) on VMR to CRD. Further, no effect of microbiota status alone was noted ($F(1,33) = 1.2$, $p = 0.281$). However, main effects of OVX ($F(1,33) = 5$, $p = 0.032$) and of microbiota status*OVX were noted ($F(1,33) = 8.1$, $p = 0.008$) **(Figure 3A)**.

For conventional females, a mixed design ANOVA with pressure as the repeated measures factor and OVX as the independent factor revealed a main effect of pressure ($F(4,68) = 37.1$, $p < 0.001$), but not OVX with respect to pressure ($F(4,68) = 1$, $p = 0.413$). However, a main effect of OVX alone was noted ($F(1,17) = 11.7$, $p = 0.003$). Post hoc analysis using Bonferroni correction revealed significant differences in the VMR to CRD between CC-Sham and CC-OVX animals at pressures of 40mmHg ($p = 0.05$) and 65mmHg ($p = 0.02$), whereby the VMR to CRD was higher in ovariectomised animals. A mixed design ANOVA with pressure as the repeated measures factor and OVX as the independent factor in GF females revealed a main effect of pressure ($F(4,64) = 25.1$, $p < 0.001$) but not of OVX with respect to pressure ($F(4,64) = 0.6$, $p = 0.65$), nor of OVX alone ($F(1,16) = 0.2$, $p = 0.65$) **(Figure 3B)**.

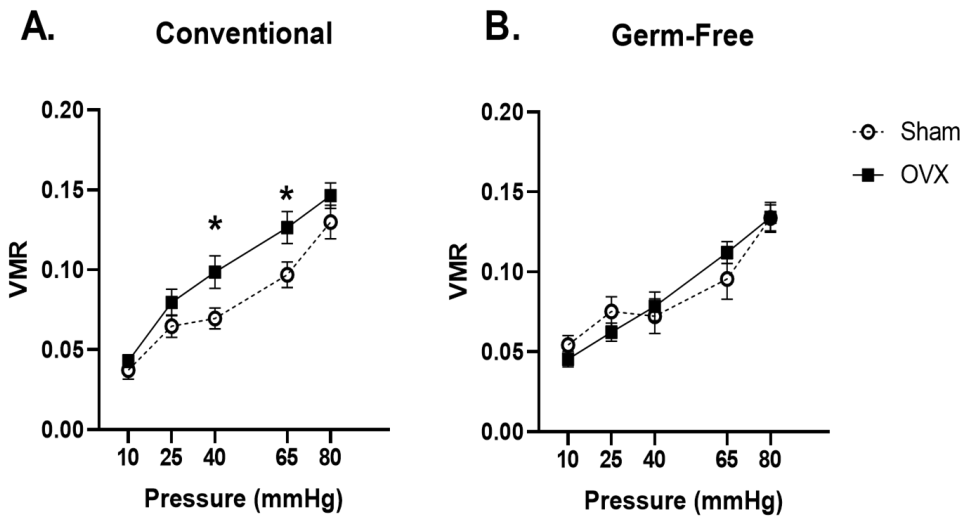


Figure 3. Ovariectomy induced visceral hypersensitivity in a microbiota-dependent manner (A) *Ovariectomy induced visceral hypersensitivity in conventional females. *p ≤ 0.05 conventionally colonised ovariectomised versus sham group.* ***(B)*** *No effect of ovariectomy on perception of visceral pain in germ-free females. Data presented as Mean ± SEM. n = 10–12 per group. VMR, visceromotor response; OVX, ovariectomy.*

Hormone replacement with 17β-oestradiol reverses ovariectomy-induced visceral hypersensitivity in conventional females

To assess the role of female sex hormones in the visceral pain response, 6-week-old CC animals were ovariectomised or underwent sham surgery. At 7 weeks old, these animals underwent 17β-oestradiol (E2) pellet or placebo implantation to investigate the role of E2 on perception of visceral pain (see methods). Finally, these animals underwent CRD at 10 weeks of age **(**see **Figure 4A** for experimental design**)**.

Finally, to investigate the impact of oestrogen in mediating the effects of female circulating sex hormones on the visceral pain response, ovariectomised E2 pellet-implanted animals underwent CRD. A mixed design ANOVA with pressure as the repeated measures factor and treatment as the independent factor revealed that E2 reduced the VMR to CRD by a main effect of pressure ($F(4,84) = 47.5$, $p < 0.001$), of

hormone replacement with respect to pressure ($F(4,84) = 2.9$, $p = 0.026$) and of hormone replacement alone ($F(1,21) = 20.6$, $p < 0.001$) **(Figure 4B)**. Significant differences in the VMR to CRD were noted by post hoc analysis using Bonferroni correction at pressures of 40mmHg ($p = 0.024$), 65mmHg ($p = 0.01$), and 80mmHg ($p = 0.005$), whereby the VMR of E2 pellet-implanted animals was lower than that of controls **(Figure 4B)**.

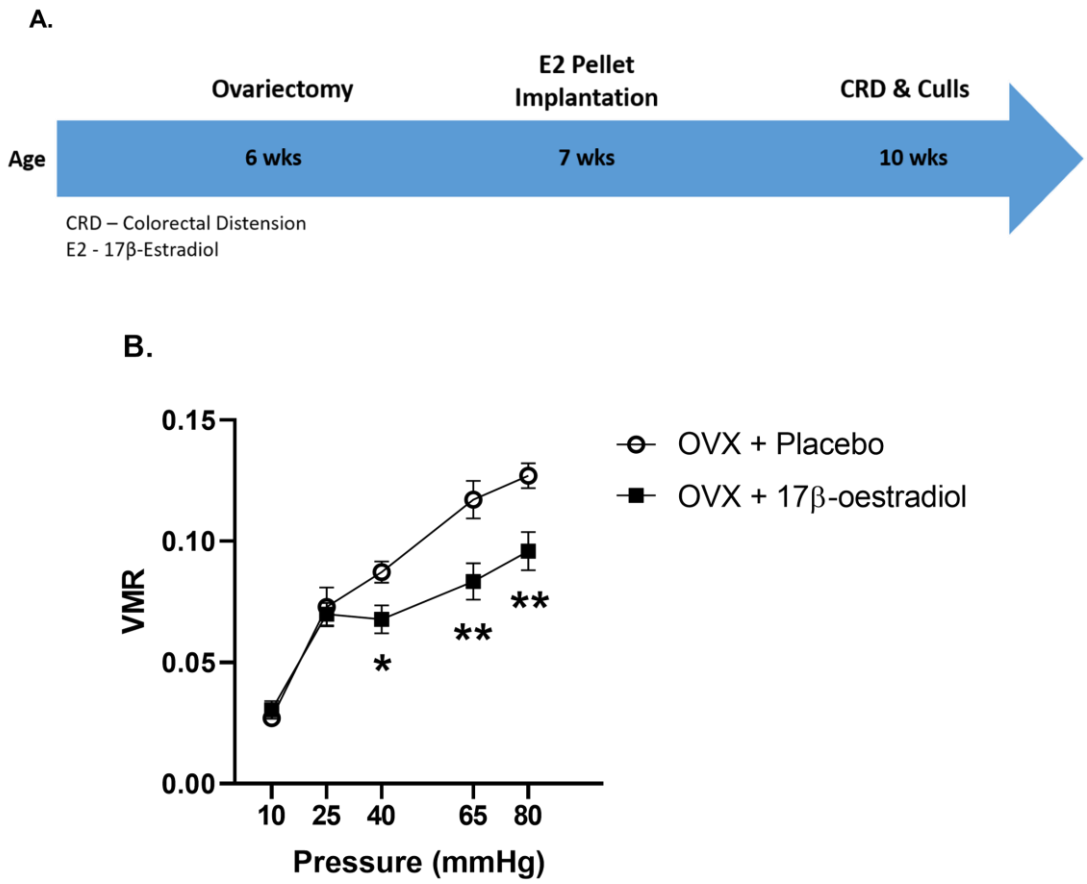


Figure 4. Hormone replacement with 17β-oestradiol reversed ovariectomy-induced visceral hypersensitivity in conventional females (A) *Experimental timeline.* ***(B)*** *17β-Oestradiol reversed ovariectomy-induced visceral hypersensitivity in conventional mice. *p ≤ 0.05, **p ≤ 0.01 ovariectomised with oestradiol versus placebo group. n = 13–14 per group. Data presented as Mean ± SEM. n = 13–14 per group. VMR, visceromotor response; OVX, ovariectomy.*

Discussion

This study aimed to investigate the role of the microbiota and female sex hormones in OVX-induced visceral pain. We show that OVX-induced VH is dependent on the gut microbiota and that visceral pain is modulated across the oestrous cycle in a microbiota-dependent manner. We also noted that OVX induced an increase in body weight regardless of microbiota status, and hormone replacement with E2 ameliorated OVX-induced increases in visceral sensitivity in conventional mice. Overall, these results highlight the major regulatory role of the gut microbiota on sensation of visceral pain, as well as the potential benefit of female sex hormones in lessening the pain response to a noxious visceral stimulus.

Here, we show that contrary to our previous work in male mice (Luczynski et al., 2017), no difference was seen between control and GF female mice in the pain response to CRD when the stage of the oestrous cycle was not accounted for. While there are several reports of sex differences in the functioning of the microbiota-gut-brain axis (Audet, 2019; Darch et al., 2021; Jaggar et al., 2020), we show for the first time that the gut microbiota is an important factor in sex differences in the visceral pain response. Here we show that the visceral pain response is modulated by the oestrous cycle in conventionally colonised control mice but not in GF mice, further supporting the role of the gut microbiota in appropriate sensation of visceral stimuli. Specifically, we show a lesser pain response to CRD during proestrus/estrus than during the metestrus/diestrus stages, and that this stage-dependent difference in the visceral pain response is reliant upon a full complement of gut microbiota.

It has been seen previously that perception of visceral pain varies across the oestrous cycle with female rats displaying a lower threshold to CRD in proestrus and estrus

versus metestrus and diestrus (Moloney et al., 2016b). Mechanisms behind this stage-dependent increase in VMR may lie in the heightened oestrogen levels seen in proestrus which has been shown to be protective against visceral pain (Cao et al., 2012). It has also been noted that GF mice have altered sex hormone levels, and colonisation of these mice increases reproductive capability, highlighting the link between the microbiota and sex hormones (Wallace et al., 2018). Furthermore, human studies have shown that women who harbour a more diverse gut microbiota display higher levels of oestradiol, supporting the relationship between the gut microbiota and gonadal hormones (Shin et al., 2019). Also, women with higher serum oestradiol levels had more *Bacteroidetes* and less *Firmicutes* than those with lower serum oestradiol, and the genera *Slackia* and *Butyricimonas* significantly correlated with serum oestradiol levels. Interestingly, the gut microbiota has been shown to significantly affect oestrogen levels whereby microbial richness and diversity correlated with circulating oestrogen levels (Flores et al., 2012). Hence, there is a clear association between gonadal hormones and the gut microbiota, which contributes to sex differences in the pain response.

Interestingly, in our study, OVX resulted in VH in control but not microbiota-deficient GF mice. Previous studies have shown that OVX results in visceral hyperalgesia to mechanical (von Frey) and thermal (hot plate) stimuli (Sanoja and Cervero, 2008), as well as to intracolonic capsaicin administration (Sanoja and Cervero, 2005). OVX has also been shown to cause shifts in the gut microbiota (Mendes et al., 2017; Wang et al., 2016). Specifically, OVX in rats resulted in increases in *Escherichia coli* and *Bacteroides fragilis* as well as a decrease in *Clostridium leptum*, *Faecalibacterium prausnitzii*, and *Lactobacillus*. As the gut microbiota plays a major role in visceral pain (O' Mahony et al., 2017), and GF mice are devoid of a gut microbiota, this could

explain why OVX in GF animals did not result in VH. Reasoning behind a more pronounced effect of OVX on the VMR at higher distension pressures may include the observation that as distension pressure increases, so too does the VMR due to increased activation of visceral nociceptors and increased pressure in the colorectal region. Thus, the effect of OVX on visceral pain perception may be more pronounced at a higher distension pressure versus a lower pressure. This finding supports the role of the microbiota in the manifestation of VH.

Oestrogen has been shown to upregulate neuronal activation in the central and peripheral nervous systems including at the level of the dorsal root ganglia in the spinal cord, GI tract, and enteric nervous system, thus exerting effects on visceral pain processing (Sun et al., 2019). The antinociceptive effects of oestrogen have also been shown previously, whereby the expression of substance P, a neuropeptide related to pain, was downregulated in lumbar dorsal root ganglia neurons in OVX rats implanted with 17β-Oestradiol pellets (Sarajari and Oblinger, 2010). Here, we show that OVX-induced VH was ameliorated by hormone replacement via E2 pellet implantation. Oestrogen receptors ERα and ERβ are capable of modulating the visceral pain response by regulation of activity of sensory neurons (Meleine and Matricon, 2014), and oestradiol has been shown to inhibit voltage-gated calcium channels of primary afferent neurons of the dorsal root ganglia in rats (Lee et al., 2002). Oestrogen receptor activation has been shown previously to have analgesic effects in a visceral pain model in mice (Zielińska et al., 2017), and the gut microbiota is also capable of metabolising oestrogens via microbial-derived β-glucuronidase, which then act via ERα and Erβ (Baker et al., 2017). ERβ knockout in mice revealed that ERβ affects gut microbiota composition (Menon et al., 2013) and that sex hormones affect the gut microbiota, thus posing a potential mechanism of action of E2 on visceral pain processing. Overall,

from the results herein and from existing literature, it is clear that the gut microbiota plays a major role in control of oestrogenic activity which modulates the visceral pain response.

An increase in body weight following OVX has been reported previously both in rats (Sharma et al., 2017) and in mice (Ding et al., 2017). Notably, here we show that this increase in body weight after OVX is independent of gut microbiota status. This OVX-induced weight gain has previously been attributed to the loss of circulating oestrogen as it is seen that exogenous addition of a phytoestrogen reduced OVX-induced weight gain (Sharma et al., 2017).

Conclusions

In summary, we report that visceral pain is modulated across the oestrous cycle in a microbiota-dependent manner. This is, to our knowledge, the first study to demonstrate reversal of OVX-induced VH to CRD by oestrogen replacement therapy by E2 pellet implantation.

Limitations

While this study provides a novel insight into the role of the microbiota in VH, further studies are needed to uncover exact molecular mechanisms behind oestrous cycle modulation of visceral pain and the role the microbiota plays in this modulation.

Chapter 5

Sex Differences in Pre-Adolescent Plasma Immune Profiles and the Impact of Early Life Stress

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Manuscript in preparation

Abstract

Both before and after birth, extensive immune system development is observed. In the pre-adolescent period in rodents, an increase in immune activation has been noted to be both required and protective against immunopathologies later in life. However, the specific changes induced by early life stress on the developing immune system in this early pre-adolescent period are not well understood. Stressors in early life can have detrimental effects on physiology and behaviour in adulthood in both rodents and humans, which extends to dysregulation of the immune system. Here, using the maternal separation (MS) model of early life stress, we investigated whether there were sex-specific pre-adolescent changes in plasma and gastrointestinal levels of pro- and anti-inflammatory cytokines using a pro-inflammatory panel 1 day prior to and 1 day after weaning. Here, we show that MS increased spleen weight in males only and report sex-dependent immune changes in the pre-adolescent period. Specifically, we noted an increase in interleukin (IL)-5 and IL-13 in males, yet a decrease in IL-5 in females in the control groups at postnatal day (PND)22 compared to PND20 Moreover, a decrease in interferon (IFN)-γ and an increase in IL-6 in males due to maternal separation was noted whereas a decrease in IL-5 in females was seen at PND20. No changes were noted in ileal cytokine levels. Overall, we report differential sex-dependent pre-adolescent changes in circulating cytokine levels which are blocked by maternal separation. Interestingly, the majority of immunological changes observed in this study are found in males. This study adds evidence for the sex differences observed in the immune literature and suggests a complex interplay between early life stress and pre-adolescent changes in immune profiles which may affect later physiology and behaviour.

Introduction

Early postnatal life is a period which sees continuation of development throughout the systems of the body which require appropriate stimulation for optimal development (Renz et al., 2012). These systems include the gastrointestinal (GI), microbial, immune, as well as central and peripheral nervous systems, which are all involved in complex crosstalk via the gut-brain axis through a myriad of both direct and indirect pathways (Cryan et al., 2019; Jena et al., 2020). The immune system is comprised of lymphoid organs such as the spleen, thymus, and bone marrow as well as immune molecules such as cytokines. Approximately 70% of the immune system is located in the gut in the form of gut-associated lymphoid tissue, a component of mucosal-associated lymphoid tissue (Vighi et al., 2008) which interacts with GI function and allows tolerance or mounting of an immune response against the luminal content. The innate immune system of the GI tract interacts with the gut microbiota and its' metabolites and includes the mucous layer, intestinal epithelial layer, and haematopoietic immune cells. In early life, the immune system undergoes major development. It is seen that in neonatal mice, the epithelium has a lower turnover, the mucous layer is much thinner, and there are very few mature Paneth cells, secretory cells that release antimicrobial molecules, versus adult mice (Kalbermatter et al., 2021). The postnatal development of the immune system has been reviewed previously (Georgountzou and Papadopoulos, 2017).

There is extensive postnatal development of the immune system, with early neonatal immunity being thought of as immature given the lack of exposure to pathogens (Jain, 2020). Early postnatal life is a time of great change for the developing immune system as it must tolerate colonisation of the GI tract. It is also seen that new-borns are highly

susceptible to infection due to the immature immune system. Sex differences in immune system responses also exist. It has been reported that females mount a stronger immune response than males, leading to a faster removal of pathogens (Klein and Flanagan, 2016). Further, it is seen that pre-pubertal males display higher levels of inflammation as well as higher levels of regulatory T cells, required for peripheral tolerance and prevention of autoimmunity, and immunoglobulin A, an antibody responsible for fighting infection. However, it is also seen that autoimmune disorders are more prevalent in women (Klein, 2012; Lotter and Altfeld, 2019).

As developmental processes are still ongoing in the early postnatal period, it is perhaps no surprise that any insult, such as stress, during this early life critical window may result in widespread detrimental effects. Depending on the type, severity, and duration of the stressor, immune responsivity may be increased or decreased (Dhabhar, 2014; Seiler et al., 2020). Other factors that play a major role in immune responsivity include genetics, prior exposure, age, and environmental factors including mode of feeding and delivery (MacGillivray and Kollmann, 2014; Taneja, 2018). Stress may also affect the release of pro- and anti-inflammatory cytokines under unstimulated conditions or in response to immune challenge (Carlsson et al., 2014).

One well-established model of stress during early life in rodents is maternal separation (MS), which involves separating pups from their mothers in the early postnatal period. The effects of MS on the immune system are generally reported to result in a pro-inflammatory state, including increased pro-inflammatory cytokine signalling in brain regions related to stress-related psychiatric disorders (Dutcher et al., 2020; Roque et al., 2016). However, some studies have reported a downregulation of cytokine genes following MS (Dimatelis et al., 2012), highlighting the need for characterisation of these effects.

Other more naturalistic events in early life that impact on physiology and behaviour include weaning from the mother in the pre-adolescent period in rodents, which may also be considered stressful for a variety of reasons. At weaning, pups are subjected to different housing conditions and are no longer in the presence of the mother. Weaning also brings about a change in diet whereby pups are solely sustained on a solid food diet and are no longer breastfed, which in turn leads to vast expansion of the microbial communities residing in the GI tract, resulting in a spike in immune activation. This cascade of events is referred to as "the weaning reaction" (Al Nabhani et al., 2019). This vigorous immune reaction described by Al Nabhani and colleagues was also shown to be necessary for optimal immune system development as inhibition of this weaning reaction led to inflammatory pathologies later in life, likely due to immune dysregulation due to blocking of proper immune imprinting. Interestingly and similarly to MS, it has been seen in piglets that weaning results in impairments in gut barrier function as marked by an increase in transepithelial permeability and reduction in transepithelial electrical resistance (Hu et al., 2013b; Moeser et al., 2007). This increased gut barrier permeability may lead to bacterial translocation and initiate an immune response. It has also been suggested that these immune changes may predispose to pathology in later life (Mahmoud et al., 2016). Similarly, it has been shown that weaning induces upregulation of cells producing IFN-γ among other cytokines for at least 2 weeks following weaning (de Groot et al., 2021). However, the specific changes in gut and circulating immune profiles in the early pre-adolescent period is unknown, as is the impact of MS on these pre-adolescent changes in immune profiles.

The objective of this study was to investigate and characterise the impact of early life stress (ELS) on pre-adolescent changes in circulating and ileal immune profiles in male and female rats.

Methods

Animals and housing

Male and female Sprague Dawley rats (approximately 10 weeks of age) were purchased from Envigo, UK and were mated in the Biological Services Unit, Western Gateway Building, University College Cork, and consequent male and female offspring were used in this study. The day of birth was designated as postnatal day 0 (PND0). Dams and littermates were housed in large plastic breeding cages (15 x 22 x 9cm) in a humidity- and temperature-controlled room set to 21°C ± 1°C. The light/dark cycle was set to 12 hours (light phase 7am-7pm).

Samples were collected 1 day prior to, and 1 day post weaning which occurred at PND21. At PND20, male and female pups were removed from the home cage and culled for sample collection. At PND21, the remaining littermates were weaned and housed separately by sex. At PND22, the remaining male and female offspring were culled for sample collection as described below. All experiments were conducted in accordance with European Directive 2010/63/EEC, the requirements of S.I No 543 of 2012 and approved by the Animal Experimentation Ethics Committee of University College Cork.

Experimental design

Male and female offspring were divided into 8 experimental groups to investigate pre-adolescent changes in circulating and gut immune profiles and the consequent impact of maternal separation (MS) on these changes. The sample collection timepoints were PND20 and PND22 (1 day prior to, and 1 day after weaning), where animals of both

sexes were divided as follows; Male MS, Male non-separated (NS), Female MS, Female NS. These same groups were formed for both the PND20 and PND22 timepoints, resulting in 8 experimental groups **(**see **Figure 1)**.

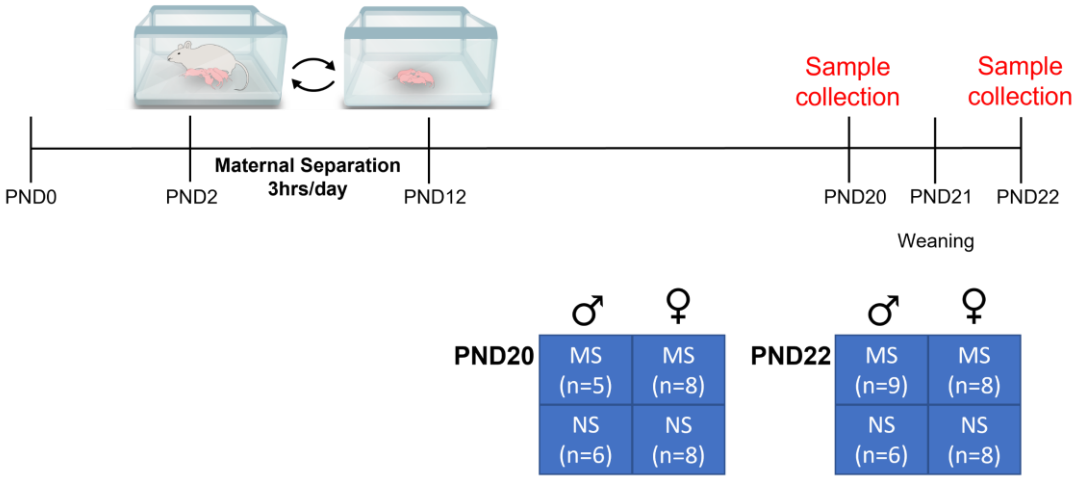

Figure 1. Experimental design. *MS: Maternal Separation, NS: Non-Separated, PND; Postnatal day.*

Maternal separation

Maternal separation was carried out as previously described (O'Mahony et al., 2009). Briefly, at PND0 litters were randomly assigned to maternally separated (MS) or non-separated (NS) groups. At PND2, the litters assigned to MS were moved from the main colony room to an adjacent room maintained at the same temperature (21 ± 2°C) and lighting conditions. The dam was first removed from the home cage and placed into a smaller holding cage, following which the pups (entire litters) were gently transferred together to a small cage where they remained for 3 hours. Cages containing the pups were placed on a heating pad set at 30–33°C and were filled with 3cm of bedding so pups could thermoregulate as needed. The dam was returned to the home cage and transferred back to the main colony room without her pups for this time period to avoid communication between the dam and her pups. After the 3-hour separation, dams were

again brought into the adjacent room and pups were returned to their original home cages. NS litters were also transported to the same room as the MS groups to avoid the confound of transportation stress but were otherwise left undisturbed in their home cages with their dams with the exception of weekly cage cleaning. This procedure was repeated daily from PND2 to PND12 inclusive. The period of separation was carried out at the same time each day (9am–12pm). At PND21, offspring were sexed and weaned, and both male and female offspring were used for the remainder of the study.

Sample collection

Animals were culled by decapitation at PND20 and PND22 (hereafter referred to as P20 and P22) and plasma and ileal samples were collected as follows:

Plasma collection: Whole trunk blood was collected into an EDTA-coated blood collection tube and placed on ice. Whole blood was spun down in a centrifuge at 3500x g at 4°C for 15 minutes. Following this, plasma was removed and frozen at -80°C until later analysis.

Ileal sample collection: The entire GI tract from stomach to anus was removed and sub-dissected into the small intestine, caecum, and colon. The small intestine was flushed with 10mM PBS to remove any luminal content and a 1.5cm segment of distal ileum, 2cm proximal to the caecum, was gently excised and snap frozen at -80°C until later analysis.

Gross anatomical measures

Gross anatomical measures including spleen weight (corrected for body weight), body weight, and measures of the gastrointestinal tract were taken at the time of cull.

Plasma cytokine measurements

Circulating cytokines in the plasma were measured using a V-PLEX pro-inflammatory panel 2 rat kit (cat no. K15059D, MSD) as per manufacturers' directions with the exception that plasma samples were not diluted prior to adding to the plate. Cytokines measured included interferon gamma (IFN-γ), interleukin (IL)-10, IL-13, IL-1β, IL-4, IL-5, IL-6, keratinocyte chemoattractant/growth-related oncogene (KC/GRO), and tumour necrosis factor alpha (TNF-α).

Homogenisation of ileal tissue

Based on the findings of (Al Nabhani et al., 2019) in changes of cytokine expression in the ileum, pre-adolescent changes in the gut immune profile were investigated in ileal tissue. To assess immune markers in gut tissue, tissue was homogenised, and the homogenate used for assessment of the levels of pro- and anti-inflammatory cytokines. 2 tablets of protease inhibitor (cat no. 589297001, Merck) were dissolved in 45ml of PBS with 5ml foetal bovine serum. Ileal tissue was carefully transferred to a PCR-grade 2ml screw cap tube containing 2.3mm beads. 500µl of the protease-PBS-FBS solution was added to each tube. Samples were then placed in a bead beater (MP FastPrep-24, MP Biomedicals) and homogenised for 30 seconds at full speed. Samples

were kept on ice for 15 minutes before the homogenate was collected. Samples were frozen and stored at -80°C until later analysis.

Ileal cytokine measurements

Levels of cytokines in the ileum were measured using a custom V-PLEX pro-inflammatory panel 2 rat kit to measure IL-10, IL-1β, IL-6, KC/GRO, and TNF-α performed as per manufacturer's instructions with the exception that the homogenate from ileum samples was not diluted prior to plating. Concentration readings were then normalised to tissue weight.

Statistical analysis

All data were assessed for normality using the Shapiro-Wilk test and Levene's test for equality of variances. Normally distributed data were analysed using three-way ANOVA, two-way ANOVA and Tukey's post hoc where appropriate. A p-value of 0.05 was set as the threshold of statistical significance and all data are presented as Mean ± SEM. All statistical testing was performed using SPSS version 28 (IBM Statistics).

Results

Early life stress induced sex-dependent increases in spleen weight, but not in body weight or gross measures of the GI tract

The weight of the spleen was used as an indicator of general immune activation. When spleen weight was normalised to body weight, two-way ANOVA revealed a significant main effect of ELS ($F(1,20) = 24.15$, $p < 0.001$) on normalised spleen weight in males **(Figure 2A)**. Further analysis using Tukey's post hoc revealed that the normalised spleen weight of MS animals at P20 and P22 was higher than that of their NS counterparts ($p = 0.014$ and $p = 0.009$ respectively) **(Figure 2A)**. In females, no differences in normalised spleen weight were noted **(Figure 2B)**.

No differences were noted in body weight or measurements of the different elements of the GI tract (data not shown).

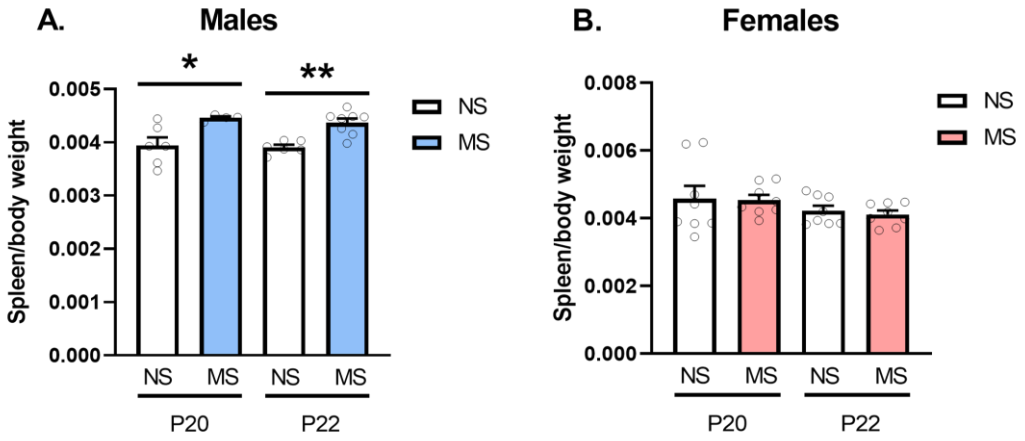

Figure 2. Sex-dependent alterations in spleen weight induced by maternal separation. ***(A)*** *Maternally separated males at P20 and P22 displayed higher normalised spleen weight versus NS controls *p ≤ 0.05, **p ≤ 0.01.* ***(B)*** *In females, no change in normalised spleen weight was noted. Data presented as Mean ± SEM. n = 4-9 per group for males and n = 8 per group for females.*

Differential sex-dependent pre-adolescent changes in circulating cytokines

To investigate the effect of MS and pre-adolescent changes on plasma cytokine levels, the data was initially split by sex. In males, a two-way ANOVA revealed a significant main effect of age ($F(1,21) = 5.202$, $p = 0.033$) and ELS ($F(1,21) = 7.286$, $p = 0.013$) on plasma levels of IFN-γ. Further analysis using Tukey's post hoc revealed a trend towards a significant decrease in plasma IFN-γ levels in the P20 MS versus P20 NS males ($p = 0.06$) **(Figure 3A)**. Two-way ANOVA revealed a significant main effect of an age*ELS interaction ($F(1,22) = 6.870$, $p = 0.016$) on plasma levels of IL-13. Tukey's post hoc revealed a significant difference between the P20 NS and P22 NS groups whereby the P22 NS males displayed higher circulating levels of IL-13 ($p = 0.03$) **(Figure 3C)**. Analysis of plasma levels of IL-1β by two-way ANOVA revealed a significant main effect of age ($F(1,16) = 11.318$, $p = 0.004$), of ELS ($F(1,16) = 7.762$, $p = 0.013$), and of an age*ELS interaction ($F(1,16) = 7.267$, $p = 0.016$). Further analysis by Tukey's post hoc revealed significant decrease in IL-1β levels in the P20 MS versus P20 NS groups ($p = 0.017$) and in the P22 NS versus P20 NS groups ($p = 0.006$) **(Figure 3D)**. Analysis of circulating levels of IL-5 by two-way ANOVA revealed a significant main effect of age ($F(1,20) = 6.308$, $p = 0.021$), and of an age*ELS interaction ($F(1,20) = 5.347$, $p = 0.032$). Tukey's post hoc revealed a significant increase in IL-5 levels in the P22 NS versus P20 NS males ($p = 0.017$) **(Figure 3F)**. Analysis of plasma levels of IL-6 by two-way ANOVA revealed a significant main effect of ELS ($F(1,18) = 11.093$, $p = 0.004$). Tukey's post hoc revealed a significant increase in plasma IL-6 levels in the P20 MS versus P20 NS males ($p = 0.015$) **(Figure 3G)**. All analyses on the other cytokines measured did not reveal significant changes.

In females, two-way ANOVA revealed a significant main effect of an age*ELS interaction ($F(1,26) = 10.182$, $p = 0.004$) on circulating levels of IL-5. Further analysis using Tukey's post hoc revealed a significant decrease in IL-5 levels in P20 MS versus the P20 NS females ($p = 0.025$), and a trend towards a decrease in the P22 NS versus P20 NS females ($p = 0.06$) **(Figure 3O)**. Again, no further differences in the other cytokines measured were noted.

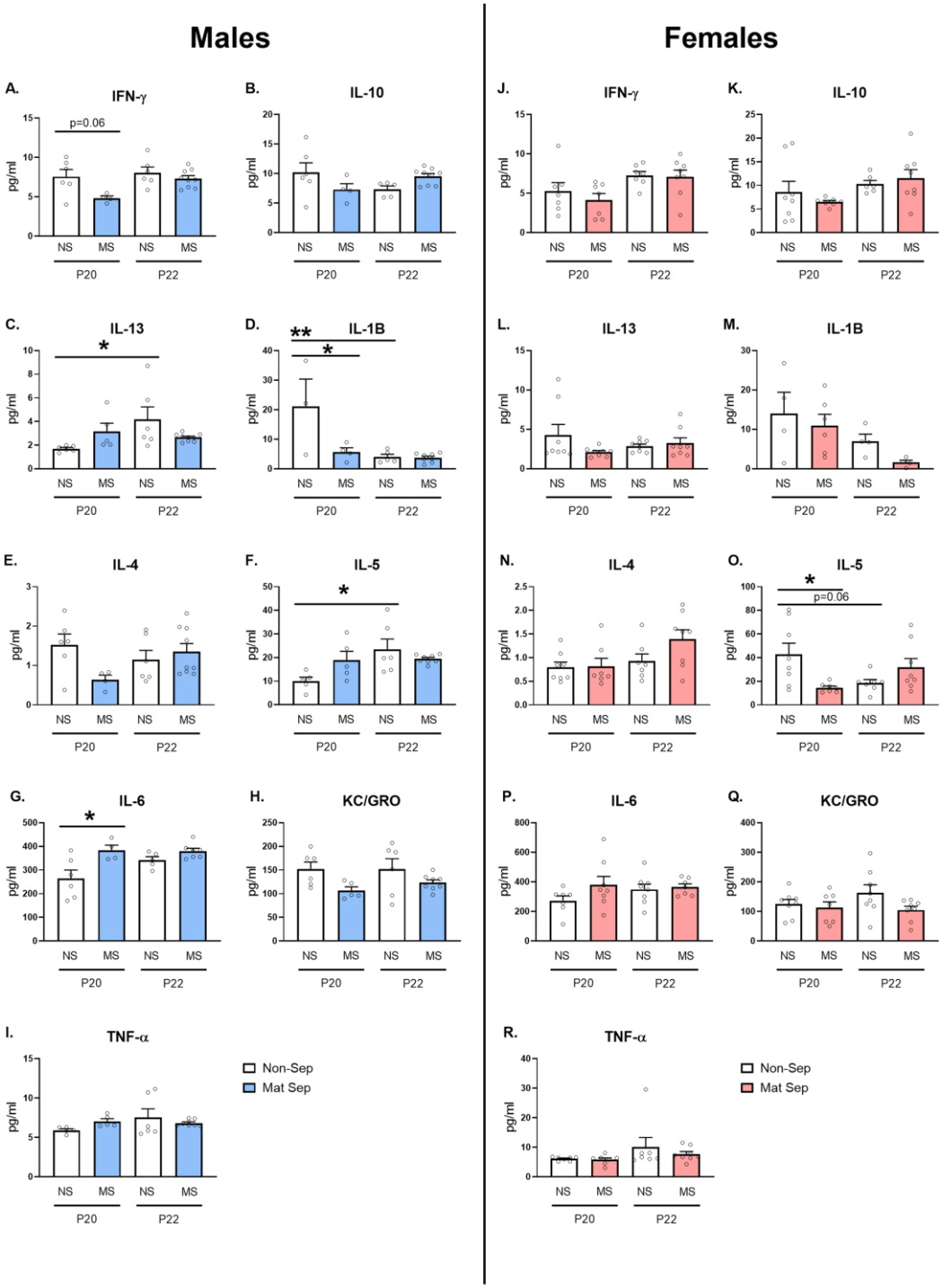

Figure 3. Differential sex-dependent pre-adolescent changes in circulating cytokines. *In males:* ***(A)*** *IFN-γ* ***(B)*** *IL-10* ***(C)*** *IL-13; higher levels of IL-13 in NS males at P22 versus P20 NS group; *p ≤ 0.05* ***(D)*** *IL-1β; P20 NS males displayed a significant increase in IL-1β plasma levels versus P20 MS and P22 MS groups; *p ≤ 0.05, **p ≤ 0.01* ***(E)*** *IL-4* ***(F)*** *IL-5; P22 NS males show higher circulating levels of IL-5 versus P20 NS group; *p ≤ 0.05* ***(G)*** *IL-6; P20 MS males show higher levels of IL-6 versus P20 NS animals; *p ≤ 0.05* ***(H)*** *KC/GRO* ***(I)*** *TNF- α. In females:* ***(J)*** *IFN-γ* ***(K)*** *IL-10* ***(L)*** *IL-13* ***(M)*** *IL-1β* ***(N)*** *IL-4* ***(O)*** *IL-5; P20 NS females showing higher circulating levels of IL-5 versus P20 MS animals; *p ≤ 0.05* ***(P)*** *IL-6* ***(Q)*** *KC/GRO* ***(R)***

TNF- α. Data presented as Mean ± SEM. n = 3-9 per group for males, n = 4-8 per group for females.

When analysed together, with sex, ELS, and age as independent variables, three-way ANOVA revealed a significant main effect of age ($F(1,30) = 16.842$, $p < 0.001$), of ELS ($F(1,30) = 7.914$, $p = 0.009$), and of an age*ELS*sex interaction ($F(1,30) = 4.199$, $p = 0.049$) on circulating IL-1β levels. Further analysis using Tukey's post hoc revealed a trend towards decreased levels of IL-1β in P20 MS versus P20 NS males ($p = 0.06$) as well as lower levels of IL-1β in P22 NS versus P20 NS males **(Figure 4D)**. Analysis of circulating IL-5 levels by three-way ANOVA revealed a significant main effect of sex ($F(1,46) = 5.247$, $p = 0.027$), and of an age*ELS*sex interaction ($F(1,46) = 12.189$, $p = 0.001$). Further analysis with Tukey's post hoc revealed significant differences between P20 NS males and P20 NS females whereby IL-5 levels were higher in the females ($p = 0.004$), between P20 NS and P20 MS females whereby the MS females displayed lower IL-5 levels ($p = 0.008$), and between P20 NS and P22 NS females whereby P22 females displayed lower levels of IL-5 ($p = 0.035$) **(Figure 4F)**. No further differences in the other cytokines measured were revealed.

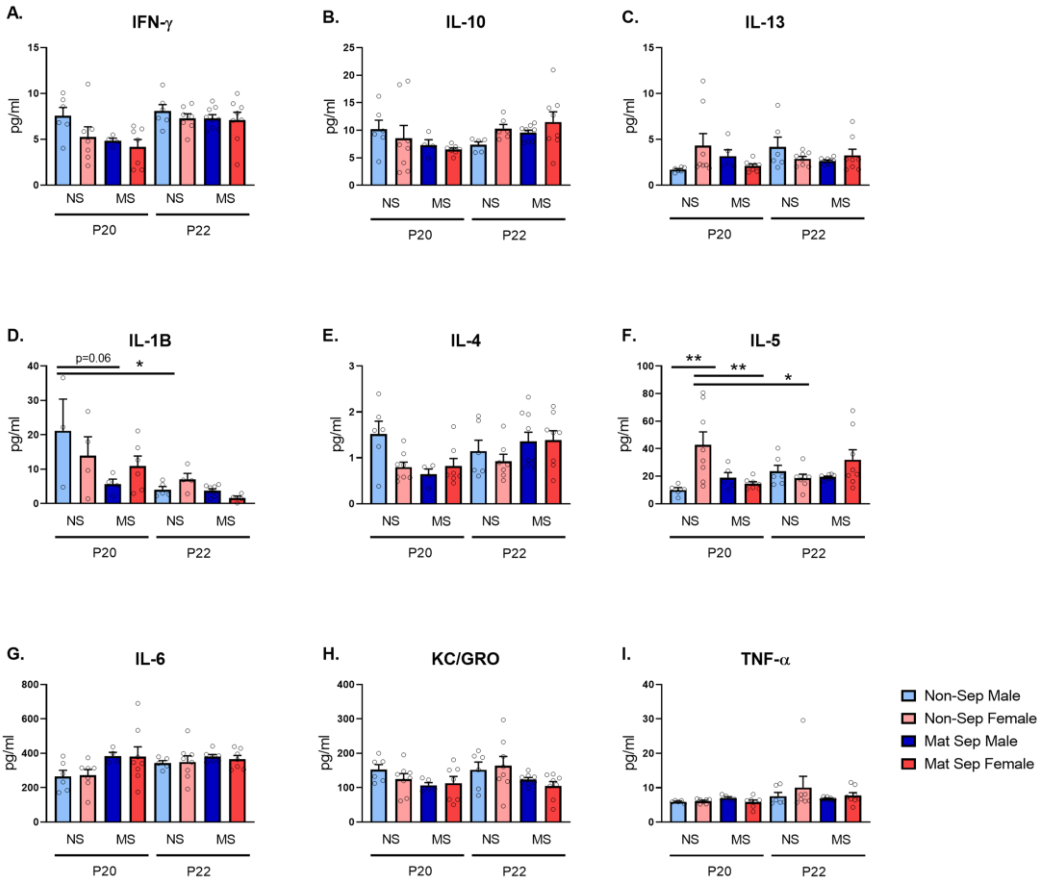

Figure 4. Differential sex-dependent effects of early life stress on circulating cytokine levels. ***(A)*** *IFN-γ* ***(B)*** *IL-10* ***(C)*** *IL-13* ***(D)*** *IL-1β; P20 NS males show higher levels of IL-1β versus P22 NS males; *p ≤ 0.05* ***(E)*** *IL-4.* ***(F)*** *IL-5; P20 NS females display higher levels of IL-5 versus P20 NS males, P20 MS females, and P22 NS females *p ≤ 0.05, **p ≤ 0.01* ***(G)*** *IL-6* ***(H)*** *KC/GRO* ***(I)*** *TNF- α. Data presented as Mean ± SEM. n = 3-9 per group.*

No pre-adolescent changes in ileal cytokines evident at P22

To investigate pre-adolescent changes in cytokines in the gut in response to maternal separation, the data was first split by sex. In males no differences in ileal levels of any of the cytokines measured were noted **(Figure 5A-E)**. Similarly in females, no changes in the levels of the cytokines measured were noted **(Figure 5F-J)**.

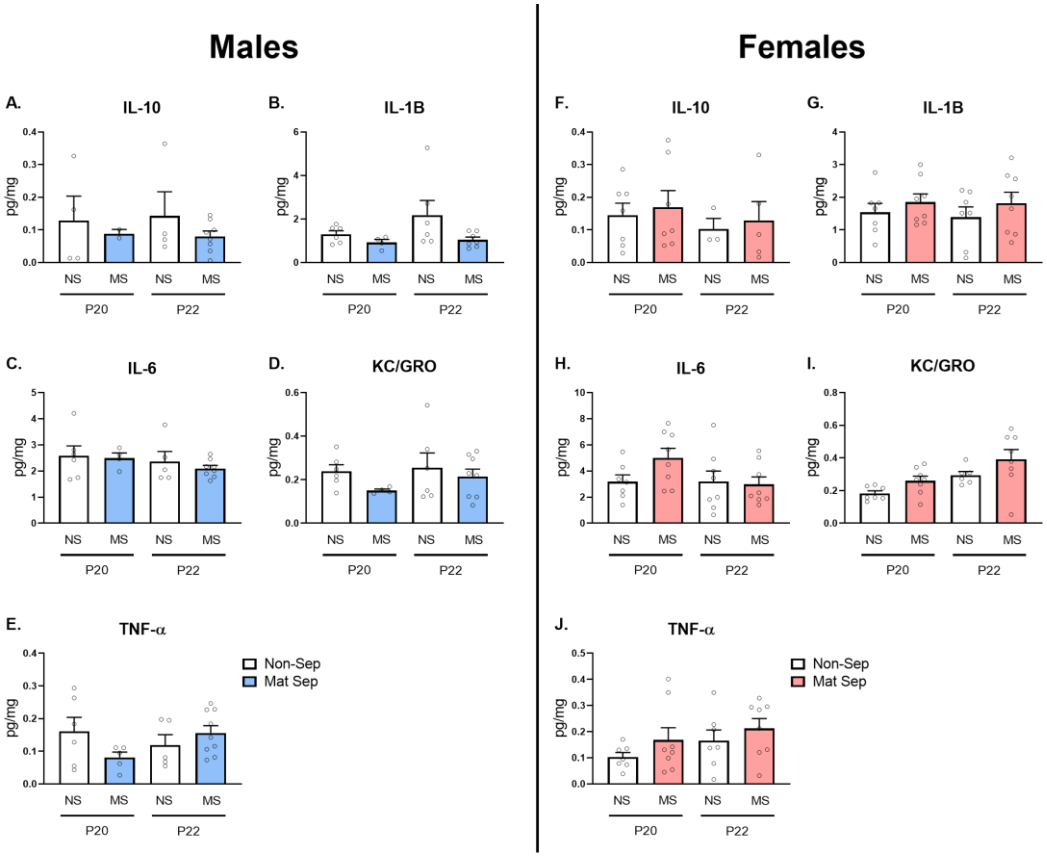

Figure 5. No pre-adolescent changes in ileal cytokines between the sexes. (A) *IL-10* ***(B)*** *IL-1β* ***(C)*** *IL-6* ***(D)*** *KC/GRO* ***(E)*** *TNF-α* ***(F)*** *IL-10* ***(G)*** *IL-1β* ***(H)*** *IL-6* ***(I)*** *KC/GRO* ***(J)*** *TNF-α. Data presented as Mean ± SEM. n = 2-9 per group for males and n = 3-8 per group for females.*

To investigate sex differences in ileal cytokine levels age, sex, and ELS were used as independent variables. When ileal levels of KC/GRO were assessed, three-way ANOVA revealed a significant main effect of age ($F(1,45) = 7.503$, $p = 0.009$), of sex ($F(1,45) = 5.146$, $p = 0.028$), and of an ELS*sex interaction ($F(1,45) = 6.576$, $p = 0.014$) **(Figure 6D)**. Further analysis using Tukey's post hoc revealed that ileal levels of KC/GRO were higher in P22 MS females versus P22 MS males ($p = 0.03$) **(Figure 6D)**. No differences in the other cytokines measures were noted **(Figure 6 A-C, E)**.

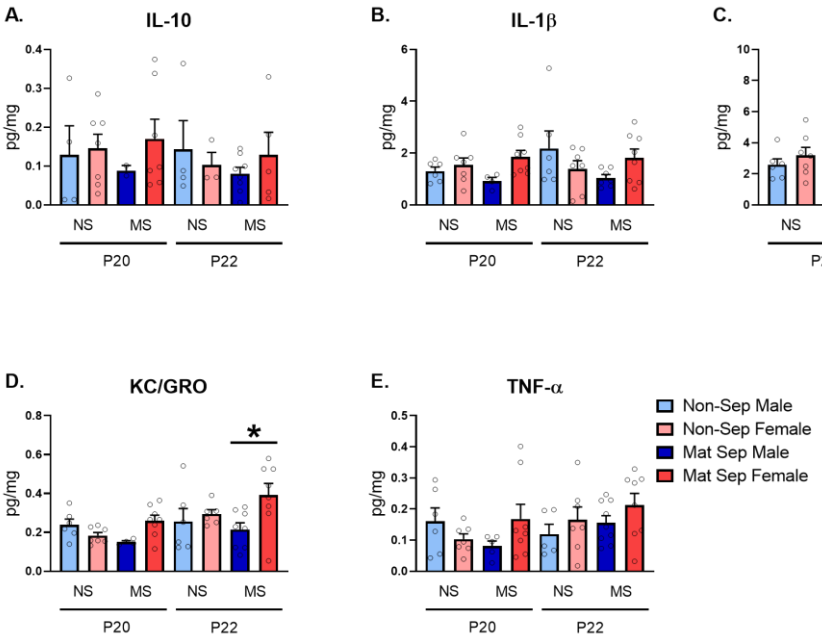

Figure 6. Sex-dependent differences in ileal cytokine levels. (A) *IL-10* ***(B)*** *IL-1β* ***(C)*** *IL-6* ***(D)*** *KC/GRO; higher levels of KC/GRO were noted in the P22 MS female group versus the P22 MS male group, *p ≤ 0.05.* ***(E)*** *TNF-α. Data presented as Mean ± SEM. n = 2-9 per group.*

A summary of the results highlighting ELS-induced and pre-adolescent-related changes on circulating and gut cytokines can be seen in **Table 1** and **2** respectively.

Table 1. Summary of pre-adolescent changes and effects of early life stress on circulating cytokines. **p = 0.06. P - Postnatal day; NS – Non-separated; MS – maternally separated; ↑ - increase; ↓ - decrease.*

Cytokine	ELS effect	Pre-adolescent Change	Sex Difference
IFN-γ	↓ in P20 MS vs P20 NS males	-	-
IL-5	↓ in P20 MS vs P20 NS females	↑ in P22 NS vs P20 NS males ↓ in P22 NS vs P20 NS females*	Higher in NS females at P20 versus P20 males
IL-6	↑ in P20 MS vs P20 NS males		-
IL-13	-	↑ in P22 NS vs P20 NS males	-

Table 2. Summary of pre-adolescent changes and changes induced by early life stress on gut cytokines. *P - Postnatal day; NS – Non-separated; MS – maternally separated; ↑ - increase; ↓ - decrease.*

Cytokine	ELS effect	Pre-adolescent Change	Sex Difference
KC/GRO	-	-	Higher in MS females versus MS males at P22

Discussion

Here, for the first time, we characterise pre-adolescent changes in circulating and gut immune profiles and investigate the effects of ELS on these changes in male and female rats. Interestingly, we report different changes in circulating cytokines depending on sex. Of interest, these pre-adolescent changes only occurred in NS animals for circulating cytokine levels, with no specific changes being seen in ileal cytokine levels, suggesting that MS blocks these normal pre-adolescent changes. Interestingly, MS induced different alterations in circulating cytokine levels in males versus females. This may suggest that MS exerts differential effects on systemic versus gut immunity or a different temporality for immune changes in the pre-adolescent period. While there are studies investigating changes in the immune system in this pre-adolescent period by using the early weaning model, the impact of appropriate weaning on the immune system in this early pre-adolescent period is not known.

MS resulted in increased spleen weight in male rats only which has been reported previously in the literature (Thornton et al., 2021), while some studies have indicated no change (Roque et al., 2014; Savignac et al., 2011). Similarly, Thornton and colleagues reported no difference induced by MS in the spleen weight of females as we also noted here. However, this is the first study to investigate these changes in this early pre-adolescent period. This increased spleen weight suggests general immune activation or an inflammatory state. This sex-dependent effect of MS has been widely reported in the literature, with males being more susceptible to the effects of MS than females.

We noted pre-adolescent changes in IL-5, a pro-inflammatory cytokine that has been suggested to play a role in eosinophilic asthma (Pelaia et al., 2019) given its role in

eosinophil differentiation, survival, recruitment, and degranulation. IL-5 is also closely linked with B-cell differentiation. Specifically, we found that IL-5 was elevated at the P22 timepoint in control male rats compared to their P20 controls, while this cytokine was decreased at this same timepoint in control females, indicating a differential sex response in these pre-adolescent changes. An increase in IL-5 production in male children with asthma has been reported following stress (Chen et al., 2006). Interestingly, IL-5 treatment in mice resulted in the production of TGF-β1 in the spleen, which resulted in a suppression of antigen-specific immune response of $CD4^+$ T cells in vitro (Nakagome et al., 2007), suggesting that this increase in IL-5 seen in males may result in suppression of the immune system, leaving the host susceptible to infection. We also noted that circulating levels of IL-13 were higher in P22 NS versus their P20 counterparts. IL-13 is an anti-inflammatory cytokine that modulates human B cells and monocytes, and may inhibit the release of inflammatory cytokines (Dembic, 2015). Similarly to IL-5, excessive amounts of IL-13 are seen in pathogenic conditions such as asthma, and anti-IL-13 drugs have been trialled against asthma (Mannon and Reinisch, 2012).

When the effect of ELS on plasma immune profiles were investigated, it was noted that IFN-γ was present at a lower level in male P20 MS rats in comparison to their control counterparts. IFN-γ is a pro-inflammatory cytokine and macrophage activator and plays a key role in protective cellular immunity (Kak et al., 2018). Low cytokine production, particularly IFN-γ in early life has been associated with an increased risk for sensitisation to allergens (Stern et al., 2007), suggesting that low levels of IFN-γ in early life may predispose to immune-related disorders.

MS was also associated with lower plasma IL-5 in female P20 MS rats relative to the NS controls at this age. The observed lower levels of IL-5, a known pro-inflammatory

cytokine, suggest that MS results in a lesser inflammatory process in females versus in males. Given that IL-5 has been shown to be increased following stress, this paradoxical decrease in MS females highlights the need for further investigations of the impacts of MS in females.

An increase in IL-6 in P20 MS males versus their NS counterparts was also seen. IL-6 is categorised as a pro-inflammatory cytokine, however, depending upon the conditions used, its effects have been described as pro- or anti-inflammatory (Scheller et al., 2011). The term "inflammation-responsive" cytokine has been used for this reason as IL-6 itself does not directly induce inflammation (Philippou et al., 2012). IL-6 has been shown previously to be decreased in the blood of MS animals under baseline conditions at PND15 (Roque et al., 2016). However, interestingly in this study by Roque and colleagues, blood IL-6 levels were shown to be increased following a consequent stressor. However, as this data was generated from rodents at PND15, 1 day after the conclusion of the stress hyporesponsive period where biological responsivity to stress is altered. Similarly, another study reported increased circulating IL-6 levels in MS samples following consequent stimulation with concanavalin A (Desbonnet et al., 2010). IL-6 has been suggested to play a role in the aetiology of depression, and has even been suggested to be suitable as a marker to classify subtypes of major depressive disorder (Ting et al., 2020). This is further reinforced by studies reporting higher IL-6 in those who were abused or neglected in early life (Munjiza et al., 2018), and it is known that ELS is associated with increased risk for development of stress-related psychiatric disorders such as depression (Syed and Nemeroff, 2017). Our data may shed some light on changes in cytokine levels due to MS outside of the stress hyporesponsive period.

Interestingly, the changes in circulating cytokine levels induced by ELS only occurred at P20, prior to weaning with no changes induced by ELS following weaning. Further, any pre-adolescent changes noted were in NS animals. This suggests a complex interplay between pre-adolescent changes and ELS whereby any pre-adolescent changes in circulating cytokine levels may have been blocked by MS as no alterations in cytokine levels due to weaning were noted in MS animals.

Interestingly, when investigating pre-adolescent changes due to weaning in the gut immune profile in the ileum, no changes in cytokines were noted. The only difference noted was that KC/GRO was higher in P22 MS females versus their male counterparts. KC/GRO is a powerful neutrophil chemoattractant and is stimulated in keratinocytes, monocytes, and macrophages in response to stimuli including TNF-α and microbial-borne signals (Shea-Donohue et al., 2008). Interestingly, mice deficient in KC/GRO-α displayed an exaggeration response to DSS-induced colitis (Shea-Donohue et al., 2008), highlighting the protective role of KC/GRO in the intestinal response to inflammatory insult. The fact that no pre-adolescent changes or impact of ELS was noted on ileal cytokine levels suggests that the circulating immune factors react initially, with any possible changes in the gut taking longer to be expressed. Given that the timepoints used were P20 and P22, this window may have been too narrow to detect changes in the gut immune response. It has also been shown that an increase in the number of lymphocytes secreting cytokines in the gut was increased at 1 and 2 weeks after weaning, supporting the slower expression of change in the gut versus in circulation (Vázquez et al., 2000). Another study reported upregulation of IL-1β, IL-6, and TNF-α mRNA along the intestine up to 2 days post-weaning, suggesting that the pre-adolescent changes in immune profiles involves changes in gene expression first, followed by changes in cytokine levels after a time (Pié et al., 2004).

The sex differences in cytokine levels reported in this study reflect the difference in immune responses between the sexes. These sex differences are thought to stem from circulating sex hormones such as oestradiol and androgens (Klein and Flanagan, 2016), however, it is not likely that variations in these circulating sex hormones may be impacting on cytokine levels at this pre-adolescent period as oestrous cycling in female rats is not thought to begin until at least PND30 (Westwood, 2008). It has been reported that cytokine production differs between the sexes whereby peripheral blood mononuclear cells from males produces more IL-10 and TNF when exposed to a viral synthetic ligand or lipopolysaccharide respectively (Moxley et al., 2002; Torcia et al., 2012). Further, the efficacy of antigen-presenting cells and phagocytic activity of macrophages are higher in females, suggesting that there are inherent sex differences in immune responses (Spitzer, 1999; Weinstein et al., 1984).

In summary, in contrast to circulating levels of cytokines, no pre-adolescent changes in ileal cytokines were noted. Further, any ELS-induced changes to circulating cytokines were only noted at the P20 timepoint in the circulation. Together, this may suggest that pre-adolescent changes in cytokines first take place in the circulation, which may then induce changes in the GI immune profile in a slower manner. Alternatively, it may be suggested that MS blocks systemic pre-adolescent changes in cytokines given the absence of circulating cytokine changes in the MS groups, which may play a role in the manifestation of MS-induced behavioural and physiological changes. These findings also support the reports of MS-induced immune dysregulation in the literature. Overall, we characterise pre-adolescent changes due to weaning on the circulating immune profile and the consequent effect of MS.

Limitations

While the current study investigates several cytokines both in circulation and in gut tissue, whether these changes in cytokine levels seen affect physiology and behaviour at these timepoints is not clear. Further, the exact cause of these changes is unclear and the question of whether it is solely the dietary change, change in housing conditions, absence of the mother, or a function of all of these is responsible requires further investigation. Future studies should further delve into the ramifications of the changes in the immune profile in this pre-adolescent period, possibly using timepoints that are further apart. It should also be noted that the apparent changes in IL-1β in the plasma and IL-10 in ileal tissue are subject to scrutiny as these cytokines in many of the samples from these groups were below the limit of detection of the inflammatory panel used.

Chapter 6

Identifying a Biological Signature of Prenatal Maternal Stress

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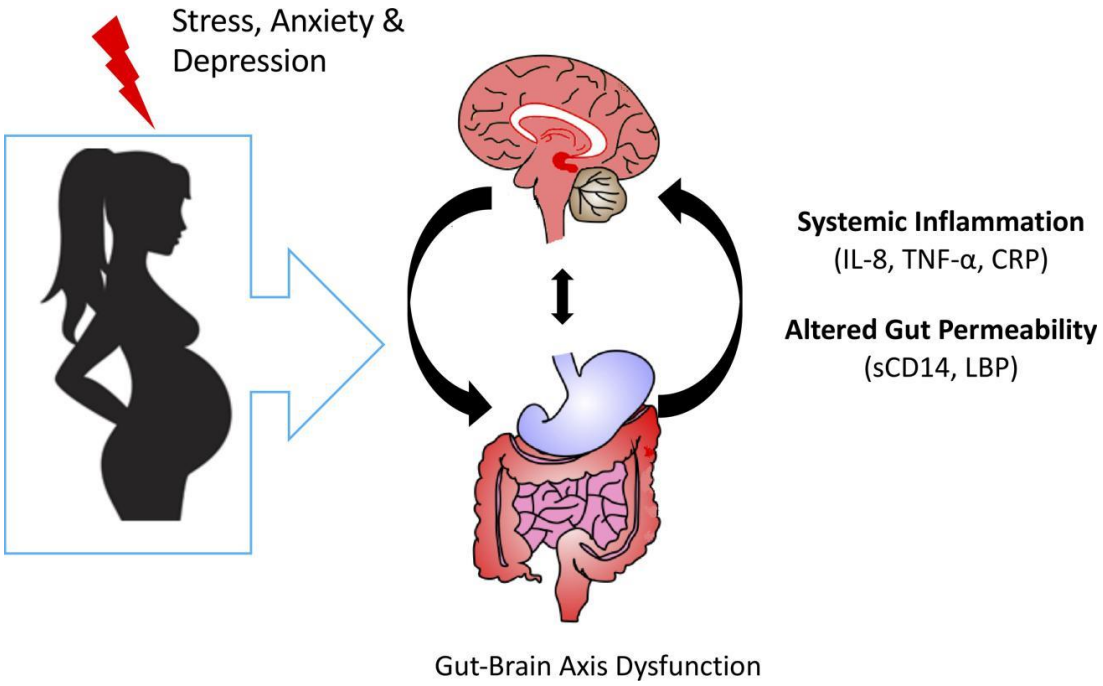
Abstract

Psychological stress affects maternal gastrointestinal (GI) permeability, leading to low-grade inflammation, which can negatively affect foetal development. We investigated a panel of circulating markers as a biological signature of this stress exposure in pregnant women with and without the stress-related GI disorder irritable bowel syndrome (IBS). Markers of GI permeability and inflammation were measured in plasma from healthy and IBS cohorts of women at 15- and 20-weeks' gestation. Biomarkers were evaluated with respect to their degree of association to levels of stress, anxiety, and depression as indicated by responses from the Perceived Stress Scale, State-Trait Anxiety Inventory, and Edinburgh Postnatal Depression Scale. High levels of stress were associated with elevations of soluble CD14, lipopolysaccharide binding protein (LBP), and tumour necrosis factor–α while anxiety was associated with elevated concentrations of C-reactive protein (CRP) in otherwise healthy pregnancies. Prenatal depression was associated with higher levels of soluble CD14, LBP, and CRP in the healthy cohort. High levels of prenatal anxiety and depression were also associated with lower concentrations of tryptophan and kynurenine, respectively, in the IBS cohort. These markers may represent a core maternal biological signature of active prenatal stress, which can be used to inform intervention strategies via stress reduction techniques or other lifestyle approaches. Such interventions may need to be tailored to reflect underlying GI conditions, such as IBS.

Graphical abstract



Introduction

Maternal prenatal stress is associated with several unfavourable pregnancy outcomes, including preterm birth (Lilliecreutz et al., 2016) and low infant birth weight (Khashan et al., 2014), while anxiety and depression early in pregnancy are considered risk factors for preeclampsia, a potentially fatal complication of pregnancy (Maher et al., 2017). Prenatal stress is also associated with altered programming of the developing foetal brain and an increased likelihood of behavioural problems manifesting during childhood and diagnosis of psychopathology in later life (Glover, 2019). Preclinical studies have demonstrated that induction of prenatal stress impedes optimum cognitive (Chan et al., 2018), behavioural (Gur et al., 2019), and psychosocial (Gur et al., 2017) development in the offspring. These findings are supported by prospective studies in humans, with neurodevelopmental ramifications encompassing temperamental and behavioural difficulties (Hartman et al., 2020), cognitive impairments (Laplante et al., 2004), and a hyperactive stress response along the hypothalamic-pituitary-adrenal axis (Van den Bergh et al., 2017).

Delineation of the molecular pathways that link prenatal stress to neurodevelopment is a critical first step in identifying biological risk factors associated with adverse outcomes. Conventional pathways of translocation of maternal glucocorticoids (Weinstock, 2008) and proinflammatory cytokines (Patterson, 2009) across the placenta and their direct action on the developing foetal brain have been the focus of many mechanistic studies in this area. It is becoming increasingly apparent, however, that alternative indirect routes may be more relevant to eliciting the downstream effects of prenatal stress, via neuroimmune interactions along the microbiota-gut-brain axis (Cryan et al., 2019).

A loss of gastrointestinal (GI) barrier integrity allows bacterial components, such as lipopolysaccharides (LPS) contained in the cell walls of gram-negative bacteria, to migrate into the general circulation and contribute to a systemic proinflammatory state (Power et al., 2014). In confirmation of this, functional and/or compositional alterations in the gut microbiota modify intestinal barrier permeability (Vaarala et al., 2008). Concomitantly, acute psychological stress has been shown to increase permeability of the small intestine (Vanuytsel et al., 2014), whereas disruption of the intestinal mucosal barrier has been implicated in the inflammatory pathophysiology of depression (Kelly et al., 2015). The state of chronic, low-grade inflammation that results from increased gut permeability is also known to negatively affect neurodevelopment (Jiang et al., 2018).

A mechanistic pathway implicated in this negative impact is the alteration in tryptophan (Trp) availability due to increased degradation of this essential amino acid along the kynurenine (Kyn) pathway (Notarangelo and Pocivavsek, 2017; O'Mahony et al., 2015). Altered gut microbiota profile, elevated gut permeability, low-grade inflammation, and Trp degradation along the Kyn pathway are all traits characteristic of irritable bowel syndrome (IBS), a highly prevalent functional disorder of the GI tract (Black and Ford, 2020; Clarke et al., 2009a; Enck et al., 2016; Kennedy et al., 2014a). Notably, stress is implicated in the pathogenesis of IBS, with its trademark symptoms, including intestinal sensitivity, motility, and permeability as well as mucosal immune activation, each shown to be exacerbated in the presence of psychological stresses (Qin et al., 2014). It is also well established that rates of psychiatric disorders, particularly anxiety and depression, are elevated among patients with IBS (Black et al., 2020a; Fond et al., 2014).

Here we investigate whether GI permeability and systemic inflammation are elevated in mothers who experience higher levels of stress, anxiety, and depression during pregnancy, and whether this stress signature is potentially augmented in women with IBS. We investigated circulating markers of these pathophysiological functions in the first and second trimester of pregnancy to identify associations that may serve as biological indicators of active prenatal maternal stress in a healthy cohort and in women with IBS. We anticipate that an assemblage of molecular indicators related to these processes, whose augmentation points to adverse pregnancy and neurodevelopmental outcomes, will aid in screening for at-risk pregnant women and informing appropriate intervention strategies aimed at counteracting the effects of prenatal maternal stress via either stress reduction techniques or other lifestyle approaches.

Methods

Study cohort.

The Screening for Pregnancy Endpoints (SCOPE) study (Kenny et al., 2014) was a collaborative project that established a unique international pregnancy biobank toward the identification of biomarkers that could be used to predict adverse pregnancy outcomes. The present study consisted of a subset (n = 209) of healthy nulliparous women with singleton pregnancies recruited to the Cork cohort of the SCOPE study (n = 1774) between November 2004 and January 2011. Women were excluded if underlying medical conditions indicated a high risk of preeclampsia, spontaneous preterm birth, or delivering a small for gestational age infant. Enrolled subjects underwent assessment by a SCOPE research midwife at 15 ± 1 (visit 1) and 20 ± 1 (visit 2) weeks' gestation. Demographic and clinical characteristics were obtained from subjects during the first visit, including the self-reported presence of IBS, defined as a combination of frequent diarrhoea and/or constipation accompanied by abdominal pain and sensation of bloating. Using this classification, all women with IBS and no exclusion criteria (n = 105) were included in the study together with an equivalent number of healthy women selected randomly from women with no exclusion criteria and no IBS (n = 104). Subject demographics, together with lifestyle characteristics collected at both visits, are presented in **Supplemental Table 2**. Heparinised blood samples were also collected before 12pm on the morning of each visit from which plasma was extracted for long-term storage in the SCOPE pregnancy biobank.

Assessment of maternal psychological status.

Subjects completed a number of clinically validated questionnaires at each visit in order to gauge prenatal levels of stress, anxiety, and depression. The Perceived Stress Scale (PSS) (Cohen et al., 1983) was used to evaluate the degree to which subjects perceived more generalised forms of stress in the month before assessment by appraising their feelings as to how they were able to handle daily hassles, how often they felt nervous and stressed, and how often they felt things were going well. The short form of the state scale of the Spielberger State-Trait Anxiety Inventory (STAI) (Marteau and Bekker, 1992) was used to assess the degree to which subjects experienced anxiety-related symptoms or emotions at the time of assessment. Finally, the Edinburgh Postnatal Depression Scale (EPDS) (Cox et al., 1996) was used to determine the presence of depressive symptoms in subjects in the week prior to assessment. Lower quartile (PSS - 7.5; STAI - 23.3; EPDS - 2.5) and upper quartile scores (PSS - 17; STAI - 40; EPDS - 9.5) of time point averages were derived from the complete Cork cohort of the SCOPE study and used as cut-offs to define low - ($<25^{th}$ percentile), moderate - (25^{th} to $<75^{th}$ percentile), and high - ($\geq75^{th}$ percentile) scoring groups for each of the psychological evaluations. Mean scores across both visits were subsequently used to stratify subjects from the present healthy and IBS cohorts into appropriate scoring groups, as outlined in **Figure 7**. A justification for the inclusion of each marker as it relates to GI permeability, systemic inflammation, and Trp metabolism is presented in **Supplemental Table 1**.

Measurement of gut permeability markers

Levels of circulating intestinal fatty acid binding protein (IFABP) and Soluble CD14 (sCD14) (Quantikine Immunoassays, R&D Systems, Bio-Techne), lipopolysaccharide binding protein (LBP) (Hycult Biotech), and anti-endotoxin core antibodies (EndoCAb IgA IgG IgM, Hycult Biotech) were determined using commercially available quantitative ELISAs. For each assay, samples were analysed in duplicate according to the manufacturers' instructions with absorbances measured at 450nm on a Synergy HT BioTek plate reader (Mason Technology). Results were calculated on a 4-parameter logistics curve generated using Gen5 BioTek Microplate Data Collection and Analysis software (Mason Technology). Inter- and intra-assay coefficients of variation for each assay are presented here respectively as follows: IFABP (5.8% and 5.5%), sCD14 (2.5% and 5.8%), LBP (5.1% and 2.7%), anti-endotoxin IgA (5.9% and 9.1%), anti-endotoxin IgG (8.8% and 9.0%), and anti-endotoxin IgM (5.2% and 9.9%).

Measurement of proinflammatory markers

Serum concentrations of 4 cytokines (IFN-γ, TNF-α, IL-6, IL-18) and 5 chemokines (IL-8, IP-10, MCP-1, SDF-1α, MIF) were determined using the Meso Scale U-PLEX platform (Meso Scale Diagnostics). This customised multiplex biomarker kit is a high sensitivity electrochemiluminescence (ECL) immunoassay. Circulating levels of C-reactive protein (CRP) were also assessed by means of ECL immunoassay using the V-PLEX Human CRP Kit (Meso Scale Diagnostics). For each assay, samples were analysed in duplicate according to the manufacturers' instructions with ECL measured on a QuickPlex SQ 120 multiplex imager (Meso Scale Diagnostics). Concentrations

were calculated from a standard curve calculated using a 4-parameter logistic fit using Workbench 4.0 software (Meso Scale Diagnostics). The inter- and intra-assay coefficients of variation are presented here respectively as follows: IFN-γ (8.7% and 9.5%), TNF-α (6% and 10.3%), IL-6 (6.4% and 9.8%), IL-18 (5.6% and 4.8%), IL-8 (5.4% and 7.2%), IP-10 (6.6% and 6.1%), MCP-1 (6.6% and 6.2%), SDF-1α (9.1% and 8.6%), MIF (12.4% and 10.9%), and CRP (1.5% and 1.8%).

Measurement of tryptophan metabolites

To determine the levels of Trp and Kyn, 198μL of plasma was spiked with internal standard (2μL) (3-Nitro l-tyrosine) before being deproteinised by the addition of 20μL of 4M perchloric acid. Samples were centrifuged at 20,000*g* on Hettich Mikro 22R centrifuge (AGB) for 15 minutes at 4°C and 100μL of supernatant transferred to an HPLC vial for analysis. Stock solutions of each standard were prepared in HPLC-grade water. Working dilutions were prepared from the stock standards, aliquoted in suitable vials, and stored at –80°C until required for analysis, at which point 20μL of 4M perchloric acid was also added and vortexed. Then 20μL of standards and sample supernatants were vortexed and 20μL of the supernatant was injected into the HPLC system (consisting of a CBM-20A system controller, a UV-Vis SPD-10A detector for Kyn, a fluorescence RF-20A detector for Trp, an LC-20AD pump, a CTO-20AC column oven at 30°C, a SIL-20AC HT autosampler, and a Prominence DGU-205R degasser). All samples were injected onto a reverse phase Luna 3μm C18(2) 100A size LC column 150 × 2mm (Phenomenex), which was protected by Krudkatcher disposable pre-column filters (Phenomenex) and SecurityGuard cartridges (Phenomenex). The mobile phase consisted of 50mM acetic acid and 100mM zinc

acetate with 3% (*v/v*) acetonitrile and was filtered through MilliporeSigma 0.45µm HV Durapore membrane filters (AGB) and vacuum degassed prior to use. Compounds were eluted isocratically over a 30-minute run time at a flow rate of 0.3mL/min after a 20µL injection. The columns were maintained at a temperature of 30°C, and samples/standards were kept a 4°C in the cooled autoinjector prior to injection. The fluorescence detector was set at an excitation wavelength of 254nm and an emission wavelength of 404nm. The UV detector was set at 330nm. l-Trp and its metabolite Kyn were identified by their characteristic retention times as determined by injection standards, which were run at regular intervals during the sample analysis. Chromatograms were analysed using the LabSolutions software (Shimadzu) and concentrations determined using analyte/internal standard peak height ratios. Results were expressed as ng/mL of supernatant.

Statistics

All data are presented as mean ± SEM, unless otherwise indicated. A 2-way repeated measures ANOVA was run to elucidate differences between healthy and IBS cohorts across gestational time points on biomarker concentrations using SPSS, version 25.0 (IBM Statistics). An equivalent analysis was also performed for each cohort individually to determine the effect of different scoring groups for each stress measure over time on each biomarker, with Tukey's HSD used for post-hoc analysis between individual scoring groups. Perceived stress, anxiety, and depression scores were also investigated as continuous response variables against each biomarker in multiple linear regression models carried out in R. Biomarker coefficients adjusted for age, BMI, socioeconomic status, smoking status, and alcohol intake prior to participation

in the study are reported. Statistical significance was accepted at the $p<0.05$ level of confidence for all models.

Study approval

The research described received approval from the Clinical Research Ethics Committee of the Cork Teaching Hospitals (protocol number: APC1004; approval number: APC-D-14). Informed consent was obtained from all participants, who were free to withdraw from the study at any time.

Results

Healthy cohort associations

Associations between maternal perceived stress and biomarker levels in the healthy cohort.

Significant differences in PSS scoring groups across gestational time points are outlined in **Figure 1**. sCD14, LBP, and TNF-α levels were found to significantly increase in the high-scoring group (110.96 ± 43.5ng/mL, p = 0.033; 4.27 ± 1.57μg/mL, p = 0.021; 0.62 ± 0.26pg/mL, p = 0.049; respectively) compared with the low-scoring group. Conversely, IL-8 concentrations were observed to be significantly lower in the high-scoring group (–0.45 ± 0.16pg/mL, p = 0.018) compared with moderate scorers. Significant differences in biomarker levels between scoring groups were not complemented by the presence of linear associations between circulating biomarkers' concentrations and PSS scores in healthy participants, as outlined in **Supplemental Table 4**.

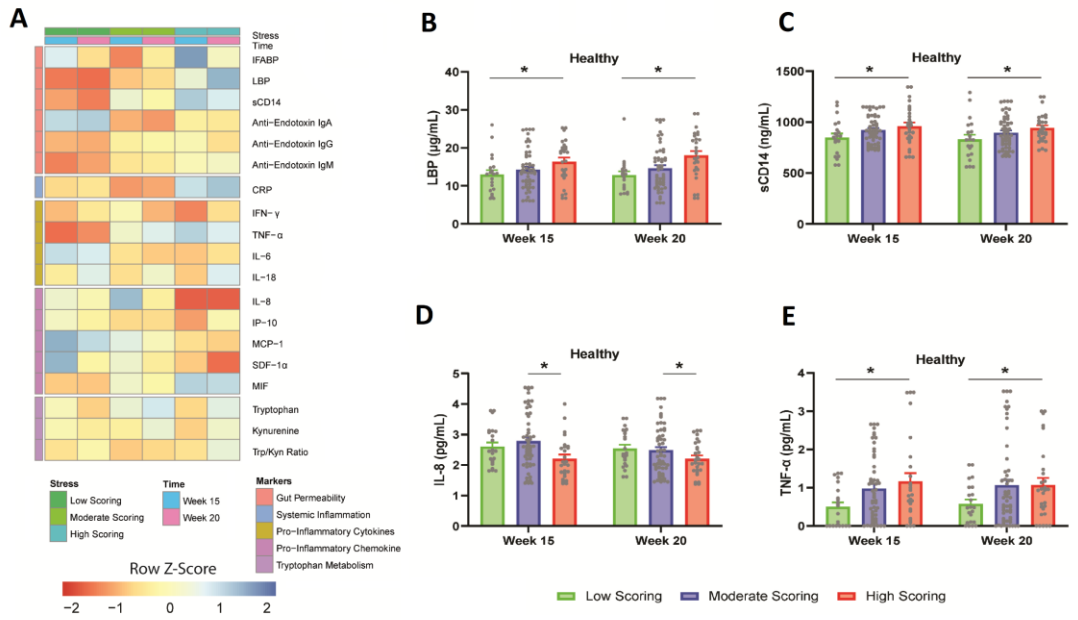


Figure 1. Differences in PSS scoring groups across gestational timepoints in the healthy cohort (n = 22/54/28 from Low/Moderate/High). (A) *Heatmap of normalised biomarker means across scoring groups and time-points. Individual boxes are represented on a scale from red to blue. Darker red boxes represent markers whose concentrations were found to be at least one standard deviation lower than the mean concentration across stress group and time point. Darker blue boxes represent markers whose concentrations were found to be at least than one standard deviation higher than the mean concentration across stress group and time point. Lower z-scores are evident for LBP, sCD14 and TNF-α in the low scoring group at 15 and 20 weeks; and for IL-8 in the high scoring group at 15 and 20 weeks. Significant differences between PSS scoring groups are illustrated for* ***(B)*** *LBP,* ***(C)*** *sCD14,* ***(D)*** *IL-8 and* ***(E)*** *TNF-α (2-way ANOVA with Tukey's HSD). *p < 0.05.*

Associations between maternal anxiety and biomarker levels in the healthy cohort

Significant differences in STAI scoring groups across gestational time points are outlined in **Figure 2**. sCD14 levels were found to significantly increase in the high- (113.02 ± 42.46ng/mL, p = 0.025) and low-scoring groups (100.72 ± 38.36ng/mL, p = 0.027) compared with moderate scorers. LBP levels were also observed to increase with high (5.56 ± 1.95µg/mL, p = 0.015) compared with moderate scores for measurements taken at 20 weeks' gestation, with concentrations significantly

increasing from 15 weeks' gestation (3.01 ± 1.14µg/mL, p = 0.019) for high-scoring participants only. Meanwhile, CRP levels were found to be significantly higher in the high-scoring group compared with moderate- (3.43 ± 1.09µg/mL, p = 0.006) and low-scoring groups (3.39 ± 1.33µg/mL, p = 0.032). Significant differences in LBP and CRP levels between scoring groups were complemented by the presence of positive linear associations between their circulating concentrations and STAI scores in both simple and adjusted models, as outlined in **Supplemental Table 5**. Additional positive associations with STAI scores are noted for simple and adjusted models of the Trp/Kyn ratio at 15 weeks' gestation, as well as for IL-6 and covariate-adjusted IP-10 and Trp/Kyn ratio at 20 weeks' gestation.

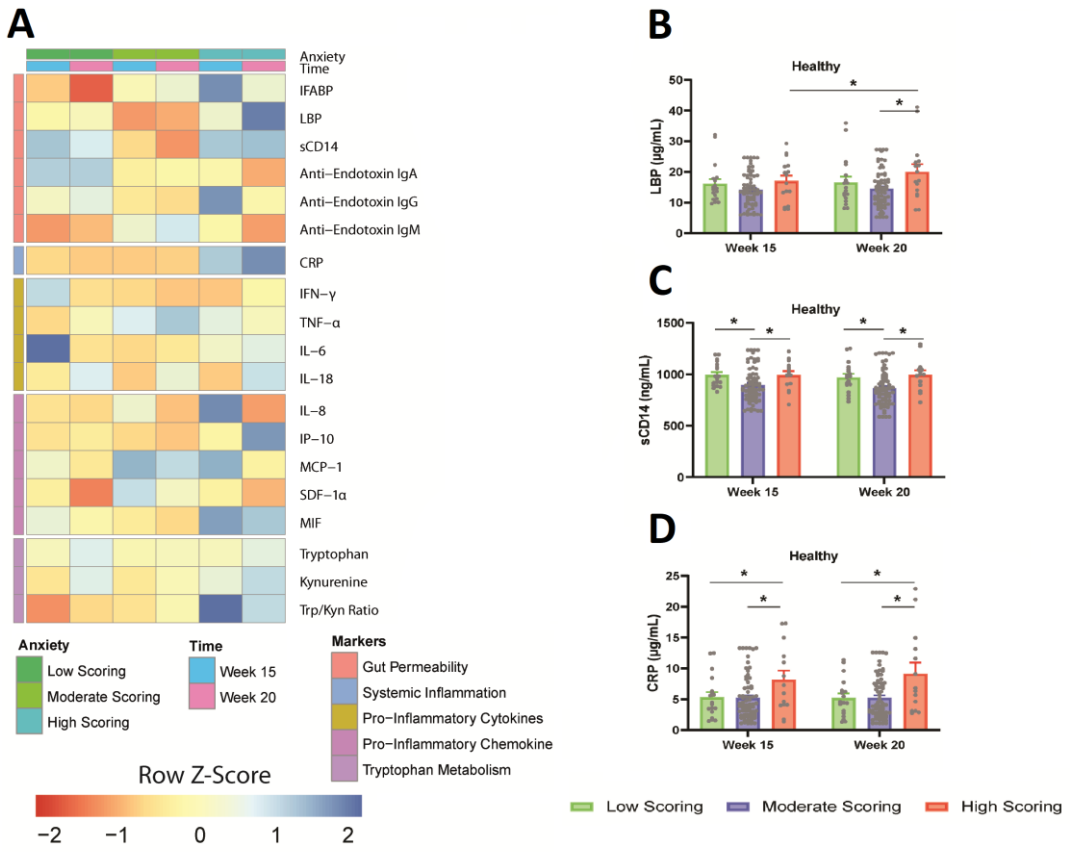


Figure 2. Differences in STAI-scoring groups across gestational time points in the healthy cohort (n = 18/70/16 from low/moderate/high). (A) *Heatmap of normalised biomarker means across scoring groups and time points. Individual boxes are represented on a scale from red to blue. Darker red boxes represent markers whose concentrations were found to be greater than 1 standard deviation lower than the mean concentration across stress group and time point. Darker blue boxes represent*

markers whose concentrations were found to be greater than 1 standard deviation higher than the mean concentration across stress group and time point. Lower z-scores are evident for intestinal fatty acid binding protein (IFABP) in the low-scoring group at 20 weeks. Higher z-scores are evident for IL-6 in the low-scoring group at 15 weeks; for IFABP, anti-endotoxin IgG, IL-8, and tryptophan/kynurenine ratio (Trp/Kyn ratio) in the high-scoring group at 15 weeks; and for LBP, C-reactive protein (CRP), and IFN-γ–induced protein 10 (IP-10) in the high-scoring group at 20 weeks. Significant differences between STAI-scoring groups are illustrated for ***(B)*** *LBP,* ***(C)*** *sCD14, and* ***(D)*** *CRP (2-way ANOVA with Tukey's HSD). *p < 0.05. MCP-1, monocyte chemoattractant protein-1; MIF, macrophage migration inhibitory factor; SDF-1α, stromal cell-derived factor 1α.*

Associations between maternal depression and biomarker levels in the healthy cohort.

Significant differences in EPDS scoring groups across gestational time points are outlined in **Figure 3**. sCD14, LBP, and CRP levels were found to significantly increase in the high-scoring group (113.87 ± 47.09ng/mL, p = 0.046; 4.41 ± 1.74μg/mL, p = 0.035; 2.71 ± 1.13μg/mL, p = 0.048; respectively) compared with low scorers, as well as compared with moderate scorers (2.56 ± 0.97μg/mL, p = 0.026) in the case of CRP. Concentrations of sCD14 and TNF-α were also observed to be elevated in the moderate-scoring group (93.02 ± 37.43ng/mL, p = 0.039; 0.66 ± 0.21pg/mL, p = 0.006; respectively) compared with the low-scoring group. Significant differences in LBP levels between scoring groups were in line with the presence of positive linear associations, as outlined in **Supplemental Table 6**, between its circulating concentrations and EPDS scores observed at 20 weeks' gestation in both simple and adjusted models. For both moderate- and high-scoring groups, IL-8 levels at 20 weeks' gestation were noted to be significantly decreased compared with 15 weeks' gestation (–0.30 ± 0.09pg/mL, p = 0.001; –0.36 ± 0.16pg/mL, p = 0.035; respectively). A negative association was also noted between circulating IL-8 levels

and EPDS scores at 20 weeks' gestation, although this association was no longer significant in the adjusted model.

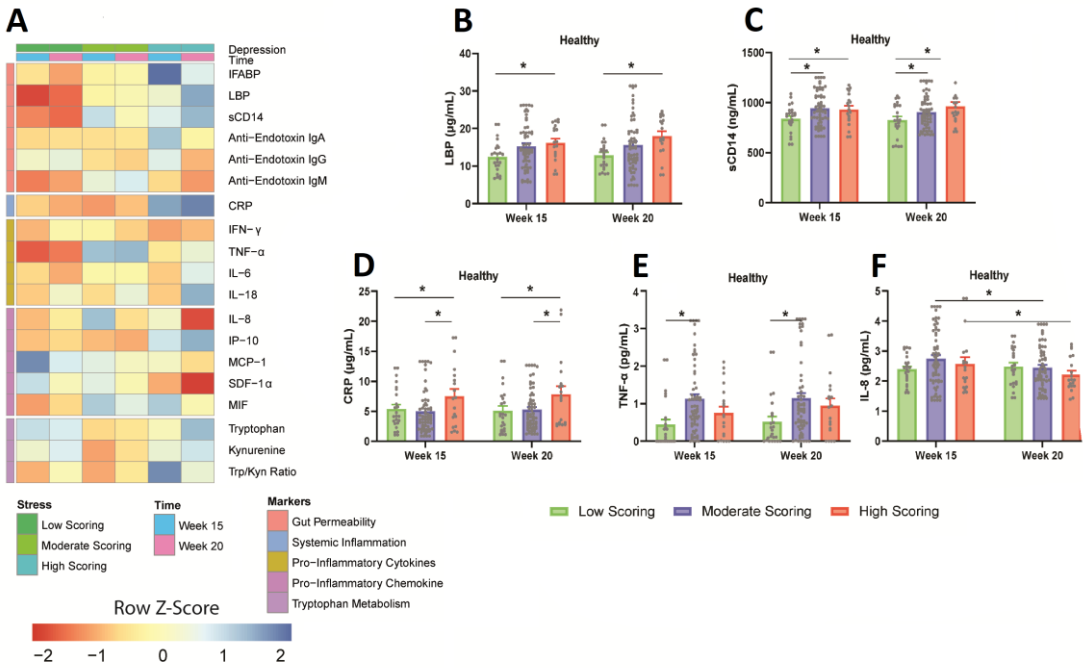


Figure 3. Differences in EPDS scoring groups across gestational time points in the healthy cohort (n = 24/61/19 from low/moderate/high). (A) *Heatmap of normalised biomarker means across scoring groups and time points. Individual boxes are represented on a scale from red to blue. Darker red boxes represent markers whose concentrations were found to be greater than 1 standard deviation lower than the mean concentration across stress group and time point. Darker blue boxes represent markers whose concentrations were found to be greater than 1 standard deviation higher than the mean concentration across stress group and time point. Lower z-scores are evident for LBP, sCD14, and TNF-α in the low-scoring group at 15 and 20 weeks and for IL-8 and SDF-1α in the high-scoring group at 20 weeks. Higher z-scores are evident for IFABP and Trp/Kyn ratio in the high-scoring group at 15 weeks and for CRP in the high-scoring group at 15 and 20 weeks. Significant differences between EPDS-scoring groups are illustrated for* ***(B)*** *LBP,* ***(C)*** *sCD14,* ***(D)*** *CRP,* ***(E)*** *TNF-α, and* ***(F)*** *IL-8 (2-way ANOVA with Tukey's HSD).* $*p < 0.05$.

IBS cohort associations

Associations between maternal perceived stress and biomarker levels in the IBS cohort

Significant differences in PSS scoring groups across gestational time points are outlined in **Figure 4**. Anti-endotoxin IgG levels were found to be significantly higher in the high-scoring group compared with moderate- (30.12 ± 9.15 IgG median units (GMU)/mL, p = 0.004) and low-scoring groups (25.69 ± 10.22 GMU/mL, p = 0.036). This is supported by the presence of a positive association between circulating IgG levels and PSS scores, although this association was no longer significant in the adjusted model. In contrast, IP-10 concentrations were found to be significantly higher in the low-scoring group compared with moderate- (40.10 ± 12.99pg/mL, p = 0.007) and high-scoring groups (39.04 ± 16.23pg/mL, p = 0.047) for measurements taken at 15 weeks' gestation. However, IP-10 levels at 20 weeks' gestation were also noted to be significantly decreased compared with 15 weeks' gestation (–29.56 ± 9.23pg/mL, p = 0.003) in low-scoring participants. No further linear relationships were observed between biomarkers' concentrations and PSS scores in the IBS cohort.

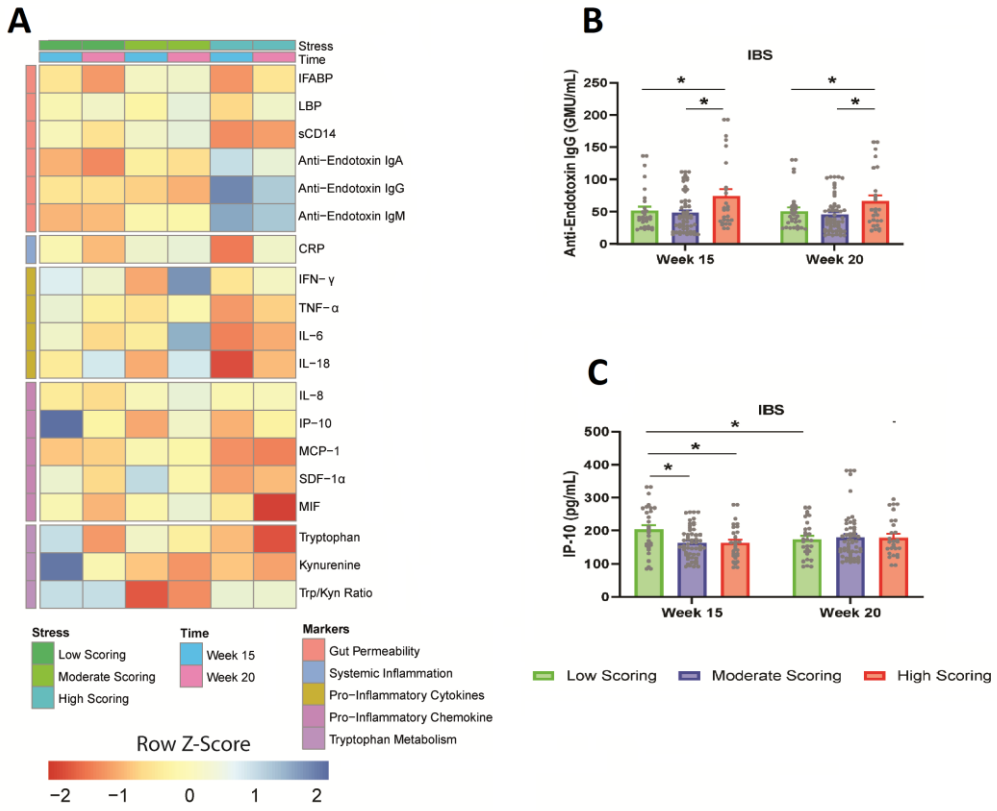


Figure 4. Differences in PSS-scoring groups across gestational time points in the IBS cohort (n = 29/55/21 from low/moderate/high). (A) *Heatmap of normalised biomarker means across scoring groups and time points. Individual boxes are represented on a scale from red to blue. Darker red boxes represent markers whose concentrations were found to be at least 1 standard deviation lower than the mean concentration across stress group and time point. Darker blue boxes represent markers whose concentrations were found to be at least 1 standard deviation higher than the mean concentration across stress group and time point.*

Higher *z*-scores are evident for IP-10 and Kyn in the low-scoring group at 15 weeks and for anti-endotoxin IgG in the high-scoring group at 15 weeks. Significant differences between PSS-scoring groups are illustrated for **(B)** anti-endotoxin IgG and **(C)** IP-10 (2-way ANOVA with Tukey's HSD). *p < 0.05. GMU, IgG median units.

Associations between maternal anxiety and biomarker levels in the IBS cohort.

Significant differences in STAI scoring groups across gestational time points are outlined in **Figure 5**. Anti-endotoxin IgA and IgG levels were observed to be significantly higher in the high-scoring group compared with the low-scoring groups

(11.59 ± 4.51 IgA median units (AMU)/mL, p = 0.031; 26.76 ± 11.11 GMU/mL, p = 0.047; respectively). Anti-endotoxin IgA differences between scoring groups were in line with the positive linear association the biomarker exhibited with STAI scores in both simple and adjusted models. A positive association also existed between circulating IgG levels and STAI scores, although this association was no longer significant in the adjusted model. Conversely, Trp levels were found to be significantly decreased in the high-scoring group compared with moderate- (–707.88 ± 289.38ng/mL, p = 0.044) and low-scoring groups (–1111.14 ± 380.08ng/mL, p = 0.013). In addition, both IFN-γ and IP-10 concentrations at 20 weeks' gestation were noted to be significantly decreased compared with 15 weeks' gestation (–7.39 ± 3.49pg/mL, p = 0.049; –34.34 ± 14.79pg/mL, p = 0.034; respectively) in low-scoring participants. In contrast, IFN-γ levels were elevated at 20 weeks' gestation compared with the first visit (8.20 ± 4.0pg/mL, p = 0.049) in the high-scoring group.

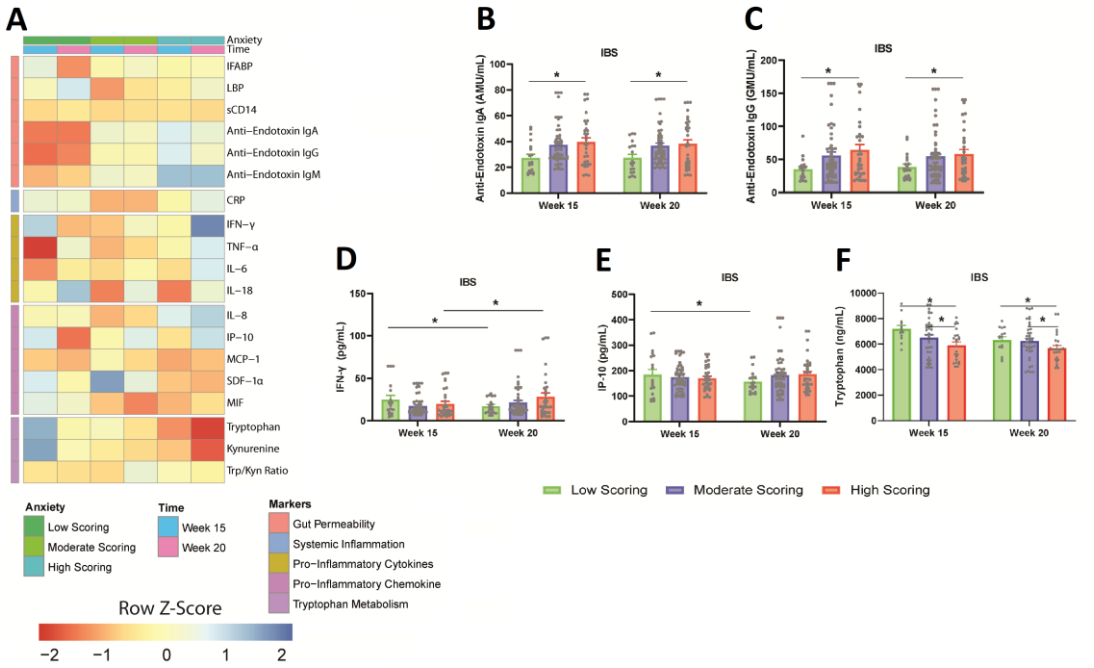


Figure 5. Differences in STAI-scoring groups across gestational time points in the IBS cohort (n = 19/53/33 from low/moderate/high). (A) *Heatmap of normalised biomarker means across scoring groups and time points. Individual boxes are represented on a scale from red to blue. Darker red boxes represent markers whose concentrations were found to be greater than 1 standard deviation lower than the*

mean concentration across stress group and time point. Darker blue boxes represent markers whose concentrations were found to be greater than 1 standard deviation higher than the mean concentration across stress group and time point. Lower z-scores are evident for anti-endotoxin IgA and IgG in the low-scoring group at 15 and 20 weeks, for TNF-α in the low-scoring group at 15 weeks, and for Trp and Kyn in the high-scoring group at 20 weeks. Higher z-scores are evident for Trp and Kyn in the low-scoring group at 15 weeks and for IFN-γ in the high-scoring group at 20 weeks. Significant differences between STAI-scoring groups are illustrated for ***(B)*** *anti-endotoxin IgA,* ***(C)*** *anti-endotoxin IgG,* ***(D)*** *IFN-γ,* ***(E)*** *IP-10, and* ***(F)*** *Trp (2-way ANOVA with Tukey's HSD). *p < 0.05.*

Associations between maternal depression and biomarker levels in the IBS cohort

Significant differences in EPDS scoring groups across gestational time points are outlined in **Figure 6**. Anti-endotoxin IgA and IgG levels were found to be significantly higher in the high-scoring group compared with moderate (9.22 ± 3.72 AMU/mL, p = 0.039; 20.74 ± 8.34 GMU/mL, p = 0.038; respectively) and low-scoring groups (10.87 ± 4.04 AMU/mL, p = 0.022; 22.13 ± 9.05 GMU/mL, p = 0.042; respectively). Differences between scoring groups were in line with a positive linear association exhibited with EPDS scores in the case of anti-endotoxin IgA, but not IgG, for both simple and adjusted models. Conversely, Kyn concentrations were found to be significantly higher in the in the low-scoring group compared with moderate- (34.2 ± 12.13ng/mL, p = 0.017) and high-scoring groups (40.89 ± 14.13ng/mL, p = 0.014). These differences between scoring groups were in line with a negative linear association exhibited with EPDS scores for the simple but not the adjusted model. Additionally, both IFN-γ and IP-10 concentrations were found to be significantly higher in the low-scoring group compared with moderate scores (12.86 ± 4.21pg/mL, p = 0.008; 32.52 ± 12.63μg/mL, p = 0.031; respectively) for measurements taken at 15 weeks' gestation. However, IFN-γ levels at 20 weeks' gestation were noted to be significantly elevated compared with 15 weeks' gestation (8.39 ± 3.97pg/mL, p =

0.040) in moderate-scoring participants, while in the low-scoring group IP-10 levels decreased at 20 weeks' gestation compared with the first visit (–26.37 ± 8.34pg/mL, p = 0.004).

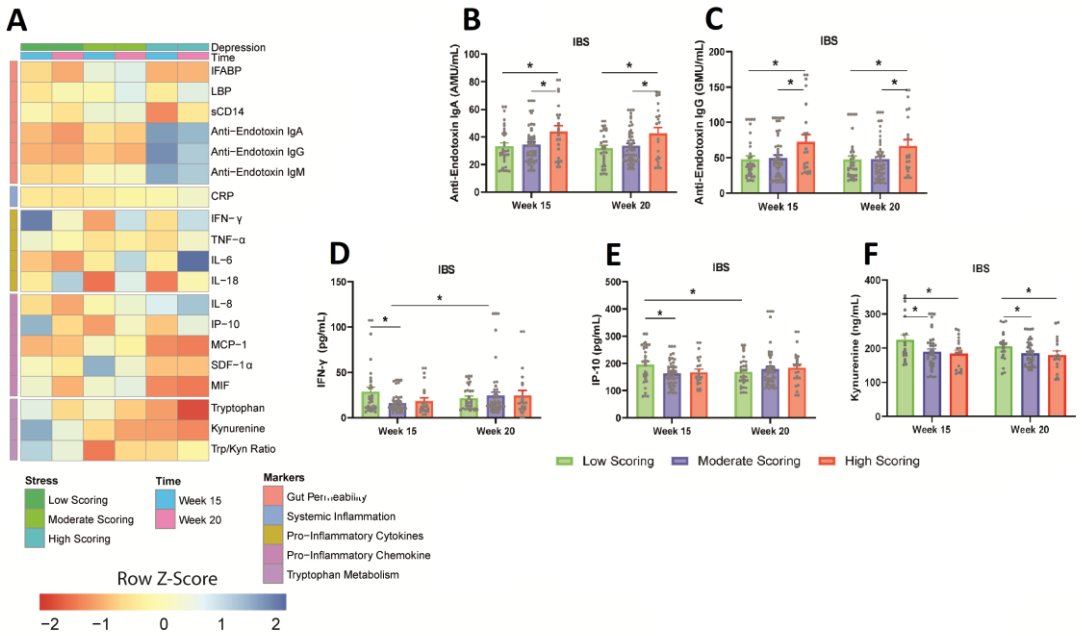


Figure 6. Differences in EPDS-scoring groups across gestational time points in the IBS cohort (n = 32/50/23 from low/moderate/high). ***(A)*** *Heatmap of normalised biomarker means across scoring groups and time points. Individual boxes are represented on a scale from red to blue. Darker red boxes represent markers whose concentrations were found to be greater than 1 standard deviation lower than the mean concentration across stress group and time point. Darker blue boxes represent markers whose concentrations were found to be greater than 1 standard deviation higher than the mean concentration across stress group and time point. Lower z-scores are evident for Trp in the high-scoring group at 20 weeks. Higher z-scores are evident for IFN-γ in the low-scoring group at 15 weeks; for IL-6 in the high-scoring group at 20 weeks; and for anti-endotoxin IgA, IgG, and IgM in the high-scoring group at 15 and 20 weeks. Significant differences between EPDS-scoring groups are illustrated for* ***(B)*** *anti-endotoxin IgA,* ***(C)*** *anti-endotoxin IgG,* ***(D)*** *IFN-γ,* ***(E)*** *IP-10, and* ***(F)*** *Kyn (2-way ANOVA with Tukey's HSD).* *$p < 0.05$.

Differences in biomarkers between healthy and IBS cohorts

Biomarker concentrations between cohorts across gestational time points are outlined in **Supplemental Table 3**. Cohort-independent effects revealing significant increases for measurements taken at 20 weeks' gestation compared with 15 weeks' gestation were noted for LBP as the mean differences (0.97 ± 0.22μg/mL, p = 0.000), TNF-α (0.07 ± 0.03pg/mL, p = 0.034), IL-6 (0.06 ± 0.03pg/mL, ± = 0.017), and IL-18 (71.09 ± 7.26pg/mL, p = 0.000). Conversely, week 20 measurements were observed to be significantly decreased compared with 15 weeks' gestation for anti-endotoxin IgG (–2.51 ± 0.91 GMU/mL, p = 0.006), MCP-1 (-2.49 ± 1.23pg/mL, p = 0.043), and SDF-1α (-38.86 ± 10.78pg/mL, p = 0.001), irrespective of cohort. Cohort-specific effects of time were observed, where levels at 20 weeks' gestation were found to be significantly decreased for IL-8 (–0.23 ± 0.07pg/mL, p = 0.001) and Trp (–460.22 ± 1477.57ng/mL, p = 0.008) compared with 15 weeks' gestation in healthy and IBS participants, respectively. For measurements taken at 20 weeks' gestation, between group effects observed include significant increases in IFN-γ (5.00 ± 2.24pg/mL, p = 0.034) as well as significant decreases in both Trp (–556.52 ± 255.57ng/mL, p = 0.031) and Kyn (–18.29 ± 8.27ng/mL, p = 0.028) in the IBS cohort compared with the healthy cohort. Significant within or between cohort effects were not observed for IFABP, sCD14, anti-endotoxin IgA, anti-endotoxin IgM, CRP, IP-10, MIF, or Trp/Kyn ratio.

Figure 7. Stratification of perceived stress, anxiety, and depression measures into low-scoring (PSS ≤ 7.5; n = 22 and 29 | STAI ≤ 23.3; n = 18 and 19 | EPDS ≤ 2.5; n = 24 and 32), moderate-scoring (PSS > 7.5 and < 17; n = 54 and 55 | STAI > 23.3 and < 40; n = 70 and 53 | EPDS > 2.5 and < 9.5; n = 61 and 50), and high-scoring groups (PSS ≥ 17; n = 28 and 21 | STAI ≥ 40; n = 16 and 33 | EPDS ≥ 9.5; n = 19 and 23) for healthy and IBS cohorts, respectively.

Discussion

Biological associations with stress during healthy pregnancy

The present study identifies several molecular candidate markers that exhibit the potential to discern expectant mothers' level of active stress during the first 2 trimesters of pregnancy in healthy individuals and patients with IBS. In the healthy cohort circulating levels of GI permeability markers sCD14 and LBP were found to be elevated in participants reporting high levels of perceived social stress and depression compared with those reporting low levels. LBP concentrations also exhibited a positive linear relationship with participants' reported level of anxiety, even after adjusting for common demographic and lifestyle confounders. Additionally, LBP levels were only found to increase across trimester visits in individuals reporting the highest levels of anxiety. A strong case is presented for the systemic inflammatory marker CRP, whose high concentrations distinguished those reporting the highest levels of anxiety and depression from all others. Further support is derived from CRP's positive linear relationship with anxiety scores, even in adjusted models. Another notable potential biomarker identified in healthy participants is TNF-α, whose circulating concentrations were found to be elevated in participants reporting the highest levels of perceived social stress compared with those reporting low levels.

Observed concentrations of sCD14 and LBP in the healthy cohort were in line with reported findings that GI integrity is undermined by high levels of psychological distress (Vanuytsel et al., 2014). It comes as no surprise that such consistent changes are observed for this biomarker in the context of active stress exposure. Indeed, both play a key role in initiating the innate immune response to the presence of gram-negative bacteria, working in tangent to present bacterial LPS to its signalling receptor

complex, MD-2/Toll-like receptor 4 in monocytes (Kitchens and Thompson, 2005). Notably, LBP concentrations are reported to fall between 5 and 15μg/mL in healthy populations (Gallay et al., 1994). Corresponding levels are observed in the present study for the healthy cohort with the prominent exception of participants falling within the high-scoring categories of all reported stress measures (range of means, 16.16–20.10μg/mL). Modest elevations in LBP levels, equivalent to those presented here in response to prenatal stress, have previously been reported to exist under conditions of chronic inflammation, such as obesity (Gonzalez-Quintela et al., 2013) and non-alcoholic fatty liver disease (Ruiz et al., 2007). Indeed, these elevations were found to correspond with an upregulated expression of proinflammatory cytokines in these studies. A notable divergence in the stress-mediated effects on sCD14 levels manifested for measures of anxiety, where moderate scores exhibited significantly lower concentrations than the lowest ($p = 0.027$) or highest ($p = 0.025$) reported levels. There is precedent within the literature for positive effects being elicited by mild to moderate levels of maternal stress, particularly in the form of anxiety (DiPietro et al., 2006).

The present study also shows that noted associations between elevated CRP levels and increased risk for psychological distress and depression among the general population (Wium-Andersen et al., 2013) hold under conditions of pregnancy.

Indeed, CRP was found to be the most robust indicator of a shift toward a proinflammatory phenotype in response to prenatal stress among healthy participants. Notably, this acute-phase reactant has been reported to reliably signify the presence of inflammation, even when interpretation from other proinflammatory markers remains ambiguous (Karadag et al., 2008). Corresponding findings in relation to TNF-α under conditions of perceived social stress have notable implications for the cytokine's

potential role as a marker of stress levels, particularly given the role it plays in regulating Trp metabolism via effects on IDO (Robinson et al., 2005). Previously, equivocal results dependent upon the stress stimulus under investigation have been reported regarding the effects of psychological distress on serum TNF-α levels (Chandrashekara et al., 2007). More recently, prenatal stress has been noted to potentiate the effects of a proinflammatory diet on maternal TNF-α concentrations (Lindsay et al., 2018). An additional, somewhat paradoxical discovery from the present study was that plasma levels of the proinflammatory chemokine IL-8 were found to be attenuated in participants reporting the highest levels of perceived social stress and depression. Interestingly, this finding concurs with recently reported observations in vitro where cortisol exposure was shown to decrease levels of IL-8 secretion from female peripheral blood mononuclear cells (Da Pozzo et al., 2018). Other inflammatory markers were not found to be associated with perceived stress, anxiety, or depression. In agreement, Brann et al. (Bränn et al., 2017) examined the association between the expression of 74 inflammation-related genes late in pregnancy and the subsequent onset of postpartum depression and failed to find any significant association for inflammatory markers examined in the present study, including IL-6, IP-10, MCP-1, and IL-18. The absence of any significant effect for IL-6 in particular further compounds the conflicting findings in the literature (Osborne and Monk, 2013), agreeing with the lack of any correlation with depression scores observed by Blackmore et al. (Blackmore et al., 2011) at either 18- or 32-weeks' gestation, but contrasting with reports of positive associations with depressive symptoms in the second trimester (Sherer et al., 2018) as well as trait anxiety and depression scales late in pregnancy (Maes et al., 2000).

Here we note increases in Trp and Kyn from 15 to 20 weeks' gestation in the healthy pregnant women. We also noted no association between inflammatory factors and Trp in the healthy cohort.

Biological associations with stress confounded by IBS.

A different picture presents itself in the case of the IBS cohort, where neither sCD14 nor LBP were found to be capable of distinguishing between incremental groupings for any of the stress measures reported in the present study. Instead, robust indications of stress-induced changes on gut permeability are provided by the anti-endotoxin antibodies IgG, whose circulating levels were found to be discernibly elevated at the highest reported levels of all stress measures, and IgA, which exhibited similar findings for reported levels of anxiety and depression. Positive linear associations were also noted between confounder-adjusted concentrations of anti-endotoxin IgA and participants' self-reported levels of anxiety and depression. Promising findings in relation to proinflammatory markers in the healthy cohort were also not found to be replicated in the IBS cohort. These obvious differences with regard to the association of stress and markers of gut permeability between IBS and non-IBS women have not been shown before (Edwards et al., 2017). While we know the microbiome and gut mucosal inflammation change during a normal healthy pregnancy, we do not know what happens with respect to permeability of the gut wall. Here we see that having IBS leads to a very different biological signature in the plasma potentially due to the already predisposed gut wall, microbiome, and stress system. While we had assumed mothers with IBS would respond to a greater degree to higher stress, anxiety, and depression scores, we did not expect this very different profile. This study indicates

the significance of the gut-associated changes in IBS and the need for specific screening and perhaps different intervention strategies.

Equivalent findings were not identified in relation to either sCD14 or LBP under conditions of IBS, a disorder traditionally posited to be characterised by an increased GI response to stress (Qin et al., 2014). It is plausible that dysregulation of immune signalling pathways under conditions of IBS pathophysiology conceal any potential effects of stress-induced enhancements in gut permeability elicited by these mediators of the innate immune response. Supporting this assertion, evidence has recently emerged establishing several autoimmune diseases, for which dysregulated signalling between immune cells is a hallmark feature (Arakelyan et al., 2017), as risk factors for IBS independent of the presence of psychological distress (Koloski et al., 2019). Indeed, serum concentrations of LBP and sCD14 have been demonstrated to decrease or remain unchanged under conditions of autoimmunity, such as type 1 diabetes mellitus, despite the presence of elevated levels of LPS (Aravindhan et al., 2015). Under such conditions the present study finds IgA and IgG antibodies targeted against LPS reliably serve as markers of endotoxin exposure, signifying changes in gut permeability under high levels of self-reported stress. Interestingly, a similar role for these antibodies has previously been reported in patients with major depressive disorder (Maes et al., 2008) where serum IgM and IgA against LPS were significantly elevated in such cases.

Here we note potential biomarkers for healthy pregnant women during active stress that are indicative of increased GI permeability whereas having the stress-related GI disorder, IBS, is a major confounder to this biomarker panel.

We do not show any evidence for increased metabolism along the Kyn pathway as previously reported in IBS in the non-pregnant state (Clarke et al., 2009b). This is not entirely unexpected because we did not note a marked inflammatory phenotype in our IBS cohort. We did see increased production of certain inflammatory markers in the IBS group with higher anxiety and depression scores, but there was not a clear association between inflammation and Trp metabolism; hence, other factors are clearly involved. Lower levels of Trp were present in the women with higher anxiety scores, which has been seen previously (Songtachalert et al., 2018). A reduction in Kyn was also seen in those with higher depression scores. These differences were observed in the IBS cohort only. This is comparative to a previous study showing an association between lower Kyn levels and depression during pregnancy (Nazzari et al., 2020).

Conclusions.

In conclusion, the present study identifies 2 circulating markers of GI permeability (LBP and sCD14) as well as 2 inflammatory markers (CRP and TNF-α) capable of differentiating low and high levels of prenatal maternal stress during otherwise healthy pregnancy. Significantly, although these distinctions were not found to hold under conditions of IBS, endotoxin core antibodies (IgA and IgG) were found to serve as reliable indicators of GI permeability in such cases. Taken together, these biomarkers demonstrate the potential to form the core of a biological signature that could serve as an early warning indicator of high active levels of prenatal maternal stress in women who do not report having IBS. Clinical application of such a signature could inform suitable intervention strategies aimed at counteracting the effects of prenatal maternal

stress either via stress reduction techniques or microbiota-targeted nutritional approaches, but it should be noted that having IBS is a major confounder to the validity of these markers. Follow-up studies investigating whether these markers demonstrate any appreciable associations with developmental consequences for the offspring are indicated.

Limitations.

As a consequence of utilising samples from a pregnancy biobank, there are several limitations associated with the present study. First, although several validated self-reported measures of stress were utilised, no physiological assessment was implemented. Indeed, developmental associations with physiological measures of stress, such as salivary cortisol, have previously been shown to be distinct from self-reported assessments (Bennet et al., 2016), and their investigation is warranted in future studies. Second, in contrast to other investigations into the developmental effects of prenatal stress, which have utilised both generalised and pregnancy-specific assessments of anxiety (Bennet et al., 2016; Davis and Sandman, 2010), only a generalised form of anxiety is reported on by the present study. This is of relevance considering that pregnancy-related anxiety measures have previously been demonstrated to be a superior indicator of certain developmental effects over broader assessments of state anxiety (Davis and Sandman, 2010). Furthermore, measurements were only taken at 2 time points, 5 weeks apart from each other, with no measurements taken in the third trimester of pregnancy. Given that developmental consequences have been associated with the onset of prenatal stress late in pregnancy (Moss et al., 2017; Simcock, 2017), investigations focusing on these parameters at later time points are

justified. Also, given all but 5 subjects included in this study were white, investigation of stress-associated changes in the circulating biomarkers presented here is warranted in other ethnic groups. It should be noted that cases of IBS among participants were self-reported, and as such the possibility cannot be discounted that results may have varied compared with a clinically diagnosed population suffering from functional GI disorders. Finally, given the exploratory nature of this study, associations between individual biomarkers and stress scores are presented on their own merits without adjustment for comparisons made for the other biomarkers investigated. Accordingly, significant associations between stress indicators and circulating biomarkers noted here require validation in future studies.

Supplementary material

Gut Permeability	
Intestinal Fatty Acid Binding Protein (IFABP)	Small, water-soluble cytosolic proteins which are easily released into the circulation upon enterocyte membrane integrity loss. Basal levels of have been reported to reflect the physiological turnover rate of enterocytes (Grootjans et al., 2010).
Lipopolysaccharide Binding Protein (LBP)	An acute phase protein involved in initiation of host defence against Gram-negative bacteria by binding to bacterial lipopolysaccharide (LPS) and presenting it to the cell surface pattern recognition receptors CD14 and TLR4 (Le Roy et al., 2001).
Soluble CD14 (sCD14)	Released by macrophages upon stimulation with endotoxin and acts as a co-factor, along with LBP, to mediate LPS recognition and initiate an innate immune response (Le Roy et al., 2001).
Endotoxin Core Antibodies (IgA; IgG; IgM)	EndoCAb assays detect immunoglobulins against the inner core of endotoxin which is highly conserved across the whole range of Gram-negative microbiota (Grootjans et al., 2010). Previously shown to be upregulated in cases of major depression indicating an increased translocation of LPS from gram negative enterobacteria into the circulation (Maes et al., 2008).
Systemic Inflammation	
C Reactive Protein (CRP)	Acute phase protein which have been shown to be a valid biomarker of low-grade systemic inflammation (Karadag et al., 2008).
Pro-Inflammatory Cytokines	
Interferon Gamma (IFN-γ)	Significantly induced in response to psychological stress (Maes et al., 1998), IFN-γ is associated with disturbances of serotonergic signalling through activation of IDO, the rate-limiting enzyme for tryptophan degradation (Myint et al., 2013).
Tumour Necrosis Factor Alpha (TNF-α)	Significantly increased in response to psychological stress, TNF-α is involved in activating the HPA axis and causing the release of cortisol (Dunn, 2000). Attenuation of TNF-α signalling was shown to prevent placental defects caused by mild maternal immune activation in early pregnancy (Carpentier et al., 2011). IFN-γ-induced IDO expression is also potentiated by TNF- α (Robinson et al., 2005).
Interleukin 6 (IL-6)	Implicated as a mediating factor in processes leading from maternal inflammation to alterations in foetal brain development (Rudolph et al., 2018).
Interleukin 18 (IL-18)	Stress induced increases in IL-18 is found to be dependent upon the presence of microbiota derived LPS in the circulation (Maslanik et al., 2012). Also found to suppress neuronal survival and differentiation in embryonic neural progenitor culture (Liu et al., 2005) .
Pro-Inflammatory Chemokines	
Interleukin 8 (IL-8 / CXCL8)	Postulated biomarker of chronic stress (Fukuda et al., 2008), whose concentrations are found to correlate between maternal and neonatal serum (Shimoya et al., 1997). Its expression was also found to correlate positively with the severity of preeclampsia (Sun et al., 2016) and high maternal serum levels were found to be indicative of preterm labour (von Minckwitz et al., 2000).
Interferon Gamma-induced Protein 10 (IP-10 / CXCL10)	Highly expressed in response to IFN-γ, with significantly high concentrations observed in patients with preeclampsia (Gotsch et al., 2007) as well as mother who deliver pre-term (Aminzadeh et al., 2012).
Monocyte Chemoattractant Protein 1 (MCP-1 / CCL2)	Significantly increased in women in response to prolonged psychosocial stress (Asberg et al., 2009) as well as populations suffering from generalised anxiety disorder (Ogłodek et al., 2015b). MCP-1 concentrations in cord blood were found to be associated with intrauterine inflammation, premature birth, and neonatal complications (Otsubo et al., 2017).
Stromal cell-derived Factor 1 (SDF-1 α / CXCL12)	Implicated in regulating interactions between the immune and nervous systems (Guyon, 2014), circulating levels are found to be elevated in populations suffering from depression (Ogłodek et al., 2014), generalised anxiety disorder (Ogłodek et al., 2015b) and post-traumatic stress disorder (Ogłodek et al., 2015a).
Macrophage Migration Inhibitory Factor (MIF)	Exhibits a similar circadian rhythm to plasma cortisol and promotes the expression of a large panel of pro-inflammatory molecules by antagonising cortisol-mediated pro-inflammatory cytokine suppression (Petrovsky et al., 2003).
Tryptophan Metabolism	
Tryptophan (Trp)	Amino acid whose inflammatory mediated breakdown is associated with enhanced sensitivity to anxiety (Kim and Jeon, 2018) and depression (Lanser et al., 2020).
Kynurenine (Kyn)	Metabolite of tryptophan whose production is implicated in both inflammatory and neurological conditions (Davis and Liu, 2015).
Kynurenine : Tryptophan Ratio (Kyn/Trp)	A measure of tryptophan degradation along the Kyn pathway which may represent a key mediator of the physiological consequences of altered immunoregulation (Clarke et al., 2009b).

Supplementary Table 1. *Explanation of how the biological characteristics investigated in the present study serve as molecular indicators of gut permeability, inflammation and tryptophan degradation associated with heightened levels of prenatal stress.*

	Healthy (*N* = 104)	IBS (*N* = 105)
Age (Years)	30.06±3.99	30.68±4.33
BMI at 15 Week Visit (kg/m2)	24.4±3.97	24.94±3.86
Alcohol Exposure in 1st Trimester (Weeks)	4.97±4.29	4.73±4.72
Alcohol Intake at 20 Week Visit (Units per Week)	0.21±0.52	0.25±0.78
Ethnicity		
Caucasian	95.19%	100%
Asian	0.96%	-
Indian	3.85%	-
Marital Status		
Partner	93.33%	10.48%
Single	6.67%	89.52%
Years in Education		
< 12yrs	0.95%	1.9%
12-13yrs	60.95%	64.76%
>13yrs	38.10%	33.33%
Employment Status		
Full Time	84.76%	76.19%
Part Time	10.48%	7.62%
Student	1.9%	-
Homemaker	0.95%	2.86%
Unemployed	1.9%	9.52%
Sickness Beneficiary	-	1.9%
Other	-	1.9%
Socioeconomic Index (SEI)*		
Low (SEI < 24)	12.50%	17.14%
High (SEI ≥ 24)	87.50%	82.86%
Type of Maternity Care		
Public	74.04%	69.52%
Private	25.96%	30.48%
Cigarettes Smoked (15 Week Visit)		
None	94.23%	89.52%
1-5 per Day	3.85%	7.62%
6-10 per Day	1.92%	2.86%
Cigarettes Smoked (20 Week Visit)		
None	92.31%	88.58%
1-5 per Day	4.81%	5.71%
6-10 per Day	2.88%	5.71%

Supplementary Table 2. *Descriptive statistics for demographic and lifestyle characteristics for healthy and IBS cohorts. Continuous variables are presented as mean ± SD. * Maternal Socioeconomic index (SEI) calculated using the New Zealand Socioeconomic Index guide (Galbraith et al., 2003).*

	Time	Healthy		IBS	
		Week 15	Week 20	Week 15	Week 20
Stress Scores					
PSS		13.9 ± 6.8	11.6 ± 6.8	13.4 ± 6.5	10.2 ± 6.6
STAI		**31.6 ± 10.6***	31.4 ± 9.9	**35.8 ± 12.7***	32.6 ± 11.9
EPDS		6.3 ± 4.7	5.3 ± 4.3	6.8 ± 4.9	4.8 ± 4.5
Gut Permeability					
IFABP (pg/mL)		5.9 ± 3.0	5.7 ± 2.5	5.7 ± 2.8	5.6 ± 2.7
LBP (μg/mL)	***	**14.9 ± 5.7**	**15.5 ± 6.3**	**15.1 ± 6.0**	**16.3 ± 6.4**
sCD14 (ng/mL)		920.3 ± 164.7	900.7 ± 167.4	897.5 ± 189.3	900.1 ± 169.1
Anti-Endotoxin IgA (AMU/mL)		35.2 ± 17.1	35.3 ± 17.8	36.3 ± 16.5	35.4 ± 15.6
Anti-Endotoxin IgG (GMU/mL)	**	**53.9 ± 38.3**	**52.4 ± 38.1**	**56.3 ± 40.3**	**52.8 ± 35.1**
Anti-Endotoxin IgM (MMU/mL)		54.5 ± 23.6	55.1 ± 22.0	56.2 ± 25.9	56.2 ± 23.7
Systemic Inflammation					
CRP (μg/mL)		5.5 ± 3.8	5.6 ± 3.8	5.8 ± 4.0	5.8 ± 4.0
Pro-Inflammatory Cytokines					
IFN-γ (pg/mL)		18.6 ± 13.7	**18.0 ± 11.9***	19.2 ± 14.3	**23.0 ± 20.7***
TNF-α (pg/mL)	*	**0.917 ± 0.914**	**0.965 ± 0.979**	**0.741 ± 0.701**	**0.833 ± 0.812**
IL-6 (pg/mL)	*	**0.564 ± 0.366**	**0.576 ± 0.352**	**0.538 ± 0.381**	**0.646 ± 0.528**
IL-18 (pg/mL)	***	**424.1 ± 147.1**	**481.2 ± 168.7**	**400.9 ± 144.6**	**485.9 ± 191.6**
Pro-Inflammatory Chemokines					
IL-8 (pg/mL)		**2.6 ± 0.9****	**2.4 ± 0.7****	2.5 ± 0.9	2.6 ± 1.0
IP-10 (pg/mL)		170.6 ± 58.9	171.6 ± 61.9	174.0 ± 57.2	177.8 ± 66.1
MCP-1 (pg/mL)	*	**118.8 ± 31.1**	**115.1 ± 27.7**	**110.2 ± 28.8**	**108.9 ± 25.5**
SDF-1α (pg/mL)	***	**939.9 ± 220.1**	**900.9 ± 232.4**	**948.6 ± 219.9**	**909.8 ± 217.7**
MIF (ng/mL)		23.2 ± 16.7	22.3 ± 14.6	21.5 ± 16.2	20.7 ± 14.6
Tryptophan Metabolism					
Trp (ng/mL)		6523.3±1786.7	**6638.9±1821.4***	**6568.5±1571.9***	**6108.3±1331.7***
Kyn (ng/mL)		197±50.9	**205.3±55.9***	198.3±55.6	**188.4±45.7***
Kyn/Trp		0.031±0.006	0.031±0.006	0.03±0.006	0.031±0.007

Supplementary Table 3. *Descriptive statistics are outlined for each stress score and biomarker across gestational time-points for healthy and IBS cohorts. Data is presented as mean ± SD. Effects between cohorts are highlighted in bold. Cohort specific effects across time-points are also highlighted in bold and underlined. Significance levels (* P<0.05; ** P<0.01; *** P<0.001) are indicated in the appropriate columns. Significance is also indicated in the Time column where biomarker levels differ across time-points irrespective of cohort.*

	Healthy		IBS	
	Week 15	**Week 20**	**Week 15**	**Week 20**
	β (95% CI)	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**
Gut Permeability				
IFABP	0.0001 (-0.0003 – 0.0005)	0.0003 (-0.0001 – 0.0007)	-0.0002 (-0.0006 – 0.0001)	0.0001 (-0.0003 – 0.0004)
	0.0001 (-0.0002 – 0.0005)	0.0002 (-0.0002 – 0.0007)	-0.0001 (-0.0005 – 0.0002)	0 (-0.0004 – 0.0004)
LBP	0.1019 (-0.0901 – 0.2939)	0.1624 (-0.0063 – 0.3311)	-0.0274 (-0.201 – 0.1462)	0.0191 (-0.1443 – 0.1825)
	0.1007 (-0.0879 – 0.2893)	0.1463 (-0.0189 – 0.3114)	-0.026 (-0.201 – 0.149)	-0.0023 (-0.1661 – 0.1615)
sCD14	0.0047 (-0.0019 – 0.0113)	0.004 (-0.0024 – 0.0105)	-0.0019 (-0.0074 – 0.0036)	-0.0016 (-0.0078 – 0.0046)
	0.0031 (-0.0036 – 0.0098)	0.0021 (-0.0045 – 0.0087)	-0.0013 (-0.0067 – 0.004)	-0.0019 (-0.0081 – 0.0042)
Anti-Endotoxin IgA	-0.0094 (-0.0728 – 0.054)	-0.0164 (-0.077 – 0.0442)	0.0476 (-0.015 – 0.1102)	0.051 (-0.0151 – 0.1172)
	-0.0158 (-0.079 – 0.0474)	-0.0101 (-0.071 – 0.0503)	0.0461 (-0.0188 – 0.111)	0.0401 (-0.0258 – 0.106)
Anti-Endotoxin IgG	0.0117 (-0.0164 – 0.0399)	0.0029 (-0.0255 – 0.0313)	**0.0288 (0.0035 – 0.0541)***	**0.0296 (0.0004 – 0.0588)***
	0.0033 (-0.0256 – 0.0322)	0.0044 (-0.024 – 0.0329)	0.0215 (-0.0037 – 0.0467)	0.0224 (-0.0071 – 0.0518)
Anti-Endotoxin IgM	0.0309 (-0.0146 – 0.0764)	0.023 (-0.026 – 0.072)	0.0297 (-0.0102 – 0.0696)	0.0299 (-0.0137 – 0.0736)
	0.024 (-0.0226 – 0.0705)	0.019 (-0.0298 – 0.0677)	0.0235 (-0.0157 – 0.0627)	0.0247 (-0.018 – 0.0675)
Systemic Inflammation				
CRP	0.1495 (-0.1345 – 0.4335)	0.1791 (-0.1069 – 0.4652)	-0.0389 (-0.3028 – 0.2251)	0.0817 (-0.1779 – 0.3413)
	0.1325 (-0.1485 – 0.4136)	0.1378 (-0.1499 – 0.4255)	0 (-0.2633 – 0.2634)	0.1287 (-0.1322 – 0.3897)
Pro-Inflammatory Cytokines				
IFN-γ	-0.0268 (-0.1056 – 0.052)	-0.0014 (-0.092 – 0.0893)	-0.0394 (-0.1118 – 0.033)	0.0178 (-0.0325 – 0.068)
	-0.0074 (-0.086 – 0.0712)	-0.0014 (-0.0903 – 0.087)	-0.0272 (-0.1016 – 0.0472)	0.0069 (-0.0429 – 0.0568)
TNF-α	0.8678 (-0.3041 – 2.0398)	0.7697 (-0.325 – 1.8644)	-0.0208 (-1.51 – 1.4683)	0.3277 (-0.9558 – 1.6112)
	0.678 (-0.4891 – 1.8452)	0.664 (-0.4226 – 1.7505)	0.1576 (-1.3264 – 1.6415)	0.4399 (-0.8199 – 1.6998)
IL-6	0.0461 (-2.9141 – 3.0063)	0.0767 (-3.0022 – 3.1557)	0.6293 (-2.106 – 3.3647)	1.1606 (-0.8009 – 3.1221)
	-0.0948 (-3.0734 – 2.8838)	0.3091 (-2.8641 – 3.4823)	0.8531 (-1.8962 – 3.6025)	1.0233 (0.9473 – 2.9939)
IL-18	-0.0013 (-0.0087 – 0.006)	0.0011 (-0.0053 – 0.0075)	-0.0055 (-0.0126 – 0.0016)	-0.0026 (-0.008 – 0.0028)
	0 (-0.0077 – 0.0077)	0.0021 (-0.0042 – 0.0084)	-0.0064 (-0.0133 – 0.0005)	-0.003 (-0.0083 – 0.0023)
Pro-Inflammatory Chemokines				
IL-8	-0.6673 (-1.8985 – 0.564)	-1.4563 (-3.032 – 0.1192)	0.5453 (-0.6327 – 1.7232)	0.7839 (-0.2466 – 1.8144)
	-0.7488 (-1.9715 – 0.4739)	-1.1998 (-2.756 – 0.3563)	0.5271 (-0.6479 – 1.702)	0.6588 (-0.3784 – 1.696)
IP-10	-0.0077 (-0.0261 – 0.0106)	0.0065 (-0.011 – 0.0239)	-0.0168 (-0.0348 – 0.0011)	0.0035 (-0.0123 – 0.0193)
	-0.0035 (-0.0221 – 0.015)	0.0094 (-0.0079 – 0.0268)	-0.0163 (-0.0345 – 0.002)	0.0021 (-0.0134 – 0.0176)
MCP-1	-0.0304 (-0.0647 – 0.0039)	-0.0293 (-0.0678 – 0.009)	-0.0136 (-0.0497 – 0.0225)	-0.0122 (-0.053 – 0.0286)
	-0.0327 (-0.0666 – 0.0012)	-0.0277 (-0.0664 – 0.011)	-0.015 (-0.051 – 0.0209)	-0.0098 (-0.0497 – 0.03)
SDF-1 α	-0.0032 (-0.008 – 0.0017)	-0.0012 (-0.006 – 0.0034)	-0.001 (-0.0057 – 0.0038)	0.001 (-0.0038 – 0.0058)
	-0.0028 (-0.0077 – 0.0021)	-0.0009 (-0.006 – 0.0039)	-0.0007 (-0.0054 – 0.0041)	0.0022 (-0.0026 – 0.0069)
MIF	0.047 (-0.017 – 0.111)	0.0389 (-0.035 – 0.1128)	-0.0217 (-0.086 – 0.0425)	-0.0178 (-0.0889 – 0.0534)
	0.0335 (-0.0298 – 0.0969)	0.0318 (-0.0406 – 0.1041)	-0.0147 (-0.0779 – 0.0485)	-0.0104 (-0.0797 – 0.0588)
Tryptophan Metabolism				
Trp	-0.0005 (-0.0012 – 0.0002)	0.0001 (-0.0006 – 0.0007)	-0.0006 (-0.0014 – 0.0002)	-0.0005 (-0.0014 – 0.0004)
	-0.0006 (-0.0013 – 0.0001)	-0.0001 (-0.0008 – 0.001)	-0.0002 (-0.001 – 0.0006)	-0.0004 (-0.0013 – 0.0005)
Kyn	-0.0097 (-0.0344 – 0.0149)	-0.0007 (-0.023 – 0.0213)	-0.0155 (-0.0372 – 0.0062)	-0.0119 (-0.0388 – 0.015)
	-0.0099 (-0.0359 – 0.0161)	0.0001 (-0.023 – 0.023)	-0.0052 (-0.0276 – 0.0172)	-0.0042 (-0.0318 – 0.0234)
Kyn/Trp	158.7 (-57.6 – 374.9)	49.5 (-184.4 – 283.4)	33.5 (-167.9 – 235.1)	21.7 (-170.2 – 213.6)
	201.5 (-28.6 – 431.6)	173.7 (-93.9 – 441.4)	36.9 (-172.6 – 246.4)	73.8 (-116.4 – 264.1)

Supplementary Table 4. *Regression coefficients and confidence intervals are presented for linear models where PSS scores are regressed against biomarker levels both in isolation (rows with clear background) as well as adjusted for age, BMI, socioeconomic index, smoking status and alcohol intake (rows with shaded background). Significant coefficients are highlighted in bold (* P<0.05; ** P<0.01; *** P<0.001).*

	Healthy		IBS	
	Week 15	**Week 20**	**Week 15**	**Week 20**
	β (95% CI)	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**
Gut Permeability				
IFABP	0.0004 (-0.0002 – 0.001)	0.0003 (-0.0003 – 0.001)	-0.0002 (-0.0009 – 0.0005)	0 (-0.0007 – 0.0007)
	0.0004 (-0.0001 – 0.001)	0.0003 (-0.0004 – 0.001)	0 (-0.0007 – 0.0006)	-0.0001 (-0.0008 – 0.0006)
LBP	**0.355 (0.0562 – 0.6537)***	**0.3785 (0.116 – 0.641)****	-0.0512 (-0.3739 – 0.2714)	-0.0011 (-0.3049 – 0.3026)
	0.3434 (0.0514 – 0.6355)*	**0.3713 (0.114 – 0.628)****	-0.048 (-0.3649 – 0.2689)	-0.0045 (-0.3062 – 0.2971)
sCD14	0.0015 (-0.0092 – 0.0121)	0.0061 (-0.0041 – 0.0164)	-0.003 (-0.0132 – 0.0073)	-0.0028 (-0.0143 – 0.0086)
	-0.0007 (-0.0113 – 0.0099)	0.0046 (-0.0059 – 0.0151)	-0.0025 (-0.0122 – 0.0072)	-0.0025 (-0.0138 – 0.0089)
Anti-Endotoxin IgA	-0.0636 (-0.1635 – 0.0363)	-0.0905 (-0.1851 – 0.004)	**0.1385 (0.0251 – 0.2518)***	**0.1429 (0.0229 – 0.2628)***
	-0.077 (-0.1754 – 0.0214)	-0.0811 (-0.1762 – 0.014)	**0.1562 (0.0423 – 0.2701)****	**0.1212 (0.0021 – 0.2402)***
Anti-Endotoxin IgG	0.0075 (-0.0373 – 0.0523)	-0.012 (-0.0571 – 0.033)	**0.0507 (0.0041 – 0.0973)***	**0.06 (0.0065 – 0.1134)***
	-0.0087 (-0.0542 – 0.0368)	-0.015 (-0.0601 – 0.0306)	0.0345 (-0.0111 – 0.0802)	0.0494 (-0.0042 – 0.1031)
Anti-Endotoxin IgM	-0.0063 (-0.0729 – 0.0666)	-0.0321 (-0.1099 – 0.046)	0.0433 (-0.0304 – 0.117)	0.0515 (-0.0289 – 0.1319)
	-0.0117 (-0.0854 – 0.0619)	-0.0296 (-0.1074 – 0.048)	0.0338 (-0.0371 – 0.1047)	0.0318 (-0.0467 – 0.1103)
Systemic Inflammation				
CRP	**0.507 (0.0651 – 0.949)***	**0.5535 (0.1092 – 0.998)***	-0.0364 (-0.5223 – 0.4496)	0.2129 (-0.2639 – 0.6897)
	0.443 (0.0077 – 0.8784)*	**0.5476 (0.0999 – 0.995)***	0.1371 (-0.3372 – 0.6113)	0.3911 (-0.0824 – 0.8646)
Pro-Inflammatory Cytokines				
IFN-γ	-0.0038 (-0.1291 – 0.1215)	0.0615 (-0.818 – 0.2048)	-0.0565 (-0.1901 – 0.077)	0.0502 (-0.042 – 0.1424)
	0.0335 (-0.0901 – 0.157)	0.0552 (-0.0862 – 0.1967)	-0.0393 (-0.1736 – 0.095)	0.0412 (-0.0497 – 0.132)
TNF-α	1.001 (-0.8678 – 2.8698)	0.4221 (-1.3294 – 2.1735)	-0.0942 (-2.8349 – 2.6465)	0.3857 (-1.9783 – 2.7497)
	0.7949 (-1.0483 – 2.6381)	0.3924 (-1.3526 – 2.1373)	0.6591 (-2.0153 – 3.3336)	0.892 (-1.4126 – 3.1966)
IL-6	3.9587 (-0.6733 – 8.5908)	**4.9178 (0.1289 – 9.707)***	-0.0487 (-5.0883 – 4.9909)	1.3612 (-2.2633 – 4.9856)
	4.1484 (-0.4653 – 8.762)	4.9628 (-0.0001 – 9.9258)	1.6192 (-3.3397 – 6.5782)	1.7171 (-1.8928 – 5.327)
IL-18	-0.0021 (-0.0137 – 0.0096)	0.001 (-0.0092 – 0.011)	-0.0101 (-0.0232 – 0.0031)	-0.0062 (-0.0162 – 0.0037)
	-0.0017 (-0.0138 – 0.0104)	0.0019 (-0.0081 – 0.012)	-0.0105 (-0.023 – 0.002)	-0.0059 (-0.0156 – 0.0038)
Pro-Inflammatory Chemokines				
IL-8	0.6362 (-1.3244 – 2.5968)	-1.3959 (-3.9218 – 1.13)	1.2011 (-0.9631 – 3.3652)	0.751 (-1.1609 – 2.6628)
	0.3766 (-1.5617 – 2.3149)	-1.3615 (-3.8592 – 1.136)	1.609 (-0.4943 – 3.7126)	0.8559 (-1.8928 – 5.327)
IP-10	0.019 (-0.0099 – 0.048)	0.0248 (-0.0025 – 0.0521)	-0.0203 (-0.0537 – 0.013)	0.0123 (-0.0167 – 0.0412)
	0.0244 (-0.0044 – 0.0532)	**0.0275 (0.0001 – 0.055)***	-0.024 (-0.0572 – 0.0091)	0.0062 (-0.0222 – 0.0346)
MCP-1	0.0109 (-0.0443 – 0.0661)	-0.0052 (-0.067 – 0.0567)	-0.0103 (-0.0769 – 0.0563)	-0.0084 (-0.0836 – 0.0668)
	0.006 (-0.0483 – 0.0603)	-0.0135 (-0.076 – 0.049)	-0.0063 (-0.0714 – 0.0588)	-0.0025 (-0.0755 – 0.0705)
SDF-1 α	-0.0006 (-0.0085 – 0.0072)	-0.0015 (-0.009 – 0.006)	-0.007 (-0.0157 – 0.0016)	-0.0038 (-0.0126 – 0.005)
	-0.0006 (-0.0083 – 0.0072)	-0.0031 (-0.011 – 0.0045)	-0.0071 (-0.0155 – 0.0013)	-0.0024 (-0.0111 – 0.0062)
MIF	0.0435 (-0.0588 – 0.1457)	0.0765 (-0.0404 – 0.1933)	-0.0181 (-0.1365 – 0.1004)	-0.0138 (-0.1449 – 0.1173)
	0.0271 (-0.0731 – 0.1273)	0.0744 (-0.0405 – 0.1893)	-0.0221 (-0.1363 – 0.092)	-0.0205 (-0.1472 – 0.1062)
Tryptophan Metabolism				
Trp	-0.0005 (-0.0018 – 0.0007)	-0.0003 (-0.0015 – 0.001)	-0.0012 (-0.0026 – 0.0002)	-0.0007 (-0.0023 – 0.001)
	-0.0009 (-0.0021 – 0.0003)	-0.0008 (-0.002 – 0.0005)	-0.0005 (-0.002 – 0.0009)	-0.0006 (-0.0022 – 0.001)
Kyn	0.0082 (-0.035 – 0.0514)	-0.0002 (-0.0388 – 0.038)	-0.0302 (-0.0695 – 0.0091)	-0.0234 (-0.0718 – 0.025)
	0.0052 (-0.0382 – 0.0487)	-0.0018 (-0.041 – 0.0377)	-0.0099 (-0.0494 – 0.0296)	-0.0086 (-0.0561 – 0.0388)
Kyn/Trp	**456.6 (88.6 – 824.6)***	209.3 (-197.9 – 616.6)	-27.2 (-393.4 – 339.02)	-88.2 (-433.4 – 256.9)
	583.6 (218.3 – 948.8)**	**481.5 (27.8 – 935.2)***	-50.6 (-419.5 – 318.3)	21.9 (-306.6 – 350.5)

Supplementary Table 5. *Regression coefficients and confidence intervals are presented for linear models where STAI scores are regressed against biomarker levels both in isolation (rows with clear background) as well as adjusted for age, BMI, socioeconomic index, smoking status and alcohol intake (rows with shaded background). Significant coefficients are highlighted in bold (* P<0.05; ** P<0.01; *** P<0.001).*

	Healthy		IBS	
	Week 15	**Week 20**	**Week 15**	**Week 20**
	β (95% CI)	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**
Gut Permeability				
IFABP	0.0002 (-0.0001 – 0.0004)	0.0003 (0 – 0.0006)	0 (-0.0003 – 0.0002)	0.0001 (-0.0002 – 0.0003)
	0.0002 (-0.0001 – 0.0004)	0.0003 (0 – 0.0006)	0.0001 (-0.0002 – 0.0003)	0 (-0.0002 – 0.0003)
LBP	0.1183 (-0.0128 – 0.2495)	**0.148 (0.0332 – 0.2632)***	-0.0019 (-0.1246 – 0.1209)	0.0167 (-0.0988 – 0.1321)
	0.1161 (-0.0136 – 0.2458)	**0.1355 (0.0217 – 0.249)***	0.0013 (-0.1199 – 0.1226)	0.0007 (-0.114 – 0.1153)
sCD14	0.0043 (-0.0003 – 0.008)	0.0039 (-0.0005 – 0.0083)	-0.0019 (-0.0058 – 0.002)	-0.0002 (-0.0045 – 0.0042)
	0.0032 (-0.0014 – 0.0078)	0.0026 (-0.0019 – 0.0072)	-0.0016 (-0.0053 – 0.0021)	-0.0002 (-0.0046 – 0.0041)
Anti-Endotoxin IgA	0.0004 (-0.0434 – 0.0441)	-0.0083 (-0.05 – 0.0335)	**0.0496 (0.006 – 0.0933)***	**0.055 (0.009 – 0.101)***
	-0.0048 (-0.0487 – 0.0392)	-0.0063 (-0.0483 – 0.036)	**0.0521 (0.008 – 0.0962)***	**0.0466 (0.0011 – 0.0921)***
Anti-Endotoxin IgG	0.0011 (-0.0183 – 0.0206)	-0.0055 (-0.025 – 0.0141)	0.0172 (-0.0007 – 0.0352)	0.0184 (-0.0023 – 0.0391)
	-0.0052 (-0.0252 – 0.0149)	-0.0055 (-0.025 – 0.014)	0.011 (-0.0066 – 0.0286)	0.0128 (-0.0079 – 0.0335)
Anti-Endotoxin IgM	0.0103 (-0.0213 – 0.0419)	0.0006 (-0.0334 – 0.0345)	0.0227 (-0.0054 – 0.0508)	0.0285 (-0.0021 – 0.0591)
	0.0065 (-0.026 – 0.0389)	-0.002 (-0.036 – 0.032)	0.0188 (-0.0083 – 0.0458)	0.0243 (-0.0054 – 0.054)
Systemic Inflammation				
CRP	0.1273 (-0.0679 – 0.3225)	0.1401 (-0.0566 – 0.3369)	-0.0102 (-0.1966 – 0.1763)	0.0491 (-0.1343 – 0.2325)
	0.1179 (-0.0767 – 0.3124)	0.1143 (-0.0856 – 0.3141)	0.0342 (-0.148 – 0.2163)	0.0925 (-0.09 – 0.2751)
Pro-Inflammatory Cytokines				
IFN-γ	-0.0223 (-0.0766 – 0.032)	-0.012 (-0.0744 – 0.0504)	-0.0335 (-0.0845 – 0.0175)	0.0154 (-0.02 – 0.0508)
	-0.0093 (-0.0638 – 0.0453)	-0.0125 (-0.074 – 0.0494)	-0.0264 (-0.0777 – 0.025)	0.0092 (-0.0256 – 0.0441)
TNF-α	0.35 (-0.4634 – 1.1634)	0.3978 (-0.3599 – 1.1555)	0.142 (-0.9091 – 1.193)	0.3703 (-0.5341 – 1.2748)
	0.2062 (-0.6087 – 1.0212)	0.314 (-0.4452 – 1.0732)	0.3116 (-0.7139 – 1.3371)	0.5047 (-0.3733 – 1.3827)
IL-6	0.038 (-2.0024 – 2.0785)	0.7396 (-1.3778 – 2.857)	0.866 (-1.0599 – 2.7919)	0.8236 (-0.5612 – 2.2085)
	-0.0574 (-2.1257 – 2.0109)	0.9197 (-1.281 – 3.1204)	1.2008 (-0.6906 – 3.0922)	0.7828 (-0.5948 – 2.1604)
IL-18	-0.0006 (-0.0056 – 0.0045)	0.0015 (-0.0029 – 0.0059)	-0.0022 (-0.0073 – 0.0028)	-0.0007 (-0.0045 – 0.0032)
	0.0002 (-0.0051 – 0.0056)	0.0021 (-0.0023 – 0.0065)	-0.0028 (-0.0076 – 0.0021)	-0.0008 (-0.0045 – 0.0029)
Pro-Inflammatory Chemokines				
IL-8	-0.1936 (-1.0462 – 0.659)	**-1.14 (-2.2208 – -0.0592)***	0.5813 (-0.246 – 1.4087)	0.4919 (-0.2374 – 1.2212)
	-0.2429 (-1.097 – 0.6112)	-0.992 (-2.0694 – 0.0854)	0.6275 (-0.1792 – 1.4342)	0.4493 (-0.2768 – 1.1754)
IP-10	0.0011 (-0.0116 – 0.0138)	0.0064 (-0.0056 – 0.0184)	-0.0116 (-0.0243 – 0.0011)	0.0057 (-0.0054 – 0.0168)
	0.0041 (-0.0088 – 0.0169)	0.0085 (-0.0036 – 0.0205)	-0.0117 (-0.0243 – 0.0009)	0.0044 (-0.0064 – 0.0152)
MCP-1	-0.0124 (-0.0363 – 0.0115)	-0.0155 (-0.042 – 0.0112)	0.0025 (-0.0231 – 0.028)	-0.0002 (-0.0291 – 0.0287)
	-0.0132 (-0.037 – 0.0106)	-0.0146 (-0.042 – 0.012)	0.003 (-0.022 – 0.028)	0.0019 (-0.026 – 0.0298)
SDF-1 α	-0.0023 (-0.0057 – 0.001)	-0.0014 (-0.005 – 0.002)	-0.0011 (-0.0044 – 0.0022)	-0.0008 (-0.0042 – 0.0026)
	-0.0021 (-0.0055 – 0.0013)	-0.0011 (-0.0044 – 0.002)	-0.0009 (-0.0042 – 0.0023)	0 (-0.0033 – 0.0033)
MIF	0.0325 (-0.0116 – 0.0766)	0.0147 (-0.0364 – 0.0648)	-0.0272 (-0.0724 – 0.0179)	-0.0152 (-0.0654 – 0.0351)
	0.0246 (-0.0193 – 0.0686)	0.0113 (-0.0392 – 0.0618)	-0.0254 (-0.0689 – 0.0182)	-0.0112 (-0.0596 – 0.0372)
Tryptophan Metabolism				
Trp	-0.0002 (-0.0007 – 0.0003)	0.0001 (-0.0004 – 0.0006)	-0.0004 (-0.001 – 0.0001)	-0.0004 (-0.0011 – 0.0002)
	-0.003 (-0.0008 – 0.0002)	0 (-0.0005 – 0.0006)	-0.0001 (-0.0007 – 0.0004)	-0.0003 (-0.0009 – 0.0003)
Kyn	-0.0041 (-0.0219 – 0.0137)	-0.0007 (-0.0166 – 0.015)	**-0.0176 (-0.032 – -0.003)***	-0.0165 (-0.0349 – 0.0019)
	-0.0045 (-0.0233 – 0.0143)	0.0001 (-0.0167 – 0.0169)	-0.0099 (-0.025 – 0.0052)	-0.0105 (-0.0291 – 0.0081)
Kyn/Trp	91.8 (-64.4 – 248.1)	-11.6 (-181.4 – 158.3)	-84.9 (-224.4 – 54.5)	-40 (-172.9 – 92.9)
	120 (-47.4 – 287.5)	58.3 (-140.5 – 257.1)	-106.6 (-246.9 – 33.8)	-4.8 (-134.5 – 125.7)

Supplementary Table 6. *Regression coefficients and confidence intervals are presented for linear models where EPDS scores are regressed against biomarker levels both in isolation (rows with clear background) as well as adjusted for age, BMI, socioeconomic index, smoking status and alcohol intake (rows with shaded background). Significant coefficients are highlighted in bold (* P<0.05; ** P<0.01; *** P<0.001).*

Chapter 7

General Discussion

1. Overview and summary

In this thesis, it was demonstrated that early life stress (ELS) induces dysfunction of the communication pathways within the gut-brain axis, specifically leading to increased risk of developing visceral hypersensitivity. Further, novel insights into potential strategies to reduce the incidence of ELS-induced visceral hypersensitivity as well as an interrogation of the role of the gut microbiota and female sex hormones in the perception of this type of pain are also provided. The immune profile in the early pre-adolescent period was then characterised and the impact of ELS on these changes was also investigated. Finally, a panel of biomarkers indicative of the impact of prenatal stress on maternal gut health was identified.

Firstly, we were able to successfully reduce ELS-induced visceral hypersensitivity in adulthood using the candidate pharmacological intervention, a β3-adrenoceptor (AR) agonist CL-316243 *(Chapter 2)*. Here, we report for the first time that CL-316243 reduced the total number of pain behaviours in response to colorectal distension (CRD) in our model of ELS. We then investigated potential mechanisms and found that this beneficial effect on visceral pain did not occur via the central serotonergic system or changes in secretomotor function.

In *Chapter 3*, again using the maternal separation (MS) model of ELS, we report that MS-induced visceral hypersensitivity was reversed by the dietary intervention with milk fat globule membrane (MFGM). We report that there was no effect of MS or the dietary intervention on either recognition memory or intestinal permeability in the colon or ileum. Further, no impact of MS was noted on either neuronal or glial networks in early life or adulthood, however, MFGM supplementation resulted in an increase in the density index of β3-Tubulin in the colonic myenteric ganglia in

adulthood. The mechanism of action of MFGM appears to be independent of changes in intestinal permeability or the enteric nervous system (ENS).

In *Chapter 4*, the role of the gut microbiota and female sex hormones in the perception of visceral pain was investigated. We report that while female germ-free (GF) mice display similar visceral pain responses to their conventional counterparts, sensitivity to CRD is oestrous cycle stage-dependent in conventional animals only. Further, ovariectomy-induced visceral hypersensitivity in conventional animals was revered by exogenous addition of 17β-oestradiol, highlighting the role of female sex hormones and the gut microbiota in the perception of visceral pain.

In *Chapter 5*, alterations in immune profiles in both male and female rats in the early pre-adolescent period as well as the consequent impact of MS were investigated. Here, we report sex-dependent pre-adolescent changes and consequent effects of MS on the circulating immune profile. Of note, these pre-adolescent changes in the circulating immune profile were only noted in non-separated (NS) groups, whereas any impact of MS on the circulating immune profile was only noted at PND20, suggesting that MS may block these pre-adolescent changes in the circulating cytokine levels given that any MS-induced changes occurred only at the earlier timepoint. Finally, MS led to an increase in normalised spleen weight in males only, which is suggestive of general immune activation, and no pre-adolescent changes or effect of MS were noted on ileal cytokine levels. Together, these findings suggest that these pre-adolescent changes begin in the circulation, with any change in the gut immune profile likely taking longer to appear, and MS blocks systemic pre-adolescent changes in the cytokine immune profile which may play a role in the manifestation of MS-induced behavioural and physiological alterations.

Finally, we investigated potential circulating biological markers of prenatal maternal stress in pregnant women with or without the disorder of gut-brain axis interactions irritable bowel syndrome (IBS) *(Chapter 6)*. Here, we report the potential use of markers of systemic inflammation and gastrointestinal (GI) permeability as biomarkers for prenatal maternal stress and anxiety in the healthy cohort. This chapter provides evidence for the use of these biological signatures as markers of prenatal maternal stress which could inform treatment strategies to negate the impacts of this stress on the foetus and the mother.

Overall, the results obtained from *Chapters 2-6* of this thesis support the use of MFGM and CL-316243 as effective treatment strategies against MS-induced visceral pain and highlight a major modulatory role of female sex hormones and the gut microbiota in visceral pain perception. Moreover, we report that markers of systemic inflammation and GI permeability are reliable biomarkers for prenatal maternal stress in a healthy cohort of pregnant women. A summary of the investigations incorporated into this thesis is available in **Figure 1**.

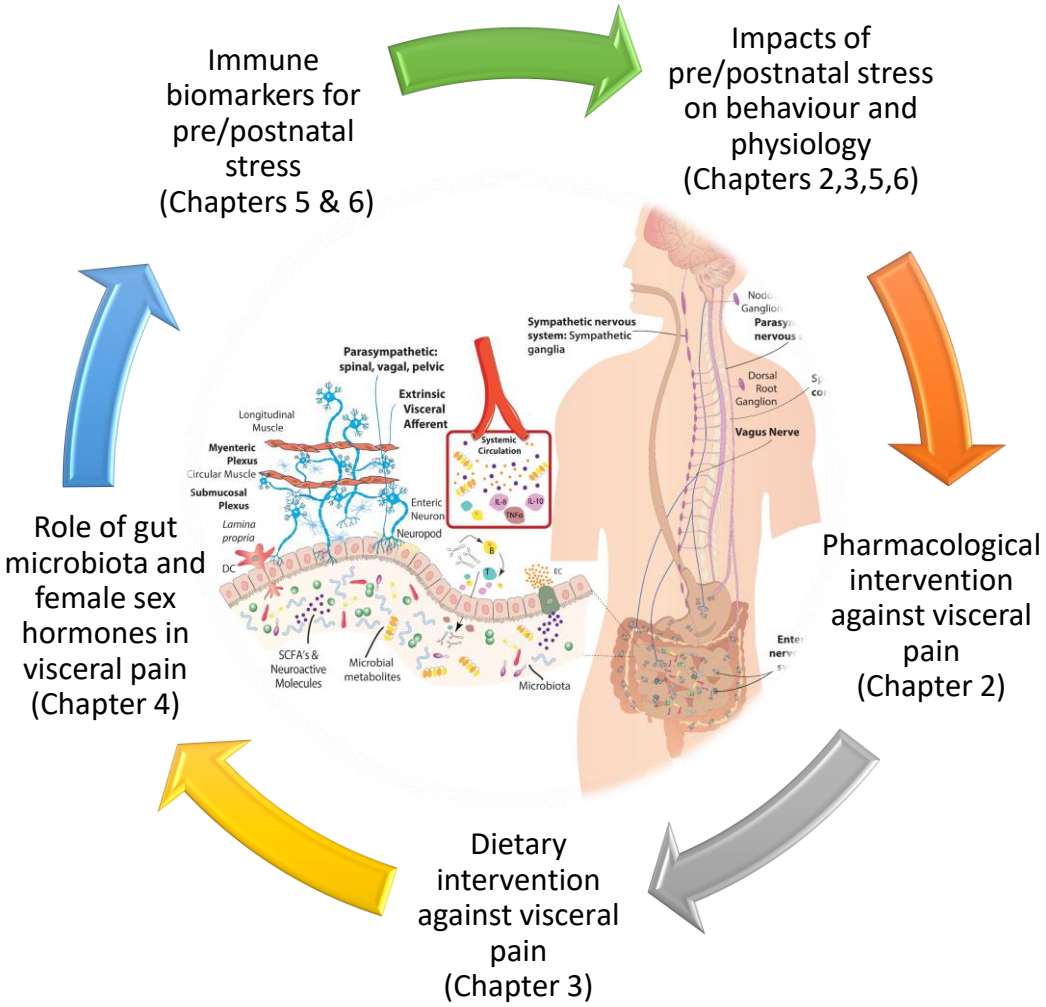


Figure 1. Summary of investigations in this thesis.

2. Strategies to treat disorders of early life stress

Stress in early life is a known risk factor for the development of many stress-related psychiatric disorders including depression and anxiety as well as disorders of gut-brain axis interactions including IBS, for which visceral hypersensitivity is a hallmark (Wilmes et al., 2021). This thesis provides insight into ELS-induced dysfunction of the gut-brain axis and potential therapeutic treatments. Given the current world climate, research into the impact of stressors in early life on the gut-brain axis is of even more importance. With the global population only now beginning to exit a pandemic brought on by COVID-19, the long-term effects of this virus both on physical and mental health is as yet unknown. However, results from studies carried out during the pandemic are already available, reporting poorer mental health in young people as a result of COVID-19 (Chadi et al., 2022; Han et al., 2021). Increasingly more studies have also reported this same negative impact on mental health in adults (Bridgland et al., 2021; Rudenstine et al., 2021; Thorndike et al., 2022).

Further, the outbreak of war in the Ukraine is having major impacts on the residents of the Ukraine as well as the world population. The Russo-Ukrainian war has already resulted in the displacement of more than 14 million people, and undoubtedly this conflict will have irrevocable consequences on physical and mental health.

It is well known that while the stress of this unnecessary brutality is having adverse effects on the people of the Ukraine, the negative consequences of this stress may be transmitted to the next generation, perpetuating the damage caused by this war.

2.1. Visceral pain

Visceral pain, or pain centred around the midline of the body originating from the viscera is a hallmark of the disorder of gut-brain axis interactions IBS and has a worldwide prevalence of between 30 and 90% in patients with IBS (Bouin et al., 2002; Ludidi et al., 2012; Posserud et al., 2007; van der Veek et al., 2008). IBS, and associated visceral hypersensitivity, displays a female predominance (Lovell and Ford, 2012b), however there is currently no satisfactory or particularly efficacious treatment, highlighting the need for further research into treatment strategies for visceral pain. Given that visceral pain is the most common form of pain resulting from disease (Cervero, 2000), there is a pressing need to identify novel and effective treatment strategies. As the overall aim of this thesis was to provide novel insights into ELS-induced dysfunction of the gut-brain axis, the impact of MS on a wide range of behavioural and physiological readouts was assessed. The implications of the findings of this thesis will be discussed in this section.

2.1.1. Relationship between early life stress and visceral hypersensitivity

The relationship between ELS and visceral hypersensitivity has been extensively explored in the literature. As previously stated, it is well known that a history of ELS is a risk factor for development of disorders of gut-brain axis interactions such as IBS (O'Mahony et al., 2017), particularly in females (Bradford et al., 2012). Rodent models of ELS such as MS have been reproducibly shown to result in visceral hypersensitivity throughout the lifespan (McVey Neufeld et al., 2020; Moloney et al., 2016b; O'Mahony et al., 2009; Vilela et al., 2017; Yi et al., 2017). In agreement with the literature, in this thesis we observed that MS resulted in visceral hypersensitivity in

adulthood *(Chapter 2 and 3)* as noted both by an increased total number of pain behaviours and a decrease in the threshold pressure to CRD. In this thesis, potential mechanisms by which MS predisposes to visceral pain in later life were also investigated. Examinations into changes at the level of central serotonergic signalling, colonic submucosal neuronal activation in response to CRD, and colonic secretomotor function did not reveal any significant differences between NS and MS groups *(Chapter 2)*. However, through observation of the results of *Chapter 2* it may be seen that although no difference in the abundance of β3-AR^{+ve} colonic submucosal neurons between NS and MS groups was noted, MS resulted in a non-significant decrease in the percentage of colonic submucosal neurons (both c-Fos^{+ve} and β3-AR^{+ve}) activated in response to CRD. A limited number of studies have investigated the impact of MS on the ENS, with reports of an increase in cholinergic enteric neurons (Gareau et al., 2007a), a decrease of neuronal nitric oxide synthase (nNOS) positive myenteric neurons (Li et al., 2017). However, we did not reveal any impact of MS on the parameters investigated including central serotonergic signalling and measures of the ENS.

Further investigations in this thesis revealed that MS also does not act at the level of intestinal permeability or ENS changes, at least under the experimental conditions we used *(Chapter 3)* suggesting that MS may be exerting effects on visceral pain perception via other mechanisms. Our finding of no effect of MS on the intestinal epithelial barrier is not in agreement with the literature reporting an increase in the permeability of this barrier following MS (Gareau et al., 2007b; Moussaoui et al., 2017), with some studies proposing that this increased permeability plays a major role in the manifestation of behavioural and biochemical alterations associated with MS (Rincel et al., 2019a). As previously mentioned, the lack of effect of MS at the levels

of intestinal permeability may be due to the inclusion of docosahexaenoic acid (DHA) in the control diet which has shown to be protective against insults that increase intestinal permeability. The same may also be said for the ENS where no impact of MS was noted. DHA has a neuroprotective capacity and may have been shielding the ENS against the deleterious effects of MS. Studies investigating these potential protective properties have shown that supplementation with DHA improved distress behaviours following intracerebroventricular administration of corticotropin releasing hormone (Takeuchi et al., 2003). Further studies have reported beneficial effects of DHA on reprogramming of the stress response, reversal of stress-induced behavioural changes and neuronal apoptosis (Pusceddu et al., 2016), and interestingly post-mortem levels of DHA are lower in the orbitofrontal cortex of patients with major depressive disorder (McNamara et al., 2007), further supporting the role of this polyunsaturated fatty acid (PUFA) in protection against stress-induced alterations in behaviour.

It is thought that visceral hypersensitivity is manifested via central or peripheral sensitisation, or dysregulation of the descending pain pathways (Sengupta, 2009). As is stated in the general introduction of this thesis, hypothalamic-pituitary-adrenal (HPA) axis activity and stress interact to affect visceral sensitivity. It is also well known that ELS results in HPA axis dysregulation. Therefore, it would be reasonable to assume that ELS, through dysregulation of HPA axis activity, feeds into the manifestation of visceral hypersensitivity. However, while this is a plausible hypothesis, the exact mechanisms behind the long-lasting impact of ELS on visceral sensitivity are unclear and thus, this thesis investigated potential mechanisms by which ELS may exert its deleterious effects on visceral sensitivity as mentioned above, while also investigating the role of the gut microbiota and female sex hormones in visceral pain perception.

2.1.2. Investigation into factors that modulate the visceral pain response

There is a litany of factors that are known to modulate the visceral pain response. These factors are outlined in detail in the general introduction of this thesis and include the gut microbiota, sex hormones, neurotransmitters including noradrenaline (NA) and serotonin (5-HT), as well as the immune system and stress. Here, we provide novel insight into the gut microbiota and female sex hormones as modulators of the visceral pain response *(Chapter 4)*. The insights into these factors that modulate the visceral pain response provide justification to exploit both the gut microbiota and female sex hormones in the treatment of visceral pain-associated disorders.

A previous study from this lab carried out in male GF mice revealed that the gut microbiota regulates the visceral pain response (Luczynski et al., 2017) whereby male GF mice display baseline visceral hypersensitivity versus their conventional counterparts. However, whether this is also true of female GF mice was heretofore unknown. We show for the first time that female GF mice do not display this same baseline visceral hypersensitivity and are insensitive to ovariectomy-induced visceral pain which was seen in their conventional controls *(Chapter 4)*. Together these findings strongly support a major role for the gut microbiota in the regulation of the visceral pain response given that in its absence, i.e. in a GF condition, an appropriate visceral pain response is lost. Moreover, these results also highlight the importance of sex differences in visceral pain that are also seen in the literature (Prusator and Greenwood-Van Meerveld, 2016). Sex differences in visceral pain are also seen following unpredictable odour-shock in early life which induced visceral hypersensitivity in female, but not male rats (Chaloner and Greenwood-Van Meerveld, 2013). Further, ovariectomy reversed this ELS-induced visceral hypersensitivity, and exogenous addition of oestradiol restored the viscerally hypersensitive phenotype,

highlighting the major role of female sex hormones in visceral sensation. Here, we show that cessation of production of ovarian hormones via ovariectomy resulted in visceral hypersensitivity which was reversed by 17β-oestradiol. Although this finding is in stark contrast with the findings of Chaloner and Greenwood-Van Meerveld, the authors performed ovariectomy following exposure to ELS whereas in *Chapter 4* of this thesis, animals were ovariectomised without being exposed to a stressor, suggesting an interaction between ELS and oestrogen on perception of visceral pain. Ovariectomy alone without the provision of an additional stressor has been shown in the literature to result in a decrease (Ji et al., 2003), or increase (Garrido-Suárez et al., 2015) in visceral sensitivity in rats, however, when paired with an additional stimulus (visceral pain-inducing stressor or compound such as capsaicin or mustard oil), an increase (Chaloner and Greenwood-Van Meerveld, 2013; Sanoja and Cervero, 2005) or decrease (Lu et al., 2007) in the visceral pain response is seen. Together, these reports suggest that stress and oestrogen exert a complex and intertwined effect on visceral pain processing. It has also been shown previously that 17β-oestradiol treatment in rats exposed to stress resulted in altered visceral nociceptive processing in the brain, resulting in visceral hypersensitivity, (Hubbard et al., 2016), further highlighting the complex interplay between female gonadal hormones and stress and their joint effects of the visceral pain response.

In the present study, we used GF mice whereas in the above cited studies, conventional animals were used. It is well known and accepted that GF animals display altered neurochemistry, HPA axis responsivity, and immune responses (Luczynski et al., 2016a); all factors that are known to play a role in the perception of visceral pain. Further, given the role of the gut microbiota and bacterial metabolites in the sensation of visceral pain as outlined in the general introduction of this thesis, the lack of the gut

microbiota in GF animals likely also plays an important role in the alterations in visceral pain perception seen in this chapter. Therefore, it may be surmised that by virtue of the use of GF animals, the alterations mentioned above may lead to a differential effect of oestrogen on visceral pain perception and may thus explain the differences seen versus other studies that report the pro-nociceptive properties of oestrogen (Ji et al., 2008; Moloney et al., 2016b). Interestingly, we show that the visceromotor response (VMR) to CRD in conventional females in the proestrus/estrus stages of the oestrous cycle when oestrogen is at its highest, is lower than that of the conventional females in metestrus/diestrus, suggesting that gonadal hormones are anti-nociceptive in our study. However, this female sex hormone-dependent decrease in VMR is not seen in the GF females, suggesting that they may be insensitive to the effects of oestrogen on perception of visceral pain, or the levels of these sex hormones present in GF animals is not sufficient to induce an effect in these animals. This finding of decreased sensitivity to pain during proestrus/estrus is in contrast with the findings of another study in rats reporting the opposite (Moloney et al., 2016b), highlighting the need for further research into the effects of female sex hormones on visceral pain perception. It has been observed that the pro-nociceptive effects of oestrogen may be mediated by activation of the G protein-coupled oestrogen receptor (ER) which results in spinal nociception (Deliu et al., 2012), or via spinal ERα (Ji et al., 2011), or at the level of the amygdala (Myers et al., 2011). Conversely, activation of ERβ results in a reduction in the response to CRD (Cao et al., 2012). Moreover, GF animals have been shown to have altered levels of female sex hormones versus conventional animals and thus the levels of oestrogen seen in the GF female mice may have been subthreshold to induce visceral pain in these animals as is reported in some studies above.

These results support a major modulatory role of both the gut microbiota and female gonadal hormones in the normal perception of visceral sensitivity. While there is no consensus on whether oestrogen is pro- or anti-nociceptive in the literature (Sun et al., 2019), future studies should fully investigate the effects of ELS on oestrogen-mediated alterations in visceral pain.

2.1.3. Novel treatment strategies for disorders of visceral pain

As mentioned previously, a wide range of microbiota-targeted and pharmacological interventions aimed at treatment of disorders of the gut-brain axis have been investigated. In the context of disorders associated with ELS such as IBS, while progress has been made in the field, there is currently no satisfactory treatment and given the increasing prevalence of disorders of gut-brain axis interactions over the past number of years (Nakov et al., 2022), there is a need for the identification of effective interventions against visceral pain-associated disorders. A table of the pharmacological interventions that have shown promise in the varying subtypes of IBS is available in the introduction of this thesis **(Table 2)**. However, prior to the development of safe and effective interventions, the complex mechanisms behind disorders of visceral pain must first be unravelled. The efficacy of two candidate interventions against ELS-induced dysfunction of the gut-brain axis will be discussed.

The efficacy of CL-316243, a pharmacological intervention aimed at agonism of the β3-AR, against ELS-induced visceral pain was assessed. Here, in agreement with the limited literature available on the use of CL-316243 against disorders of visceral pain, we found that CL-316243 was effective in ameliorating the ELS-induced visceral pain as noted by a decrease in the total number of pain behaviours and an increase in the

threshold pressure before a pain behaviour was displayed. Previous studies reporting the efficacy of β3-AR agonists against visceral pain have proposed that the mechanism of action of this pharmacological intervention is mediated by the release of somatostatin (Cellek et al., 2007). Somatostatin, a regulatory neuropeptide involved in control of motility and secretomotor activity released from enteroendocrine cells and enteric neurons, has been suggested to play a role in the visceral pain response. Studies administering somatostatin analogues such as octreotide report a decrease in visceral pain perception preclinically (Mulak et al., 2015; Su et al., 2001) and in both healthy (Hasler et al., 1993; Plourde et al., 1993), and IBS cohorts (Bradette et al., 1994; Schwetz et al., 2004a). Moreover, β3-AR agonists have been shown to be effective against diarrhoea (Cellek et al., 2007), and slow GI transit in mice (Fletcher et al., 1998), which when coupled with its analgesic effects further support their use as therapies for disorders of gut-brain axis interactions such as IBS which displays visceral hypersensitivity as well as alterations in GI motility.

Although one potential mechanism of action of β3-AR agonists is proposed to be somatostatin-mediated, we aimed to investigate other signalling pathways and physiological changes that are known to be relevant to visceral pain perception. Overall, we noted that CL-316243 reduced the percentage of c-Fos^{+ve} and β3-AR^{+ve} colonic submucosal neurons in response to CRD, altered central and peripheral tryptophan levels, and did not affect secretomotor activity in the colon. While alterations in central and peripheral tryptophan were observed, these changes did not translate to increased central 5-HT, a known regulator of the pain response, suggesting that CL-316243 acts independently of the central serotonergic system. Moreover, we did not note an effect of CL-316243 alone on colonic secretomotor activity, however in the presence of tetrodotoxin, a resultant increase in short circuit current was found.

This finding suggests that there may be an alleviation of inhibitory ENS-mediated input which when alleviated allows for modulation of secretomotor activity via activity at the β3-AR. From the above results, we can conclude that the candidate pharmacological intervention CL-316243 is sufficient to reduce visceral hypersensitivity resultant of exposure to ELS, and its mechanism of action does not involve the central serotonergic system, nor alterations at the level of secretomotor activity. This pharmacological intervention may also have relevance in the treatment of IBS given its analgesic properties and its ability to modulate motility.

Given the increasing awareness of the accessibility of the gut microbiota as an avenue for treatment of disorders of the gut brain axis, dietary interventions are receiving increasingly more attention. We explored the potential of MFGM against ELS-induced dysfunctions in behaviour with a particular focus on visceral pain *(Chapter 3)*. We report that life-long dietary supplementation with MFGM ameliorates MS-induced visceral hypersensitivity and reduces the impact of MS on spatial memory. While very few studies have investigated the efficacy of MFGM in the amelioration of ELS-induced visceral hypersensitivity, we have shown previously in this lab that post-weaning administration of MFGM to rats exposed to MS reduced visceral hypersensitivity (O'Mahony et al., 2020). A similar degree of reduction in the total number of pain behaviours and increase in threshold pressure seen between *Chapter 3* of this thesis and the above cited study, suggesting that the benefit of MFGM was not augmented by the prolonged exposure to the diet pre-weaning. We also investigated possible mechanisms of action of MFGM against MS-induced visceral hypersensitivity. We reported that this mechanism is independent of changes at the level of the ENS or intestinal permeability. Again, these findings have the caveat of the inclusion of DHA in the control diet, which has protective effects on the two

parameters as mentioned above, which may have masked any true effects of MFGM on these GI measures. Dietary supplementation with MFGM has also been suggested to aid in the reduced prevalence of diarrhoea in children (Zavaleta et al., 2011), and ameliorate *Escherichia coli*-induced changes in stool frequency and GI complaints (Ten Bruggencate et al., 2015), however, other studies also report no beneficial effect of MFGM on diarrhoea predominance (Billeaud et al., 2014), highlighting the need for further research into this dietary intervention as a treatment for symptoms associated with IBS.

While the exact mechanisms of action behind the beneficial effect of MFGM on visceral pain and infection are unknown, it has been shown that MFGM modulates the gut microbiota (O'Mahony et al., 2020). Previous studies have also proposed that this dietary intervention plays a role in shaping the gut microbiota in early life (He et al., 2019). Specific effects of MFGM on the gut microbiota include an increase in the abundance of both *Barnsiella* and *Flavonifractor* and a decrease in *Lachnospiraceae*. Functionally, *Barnsiella* has been linked with beneficial effects in the gut due to the conferring of pathogen resistance, while *Flavonifractor* consumes GABA which may impact upon neurotransmission relevant to pain while *Lachnospiraceae* is associated with GI-related diseases such as Crohn's disease and colitis. Given the major role of the gut microbiota in the perception of visceral pain as evidenced in this thesis, it would stand to reason that the modulation of the gut microbiota by MFGM may prove to be a mechanism of action against MS-induced visceral hypersensitivity.

2.2. Biomarkers for disorders of early life stress

Biomarkers are defined as "A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions" (FDA-NIH, 2016). The use of these biological markers is crucial in the development of treatment strategies for a wide range of disorders including anxiety and depression as mentioned in the general introduction of this thesis. Through this thesis, a number of biomarkers for prenatal maternal stress in humans are proposed (*Chapter 6*) as well as a characterisation of immune changes in the early pre-adolescent period and the consequent impact of ELS on these changes in rats (*Chapter 5*).

We report the suitability of markers associated with GI permeability and inflammation as biomarkers for prenatal maternal stress and depression in healthy pregnant females. Stress during pregnancy can exert detrimental effect on both the mother and the developing foetus, resulting in complications of pregnancy including preeclampsia, preterm birth, low infant birth weight, as well as predisposing the offspring to the development of depression, anxiety, and autism spectrum disorders. Given the many complications that arise as a result of a stressful maternal experience, there is a great need for the development of biomarkers to aid in the early identification and treatment of these disorders, which could prevent any negative consequences on foetal development and maternal health. We propose that sCD14, LBP, and TNF-α together are promising biomarkers for prenatal maternal stress, while CRP may be useful in the identification of maternal anxiety in healthy pregnancies. Further, we found that sCD14, LBP, and CRP together were positively associated with prenatal maternal depression, highlighting their possible use as a biomarker for maternal depression.

It is well known that the experience of stress may result in inflammation, however, the mechanisms by which prenatal maternal stress may affect foetal programming are still not clear. The experience of stress during pregnancy and the resultant inflammation has been suggested to be a major contributor to alterations in foetal programming, leading to possible neuropsychiatric disorders in the offspring (Hantsoo et al., 2019). Further, prenatal maternal stress has been shown to result in inflammation in the uterine environment and lead to alterations in serotonergic signalling which has been proposed to play a causative role in the apparition of neuropsychiatric disorders in the offspring (Chen et al., 2020). Moreover, the connection between stress and gut barrier dysfunction has been extensively explored (Kelly et al., 2015), as has the link between both of these with depression (Cruz-Pereira et al., 2020; Trzeciak and Herbet, 2021). From the evidence outlined herein, it stands to reason that biomarkers for depression, anxiety, and possibly other stress-related neuropsychiatric disorders should include markers of the major contributors to the disorder; these being markers of GI permeability and inflammation.

While the mechanisms by which prenatal maternal stress affects foetal programming are still being uncovered, it is known that inflammation may directly alter foetal neurodevelopment via cytokine signalling (Dammann and O'Shea, 2008; Ratnayake et al., 2013).

In early life there is a priming of the immune system due to the vast expansion of the gut microbiota, and this vigorous immune reaction has been shown to be required for normal immune imprinting and protection against immunopathologies later in life (Al Nabhani et al., 2019). However, the specific changes in the immune system in the early pre-adolescent period are not known, nor is the impact of early life stress on this immune priming. We aimed to characterise both these changes in the immune system

in the early pre-adolescent period as well as the consequent impact of our model of ELS *(Chapter 5)*. We report moderate pre-adolescent changes in circulating cytokine levels involving an increase in both IL-5 and IL-13 in males, whereas a decrease in IL-5 was seen in females. MS resulted in an increase in normalised spleen weight and plasma IL-6, and a decrease in plasma IFN-γ in males, and a decrease in IL-5 in females. Interestingly, any changes in circulating cytokines induced by MS were only noted at PND20, whereas any pre-adolescent changes in circulating cytokines were noted in NS groups only, suggesting that MS blocks these pre-adolescent changes. While the functional effect of these noted changes cannot be extrapolated, it appears that MS results in a state of general immune activation as noted by the increased spleen weight and higher levels of IL-6 and leads to alterations in the normal pre-adolescent changes in the immune system at this early timepoint. This may have ramifications on later life immunity.

2.3. Methods to reduce the impacts of stress on mother and child

We have shown that ELS results in visceral hypersensitivity. Therefore, one major avenue to ameliorate or even prevent these negative effects would be to reduce stress in both the mother and the offspring. Research into methods of accomplishing this are far-reaching and include interventions based on music (Corbijn van Willenswaard et al., 2017), mindfulness, and exercise (Alderdice et al., 2013) to reduce maternal stress and anxiety during pregnancy. While the results of such studies investigating these interventions as methods to reduce maternal stress are mixed, it has been seen that the above-mentioned interventions as well as psychosocial interventions have shown efficacy in women displaying mental health issues (Song et al., 2015; Stoll et al.,

2018). Given the reported efficacy of these interventions against maternal stress, further studies should investigate whether such interventions are also capable of negating the deleterious effects of stress on foetal development.

Further, given that data from this thesis suggests the use of biomarkers related to both inflammation and heightened GI permeability, this could suggest that increased maternal GI permeability could be playing a role in the psychological effects of stress. Increased GI permeability is seen in patients with major depressive disorder (Ohlsson et al., 2019) and is thought to be a causative mechanism by which systemic inflammation occurs in cases of disease (Mokkala et al., 2016). An obvious intervention to aid in the reduction of this inflammation would be to return GI permeability to normal physiological levels. This may be achieved through the use of probiotics such as *Lactobacillus* and *Bifidobacterium* which have been shown to improve gut barrier function (Rose et al., 2021). Restoration of gut barrier function could prevent the systemic inflammation seen, which would also protect the developing foetus. These observations suggest that targeting the mother by reducing the impacts of ELS may be a more effective strategy for protecting both the mother and developing foetus from the deleterious effects of ELS.

3. Beyond visceral pain: other applications of treatment strategies identified in this thesis

While both the dietary and pharmacological interventions explored in this thesis display efficacy against ELS-induced visceral pain, they have been shown to be beneficial in the treatment of other disorders or dysfunction of the gut-brain axis. The concept of repurposing existing medications and treatment strategies is not new, and this process saves time and money and given that the candidate intervention already has proven safety in humans, the likelihood of adverse reactions related to health is lessened (Pushpakom et al., 2019). Examples of some treatment strategies that have undergone successful repurposing against disorders of pain include gabapentin, originally indicated for epilepsy but has since been repurposed to treat neuropathic pain, ibudilast, used in the treatment of asthma and now for neuropathic pain, and topiramate which was originally used in the treatment of fungal infections but is now also used for treatment of irritable bowel disease (Rudrapal et al., 2020). Here, the potential other applications of the dietary and pharmacological interventions investigated in this thesis will be discussed.

3.1. β-3 adrenoceptor agonists

The β3-AR agonist CL-316243 displayed efficacy in reversing ELS-induced visceral pain. However, pharmacological interventions targeting the β3-AR have seen many other uses over the past number of decades. CL-316243 has been shown to impact on metabolism by increasing energy expenditure and improving glucose tolerance (Himms-Hagen et al., 1994; Xiao et al., 2015). Moreover, CL-316243 ameliorated

high fat diet-induced metabolic dysfunction in rats (Ding et al., 2021). In healthy women, the β3-AR agonist mirabegron increased resting energy expenditure and increased brown adipose tissue metabolic activity (O'Mara et al., 2020) which has relevance for metabolic disorders related to obesity. Beneficial effects as an anti-obesity drug have also been reported. Through the expression of the β3-AR in white and brown adipocytes, CL-316243 leads to increased thermogenesis in the brown adipose tissue (Atgié et al., 1997) and a reduction in fat stores in obese mice (Ghorbani et al., 1997). Further anti-diabetic and anti-obesity effects of β3-AR agonists have been noted in rodents (Edwards et al., 2021; Ghorbani et al., 2012; Xie et al., 2021), as well as some promising results via improvement of glucose homeostasis in obese insulin-resistant humans (Finlin et al., 2020).

Other agonists of the β3-AR including mirabegron are also used in the treatment of overactive bladder syndrome, a symptom set categorised by urinary urgency and/or incontinence. While the exact aetiology of overactive bladder syndrome is unclear and likely multi-factorial in nature, it has been suggested that it is related to either increased sensory afferent signalling or abnormal detrusor muscle excitability (Palmer and Choi, 2017). It is known that activation of the β3-AR results in detrusor muscle relaxation, therefore treatment strategies aimed at improving the symptoms of overactive bladder syndrome are agonists of the β3-AR. CL-316243, the pharmacological intervention investigated in *Chapter 2* of this thesis has been shown to decrease purinergic nerve-mediated contractions of the detrusor muscle in mice (Fong et al., 2019). Mirabegron has been shown to decrease the frequency of rhythmic bladder muscle contractions, reducing the symptoms of overactive bladder syndrome including urinary frequency and episodes of urgency (Chapple et al., 2014; Kelleher et al., 2018; Takasu et al., 2007). Another agonist of the β3-AR, solabegron has also

shown promise in the treatment of overactive bladder syndrome in a similar manner to mirabegron (Ellsworth and Fantasia, 2015; Ohlstein et al., 2012). Overall, agonists of the β3-AR either alone or in combination with anti-muscarinic agents have been suggested to be favourable treatments for overactive bladder syndrome.

3.2. Milk fat globule membrane

As alluded to in the discussion on *Chapter 3*, MFGM has been shown to have beneficial effects of several parameters related to both health and disease. Supplementation of the infant's diet with MFGM has been shown to lessen the prevalence of infection including acute otitis media and associated fever (Timby et al., 2015) as well as reduce the number of febrile episodes in children (Veereman-Wauters et al., 2012). Interestingly, dietary supplementation with MFGM significantly reduced the oral level of *Moraxella catarrhalis* (Timby et al., 2017), a pathogen commonly observed in otitis media, suggesting that there may be a microbiome-mediated mechanism of action.

Moreover, MFGM supplementation narrows the differential in cognitive development between breastfed and formula-fed infants (Timby et al., 2014) and improved intelligence quotient scores in hand and eye coordination (Gurnida et al., 2012), supporting its role in promoting neurodevelopment. Other studies have reported beneficial effects of MFGM on metabolic changes seen in poorly nourished children (Lee et al., 2018a). Further beneficial effects of MFGM supplementation are noted in adulthood; MFGM in combination with exercise improved agility in middle aged adults (Ota et al., 2015) as well as improved measures of frailty (weight loss, exhaustion, slow walking speed) (Kim et al., 2015) and agility (Yoshinaka et al., 2018)

in elderly adults. These observations support the benefit of MFGM supplementation on measures of health and has been extensively reviewed (Abd El-Salam and El-Shibiny, 2020; Ambrożej et al., 2021; Brink and Lönnerdal, 2018; Herrmann et al., 2021).

The positive effects of MFGM seen in *Chapter 3* also provide further evidence for the benefits of breastfeeding. Breastfeeding is a highly effective method of providing MFGM and other nutrients to the developing infant, and as such is the gold standard for infant nutrition.

Overall, MFGM has displayed promising results in promoting neurodevelopment, reducing infection severity, as well as promoting health in older adults.

4. Future directions

This section proposes alternative experimental approaches to better understand how these treatments and novel factors modulate the visceral pain response as well as further investigations into biomarkers for prenatal maternal stress.

4.1. Efficacy of these novel treatments in other models of visceral pain

This thesis supports the efficacy of both MFGM and CL-316243 against MS-induced visceral pain. However, it is not known whether these treatment strategies are effective in other models of visceral pain. As outlined in the introduction of this thesis, there are many preclinical models of visceral pain which could be used to further characterise the effect and potential mechanisms behind the action of these therapies. Firstly, the activity of both MFGM and CL-316243 in a spontaneously viscerally hypersensitive rat strain, the Wistar-Kyoto strain would be of interest as there is a known difference in visceral sensitivity between Sprague Dawley rats used in the course of the work of this thesis and the Wistar-Kyoto strain (O'Mahony et al., 2013). As it has been proposed that there is a difference in endocrine and molecular pathways between Wistar-Kyoto and Sprague Dawley rats, the investigation of the ability of the treatments investigated in this thesis against other models of visceral pain would allow for a better understanding of the mechanism of action. The same could be said for the water avoidance stress model of visceral pain, which may involve different pathways than seen in the Wistar-Kyoto rats.

To investigate whether the mechanisms of action of these treatments is mediated by the gut microbiota, the use of antibiotics would be of benefit. The efficacy of the

dietary intervention MFGM in a microbiota-depleted state would inform whether the effects of this intervention are mediated by the gut microbiota as it has been seen that MFGM results in alterations in gut microbiota composition (O'Mahony et al., 2020). However, whether these changes in the gut microbiota are causative or correlative in the mechanism of action of MFGM is unknown. Further, by carefully choosing the type of antibiotics to be used and targeting with gram-positive or gram-negative bacteria, one could narrow down the classification of the bacteria that may be playing a role in the mechanism of action. However, studies have shown that antibiotic administration in itself results in visceral hypersensitivity (O'Mahony et al., 2014), or reduced visceral sensitivity (Hoban et al., 2017), so caution must be used when interpreting results. An alternative method for delineating the role of the gut microbiota in the mechanism of action of MFGM against stress-induced visceral pain could involve the use of GF animals. As shown in this thesis, female GF mice do not display the same baseline visceral hypersensitivity that male GF mice do, therefore this experimental approach would provide important insight into the differential sex-dependent regulation of the visceral pain response by the gut microbiota and potentially MFGM.

If the efficacy of MFGM and CL-316243 in the above models was to be assessed, it would allow for a better characterisation of the effects and potential mechanisms of these treatment strategies.

4.2. Translational value of these interventions

In the course of this thesis, research was carried out both *in vivo* and in humans. As already stated in this thesis, MS is a well-established, reproducible rodent model of

ELS and a similar phenotype that is observed in rats following exposure to MS can be seen in humans who experience stress in early life (Syed and Nemeroff, 2017). This observation supports the face validity of this model. While the ultimate test of an interventions capacity to ameliorate these stress-induced deficits is its effect in a clinical population, preclinical research provides a strong foundation upon which to build these clinical studies. Here, we report that a dietary intervention provided from early life throughout the lifespan is sufficient to reduce MS-induced visceral hypersensitivity, while the candidate pharmacological intervention, which was administered in adulthood, also showed benefit in reducing visceral hypersensitivity resultant from exposure to ELS. These 2 interventions provide different avenues of treatment; the dietary intervention as an intervention to undertake during the experience of this stress and the pharmacological intervention to negate these effects in later life. Evidence from this thesis further supports the value of preclinical models as methods to inform future preclinical and clinical studies.

4.3. Alternative methodological approaches to further develop the findings of this thesis

Aside from the use of alternative preclinical models of visceral pain to assess the efficacy of these therapeutic approaches, alterations in the methodology of how these experiments could be carried out may also provide mechanistic insight.

The efficacy of CL-316243 against ELS-induced visceral hypersensitivity was assessed in *Chapter 2*. In this chapter, the pharmacological intervention was delivered via oral gavage to recapitulate the normal route of administration in humans using other β3-AR agonists for treatment of a range of disorders as mentioned above. Another more mechanistic approach would be the utilisation of β3-AR knockout

animals to investigate the phenotype in the absence of the β3-AR. Given that one potential mechanism of action of β3-AR agonists against visceral pain involved the β3-AR-mediated release of somatostatin, one could hypothesise that these β3-AR knockout animals would likely not display heightened visceral pain, but rather would have impaired NA-induced inhibitory input on visceral pain, leading to altered visceral sensitivity. However, there are other mechanisms of visceral pain modulation that are independent of the β3-AR, so whether a knockout of this receptor would majorly alter visceral sensation is as yet unknown. While no study to date has investigated the visceral sensitivity of animals with a β3-AR knockout, it has been seen that these animals display an increased susceptibility to diet-induced obesity (Preite et al., 2016) and decreased occurrence of brown adipocytes in white fat, indicative of altered thermogenesis (Jimenez et al., 2003).

In *Chapter 3* of this thesis, leading on from a previous study in this lab investigating the effect of post-weaning administration of MFGM (O'Mahony et al., 2020), we administered MFGM in the diet throughout the lifespan of the animals. While this experimental approach provides novel insight into the impact of life-long dietary supplementation with MFGM on ELS-induced visceral hypersensitivity, to be more clinically relevant the impact of supplementation with MFGM in early life only should be investigated. Rationale for this is based on the observation that MFGM is being supplemented to infant formula in order to minimise the gap in cognition between breastfed and formula-fed infants as mentioned above. To better recapitulate this real-world situation, the diet of the neonatal rats should be supplemented with MFGM from birth until weaning. Focusing on this critical time period from the lifespan may provide valuable insight into the structural, behavioural, and neurochemical changes induced by this dietary intervention. Further studies should also aim to assess the efficacy of

this novel dietary intervention against visceral pain in humans through clinical trials in patients with IBS given the beneficial effects of this treatment on GI symptoms as discussed above.

In *Chapter 4* of this thesis, the role of the gut microbiota and female sex hormones in perception of visceral pain was investigated. To build on these findings further, the levels of circulating gonadal hormones should be measured and compared to a conventional control to fully investigate the hormonal alterations induced by the GF status. Once assessed, the levels of these circulating gonadal hormones may inform further on the differences found between our results and the findings of other studies reporting pro-nociceptive effects of oestrogen.

A characterisation of changes in the immune profile of pre-adolescent rats and the impact of MS on these changes was carried out in *Chapter 5*. While this chapter was purely descriptive in nature, it provides a strong basis for further investigations into the sex- and age-specific changes in the immune system in the early pre-adolescent period and its shared interaction with ELS. Future studies should investigate these changes further to provide information on specific immune changes in this early stage of life. Building on these changes, the behavioural and physiological correlates of these alterations in cytokine levels should also be investigated. These further experiments would advise on whether these cytokine changes are simply in response to different housing and feeding conditions, or whether this immune reaction impacts upon both the GI tract and CNS structurally to exert changes at a behavioural and molecular level to confer this protection against pathologies in later life as reported (Al Nabhani et al., 2019).

While several potential biomarkers for prenatal maternal stress in healthy women were proposed in *Chapter 6*, only self-reported measures of stress were used. Further studies should assess biological readouts of stress such as cortisol to standardise the amount of stress these women are experiencing. Further, the findings of this chapter are from samples taken at 15- and 20-weeks gestation, the applicability of these biomarkers both in the first and third trimester of pregnancy would also be of importance given the vast endocrine changes that occur across pregnancy (Moya et al., 2014; Soma-Pillay et al., 2016).

5. Overall conclusions

This thesis provides data that supports the use of novel dietary and pharmacological interventions against ELS-induced visceral pain using the MS model (*Chapters 2 and 3*). Further, we highlight the role of female sex hormones and the gut microbiota in the appropriate sensation of visceral pain (*Chapter 4*) as well as characterise the impact of MS on the pre-adolescent immune profile (*Chapter 5*). Finally, we propose the utilisation of specific markers related to GI permeability and inflammation as biomarkers for prenatal maternal stress (*Chapter 6*).

Initially, we characterised the effect of MS on GI, CNS, and behavioural parameters and found that MS resulted in visceral hypersensitivity to CRD. This heightened visceral sensitivity was ameliorated by agonism of the β3-AR and life-long dietary supplementation with MFGM, mechanisms of which appear to be independent of changes at the level of the central serotonergic signalling and secretomotor activity, as well as at the level of the ENS and intestinal permeability changes respectively.

Subsequently, the role of the gut microbiota and female sex hormones in the visceral pain response were assessed. Here, we found that the absence of the gut microbiota in female mice does not impact on visceral sensitivity, however the oestrous cycle modulated the visceral pain response in a microbiota-dependent manner. Further, female GF mice were insensitive to ovariectomy-induced visceral pain, and addition of exogenous oestradiol to conventional mice reversed this ovariectomy-induced visceral pain. Together, these results support a major modulatory role for the gut microbiota and female sex hormones in the perception of visceral pain.

Next, given the important role of the immune system in the pain response, we sought to investigate the specific changes in the immune profile of pre-adolescent pups. Here

we report modest changes in cytokine levels in the circulation as well as an increase in spleen weight in males and suggest that MS blocks this immune response which may be required for normal development.

Finally, we propose the use of markers of GI permeability and inflammation as biomarkers for maternal stress, anxiety, and depression with the idea that they may be used clinically to aid in the prevention of complications of pregnancy and foetal development arising from prenatal stress exposure.

The findings of this thesis present a need for further investigations into ELS-induced dysfunction of the gut-brain axis as well as potential treatment options. Given the safety of both MFGM and CL-316243 in humans as outlined in this discussion, this thesis presents an opportunity for the use of these interventions against disorders of visceral pain. Further, the work carried out in the course of this thesis may aid future studies exploring therapeutic options for treatment of disorders of gut-brain axis interactions.

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MINI-REVIEW

Of bowels, brain and behavior: A role for the gut microbiota in psychiatric comorbidities in irritable bowel syndrome

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Abstract

Background: The gastrointestinal microbiota has emerged as a key regulator of gut-brain axis signalling with important implications for neurogastroenterology. There is continuous bidirectional communication between the gut and the brain facilitated by neuronal, endocrine, metabolic, and immune pathways. The microbiota influences these signalling pathways via several mechanisms. Studies have shown compositional and functional alterations in the gut microbiota in stress-related psychiatric disorders. Gut microbiota reconfigurations are also a feature of irritable bowel syndrome (IBS), a gut-brain axis disorder sharing high levels of psychiatric comorbidity including both anxiety and depression. It remains unclear how the gut microbiota alterations in IBS align with both core symptoms and these psychiatric comorbidities.

Methods: In this review, we highlight common and disparate features of these microbial signatures as well as the associated gut-brain axis signalling pathways. Studies suggest that patients with either IBS, depression or anxiety, alone or comorbid, present with alterations in gut microbiota composition and harbor immune, endocrine, and serotonergic system alterations relevant to the common pathophysiology ofthese comorbid conditions.

Key results: Research has illustrated the utility of fecal microbiota transplantation in animal models, expanding the evidence base for a potential causal role of disorder-specific gut microbiota compositions in symptom set expression. Moreover, an ex- citing study by Constante and colleagues in this issue highlights the possibility of counteracting this microbiota-associated aberrant behavioral phenotype with a pro- biotic yeast, Saccharomyces boulardii CNCM I-745.

Conclusions and inferences: Such data highlights the potential for therapeutic targeting of the gut microbiota as a valuable strategy for the management of comorbid psychiatric symptoms in IBS.

KEYWORDS
anxiety, comorbidity, depression, IBS, microbiota-gut-brain axis

Abbreviations: AhR, aryl hydrocarbon receptor; ANS, autonomic nervous system; CNS, central nervous system; ENS, enteric nervous system; FMT, fecal microbiota transplantation; HPA, hypothalamic-pituitary-adrenal; IAA, indole-3-acetic acid; IBS, irritable bowel syndrome; IBS-D, diarrhea-predominant irritable bowel syndrome; IPA, indole-2,3-dioxygenase; PFC, prefrontal cortex; *S. bou*, *Saccharomyces boulardii*; SCFA, short-chain fatty acids; TLR, toll-like receptor.

Lars Wilmes and James M. Collins contributed equally to this work.

1 | INTRODUCTION

Irritable bowel syndrome (IBS), now regarded as a disorder of gut-brain axis interactions, is one of the most prevalent gastrointestinal disorders, with varying incidence rates around the globe, constituting 20–50% of the gastrointestinal workload.[1,2] IBS is characterized by abdominal pain and altered bowel movement without overt structural or biochemical abnormalities.[3] While the understanding of IBS has been improved in recent years, concurrent with some effective therapeutic options becoming available, many IBS patients present with psychiatric comorbidities, a subset that is much more difficultto treat. This significant cohort includes approximately 44% and 25%of IBS patients presenting at gastroenterology clinics with comorbid anxiety and depression respectively.[4] Moreover, the co-occurrence of psychiatric comorbidities is associated with IBS symptom sever- ity[5,6]), while some studies show the efficacy of specific antidepressants in reducing IBS symptomatology.[7]

Psychiatric disorders, such as anxiety disorders (hereafter referred to as anxiety) and major depressive disorder (hereafter referred to as depression) are among the most prevalent mental health problems worldwide. It is estimated that approximately 10% of the global population suffers from these disorders each year.[8,9] Althoughthere has been extensive research into the pathophysiology of depression and anxiety, their diagnosis is still symptom based, with treatment options remaining suboptimal and stubbornly focused on targeting monoamine neurotransmitter pathways.[10] Independently, IBS, depression, and anxiety are complex heterogenous disorders with an already difficult clinical management profile made more challenging when combined in comorbid gastrointestinal and psychiatric phenotypes.[3,8,9]

Research in the last decade or more points toward a role of the gut-brain axis in both IBS and psychiatric disorders.[11–13] The gut is in continuous bidirectional communication with the brain through neuronal, endocrine, and immune signalling pathways. The important role the gut microbiota plays in regulating these routes of communication to influence brain function and behavior has seen this axis renamed to reflect this and it is now termed the microbiota-gut-brain axis.[14]

The clinical care of IBS patients with psychiatric comorbidity is complex with treatment failure common. Repositioning IBS as a disorder of gut-brain axis interactions, along with recognition of the important role played by the gut microbiota in symptom expression, has led to calls for integrated clinical management models that blend medical management with behavioral and dietary interventions.[15] Here, we outline why the success of this approach for this particular subset of comorbid IBS patients demands greater focus on the common ground, and the diverging routes, that might explain why particular microbiota configurations lead to distinct clinical representations of IBS. As of now, the mechanisms underpinning these co-morbidities are not fully known. A recent study also highlighted the bidirectional nature of this comorbidity by showing that psychiatric symptoms are predictive for the development of IBS, while IBS isalso predictive of depression and anxiety later in life.[16] Interestingly,

Key Points

- Gut microbiota alterations are a feature of IBS. However, the functional implications of these compositional reconfigurations remains unclear.
- There are common and disparate features of these microbial signatures associated with IBS and IBS with psychiatric comorbidities but the neurobiological implications require elaboration.
- Improving our understanding of the role of gut microbiota in driving specific gut-brain axis signalling pathways important for the cardinal gastrointestinal and psycho- logical features of IBS will be critical to accrue therapeutic benefits.

most comorbid IBS patients develop gastrointestinal symptoms be- fore psychiatric comorbidities.[16] After a summary of the communication pathways of the microbiota-gut-brain axis, we will analyze thelatest literature on psychiatric comorbidities in IBS. This review will focus in particular on alterations in the gut microbiota reported in IBS, depression, and anxiety and in IBS with comorbid anxiety and depression. We will discuss how gut microbiota signatures associated with these disorders might impact on gut-brain axis signalling pathways and the therapeutic implications of these observations.

2 | SIGNALLING PATHWAYS OF THE MICROBIOTA-GUT-BRAIN AXIS

Understanding the role of the gut microbiota in IBS and its psychiatric comorbidities requires an appreciation of the signalling pathways of the microbiota-gut-brain axis. The main routes of communication are summarized in Figure 1 and include neuronal, immune, and endocrine host signalling pathways, as well as the microbial production or regulation of bioactive molecules such as neurotransmitters, their precursors and short-chain fatty acids (SCFAs).

Neuronal communication along the microbiota-gut-brain axis is mostly mediated by the autonomic nervous (ANS), with the enteric nervous system (ENS) arm regulating important mechanisms locally in the gastrointestinal tract. One of the most important routes of communication is the vagus nerve. The vagus nerve connects the brain to all visceral organs among others and relays information via 80% afferent and 20% efferent fibers.[17–19] A portion of afferent axonal endings are located in the mucosa of the GI tract. These afferents are thought to contain a wide array of receptors, making them able to detect signals such as gut hormones, neurotransmitters, andbacterial metabolites.[14]

A major player in endocrine signalling of the microbiota-gut- brain axis is the hypothalamic-pituitary-adrenal (HPA) axis, the majorstress axis of the body, whose activation results in the release of glucocorticoids. This endocrine signalling pathway can be restrained

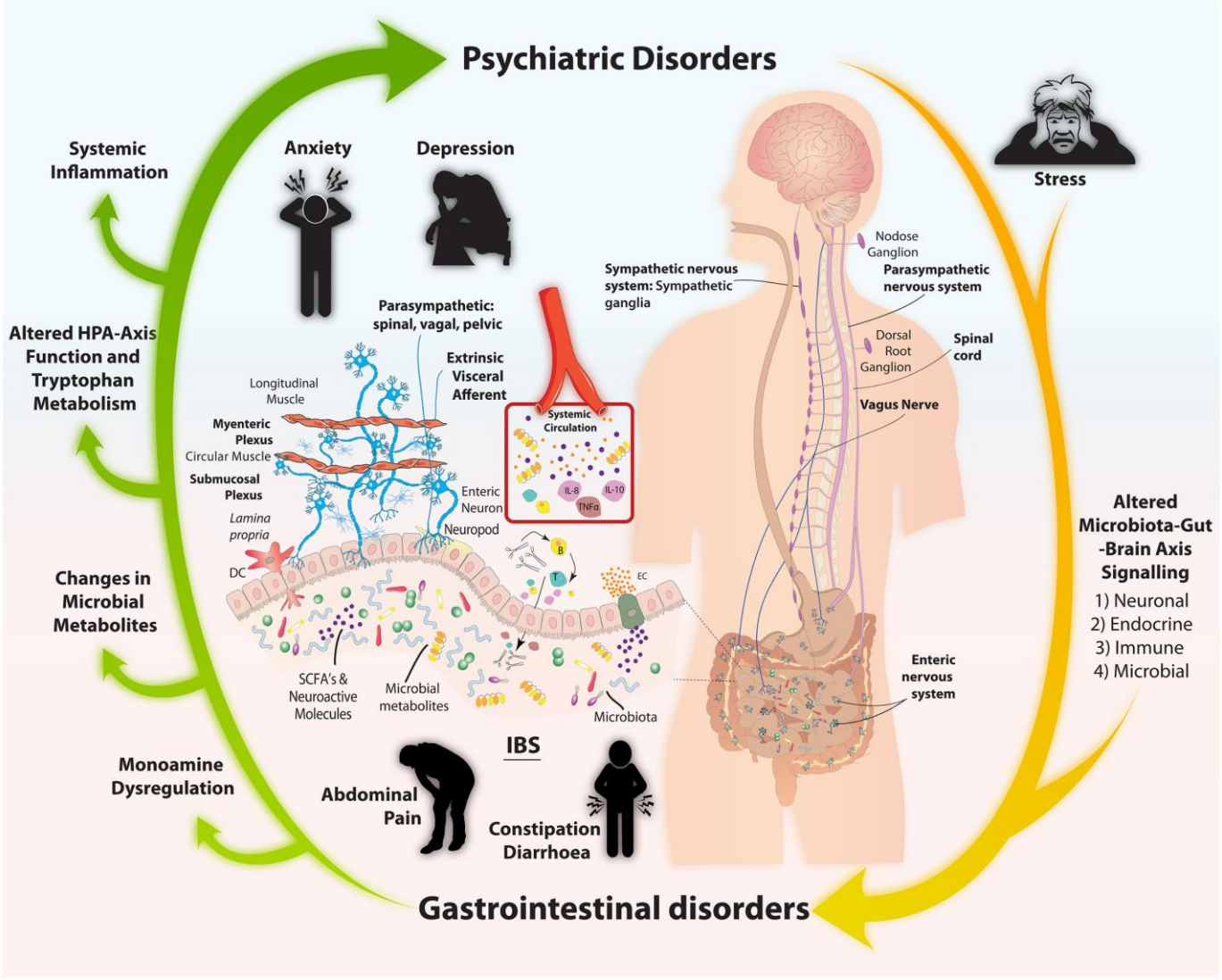

FIGURE 1 Summary of microbiota-gut-brain axis signalling pathways. There are a number of important routes of communication in the microbiota-gut-brain axis that may be relevant for the expression of gastrointestinal and psychiatric symptoms in IBS. It is well known that stress, a major predisposing factor for the development of both IBS and depression in later life, may also impact on gut microbiota composition and function. (1) Neuronal, (2) Endocrine, (3) Immune and (4) Microbial signalling pathways are also associated with specific symptom sets. Alterations in the composition and function of the microbiota have been reported in IBS, depression, and anxiety. These alterations can, for example, result in dysregulation of monoamine signalling and alterations in microbial metabolites which may be related to systemic inflammation. It is also now appreciated that the prominent gastrointestinal features of IBS including constipation, diarrhea and visceral pain may worsen the associated comorbid psychiatric symptoms such as anxiety and depression. It is still unclear if IBS with psychiatric comorbidity represents a distinct clinical entity that can be explained on the basis of converging gut-brain axis signalling pathways.

at brain-level by negative feedback of glucocorticoids acting on glucocorticoid receptors. Both IBS and psychiatric disorders show dysregulation of the HPA axis.[20,21] It is now appreciated that the microbiota plays a key role in the priming and regulation of this axis, shown initially by increased stress response in germ-free animals, which is reversed by colonization with specific bacteria or a more complete microbiota.[22,23] In turn, it has long been known but recently reinforced in the preclinical literature that stress exposures can also modify gut microbiota composition and function.[14,24]

The crosstalk between the microbiota and the hosts' immune system mostly takes place at the mucosa either by direct contact or through molecules secreted by the microbiota and is essential for priming and education of the immune system.[25] The communication is facilitated by microbe-associated molecular patterns, which are sensed by colonocytes and immune cells through pattern recognition receptors such as toll-like receptors (TLRs), triggering an immune response by the secretion of cytokines. The impact of the gut microbiota on the immune system extends to the brain, shown by changes in microglia morphology and gene expression profile in germ-free animals.[26,27]

An important topic in the context of inflammation in the gut-brain axis is the integrity of the intestinal barrier. Changes in intestinal

TABLE 1 Gut signatures associated with IBS, depression, anxiety, and comorbid IBS.

Taxonomic rank	IBS	Depression	Anxiety	Comorbid IBS andDepression
Phylum	*Firmicutes*: *Bacteroidetes* ↑[58]	Actinobacteria↑[51] Bacteroidetes↓[51]	Firmicutes↓[45]	
Order			Enterobacterales↑ [51]	
Family	*Lactobacillaceae*↑[52] *Enterobacteriaceae*↑[46]	*Prevotellaceae*↓[51]	*Enterobacteriaceae*↑[51] *Ruminococcaceae*↓[45]	
Genus	*Bacteroides*↑[52] *Bifidobacterium*↓[52] *Faecalibacterium*↓[52] *Eubacterium*↑[58]	*Faecalibacterium*↓[51] *Sutturella*↓[51] *Coprococcus*↓[51] *Eggerthella*↑[51]	*Escherichia/Shigella*↑[51] *Subdoligranulum*↓[51] *Dialister*↓[51]	*Bacteriodes* ↑[63] *Faecalibacterium* ↑[63]*Lachnospiraceae*↑[63]

↑ indicates increase, ↓ indicates decrease.

permeability creates a passage for bacteria and their products from the lumen to the ENS, immune cells and systemic circulation, which can evoke an immune response. Increased intestinal permeability is associated with low-grade inflammation, a neurobiological featureof both IBS and depression.[28,29]

Another form of communication in the microbiota-gut-brain axis is via microbial metabolites, such as SCFAs and neurotransmitters. SCFAs are mostly used as an energy source by the host,for example, butyrate is the primary energy source for colonocytes. The SCFAs not utilized by colonocytes enter the systemic circulation and other tissues including the brain.[30] SCFAs can activate a set of G-protein coupled receptors, FFAR2 and FFAR3 being the most investigated. They are found in tissues such as the colon, the heart, and immune cells. FFAR3 is also expressed in the peripheral nervous system in enteric plexi, the portal nerve and autonomic and sensory ganglia,[31] which further implicates their involvement in gut-brain signalling.[32]

The microbiota can produce a wide range of neuroactive molecules that have implications for behavior, mood, and cognition. Many of these neurotransmitters (GABA, noradrenaline, serotonin) are involved in both gastrointestinal and brain function. One of the most important neurotransmitters in terms of the microbiota-gut- brain axis is serotonin. Serotonin is an important signalling molecule in both the central nervous system (CNS) and the ENS and is produced from the precursor tryptophan, an essential amino acid.[33] The majority of serotonin is synthesized by enterochromaffin cells. However, most tryptophan is metabolized along the kynureninepathway, whose end products have neuroactive properties and are N-methyl-D-aspartate (NMDA) receptor antagonists and agonists.[34] In contrast to serotonin, both tryptophan and kynurenine can cross the blood brain barrier and are further metabolized in the brain by glial cells.[35]

The microbiota can directly modulate the levels of tryptophan and its metabolites by producing or utilizing tryptophan them- selves.[36] The third major pathway of tryptophan metabolism is microbial and results in indoles and its derivates, such as indole-3- acetic acid (IAA), indole-3-propionic acid (IPA) ligands of the aryl hydrocarbon receptor (AhR).[37] AhR is a key regulator of the immune system, involved in the function of macrophages, dendritic cells, and neutrophils.[38] For example, a lack of AhR ligand-producing bacteria is associated with increased intestinal inflammation.[39]

Although the majority of serotonin is synthesized by the host, its production is strongly modulated by gut bacteria. Studies in germ- free animals showed that the levels of tryptophan, serotonin, and kynurenine are significantly different from conventional animals in the gut lumen, plasma, and the brain, both at baseline and following acute stress exposures.[23,40–32] One of the theories involving the role of tryptophan in affective disorders is that the more tryptophan is converted into its alternative metabolites, the less tryptophan can enter the brain via the circulation, decreasing central levels of serotonin.[43]

3 | GUT MICROBIOTA COMPOSITIONAL ALTERATIONS ASSOCIATED WITH DISORDERS OF THE GUT-BRAIN AXIS

There is a growing body of evidence suggesting alterations in gut microbiota composition or function in psychiatric disorders,[44] which has been associated with increased levels of inflammation.[45] It is generally thought that gastrointestinal and psychiatric disorders are associated with decreased alpha diversity (richness, evenness, and biodiversity of the microbiome).[46–48] However, while some published articles show reduced alpha diversity in these disorders, other studies found no changes.[49,50]

Table 1 summarizes changes in relative abundance of specific bacteria associated with IBS, depression, and anxiety, based onthe findings in these systematic reviews, in comparison with the relatively few studies looking at IBS with comorbid anxiety and de- pression. Overall, these disorders present an altered gut microbiota signature but likely due to the heterogeneity of these disorders, conflicting results are common. However, two recent metanalyses identified the gut microbiota signatures most consistently found in depression, anxiety, and IBS.[51,52] These changes in microbial abundance were hypothesized to play functional roles in these disorders. For example, the increased abundance of strains such as *Escherichia* in anxiety has been hypothesized to lead to increased secretion of exotoxins potentially inducing inflammatory processes impacting on

the CNS.[53] In relation to IBS, it was hypothesized that the metabolic products of the strains *Lactobacillaceae* and *Bacteroides*, such as organic acids or toxins respectively, may contribute to the IBS pathology by causing bloating or inflammation peripherally.[52]

Fewer studies have investigated the microbiota using the more informative shotgun metagenomic approach. One such study found that numerous species of the genus *Bifidobacterium* such as *B. adolescentis*, *B. longum*, *B. dentium* are increased in depressed patients.[54] This was unexpected because *Bifidobacterium* strains are commonly used as probiotics with preclinical evidence supporting their possible use for the treatment of psychiatric disorders,[55] although whether a particular microbial member of the gut microbiota should be considered beneficial or harmful depends on context. The most recent study using metagenomic assessment identified 47 species with altered relative abundances in patients with depression comparedto healthy controls. Most of the enriched species belonged to the genera *Bacteroides*, whereas the depleted species belonged to the genera *Blautia*, *Eubacterium* and *Clostridium*.[53] The largest study to date, which included a discovery and validation cohort, showed that *Coprococcus* spp. and *Dialister* are both depleted in depression.[56] Inaddition, the study by Valles-Colomer and colleagues[56] conducted a module-based analysis, profiling microbial pathways with neuroactive potential involved in microbiota-gut-brain axis communication. They showed that depression and quality of life were associatedwith GABA and DOPAC, a metabolite of dopamine. Interestingly, GABA has also been linked to visceral pain perception.[57]

Some studies aimed to subdivide IBS patients with and without distinct microbial signatures. For example, IBS patients characterized by an increased Firmicutes:Bacteroidetes ratio show increased abundance of strains of SCFA-producing eubacteria as well as flagellin producing bacteria,[58] which are associated with increased visceral hypersensitivity and low-grade inflammation.[59,60] Interestingly, it was the patients showing a similar gut microbiota signature com- pared to healthy controls were linked to comorbid depression.[58] Similarly, a distinct gut microbiota signature was shown with in- creased IBS symptom severity. However, in this study, psychiatric comorbidities were associated with the gut microbiota signatures reported in severe cases of IBS.[61]

Relatively few studies have directly assessed the gut microbiota signatures associated with psychiatric comorbidities in IBS. A recent study, analyzing the therapeutic effect of fecal microbiota transplantation (FMT), showed that IBS patients and healthy controls show higher alpha diversity compared to IBS with comorbid depression. Similarly, comorbid IBS patients clustered differently from IBS patients and healthy controls in a beta-diversity analysis.[62] Research has also suggested that patients with IBS and depression show a similar gut microbiota imbalance characterized by either high levels of *Bacteriodes* or *Prevotella*.[63] Further analysis showed that comorbid patients show a similar enterotype to healthy controls, characterized by dominant genera including *Bacteroides*, *Faecalibacterium*and *Lachnospiraceae*. However, differences were shown in thecomposition of non-dominant bacteria. Of note is that the presence of depression at baseline was associated with lasting effect of FMT in IBS-related quality of life and fatigue in patients with non-constipated IBS.[64]

There have not yet been extensive attempts to address the gut microbiota in IBS patients with comorbid anxiety. De Palma and colleagues identified indicator species, rather than taxonomic differences in relative abundances *per se*, of the genera *Eggerthella*, *Blautia*, *Copcrococcus*, *Streptococcus* and *Clostridium*, which were associated with the disease state of comorbid anxiety.[65] However, this was based on a small number of IBS subjects with and without anxiety, making definitive conclusions about a distinct comorbid anxiety related gut microbiota signature difficult.

While it is hard to confidently compare results derived fromsingle studies to that of meta-analyses, it does appear possible that comorbid patients cluster differently than patients with one of the disorders alone. However, there is a greater need for studies including a clinical diagnosis of IBS patients with comorbid depression andanxiety rather than the more common approach of assessing depression and anxiety scores.

4 | SIGNALLING PATHWAYS ALTERED IN GASTROINTESTINAL AND PSYCHIATRIC DISORDERS

It has been theorized that the low-grade inflammation, such as increased cytokine levels[66] associated with depression, stems from increased intestinal permeability[67,68] which in turn results in increased contact of the immune system to bacteria. Similarly, anxiety is associated with a distinct inflammatory state.[69,70] Increased inflammatory signalling may dysregulate the HPA axis, which is associated with symptoms of anxiety and depression.[71] Bacteria showing a higher relative abundance in depression and anxiety, including *Eggerthella* and Enterobacterales, are associated with increased intestinal inflammation and permeability.[72] This low-grade inflammation can be further exacerbated by the loss of SCFA-producing bacteria, such as *Faecalibacterium*, which have anti-inflammatory properties.[73]

IBS is similarly associated with low-grade intestinal and systemic inflammation.[74] Studies showing an increased production of pro-inflammatory cytokines in IBS patient-derived PBMCs also indicate an association with anxiety symptoms.[75] Low-grade intestinal inflammation characterized by increased eosinophil and mast cell numbers in the descending colon may drive the gastrointestinal pathology of IBS.[76] Mucosal inflammation driven by changes in microbiota composition and strains including *Prevotella* is associated with overall immune dysregulation.[77] In conjunction with this, ithas been shown that IBS patients show altered tryptophan metabolism with a shift toward the kynurenine pathway.[78] This changehas been linked to an altered pro-inflammatory state via activation of TLRs.[79] Kazemi et al.[80] additionally showed an improved kynurenine/tryptophan ratio in the blood of the subjects using a probiotic mix containing *L. helveticus* and *B. longum*. Psychiatric comorbidities in IBS can potentially be linked to increased neuroinflammation triggered by the systemic inflammation seen in these

disorders.[66,69,74] These changes are thought to also be in part modulated by changes in SCFA production.[32] In addition, microglia activation has been observed in animal models of stress-induced changes in the microbiota-gut-brain axis.[81] Changes of the gut microbiota signature in these disorders could potentially evoke similar changes relevant for the pathophysiology.

Affective disorders are believed to be mainly caused by dysregulation of neurotransmitters in the brain. For example, the majority of current medications for depression and anxiety act by increasing the level of monoamines in the synapses.[8,9] The level of these neurotransmitters in the brain is also strongly affected bythe gut microbiome. Germ-free animals show altered neurotransmitter concentrations in the brain in addition to reduced anxiety-like behaviors[23,82] and these serotonergic system alterations aredifferentially modulated by acute stress.[42] Interestingly, one of the common therapeutic interventions for IBS are antidepressants. While tricyclic antidepressants (TCAs) are recognized to be an effective treatment in IBS, selective serotonin reuptake inhibitors are not as efficacious.[83] However, serotonin plays an important role in gastrointestinal motility whereby antagonism of the serotonin 5-HT3 receptor improves stool quality[84] and decreases motility in diarrhea-predominant IBS (IBS-D) patients.[85] 5-HT4 receptor agonist have also proven useful in relief of constipation.[86] Serotonin is also a modulator of visceral pain as 5HT-3 antagonism increased colonic compliance[87] and agonism of 5HT-4 reduced sensitivity to rectal distension.[88] The involvement of serotonin in mood disorders has also been extensively studied, particularly in depression, however, its precise neurobiological role in psychiatric disorders is likely of greater complexity than heretofore appreciated.[89] Overall, serotonin is a key signalling molecule in the gut- brain axis implicated in the core symptoms experienced by IBS and patients with psychiatric comorbidity.

IBS has frequently been associated with structural brain changes. For example, one study showed reduced volumes in multiple cortical and limbic structures in female IBS patients compared to healthy controls.[90] However, the majority of the differences were associated with early-life trauma and not IBS alone *per se*, highlighting the importance of early-life stress inthis disorder.[90] Other studies showed alterations in white matterof IBS patients between basal ganglia, thalamus, and prefrontal cortex (PFC).[91] Interestingly, when grouping IBS patients based on the microbiota profile, patients characterized by a reduced Firmicutes:Bacteriodetes ratio present alterations in the anteriorinsula, the motor cortex and the ventral PFC. Furthermore, increasing volume of the posterior insula, was associated with changes in SCFA metabolism and glutamate metabolism.[92] Similarly, patients with depression show structural brain alterations such as reduced hippocampal volume.[9,93] These alterations are accompanied by reduced expression of BDNF in the corresponding brain regions and in the serum.[94,95] Interestingly, reduced serum levels of BDNF have also been reported in comorbid IBS patients.[96] It has been shown that brain BDNF levels are modulated by the microbiotawith germ-free animals showing reduced BDNF expression in the hippocampus.[22,23] However, mechanisms behind the regulation of BDNF by the microbiota are still unclear. Future studies should identify brain regions and circuits, such as the thalamus or pre-frontal areas important for the modulation of sensory information and emotions, common across these pathologies responsible for the symptom presentation.

5 | PRECLINICAL MODELS FOR COMORBID IBS

Part of the difficulty in gaining mechanistic insights in IBS with psychiatric comorbidity pertains to the limited availability of pre- clinical animal models of complex heterogenous behavioral phenotypes. Nevertheless, some options do go some way toward recapitulating a relevant constellation of gastrointestinal and psychiatric symptoms.

5.1 | MATERNAL SEPARATION

Maternal separation is a well-established rodent model of early-life stress and results in widespread changes across the microbiota-gut-brain axis.[97] The maternal separation paradigm does not just model the animal behavioral correlates of one specific disorderbut rather recapitulates several aspects of stress-induced psychiatric disorders and produces robust and reproducible changes across the microbiota-gut-brain axis. These alterations include perturbations in gut microbiota,[97] which are detectable in adulthood, increases in anxiety- and depressive-like behaviors[98,99] aswell as development of visceral hypersensitivity,[100] a hallmark of IBS thought to explain the abdominal pain which is a dominant characteristic of this disorder.[101]

In terms of maternal separation-induced alterations in signalling pathways of the microbiota-gut-brain axis, this early-life stress exposure has also been shown to alter central neurotransmitter levels, particularly monoamines such as serotonin and noradrenaline.[102,103] As serotonin plays an important role in gut to brain communication with respect to mood[104] and descending pain pathways,[105] changesin levels may adversely affect gut function and communication with the CNS. Interestingly, maternal separation has also been shown to alter central serotonin transporter expression.[106] It has been seen that maternal separation also results in upregulation of TLR4 in the paraventricular nucleus of mice as well as visceral hypersensitivity, which is blocked by inhibition of TLR4 signalling,[107] supporting the notion of stress-induced dysregulation of gut-brain axis signalling.

Maternal separation has also been shown to cause reprogramming of the HPA axis, leading to profound effects on endocrine signalling whereby both baseline[97,108] and stress-induced[109] corticosterone levels are increased. Dysregulation of the HPA axis by ma- ternal separation may be likened to clinical cases of IBS where stressreactivity and recovery is altered, and early-life stress is a known risk factor.[110,111]

5.2 | FECAL MICROBIOTA TRANSPLANTATION

FMT studies in rodents currently provide the strongest evidencefor an involvement of the gut microbiota, both in the expression of specific symptoms and the alterations in gut-brain axis signalling pathways of relevance to the pathophysiology of IBS and affective disorders. Multiple studies have used FMT to investigate gastrointestinal, behavioral and molecular alterations associated with IBS. Animals in receipt of a microbiota transplant from IBS patients with predominant constipation or patients with chronic constipation developed delayed GI transit and alterations in intestinal contractions, which was accompanied by decreased levels of SCFAs.[112,113] Conversely, a study using fecal material from diarrhea-predominant IBS patients (IBS-D) developed increased gastrointestinal transit. Furthermore, they showed associations between the gut microbiota, IBS, and psychiatric comorbidities.

Studies showing a disturbed gut microbiota profile in patients with depression linked these alterations to disrupted tryptophan metabolism and intestinal low-grade inflammation.[47] This was achieved by FMT of depressed patients to rats, which induced a similar behavioral and molecular phenotype to the donors. Two studies using a similar approach linked the microbiota-induced depression in mice to alterations in the CREB signalling pathway in the olfactory bulb[114] and alterations of carbohydrate and amino acid metabolism.[115] The latest study transferring the microbiome of depressed patients into mice showed alterations in neurotransmitter levels in the brain and inflammatory markers in the serum.[116]

Earlier studies indicated the rodent-to-rodent transfer ofanxiety-like behaviors[117] and human-to-rat transfer of visceral hypersensitivity.[118] Taken together, FMT studies confirm the individual adoptive transfer of both the cardinal features of IBS (visceral hypersensitivity, altered motility) as well as the psychiatric comorbidity (depression and anxiety-like behaviors).[14] Germ-free micecolonized with fecal microbiota of IBS-D patients with comorbid anxiety showed, in addition to gastrointestinal motility alterations, increased anxiety-like behavior,[65] which was absent in mice receiving the donor material from patients with IBS only and associated with increased immune activation in the colon. This study confirms the simultaneous transfer of multiple phenotypes via the gut micro- biota, positioning FMT studies as a useful preclinical approach to study IBS with psychiatric comorbidity.

In this issue, leading on from their previous study,[65] Constante and colleagues[119] investigated the treatment of comorbid anxiety in IBS using FMT in germ-free mice treated with the probiotic *Saccharomyces boulardii* CNCM I-745 (*S. bou*). Treatment with *S. bou* improved anxiety-like behavior, but not gastrointestinal motility alterations in mice. These results go a step beyond implicating this microbiota configuration in comorbid symptom expression by confirming that an intervention targeting this microbiota can improve symptoms relevant to anxiety. The microbiota profiles revealed differences between the mice transplanted with material from the IBS patient and the healthy control, which were in part normalized by *S. bou* treatment. On the molecular level, they showed a role of indoles (microbial metabolites of tryptophan) and immune activation in IBS with comorbid anxiety. While no clear association was shown between the gut microbiota compositional differences and alterations in indole levels, they nicely linked the anxiolytic effect of *S. bou* to increased indole production. *S. bou* increased both the levels of IAA in the feces as well as the expression of bacterial genes relevant for indole alkaloid synthesis, possibly by increasing the abundance of indole producing bacteria, such as *Lactobacillus*. However, the associated increase in AhR activity failed to reach significance posing the questions of if, and by which mechanisms, the increased indole production induces the anxiolytic effects. Conversely, the authors reported increased expression of the capsaicin receptor TRPV1 in colonic tissue of mice with comorbid IBS- associated microbiota. This receptor, important for the modulationof nociception, is mainly found on neurons of the peripheral nervoussystem. While TRPV1 expression was associated with the anxiety- like behavior, it was not modulated by *S. bou*. Altogether, this studyreports some interesting observations which are potentially relevant to comorbid IBS treatment.

As provocative and timely as the study is, the authors use a single donor for FMT into mice, in contrast to recommendations for the use of multiple individual donors made recently by Walter and colleagues.[120] The authors previous work showed the successful transplantation of phenotypes via the use of multiple donors, providing strong evidence for the gut microbiota in both IBS specifically andits comorbidities.[65] It is not clear from the current study whetherthe beneficial effects of *S. bou* are applicable to a wider range of microbiome compositions of different IBS patients or indeed how well it applies to different comorbidities such as depression. Gut microbial signatures of different donors could be differentially affected by *S. bou*, leading to different outcomes. It would also be interesting to see how effective *S. bou* treatment is against IBS patients without psychiatric comorbidities and whether some of the other cardinal features of IBS including visceral hypersensitivity were impacted. This raises the question of whether the mechanisms described are exclusively altered in comorbid patients or if they also generalize to other subgroups of IBS. The authors recommend that the first point of study in future clinical trials in IBS should be in the subpopulationwith this psychiatric comorbidity. These considerations aside, this study brings important additional insights, expanding on the results reported in previous studies with mechanistic insights and highlighting the therapeutic possibilities of *S. bou*.

6 | THE GUT MICROBIOTA: A NOVEL TARGET FOR TREATING PSYCHIATRIC COMORBIDITIES IN IBS

Currently, treatment options for IBS revolve around symptom control. Some of the more common medications in the treatment ofIBS are antispasmodics or TCAs.[121] Antispasmodics, exerting their effects by relaxation of intestinal smooth muscle, are currently not recommended by the new clinical guidelines by the American

college of gastroenterology although only those currently availablein the United States were evaluated.[122] TCAs such as amitriptyline mainly improve visceral pain, possibly by acting on the -, dopamine- and acetylcholine system.[123] The dose of TCA used is often below that employed in the treatment of depression so the extent to which psychiatric comorbidities are potentially treated by gut-brain neuromodulatory agents is unclear.[121] The integration of psychological behavioral approaches into gastroenterology practice is now more routinely considered,[124] building on the success of gut-focused hypnotherapy as an option in treatment-refractory IBS.[125] The use of food supplements and diet as treatment options has recently been evaluated in this journal.[126] These varied approaches reflect a willingness to target multiple levels of the gut-brain axis to deliver gastrointestinal symptom relief.

One important implication of the study from Constante and colleagues[119] is the potential for therapeutic targeting of the gut microbiota to alleviate the comorbid psychiatric symptoms. Does this mean that specific features of the comorbid gut microbiota lead independently to the cardinal and behavioral features of IBS? The useof a single probiotic strain then, based on the results of this study,is unlikely to be sufficient to improve the global symptom profile in IBS. It has of course long been appreciated that the beneficial effectsof specific probiotics are strain specific and a number of therapeutic options can be considered for targeting the gut microbiota to im- prove gut-brain axis signalling pathways.[127]

6.1 | PREBIOTICS AND PROBIOTICS

Consideration of probiotic (defined as "live microorganisms which when administered in adequate amounts confers a health benefit on the host"[128]) and prebiotic (defined as "a substrate that is selectivelyutilized by host microorganisms conferring a health benefit"[129]) use for treatment of IBS symptoms and associated psychiatric comorbidities has increased in recent years (for review see[130]). Although the exact mechanisms of action of specific prebiotics and probiotics have not been fully elucidated, it has been seen that different prebiotic blends such as polydextrose, galactooligosaccharide and probiotics such as *Lactobacillus rhamnosus GG* ameliorated ma- ternal separation-induced anxiety-like behavior as well as altering hippocampal levels of stress-related genes.[131] Similarly, a prebiotic blend combined with milk fat globule membrane, the bioactive fraction of breastmilk, attenuated maternal separation-induced visceral hypersensitivity and facilitated faster return to baseline of stress- induced corticosterone levels.[132]
Evidence supporting the role of prebiotics and probiotics against IBS symptoms is not purely preclinical whereby IBS patients administered *B. longum* subsp. *longum 35624* (formerly *B. infantis 35624)* for 8 weeks reported a reduction in IBS symptomatology with respect to abdominal pain, bloating and bowel movement difficulty as wellas normalization of the anti-inflammatory: pro-inflammatory cytokine ratio.[133] Several other studies have assessed the efficacy of this treatment with varying degrees of success (for review see[130]). It canbe seen above and from recent technical reviews and clinical guide- lines that while some prebiotic and probiotics have shown promisein the symptomatic treatment of IBS specifically in the context ofa single trial, the jury remains out on making strong recommendations.[134,135] Additional and robust clinical studies are required to determine if we can achieve benefits for associated comorbid psychiatric conditions.

6.2 | THERAPEUTIC FECAL MICROBIOTA TRANSPLANTATION

In recent years, evaluation of the use of FMT from healthy donors as a treatment option for gastrointestinal disorders has increased. There are multiple studies showing the benefits of FMT as a treatment for IBS, further supporting the role of the microbiota in this disorder. The use of a single FMT in IBS patients improved the gastrointestinal symptoms in a subset of patients for a prolonged duration.[136,137] Furthermore, studies showed that the use of FMT additionally improved symptoms of affective disorders, providing evidence for a causal role of the microbiota in psychiatric comorbidities in IBS.[62,138] A double-blind, randomized, placebo- controlled study investigating the effect of FMT in IBS patients showed the effectiveness of FMT as a treatment option and determined that the presence of depression at baseline is predictive of successful treatment.[64] While these studies look promising for treatment, FMT is currently not recommended as a treatment option, as evidence is still limited and large double-blind, placebo-controlled trials are required to determine the treatment efficacy.[122,139] There are a number of important factors to consider in the selection of suitable donors, including microbiota pro- file, in addition to FMT dose that may be critical to a successful FMT.[140] Interestingly, European guidelines on donor selection for the use of FMT in clinical practice does recommend exclusion of subjects with a history of psychiatric conditions.[141]

7 | CONCLUSION

Evidence continues to accumulate in support of the view that the strong link between gastrointestinal and psychiatric disordersis mediated by the microbiota-gut-brain axis. Individually these disorders share similar pathophysiological mechanisms, such as increased pro-inflammatory states or changes in monoamine levels. Many questions remain surrounding the nature of the clinical entity that sits at the intersection between IBS, depression, and anxiety (Figure 2). It is plausible to conceptualize common dysfunctions in gut-brain axis signalling pathways that define this troublesome subset of patients. While this may be a preferable conclusion from a treatment perspective, the reality hinted at by Constante and colleagues[118] is more complex and may developaround a number of diverging targets.

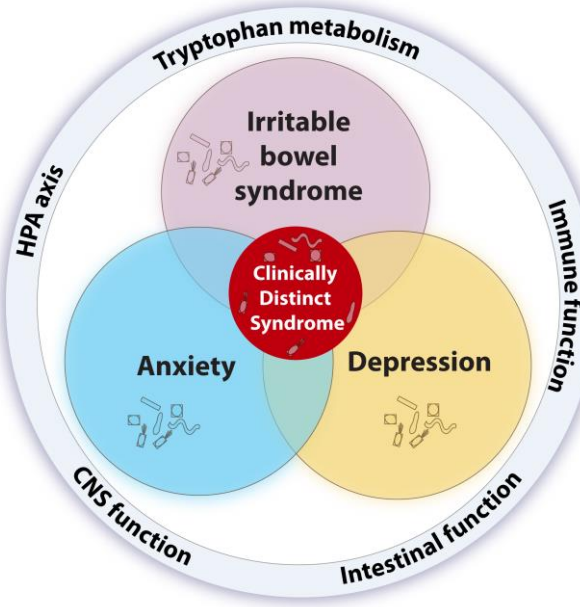

FIGURE 2 A microbial perspective on the intersection between IBS, depression, and anxiety. There is currently a poor understanding of the nature of the clinical entity that sits at the intersection between IBS, depression, and anxiety. One possibilityis that a comorbid gut microbiota drives aberrant signalling along the gut-brain axis, leading to the manifestation of both gastrointestinal and behavioral symptom sets. Increased researchefforts are required to understand why specific microbiota configurations lead in some cases to IBS and in others IBS with psychiatric comorbidity.

What this intriguing study does not answer is why specific microbiota configurations, compositional or functional, lead in some cases to IBS and in others IBS with psychiatric comorbidity. This is an important missing piece in the puzzle that requires increased re- search focus as the current evidence is insufficient to draw definitive conclusions. Animal models of IBS with psychiatric comorbidity hold promise to help disentangle the molecular mechanisms at play and to expand on the associations identified between the gut microbiota, pain pathways and indole production.[119] It will be important to treadcarefully in this regard and not to assume that the signalling pathways implicated in the benefits of particular interventions automatically double as a neurobiological basis for psychiatric comorbidityin IBS. Improving our understanding of how the relevant signalling pathways for depression and anxiety overlap with, or deviate from, those important for the cardinal gastrointestinal features of IBS will be critical. Despite the complexity of these interactions, therapeutic targeting of the gut microbiota for the management of comorbid psychiatric symptoms in IBS may be a strategy worth the effort involved.

DISCLOSURE

GC has spoken at meetings sponsored by food and pharmaceu tical companies and received research funding from Pharmavite. JFC has received research support from Mead Johnson, Cremo, 4D Pharma, Suntory Wellness, Pharmavite, and Nutricia and has spoken at meetings sponsored by food and pharmaceutical companies.

AUTHOR CONTRIBUTIONS

All authors devised the content of the review. LW and JMC carried out literature searches and wrote the manuscript. KJOR designedthe figures with input from JMC, LW, GC and JFC. GC, SMOM and JFC edited the manuscript. All authors approved the final version.

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