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Novel Insights into the Role of the Gut Microbiome on Hippocampal Synaptic Plasticity

Thesis presented by

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Under the supervision of

Prof. John F. Cryan

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For the degree of

Doctor of Philosophy

November 2024

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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: The author and Dr. Henry Darch carried out equal amounts of electrophysiological experiments and data analysis for this study.

Chapter 3: Dr. Henry Darch assisted with the electrophysiological experiments for this study.

Chapter 4: Dr. Cristina Rosell-Cardona assisted with some of the electrophysiological analyses for the male animals of this study. Meghan McDermott carried out the quantitative PCR experiment.

Chapter 5: Dr. Thomaz Bastiaanssen worked with the author on the transcriptomic and metabolomic analysis. Dr. Caoimhe Lynch and Dr. Emily Knox aided in the collection of hippocampal samples.

Signed

_____

Michael Collins

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Publications and Presentations

Published Manuscripts

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Kenneth J O'Riordan, Michael K Collins, Gerard M Moloney, Emily G Knox, María R Aburto, Christine Fülling, Shane J Morley, Gerard Clarke, Harriët Schellekens, John F Cryan. **Short chain fatty acids: Microbial metabolites for gut-brain axis signalling**

Manuscripts in preparation / submitted

Michael K Collins, Henry Darch, Loreto Olavarría-Ramírez, Cian McCafferty, Kenneth J O'Riordan, John F Cryan. **Robust Hippocampal Synaptic Plasticity Despite Gut Microbiota Depletion in Adult Mice**
In preparation

Michael K Collins, Cristina Rosell-Cardona, Meghan M. McDermott, Amiee M. Cronin, Gerard Clarke, Kenneth J. O'Riordan, John F. Cryan. **Modulation of Murine Hippocampal Synaptic Plasticity by Microbial Metabolites: Sex-Specific Effects of the Short-Chain Fatty Acid Butyrate**
In preparation

Michael K. Collins, Thomaz F.S. Bastiaanssen, Caoimhe M.K. Lynch[1,2], Emily G. Knox[1,2], Gerard Clarke[1, 3], Kenneth J. O'Riordan[2], John F. Cryan. **The Gut Microbiota Defines the Early Life Developmental Trajectory of Hippocampal Synaptic Plasticity in a Sex-Dependent Manner**
In preparation

Caoimhe M.K. Lynch, Emily G. Knox, Daniel Soong, Thomaz F.S. Bastiaanssen, Kalevi Trontti, Gabriel Tofani, Sophia Ivaschuk, Michael K. Collins, Donia Arafa, Jatin Nagpal, Iiris Hovatta, David A. Lyons, Gerard Clarke, John F. Cryan. **The Gut Microbiota Shapes Myelination Dynamics During Critical Windows in Early Life**
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Oral presentations

Michael K Collins, Cristina Rosell-Cardona, Amiee M. Cronin, Gerard Clarke, Kenneth J. O'Riordan, John F. Cryan **The Importance of Gut Microbial Metabolites in Hippocampal Synaptic Plasticity.** CoMH Futures, September 2023, University College Cork, Ireland.

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Michael K Collins, Henry T Darch, Cristina Rosell-Cardona, Loreto Olavarría-Ramírez, Cian McCafferty, Amiee M. Cronin, Gerard Clarke, Kenneth J. O'Riordan, John F. Cryan **How Important are our Gut Microbiota in Hippocampal Synaptic Plasticity.** APC symposium. September 2022, University College Cork, Ireland.

Poster presentations

Michael K Collins, Cristina Rosell Cardona, Kenneth J O'Riordan, John F Cryan. **Modulation of Murine Hippocampal Synaptic Plasticity by Microbial Metabolites: Sex-Specific Effects of the Short-Chain Fatty Acid Butyrate** ECNP Milan, Italy. September 2024

Michael K Collins, Cristina Rosell-Cardona, Amiee M. Cronin, Gerard Clarke, Kenneth J. O'Riordan, John F. Cryan **The Microbial Metabolite Butyrate Alters Hippocampal Synaptic Electrophysiology.** Neuroscience Ireland. August 2023. Dublin, Ireland.

Michael K Collins, Henry T Darch, Loreto Olavarría-Ramírez, Ken J O'Riordan, Cian McCafferty, John F Cryan**. Hippocampal Synaptic Electrophysiology in a Murine Model of Gut-Microbiota Depletion.** March 2022. ECNP Workshop for Early Career Scientists in Europe. Nice, France.

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Michael K Collins, Henry T Darch, Loreto Olavarría-Ramírez, Kenneth J O'Riordan, Cian McCafferty, John F Cryan. **Hippocampal Synaptic Electrophysiology in a Murine Model of Gut-Microbiota Depletion.** Brain Therapeutics, October 2021, University College Cork, Ireland

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Abbreviations

Antibiotic (ABX)

Adrenocorticotropin Hormone (ACTH)

Alzheimer's Disease (AD)

Area under the curve (AUC)

Artificial cerebrospinal fluid (aCSF)

Autism spectrum disorder (ASD)

Beta-hydroxybutyrate (BHB)

Brain-derived neurotrophic factor (BDNF)

Blood-brain-barrier (BBB)

Caesarean-section (C-section)

Cerebrospinal fluid (CSF)

Central nervous system (CNS)

Corticotrophin-releasing hormone (CRH)

Enteric nervous system (ENS)

Enteroendocrine Cells (EECs)

Excitatory/Inhibitory (E/I)

False discovery rate (FDR)

Farnesoid X receptor (FXR)

Fibre volley (FV)

Free fatty acid receptor (FFAR)

Faecal microbiota transplantation (FMT)

Field excitatory post-synaptic potential (fEPSP)

Gamma-aminobutyric acid (GABA)

Gene Ontology (GO)

Germ free (GF)

Glucocorticoids (GCs)

G-protein-coupled receptor (GPCR)

Gut-brain axis (GBA)

High Frequency Stimulation (HFS)

Histone acetyl transferase (HAT)

Histone deacetylase (HDAC)

Hypothalamic-pituitary-adrenal (HPA)

Indole-3-lactic acid (ILA)

Indole-3-propionic acid (IPA)

Irritable bowel syndrome (IBS)

Lipopolysaccharide (LPS)

Long-term depression (LTD)

Long-term potentiation (LTP)

Major depressive disorder (MDD)

Monocarboxylate transporter (MCT)

Multielectrode array (MEA)

Paired-pulse facilitation (PPF)

Parkinson's disease (PD)

Postnatal day (P)

Short-chain fatty acids (SCFAs)

Sodium-coupled monocarboxylate transporter (SMCT)

Toll-like receptor (TLR)

Trimethylamine N-oxide (TMAO)

Tumour necrosis factor alpha (TNF-α)

Abstract

Synaptic plasticity especially within the hippocampus is a central process of the learning machinery of the brain. The microbiota gut-brain axis has emerged as an important regulator of hippocampal function yet there exists a paucity of information on how and when this axis can affect hippocampal synaptic plasticity. New strides need to be taken to develop successful interventions for human diseases which feature dysregulation of synaptic plasticity and to understand when normal synaptic plasticity may be susceptible to altered regulation from the microbiota-gut brain axis. In this thesis we focused primarily on how adult synaptic plasticity in the hippocampus may be affected by microbiome interventions. We also assessed the microbiome's role in altering the developmental trajectory of the hippocampal transcriptome with a special focus on plasticity-related pathways.

Firstly, we used germ free mice to examine how growing up without a gut microbiome has altered synaptic plasticity with hippocampal slice electrophysiology. While basal synaptic efficacy and pre-synaptic short-term plasticity appear normal, we found a deficit of hippocampal long-term potentiation in slices from male germ-free mice whereas slices from female animals produced this plasticity process relatively normally. However, the spike output of these neuronal populations remained normal, possibly indicating an altered excitation-inhibition balance or a homeostatic compensatory mechanism.

Following this, we began to interrogate whether the microbiota's ability to alter synaptic plasticity is developmentally wired or remains susceptible in adulthood. To this end we depleted the microbiome in adult life using either an antibiotic cocktail or a single antibiotic. This two-week microbiota intervention in adulthood produced only subtle effects on short-term plasticity and left LTP unchanged, demonstrating the robustness of hippocampal plasticity to the effects of microbiome depletion in adulthood.

We then investigated how a local exposure to short-chain fatty acids (SCFAs), an abundantly produced class of microbial metabolite, may affect hippocampal synaptic plasticity in adulthood. Three SCFAs were tested, acetate, propionate, and butyrate at physiologically relevant concentrations. While no effect of acetate or propionate was found, butyrate-treated slices produced a greater degree of LTP in slices from both male and female mice. We hypothesized this effect was occurring through butyrate's action at free fatty acid receptor 3 (FFAR3). However, when slices were pre-treated with an inhibitor of this receptor only slices from female mice reverted to producing normal levels of LTP, showing once again a sex-specific response to microbiome signals.

Finally, we returned to the GF mouse model, examining four putative critical windows in early life, to establish how the microbiota changes the hippocampal transcriptome and metabolome. Metabolomic analysis revealed post-natal day (P) 21 to be the most differentially regulated in GF mice regardless of sex. GF status also significantly altered the hippocampal transcriptome with effects culminating at the oldest examined timepoint, P21. This effect was mirrored in a targeted enrichment analysis of pathways relating to synaptic plasticity. Unlike the metabolomic analysis however, transcriptomic analysis revealed a strong interaction of microbiome and sex with females showing the greatest differences despite a similarly altered metabolome.

Overall, the results of this thesis provide novel insights into how the microbiome may alter hippocampal plasticity in adulthood and early life, with microbiome-induced effects often emerging in a sex-specific manner.

Chapter 1: General Introduction

1 Background and Significance

1.1 Humans and Microbes

“IN NATURE, NOTHING EXISTS ALONE.” – Rachel Carson. Humans exist in a world surrounded by other living organisms. We live in tandem with them, all affecting each other. An often-overlooked part of this wide relationship is the connection of human beings and microorganisms such as bacteria (McFall-Ngai et al., 2013; Wilde et al., 2024). We have never lived in a world without microorganisms, indeed while humans evolved around 300,000 years ago, microbes may be 3.7 billion years old. Microbes are found in almost every habitat in nature and one of these habitats is humans. We are intimately connected with one another, they live on our skin, in our mouths, our airways, and in our gut (Byrd et al., 2018; Cryan et al., 2019a; Deo & Deshmukh, 2019; Natalini et al., 2023). Even the mitochondria in our cells are of bacterial origin according to prevailing theory (Muñoz-Gómez et al., 2022). In the past few decades, science has turned a spotlight onto the microbes that live in our gut, discovering that these microbes affect our health, our bodies’ various systems, and the very way we behave. This thesis focuses on the connection our gut bacteria have with our brain, shedding light on how they affect processes underlying learning and memory, through early life development and the everyday crosstalk between our gut microbes and us.

1.2 Overview of the Gut-Brain Axis

The complex, bidirectional communication network that links the gastrointestinal tract and the central nervous system is called the gut-brain axis. One of the critical regulators of this axis is the gut microbiota which is composed of trillions of microorganisms that live in our gut (Cryan et al., 2019a) (Figure 1.1). The gut microbiome and the gut microbiota are terms that are often used interchangeably but for specificity, the gut microbiota refers to the

microorganisms themselves that make up the gut community, while the gut microbiome refers to the full collection of genomes of these microorganisms. Gut bacteria are the most well-studied members of this community, however it is also made up of viruses as well as other microorganisms, including fungi, archaea, and protozoa (Garcia-Bonete et al., 2023). Through the production of various metabolites, modulation of the immune system, and direct interaction with the vagus nerve, the gut microbiota is a fundamental part of this axis, influencing neural development, behaviour, and cognitive processes, and establishing a fundamental connection between gut health and brain function (Cryan et al., 2019a). One way in which studies have convincingly established the connection between gut microbes and the brain is through utilization of the germ-free rodent model. Isolator cages are used to breed germ-free rodents, fully blocking them from exposure to microorganisms (E. A. Kennedy et al., 2018). This allows for the study of how the complete absence of microbes can affect an organism. These rodents have been observed to have numerous behavioural changes including altered anxiety-like behaviour, sociability, and memory (Cryan et al., 2019a).

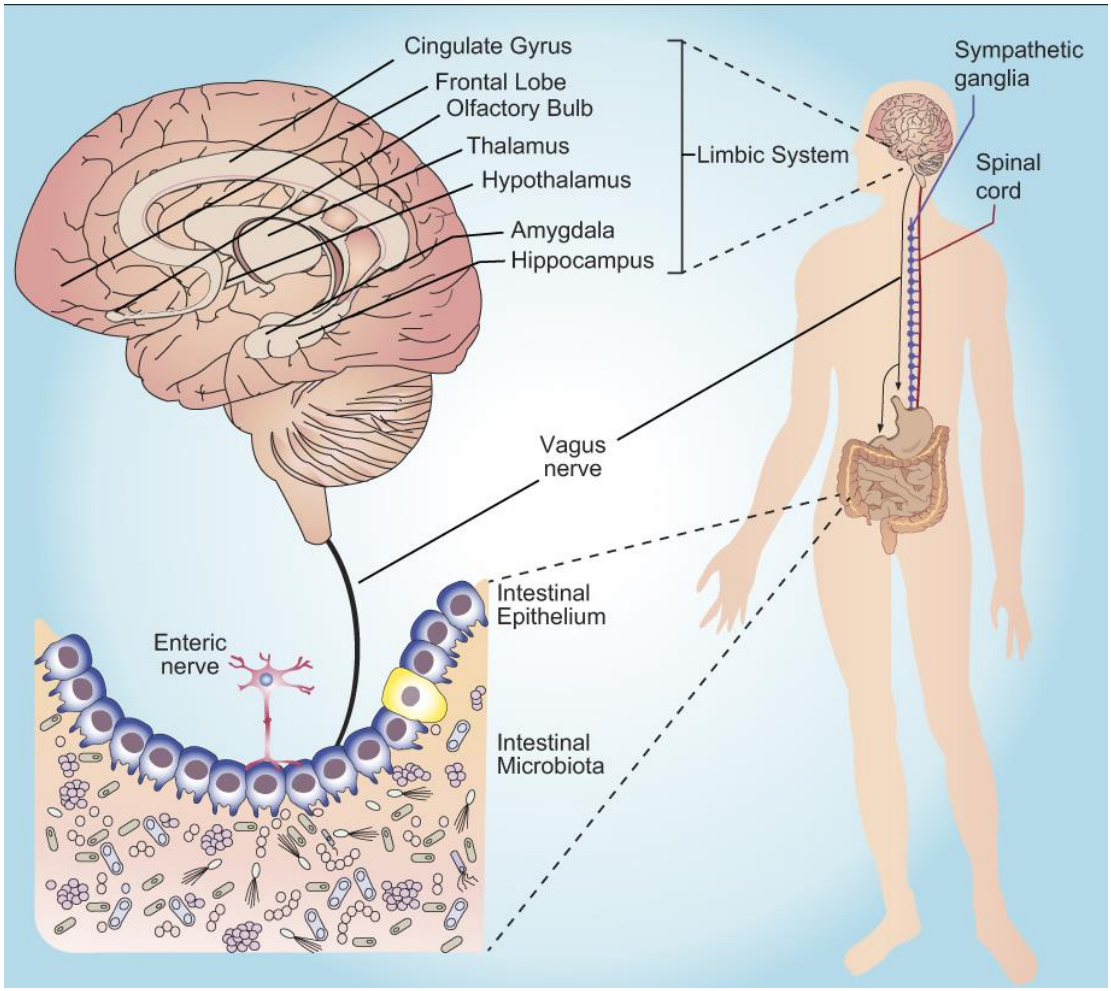


Figure 1.1 Overview of the Gut-Brain Axis (Cryan et al., 2019a). Illustration of the bidirectional communication pathways between the brain and the gut, commonly referred to as the gut-brain axis. The brain is depicted with key areas involved in emotional and physiological regulation. These regions are part of the limbic system and play significant roles in emotional processing, stress response and homeostatic control. The vagus nerve, a primary communication link between the gut and brain extends from the brainstem to the intestinal tract. An enteric nerve within the intestinal wall connects with the intestinal epithelial layer and surrounding structures, symbolizing the enteric nervous system (ENS), which is often called the "second brain" due to its complex, autonomous control of gut function. Beneath the gut epithelial cells, various intestinal microbiota are shown.

This gut microbiota impacts the lives of human beings in both health and disease. These microorganisms may promote health and normal physiological function, aiding us in numerous ways such as protecting the host from pathogens, recovering energy from indigestible food components such as dietary fibre, and modulating the immune system (Bull & Plummer, 2014). The gut microbiome's influence is evident in a wide variety of diseases ranging from irritable bowel syndrome (IBS) to cardiometabolic disease; from infections to neuropsychiatric

disorders (Bonnechère et al., 2022; Chakaroun et al., 2023; Raskov et al., 2016). For example there is increasing evidence for a role of the microbiota-gut-brain axis in major depressive disorder (MDD) (Bastiaanssen et al., 2020). These changes include a reduction in bacteria that produce metabolites known as short chain fatty acids (SCFAs) (McGuinness et al., 2022; Nikolova et al., 2021; Skonieczna-Żydecka et al., 2018; P. Zheng et al., 2016) and increased bacterial production of compounds that contribute to additional activation of the HPA axis (Makris et al., 2021). The gut microbiome also contributes to the pathogenesis of neurodegenerative disorders (Cryan et al., 2020; Loh et al., 2024). At a fundamental level, such diseases are disorders of the brain, yet early microbiome changes are detected in prodromal Parkinson's Disease (PD) patients and preclinical Alzheimer's Disease (AD) patients (Ferreiro et al., 2023a; B. Huang et al., 2023; Palacios et al., 2023). These examples help build a picture of brain function being intricately connected to our gut and the microorganisms in residence there.

2 The Gut Microbiome and Brain Health

2.1 Composition and Function of the Gut Microbiome

Our understanding of the gut microbiota is consistently growing and changing. It was previously estimated that the human body holds 10^{14}–10^{15} microbial cells, outnumbering the human cells of the body by a factor of 10 (Berg, 1996; Savage, 1977; Sender et al., 2016). This was refuted in 2016 however, when the estimated ratio was changed to 1:1, meaning our bodies contain as many microbial cells as human cells (Sender et al., 2016). This substantial community hosts great complexity. It is largely composed of the following Phyla, Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia, of which Firmicutes and Bacteroidetes are the most numerous (Laterza et al., 2016). Factors which influence the composition of the gut microbiota are of great interest as the composition of the microbiota is wholly linked to the functions that it carries out in the host. These factors include diet,

genetics, environment, medication use, and age, each helping to create a unique signature in each individual's gut over time (Figure 1.2). The microbial colonisation of the gut early in life is especially important as it carries a disproportionate effect on the long-term trajectory of an individual's gut microbiome signature compared to many later life influences (Borre et al., 2014; Palmer et al., 2007). Different birth modes promote vastly different populations of microbes to seed the gut during this initial colonisation and can therefore confer stark differences to an individual's long-term microbiome signature (Dunn et al., 2017). Those born by Caesarean-section (C-section) have a different microbial signature, often being colonised with bacteria from epidermal or hospital environment rather than of vaginal origin (Dominguez-Bello et al., 2010; Shao et al., 2019). This difference perfectly serves as an initial clue to the importance of gut microbiota composition as C-section births are linked to a higher risk of allergy and asthma development compared to children born vaginally (Darabi et al., 2019).

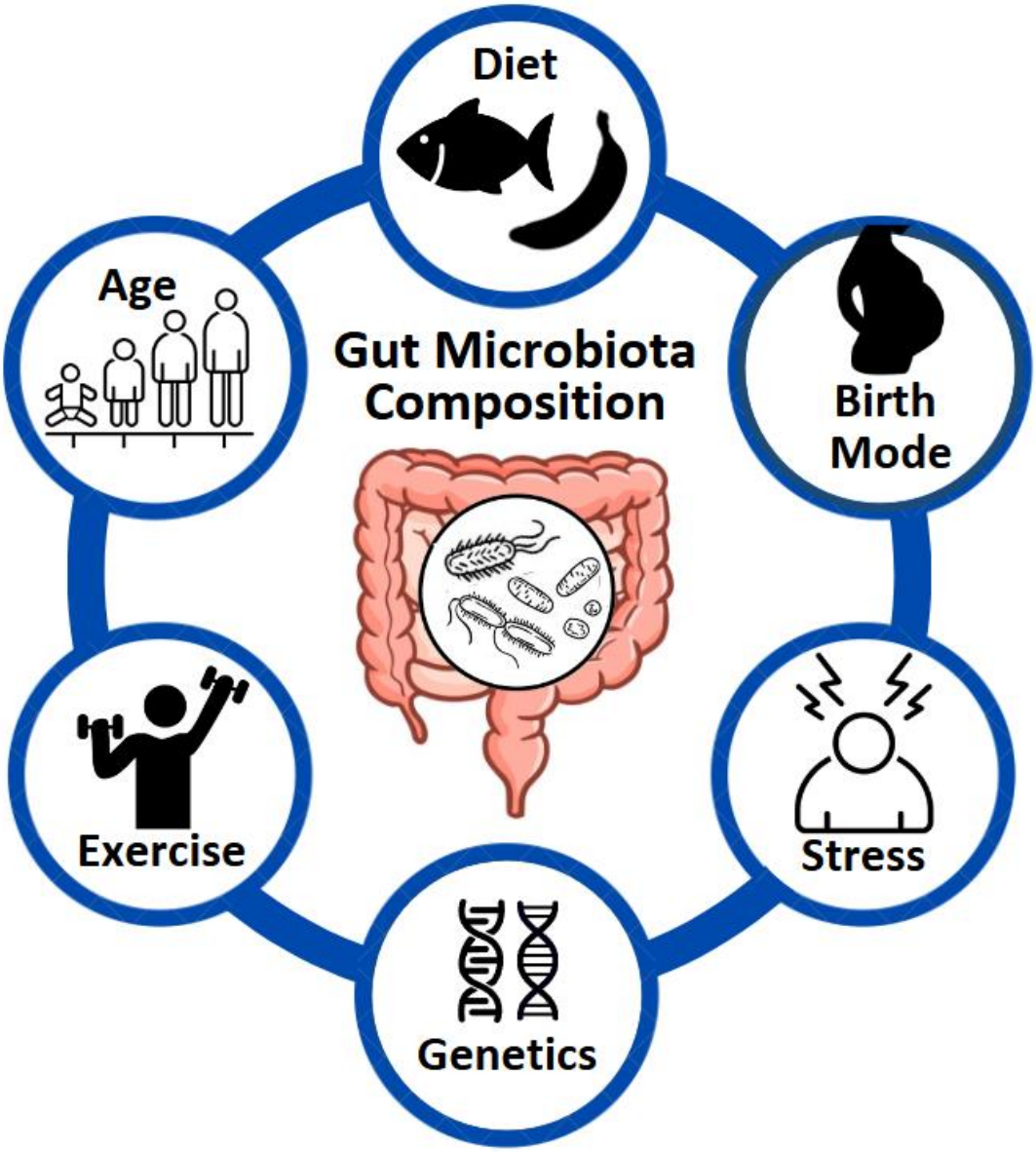


Figure 1.2 Gut microbiota composition is affected by various factors including Age, Host Genetics, Birth Mode, Diet, Exercise, Stress.

In its healthy state the gut microbiome functions symbiotically with its host, contributing to host health in numerous ways, aiding in digestion through fermentation of food, synthesizing vitamins, stimulating or dampening the immune response, and protecting against pathogens (E. T. Hillman et al., 2017). However, other microbiome states featuring certain compositional factors are linked to conditions such as inflammatory bowel disease, obesity, and neuropsychiatric disorders. Obesity for example, sees an overgrowth of bacteria

which produce lipopolysaccharide (LPS), a component of the outermost membrane of Gram-negative bacteria, and a lack of the bacteria that are essential for preserving the proper integrity of the intestinal mucosal barrier (Zhuang et al., 2023). Meanwhile, inflammatory bowel disease sees a depleted diversity of gut bacteria and a lack of bacteria such as *Faecalibacterium prausnitzii* which are producers of metabolites that are important to our health (Dahal et al., 2023). The gut microbiome therefore can serve as an integral part of the homeostasis of the human body, working in varied ways to maintain normal bodily function but can also take on functions that are deleterious to the host.

2.2 Mechanisms of Gut-Brain Communication

There exist multiple pathways for the gut and its resident bacteria to communicate with, and exert effects on, the nervous system (Figure 1.3). These include the release of circulating metabolites, stimulation of the vagus nerve, and activation of the immune system (Cryan et al., 2019a). While I have separated these communication pathways for describing them here, it must be emphasised that there is also a complex mechanistic interplay between the routes themselves that likely vary across the lifespan.

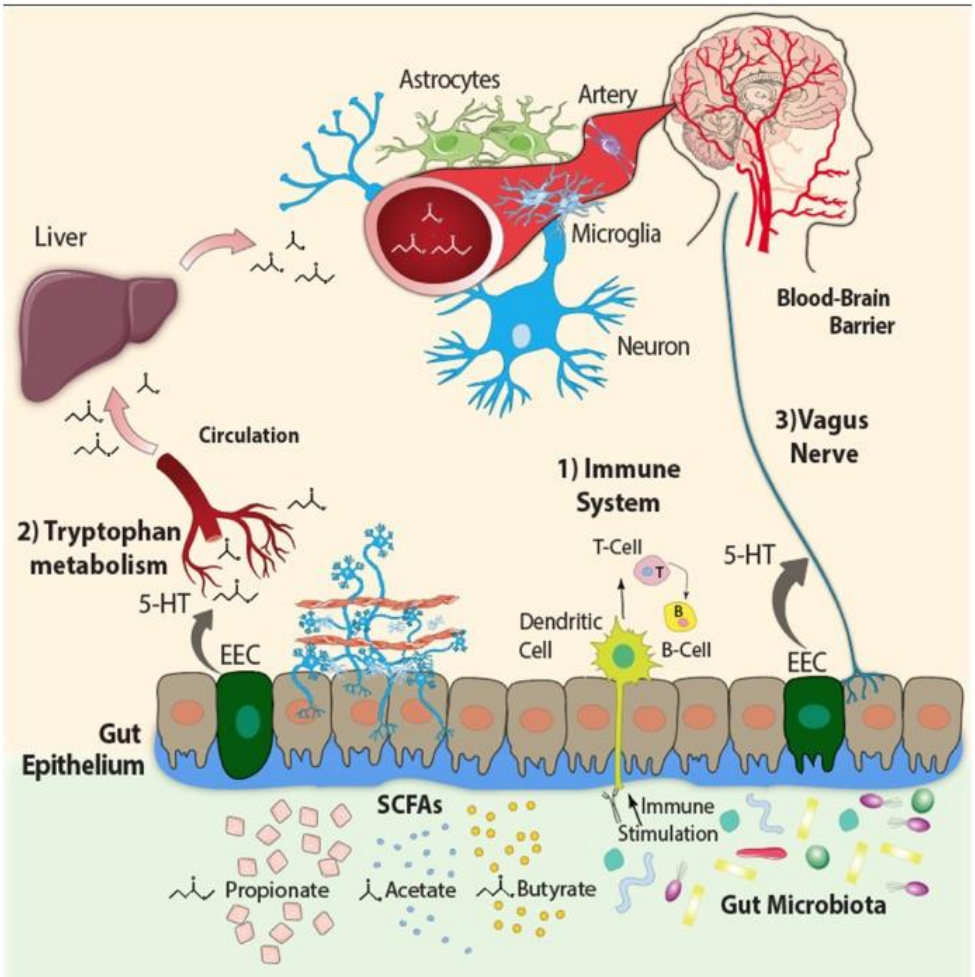


Figure 1.3 Mechanisms of Microbiota-Gut-Brain Communication. Communication between the gut microbiome and the brain occurs along the gut-brain axis, a bidirectional signalling pathway, comprising the 1) immune system, 2) tryptophan metabolism, 3) vagus nerve activity, as well as the bioactive microbial metabolites produced by the gut microbiome; SCFA = Short-chain fatty acids; EEC = Enteroendocrine Cell; 5-HT -5-hydroxytryptamine/Serotonin.

2.2.1 The Immune System

The host immune system plays a vital role in defence against harmful external agents. The gut microbiota helps to train and develop major components of the host's adaptive and innate immune system. Through this mutualistic partnership the host coordinates metabolic signalling, microbial sensing, and immune response pathways to aid survival (Rooks & Garrett, 2016; D. Zheng et al., 2020).

The gut microbiota can activate immune cells in both the intestine and the brain. In the intestine this occurs through pattern recognition receptors such as toll-like receptors (TLRs) and NOD-like receptors, on the surface of immune cells (C. Lin

et al., 2019; Pusceddu et al., 2019). These immune cell receptors recognise a class of molecules that have a structure unique to microbes called microbial-associated molecular patterns. This includes peptidoglycan from bacterial cell walls and LPS from the outer membrane of gram-negative bacteria (Kasarello et al., 2023). This direct stimulation of gut immune cells further stimulates the production of cytokines, a class of small immune signalling proteins, such as IL-1β, and TNF-α, which can affect brain function and behaviour following signalling through the vagus nerve in the gut or crossing into the circulation and from there through the blood-brain-barrier (BBB) (Foster et al., 2021; Giannoni et al., 2020; Logsdon et al., 2018). Finally, aside from direct stimulation of resident gut immune cells the gut microbiota can also secrete immunomodulatory metabolites and molecules to influence the gut-brain axis (Erturk-Hasdemir et al., 2021).

2.2.2 The Vagus Nerve

The vagus nerve plays an important role in the gut-brain axis. It is the 10th cranial nerve and links the brain and gut directly. It contains both motor (efferent) and sensory (afferent) neurons. Vagal afferents terminate in the muscle layer of the gut as well as in the mucosa. In the muscle layer these afferents may form intramuscular arrays or intraganglionic laminar endings. Some afferents also synapse onto neurons from the enteric nervous system, which governs the function of the gut. Meanwhile vagal efferents have a major role in the regulation of inflammation (Pavlov & Tracey, 2012). The vagus nerve also plays a role as a parasympathetic branch of the autonomic nervous system. Changes to vagal nerve properties such as excitability, and synapse remodelling, alter vagal tone, which describes the strength of parasympathetic vagal activation in the changing interaction between sympathetic and parasympathetic branches of the ANS (Bonaz et al., 2016).

This nerve is a clear example of the bi-directional communication of the microbiome gut-brain axis as it tonically transmits information to and from the gut and brain. Evidence of gut vagal neurons being involved in brain function

dates to 20th century gastrectomy (surgical removal of part or all the stomach) for the treatment of peptic ulcers. These surgeries also ablated vagus nerve activity in the lower intestine, cutting communication between this area and the brain, unfortunately resulting in increased occurrence of psychiatric-related disorders. These findings were supported by subsequent animal studies which cut gut-related vagal communication and saw diverse changes in the brain in stress reactivity, cognition, and adult neurogenesis (Fülling et al., 2019a). Electrophysiological experiments found that vagal afferents respond to a variety of microbial-associated stimuli such as gut peptides, nutrients, hormones and cytokines from immune cell stimulation. The gut microbiota alter vagal signalling through influencing the concentration or inducing the release of these factors from gastrointestinal or enteroendocrine immune cells (Fülling et al., 2019a). For example, the gut microbiota may stimulate the release of gut hormones and peptides from enteroendocrine cells (Covasa et al., 2019; Woźniak et al., 2021)

2.2.3 Hypothalamic-Pituitary-Adrenal Axis

The hypothalamic-pituitary-adrenal (HPA) axis is a system that allows for successful physiological adaptation of an organism to stress. During stress this axis induces a cascade of hormone release from the hypothalamus to the anterior pituitary, and on to the adrenal cortex. Firstly, the hypothalamus releases corticotrophin-releasing hormone (CRH) and arginine vasopressin. CRH stimulates the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary into the systemic circulation. Finally, ACTH leads to the secretion of glucocorticoids (GCs), the final effectors of the HPA axis, from the adrenal cortex. The main GC differs by species, with cortisol having the main role in humans while in rodents this role is filled by corticosterone (Sudo, 2016).

The HPA axis is often highlighted as one of the major pathways of communication between the brain and the gut microbiota (de Weerth, 2017; Tetel et al., 2018). Glucocorticoids impact the gut-brain axis through multiple pathways and thus the receptors for these steroid hormones are expressed by epithelial, immune,

and enteroendocrine cells in the gut, demonstrating a direct effect of GCs on gut function (Carabotti et al., 2015). Their effects on gut function, namely changing gut transit time, nutrient availability, and intestinal permeability, exercise an effect over the gut microbiota, changing its diversity and composition (Mayer, 2000). In the brain, these stress hormones bind to glucocorticoid receptors in such areas as the hippocampus, the brain's centre of learning and memory (Zhu et al., 2014). However, in turn, gut microbes can influence the levels of circulating glucocorticoids in both times of stress and in the baseline state (Mukherji et al., 2013; Sudo et al., 2004). Given the role of glucocorticoids in neuronal function, changes in these compounds underly alterations in multiple behaviours including anxiety- and depression-like behaviour associated with gut microbiota changes (Luo et al., 2018).

2.2.4 Microbial Metabolites

Finally, the substantial variety of genes within the gut microbiome—including many acquired through horizontal gene transfer—allows gut bacteria to produce enzymes capable of interacting with a wide variety of substances in the gut, leading to the generation of microbial metabolites that play an important role in the gut-brain axis (Soucy et al., 2015). These microbial products can be divided into three main types when classified by origination. Firstly, metabolites produced directly from the diet as gut microbes ferment many compounds either missed by the host digestion process or those outside the remit of human digestion such as dietary fibres. Products of this fermentation include SCFAs. Secondly, host-generated metabolites can be modified by the microbiota, such as secondary bile acids. Finally, gut microbes also produce some compounds *de novo,* such as vitamin K. This wide variety of metabolites may have direct and/or indirect effects on the gut-brain axis.

Starting in the gut, microbial metabolites engage with the other routes of gut-brain axis communication such as the vagus nerve. These metabolites interact with the vagus nerve via enteroendocrine cells (EECs). EECs detect microbial products such as LPS and in response activate receptors on vagal afferents

through the release the neurotransmitter serotonin and hormones such as cholecystokinin (Bonaz et al., 2018). This indirect microbial metabolite activation of the vagus nerve allows for regulation of food intake at the level of the brain, and gastrointestinal motility, and secretion in the gut. Microbial metabolites such as the SCFA butyrate, can also act directly on the vagus nerve, stimulating vagal nerve activity (Lal et al., 2001).

The presence of microbial metabolites is however not confined solely to the gut. Many microbial metabolites can leave the intestinal lumen and enter the circulation. From there, some microbial metabolites including TMAO and p-cresol sulphate can cross the BBB and influence central nervous system functions locally (Kärkkäinen et al., 2021). These influences include modulation of neurotransmitter signalling, neuroinflammation, and neuroplasticity (Dalile et al., 2019). The SCFA acetate for example when taken up by the BBB alters the levels of the neurotransmitters such as glutamate and gamma-amino butyric acid (GABA) in the hypothalamus (Frost et al., 2014). Furthermore, microbial metabolites can impact the central nervous system indirectly by modulating systemic metabolism and hormone release, further highlighting the intricate web of communication between the gut and the brain (J. Liu et al., 2022).

3 Microbial Metabolites and Brain Function

3.1 Types of Microbial Metabolites

The human gut microbiota produces a wide variety of metabolites that play critical roles in the microbiota-gut-brain axis. These microbial metabolites have the potential to influence our behaviour and brain function through various mechanisms mentioned thus far, including modulation of the immune system, alteration of neurotransmitter synthesis, and direct signalling to the brain. Key types of metabolite include SCFAs, neurotransmitters and their precursors, bile acids, and indole derivatives, which will be discussed in more detail below (Banfi et al., 2021).

3.1.1 Short-Chain Fatty Acids (SCFAs)

SCFAs are primary products of the fermentation of host-indigestible dietary fibres by gut bacteria. They are volatile fatty acids with a carbon chain length of two to five (acetate, propionate, butyrate, and valerate, in order of increasing size) (Koh et al., 2016) (Figure 1.4). These metabolites serve multiple functions in the gut-brain axis. At the level of the gut they maintain gut barrier integrity, exert anti-inflammatory effects and provide energy to intestinal cells (Cook & Sellin, 1998; Seethaler et al., 2022; Vinolo et al., 2011). They are transported into gut surface epithelial cells through monocarboxylate transporters such as monocarboxylate transporter (MCT) 1 and 4, and sodium-coupled monocarboxylate transporter (SMCT) 1 (Miyauchi et al., 2004; Ritzhaupt et al., 1998). Here, SCFAs such as butyrate, undergo β-oxidation to generate cellular fuel, providing energy to gut epithelial cells. Butyrate provides an estimated 60-70% of the energy needs of colonocytes and as well as 5-15% of the total caloric requirement of humans (Brahe et al., 2013). Intestinal epithelial cells also express specific receptors which bind SCFAs e.g. GPR41/FFAR3, GPR43/FFAR2, and GPR109A (Kimura et al., 2020), activating various signal pathways.

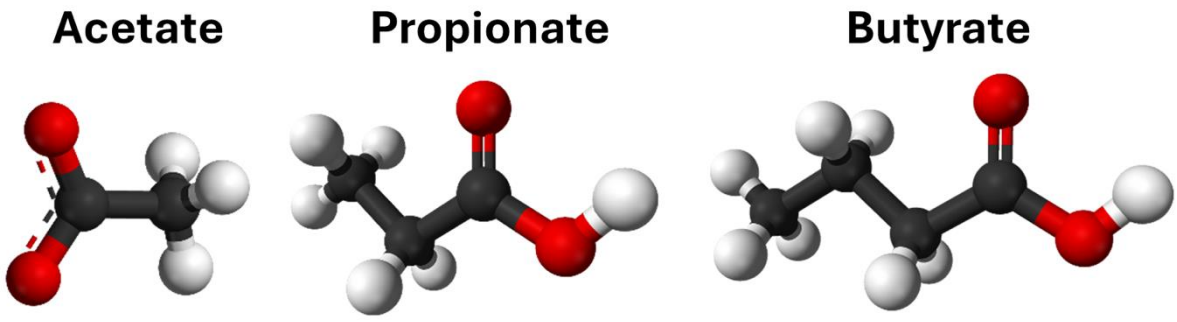


Figure 1.4 SCFA chemical structures; Acetate, Propionate, Butyrate (from left to right in order of increasing carbon chain size). All images have been released into the public domain by their author, Benjah-bmm27.

Due to the importance of SCFAs as a metabolite, their ubiquity in the body and the focus on them for this thesis, a more detailed discussion of the function of SCFAs will follow in subsequent sections. However, it is important to note here

that SCFAs are among the metabolites that continue their function beyond the level of the gut to other areas of the body through passage into the circulatory system. The passage of SCFAs through the BBB is permitted through the expression of transporters such as MCT1, SMCT1, and MCT4 (Dehouck et al., 2022; Gerhart et al., 1997; Leino et al., 1999; Vijay & Morris, 2014). Once through the BBB they may influence brain function in numerous ways such as reducing inflammation and promoting neuroplasticity (Dalile et al., 2019). SCFAs also affect the BBB itself (Figure 1.5), protecting it from lipopolysaccharide-induced disruption (Hoyles et al., 2018). SCFAs also decrease barrier permeability while germ free (GF) mice have been observed to have a disrupted BBB (Braniste et al., 2014).

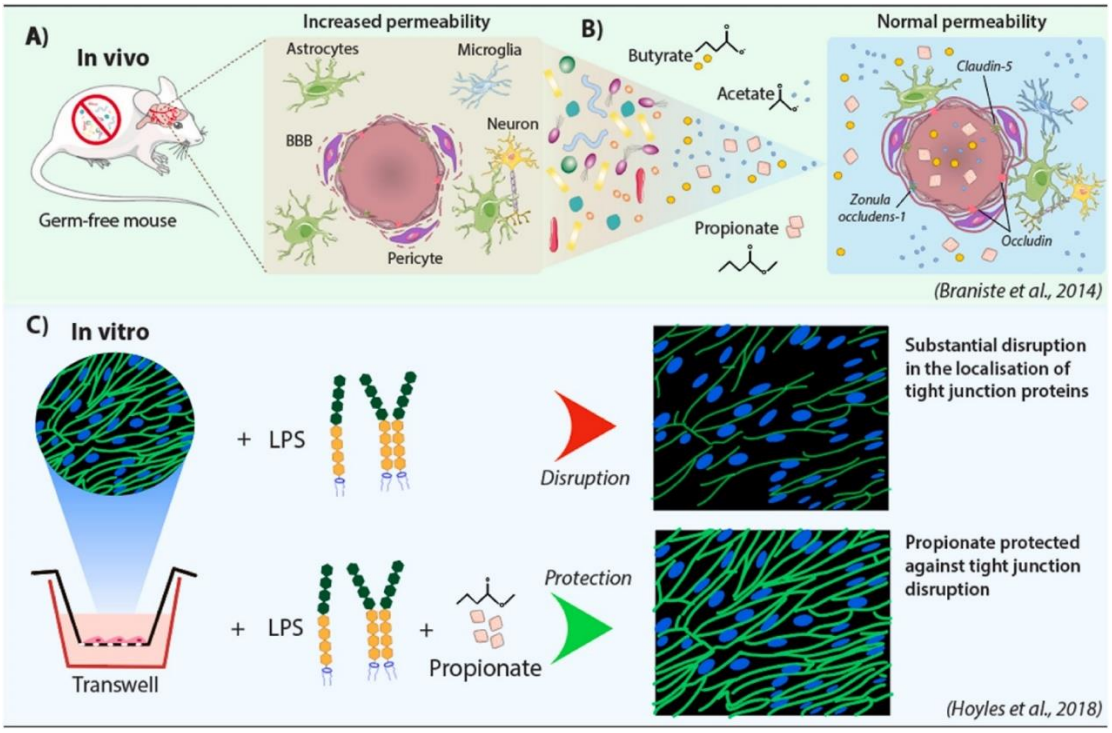


Figure 1.5 (O'Riordan et al., 2022) Short chain fatty acids protect blood brain barrier integrity and structure. A) Germ-free mice display blood-brain barrier (BBB) disruption and, B) monocolonisation with SCFA-producing bacterial strains as oral gavage of sodium butyrate, improves BBB integrity. C) Cell culture BBB models display disrupted BBB integrity when treated with LPS, but pre-treatment with propionate protects against this disruption. BBB = Blood-brain barrier. LPS = Lipopolysaccharide.

3.1.2 Neurotransmitter Precursors and Neuroactive Compounds

Gut bacteria can produce or influence the levels of various neurotransmitter precursors and neuroactive compounds. Certain *Lactobacillus* and *Bifidobacterium* species for example produce GABA, the major inhibitory neurotransmitter of the human body (Tette et al., 2022; Yunes et al., 2016). Microbial-derived GABA can cross into the circulation then into the CNS through specific GABA transporters expressed in the BBB (Takanaga et al., 2001). To bolster the role of microbial influence on behaviour and neurotransmitter function it has been observed that colonisation of mice with GABA-producing bacteria changed cerebral GABA activity alongside a vagus nerve-dependent reduction in depressive- and anxiety-like behaviours (Strandwitz, 2018).

Gut bacteria also influence the metabolism of tryptophan, an amino acid which cannot be synthesised by the host but must be acquired through diet (Kałużna-Czaplińska et al., 2019). Tryptophan metabolism is the host's only source of the neurotransmitter serotonin, conferring high importance on gut bacterial influence over this metabolism. 95% of the body's serotonin is synthesized in the gut and can pass into the circulation but unlike tryptophan, cannot pass through the BBB. SCFAs, produced by gut bacteria, have been found to influence enterochromaffin transcription of tryptophan hydroxylase 1, the rate-limiting enzyme in the production of serotonin, influencing its rate of production in the gut (Reigstad et al., 2015). In this way, gut microbes greatly influence the levels of available serotonin and tryptophan in the gut and circulation, governing how much tryptophan is able to reach the brain where it may be synthesized to serotonin for CNS neurotransmission. Gut-derived serotonin can modulate gut motility and gut barrier integrity, while microbially-regulated levels of circulating tryptophan cross the BBB and can be converted into serotonin in the brain, affecting mood, cognition, and sleep (Roth et al., 2021; Strandwitz, 2018). Additionally, microbial fermentation of amino acids like tryptophan leads to the production of indole and its derivatives, which can affect mood and cognition (Y. Zhou et al., 2023).

3.1.3 Bile Acids and Derivatives

Bile acids are made in the liver through the clearance of cholesterol and stored in the gall bladder conjugated with glycine or taurine as bile salts. They are secreted into the duodenum following food consumption where they are critical for the digestion and absorption of dietary fats. While most are reabsorbed before reaching the terminal ileum, the bile acids left are metabolised by gut bacteria into secondary bile acids (Collins et al., 2023; Ramírez-Pérez et al., 2017). Microbial transformation of primary bile acids into secondary bile acids results in compounds that interact with various receptors in the gut and brain (Ferrell & Chiang, 2021). These bile acids activate the nuclear farnesoid X receptor (FXR) and other receptors such as G protein-coupled bile acid receptor 1 (GPBAR1), influencing satiety, neuroinflammation, gut motility, energy metabolism, and immune responses. Additionally, bile acids can affect brain function indirectly through systemic metabolic effects (Wahlström et al., 2016).

3.1.4 Indole and Indole Derivatives

Indole is produced from bacterial degradation of the dietary amino acid tryptophan. Indole and derivatives, such as indole-3-propionic acid (IPA), have significant neuroactive properties. IPA is a potent antioxidant that can protect neurons from oxidative stress (C.-S. Kim et al., 2023). Indole derivatives influence gut barrier function, immune modulation, and even central nervous system health by interacting with the aryl hydrocarbon receptor and other signalling pathways (Roager & Licht, 2018). The importance of indole derivatives has been noted in clinical studies. For instance the ratio of indole derivatives such as indole-3-lactic acid (ILA) and IPA ratios with another derivative, indoxyl sulphate, are negatively correlated with symptom severity in clinical depression (Brydges et al., 2021) and can be used to regulate proinflammatory signals and improve prognosis in patients with AD (Platten et al., 2019).

3.1.5 Other Metabolites

Other important microbial metabolites include vitamins (e.g., B vitamins and vitamin K), which are crucial for normal cellular functions and can have indirect effects on brain health (McCann et al., 2019). Microbial metabolites like polyamines (e.g., spermidine) are involved in cellular growth, differentiation, and apoptosis, and have been linked to longevity and neuroprotection (Makletsova et al., 2022). Some microbial metabolites have obvious deleterious effects such as trimethylamine N-oxide (TMAO), which can cross the BBB where it promotes hippocampal neuroinflammation through cytokine release and is closely related to neurological disease development (Quan et al., 2023; L. Xie et al., 2024).

In summary, the diverse array of metabolites produced by the gut microbiota illustrates the complexity and importance of the microbiota-gut-brain axis. These metabolites influence brain function and behaviour through various direct and indirect mechanisms, highlighting the intricate and essential role of gut microbiota in maintaining brain health as well as general health.

3.2 Production of SCFAs by the Gut Microbiota

SCFA production begins with the intake of dietary fibres which are resistant to digestion in the upper gastrointestinal tract. These fibres undergo fermentation by resident gut microbiota in the colon with SCFA concentrations peaking in the caecum for acetate and butyrate and the ascending colon for propionate (Cummings et al., 1987). However, SCFA quantity and type of production depends on the bacterial species present, and their metabolic pathways. Concentrations of SCFAs also vary considerably along the GI tract with concentrations peaking in the large intestine (Song et al., 2022).

Acetate is the most abundant SCFA in the colon, typically produced by a broad range of bacterial species, including *Bacteroidetes*, *Prevotella* spp., *Bifidobacterium* spp., and *Akkermansia muciniphila* (Rey et al., 2010). These bacteria use certain types of dietary fibre, including inulin, in two main pathways, acetogenesis (which uses H^2 and CO^2) and carbon fixation (which uses solely

CO^2) (Fusco et al., 2023). Acetate serves as a precursor for the synthesis of other SCFAs and for utilisation as a carbon or energy source by other bacteria, such as those that produce butyrate (den Besten et al., 2013). Propionate is mainly produced by Bacteroides, Veillonella, and specific Firmicutes (Louis & Flint, 2017). It is formed through several metabolic pathways, including the acrylate pathway (conversion of lactate to propionate) and the succinate pathway (conversion of succinate to propionate) (Louis et al., 2014). Lastly, butyrate's main producers include *Faecalibacterium prausnitzii*, *Eubacterium rectale*, and *Roseburia* spps. It is produced via the acetyl-CoA pathway, which involves the condensation of two acetyl-CoA molecules to form butyrate (Louis & Flint, 2009).

Concentrations of gut SCFAs may vary significantly based on factors such as diet and microbiota composition (den Besten et al., 2013). Concentrations vary significantly throughout the body, being most concentrated in the stool and far less so in the circulatory system and brain. Blood concentrations of SCFAs have been found to vary significantly depending on the origin of the blood sample in a study featuring six sudden death autopsies. Portal vein concentrations of butyrate being measured as approximately 29µmol/L, while samples from the peripheral blood stream were lower at 4µmol/L (van Deuren et al., 2022). Typical SCFA concentrations in the body have been reviewed elsewhere (O'Riordan et al., 2022). The three most abundant SCFAs, acetate, propionate, and butyrate, comprise approximately 95% of total SCFAs in the body, and exist in an approximate ratio of 3:1:1 respectively (Canfora et al., 2015). These concentrations can fluctuate with changes in dietary fibre intake and other dietary components including protein. For instance, a diet high in resistant starch and fibre increases the production of butyrate, which has been linked to various health benefits (Akagawa et al., 2021; De Vadder et al., 2014). One study attempting to increase blood plasma SCFA content was able to raise plasma butyrate from a median 2.02 µmol/L to 2.85 µmol/L following a 21 day diet intervention (Gill et al., 2022). Western diets are typically lower in dietary fibre, resulting in lower overall SCFA production, particularly butyrate. This is associated with a higher prevalence of metabolic and inflammatory diseases

(David et al., 2014). Meanwhile, populations consuming traditional high-fibre diets, such as those rich in fruits, vegetables, and whole grains, show higher SCFA production. The ability of dietary fibre to change blood and faecal SCFA concentrations is dependent on fibre type and quantity (Ilyés et al., 2022). As a consequence, high-fibre diet populations tend to have lower rates of inflammatory and metabolic disorders (J.-P. Chen et al., 2017; De Filippo et al., 2010). In summary, SCFA production by gut bacteria may play a crucial role in maintaining health and is highly influenced by dietary fibre intake.

3.3 SCFAs as Modulators of Brain Function

Acetate, propionate and butyrate, are known to influence brain function and host behaviour through modifying neuroinflammation, regulating hormones, interacting with vagal afferents, acting as a HDAC inhibitor, and activating free fatty acid receptors (Dalile et al., 2019). The various effects of butyrate on the nervous system has been well reviewed by Stilling and colleagues (Stilling et al., 2016).

3.3.1 Neuroinflammation Modulation

Neuroinflammation is a critical process in CNS diseases such as Parkinson's Disease and AD, as well as in cognitive impairment such as that experienced during recovery from surgical procedures and that seen in sickle cell disease (DeVeaux et al., 2024). The regulation of the brain's immune system and thus neuroinflammation is therefore of great interest. SCFA's may play an important role in this process. Butyrate is a strong immune modulator and anti-inflammatory agent. Upon reaching the brain butyrate inhibits neuroinflammatory markers such as Tumour Necrosis Factor alpha (TNF-α), interleukin 1β and interleukin 6, as seen in animal models (Cavaliere et al., 2022). SCFAs boast beneficial effects on microglia, the resident immune cells of the brain. Mice fed a diet that increased levels of SCFAs in the gut exhibited reduced microglial TNF-α secretion, an effect reproduced *in vitro* with butyrate and/or acetate exposure (Caetano-Silva et al., 2023). This study also saw an overall reduction of microglial activation through targeting the nuclear factor-κB

pathway. These effects likely occur through epigenetic mechanisms inside microglia following diffusion into these cells (Caetano-Silva et al., 2023). This effect of SCFAs on microglia is of great importance as it is these cells that sculpt our neuronal networks, eliminating unnecessary synaptic connections. The crucial nature of microbial influence on these cells has been demonstrated in studies using GF mice, a model mentioned earlier in this introduction. These are mice that are raised in a sterile environment and develop in the absence of any microbiota. Young GF mice display microglia that are stunted. However, this phenotype can be completely recovered with the supplementation of a cocktail of acetate, propionate, and butyrate (Erny, Hrabě de Angelis, et al., 2015). Interestingly activation of microglial FFAR2 may be at the root of these effects as mice deficient in FFAR2 display similar microglial phenotypes as those seen in GF mice (Gautier et al., 2012). These neuroprotective properties give SCFA-focused therapies great potential for the treatment of neurodegenerative diseases. Indeed, dietary butyrate supplementation has been demonstrated in animal models of multiple sclerosis and AD to ameliorate these conditions through regulation of inflammatory mediators and neurogenesis (Qian et al., 2022; Saadh et al., 2024).

3.3.2 HDAC inhibition

In several areas here we have seen that SCFAs exert effects on human health through HDAC inhibition. Of the three main SCFAs, butyrate is the most potent in this regard (Waldecker et al., 2008). HDAC inhibition increases the level of acetylation in chromatin, increasing the accessibility of the genetic material of the cell to transcription processes. As the cell will be actively transcribing genes relevant to the behaviour taking place this enhances the level of protein transcription relevant to the behaviour in question. This effect has been demonstrated previously to affect learning and memory. For instance butyrate was found to enhance memory in rats with weak memory as a result of fear conditioning programme, allowing for the reinstatement of impaired contextual memory (Vinarskaya et al., 2021). Similar effects of butyrate on fear memory and LTP have been seen previously and reinforced with electrophysiological

demonstrations of *ex vivo* enhancements of long-term potentiation (LTP), a synaptic plasticity process in which synaptic connections between neurons become stronger with frequent activation (Levenson et al., 2004). Further, in the context of models of neurodegeneration, it has been seen in AD mouse models that butyrate's HDAC inhibition partly rescues neurodegeneration and improves memory performance (T. Cao et al., 2018).

3.3.3 Immune Modulation for the Benefit of the Brain

SCFAs serve to maintain the integrity of the blood-brain barrier, an essential process in brain health. This effect was hinted at in research into antibiotic-treated and GF mice which display a BBB with greater permeability compared to controls along with dysregulated inter-endothelial cell tight junctions (Braniste et al., 2014; Fröhlich et al., 2016). Propionate was shown to boost the integrity of the BBB and thus reduce the unwanted infiltration of peripheral immune cells into the CNS, protecting against inflammatory disease (Braniste et al., 2014). Finally, SCFAs can modulate immune responses by binding to G-protein-coupled receptors (GPCRs) like GPR41 and GPR43, reducing systemic inflammation (Silva et al., 2020). Lower inflammation levels have been associated with improved cognitive functions and a reduced risk for the development of neurodegenerative diseases (Ozawa et al., 2017).

4 Rodent Models in Gut-Brain-Axis Research

4.1 Germ-Free, Antibiotics, FMT, Prebiotics, Probiotics, Psychobiotics

Mouse models have been essential for studying the complex interactions of the gut-brain axis, as they can be housed in controlled environments and share mammalian status, phylogenetic proximity, and physiological similarities with humans. The models generally used include conventional mice, germ-free mice, specific-pathogen free mice, antibiotic-exposed mice, and mice "humanised"

through gut colonisation with human microbiota following a microbiota depletion.

Germ-free mice have provided some of the most influential findings in gut-brain axis research to date (Kundu et al., 2019a; Luczynski, McVey Neufeld, et al., 2016a; Spichak et al., 2018a) (Figure 1.6). As previously mentioned, these are mice raised without any exposure to microorganisms, providing a valuable insight into how the microbiome affects mammal development. However, it also poses a limitation in the quality of evidence it can provide for microbiome effects in adult animals when studying non-developmental conditions due to the massive influence of the microbiome in early neurodevelopment. This upbringing induces enduring changes offering a highly informative model to early life questions into conditions such as ASD and obsessive-compulsive disorders (Spichak et al., 2018a). Notable changes seen in GF animals include changes to anxiety-like behaviour, learning and memory, BBB integrity, stress hormone signalling, and exercise capacity (Braniste et al., 2014; Hoban et al., 2018a; H. J. Kim et al., 2023; Luczynski, McVey Neufeld, et al., 2016a; Neufeld et al., 2011a).

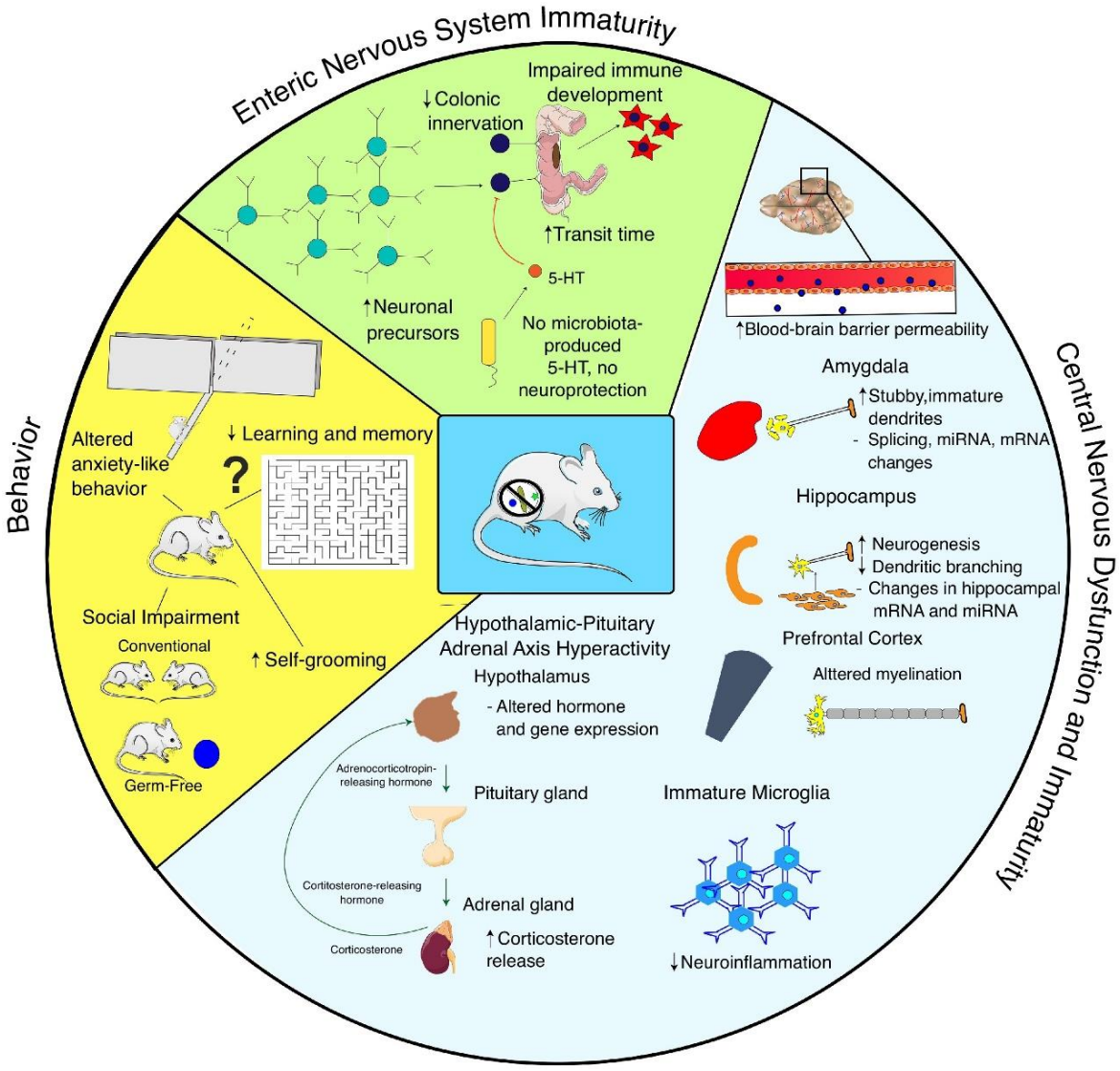


Figure 1.6 Phenotype of the GF rodent. Summary of the most common findings across species and strain of GF rodent. GF rodents have learning and memory, social, anxiety-like and grooming behaviours. Gut and peripheral immunity is severely compromised. The blood-brain barrier possesses increased permeability while the microglia have an immature phenotype. GF rodents possess many transcriptional changes in the amygdala and hippocampus contributing to an immature neuronal morphology, prefrontal cortex hypermyelination, and a hyperactive hypothalamic-pituitary adrenal axis. Adapted from (Spichak et al., 2018a)

Antibiotic-treated mice provide a window into the function of the gut-brain axis where germ-free animals cannot, namely a view into microbiota disturbance and subsequent recolonisation at a point in an animal's life, as dictated by the research question(s). Antibiotic treatment leads to changes in anxiety-like behaviour and cognitive functions as well as sociability (Hayer et al., 2023). Early-life antibiotic administration has led to altered myelin-related gene expression

and microglial morphology in adolescent mice, accompanied by anxiety-like and compulsive behaviours (C. M. K. Lynch et al., 2023). Similar antibiotic-induced microglial changes have been noted before. Erny and colleagues note that antibiotic-treated mice were subject to impaired microglial immune responses when challenged with LPS and lymphocytic choriomeningitis virus infection (Erny, Hrabě de Angelis, et al., 2015). These changes could be partially restored by microbiota recolonisation and SCFA supplementation. Notably, antibiotic treatment from early adolescence prevented the development of depression- and anxiety-like behaviours, as well as HPA axis dysregulation, and the oxidative stress that is seen in AD-induced male mice (Mosaferi et al., 2021). Antibiotic treatment has also been seen to promote Ly6C^{hi} monocytes towards a pro-inflammatory state which was coupled with impaired hippocampal synaptic transmission (Çalışkan et al., 2022), an impact of great relevance for this thesis which focuses on hippocampal synaptic plasticity.

Faecal microbiota transplantation (FMT) is the administration of a solution of donor faecal matter into a recipient's intestinal tract to enact a direct change on the recipient's gut microbial composition, usually with the aim of conferring a health benefit (Gupta et al., 2016). In animal models, it has been used extensively to investigate the role of the microbiome in the possible amelioration or exacerbation of a variety of disorders and diseases. FMT has been used to establish the connection between the microbiota-gut-brain axis and AD for example. In male mice, a microbiome perturbed by antibiotics was associated with a reduction in Aβ pathology, one of the hallmarks of AD (Minter et al., 2016). FMT has been found to restore the gut microbiome along with a partial restoration of Aβ pathology (Dodiya et al., 2019). GF 3xTg mice, a genetic model containing three mutations associated with familial AD, upon receiving FMT from AD patients, underwent increased microglial activation and exacerbation of their AD pathologies (C. Chen et al., 2022). Improvements to AD symptomatology through FMT has also been achieved. For instance FMT from wild type mice into an APPswe/PS1dE9 transgenic mouse model improved cognitive function, synaptic plasticity, and decreased Amyloid-beta brain deposition (J. Sun et al., 2019).

Conversely certain disease phenotypes can be transferred via FMT in mouse models, displaying the possible causal role of the microbiome in the condition's symptomatology. FMT has been used to investigate the role of the microbiome in symptoms associated with sleep deprivation, which has been found to induce changes to the microbiome as well as intestinal barrier disruption, which lead to cognitive decline and microglial activation (N. Li et al., 2023; Z. Wang et al., 2021; N. Zhao et al., 2023). FMT from chronically sleep-deprived mice to control mice has been found to produce similar pathological changes observed in chronic sleep deprivation (N. Zhao et al., 2023) such as an impaired BBB, and an increased number of IBA-1 positive microglia in the hippocampus, a marker of microglial activation.

Prebiotics and probiotics are classes of further intervention aimed at altering the gut microbiome. Prebiotics are ingested substrates, mainly types of non-digestible fibre, that are selectively utilised by the host microorganisms to confer a health benefit (Gibson et al., 2017; Mukherjee et al., 2018). Some of the main prebiotics include glucans and fructans such as galacto-oligosaccharides and fructo-oligosaccharides respectively (Sherwin et al., 2016). Prebiotic supplementation of fructo-oligosaccharides in rodents has produced a myriad of effects; for example, increased expression of brain-derived neurotrophic factor (BDNF), a prevalent central nervous system growth factor, was reported in the dentate gyrus of rats gavaged with prebiotics (Savignac et al., 2013). BDNF is a potent modulator of synaptic plasticity, particularly in the hippocampus during neurogenesis (Cowansage et al., 2010). Increases in hippocampal BDNF have been noted in other probiotic studies which supplemented *Bifidobacterium longum* (Bercik et al., 2011). Prebiotic administration in the context of disease models has also demonstrated the capacity of prebiotics to improve behaviours such as learning and memory, with fructo-oligosaccharide administration ameliorating spatial learning and memory dysfunction brought about in an $A\beta_{1\text{-}42}$ rat model of AD (Chen et al., 2017).

Probiotics are species of live bacteria that confer health benefits on the host when ingested in adequate amounts through interaction between the

commensal gut bacteria and intestinal epithelium (Butel, 2014). Preclinical studies have demonstrated the efficacy of probiotics in a wide range of areas with various strains improving endocrine function, immunity, metabolism, and even slowing ageing (Aidy et al., 2015; Patterson et al., 2016). When administered as a probiotic *Limosilactobacillus reuteri* has been found to rescue social deficits in various mouse models of ASD, an effect which was found to be dependent on vagal nerve signalling rescuing social interaction-induced synaptic plasticity in the ventral tegmental area of these mice (Sgritta et al., 2019). Probiotic influence over synaptic plasticity was also seen in a study in a chemically induced mouse model of PD. In this study, synapsin I, a synaptic plasticity marker was found to be reduced in the model, an effect which could be recovered through oral administration of the probiotic *Clostridium butyricum* (J. Sun et al., 2021).

Finally, psychobiotics are a subclass of probiotics which confer mental health benefits on the host through interaction between the commensal gut bacteria (Del Toro-Barbosa et al., 2020). Administration of these bacteria in mice can change emotional behaviours. *Limosilactobacillus plantarum* administration reduced depression-like behaviours in mice that were subject to early-life stress, and reduced anxiety-like behaviours in normal adult mice (Y.-W. Liu et al., 2016). *Bifidobacterium breve* also helped to reduce depression- and anxiety-like behaviours (Tian et al., 2020). These changes were accompanied by reduced levels of IL-6 and circulating TNF- α. A later clinical study served to confirm the possible psychiatric benefit of this psychobiotic (Tian et al., 2022). However, some psychobiotic studies have experienced difficulties during translation from preclinical to clinical studies. For example *Lacticaseibacillus rhamnosus,* a bacterium which was found to reduce stress-related behaviours in an anxious mouse strain failed to produce an effect on mood, stress, anxiety, or sleep quality in healthy male humans (Kelly et al., 2017). This highlights the potentially fraught path in between microbiome discoveries in animal models and their eventual translation into the human population.

4.2 Relevance of Mouse Models to Studying the Gut-Brain Axis

Naturally there is no perfect animal model to study the gut-brain axis, however it is important to consider suitability and limitations of the animal models we do choose. A variety of measures are used to measure certain qualities of the gut microbiome. For broad measures of diversity, alpha and beta diversity are commonly used. Alpha diversity describes the observed number of taxa or the relative abundance of those taxa (Walters & Martiny, 2020). Beta diversity is the variability of community composition among different samples in (Walters & Martiny, 2020)

4.2.1 Microbiome Differences, Humans and Animal Models.

Chao1, a measure of alpha diversity in the gut microbiome has been reported to be higher in humans vs. laboratory mice. In term of β-diversity, one often used measure is unique fraction or UniFrac (Lozupone et al., 2011) which measures the phylogenetic distance between sets of taxa. The use of these measures shows that while non-human primates are the most-humanlike animal model with regard to microbiome makeup, when considering rodent models only, mice are more humanlike than rats (Nagpal et al., 2018). Murine SCFA concentrations are somewhat comparable to that of humans, displaying similar ratios to humans of faecal concentrations of propionate and butyrate (1:1 for each in mice and 4:3 respectively in humans) (Nagpal et al., 2018). This study revealed however that the relative proportion of acetate is double in mice compared to what it is in humans. When Nagpal and colleagues looked at exact faecal concentrations they found that while butyrate concentrations were similar between mice and humans, the concentrations of propionate in human faeces tended to be higher (Nagpal et al., 2018).

The potency of free fatty acid receptors (FFAR) to which SCFAs bind has been well reviewed elsewhere (Carretta et al., 2021). Some differences exist between mice and humans when quantifying SCFA potency and selectivity at FFARs. In humans, acetate was more potent at FFAR2 than FFAR3. Propionate is reported to be equipotent and non-selective at FFAR2 and FFAR3. Butyrate is

approximately equipotent and non-selective at both mouse and human FFAR2 and FFAR3. In mice, the potency of the two shorter chain SCFAs differs. Acetate was equipotent at mouse Ffar2 and Ffar3. Propionate was found to be significantly more potent at mouse Ffar3 over Ffar2 (Hudson et al., 2012). Analysis of this data also concludes that shorter-chain SCFAs are more potent at mouse Ffar3 when compared with human FFAR3. However, all SCFAs tested were more potent at human FFAR2 than mouse Ffar2. Exact comparison of FFAR2 and FFAR3 expression in the human and mouse brain has so far not been performed however the expression of FFAR3 transcripts has been found in in both (Zamarbide et al., 2022).

5 Neurodevelopment and the Gut Microbiome

5.1 Influence of Gut Microbiota on Brain Development: Critical Windows

Growing evidence suggests that the gut microbiota are highly important for brain development (Borre et al., 2014; Ratsika et al., 2021). This has been seen in cases where early-life gut microbiota composition changes have been associated with neurodevelopmental and psychiatric disorders (Hashimoto, 2023). Gut microbiome development is even associated with fine motor skill development (Acuña et al., 2021). Neurodevelopmental psychiatric disorders arising from nonstandard brain development include attention deficit hyperactivity disorders (ADHD), schizophrenia, and autism spectrum disorders (ASD). Clinical and preclinical studies have brought increased focus to the interaction between gut microbes and brain development and their association with neurodevelopmental psychiatric disorders, as reviewed by (Dash et al., 2022). Factors impacting the early life microbiome that are associated with neurodevelopmental psychiatric disorders include maternal immune activation, antibiotic use, disease and infections, as well as poor diet. The microbiome therefore is posited to be of great importance for regulating neurodevelopmental trajectories. This phenomenon appears to be largely due to the alteration of

immune system regulation brought about by early life microbiome insults (Dash et al., 2022).

Development of the brain begins *in utero* but continues post-birth, with the first 2-3 years of life being crucial for neurodevelopment. The microbiome also sees a high degree of development and fluctuation in early life, especially within the first twelve months (Bäckhed et al., 2015; Gensollen et al., 2016). Indeed, the co-development of these two features doesn't just occur at similar times but is also interdependent during this early-life period (A. Jena et al., 2020). This interdependence has led to the gut microbiome being implicated in a range of neurodevelopmental disorders such as ASD, ADHD, and schizophrenia (Cryan et al., 2020). Studies conducted with GF and antibiotic-treated mice have demonstrated the key role played by the microbiome in neurodevelopmental processes including the development of the BBB (Braniste et al., 2014), neuroinflammation (Erny, Dokalis, et al., 2021), and neuroplasticity (Luck et al., 2020). The deficits seen in these animal models can be restored with gut microbiota interventions; however, attempts at restoration is often ineffective when the recolonisation occurs in later life. Early life recolonisation however, is of much greater efficacy for restoring deficits brought about by the germ-free state for example (Buffington et al., 2016; Heijtz et al., 2011; Olszak et al., 2012; Sudo et al., 2004). This suggests the existence of early life critical windows with particular focus on weaning. These critical windows of neurodevelopment correspond to points during which the microbiome is exceptionally plastic and also the onset times of multiple neurodevelopmental disorders (Borre et al., 2014; Cowan et al., 2020). During these periods the gut bacteria can exert great influence over neurodevelopmental processes (Q. Wang et al., 2023).

Several putative critical windows of neurodevelopment are hypothesised to exist. In mice, these are divided into the post-natal, pre-weaning, and post-weaning periods. Insults to the gut microbiome during these windows has been found to have unique effects on neurophysiology and circulating immune cells. These effects carry on into adulthood and are seen as behavioural phenotypes (C. M. K. Lynch et al., 2023). In humans, two studies of childhood exposure to

antibiotics found that exposure in the first year of life, but not in later periods, had an enduring negative impact on cognitive development (Slykerman et al., 2017, 2019). This antibiotic use was seen to impact overall IQ and reading ability during primary school years. To another example of the importance of the microbiome on neurodevelopment one can look at birth mode. C-section which has been seen to change gut microbiome composition and trajectory, also results in significantly lower white matter development in the CNS combined with lower functional connectivity in the brain default mode network (Deoni et al., 2019). The gut microbiota influences various features throughout neurodevelopment including myelination, neuroplasticity and axonogenesis (Hoban, Stilling, et al., 2016a; Luck et al., 2020; Vuong et al., 2020).

5.2 Neurodevelopmental Processes: Influence of the Microbiome

5.2.1 The Blood Brain Barrier

The BBB controls the exchange of cells and molecules between the CNS and circulating blood, maintaining homeostasis and preventing the passage of pathogens, toxins, and cells into the brain parenchyma (Daneman & Prat, 2015). The structure establishes early *in utero*, forming a restrictive barrier between the brain and systemic circulation, while facilitating the transfer of nutrients and molecule exchange that's needed for healthy brain function and maintenance (Braniste et al., 2014; Obermeier et al., 2013). The presence of the gut microbiota along with SCFAs and other microbial metabolites is essential for the proper development and maintenance of this structure (Braniste et al., 2014; Michel & Prat, 2016; Obermeier et al., 2013). The expression of junctional proteins such as claudin-5 and occludin in the brain's endothelial layer has been seen to be decreased in GF mice, providing maladaptive increased BBB permeability (Braniste et al., 2014). As was mentioned previously, the supplementation of butyrate has been shown to reduce this permeability in GF mice (Braniste et al., 2014). *In vitro* BBB models have shown butyrate and propionate to be capable of altering the actin cytostructure of the BBB as well as increasing tight junction

proteins and improving overall BBB integrity (Knox et al., 2022). Other microbial metabolites such as TMAO can also enhance BBB integrity, firmly placing the microbiota as a regulator of the BBB (Hoyles et al., 2021). Neurodevelopmental disorders such as ASD and schizophrenia have been found in preclinical and clinical studies to be associated with BBB disruption (Fiorentino et al., 2016; Goldwaser et al., 2022; Gozal et al., 2021; Q. Zhao et al., 2022) with accompanying microbiota changes (Fiorentino et al., 2016; Pellegrini et al., 2023; Xu et al., 2020). Mouse models of schizophrenia and ASD show BBB disruption (Crockett et al., 2021; Q. Zhao et al., 2022), suggesting that these neurodevelopmental disorders share barrier disruption as part of their complex aetiology.

5.2.2 Neurogenesis

Neurogenesis is the creation of new neurons through neural stem/progenitor cell differentiation (Gage, 2019). This process is important for learning and memory, stress responsivity, and cognition, and the gut microbiota play a large role (Sarubbo et al., 2022). For example, a range of gut microbial metabolites cross through the placenta into the foetal compartment, inducing and regulating the prenatal development process (Pessa-Morikawa et al., 2022). Furthermore, the expression of FOXG1, an essential transcription factor in neuroproliferation and regulation of neurogenesis and wider development can be triggered through the activation of Toll-like receptor 2, which can occur when peptidoglycan, a component of the bacterial cell wall reaches the foetal brain after crossing through the placenta (Humann et al., 2016; Kaul et al., 2012). Evidence for the microbiota's effect on neurogenesis is bolstered by various studies. The usage of antibiotics for instance which has a clear negative impact on the gut microbiota is associated with decreased neurogenesis (Möhle et al., 2016). Later in life the microbiome is also reported to influence neurogenesis. GF mice exhibit increased hippocampal neurogenesis, particularly in the dorsal hippocampus. This effect is not prevented by colonisation post weaning, demonstrating the crucial nature of the microbiota in shaping this process from early life (Ogbonnaya et al., 2015)

5.2.3 Myelination

Myelin serves as insulation for axons in the nervous system, allowing for the fast propagation of action potentials. Humans are born with primarily unmyelinated axons. As axons mature they undergo rapid myelination which sees a variable rate of myelin content and myelination over time until early adulthood (Benes, 1989; Lebel et al., 2012; Williamson & Lyons, 2018). Dysregulation of immune responses and neuronal differentiation due to altered microbiome signalling can impact proper myelination during early life. Indeed the developmental trajectory of the microbiota coincides with both the onset of neurodevelopmental disorders and CNS myelination (C. M. Lynch et al., 2021). GF mice have been found to go through a marked upregulation of myelination related genes, which is accompanied by hypermyelinated axons in the prefrontal cortex (Hoban, Stilling, et al., 2016a). Diving further into the effects of the microbiota on myelination in early life it has been found that daily treatment of neonatal mice with antibiotics until weaning affected myelin-related gene expression in adulthood, particularly in the prefrontal cortex (Keogh et al., 2021a). Notably this alteration could be restored with administration of the microbial metabolite butyrate post antibiotic treatment. The timing of microbiota influence over myelination in early life has also been explored in mice. When antibiotics were given for short periods at various points in early life it was found that the time periods directly post-birth and right before weaning showed the most vulnerability to enduring myelination changes (C. M. K. Lynch et al., 2023). Microbiota influence over myelination has also been seen in later life. Myelination changes and accompanying social avoidance behaviours induced by oral gavage in nonobese diabetic mice can be prevented by antibiotic administration (Gacias et al., 2016). This study also found that FMT from gavaged nonobese diabetic mice to controls was also sufficient to reproduce the observed changes to myelination and social behaviour. Interestingly for this thesis, the scale of myelination has also been linked to neuroplasticity (Almeida & Lyons, 2017).

5.2.4 HPA Axis

The HPA axis is the bodies' major physiological stress system. Like the gut microbiota it undergoes rapid and profound developmental changes during the first few years of life (de Weerth, 2017). Dysfunctions in the HPA axis have also been linked to the neurodevelopmental disorders ASD and schizophrenia (A. J. Bradley & Dinan, 2010; Taylor & Corbett, 2014). The neuroendocrine interactions seen in the response to stress is developed in early life and is subject to the influence of the gut microbiota (Hantsoo & Zemel, 2021). In GF mice stress responsivity is upregulated (Luczynski, McVey Neufeld, et al., 2016a). The improper regulation of stress and anxiety behaviour that is observed in these mice has also been linked to a dopamine deficiency (Zarrindast & Khakpai, 2015). When the gut microbiota's influence on HPA axis development in early-life was investigated through early-life antibiotic administration until weaning, it was found that serum corticosterone levels in antibiotic treated mice were elevated at P21 and P42 (Farzi et al., 2018). Further regulation of this axis by the gut microbiota has been observed in probiotic administration studies where *Lactobacillus helveticus* and *Bifidobacterium longum* decreased anxiety significantly following social defeat stress (De Oliveira et al., 2023; Partrick et al., 2021).

5.2.5 Microglia

Microglia are the resident immune cells of the brain, providing immune defence and maintenance of the CNS (Colonna & Butovsky, 2017). In GF mice these cells are severely altered, exhibiting aberrant morphology, overlapping territories and longer processes (Erny, de Angelis, et al., 2015; Erny, Dokalis, et al., 2021). These microglia display a limited response to viral infections and microbe-associated molecular patterns (Bettag et al., 2023). Interestingly, this abnormal state can be rescued by SCFA administration (Hanamsagar et al., 2017). Antibiotic studies have also made further discoveries on the influence of the microbiome on microglia in development. Early-life ABX administration alters microglial morphology in adolescent mice (C. M. K. Lynch et al., 2023).

6 Microbiome and Higher Brain Function: Cognition, Learning, and Memory

I have detailed the various ways in which the gut microbiome influences fundamental brain functions and body systems that directly and/or indirectly impact brain function. These interactions are significant primarily due to their potential to alter host behaviour. In this thesis, the focus now shifts to examining how these microbiome effects extend to cognitive processes, in particular learning and memory, which are critical subsets of cognitive functioning. Studies point to the microbiota as an avenue to improve cognition (Ni Lochlainn et al., 2024). Microbiome influence on cognition may occur through various direct and/or indirect routes such as the alterations to neurotransmitter function or the HPA axis (Y. Chen et al., 2021; Rusch et al., 2023). Indeed correlations exist between higher microbial diversity and executive function and enhanced cognitive flexibility (Tooley, 2020). GF mice have been demonstrated to have a deficit in non-spatial memory and working memory compared to specific pathogen free mice (Gareau et al., 2011a). This altered cognition was accompanied by reductions in BDNF and c-Fos, two proteins involved in hippocampal-dependent memory. Studies have also used metabolomics to expand knowledge surrounding the effect of germ-free status on brain function. The cerebral metabolome of GF mice has been found to have altered biosynthetic pathways for dopamine and serotonin compared to conventionalised controls (Matsumoto et al., 2013). This indicates the possibility of changed brain health, development, and behaviour due to microbiota-impacted neurotransmitter levels. This is of great interest for research into the ability of the microbiome to affect cognition as serotonin can change cognitive function independent of changes in overall mood (Schmitt et al., 2006). While there exists a gross effect of the microbiome on cognitive processing, it is of particular interest for this thesis on how short-chain fatty acids may affect cognitive processing.

There exists rousing evidence that points toward SCFAs as a good candidate for the treatment of cognitive impairment, or even perhaps as a supplement for boosting cognition. As previously described, neuroinflammation is seen as a function of cognitive impairment in obesity, a condition which also sees reduced concentrations of faecal butyrate and butyrate-producing bacteria compared to healthy controls. (Duncan et al., 2007; Elli et al., 2010). However butyrate supplementation in a mouse model of obesity prevented the deterioration of short-term memory commonly seen in the model (Ge et al., 2023). AD, a neurodegenerative disorder characterised by progressive cognitive decline has also been shown to benefit from microbiome manipulations, seeing a reduced rate of progression (Cryan & Dinan, 2012; J. M. Hill et al., 2014; Hoffman et al., 2019). In addition, SCFA levels have been noted to be reduced in AD mouse models (L. Zhang et al., 2017). Here, the beneficial functions of SCFAs are varied, most dramatically is their ability to disrupt the assembly of amyloid-β peptides (Aβ) into neurotoxic oligomers which are the cause of cognitive deficits in AD and one of the main hallmarks of the disease (Ho et al., 2018). intriguingly, the addition of butyrate to the APP/PS1 mouse model of AD improved memory function, and increased the expression of associative learning genes in the hippocampus through its capacity as a HDAC inhibitor. (Govindarajan et al., 2011).

6.1 Hippocampus and the Microbiome

As we lean into the topic of the gut microbiome and higher brain processes this thesis focuses its attention on learning and memory and the gut microbiome's ability to affect these processes. Learning and memory are largely dependent on a part of the brain called the hippocampus (Figure 1.7 (A)). The hippocampus is crucial for the formation and storage of semantic, declarative and episodic memories. Decades of research has provided substantial evidence of the cellular and molecular mechanisms of this process. The hippocampus is a relatively conserved structure across species with similar function,

unidirectional summative circuits that are relatively easy to assay in a rodent model (Insausti, 1993; Manns & Eichenbaum, 2006; Zemla & Basu, 2017). Throughout this introduction it has been noted that microbiome interventions can impact the hippocampus and learning and memory. These interventions include growing up GF and the alteration of gut microbial metabolites such as butyrate.

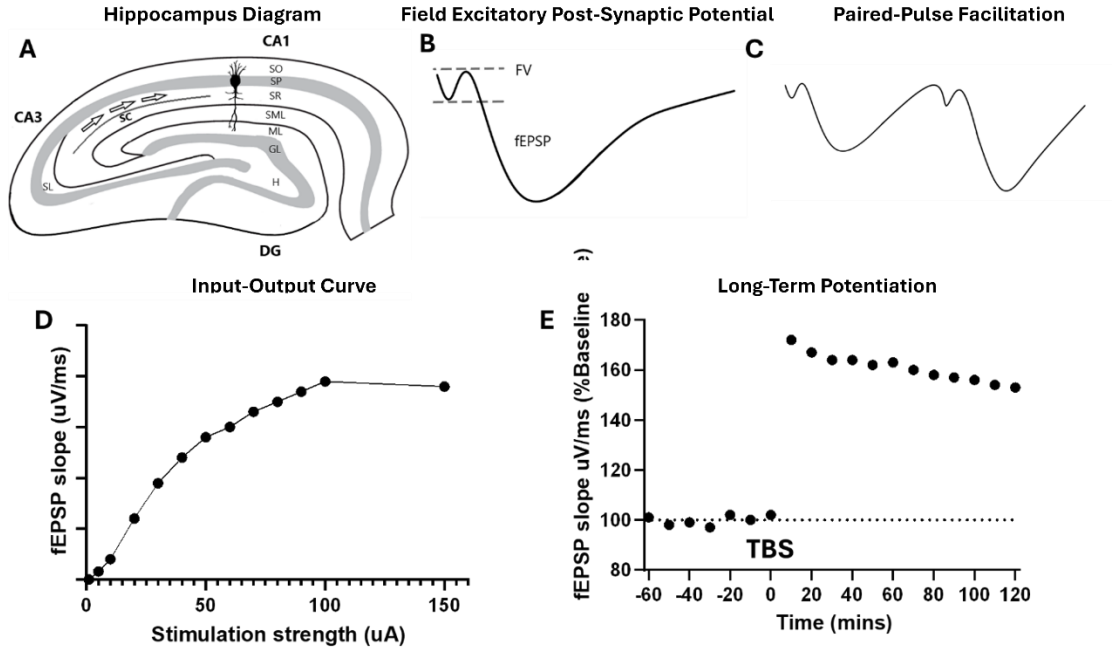


Figure 1.7 (A) Diagram of hippocampus. SO: Stratum Oriens, SP: Stratum Pyramidale, SR: Stratum Radiatum, SML: Stratum Lacunosum Moleculare, ML: molecular layer of the dentate gyrus, GL: Granule cell layer, H: Hilus of the dentate gyrus, SL: Stratum lucidum, DG: Dentate gyrus. (B) Stylised field excitatory post synaptic potential (fEPSP), featuring the presynaptic component of the elicited response, the fibre volley (FV), and the summated action potentials (the fEPSP). (C) Stylised paired-pulse facilitation response. When two fEPSPs are induced within a short amount of time the second response is potentiated. (D) A sample input-output curve, used to measure the basal synaptic efficacy of a slice, featuring increasing strengths of stimulation eliciting greater response size. (E) A sample long-term potentiation (LTP) dataset featuring a 60 minute baseline and a two hour recording post LTP induction through theta burst stimulation (TBS).

6.2 Synaptic Plasticity, Behind Learning and Memory

In 1957 a report on the now famous patient H.M. case was brought to light. This case referred to a man that had lost the ability to form new declarative memories after he went through the surgical removal of his hippocampus in the hopes of treating his intractable epilepsy (Squire, 2009). Since then, the hippocampus has

been at the forefront of research into learning and memory. Further, the wealth of information on learning and memory was improved greatly through the discovery of hippocampal place cells which are important for an animal to navigate its spatial position (O'Keefe & Dostrovsky, 1971) resulting in the Nobel Prize being awarded in 2014. These discoveries have firmly placed this brain region as the centre of memory formation and spatial navigation.

Decades of research has informed us that the brain's ability to adapt to the host's environment throughout our lifetime is the foundation upon which cognitive processes such as learning and memory are built. The most common of these changes are at the level of individual connections or synapses between neurons. The weakening or strengthening of these connections is known as synaptic plasticity (T. V. P. Bliss & Lømo, 1973; Magee & Grienberger, 2020). This is an activity-dependent process as it changes based on the level of activity between the neuronal populations, requiring presynaptic neurons firing on postsynaptic neurons in a specific fashion to produce the physical changes needed for the strengthening or weakening of the connection.

Synaptic plasticity includes short-term transient changes that last from several milliseconds to several minutes (B. Pan & Zucker, 2009; Powell, 2006), as well as longer lasting changes that have durations of hours, days, or even longer time periods (Abraham, 2003). Types of long-term synaptic plasticity include long-term potentiation (LTP) and long-term depression (LTD). The discovery of long-term potentiation started in 1964, when research by Lømo discovered brief trains of stimuli to neurons in the perforant pathway of the hippocampus of rabbits increased the efficiency of transmission of stimuli in the stimulated pathway (P. Andersen & Lomo, 1967; Lømo, 2003). Since this discovery thousands of papers have been published on this phenomenon as it provides an engaging cellular model of learning and memory. While there still exists debate over whether these processes are what actually underlie learning and memory it is clear at a cellular level that many forms of memory rely on the similar molecular mechanisms as LTP and LTD (T. V. P. Bliss & Cooke, 2011). The bulk of the debate falls not on the

notion that learning and memory need synaptic plasticity to occur but on whether these processes are sufficient for it to occur (Martin et al., 2000).

6.3 Synaptic Plasticity Subtypes

6.3.1 Long-Term Potentiation

LTP is the most widely studied form of synaptic plasticity and is considered a primary cellular mechanism that underlies learning and memory. It has now become a standard model for investigating the synaptic and cellular mechanisms of learning and memory. It involves the persistent strengthening of synapses based on recent patterns of activity. When a high-frequency train of stimuli is delivered to a synapse, the post-synaptic neuron subsequently produces an enhanced response to the stimuli, indicating greater efficiency in the transmission of a signal (Figure 1.7 (E)). These high-frequency trains of stimuli cause high influx of postsynaptic calcium, which is followed by alterations in the shape of dendritic spines and the formation of new synapses, along with functions which increase the probability of neurotransmitter release (Citri & Malenka, 2008a; G. Lynch et al., 1988).

The molecular basis of LTP involves several key steps; high-frequency stimulation induces the release of the neurotransmitter glutamate from the presynaptic neuron. This glutamate binds to the AMPA receptors on the postsynaptic neuron which leads to depolarization. If a sufficient threshold of depolarization is reached a magnesium block is removed from NMDA receptors on the postsynaptic neuron, allowing calcium ions to flow into the postsynaptic cell. This influx of calcium triggers signalling cascades including the activation of protein kinases such as CaMKII and PKC. The activation of these kinases facilitates the insertion of additional AMPA receptors into the membrane of this cell which thereby increase the sensitivity of the synapse to glutamate. The ensuing long-term changes in synaptic strength are coupled with structural remodelling including the growth of new dendritic spines to reinforce a particular pathway (Chonchaiya et al., 2009; Purves et al., 2001b, 2001a).

6.3.2 Long-Term Depression

LTD, in contrast, is a process by which synaptic strength is persistently weakened. If synapses simply continued to increase in strength as a result of LTP a maximum efficacy would be reached, making the encoding of new information arduous or even producing a possible ceiling effect. LTD is one answer to this problem, allowing for the selective weakening the synapse or circuit in question. However, the functions of LTD extend beyond this, indeed the process also appears to be involved in the encoding of new memory (Ashby et al., 2021; Cp et al., 2016; Sasaki-Hamada et al., 2021). There are multiple pathways for the induction of LTD, such as the activation of NMDAR or mGluR, ATP-induced LTD, or insulin-induced LTD (Beattie et al., 2000; Cavaccini et al., 2020; Oliet et al., 1997; Pougnet et al., 2014). Each of these forms however results from the a specific physiological stimulus, the most well-known of which being low-frequency stimulation (LFS), which is usually driven over an extended time period relative to high frequency stimulation (HFS) induction protocols (Gonzalez et al., 2014). This process mainly involves NMDA receptors (Dudek & Bear, 1992). These pathways all however lead to a rapid increase in the endocytosis of AMPA receptors. This weakening of synapses is equally as important as LTP for learning and memory and allows for the removal of old memories and the fine-tuning of neural circuits (Fox, 2014; Nabavi et al., 2014).

The molecular mechanisms of LTD include the following: in a similar fashion to LTP, LTD begins with the release of glutamate into the synapse where is binds to AMPA and NMDA receptors. However, the rate at which glutamate binds is slower in low-frequency stimulation resulting in a modest and prolonged increase in intracellular calcium levels as opposed to the brief and large influx seen in LTP. This sustained but low level of calcium activates protein phosphatases such as calcineurin and PP1 (Isaac, 2001). These phosphatases lead to the dephosphorylation and subsequent internalisation of AMPA receptors, reducing the sensitivity of the synapse to glutamate. Further long-term changes of LTD include the retraction or pruning of dendritic spines (Stein & Zito, 2019).

Dysregulation of LTP and LTD producing dysfunctional plasticity has been implicated in various neurological and psychiatric disorders such as depression, schizophrenia, and posttraumatic stress disorder (Appelbaum et al., 2023). For instance, aberrant LTP is associated with conditions like epilepsy (Yehezkel, 2008), while impaired or excessive LTD are linked to cognitive deficits observed in AD and other forms of dementia (Mango et al., 2019; Tamagnini et al., 2012). Despite LTD being an interesting phenomenon to study it is not covered in this thesis.

6.3.3 Short-Term Facilitation

Short-term facilitation is a phenomenon where the response to a second synaptic stimulus is enhanced due to its closeness in time to an initial stimulus (Figure 1.7 (C). These processes occur on a millisecond-to-minute timescale. Similar to long-term plasticity phenomena they modulate synaptic efficacy in an activity-dependent manner. Several factors account for short term depression such as vesicle depletion, inactivation of release sites, and AMPA receptor desensitisation (Fioravante & Regehr, 2011; J, 2022). Meanwhile short-term potentiation occurs through factors such as residual calcium in the presynaptic terminal and saturation of endogenous calcium buffers (Fioravante & Regehr, 2011). Strong support now exists for the hypothesis that short-term plasticity is involved in information processing, decision making, and working memory (P.-Y. Deng & Klyachko, 2011).

In short-term facilitation for example, the enhancement occurs due to residual calcium in the presynaptic terminal, the presence of which is brought about by the first stimulus. This allows an augmentation of neurotransmitter release during the second stimulus as the residual calcium from the first combines with that from the second to produce a stronger signal and increase the probability of neurotransmitter release from the presynaptic neuron (Regehr, 2012).

Synaptic plasticity in the hippocampus is a dynamic and complex process essential for the brain's ability to learn and form memories. Through mechanisms like LTP and LTD, the hippocampus adapts to new information and experiences,

reinforcing and fine-tuning neural connections. Understanding these processes not only provides insights into fundamental brain functions but also offers potential avenues for addressing neurological diseases and cognitive disorders.

6.4 Techniques for Measuring Synaptic Plasticity

Understanding synaptic plasticity is crucial for deciphering the mechanisms through which learning and memory occur and are affected by factors such as the gut microbiome. There exists a wide array of technologies that have been developed to examine this phenomenon, each offering their own advantages and disadvantages. Methods of examining electrophysiological measures in animals include a host of variations; for example, electrophysiology can be performed *in vivo*. Here, the animal is anaesthetised, head-fixed in position, or awake and freely moving during certain behavioural tests. Tests can also be performed *ex vivo,* in brain slices from animals or even cultures of neurons. One can measure from a single area of the brain or measure whole circuits or correlations between activity in different brain areas. Variations exist within these methods again, deciding whether one examines electrophysiological activity at individual ion channels or receptors in neurons, to whole cell recordings, to taking summated responses from whole populations of neurons at once, termed field recordings. These methods include patch clamp, voltage clamp, lipid bi-layer recording, and multielectrode array (MEA) recordings (Wickenden, 2014). These recordings setups are sensitive enough to capture processes such as LTP and LTD as well as types of short-term plasticity such as short-term facilitation. Single unit recordings also exist, measuring spike responses of single neurons using microelectrodes. The pipette is filled with an ionic solution to allow the conduction, forming an electrical terminal. Similarly, with patch-clamp electrophysiology a micropipette is used to record from a single cell or even part of a neural cell membrane. Multiple subtypes of this method exist, including inside-out, whole-cell, and perforated (Cuevas, 2014; Linley, 2013). This method can be used to characterise whole cells or even just specific receptors. The recording of excitatory or inhibitory post-synaptic currents or potentials can also

be carried out with this method, giving insights in the mechanisms of synaptic plasticity at the level of individual synapses (C. L. Hill & Stephens, 2021).

In vivo electrophysiology is a highly involved and invasive method of recording neuronal electrical activity in a living organism. It allows for the invaluable study of synaptic plasticity within the natural context of the intact, live, functioning brain. This allows researchers to observe neural circuits in real time and in physiological conditions as they respond to the behaving animal. This can provide great insight into the firing patterns of neurons during various behavioural tasks (Halladay, 2023).

E*x vivo* electrophysiology on the other hand involves the removal of brain tissue usually in the form of a brain slice. This allows the investigation of a synapse or circuit in isolation from the rest of the brain (Wickenden, 2014). One such *ex vivo* technique is Multielectrode Array (MEA) recordings which represent a powerful technique to record electrical activity simultaneously from multiple neurons or synapses across a neural network. This method is nonspecific and uses extracellular field recordings taking the input from all the cells in an area. This is particularly useful for studying network-level dynamics and the spatial distribution of synaptic plasticity. This wider view can be useful for observing the synaptic plasticity of whole neuron populations/ circuits. MEAs consist of arrays of electrodes embedded in a substrate, which can be placed in contact with recently excised neural tissue or cultured neurons. This provides a nonperturbative way to measure the activity of many neurons with high temporal resolution for extended periods. The spatial resolution is determined by the spacing of the electrodes which must strike a balance to allow for signal propagation and minimal noise levels among other factors. MEAs can be used to both stimulate and record from tissue, allowing precise control of synaptic inputs and observing the resulting network activity changes, making it ideal for the study of LTP and LTD. MEAs therefore pose the advantage of understanding the coordinated activity of neural circuits and propagation of plastic changes across networks (Manz et al., 2021).

MEAs are versatile, with applications in both *in vitro* and *in vivo* settings (Abu Shihada et al., 2024). *In vitro* MEAs are commonly used with cultured neurons or brain slices, facilitating detailed mechanistic studies under controlled conditions. The use of MEA field recordings in brain slices allows for the specific control of the slice environment including the addition of pharmacological agents or even microbial metabolites to study the direct effect of these agents on brain activity in an isolated circuit. In the case of the gut microbiome where inputs can come from a variety of sources such as the vagus nerve or peripheral immune signalling it can be difficult to gain understanding into a single route of communication without obfuscation. Therefore, the opportunity to study the isolated local effects of microbial agents on neuronal physiology is invaluable.

To conclude, different techniques for measuring synaptic plasticity such as *in vivo* electrophysiology or MEA field recordings offer the opportunity to provide critical insights into neuronal physiology in synaptic plasticity.

7 Synaptic Plasticity and the Microbiome

7.1 Linking Gut Microbiome to Hippocampal Synaptic Plasticity

The microbiota gut brain axis has been suggested to have substantial impact on cognition and learning and memory. There exists considerable evidence from preclinical studies demonstrating the impact of the microbiome on hippocampal synaptic plasticity and behavioural readouts of learning and memory. In animals, hippocampal plasticity may be measured indirectly through the quantity of plasticity related mRNA transcripts and proteins such as BDNF, or directly through electrophysiological study which provides an accessible route to link the gut microbiome to the synaptic plasticity that underlies learning and memory. In humans this route is not as clear. Despite progress in the measurement and inference of synaptic plasticity in humans, neuronal circuits are not readily accessible as in animal models (Nathan et al., 2011). However techniques such as electroencephalography and deep brain stimulation can be used (Krothapalli et al., 2024; Nathan et al., 2011). The human gut microbiota has however been

explored as a target in the hopes of improving or preserving cognitive performance. Correlations have previously been observed between greater cognitive flexibility and executive function and gut microbiota intervention studies have produced positive results in the improvement of cognition and brain function such as visuospatial memory and verbal learning (Tooley, 2020). For instance, the consumption of a *B. longum* psychobiotic in healthy human volunteers has been found to produce improvements in hippocampus-dependent visuospatial memory (A. P. Allen et al., 2016). *Lactobacillus plantarum* has also been found to enhance memory and cognition in stressed adults (Lew et al., 2019, p. 8). The gut microbiome may affect hippocampal synaptic plasticity through any of the usual channels which gut-brain communication usually takes place e.g. vagus nerve signalling, immune modulation or circulating metabolites (Kuijer & Steenbergen, 2023).

7.2 Gut Microbiome and Hippocampal Plasticity in Animal Studies – Indirect Measures.

SCFAs in the CNS are believed to exert effects on memory directly through BDNF enhancement and histone deacetylase modulation. Hippocampal BDNF is associated with neurogenesis and has shown to be critical to memory processes (Bathina & Das, 2015). BDNF regulates activity-dependent synaptic plasticity and psychiatric disorders (Sharma et al., 2019). Gut microbiota changes have been shown to alter BDNF levels. For example, alterations to the gut microbiota brought about by antibiotic-treatment in early life has been seen to decrease BDNF levels, specifically in the hippocampus (Bistoletti et al., 2019; Desbonnet et al., 2015). In a similar fashion, GF mice display reduced BDNF along with deficits in non-spatial and working memory compared to their conventional counterparts (Gareau et al., 2011b). In contrast, a probiotic such as *Bifidobacterium longum* can enhance hippocampal BDNF mRNA (Bercik et al., 2010). Similarly treatment of cholinergic memory impairment in mice by *Lactobacillus johnsonii* increased BDNF expression (H.-J. Lee et al., 2018) which potentiates excitatory synapses via pre-and post-synaptic

mechanisms(Minichiello, 2009; Waterhouse & Xu, 2009). Changes in SCFA levels are touted the possible mechanism behind some of these effects. GF mice receiving a faecal matter transplant from older conventional donors were found to have increased BDNF and neurogenesis (Kundu et al., 2019a). Metagenomic analysis in this study revealed the potential role of butyrate-producers in the mediation of this effect (Kundu et al., 2019a). In a similar study involving FMT from aged donor mice into young adult mice it was found that 87 learning-related proteins were downregulated in the recipient mice (D'Amato et al., 2020a). The case for butyrate producers increasing BDNF levels is made stronger by more studies such as that of (J. Liu et al., 2015a) which showed *Clostridium butyricum* increasing butyrate production, enhancing hippocampal BDNF and counteracting cognitive decline. Some hippocampal BDNF expression has also been found to be modulated by the vagus nerve as subdiaphragmatic vagotomy has been found to regulate it (O'Leary et al., 2018).

Learning and memory behavioural outcomes of microbiota-based interventions also boast clear evidence for the ability of the microbiome to modulate these hippocampal dependent processes. Spatial navigation and long-term object recognition memory showed improvement in 15-17 month old mice when given a probiotic of *Lactobacillus* and *Bifidobacteria* (O'Hagan et al., 2017). While AD mice show impaired behaviour in Y-maze tests, B. breve administration can reverse these effects (Kobayashi et al., 2017). In conclusion, the growing body of evidence highlights the significant role of gut microbiota and SCFAs in modulating hippocampal BDNF levels and, consequently, memory and cognitive function. These findings underscore the potential of microbiota-based interventions as therapeutic strategies for improving cognitive health, particularly in the context of aging and neurodegenerative disorders.

7.3 Electrophysiological Changes in Response to Microbiome Alterations

A variety of electrophysiological measures have demonstrated the power of the microbiome to impact synaptic plasticity, which sees changes across the lifespan like the gut microbiota. For example, hippocampal LTP is reduced in middle-aged and aged rats(Barnes & McNaughton, 1980; Ojo et al., 2015; Rex et al., 2005; Shivarama Shetty & Sajikumar, 2017). Distrutti and colleagues discovered that it is possible to attenuate the age-related deficit in LTP and cause a concurrent increase in BDNF expression by targeting the microbiome through administration of a cocktail of eight Gram-positive bacterial strains (Distrutti et al., 2014). Notably, the ratio of Gram-positive to Gram-negative bacteria has been seen to alter the ratios of SCFAs in the gut, with decreases in Gram-positive bacteria producing reductions in the relative abundance of pathways involved in acetate and butyrate synthesis (O'Reilly et al., 2023). The microbiome also features in synaptic plasticity deficits that are a feature of neurodegenerative disease. Probiotics have also been found to produce electrophysiological changes in a rodent model of AD (Rezaei Asl et al., 2019). This study found hippocampal LTP impaired in the rat model of AD was restored by treatment with a cocktail of probiotics (*Lactobacillus acidophilus, Bifidobacterium bifidum and Bifidobacterium longum*). At the other end of life, from a neurodevelopmental perspective, it has been found that antibiotic treatment of newborn mice decreased adult neurogenesis and hippocampal long-term potentiation induced by HFS (G. Liu et al., 2022). This impacted LTP could be restored by FMT from control mice, demonstrating that a microbiome trajectory set from early life could be behind the observed effect.

Interaction between synaptic plasticity and environment has begun to be explored. Treating mice living in an enriched environment while being fed antibiotics prevented enrichment-driven enhancements in cortical plasticity (Lupori et al., 2022). In this study it was also found that FMT from environmentally enriched donors to control mice reactivated cortical plasticity. Diet appears to

be another major factor which can influence synaptic plasticity and the gut microbiota. Diets high in fat and sugar, sometimes referred to as Western diets, have been linked in humans to cognitive decline and imbalanced microbial population in the gut (Proctor et al., 2017). This Western diet, which affects the gut microbiome in mice and induces systemic inflammation, has also been shown to reduce LTP (P. K. Jena et al., 2022). Supplementation with the probiotic *Bifidobacterium infantis* was shown to largely recover the impact of this diet on LTP accompanied by an increase in brain BDNF levels. Microbiome intervention in male and female mice through the supplementation of PEA-OXA, a natural compound with “precognitive” properties and capability to affect microbiome function (Belardo et al., 2023; C et al., 2023; Izzo & Sharkey, 2010), has been utilised to restore hippocampal plasticity in the dentate gyrus in a vitamin D deficiency model (Guida et al., 2024). This intervention also saw an increase in butyrate-producing bacteria. Similarly NMDAR mediated currents and spontaneous synaptic events of hippocampal CA1 neurons have been found to be altered by administration of prebiotic, bimuno galacto-oligosacharides, to neonatal rats (Spitzer et al., 2021). This was accompanied by a difference in colonic butyrate levels between control and prebiotic-treated animals (Spitzer et al., 2021). Other electrophysiological changes include changes in excitatory/inhibitory (E/I) balance in the brain from microbiota interventions. Chd8+/− mice which are used to model ASD have been shown to display an E/I imbalance which can be partially restored by supplementation with *B. uniformis* (Y. Yu et al., 2022). The electrophysiological changes seen here demonstrate the microbiome’s effect on synaptic plasticity and brain function. However, many questions remain unanswered regarding the microbiome's impact on synaptic plasticity, which this work seeks to address.

8 Thesis Goals and Aims

The capacity of the microbiota-gut-brain axis to influence brain function and behaviour has been explored and its role implicated in many psychiatric and neurodevelopmental disorders (Cryan et al., 2019a; Warner, 2019). This growing body of research suggests that our gut microbes are an important modulator of hippocampal function and learning and memory (Tang et al., 2021a; Tooley, 2020). Although the scientific community is coming to understand that the gut microbiome can affect learning and memory and thus synaptic plasticity, there is foundational knowledge that is yet to be gathered. The work presented here aims to explore how the gut microbiota can affect hippocampal synaptic plasticity in various stages of development.

Aim 1: Uncover the effect of microbiome perturbations on hippocampal plasticity across the lifespan.

It is not yet known whether hippocampal synaptic plasticity is vulnerable to an interruption to the normal microbiome signals during development and across the lifespan. We aimed to use germ-free and antibiotic-treated adult mice to discern the degree to which hippocampal synaptic plasticity may be vulnerable to microbiome insults and to what extent this vulnerability may be a function of neurodevelopment or a consistent communication between the microbiome and the hippocampus across the lifespan.

Aim 2: Gain insight into the ability of primary microbial metabolites to impact synaptic plasticity in the local environment of the hippocampus.

As circulating microbial metabolites are one of the routes of the gut-brain axis we sought to explore the extent to which the most common SCFAs could affect the hippocampus when they pass through the BBB and reach the hippocampus itself. We also aimed to gain insight into the mechanism through which these SCFAs may exert their effects on hippocampal synaptic plasticity, locally, at physiologically relevant concentrations *ex vivo*.

Aim 3. Determine the influence of the gut microbiome on the neurodevelopment of the hippocampus metabolome and transcriptome.

The ability of the microbiome to exert long-standing effects on the brain is highly evident in neurodevelopment. Yet little is known about the potential metabolic and transcriptomic effects that the microbiome exerts in the hippocampus during critical windows of development in early life. We sought to compare GF and conventional mice to gain insight into this at 4 early life timepoints with a focus on plasticity related pathways.

Chapter 2

Microbial memories: Sex-dependent impact of the gut microbiome on hippocampal plasticity

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Abstract

Germ-free rodents, raised in the absence of a measurable gut microbiome, have been a key model to study the microbiome-gut-brain axis. Germ-free mice exhibit marked behavioural and neurochemical differences to their conventionally raised counterparts. It is as yet unclear how these neurochemical differences lead to the behavioural differences. Here, we test the electrophysiological properties of hippocampal plasticity in adult germ-free mice and compare them to conventionally raised counterparts. Whilst basal synaptic efficacy and pre-synaptic short-term plasticity appear normal, we find a striking alteration of hippocampal long-term potentiation specifically in male germ-free slices. However, the spike output of these neurons remains normal along with altered input-output coupling, potentially indicating homeostatic compensatory mechanisms, or an altered excitation/inhibition balance. To our knowledge this is the first time the electrophysiological properties of the hippocampus have been assessed in a microbiome deficient animal. Our data indicate that the absence of a microbiome alters integration of dendritic signalling in the CA1 region in mice.

1 Introduction

Growing evidence suggests that the commensal gut microbiota modulate our behaviours and may contribute to certain disease processes (Cryan et al., 2019b). One of the principle animal models in the field has been the germ-free (GF) rodent (Spichak et al., 2018b). In this model, animals are born into a sterile environment, depriving them of a microbiome, thereby allowing us to observe how they develop in the absence of microbial influence, and make inferences about what role the microbiome plays in normal physiology.

GF animals have been shown to exhibit altered central neurochemistry and behaviours (Bercik et al., 2011; Clarke et al., 2013; De Palma et al., 2015; Neufeld et al., 2011a). Both cortical and hippocampal BDNF expression has been reported lower than normal in GF mice (Clarke et al., 2013; Heijtz et al., 2011). GF mice exhibit an altered transcriptional profile, combined with an increase in immediate-early gene (Fos, Egr2, Fosb, Arc) expression in the amygdala (Hoban et al., 2018b), enhanced expression of gene splicing factors and exon usage (Stilling et al., 2018), and altered expression of genes implicated in neurophysiology in the amygdala (Stilling et al., 2015) and hippocampus (J.-J. Chen et al., 2017). Recent evidence has also implicated Lactobacilli in the expression of GABA in the hippocampus (Mao et al., 2020). Hippocampal Fos expression following a water avoidance stress paradigm was lower in GF mice compared to colonized counterparts (Gareau et al., 2011a). Swiss-Webster GF mice also exhibit altered hippocampal morphology with increased hippocampal volume (10%) and altered pyramidal neuron morphology when compared to conventionally raised controls (Luczynski, Whelan, et al., 2016).

Furthermore, GF mice have been shown to exhibit altered anxiety-like behaviours in both the open-field and elevated plus maze, and that this is mediated via microbial regulation of NMDA receptors (Neufeld et al., 2011b), important in brain development and neural plasticity (Bercik et al., 2010; O'Sullivan et al., 2011; Sudo et al., 2004). GF mice exhibit other cognitive deficits including hippocampus dependent learning and memory processes (Gareau et al., 2011a;

Hoban et al., 2018a; J. Lu et al., 2018; Luk et al., 2018; J.-X. Pan et al., 2019), as well as social behaviours (Buffington et al., 2016; Desbonnet et al., 2014; Sarkar et al., 2020; Sgritta et al., 2019). Together, this evidence implies that the microbiome has the potential to alter a host's neuronal activity, either directly or indirectly.

There are limited published studies directly measuring neuronal activity in a model of microbiome disruption, and the functional consequence to neuronal activity has yet to be shown. However, long-term dietary supplementation of a probiotic mixture containing *Lactobacillus acidophilus, Bifidobacterium lactis, and Lactobacillus fermentum,* rescued diabetes induced CA3-CA1 LTP deficits in rats (Davari et al., 2013), chronic treatment with a *Lactobacillus acidophilus, Bifidobacterium bifidum, and Bifidobacterium longum* probiotic mix enhanced CA3-CA1 LTP in a rat model of Alzheimer's disease and control animals (Rezaei Asl et al., 2019), and a probiotic mixture of a probiotic mixture comprising 8 Gram-positive bacterial strains ameliorated age-related LTP deficits (Distrutti et al., 2014). Furthermore, *Lactobacillus reuteri* restored social interaction induced plasticity in the ventral tegmental area of a mouse model of autism, through an oxytocin dependent mechanism (Sgritta et al., 2019). These provide evidence that the microbiome can indeed modify functional neuronal activity.

Here, we have tested whether the absence of a microbiome alters basal synaptic efficacy, and inducibility of plasticity, in the adult mouse hippocampus.

2 Methods

2.1 Animals

C57/Bl6 mice, purchased from Taconic (NY, USA), were bred either as GF (*n* = 14, 6 female), or conventionally raised (*n* = 13, 6 female) in University College Cork. Breeding was performed according to supplier guidelines, and we used animals ranging from F8-F11. GF animals were housed 4–5 per cage in individually ventilated cages (area: 420cm^2. Arrowmight, UK), conventionally raised animals were raised 4–5 per cage in standard cages (area:330cm^2. NKP isotech, UK). Both had paper bedding/nesting material and were housed in identical controlled conditions (20–21°C, 55%–60% humidity) on a 12-hr light/dark cycle, with access ad libitum to autoclaved chow and water. On each recording day, one animal (8–12 weeks old) was separated into its own cage and brought to the laboratory for use in the experiments. Experiments were undertaken in accordance with National and European legislation (Directive 2010/63/EU and Irish SI No 543 of 2012).

2.1.1 Slice preparation

Hippocampal slice electrophysiology is a well-used technique and our methods followed common practices (Papouin & Haydon, 2018). Fresh artificial cerebrospinal fluid (aCSF) was made (see below) and bubbled with carbogen (95% O_2/5% CO_2) for at least 40 min prior to use, and continually throughout recordings.

Mice were rapidly decapitated, and the brain removed and cooled with ice cold 'cutting' aCSF (see protocol below). The brain was quickly hemisected, the cerebellum and midbrain removed, and the remaining tissue affixed to the stage of a Leica VT1200 vibratome to enable 300µm transverse slices of the hippocampus and attached cortex to be cut, under ice-cold cutting aCSF. It is important to note that GF mice exhibit altered acute stress responses and HPA axis function (Sudo et al., 2004; Vagnerová et al., 2019). To minimize any effect of differential stress response, all animals were left to acclimatize to the

experimental room for at least 20 min and were all observed to be in a non-stressed state.

Slices were transferred to a chilled glass petri-dish, filled with cutting aCSF, and the cortex were removed from the hippocampal slices. A selection of slices spanning the central portion of the dorso-ventral axis (approximately 1.5–2.1 mm from dorsal end) were transferred to mesh holding wells in a bath of standard aCSF held at 32°C for 20 min, and then at room temperature for a further hour.

After this recovery period, slices were transferred to a multi-electrode array (MEA) chip (Multichannel Systems, Germany), perfused with aCSF. Slices were viewed under an inverted microscope (4x magnification, Olympus IX70) and compared for structural integrity and general slice health. The best candidate slice was used for electrophysiological testing.

2.1.2 Electrophysiology recording

The slice was placed onto an MEA chip (Menigoz et al., 2016; Shaban et al., 2017) and aligned so that a stimulating electrode was located over the Schaffer collateral path leaving the CA3, heading towards the CA1, and a recording electrode was situated in the middle of the CA1 striatum radiatum (200 µm 'downstream' of the stimulus electrode) (O'Dell et al., 1991). The slice was held with a nylon mesh anchor. A benefit of using an MEA chip is that it allowed for an additional recording electrode to be sited in the striatum oriens, to record the somatic population spike of the stimulated neurons.

Perfusion rates of the aCSF (heated to 31–32°C) were 2.5 ml/min in, 4 ml/min out, which optimally maintained fluid volume while minimizing turbulent flow forces.

After testing the presence of field excitatory post-synaptic potential (fEPSP) responses with a small bipolar current (10–20 µA), the slice was left to recover in the MEA chip for another hour.

Once fEPSP slope measurements were stable (±15%) for approximately 15 min (typically 1-hr after placement), an input/output curve was computed by increasing the stimulation current from 1 µA to 100 µA and measuring the slopes

of the resultant fEPSPs, taking an average of three sequential stimulation ramps. All subsequent stimulation was performed at an intensity eliciting approximately 40% of the maximum response of each slice. Subsequently, a test of short-term synaptic plasticity was performed with a paired-pulse stimulation protocol (PPF). Inter-pulse intervals were; 25, 50, 75, 100, 150 and 200ms. A 30 s interval between each of these paired pulse events was maintained, and this train of 6 paired pulses was repeated three times.

Once both input/output, and PPP tests were performed, the slice was again stimulated using single bipolar pulses every 30s. After a minimum period of 30 min in which the fEPSP slope did not exceed ± 15% of itself (defined here to be the baseline period), the Schaffer collateral pathway was stimulated with a theta burst (TBS) protocol to induce long-term potentiation (LTP). Each TBS consisted of four 100Hz pulses, repeated 10 times at 5Hz. Three theta bursts (3xTBS) were delivered with an inter-burst interval of 30 s. After this induction protocol, the stimulator reverted back to a single pulse every 30 s as in baseline.

All stimulation protocols were designed in MCStimulusII (Multichannel Systems, Germany) and played during recordings via MCRack (Multichannel Systems, Germany) software.

2.1.3 aCSF content

The aCSF was made up of (in mM): NaCl (124), KCl (2.7), NaH2PO4 (1.25), $CaCl_2$ (2), $MgSO_4$ (1.3), D-Glucose (18) and Ascorbic acid (2).

Cutting aCSF was made up of the same aCSF, with excess $MgSO_4$ (8.3 mM).

2.1.4 Data analysis and statistical methods

Data files were converted to '.abf' files and the slope (capturing 80%–90% of the inward current slope) of fEPSPs was extracted using semi-automated procedures within Clampfit software. To extract the amplitude of the population spike, we subtracted the trough of the spike from an average of the adjacent peaks. For input/output relationships and PPP, the average of three responses at each stimulus intensity/interval was used. For the test of LTP, data values of

fEPSP slopes were expressed as a percentage of the average of the baseline period fEPSPs (30 min prior to TBS), and an average of 10 min (20 stimuli) were used for statistical analysis.

Parametric tests were utilised for all statistical analyses after appropriate checks for data normality. Specific test details are given in the results. Individual slices (each originating from an individual animal) were treated as subjects, sex and GF status of the donor animals was treated as fixed factors. Data originating from individual slices were treated as repeated measures (e.g. pre and post TBS data).

2.2 Results

2.2.1 Germ-free mice display normal basal synaptic excitability

To test basal synaptic efficacy of GF hippocampal slices, we computed 'input-output' characteristics of the fibre volley and resultant fEPSP at a variety of stimulus intensities (Figure 2.1b and c). We summarized each slice's data by calculating the linear slope of this relationship (C.-H. Kim et al., 2005; Woo et al., 2005). ANOVA revealed no significant difference between GF and Conv groups ($F_{1,23} = 1.326$, $p = .261$), and no interaction between GF status and sex ($F_{1,23} = 0.644$, $p = .431$). Nor was there a main effect of sex ($F_{1,23} = 0.004$, $p = .953$). This suggests that GF and Conv slices exhibit the same basal synaptic excitability, although Figure 2.1d reveals that male GF slices had a tendency for lower values (indicating smaller fEPSP responses for the same fibre volley stimulus).

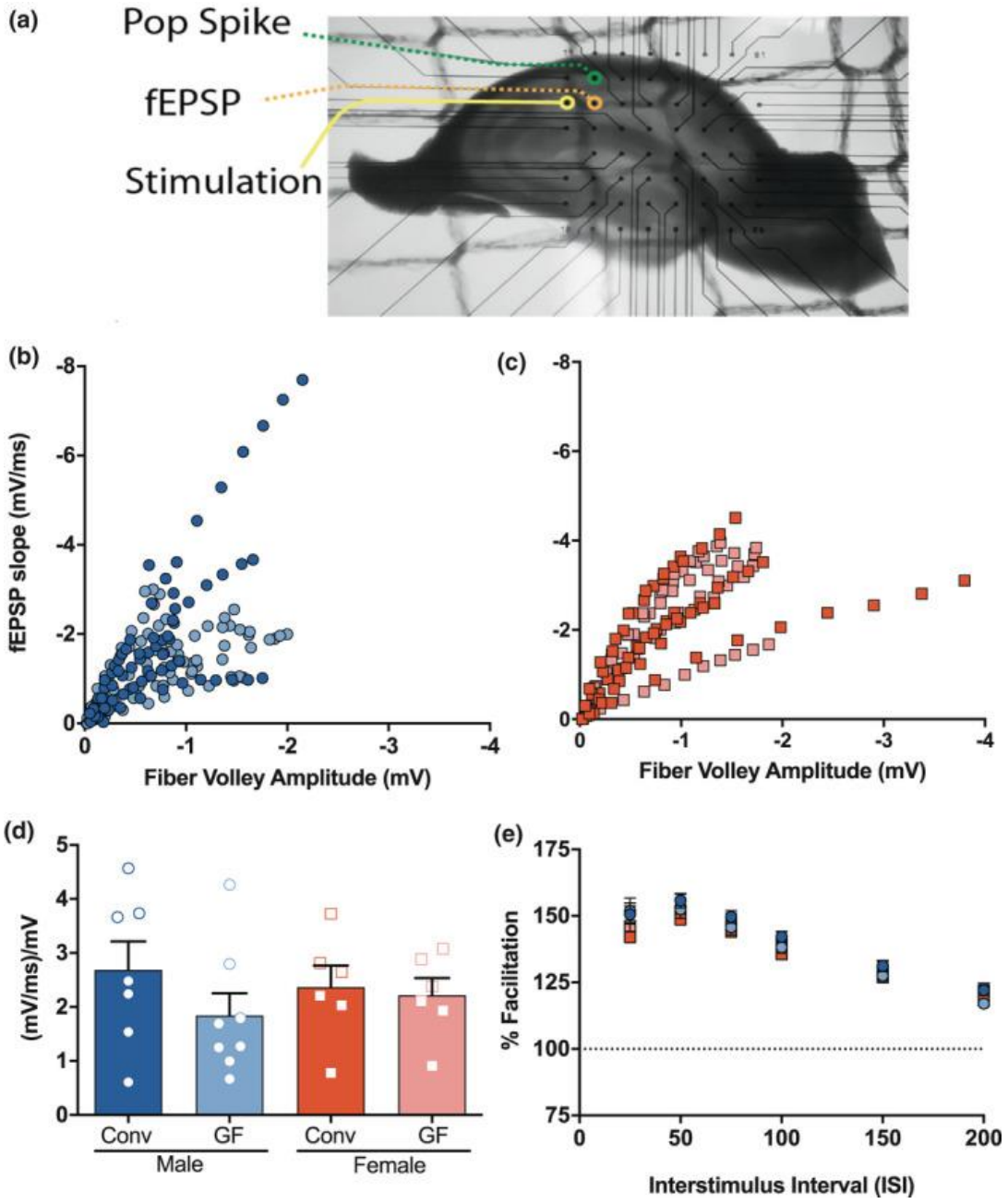


Figure 2.1 (a) Representative image of hippocampal slice on MEA, with target locations of stimulation and recording shown. The relationship between fibre volley amplitude and fEPSP slope of male (b) and female (c) slices. (d) Basal excitability summarised with the linear slope of each slice. (e) Paired-Pulse facilitation of fEPSPs. No statistically significant differences were found between germ-free and conventionally raised control slices. Error bars represent standard error of the mean. Male $N_{Conv/GF}$ *= 7/8, Female* $N_{Conv/GF}$ *= 6/6*

2.2.2 Germ-free mice display normal pre-synaptic function

If two excitatory stimuli occur within a short space of time of each other, the second post-synaptic response, in conventional animals and under normal conditions, will be enhanced (Creager et al., 1980). This short-term facilitation is a result of pre-synaptic mechanisms leading to an increase in the amount of neurotransmitter release (Jackman & Regehr, 2017).

To establish whether GF hippocampal slices exhibited normal pre-synaptic function, we tested GF short-term plasticity using paired-pulse stimulus protocols. Slices from both GF and conventionally raised animals exhibited paired-pulse facilitation with inter-pulse intervals spanning 25–200ms, with maximal facilitation of around 150% at an inter-pulse interval of 50ms (Figure 2.1e), similar to that reported previously in the literature (Chong et al., 2011; Menigoz et al., 2016; Rohan et al., 2015). Mixed model analysis found no significant interaction between the sex and GF status ($F_{1,96.776} = 3.307$, $p = .072$), or main effects of either sex ($F_{1,96.776} = 1.392$, $p = .241$), or GF status ($F_{1,96.776} = 0.000$, $p = .995$).

2.2.3 Germ-free males exhibit reduced Long-Term Potentiation after Theta-Burst Stimulation

After a minimum 30-min baseline recording period, LTP was induced using three trains of theta-burst stimulation (3xTBS). In conventionally raised animals, this led to a rapid potentiation of fEPSPs that lasted for 2 hr (males: mean = 153.0% *SEM* 8.5, females: mean = 138.6% *SEM* 5.4). Female GF slices matched this potentiation (mean = 140.9% *SEM* 5.4). Conversely, slices from male GF mice showed no immediate potentiation, with a slow upward trend for the duration of the recording, finishing at a mean of 125.7% *SEM* 7.9 (Figure 2.2).

Mixed-model analysis revealed a significant 3-way interaction between Time, Sex and GF status ($F_{2,38.163} = 5.002$, $p = .012$). Subsequent analyses on each sex independently revealed no significant interaction between GF status and Time ($F_{2,20.669} = 0.476$, $p = .628$), nor main effect of GF status ($F_{1,17.733} = 0.162$, $p = .692$),

leaving only the main effect of Time ($F_{2,20.669} = 86.924$, $p < .0005$) in female slices. Bonferroni corrected pairwise comparisons revealed that pre-TBS data were significantly different from both 1-hr ($p < .0005$) and 2-hr time points ($p < .0005$).

Conversely, for male slices, there was a significant interaction between GF status and time ($F_{2,18.198} = 16.371$, $p < .0005$), suggesting GF status impacts LTP processes in male slices. Subsequent ANOVA found significant differences between GF and Conv. slices at both 1-hr ($p < .0005$) and 2-hr time points ($p = .035$).

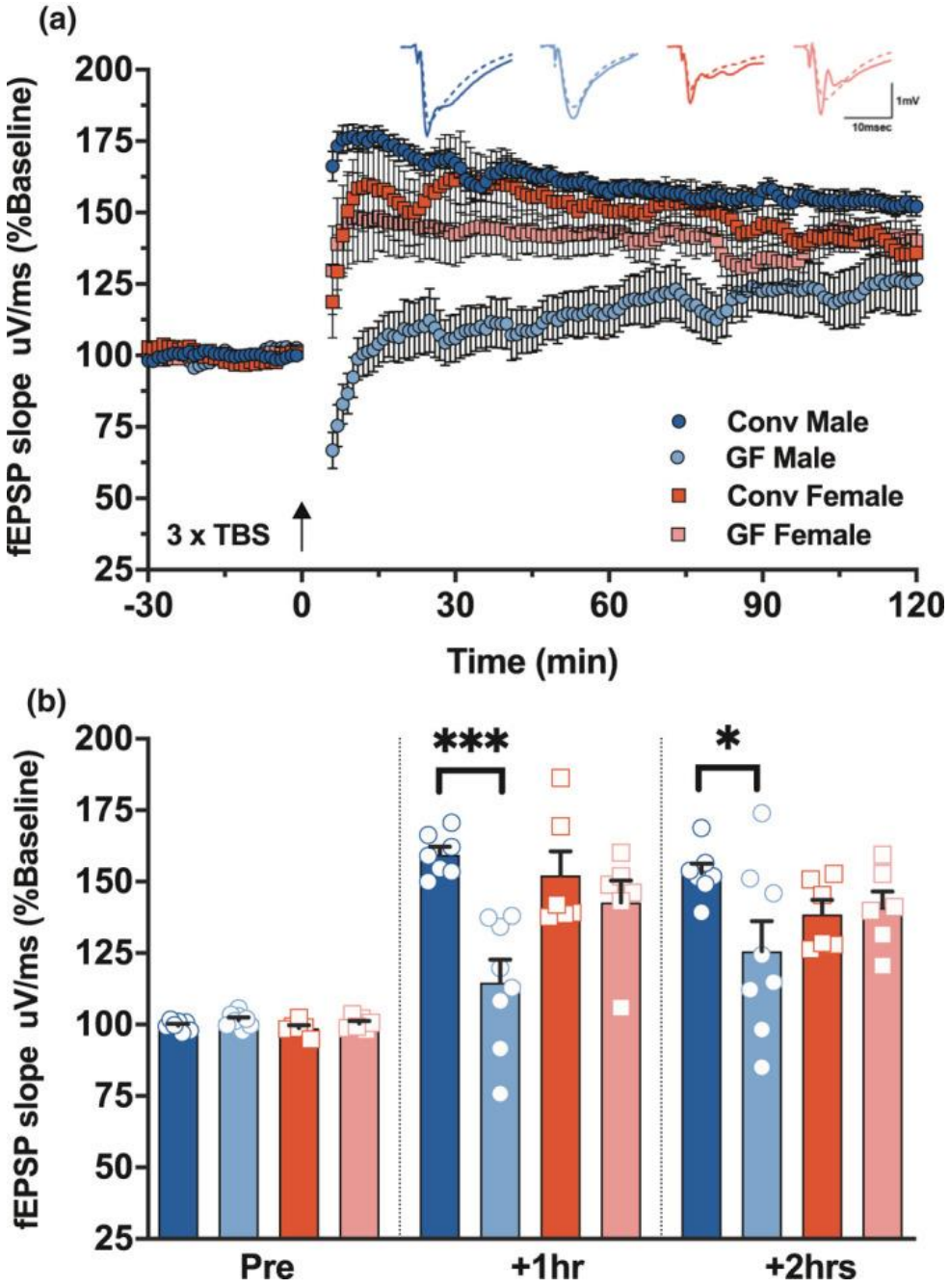


*Figure 2.2 (a) Timeline of normalised CA1 fEPSP slopes for conventionally raised (dark shading) and germ-free (light shading) mice. Female (red) germ-free slices exhibited normal potentiation at 2 hr post LTP induction. Male (blue) germ-free slices exhibited a markedly decreased potentiation, that remained up to 2 hr post induction. Representative traces at both pre (dashed lines) and post (solid lines) LTP induction for each group. Scale bar is 1 mV × 10ms. (b) Bar chart summarising pre and 2 hr post LTP induction fEPSP responses. Error bars represent standard error of the mean. Male $N_{Conv/GF}$ = 7/8, Female $N_{Conv/GF}$ = 6/6. *Mixed model p = .035. ***Mixed model p < .005*

2.2.4 Theta-Burst stimulation induces normal potentiation of somatic responses in germ-free mice

With the advantages of multiple recording sites with the MEA system, we were able to concurrently record the population spike responses to TBS (Figure 2.3). In both groups, TBS led to sustained potentiation of pop. spike amplitude for 2-hr post induction (Conv.: $Mean_{male}$.= 236.5% *SEM* 37.7, $Mean_{female}$ = 275.4% *SEM* 31.8, GF: $Mean_{male}$=230.9% *SEM* 31.3, $Mean_{female}$ = 227.3% *SEM* 23.7).

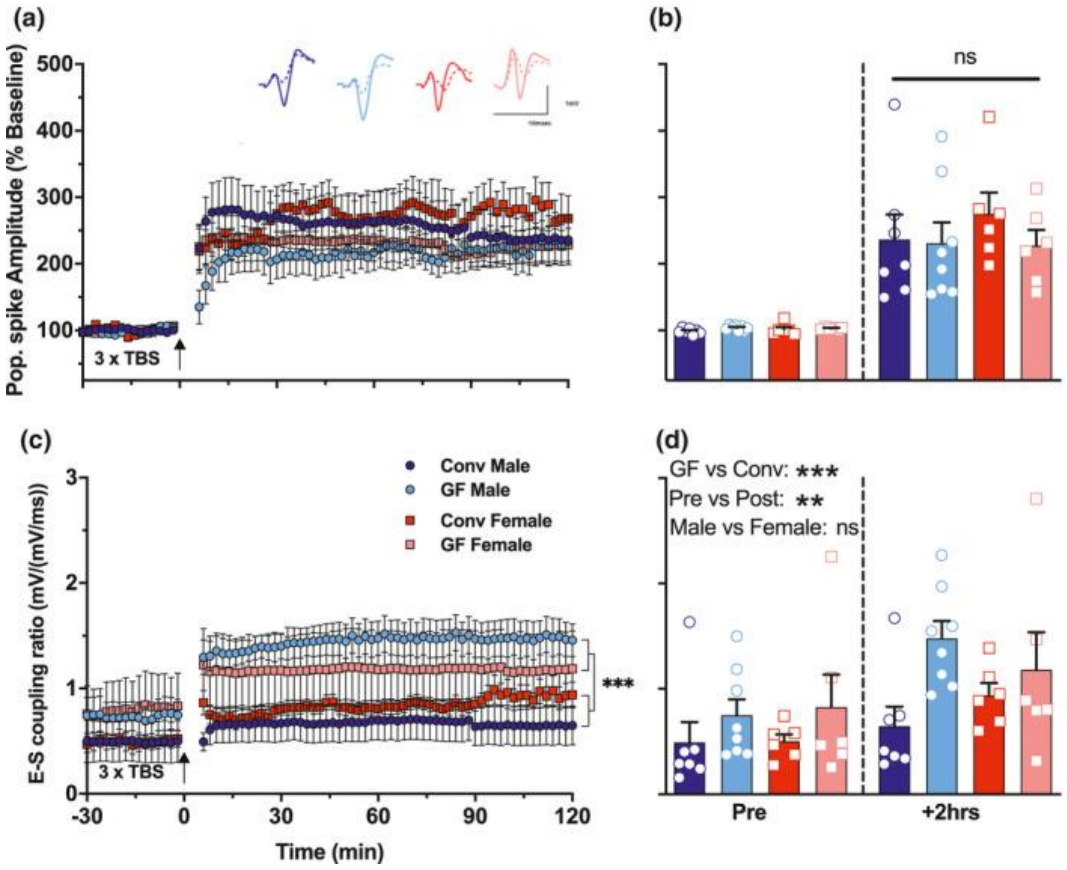


*Figure 2.3 (a) Timeline of normalised CA1 pop. spike amplitudes for conventionally raised (dark shading) and germ-free (light shading) mice. Germ-free slices exhibited normal potentiation compared to conventionally raised slices. Representative traces at both pre (dashed lines) and post (solid lines) LTP induction for each group. Scale bar is 1 mV × 10ms. (b) Bar chart summarising pre and 2 hr post LTP induction pop. spike responses. (c) Timeline of CA1 E-S coupling for conventionally raised (dark shading) and germ-free (light shading) mice. Both male and female germ-free slices exhibited markedly increased E-S coupling both pre and post LTP induction. (d) Bar chart summarising pre and 2 hr post LTP induction E-S coupling. Error bars represent standard error of the mean. Male $N_{Conv/GF}$ = 7/8, Female $N_{Conv/GF}$ = 6/6. ***Mixed model p < .0005. **Mixed model p = .008*

Mixed model analysis revealed no significant 3-way or 2-way interactions, and only a main effect of Time ($F_{2,40.211} = 71.787$, $p < .0005$). Bonferroni corrected pairwise comparison showed significant differences between pre-TBS and both 1-hr ($p < .0005$) and 2-hr ($p < .0005$) post-TBS. Together, this shows that population spike potentiation in response to 3xTBS is comparable between GF and Conv. groups.

2.2.5 GF slices exhibit altered E-S coupling

The stark contrast between the effects of TBS on the dendritic and somatic responses in GF slices suggested a difference in the E-S coupling relationship between conventionally raised and GF mice. After TBS stimulation, both conventional and GF slices exhibit an increase in the E-S coupling ratio (here representing an increase in pop. spike amplitude for a given fEPSP response) that is maintained for the duration of the recordings (Figure 2.3c). Notably, GF slices appear to have a greater E-S ratio both before and after LTP induction, with male slices exhibiting the greatest differences to conventional slices (Mean difference = 0.832 mV/(mV/ms)). Mixed model analysis revealed no significant 3-way or 2-way interactions, but significant main effects of GF status ($F_{1,68.466} = 15.419$, $p < .0005$), and time ($F_{2,45.008} = 5.446$, $p = .008$).

In summary, GF slices exhibited a greater E-S ratio than Conv. slices, and this difference was not markedly altered by the effect of TBS.

2.3 Discussion

In the brain, activity-dependent modification of synapses has long been regarded as the brain's principal mechanism of assimilating transient experiences into perseverant memory engrams (T. V. Bliss & Lomo, 1973; Josselyn & Tonegawa, 2020), where an activity-dependent persistent strengthening of synaptic efficacy can be described as long-term potentiation (LTP) (Madison et al., 1991). Here, we show that the microbiome may play an important role in the expression of these processes.

In the unidirectional tri-synaptic excitatory pathway of the rodent hippocampus, any significant enhancement in synaptic activity lasting at least one hour post-tetanus is understood to be LTP (Nicoll, 2017). Therefore, LTP has been used as a reliable indicator of healthy brain function, and its impairment has been seen in rodent models of neurodegenerative disease. Deficits in LTP have been seen accompanying cognitive dysfunction (Y. Li et al., 2015; N. Lin et al., 2014; Wieschollek & Manahan-Vaughan, 2013), and it is regarded as a reliable, functional readout for neurophysiological processes. Indeed, neuronal synaptic plasticity is particularly vulnerable to disruption by factors that alter cognition in psychiatric and neurological disease, including those that alter synaptogenesis (Bednarek & Caroni, 2011), axonal sprouting or pruning, and dendritic remodelling (Selemon, 2013; Ziv & Brenner, 2018), as well as neurogenesis (Ganguly & Poo, 2013), many of which have been shown to be influenced by gut-microbiota (Cryan et al., 2019b). Furthermore, the hippocampus is highly responsive to glucocorticoids (Lupien et al., 2005), and it has been shown that hormone interaction with CA1 receptors in the hippocampus leads to a variety of different cellular responses, including changes in synaptic function, including neuronal injury (Y. Chen et al., 2006).

GF animals have been utilized in examining the involvement of the microbiota in central synaptic plasticity, and here, we show that the absence of a microbiome significantly alters fundamental hippocampal neuronal plasticity processes. This fits with the evidence that GF animals exhibit altered molecular chemistry,

morphology of the hippocampus as well as an altered behavioural phenotype associated with hippocampal activity (Gareau et al., 2011a; Luczynski, Whelan, et al., 2016).

Specifically, the GF mice tested here appear to have normal ability to produce fEPSPs, and short-term potentiation (Figure 2.1), although male GF slices appear to have a small (nonsignificant) trend for lowered basal excitability (Figure 2.1d). However, testing post-synaptic LTP mechanisms (with 3xTBS) reveals a sex-dependent functional shift, with males showing greater deficits than females (Figure 2.2). Sex-specific impacts of the gut microbiome have been reported, however, there is no clear consensus with heterogeneous results possibly resulting from strain specificity of both microbiome and host (reviewed in Jaggar et al., 2020).

Whilst we have not investigated the molecular mechanisms underpinning this phenomenon, it is plausible that the altered genetic/transcriptional profile of immediate early genes (Gareau et al., 2011a) leads to a block of the dendritic potentiation signals. Additionally, a recent study has shown that microbial metabolites can translocate from the gut to the brain during early developmental periods (Swann et al., 2020). Interestingly, this included imidazole propionate, which activates mammalian target of rapamycin complex 1 (mTORC1), which is known to be involved in hippocampal LTP (Hoeffer & Klann, 2010). Furthermore, altered inflammatory signalling, widely implicated as a route of gut-brain interaction, has been shown to reduce hippocampal long-term potentiation via an interleukin-1 receptor dependent pathway (York et al., 2021).

When examining the spiking output from the CA1 area we find a normal potentiation of the pop. spike in response to 3xTBS in both male and female GF slices (Figure 2.3a). Moreover, when examining the input–output relationship between the dendritic fEPSP and somatic pop. spike (E-S coupling), we find that GF slices (both male and female) exhibit a significantly enhanced coupling (Figure 2.3b). This could suggest homeostatic mechanisms in the somatic integration of dendritic signals (E-S coupling) acting to rescue the functional

deficits observed at the dendritic regions. Overall, this compensation may mask more severe microbiome driven influences, resulting in the milder behavioural phenotypes.

Whilst E-S coupling is a relatively understudied facet of neuronal plasticity, it is believed that it may be driven by a mixture of intrinsic factors as well as the excitation/inhibition balance, with one study suggesting around 40% of E-S coupling in hippocampus CA1 was driven by cell intrinsic factors (Daoudal et al., 2002). Interestingly, GF mice have previously been suggested to have a disrupted excitatory/inhibitory balance. This is supported by the finding that GF mice exhibit a reduced concentration of GABA in the hippocampus compared to specific-pathogen free mice (Kawase et al., 2017), and colonization of GF mice with *Lactobacillus reuteri*, *Lactobacillus plantarum*, and *Lactobacillus brevis* probiotics leads to increased GABAergic cells in the hippocampus (Mao et al., 2020). Disruption of the inhibitory/excitatory balance is thought to underpin many physiological disorders, including epilepsy. GF mice have not, to our knowledge, been reported to exhibit an epileptic phenotype, however, the microbiome has been implicated in disruption of the excitatory/inhibitory balance. Specifically, whilst a ketogenic diet was able to confer protective effects against a 6Hz electrical stimulation epilepsy paradigm, GF mice did not benefit from the same diet, but the protection was restored when supplementation of *Akkermansia muciniphila, Parabacteroides merdae*, and *Parabacteroides distasonis* was given (C. A. Olson et al., 2018). This microbiome derived protection has been attributed to elevation of GABA inhibition (Dahlin & Prast-Nielsen, 2019). The morphological differences in hippocampal pyramidal neurons of GF mice (Luczynski, Whelan, et al., 2016) also suggests an immaturity of the glutamatergic synapses, which would most likely alter the inhibitory/excitatory balance of the network. Furthermore, microbiome/dietary based interventions have been proposed as an interesting approach to address epilepsy (De Caro et al., 2019).

GF mice often display altered measures of motor behaviour, as well as social interaction/cognition (reviewed in Spichak et al., 2018). Experience is well-known

to modulate hippocampal plasticity processes. Whilst housing conditions between the Conv. and GF animals in this study were identical as far as possible, it is possible that the differences observed in our study are as a result of altered social experiences/interactions of the animals (driven by GF status) rather than as a direct result of any microbiome metabolite or signalling process. Further experiments will be needed to disentangle these possibilities.

Although translation of our findings to the human setting is not yet possible, there are still clear differences in the functional properties of the hippocampal circuit in vitro, supporting the idea that neurochemical and morphological alterations found in GF mice lead to altered functional activity. Further examination will be important to characterize changes in electrophysiological properties of circuits from animals with perturbed gut-microbiomes. Some principal questions that arise from this study are: Can hippocampal properties be altered with antibiotic knockdown of the microbiome? Are differences observed earlier in the lifespan? Are other forms of plasticity (i.e. long-term depression) influenced by the microbiota? Can these effects be reversed with pre/probiotic administration?

We conclude that the absence of a gut-microbiome leads to a selective alteration of dendritic LTP mechanisms, with males more severely affected, which may be compensated for by an E-S coupling driven hyperexcitability. This functional effect of hippocampal activity is likely to be important in the behavioural phenotype of GF animals and further implicates the microbiome in molecular memory processes.

2.4 Conflicts of Interest

J.F.C has been an invited speaker at meetings organized by Yakult, Alkermes, Ordesa, Servier, Lundbeck, Janssen, and AstraZeneca, and have received research funding from Mead Johnson, Cremo, Nutricia, Dupont, Pharmavite, and 4D Pharma. All other authors declare no competing interests.

2.5 Author's Contributions

H.T.D., K.J.OR., and J.F.C. conceived and designed the experiment. H.T.D., M.K.C., and K.J.OR. performed experiments, and data analysis. K.J.OR. prepared figures. H.T.D., K.J.OR., and J.F.C. prepared the manuscript.

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Chapter 3

Robust Hippocampal Synaptic Plasticity Despite Gut Microbiota Depletion in Adult Mice

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Abstract

The microbiota-gut-brain axis describes the bidirectional communication between the brain and the trillions of microorganisms living in the gut. Moreover, current evidence suggest that this axis can influence host behaviour and brain physiology. Previously we have shown that adult mice that have not been exposed to microbes throughout their lives display sex-specific deficits in hippocampal synaptic plasticity. However, it is not known whether this phenomenon originates in neurodevelopment or whether similar effects could be recreated with microbiome depletion in adulthood. Therefore, we explored the vulnerability of hippocampal synaptic function to altered microbiome signals, depleting the microbiome of male and female mice for two weeks with either an antibiotic cocktail or a single antibiotic in drinking water. The antibiotic cocktail contained a variety of antibiotics including broad-spectrum antibiotics to ensure widescale microbiota depletion (ampicillin, vancomycin, and imipenem). In addition, a more targeted depletion of gram-positive gut bacteria was conducted using the gut-restricted antibiotic vancomycin. *Ex vivo* hippocampal electrophysiology measures of basal synaptic efficacy, short-term plasticity, and long-term potentiation (LTP) were examined.

We found that there was no effect of antibiotic administration on any of these measures, demonstrating the robustness of these hippocampal circuits to microbiome depletion during early adulthood. Taken together this shows the ability of adult hippocampal plasticity to withstand a gut microbiome insult.

1 Introduction

Emerging evidence indicates that the gut microbiota play a crucial role in modulating host behaviour and contribute to certain disease processes (Cryan et al., 2019a). Among the various behaviours and processes that appear to be influenced are cognitive functions including learning and memory (Kuijer & Steenbergen, 2023). Notably, the composition of the gut microbiota has been correlated with the functioning of the hippocampus, a key brain region involved with learning and memory, in older adults (Manderino et al., 2017; Renson et al., 2020). Microbiome depletion has been the subject of extensive study and has been found to affect social and anxiety-related behaviours (Olavarría-Ramírez et al., 2023), both of which are hippocampal-related behavioural types (Ghasemi et al., 2022; Rubin et al., 2014). Furthermore, significant differences in gut microbiota composition have been discovered between healthy individuals and Alzheimer's disease patients, suggesting a potential role of the gut microbiota in cognition and memory (Chandra et al., 2023).

Synaptic plasticity, a major cellular mechanism underlying learning and memory (M. B. Kennedy, 2016), is an activity-dependent process that involves enduring changes to the synaptic strength (T. V. Bliss & Lomo, 1973). The microbiome has demonstrated a significant capacity to affect this process in the hippocampus. The administration of probiotics, in this case a cocktail of eight Gram-positive bacterial strains, has been found to mitigate age-related deficits in long-term potentiation (LTP), a specific form of synaptic plasticity (Distrutti et al., 2014). Additionally, the gut microbiome transplanted from young to old mice has also been found to attenuate age-related deficits in cognitive behaviour alongside the hippocampal transcriptome (Boehme et al., 2021). Finally, we have previously discovered a sex-dependent deficit in LTP in germ-free mice, which are raised in a sterile environment devoid of any microbial influence (Darch et al., 2021). However, it remains unclear whether this deficit in germ-free animals is due to neurodevelopmental alterations resulting from their germ-free upbringing, or whether continuous microbial signalling is necessary for maintaining normal

hippocampal synaptic plasticity.

There is evidence that antibiotic exposure can impact learning and memory, although studies often examine only one sex. Previous research has shown that antibiotic treatment decreases memory retention in female mice (Möhle et al., 2016) while in male mice, it disrupts novel object recognition but not spatial memory (Fröhlich et al., 2016). Further studies have found evidence supporting the potential of antibiotics to alter synaptic plasticity (Bercik et al., 2011) discovered in male mice that microbiome perturbation through antibiotic treatment increased hippocampal BDNF, a factor strongly associated with synaptic plasticity (B. Lu et al., 2014). Hippocampal BDNF was also found to be increased in germ-free male mice, further strengthening the link between the microbiome and this synaptic plasticity associated factor (Neufeld et al., 2011b).

Given the sex-dependent deficits in synaptic plasticity observed in germ-free mice and the impact of antibiotics on learning and memory processes in adulthood we pose the following question: Can deficits in synaptic plasticity be replicated with microbiome depletion in adulthood, and will this reveal similar sex differences? To address this question, we aimed to deplete the microbiome of adult male and female mice using different antibiotic regimens and subsequently perform *ex vivo* hippocampal electrophysiology.

2 Methods

2.1 Animals

Adult male and female C57BL/6 mice (n = 10; 8 weeks; ENVIGO, UK) were used in this study. All experimental procedures on live animals were conducted in accordance with the European Communities Council Directive 2010/63/EC, the requirements of the S.I No 543 of 2012 and approved by the Animal Experimentation Ethics Committee of University College Cork and the Health Products Regulatory Authority (HPRA AE19160/P118).

Animals were habituated in the animal facility for at least one week before experiments started and kept under a 12-h light/dark cycle, with a temperature of 21 ± 1 °C and humidity of 55 ± 10%. Food and water were given *ad libitum*.

2.2 Study design

The gut microbiota from the experimental animals was depleted using one of two approaches. The first approach used a wide-spectrum antibiotic cocktail (ABX) consisting of ampicillin (1 g/L, CAS no. 69-52-3), vancomycin (0.5 g/L, CAS no. 1404-93-9), and imipenem (0.25 g/L, CAS no. 74431-23-5). The second approach used a single antibiotic, vancomycin (Vanco; 0.5 g/L, CAS no. 1404-93-9), which targets gram-positive bacteria. Both treatments were prepared freshly with tap water every second day for 2 weeks (Frohlich et al. 2016). Control mice (Ctrl) received tap water. Water consumption in the experimental groups (ABX and Vanco) was recorded to ensure the animals were drinking the antibiotics. Each cage housed two to three mice. Mice were then divided into the three experimental groups: 1. Drinking water (Ctrl), 2. Antibiotic cocktail (ABX), and 3. Vancomycin (Vanco), in both males and females. After 2 weeks of treatment, the mice were culled, and the brain was dissected for electrophysiological examination.

2.3 Artificial Cerebrospinal Fluid Content

The artificial cerebrospinal fluid (aCSF) was made up of (in mM): NaCl (124), KCl (2.7), NaH2PO4 (1.25), $CaCl_2$ (2), $MgSO_4$ (1.3), D-Glucose (18) and Ascorbic acid (2).

Cutting aCSF was made up of the same aCSF, with excess $MgSO_4$ (8.3 mM) and cooled to between 0-4°C.

2.4 Slice Preparation

Hippocampal slice electrophysiology is a well-used technique and our methods followed common practices (Papouin & Haydon, 2018) and those of our previous publications (Darch et al., 2021). Fresh aCSF was made (see below) and bubbled with carbogen (95% O_2/5% CO_2) for at least 40 min prior to use, and continually throughout recordings.

To minimize any effect of differential stress response, all animals were left to acclimatise to the experimental room for at least 1 hour following transfer from the animal unit. Ice-cold cutting aCSF (see above protocol) was used for the entirety of the dissection. Mice were rapidly decapitated, and the brain removed and cooled with aCSF. The caudal third of the brain was removed with a razor blade, making a cut just rostral to the cerebellum. This created a flat surface for the brain to be glued to the stage of a Leica VT1200 vibratome. An agar block support was also glued on its ventral side to aid slicing stability. 300µm coronal slices of the brain were cut under oxygenated aCSF.

Slices were transferred to a chilled petri-dish, filled with oxygenating aCSF, and the hippocampus was isolated from the slices. A selection of slices spanning the central portion of the dorso-ventral axis (approximately 1.5–2.1 mm from dorsal end) were transferred to mesh holding wells in a bath of standard aCSF held at 32°C for 45 min, and then at room temperature for a further 45 minutes.

After this recovery period, slices were transferred to a multi-electrode array (MEA) chip (Multichannel Systems, Germany), perfused with aCSF at 31-32°C.

Slices were viewed under an inverted microscope (4x magnification, Olympus IX70) and compared for structural integrity and general slice health. The best candidate slice was chosen based on clearly observable stratum pyramidale and best initial response to test stimulation. This slice was therein used for electrophysiological testing.

2.5 Electrophysiology recording

The chosen slice was placed onto an MEA chip (Menigoz et al., 2016; Shaban et al., 2017) and aligned so that a stimulating electrode was located over the Schaffer collateral path leaving the CA3, heading towards the CA1, and a recording electrode was situated in the middle of the CA1 striatum radiatum (200 µm 'downstream' of the stimulus electrode) (O'Dell et al., 1991). The slice was held with a nylon mesh anchor. A benefit of using an MEA chip is that it allowed for an additional recording electrode to be aligned in the striatum oriens, to record the somatic population spike of the stimulated neurons (Figure 3.1).

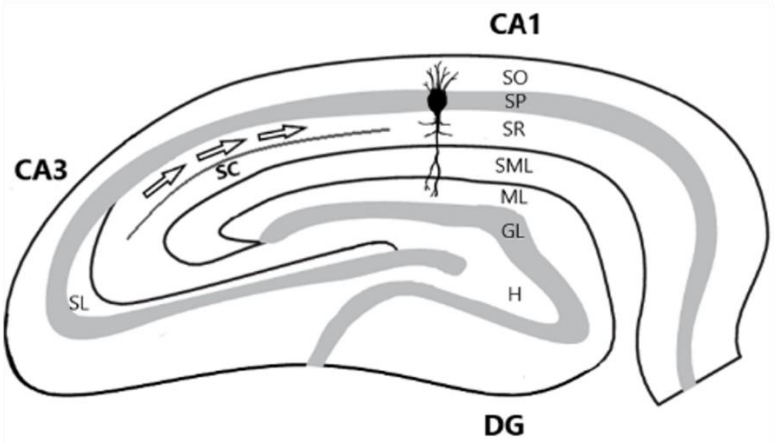


Figure 3.1 Diagram of hippocampal section featuring hippocampal layers and Schaffer Collateral (SC) pathways from CA3 to CA1. SO: Stratum Oriens, SP: Stratum Pyramidale, SR: Stratum Radiatum, SML: Stratum Lacunosum Moleculare, ML: molecular layer of the dentate gyrus, GL: Granule cell layer, H: Hilus of the dentate gyrus, SL: Stratum lucidum, DG: Dentate gyrus.

Perfusion rates of the aCSF (heated to 31–32°C) were 2.5 ml/min in, 4 ml/min out, which optimally maintained fluid volume while minimising turbulent flow forces.

After testing the presence of field excitatory post-synaptic potential (fEPSP) responses with a small bipolar current (20 μA), the slice was left to recover in the MEA chip with bipolar test pulse stimulation every 5 minutes to monitor recovery.

Once fEPSP slope measurements were stable (±15%) for approximately 15 min (typically 1.5 hrs after placement), an input/output curve was computed by increasing the stimulation current from 1 μA to 150 μA with a pulse every 30s and measuring the slopes of the resultant fEPSPs, taking an average of three sequential stimulation ramps. All subsequent stimulation was performed at an intensity eliciting approximately 35+/-5% of the maximum response of each slice. Subsequently, a test of short-term synaptic plasticity was performed with a paired-pulse stimulation protocol (PPS). Inter-pulse intervals used were: 25, 50, 75, 100, 150 and 200ms. A 30s interval between each of these paired pulse events was maintained, and this train of six paired pulses was repeated three times.

Once both input/output and paired-pulse facilitation (PPF) tests were performed, the slice was again stimulated using single bipolar pulses every 30s. To test LTP, a baseline consisting of at least 30 minutes of stable recordings was acquired, during which fEPSP slope did not exceed ± 10% of itself for more than 2 minutes. Immediately following this 30-minute baseline the Schaffer collateral pathway was stimulated with a theta burst (TBS) protocol to induce LTP. Each TBS consisted of four 100Hz pulses, repeated 10 times at 5Hz. Three theta bursts (3xTBS) were delivered with an inter-burst interval of 30s. After LTP induction, the stimulation frequency reverted to a single pulse every 30s as in baseline for total of two hours post-tetanus.

All stimulation protocols were designed in MCStimulusII (Multichannel Systems, Germany) and played during recordings via MCRack (Multichannel Systems, Germany) software.

2.6 Data Analysis and Statistical Methods

Data files were converted to '.abf' files and the slope (capturing 80%–90% of the inward current slope) of fEPSPs was extracted using semi-automated procedures within Clampfit software. To extract the amplitude of the population spike, we subtracted the trough of the spike from an average of the adjacent peaks. For input/output relationships and PPF the average of three responses at each stimulus intensity/interval was used. For the test of LTP, data values of fEPSP slopes were expressed as a percentage of the average of the baseline period fEPSPs (30 min prior to TBS), and an average of the final 10 min of this baseline (20 stimuli) were used for statistical analysis (and used for representation in LTP figures).

Parametric tests were utilised for all statistical analyses after appropriate checks for data normality. Specific test details are given in the results. Individual slices (each originating from an individual animal) were treated as subjects, sex and treatment group status of the donor animals was treated as fixed factors. Data originating from individual slices were treated as repeated measures (post TBS data).

3 Results

3.1 Microbiome Depletion does not Affect Basic Synaptic Efficacy or Short-Term Plasticity in Adult Mice

To test whether microbiome depletion affected basal synaptic efficacy in hippocampal slices we computed "input-output" characteristics of the fibre volley and resulting fEPSP at a variety of stimulus intensities (Figure 3.2 a, b). We summarised each slice's data through calculating the linear slope of this relationship (C.-H. Kim et al., 2005; Woo et al., 2005) (Figure 3.2 a, b). A univariate general linear model revealed that microbiome depletion with either an antibiotic cocktail or vancomycin on its own affected basal synaptic efficacy ($F_{2, 48} = 0.6$, $p = 0.692$). No effect of sex was found ($F_{1, 48} = 0.6$, $p = 0.330$). This suggests that microbiome depletion does not exert any effect over basal synaptic excitability despite male Vancomycin-treated slices exhibiting a tendency toward increased values (Figure 3.2 a).

In conventional animals and under normal conditions, when two excitatory stimuli occur within a short space of time of each other the second post-synaptic response will be enhanced (Creager et al., 1980). This short-term facilitation is a result of pre-synaptic mechanisms leading to an increase in neurotransmitter release (Jackman & Regehr, 2017). To establish whether microbiome depletion had any effect on presynaptic function, we tested short-term plasticity using paired-pulse stimulation protocols. Maximal facilitation reached approximately 170% in male slices and 150% in female slices at an inter-pulse interval of 50ms. Linear mixed model analysis revealed no main effect of microbiome depletion was found at any inter-stimulus interval. However, the greatest effect size difference was seen between male control and vancomycin-treated groups at the 25ms inter-stimulus interval (Figure 3.2 c). A main effect of sex was found however, at 200ms ($F_{1, 43} = 11.185$, $p = 0.002$). Pairwise comparisons revealed this effect was only present between males and female of Antibiotic Cocktail treated groups ($F_{1, 43} = 9.236$, $p = 0.004$). While no interaction effect was present between

sex and treatment ($F_{1, 43} = 0.964$, $p = 0.389$), the lack of a sex difference between control and vancomycin-treated groups while one exists in antibiotic treated groups does indicate a subtle sex-dependent effect of a more restricted microbiome depletion as opposed to broad knockdown of the microbiota.

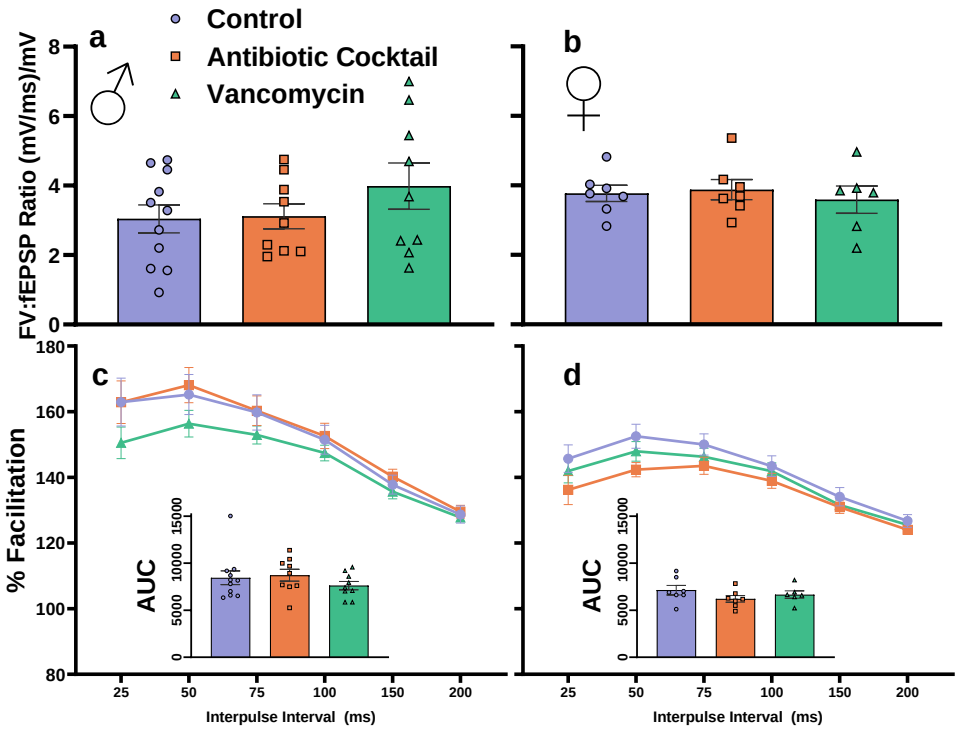


Figure 3.2 (a, b) Basal excitability summarised with the linear slope of each slice through an "input-output" protocol measuring responses through a series of increasing stimulation strengths. (c, d) Paired-pulse facilitation of fEPSPs. No statistically significant differences were found between slices pre- and post-SCFA treatment. Error bars represent standard error of the mean. Male N Control/Antibiotic Cocktail/Vancomycin = 11/9/9, Female Control/Antibiotic Cocktail/Vancomycin = 7/7/6.

3.2 Microbiome Depletion Leaves Hippocampal Long-Term Potentiation Unchanged

After a 30-minute baseline recording period, LTP was induced using three trains of theta burst stimulation (3xTBS) (Figure 3.3). This produced a rapid potentiation of fEPSPs that lasted for a recording period of two hours (Control: $Mean^{male}$ = 157.1% SEM 4.98, $Mean^{female}$ = 152.9% SEM 6.2, Antibiotic Cocktail: $Mean^{male}$ =156.6% SEM 5.5, $Mean^{female}$ = 141.9% SEM 6.2, Vancomycin: $Mean^{male}$ = 154.8% SEM 5.5, $Mean^{female}$ = 141.7% SEM 6.7). The degree of LTP did not change in

antibiotic cocktail nor vancomycin treated groups when compared to control groups. General Linear Model with Repeated Measures at two hours post TBS revealed no significant effect of antibiotic treatment ($F(2, 43) = 0.809$, $p = 0.452$), but did reveal a significant effect of sex ($F(1, 43) = 4.992$, $p = 0.031$). No interaction between sex and antibiotic treatment was found ($F(2, 43) = 0.487$, $p = 0.618$).

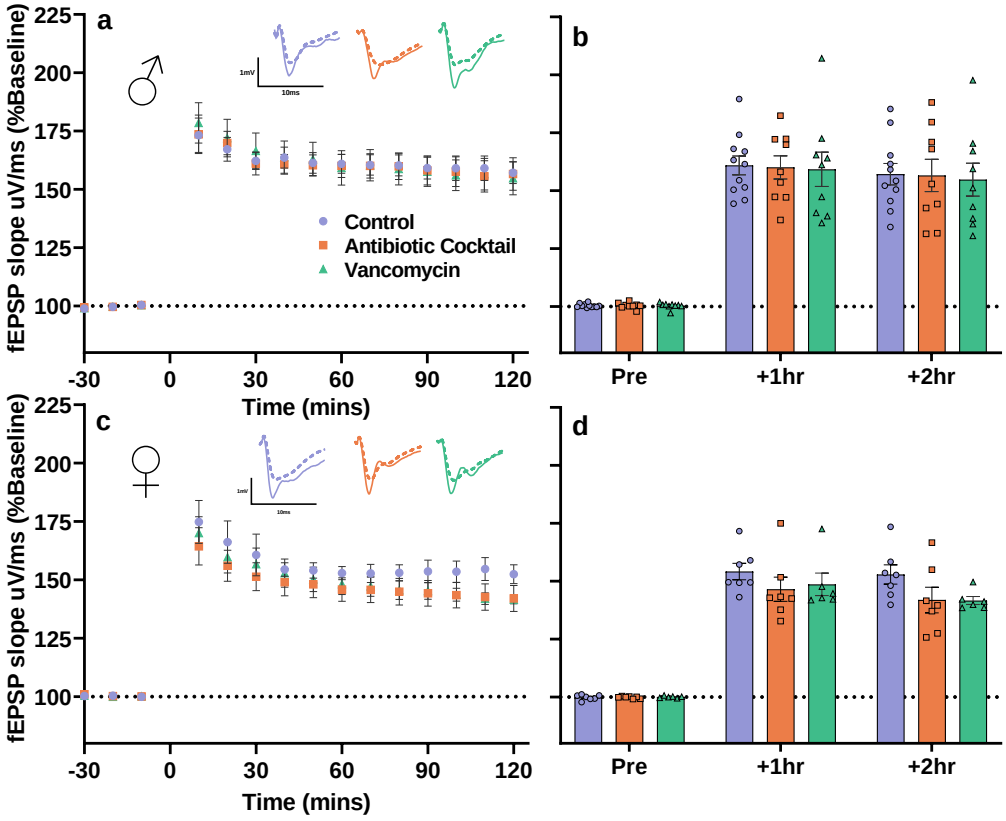


Figure 3.3 (a and c) Timeline of normalised CA1 fEPSP slopes for control, antibiotic cocktail, and vancomycin-treated groups with male slices featured in (a) and female slices featured in (c). No difference was noted between treatment groups. Representative traces at both pre (dashed lines) and post (solid lines) for each group. Scale bar is 1mV x 10ms. (b and d) Bar chart summarising pre, 1hr post, and 2hr post LTP induction fEPSP responses. Error bars represent standard error of the mean. Male N Control/Antibiotic Cocktail/Vancomycin = 11/9/9, Female Control/Antibiotic Cocktail/Vancomycin = 7/7/6.

3.3 Microbiome Depletion does not Affect Somatic Responses after Induction of Hippocampal Long-Term Potentiation.

We were able to concurrently record the population spike responses to TBS stimulation (Figure 3.4). In all groups, 3xTBS led to sustained potentiation of pop. spike amplitude for 2hr post induction (Control: $Mean^{male}$ = 294.5% SEM 23.1, $Mean^{female}$ = 210.1% SEM 29.0, Antibiotic Cocktail: $Mean^{male}$ = 268.5% SEM 25.6, $Mean^{female}$ = 214.0% SEM 29.0, Vancomycin: $Mean^{male}$ = 247.7% SEM 25.6, $Mean^{female}$ = 228.5% SEM 31.3). General Linear Model with Repeated Measures at two hours post TBS stimulation revealed no significant effect of microbiome depletion (F 2, 43 = 0.152, p = 0.860) and no interaction effect (F 2, 43 = 0.706, p = 0.499), but did reveal a significant effect of sex (F 2, 43 = 5.548, p = 0.023). Pairwise comparisons revealed that the significant effect of sex only existed between control animals (p = 0.028). This shows that while population spike potentiation in response to 3xTBS is comparable between control and antibiotic-treated adult mice, there may exist a subtle effect of microbiome treatment which obfuscates the sex differences otherwise present.

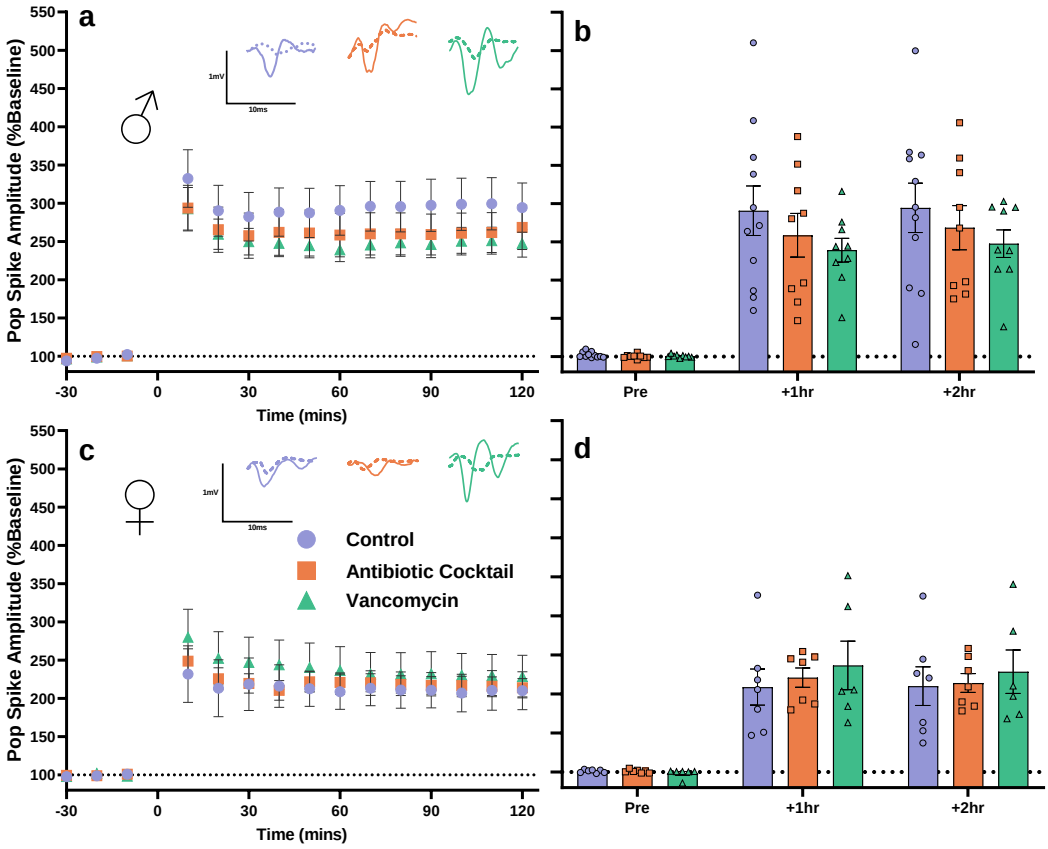


Figure 3.4 (a and c) Timeline of normalised CA1 pop. Spike amplitudes for control, antibiotic cocktail-, and vancomycin-treated groups with male slices featured in (a) and female slices featured in (c). No difference was noted between treatment groups. Representative traces at both pre (dashed lines) and post (solid lines) for each group. Scale bar is 1mV x 10ms. (b and d) Bar chart summarising pre, 1hr post, and 2hr post LTP induction pop. spike responses. Error bars represent standard error of the mean. Male N Control/Antibiotic Cocktail/Vancomycin = 11/9/9, Female Control/Antibiotic Cocktail/Vancomycin = 7/7/6

3.4 Microbiome Depletion does not alter Excitatory Postsynaptic Potential-Spike Coupling

While not significant, the contrast between the effects of TBS on the dendritic and somatic responses of slices from control and antibiotic-treated mice suggested a difference in the ability of the neuronal populations examined to integrate synaptic excitation to generate an action potential (E-S coupling). Before TBS stimulation general Linear Model with Repeated Measures revealed no significant effect of microbiome depletion (F 2, 43 = 1.415, p = 0.254), no significant effect of sex (F 1, 43 = 0.819, p = 0.370) and no interaction effect (F 2, 43 = 0.804, p = 0.454). All groups exhibit an increase in the E-S coupling ratio

(here representing an increase in pop. spike amplitude for a given fEPSP response) that is maintained for the duration of the recordings (Figure 3.5). However, general Linear Model with Repeated Measures at two hours post TBS stimulation also revealed no significant effect of microbiome depletion (F 2, 43 = 1.345, p = 0.271), no significant effect of sex (F 1, 43 = 1.976, p = 0.167) and no interaction effect (F 2, 43 = .238, p = 0.789).

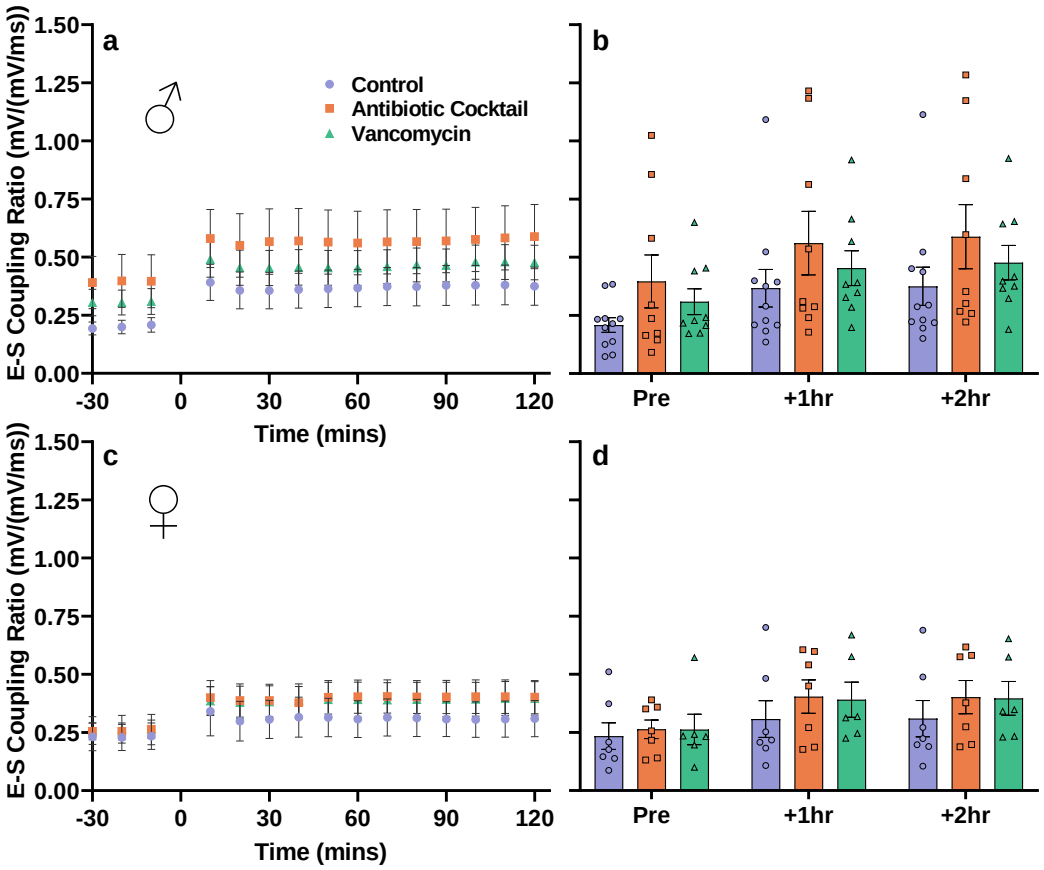


Figure 3.5 (a and c) Timeline of normalised CA1 E-S coupling for control, antibiotic cocktail, and vancomycin-treated groups with male slices featured in (a) and female slices featured in (c). No difference was noted between treatment groups (b) and (d). Bar chart summarising pre, 1hr post, and 2hr post LTP induction E-S coupling. Error bars represent standard error of the mean. Male N Control/Antibiotic Cocktail/Vancomycin = 11/9/9, Female Control/Antibiotic Cocktail/Vancomycin = 7/7/6

4 Discussion

Previously, this lab has found that a germ-free upbringing produces deficits in hippocampal plasticity that affect male but not female mice (Darch et al., 2021). Additionally, other studies show that the behavioural correlates of LTP - learning and memory - can be disrupted by microbiome depletion in early adulthood (Fröhlich et al., 2016; Möhle et al., 2016). Indeed, similar antibiotic regimens in adult male mice have been found to lead to hippocampal dysfunctions including reduced synaptic transmission and gamma oscillations (Çalışkan et al., 2022). This led us to hypothesise that hippocampal plasticity processes may be vulnerable to microbiome depletion during adulthood. LTP is widely considered to be one of the primary cellular mechanisms underlying learning and memory. In this study, we find that a 2-week antibiotic treatment did not affect hippocampal LTP, short-term plasticity as measured by paired-pulse facilitation, or basic synaptic efficacy in the hippocampus of male and female mice. These findings suggest that hippocampal plasticity is resilient to microbiome depletion in adulthood.

Given the vulnerability of learning and memory to microbiome depletion in adulthood in previous studies, these findings were unexpected. Several factors may account for this discrepancy, including variations in microbiome depletion protocols, differences in the specific learning and memory processes evaluated, and potential sex differences. Previous data reported that antibiotic treatment reduced memory retention, as assessed by novel object recognition (Möhle et al., 2016). It is worth noting that this study involved female mice exclusively, and their antibiotic treatment lasted 7 weeks, compared to the 2-week treatment used in our study (Möhle et al., 2016). Moreover, we have previously shown in male rats that treatment with a combination of antibiotics (including metronidazole) in adulthood (10 weeks old at start of treatment) that continued throughout behavioural testing for a total of 13 weeks, led to spatial memory deficits in the Morris Water Maze (Hoban, Moloney, et al., 2016). This suggests that a longer treatment duration may be necessary for functional changes in

hippocampal plasticity to manifest. It is also worth noting that many early studies included metronidazole in their cocktail. Now there is a greater appreciation that this antibiotic readily penetrates the blood brain barrier following oral administration (Jokipii et al., 1977; E. J. Olson et al., 2005). This may induce local effects in the brain including neurotoxicity (K.-I. Park et al., 2011) through inhibition of protein synthesis (W. G. Bradley et al., 1977), a process upon which LTP is dependent (Abraham & Williams, 2008).

In contrast, some studies employed an antibiotic regimen more comparable to ours (Fröhlich et al., 2016). In this study, which examined only adult male mice, differences were also found in novel object recognition. While this test examines behaviour that is reliant on the hippocampus (Broadbent et al., 2010), it also involves other regions such as the perirhinal cortex (Cinalli Jr. et al., 2020). It is possible that electrophysiological examination of this region might have revealed changes to LTP in our study. Further, there exists conflicts regarding the role of the hippocampus in object recognition memory (Barker & Warburton, 2011). Spatial memory, a process dependent on the dorsal hippocampus (Duda & Węsierska, 2021) was insensitive to antibiotic administration (Fröhlich et al., 2016). Our study which examined the mid-dorsal hippocampus aligns with this.

Finally, other studies found long-term memory to be affected in males by microbiome depletion by antibiotics starting at 6 weeks (Takahashi et al., 2024). However, this was measured by the passive avoidance test which is once again more associated with the ventral hippocampus (Bryant & Barker, 2020). In agreement with the lack of changes seen in synaptic plasticity in this study they found spatial working memory as examined via the y-maze test to be insensitive to antibiotic administration (Fröhlich et al., 2016).

This comparison of our work with that of other studies leads us to question where future studies should direct their attention. There exists a clear bias in studies concerning microbiome depletion and learning and memory toward the examination of only a single sex. This may occlude the discovery of potential sex differences considering the previous microbiome-associated sex differences

noted by this lab (Darch et al., 2021) and others (Geary et al., 2021; Jaggar et al., 2020). Furthermore, longer microbiome depletion interventions and those starting at a younger age appear to have greater capacity to induce changes in learning and memory (Fröhlich et al., 2016; Möhle et al., 2016; Takahashi et al., 2024).

While hippocampal plasticity has shown resilience to microbiome depletion in adulthood, in the current study this does not preclude the possibility that microbiome depletion at earlier stages of development may affect this process. Evidence now points to learning and memory and synaptic plasticity being more vulnerable to insults during neurodevelopment; previous work by this lab shows that maternal antibiotic administration can affect novel object recognition in adult offspring (O'Connor et al., 2021) and that germ-free adult mice display synaptic plasticity deficits (Darch et al., 2021). Early-life microbiome interventions during various critical windows of neurodevelopment may then help paint a picture of learning and memory and synaptic plasticity being especially vulnerable during this period. Indeed, early-life antibiotic administration has revealed gene expression changes in the amygdala and prefrontal cortex accompanied by enduring changes to anxiety-like and compulsive behaviour (C. M. K. Lynch et al., 2023). Despite a prior study on early-life antibiotic administration in rat pups reporting no spatial memory impairments that would be indicative of hippocampal plasticity deficits (O'Mahony et al., 2014), there is a large degree of variance for susceptibility of behavioural changes to antibiotic administration (Olavarría-Ramírez et al., 2023). Thus, the possibility of a critical window of plasticity development that is vulnerable to microbiome insult remains.

We conclude that microbiome depletion does not lead to any robust alteration of hippocampal LTP or electrophysiological measures in adulthood. Given that we have shown that antibiotic depletion in adolescence leads to more robust changes than that in adulthood, when examining behavioural and transcriptomic measures in adulthood (Lach et al., 2020), future work should target ages at Chapter 5.

Chapter 4

Modulation of Murine Hippocampal Synaptic Plasticity by Microbial Metabolites: Sex-Specific Effects of the Short-Chain Fatty Acid Butyrate

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In Preparation

Abstract

The microbiota-gut-brain-axis refers to a system of bidirectional communication between the trillions of microbes within the gastrointestinal tract and the brain. This communication influence on human health and disease and has been implicated in brain processes such as learning and memory. However, the mechanisms of communication between the microbiota and brain are not fully resolved. Microbial metabolites such as short-chain fatty acids (SCFAs) produced mainly through the fermentation of dietary fibre by gut microbes, are one of the key potential mediators of this communication network. However, the impact they have on brain physiology remains largely unexplored. Here, we examined the effects of the three most abundantly produced SCFAs in the gut, acetate, butyrate, and propionate, on *ex vivo* hippocampal slice electrophysiology to explore potential sex-specific mechanisms underlying their action. Importantly, we used concentrations considered to be physiologically relevant to ensure the translatability of this study.

Our findings revealed that a 40-minute exposure to 3µmol butyrate enhanced long-term potentiation (LTP) in both male and female mice. As butyrate preferentially activates the free fatty acid receptor 3 (FFAR3) we showed that its inhibition using β-Hydroxybutyrate (BHB) abolishes the enhanced potentiating effect of butyrate on LTP in slices (from female mice while leaving its efficacy intact in slices from male counterparts. Notably BHB alone did not affect LTP in either sex. To elucidate potential sources of this dimorphism we examined mRNA expression levels of FFARs in CA1 hippocampal tissue from male and female animals. Regarding other SCFAs there was no major effects of acetate or propionate on LTP under the same stimulation paradigms, or over basic synaptic efficacy and short-term plasticity as measured by paired-pulse facilitation.

To conclude, our study provides novel insights into the sex-specific modulation of hippocampal synaptic plasticity by butyrate. Moreover, our data suggests that FFAR3 activation is key to such effects in females. This data highlights the potential ability of the gut microbiota to shape hippocampal function. Studies

investigating the behavioural consequences of enhancing hippocampal butyrate are now warranted, as are mechanistic studies parsing the underpinning of the sex differences unmasked here.

1 Introduction

Growing evidence suggests that the gut microbiota can modulate brain and behaviour in both health and disease (Cryan et al., 2019). The mechanisms of communication between the gut microbiota and brain are slowly being deconvoluted (Mayer et al., 2022). One of the key routes of this bi-directional communication between the gut microbiota and the brain is through microbial metabolites (Cryan et al., 2019a; O'Riordan et al., 2022; Rahman et al., 2023). These are primarily produced in our gut by microbial fermentation of the food we eat. Microbial metabolites exert their effect not only in the gut but can make their way into the systemic circulation and from there a limited subset passes through the BBB into the brain (Dalile et al., 2019; Logsdon et al., 2018).

SCFAs are some of the most abundantly produced microbial metabolites in the gut and one of the most well-studied. Three of these, acetate, propionate, and butyrate, make up 95% of the SCFAs produced in a 60:20:20 ratio respectively (Binder, 2010). SCFAs have been found to be required for the normal functioning of physiological processes such as innate and adaptive immunity, energy expenditure, metabolism, circadian rhythms, appetite, and sleep (He et al., 2020; C. H. Kim, 2023; Magzal et al., 2021; Sukkar et al., 2019; Tahara et al., 2018). Importantly, they potentially play a role in cognition and learning and memory (Levenson et al., 2004; Liao et al., 2022). Many neuropsychiatric and neurodevelopmental disorders which feature changes in cognition and learning and memory see altered levels of SCFAs (Lan et al., 2024). These conditions include stroke, multiple sclerosis, traumatic brain injury, Parkinson's disease, and Alzheimer's disease (Barcutean et al., 2024; Kwon et al., 2024; Opeyemi et al., 2021; Qian et al., 2022; W. Zhang et al., 2024).

SCFAs have multiple possible mechanisms through which they influence the gut-brain axis, including provision of energy, histone deacetylase (HDAC) inhibition and as endogenous ligands for G-protein coupled receptors (GPCR), mainly FFAR3, FFAR2, and GPR109A (Binder, 2010; Licciardi et al., 2011; M. Sun et al., 2017). While some studies exist demonstrating propionate can affect

learning and memory processes (Lang et al., 2022; Wu et al., 2022), there seems to be a paucity of similar data concerning acetate. Butyrate on the other hand, is extensively studied at high doses, particularly in its capacity to affect these processes through HDAC inhibition as it is the most potent HDAC inhibitor of the three main SCFAs (Waldecker et al., 2008). Butyrate injections at supraphysiological doses have been found to improve and restore learning and memory through HDAC inhibition in various mouse models (Fischer et al., 2007; Levenson et al., 2004; Stafford et al., 2012; Vinarskaya et al., 2021). Some of this research has translated to humans where colonic butyrate supplementation was found to alter subjective fear memory (Dalile et al., 2024).

Synaptic plasticity, the activity-based process which underlies learning and memory by producing lasting changes to synapses (T. V. Bliss & Lomo, 1973; Magee & Grienberger, 2020) is affected by butyrate. Butyrate supplementation recovered deficits in plasticity-related protein expression (Jiang et al., 2021). Meanwhile in electrophysiological studies of the hippocampus, the key area of the brain involved in learning and memory, (Anand & Dhikav, 2012), butyrate has been found to change functional synaptic plasticity (Belén Sanz-Martos et al., 2022; Levenson et al., 2004). However, these studies focus primarily on butyrate's action as a HDAC inhibitor and thus at relatively high supraphysiological doses. Less exploration has been undertaken into butyrate and other SCFA's in their capacity to change learning and memory and synaptic plasticity as agonists of FFARs.

As previously mentioned, SCFAs bind to certain GPCRs. These receptors include FFAR3, FFAR2, and Hydroxycarboxylic acid receptor 2 are expressed in a wide variety of tissues such as the colon (Karaki et al., 2006; Tazoe et al., 2009), vasculature (Nooromid et al., 2020; F. Yu et al., 2024), and the brain of humans and rodents including mice (Bonini et al., 1997; Erny, Hrabě de Angelis, et al., 2015; Razazan et al., 2021; Tran & Mohajeri, 2021; Zamarbide et al., 2022; Z. Zhou et al., 2021). FFAR3 receptor signalling changes a variety of synaptic plasticity-associated factors (Ang et al., 2018).

Given the extensive influence of SCFAs, particularly butyrate, on learning and memory we sought to characterise the possible local effects of SCFAs on hippocampal synaptic plasticity processes following their passage through the BBB into the brain. Thus far SCFAs have been used in supraphysiological concentrations. Here we explored the neurobiological effects of SCFAs at physiological relevant concentrations (Perry et al., 1970). To do this we used *ex vivo* electrophysiological methods with direct exposure of hippocampal brain slices to these physiological levels of SCFAs.

2 Methods

2.1 Animals

C57BL/6 mice were purchased from Envigo. Animals were housed in cages of 2-3 per cage (area:330cm^2. NKP isotech, UK). Cages had paper bedding/nesting material and were kept in controlled conditions (20–21°C, 55%–60% humidity) on a 12-hr light/dark cycle, with access *ad libitum* to autoclaved chow and water. On each recording day, one to two animals (8–13 weeks old) were separated into their own cage and brought to the laboratory for use in the experiments, which were undertaken in accordance with National and European legislation (Directive 2010/63/EU and Irish SI No 543 of 2012).

2.2 aCSF Content

The aCSF was made up of (in mM): NaCl (124), KCl (2.7), NaH2PO4 (1.25), $CaCl_2$ (2), $MgSO_4$ (1.3), D-Glucose (18) and Ascorbic acid (2).

Cutting aCSF was made up of the same aCSF, with excess $MgSO_4$ (8.3 mM) and cooled to between 0-4°C.

2.3 Slice Preparation

Hippocampal slice electrophysiology is a well-used technique and our methods followed common practices (Papouin & Haydon, 2018) and those of our previous publications (Darch et al., 2021). Fresh artificial cerebrospinal fluid (aCSF) was made (see below) and bubbled with carbogen (95% O_2/5% CO_2) for at least 40 minutes prior to use, and continually throughout recordings.

To minimize any effect of differential stress response, all animals were left to acclimatize to the experimental room for at least 1 hour following transfer from the animal unit. Ice-cold cutting aCSF (see above protocol) was used for the entirety of the dissection. Mice were rapidly decapitated, and the brain removed and cooled with ice-cold aCSF. The caudal third of the brain was removed with a razor blade, making a cut just rostral to the cerebellum. This created a flat

surface for the brain to be glued to the stage of a Leica VT1200 vibratome. An agar block support was also glued on its ventral side to aid slicing stability. 300µm coronal slices of the brain were cut under aCSF.

Slices were transferred to a chilled petri dish, filled with ice cold cutting aCSF, and the hippocampus was isolated from the slices. A selection of slices spanning the central portion of the dorso-ventral axis (approximately 1.5–2.1mm from dorsal end) were transferred to mesh holding wells in a bath of standard aCSF held at 32°C for 45 min, and then at room temperature for a further 45 minutes.

After this recovery period, slices were transferred to a multi-electrode array (MEA) chip (Multichannel Systems, Germany), perfused with aCSF. Slices were viewed under an inverted microscope (4x magnification, Olympus IX70) and compared for structural integrity and general slice health. Following elicitation of test pulses, the best candidate slices were used for electrophysiological testing.

2.4 Electrophysiology Recording

The slices were oriented on the MEA chip (Menigoz et al., 2016; Shaban et al., 2017) and aligned so that a stimulating electrode was located over the Schaffer collateral path leaving the CA3, heading towards the CA1, and a recording electrode was situated in the middle of the CA1 striatum radiatum (200µm 'downstream' of the stimulus electrode) (O'Dell et al., 1991). The slice was held in place with a nylon mesh anchor.

Perfusion rates of the aCSF (heated to 31–32°C) were 2.5 ml/min in, 4 ml/min out, which optimally maintained fluid volume while minimizing turbulent flow forces.

After testing the presence of field excitatory post-synaptic potential (fEPSP) responses with a small bipolar current (20µA), the slice was left to recover in the MEA chip with bipolar test pulse stimulation every 5 minutes.

Once fEPSP slope measurements were stable (±15%) for approximately 15 min (typically 1.5 hrs after placement), an input/output (I/O) curve was computed by increasing the stimulation current from 1μA to 150μA with a pulse every 30s and measuring the slopes of the resultant fEPSPs, taking an average of three sequential stimulation ramps. All subsequent stimulation was performed at an intensity eliciting approximately 35+/-5% of the maximum response of each slice. Subsequently, a test of short-term synaptic plasticity was performed with a paired-pulse facilitation protocol (PPF). Inter-pulse intervals were 25, 50, 75, 100, 150 and 200ms. A 30s interval between each of these paired pulse events was maintained, and this train of 6 paired pulses was repeated three times. For testing possible SCFA effects on I/O curves and PPF, SCFA was added to perfusing aCSF 20 mins prior to I/O start. SCFA aCSF perfusion continued for subsequent PPF tests. SCFA-exposed I/O and PPF tests were performed on different animals and different days to LTP experiments.

Once both I/O, and PPF tests were performed, the slice was again stimulated using single bipolar pulses every 30s. To test LTP a baseline consisting of three components was acquired, these will be described in chronological order; **1.** A period of 30 min in which the fEPSP slope did not exceed ± 10% of itself for more than 2 minutes (defined here to be the baseline period). **2.** A ten-minute period during which an inhibitory compound was added for test or no further addition was made for controls. **3.** A 20-minute period, at the start of which either an SCFA was added to the source aCSF, or no further addition was made for controls. Immediately following this baseline which totalled 1 hour, the Schaffer collateral pathway was stimulated with a theta burst (TBS) protocol to induce LTP. Each TBS consisted of four 100Hz pulses, repeated 10 times at 5Hz. Three theta bursts (3xTBS) were delivered with an inter-burst interval of 30 s. SCFA infused aCSF continued to perfuse the slice for 20 mins post LTP induction after which normal aCSF perfusion was resumed. After LTP induction, the stimulator reverted to a single pulse every 30 s as in baseline for total of two hours.

All stimulation protocols were designed in MCStimulusII (Multichannel Systems, Germany) and played during recordings via MCRack (Multichannel Systems, Germany) software.

2.5 Concentrations

To investigate the local action of acetate, propionate, and butyrate in the hippocampus we searched the literature for appropriate concentrations. Here we find that data on SCFA concentrations is limited. Ranges are found for "normal values", but as the information is collected entirely from Canadian patient populations, they are normal for one part of the world in a limited demographic. Furthermore, we know SCFAs to be manipulable through a range of factors including diet (Ilyés et al., 2022). Concentrations chosen for acetate, propionate, and butyrate match the approximate high end of the range found in (Perry et al., 1970) at 171, 6, and 3uM respectively.

In choosing the concentration of acetate, propionate, and butyrate we searched for studies that report human physiological brain concentrations of SCFAs. We found the literature to be limited and included several stipulating factors that must be accounted for. Ranges are found for "normal values", but the information is collected entirely from Canadian patient populations (Commodari et al., 1991; Perry et al., 1970; Wishart et al., 2008). These samples came from a variety of sources including patients with no known metabolic, neurological, or mental disease that were undergoing elective surgery for benign prostatic hypertrophy, inguinal hernia, varicosities of leg veins, and sterilisation by tubal ligation (Perry et al., 1970); patients undergoing neurological investigation with normal protein, glucose and immunoglobulin levels and/or patients with a final diagnosis that was generally associated with normal CSF (Commodari et al., 1991); and patients screened for meningitis (Wishart et al., 2008). Further, as SCFA levels rise postprandially and start to drop 30 minutes post food ingestion (Nogal et al., 2023) these SCFA concentrations may be lowered due to CSF sampling taken during a time where the patient may be fasted pre-surgery. We must

acknowledge therefore that these values taken from the literature offer a limited view of brain SCFA concentrations. Further, we know SCFAs to be manipulable through a range of factors including diet and therefore brain SCFA concentrations may be quite different in other populations (Ilyés et al., 2022). Notably, the one source for acetate CSF concentrations in healthy children shows them to be much higher than the adult range at 284.0 +/- 126.0μM (Subramanian et al., 2005). Quantification of SCFAs in human brain tissue by another source lists them as 17.0pmol/mg of tissue for butyrate and 18.8pmol/mg of tissue for propionate (Bachmann et al., 1979). This data was recalculated to molarity in brain water in a review by Fock and Parnova as 21μM for butyrate and 23μM for propionate, making brain concentrations of SCFAs in the same order of magnitude as those in the plasma (Fock & Parnova, 2023) and 4-9 times higher than our chosen concentrations.

2.6 Quantitative PCR

RNA was reverse transcribed to cDNA using a High-capacity cDNA reverse transcription kit (Thermo Fischer Scientific). Gene expression was assessed by TaqMan Gene Expression Master Mix (Applied Biosystems) using the LightCycler 480 Real-Time PCR System. Taqman primer sequences for Ffar3 and Ffar2 were used. Each sample was run in duplicate and cycle threshold (CT) values were used for gene expression calculations, and all CT values were normalised to the expression of Gadph. Female values were then normalised to average male expression. Relative gene expression was calculated using the equation 2-ΔΔCT.

2.7 Data Analysis and Statistical Methods

Data files were converted to '.abf' files and the slope (capturing 80%–90% of the inward current slope) of fEPSPs was extracted using semi-automated procedures within Clampfit software. To extract the amplitude of the population spike, we subtracted the trough of the spike from an average of the adjacent peaks. For I/O relationships and PPF, the average of three responses at each stimulus intensity/interval was used. For the test of LTP, data values of fEPSP

slopes were expressed as a percentage of the average of the baseline period fEPSPs (30 minutes prior to TBS), and an average of 10 minutes (20 stimuli) was used for statistical analysis.

Parametric tests were utilised for all statistical analyses after appropriate checks for data normality. Specific test details are given in the results. Individual slices (each originating from an individual animal) were treated as subjects, sex and treatment group status of the donor animals was treated as fixed factors. Data originating from individual slices were treated as repeated measures (e.g. pre and post TBS data). Data originating from individual slices pre- and post-SCFA treatment for I/O and PPF testing were also treated as repeated measures.

3 Results

3.1 Butyrate Addition Increased Long-Term Potentiation: Effects of FFAR3 Antagonist, β-Hydroxybutyrate.

After a 60-minute baseline recording period, LTP was induced using 3xTBS. This produced a rapid potentiation of fEPSPs that lasted for a recording period of 2 hours (2hrs-post, males: mean 139.19% SEM 4.79, females 144.78% SEM 5.081). In both male and female slices the presence of added butyrate (3μM) for a 40-minute period surrounding LTP induction increased potentiation (2hrs-post, males: mean 165.75% SEM 5.43, females 167.99% SEM 5.43). However, when β-Hydroxybutyrate, an FFAR3 antagonist, was added 10 minutes before butyrate and kept in the solution until 20 minutes after LTP induction, slices from female animals did not display the increased LTP seen with butyrate alone (2hrs-post, females: mean 146.42% SEM 4.55). Slices from male animals however exhibited much the same level of LTP with butyrate and BHB as they did with just butyrate alone (2hrs-post, males: mean 158.60% SEM 5.08). Notably, no change in LTP due to BHB addition was found (2hrs-post, males: mean 134.87% SEM 5.08, females 46.78% SEM 5.43) (Figure 4.1).

General Linear Model with Repeated Measures revealed a significant effect of treatment group ($F(3, 56) = 10.395$, $p < 0.001$). No significant effect of sex was found ($F(1, 56) = .272$, $p = 0.604$), nor any significant interaction effect ($F(3,56) = 2.114$, $p = 0.109$). Butyrate-treated slice LTP showed increased LTP compared to control slices in both male and female slices (Male: $p = 0.003$, Female: $p = 0.017$). No effect was found of BHB on LTP compared to control slices ($p = 1.00$). Butyrate+BHB treatment was statistically significant to Butyrate treatment in female slices ($p = 0.021$). No effect was found between butyrate+BHB and butyrate treatment in male slices ($p = 1.00$). Šídák correction for multiple comparisons was used.

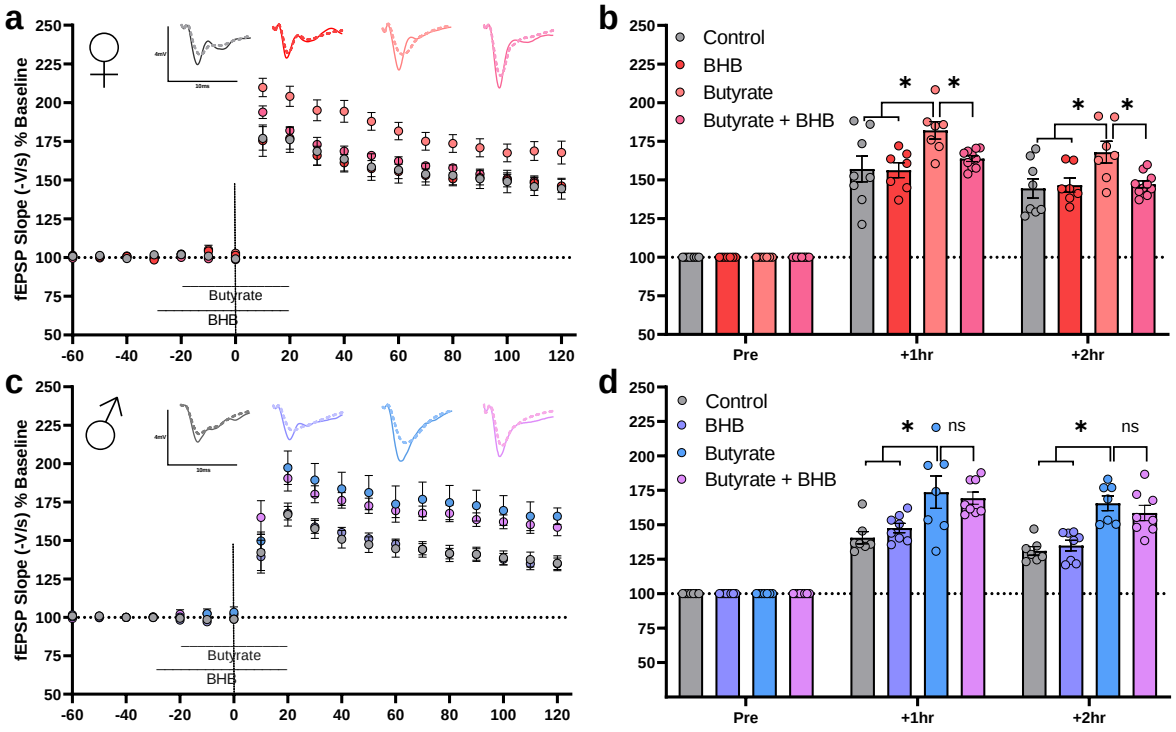


*Figure 4.1 (a and c) Timeline of normalised CA1 fEPSP slopes for control, BHB-, Butyrate-, and Butyrate+BHB-treated male and female slices. Male and female butyrate-treated slices exhibited increased potentiation at 1 and 2hrs post LTP induction compared to control slices. Female slices which underwent a preincubation with BHB before butyrate addition exhibited LTP similar to control slices at 1 and 2hrs post LTP induction. No change in LTP was seen to LTP with the sole addition of BHB. Representative traces at both pre (dashed lines) and post (solid lines) for each group. Scale bar is 1mV x 10ms. (b and d) Bar chart summarising pre, 1hr post, and 2hr post LTP induction fEPSP responses. Error bars represent standard error of the mean. Male N Control/BHB/Butyrate/Butyrate+BHB = 7/8/7/8, Female Control/BHB/Butyrate/Butyrate+BHB = 8/7/7/9. General Linear Model with Repeated Measures *p < .05.*

3.2 Neither Acetate nor Propionate Altered Long-Term Potentiation.

After a 60-minute baseline recording period, LTP was induced using 3xTBS. This produced a rapid potentiation of fEPSPs that lasted for a recording period of 2 hours (2hrs-post tetanus, males: mean 143.41% SEM 5.70, females 139.93% SEM 6.24) (Figure 4.2). The addition of acetate (171μM) or propionate (6μM) failed to change LTP (Acetate (2hrs-post, males: mean 143.72% SEM 6.24,

females 141.68% SEM 6.24), Propionate (2hrs-post, males: mean 145.54% SEM 5.69, females 142.35% SEM 5.69).

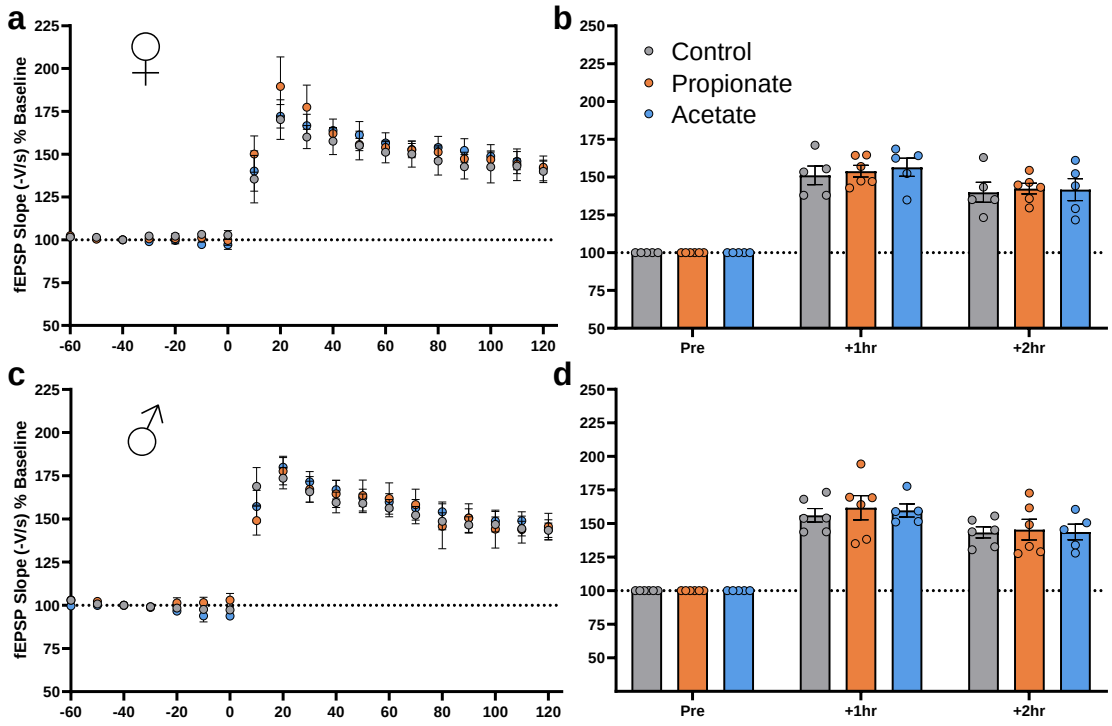


Figure 4.2 (a and c) Timeline of normalised CA1 fEPSP slopes for control, acetate- and propionate-treated male and female slices. No difference was noted between treatment groups. Representative traces at both pre (dashed lines) and post tetanus (solid lines) for each group. Scale bar is 1mV x 10ms. (b and d) Bar chart summarising pre, 1hr post, and 2hr post LTP induction fEPSP responses. Error bars represent standard error of the mean. Male N $_{Control/Propionate/Acetate}$ = 5/6/5, Female $_{Control/Propionate/Acetate}$ = 6/6/5. General Linear Model with Repeated Measures.

General linear model with repeated measures found no significant effect of slice treatment group ($F(2,27) = 0.076$, $p = 0.927$). No significant effect of sex was found ($F(1,27) = 0.345$, $p = 0.557$), nor any significant interaction effect ($F(2,27) = 0.008$, $p = 0.992$).

3.3 SCFA Exposure was not Found to Affect Basal Synaptic Efficacy or Short-Term Plasticity.

To test whether SCFA exposure affected basal synaptic efficacy in hippocampal slices we computed “input-output” characteristics of the fibre volley relative to

the resulting fEPSP slope at a variety of stimulus intensities (Figure 4.3). We summarised each slice's data through calculating the linear slope of this relationship (C.-H. Kim et al., 2005; Woo et al., 2005). Paired t-test with parametric datasets and Wilcoxon Signed Ranks test with non-parametric datasets revealed no significant difference between slices before and after exposure to either acetate, propionate, or butyrate which started 20 minutes pre "input-output" test and continued throughout the test (Acetate (Female, Mean Difference= -0.29, SEM 0.16, $^{2\text{-sided}}p = 0.09$), (Male, Mean Difference = -0.57, SEM 0.27, $^{2\text{-sided}}p = 0.13$), Propionate (Female ($Z = -0.73$, $p = 0.46$), Male ($Z = -1.36$, $p = 0.17$)) Butyrate (Female ($Z = -1.79$, $p = 0.75$), Male ($Z = -.73$, $p = 0.46$)) (Figure 4.3). This suggests that SCFA exposure does not affect basal synaptic excitability at the indicated concentrations.

In conventional animals and under normal conditions, when two excitatory stimuli occur within a short space of time of each other the second post-synaptic response will be enhanced (Creager et al., 1980). This short-term facilitation is a result of pre-synaptic mechanisms leading to an increase in neurotransmitter release (Jackman & Regehr, 2017). To establish whether SCFAs had any effect on presynaptic function, we tested short-term plasticity using paired-pulse facilitation protocols. Paired t-tests with parametric datasets and Wilcoxon tests with non-parametric datasets were employed to reveal whether there was any significant difference between slices before and after exposure to either acetate, propionate, or butyrate. One SCFA was added to the perfusate 20 minutes prior to "Input-output" tests which were run prior to paired-pulse facilitation tests in the same slices. Thus, slices were exposed to acetate, propionate, or butyrate only for 40-50 minutes prior to the test commencing. Acetate was found to reduce the amount of short-term facilitation at a 25ms inter-stimulus interval in female slices only ($z = -2.201$, $p = 0.028$) (Figure 4.3g). However, no corrections for multiple comparisons have been applied to this result so the possibility of type-1 error is relatively high (Figure 4.3i). No other significant difference in short-term plasticity was found.

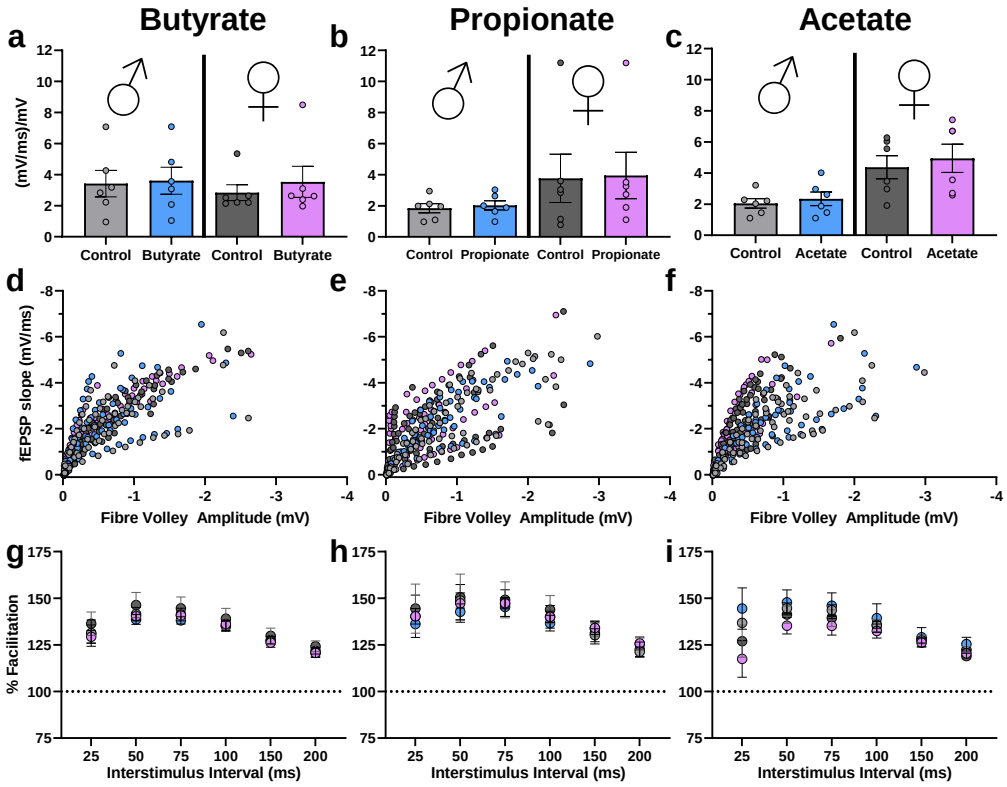


Figure 4.3 (a, b, and c) Basal excitability summarised with the linear slope of each slice through an "input-output" protocol measuring responses through a series of increasing stimulation strengths. (d, e, and f) The relationship between fibre volley amplitude and fEPSP slope of (d) acetate-, (e) propionate-, and (f) butyrate-untreated/treated slices. (g, h, and i) Paired-pulse facilitation of fEPSPs at six inter-pulse intervals (25, 50, 75, 100, 150, and 200ms). No statistically significant differences were found between slices pre- and post-SCFA treatment in all slices used for this thesis. Error bars represent standard error of the mean. (N = 6 mice/group).

3.4 Ffar3 RNA Levels do not Differ Between Sexes

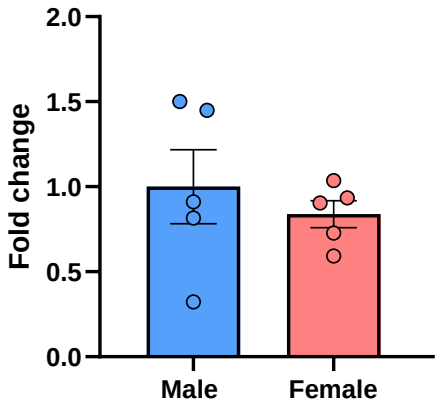


Figure 4.4 Ffar3 gene expression is not sex-dependent. Relative gene expression of Ffar3, normalised to expression of GAPDH in the mid-dorsal hippocampus (n = 5 mice/group) as measured by quantitative PCR. Data expressed as mean ± SEM; analysed by general linear model.

To test whether expression levels of Ffar3 and Ffar2 were different between sexes we conducted a quantitative PCR in control mice. One-way ANOVA revealed mid-dorsal hippocampal expression of Ffar3, is not affected by sex ($F(1, 0.482)$ $p = 0.507$) (Figure 4.4). Despite a lack of significant difference between males and females the variability from the mean in males tended to be greater (Standard deviation Male: 0.49, Female: 0.17). Ffar2 expression was not detected.

4 Discussion

LTP is widely considered to be one of the primary cellular mechanisms underlying learning and memory. Here, we show that the gut microbial metabolite butyrate has the capacity to alter this process in the murine hippocampus at a concentration relevant to that found in human cerebrospinal fluid. Meanwhile two other SCFAs, acetate and propionate failed to produce an effect on LTP.

We have previously shown that the absence of microbiota leads to deficits in LTP (Darch et al., 2021, **Chapter 2**). Here we show that absence of SCFAs may be responsible in part for such effects. In our investigation of SCFAs' impact on synaptic plasticity, we observed that butyrate elicited similar enhancements in hippocampal LTP in both male and female slices. The concentrations of SCFAs used were informed by prior research showing that this represents the approximate upper range of known physiological CSF levels in the human brain (Perry et al., 1970). However, as stated in the methods, quantification of SCFAs in human brain tissue by another source lists them as 17.0pmol/mg of tissue for butyrate and 18.8pmol/mg of tissue for propionate (Bachmann et al., 1979). This data was recalculated to molarity in brain water by Fock and Parnova as 21μM for butyrate and 23μM for propionate, making brain concentrations of SCFAs in the same order of magnitude as those in the plasma (Fock & Parnova, 2023) and 4-9 times higher than our chosen concentrations. This finding suggests that butyrate, a gut-derived microbial metabolite, at a concentration it exists at in the brain, may play a role in modulating hippocampal plasticity, and therefore learning and memory, under normal physiological conditions.

Given butyrate's pleiotropic effects on molecular targets in the body (Stilling et al., 2016) it was important to parse the mechanism that may underpin such effects. One likely candidate is through activation of the free fatty acid receptor FFAR3. We thus employed BHB, a compound that has been reported to function variably as either an antagonist or agonist of FFAR3 depending on the experimental context (Kimura et al., 2011; Won et al., 2013). Intriguingly, when

slices were pretreated with BHB in the perfusate, the butyrate-induced increase in LTP in female hippocampal slices was abolished. However, BHB only produced a relatively small non-statistically significant reduction in male slices.

This observed sex-specific difference raises important questions about the role of FFAR3 in mediating butyrate's effects on LTP. While analysis of hippocampal FFAR3 mRNA expression did not reveal a sex difference, expression did exhibit a tendency towards increased variability in males which may help to the other results seen here. It is also possible that differences in receptor expression levels or conformation exist between male and female animals. Previous studies have suggested that FFAR3 can form a heteromer with FFAR2 which can modify SCFA signalling (Ang et al., 2018) and may result in distinct downstream signalling pathways. The presence and composition of such heteromers could differ between sexes, potentially influencing the response to butyrate and BHB. Previously sex-differences in receptor heteromerization have been observed in kappa opioid receptors (Chartoff & Mavrikaki, 2015) and dopamine D1-D2 receptor complex expression (Hasbi et al., 2020). Receptor phosphorylation is another factor that could contribute to the observed sex differences. Sex-specific variations in GPCR phosphorylation have been documented in other receptor systems such as in the previously mentioned kappa opioid receptor (P. Huang et al., 2022). Their role in FFAR3 signalling however, remains unexplored. This presents an intriguing avenue for future research, as differential phosphorylation could potentially explain the differential effects of butyrate and BHB on LTP in male versus female slices. Interestingly, we did not find hippocampal expression of Ffar2 which is backed up by the Allen Mouse Brain Atlas showing expression of Ffar3 but not Ffar2 in the adult hippocampus (*Allen Brain Atlas, Ffar2*, 2024; *Allen Brain Atlas, Ffar3*, 2024). Other studies have previously confirmed expression of Ffar3 and Ffar2 in both human and mouse hippocampus (Zamarbide et al., 2022). This may indicate inter-strain differences in mice expression of FFARs.

Additionally, the differential expression of accessory proteins or signalling partners in male and female tissues might further modulate FFAR3 activity.

Accessory proteins that stabilise the active conformation of FFAR3 or preferentially couple it to specific signalling pathways could render BHB more effective in one sex over the other. These proteins could play a critical role in determining the sex-specific outcomes observed in our study. One case study which exemplifies a sex-selective GPCR signalling is that of mGLUR5 signalling in Alzheimer's disease. Soluble Aβ oligomers disrupt mGluR5 signalling male but not female murine brains (Abd-Elrahman et al., 2020, 2022). This sex-dependent Aβ-mGluR5 interaction was dependent not on oestrogen, but the ability of mGluR5 to interact with cellular prion protein which was only detectable in male brains (Abd-Elrahman et al., 2020, 2022). This resulted in the pharmacological allosteric modulator of mGluR5, CTEP, being effective in male brains only.

Lastly, it is important to acknowledge that the dual role of BHB as both an antagonist and agonist of FFAR3 across different studies and experimental models suggests a complex interaction that might be context dependent. In a human embryonic kidney cell line, HEK293, BHB had a potent antagonistic effect on FFAR3 (Kimura et al., 2011) with similar antagonism found in other studies (J. Liu et al., 2015a; Nagliya et al., 2024; Robles-Vera et al., 2020; Y. Zhang et al., 2021). Meanwhile in rat sympathetic neurons BHB acted as an agonist (Won et al., 2013). The variability in BHB's effects underscores the need for a more nuanced understanding of its role in modulating FFAR3 activity. Taken together, these factors could collectively explain why BHB was able to antagonise butyrate's Ffar3-mediated enhancement of LTP in female hippocampal slices but not in male slices. Further investigation into the sex-specific regulation of Ffar3, including its expression, phosphorylation status, and interactions with accessory proteins, is essential to fully elucidate the mechanisms underlying these differential effects. Moreover, harnessing genetic tools for FFAR3 will be of interest for future exploration.

This study also found that while butyrate enhanced hippocampal LTP both propionate and acetate had no effect on the same process. This is an intriguing result when one considers the possibility that the effect is FFAR3 mediated. While some studies report propionate to be more potent than butyrate at FFAR3

with pEC_{50} values of 4.87 ± 0.12 and 3.67 ± 0.17 respectively (Hudson et al., 2012). Other studies report contrasting results with butyrate's EC_{50} at rat FFAR3 as 33 ± 9 micromolar while propionate's EC_{50} at the same receptor is 41 ± 17 micromolar (Brown et al., 2003). If indeed butyrate does activate FFAR3 at a lower concentration this could explain the effect seen on LTP following butyrate exposure while propionate produces no effect at a higher but comparable concentration. One must also consider the possibility that the increase in LTP in male slices seen in this study could occur through another receptor such as FFAR2 or HCAR2, both activated by the SCFAs tested in this paper. However, as reported pEC_{50} values are similar for propionate and butyrate at FFAR2 (Hudson et al., 2012) it may be unlikely that this is the avenue through which the observed effect is occurring.

We must also consider the possibility that some of the observed effects of butyrate could occur through HDAC inhibition especially as the SCFA which affected LTP, butyrate, is considered to the most potent SCFA HDAC inhibitor (Waldecker et al., 2008). However, the IC_{50} of butyrate as a HDAC inhibitor ranges from 1.3-2.5mM in human colon cancer cells (Zeng et al., 2020). This brings expected HDAC inhibition to occur in the millimolar range in the cellular context. This does however leave HDAC inhibition as a doubtful mechanism of action in the brain where concentrations of butyrate have so far found to be in the 0-2.8μM range in CSF (Perry et al., 1970) and 21μM in brain tissue water (Fock & Parnova, 2023).

To further elucidate the potential mechanisms involved in these observations, several investigative steps could be undertaken. One approach would be to examine the expression of additional genes, particularly well-known markers of synaptic plasticity such as BDNF (B. Lu et al., 2014). Measuring BDNF levels could provide valuable insights into the underlying processes, as previous studies have shown that SCFAs can modulate hippocampal BDNF expression (Marrocco et al., 2022). A pertinent question is whether pretreatment with the FFAR3 antagonist, BHB, might occlude any SCFA-induced increases in BDNF levels.

Of note, previous research has identified sex differences in the expression of SCFA transporters in the liver (J. Cao et al., 2017). If similar differences are present in the hippocampus, they could result in varying intracellular SCFA levels between sexes. This variance might influence the degree of HDAC inhibition, potentially contributing to the observed disparities in LTP. Recent insights from studies in metabolism and cancer research have shown that SCFAs directly modify specific genomic regions, increasing chromatin accessibility and promoting the expression of genes involved in differentiation and growth (Nshanian et al., 2025). These findings could inspire further exploration of similar mechanisms in brain cells. Assessing SCFA transporter expression in the hippocampus, coupled with a chromatin immunoprecipitation (ChIP) assay to measure HDAC inhibition, could provide additional clarity.

Finally, the downstream signalling of the FFAR3 receptor should be thoroughly investigated. For instance, changes in intracellular calcium resulting from FFAR3 activation could be measured using a fluorometric imaging plate reader (Hansen & Bräuner-Osborne, 2009). However, given the potential for signal amplification and crosstalk to skew results, it may be useful to complement this approach with biosensor-based methods that directly measure G protein activation, such as the ONE-Vector G protein Optical biosensor (Janicot & Garcia-Marcos, 2024). Further downstream signalling events, including cAMP production, could also be explored. Considering the potential involvement of heteromeric FFAR2/FFAR3 receptors, alternative pathways, such as p38 phosphorylation and β-arrestin-2 recruitment, could be analysed to provide a more comprehensive understanding (Ang et al., 2018).

For future research, it is essential to obtain precise measurements of SCFA concentrations in the cerebrospinal fluid (CSF) and brain tissue of mice. Additionally, investigating the potential sex-specific expression patterns of FFAR2 and FFAR3 receptors, along with evaluating the impact of FFAR2 inhibition on butyrate-enhanced LTP, will be critical for understanding the underlying mechanisms of these observed effects.

Translation of our findings to the human setting is not yet possible. However, there is still a clear effect of butyrate in the functional properties of the hippocampal circuit *in vitro*, supporting the idea that SCFAs can impact functional properties of the hippocampus at the concentrations that cross the BBB and exist in the local environment of the brain. Further examination is warranted to further characterize the ability of gut microbial metabolites in shaping the electrophysiological properties of circuits. Some principal questions that arise from this study are: Can other hippocampal properties be altered with exposure to gut microbial metabolites at the concentrations they exist at in the brain? Are similar differences observed earlier in developmental critical windows? Given previously seen associations between microbiome depletion, lowered faecal SCFA levels, and impaired cognitive function in early neonatal development in mice (Q. Zhang et al., 2024), such developmental stages may reveal changed hippocampal properties induced by SCFAs.

We conclude that butyrate leads to an alteration of hippocampal LTP which can be precluded in female mice by exposure to the FFAR3 inhibitor, BHB. This functional effect of hippocampal activity is likely to be important in aspects of learning and memory affected by the gut microbiome.

Chapter 5

The Gut Microbiota Defines the Early Life Developmental Trajectory of the Hippocampus

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Abstract

Developmental maturation of the gut microbiota aligns with critical windows of neurodevelopment. The microbiota-gut-brain axis, the bidirectional communication network between the vast microbial community in the gastrointestinal tract and the brain, is increasingly recognized for its role in human health and disease. Of all brain regions the hippocampus has been the one most studied in the context of the microbiota-gut-brain axis. Emerging evidence implicates this axis in the modulation of hippocampal synaptic plasticity in adulthood and associated behaviours such as learning and memory.

However, we lack a mechanistic understanding of the trajectory of microbiome-driven effects on early life hippocampal neurodevelopmental trajectories. Specifically, it is unclear whether certain periods represent heightened sensitivity, or critical windows, during which the microbiome exerts pronounced effects on synaptic plasticity-related processes. To this end, we performed transcriptomic and metabolomic analyses of the hippocampus across four postnatal developmental stages (postnatal day (P)2, 8, 14, and 21, approximately 10 male and females per group) in both germ-free and conventionally raised SPF C57BL/6 mice.

Using untargeted metabolomic analyses we found metabolomic shifts between germ-free and conventional mice which were most pronounced at P2 and P21 which was not dependent on sex. Targeted metabolomic analysis of metabolites with specific relation to cognition and synaptic plasticity revealed a similar substantial effect of GF status at P21. Parallel untargeted transcriptomic analyses of the hippocampus across these developmental stages in both GF and CON animals uncovered an altered sex-dependent trajectory with the greatest effect seen at P21 in females. This effect continued into targeted synaptic plasticity focused transcriptomic analysis. Finally, integrated multi-omic analysis found several significant correlations between metabolite abundance and gene expression that was dependent on both sex and microbiome status.

This multi-omics approach underscores the critical role of the gut microbiota in the early life development of hippocampal synaptic plasticity-related processes.

1 Introduction

Synaptic plasticity, the ability of synapses to strengthen or weaken over time, is fundamental to learning and memory processes (Citri & Malenka, 2008b). In the early stages of development, synaptic plasticity undergoes critical windows where neuronal circuits are particularly malleable, allowing for the formation of essential neural networks (Hensch, 2005). During these periods, achieving a balance in synaptic plasticity is crucial; indeed it is hypothesized that neurodevelopmental disorders such as autism spectrum disorder feature dysfunctional synaptic plasticity and synaptic homeostasis at their root (Huguet et al., 2016).

Recent studies from our lab have provided compelling evidence of the microbiota's role in modulating hippocampal synaptic plasticity (Darch et al., 2021, **Chapter 2**), a key region of the brain associated with learning and memory (Neves et al., 2008). While the entire brain is exceptionally vulnerable during these critical periods of development, the hippocampus is known to be particularly susceptible to interruptions in normal development in the perinatal period, which can arise from various factors, including environmental influences and genetic predispositions (White et al., 2024). Emerging evidence suggests that the gut microbiota plays a pivotal role in this developmental process, acting as a mediator through secretion of neuroactive microbial metabolites such as tryptophan into the bloodstream which can affect brain function and behaviour (Clarke et al., 2013).

The microbiota-gut-brain axis, a complex communication network linking the gut microbiota and the central nervous system, has been shown to influence various developmental processes such as myelination (Cryan et al., 2019a; Cryan & Dinan, 2012; Darch et al., 2021; Keogh et al., 2021b; C. M. K. Lynch et al., 2023). Not only has gut microbiota perturbation been shown to disrupt hippocampal function in adulthood (Çalışkan et al., 2022; Hoban, Moloney, et al., 2016), the case for the gut microbiota's role in developmental regulation of this region is notably stronger, as evidenced by the greater susceptibility of learning and

memory changes brought about by microbiome perturbation in early life (Diamanti et al., 2022; Hickey et al., 2021; Z. Liu et al., 2022). Importantly, the maturation of the gut microbiota appears to coincide with potentially sensitive periods for synaptic plasticity, suggesting a temporal alignment that may allow for a disproportionate effect on synaptic plasticity due to microbiome disruption or absence (Cowan et al., 2020; Sakimoto et al., 2022). The adult consequences of a lack of gut microbiota signals, as seen in germ free (GF) mice, has a surprising sex-dependent consequence, producing a male-specific disruption in hippocampal synaptic plasticity (Darch et al., 2021, **Chapter 2**). Indeed previous examination of hippocampal miRNA in GF mice revealed a sex-specific role for the regulation of miR-294-5p expression (Moloney et al., 2017). These results echo the wealth of associations between sex and the microbiome previously seen throughout the lifespan (Jaggar et al., 2020).

Given these insights, we hypothesize that the gut microbiota, through joint host-microbial co-metabolism, is necessary for the normal early life development of hippocampal synaptic plasticity. To test this hypothesis, we investigated the effects of gut microbiota absence on synaptic plasticity during four early life timepoints. Our approach integrated metabolomic and transcriptomic analyses, revealing that the absence of microbiota disrupts essential pathways associated with synaptic plasticity.

This study represents to our knowledge the first in-depth exploration of microbiota-mediated modulation of hippocampal synaptic plasticity-related transcriptomic and metabolomic processes during early life and the role of sex herein.

2 Materials and Methods

2.1 Mouse Husbandry

C57/BL6 mice breeding pairs were obtained from Taconic Biosciences and subsequent generations were used for experiments. Germ-free and conventionally raised mice were initially housed in breeding pairs for two weeks, after which females were individually housed until birth (designated postnatal day P0). Mice were maintained on a 12 hr light:dark cycle under controlled temperature and humidity conditions (21 ± 1°C, 55%– 60%), with *ad libitum* access to standard rodent chow (Special Diet Services, Product Code 801010) and autoclaved water. Experimental analyses included no more than two animals of each sex from any given litter. All experiments adhered to the European Directive 2010/63/EC, met the requirements of the S.I No 543 of 2012, and were approved by the Animal Experimentation Ethics Committee of University College Cork and the Health Products Regulatory Authority (HPRA AE19130 P047/118).

2.2 Tissue Collection

Mice designated for fresh culls were sacrificed by decapitation between 10 am and 12 pm. The hippocampus was rapidly dissected out and put on ice. All samples were promptly snap-frozen on dry ice and stored at -80°C until further analysis.

2.3 RNA-Sequencing

Total RNA was extracted from the hippocampus using the RNeasy Plus Universal Mini Kit (Qiagen). RNA quality and concentrations were measured using a Nanodrop ND-1000 (Thermo Scientific). mRNA sequencing was conducted by Azenta (Strand-specific RNA-Seq) on the Illumina NovaSeq 6000 (S4 flow cell, 2x 150bp Pair-Ended (PE) configuration). Quality filtering and mapping were performed using FASTQC v.0.11.8 (Andrews, 2018). Reads were aligned to the UCSC Genome Browser assembly ID: GRCm39 (Chinwalla et al., 2002).

Bioinformatics analysis was conducted using R (version 4.3.3) with the RStudio GUI (version 2022.07.2 build 576).

To control for multiple comparisons the Benjamini-Hochberg post hoc procedure was applied with a false discovery rate (FDR) q-value of 0.1 as a threshold (Benjamini and Hochberg, 1995). For targeted pathway enrichment analysis, we selected terms (Table 5.2) from the Gene Ontology Database that were associated with synaptic plasticity (Ashburner et al., 2000) to identify candidate genes. These GO terms were picked due to their direct relevance to synaptic plasticity. We then evaluated enrichment using the hypergeometric test implemented as phyper in the base R stats package.

See the following table for n numbers of hippocampal samples used in transcriptomic analysis.

Table 5.1 N numbers of whole hippocampal samples used in transcriptomic analysis of Conventional (CON) and germ-free (GF), male and female mice at four early life timepoints; Postnatal day (P)2, 8, 14, 21. N numbers formatted with CON n number before the forward slash and GF n number after the forward slash e.g. x/y.

CON/GF				
	P2	P8	P14	P21
Male	9/10	9/10	10/10	10/10
Female	10/10	10/10	10/10	10/10

Table 5.2 Gene Ontology (GO) term titles and IDs used in targeted transcriptomic analysis.

GO Term	ID
long-term synaptic depression	GO:0060292
long-term synaptic potentiation	GO:0060291
negative regulation of synaptic plasticity	GO:0031914
positive regulation of long-term neuronal synaptic plasticity	GO:0048170
positive regulation of synaptic plasticity	GO:0031915
regulation of long-term synaptic depression	GO:1900452
regulation of long-term synaptic potentiation	GO:1900271
regulation of neuronal synaptic plasticity	GO:0048168
regulation of short-term synaptic potentiation	GO:1905512
regulation of synaptic scaling	GO:0150092
regulation of translation at synapse, modulating synaptic transmission	GO:0099547
short-term synaptic potentiation	GO:1990926

2.4 Metabolomics

Metabolomics was conducted by MS-Omics (Copenhagen). Briefly, samples of hippocampus were acidified using hydrochloric acid, and deuterium-labelled internal standards were added. All samples were analysed in a randomised order using a UPLC system (Vanquish, Thermo Fisher Scientific) coupled with a high-resolution quadrupole-orbitrap mass spectrometer (Orbitrap Exploris 240 MS, Thermo Fisher Scientific). Ionization was achieved with an electrospray ionization interface operated in positive and negative ionization mode under polarity switching. A QC sample was analysed. Data were processed using Compound Discoverer 3.3 (ThermoFisher Scientific) and Skyline 22.1 (MacCoss Lab Software). Peaks were quantified using area under the curve (AUC). Further bioinformatics analysis was conducted using R (version 4.3.2) with the RStudio GUI (version 2022.07.2 build 576).

Untargeted enrichment analysis was performed on differentially abundant metabolites ($p < 0.05$) using MetaboAnalyst with the murine KEGG library as a

reference (Pang et al., 2022).

Further bioinformatics analysis was conducted using R (version 4.3.3) with the RStudio GUI (version 2022.07.2 build 576). To control for multiple comparisons the Benjamini-Hochberg post hoc procedure was applied with a false discovery rate (FDR) q-value of 0.1 as a threshold (Benjamini and Hochberg, 1995).

Table 5.3 N numbers of whole hippocampal samples used in metabolomic analysis of Conventional (CON) and germ-free (GF), male and female animals at four early life timepoints; Postnatal day (P)2, 8, 14, 21. N numbers formatted with CON n number before the forward slash and GF n number after the forward slash e.g. x/y.

CON/GF				
	P2	P8	P14	P21
Male	10/10	9/10	10/10	9/10
Female	10/10	8/10	10/10	9/10

2.5 Multi-Omics Integration Analysis

Using Anansi (ANnotation based ANalysis of Specific Interactions) associations between transcriptomics and metabolomics were estimated ($q < 0.1$) (Bastiaanssen, 2023). In summary, all transcripts relevant to GO terms mentioned in 3.3.2 as well as metabolites that showed associations in the literature with synaptic plasticity and learning and memory. Subsequently, linear mixed-effects models were fitted to estimate the associations between converted transcript-metabolite pairs that were adjacent in a KEGG pathway.

3 Results

3.1 Gut Microbiota Shapes Developmental Hippocampal Neurochemistry.

Considering the established role of early gut microbiota in various neurodevelopmental processes, we hypothesized that gene expression differences observed in germ-free (GF) animals might be linked to alterations in key brain metabolites. To investigate this, we conducted untargeted metabolomic profiling of the hippocampus in both GF and conventionally raised (CON) SPF mice across four developmental stages (postnatal days [P]2, P8, P14, and P21).

Principal Component Analysis (PCA) followed by PERMANOVA revealed a significant microbiome effect at each developmental time point. In total, we identified 102 metabolites with altered abundance in GF mice at one or more stages ($p < 0.05$) (Figure 5.1). The greatest number of altered metabolites were observed at P2 (41 differentially abundant metabolites) and P21 (39 altered) (Figure 5.1). At P2, all significantly altered metabolites (41 altered) were found to be reduced in the GF condition, whereas later stages exhibited a mix of both increased and decreased metabolite levels. Importantly, no significant changes were detected between sexes nor were there any interactions between microbiome status and sex. Individual abundance profiles of differentially abundant metabolites are provided in Supplementary along with statistical output tables (Supplementary Figure 5.1).

Untargeted enrichment analysis using MetaboAnalyst further indicated that the absence of gut microbiota during early development disrupted several pathways critical to brain function (Figure 5.1 A), with pathways involved in histidine metabolism, glycine, serine, and threonine metabolism, and beta-alanine metabolism showing the most significant alterations.

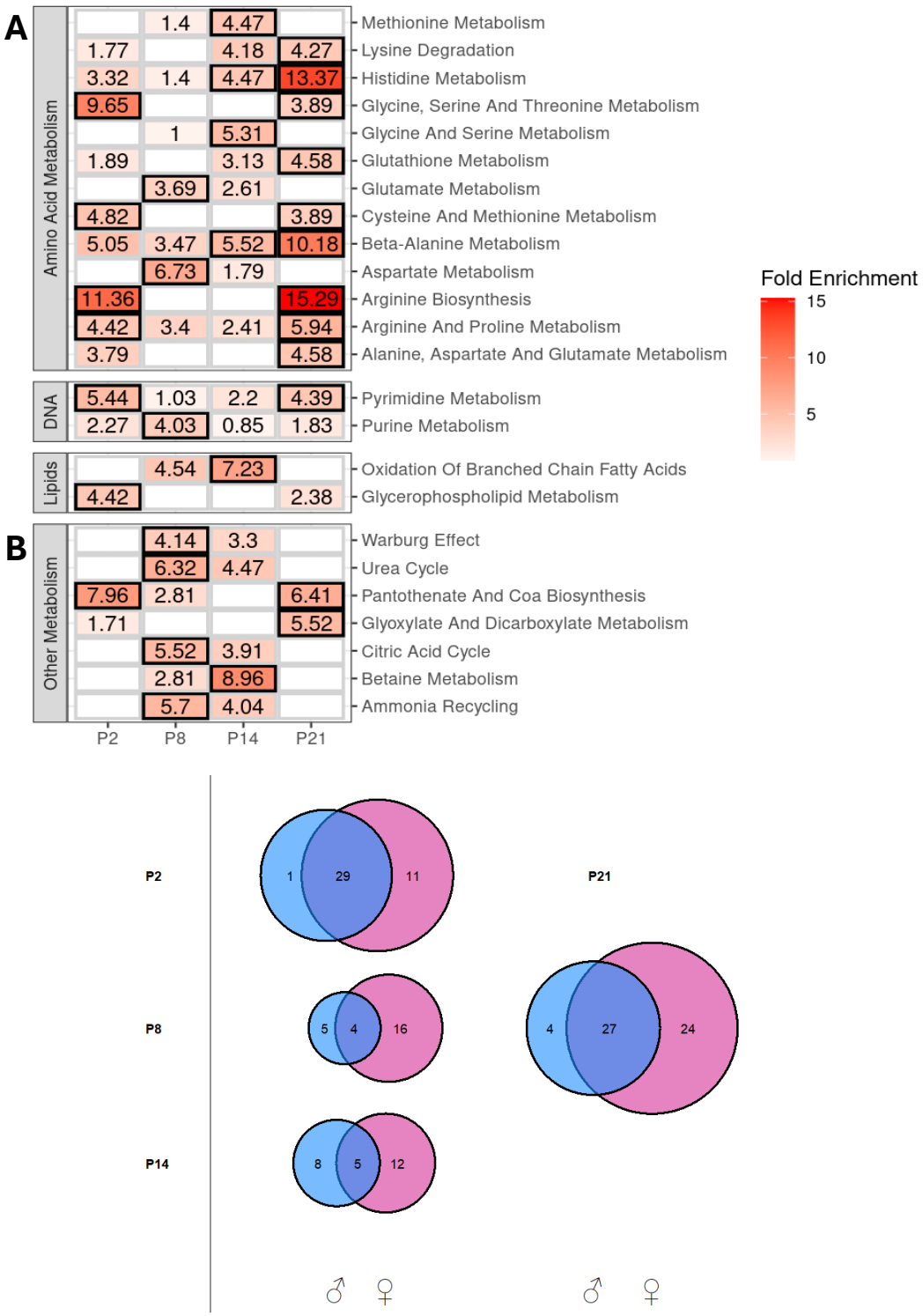


Figure 5.1 (A) Disrupted metabolism pathways in germ-free (GF) mouse hippocampus at four stages of post natal (P) development. P2, P8, P14, P21. Pathways are further categorised into their relation to amino acids, DNA nucleotides, lipids, and miscellaneous. (B) Venn diagrams of the number of altered metabolites in GF hippocampus at four early life developmental timepoints.

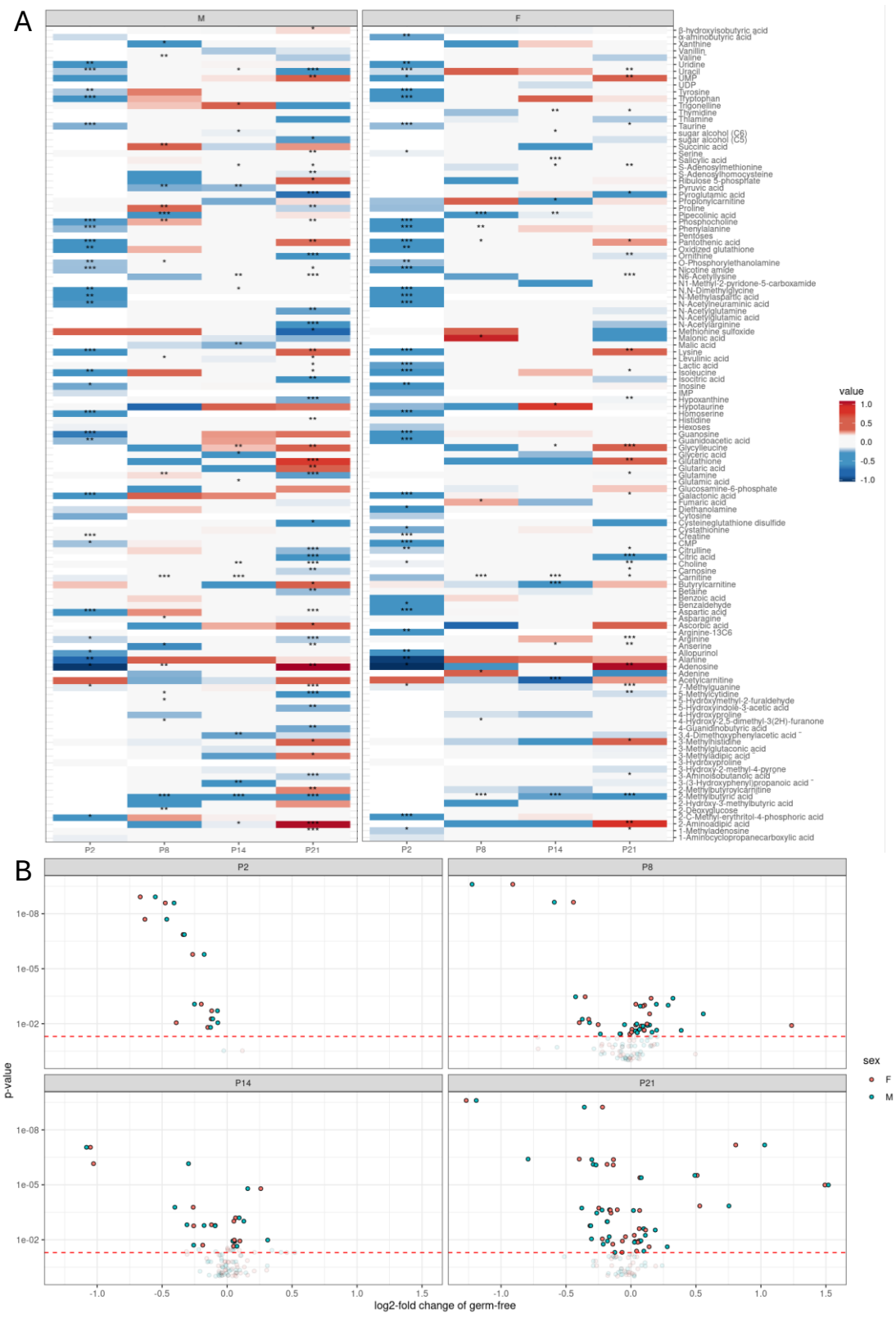


*Figure 5.2 (A) Untargeted heatmap featuring differentially abundant metabolites in male and female germ-free (GF) and conventional (CON) mice at four early life developmental stages (Post natal day (P)2, P8, P14, P21. * p < 0.05, ** p < 0.01, ***p <0.001. (B) Volcano plots of differentially abundant metabolites in GF mice at early developmental timepoints.*

3.2 The Microbiome Creates a Critical Window in Hippocampal Metabolomics at Post-Natal Day 21.

To further investigate the developmental underpinnings of synaptic plasticity alterations observed in adult GF animals (Darch et al., 2021 **Chapter 2**), we conducted a targeted analysis using a curated list of metabolites associated with synaptic transmission and synaptic plasticity based on existing literature see (Supplementary Figure 5.2). In contrast to findings from untargeted metabolomics, the first three age groups (P2, P8, and P14) exhibited similar numbers of changed metabolites, while P21 still showed the greatest number of significantly altered metabolites (13 altered). Echoing the untargeted analysis, while there were minor differences in the number of metabolites that reached Tukey's significance threshold between females and males, no significant effect of sex was observed. Similarly, no interactions were observed between sex and microbiome status, indicating a sexually monomorphic hippocampal metabolome.

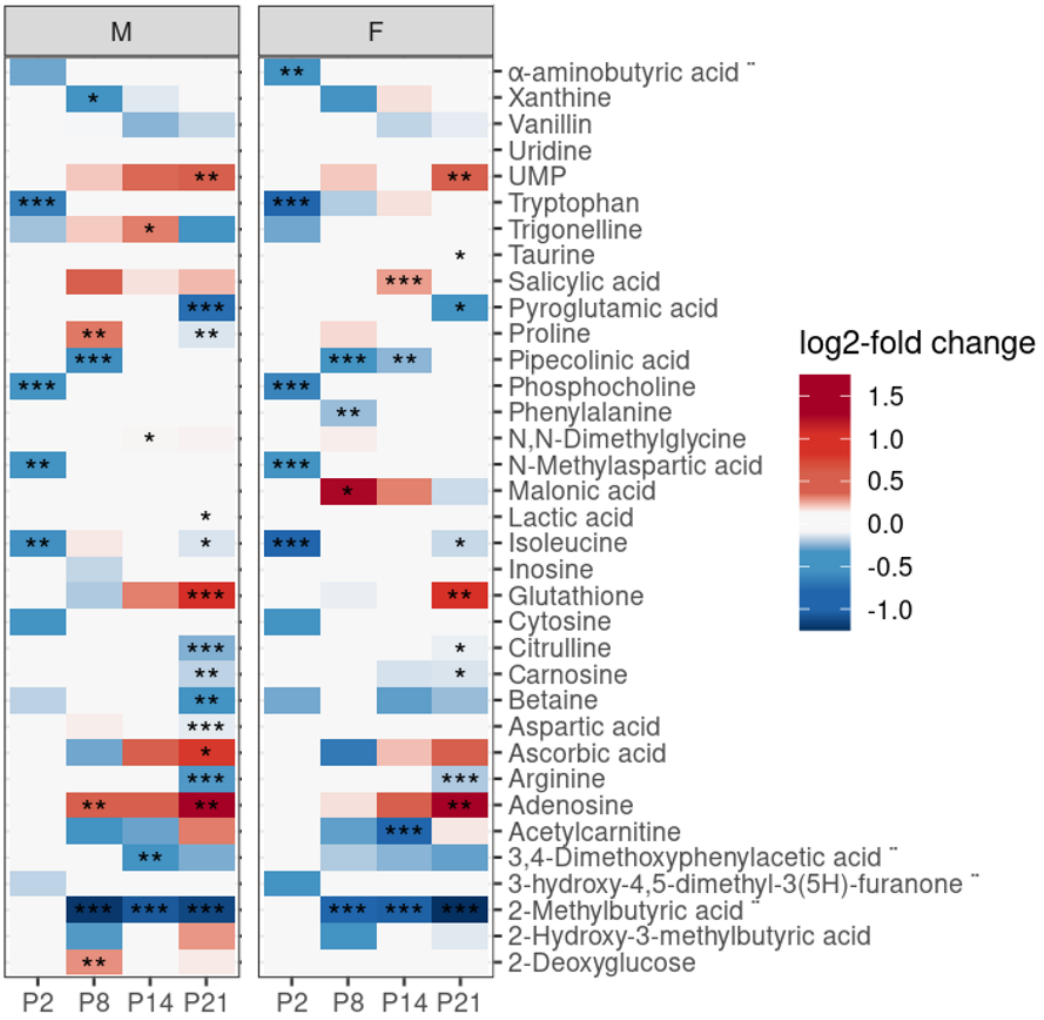


*Figure 5.3 Heatmap of differential abundance of targeted metabolites in the hippocampus of male and female germ free and conventional mice at four early life developmental timepoints, post-natal day (P)2, P8, P14, P21. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.*

3.3 Gut Microbiota Shapes Early Life Hippocampal Transcription, Effects Increasing with Age

Building on the observed changes in hippocampal metabolites linked to synaptic plasticity as well as learning and memory, we performed untargeted hippocampal transcriptomic analysis at the same four early developmental stages to map the functional trajectory of hippocampal processes. Notably, in a gene-wise differential expression analysis, the influence of microbiota and sex, as well as their interaction effect, intensified progressively in concert with age, with P21 showing the most pronounced microbiome effects (Supplementary

Figure 5.2). Across all age groups, GF status affected the expression of 649 genes, with P21 exhibiting the highest number of differentially expressed genes irrespective of sex. Interestingly, females displayed both greater effect sizes and a greater number of genes which reached the significance threshold than their male counterparts at this age, whereas effect sizes were similar between sexes at earlier ages (Figure 5.4 C).

Untargeted enrichment analysis revealed disruptions in several pathways related to endoplasmic reticulum stress, alongside endothelial cell migration, and negative regulation of nucleic acid-templated transcription. The greatest amount of differentially expressed genes was at P21 with females more affected at every timepoint except for P8. A clear increase over time was observed in this differential expression. Interestingly the dysregulated genes were largely unique in males and females with only a small portion of differently expressed genes being shared between the sexes at any timepoint.

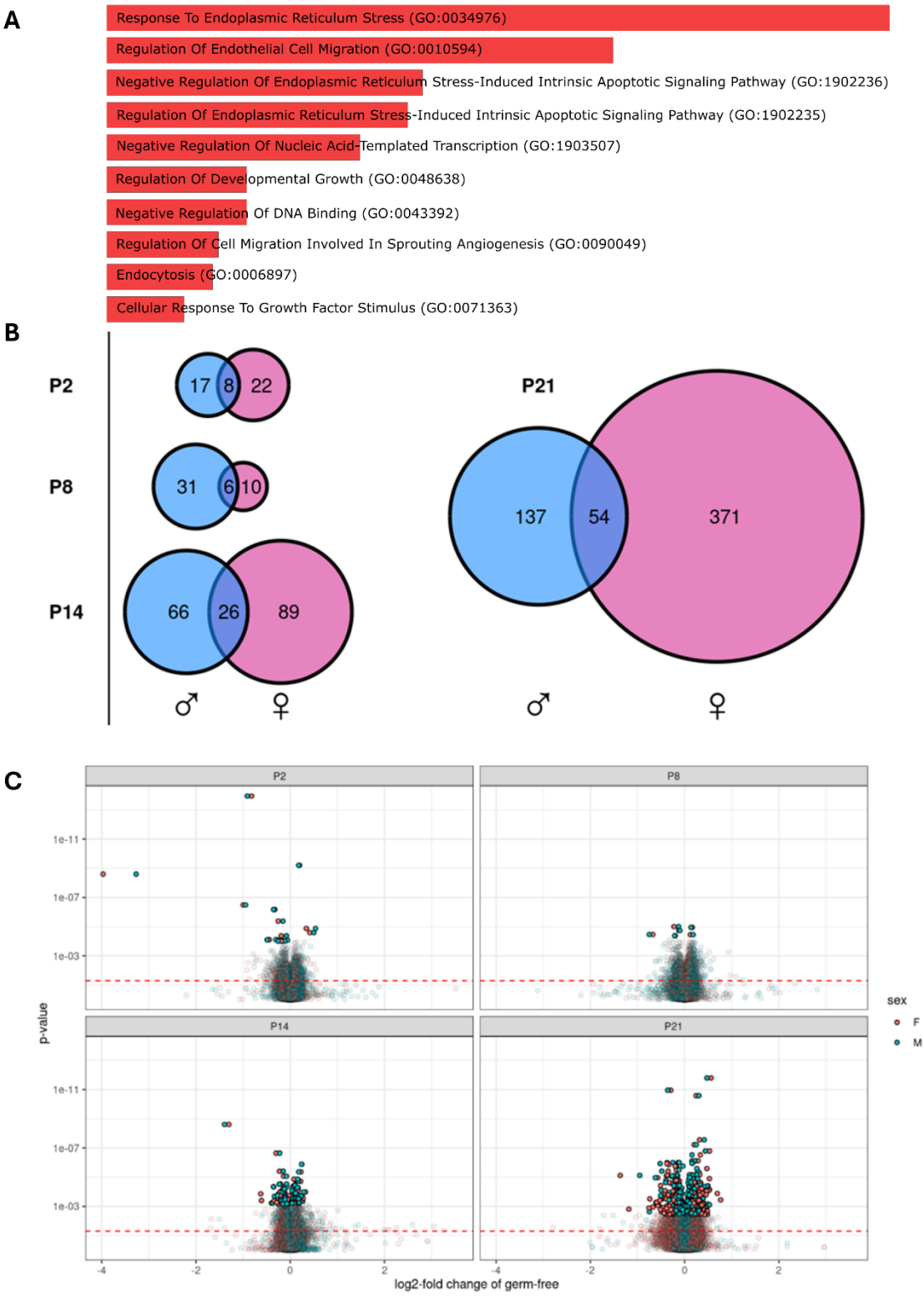


Figure 5.4 (A) Differentially altered Gene Ontology (GO) terms from an untargeted analysis of all timepoints. Made using EnrichR (B) Venn diagrams of differentially expressed genes in male and female germ free (GF) mice across four early-life developmental timepoints. (C) Volcano Plot of differentially expressed genes across four early-life developmental timepoints.

The microbiota alters the developmental trajectory of the hippocampal transcriptome associated with synaptic plasticity pathways.

Previous research has implicated gut microbiota in the regulation of hippocampal synaptic plasticity and hippocampal-associated learning and memory in adulthood (Darch et al., 2021, **Chapter 2**). To explore the developmental roots of these synaptic plasticity changes, we conducted targeted transcriptomic analyses on the hippocampi of CON and GF mice at four early-life stages.

Targeted enrichment analysis using Gene Ontology (GO) terms (Table 5.1) revealed significant enrichment of genes involved in the regulation of neuronal synaptic plasticity (0.4 $\log^2$fold enrichment), as well as specific changes to long-term synaptic depression (LTD) (0.78 $\log^2$fold enrichment) (Figure 5.5 A). These enriched GO terms comprised 123 and 49 genes, respectively, with 20 and 12 of these genes altered at least once across the developmental stages. P21 demonstrated the highest number of altered genes, closely followed by P14. By contrast, P8 showed only a single statistically significant gene alteration, and P2 exhibited no significant changes (Figure 5.5 B, C).

The largest effect sizes were also observed at P21, where most differentially expressed genes showed downregulation. Gene expression changes were more pronounced in females compared to males, both in terms of number and effect size, with males showing only one differentially expressed gene at any time point. Indeed, 40 and 16 genes in each respective category displayed significant microbiome-sex interactions, underscoring a clear sex-dependent response to microbiome status. The most frequently altered GO sub terms were related to Cell Structure, Enzymes, and Receptor Function, including key genes from these sub terms such as *Arc*, *Kmt2a*, and *Kit*, respectively.

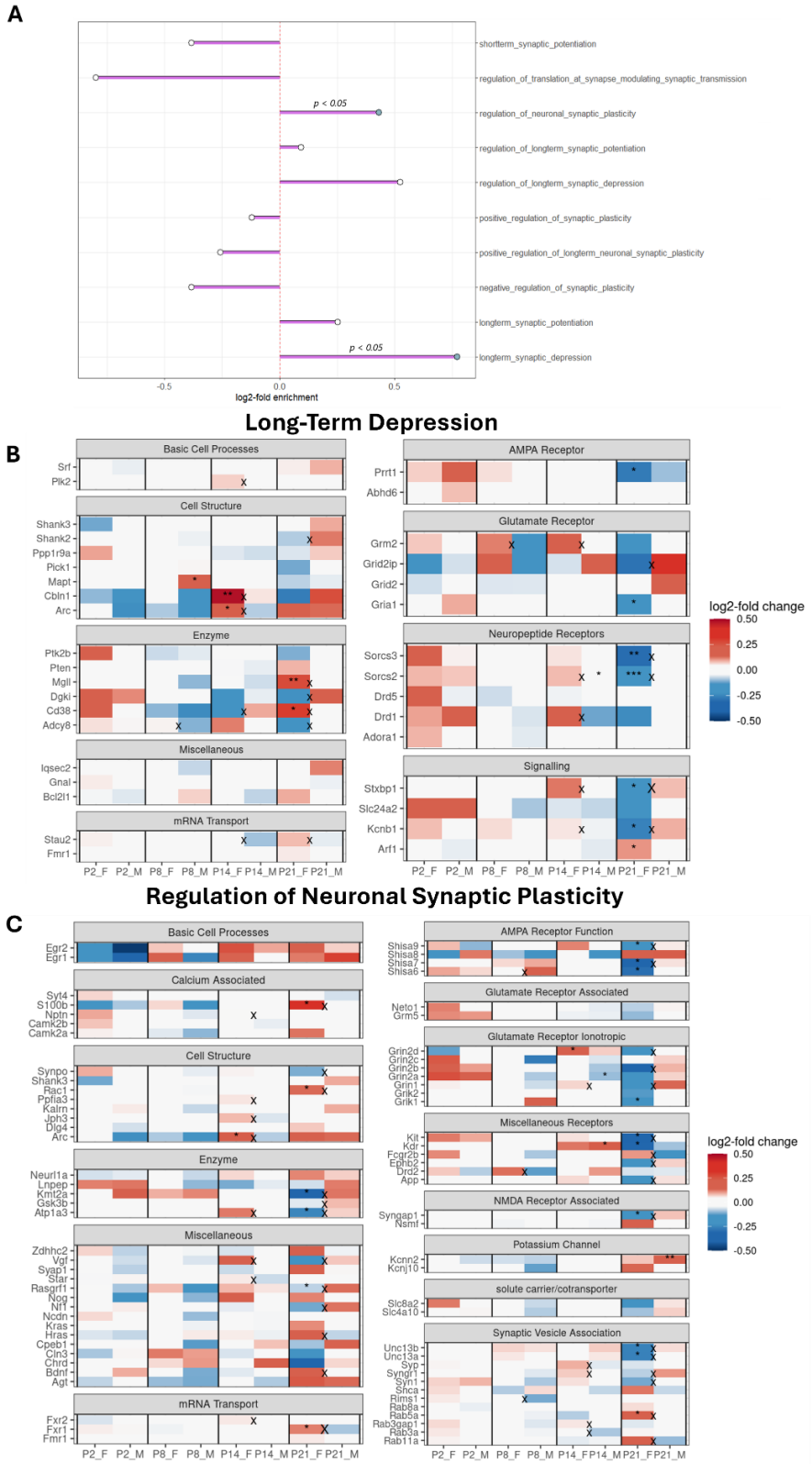


*Figure 5.5 Targeted Enrichment Analysis of the hippocampal transcriptome in male and female germ free (GF) and conventional (CON) mice at four early life developmental timepoints, post-natal days (P)2, P8, P14, P21. Synaptic plasticity associated Gene Ontology (GO) Terms used (A) Enriched and non-enriched GO terms. (B) Heatmap of changes in genes nested in the enriched Long-Term Depression GO term. (C) Heatmap of changes in genes nested in the enriched Regulation of Neuronal Synaptic Plasticity GO term. * p < 0.05, ** p < 0.01, ***p <0.001*

4 Integrated Multi-Omics Reveal Microbiome Control over Metabolite-Gene Interactions

The absence of the microbiota in early life disrupts metabolic and transcriptomic profiles in the developing brain (Pronovost et al., 2023; Rea et al., 2022; Vuong et al., 2020). We pursued only the differences found thus far in this study, looking at affected GO terms and metabolites previously found to be significantly altered by the GF state, with relevance to synaptic plasticity. Having found marked differences in both synaptic plasticity associated transcription and microbial metabolites, we sought to understand the degree to which these features were related with each other with a multi-omics approach. To this end we integrated these datasets through application of an annotation-based analysis of specific interactions (Anansi) (Bastiaanssen et al., 2023). Through this joint-pathway analysis we found interactions between the hippocampal metabolome and transcriptome at key developmental timepoints. Strikingly, we found differences in how sexes react to GF status at each developmental stage. These interactions arose for proline, tryptophan, 2-hydroxy-3-methylbutyric acid, and pipecolinic acid which are all microbially associated (Gao et al., 2020; Mayneris-Perxachs et al., 2022; Procházková et al., 2024; Qiao et al., 2022) (Figure 5.6). The greatest number of significant effects were seen ats P2 with four significant metabolite-gene correlations. Correlations were tied to expression of genes including Bdnf, Grin2c, and Syp. Most of these effects occurred at only one timepoint, besides one interaction between proline and Ptk2b which occurred at both P2 and P8. This shows sex-dependent changes occurring with these metabolites, with different effects occurring at different times. This may allude to the existence of a critical window not just surrounding one specific timepoint examined here but multiple timepoints spanning the duration of early life development.

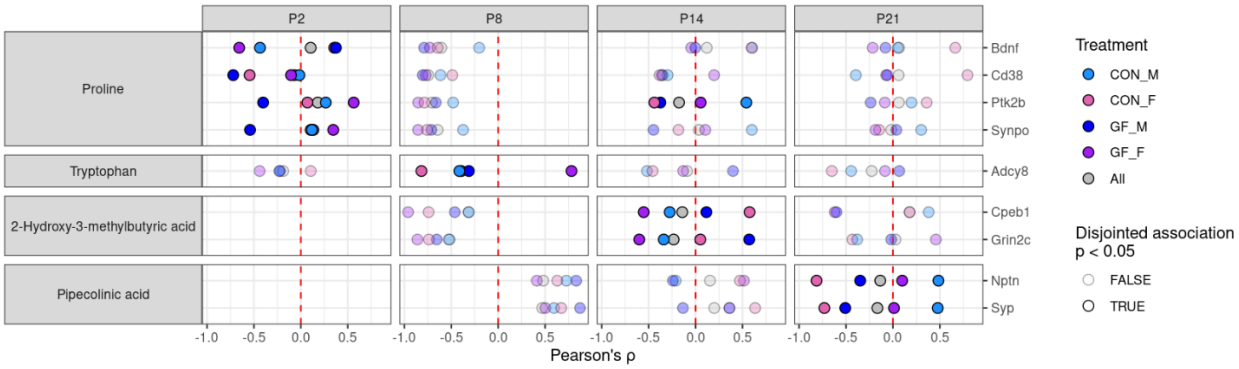


Figure 5.6 Integrated multi-omics analysis of the murine hippocampal transcriptome and metabolome of germ-free vs conventional mice, at four early life developmental timepoints, depicting interactions that are significant for both microbiome and sex. X-axis depicts Pearson's correlation coefficient. Dots are coloured by treatment group with opaque dots signifying different reactions to germ free status between sexes (interaction effect) ($q < 0.1$, $p < 0.05$). Significant metabolite-gene association not differing by sex are depicted with transparent datapoints. Where correlations are not depicted in the figure i.e. 2-Hydroxy-3-methylbutyric acid and pipecolinic acid, the metabolites in question were not detected in the post-natal day (P) 2 brain.

These metabolites showed significant correlations with functionally relevant genes identified in our synaptic plasticity targeted enrichment analysis, particularly relevant to cell structure, enzymes, and calcium signalling. Notable significant interactions include proline and Bdnf. These findings strengthen the notion that microbial metabolites are key to regulation to hippocampal synaptic plasticity, however this would need to be validated in mechanistic follow-up experiments in vitro. show that the germ-free state produces alterations to the abundance of several structural and synaptic plasticity regulatory genes, potentially impacting the development of hippocampal synaptic plasticity at these early developmental timepoints.

5 Discussion

In this present study, we aimed to characterise the impact of the gut microbiota in shaping the transcriptome and metabolome of the hippocampus critical windows of early life development. Here, we show that microbiota-derived signals in early life are necessary for development of regular gene and metabolite production in a sex and time-dependent manner. To our knowledge, we demonstrate for the first time, an integrated multi-omics approach to examining the developmental trajectory of synaptic plasticity associated transcriptomic signature.

Our study reveals striking and consistent changes in the hippocampal metabolome associated with germ-free status. Using untargeted metabolomic analyses we found that these shifts are most pronounced at P2 and P21, suggesting that these time points could represent key periods during which microbiome-induced metabolic alterations may influence general hippocampal function. Interestingly no sex-dependent effects were statistically significant.

When examining metabolites with specific relation to synaptic plasticity, and learning and memory, a similar trend is observed, with some notable differences. Specifically, the metabolic changes at P2 appear somewhat reduced compared to those seen in wider hippocampal function, while those at P21 remain substantial. These findings suggest that the impact of germ-free status on metabolomic changes relevant to synaptic plasticity is most pronounced at P1.

Parallel transcriptomic analyses of the hippocampus across these developmental stages in both GF and CON animals reveal a trajectory that somewhat mirrors the trends observed in targeted metabolomics. The transcriptomic changes increase progressively, reaching their highest level at P21. Unlike the metabolomic alterations, however, these transcriptomic shifts are sexually dimorphic, with females exhibiting a higher number of gene expression changes. This suggests that the hippocampal transcriptome exhibits a sex-specific response despite the metabolomic environment remaining largely

the same in both sexes. The observation of significant alterations in the metabolome at both the earliest and latest timepoints, which are consistent between males and females, alongside transcriptomic changes that exhibit pronounced sex differences, affecting females more than males, suggests a potential temporal relationship between metabolic and transcriptomic differences. Some commonality is found between these findings and previous studies. Exploration of hippocampal miRNAs in GF mice found various sex-specific effects on gene expression resulting from GF status (Moloney et al., 2017). Notably this study found female GF mice to be more sensitive to recolonisation at P21 and its effect on transcription. Given P21 shows the greatest number of affected genes in our data and females of this timepoint have a far greater number of changed genes compared to males, this data and the study by Moloney and colleagues centre P21 as an important age for microbiome influence of hippocampal gene expression (Moloney et al., 2017). This pattern may indicate that females are more sensitive to metabolome-driven disruptions, or alternatively, that females deploy compensatory mechanisms in response to metabolomic changes, mitigating their impact and reflecting a protective adaptation. This possible distinction is important to note in the absence of a clear causal relationship (Walter et al., 2020). Our targeted transcriptomic analysis further corroborates these observations, highlighting disrupted pathways (GO terms) associated with synaptic plasticity, specifically the regulation of neuronal synaptic plasticity and long-term synaptic depression, in germ-free animals in a sex-dependent manner, with almost the entirety of the effect being in female animals. Consistent with previous findings relating to the peri-weaning period being particularly susceptible to immune insults and microbiota modulation (Al Nabhani et al., 2019; Dziabis & Bilbo, 2022; Spencer et al., 2006), P21 is identified here as the most affected time point, suggesting a potential critical window for synaptic plasticity, particularly in female animals. These sensitive periods are important as the effects of microbiota modulation on developmental processes can persist into later life (Desbonnet et al., 2015; C. M. K. Lynch et al., 2023).

The identification of a sex-dependent critical window for the modulation of the hippocampal synaptic plasticity transcriptome by microbiome status is novel. Previous hippocampal electrophysiology research from our lab demonstrated dysregulated synaptic plasticity in adult germ-free animals, with males primarily affected rather than females (Darch et al., 2021). Long-term potentiation (LTP) was the specific form of synaptic plasticity found to be disrupted in this previous study, contrasting somewhat with the present findings where LTD dysregulation is observed predominantly in females. This distinction leads us to speculate that an electrophysiological investigation of LTD in female germ-free animals at P21 or later could uncover further dysregulation. Interestingly, no comparable male-specific changes in synaptic plasticity-associated gene expression were observed between germ-free and conventional animals at these early ages. While this does not preclude the possibility of synaptic plasticity transcriptome dysregulation in germ-free males at these ages, it may indicate a later critical window for male hippocampal synaptic plasticity vulnerability, potentially occurring post-weaning. Previous work from our lab has shown that microbiome depletion in adulthood (beginning at 8 weeks) does not produce synaptic plasticity deficits (Chapter 3), suggesting that the vulnerability of male hippocampal synaptic plasticity to microbiome status may occur between P21 and 8 weeks, delineating a later developmental window for these effects.

Our use of a targeted transcriptomic approach, specifically investigating GO terms relating to synaptic plasticity allowed us to uncover these striking effects. It is of great importance that the P21 transcriptome and metabolome is the timepoint most affected by age as this early life timepoint is integral to synaptic plasticity and learning and memory development. Previously it has been found that it is at this period that reliable LTP induction is developing in mice (Ostrovskaya et al., 2020). Similarly in rat studies learning performance starts to improve at 3 weeks while it is still poor at 2 weeks of age (Sakimoto et al., 2022). Furthermore, from P15 to P28, in the middle of which we see the greatest dysregulation in the synaptic plasticity transcriptome, neuronal spines are highly dynamic and synapse numbers increase rapidly (Lohmann & Kessels, 2013). This

increase is almost entirely driven by the emergence of spine synapses and is dependent on NMDAR function (Ultanir et al., 2007). At this point NMDAR receptors are dominantly composed of Glu2nB/Grin2B (Gray et al., 2011), a subunit seen in the present study to show a significant microbiome*sex interaction at P21. The concurrent disruption of the hippocampal metabolome and transcriptome complements previous research highlighting the importance of metabolites in regulating brain function and behaviour (Erny, N, et al., 2021; Needham et al., 2022; Philip et al., 2021; Vuong et al., 2020). Furthermore, given the previous findings that the hippocampus of GF mice in adulthood exhibits differences in spine morphology (Luczynski, Whelan, et al., 2016) the dysregulation of this morphology may begin to occur at this timepoint, fuelled by the metabolomic changes that exist in the region. Indeed we see differences at P21 in the transcription of Rac1 for example, a gene that is necessary for normal spine development (Bongmba et al., 2011). Investigation into the timing of spine changes in development should be considered a pressing area for future study. This timepoint may also represent an effective opportunity for the administration of a postbiotic or probiotic to confer long-term beneficial neurodevelopmental outcome. A similar opportunity has previously been exploited from gestational day six until birth (Siegler Lathrop et al., 2024).

Our multi-omics integration of these datasets allowed us to identify metabolite-gene interactions which significant at the level of both microbiome and sex. The genes in question have functional relevance to synaptic plasticity and fall under categories associated with cell structure, glutamate receptors, and calcium signalling. These findings that the early life microbiome is important for neurodevelopment and can alter hippocampal synaptic plasticity related processes in a sex dependent manner aligns with previous research emphasizing the importance of the microbiome in the development of the hippocampus and hippocampal synaptic plasticity processes (Jaggar et al., 2020; G. Liu et al., 2022; Luczynski, Whelan, et al., 2016; Salami & Soheili, 2022; C. Zhou et al., 2020). Furthermore, it adds to previous findings which give evidence toward the role of metabolites in regulating neurodevelopment, brain function, and behaviour

(Erny, N, et al., 2021; Gacias et al., 2016; Needham et al., 2022; Vuong et al., 2020). From this new evidence we see genes which are well known in synaptic plasticity studies. Proline significantly correlates with expression of Bdnf, a neurotrophic factor with potent effects on synapses that plays a critical role in hippocampal long-term potentiation (B. Lu et al., 2014). Proline supplements in the form of proline-rich peptides have previously been explored with the aim of increasing serum BDNF with some success (Banasiak-Cieślar et al., 2023). Proline also saw significant correlation with Synpo, a cytoskeletal protein upon which spine morphology is critically dependent (Falahati et al., 2022; Kremerskothen et al., 2005). This adds further evidence to the possibility that differences seen in dendritic spine morphology in germ-free animals is developmentally regulated (Luczynski, Whelan, et al., 2016).

The observed sex difference in neurodevelopmental trajectory caused by the GF condition is reminiscent of the sex-differences observed in human microbiome-associated neurodevelopmental conditions such as autism spectrum disorders (ASD) (Baron-Cohen et al., 2011; Lai et al., 2014) and attention deficit hyperactivity disorder (Stibbe et al., 2020). In the case of ASD, a condition which has a foundation in altered synaptic plasticity (Bourgeron, 2015), males are diagnosed at a higher rate (Zeidan et al., 2022). The application of our results which saw a strong effect in female, but not male mice may then seem counterintuitive. However, as previously mentioned the effect we see in female mice at P21 may be a compensatory effect exerted to counteract the effects of the GF state such as the altered hippocampal metabolome we see at the same timepoint.

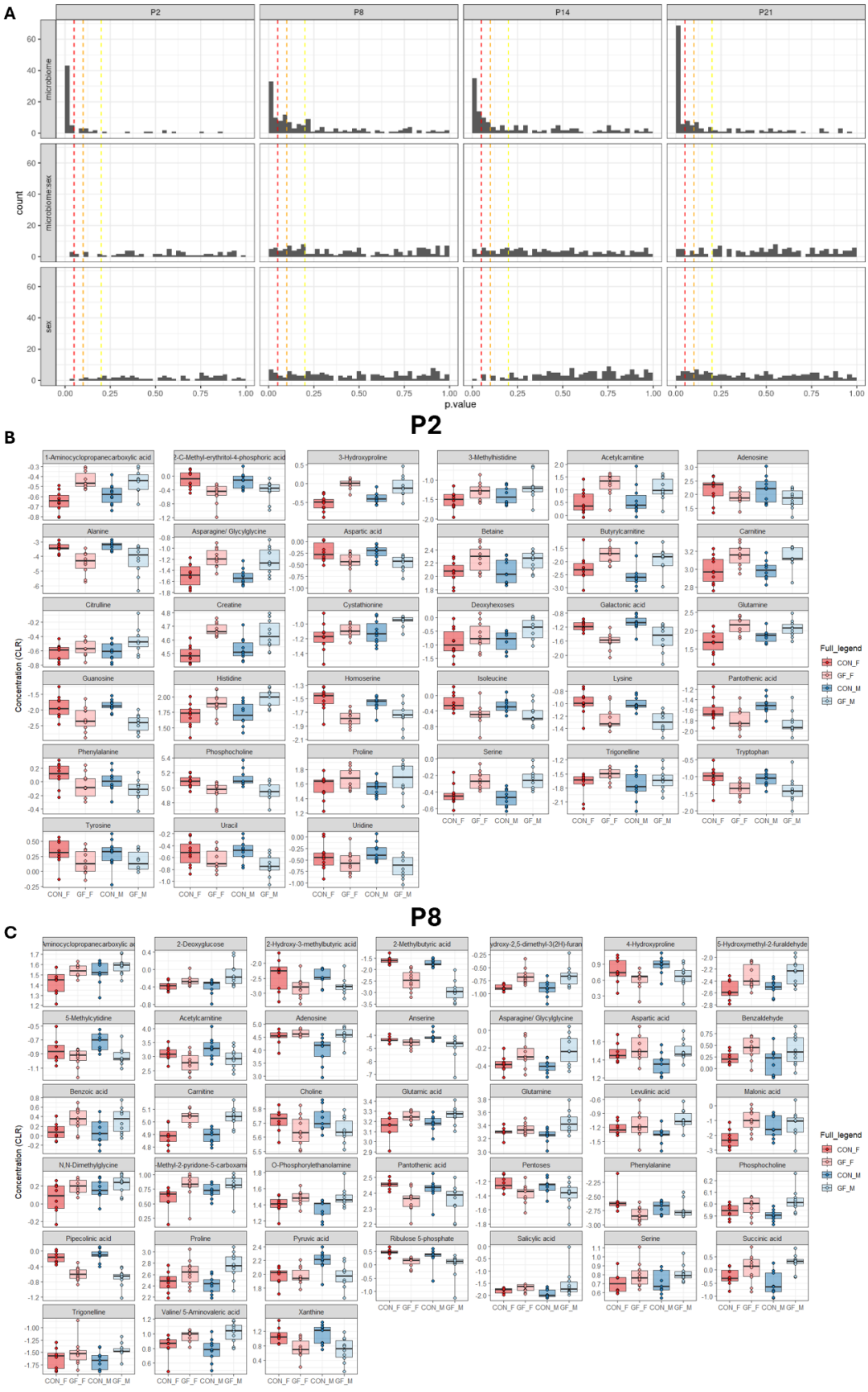
It is worth considering some of the limitations of the current study. Due to the extreme nature of the GF model, direct translation to clinical studies is not possible. To follow a similar thread, the mapping of developmental stages of mice to those of humans is an inexact science (Semple et al., 2013). Despite this, our study does emphasise the potential for microbiota perturbations in early life to impact a variety of developmental processes in the hippocampus, including development of normal synaptic plasticity. Secondly, this study follows in part

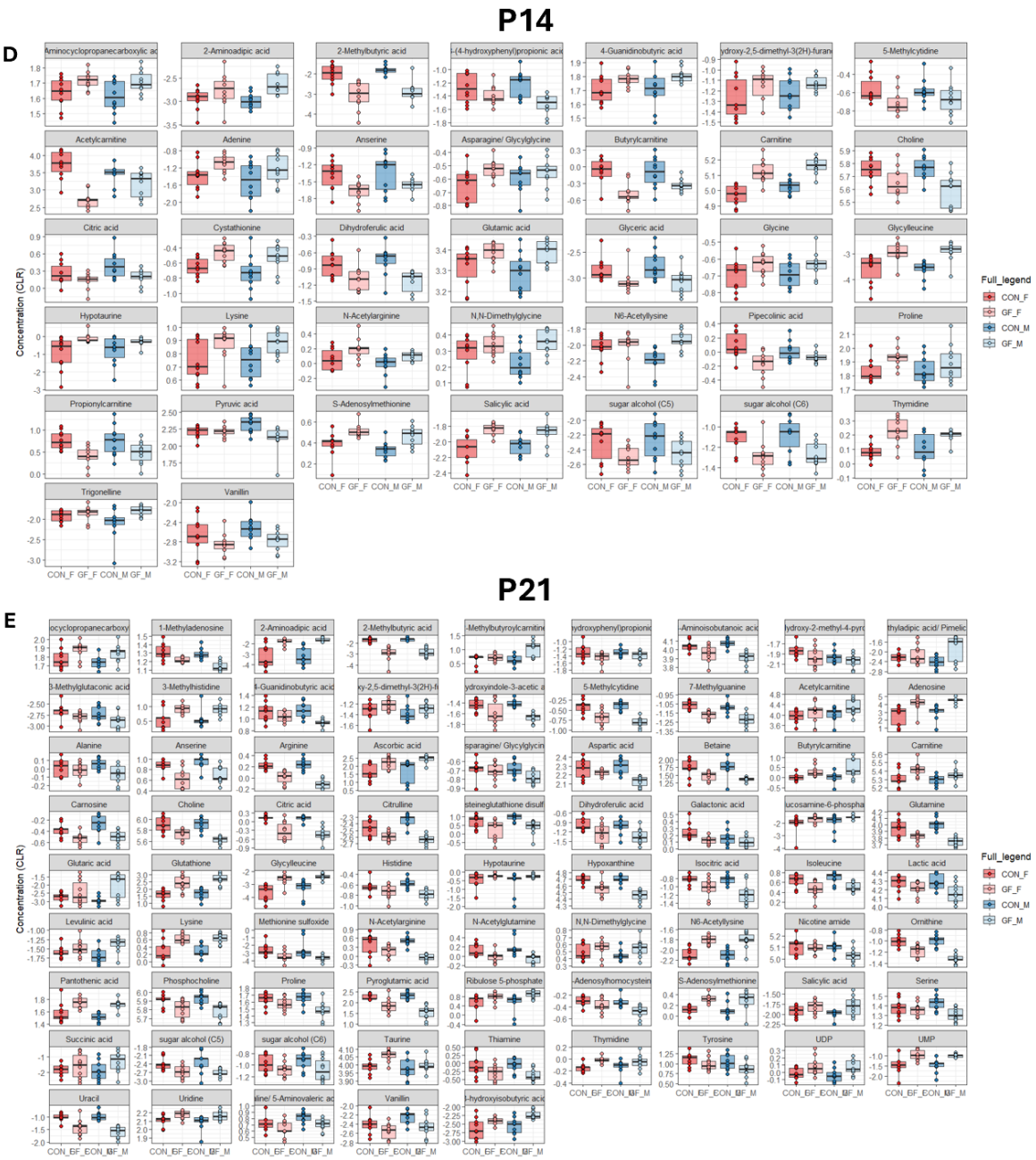
from previous electrophysiological examinations of hippocampal synaptic plasticity. Optimally this study would have included similar measures and compared those to the transcriptomic and metabolomic differences we observed. Inducibility of long-term potentiation is not dependable however at the majority of early life stages examined in this study (Ostrovskaya et al., 2020). Further, while the CON mice included are SPF, we do not have microbiome measures of these mice that would allow us to characterise the specific microbiome profiles we are comparing the GF state to, but we expect no major differences in the control microbiome.

6 Conclusion

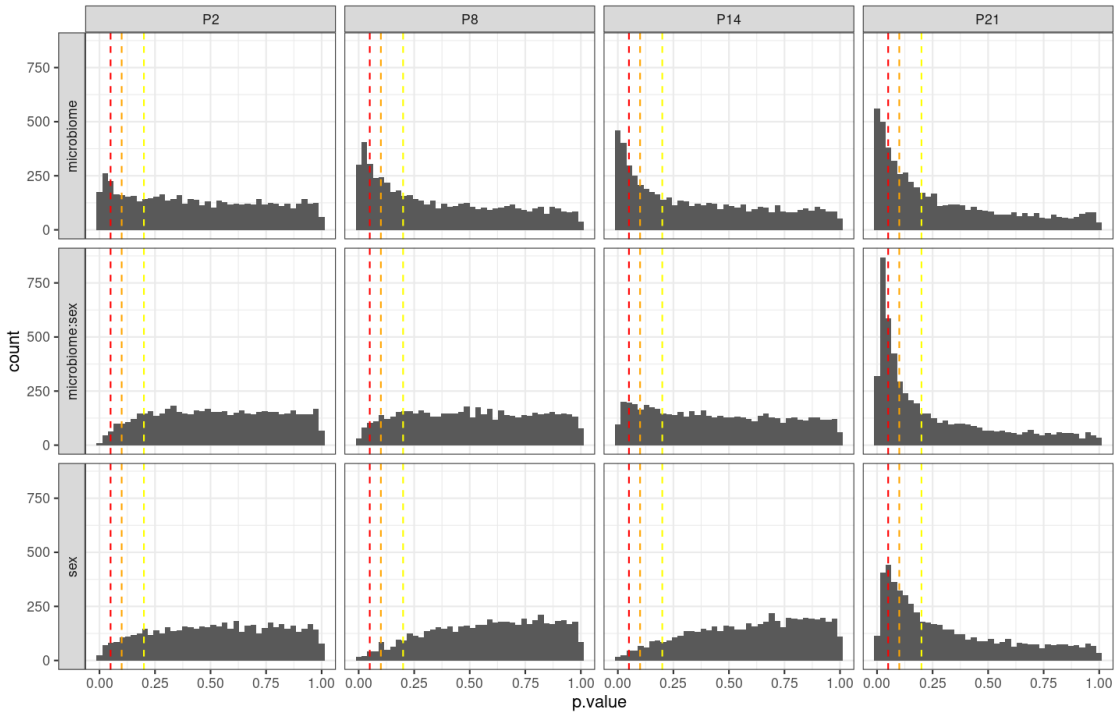
This study presents a multi-omics approach to give evidence to the likely presence of a critical window for the development of gene expression associated with hippocampal synaptic plasticity. We confirm early life origin of often-described microbiome mediated sex effects and give a mechanistic view of how these effects may be caused; These changes are microbial metabolite driven and manifest in the differential reaction of the male and female murine hippocampal transcriptome. Further we set the possible developmental origin of the altered structural plasticity seen in dendritic spines in adult GF mice. This study sows the seeds for several lines of further research including investigations into the timing of the appearance of structural changes to the germ-free brain.

7 Supplementary





Supplementary Figure 5.1 (A) P-value count charts comparing the hippocampal transcriptome of male and female germ free (GF) to conventional (CON) mice at four early life developmental timepoints, post-natal day (P)2, P8, P14, P21. P-value counts calculated at each timepoint across the effect of microbiome status, sex, and the interaction between microbiome and sex. A steadily growing effect of microbiome status is seen in the top row. The bottom row reveals a mild effect of sex at the three earliest timepoints which spikes at P21. The interaction effect of microbiome and sex follow a similar pattern to that of sex.



Supplementary Figure 5.2 P-value count charts comparing the hippocampal transcriptome of male and female germ free (GF) to conventional (CON) mice at four early life developmental timepoints, post-natal day (P)2, P8, P14, P21. P-value counts calculated at each timepoint across the effect of microbiome status, sex, and the interaction between microbiome and sex. A steadily growing effect of microbiome status is seen in the top row. The bottom row reveals a mild effect of sex at the three earliest timepoints which spikes at P21. The interaction effect of microbiome and sex follow a similar pattern to that of sex.

Chapter 6: General Discussion

1 Overview and Summary

This thesis has demonstrated that the gut microbiome plays a crucial role in modulating hippocampal synaptic plasticity, often in a sex- and age-dependent manner. The presence of the microbiota was found to be essential for the normal development of synaptic plasticity (**Chapter 2**). The ability of the microbiome to affect hippocampal plasticity was developmentally wired, decreasing in adulthood but does not disappear completely as we found that adult hippocampal synaptic plasticity (**Chapter 3**) remains sensitive to some microbial signals (**Chapter 4**). Furthermore, novel insights are provided into the development of synaptic plasticity-related gene expression during early life (**Chapter 5**).

Firstly, in Chapter 2, we observed a sex-specific effect of growing up germ-free, where male, but not female, mice exhibited a deficit in a form of synaptic plasticity known as LTP within the Schaffer Collateral pathway between the hippocampal CA3 and CA1 regions. This finding aligns with previous research demonstrating sex-dependent neural responses to environmental factors during development (Rosenfeld & Trainor, 2014), but uniquely implicates the microbiome as a key modulator of these differences. Notably, this work extends the understanding of how early-life microbial exposure shapes neurodevelopment, highlighting the critical role of the gut-brain axis in male-specific hippocampal plasticity. Furthermore, the observed reduction in the integration of synaptic excitation into action potential generation in germ-free slices underscores the microbiome's role in fine-tuning synaptic function, independent of sex-specific effects.

Building on these findings, we considered the possibility that hippocampal plasticity in adulthood may either be developmentally altered due to a lack of microbial exposure during critical periods or that the adult hippocampus

requires ongoing gut microbial signalling to sustain normal synaptic plasticity. To explore the latter hypothesis, as presented in **Chapter 3**, we administered a 2-week antibiotic treatment to adult male and female mice. Interestingly, our results indicate that adult hippocampal properties and synaptic plasticity, as measured by LTP, paired-pulse facilitation, and basic synaptic efficacy, remains robust despite microbiome depletion. This result contrasts with the pronounced effects of germ-free status observed in **Chapter 2**, suggesting a developmental window during which microbial signals are essential for establishing hippocampal plasticity, after which these processes become more independent of the microbiome. These findings provide important new evidence for the temporal specificity of gut-brain interactions in shaping neural plasticity and resilience in adulthood.

In **Chapter 4**, we further investigated the potential influence of the microbiome on adult hippocampal synaptic plasticity, this time treating hippocampal slices *ex vivo* with physiological concentrations of SCFAs. While acetate and propionate did not alter synaptic plasticity, butyrate significantly enhanced LTP in both male and female mice. Strikingly, mechanistic investigation revealed a sex-specific component to this effect, reminiscent of the sex-specific effect of the microbiome observed in **Chapter 2**; pre-incubation with an FFAR3 inhibitor abolished the butyrate-induced LTP enhancement in female slices, while male slices exhibited only a small, non-significant reduction. This result demonstrates the ability of the microbiome to exert influence over adult hippocampal plasticity. Our discovery that butyrate enhances LTP via a sex-specific mechanism **(Chapter 4)** provides the first direct evidence of a microbial metabolite influencing synaptic plasticity in a sex-dependent manner. While previous work has implicated SCFAs in brain function (Dalile et al., 2019), the specific role of FFAR3 in modulating synaptic plasticity, represents a novel mechanism that had not been previously described.

In **Chapter 5**, we shifted our focus to the developmental influence of the microbiome on the hippocampal transcriptome and metabolome, with a

particular emphasis on pathways related to synaptic plasticity. By examining germ-free and conventionally raised mice at four early life stages (post-natal day (P) 2 to P21), we leveraged a multi-omic approach to investigate how the microbiome shapes hippocampal development during critical windows. Our metabolomic analysis identified P21 as the most differentially regulated timepoint in germ-free mice, irrespective of sex, suggesting that this stage may represent a critical timepoint in microbiome-dependent metabolic programming.

In parallel, transcriptomic analysis revealed significant microbiome-induced alterations in the hippocampus, with effects peaking at P21 and showing strong enrichment in pathways associated with synaptic plasticity. Importantly, unlike the metabolomic changes, the transcriptomic data highlighted a pronounced interaction between microbiome and sex, with females displaying the greatest differences in gene expression. These findings extend previous research demonstrating sex-specific effects on neurodevelopment (Breach & Lenz, 2023; Edlow et al., 2022), by identifying the microbiome as a key regulator of these processes at the transcriptomic level. Moreover, the observed dissociation between metabolomic and transcriptomic sex-specificity underscores the complexity of gut-brain interactions and suggests that distinct mechanisms underlie microbiome influence on different aspects of hippocampal development.

Collectively, this chapter provides novel insights into how the microbiome orchestrates hippocampal development at molecular and metabolic levels. The identification of P21 as a critical timepoint for microbiome-dependent regulation of synaptic plasticity-related pathways highlights a developmental window during which microbial interventions may be particularly impactful. These results further underscore the sex-dependent nature of microbiome-neurodevelopment interactions, revealing potential pathways for targeted therapeutic strategies.

Across the chapters, a unifying theme emerges: the influence of the microbiome on hippocampal synaptic plasticity is both developmentally dynamic and context dependent. In early life, the microbiome is critical for normal synaptic plasticity development **(Chapters 2 and 5)**, with notable sex differences in its effects. In adulthood, while synaptic plasticity becomes less dependent on microbial signals **(Chapter 3)**, specific microbial metabolites like butyrate retain the ability to enhance plasticity in a sex-specific manner **(Chapter 4)**. This developmental trajectory underscores the microbiome's capacity to shape neural function across the lifespan, revealing mechanisms that may be leveraged for therapeutic interventions in neurodevelopmental and neuropsychiatric disorders.

Collectively, these findings underscore the changing ability of the microbiome to shape hippocampal synaptic plasticity at varying life stages, and shed light on some of the underlying mechanisms, including the involvement of FFAR3 and the modulation of synaptic plasticity-related gene expression and metabolite biomarkers in neurodevelopment.

1.1 Hippocampus and Microbiome: What Do We Know, What Have We Learned, and Where Are We Going?

The gut microbiome has emerged as a significant modulator of central nervous system (CNS) function, including the hippocampus (Tang et al., 2021b). The hippocampus is pivotal for cognitive processes like learning and memory (Hainmueller & Bartos, 2018; Lisman et al., 2017) and also plays a key role in the regulation of mood, influencing conditions such as depression and anxiety (Campbell & MacQueen, 2004; Ghasemi et al., 2022). Processes like hippocampal neurogenesis and synaptic plasticity are essential for these functions (Akhondzadeh, 1999; Toda et al., 2019). Given these wide-ranging functions of the hippocampus, understanding its relationship with the microbiome is of considerable importance. This is underscored in

neurodegenerative diseases, where hippocampal dysfunction is prominent. The hippocampus is among the earliest brain regions affected in Alzheimer's disease (AD) (Rao et al., 2022), and evidence suggests that the gut microbiome may contribute to the pathogenesis of this condition (Chandra et al., 2023). The link between the gut microbiome and AD is among the connections which have catalysed further exploration into how microbial communities can influence hippocampal structure and function.

1.1.1 Structural and Functional Implications

One of the key findings in this area is the impact of the microbiome on hippocampal morphology. Studies using GF mice have shown increases in hippocampal volume, accompanied by changes in dendritic spine morphology of pyramidal neurons and altered branching patterns of granule cells in the ventral hippocampus (Luczynski, Whelan, et al., 2016). Colonisation of GF mice post-weaning between P21 and P28 has been found to return hippocampal size to SPF levels, despite the volume of most regions being unaffected by conventionalisation (Yeo et al., 2023). This observation by (Yeo et al., 2023) shows the particular sensitivity of the hippocampus toward metabolic alterations and importantly lines up with the timepoint at which the greatest transcriptomic alterations were noted, as covered in **Chapter 5**. These previous studies highlight the microbiome's role in shaping hippocampal architecture during development.

Microbiome-induced changes are not limited to structure alone but are paralleled by functional implications. These include sex-dependent changes in hippocampal serotonergic signalling (Clarke et al., 2013), and changes in hippocampal neurogenesis (Ogbonnaya et al., 2015). Our research has expanded on these findings concerning hippocampal serotonergic signalling. The study by Clarke and colleagues (Clarke et al., 2013) found that six to nine week old GF Swiss Webster mice showed significant elevation in hippocampal concentrations of the main metabolite of serotonin, 5-hydroxyindoleacetic acid (5-HIAA), in male but not female mice. This sex-specific finding is echoed in

Chapter 5 where we found that GF C57BL/6 male but not female mice had reduced concentrations of hippocampal 5-HIAA at P21. This contrasting finding may speak to host-microbiome interactions differing due to age or genetics. Work from this thesis further adds to discoveries involving the GF hippocampal phenotype, demonstrating that the absence of microbiota during critical developmental periods leads to long-lasting changes in hippocampal synaptic plasticity in a sex-dependent manner (Darch et al., 2021 (**Chapter 2**)).

1.1.2 Early Life and Adulthood Hippocampal Susceptibility

Previous antibiotic research gives further sway to the concept of critical developmental periods during which insults to the gut microbiome may cause cognitive deficits. Early life exposure to maternal antibiotics has been found to have wide-reaching behavioural effects that include deficits in social recognition and novel object recognition in offspring (O'Connor et al., 2021). Not all early-life antibiotic exposure produces similar deficits, however. Early life microbiome depletion from P4-13 has previously been found not to cause spatial memory deficits in adulthood (O'Mahony et al., 2014). This result potentially aligns with the results presented in **Chapter 5** in which transcriptomic targeted enrichment analysis of synaptic plasticity pathways found that out of four early life timepoints, P2, 8, 14, and 21, the two earliest timepoints, P2 and 8 showed little to no difference brought about by GF status. Together these results reinforce the possible existence of specific critical windows, during which cognition and its associated synaptic plasticity may be vulnerable to microbiota depletion.

The development of the hippocampus is tightly interwoven with the presence and composition of the gut microbiota. Early-life microbial colonisation coincides with critical periods of hippocampal development, suggesting a potential influence on neurodevelopmental trajectories. There is a need to delineate the specific microbial signals and mechanisms involved in this process, as well as the long-term functional outcomes of early-life microbiome alterations. In **Chapter 5** we provided transcriptomic and metabolomic evidence for the existence of a critical window for the microbiome influence over the

development of hippocampal synaptic plasticity as well as a variety of other processes. Previous studies have found, for example, that germ free status produces strong changes in cellular metabolism in the nucleus accumbens in adulthood (Sens et al., 2023), however, they do not pinpoint when in life the microbiome exerts these changes. Our work in **Chapter 5** helps to map when these changes may occur. A previous study which did aim to piece together microbiome influence over neurodevelopment conventionalised GF mice at P21 and examined the transcriptomic profile of the amygdala at adulthood (Stilling et al., 2015). Similar to our hippocampal transcriptomic work they found differential gene expression implicated in processes such as neuronal plasticity and neurotransmission; despite the specific genes involved largely differing (Stilling et al., 2015). Nonetheless, we did demonstrate non-significant changes in several more of the genes found to be differentially altered in the study by Stilling and colleagues (Stilling et al., 2015). This suggests that our transcriptomic data may underestimate the degree of change for individual genes, potentially due to limitation of sample size.

Moving on from early life, antibiotic administration in adulthood has also caused cognitive deficits. Microbiome depletion in 10 week old rats caused deficits in Morris Water Maze performance (Hoban, Stilling, et al., 2016b), a spatial memory test associated with the dorsal hippocampus (Othman et al., 2022). This result is strangely opposite to the lack of synaptic plasticity deficits we saw in the dorsal hippocampus in **Chapter 3**. This may be due to the length of antibiotic treatment (12 weeks at behavioural testing in comparison to the two-week depletion we carried out). The extent to which longer or indeed acute microbiome depletion paradigms affect hippocampal plasticity remains an area requiring further investigation.

1.1.3 Microbial Metabolites and the Hippocampus

As laid out in the introduction to this thesis there are many potential pathways for communication between gut microbes and the hippocampus. An emerging area of interest is the role of microbial metabolites, such SCFAs, in modulating

hippocampal function. In **Chapter 4** we showed that hippocampal synaptic plasticity was enhanced upon exposure to butyrate through a sex-dependent mechanism, meaning the ability of these metabolites to cross the blood-brain barrier (BBB) and exert effects on the brain locally could be crucial. Prior studies have identified the expression of specific SCFA transporters, such as the monocarboxylate transporters (MCTs), in the BBB (Gerhart et al., 1997; Leino et al., 2001; Vijay & Morris, 2014). The expression of these transporters can be modulated by factors such as acute exercise, which has been shown to enhance MCT levels in the hippocampus, potentially further facilitating the passage of butyrate into the brain (Takimoto & Hamada, 2014). Given the many sex-dependent microbiome findings it is important to note that the regulation of MCT1 on the blood brain barrier is also sex hormone-dependent (Q. Zhang et al., 2022). Understanding the regulation of these transporters and their impact on hippocampal physiology may help elucidate gut microbiome-brain interaction. While local exposure following passage through the BBB has significant potential importance, other routes of communication between the microbiota and the brain are more established, namely the vagus nerve.

The vagus nerve is the primary and fastest means of neural communication between the gut and the brain (Fülling et al., 2019b). Gut-derived signals mediated by the vagus have been found to change hippocampal activity (Larsen et al., 2016). Foundational studies for this concept found electrical stimulation of the vagus nerve potentiates hippocampal LTP in freely moving rats (Y. Zuo et al., 2007) and facilitates learning and memory in clinical and preclinical studies (Clark et al., 1998, 1999). Conversely, selective vagal sensory/afferent ablation in rats has been found to impair hippocampus-dependent episodic memory and spatial memory (Suarez et al., 2018). This study by Suarez and colleagues mapped the neural pathway connecting the gut to the hippocampus, finding the medial septum relays vagal-derived signals from the nucleus tractus solitarius to dorsal hippocampal neurons (Suarez et al., 2018). Progress has since been made in microbiota-centred examinations of the connection between the vagus nerve and the hippocampus and pharmacogenetic vagus nerve stimulation has even

been identified as a potential therapeutic strategy to reduce microbiota-dependent age-associated impairments in hippocampal function (Rei et al., 2022). Vagotomy has been found to reduce and or prevent cognitive deficits in mice brought about by exposure to the pathogenic *Paenalcaligenes hominis* (K.-E. Lee et al., 2020), and abrogate hippocampal neurogenesis deficits, neuroinflammation, and depressive-like behaviours following faecal matter transplant from mice that underwent chronic unpredictable mild stress (Siopi et al., 2023). Prebiotic supplementation of 2´-fucosyllactose has also been found to potentiate hippocampal LTP in behaving rats in a vagus-nerve dependent manner (Vazquez et al., 2016). While no studies currently exist to our knowledge showing vagal influence over SCFA-mediated changes in hippocampus-associated behaviours, SCFAs do signal to the brain through the vagus nerve; SCFAs have been found to suppress central control of food intake by activating vagal afferents (Goswami et al., 2018). Together these studies illustrate the importance of the vagus nerve in microbiota-hippocampal interactions and the potential for hippocampus-dependent behaviours to be mediated in part through the vagus nerve. Despite this, need still exists for a targeted examination of whether SCFAs may affect hippocampal activity through the vagus nerve.

In conclusion, the interaction between the hippocampus and the microbiome is a rapidly evolving field with significant implications for understanding brain health and disease. By unravelling the complexities of this relationship, we can advance our knowledge of fundamental brain processes and identify novel strategies for promoting cognitive and emotional well-being.

1.2 Cognitive Impairment - The Role of the Microbiome.

The global population is aging, leading to a rising prevalence of cognitive impairment and age-related diseases such as AD which feature such deficiencies (“2023 Alzheimer’s Disease Facts and Figures,” 2023). This age-related impairment is associated with increased mortality, decreased quality of life, and functional disability (Panza et al., 2018). Furthermore, impaired cognition is associated with malnutrition, mortality in hospital, and increased

hospital admission rate (Amini et al., 2019; Fogg et al., 2019; Malara et al., 2014). The development of therapeutic solutions to this problem should be prioritised. It is now well known that changes in the gut microbiome accompany aging (Buford, 2017; Vaiserman et al., 2017). Chronic low-level inflammation is one of aging's hallmarks and is associated with various age-related diseases and frailty (L. Zuo et al., 2019). Alterations in the gut microbiota have been implicated in this process, contributing to the observed inflammation and decline in mental function (Buford et al., 2018). Nevertheless, this leaves open the possibility that the microbiome can be harnessed to treat these cognitive outcomes.

1.2.1 Alzheimer's Disease, the Microbiome, and the Hippocampus

Emerging research highlights the significant influence of the gut microbiome on brain function in diseases which affect cognitive function such as AD. The microbiome signature of AD patients is markedly different (Vogt et al., 2017) and some of the changes to the gut microbiome begin before symptoms such as memory loss appear (Ferreiro et al., 2023b). Further evidence of the microbiome-AD link appeared in recent studies, demonstrating that young adult rats receiving gut microbiota transplants from AD patients exhibited deficits in memory tasks, such as the Morris water maze, and showed decreased neurogenesis (Grabrucker et al., 2023).

Concurrently, studies using transgenic mouse models of AD have demonstrated significant alterations in microbiota composition (Dodiya et al., 2019; Harach et al., 2017; L. Zhang et al., 2017). Foundational research in germ-free mice revealed a substantial reduction in amyloid plaque formation and neuroinflammation in the absence of microbial communities (Harach et al., 2017). More recent research has also shown the protective nature of the GF condition against another hallmark of AD, tau-mediated neurodegeneration (Seo et al., 2023). Similarly, prolonged administration of an antibiotic cocktail to APPPS1-21and APPSWE/PS1ΔE9 transgenic mice respectively, reduced the accumulation of microglia and astrocytes surrounding amyloid plaques in the hippocampus, as well as decreased presence of insoluble amyloid-β plaques

(Dodiya et al., 2019; Minter et al., 2016). Seo and colleagues also found that antibiotic treatment was neuroprotective in AD model mice, but only in males and not females (Seo et al., 2023). Collectively, these findings underscore a regulatory role of the gut microbiota in modulating key molecular mechanisms associated with AD.

The putative anti-AD agent, icariin, further underscores the links between AD, the hippocampus, and the microbiome. In preclinical studies icariin has been shown to significantly improve cognitive dysfunction and typical AD pathologies (Y. Liu et al., 2023). Furthermore, icariin reversed AD-induced metabolic disorder associated with sphingolipid and glycerophospholipid metabolism. Icariin-induced microbiome changes affected the abundance of *Alistipe* and *Akkermansia,* which were found to be closely related to glycerophospholipid and sphingolipid metabolism, potentially demonstrating a microbiome-mediated mechanism of action (Y. Liu et al., 2023). With the changes to hippocampal LTP reported here in **Chapter 4,** and gut microbiome-mediated therapies in mind, it is important to note the potential role of SCFA-targeted treatments in AD. The shuttling of glutamate-glutamine from astrocytes to neurons, a process promoted by SCFA supplementation, has been shown to be instrumental for neuroprotective activity in a preclinical AD mouse model (Y. Sun et al., 2023). However, the effects of SCFAs in mouse models is nuanced. In the study by Seo and colleagues mentioned above, while GF mice were protected against tau-pathologies, SCFA supplementation increased gliosis and tau pathology in these mice (Seo et al., 2023), potentially demonstrating a negative role for these metabolites in AD.

Overall, these findings prompt critical questions about which specific features of the microbiome can affect cognition and its underlying processes and what microbiome interventions can be used to benefit those experiencing impairments in these processes. Our own research, where antibiotics were used to modulate the gut microbiome, revealed no significant differences in LTP, a neural correlate of learning and memory (Jeffery & Morris, 1993; Moser et al., 1998), underscoring the nuanced relationship between the microbiome and

synaptic plasticity. In **Chapter 4**, we demonstrated that the SCFA butyrate, at known physiological concentrations, has the potential to alter hippocampal synaptic plasticity. This finding strengthens the possibility that microbial metabolites may be an important part of microbiome-based interventions harnessed to treat disease-associated deficits in processes such as learning and memory. Given that synaptic plasticity is a fundamental cellular mechanism underpinning learning and memory, such interventions could be of significant therapeutic value in the context of cognitive impairment.

1.2.2 Microbiome Interventions: Effects on Cognition

1.2.2.1 Probiotics

Probiotics have emerged as a potential intervention for cognitive decline, primarily due to their anti-inflammatory properties (Virk et al., 2024). The effects of probiotics on impairments of mental function are not yet fully understood. Several preclinical studies have reported beneficial outcomes in models of AD (Athari Nik Azm et al., 2018; Bonfili et al., 2017; Nimgampalle & Kuna, 2017), neuroinflammation (Musa et al., 2017), and other conditions such as vascular dementia (J. Liu et al., 2015b) and diabetes (Davari et al., 2013). A study using the probiotic *Clostridium butyricum* in a vascular dementia mouse model demonstrated that the probiotic could ameliorate spatial learning deficits, an effect they concluded was mediated by increased butyrate production (J. Liu et al., 2015b). This underscores the potential of butyrate-producing bacteria in supporting brain health through reduction of neuroinflammation due to its anti-inflammatory properties (Caetano & Castelucci, 2022). Our own research into the effect of butyrate fits neatly in amongst these studies as our work in **Chapter 4** demonstrated the capacity of butyrate to alter the cellular correlates of learning and memory.

1.2.2.2 Prebiotics

Prebiotics have also emerged as a potential intervention for cognitive decline. In a human study on elderly Americans an interesting sex dependent effect was discovered, where for elderly men but not women, general prebiotic use seemed

to be an effective method to protect against this decline (J. Chen et al., 2023). This result is reminiscent of the sex difference observed here in **Chapter 2**, perhaps pointing toward male cognitive health being more easily treatable with microbiome focused interventions. Other fibre studies contrast this sex-dependent effect somewhat. In a four-week dietary intervention which supplemented polydextrose fibre to the diet of adult women it was found that fibre supplementation provided improvements a task requiring sustained attention (Berding et al., 2021). These sex differences in cognitive effects of prebiotic supplementation have not yet been properly replicated in preclinical studies leaving the possible mechanism at play unknown. Preclinical studies have however shown similar benefits of prebiotic supplementation. In adult male rats, supplementation with 2´-fucosyllactose enhanced LTP to improve learning and memory in a vagus dependent fashion (Vazquez et al., 2016) while in other studies prebiotic supplementation of fucoidan has been shown to reduce LPS-induced deficits in spatial memory through reducing neuroinflammation and BBB permeability among other mechanisms (Y. Wang et al., 2022). Some interventions employ the use of both pre- and probiotics together, termed a synbiotic, to improve cognitive measures. In one study in male rats a synbiotic treatment of *Enterococcus faecium* and agave inulin was shown to reduce age-related decline in spatial and associative memory (Romo-Araiza et al., 2018).

1.2.2.3 Fecal Microbiota Transplantation in Cognitive Decline

FMT has been explored as a therapeutic intervention for various conditions, including ulcerative colitis and neurodegenerative diseases like PD (Keshavarzian et al., 2015; Konturek et al., 2015). FMT has shown great promise as an intervention in age-related cognitive decline in preclinical work. Several studies have found that transplant of the microbiota from young donors into an aged host can attenuate selective age-associated impairments such poor spatial memory (Boehme et al., 2021; Mossad et al., 2021; Parker et al., 2022). The changes seen by Boehme and colleagues were accompanied by reversal of age-related transcriptomic and metabolomic differences in the hippocampus (Boehme et al., 2021). Similarly, the age-related cognitive decline reversed by

FMT in the study by Mossad was associated with metabolomic differences such as an increase in δ-valerobetaine in aged mice (Mossad et al., 2021). These results help to emphasise the relevance of the transcriptomic and metabolomic differences found in **Chapter 5**. The power of FMT to change cognitive ability has also been seen in the converse of these studies, namely the FMT from old to young rats. One such study found that the FMT brought about a strong decline in performance in spatial and working memory akin to what is normally seen in the aging process, accompanied by decreased regional homogeneity in the hippocampus, decreased BDNF expression, and increased neuroinflammation (Y. Li et al., 2020). Similar results with learning and memory and synaptic plasticity have been replicated elsewhere (D'Amato et al., 2020b). Taken together offer the promise that plasticity and cognition are highly manipulable targets through microbiome intervention.

In PD, a pro-inflammatory microbiome state is characterised by low counts of butyrate-producing bacteria (A. Xie et al., 2022). Despite this, evidence on whether FMT can improve cognitive function in PD is mixed. Some studies report FMT brings about no improvement in these measures (Scheperjans et al., 2024), while others suggest a positive effect (Cheng et al., 2023), particularly in patients showing enrichment of butyrate-producing bacteria post-FMT. In a study where young GF mice received an FMT from aged mice, adult hippocampal neurogenesis increased (Kundu et al., 2019b), which is usually associated with positive cognitive outcomes (Toda et al., 2019). Similar to the previously mentioned study by Cheng and colleagues (Cheng et al., 2023), this study concluded that microbial butyrate was central to the changes seen following a recreation of the phenotype through exogenous butyrate treatment (Kundu et al., 2019b). These findings highlight the complexity of the microbiome's role in neurodegenerative diseases and suggest that increased butyrate production and the successful colonisation of the gut by butyrate-producing bacteria may be key factors in the therapeutic efficacy of FMT.

FMT has shown promise in ameliorating cognitive deficits in other scenarios; for example, in improving the cognitive deficits induced by a high-fat diet in mice

(Pereira et al., 2024). In a preliminary study, FMT from healthy donors into AD patients with a relapsed *Clostridioides difficile* infection was found to correlate with an increase in the faecal expression of genes involved in the metabolism of BHB, a ketone body related to butyrate and FFAR3 functioning (J.-S. Kim et al., 2024). Additionally, treatment with BHB has been shown to rescue LTP deficits in AD model mice (Di Lucente et al., 2024). While these studies may not implicate butyrate directly, they do potentially implicate FFAR3 signalling which was a core message of our findings in **Chapter 4**. Curiously we found that BHB reversed butyrate's enhancement of LTP through a blockade of FFAR3 signalling, a seemingly opposite effect to that seen by Di Lucente and colleagues (Di Lucente et al., 2024). While these effects may seem to work in opposite directions they help to build a picture of cognitive decline being centred around abnormalities in microbiota/FFAR3 signalling, a topic receiving increasing attention (Mishra et al., 2024).

1.2.2.4 Microbiome Depletion – Effects on Cognition

Microbiome depletion is an intervention that has previously been used in the hopes of slowing progression of microbiome-associated diseases. For example, antibiotic depletion was used unsuccessfully in an attempt to slow disease progression in AD patients (Molloy et al., 2013). Given the role of the microbiome in cognition one may assume that the cognitive effects of microbiome depletion would be obvious, but in adulthood at least there seems to be only a tentative link. A low rate of cognitive impairment has been reported as a side effect of antimicrobial use (Warstler & Bean, 2016). Alternatively, there is also limited evidence that antibiotics use can improve cognition. A single administration of minocycline, an antibiotic which also crosses the blood brain barrier where it may have local effects on microglia (S & G, 1988), improved implicit learning in healthy adult volunteers (Chan et al., 2020). Furthermore, in middle-aged women, long-term antibiotic use correlated with minor cognitive declines after seven years (Mehta et al., 2022). Regarding amygdala-dependent learning it has been shown that minocycline has inhibitory effects whereas the recall of threat memory is significantly attenuated in individuals who were under doxycycline

during acquisition (Wehrli et al., 2024). Moreover, doxycycline slightly reduced motor learning but strengthened long-term motor memory, meaning in the Finger Sequence Tapping Task, the doxycycline-treated group acquired the sequence slower but retained the sequence better seven days post-acquisition. Despite these, albeit indirect links of microbiome depletion to cognition, we found a lack of effect of antibiotic treatment on hippocampal plasticity processes in adulthood in **Chapter 3**. This result speaks to the stability of the microbiome in adulthood. Taking into account the clear deficits in adult synaptic plasticity observed in **Chapter 2** when the microbiome was depleted throughout development in early life, we then turned our sight to the greater potential for synaptic plasticity to be changed by microbiome depletion in early life.

Early-life adversity can have profound impacts on brain development and function (Z. Huang et al., 2023). The microbiome plays a crucial role during this period, and disruptions can lead to long-term effects on cognition and behaviour. Notably, SCFAs such as butyrate contribute to maintaining the integrity of the gut mucosal barrier and may help mitigate the adverse outcomes of early-life stress (Ma et al., 2021). Investigating how early-life microbiome composition and interventions can influence hippocampal development and cognitive outcomes is a crucial area for future research. Chronic antibiotic use, particularly in early life, has been associated with an increased risk of cognitive impairment in later life (Z. Liu et al., 2022). Additionally, preclinical studies show that long-term gut microbiota depletion during early life can lead to memory impairments in adulthood (J. Li et al., 2022). These are similar to the cognitive deficits noted in adult germ-free mice (Gareau et al., 2011a). These observations support our findings from **Chapter 2**, where the absence of microbiome signals during development led to changes in synaptic plasticity in adulthood.

1.3 Biomarkers of Synaptic Plasticity Deficits - Could Circulating Metabolites be Markers of Future Cognitive Deficits

In **Chapter 5** we explored the interplay between metabolites and gene expression in the developing hippocampus. Here, we found that hippocampal synaptic

plasticity pathways disrupted by GF status correlated with disruption to the metabolome, helping to strengthen the evidence for metabolites as markers of cognitive deficits and the evidence for the gut microbiome being a mediator of such. Previous studies have used MR spectroscopy in children to find several metabolites that are associated with cognition (Perdue et al., 2023).

There now exists numerous studies providing evidence that links specific metabolites with lower cognitive function or cognitive decline. For example, alpha-aminoadipic acid, found in greater abundance in GF animals (see **Chapter 5**) is associated with faster cognitive decline (D. Li et al., 2017). Another study in adult humans, linked levels of glycoprotein acetyls, ornithine, and glutamine present in the blood with lower cognitive ability and dementia (van der Lee et al., 2018). Importantly ornithine and glutamine were found to be downregulated in GF mice (**Chapter 5**), demonstrating the potential of the microbiome to alter metabolites with relevance to cognitive ability. While it curiously points toward GF status perhaps having a protective effect over cognitive ability this is likely due to metabolites having age-specific effects. In children, low glutamine levels are associated with lower cognitive ability (Rogers & Pelton, 1957), an effect somewhat replicated in rodents (Son et al., 2019). Similar positive effects of glutamine, mediated by the microbiome were found in a study which conducted FMT from young children into mice, raising plasma glutamine and cognitive and memory related parameters (Guzzardi et al., 2023). Other metabolites seem to support this hypothesis. Low levels of acylcarnitine, as seen in GF animals at P21 in (**Chapter 5**), are associated with a faster cognitive decline as measured in an adult human population over seven years (D. Li et al., 2017), an observation which fits well with the lower cognitive ability found in GF animals (J. Lu et al., 2018).

These results show that a nuanced perspective is needed in linking metabolomics biomarkers of cognition and their connection to the microbiota The metabolite level changes observed in GF mice (**Chapter 5)** do not always align with the directionality of metabolite changes associated with cognitive decline as reported in the literature. More studies are needed describing the age-

sensitive nature of these metabolic biomarkers for cognitive ability. There is potentially limited value in looking at levels of individual metabolites while overall metabolic pathway analysis may be a more accurate predictor of cognitive ability. Furthermore, with current available technology one can analyse brain metabolites in living humans through MR spectroscopy (Perdue et al., 2023). Coupling this with microbiome analysis and cognitive testing, there exists the opportunity to trace the link between the brain metabolome, the microbiome, and cognition in human development.

1.4 Neuroplasticity: Further Mechanisms of Microbiome-Mediated Modulation

1.4.1 Epigenetic Regulation

The gut microbiota plays a significant role in epigenetic modifications such as histone acetylation and sumoylation, as well as DNA methylation (Stilling et al., 2014). These epigenetic mechanisms are associated with altered neural plasticity as they coordinate several translation pathways. The gut microbiota and can influence histones through inhibiting histone deacetylases (HDACs) via microbial-derived metabolites such as butyrate (Modoux et al., 2022; Yuille et al., 2018). Through this process, these metabolites may modulate the onset and progression of disease (Yuille et al., 2018). Histone deacetylase enzymes repress gene expression by removing acetyl groups bound to chromatin, driving histone acetyl transferase (HAT) activity, resulting in a tighter chromatin complex that becomes less accessible to RNA polymerase (S.-Y. Park & Kim, 2020). DNA methylation achieves a similar result in a different way. It is a process in which methyl groups are added to cytosine in DNA, thereby recruiting proteins involved in gene repression or inhibiting the recruitment of transcription factors to DNA (Moore et al., 2013). The gut microbiota once again has a hand in this process, being an important mediator between diet and host DNA methylation for example (D'Aquila et al., 2020). Probiotic supplementation (Lactobacillus rhamnosus) in zebrafish produced changes to DNA methylation of BDNF and tryptophan 5-monooxygenase genes (Cuomo et al., 2021). The gut microbiota

can influence this methylation through direct suppression of enzymes involved in methylation, and by altering the availability of essential cofactors and substrates required by epigenetic enzymes (Allison et al., 2021). For example, Bifidobacterium produces folate, a methyl donor required by DNA methyltransferase (DNMT) (Crider et al., 2012).

Epigenetic reprogramming is an established modulator of neural plasticity (Nayak et al., 2022). For example, loss of Dnmt3a, a DNMT, from excitatory neurons in mice alters the expression of synapse associated genes leading to impaired learning and memory (S. Li et al., 2020). In the context of the gut microbiome, links between DNA methylation and neural plasticity have been noted. In a preclinical model of traumatic brain injury, BDNF, a well characterised neurotrophin instrumental to neural plasticity (Miranda et al., 2019), is dysregulated due to the formation of a transcriptional co-repressor following histone deacetylase and DNA methylation activity at the BDNF promoter (Jadhav et al., 2023). Treatment with *Lactobacillus rhamnosus*, a potent SCFA producer, or FMT from an injury-naïve animal was found to alter these processes, normalising BDNF expression (Jadhav et al., 2023). Butyrate supplementation was able to recapitulate such effects leading to the conclusion that the effect is mediated by microbial butyrate's action as a HDAC inhibitor.

Moving forward, future studies should focus on the relative contribution of epigenetic pathways to microbial influences on LTP and plasticity across development and in the actions of SCFAs.

1.4.2 Adult Hippocampal Neurogenesis

Adult neurogenesis is a form of structural plasticity in the brain. It is the ability of the adult brain to produce new neurons in certain brain areas including the hippocampus (Kempermann et al., 2015) and has come to be regarded as an essential component for learning and long-term memory formation (Toda et al., 2019). This important process is regulated in part by the microbiome and has been explored in many different studies focusing on stress, old age, and AD for example (Cruz-Pereira et al., 2020; Guzzetta et al., 2022).

Simple foundational models such as antibiotic-treated animals, and the GF model have given clear evidence of the microbiota's influence over neurogenesis. In GF mice, neurogenesis is disrupted with an increased rate of neurogenesis in the dorsal hippocampus (Ogbonnaya et al., 2015). While this change may help to explain the altered cognition seen in GF mice (Luczynski, McVey Neufeld, et al., 2016b) increased neurogenesis is usually associated with better cognitive outcomes (W. Deng et al., 2010). Given these effects in GF mice were predominantly observed in the dorsal hippocampus this may have important implications for cognitive functions that primarily engage the same region such as spatial learning and memory (O'Leary & Cryan, 2014). Alternatively, microbiome depletion through long-term antibiotic administration induced a significant deficit in the process, a phenotype which could be restored with probiotic administration (a mixture of *Streptococcus thermophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, and Lactobacillus delbrueckii subspecies Bulgaricus*) or exercise (Möhle et al., 2016). A similar reduction in adult neurogenesis was brought about by early life antibiotic administration (G. Liu et al., 2022). These changes are cohesive with negative cognitive effects associated with long-term or early life antibiotic administration (Ye et al., 2024). Decreased neurogenesis has also been found to be a behavioural phenotype that is transferable by the microbiome. FMT from chronically stressed animals into healthy controls produced deficits in adult hippocampal neurogenesis (Siopi et al., 2023). While the gut microbiome can transfer this phenotype, microbiota based interventions such as treatment with a probiotic mixture of *Lactobacillus helveticus* and *Bifidobacterium longum* can also be used to prevent the deleterious effect of stress on neurogenesis (Ait-Belgnaoui et al., 2014). FMT can also transfer a reduced neurogenesis phenotype as seen in aged animals (Rei et al., 2022). Similarly a preclinical model of AD, created through FMT from AD patients into young adult rats, found decreased adult neurogenesis as part of its phenotype (Grabrucker et al., 2023). Despite seeing decreased neurogenesis in older animals, FMT from aged mice into young

GF mice has been shown to produce unexpected results, increasing the degree of neurogenesis present (Kundu et al., 2019b). In spite of the somewhat mixed effects of the microbiome on neurogenesis it is clear that microbiome interventions act as strong mediators of the process. These effects likely occur through a variety of pathways including immune modulation, as well as microbial metabolite and endocrine signalling (Guzzetta et al., 2022).

1.4.3 Neuroplasticity and the Microbiome: Lessons from the Visual Cortex

Other notable microbiome influences over neural plasticity have come from studies that have yielded intriguing results. Ocular dominance plasticity is a change in eye preference by cortical cell responses induced by monocular deprivation (Hensch, 2005). This process is normally only observed in development, but it can be induced in adult rodents through exposure to environmental enrichment (Greifzu et al., 2014). The reinstatement of this form of plasticity in adulthood appears to be microbiome-dependent, with depletion of the gut microbiota through antibiotic administration preventing activation of ocular dominance plasticity (Lupori et al., 2022). Ocular dominance plasticity was also found to be inducible in adulthood by Lupori and colleagues through SCFA supplementation and FMT from environmentally enriched animals, an effect possibly driven by SCFA remodelling of microglia (Lupori et al., 2022). This study is important as it shows that the microbiota can potentially trigger more juvenile forms of plasticity which could be more capable of inducing recovery from serious conditions such as traumatic brain injury and stroke. This stark plasticity reversion is reminiscent of studies which conduct FMT from aged mice to young or vice versa.

1.5 Modifying the Microbiome for Cognitive Health

Modifying the gut microbiome offers promising avenues for enhancing learning and memory function and maintaining hippocampal health. Two primary lifestyle factors, diet and exercise—are well-recognised for their influence on the gut microbiome and their potential benefits for learning, memory. These factors can

be modified to lower the risk of cognitive decline and impairment by promoting a beneficial microbiome composition.

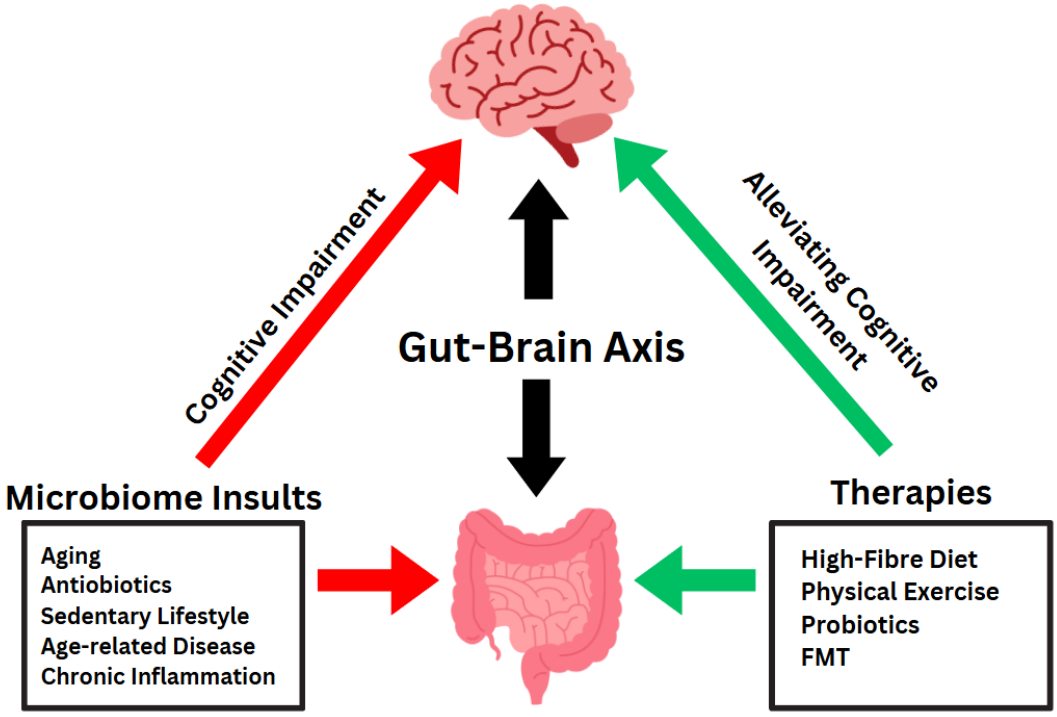


Figure 6.1 Adapted from(Y. Sun et al., 2020). Multiple factors including aging, and a sedentary lifestyle mediate cognitive impairment through the gut microbiome. Gut-microbiome associated therapies such as a high-fibre diet can work to ameliorate this impairment.

1.5.1 Diet and the Microbiome

Diet is a key modulator of the gut microbiome. Although the adult microbiome is relatively stable, dietary choices can significantly alter its composition (Mansour et al., 2021). Diet and eating habits impact cognitive function both in the short and long term (Kanarek et al., 2005; Prado & Dewey, 2014). While many effects on processes such as learning and memory are mediated without the direct influence of the microbiome by essential nutrients like omega-3 fatty acids, vitamins, and antioxidants (Dighriri et al., 2022; Fekete et al., 2023), the role of the microbiome in this context is gaining attention.

1.5.2 Western Diet vs. Mediterranean Diet

The Western diet, characterised by high sugar, saturated fats, and low fibre intake, is associated with cognitive impairment and increased risk of dementia (Francis & Stevenson, 2013; Parrott et al., 2021). In contrast, the Mediterranean diet, rich in fibre from whole grains, fruits, and vegetables and low in red meat and processed foods, is linked to improvements in the same measures (van den Brink et al., 2019). Given the focus of this thesis on the hippocampus it is of immediate relevance that adherence to the Mediterranean diet has been associated with a larger left hippocampal volume (Jacka et al., 2015). This association between the Mediterranean diet and improved cognition and changes to the hippocampus has not been consistently replicated, suggesting that the beneficial effects of the Mediterranean diet on hippocampal structure may not be robust (Gregory et al., 2023). Going deeper into this phenomenon it has been found that adopting the Mediterranean diet later in life may not be effective in preventing neurodegeneration or improving cognitive function, indicating that lifelong adherence might be necessary for maximal benefit (Gregory et al., 2024). This may hint at the importance of diet in early life specifically, underscoring the importance of healthy microbiome signals during the developmental periods examined in **Chapter 5**. While these studies sow reasonable doubt as to whether the later life adoption of a healthier diet can be used to alter the gut microbiome to improve cognition, other studies give some hope, including one study which found that later-life adoption of the Mediterranean diet did lead to increased learning and memory scores in older adults (Karstens et al., 2019). Diets which are more focused on simply raising fibre intake have perhaps shown greater promise for employing the microbiome to treat impaired cognitive function. This is backed by evidence from preclinical studies demonstrating that mice given a diet deficient in fibre rapidly develop deficits in object location memory, temporal order memory, and the ability to build a nest as measured by the deacon nest score (Shi et al., 2021). A modified Mediterranean ketogenic diet which focuses on both intake of fibre through fruit and vegetable consumption, and intake of healthy fats and proteins, has also

been found to increase SCFA levels, particularly butyrate and propionate (Nagpal et al., 2019). Given that lower levels of SCFAs have been observed in individuals with mild cognitive impairment, this diet's ability to boost faecal SCFA levels may be a key factor in its potential benefits. Butyrate plays a crucial role in maintaining gut health and has anti-inflammatory properties (Caetano & Castelucci, 2022). Aging is associated with reduced butyrate production alongside dysfunction in learning and memory, but supplementation with butyrate has been shown to alleviate these deficits in mice (Mishra et al., 2024). This aligns with findings in **Chapter 4** of our research, where butyrate exposure was able to modify hippocampal synaptic plasticity. Therefore, a high-fibre diet, which promotes the production of butyrate, could be an effective strategy for mitigating age-related cognitive decline.

1.5.3 Exercise and the Microbiome

Exercise is another potent modulator of the gut microbiome, capable of increasing the diversity of gut bacteria and enhancing the production of SCFAs in normal healthy individuals (J. M. Allen et al., 2018) and showing similar results in individuals with conditions such as irritable bowel syndrome (C. Li et al., 2024). Preclinical studies echo this result, showing that exercise can enhance the levels of butyrate-producing bacteria in the gut (Matsumoto et al., 2008; C. Yu et al., 2019) However, similar to the results of the dietary interventions noted above, the positive effects of exercise on the microbiome are not entirely dependable. For instance, exercise-induced changes to the microbiome may be more pronounced in lean individuals compared to those with obesity (J. M. Allen et al., 2018). In older populations, exercise increases the abundance of butyrate-producing bacteria but does not necessarily increase faecal butyrate concentrations (Torquati et al., 2023). Despite these limitations, exercise has demonstrated significant potential in mitigating cognitive deficits in older adults (C. H. Hillman et al., 2008). Additionally, exercise has been shown to enable hippocampal-dependent learning in rodents under conditions that are typically subthreshold for memory encoding and formation, as measured by Intlekofer and colleagues (Intlekofer et al., 2013). This effect was mediated through

butyrate-induced HDAC inhibition and the subsequent upregulation of brain-derived neurotrophic factor (BDNF). The mechanism is different from the pathway identified in **Chapter 4**, where butyrate acted through free fatty acid receptor 3 (FFAR3) to modulate synaptic plasticity. Exercise-induced changes to BDNF have also been noted elsewhere. A short period of high intensity cycling was seen to enhance circulating BDNF as well as performance in the hippocampus-recruiting face-name matching task (Griffin et al., 2011). Notably, other studies have found that exercise-induced changes in BDNF levels occur independent of histone acetylation (Kitahara et al., 2021), potentially aligning more closely with the FFAR3-mediated effects we observed. These effects may also be completely uninvolved with the microbiome as platelets are reported to be a source of BDNF (Yamamoto & Gurney, 1990), and are proposed to be the possible cause of exercise-induced increase in BDNF (Griffin et al., 2011). This BDNF source is relevant for the brain as BDNF is reported to be able to cross the BBB (W. Pan et al., 1998). Importantly, preclinical studies have also provided evidence for a BDNF related mechanism for enhanced hippocampal-dependent learning in rats (Bechara et al., 2014; Griffin et al., 2009). Other exercise-induced enhancements of age-related cognitive decline have been shown to act through the microglia (Mela et al., 2020). Here, we once again see links between the beneficial effects of exercise on cognition and the gut microbiota. FMT from exercised rats decreased microglia activation and neuroinflammation in a model of spontaneous hypertension (Xia et al., 2021). Curiously exercise has been shown to somewhat mitigate some aspects of gut-microbiota mediated deficits in spatial pattern separation and memory in rats caused by lifelong antibiotic treatment (Nicolas et al., 2024). This study found that exercise could fuel a net increase in neurogenesis in the hippocampus regardless of the anti-neurogenic effect of antibiotics. This effect was still somewhat attenuated by the disrupted microbiome, possibly due to the continued antibiotic-induced presence of low-grade inflammation in the hippocampus (Nicolas et al., 2024).

1.5.4 Timing and Synergy with Diet

Exercise-induced changes in the microbiome may be more robust in early life. Early-life exercise increases the abundance of *Lactobacillus* spp. and butyrate-producing bacteria, with increased faecal butyrate levels observed in rats which underwent an exercise intervention starting at a young age (Mika et al., 2015; Mika & Fleshner, 2016), an effect not replicated with the same intervention at an older age. In older individuals, combining exercise with a probiotic or prebiotic diet may be necessary to achieve similar microbiome changes (Mika & Fleshner, 2016).

Studies also indicate that the dose and type of exercise, as well as the breaking of sedentary behaviour, influences the composition of the gut microbiota (Bressa et al., 2017). Moreover, functional differences in the microbiome, such as enhanced SCFA production, have been observed in athletes compared to controls (Barton et al., 2018). Interestingly, FMT from exercised mice into sedentary mice on a high-fat, high-cholesterol diet increased SCFA content and mitigated diet-induced deficits in spatial memory (R. Li et al., 2023).

1.5.5 Are SCFAs the Link between Exercise and Cognitive Benefits?

The evidence suggests that SCFAs are likely one of the mechanisms through which exercise confers cognitive benefits. Exercise has the capacity to increase SCFA levels and butyrate-producing bacteria in the gut. The connection between exercise-induced microbiome changes and enhanced cognitive function is complex and influenced by various factors, including diet, genetics, and the type and duration of exercise. The findings that exercise-induced microbiome changes are more prominent in early life further emphasise the importance of timing when considering lifestyle interventions for cognitive health. The focus on early life intervention in these exercise- and diet-focused studies underscores the results seen in **Chapters 2** and **3** of this thesis which demonstrate the increased vulnerability of hippocampal synaptic plasticity to microbiome influence in early life. The strong emphasis for the mediating effect of butyrate in

these studies gives further relevance to the effect of butyrate on hippocampal synaptic plasticity in **Chapter 4**.

1.6 The Microbiome, Beyond Cognition

The Multifaceted Role of SCFAs

SCFAs, and their metabolites, play a significant role in maintaining cellular homeostasis with their anti-inflammatory, antioxidant, and anticarcinogenic properties (González-Bosch et al., 2021; Hinnebusch et al., 2002). This wide range of actions has implications beyond cognitive health and hippocampal function, potentially impacting various diseases and overall cellular health (H. Liu et al., 2018).

SCFA-targeted treatment has been explored in cancer contexts, such as colorectal cancer, where it may improve outcomes by modulating the tumour environment and promoting anti-carcinogenic activity (Kaźmierczak-Siedlecka et al., 2022; K. Kim et al., 2019; J. Sun et al., 2024). The anti-inflammatory and antitumor properties of butyrate may enhance the effectiveness of traditional cancer treatments, providing a supportive therapeutic strategy. In cerebral ischemia models, sodium butyrate administration has been shown to increase neurogenesis. It enhances the expression of neural proteins like polysialic acid-neural cell adhesion molecule, nestin, glial fibrillary acidic protein, and BDNF (Kim et al., 2009). These proteins are crucial for neural repair and regeneration, suggesting that butyrate could be a potential therapeutic agent for ischemic brain injuries and neurodegenerative conditions. The anti-inflammatory properties of butyrate extend to various body systems. By modulating immune responses, butyrate may help manage systemic inflammation, which is linked to numerous chronic conditions, including cardiovascular disease, metabolic disorders, and neurological diseases (Peng et al., 2023; Siddiqui & Cresci, 2021).

1.7 Outside The Hippocampus

1.7.1 Prefrontal Cortex and the Microbiome

Future research into how the microbiome affects synaptic plasticity in the brain should also be directed toward other brain areas such as the prefrontal cortex (PFC). Recent studies from the host lab (Hoban, Stilling, et al., 2016b) and elsewhere have shown that there is altered gene expression in the PFC of antibiotic-treated mice (Chu et al., 2019). How such changes affect neural plasticity remains completely unexplored at an electrophysiological level. The rodent PFC is an area of the brain that is crucial to functions such as fear, anxiety, emotional learning, and the regulation of the HPA axis (Morgan et al., 1993; Sullivan & Gratton, 2002). Dysfunction in the human and primate PFC is profoundly evident in various psychiatric and neurodevelopmental disorders such as bipolar disorder and autism (Amaral et al., 2008; Arnsten, 2009), disorders that have in turn shown altered gut microbiome in their pathology (Flowers et al., 2019; Oh & Cheon, 2020). The PFC is involved in reversal learning, attentional set-shifting, extinction of fear and social cognition, processes which are found to be altered in conditions such as schizophrenia and autism (Bicks et al., 2015; Godsil et al., 2013; Kehagia et al., 2010; Waltz & Gold, 2007; Worley et al., 2020). More recently there has been an accumulating interest on microbial metabolites as key regulators of brain function with implications in stress and neurodevelopment (Jaggar et al., 2020; Needham et al., 2020). Among the most studied are the SCFAs as well as tryptophan metabolites such as indole (Caspani et al., 2019). There is a complete absence of knowledge on if and how any of these metabolites, at physiologically relevant doses, can affect synaptic plasticity in the brain.

1.7.2 Amygdala and the Microbiome

The amygdala plays a central role in regulating stress responses and processing emotional stimuli, making it a critical focus for advancing treatments targeting emotional regulation disorders (Šimić et al., 2021). Neural pathways connect it with regions such as the PFC and hippocampus across limbic, subcortical, and

cortical networks. Emerging evidence shows that the gut microbiota may influence a variety of amygdala-dependent brain functions, including pain perception, social behaviour, and emotional regulation, which are closely tied to psychiatric health (Ressler, 2010). Moreover, current findings suggest that the gut microbiota is essential for the amygdala's normal structural and functional development (Fernandez-Real et al., 2015; Hoban et al., 2018b; Luczynski, Whelan, et al., 2016). Although further research is required to clarify the mechanisms of microbiota-to-amygdala communication, current research suggests the gut microbiota as a promising therapeutic target for conditions involving amygdala dysregulation, such as visceral pain and post-traumatic stress disorder.

Both clinical and preclinical research has demonstrated that the gut microbiota significantly influences the amygdala's functional connectivity, genetic expression, and overall function (Hoban et al., 2017, 2018b; Stilling et al., 2015). Thus it is not surprising that it is one of the most studied regions in the context of microbe-brain signalling (Cowan et al., 2018). Studies involving GF mice have revealed an altered amygdala transcriptome along with heightened neuronal activity (Stilling et al., 2015). These GF mice also exhibit increased amygdala volume and greater excitatory spine density within the basolateral nuclei (Luczynski, Whelan, et al., 2016). Transcriptomic modifications in the GF state involve genes linked to neural activity and synaptic transmission (Hoban et al., 2017).

Mice which received a traumatic brain injury had normal amygdalar BDNF expression restored through butyrate-centred microbiome intervention (Jadhav et al., 2023). Consistent with other brain regions, the amygdala may be further susceptible to microbiome intervention in early life, where antibiotic exposure produced significant alterations to adolescent microglial morphology (C. M. K. Lynch et al., 2023), while neurodevelopmental gene expression in the region is also significantly impacted by early life antibiotic exposure. This is in contrast to more subtle alterations brought about by the same intervention in adulthood (Lach et al., 2020). The amygdala has also shown itself to be an important top-

down regulator of the microbiome through a vagally mediated pathway from the amygdala to Brunner's glands in the duodenum (Chang et al., 2024). This study found that excitation of the central amygdala activates Brunner's glands which cause enrichment of *Lactobacillus* species in the gut, thereby reversing the effects of stress on the gut microbiome (Chang et al., 2024). Taken together these studies not only place the gut microbiota as an important regulator of the amygdala but also the amygdala as an important regulator of the gut microbiota.

Given the microbiome's important role in fear conditioning (Chu et al., 2019) and the role of amygdalar LTP in fear conditioning (Maren, 1999) it will be important moving forward to study plasticity-related correlates of microbiome-related amygdala-dependent behaviours changes.

2 Future Directions

The relationship between the microbiome and hippocampal function is a burgeoning field of study, offering promising insights into brain health and disease. Despite significant progress, several critical questions remain unanswered. Addressing these gaps is essential to fully understand the mechanisms involved and to develop effective interventions.

2.1 Mechanisms of Microbiome-Induced Hippocampal Synaptic Plasticity

A deeper understanding of how the microbiome influences synaptic plasticity and neurogenesis in the hippocampus is required. This includes identifying other microbial metabolites that may affect synaptic plasticity. Following characterisation of the effects of individual metabolites more work should follow into how these metabolites work in tandem with each other. For example, the specific ratios of SCFAs present in the body seem to have important implications for health (Müller et al., 2021). Acetate, propionate, and butyrate are present in an approximate molar ratio of 60:20:20 in the colon and stool (Besten et al., 2013). These ratios have been found to altered alongside the presence of depressive and gastrointestinal symptoms (Müller et al., 2021). While an isolated view of how these individual metabolites affect synaptic plasticity is necessary for our knowledge of fundamental microbiota-brain interactions there may exist synergistic effects that will only be revealed when metabolites are investigated in tandem with one another. Investigating how these normal and altered ratios affect synaptic plasticity is a logical next step to gain a more holistic view into how the gut microbiome affects synaptic plasticity. Further, while knowledge surrounding the signalling pathways of these metabolites is slowly growing there is still a lack of research into how different microbiome interventions exert their effects on hippocampal plasticity. Studies into topics such as the rate of expression of FFAR3 and FFAR2 heteromers and how this dimerisation affects signalling pathways is lacking (Ang et al., 2018). Further research should focus on elucidating how these interactions contribute to changes in synaptic strength,

neuronal growth, and overall cognitive function. SCFAs and other microbial metabolites play a significant role in modulating other brain functions also. Future studies should aim to pinpoint the exact mechanisms through which these compounds impact other processes such as neurogenesis. Understanding the influence of SCFAs on gene expression and signalling pathways within the hippocampus will lay the groundwork for their potential therapeutic applications.

2.2 Impact of Microbiome on Disease-Associated Hippocampal Changes

Investigating how microbiome changes contribute to hippocampal dysfunction in neuropsychiatric and neurodegenerative disorders is crucial. The establishment of a causal relationship between the microbiome and these diseases is difficult but is an essential part of where future research should focus. Progress is being made in this area, particularly through the randomised controlled trials in conditions such as cancer immunotherapy (Prosty et al., 2024). Aspects of good practice such as the gathering of long-term data following administration of microbiota-based therapeutics will aid this. Unravelling the role of microbiome disturbances in the progression of these diseases could reveal novel therapeutic targets and inform treatment strategies. There is a need to understand how microbiome-induced neuroinflammation affects hippocampal integrity and contributes to neurodegenerative processes. This includes examining the potential of specific microbial profiles and the presence or absence of certain functions in an individual's gut microbiome that may promote or mitigate inflammation and neuronal damage.

2.3 Intervention Strategies and Therapeutic Potential

Future research should explore and compare the therapeutic potential of microbiome interventions, such as dietary modifications, probiotics, and FMT, in restoring or enhancing hippocampal function. Key questions include determining whether antibiotic depletion pre-therapeutic administration is beneficial, determining optimal strains, doses, and treatment durations for these

interventions, as well as assessing if the characteristics of an individual's gut microbiota will affect the reliability of their impact on neuroplasticity and cognitive resilience. Investigating possible synergistic effects of combination intervention strategies, such as exercise with high-fibre diets or probiotic supplementation, could enhance benefits on the microbiome and cognitive function. This is particularly relevant for older individuals, where a multi-faceted approach may be necessary to achieve significant improvements.

Given the variability in individual responses to interventions, future studies should consider the development of personalised approaches based on genetic background, baseline microbiome composition, and lifestyle factors. Tailoring interventions to the individual's unique microbiome profile could improve efficacy and outcomes.

While research has shown that interventions like a high-fibre diet and regular exercise can benefit cognition in part through the microbiome alteration, the greatest potential benefit microbiome studies can have on human health cannot come solely from further study into these mechanisms but from public health initiatives. Although general awareness exists of the benefits of a healthy diet and exercise, many still struggle to adopt these habits due to barriers like convenience, access, and socio-economic factors. Rather than focusing solely on the biological effects of these interventions, efforts to promote behavioural change through public education, policy changes such as tax incentives, and environmental improvements are needed alongside scientific research to assure quality research has an appropriate impact. Governmental policies that make healthy food and exercise more accessible, through subsidisation of healthy foods, improvement of urban design for physical activity, or offering workplace wellness programs. These measures, alongside an increase of causal and mechanistic evidence on the link between microbiome and health may allow scientific knowledge to come into practice and realise benefits at a population level (Higgs et al., 2024).

2.4 Sex-Dependent Effects and Hormonal Interactions

There is considerable emerging evidence of sex-specific effects of the microbiome on hippocampal function. Research should focus on understanding how sex hormones interact with microbial signals to influence hippocampal function and disease susceptibility. Investigating these interactions will provide insight into the differential impact of microbiome interventions in males and females. Given the clear interactions between the microbiome and sex seen in this thesis (Darch et al., 2021, **Chapter 2, Chapter 4, Chapter 5**) and in other studies, research should be considered lacking if only males or females are examined. Understanding these differences will be crucial for developing sex-specific treatment strategies and for optimising therapeutic outcomes.

2.5 Early-Life Interventions and Long-Term Outcomes

Research into how early-life modulation of the microbiome through diet, exercise, or probiotic supplementation affects long-term cognitive outcomes is essential. These studies could inform strategies to prevent or mitigate neurodevelopmental disorders and support optimal brain development. Long-term, longitudinal studies in humans are necessary to establish causal links between early-life microbiome composition, SCFA levels, and cognitive function across the lifespan. Such studies will also help to confirm preclinical evidence of critical windows for intervention, the potential long-term benefits of early microbiome modulation, and in later life identify potential biomarkers for cognitive decline.

2.6 Alternate Methodologies – Advancing the Findings of this Thesis

To further enhance the findings of this thesis, the incorporation of more diverse electrophysiological techniques could provide valuable insights into synaptic plasticity within similar experimental contexts. For instance, exploring the transformation from early to late LTP could unearth differences where we have only seen subtle hints of changes such as those seen in **Chapter 3.** Exploring

other electrophysiological process such as spontaneous spiking activity, various LTD induction paradigms, alternate LTP induction paradigms such as those featuring a more subtle stimulus tetanus, and depotentiation would provide a more complete profiling of how the microbiome can affect synaptic plasticity in the brain. Additionally, integrating behavioural assessments that focus on hippocampal-dependent tasks in murine models would offer critical data regarding the functional implications of synaptic changes observed. Such behavioural paradigms, including spatial navigation tasks and memory assessments, would allow for a comprehensive understanding of the relationship between the microbiome, synaptic plasticity, and cognitive performance, thereby elucidating the translational significance of the neurophysiological findings. Additionally, techniques such as optogenetics would allow us to dissect the specific contributions of different populations of neurons in microbiome-influenced synaptic plasticity. Finally, a highly important topic which this thesis has not interrogated is the contribution of the vagus nerve in microbiome-associated synaptic plasticity. The use of vagotomy and the resulting lack of direct gut-brain communication would be highly useful in parsing out the relative contributions of different microbiome pathways to synaptic plasticity in the brain.

3 Limitations

As with all studies there are of course obvious limitations to the scope of what we have covered in this thesis. The focus of this thesis was centred on the hippocampus, a brain region integral to learning and memory. While this focus is justified, it is somewhat limited in scope, as other brain areas involved in related functions, such as the amygdala and prefrontal cortex, were not examined. Including these regions would have provided a more comprehensive understanding of how the microbiome influences neural circuits underlying complex behaviours.

In **Chapters 2** and **5**, recolonization experiments were not performed, limiting our ability to evaluate the potential of the microbiome to normalize changes in synaptic plasticity, gene expression, and metabolite abundance. Including recolonization would have provided stronger evidence for the causal role of the microbiome in these observed changes, thus future studies should focus on such approaches. Similarly, the decreased abundance of specific metabolites identified in **Chapter 5** represents an opportunity for further exploration going forward. Future studies could supplement these metabolites in GF animals, either during early life or adulthood, to determine whether such interventions can rectify the effects of microbiome depletion.

Additionally, extending the experimental window of **Chapter 5** from early life into adulthood would have provided critical insights into whether the observed early-life changes persist into later developmental stages. Incorporating electrophysiological measurements, like those used in **Chapter 2**, would have further enhanced the mechanistic understanding of microbiome-induced changes presented in **Chapter 5**.

The translational relevance of some findings in this thesis is another limitation. The GF model used in **Chapters 2** and **5**, while invaluable for elucidating the microbiome's role in neurodevelopment, is inherently extreme and may not reflect clinically relevant scenarios. **Chapters 3** and **4** offer more translational potential, but limitations remain. For example, while the supplementation of

individual SCFAs is clinically feasible, the effects of naturally occurring SCFA cocktails or endogenous SCFA dynamics remain unexplored, limiting direct applicability.

Lastly, all studies included in this thesis lack a behavioural component. While the focus on synaptic plasticity and molecular mechanisms provides important insights, behavioural assays would have been valuable in linking the observed changes in synaptic function and gene expression to functional outcomes, such as learning and memory. Including these assessments in future studies would strengthen the translational impact of this research.

4 Conclusion

This thesis comprehensively examined the ability of the microbiome to influence hippocampal synaptic plasticity, revealing a dynamic and context-dependent relationship that varies by sex and developmental stage. It was established that the microbiome is essential for the normal development of hippocampal synaptic plasticity, particularly in early life, with a pronounced sex-dependent effect on LTP in GF mice **(Chapter 2)**. This finding extends previous research on sex-specific vulnerabilities in neurodevelopment, identifying microbial influences as a novel determinant of these differences.

As hippocampal plasticity matures, its sensitivity to microbiome signals decreases, reflecting a shift in its reliance on microbial inputs. However, this thesis demonstrates that adult hippocampal synaptic plasticity retains the capacity to respond to specific microbial signals, such as butyrate, in a sex-dependent manner **(Chapters 3 and 4)**. Mechanistic insights, including the involvement of FFAR3 signalling pathways, provide new understanding of how microbial metabolites exert their effects, adding to the growing body of evidence linking gut-brain interactions to sex-specific neural function.

During early development, the microbiome was shown to regulate hippocampal transcriptomic and metabolomic profiles in a time-sensitive manner **(Chapter 5)**. These findings underscore the critical role of the microbiome in shaping

synaptic plasticity-related pathways during key developmental windows, with notable sex-specific interactions that may have lasting effects on neural function.

Collectively, this thesis positions the microbiome as a pivotal modulator of hippocampal synaptic plasticity across the lifespan, integrating evidence from molecular, cellular, and systems-level analyses. It builds upon existing literature by bridging gaps in our understanding of microbiome-neurodevelopment interactions, particularly regarding sex differences and age-dependent shifts in microbial influence. These insights not only deepen our fundamental knowledge of brain plasticity but also highlight potential therapeutic targets for microbiome-based interventions in disorders characterized by disrupted synaptic plasticity, such as autism and depression.

In conclusion, the findings presented here emphasize the developmental regulation of hippocampal synaptic plasticity by the microbiome while demonstrating its retained capacity for modulation in later life. This work provides an important new contribution linking the microbiome's influence from early development through to adulthood, offering new perspectives on the dynamic interplay between microbial signals and brain plasticity.

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